Genetic Mapping of Adult Plant Stripe Rust Resistance in the Wheat Cultivar 'Kariega'

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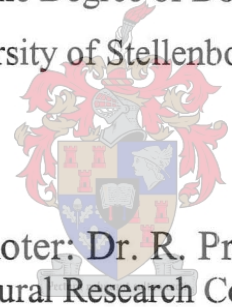

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I, the undersigned, do hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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Date.

SUMMARY

Stripe (yellow) rust of wheat, caused by *Puccinia striiformis* f.sp. *tritici*, was first detected as a single introduction into South Africa in 1996. Two additional pathotypes have since been identified. Control of the disease may be achieved by use of genetic adult plant resistance (APR) as is present in the local cultivar 'Kariega'. The aim of this project was to understand the genetic basis of the APR in 'Kariega' to facilitate breeding of new varieties with genetic resistance to stripe rust.

A partial linkage map of a 'Kariega X Avocet S' doubled haploid population covering all 21 wheat chromosomes was generated using 208 DNA markers, viz, 62 SSR, 133 AFLP, 3 RGA and 10 SRAP markers, and 4 alternative loci. The different marker techniques detected varying polymorphism, viz, overall SSR: 46%, AFLP: 7%, SRAP: 6% and RGA: 9%, and the markers produced low levels of missing data (4%) and segregation distortion (5%). A significant feature of the linkage map was the low polymorphism found in the D genome, viz, 19% of all mapped DNA markers, 11% of all AFLP markers and 30% of the total genome map distance. A region exhibiting significant segregation distortion was mapped to chromosome 4A and a seedling resistance gene for stem rust (*Puccinia graminis* f.sp. *tritici*), *Sr*26, mapped to chromosome 6A close to three SSR markers. The leaf tip necrosis gene, *Ltn*, which was also segregating in the population, mapped to chromosome 7D. Protocols for SRAP and RGA were optimised, and SRAP marker use in wheat genetic linkage studies is reported for the first time.

The linkage map was used together with growth chamber and replicated field disease scores for QTL mapping. Chromosomes showing statistically significant QTL effects were then targeted with supplementary SSR markers for higher resolution mapping. The quality of disease resistance phenotypic data was confirmed by correlation analysis between the different scorers for reaction type (0.799±0.023) and for transformed percentage leaf area infected (0.942±0.007).

Major QTL were consistently identified on chromosome 7D (explaining some 25-48% of the variation) and on chromosome 2B (21-46%) using transformed percentage leaf area infected

and transformed reaction type scores (early and final) with interval mapping and modified interval mapping techniques. Both chromosomal regions have previously been identified in other studies and the 7D QTL is thought likely to be the previously mapped APR gene *Yr18*. Minor QTL were identified on chromosomes 1A and 4A with the QTL on 4A being more prominent at the early field scoring for both score types. A QTL evidently originating from 'Avocet S' was detected under growth chamber conditions but was not detected in the field, suggesting genotype-environment interaction and highlighting the need for modifications of growth chamber conditions to better simulate conditions in the field.

The genetic basis of the APR to stripe rust exhibited by 'Kariega' was established by mapping of QTL controlling this trait. The linkage map constructed will be a valuable resource for future genetic studies and provides a facility for mapping other polymorphic traits in the parents of this population with a considerable saving in costs.

OPSOMMING

Streep of geelroes van koring word veroorsaak deur *Puccinia striiformis* f. sp *tritici*, en is die eerste keer in 1996 in Suid-Afrika na introduksie van 'n enkele patotipe waargeneem. Twee verdere patotipes is sedertdien in Suid-Afrika geïdentifiseer. Beheer van die siekte word veral moontlik gemaak deur die gebruik van genetiese volwasseplantweerstand soos geïdentifiseer in die plaaslike kultivar 'Kariega'. Die doel van hierdie studie was om die genetiese grondslag van die streeproesweerstand te ontrafel ten einde die teling van nuwe bestande kultivars moontlik te maak.

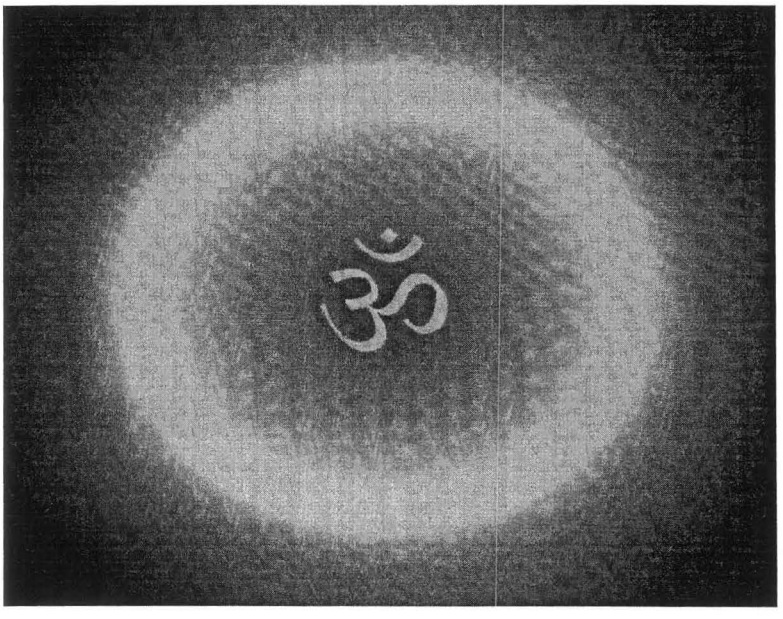
'n Verdubbelde haploïede populasie uit die kruising 'Kariega X Avocet S' is aangewend om 'n gedeeltelike koppelingskaart vir die volle stel van 21 koring chromosome saam te stel. Die kaart het uit 208 DNA merkers, nl., 62 SSR, 133 AFLP, 3 RGA, 10 SRAP merkers en 4 ander lokusse bestaan. Totale polimorfisme wat deur die verskillende merkersisteme opgespoor is, was as volg: SSR: 46%, RGA: 9%, AFLP: 7% en SRAP: 6%. Die mate van ontbrekende data was gering (4%) asook die mate van segregasie distorsie (5%) van 'n enkele geval wat op chromosoom 4A gekarteer is. 'n Prominente kenmerk van die koppelingskaart is die relatiewe gebrek aan polimorfiese merkers op die D-genoom, nl., slegs 19% van alle DNA merkers en 11% van alle AFLP merkers wat slegs 30% van die totale genoom kaartafstand bestaan het. Die stamroes (*Puccinia graminis* f. sp. *tritici*) saailingweerstandsgeen, *Sr*26, karteer op chromosoom 6A naby drie SSR merkers. Die geen vir blaartipnekrose, *Ltn*, karteer op chromosoom 7D. Protokolle vir SRAP en RGA merkers is ge-optimiseer en gebruik van SRAP merkers in koppelings-analise word vir die eerste keer in koring gerapporteer.

Die koppelingskaart is in kombinasie met groeikamerdata en gerepliseerde veldproefdata gebruik om die gene (QTL) vir volwasseplant streeproesweerstand te karteer. Chromosome met statisties betekenisvolle QTL is met aanvullende SSR merkers geteiken om die resolusie van kartering verder te verhoog. Die kwaliteit van fenotipiese data, soos in die proewe aangeteken, is bevestig deur korrelasies te bereken tussen lesings geneem deur onafhanklike plantpataloë (0.799 ± 0.023 vir reaksietipe en 0.942 ± 0.007 vir getransformeerde persentasie blaaroppervlakte besmet).

Hoofeffek QTL vir die twee maatstawwe van weerstand is deur middel van die metodes van interval QTL kartering en gemodifiseerde interval QTL kartering konsekwent op chromosome 7D (25-48% van variasie verklaar) en 2B (21-46% van variasie verklaar) geïdentifiseer. In vorige studies is aangetoon dat beide chromosome 7D en 2B QTL vir volwasseplant streeproesweerstand dra. Die 7D QTL is waarskynlik die weerstandsgeen, *Yr18*. QTL met klein effekte op weerstand is op chromosome 1A en 4A geïdentifiseer. Die effek van laasgenoemde geen was meer prominent in die velddata in die vroeë datum van weerstandsbeoordeling. Een QTL, afkomstig van 'Avocet S', is slegs onder groeikamertoestande identifiseerbaar. Dit dui op moontlike genotipe-omgewing wisselwerking en beklemtoon die noodsaaklikheid om aanpassings te maak in groeikamertoestande vir beter simulasie van veldproeftoestande.

Die genetiese grondslag van volwasseplantweerstand teen streeproes in die kultivar 'Kariega' is deur QTL kartering bepaal. Die 'Kariega X Avocet S' koppelingskaart kan as 'n waardevolle basis dien vir toekomstige genetiese ontledings van ander polimorfiese kenmerke in die populasie.

To My Parents



'It is what we make of what we have, not what we are given, that separates one person from another'

Nelson R. Mandela

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LIST OF ABBREVIATIONS AND ACRONYMS

a	Effect of the QTL
α	Alpha
A	Adenine
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
APR	Adult plant resistance
ARC	Agricultural Research Council
ATP	Adenosine tri-phosphate
AUDPC	Area under disease progress curve
Avr	Avirulence gene
bp	Base pair
BSA	Bulk segregant analysis
°C	Degrees celsius
C	Cytosine
c	Recombination fraction
CDL	Cereal disease laboratory (www.crl.umn.edu)
cDNA	Complementary DNA
CFA	Microsatellites developed at INRA, France
CFD	Microsatellites developed at INRA, France targeting the D genome
Chrom.	Chromosome
CIM	Composite interval mapping
cM	Centimorgan
CS	Chinese Spring
CSNT	Chinese Spring nulli-tetrasomic
CTAB	Cetyl tri-methyl ammonium bromide
DH	Doubled haploid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphophate
DR	Defense response
E	Multiplex ratio
EDTA	Ethylene diamine tetra-acetic acid
EM	Expectation maximization
EST	Expressed sequence tag
FISH	Flourescence *in situ* hybridization
G	Guanine
γ	Gamma
GDM	Gatersleben D-genome microsatellite
Glu-1A	High molecular weight glutenin subunit on 1A
Glu-1B	High molecular weight glutenin subunit on 1B
GWM	Gatersleben wheat microsatellite
H	Expected heterozygosity
HCl	Hydrochloric acid
HSB	Hybridization buffer
IDT	Integrated DNA Technologies
IM	Interval mapping
IRAP	Inter-retrotransposon amplified polymorphism
ITMI	International Triticeae Mapping Initiative
IX	Interaction effect statistic produced by Map Manager QTX
JIC	John Innes Centre
LAI	Leaf area infected
LB	Lesley A. Boyd

Ltn	Leaf tip necrosis
LOD	Log_{10} function of the ratio L_1/L_2
LRR	Leucine rich repeat
Lrstat	Likelihood ratio statistic
Ltn	Leaf tip necrosis
MAS	Marker-assisted selection
MIM	Multiple interval mapping
mIM	modified Interval Mapping
ml	Milliliter
MLE	Maximum likelihood estimate
MQM	Multiple QTL Mapping
N	Resistance gene in tobacco
No.	Number
NBS	Nucleotide binding site
NILs	Near-isogenic lines
NWMMP	National Wheat Molecular Marker Programme
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PIC	Polymorphic information content
PSP	Microsatellites developed at the John Innes Centre
PST	*Puccinia striiformis* f.sp. *tritici*
QTL	Quantitative trait loci
R	Resistance gene
RPS2	Resistance gene in *Arabidopsis thaliana*
RAPD	Random amplified polymorphic DNA
REMAP	Retrotransposon-microsatellite amplified polymorphism
RFLP	Restriction fragment length polymorphism
RGA	Resistance gene analogue
RILs	Recombinant inbred lines
RNA	Ribonucleic acid
SCAR	Sequence characterized amplified region
SDS	Sodium dodecyl sulfate
sec	seconds
SGI	Small Grain Institute
SNP	Single nucleotide polymorphism
SRAP	Sequence related amplified polymorphism
SS	Sum of Squares
Sr26	Seedling resistance gene for resistance to *Puccinia graminis*
SSC	Sodium chloride/ sodium citrate
SSR	Simple sequence repeat
STS	Sequence tagged site
T	Thymine
TBE	Tris-borate EDTA
TE	Tris-EDTA
U	Units
USA	United States of America
WB	Willem H.P. Boshoff
WMC	Wheat microsatellite consortium
WMS	Wheat microsatellite
Yr	Yellow rust
µg	Microgram
µl	Microlitre
µm	Micrometre
ZP	Z.A. Pretorius

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PREFACE

The results of this study as presented in this dissertation may be divided in to three focus areas, viz, the marker techniques, the development of the linkage map, and quantitative trait locus (QTL) mapping. These are presented in chapters 3, 4, and 5, respectively. A list of tables, figures and definitions of all acronyms used, together with a summary of the dissertation, is also provided.

Chapter 1 is a literature review ending with a statement of the purpose of the study and the study objectives. The literature review consists of four parts: (i) a general introduction to genetic resistance mechanisms and definition of terms used; (ii) a discussion of stripe rust and adult plant resistance in wheat, including resistance genes identified to date; (iii) a review of molecular markers used, together with descriptions of the techniques and their relevance to studies in wheat and (iv) QTL mapping theory covering the construction of linkage maps, QTL mapping methods and populations used, and associated problems.

Chapter 2 provides a detailed description of all protocols used in the study as well as descriptions of linkage and QTL analyses. This chapter is supplemented with appendices at the end of the dissertation listing chemicals and primers used.

Chapter 3 provides results of polymorphism studies using the different marker techniques. SSRs developed in different laboratories were used in this study and these are also compared in this chapter.

Chapter 4 describes the development of the linkage map, as well as some of the data checks performed, the distribution of the various markers mapped, and the mapping of additional genes segregating in the population. Maps for the chromosomes covered by the linkage groups are given at the end of this chapter.

Chapter 5 discusses the results of QTL analyses of the different disease scores with the different QTL mapping techniques. QTL results are then compared with those obtained by different groups working on the same disease trait.

Chapters 6 and **7** point out the conclusions made and possible future work that can be initiated based on the results of this study, respectively. The dissertation ends with list of references cited in the text and appendices.

Data obtained from co-workers that were used in this study is noted in the relevant sections.

CHAPTER 1: LITERATURE REVIEW

1.1 General Introduction

The defence systems that plants employ against pathogens differ from the immune system in vertebrates, but there are similarities in the defence strategies adopted. In both plants and vertebrates, active defence mechanisms are involved following pathogen infection. In vertebrates this response is produced by the various components of the immune system, whereas in plants a hypersensitive response is mostly produced (Keen, 1990). The hypersensitive response is associated with rapid cell death (necrosis) and synthesis of an antimicrobial compound at the site of infection which restricts growth of the pathogen (Dong, 1995). This response has been observed as the most common plant resistance response to viruses, bacteria, fungi, nematodes and insects (De Wit, 1997).

1.1.1 Genetics of the host-pathogen relationship

Knowledge of host-pathogen genetics requires an understanding of certain terms and concepts that are directed separately at the host and the pathogen. The host phenotype is described as the disease reaction, and this may be described as resistant or susceptible. The pathogen phenotype is described as avirulent when it is of low pathogenicity, and virulent in cases of pathogenicity (Johnson, 1992). Pathogens are variable with respect to host resistance but the variation is, in itself, a variable quality (Johnson, 1992). This means that in some cases, highly developed specificity occurs with regard to host resistance e.g. in rusts and powdery mildew. In other cases, a number of pathogens may differ in their ability to infect a number of hosts if each pathogen is used separately (Johnson, 1992).

Flor (1971) demonstrated that this observed variation was due to a gene-for-gene interaction between host and pathogen. In his work with flax and the flax rust pathogen *Melampsora lini*. Flor (1971) found that dominant avirulence, *Avr,* genes (*Avr* resulting in avirulence, and *avr* resulting in virulence) in the fungus, determined avirulence on flax, which contained corresponding dominant *R* genes (*R* resulting in resistance, and *r* resulting in susceptibility). The four possible combinations of alleles give resistance (avirulence) when *R* in the host coincides with *Avr* in the pathogen, and susceptibility (virulence) for all the other

combinations. It was hypothesized by De Wit (1997) that in some plant-pathogen interactions, the products of *R* genes recognize *Avr* gene products, and this provokes resistance. The minimum number of gene pairs to depict a gene-for-gene interaction is two, in order to confirm the specificity of the *Avr/R* interactions (Table 1.1).

Table 1.1. A gene-for-gene interaction between two host loci for resistance, each with two alleles (*R1* or *r1*, *R2* or *r2*) and the corresponding pathogen loci for pathogenicity (*Avr1* or *avr1*, *Avr2* or *avr2*) (Johnson, 1992).

Pathogen Alleles	Host Alleles			
	R1R2	*R1r2*	*r1R2*	*r1r2*
Avr1, Avr2	I	I	I	C
Avr1, avr2	I	I	C	C
avr1, Avr2	I	C	I	C
avr1, avr2	C	C	C	C

I - incompatible interaction (resistant/avirulent)
C- compatible interaction (susceptible/virulent)
C - shows that *R2* does not give incompatibility with *Avr1* and *R1* does not give incompatibility with *Avr2*, thus demonstrating the specificity of the *R1r1/Avr1avr1* and *R2r2/Avr2avr2* interactions

1.1.2 Resistance Genes

Support for Flor's gene-for-gene hypothesis has been provided by molecular genetics with the isolation and characterization of many virulence and avirulence genes (Keen, 1990). Two classes of genes, genes for resistance to plant pathogens involved in the recognition process (R genes), and genes involved in defence response (DR genes) have been cloned from diverse plant species and sequence comparisons among these genes reveal structural similarities (Chen *et al.,* 1998).

DR genes encode a diverse array of enzymes and their expression is induced by a range of offensive stimuli, e.g. pathogen challenge, insect attack and wounding (Li *et al.,* 1999). Map locations indicate that DR gene loci are not randomly distributed throughout the wheat (*Triticum aestivum* L.) genome but rather are located in clusters and/or in distal gene-rich areas of the chromosomes (Li *et al.,* 1999). Group 7 chromosomes were found to carry most of the DR genes. Faris *et al.* (1999) and Boyko *et al.* (2002) used DR genes as molecular

markers in candidate gene analysis in wheat which indicated that many of these loci map to regions associated with resistance to several pathogens.

The major plant disease resistance genes (R genes) that have been cloned can be divided into four structural categories with the majority of the R genes encoding leucine rich repeat (LRR) domains. Other categories include: the *Pto* gene in tomato that encodes a serine threonine kinase; *mlo* in barley (*Hordeum vulgare* L.) that produces a trans-membrane protein perceived to function as a negative regulator of cell death and the maize (*Zea mays* L.) *Hm1* gene encoding an active reductase enzyme that confers resistance to a fungal pathogen. The LRR class of resistance genes can be further divided into (i) those that encode a nucleotide binding site (NBS) LRR repeat; (ii) the *Cf* genes that confer resistance to *Cladosporium fulvum* and encode extracellular LRR and (iii) *Xa21* in rice (*Oryza sativa* L.) that encodes a receptor kinase domain at the C terminus (Langridge *et al.,* 2001).

The majority of cloned resistance genes encode proteins that resemble intracellular receptors with a nucleotide binding site (NBS) followed by a LRR domain at the C-terminal end of the protein (Spielmeyer *et al.,* 2000). These may be further divided into two sub-classes based on the presence or absence of a toll interleukin receptor domain at the N terminus. The LRR domains of these proteins may enable the plant to detect *Avr*-gene-specific pathogen molecules, while the NBS and associated structures may be involved with signalling and triggering plant defence reactions.

Lagudah *et al.* (1997) isolated NBS-LRR sequences of the *Cre3* locus for resistance to cereal cyst nematode, from *Aegilops tauschii,* and these were used to develop markers for other cereal cyst nematode resistance genes. Spielmeyer *et al.* (2000) used a resistance gene analogue (RGA) clone that produces markers co-segregating with a stripe rust resistance gene, to detect markers co-segregating with leaf rust resistance genes. Analysis of the super-family of NBS-LRR encoding genes revealed that they are present in all homoeologous groups (Spielmeyer *et al.,* 1998). Feuillet *et al.* (1997) isolated a kinase domain at the *Lr10* wheat leaf rust resistance locus using oligonucleotides corresponding to the serine/threonine protein kinases. Different parts of the wheat genome encode different rust resistance genes with varying specificity against different races, and molecular basis of this specificity remains unknown until these genes have been cloned.

1.1.3 Resistance classification

The gene-for-gene hypothesis serves as the basis for much of the work of plant pathologists, geneticists and plant breeders, and from it a number of different concepts with regards to resistance have been developed. The term race specific resistance may be used to describe resistance that interacts differentially with pathogen races, and may be applied to both complete resistance, and to components of incomplete resistance that so interact (Johnson, 1984). The detection of race specificity may be simple where reactions are qualitative, but may often involve the interpretation of statistical analyses for quantitative interactions (Scott *et al.,* 1980). Vanderplank (1982) defined race specific resistance as vertical resistance, and race non-specific resistance as horizontal resistance. Resistance provided by single genes (vertical resistance) mostly remains effective only until a race of pathogen that is able to circumvent it becomes established and, as a result, such resistance is likely to be useful only for a few years.

Seedling resistance is characterized by race specificity and a low infection type at all stages of plant growth and over a wide range of temperatures (Line and Chen, 1995). Race specific resistance, expressed as low infection type in seedling tests, often proves short-lived in the field owing to genetic shift and the emergence of new virulence in the pathogen population in response to host selectivity for race specific genes (Singh *et al.,* 2000). New races usually circumvent the seedling resistance of cultivars within 3-4 years after release when cultivars are used extensively over time and space (Line and Chen, 1995). However, the use of race specific seedling resistance in multi-line cultivars and cultivar mixtures has extended the duration of the effective use of seedling resistance (Chen and Line, 1995).

The alternative to race specificity, where resistance does not interact differentially with races, is termed race non-specific or horizontal resistance (Vanderplank, 1982), considered to be polygenically controlled and difficult to identify and evaluate (Sawhney, 1995). Scott *et al,* (1980) defined non-specific resistance as that which does not show any specificity after prolonged testing.

The term slow rusting was used by Caldwell *et al.* (1970) for leaf rust, caused by *Puccinia triticina* Eriks., to describe host-pathogen combinations in which rust develops slowly but

never reaches a high degree of severity (also incomplete resistance) despite the cultivars being susceptible. Vanderplank (1984) suggested that slow rusting was a form of partial resistance partitioned into vertical and horizontal components, with emphasis on the horizontal component. However, Parleviet (1981) differentiated slow rusting and partial resistance. According to him, all incomplete resistance (producing intermediate infection types) to rusts produces slow rusting, while partial resistance is a form of incomplete resistance characterized by susceptible or high infection types (excluding intermediate infection types).

Durable resistance to a disease is that which remains effective during its prolonged and widespread use in an environment favorable to the disease (Johnson and Law, 1975; Johnson, 1984). Durability can only be established retrospectively and tests for the presence of durable resistance include testing the cultivar at a number of locations and years, and with many races from an existing collection (Johnson, 1984). The focus of disease control is now durable genetic resistance (Johnson, 1981) that often involves race non-specific, slow rusting mechanisms, and is best identified in the adult stages. Adult plant resistance (APR) has been defined as resistance that is absent in young seedlings, but develops as the plant matures. APR have been reported to be involved in the durable stripe rust resistance of 'Cappelle Desprez' (McIntosh *et al.*, 1995). Cases have been reported where durable resistance in cultivars has been attributed to single genes (Russell, 1978; Sharp, 1983), providing a contrast to the idea that resistance must be under polygenic (more than one gene) control to be effective. In relation to resistance against stripe rust (*Puccinia striiformis* Westend. f. sp. *tritici* Eriks.), seedling resistance and APR are considered to be the most important types of resistance (Quayom and Line, 1985; Milus and Line, 1986; Chen and Line, 1995).

1.2 Stripe rust

Wheat (*Triticum* spp.) is widely cultivated throughout the world. The most commonly cultivated species include *T. aestivum* (bread wheat), *T. dicoccum* L. (einkorn) and *T. durum* L. (durum). The grain of wheat is utilized for the production of bread, pastries, biscuits, breakfast cereals, semolina products, as well as animal products and feeds. Approximately 718 000 ha is allocated toward wheat production in South Africa from which about 2.4 tons per hectare is harvested (FAO, 2000-2001). Wheat production is affected by the presence of several rust diseases.

Stripe rust (also known as yellow rust) of wheat, caused by the biotrophic fungal pathogen *P. striiformis* f. sp. *tritici* (PST), is one of the most damaging diseases in many areas of the world (Zadoks, 1961). The fungus *P. striiformis* West. also consists of the formae specialis (f. sp.) *hordei* that infects barley. The life cycle of the rust consists of three spore stages (Fig. 1.1), as opposed to the five spore stages in leaf and stem (*Puccinia graminis* Pers. f. sp. *tritici* Eriks & Henn.) rust, and no alternate host has been identified (McIntosh *et al.,* 1995). Stripe rust develops in cooler temperatures than the other rust diseases and this low temperature requirement of the pathogen restricts it as a major disease in the more temperate areas of the world.

Urediniospores of PST are the only known source of inoculum for wheat. These are vegetative spores that may be repeatedly produced during the growing season of the host (Roelfs *et al.*, 1992). Urediniospores occur in a dikaryotic stage and initiate dikaryotic mycelia and teliospores, which are at first dikaryotic. Nuclear fission (karyogamy) occurs in the spore to produce diploid nuclei. Teliospores produce basidia, and meiosis takes place within basidia to produce four haploid basidiospores (Hiratsuka and Sata, 1982).

Typical symptoms of stripe rust are long yellow stripes on leaves. A single infection on a leaf can produce a long stripe containing many uredia that produce orange-yellow urediospores. McGregor and Manners (1985) found that urediniospore production on wheat per unit leaf area infected was much lower at low light intensities than at high light intensities. Colonization rate and frequency of pustules per unit area of infected leaf increased between 7 and 15°C, but declined markedly at 20°C (McGregor and Manners, 1985). This foliar pathogen of wheat can reduce grain yield by up to 84%, kernel mass by up to 43%, and kernel number by up to 72% (Murray *et al.,* 1995).

Different races (or pathotypes) of PST exist. Understanding the relationship among the races, and how virulence evolves, is useful for developing management strategies for the disease (Chen *et al.,* 1993). Stripe rust prevalence, severity and distribution are monitored by trap plots consisting of different cultivars. Virulence of the pathogen is determined from trap plot data, and by the testing of rust collections with a set of differential cultivars that have different genes for resistance under controlled conditions (Chen *et al.,* 1993). Disease is usually assessed by the methods of infection type and reaction type (host response and symptoms),

assessed by the methods of infection type and reaction type (host response and symptoms), latent period (time for the disease to establish itself), and area under the disease progress curve (AUDPC) values.

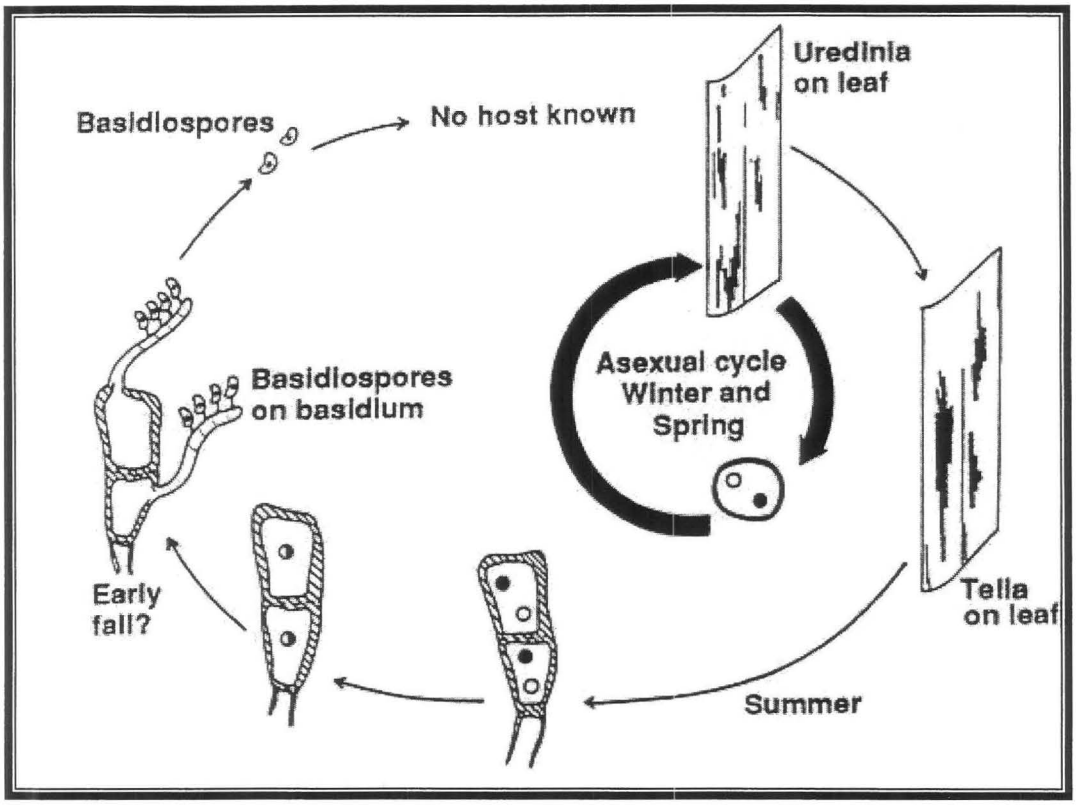


Figure 1.1. A diagrammatic representation of the life and disease cycles of *Puccinia striiformis* f. sp. *tritici* (Roelfs *et al.,* 1992).

The Cobb-scale (0-100% infected leaf area) or modifications thereof is used as a quantitative measure of disease infection (Peterson, 1948, McIntosh *et al.,* 1995 p11, Broers *et al.,* 1996). Host reaction type is widely scored on the classical ordinal scale R (resistant), MR (moderately resistant), MS (moderately susceptible) and S (susceptible), sometimes augmented (as in the present study) by three classes through practical experience of scoring the disease, *viz*, RMR between R and MR, MRMS between MR and MS and MSS between MS and S. This corresponds exactly to the order of the seven classes in the Australian Plant Breeding Institute adaptation of the original 10 class scale of McNeal *et al.* (1971).

1.2.1 Mutation rate of *P. striiformis* f.sp *tritici*

Virulence frequency dynamics denotes the phenomenon of change of frequency of virulence in populations of rust fungus, usually due to manipulation of the population of host plants by humans (Groth, 1984). Selection for the virulence allele within the pathogen population results in an increase in its frequency, and the host plants infected incur reduced fitness as this occurs. Genetic variation in rust fungi which gives rise to new pathotypes is believed to be controlled by the combined effects of mutation and migration with subsequent sexual reproduction and possibly somatic hybridization within the migrant and local populations (Watson, 1981, cited in Steele *et al.,* 2001). Wright and Leonard (1980) produced a novel pathotype of stripe rust by somatic recombination, but there is apparently no other evidence that new pathotypes arise in the field by this mechanism.

Molecular techniques have been used to examine the variation within rust species and between races. Newton *et al.* (1985) examined the variation among isolates of PST and two other cereal rusts using isozyme and dsRNA analysis. They reported that PST and *P. striformis* f. sp. *hordei* that infects barley, produced different isozyme and dsRNA patterns, but no variation within the diverse isolates of PST was found. DNA sequence analysis of the internal transcribed spacer region of ribosomal DNA was used successfully by Zambino *et al.* (1992) to differentiate PST from other rust species. Chen *et al.* (1993) used RAPD assays on 23 collections of PST and found 47% of the primers tested to be polymorphic in North American stripe rust populations. Steele *et al.* (2001) used AFLP and RAPD to examine molecular variation in New Zealand and Australian pathotypes of PST but found no molecular variation within these isolates.

Monitoring of pathotypes in Australia and New Zealand has revealed that new pathotypes appear to differ from existing ones only at single avirulence loci, suggesting a sequential pattern of single gene mutations for virulence (Wellings and McIntosh, 1990). The Stepwise Mutation Model, implying a spontaneous mutation from avirulence to virulence, is most likely an important mechanism for generating variation in virulence (Steele *et al.,* 2001). In Australia, the introduction of a single pathotype led to the development of some 15 to 20 new pathotypes in 10 years (Wellings and McIntosh, 1990). Further analysis is required to

ascertain whether the evolution of new pathotypes at avirulence loci is caused by random mutation/deletion events or by more specific events (Steele *et al.,* 2001).

1.3 Stripe rust resistance in wheat

Less is known about host resistance to stripe rust than resistance to the other wheat rusts. This can be attributed to many factors. Stripe rust investigations require specialized control in the greenhouse, such as temperature control to maintain growth of the fungus due to its high sensitivity (Roelfs *et al.,* 1992). Furthermore, the infection types are less discrete, there are numerous recessive host genes, the resistance genes have additive effects, there are temperature sensitive genes and many genes function only in the adult plant stages (Roelfs *et al.,* 1992). Despite these difficulties associated with working with the disease, progress has been made toward breeding cultivars with host resistance.

Biffen (1905) cited in Johnson (1992) reported that resistance to stripe rust in 'Rivet' wheat was controlled by a single recessive gene. He produced the cultivars 'Little Joss' and 'Yeoman' which remained resistant to stripe rust for many years while grown in Britain (Johnson, 1992). Physiologic specialization of PST was first demonstrated by Allison and Fisenbeck (1930) (cited in Johnson, 1992). Thereafter, there was an increase in the number of races identified, but only a small number of differential cultivars were used. Little information regarding the genetics of resistance was available, and these reported the presence of single dominant or recessive genes (Robbelen and Sharp, 1978, cited in Johnson, 1992). Lupton and Macer (1962) studied the inheritance of resistance in the seedlings of cultivars, and introduced the symbolism of *Yr* genes, initially identifying four *Yr* genes.

Chromosomal location is an intermediate step in establishing the identity and allelic relationships of a new rust resistance gene. A common method for locating a dominant resistant gene is the F_2 method whereby the resistant line is crossed to a monosomic series compromising each of the 21 chromosome pairs in a susceptible genotype. With a single dominant gene, segregation in the F_2 generation will be 3:1 (resistant: susceptible) for 20 of the 21 crosses. In the critical single cross, there will be a significant excess of resistant plants and the chromosomal location of the gene can then be established depending on the monosomic parent used in the cross. Molecular and cytological markers, such as C-banding,

and fluorescence *in situ* hybridization may circumvent the time consuming process of chromosome location using aneuploids (McIntosh *et al.,* 1995). Some of the currently characterized resistance genes are listed in Table 1.2. Most of the information was obtained from McIntosh *et al.* (1995) and the Cereal Disease Laboratory (CDL) web site (http://www.crl.umn.edu), but where additional information is included, the appropriate reference is noted. In addition to the genes listed there is also a set of differentials described by Johnson and Minchin, (1992). Stubbs (1985) refers to three distinctive specificities for adult plant resistance that is presumed to be different from those typified by the genes *Yr11-Yr14* and *Yr16* and *Yr18*. These were designated '12' ('Carstens V'), '13' ('Arminda'), '14' ('Alba', 'Apollo'), and '15' ('Dippes Triumph').

1.3.1 Adult Plant Resistance (APR)

APR genes are expressed at various growth stages in the plant life cycle. The results of Ma and Singh (1996) suggest that expression of adult plant resistance begins at the mid-tillering stage of wheat development. Their results agree with those of Qayoum and Line (1985) who worked with cultivars with genes for high temperature adult plant resistance (HTAP). Park and Rees (1989) also found that the percentage leaf area affected by stripe rust at mid-tillering was significantly less than the controls used. However, in some plants exhibiting intermediate levels of resistance, expression of resistance occurred later, and plants were less susceptible to stripe rust at the stem elongation growth phase (Ma and Singh, 1996). This may be due to translocation of nutrients by the plant at this stage. This would reduce the nutrient supply available to the rust to develop. Assessments of stripe rust are usually based on upper leaves, although other areas of the plant may be infected (McIntosh *et al.,* 1995).

HTAP resistance is influenced by temperature. In the field, as the season progresses and temperatures increase, rust develops slower on APR cultivars than on susceptible cultivars. Under controlled conditions, seedlings of APR cultivars are susceptible to the prevalent races over a wide range of temperatures. As these plants mature, they become more resistant when grown at high temperatures (diurnal temperatures of 10-30°C or higher). At lower temperatures (diurnal temperatures of 6-21°C or lower), the plants remain susceptible (Quayom and Line, 1985). APR, which is more desirable than seedling resistance, can also have race specific genes (Johnson, 1981).

Chen and Line (1995) conducted studies on HTAP resistance with the wheat cultivars 'Stephens' and 'Druchamp'. Both cultivars have durable, non-race specific HTAP resistance to

Table 1.2. Named genes for stripe rust resistance, their source, genome location and tester lines (adapted from Roelfs *et al*, 1992; Chen *et al*, 1996; Cereal Disease Laboratory).

Yr Gene	Genome location	Source	Tester	Reference
1	2A	Chinese 166	Chinese 166	Lupton and Macer (1962)
2	7B	Heines VII	Heines VII	Lupton and Macer (1962)
3a	1B	Vilmorin 23	Vilmorin 23	Lupton and Macer (1962)
3b	1B	Hybrid 46	Hybrid 46	Lupton and Macer (1962)
3c	1B	Minister	Minister	Lupton and Macer (1962)
4a	6B	Cappelle Desprez	Cappelle Desprez	Lupton and Macer (1962)
4b	6B	Hybrid 46	Hybrid 46	Lupton and Macer (1962)
5	2BL	*T. speltum album*	*T. speltum album*	Macer (1966)
6	7BS	Heines Kolben	Heines Kolben	Macer (1966)
7	2BL	Lumillo durum	Lee	Macer (1966)
8	2D	*T. comosa*	Compair	Riley *et al.*, (1968)
9	1Bl-1RS	Rye	Riebesel	Macer (1966)
10	1BS	Moro	Moro	Macer (1966)
11		Joss Cambier	Joss Cambier	Priestley (1978)
12		Caribo	Mega	Priestley (1978)
13		Ibis	Maris Huntsman	Priestley (1978)
14		Falco	Maris Bilbo	Priestley (1978)
15	1B	Dippes Triumph	*T. dicocc.* G25	Amitai *et al.* (1970)
16[a]	2DL	Cappelle Desprez	Cappelle Desprez	Worland *et al.* (1986)
17	2AS	*T. ventricosa*	VPM1	Bariana *et al.* (1993)
18[a]	7DS	Frontana	Jupateco 73R	Singh (1992a)
19	5B	Compare	-	Chen and Line (1995)
20	6D	Fielder	-	Chen and Line (1995)
21	1B	Lemhi	-	Chen and Line (1995)
	4D	Lee	Lee	Chen and Line (1995)
23	6D	Lee	Lee	Chen and Line (1995)
24	1B	K733 (durum)	-	McIntosh (CDL)
25	1D	TP1295	StrubesDickkopf	Calonec *et al.* (CDL)
26	6AS	Haynaldia villosa	Yangmai 5	Jones (CDL)
27	2BS	Selkirk	-	McIntosh (CDL)
28	4DS	*T. tauschii* W219	-	Singh *et al.* (2000)
29[a]	1BL	Lalbahadur	Lalbahadur	Singh (CDL)
30[a]	3BS	Opata 85	Opata 85	Singh (CDL)
YrA	3D	Avocet	Funo, Inia 66	McIntosh *et al.* (1995)
YrSelkirk	-	Bluejay S	Opata 85	McIntosh *et al.* (1995)

[a]Indicates APR genes

PST. They estimated that 2-3 HTAP resistance genes were involved which differed between the cultivars. Estimated broad sense heritabilities were 95-97%, and narrow sense heritabilities were 86-89%. The high heritability values obtained indicate that the effect of the APR genes is mostly additive and has important implications for selection in breeding programmes. Chen and Line (1995) concluded that combining these genes for HTAP resistance should produce new cultivars with improved stripe rust resistance.

Johnson (1992) argues that temperature sensitivity is extremely common in resistance to rust diseases. Therefore, a particular type of temperature-sensitive resistance (e.g. at high temperatures) is not necessarily indicative of durability. Schultz and Line (1992b) have shown that the level of effectiveness of HTAP resistance is location/environment specific. An example of this is the HTAP resistant cultivar 'Waner' from the USA, found to be more susceptible than other HTAP resistant cultivars from the USA ('Gaines', 'Nugaines', 'Luke') when tested in the United Kingdom (Johnson, 1992).

Sources of durable resistance carry both APR and HTAP resistance genes, so there has been increasing interest in understanding the host-parasite interaction involving these types of resistance. HTAP in wheat has remained durable for at least 30 years in the USA (Chen and Line, 1995) and increasing reliance is therefore being placed on both APR and HTAP resistance in breeding programmes. However, strains of PST with increased virulence on cultivars with APR have also been detected (Stubbs, 1985). In general plant pathologists and breeders need to know more about the diversity and genetic control of APR so they can manipulate it confidently and successfully in breeding programmes.

Broers *et al.* (1996) identified quantitative variation on a continuous scale between the two extremes of resistant and susceptible cultivars in replicated field assessments of spring wheat cultivars. This calls for an analysis of the variability in progeny groups of crosses using the methods of quantitative genetics, such as mixture model fitting and QTL mapping, where the latter utilises genetic maps of molecular markers. These approaches do not pre-suppose any basis of inheritance in terms of the number of genes segregating (i.e., monogenic or multigenic) in any particular segregating population (Young, 1996; Lynch & Walsh, 1998).

Apart from their own findings on the important roles of chromosomes 2AL, 2B and 7DS in stripe rust APR in the International Triticeae Mapping Initiative (ITMI) reference population 'Opata85 X W-7984', Boukhatem *et al.* (2002) point out that several other studies indicated the importance of chromosomes 2D (Worland and Law, 1986), 7DS and 7BS (Pink and Law, 1985), chromosomes 5AS and 5DS (Pink *et al.,* 1983) and the 5BS-7BS translocation (Johnson and Law, 1975; Law and Worland, 1996, 1997). Bariana *et al.* (2001) used the QTL mapping approach in identifying *Yr18* (chromosome 7D), *Yr29* (seedling resistance: chromosome 1BL), and *YrKat and YrCK* (APR: chromosome 2DS) in different mapping populations. Boukhatem *et al.* (2002) identified highly significant QTL on chromosomes 2B in the ITMI and 'Gb X 7' ('Camp Remy X Michigan Amber') mapping populations and on 7D (ITMI). Their QTL map of chromosome 7D covers the region of the location of *Lr34* (Nelson *et al.*, 1997) which is also closely linked to *Yr18* (Dyck PL, 1991; Singh, 1992a, b).

Yr16 is a single, dominant, APR gene, originating in common wheat and located on chromosome 2DL (Worland and Law, 1986). 'Cappelle Desprez' is a reference stock for this gene. *Yr16* probably contributes to the durable resistance of ' Cappelle Desprez ' that is a combination of *Yr3a, Yr4a* and *Yr16.* 'Cappelle Desprez' also carries the reciprocal translocation 5BL-7BL and 5BS-7BS, and Worland and Law (1997) report a correlation between the presence of the 5BS-7BS translocation and stripe rust resistance. Background chromosomes reportedly play a role in the APR of 'Cappelle Desprez', but this still remains incompletely characterized.

Mature plants with the *Yr18* APR gene are distinctly more resistant than related counterparts not possessing it. *Yr18* is located on chromosome arm 7DS, and is possibly completely linked to *Lr34* (a gene for APR to leaf rust) and also *Ltn*, which controls a distinct leaf tip necrosis (Singh, 1992a; 1992b; McIntosh, 1992). *Yr18* is widely used in spring wheats and some winter wheats. The genes *Yr18* and *Lr34* are characterized by moderately high or compatible infection types in the seedling stage and a disease lowering, or slow rusting effect, with increasing plant maturity (Singh, 1992a).

Yr18 has been identified as a key contributor to the slow rusting of wheat cultivars of CIMMYT origin (Singh and Rajaram, 1994). Results obtained by Singh (1992a) and McIntosh (1992) show that the durable APR in the cultivars 'Anza' and 'Bezostaja' is

controlled in part by *Yr18*. Studies at CIMMYT under growth chamber conditions have shown that *Yr18* increases latent period, and decreases infection frequency and the length of infection lesions (stripes). In 1996/1997 trials in New Zealand with lines containing *Yr18*, presence of the gene reduced AUDPC by up to 77%, and maximum disease severity from 88% to 35% (Viljaren-Rollinson and Cromey, 1998). Under field conditions, *Yr18* exerts partial control, and is best when combined with other slow rusting genes (Ma and Singh, 1996, cited in Singh *et al.,* 2000). Singh and Rajaram (1994) also reported that *Yr18* in combination with 2-4 additional slow rusting genes (*Yr18* complex) produces adequate levels of resistance in most environments.

Singh *et al.* (2000) identified regions on chromosomes 3BS, 3DS and 5DS associated with APR and the 3BS region originating from the cultivar 'Opata', was designated *Yr30*. The cultivar 'Lalbahadur' also exhibits an APR gene *Yr29* on chromosome 1BL (R.P. Singh, CDL) the chromosomal location of which was confirmed by Bariana *et al.* (2001).

In general it appears that only a limited number of APR *Yr* genes are available to plant breeders, and plant pathologists need to examine existing gene pools for additional sources. A wide range of genetic diversity is critical to successful breeding programmes and such a wide range is not as yet available. Existing resistance genes must be managed carefully to prevent rapid development of pathogen virulence as has happened in the case of many *Yr* genes. Disease management will play an increasingly important role to ensure that genetic resistance is maintained.

1.4 DNA marker informativeness

In the past, experiments in plant genomics have been based on morphological characteristics that have limitations including subjectivity and the influence of the environment. With the aid of DNA molecular markers scientists are now able to map the locations of genes on chromosomes more accurately. Genetic maps define the order of genes and other structures (centromeres, telomeres, etc.) along chromosomes in terms of recombination distances. Recombination distances are determined from recombination frequencies, which are converted to map distances using either the Haldane or Kosambi mapping function (Haldane,

1919; Kosambi, 1944). Many characteristics of DNA markers make them ideally suited for studying genomes.

Molecular markers are phenotypically neutral, and because most of the allelic variation is in the non-coding portion of the genome, this does not influence the region of interest (Tanksley, 1993). Allelic variation produces segregation that is required for linkage analysis. Low selection pressures and high mutation rates (in some DNA markers) cause allelic variation to be higher at molecular marker loci than at morphological marker loci (Tanksley, 1993). With a large number of DNA markers, it is theoretically possible to map all the genes affecting a particular trait. Some DNA markers are co-dominant so it is possible to infer all possible genotypes from the phenotypes in each generation. Molecular markers also do not exhibit epistatic or pleiotropic effects that are common with morphological markers.

DNA markers may be broadly classified into: (i) hybridization-based markers. e.g. restriction fragment length polymorphism (RFLP); (ii) polymerase chain reaction (PCR) based markers e.g. amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD); and (iii) DNA chip markers such as single nucleotide polymorphisms (SNP) (Gupta *et al.,* 1999). Ultimately the amount of information and the accuracy of the information required in an experiment determine the markers to be used.

Most DNA markers rely on PCR technology. The polymerase chain reaction (PCR) employs the enzymatic amplification of DNA *in vitro*. By synthesizing copies of a selected DNA sequence, PCR substantially increases the quantity of the selected DNA sequence in a sample. Advantages of the technique include (Morrel *et al.,* 1995): (i) PCR requires only small amounts of DNA; (ii) PCR is relatively quick to perform and technically straightforward once optimized conditions have been established; (iii) PCR is easily automated; and (iv) the range of primer sequences available gives the technique great diagnostic power. There are a number of DNA markers in use today.

1.4.1 Restriction fragment length polymorphism (RFLP)

RFLPs have been used successfully in a wide variety of plant species (Beckmann and Soller, 1986). These markers detect variation by monitoring changes in the length of defined DNA fragments produced by digestion of the DNA sample with restriction endonucleases (Beckmann and Soller, 1986). RFLP, or length differences in homologous fragments of different DNA, are caused by changes in the primary sequence of nucleotides which can be a result of (Grant and Shoemaker, 1997): (i) a point mutation resulting in the loss or gain of a restriction enzyme site; (ii) an insertion or deletion of nucleotides of restriction enzyme sites; (iii) a deletion which overlaps a restriction enzyme site; or (iv) a DNA rearrangement where the end of the rearranged segment resides between two restriction enzyme sites. Methylation, unequal crossing over and replication slippage also create new alleles through differences in restriction fragment lengths. RFLPs are useful genetic markers, are well suited for the construction of linkage maps and allow for synteny studies due to their 'cross species' transferability.

The procedure involves cleaving DNA with site-specific restriction endonucleases, wherever a recognition sequence occurs, usually a 4-6 base pair (bp) palindromic sequence is present (Grant and Shoemaker, 1997). The DNA used for RFLP detection must be of high average molecular weight, as autoradiogram signal strength is determined to a large extent by the quality and amount of DNA. The distribution of cleavage sites will differ between individuals resulting in a somewhat different matrix of restriction products (Beckmann and Soller, 1986). Up to five times of excess enzyme is suggested due to impurities which may be present in the DNA sample. These impurities can influence enzyme activity by influencing buffer conditions which are essential for enzyme activity. The restriction fragments are separated by agarose gel electrophoresis. A Southern blot (Southern, 1975) and hybridization is then performed. DNA hybridization involves exposing the membrane to a labeled DNA probe. When the membrane is incubated with the probe under conditions that promote the formation of hydrogen bonds between complementary base pairs, the probe will hybridize wherever it finds a complementary DNA sequence on the membrane (Grant and Shoemaker 1997). The probe may be radioactively or non-radioactively labeled and detected respectively by autoradiography and chemi-luminescence.

The genetic maps of a number of economically important crop species include a large number of RFLP markers. This makes them important anchor markers in any mapping experiment. Their co-dominant inheritance allows for all possible genotypes to be inferred from observed phenotypes and as anchor markers, they are necessary to assign additional markers to particular chromosomes. Self-pollinated crops, such as wheat have low levels of genetic diversity, so informative RFLP probes are difficult to find. From a practical viewpoint, a large quantity of DNA is required, substantially more DNA than required for PCR-based techniques. The DNA must also be of good quality for the restriction digests. The technique itself is laborious, and automation is difficult. Furthermore, low throughput means that a large number of samples cannot be processed per day.

1.4.2 Microsatellites (SSRs)

Simple sequence repeats (SSRs) were detected in eukaryotic genomes over 15 years ago. They were regarded as sequences of no particular interest, but making up a large portion of 'silent' eukaryotic DNA (Jarne and Lagoda, 1996). Microsatellites are a class of repeat sequences consisting of short, tandemly repeated nucleotide motifs, scattered throughout the genomes of eukaryotes (Depeiges *et al.,* 1995). With the discovery of the PCR technique scientists realized the potential of microsatellites as valuable molecular markers and they have since been used in a number of fields ranging from the amplification of microsatellite loci in semen cells and feathers to the amplification of microsatellite loci in an 1850-year-old Egyptian mummy (Jarne and Lagoda, 1996). Microsatellite markers have also been used in the fields of variety discrimination, phylogenetics and mapping studies.

The regions flanking microsatellite sequences are generally conserved. PCR primers, designed for these regions, are then used to amplify the microsatellite containing DNA fragment (Cregan and Quigley, 1998). Length polymorphism is detected when PCR products from different individuals vary in length as a result of variation in the number of repeat units in the SSR (Cregan and Quigley, 1998), usually a result of mutation events. This length polymorphism can be visualized by electrophoretic separation of the fragments, via agarose or polyacrylamide gels. The technique is also suitable for automation using fluorescence-based detection in DNA sequencing reactions.

According to Saghai-Maroof *et al.* (1994), microsatellites are ideal DNA markers for genetic mapping for the following reasons: (i) they are abundant and evenly distributed across genomes; (ii) they are highly polymorphic. Saghai-Maroof (1994) detected 37 alleles at individual loci in barley and Rongwen *et al.* (1995) reported 26 alleles at a locus in soybean; (iii) they exhibit co-dominant characteristics; (iv) they are easily assayed by PCR and (v) they are easily accessible to other laboratories via published primer sequences.

The primary disadvantage of microsatellites as molecular markers is the cost and research effort required to clone and sequence SSR containing DNA fragments from species of interest (Brown *et al.,* 1996). This is the only way to create PCR primers that can be used to amplify the SSR. High-resolution electrophoresis is required, and this is costly and time consuming, especially if radioactivity is used. It is clear however that for important crops, the cost of developing microsatellite primers is likely to be outweighed by the high information content and reliability of this marker system (Morrel *et al.*, 1995).

1.4.3 Amplified fragment length polymorphism (AFLP)

AFLP (Zabeau and Vos, 1993) is a very powerful DNA marker technique and was originally conceived to allow the construction of very high density DNA marker maps for application in genome research and positional cloning of genes (Vos and Kuiper, 1997). AFLP has been used to identify markers linked to disease resistance loci (Becker *et al.,* 1995), to fingerprint DNA and to assess relationships between molecular polymorphism and hybrid performance in maize (Ajmone-Marsan, 1998, cited in Pejic *et al.,* 1998).

With the basic AFLP technique, DNA is cleaved with two restriction enzymes, a frequent base cutter and a rare base cutter. Adaptors are ligated to the restriction fragments and PCR is carried out with generic primers which comprise a common part corresponding to the adaptors and restriction site, and a unique part corresponding to the selective bases (Karp and Edwards, 1997). The sequence of the adaptors and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. The amplified fragments are visualized by denaturing polyacrylamide gel electrophoresis and autoradiography or silver staining.

A preamplification reaction may be performed in addition to a selective amplification procedure. In this procedure primer sequences corresponding to the restriction site and adaptor sequences are used for the preamplification reactions. Amplification in two steps warrants optimal primer selectivity (Vos *et al.,* 1995). The preamplification reaction can also be used to increase the amount of template, as the preamplification primers have no selective nucleotides. Competition between the template molecules during the pre-selective and selective amplification stages can be a problem in species with large genomes (e.g., wheat) and can compromise AFLP comparisons, because not all fragments that are theoretically amplifiable are visualized in practice (Koebner *et al.,* 2001).

The molecular basis of the polymorphism is sequence polymorphism at the nucleotide level. These will be detected by AFLP when the restriction sites or the nucleotides adjacent to the restriction sites are affected, causing the primers to mis-pair at the 3' end and thus preventing amplification (Vos and Kuiper, 1997). Deletions, insertions and rearrangements affecting restriction fragments will then be detected by the procedure. Most markers are mono-allelic (dominant). However, co-dominance has been reported (Vuylsteke *et al.,* 1999).

The effect of restriction enzymes

The use of two restriction enzymes allows for flexibility in primer selection which ultimately determines the number of amplified fragments. Combinations of a frequent base cutter and a rare base cutter are used. The frequent base cutter generates small fragments which can be separated on denaturing polyacrylamide gels. The rare base cutter limits the number of fragments amplified, and thereby allows for preferential amplification of restriction fragments with two different adaptors (Vos *et al.,* 1995). The number of restriction sites available for the rare base cutter determines the number of effective fragments.

Different combinations of restriction enzymes can also be used. Vuylsteke *et al.* (1999) generated high-density linkage maps in maize using a recombinant inbred population and an immortalized F_2 population and found that *Pst*I/*Mse*I primer combinations minimized the mapping efforts and maximized the number of markers mapped. Castiglioni *et al.* (1999) also found that *Pst*I/*Mse*I primer pairs were more efficient in detecting polymorphism in maize than *Eco*RI/*Mse*I primers. Young *et al.* (1999) reported that the *Pst*I enzyme is notably sensitive to cytosine methylation, and this may effect the distribution of markers generated

using this enzyme. The *Sse*I enzyme is also sensitive to cytosine and adenine methylation (Donini *et al.*, 1997). The AFLP technique is most effective in wheat with *Pst*I because of the high GC content of *Pst*I recognition sites, which preferentially targets low copy regions of the genome, thus providing less clustering of markers and better genome coverage (Langridge and Chalmers, 1998).

Detection of fragments

AFLP fragments may be detected by silver staining, autoradiography or fluorescent labeling. In the presence of a reducing agent, and at high pH values, silver ions form coloured complexes with charged side groups of nucleic acids. However, the pH change required by most silver staining protocols causes the non-specific formation of insoluble silver salts on gels thereby impairing contrast. As a result the contrast of the fingerprint obtained is poor. Silver staining is the least costly of the detection methods currently used. Another method used is radioactive labeling with $\gamma\ ^{33}$P ATP. Only one of the primers used in the selective amplification is labeled by phosphorylating the 5' primer ends with $\gamma\ ^{33}$P with the aid of polynucleotide kinase. After electrophoresis, the gels are dried and exposed to X-ray films. Alternatively phosphorimaging technology may be used. With silver staining and autoradiography, throughput is slow because only one sample per lane can be analyzed. Poor resolution may also prevent accurate allele typing. Manual scoring, which is necessary with these systems, is very time-consuming and mistakes are easily made.

Schwarz *et al.* (2000) compared fluorescent labeling to the techniques reviewed above. Higher resolution of AFLP fragments was obtained with fluorescent labeling so more fragments were distinguished. Hartl and Seefelder (1998) detected 523 AFLP fragments from just eight fluorescent-labeled primer pairs in an assay of eight hop cultivars. Electrophoresis of different AFLP samples in one lane was possible (multiplexing) and consistent results with regard to fragment number and polymorphic peaks were observed. The fluorescent-labeled AFLP system appears to be the recommended system for future use. Using high-throughput instruments, and infrared dye labeled AFLP primers, Myburgh *et al.* (2001) generated up to 70 000 polymorphic markers in one week in a *Eucalyptus* spp. One limitation is that subsequent development of sequence characterized amplified regions (SCARs) is not possible as access to fragments is not possible using automated DNA sequencers.

The AFLP approach is now widely used for developing molecular markers. No prior knowledge of genome sequence is required, so no development costs are involved. High reproducibility and high frequency of identifiable fragments makes AFLP an attractive tool for detecting polymorphism (Ridout *et al.,* 1999). The polymorphic bands may be converted into probes or locus specific markers. Problems have been encountered while converting them into SCARs because of the presence of a mixture of sequences of the same size in individual bands (Gupta *et al.,* 1999).

1.4.4 Resistance gene analogs

The structures of cloned resistance genes (see 1.1.2) have highlighted numerous related gene families. Genes for resistance to pathogens derived from diverse plant species have sequence similarity in domains of the protein probably involved in pathogen recognition and signal transduction in triggering the defense response (Chen *et al.,* 1998). The conserved domains of these genes offer opportunities for PCR amplification and the isolation of similar sequences in other plant species, i.e., resistance gene analogs (RGAs) (Chen et *al.,* 1998).

Degenerate primers targeting conserved regions in the nucleotide binding site (NBS) and neighbouring regions of resistance genes, are amplified by PCR giving rise to RGA PCR products. Leister *et al.* (1996), Yu *et al.* (1996) and Chen *et al.* (1998) observed that these PCR products, when cloned from agarose gels were highly heterogenous. Chen *et al.* (1998) then separate these heterogenous PCR products with high-resolution polyacrylamide gel electrophoresis. Alternatively, the PCR products may be cloned to produce RFLP probes which then assay for the RGA by RFLP hybridization reactions.

Leister *et al.* (1996) developed degenerate primers based on the LRR protein domains of the *RPS2* gene in *Arabidopsis thaliana* and the *N* gene in tobacco, and obtained PCR amplification products from potato (*Solanum tuberosum*) that were linked to the nematode resistance locus *Gro1* and the late blight locus *R7.* Thereafter, similar studies have produced a number of degenerate RGA primers (Table 1.3). A kinase domain at the wheat leaf rust resistance gene *Lr10* was isolated by Feuillet *et al.* (1997) using oligonucleotides corresponding to the serine/threonine protein kinases.

PCR RGAs have been used by Toojinda *et al.* (2000) to develop linkage maps in barley. They concluded that RGAs markers are useful tools for mapping quantitative and qualitative resistance genes, and recommend the sequencing of the RGA PCR products before inferring any relationship to disease resistance genes. However, the RGA technique has been used to detect markers close to resistance genes. Shi *et al.* (2001) detected 30 RGA markers linked to the stripe rust resistance gene *Yr9* by employing near isogenic lines (NILs) together with bulk segregant analysis (BSA). The RGA RFLP locus *XksuD14* was identified as a candidate for the *Lr21/Lr40* gene on chromosome 1DS (Li *et al.*, 2001).

Table 1.3. A list of some RGA primers, the cloned resistance gene source and the appropriate reference.

Primer	Cloned resistance gene	Domain	Reference
NLRR	*N*	LRR	Chen *et al.* (1998)
RLRR	*Rps2*	LRR	Chen *et al.* (1998)
Ptokin	*Pto*	Kinase	Chen *et al.* (1998)
Ptokin1IN	*Pto*	Kinase	Shi *et al.* (2001)
CLRRINV1	*Cf9*	LRR	Shi *et al.* (2001)
XLRRINV1	*Xa21*	LRR	Shi *et al.* (2001)
Xa1NBS	*Xa21*	NBS	Shi *et al.* (2001)
Xa1LR	*Xa1*	LRR	Shi *et al.* (2001)
RLK	*Lr10*	Kinase	Feuillet *et al.* (1997)
AS3	*Rps2/N*	LRR	Leister *et al.* (1996)

1.4.5 Marker choice and information content

A number of DNA markers and applications thereof are available for the molecular biologist, and the number of new DNA markers is increasing. The scientist is faced with the problem of choosing appropriate molecular markers for a specific investigation, as each has different features (Table 1.4). The different DNA marker techniques have a number of advantages and disadvantages (Table 1.5). Most importantly, the information obtained from the marker must address the requirements of the investigation.

In order to be able to choose among different marker systems, a measure of information content is required. Several metrics are used to compare different marker systems. Expected heterozygosity (H) is a function of the ability of a marker system to distinguish between

different genotypes, and corresponds to the probability that two alleles taken at random from a population can be distinguished using the marker in question (Powell *et al.*, 1996). Expected heterozygosity is a valuable metric if researchers are interested in fingerprinting genomes and looking for differences. The metric is also important in phylogeny reconstruction and other evolutionary studies. The multiplex ratio (*E*) of a marker system is defined as the number of loci or bands simultaneously analyzed in an experiment, e.g., in a gel lane (Powell *et al.*, 1996). Multiplex ratio is important to generate a number of markers with modest effort in a relatively short period of time. AFLPs are popular markers because of their high multiplex ratio.

Table 1.4. Comparison of the main features of AFLP, RFLP and SSR (adapted from Karp *et al.*, 1997 and Ridout *et al.*, 1999).

FEATURE	AFLP	RFLP	SSRs
Development costs (per sample)	None	Medium	High
Running cost (per sample)	Medium	High	Low
DNA required (μg)	0.5-1.0	10	0.05-0.1
Reproducibility	Very high	Very high	Very high
Ease of use	Difficult initially	Labour intensive	Easy
Dominant/Co-dominant	Dom/Co-dom	Co-dominant	Co-dominant
Polymorphism	Medium	Medium	High

The utility of a marker system is a balance between the level of polymorphism detected, and the extent to which an assay can identify multiple polymorphisms (Powell *et al.*, 1996). The numerical values obtained for metrics will change when different species are analyzed. If the mechanisms that generate DNA polymorphisms remain constant across species, then the ranking of the markers in terms of the calculated metric values should remain the same, but this issue has not really been examined (Powell *et al.*, 1996).

There are clearly a number of ways to measure marker informativeness. Marker informativeness is not just about dominance and polymorphism. Phylogenetic, fingerprinting and evolutionary studies have different requirements compared to mapping experiments. It is important to know what the data will be used for, the facilities available in the laboratory to process samples, and the means by which the data will be analyzed. The accuracy of the

information is extremely important. With this in mind, the characteristics of the different marker systems are compared and the one satisfying most of the criteria is usually favoured.

Assuming reliable data, factors which influence the choice of marker system in mapping experiments include the time frame of the experiment, the availability of probes/primers and the availability of software packages to aid scoring and mapping. The amount of DNA required and the convenience and technical difficulties are also considered, especially when radioactive detection is required. These factors must be taken into consideration together with characteristics of the markers, such as expected heterozygosity and multiplex ratio.

Table 1.5. Advantages and disadvantages of RFLP, AFLP, SSR (adapted from Karp *et al.*, 1997, Ridout *et al.*, 1999).

TECHNIQUE	ADVANTAGES	DISADVANTAGES
RFLP	Reliable and robust. Filters can be re-used for many probes. Locus locations translate across crosses and species. Can convert to PCR based STS. Co-dominant.	Slow assay. Requires large amounts of DNA. Low levels of polymorphism in some species.
AFLP	Quick assay. Requires small amounts of DNA. No sequence information required. Several markers revealed on same gel. Can be adapted for special uses e.g. cDNA AFLP. Highly polymorphic.	Generally dominant. Locus locations do not translate across species. Need a commercial license.
SSR	Quick assay if primers are available. Requires very small amount of DNA. Usually single locus, even in polyploids. High polymorphism.	High development costs. Interpretation sometimes difficult because of stuttering.

1.4.6 Markers and the wheat genome

Wheat contains three distinct but genetically related (homoeologous) genomes, labeled A, B and D according to reconstructed evolutionary ancestry. The total haploid DNA content of the bread wheat genome is approximately 1.7×10^{10} bp (Gupta *et al.*, 1999). The average wheat chromosome is 25-fold longer than the average rice chromosome (Gupta *et al.*, 1999). Thus, three wheat chromosomes are equivalent to the total haploid maize genome, and one-half of

an average wheat chromosome is equivalent to the total haploid rice genome (Gill and Gill, 1994).

The complexity of the wheat genome, its hexaploid nature and the three different genomes make breeding and selection difficult. Furthermore, some genomes, particularly the D genome, are less diverse than others, thus requiring extensive testing of markers in order to identify useful polymorphisms. Diverse end-uses and strict quality constraints results in conservative breeding programmes with a narrow germplasm range and lower levels of genetic diversity (Langridge *et al.,* 2001). As a result, the genetic diversity present in the world's wheat has not been fully exploited, and the germplasm used in breeding programmes has a low level of polymorphism in comparison to other cereals (Langridge and Chalmers, 1998).

Problems in the preparation of molecular maps, and in the development of molecular markers for marker-assisted selection (MAS), have been encountered in wheat (Gupta *et al.,* 1999). A summary of existing linkage maps developed for wheat is given in Langridge *et al.* (2001). A common feature of most of the contemporary maps is an apparent low level of polymorphism in the D genome and the scarcity of markers in many regions of the total genome. According to Gupta *et al.* (1999), over 80% of the genome consists of repetitive DNA sequences with the majority of wheat genes present in clusters. However, the small chromosome regions containing these gene clusters are highly recombinogenic, making them suitable for molecular manipulation (Gupta *et al.,* 1999). Targeting these gene clusters with the aid of molecular markers offers the opportunity of indirect genotypic selection without the interference of non-genetic variation.

Chao *et al.* (1989), and Kam-Morgan *et al.* (1989) reported less than 10% allelic variation with RFLPs in wheat. Liu *et al.* (1991) also reported low levels of RFLP polymorphism in wheat. RFLP maps that have been constructed show that RFLP markers are essentially co-linear within a homoeologous group i.e. in the same linear genetic order on all three homoeologous chromosomes. A high level of synteny between cereal crops means that information on the location of RFLP loci can be from barley, rye or related species. The cross species use of RFLP has been used to demonstrate the relationships between different chromosomes of the grass species (Moore *et al.,* 1995a).

Gale *et al.* (1995) found the RFLP technique to be too costly and too slow for the rapid evaluation of the large number of progeny commonly used in commercial breeding programmes. RFLP technology has been found to be more useful for the selection of chromosome regions carrying useful genes from wild relatives (Koebner *et al.,* 1998). This property of RFLP may also be exploited in the process of comparative mapping, involving the detection of similarities across genomes. The technique has also been used for genome mapping, varietal identification, characterization of wheat-rye recombinants and identification of homoeologous chromosome arms in wheat (Gupta *et al.,* 1999).

Röder *et al.* (1995) found that with microsatellites, in contrast to RFLP, one locus usually corresponds to one genome amplified. However, microsatellites are not exclusively derived from single copy sequences but may also be derived from repetitive sequences. As a result only 36% of all primer pairs flanking wheat microsatellites amplified fragments were of the expected size range (Röder *et al.,* 1998), with lower polymorphism levels recorded for the D-genome (Röder *et al.,* 1995). SSRs targeting the less diverse D-genome of wheat were developed to increase the number of available markers for this genome (Pestsova *et al.,* 2000; Guyomarc'h *et al.,* 2002).

The Wheat Microsatellite Consortium (WMC), an international collaborative programme, was formed and approximately 500 SSR markers developed through the efforts of this organization. This has contributed in part to an increase in the identification of polymorphic SSR markers (Bryan *et al.,* 1997; Pestsova *et al.,* 2000; Harker *et al.,* 2001; Gupta *et al.,* 2002; Guyomarc'h *et al.,* 2002). The availability of expressed sequence tags (ESTs) for wheat is a potentially valuable source of SSR markers (Langridge *et al.,* 2001). Microsatellites are fairly evenly distributed along the linkage groups, with no reports of clustering of markers (Röder *et al.,* 1998). Microsatellites have been linked to stripe rust resistant accessions of *T. dicoccoides* (Fahima *et al.,* 1998) and the stripe rust resistance gene *YrH52* (Peng *et al.,* 1999). With the large number of SSR primer sequences available the use of SSR markers in wheat genome research is increasing.

The high multiplex ratio of AFLPs is being exploited in the construction of genetic linkage maps of wheat, thereby reducing costs. Although evenly distributed across the A and B genomes, there appears to be a reduction in AFLP polymorphism in the D genome. Some

clustering of AFLP and RFLP markers have been reported near centromeric regions, but this is consistent with most of the cereal maps and has been attributed to suppression of recombination in these regions (Hart, 1994; Langridge *et al.,* 2001). In wheat, AFLP loci are dispersed throughout the genome and their incorporation into existing RFLP maps has been found to significantly increase mapped marker density (Ridout *et al.,* 1999). A single primer combination detects up to eight times more polymorphism than a polymorphic RFLP marker (Gupta *et al.,* 1999). The high frequency of identifiable AFLPs coupled with high reproducibility makes the technique attractive and popular for future mapping studies.

In studies using the RGA technique, up to 27% polymorphic bands have been found (Chen *et al.,* 1998). RGAs are distributed throughout the wheat genome and have been shown to be associated with known resistance phenotypes. Spielmeyer *et al.* (2000) used RGA clones derived from a candidate gene for stripe rust to detect candidate genes for leaf rust. NBS-LRR sequences derived from *Ae. tauschii* (Lagudah *et al.,* 1997) were found to be useful for developing markers for cereal cyst nematode resistance genes. Shi *et al.* (2001) used the RGA polymorphism technique to identify molecular markers for *Yr9*. The technique has also been used to develop high-density linkage maps for *Ae. tauschii*, the D genome donor of bread wheat (Boyko *et al.,* 2002).

1.4.7 Sequence related amplified polymorphism (SRAP) and other new marker techniques

The sequencing of plant genomes such as that of *Arabidopsis thaliana* has produced genomic information that can be exploited using bio-informatics tools, to produce new marker techniques such as sequence-related amplified polymorphism (SRAP) (Li *et al.,* 2001). The SRAP technique targets open reading frames (ORF's), promoters and introns. Li *et al.* (2001) developed primers with a core 'CCGG' region to target exons which are normally GC rich regions. They also developed primers with a core 'AATT' region to target AT rich promoter and intron regions. Exon sequences are generally conserved among different species (Quiros *et al.,* 2001), and introns and promoters are variable thus making it possible to generate polymorphic bands.

The forward and reverse primer of the SRAP technique aim to target exons and ORFs (forward primers) and promoters and introns (reverse primers) through PCR amplification.

An initial annealing temperature of 35°C for 5 cycles of PCR is used to ensure the binding of both primer sites with a partial match, in the target DNA. Subsequently, the annealing temperature is increased to about 50°C, to ensure that the DNA products amplified in the first five cycles are consistently and efficiently amplified in an exponential fashion.

Li *et al.* (2001) tested the SRAP technique on *Brassica* spp. (cauliflower, collard and broccoli), and bands were found to be fully reproducible with up to 10 polymorphic bands per primer combination. Forty five percent of the amplified PCR products that were excised from the gels and sequenced were found to match known genes in the Genbank database, suggesting that SRAP could be useful for tagging genes as demonstrated by Li *et al.* (2001). The technique is simple and marker efficient, and can be adapted for a variety of purposes including map construction, gene tagging, genomic DNA fingerprinting and map-based cloning. Furthermore, the technique has a reasonable throughput rate, produces co-dominant markers, targets ORFs and allows for the isolation of bands for sequencing (Li *et al.,* 2001).

Recently SNP analysis was developed where markers are scored in a plus-minus assay so that automation is easily possible. Their abundance (about one in every 100 bp) makes them very attractive tools as markers. ESTs are DNA segments representing the sequence from a cDNA clone that corresponds to an mRNA molecule or part of it (Gupta *et al.* 1999). They may be matched with sequences in sequence databases and thus be assigned to specific genes. ESTs only target expressed sequences, so markers developed from them could be valuable tools when studying the wheat genome as the large amount of repetitive DNA is not a factor. The presence of retrotransposons in plant genomes has also initiated the use of marker techniques such as Inter-retrotransposon amplified polymorphism (IRAP), and Retrotransposon-microsatellite amplified polymorphism (REMAP) (Kalendar *et al.,* 1999; Boyko *et al.,* 2002).

The development of new marker techniques reviewed above and improvements or modifications of existing marker techniques, together with the high throughput of these techniques, will accelerate the process of finding markers linked to traits of interest. The establishment of markers such as SNPs will provide DNA array-type technologies to wheat which will increase throughput. More markers will then be available to create high-density linkage maps. Markers closely linked to traits of interest will be more easily mapped which

may then be available for use in MAS. MAS has the potential to improve the gains from selection of desirable traits, currently produced by conventional plant breeding.

1.4.8 Marker-assisted selection

Genes are tagged with molecular markers with the aim of using them in MAS programme. The essential requirements for MAS in a plant breeding programme are: (i) marker or markers should co-segregate with or be closely linked to the desired trait, (ii) an efficient means of screening large populations for the markers should be available and (iii) the screening technique should have high reproducibility across laboratories, be economical to use and user-friendly (Gupta *et al.,* 1999). It is important therefore to convert AFLP and RFLP markers, so that they can be screened with a simpler, more economical assay.

Loci accounting for a large proportion of the total variance in the trait of interest in one cross, might not have the same effect in a different genetic background. If there is genotype x environment interaction, or epistatic interaction, it may be possible to identify the extent to which the variation in the trait is controlled by the interaction. When quantitative trait loci (QTL) cannot be extrapolated from one cross to the next, this may seriously hamper applications in breeding programs. Marker validation is therefore important before a marker can be implemented in a MAS breeding program, and this involves studying the behaviour of markers and the associated polymorphism in different genetic backgrounds (Langridge and Chalmers, 1998).

The implementation of MAS involves marker validation and communication between plant breeders in (i) the identification of potential markers, (ii) identification of the genetic material from the breeding programme which segregates for the gene of interest, (iii) examining the effectiveness of the marker trait linkage in the breeding material and (iv) critical discussion of the results and verification of the marker-trait combination (Gupta *et al.,* 1999). Approximately 15-20% of the total MAS resources of the Australian National Wheat Molecular Marker Program (NWMMP) go into marker validation and Langridge *et al.* (2001) suggest that a reduction of some of the costs involved with MAS may enhance its attractiveness.

Lande and Thompson (1990) studied the efficiency of MAS and they proposed to construct an index in which phenotype and marker genotype are given optimal weights. They concluded that the lower the heritability the more benefit would be derived from MAS. MAS is another form of indirect selection with a number of regression coefficients being used to construct the index. Selection pressures that aim directly at the genes underlying quantitative traits can change MAS from 'blindfold' statistical selection to real applications of Mendelian genetics.

MAS offers exciting prospects for agricultural production in the near future. However, many of the theories proposed (some mentioned above) still need to be put into practice, and as a result the promise offered by MAS, in the form of practical benefits and improved cultivars, is taking longer then expected as the obstacles have turned out to be much bigger than originally thought (Young, 1999). Databases and management systems need to be optimized to cater for the large amount of molecular data (of all types) being generated for the different crops. It should be possible to link this available data to agronomic and quantitative data in a user-friendly environment with appropriate software. The development of reliable software packages for genetic mapping, QTL analysis and prediction procedures, based upon reliable statistical procedures and theories is a major aspect of MAS and presents a major challenge for population and biometrical geneticists.

1.5 QTL Mapping

Quantitative traits most often follow a continuous normal distribution. The inheritance of quantitative traits is determined by loci that act collectively on the expression of a trait, but variability is typically also due to environmental factors. QTL is a term widely used for genes that underlie quantitative traits. The ability to manipulate genes responsible for quantitative traits is a prerequisite for sustained improvement of crop plants. The inability to identify the genes controlling variation in a quantitative trait complicates the location of the genes by linkage mapping (Tanksley and Nelson, 1996) and makes manipulation difficult.

Quantitative trait variation has been studied since the early twentieth century. Sax (1923) was the first to detect specific genes by studying their association with specific marker genes. He detected associations between seed size differences (a complex trait) and seed coat pigmentation (a simple monogenic trait) in *Phaseolus vulgaris* L. Thoday (1961) used single

morphological marker genes to conduct studies on quantitative traits in *Drosophila melanogaster* and suggested that if segregation of simply inherited markers could be used to detect linked QTL, then it should be possible to map and characterize QTL involved in complex traits, provided single marker genes are scattered throughout the genome of the organism (Young, 1996). Most of the initial work was limited by the availability of adequate numbers of polymorphic morphological or qualitative markers spanning the entire genome. This limitation has been largely overcome with the advent of molecular markers.

1.5.1 Constructing linkage maps

The development of a linkage map is the first step before QTL analysis can be attempted. With molecular markers, the variation at DNA level can be very high for any particular cross, so there are potentially a very large number of markers segregating. These markers can be used to create genetic linkage maps that are representations of the position of markers or genes within linkage groups.

Linkage groups are established by searching through all pairs of markers using a certain threshold value as criterion for placement in a group. The number of linkage groups should ideally be equal to the haploid chromosome number of the species. The use of less stringent threshold values may generate more linkage groups than expected. The markers in the linkage groups are then ordered by software packages which employ various algorithms to achieve this, e.g., the 'greedy algorithm' described by Jansen (2001). Software packages calculate a value indicating the 'goodness of fit' for a particular order and this value may be calculated using various methods, e.g., the Joinmap® linkage software package uses the weighted least squares method.

The genetic map distance between any two markers is a function of the number of recombination events between the markers and may be influenced by the degree of interference between recombination events. The genetic mapping function describes the relation between the recombination frequency (r) and the map distance (x). Interference occurs when recombination events in one interval reduces or suppresses recombination events in a neighbouring interval (positive interference), or enhances these events in neighbouring intervals (negative interference).

Two mapping functions are commonly used to develop linkage maps: In the absence of interference, the Haldane mapping function (Haldane, 1919) is used:

$r = ½ (1-e^{-2x})$

The Kosambi mapping function (Kosambi, 1944) accounts for the interference component compared to the Haldane mapping function, and leads to shorter map distances for a given recombination frequency:

$r = ½ \tanh (2x)$

Publicly available software packages such as MapMaker (Lander *et al.*, 1987; Lincoln *et al.*, 1992a), and Map Manager (Manly and Olson, 1999) that incorporate these algorithms and mapping functions make the development of linkage maps a much easier task.

1.5.2 Procedures for mapping QTL

Methodologies for mapping QTL involves making a cross between inbred (usually) lines differing substantially in a quantitative trait, thus creating a mapping population. Individuals in the mapping population are analysed in terms of DNA marker genotypes and the phenotype of interest (Young, 1996). For each DNA marker the individuals are split into classes according to the marker genotype. Mean and variance parameters for the trait of interest are estimated among the different marker genotype classes. A significant difference between the phenotypes of the marker classes may be interpreted as the DNA marker being linked to the QTL (Figure 1.2). Designs and procedures for estimating effects and positions of QTL are based on linkage disequilibrium between the alleles at the marker locus and alleles at the linked QTL. Procedures include single marker analysis, flanking marker analysis, and multiple marker analysis.

Single marker analysis

This is the simplest approach for detecting a QTL. The phenotypic means of two classes of progeny are compared, i.e., those with marker genotype AA, and those with marker genotype AB. The data are analyzed using one marker at a time. Ordinal data such as disease scores and mapping scores do not always follow a normal distribution so statistical tests requiring the assumption of normality cannot be employed.

Furthermore grouping of ordinal data leads to loss of information. Van Ooijen (1994) proposed the Kruskal Wallis (KW) test of Lehman (1975) to analyze such ordinal data. The Kruskal-Wallis test, employed by the MapQTL® mapping software package can be regarded as the non-parametric equivalent to the one-way analysis of variance (ANOVA), i.e., no assumptions are required for the probability distribution of the quantitative trait in this test, but the statistical power of the test is generally lower.

Single marker analysis has shortcomings related to the size of the effect of the gene (a) and the recombination fraction (c): (i) Mis-classification may occur if a QTL is located far away from a marker and the further away a QTL is from a marker gene the less likely it is to be detected statistically (Tanksley, 1993). The phenotypic effect diminishes relative to the true effect of the QTL as the distance (c) between the marker locus and the QTL increases (Stuber, 1992); (ii) the approach makes the assumption that QTL can occur exactly at the marker locus. Linkage is, however, hardly ever complete. Therefore, conclusions regarding the existence of the QTL can be inaccurate, and the size of the effect can be underestimated; (iii) the recombination fraction between a marker locus and a QTL is an unknown variable and (iv) individuals missing genotypic data cannot be used in the analysis. Recombination between the marker and the QTL will increase the number of progeny required to detect linkage to the QTL. This can be summarized by the fact that the difference between the marker means, $\delta = \mu_{AA} - \mu_{AB} = (1-2c)\ a$. Some of these disadvantages may be minimized when more than one marker is used at a time (Lander and Botstein, 1989; Utz and Melchinger, 1994).

Interval Mapping

Lander and Botstein (1989) proposed interval mapping as a tool for exploiting the full power of linkage maps by adopting the approach of LOD score analysis that was developed in human linkage studies and relies on maximum likelihood estimation. Instead of analyzing the population one marker at a time, sets of linked markers are analyzed simultaneously with regard to their effect on quantitative traits through linkage (Tanksley, 1993). Pairs of neighbouring linked markers are used to infer the position and effect of the QTL which may lie between them. This is a systematic strategy for searching for QTL and can be performed on any portion of the genome provided that it is adequately covered by markers (Zeng, 1994).

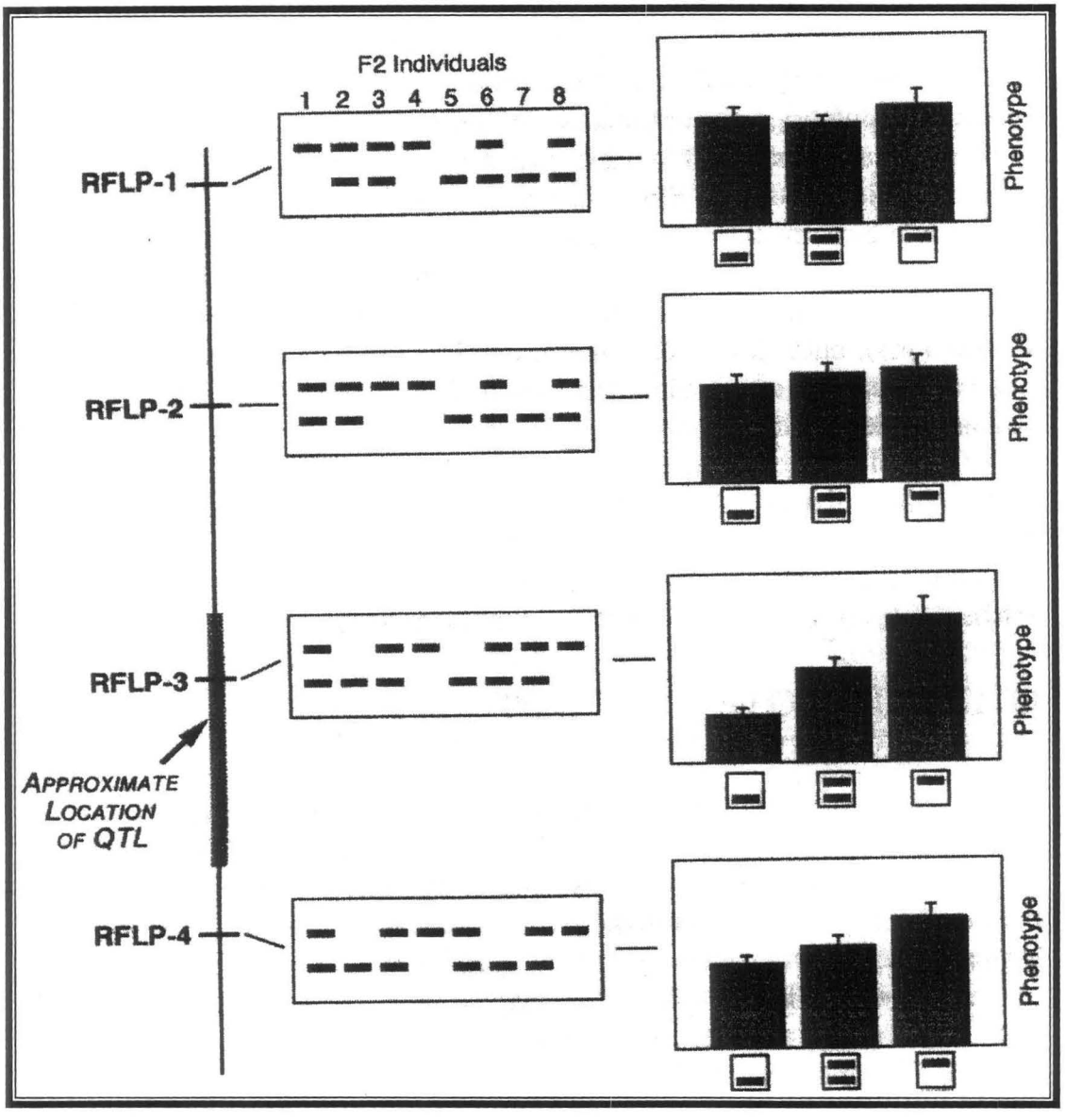


Figure 1.2. The conceptual basis of QTL mapping in an F_2 population. RFLP DNA markers are tested throughout the genome for the likelihood that they are associated with a QTL. A significant difference in mean phenotype among marker genotypic classes as observed at RFLP-3 and RFLP-4 indicate that a QTL is probably located close to these markers (after Young, 1996).

The LOD score is the log10 function of the likelihood ratio L1/L2. L1 is the likelihood of the data for a specified recombination fraction c ($0 \leq c \leq 0.5$) and L2 the likelihood of the data for c=0.5, i.e., no linkage. If the calculated LOD value exceeds a certain threshold value, usually 3-4, then it is an indication of linkage. LOD score is related to classical maximum likelihood

estimation. Lander and Botstein (1989) describe maximum likelihood as parameter estimates that maximize the probability of the observed data for a given underlying probability distribution. The maximum likelihood estimates are compared to constrained maximum likelihood estimates (MLE), i.e., linked QTL in this application. According to maximum likelihood theory a 95% confidence interval for QTL locations is bounded by the map positions where the profile is 0.84 LOD less than the peak of the QTL, so a 1 LOD support interval is used as a 95% confidence interval for the QTL, in a marker interval.

Marker regression is used in interval mapping which relies on the linear relationship between the size of the differences in the marker means and the recombination frequency between the QTL and the individual markers (Kearsney and Pooni, 1996). The following model represents this:

$$\delta_i = a\,(1\text{-}2R_i), \text{ where}$$

a is the effect of the QTL, i.e., half the true difference between homozygotes at the locus and δ_i is half the difference between the means at the i^{th} marker. When the marker and the QTL are unlinked, R=0.5, and δ=0. When the marker is closely linked ($R \rightarrow 0$), then $\delta=a$ and R_i is the recombination frequency between the QTL and the i_{th} marker.

At the true position of the QTL there should be a linear regression of δ_i on $(1\text{-}2R_i)$, with a slope=a which passes through the origin. This is equivalent to the normal linear regression $y=c+bx$, were $y=\delta_i$, $c=0$, $b=a$, and $x = (1\text{-}2R_i)$. The true location of the QTL is of course not known, so the regression is carried out at regular intervals (e.g. every 2 cM) with continuous calculation of the residual sum of square (SS). The interval with the minimum SS indicates the most likely position of the QTL. Haley and Knott (1992) developed a method of analysis for inbred lines from a cross using flanking markers that is based on this approach. Although the method can save time in computation and produce similar results to that obtained by interval mapping, the estimate of the residual variance is biased, and thus the power of QTL detection can be affected (Xu, 1995). The regression method of QTL mapping is employed by the Map Manager QTX software package for QTL mapping (Haley and Knott, 1992; Zeng, 1993; 1994).

Interval mapping offers several advantages over single marker analysis (Zeng, 1993). The genotype at the QTL can be inferred more accurately with flanking markers, so somewhat

fewer progeny and generations are required. Individuals with missing data are not discarded from the analysis since information about the QTL can be extracted from the nearest, flanking informative markers.

Although widely used, interval mapping has some associated shortcomings. The test statistic on an interval with markers may be affected by QTL located in other regions of the chromosome. Zeng (1993) states that it is not efficient to use only two markers at a time to carry out the test as information from other markers is not taken into account. When two closely linked QTL are found, they can often be identified as a single 'ghost' QTL located in an intermediate position. According to Utz and Melchinger (1994), QTL with effects of opposite signs may cancel each other out so that no QTL is detected. However, with more markers in the region it may be possible to separate QTL with effects of opposite sign. Unlinked major QTL tend to inflate the test statistic, producing incidental association. Interval mapping also detects the average effect of all QTL in a region, which may be the sum of smaller QTL effects as opposed to a single isolated QTL (Jansen, 2001). The precision of estimation of the position of QTL by interval mapping is often likely to be too low for gene cloning purposes, but seems nevertheless adequate for breeding purposes. Improved resolution may be obtainable by fine mapping techniques such as multiple marker analysis.

1.5.3 Multiple Marker Methods

The methods described above assume a single QTL linked to a marker or markers of interest. These methods cannot determine whether significant effects at several linked markers or intervals are due to a common QTL or to several linked QTL. Lander and Botstein (1989) cited by Zeng (1993) proposed a simultaneous search strategy for multiple QTL on multiple intervals. Simultaneous mapping of multiple QTL is more efficient and more accurate (Jansen and Stam, 1994). Haley and Knott (1992) argue that parameter estimation and model identifiability becomes difficult when the search for QTL becomes multidimensional, largely because the number of QTL on a chromosome is unknown. However, methods have been suggested to reduce the bias in mapping caused by the effects of associated QTL. These include Composite Interval Mapping (CIM) (Zeng, 1993) Multiple QTL Mapping (MQM) (Jansen and Stam, 1994), and Multiple Interval Mapping (MIM) (Kao and Zeng, 1997).

Composite Interval Mapping and Multiple Interval Mapping

These approaches to dealing with multiple QTL involve the modification of standard interval mapping to include additional markers as co-factors in the analysis (Lynch and Walsh, 1998). It is assumed that the selected co-factors absorb the genetic effects of closely linked QTL, thereby enhancing the power to detect other QTL (Van Ooijen, 1994). With CIM, a genome scan is performed moving a QTL along the chromosome while using all markers as co-factors except those flanking the interval under study. A shortcoming of the technique is the large number of parameters (marker co-factors) being considered which results in high precision but low power to detect QTL (Jansen, 2001). As a result, the presence of a QTL is not falsely declared, but most of the QTL can be missed.

There are some advantages associated with the CIM strategy (Zeng, 1994): (i) by confining the test to one region, the search for multiple QTL becomes a one-dimensional problem; (ii) the inclusion of linked markers can greatly improve the efficiency of mapping; (iii) by including multiple markers in the test the method utilizes more information from the data and should therefore be more efficient and (iv) the method can still use the likelihood profile to present the strength of the evidence for a QTL and so preserves the main feature of interval mapping.

MIM uses multiple marker intervals simultaneously to construct multiple putative QTL in the model for QTL mapping (Kao *et al.,* 1999). MIM encompasses a stepwise selection phase to choose marker co-factors to be used in the analysis. The model was proposed to address the limitations of CIM. CIM uses information from other markers as co-factors in the search for QTL, but not information from selected marker intervals. Compared with the current methods, MIM tends to be more powerful and precise in detecting QTL (Kao *et al.,* 1999).

Multiple QTL Mapping

If a QTL explains a large proportion of the total variance in the trait, then the use of a linked marker as a co-factor in MQM will enhance the power in the search for other segregating QTL (Van Ooijen, 1994). The MQM procedure encompasses both the properties of multiple regression and interval mapping. As with CIM, the QTL is moved along the chromosome, but using a pre-selected set of markers as co-factors as opposed to all markers. Again markers on the same flanking interval on which a QTL is located are temporarily excluded from the

analysis. The Map Manager QTX software package (Manly and Olson, 1999) allows the user to choose a set of marker co-factors and then perform CIM with this selected subset.

The markers to be used as co-factors must be chosen carefully. The two markers flanking the QTL interval must be used, the use of appropriate unlinked markers can partly account for the segregation variance generated by unlinked QTL (Zeng, 1993; 1994). The inclusion of too many factors reduces the power of the test (Zeng, 1994). Jansen and Stam (1994) suggest performing multiple regressions on all markers and eliminating those that are not significant. They recommend that the number of co-factors should not exceed $2\sqrt{n}$, where n is the number of individuals in the analysis. The gain through the use of MQM mapping is expected to be small if the genetic effect of the QTL is small (Van Ooijen, 1994).

Epistasis and environmental effects

Complex phenotypes and epistatic effects of QTL can influence the analysis. Marker co-factors are used for controlling the effect of background variation in mapping QTL excluding epistasis (Jansen and Stam, 1994; Zeng, 1993; 1994). Epistasis or genotype-by-genotype interaction can be determined using two-way ANOVA with two unlinked QTL as the independent variables, and the quantitative character as the dependent variable. Theoretically, it should be possible to measure up to n-way interactions among QTL using n-way related ANOVA, however, Tanksley *et al.* (1993) found some limitations to this approach.

Wang *et al.* (1999) proposed a mixed linear model approach for mapping QTL involved in digenic epistasis and QTL x environment interactions, and suggested that the use of main effects and interaction effects of markers would be superior to using just main effects alone. Markers close to the QTL (main effect markers) are important sources of background variation control, but the use of too many markers (as discussed above), or irrelevant markers, could reduce precision. A QTL mapping software package, QTLMapper, suitable for the mapping of QTL with additive epistatic and environmental interactions, was developed by Wang *et al.* (1999) and could be useful in determining the variation explained by epistatic and environmental factors.

The phenotype of an individual is conditioned by the genotype and interaction with the environment. Breeders prefer QTL that perform consistently over a range of environments,

however, the use of environment-specific QTL can have its advantages (Lander and Botstein, 1989). Studies described by Tanksley *et al.* (1993) suggest that a substantial portion of QTL affecting a quantitative trait in one environment, should be active in other environments, and this is especially true for QTL with major effects to the benefit of plant and animal breeders attempting to use linked markers to transfer potentially valuable QTL into modern varieties or breeds.

The techniques described above are powerful in the sense that they can extract important information from the data. The data need to portray accurately the nature of the trait under study. For this reason, the data generated are largely dependent on the population chosen or created for the study. Various options are available to the geneticist, and some of these are discussed in the following section.

1.5.4 Populations used to locate and study QTL

QTL location in plants has involved the association of quantitative variation for a trait with alleles at particular marker loci in segregating populations derived from an F_1. The parents used to create the F_1 are generally selected inbred lines offering an ideal setting for mapping QTL by marker-trait association. By crossing two inbred lines, linkage disequilibrium is created between loci, QTL and marker, that differ between the lines and can be exploited to search for linkages. Populations used to exploit linkage disequilibrium to determine position and effects of QTL include F_2, and backcross generations, recombinant inbred lines (RILs), near isogenic lines (NILs) and doubled haploids (DH) and are described in Table 1.5.

Doubled haploids

With DH lines, plantlets derived from haploid gametes from F_1 plants are chemically treated to double the chromosome number, instantly producing homozygous individuals. DH lines are usually produced by anther culture, and they are becoming the design of choice in many QTL experiments. Experiments can be conducted with smaller numbers for a given power and accuracy of estimation of recombination fraction and QTL effects since heterozygotes are absent (Carbonell *et al.*, 1993). The design is capable of detecting QTL with heritabilities as low as 5% and sample sizes of 250 individuals. The wheat × maize cross is used to generate doubled haploid plants via chromosome elimination (Laurie and Bennett, 1988). Other

advantages over other mapping populations are that they are genetically sustainable since they are homozygous, material can be shared between collaborating labs with no problem of genetic sampling, they allow for replicated measurement and possible distortions of segregation ratios of markers is easily testable (Tixier *et al.,* 1998; Kammholtz *et al.,* 2001). DH mapping populations developed in the Australian NWMMP showed low levels of segregation distortion highlighting the suitability of the technique for mapping studies.

Genome targeting strategies

Mapping populations should consist of at least 100 individuals and scoring individuals for a marker can then become very expensive depending of course on the types of markers being used. To reduce some of the cost involved, techniques such as bulk segregant analysis (BSA) (Michelmore *et al.,* 1991; Giovannoni *et al.,* 1991), and selective genotyping (Lander and Botstein, 1989) are used. Replication by progeny testing may also be used to reduce the effects of environmental variation.

BSA involves combining individuals (bulks or pools) into groups based on trait value, with unlinked markers (in linkage equilibrium with the QTL) randomly distributed across the bulks. Marker alleles in linkage disequilibrium with QTL are expected to have a particular allele present only in one bulk, and the alternative allele present in the other (Lynch and Walsh, 1998). When individuals are sorted into pools based on alternate alleles at the marker, these pools are enriched for additional linked markers. This increase in marker density is required for the fine mapping of QTL position. BSA requires very high enrichment of the alternative QTL alleles in the tails of the distribution, and is therefore not expected to work well for QTL of small to modest effect. The BSA strategy may be enhanced by the use of large mapping populations, which may provide a more accurate distinction between genotypes.

Some disadvantages are associated with BSA. (i) The method is not effective for complex traits controlled by several unlinked loci. (ii) The actual location of the gene of interest is not revealed, one detects only linked markers. (iii) The technique can only be used effectively with PCR-based methods. (iv) Markers that are identified cannot be used to identify heterozygotes. (v) Conversion of markers to sequence tagged sites is often necessary (Langridge and Chalmers, 1998).

When interest lies in a single trait the individuals may be scored for the trait, but only a selected subset of these will be genotyped. This is selective genotyping. The strategy can result in a large increase in power as much of the linkage information resides in individuals with extreme phenotypes (Lander and Botstein, 1989; Darvasi and Soller, 1992 cited by Lynch and Walsh, 1998). However, in selecting only a portion of the individuals to be genotyped, biased estimates of the effects of the QTL may be generated.

1.5.5 Factors affecting QTL mapping

The actual power and precision of QTL mapping approaches may be debated and a scan of the literature reveals numerous reports of the presence of QTL in a variety of important crops. The results of Beavis (1994) cited in Young (1999) provide a contrast to the encouraging ideas associated with QTL mapping. Using Monte Carlo simulation studies and mapping populations of between 100-200 individuals, Beavis (1994) found that only a fraction of the true QTL were detected. In populations of 100 individuals the power to identify QTL was only 0.117. QTL mapping must be accurate, and this is influenced by factors such as markers and sample size, significance levels, gene effects, epistasis and genotype by environment interaction. However, the use of enhanced statistical tools and devised breeding strategies can improve the results obtained.

Table 1.6. A summary of some of the population types used for QTL mapping, and the advantages and disadvantages of each.

POPULATION TYPE	DEVELOPMENT	ADVANTAGES	DISADVANTAGES
F_2 population	F_2 is produced by crossing or selfing the F_1 generation.	May already be present in many breeding programmes. Generates three genotypes at the marker locus. Allows for detection of dominance associated with QTL	Large numbers of individuals need to be genotyped, typically between 500 to 2000 individuals. Unless asexually propagated, it can be grown only once.
Backcross (BC)	Backcrossing the F_1 to one of the parental lines for a number of generations forms backcross populations.	The QTL effect is described in terms of allelic substitution. May already be present in many breeding programmes.	The backcross generation can only be grown once unless it is asexually propagated. Good resolution mapping using a backcross population requires a large population size.
RILs	RILs are formed by taking F_1 lines (derived from well established progenitor inbreds) through multiple rounds of selfing, or multiple generations of brother-sister matings.	RILs make up a perpetual population with fixed segregation ratios, which can be propagated by many investigators over a wide range of environments. They give estimates of map distance with small confidence limits, and a small population size is required for mapping.	Considerable time is required to develop these lines. There are a limited number of RIL populations available.
NILs	NILs are constructed by crossing a donor parent to an inbred line (recurrent parent) to form an F_1. The F_1 offspring are then backcrossed to the recurrent parent for several generations and then selfed.	F_1's contain about 50 % donor DNA, due to crossing of the descendant lines. Five to seven generations of backcrossing give the expected portion of donor genome of NILs as 1.2 % to 0.5 %.	Development of these lines is a timely process.
DHs	Derived from the haploid gametes of F_1 plants, chemically treated to double the chromosome number.	Homozygous, genetically sustainable, allows For easy sharing of material between labs, and for replicated measurement. Generally quicker to generate than other mapping populations.	The development of DH requires specialised laboratory facilities compared to the other mapping populations.

Modern molecular biology has facilitated an explosion in the use of marker-based methods. Marker spacing and density influence QTL detection. The sample size required to detect a QTL depends largely on the effect of the QTL. This makes it difficult for researchers, because the effect of the QTL is usually not known. If no markers are situated between two linked QTL, they will be indistinguishable from each other. Increasing the number of markers will increase the probability of detecting each QTL. Darvasi and Weller (1992) showed that a reduction in marker spacing would lead to an increase in mapping accuracy. Using large mapping populations may facilitate this. Moreno-Gonzalez (1992) showed that a higher density of markers led to an increase in the percentage of significant estimates of linked QTL. Greater marker densities and large population sizes tend to reduce the confidence intervals of distances between QTL.

A large number of tests for marker-trait associations are performed in a mapping experiment. With a small α significance level set for each test, the probability of a false positive is small. However, the probability of a false positive in the entire mapping experiment using the same significance level is relatively high as a number of individual tests are conducted. Genome wide significance thresholds can be set by using techniques such as the Bonferroni correction (Lynch and Walsh, 1998) to determine overall significance levels for the entire experiment. Other techniques include permutation tests (Doerge and Churchill, 1996) or bootstrap strategies. In the permutation test approach, the observed trait data are reshuffled over the different individuals so as to break any marker-trait associations. This artificial data set is then analyzed for QTL with the maximum test statistic calculated and stored. The procedure is then repeated, usually up to 1000 times to create a cumulative distribution of the test statistic from which the relevant percentiles are read off (usually 95% and 99%) to determine the critical threshold values.

The power to detect QTL is also related to heritability of the quantitative trait. The probability of detecting a QTL increases with increased heritability of the QTL (Hyne and Kearsney, 1995). Low QTL heritabilitites cause estimates of QTL location to have large confidence intervals and so the position may be estimated over a large range. Not all QTL have equal effects, though the trend emerging for diversely different traits is one in which a few QTL with relatively large effects account for most of the divergence between parental strains

(Falconer and Mackay, 1996). Matters may be further complicated by the presence of epistatic and environmental effects.

In general, information obtained from molecular data can only be exploited with statistical packages that can accurately map the position of markers on the genome of interest. Young (1999) questions the detection capabilities of these statistical packages. In order to provide accurate estimates of the locations of genes being expressed, these packages must take into account the various factors affecting QTL such as epistasis, interactions, sample size and pleitropic effects. The effects of these factors must be studied to ascertain their effect on QTL so that provision can be made for them when mapping QTL. The development of new procedures to detect QTL is promising and these appear to be more effective in detecting QTL, even those with minor effects. Such techniques can facilitate accurate mapping of QTL with the ultimate goal of isolating the exact position of QTL taking non-genetic factors into account and identifying closely linked markers for MAS.

1.6 Stripe rust in South Africa

Stripe (yellow) rust was first reported in South Africa in 1996 with the introduction of a single pathotype, 6E16A- (Pretorius *et al.,* 1997; Boshoff *et al.,* 2002). Prior to that no breeding or selection had been undertaken, so several cultivars were susceptible to the disease. This led to widespread epidemics in the growing areas of the Western Cape in 1996 and 1997, resulting in severe yield losses, so farmers had to resort to fungicide control, thereby increasing production costs (Pretorius *et al.,* 1997). No accurate figures of yield losses due to stripe rust in commercial farming situations are available, but Boshoff (2000) has recorded yield losses as high as 56% in experimental plots. The alternative to breeding for genetic resistance is the use of chemical control, which can become very expensive, as was reflected in the increased production costs resulting from fungicide applications following PST infection in South Africa in the 1996 and 1997 growing seasons (Boshoff *et al.,* 2002). In addition to being expensive and causing damage to the environment, the application of fungicides contributes little to the problem of controlling inoculum levels between cropping cycles (McIntosh *et al.,* 2001), which ultimately results in the development of rust in the next growing season. The development of cultivars with genetic resistance to stripe rust is an important step to ensure cost effective and environmentally friendly control of the disease.

With the development of a new pathotype, 6E22A- (Pretorius, 1998), an early breakdown of monogenic resistance in the local cultivars 'Hugenoot' and 'Carina' during 1998 has been reported by Boshoff and Pretorius (1999). These cultivars may have little potential for durability according to current views on resistance breakdown (McIntosh & Brown, 1997). In Australia and New-Zealand more than 20 pathotypes have been detected since the original single introduction in 1979 (Wellings and McIntosh, 1990). McIntosh *et al.* (2001) report that breeding for rust resistance has been highly successful in rust-prone areas in Australia, with the presence of stable pathotypes and cultivars with durable resistance. However, greater problems occur in rust prone areas where both resistant and susceptible cultivars may be grown, which could lead to damaging levels of rust following a favourable sequence of seasons for the disease with yield losses as high as 84% recorded by Murray *et al.* (1994). McIntosh and Brown (1997) have reported that stripe rust control in Australian wheat has been achieved mainly through selection for APR.

Considering that stripe rust was only detected for a few years in South Africa, and many cultivars are susceptible, it is of critical importance to identify useful sources of resistance and incorporate these into existing breeding programmes. Pretorius (1998) identified several sources of resistance that may be utilised in South African breeding programmes, but limited information is available on the genetic basis of these sources. An example of this is the cultivar 'Kariega' which has excellent APR, and knowledge about the genetic basis of this existing host resistance is required to better understand and manipulate these genes in breeding programmes, to minimise losses due to stripe rust, and to develop cultivars with durable resistance in South Africa.

1.7 Objectives of this study

This project was undertaken with the aim of mapping the number of genes in 'Kariega' involved in APR to stripe rust by adopting a QTL approach, using a DH mapping population and DNA markers. This would be achieved by developing a partial linkage map that is faster to construct than complete linkage maps, and does not require extensive mapping efforts. This map would be used to detect at least linkage to the trait. The aims of the project would be achieved through the following:

(i) Developing a partial linkage map of the 'Kariega X Avocet S' mapping population.

- Parental screening using RFLP, SSR and AFLP markers. RFLP and SSR markers would serve as anchor markers to assign linkage groups to chromosomes. AFLP markers would provide adequate genome coverage.
- Optimization of additional marker strategies, e.g.. RGA and SRAP techniques and tests for application in extension of the linkage map.
- Mapping of polymorphic markers and the construction of a partial linkage map of 150-200 markers, with data checks for possible sources of error.
- Targeting of chromosomes not represented in the initial maps by finding additional SSR markers in order to extend the partial linkage map to cover all wheat chromosomes.

(ii) Mapping of QTL for APR to stripe rust.

- Identification of linkage of markers with QTL for stripe rust resistance and the chromosomes involved, with single marker, interval mapping, and multiple marker techniques.
- Examination of the effect of two different field scoring dates (early, final) and field vs. growth chamber testing on QTL detection.
- Targeting important chromosomes identified with QTL mapping with SSR markers in order to obtain more markers close to the QTL regions.
- Identification of candidate markers that can be validated and if suitable, used for MAS for the chromosomal regions with APR to stripe rust.

In addition to the aims described above, the linkage map would also be used to map the *Ltn* gene for leaf tip necrosis, and *Sr26,* a seedling resistance gene for resistance to stem rust (*P. graminis* f. sp. *tritici*), for which the DH population was also segregating.

CHAPTER 2: MATERIAL AND METHODS

2.1 Germplasm and data made available for this study by co-workers

R Prins generated a DH mapping population consisting of 150 lines derived from the F_1s of the 'Kariega X Avocet S' cross using the wheat x maize technique (Laurie and Bennett, 1988). 'Kariega' [pedigree:SST44{CI13523(Agent)/3*T4(Anza)}//K4500.2/Sapsucker S] is a hard-red spring wheat which exhibits flag leaf tip necrosis and adult plant resistance (APR) to stripe (0-10R for 6E16A- and 6E22A-; Pretorius, 1998) and leaf rust, suggesting presence of the linked *Yr18/Lr34* rust resistance genes (Singh 1992a; b). Leaf tip necrosis is characterised by symptoms of 2 to 3 cm of necrosis at the tips of leaves extending to 2 to 4 cm at the tips of leaves. The dominant gene, designated *Ltn* reportedly linked to *Lr34* a gene for leaf rust resistance (Singh, 1992a) and *Yr18* is involved in the expression of leaf tip necrosis. The moderate level of durable APR to stripe rust in 'Anza' has partly been ascribed to the presence of *Yr18* (Singh, 1992a; McIntosh, 1992). Kariega also carries the leaf rust genes *Lr1* and *Lr3a* for seedling resistance to *P. triticina* (ZA Pretorius and RP Singh, unpublished). The cultivar has excellent baking quality but is susceptible to stem rust. 'Avocet S' is a white-seeded stripe rust-susceptible selection (100S for 6E16A- and 6E22A-; Pretorius, 1998) from the Australian spring wheat cultivar 'Avocet'. 'Avocet' carries *Sr26* derived from *Thinopyrum elongatum* (Knott, 1961), *Lr13* and is heterogeneous for the seedling resistance gene *YrA* and for adult plant stripe rust resistance (Wellings *et al.,* 1988; McIntosh *et al.,* 1995). The presence of *Sr26* and *Lr13* has not been confirmed in the 'Avocet S' selection (Wellings, personal communication). *Sr26*, a dominant, seedling resistance gene conferring resistance to stem rust has been mapped on chromosome 6A (McIntosh *et al.,* 1995). Despite apparent yield penalties associated with use of the gene, it has contributed to the establishment of stem rust resistance in Eastern Australia (McIntosh *et al.,* 2001).

The DH mapping population was planted in a field trial and artificially infected with stripe rust (described in 2.1a), and the phenotypic scores obtained for the individual DH lines and leaf tip necrosis were made available by co-workers (Z.A. Pretorius; L.A. Boyd and W.H.P. Boshoff) for this study. To assess the feasibility of quick detection of APR and thereby avoiding extensive field trials, the DH lines were also artificially inoculated and scored under growth chamber conditions (described in 2.1a). Z.A. Pretorius made the growth chamber data as well as the *Sr26* phenotypic scores available for use in this study. J.H. Louw made

available transformed phenotypic data for QTL analysis. B. Wentzel made data for storage protein loci mapped in the DH population available. The data for SSR markers that are not listed in Appendix 2 was captured by R. Prins, and was used for linkage mapping in this study. R. Prins also screened 300 AFLP markers for polymorphism.

2.2 Disease evaluation (data provided by co-workers)

Field evaluation of stripe rust APR

The full set of DH lines was planted in a field experiment with four randomized complete blocks on June 2, 2000 at the PANNAR Research Station near Greytown in KwaZulu-Natal. Entries were planted in 1m row plots spaced 90 cm apart. Each block contained six 'Kariega' and two 'Avocet S' control plots. The entire trial area was surrounded by two rows of a mixture of stripe rust susceptible spreader wheats. Spreader rows were also sown in paths running perpendicular to all experimental plots. The field trial was artificially infected with a spore suspension of pathotype 6E22A- of *P. striiformis* f.sp. *tritici* and was scored by three independent scorers. Severity of infection was scored on September 11 (early) and September 28 (final) using the modified Cobb-scale (0-100% infected leaf area) as a quantitative measure of disease infection (McIntosh *et al.,* 1995 p11; Broers *et al.,* 1996). Host reaction type was scored on the classical ordinal scale R (resistant), MR (moderately resistant), MS (moderately susceptible) and S (susceptible), augmented with three classes through practical experience of scoring this disease, *viz*, RMR between R and MR, MRMS between MR and MS and MSS between MS and S., corresponding exactly to the order of the seven classes in the Australian Plant Breeding Institute adaptation of the original 10 class scale of McNeal *et al.* (1971) (McIntosh *et al.* 1995 p10). One evaluator (ZP) undertook the early assessment in three of the four blocks, and the assessment by LB was for percentage leaf area infected only.

The *Ltn* typing of the DH population was done in the field trial at the same time as the final stripe rust scoring (28 September 2000), but was found to be very difficult as has been reported before (Messmer *et al.*, 2000; William *et al.*, 1997). One scorer (LB) scored the presence or absence of *Ltn* for all entries, whilst two scorers (ZP, WB) scored only for the presence of *Ltn*, which resulted in a large number of missing values.

Growth chamber evaluation

To assess the feasibility of quick detection of APR and thereby avoiding extensive field trials the DH population and control entries were evaluated under growth chamber conditions. Plants were grown at 25°C in a growth chamber where continuous light (200 µmol/m²/second) was provided by fluorescent tubes and incandescent bulbs situated 70 cm above the chamber floor. Plants were inoculated 30 days after planting when most entries were heading (Zadoks growth stage 55) by spraying them with a fine mist of sterile distilled water containing spores of *P. striiformis* f. sp. *tritici* pathotype 6E22A- and a surfactant. Following incubation at 6°C in a high humidity chamber for 48 h, plants were returned to the growth chamber where a temperature of 18°C and 14 h of light per day were maintained. Reactions were assessed 12 days after inoculation when stripe rust development on susceptible plants appeared maximal. Disease assessment was based on whole plant host reaction type (R to S scale) converted to numerical values on the 1-7 ordinal scale as in the case of the field trial.

In a preliminary experiment where 'Kariega', 'Avocet S' and the *Sr26* controls were inoculated with four pathotypes of *P. graminis* f. sp. *tritici*, UVPgt50 was selected on the basis of the typical *Sr26* phenotype (;1c infection type) expressed by 'Avocet S' and susceptibility in 'Kariega' (3^{++}). DH and parental lines were grown in a sterilized soil-peat mixture in 10-cm diameter plastic pots at 18-25 °C in a greenhouse. Eight days after sowing seedlings were spray-inoculated with fresh urediniospores of UVPgt50 suspended in light mineral oil. Plants were then incubated in the dark at 22° C in a dew chamber for 16 h. Upon removal from the chamber seedlings were placed directly below fluorescent tubes emitting 200 µE/m²/s for 3 h before being transferred to a greenhouse cubicle maintained at 20-22 °C. Seedlings were fertilized with a 3:2:1 N-P-K mix (10 g/L and 50 ml/pot) 7 and 14 days after sowing. Infection types were scored on a 0 to 4 scale (McIntosh *et al.*, 1995) 14 days after inoculation when resistant and susceptible reactions on primary leaves could be clearly determined.

2.3 Storage protein loci (data provided by co-worker)

'Kariega' and 'Avocet S' were tested for polymorphisms at the *Glu-1A, Glu-1B and Glu-1D* (high molecular weight glutenin subunits) loci using the technique described in Randall *et al.* (1992). Polymorphic *Glu-1A* and *Glu-1B* alleles were mapped in the DH population.

2.4 Plant DNA extraction

The parents, 'Kariega' and 'Avocet S' were used for parental screens in RFLP SSR, AFLP, RGA and SRAP reactions. 'Chinese Spring' nulli-tetrasomic lines were used as controls in the RFLP and SSR analysis. Protocols for the preparation of all solutions referred to in what follows are given in Appendix 1.

For DNA extractions, seeds were planted directly into pots in the greenhouse. Seeds of DH plants, 3 weeks post harvest, were germinated in petri dishes before being transferred to the greenhouse. The seeds were placed in 70% ethanol for 60 seconds, and then in 30% JIK (household bleach, sodium hypochlorite, 3.5% (m/v)) for 5 minutes. Seeds were washed thoroughly with distilled water before being placed in sterilized petri dishes lined with two pieces of Whatman No. 1 filter paper and sprayed with gibberellic acid (0.01 g/ml) and germinated at 24°C. Seeds were transferred to the greenhouse when roots appeared and planted about 5-10 cm below the soil surface. DNA was extracted when the first tillers appeared after 3-4 weeks.

The DNA isolation protocol used was adapted from Doyle and Doyle (1990).

Day 1

10 ml CTAB solution was added to a 50 ml centrifuge tube (Nalgene). Fresh leaf material (4-6 g) was grinded with liquid nitrogen in a mortar and pestle. Grinded leaf material was added to the centrifuge tubes. 20 μl of β-mercaptoethanol (Merck) was added. The tubes were shaken at 60°C for 60 minutes. 15 ml chloroform-isoamylalcohol (24:1 (v/v)) was added and the contents were mixed thoroughly. Tubes were balanced and centrifuged at 7000 rpm in a Beckman Avanti™ centrifuge for 10 minutes at 25°C, using a JA-20 rotor. The upper phase, obtained after centrifugation, was transferred to clean 50 ml tubes, and to the determined volume, ⅔ volume of ice-cold isopropanol was added. Tubes were mixed gently to precipitate the nucleic acids, and left at -20°C for 1-2 hours. The tubes were centrifuged at 10 000 rpm for 5 minutes at 4°C. The supernatant was poured off, and the pellet allowed to air dry briefly. 15 ml wash buffer was added, and the tubes were left at room temperature for an hour. Tubes were then spun at 10 000 rpm for 10 minutes at 4°C.

The supernatant was poured off, and the pellet allowed to air dry. The pellet was resuspended in 1 ml of distilled water overnight.

Day 2

5 µl of RNAse (1 µg/ul stock) was added to each tube. Tubes were incubated at 37°C for 30-60 minutes. 2 ml of distilled water was added, followed by 1 ml of 7.5 M ammonium acetate (pH 7.6) to give a concentration of 2.5 M, and 10 ml of ice-cold ethanol. The tubes were mixed gently to precipitate the DNA, and left at -20°C for 1-3 hours followed by centrifugation at 10 000 rpm for 10 minutes at 4°C. The supernatant was poured off and the pellet air-dried briefly. Pellets were then resuspended in 1 ml distilled water overnight.

Day 3

Samples were transferred to 2.2 ml microfuge tubes (Eppendorf), followed by a phenol-chloroform:isoamylalcohol (24:1(v/v)) extraction to remove proteins. The volumes in the tubes were determined, and separated into two 2.2 ml tubes. 7.5 M ammonium acetate (pH 7.6) was added to a concentration of 2.5 M, followed by double the volume of ice-cold ethanol. The tubes were shaken gently to precipitate the DNA, and left at -20°C for a few hours, or overnight. The samples were centrifuged in a Beckman GS-15R centrifuge at 14 000 pm at 4°C using a F2402 rotor for 30 minutes. The pellet obtained was washed twice with 70% (v/v) ethanol by performing the same centrifugation. Finally the pellet was speedi-vac dried for 15-20 minutes in a Savant SC110 Speed Vac. Pellets obtained were resuspended in 50-100 µl of distilled water. The quantity and quality of DNA was checked by agarose gel electrophoresis (0.8%) using lambda DNA standards (Roche) of 0.1 to 0.5 µg DNA.

2.5 Agarose gel electrophoresis

Agarose gels were used to determine DNA concentration, test restriction-ligations, preamplification reactions, RFLP analysis, SSR analysis, and to test other PCR products. Agarose gels ranging from 0.8% to 2% (w/v) were prepared in 0.5X TBE, diluted from a 10X TBE stock. Seakem LE agarose was used. A Pharmacia LKB GNA 200 gel apparatus was used together with a Pharmacia LKB GPS 200/400 power pack, or a Bio-Rad Mini-sub®

cell GT apparatus with a Bio-Rad 300 power pack. The voltage and duration of the electrophoresis depended on the samples being electrophoresed.

2.6 Denaturing polyacrylamide gel electrophoresis (PAGE)

Glass plates used for the electrophoresis were cleaned thoroughly using dilute Alconox® solution. Plates were rinsed with tap water and rinsed thoroughly in distilled water and allowed to dry. The plates were wiped clean with 100 % ethanol. Plates were set up using 0.4 mm spacers (GIBCO BRL) and a Model S2 gel-casting clamp (GIBCO BRL).

The gel was prepared by adding 300 µl 10% (w/v) ammonium persulphate and 60 µl TEMED (Sigma) to 60 ml of 6% (w/v) gel mix (19:1 acrylamide: bis-acrylamide; 7 M Urea) in a 100 ml Erlenmeyer flask. The gel was poured carefully between the glass plates, trying to avoid air bubbles. The wells were formed by inserting the sharks-tooth comb (GIBCO BRL) upside down into the gel, and clamping it fast with double clips to ensure that the combs remained stationary. The gel was left flat down on the lab bench to polymerize for at least 1 hour.

Thereafter, the combs and clamps were removed and the glass plates washed clean. The gel was placed into a Model S2 sequencing gel apparatus (Life Technologies) and clamped tightly into position. 1X TBE buffer was poured into the buffer trays and excess urea and gel pieces were removed by rinsing using a syringe. The gel was then pre-electrophoresed at 80 W for 30 minutes with the comb removed. Thereafter the wells were flushed to remove any urea that may have been released and the combs were positioned in the gel. Samples were denatured following addition of AFLP loading dye (Appendix 1) at 94°C for 4 minutes, immediately placed on ice and 4-6.5 µl of sample was loaded. The gel was electrophoresed at 80 W for 2.5 hours.

After electrophoresis, one glass plate was removed carefully and the gel transferred to 3 MM Whatman filter paper, or chromatography paper. The gel was covered with cling wrap and dried for 2 hours on a Savant SGD4050 slab gel drier at 80°C and then exposed to an X-ray film (Kodak Biomax MR).

Alternatively, at the John Innes Centre (JIC), Sequagel XR high-resolution rapid sequencing solution (64 ml) and Sequagel complete buffer (16 ml), both supplied by National Diagnostics, were used. 640 µl of 10% (w/v) ammonium persulfate was added to this to polymerize the gel. A BioRad Sequigen®GT sequencing cell with a BioRad 3000 Power Pack was used for the electrophoresis step. The gels were pre-electrophoresed until a temperature of 50°C was reached and samples were loaded. The gels were electrophoresed at 150 W for 105 minutes. Gels were dried on a BioRad Model 583 dryer with a BioRad Hydrotech vacuum pump.

2.7 Isolation of RFLP probes

Probes that were used in this study were provided by M.D. Gale (John Innes Centre, Norwich, UK). These PSR probes (Table 2.1a) were supplied as wheat leaf cDNA or genomic DNA inserts into the various restriction sites of the pUC 18 plasmid vector. These probes were not transformed in this study. Probes (PA) obtained from the Graingenes database (http://www.genome.cornell.edu) (Table 2.1b.) were transformed.

Preparation of competent cells

8 ml of LB media was inoculated with *Escherichia coli* DH5α strain, and incubated overnight at 37°C on a shaker. The next day, 1 ml of this culture was inoculated into 100 ml LB and incubated on a shaker at 37°C for about 4 hours. The culture was placed on ice for 30 minutes. 40 ml of culture was transferred to 50 ml centrifuge tubes (Nalgene) and centrifuged at 5000 rpm for 5 minutes at 4°C, in a Beckman Avanti™ centrifuge using a JA-20 rotor. The supernatant was poured off and the cells resuspended in 20 ml 50 mM $CaCl_2$ (sterile and ice-cold). The tubes were kept on ice for 15 minutes, and then centrifuged at 5000 rpm for 5 minutes as before. The supernatant was poured off and the cells resuspended in 4 ml 50 mM $CaCl_2$. The tubes were kept on ice for at least 2 hours, then aliquoted into 1.5 ml microfuge tubes (200 µl aliquots).

Table 2.1a. RFLP probes tested for polymorphism, but not transformed in this study.

PROBE	SOURCE	CHROMOSOME	SIZE (kB)
PSR112	Leaf cDNA	2	1.8
PSR102	Leaf cDNA	2	0.57
PSR1196	Genomic DNA	3	0.4
PSR128	Leaf cDNA	5	0.56
PSR154	Leaf cDNA	6	0.4
PSR152	Leaf cDNA	7	0.85
WG834	Genomic DNA	7	Not known

Table 2.1b. RFLP probes obtained from the Graingenes database, which were transformed in this study.

ID Number	Clone Name	Vector
PA280	BCD0828	PBssk
PA744	BCD0707	PBssk
PA783	BCD1872	PBssk

Transformations

1 µl of plasmid was added to 200 µl competent cells and kept on ice for 30 minutes. The cells were heat shocked for 2 minutes at 42°C to stimulate the uptake of plasmids. 1 ml of LB was added (no antibiotic) to the tubes and this was shaken at 37°C for 1 hour. The tube was then centrifuged briefly to collect the contents, and the supernatant was poured off. The cells were then mixed with a pipette tip, transferred to agar plates containing the appropriate antibiotic, distributed with a hockey stick, and incubated overnight at 37°C. Transformed colonies were picked with a sterilized toothpick and rubbed on the inside of a microfuge tube containing 50 µl distilled water. The same toothpick was used to inoculate 5 ml of LB containing the appropriate antibiotic. The LB was shaken overnight at 37°C and 50% glycerol freezer stocks were made of the cultures. The microfuge tubes were boiled at 100°C for 5 minutes, and placed at -20°C. These cells were used in PCR reactions to obtain the RFLP probes.

PCR of probes

PCR was performed to obtain the RFLP probes from the boiled cells. The PCR reaction used 10 µl of boiled cells and comprised 50 ng M13 forward primer (50 ng/ul stock-Bioline),

50 ng M13 reverse primer (50 ng/ul stock-Bioline), 1.5 mM $MgCl_2$ (25 mM stock), 200 μM dNTP (5 mM stock), PCR reaction buffer to 1X concentration (Bioline), 1 unit of *Taq* DNA polymerase (Bioline), and distilled water to 50 μl. The reactions were performed using a DNA engine, MJ Research PTC-200 thermal cycler using the following program:

94°C for 30 sec
55°C for 30 sec } for 35 cycles
72°C for 1 min

72°C for 5 min and 4°C soak temperature

The probes were then cleaned using a PCR clean-up kit (Promega), following the manufacturers' instructions and checked by electrophoresis on 2% agarose gels.

2.8 RFLP Analysis

The protocol used for RFLP analysis was derived from Devos *et al.* (1992).

Restriction digests

Test restrictions were first performed to test if the genomic DNA restricts properly, and to assess the quality of the DNA. The test restrictions were performed with 250 ng of test DNA, 1 unit of *Hind*III restriction enzyme (Promega- 10U/μl), *Hind*III restriction buffer to 1X concentration, 0.1 μg/μl BSA (Promega), and distilled water to 10 μl. The samples were mixed and centrifuged to collect the contents, and then incubated at 37°C for 3 hours. 1 μl of loading dye was added and the samples checked by agarose gel electrophoresis. 0.8% (w/v) gels were used with electrophoresis at 80 V for 45 minutes. Based on the quality of the test restrictions obtained, the DNA samples in question were either used for RFLP or discarded.

1.5 ml microfuge tubes were used for the restriction digests. Genomic DNA was digested with each of the enzymes *Hind*III (Promega- 10 U/μl), *Dra*I (Promega- 10 U/μl), *Eco*RI (Promega- 12 U/μl), and *Eco*RV (Promega- 10 U/μl) respectively. 60 units of enzyme were used for the *Hind*III, *Dra*I, and *Eco*RV restriction digests while 72 units of enzyme were used for the *Eco*RI digest. The restriction enzymes were used in excess to counteract the effects of impurities that might be present in the DNA samples and might influence enzyme activity. 4 μl (40 U) of restriction enzyme was added to the reactions overnight, and 2 μl

(20 U) was added the next morning to ensure complete restriction of the genomic DNA. In addition to the restriction enzyme, the restriction reactions consisted of 1-2 µg of genomic DNA, the buffer of the respective enzyme to 1X concentration, 0.1 µg/µl BSA (Promega) and distilled water to a total volume of 40 µl. The restriction reactions were left at 37°C overnight. A precipitation step was included to reduce the sample volume for loading. Distilled water was added to the restricted DNA sample to 80-100 µl. The DNA was precipitated with a final concentration of 2.5 M ammonium acetate (pH 7.6), followed by two volumes of ethanol. The samples were left at -20°C for at least 2 hours, followed by centrifugation, wash steps with 70% (v/v) ethanol and drying steps.

Gel electrophoresis

A 300 ml 0.8% (w/v) agarose gel (20 cm X 20 cm) was prepared in 0.5 X TBE with no ethidium bromide added. Two 22 well combs were used so that two blots could be prepared from one gel. The gel was allowed to set for 45 minutes to 1 hour. The top half of the gel was first loaded and the samples electrophoresed into the gel at 100V for 10 minutes followed by the bottom half. The gel was electrophoresed between 35 V and 45 V overnight. Electrophoresis was then stopped, and the gel seperated, stained in ethidium bromide (0.5 µg/ml) for 20 minutes, photographed, and transferred to nylon membranes through Southern blotting.

Southern blotting

An alkaline transfer method was used. The gels were placed in 0.2 N HCl until the loading dye changed colour, and then left for another 10 minutes (~ 20 minutes) (depurination). The HCl was then poured off and the gels rinsed with distilled water. Gels were then placed in 1.5 M NaCl; 0.5 N NaOH (Appendix 1) for two 15-minute washes with rinse steps in between. The gels were finally covered with 0.4 N NaOH for 30 minutes. The blotting stack consisted of a microwave dish filled with 0.4 N NaOH with a glass plate across the ends. Two sheets of 3MM Whatman paper were soaked in the buffer and used to make a wick. The gel was placed on the wick with the open ends of the wells against the wick. Hybond N^+ membrane (Amersham) was cut to the size of the gel and placed over it. Three sheets of 3MM Whatman paper, saturated with transfer buffer were placed on the membrane, and a stack of absorbent towels on top of the Whatman paper. Cling wrap was used to surround the gel and membrane and ensure that the buffer was not absorbed directly by the towels. A

glass plate was placed on the paper towels, and a weight was placed on it. DNA transfer was allowed to take place overnight. The blotting stack was disassembled the next day. The membranes were marked and washed in 2X SSC to remove any adhering agarose. The membranes were then blotted dry, covered in plastic, and stored at 4°C.

Probe labeling

1.5 ml Safe-lock microfuge tubes (Eppendorf) were used for the probe labeling reactions. 60-80 ng of probe was labeled using the high prime DNA labeling reaction mix (Roche). The probe was first denatured for 10 minutes at 100°C, and then placed on ice for 2 minutes. 5 µl of α ^{32}P ATP (Amersham) was used in each labeling reaction. The tube was centrifuged to collect the contents and incubated at 37°C for 60 minutes. One-tenth volume 3M NaOH (2 µl) was added to stop and denature the reaction. The labeling reaction was then placed at room temperature for 5 minutes before being added to the prehybridisation solution containing the blot.

Prehybridisation and wash steps

60 ml of prehybridisation solution was added to a plastic container with an airtight seal. The lid of the container was covered with cling wrap to prevent contamination with radioactivity. The membrane was added to the container and placed in a shaking incubator at 65°C. New membranes were pre-hybridized overnight, and membranes being reused were pre-hybridized for 6 hours. Membranes were washed first with wash buffer 1 for 15 min in the shaking incubator at 65°C. Based on the radioactivity counts per second, more wash steps might be performed. Each wash step was performed at 65°C in a shaking incubator for 15 minutes. Usually two washes were performed with wash buffer 1, followed by two washes with wash buffer 2 (Appendix 1). However the number of washes was dependant on the radioactivity counts per second observed. The wash steps were stopped when 2-5 counts per second were observed with a Geiger Mueller counter. The membranes are then exposed to Kodak X-O Matic film and subsequently developed.

2.9 SSR Analysis

The SSR protocol followed was that of Bryan *et al.* (1997). SSR primers were produced by Integrated DNA Technologies (IDT). The GWM and GDM primers were developed at the

Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) (Gaterslaben, Germany) (Röder *et al.,* 1998; Pestsova *et al.,* 2000). WMC primer sequences were obtained from Gupta *et al.* (2002) and CFD primer sequences were provided courtesy of Institit National De La Recherche Agronomique (INRA), France (Guyomar'ch *et al.,* 2002). A list of SSR primers used in this study is provided in Appendix 2.

Primer labeling

Labeling reactions consisted of 10 pmol forward primer, One-Phor-All buffer to 1X concentration, 0.05 units T4 polynucleotide kinase (Pharmacia- 7.9 U/μl), 0.5 μCi γ^{33}P ATP (NEN Life sciences), and AFLP grade water. Labeling reactions were incubated at 37°C for 2 hours. Reactions were stopped by incubating at 65°C for 10 minutes, and then stored at - 20°C.

Amplification reactions

100 ng of template DNA was used in 10 μl reaction volumes. PCR reactions consisted of 10 pmol of each primer, 0.5 units *Taq* DNA polymerase (Bioline-5U/μl), PCR buffer (Bioline) to 1X concentration, 200 μM dNTPs, 1.5 mM $MgCl_2$ (Bioline), and distilled water. The $MgCl_2$ concentration had to be adjusted in some cases. PCR amplification was done in a MJ Research DNA Engine thermocycler with the relevant SSR program which included a ramping step of 0.5°C /second. 3 μl of loading dye was added to the samples followed by electrophoresis using denaturing PAGE for 90 minutes and visualization with autoradiography as described in section 2.4.

2.10 AFLP analysis

A large portion of the AFLP work in this study was completed in the laboratory of L.A. Boyd under the guidance of P.H. Smith at the JIC in the United Kingdom. Keygene owns the copyright for the AFLP process. The protocol of Donini *et al.* (1997) was used, with some modifications. Lists of the AFLP primers used in this study are given in Appendices 3 and 4.

Dilution of primers

The primers were obtained from IDT, USA. Primers used at the JIC were obtained from GIBCO-BRL and a working concentration of 50 ng/μl was used.

The adaptors were handled differently. The oligonucleotides that were received were diluted to 100 pmol/μl. With the *Mse*I adaptor, equal volumes of each oligonucleotide (MA1 and MA2) were added together in a single microfuge tube, to a concentration of 50 pmol/μl. With the *Sse*I adaptor each oligonucleotide (SA1 and SA2) was diluted with AFLP grade water in a single microfuge tube to 5 pmol/μl. The tubes with the adaptors were then heated at 65°C for 10 minutes to separate the oligonucleotides, and the block was then switched off and allowed to reach room temperature.

The sequence of the adaptors used were:

*Sse*I adaptor 1 (SA1)	5' CTCGTAGACTGCGTACATGCA 3'
*Sse*I adaptor 2 (SA2)	3' CATCTGACGCATGT 5'

*Mse*I adaptor 1 (MA1)	5' GACGATGAGTCCTGAG 3'
*Mse*I adaptor 2 (MA2)	3' TACTCAGGACTCAT 5'

Restriction digestion of genomic DNA

Genomic DNA was diluted to 250 ng/μl in distilled water. The *Sse*I and *Mse*I restriction enzyme system was used, and 1.5 ml microfuge tubes were used as reaction vessels. 500 ng of genomic DNA was restricted with 5 units of *Sse*I (Amersham 10 U/μl), and 5 units of *Mse*I (New England Biolabs 4 U/μl), in a reaction volume of 40 μl. The reaction volume also consisted of One-Phor-All buffer (Pharmacia) at 1X concentration, BSA (New England Biolabs 10 mg/ml) at 0.1 μg/μl, and AFLP grade water. The reaction vessels were tapped gently to mix the contents and then centrifuged to collect the contents. The microfuge tubes were then incubated at 37°C for 2-4 hours.

Ligation of adaptors

After 2-4 hours, 10 μl of ligation mix was added to the reaction. The ligation mix consisted of 5 pmol *Sse*I adaptor (5 pmol/μl stock solution), 50 pmol *Mse*I adaptor (50 pmol/μl stock), 1 mM ATP (10 mM stock), 1 unit T4 DNA ligase (Amersham 7.5 U/μl), One-Phor-All-buffer (Pharmacia) to 1X concentration, BSA (New England Biolabs 10 mg/ml) to 0.1 μg/μl, and AFLP grade water to a final volume of 10 μl. Again, the contents of the tubes were mixed gently, and centrifuged to collect the contents. The tubes were then incubated

overnight at 37°C. Thereafter 45 µl of the restriction-ligation reaction was diluted with 405 µl 1X $TE_{0.1}$ and stored at -20°C.

At the JIC the restriction and ligation reaction were performed in one step. Genomic DNA was diluted to 100 ng/µl, and 500 ng of DNA was restricted. The *Sse*I and *Mse*I restriction enzyme system was used. Genomic DNA was restricted with 5 units of *Sse*I (MBI fermentas-5 U/µl), and 5 units of *Mse*I (New England Biolabs-4 U/µl). 5X restriction-ligation (RL) buffer was used at 1X concentration together with 5 pmol *Sse*I adaptor (5 pmol/µl stock solution), 50 pmol *Mse*I adaptor (50 pmol/µl stock), 1 mM ATP (10 mM stock), 1 unit T4 DNA ligase (GIBCO-BRL-1 U/µl), and AFLP grade water to make up 40 µl. Again, the contents of the tubes were mixed gently, and centrifuged to collect the contents. The tubes were then incubated overnight at 37°C.

Pre-amplification reactions (Cold PCR)

Pre-amplification reactions, with non-selective primers (M_{00} and S_{00}) were performed using 13-15 µl of the diluted restriction-ligation reaction in a reaction volume of 50 µl. The pre-amplification reactions consisted of 75 ng S_{00} primer (50 ng/µl stock), 75 ng M_{00} primer (50 ng/µl stock), 200 µM dNTPs (5 mM stock), 1.5 mM $MgCl_2$ (50 mM stock), PCR buffer (Bioline) to 1X concentration, 1 unit of *Taq* DNA polymerase (Bioline 5 U/µl), and AFLP grade water to 50 µl. A DNA Engine MJ Research PTC-200 thermal cycler was used.

At the JIC 5 µl of undiluted restriction-ligation reaction was used in 20 µl pre-amplification reactions. The pre-amplification reactions consisted of 50 ng S_{00} primer (50 ng/µl stock), 50 ng M_{00} primer (50 ng/µl stock), 200 µM dNTPs (2.5 mM stock), PCR buffer with $MgCl_2$ (Roche) to 1X concentration, 1 unit of *Taq* DNA polymerase (Roche-5U/µl), and AFLP grade water to 20 µl. A DNA Engine, MJ Research, PTC-200 thermal cycler was used. The following PCR program was used for the pre-amplification reactions:

72°C for 5 min
94°C for 30 seconds
56°C for 60 seconds
72°C for 60 seconds
} 30 cycles
72°C for 5 min and 4°C soak temperature

45 µl of the pre-amplification reaction was diluted with 405 µl of 1X $TE_{0.1}$ buffer, and stored at -20°C. At JIC 16 µl of the pre-amplification reaction was added to 784 µl of 1X $TE_{0.1}$. The pre-amplification reactions were then checked on 2% agarose gel by electrophoresing for 2 hours.

Primer labeling

Safe-lock 1.5 ml microfuge tubes were used for the labeling reactions. Primer labeling reactions consisted of 2.5 ng *Sse*I primer (50 ng/µl stock), One-Phor-All-buffer (Pharmacia) to 1X concentration, 0.05 units T4 polynucleotide kinase (Pharmacia 7.9 U/µl), 0.5 µCi γ^{33}P ATP (NEN Life sciences), and AFLP grade water. The contents were mixed, and the tubes centrifuged to collect the contents. Labeling reactions were then incubated at 37°C for 2 hours. Incubation at 65°C for 10 minutes inactivated the polynucleotide kinase. The labeled primer was then stored at -20°C until required. 5 ng *Sse* primer and 0.1 U of T4 polynucleotide kinase (MBI fermentas) with 0.07 µl γ^{33}P ATP (Amersham) per reaction was used for labeling reactions at the JIC.

Selective amplification reactions (Hot PCR)

For the selective PCR reactions, PCR plates (ABgene) and sealers (ABgene) were used. 5 µl of the diluted pre-amplification reactions were used for the selective PCR. 20 µl reaction volumes were used. Reactions consisted of 15 ng unlabeled *Mse*I primer (50 ng/µl stock), 2.5 ng labeled *Sse*I primer, 200 µM dNTPs (5 mM stock), PCR buffer (Bioline) to 1X concentration, 1.5 $MgCl_2$ (50 mM stock), 0.05 units *Taq* DNA polymerase (Bioline- 5U/µl), and AFLP grade water. A DNA engine MJ Research PTC-200 thermal cycler was used.

2.5 µl of diluted pre-amplification reaction was used for the hot PCR reactions at JIC with 30 ng *Mse* primer. 0.1 U of Sigma Red *Taq* DNA polymerase (1U/ul) was used with Sigma buffer and magnesium to 1.5 mM concentration using a MJR PTC-200 thermal cycler and the following PCR program.

94°C for 30 sec 65°C for 30 sec 72°C for 1 min 1 cycle

The annealing temperature is lowered by 0.7°C for 13 cycles

94°C for 30 sec
-0.7°C for 30 sec
72°C for 1 min
] for 13 cycles

Then,
94°C for 30 sec
56°C for 30 sec
72°C for 1 min
] for 23 cycles

4°C soak temperature

Amplification reactions were stored at -20°C. 10 µl AFLP loading dye (Appendix 1) was added to the sample prior to denaturing and loading. AFLP profiles were visualized using denaturing PAGE and autoradiography as described in 2.4.

2.11 RGA technique

The RGA protocol first attempted was modified from Chen *et al.* (1998). This protocol was developed for silver staining detection of fragments in wheat and barley. The protocol given was optimised for detection of PCR products using radionucleotides. A list of the RGA primers used is given in Appendix 5. Oligonucleotide primer sequences were kindly provided by Xianming Chen and primers were manufactured by IDT.

Primer labeling

Labeling reactions consisted of ~5 ng forward primer, One-Phor-All buffer to 1X concentration, 0.05 U T4 polynucleotide kinase (Pharmacia- 7.9 U/µl), 0.04 µl γ ^{33}P ATP (NEN Life sciences), and AFLP grade water. Labeling reactions were incubated at 37°C for 2 hours. Reactions were stopped by incubating at 65°C for 10 minutes, and then stored at -20°C.

Amplification reactions

PCR reactions consisted of 5 ng unlabeled reverse primer, 5 ng labeled forward primer, 0.5 U *Taq* DNA polymerase (Bioline-5U/µl), 10X PCR buffer (Bioline) to 1X concentration, 200 µM dNTPs, 1.5 mM $MgCl_2$ (Bioline), and AFLP grade water. Approximately 35 ng of

template DNA was used in 10 µl reaction volumes. PCR amplification was done in a MJ Research DNA Engine thermocycler using the following program:

94°C for 5 min
94°C for 1 min ┐
45°C for 1 min │ 45 cycles
72°C for 2 min ┘
72°C for 7 min and 15°C soak temperature

3 µl of AFLP loading dye (Appendix 1) was added to the samples. PCR products were separated using denaturing PAGE (6% (w/v)) for 90 minutes, and visualized using autoradiography as described in section 2.4.

2.12 SRAP technique

The SRAP marker technique was originally developed by Li *et al.* (2001) and was optimized in *Brassica* spp. The primer sequences used in this study were obtained from the original publication, and manufactured by IDT and are given in Appendix 6. Primer labeling and PCR amplification reactions were as described in Li *et al.* (2001). PCR amplification was done in a MJ Research DNA Engine thermocycler using the following program:

94°C for 5 min
94°C for 1 min ┐
35°C for 1 min │ 5 cycles
72°C for 1 min ┘

94°C for 1 min ┐
52°C for 1 min │ 35 cycles
72°C for 1 min ┘
72°C for 5 min and 15°C soak temperature

3 μl of AFLP loading dye (Appendix 1) was added to the samples. PCR products were separated with denaturing PAGE (6% (w/v)) for 150 minutes, and visualized using autoradiography as described in section 2.4.

2.13 Data analysis

Marker segregation and linkage map construction

The standard Chi-square test was used to test the goodness of fit of observed marker ratios to theoretical expectations. Markers were tested for 1:1 segregation (as is expected with a DH population) using a Chi-square test with 1 degree of freedom. Markers with significantly distorted segregation ratios ($P<0.01$) were not used in the subsequent analysis. The distribution of alleles among the individuals of the DH population was also examined using standard Chi-square tests.

The program Map Manager QTX ver. 15.0 (Manly and Olson, 1999) was used to generate linkage maps. Linkage maps were generated at a threshold value of $P=0.001$ using the Kosambi mapping function (Kosambi, 1944) and drawn with MapChart 2.1 (Voorrips, 2002). Markers mapped at a later stage were placed in linkage groups using the 'distribute' function of Map Manager to determine the best order and map position of the markers added to the linkage groups. The linkage maps developed were checked for double crossovers and markers with more than 3 double crossovers, especially those in clusters, were excluded and the maps re-drawn. Large numbers of double crossovers are often artifacts produced by marker mis-classification. Double crossovers occurring in clusters have been reported to inflate map length (Castiglioni *et al.,* 1998). At the JIC, the Joinmap® linkage software was used (Stam *et al.,* 1995). MAPMAKER/EXP ver. 3.0b (Lander *et al.*, 1987; Lincoln *et al.,* 1992a) was used to compare the results of different software packages for linkage analysis.

QTL analysis

Various transformations of the data were attempted to normalise the disease scores as is appropriate for QTL analysis. Field scores on the percentage of leaf area infected were converted to proportions, p, and transformed to *arcsin* ($\sqrt{p}$) (angular transformation) as is appropriate for percentage data (Sokal and Rohlf, 1995). Host reaction type scores on the ordinal scale were assigned numerical values from 1 (resistant) to 7 (susceptible) and

transformed to ln (score + 1) (Sokal and Rohlf, 1995; Lynch and Walsh, 1998) for the QTL mapping analysis.

Map Manager QTX ver. 15.0 (Manly and Olson, 1999) was used to perform QTL analysis. QTL were characterized using single marker interval mapping and interaction analyses using the regression procedures of Zeng (1993; 1994). Pre-selected marker co-factors (Appendix 7) were chosen as control for background variation (referred to here as modified interval mapping (mIM)) based on significant main and interaction effects (Wang *et al.*, 1999) for each of the disease scores. A marker-cofactor was dropped when the chromosome harbouring the marker was analysed using mIM. A permutation test (Doerge and Churchill, 1996) set at 1000 iterations was used to calculate the likelihood ratio statistic (Lrstat) threshold for declaring statistical significance.

CHAPTER 3: MOLECULAR MARKER ANALYSIS

Polymorphism screens were performed using the parent cultivars 'Kariega' and 'Avocet S' as controls and the levels of polymorphism observed with the different marker techniques are presented and discussed in this chapter. Polymorphic markers were selected for mapping in the DH population in order to generate a partial linkage map.

3.1 DNA isolations

DNA obtained from the large-scale extraction technique of Doyle and Doyle (1990) was used in all of the molecular marker analyses in this study. The yield of DNA obtained varied from 100 ng/µl to 600 ng/µl from 4 to 6 g leaf material. Electrophoresis of the extracted DNA on 0.8 % (w/v) agarose gels produced a dense band near the top of the lanes with little smearing. RNA was visible as a smear near the bottom of the gel for some samples.

3.2 RFLP markers

RFLP nylon blots were prepared for each of the seven groups of chromosomes. The parental screens included 'Chinese Spring' and 'Chinese Spring' nulli-tetrasomic or nullisomic controls for each of the A, B and D genomes. These controls were used for each of the different restriction enzymes, and would confirm the genome on which any polymorphism occurred. Seven RFLP markers were screened for polymorphism in this study and none of the RFLP probes (Table 2.1b) were polymorphic.

DNA samples to be used for Southern analysis were first checked by performing test restriction digests. The test digests usually looked good on agarose gels, but larger quantities of the same sample often produced poor blots. Impurities in the DNA samples would have been more concentrated in the larger quantities to adversely affect the digestion. DNA used to produce Southern blots should be free of impurities, of high molecular weight, and with little or no degradation. Autoradiogram signal strength is determined to a large extent by the amount and quality of DNA used for the blots (Grant and Shoemaker, 1997). The effect of DNA quality on the resolution of RFLP was seen in this study. A blot of the group seven chromosomes obtained using DNA of good quality is shown in Fig. 3.1.

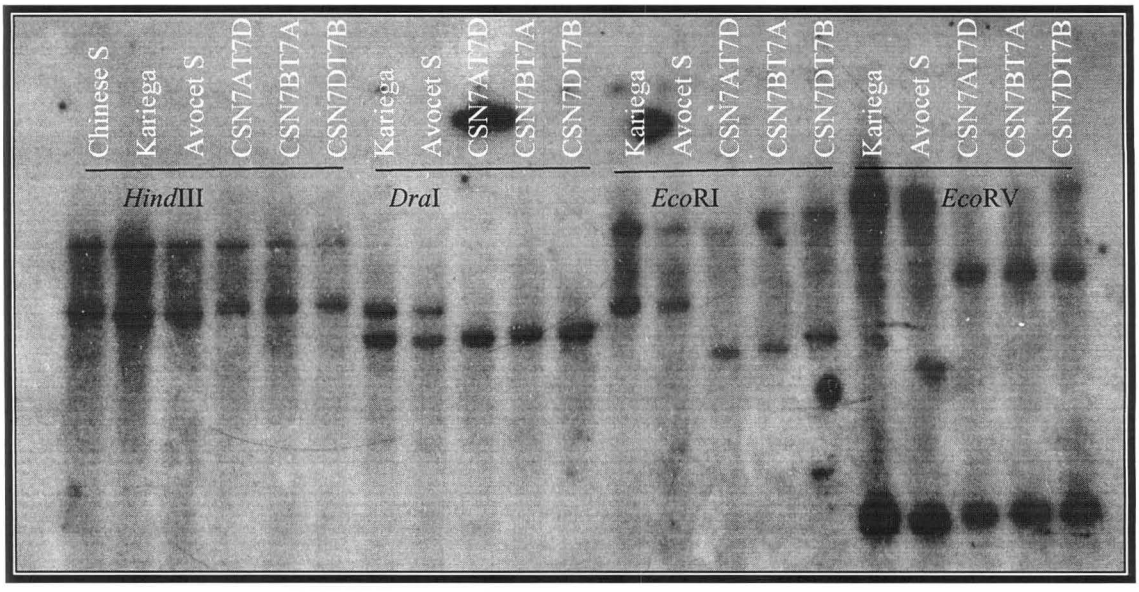


Figure 3.1. RFLP blot using probe WG834 which maps to chromosome 7D. 'Chinese Spring' and 'Chinese Spring' group 7 nulli-tetrasomic lines (CSN7AT7D; CSN7BT7A; CSN7DT7B) were used as controls. The probe was monomorphic with the different restriction enzymes used. There is no band missing in the CSN7DT7B so the probe appears to map to the group 7 chromosomes in general. Some bands appear to be missing or faint and these could be due to stringent wash steps (see 2.8).

The RFLP technique did not produce any polymorphism and this is not surprising, as a low level of RFLP polymorphism has generally been found in wheat (Kam-Morgan *et al.,* 1989; Chao *et al.,* 1989). The low level of polymorphism, the time and effort involved in generating RFLP markers and the limited use of these markers in actual breeding programmes have contributed to a decrease in use. The conversion of RFLP markers into STS markers that can be screened for by using quick, simple assays has been reported, but with varying levels of success (Talbert *et al.,* 1994; Adlam *et al.,* 1999).

In this study it was found that RFLP markers were expensive to use, and technically very demanding. Furthermore, results could only be obtained after about 10-15 days, which is far from ideal. The RFLP assay was found to be too slow and it was clear at an early stage that many more AFLP and SSR markers could be screened for in the same time required to search for polymorphic RFLP. Langridge *et al.* (2001) highlighted some of the difficulties associated with RFLP markers. RFLPs are important as anchor markers on many of the chromosomes in wheat, and are useful in comparative mapping studies where they can be

translated across species. They were not favoured in this study largely due to the technical difficulties and to the time required for the assay.

3.3 SSR markers

It is important in the development of partial linkage maps to have anchor markers that are well spaced and cover all chromosomes. SSR markers that could serve as anchors on the 21 chromosomes of wheat were screened and mapped by R. Prins, but not all chromosomes were covered. Further GWM, GDM, WMC, CFD and CFA SSR primers (Appendix 2, Table 3.1, Fig.3.2) aimed at the remaining chromosomes were tested in this study in order to develop a linkage map with good genome coverage. In addition SSR markers targeting important chromosomes (identified with QTL mapping-see Chapter 5) were also tested (Appendix 2, Table 3.2, Fig 3.2). 'Chinese Spring' and 'Chinese Spring' nulli-tetrasomic and nullisomic lines were used as controls to confirm chromosomal locations of most SSRs (Fig.3.2). Both co-dominant (Fig 3.2 A, B) and dominant (Fig 3.2 C) were detected on agarose (Fig. 3.2 C) and denaturing polyacrylamide gels (Fig 3.2 A, B).

Some SSR primer combinations gave a number of amplification products in addition to the target SSR amplified. In some cases these were polymorphic bands that always co-segregated or they were other monomorphic bands. Microsatellites usually remain specific to the genome where they were identified and mapped but a duplication of one of the

Table 3.1. Summary of GDM, GWM, WMC, CFD and CFA primers tested in this study.

SSR	No.[a]	Pol.[b]	Not Pol.[c]	Not Clear	Poly-morphism[d]	Reported polymorphism and source reference
GDM	7	1	3	3	25 %	74 %-Pestsova *et al.* (2000)
GWM	84	29	36	19	45 %	80.0 %-Röder *et al.* (1998)
WMC	12	3	7	2	30 %	15-30 %-Harker *et al.* (2001)
CFD	12	4	7	1	36 %	91 %-Guyomar'ch *et al.* (2002)
CFA	10	2	6	2	25 %	Not reported
Total	125	39	59	27	40 %	

[a] Number of relevant SSRs tested
[b] Number of polymorphic SSR loci
[c] Number of monomorphic SSR loci
[d] Calculated as (No. polymorphic SSR/(No. polymorphic + No. monomorphic SSR) × 100)

flanking sequences may produce multiple products. These PCR products could be derived from moderately repeated DNA sequences, such as inactive retrotransposons that are found in Gramineae genomes (Röder *et al.,* 1998). If an SSR primer combination is sufficiently specific to amplify a locus, and multiple copies of the locus exist, then multiple bands may be produced which co-segregate. Sequencing these different bands will confirm whether they are multiple copies of the same sequence or not.

SSR polymorphism

An average of 40% polymorphism was obtained using the SSRs tested in this study. Prins *et al.* (unpublished) reported an average polymorphism of 46% for SSRs (GWM, PSP, WMC) representing all three wheat genomes, while those derived from *Aegilops tauschii* D-genome were clearly much less polymorphic (24%) in the 'Kariega X Avocet S' cross. A low polymorphism level (25%) was also obtained for the GDM SSRs in this study although the 36% polymorphism observed for the CFD SSRs is promising. The high level of polymorphism reported by Pestsova *et al.* (2000) and Röder *et al.* (1998) (Table 3.1) was obtained with the ITMI RIL population which has a high level of polymorphism as one of the parents in the cross, 'W7984', is a synthetic hexaploid. Eujayl *et al.* (2002) obtained a 52% polymorphism level for the GWM SSRs in durum wheat. The polymorphism level obtained with the WMC SSRs compares well with the 15-30% range of polymorphisms found for SSRs in four Australian mapping populations derived from common wheat (Harker *et al.,* 2001). A 61% polymorphism level was reported by Guyomar'ch *et al.* (2002) for the CFD SSRs in the 'Chinese Spring X Courtot' population. Genetic maps generated using locally adapted wheat cultivars as parents usually reveal a lower level of polymorphism when compared to the ITMI population, as is evident in the results of Guyomar'ch *et al.* (2002), Harker *et al.* (2001) and this study.

The low polymorphism observed on chromosome 7D (Table 3.2) is consistent with the generally low polymorphism reported for the D genome (Chalmers *et al.,* 2001; Röder *et al.,* 1998) while the results for the other chromosomes are in general agreement with the overall polymorphism level of 40%. Genes for resistance to leaf rust (Nelson *et al.,* 1997), powdery mildew (Börner *et al.,* 2002), Russian wheat aphid (Ma *et al.,* 1998) and stripe rust (Singh, 1992a), all of which are economically important traits, have been mapped to chromosome 7D, so it is important to target this region with more markers. Improving the marker density in this region could have associated advantages for MAS for these traits.

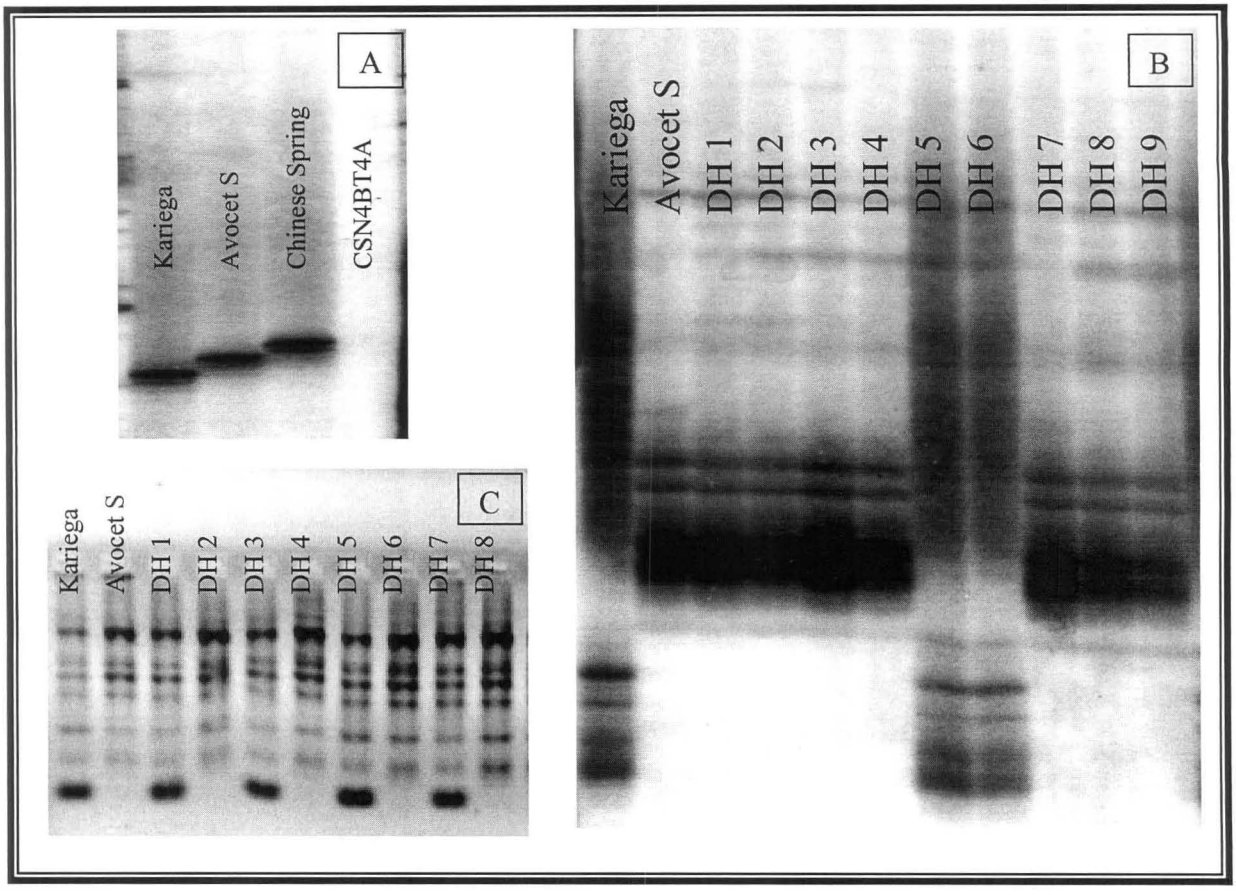


Figure 3.2. SSR polymorphism screens and mapping gels. A: An example of an SSR polymorphism screen with GWM 495 and 'Chinese Spring' and CSN4BT4A control. This marker is co-dominant and maps to chromosome 4B as no band is seen in the CSN4BT4A control. B: A section of an autoradiogram of an SSR mapping gel on a 6% denaturing polyacrylamide gel with GWM 742. This marker is also co-dominant and both alleles are segregating in the DH lines. C: A section of an SSR mapping gel on 3% agarose with GWM 735. This marker is dominant and can easily be scored on an agarose gel.

Table 3.2. Summary of the SSRs targeting specific chromosomes important in this study.

Chromosome	No.[a]	Pol.[b]	Not Pol.[c]	Not Clear	Polymorphism[d]
7D	22	7	12	3	37 %
2B	16	5	6	5	45 %
4A	14	5	7	2	42 %
1A	6	2	3	1	40 %

[a] Indicates the number of relevant SSRs tested
[b] Indicates the number of polymorphic SSR loci
[c] Indicates the number of monomorphic SSR loci
[d] Calculated as (No. polymorphic SSR/(No. polymorphic + No. monomorphic SSR) × 100)

SSRs proved to be very valuable markers and were used to anchor all linkage groups formed in this study, and the technique is not as time consuming as RFLP. Microsatellite maps of hexaploid wheat reveal an even distribution of SSR markers along the linkage groups with no reports of any significant clustering (Gupta *et al.,* 1999), with the highest proportion occurring in the B genome (Röder *et al.,* 1998). The majority of SSR primers will amplify a single band representing a locus. This makes microsatellites ideal markers to anchor linkage groups. SSRs have largely replaced RFLPs as anchor markers due to easy automation of the technique, in addition to the high levels of polymorphism generally detected and the ability to detect heterozygotes and heterogeneity. Most of the SSRs tested in this study mapped to the chromosomal location identified in the original publication, (exceptions mentioned in section 4.2) thus indicating reproducibility across different laboratories.

Microsatellites can be profitably utilized in wheat for tagging genes, detecting polymorphism, genotypic identification and for estimating genetic diversity (Prasad *et al.,* 2000). The easy transferability of primer sequences allows for verification of SSR alleles in different laboratories, and has resulted in the construction of large molecular-marker databases for wheat varieties (Röder *et al.,* 2002). The technique has an added advantage in that SSRs may also serve as STS markers (requiring no conversion for a suitable assay) if mapped in a region of interest. The exploitation of SSRs in wheat genetics generally appears most promising for future research studies due to the many advantages associated with the technique which also justifies the costs involved in their initial development.

3.4 AFLP markers

A total of 900 AFLP primer combinations were tested for polymorphism of which R Prins tested 300 and 600, consisting of both S+2 and M+2/M+3 (Appendices 2 and 3) primer were tested in this study. Loci from 65 primer combinations were mapped in this study. Polymorphic fragments on each gel were labeled alphabetically from top to bottom. Markers were named according to the restriction enzymes used with a letter indicating either the *Sse*I (S) or *Mse*I (M) adaptor, and a number indicating the identity of selective nucleotides.

Reproducibility of AFLP

One of the essential requirements of a molecular marker is reproducibility. Markers should be reproducible across experiments and across laboratories when using the same samples.

AFLP reactions were performed in different laboratories in this study, and the DNA profiles obtained were highly reproducible. Restriction fragment ligations and pre-amplification reactions prepared were compared and revealed the same bands segregating consistently in the case of both parents, and in a subset of the DH population (Fig 3.3).

AFLP polymorphism

Of the 900 AFLP primer combinations tested on the parental lines, 65 were selected for mapping based on their profile quality and the number of polymorphisms generated. These 65 primer pairs amplified between 35-50 bands per combination, of which 2-3 were polymorphic (i.e., approximately 7% polymorphic bands per total number of bands). These generated a total of 180 polymorphic markers. Huang *et al.* (2000) obtained a 8.2% polymorphism rate per primer combination between a Chinese landrace 'Chiyacao' and 'Chinese Spring', and Chalmers *et al.* (2001) obtained polymorphisms of 12.8% in the parental lines used to generate 3 DH populations and the ITMI population. Polymorphism levels are largely a function of the genetic diversity between the parental lines in which respect combination of 'Kariega' with 'Avocet S' has proven to be a very fortunate choice. In accordance with other studies, the present AFLP marker results highlight the value of AFLPs as a reproducible and cost effective way to generate genetic linkage maps (Ma and Lapitan, 1998; Huang *et al.,* 2000).

The *Sse*I/*Mse*I restriction enzyme system, shown to be suitable for AFLP analysis in wheat (Donini *et al.,* 1997) was available, and therefore used in this study. Although it is an eight base pair cutting enzyme, the restriction sites generated by *Sse*I, are compatible with *Pst*I (six base pair cutter) adaptors and primers The *Sse*I restriction enzyme will not cut at recognition sequences where any C5 methyl cytosine or N6 methyl adenine is present. 90% of the wheat genome consists of repetitive and highly methlyated DNA (Donini *et al.,* 1997). This fraction of the genome is not represented in the AFLP profiles obtained using methylation sensitive enzymes and only un-methylated portions of the genome are represented. A methlylation insensitive isoschizomer of *Sse*I will produce a more complex AFLP profile due to the larger proportion of restriction sites covered by the enzyme. Donini *et al.* (1997) found that AFLP profiles of root samples produced more complex AFLP profiles, suggesting that the DNA present in roots is less methylated than that in the leaves.

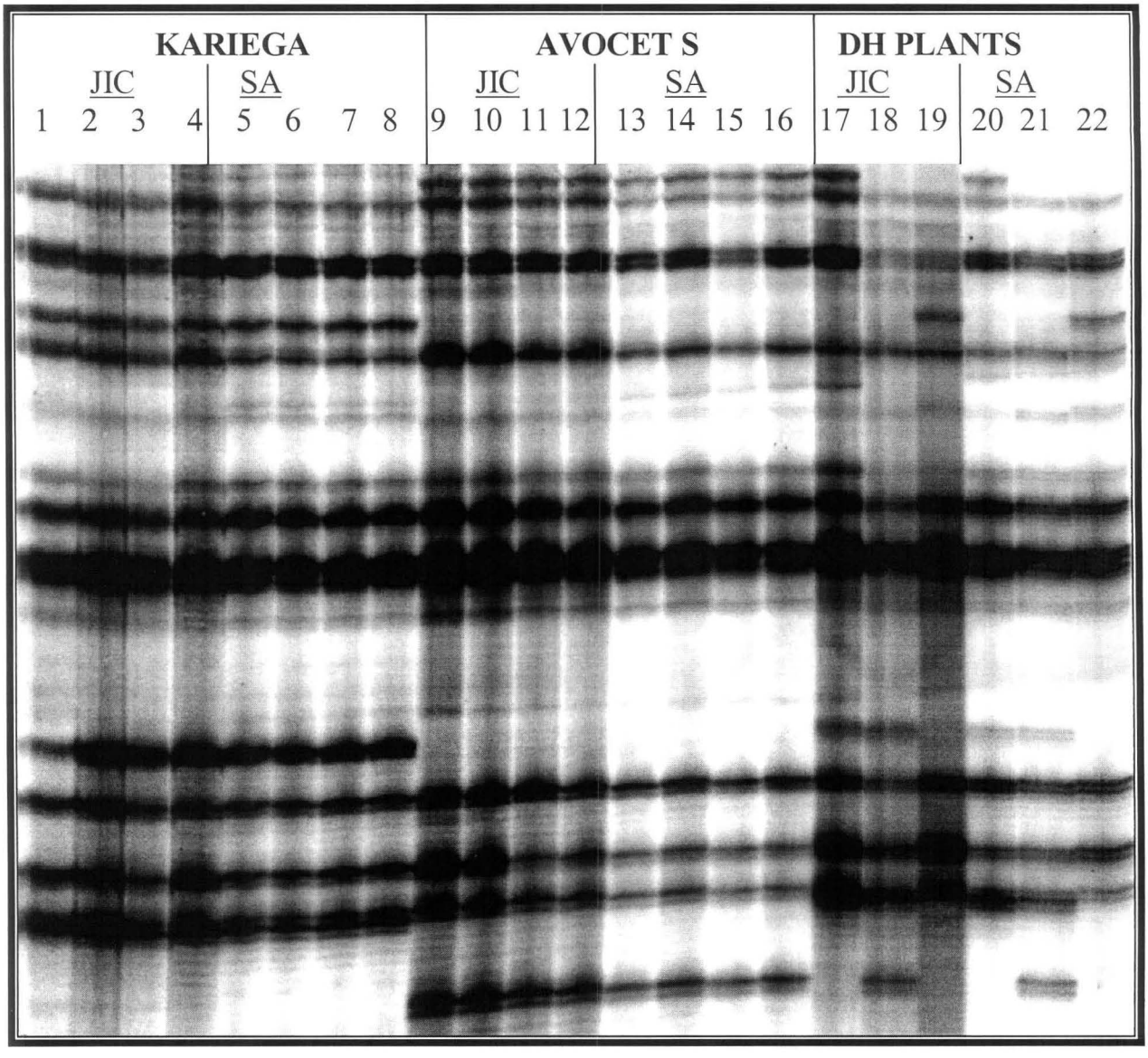


Figure 3.3. Segment of an autoradiogram showing the results of AFLP reproducibility studies. Lanes 1-8 ('Kariega' parent), and lanes 9-16 ('Avocet S' parent) show similar profiles for each parent. DH pre-amplification reactions produced at JIC (lanes 17-19) and in South Africa (lanes 20-22) showed similar inheritance of bands polymorphic in the parents.

AFLP was the most efficient technique in generating large numbers of markers in this study and the level of polymorphism observed was comparable to other studies. The use of AFLP in linkage map studies has been reported previously (Boyko *et al.,* 1999, Parker *et al.,* 1999, Chalmers *et al.,* 2001). BSA in combination with AFLP may also be used and Campbell *et al.* (2001) found that regions of the wheat genome containing telomere sequences could be targeted with AFLPs and a telomere specific anchor primer. AFLPs have also been used to assess genetic diversity in wheat (Barrett *et al.,* 1998; Donini *et al.,* 2000). The high

multiplex ratio of AFLP makes it a very attractive marker technique, and the increasing use of fluorescent detection combined with the availability of commercial kits for fluorescent detection is contributing toward an increase in its use in wheat genetics.

3.5 RGA markers

Of the 52 RGA primer combinations tested in this study (Appendix 5), 45 (87%) produced PCR amplification products (Fig. 3.4). A total of 783 bands were amplified with an average of 17 bands per primer combination, with the least number of bands amplified being 5 (Pto kin 2IN/RLK) and the most 33 (Xa1NBS). The 74 polymorphic bands identified represent a polymorphism level of 9.5% (polymorphic bands per total number of bands amplified) and an average of 1 polymorphic band per primer combination. The high polymorphism rate (21%) reported by Chen *et al.* (1998) was obtained using the silver staining technique. It is possible that the use of radioisotope labelled primers prevents the detection of all possible PCR amplification products and as a result fewer bands are visible.

Successful use of RGAs in the detection of resistance genes has been reported in many studies. Shi *et al.* (2001) detected 30 RGA markers linked to stripe rust resistance using 117 RGA primer pairs in combination with NILs and BSA. An RGA clone that produces a marker co-segregating with a stripe rust resistance gene was used to detect markers co-segregating with leaf rust resistance genes (Spielmeyer *et al.,* 2000). Mohler *et al.* (2002) demonstrated high similarity at the nucleotide level among members of the *Triticeae* of the RGA marker aACT/CAA and demonstrated applications in the genetic mapping of disease resistance loci. Li *et al.* (2001) identified an RGA locus *XksuD14* as candidate for the *Lr21/Lr40* gene on chromosome 1DS. Mapping of markers related to RGAs is important for map-based cloning, marker assisted selection, and candidate gene analysis of qualitative and quantitative disease resistance loci (Li *et al.,* 1999; Faris *et al.,* 1999).

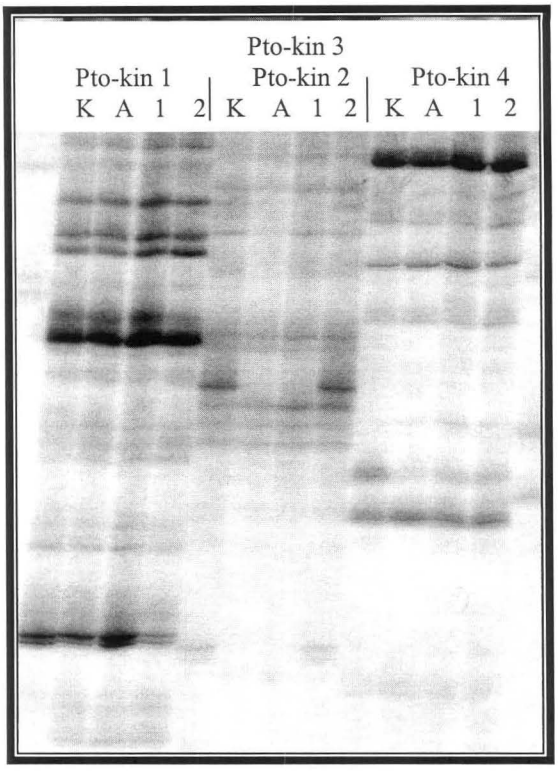


Figure 3.4 A segment of an autoradiogram showing the results of a RGA polymorphism screen with the Pto-kin primer set. K and A represent the parental lines 'Kariega' and 'Avocet S' respectively, and 1, 2 represent selected DH lines.

3.6 SRAP markers

Five SRAP forward, and 6 SRAP reverse primers (Appendix 6) were used in SRAP PCR amplification. Combinations of SRAP and RGA primers were also tested for PCR amplification.

SRAP markers have been successfully amplified in rice (Li *et al.,* 2001) but as yet not in wheat. The SRAP technique was therefore optimised for utilization in the present study. The profiles consisted of a number of bands near the wells, closely clustered together and visible as dark smears. This may be due to non-specific amplification as a result of the degenerate primers used in the technique. The bands of about 330 to 50 bp were more easily discernable, and could be checked for polymorphism.

Thirty primer combinations were tested (Appendix 6) of which 25 gave PCR amplified products (Fig. 3.5). Some primer combinations, including the em3 reverse primer, did not

give amplification products. It is likely that a lower annealing temperature is required for primer combinations involving em3, or that the primer is of poor quality. The highest number of bands amplified was 49 (primer combination me4/em1) and the lowest 9 (primer combination me2/em4).

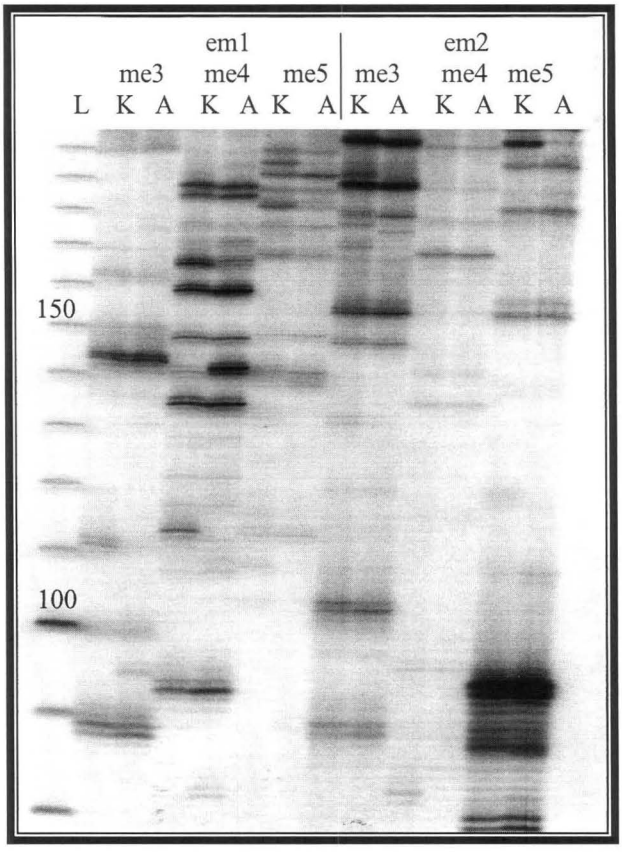


Figure 3.5. A segment of an autoradiogram showing the results of a SRAP PCR polymorphism screen. L represents the ladder, with the sizes given in base pairs. K, and A represents the 'Kariega' and 'Avocet S' parents respectively.

A total of 539 SRAP PCR products were amplified with 25 primer combinations. Thirty-two bands (6%) were identified as polymorphic. Primer combinations involving the em1 and em2 reverse primers amplified the highest number of PCR products and consequently produced the most polymorphism. Seven primer combinations were chosen for mapping in the DH populations and these produced 16 SRAP markers.

The SRAP technique is based on degenerate primers and may be prone to problems similar to those of RAPD, i.e., poor reproducibility (Devos and Gale, 1992). In order to test the reproducibility of the technique 'Kariega', and 'Avocet S' parental lines were used as controls

in experiments conducted independently and using the primer combination me1, me2, em1 and em2. It was found that polymorphisms observed between the parental lines were reproducible among PCR reactions in the same experiment as well as among PCR reactions between repeated experiments.

Considering that the primer pairs were originally designed to target open reading frames and promoter and intron regions, it will be of interest to sequence these SRAP products and compare them to sequences in the recently sequenced rice genome. RFLPs, derived from cDNA clones represent expressed genes, and Moore *et al.* (1995a) have demonstrated the conservation of gene order among the grasses, implying a likely conservation in DNA sequences as well. Li *et al.* (2001) found that 45% of SRAP PCR amplified bands from *Brassica* spp. matched known genes in the Genbank database.

One of the requirements for MAS is close linkage between markers and the gene of interest. A gene-based marker technique will only target gene-rich regions and not the large percentage of repetitive DNA in some species, e.g., up to 90 % in wheat (Donini *et al.*, 1997). It is known that gene-rich regions are found in clusters in wheat (Gupta *et al.*, 1999), so that a marker technique capable of targeting these gene clusters will save time involved in developing extensive linkage maps and facilitate the identification of markers associated with genes of interest.

The SRAP-RGA technique was also attempted with RGA forward primers (labelled with radio-isotope) and SRAP reverse primers. The profiles obtained were clear and similar to that of the SRAP technique, producing bands in the same size range and high polymorphism levels (data not shown). However, this technique was not reproducible in the same DNA samples and was not used further in this study. The SRAP technique may also be attempted in combination with SSR, AFLP, or retrotransposon based methods.

3.7 Comparing the marker techniques

Varying levels of polymorphism were found with the different marker techniques used (Table 3.3). The numbers of bands per assay, and the number of polymorphism per assay, depend to a large extent on the primer pair being used as well as the genetic variation between parents. Wide crosses and genetically diverse parents are commonly used to

increase the levels of polymorphism in wheat. The choice of marker method for genome analysis is dependent largely on the purpose for which the data generated is to be used. Metric measures such as expected heterozygosity (H) and multiplex ratio (E) are often used to measure information content. In a mapping study the technique used must be reproducible, easy to use, economically feasible, efficient and quick in generating markers.

In the present study the AFLP technique generated the largest number of markers per assay, as well as the largest total number of markers, thus proving to be a very effective technique for augmenting genetic linkage maps. Reported polymorphism levels in cereals vary from 26.6% in rice (Cho *et al.,* 1998), 48% in maize (Vuylsteke *et al.,* 1999) and 11.3% in barley (Becker *et al.,* 1995). The quality requirement constraints on wheat cultivars, the complexity of the wheat genome and the generally low level of polymorphism in wheat (Langridge *et al.,* 2001) compared to other cereals is reflected in the results of this study.

Table 3.3. Comparison of the marker methods used in this study. Percentage polymorphism is calculated as (RFLP, SSR) or (AFLP, SRAP, RGA). Estimates obtained in this study are given in bold.

FEATURE	RFLP[a]	SSR[a]	AFLP[b]	SRAP[b]	RGA[b]
BANDS PER ASSAY	2.46[c] 3-7[d] **3-4**	2.24[c] **Variable**	50.82[c] **35-50**	**21**	49.4[g] **17**
POLYMORPHISM PER ASSAY (%)	1.91[c] 3[d] **0**	15-30[f] **40 (overall)**	12.8[e] **7**	**6**	21[g] **9.5**
DNA REQUIRED	10-12 μg	100 ng	500 ng	35 ng	35 ng

[a] Polymorphism calculated as (number of polymorphic probes or primers tested)/(total number tested)
[b] Polymorphism calculated as (number of observed polymorphic bands)/(total number of bands)
[c] Bohn *et al.* (1999)
[d] Ma and Lapitan (1998)
[e] Chalmers *et al.* (2001)
[f] Harker *et al.* (2001)
[g] Chen *et al.* (1998)

The high level of polymorphism detected by the SSR technique is consistent with the findings of other studies (see Table 3.1) and offers a solution to the generally low polymorphism levels found in wheat. Furthermore, SSR targeting of the less diverse D-genome of wheat (Pestsova *et al.,* 2000; Guyomar'ch *et al.,* 2002) should increase the number

of markers in this genome. The technique is currently the most popular marker system in wheat (Langridge *et al.,* 2001).

The data reported for the RGA technique shows promise for future work. The polymorphism level was comparable to AFLP and SRAP techniques (Table 3.3) and RGAs have recently also been employed in other studies (Chen *et al.,* 1998; Spielmeyer *et al.,* 2000; Shi *et al.,* 2001; Li *et al.,* 2001; Boyko *et al.,* 2002). Only limited use of PCR-RGA techniques has been reported in genetic linkage mapping (Toojinda *et al.,* 2000). A small number of markers was therefore mapped in the 'Kariega X Avocet S' DH population in this study to test for Mendelian segregation and to investigate their suitability for mapping studies.

The sequence related amplified polymorphism (SRAP) technique targets open reading frames, promoters and introns, which are mostly expressed sequences. The technique has been applied successfully in rice (Li *et al.*, 2001) and may have application in wheat. The results obtained are comparable to AFLP in terms of the number of markers generated per assay and the polymorphism level (Table 3.3). The technique offers an exciting prospect as it targets expressed sequences and therefore can theoretically improve the chances of finding a marker close to an expressed gene. This is in contrast to AFLP which is reportedly based on repetitive DNA (Reamon-Buttner *et al.,* 1999) and is also sensitive to DNA methylation (Donini *et al.,* 1997). The results of this study show that SRAP is as effective as AFLP in the detection of polymorphism. However, the AFLP technique does detect more bands per assay, which contributes to the observed polymorphism levels. The fact that the technique targets expressed sequences (Li *et al.,* 2001) is promising, but application in the development of linkage maps in cereal crops is still to be demonstrated. SRAP was recently used by Riaz *et al.* (2001) to study genetic diversity in *Brassica napus,* suggesting additional uses for this marker technology.

Comparing the amount of DNA required by the different techniques (Table 3.3) it is clear that the SRAP and RGA protocols developed in this study require less DNA. This has important implications for mapping experiments, where it is preferable that a single DNA extraction provides enough DNA for all analyses in order to reduce time and cost.

In general level of polymorphism detected is an important factor to consider when generating a linkage map. SSRs exhibit a high level of polymorphism (Table 3.3) and can anchor

linkage groups, and were therefore very important markers in this study. Markers that generate a large number of bands per assay are useful to augment linkage maps and provide genome coverage, and AFLP has been used previously for this purpose (reviewed in Gupta *et al.,* 1999; Langridge *et al.,* 2001) and in this study. This combination of markers proved to be successful at generating the partial linkage maps required for this study. In addition the SRAP and RGA techniques detect levels of polymorphism comparable to AFLP and may be important markers for future studies.

CHAPTER 4: DEVELOPING A LINKAGE MAP FOR THE 'KARIEGA X AVOCET S' CROSS

The polymorphic markers identified were mapped in the DH population generated by R. Prins and data checks were done to identify possible sources of error. SSR markers mapped in the linkage groups, but not in listed in Appendix 2, were mapped by R. Prins, and data were made available for this study. The maps generated in this study are given and discussed in this chapter and the mapping of *Sr26* and *Ltn* is also described. The *Sr26* and *Ltn* phenotypic data were not generated in this study, but were made available for linkage analysis and generation of the maps.

4.1 Data checks and segregation distortion

Data verification is an important step before constructing a linkage map as errors in the initial data may be carried over to subsequent analyses. It is important to check that marker genotypes are coded correctly for the population type involved, and that the notation being used is compatible with the linkage software. Checking the data 'by eye' may spot errors in the values or notation. By re-examining double crossover events carefully, the errors introduced by poorly scored markers may be reduced. Typing errors created during data entry can lead to apparent crossing over resulting in large increases in map length (Lincoln and Lander, 1992). In the present study, data was entered in Microsoft Excel® spreadsheets with '0' and '1' denoting 'Kariega' and 'Avocet S' alleles respectively, and then adjusted accordingly for the different linkage software packages used.

DH populations have successfully been utilised in linkage and quantitative trait analyses in wheat (Chalmers *et al.*, 2001; Peretrant *et al.*, 2000; Sourdille *et al.*, 2000; Tixier *et al.*, 1998). With DH lines complete homozygosity is achieved in one generation following the F_1, so loss of genotypes by chance is smaller than for lines derived from repeated selfing. Markers exhibiting dominance are as informative as co-dominant markers and DH germplasm can be replicated in blocks over years and locations, allowing for replicated measurements on quantitative traits and hence better control of environmental variation. Skewness of loci segregation might be a potential problem in DH populations (Lu *et al.*, 1996; Voorrips *et al.*, 1997) but in general it has been found that marker loci segregate in a Mendelian fashion in DH lines produced by the wheat x maize system (Laurie and Bennett,

1988), with segregation distortion occurring only in certain 'hot spots' or when heterogeneous parents are used (Kammholz *et al.,* 2001).

The 1:1 segregation ratio of markers expected with a DH population was checked before constructing the linkage maps. The Chi-square test (1 df) using a 1% significance level was used to test for possible segregation distortion and markers were discarded if the test was significant. Small significant segregation distortion can result in a reduced estimate of the recombination fraction and may serve as a source of spurious linkage (Kammholtz *et al.,* 2001).

A general Chi-square for all marker loci revealed that the DH population was not significantly skewed with 50.6% of the marker alleles from the 'Kariega' parent and 49.4% from 'Avocet S' (P=0.99). Chi-square segregation tests on the individual loci indicated that 14 (5%) deviated significantly from the expected 1:1 ratio at P=0.01 with χ^2 values ranging from 8.0 (P=0.005) to 17.69 (P<0.0001). One out of every 100 markers was expected to produce significant genotype distortion by chance, so those with intermediate levels of significance i.e. 0.01<P<0.05, were retained in the analysis. Skewed ratios can occur for several reasons including inaccurate scoring, errors made during data entry, heterogeneity within the parents, selection during DH production, presence of an alien chromosomal segment or location of a chromosome region that is associated with segregation distortion (Cadalen *et al.,* 1997; Kammholz *et al.,* 2001; Nachit *et al.,* 2001). Distorted segregation ratios have been reported for SSRs (30%), RFLPs (11.6%) and AFLPs (9.7%) in *T. turgidum* (Nachit *et al.,* 2001).

Checks on the DH individual lines are less common than checks on the markers, and are used to check if any of the DH individuals carry unusually few alleles from either parent. This is done by examining the number of marker bands of each parent in each DH line. Markers discarded in the previous step (1:1 marker segregation ratio tests) were not used for checks on the individuals. It was found that parents contributed an equal number of bands to the offspring on average as expected.

The linkage data was checked for marker groups exhibiting excessive numbers of double crossovers using Map Manager QTX. The gel profiles of markers involved in more than 3 double crossovers, particularly those occurring in clusters, were re-examined and removed if

necessary and the maps re-drawn. It was noted that this often caused a reduction in map length for the particular linkage group affected. Double crossovers occur naturally at very low frequency. However, the presence of clusters of double crossovers has been reported to inflate map length (Castiglioni *et al.,* 1998). The issue of the treatment of double crossovers is complex because (i) double crossovers are usually ignored in the likelihood function for the genetic map, (ii) double crossover frequency strictly depends on locus order, and may differ with changing locus orders and, (iii) expected double crossovers cannot be distinguished from unexpected double crossovers (Liu *et al.,* 1998). Although removal of double crossovers may result in linkage maps with high LOD score support for the locus order, mis-interpretation of the data may result in biased maps due to incorrect locus order, underestimation of map distances or unrealistic LOD score support for a particular locus order (Liu *et al.,* 1998).

Missing data made up 4% of the total mapping data in the present study and arose mainly as a result of ambiguities during the scoring process. If markers have many missing observations their recombination frequencies with other markers can be considerably inflated (Jansen *et al.,* 2001). The reason for this is that the occurrence of missing data might not be random, thus causing erroneous estimates of recombination frequency. This has the effect of combining distinct linkage groups and increasing the map length. Jansen *et al.* (2001) suggested using the Gibbs sampler, followed by simulated annealing, to reduce the adverse effects of missing data. These features are available in versions of the Joinmap® software.

4.2 Linkage analysis

Construction of the linkage map

Of the 230 marker loci mapped, 219 were assigned to 31 linkage groups (Fig. 4.4) with 11 (5%) of the loci remaining unlinked. Markers which could not be placed in linkage groups at P=0.001 were mapped at P<0.01. The exclusion of markers forming double crossover clusters caused a reduction in total map length as in Castiglioni *et al.* (1998). Three linkage groups (un1, un2 and un3, Fig. 4.4) could not be assigned to specific chromosome locations. The remaining 28 groups covered the 21 chromosomes with 212 markers. The linkage groups were assigned to individual chromosomes using SSR markers as anchors. There were two or more linkage groups for chromosomes 3B, 3D, 5D, 7A, 7B and 7D (Fig. 4.4). In

those cases where more than one anchor SSR locus was present in a linkage group, the orientation of the chromosome was established according to existing maps of Bryan *et al.* (1997), Gupta *et al.* (2002), Pestsova *et al.* (2000), and Röder *et al.* (1998) (Fig 4.4). The ultimate map (total size 2155.4 cM) covered the entire wheat genome of 21 chromosomes using a relatively small number of molecular markers (Fig. 4.4).

Several linkage maps of wheat have been published and the major ones are summarised in Langridge *et al.* (2001) of which the ITMI RIL linkage map spanning 3700-6542 cM is the most significant. Maps with fewer markers, similar to the one developed in this study, have also been developed. Using 230 markers (RFLP and SSR) Messmer *et al.* (1999) generated a map covering 2469 cM. Cadalen *et al.* (1997) developed a map covering 1772 cM with 266 markers, but covering only 18 of the 21 chromosomes. More recently, Chalmers *et al.* (2001) developed 3 linkage maps in DH mapping populations with 919, 502 and 363 markers, respectively, providing fairly comprehensive genome coverage. The linkage maps in this study highlight the contribution of anchor loci in the creation of partial linkage maps since 212 markers were adequate to obtain linkage groups for all 21 chromosomes (Fig. 4.4). Partial linkage maps do not provide extensive genome coverage but may be adequate to detect linkage to traits of interest (Langridge and Chalmers, 1998).

In the 'Kariega X Avocet S' cross the segregation ratios of SSRs *Xgwm742-4A*, *Xgwm160-4A*, *Xgwm832.1-4A*, *Xwmc219-4A* and several AFLP markers all mapping to a region on 4A appeared to be slightly distorted ($P<0.05$) (Figure 4.4). Guyomarc'h *et al.* (2002) reported distorted segregation ratios for 15 of 84 (18%) *Ae. tauschii* D-genome derived (CFD) SSRs in the 'Courtot X Chinese Spring' DH population and found that these markers mapped to the same regions as had previously been shown to be associated with segregation distortion in this cross (Cadalen *et al.* 1997) i.e., 4A and 6B. The present study confirmed the presence of a region showing segregation distortion identified previously on chromosome 4A (Cadalen *et al.*, 1997; Guyomarc'h *et al.*, 2001). Kammholtz *et al.* (2001) reported segregation distortion in 'hot spots', where distorted markers appeared to group together in the Australian NWMMP maps, but these varied between the mapping populations used.

Distribution of markers

The markers in this study were not evenly distributed across the three genomes, the order being A>B>D with the D genome being markedly under-represented (Table 4.1). Compared to other studies the inconsistency of more markers mapping to A than to the B genome may be attributed to the many markers (±17%) located on chromosome 6A (Table 4.2) as a result of the presence of an alien segment on this chromosome in the cultivar 'Avocet' from which 'Avocet S' is a selection (Friebe *et al.,* 1994; McIntosh *et al.,* 1995). The presence of alien chromatin in parental lines may increase polymorphism levels in the introgressed region, a phenomenon reported previously by Kammholtz *et al.* (2001).

The scarcity of polymorphic markers on the D genome is a significant feature of the linkage map with only 19% of the total number of markers, covering 30% of the total cM distance, mapping to this genome (Table 4.1). This is consistent with several studies using RFLPs, SSRs and AFLPs (Bryan *et al.,* 1997; Cadalen *et al.,* 1997; Chalmers *et al.,* 2001; Chao *et al.,* 1989; Gupta *et al.,* 2002; Harker *et al.,* 2001; Kammholz *et al.,* 2001; Kam-Morgan *et al.,* 1989; Marino *et al.,* 1996; Röder *et al.,* 1998). Taking this into consideration, an increased effort was put into mapping D genome specific SSRs in the present study. It was anticipated that the *Ae. tauschii*-derived SSRs would increase the number of useful markers for the D genome (Guyomarc'h *et al.,* 2002; Pestsova *et al.,* 2000). However, it was found that the D genome specific GDM SSRs have relatively low levels of polymorphism (25%) with the CFD SSRs relatively higher at 36% polymorphism (see Chapter 3).

A high-density linkage map for *Ae. tauschii,* the D-genome donor of bread wheat of 732 loci was generated by Boyko *et al.* (2002) consisting of RFLP (R and DR genes), SSR, ISSR and retrotranposon based (IRAP, REMAP) markers. The *Ae. tauschii* genome exhibits almost complete homology to the D genome of bread wheat, and its high polymorphism makes it ideal for genetic mapping. Boyko *et al.* (2002) found that retrotransposon clusters occur in the peri-centromeric regions, and R and DR gene clusters in the distal/telomeric regions in their *Ae. tauschii* map. The results obtained by Boyko *et al.* (2002) may be extrapolated to bread wheat, and in so doing provide a supplement to the D genome markers mapped in bread wheat. Using this approach Li *et al.* (2001) found a candidate RGA locus on chromosome 1DS for the *Lr21/Lr40* gene.

A total of 66 polymorphic SSR loci were mapped (Table 4.2). In many cases the chromosome location of the SSR loci could be verified with the CS and CSNT controls, but in some cases polymorphic SSR loci with unverified chromosome positions were included and their positions, as published, were then accepted as correct. Some SSR markers mapped to positions different to those originally reported, underlining the fact that multiple loci might exist and that different polymorphisms may be mapped in different populations (Kammholz *et al.*, 2001). Polymorphic loci detected by WMC149 (2B), GDM5 (2D), GDM33 (1A) and GDM98 (6D) remained unlinked.

Table 4.1. Distribution of markers across genomes, and the cM distances covered. Percentages are given in parentheses.

	A GENOME	B GENOME	D GENOME	TOTAL
NO. OF MARKERS	92 (43)	79 (38)	41 (19)	212
cM DISTANCE	738.1 (34)	774.5 (36)	642.8 (30)	2155.4
NO. OF AFLP MARKERS	65 (49)	53 (40)	15 (11)	133

Multiple loci were detected in the 'Kariega X Avocet S' population for the SSRs *Xgwm111, Xgwm832, Xgwm885, Xgwm974, Xcfd66* and *Xwmc149*. The locus detected by *Xgwm372* mapped to 2B and not to 2A (Röder *et al.*, 1998), *Xgwm111.2* mapped to 5B and not 7D (Sourdille *et al.*, 2001), *Xgwm192* mapped to 4D not 4A (Röder *et al.*, 1998), *Xcfd31* mapped to 3D and not 7D (Guyomar'ch *et al.*, 2002), *Xcfd66.1* mapped to 3D and not 7D (Guyomar'ch *et al.*, 2002), *Xcfd66.2* mapped to 7A and not 7D (Guyomar'ch *et al.*, 2002) and *Xgwm832.2* mapped to 7A and not 4A (Röder *et al.*, unpublished). Three markers were mapped with the *Xgwm974* SSR, of which two mapped to the same position (Fig. 4.4).

As with the SSR markers only a few of the AFLP markers (*Sse*I/*Mse*I) mapped to the D genome (11%) (Table 4.1). Chalmers *et al.* (2001) also found that fewer AFLP markers (*Pst*I/*Mse*I) (16%) mapped to the D genome with the B genome containing more AFLP markers than the A genome. The higher number of AFLP markers on the A genome may again be due to the alien translocation on chromosome 6A to which 29 AFLP markers were mapped (Table 4.2). Some aspects of the AFLP technique may bias markers detected towards regions of suppressed recombination in the genome, e.g., the high frequency of

Table 4.2. Summary of the number and type of markers in each linkage group and the cM distance covered.

Chrom.	No. of Markers/ Genes	SSR Loci	AFLP Markers	RGA Markers	SRAP Markers	cM Distance
1A	10 (4.72)[a]	2	7			10.24
1B	19 (8.96)	3	14		1	146.2
1D[b]	4 (1.89)	2	2			92.4
2A	7 (3.30)	2	5			33.1
2B	20 (9.43)	5	14		1	110.2
2D	2 (0.94)	2	0			3.4
3A	3 (1.42)	1	2			23.8
3B-1	9 (5.19)	2	5		2	71.3
3B-2	2	1	1			3.5
3D-1	2 (2.83)	1	1			36.3
3D-2[c]	2	4	0			85.5
4A	14 (6.60)	5	9			128.4
4B	4 (1.89)	1	3			84.7
4D	4 (1.89)	3	1			62.3
5A	4 (1.89)	1	2		1	74.2
5B	13 (6.16)	2	9		2	98
5D-1	3 (5.66)	2	1			61.8
5D-2	6	1	5			68.3
5D-3	3	1	2			27.9
6A	35(16.51)	3	29	2		134.9
6B	7 (3.30)	2	4		1	92.5
6D	4 (1.89)	1	2	1		54.7
7A-1[c]	10 (8.96)	4	4		2	114.1
7A-2[c]	9	3[d]	5			127.2
7B-1	2 (2.36)	1	1			58.9
7B-2	3	1	2			109.2
7D-1	7 (4.25)	5	1			123.2
7D-2	2	1	1			27
Total	212	62	133	3	10	2155.4
un1	2		2			35.2
un2	2		2			6.9
un3	3		2		1	23.1
Mapped	219	62	139			2220.6
Unlinked	11	4	4	1	1	
Total	230[e]	66	143	4	12	

[a]Percentage of the total number of markers
[b]Linkage groups created at P = 0.01
[c]Linkage groups created at P = 0.05
[d]*Xgwm974.2* and *Xgwm974.3* map to the same locus
[e]Total including *Sr26, Ltn, Glu-A1-1, Glu-B1-1,Xgwm974.3*

polymorphism in these regions as they are usually non-coding sequences (Young *et al.*, 1999). No significant clustering of AFLP markers was observed in the maps produced, other than those on 6A (Fig. 4.4). It has been found previously that AFLP markers differ in both frequency and distribution throughout the wheat genome and the choice of primer sets used directly influences the distribution of loci identified (Langridge *et al.*, 2001).

AFLP markers produced from the same primer combination mapping to the same position are likely to be the result of large polymorphic regions being digested into smaller fragments of different sizes. A low level of AFLP co-dominance was detected (0.04%) as has also been reported by Vuylsteke *et al.* (1999) for AFLP markers in maize. They were classified as such if they (i) originate from different parents but amplify from the same primer combination; and (ii) they map to the same locus (Vuylsteke *et al.*, 1999). It is interesting that AFLP markers used to generate maps of the Australian NWMMP did not map consistently to the same chromosome regions (Chalmers *et al.*, 2001).

Only four of the seven RGA markers were placed in the 'Kariega X Avocet S' linkage map (Fig. 4.4). Three RGA markers showed a number of double crossovers and were omitted from the linkage groups. On re-examination of the mapping gels it was noticed that the autoradiograms of the markers in question were difficult to score, implying possible errors during the scoring process. The high polymorphism and Mendelian segregation observed with PCR RGA markers suggest that they do have some use in the development of genetic maps in wheat. Boyko *et al.* (2002) mapped RGA loci (RFLP markers) in an *Ae. tauschii* cross and reported that clusters of RGA loci coincide with various resistance phenotypes. A targeted PCR RGA approach (as used by Shi *et al.*, 2001) as opposed to entire genome scans may identify markers linked to resistance genes. The PCR RGA protocol developed in this study incorporating the use of radioisotopes was shown to have use in the identification of polymorphism (see Chapter 3) and the development of the maps (Fig 4.4).

The SRAP markers mapped were randomly distributed with no evident clustering (Fig 4.4). Similar to the RGA technique, four markers showed a number of double crossovers that may again be due to difficulties in scoring. Additional markers will have to be mapped in order to ascertain whether the SRAP technique targets genomic regions different to those of the AFLP technique. The high polymorphism and large number of bands per assay obtained makes the technique suited for a targeted mapping approach such as a targeted BSA approach

(Campbell *et al.,* 2001) which uses information from flanking markers. The SRAP results obtained in this study are very encouraging, and the linkage map developed here may be extended with additional SRAP markers in order to demonstrate whether wide-scale use of the SRAP technique is feasible. This study is the first report of the use of SRAP marker in wheat linkage mapping.

Linkage maps can be used to determine the chromosomal location of genes and closely linked markers for marker-assisted selection. The presence of three genomes, the large overall genome size and the lack of polymorphism have made this difficult in wheat (Gupta *et al.,* 1999) and the construction of complete linkage maps has thus been generally difficult, expensive and labour-intensive, requiring some 300-400 mapped markers for reasonable total genome coverage. The most comprehensive linkage map for wheat, the ITMI RIL mapping population comprises some 1200-1500 loci (6542-3700 cM) yet still contains regions which are sparsely covered (Gupta *et al.,* 1999, 2002; Langridge *et al.,* 2001). Recently, Chalmers *et al.* (2001) developed a linkage map covering all 21 chromosomes with 902 markers but again showing sparse coverage in some regions. Considering the costs and resources involved, the alternative of developing partial but functional maps appears to be the only viable alternative especially in small laboratories.

The 'Kariega X Avocet S' map represents the first linkage map for wheats in South Africa that covers all 21 chromosomes, and can thus serve as a reference for future linkage and QTL studies. It may also aid comparative mapping in different wheat mapping populations using suitable techniques such as AFLP and SRAP. As initially demonstrated by Waugh *et al.* (1997) in three mapping populations of barley, the comparative mapping approach can provide information on the chromosomal location of markers segregating in populations of the same species, and thereby obviate some of the costs involved in generating full maps for each cross. Williams *et al.* (2002) identified an AFLP marker linked to root lesion nematode resistance, and this marker was placed on a dense wheat map described in Chalmers *et al.* (2001), where it was found to be closely linked to RFLP and SSR markers. Comparative linkage mapping thus identified the chromosomal location of a root lesion nematode resistance gene in addition to identifying potential markers for marker validation and MAS. The 'Kariega X Avocet S' map may be used in a similar manner for genes that could be employed in South African breeding programmes.

4.3 Mapping of the stem rust resistance gene, *Sr26*

The dominant *Sr26* gene confers resistance to a wide range of races of *Puccinia graminis* f. sp. *tritici* and is used extensively in breeding for stem rust resistance in wheat (Zhang *et al.*, 1999). Stem rust resistance has been established in Eastern Australia through *Sr26* and other stem rust resistance genes, despite apparent yield penalties associated with use of the gene (McIntosh *et al.*, 2001). *Sr26* segregates in the 'Kariega X Avocet S' DH population (Fig. 4.1) and thus provided an opportunity to map the gene to chromosome 6A. 'Avocet S' is a selection from 'Avocet', known to carry *Sr26* (McIntosh *et al.*, 1995). This study provides the first report of the presence of *Sr26* in 'Avocet S' (Wellings, personal communication via Z.A. Pretorius).

A linkage map (134.9 cM) was obtained for chromosome 6A with 34 DNA markers (Fig 4.4). The map accounted for 17% of all mapped markers, indicating a highly polymorphic region (Table 4.2). Considering the *Thinopyron elongatum* origin of *Sr26* (Knott, 1961), and the location of the gene on the T6AS.6AL-6Ae#1 translocation chromosome (Friebe *et al.*, 1994), it seems likely that the region represents an introgressed segment of *Thinopyrom*. Working with wheat line 'K2046', which carries the above mentioned translocation and using C-banding and *in situ* hybridization, Friebe *et al.* (1994) established that the segment transferred from *Th. elongatum* measures 2.48 µm in length. The presence of alien chromatin may explain the highly polymorphic nature of chromosome 6A as has been reported previously for chromosome 2B (Kammholtz *et al.*, 2001) in the Australian NWMMP 'Sunco X Tasman' DH population.

Dense marker clusters were detected on chromosome 6A (Fig 4.4). Vuylsteke *et al.* (1999), working with maize, reported AFLP markers clustering near centromeric regions. Nachit *et al.* (2001) identified marker clusters in a map developed for *T. turgidum*, and reported that the marker clusters were associated with regions of reduced recombination. The distribution of recombination over chromosomes is highly variable in wheat with 80% of the recombination events occurring in only 18% of the arm-length, and no recombination within the closest 20% of the arm surrounding the centromere (Gill *et al.*, 1996). Reduction in the recombination frequency of both tomato and soybean (similar in genome size to wheat) has been recorded in the centromeric region (Young *et al.*, 1999). The SSR *Xpsp3152-6A* has been mapped close to the centromere of chromosome 6A (Bryan *et al.*, 1997). Furthermore,

Prabhakara Rao (1996) mapped *Sr26* to chromosome 6A in 'Kite' and reported no crossovers between *Sr26* and the 6A centromere. It is therefore likely that this region maps close to the centromere of 6A.

The *Sr26* gene originates from *Th. elongatum* and Prabhakara Rao (1996) reported no translocation between the introgressed region of *Thinopyron* and the homoeologous chromosome segment in wheat. The linkage block of markers obtained (Fig 4.4) is therefore expected, and can be attributed to no recombination between the *Th. elongatum* introgressed region and the region on chromosome 6A in wheat. However Prabhakara Rao (1996) reported that the introgressed region occupies most of the long arm of 6AL, so the linkage block of markers is expected to span a much larger region on this chromosome, including the *Sr26* gene. Recombination was detected between markers mapped to the long arm of 6A, contradictory to what was expected, and this requires further investigation. This recombination may be an artifact of marker mis-classification.

It is known that dense maps often result in map expansion (Heun *et al.,* 1991) for which typing errors are partly responsible (Lincoln and Lander, 1992), as well as the increased detection of 'singletons' i.e. caused to a degree by the occurrence of clusters of double crossovers as reported by Castiglioni *et al.* (1998). However it is likely that the dense marker map obtained for chromosome 6A is largely due to the presence of introgressed *Th. elongatum* chromatin.

Zhang *et al.* (1999) attempted to tag *Sr26* using RAPD markers and a polymorphic fragment OPH11 (850 bp), generated by OPH11 was found to be closely linked (5.6 cM) to the gene. In this study *Sr26* mapped close to three SSR loci (Figure 4.4), which spans the region containing it, i.e., *Xgwm427-6A*, *Xgwm169-6A* and *Xpsp3152-6A*. AFLP loci *s11m16F, s11m60B* and *s11m60C* also mapped close to the gene. Validation of SSR markers in other breeding material is required before being adopted for MAS, while AFLP markers require more user-friendly detection assays. The introgression of *Sr26* into new cultivars using these markers is therefore a strong possibility.

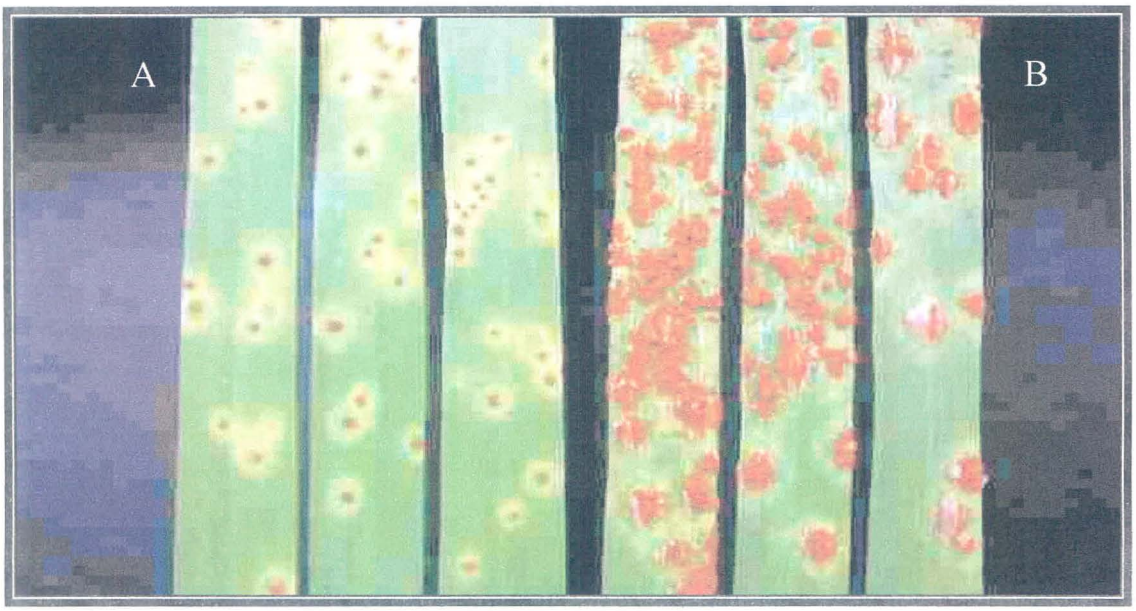


Figure 4.1. Inoculations of (A) 'Avocet S' and (B) 'Kariega' with *Puccinia graminis* f. sp. *tritici* UVPgt50 (Photo provided courtesy of Z.A. Pretorius).

Figure 4.2. The symptoms of leaf tip necrosis (*Ltn*) as observed in 'Kariega' (Photo provided courtesy of Z.A. Pretorius).

4.4 Mapping of the gene for leaf tip necrosis

Symptoms of leaf tip necrosis include 2 to 3 cm of necrosis at the tips of the leaves, extending to 2 to 4 cm on the edges (Fig. 4.2). The gene, designated *Ltn* is dominant and reported to be linked to *Lr34,* a gene for resistance to leaf rust (Singh 1992a). Singh (1992b) and McIntosh (1992) have independently reported that *Lr34* is genetically associated with *Yr18* which confers APR to stripe rust, and with *Bdv1,* a gene for tolerance to barley yellow dwarf virus (also called the *Yr18* complex).

Messmer *et al.* (2000) observed leaf tip necrosis in the European winter wheat cultivar 'Forno', and reported that this differed from that reported by Singh (1992a). Typing of the DH population in this study was done in the field trial at the same time as the final stripe rust scoring (28 September 2000) but was found to be dicey as has been previously reported by Messmer *et al.* (2000) and by William *et al.* (1997). One scorer (LB) scored the presence or absence of symptoms in all entries, while two scorers (ZP, WB) scored only for presence and this resulted in many missing values. Preliminary linkage analysis demonstrated that despite the different scoring methods used, *Ltn* still mapped to the same region on chromosome 7D (Fig. 4.4) consistent with previously published work (Nelson *et al.,* 1997). However, only the field scores of LB were used in the final analysis.

Difficulties in scoring *Ltn* may preclude its use as the only selection tool for the *Yr18* complex and resulted in a further search for markers in this region. Mapping in the 'Avocet X Parula' population enabled the identification of an SSR marker, *Xwms130,* closely linked to *Ltn* (4.5 cM) (William *et al.,* 2001). Maintaining leaf tip necrosis should be advantageous in breeding programmes due to its close association with *Yr18* and *Lr34* (Singh, 1992; McIntosh, 1992).

4.5 Comparison of different software packages for linkage analysis

Linkage maps were compared in this study using MAPMAKER\EXP, Map Manager QTX and Joinmap®, (Fig. 4.3) with a total of 180 mapped markers, and not the complete data set used to generate the maps in Figure 4.4, as Joinmap® has not been licensed for use by the Small Grain Institute. The map of the linkage group of chromosome 1B is provided as an example, with only 13 markers mapping here (Fig. 4.3). The 'error detection' function of

MAPMAKER/EXP, which excludes loci mapping very close together, was on, resulting in only 10 markers being mapped.

Maps produced by Joinmap® were shorter than those produced by MAPMAKER\EXP and Map Manager QT (Fig. 4.3) and this may be attributable to the different algorithms used in these programs. Maps produced by Map Manager QTX are very similar to those produced by MAPMAKER\EXP. To estimate the distance between a pair of markers, MAPMAKER\EXP uses information from those 2 markers alone, whereas Joinmap uses pair wise recombination estimates from all markers in the dataset simultaneously (the 'greedy' algorithm described earlier in section 1.5) and calculates map distances by applying a mapping function to the recombination estimates. The order of markers is generally in good agreement among the maps, except for loci showing very close linkage (Fig. 4.3).

Essentially, the methods used for simple linkage detection and recombination fraction estimation are the same in the different software packages, with differences arising in data format, computer platforms, user interface, graphical outputs and algorithms for locus ordering (Liu *et al.,* 1998). The choice of which package to use most often depends on the computer platforms available, the accessibility to the software, user friendliness and exchangeability of information between laboratories.

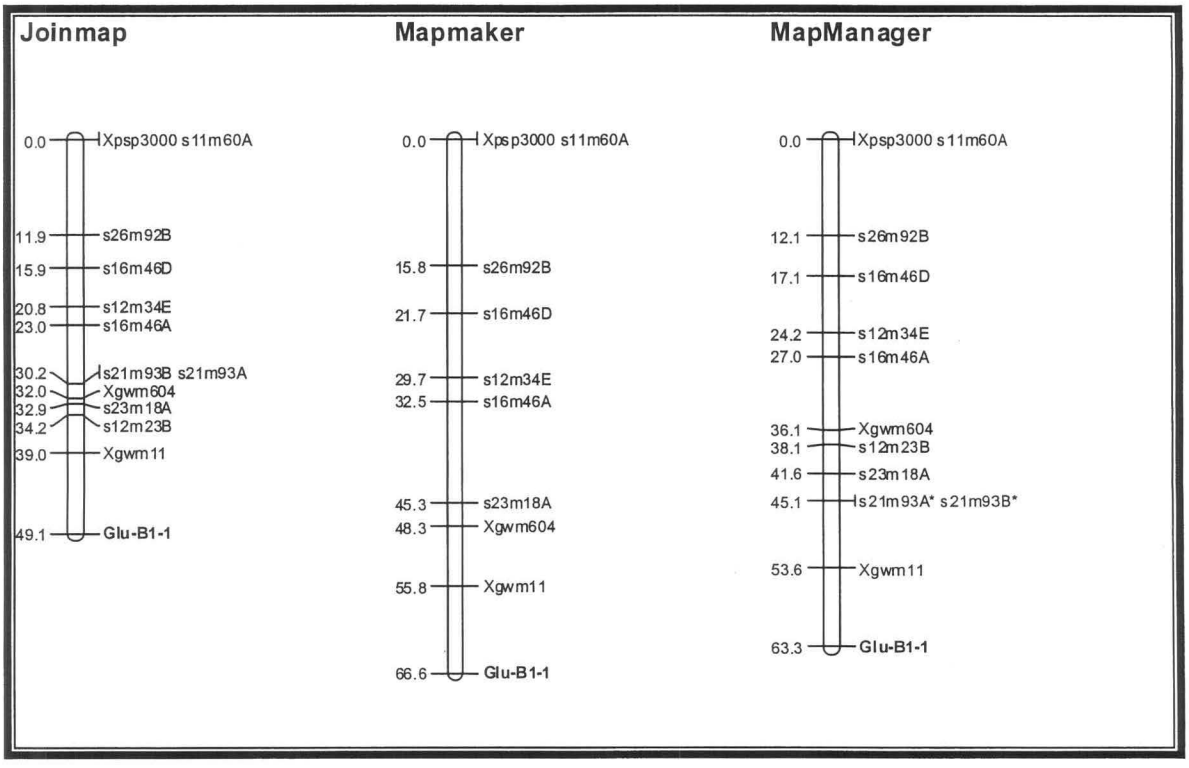
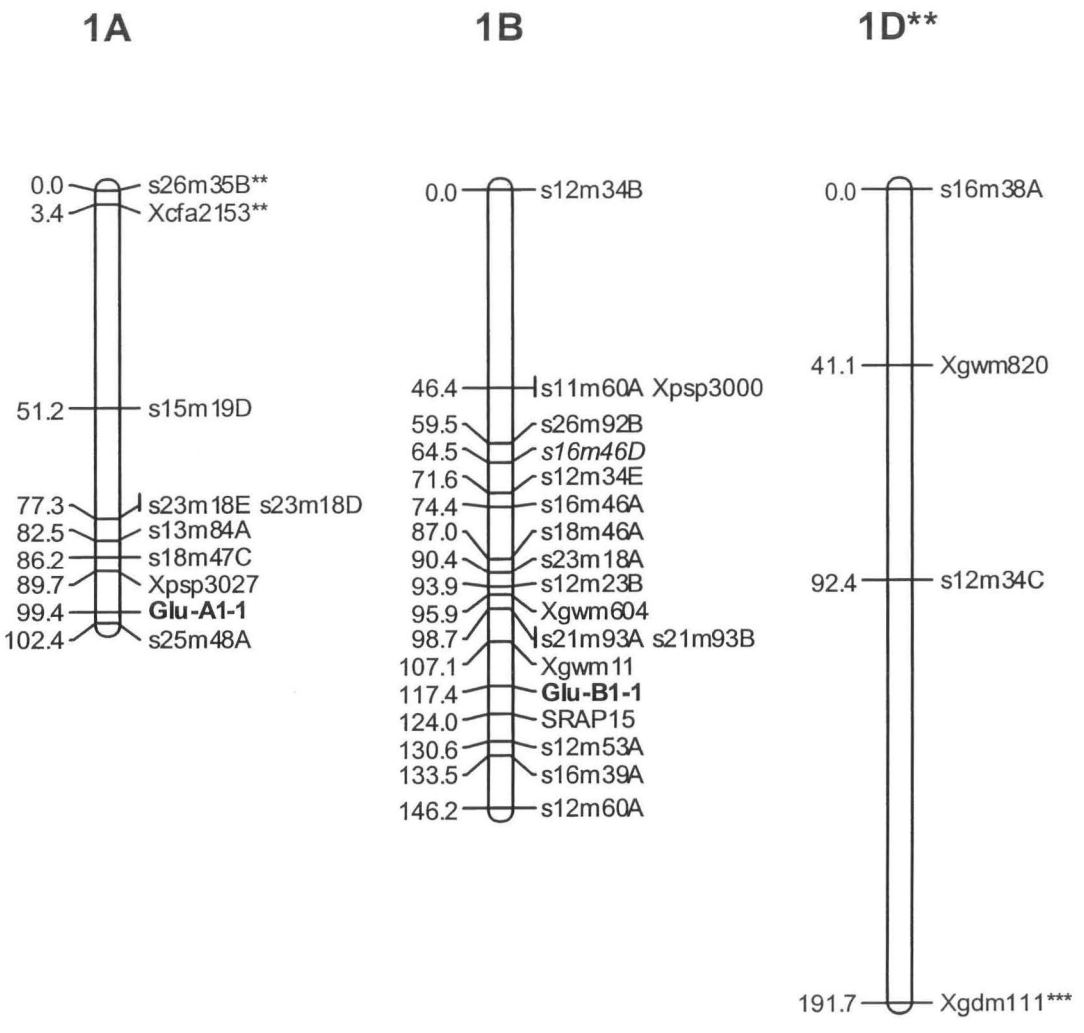
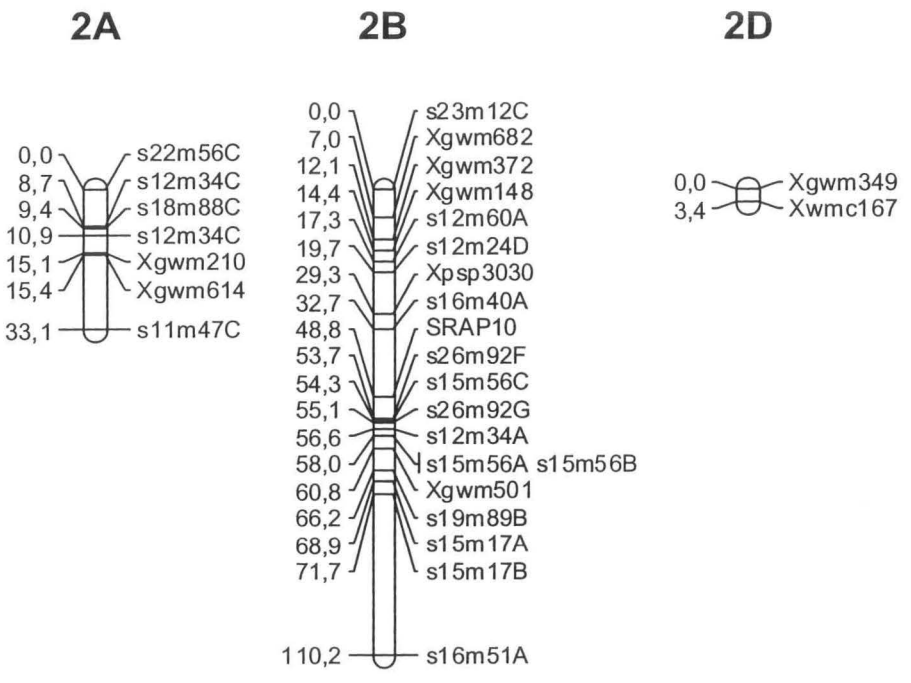
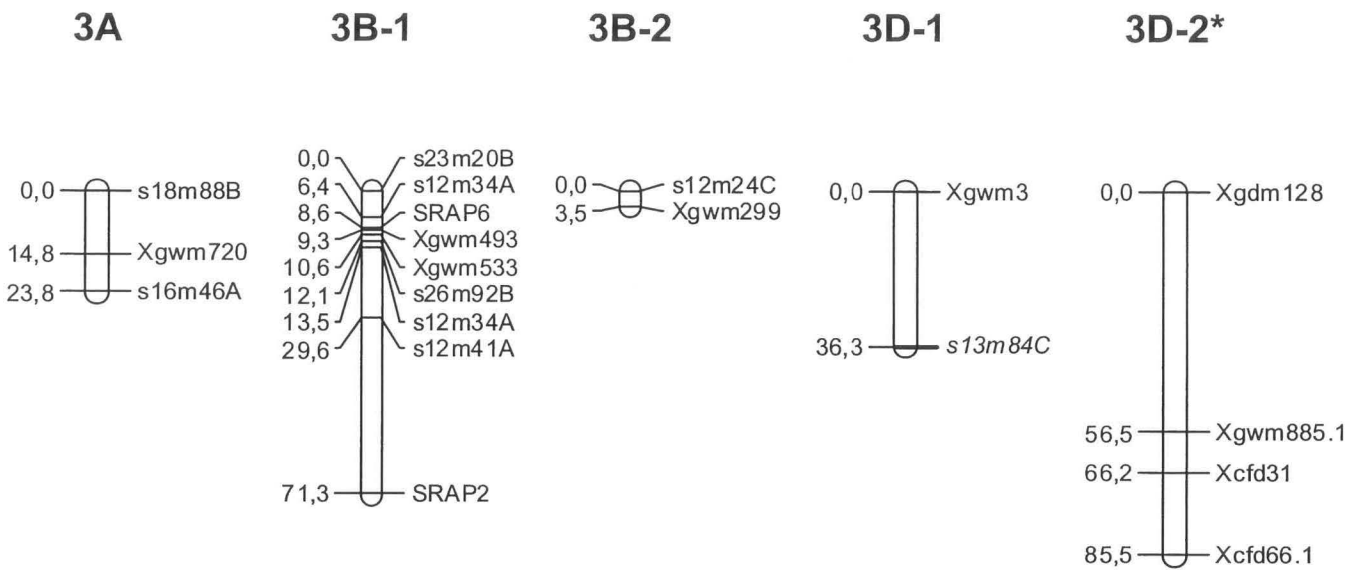


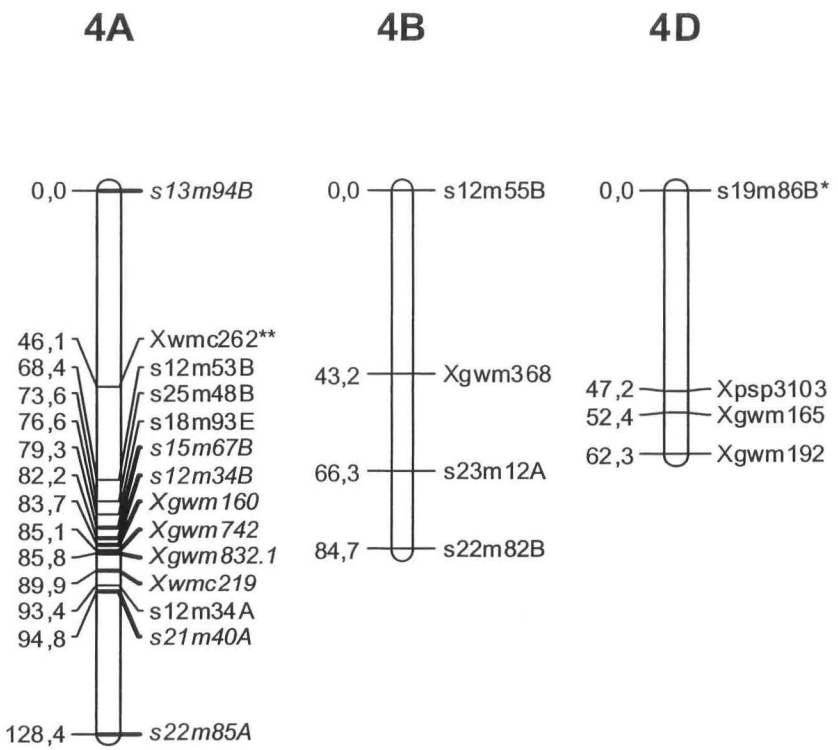
Figure 4.3 Comparison of the linkage groups of chromosome 1B produced by Joinmap®(LOD=3), MAPMAKER/EXP (LOD=3) and Map Manager QTX (P=0.001) using a subset of mapped markers. *Indicates a marker mapped at P=0.01 with Map Manager QTX.

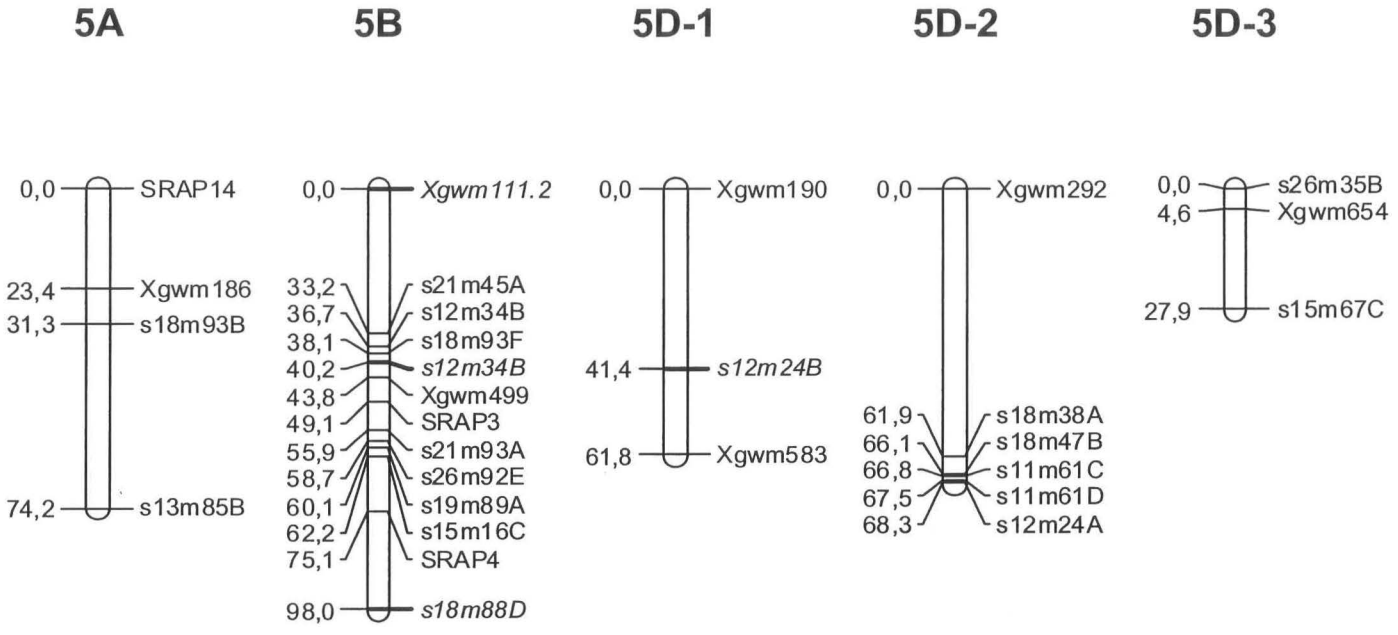
Figure 4.4. Genetic linkage map of the 'Kariega X Avocet S' cross with mapped markers and linkage groups created at P=0.001 (LOD > 3.0). Any deviations from P=0.001 are indicated as follows: *mapped at P=0.05, **mapped at P=0.01, ***mapped at P<0.01. Linkage groups were named according to the chromosomes to which they were anchored by SSR markers. Where more than one linkage group exists for a chromosome, these are numbered accordingly. The cumulative distance between markers (cM) is shown on the left, with the marker indicated on the right. Markers in italics show distorted segregation (P<0.05) (Next 8 pages).

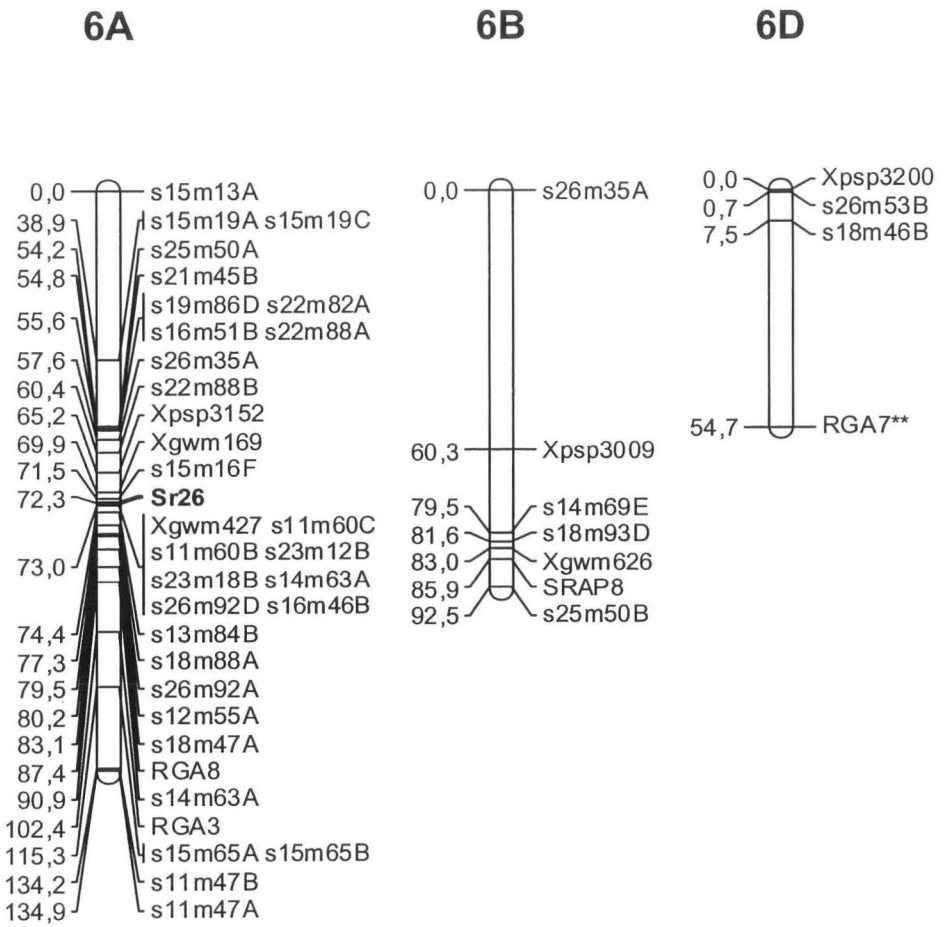


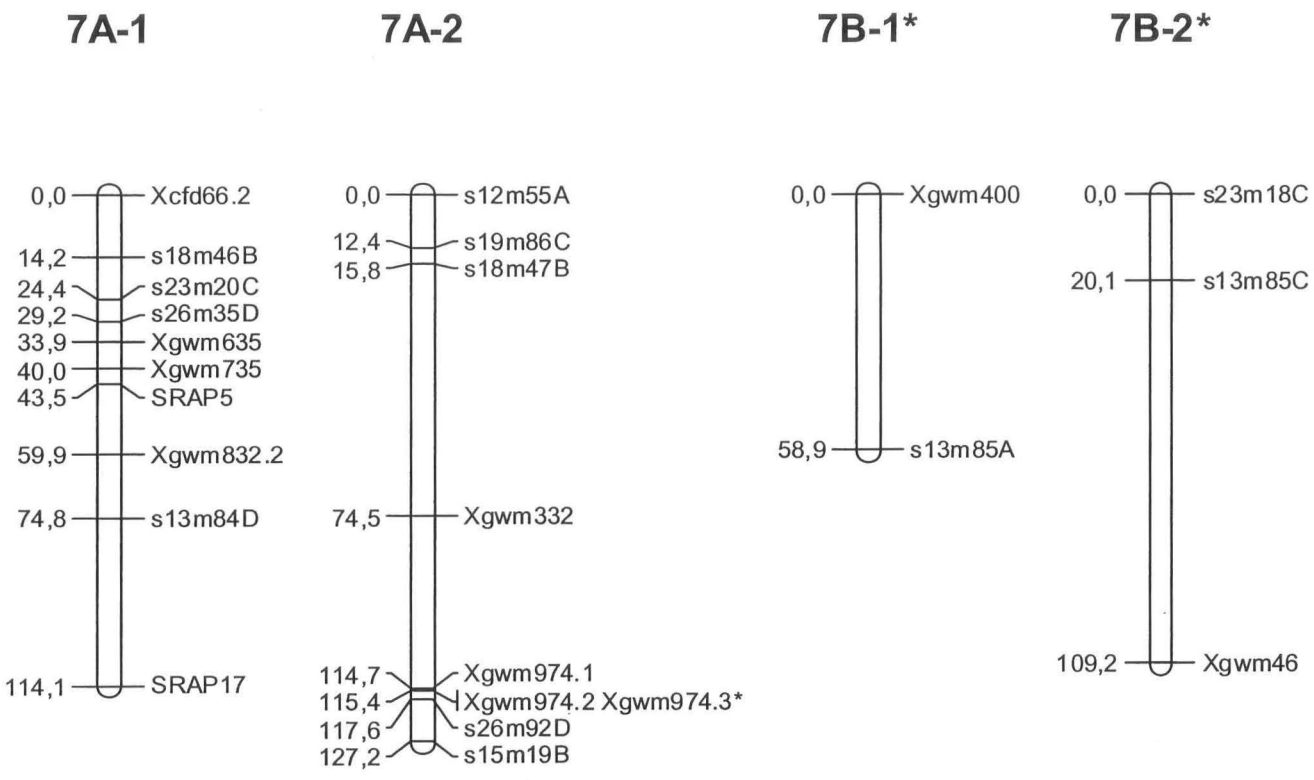


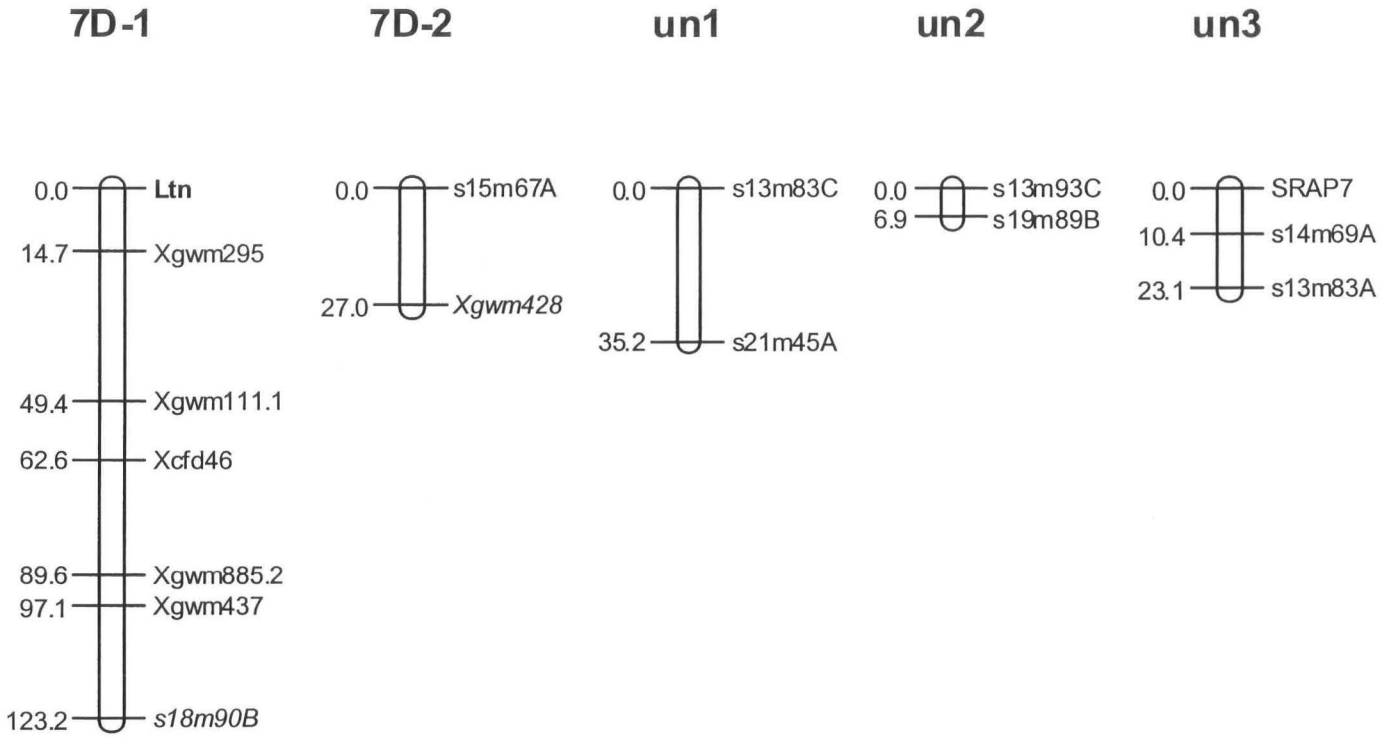












CHAPTER 5: QTL ANALYSIS OF APR TO STRIPE RUST

The linkage maps described in Fig. 4.4 were used together with field and growth chamber data to map QTL. The field and growth chamber data were not generated in this study, but were made available by co-workers for QTL analysis. In this chapter, details of the QTL analysis is represented and discussed.

5.1 Field data analysis and transformation

A severe and uniformly distributed epidemic developed among plots in the field trial. Occurrence of the pathotype 6E22A- was confirmed by susceptible reactions on control phenotypes in adjacent plots. At the early assessment 'Avocet S' displayed reactions of 70S to 80S whereas 'Kariega' showed no signs of infection. At the final assessment 'Avocet S' consistently scored 100S and 'Kariega' tR to 10R (Fig. 5.1), depending on the scorer. Extreme responses of the DH lines were similar and scores recorded covered the full range between these extremes.

The DH lines were scored in the growth chamber to assess the feasibility of quick detection of adult plant resistance, thereby avoiding the need for extensive field trials. The range of variation detected in the field was also found in the growth chamber scores. Flag leaves of plants in the growth chamber were small (5 to 10 cm in length) but provided adequate leaf tissue for scoring and infection types ranged from flecking to large susceptible type pustules.

Departure from a normal distribution and non-additive interactions sometimes complicate data analysis, and this can often be eliminated by transformation of the data to a new scale, which changes the relationship of character values to one another, but does not alter the information content of the original data (Lynch and Walsh, 1998). Field scores on the percentage of leaf area infected (LAI) were converted to proportions, p, and transformed to arcsin ($\sqrt{p}$) (angular transformation) and host reaction type scores on the ordinal scale were transformed to ln (score+1) (Sokal and Rohlf, 1995; Lynch and Walsh, 1998). In the case of reaction type, the data transformation did not affect the chromosomal location of QTL identified, in both the field and growth chamber studies, when compared to using the raw data. The raw percentage LAI data were not used for any analyses, and only the transformed data were employed for QTL and correlation analyses.

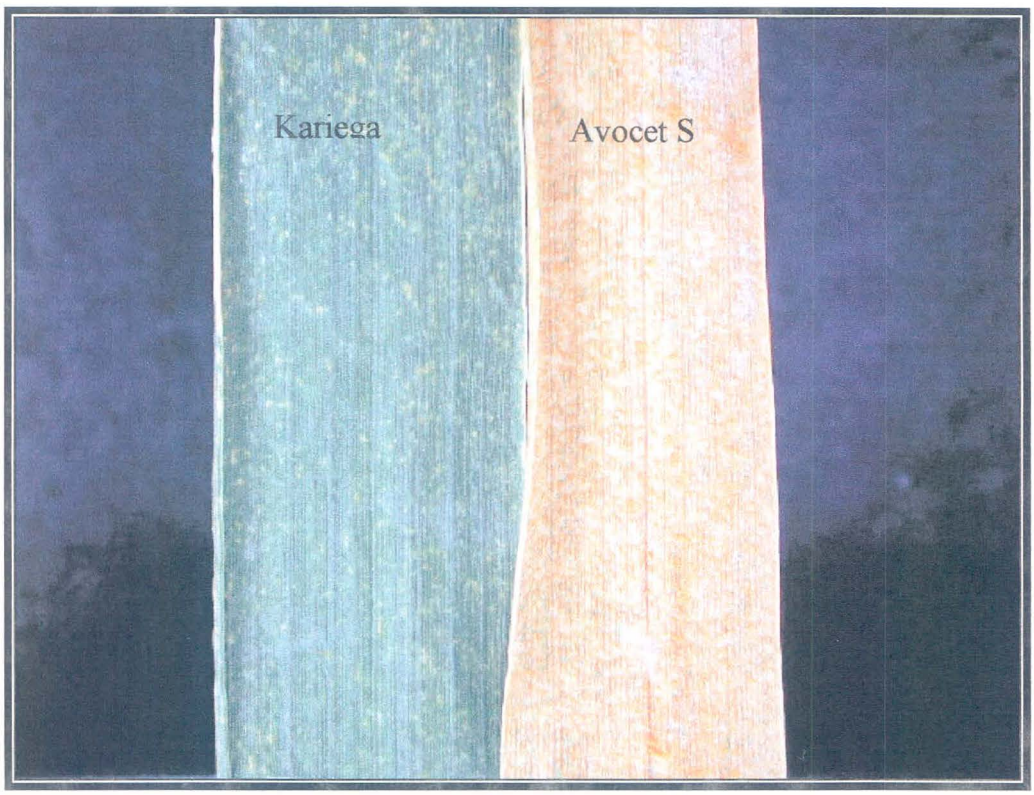


Figure 5.1. An example of the response to stripe rust infection observed in the parental lines under field conditions (Photo provided courtesy of Z.A. Pretorius).

An examination of the transformed phenotypic data distributions (Fig. 5.2) for both the early and final scores suggests the absence of significant transgressive segregation as the disease scores of the DH progeny fell between the range of the parent lines. The frequency distributions obtained (Fig. 5.2) indicated that a monogenic model could not explain the observed resistance. Falconer and McKay (1996) indicated that a multimodal distribution may indicate of the presence of major genes, since the genes have an effect large enough relative to the background genetic and environmental variation to produce this distribution. A non-normal distribution (Fig. 5.2) may indicate the presence of a major gene or genes (Falconer and Mackay, 1996), but may also reduce the power of QTL detection via interval mapping (Lander and Botstein, 1989).

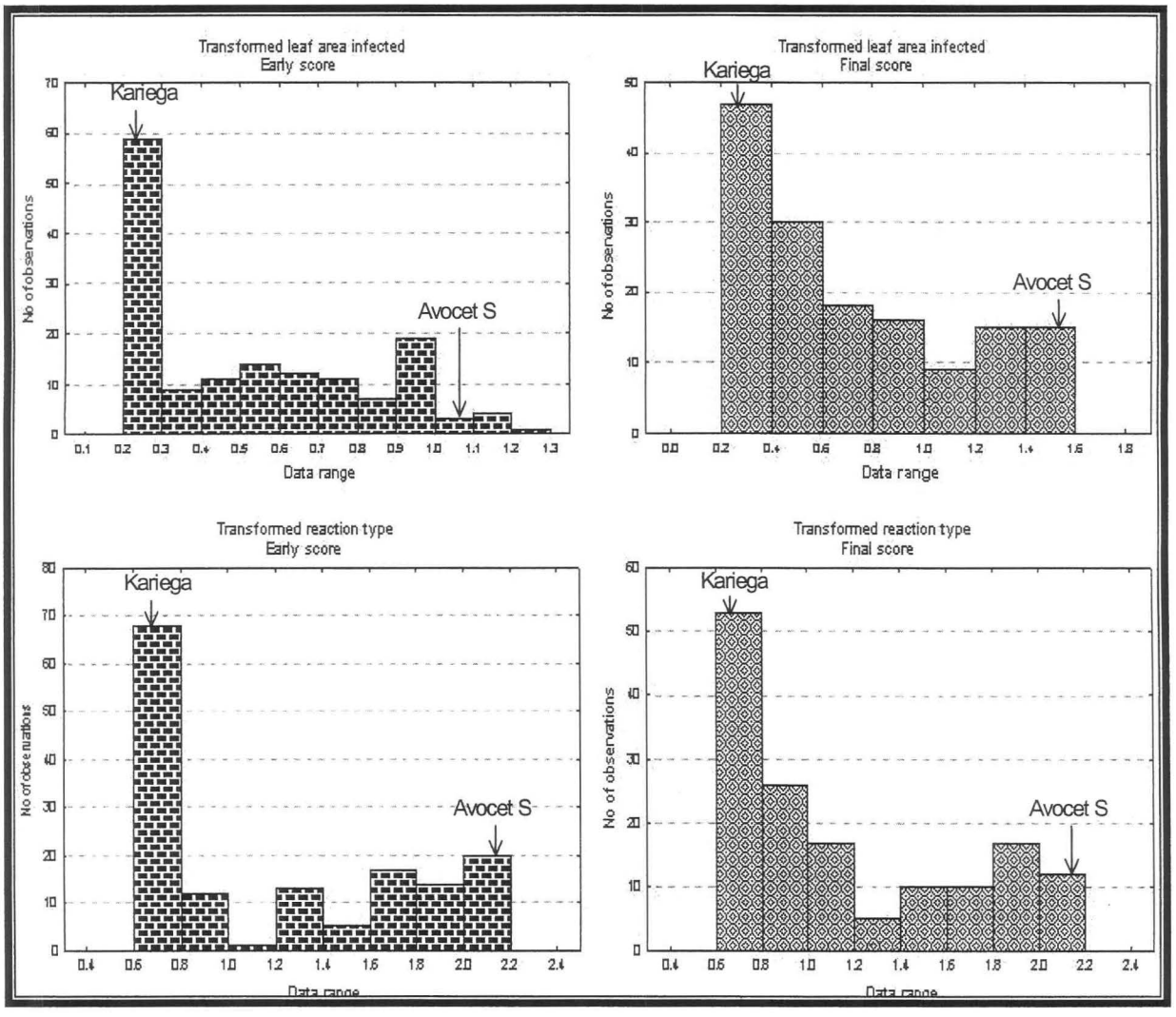


Figure 5.2. Histogram distributions of transformed LAI and transformed reaction type for early and final field scores. The approximate scores obtained for the parent lines are indicated relative to the scores of the segregating DH population.

The repeatability of disease scoring is statistically measurable in terms of coefficients of correlation at various levels as summarised in Table 5.1, where it should be recalled that the adjusted mean per replication at the final scoring was calculated from means averaged over scorers (ZP, WB and LB in the case of transformed leaf area scores, and ZP and WB in the case of reaction type) while the early score was evaluated by ZP for three replications only. This repetition of scoring, and the highly positive correlation between scorers (Table 5.1), made a major contribution to the power of resolution of the QTL analysis, which followed.

The high correlation observed between the early and final score (Table 5.1) suggests that these are very similar. More information may have been obtained had the data been scored at regular intervals, e.g. every 2-3 weeks, to monitor the changing levels of APR in the field trial. However, the manpower required for this, did not make it feasible. The low correlation of both early and final field scores with growth chamber scores (Table 5.1) highlights that improvements are required to synchronize growth chamber results with those obtained in the field.

J.H. Louw proposed a new score that involved the combination of reaction type and transformed percentage of LAI to obtain a scale for the purposes of ranking and selection of lines, and for identifying and mapping QTL. Transformed LAI was subjected to standard analysis of covariance (Sokal and Rohlf, 1995) treating blocks and lines as main effects and reaction type (untransformed) as covariate after averaging over scorers, to obtain for each line a mean transformed leaf area adjusted for host reaction type, referred to as the *adjusted mean score.* The angular transformation and adjustment by covariance are biometrically justifiable and yield a continuous variable with properties appropriate for fitting quantitative trait models.

Table 5.1. Coefficients of correlation (intraclass, t, and product-moment, r) measuring repeatability of disease scores and scorers in the field trial at two dates (Early and Final) ZP, WB and LB refer to individual independent scorers. Early scores by ZP were for 3 replications (Table provided courtesy of J.H. Louw).

Scorers and scores	Coefficient of correlation ± Standard error		
	Reaction type	Transformed LAI	*Adjusted mean score*
Early score (ZP):			
Between blocks	$t = 0.911 \pm 0.012$	$t = 0.929 \pm 0.010$	-
Final score (ZP, WB, LB):			
Between scorers within blocks	$t = 0.799 \pm 0.023$	$t = 0.942 \pm 0.007$	-
Between blocks (averaged over scorers)	$t = 0.965 \pm 0.005$	$t = 0.928 \pm 0.009$	-
Early score – Final score	$r = 0.865 \pm 0.021$	$r = 0.886 \pm 0.018$	$r = 0.855 \pm 0.022$
Early – Growth Chamber	$r = 0.372 \pm 0.070$	$r = 0.220 \pm 0.078$	$r = 0.165 \pm 0.079$
Final – Growth Chamber	$r = 0.482 \pm 0.063$	$r = 0.380 \pm 0.07$	$r = 0.341 \pm 0.072$

The *adjusted mean score* showed very high correlation with the transformed percentage LAI scores i.e. 0.989 for the early score and 0.991 for the final score, and this is reflected in the similar QTL results obtained for these two scores (data not shown). However, the high correlation observed between scorers (Table 5.1) and between the transformed LAI and *adjusted mean score* obviates the use of this composite score in this study (J.H. Louw, personal communication). The composite score is worth mentioning as it provides a means of usefully utilizing a variate scored on a nominal scale (reaction type) and may be of interest to plant pathologists and other researchers.

5.2 Establishing significance level thresholds

Regressions on a set of linked markers for association with a quantitiative trait cannot be considered independent tests, which makes establishing significance levels difficult (Hackett *et al.*, 2002). If one adopts a significance level of 0.05 for marker regression tests, there is a 1 in 20 chance that we declare a spurious association significant due to random variation, i.e., a type I error. With a large number of markers significance levels need to be stringent to avoid falsely declaring associations with the trait of interest. Permutation tests may be used to establish empirical significance thresholds for QTL mapping experiments and were used to calculate suitable significance thresholds for both types of field scores used in this study (Table 5.2).

Separate permutation tests were done for modified interval mapping (mIM) (results not shown), and these produced significance level thresholds very similar to those obtained for interval mapping (Table 5.2). Map Manager QTX produces a likelihood ratio test statistic (Lrstat), which can be converted to the more conventional base 10 LOD score by dividing by 4.61 (Table 5.2). The suggestive, significant and highly significant values given (Table 5.2) correspond to genome-wide probabilities proposed by Lander and Kruglyak (1995).

5.3 Single marker regression

Single marker regression generally involves, for each marker, a classification of the offspring into marker classes depending on the genotype at the marker locus, calculating the mean trait value associated with each class and comparing them to see if they are significantly different.

Despite some of the problems associated with single marker tests (Lander and Botstein, 1989; see section 1.4) it is a good starting point for QTL analysis.

Table 5.2. Threshold Lrstat values, converted to the equivalent LOD scores obtained via a permutation test set at 1000 iterations for the different disease scores and scoring times.

		Growth chamber		Transformed reaction type		Transformed LAI	
		Lrstat	LOD	Lrstat	LOD	Lrstat	LOD
Early	Suggestive[a]			7.5	1.63	7.3	1.58
Score	Significant[b]			13.7	2.97	13.5	2.93
	Highly significant[c]			20.2	4.38	19.4	4.21
Final	Suggestive[a]	7.3	1.58	7.3	1.58	7.3	1.58
Score	Significant[b]	13.4	2.91	14.1	3.06	13.6	2.95
	Highly significant[c]	18.9	4.1	22.5	4.88	20.6	4.47

[a]Corresponds to the 37th percentile (P = 0.63)
[b]Corresponds to the 95th percentile (P = 0.05)
[c]Corresponds to the 99th percentile (P = 0.01)

Markers that were unlinked to any of the linkage groups created were tested for associations with the disease score types using single marker regression and two markers (*Xwmc149.2* and s13m83A) were consistently found to be associated with both score types (Table 5.3). Unlinked markers associated with the trait imply that the linkage groups developed do not represent all the regions of the genome that affect the trait as is the case with AFLP marker s13m83A, as it is impossible to say from which chromosome this marker originated. It is interesting to note that SSR WMC149 was mapped to chromosome 2B (Harker *et al.*, 2001), and that other single markers on 2B showed significant single marker regressions (Table 5.4), suggesting that the linkage map developed for 2B may be incomplete.

Single marker regressions are useful to provide an indication of chromosomes or linkage groups that may be important. Markers placed in linkage groups, that produced significant single marker regressions are listed in Table 5.4. In this study, markers on chromosomes 2B and 7D were consistently found to have significant single marker regression results using the different scoring methods (Table 5.4). These chromosomes are clearly involved in APR to stripe rust and this has been reported previously in other studies (Bariana *et al.*, 2001; Boukhatem *et al.*, 2002).

Table 5.3. Single marker regression likelihood ratio statistics (Lrstat) of markers not placed in any linkage groups.

Marker	Growth chamber	Transformed reaction type		Transformed LAI	
		Early score	Final score	Early score	Final score
s14m69D	0.5	0	0	0.3	0
Xgdm98	0.3	0.1	0.2	0.5	0.8
s14m69C	0	0	0.2	0.3	0.6
s13m83A	0.4	8.5	4.8	9.6	12.0[b]
Xwmc149.1	1.9	0.1	1.1	0	0.1
Xwmc149.2	0.1	10.5[b]	3.6	16.6[a]	7.0
Xgdm5	1.9	0.7	0.7	0	0.2
s26m35C	2.1	0.9	1.9	0.9	0.2
Xgdm33	0.1	0.3	2.1	0.9	1.4
SRAP1	0.7	0.3	0	0.2	0.2

[a]Significant at P = 0.0001
[b]Significant at P = 0.001

A number of markers on 4A were significant with the early LAI scores only (Table 5.4). Based on these results it appears that chromosome 4A may be involved in APR to stripe rust in the early development of the disease. Perhaps an early scoring date may have ascertained this more conclusively. The SSR locus *Xcfd31* was previously mapped to 7D (ITMI population), and to 4A ('Chinese Spring X Courtot') (Guyomar'ch *et al.*, 2002), but to 3D in this study ('Kariega X Avocet S') (Fig. 4.4). The mapping gels for this SSR were re-examined to check for errors during the scoring of the marker profile that may have incorrectly placed this marker on the linkage map. However, the profile was easy to score and it is therefore likely that an additional locus to the ones previously identified, was mapped in the 'Kariega X Avocet S' population. No other markers on 3D were significantly linked to the trait.

Markers on chromosome 1A were detected at a suggestive level of significance (Table 5.2) (data not shown). Lander and Kruglyak (1995) suggest that markers detected at this level of significance be recorded. If the region is important then it is likely that this will be reflected in the results of interval mapping as was the case with this region.

Table 5.4. Single marker regression likelihood ratio statistics (Lrstat) of markers in linkage groups. Marker chromosomal locations and data for both diseases scoring methods and times are provided. Only markers showing significant linkage are tabulated.

Marker	Chrom.	Growth Chamber	Transformed Reaction type		Transformed LAI	
			Early score	Final score	Early score	Final score
s23m12C	2B		32.1*	47.4*		30.2*
Xgwm682	2B		57.0*	84.4*	27.0*	52.2*
Xgwm372	2B		55.9*	83.8*	26.4*	49.6*
Xgwm148	2B		60.1*	93.1*	28.6*	55.2*
s12m60A	2B		53.9*	85.8*	24.1*	49.1*
s12m24D	2B		60.2*	86.3*	28.2*	53.2*
Xpsp3030	2B		39.6*	76.1*	18.3	36.4*
s16m40A	2B		30.9*	62.2*		29.5*
SRAP10	2B		18.3	34.9*		20.8*
s26m92G	2B		16	31.4*		19.2
s15m56C	2B		17.2	30.5*		19.9
s26m92F	2B		16	31.4*		19.1
s15m56A	2B		17.2	30.5*		19.9
Xgwm501	2B			29.0*		17.8
s19m89B	2B		16.2	31.5*		19.4
s15m17A	2B		15.6	32.2*		18.4
s15m17B	2B		18.6	35.7*		20.7*
Ltn	7D	22.5*	51.2*	45.5*	49.8*	77.8*
Xgwm295	7D		26.6*		43.1*	47.0*
Xgwm111.1	7D				15.5	20.9*
Xgwm437	7D					15.2
s15m53B	4A	38.5[a]				
s25m48B	4A	31.7[a]			15.4	
s18m93E	4A	31.6[a]			22.6*	
s15m67B	4A	35.9[a]			20.5*	
s12m34B	4A	32.9[a]			21.6*	
Xgwm160	4A	36.9[a]			18.4	
Xgwm742	4A	32.2[a]			17.7	
Xgwm832.1	4A	28.0[a]			16.3	
Xwmc219	4A	24.5[a]			25.9*	
s12m34A	4A	37.0[a]			17.2	
s21m40A	4A	32.3[a]				
s19m89C	7A	18.0				
Xcfd31	3D					16.6

*Highly significant (see Table 5.3)

[a]Highly significant effect originating from the 'Avocet S' parent

Single marker regressions were also done on the data obtained from the growth chamber studies and some interesting results were obtained. With the exception of *Ltn*, no other markers on 2B or 7D were significant and one marker on 7A was also significant (Table 5.4). It is interesting that markers significant on 4A may be a result of QTL originating from the 'Avocet S' parent and not the resistant parent 'Kariega' (Table 5.4). The results of the growth chamber studies are not in agreement with those obtained in the field, as was also reflected in the low correlations observed between the growth chamber and field scores (Table 5.1). The growth environment of the plants under field and growth chamber conditions differs markedly, and this may influence the plants response to infection, thus explaining the inconsistent results observed.

The distance between the marker and the QTL, and individuals with missing data, influence single marker analysis and the effect of the QTL (a) is confounded with the recombination fraction (c), as both are unknown. The exact location of the QTL on a linkage group cannot be determined with single marker analysis, only association of a marker with a QTL.

5.4 Interval Mapping (IM) and modified Interval Mapping (mIM)

Six QTL were identified in this study (Table 5.5). QTL were consistently detected on chromosomes 7D (QTL-1) and 2B (QTL-2) similar to the results obtained for single marker regression. QTL were also detected on 1A (QTL-3), 4A (QTL-4, QTL-5) and 7A (QTL-6). QTL-1, QTL-4, and QTL-6 were detected under growth chamber conditions. In general the interval mapping results coincide with those obtained with single marker regression.

IM analysis detected QTL on chromosomes 7D and 2B across the different field phenotypic data treatments and scoring times (Fig. 5.3). Utz and Melchinger (1994) and Van Ooijen (1994) found substantial improvements in the power of detecting a QTL when selected co-factors are used in combination with interval mapping. QTL that remain undetected with IM are detected with multiple QTL methods, though not very accurately, and improvement by the use of multiple marker methods is only possible when there are more than one segregating QTL (Van Ooijen, 1994). If one of the QTL segregating explains a large proportion of the total variation in the trait, the use of a linked marker in subsequent multiple QTL mapping will enhance the power in the search for other segregating QTL (Van Ooijen and Maliepaard, 1996).

The results of mIM highlight the importance of the use of markers as co-factors to control background variation (Table 5.5). With the major QTL (see 5.4.1) mIM reduced the variance explained while the detection of QTL-5 was improved with an increase in the variance explained (Table 5.5). The findings of Wang *et al.* (1999) highlight the fact that using both main effect markers and interaction effect markers were superior to using main effects markers alone to control background variation. The results obtained using mIM with specific marker co-factors were used in this study to confirm the chromosomal location of major QTL and improve the detection of other minor QTL (Table 5.5).

Only a portion of the total variance is accounted for by using the different disease scores and QTL mapping techniques (Table 5.5). Variation not accounted for by the QTL detection techniques in this study may be attributed to the presence of undetected minor QTL in regions of the genome not covered by markers, epistasis, QTL interactions, or QTL-environment interactions (Toojinda *et al.,* 2000). This highlights the need for linkage maps with more complete genome coverage, and more effective QTL mapping software as well as replicated, accurate field trials adjusted for spatial variation.

5.4.1 Major QTL

Major QTL as defined in this study refer to those QTL which explain a large proportion of the variation in the trait, i.e., 30-50%. The two major QTL (QTL-1, QTL-2) were consistently detected using the different disease scores, taken at the different times (Table 5.5; Fig. 5.3).

The SSR *Xgwm295-7D* was located close to QTL-1 (~10 cM) (Fig 5.3). The lack of polymorphism in the 7D chromosome prevented the construction of a dense linkage map for this chromosome, with only 7 markers mapping to this linkage group (Fig. 5.3). Boukhatem *et al.* (2002; ITMI population) and Bariana *et al.* (2001;'CD87 X Katepwa') also identified QTL for APR to stripe rust on chromosome 7D using untransformed infection type and percentage leaf area infected, respectively. Börner *et al.* (2002) also mapped QTL for leaf rust and powdery mildew on the short arm of 7D in the ITMI population.

The *Yr18* gene located on chromosome 7D is reportedly linked to the leaf rust resistance gene *Lr34*, and the leaf tip necrosis phenotypic characteristic (Singh 1992a; b). In this study,

Ltn was mapped 14 cM away from the closest marker (*Xgwm295-7D)* to QTL-1 (Fig. 5.3). *Lr34* could not be mapped in this study due to the presence of other *Lr* genes, which would have confounded the results obtained with *Lr* pathotypes prevalent in South Africa. However, Nelson *et al.* (1997) mapped *Lr34* (derived from 'Opata85') to the chromosome 7DS region flanked with RFLP loci *Xbcd1872-7D* and *Xbcd1438/Xrz2-7D* (~15 cM) in the ITMI population and Röder *et al.* (1998) mapped *Xgwm295-7D* (also mapped in this study) close to *Xbcd1438-7D* (~3 cM) in the same population. Durability of the *Lr34* APR leaf rust resistance (closely linked to *Yr18*) is due to its combination with other minor leaf rust resistance genes (Roelfs, 1988, Singh and Rajaram, 1994) and it is therefore not unexpected that more than one QTL for APR to stripe rust is detected in 'Kariega'.

QTL-2 explained more variation in terms of host reaction type (33-46%) than percentage LAI (17-30%) severity (Table 5.5). In this case the reaction was more clearly detected at the final evaluation (IM, 46%) compared to the early evaluation (IM, 33%), presumably due to environmental conditions conducive to the moderately resistant (MR) moderately susceptible (MS) range of phenotypes. Qayoum and Line (1985) described the expression of high-temperature APR to stripe rust and it is possible that some of the genes in 'Kariega' function in a similar way, therefore the more pronounced manifestation as the season progressed and temperature increased.

QTL-2 is detected in a large region (~25 cM) spanning chromosome 2BS with *Xgwm148-2B* closest to QTL-2 (Fig. 5.3). Boukhatem *et al.* (2002) also identified QTL for APR on chromosome 2B, (2002; ITMI, ~30 cM and 'GB X 7') using log transformed AUDPC and untransformed infection type (IT). QTL for stripe rust resistance on 2B were also detected across environments over 2 years by Börner *et al.* (2002) when assessing adult plants of the ITMI population. Catalogued genes for stripe rust resistance on 2B include *Yr27* (unmapped), and *Yr5* and *Yr7* on 2BL (mapped by Bariana *et al.,* (2001)). A stripe rust seedling resistance gene from the cultivar 'Spaldings Prolific' designated *YrSp* has also been mapped to 2B (McIntosh *et al.,* 2001).

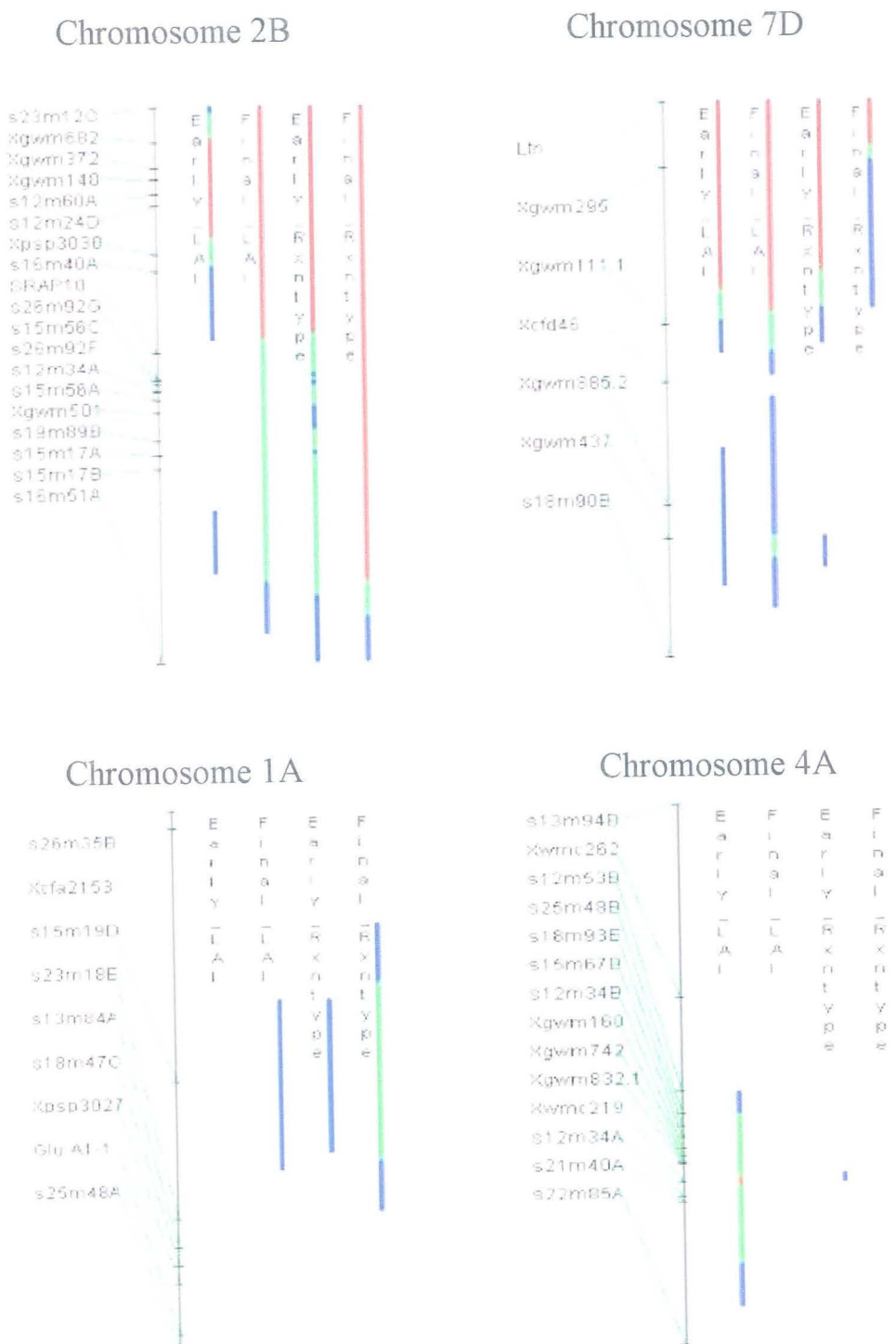


Figure 5.3. A diagrammatic representation of the results of interval mapping for chromosomes 1A, 4A, 2B and 7D for transformed LAI and transformed reaction type (early and final score). The colours blue, green and red indicate regions of suggestive, significant and highly significant probability of QTL, with marker positions indicated on the left.

Table 5.5. Summary of the IM and mIM analysis of chromosomes involved in APR to stripe rust with the different disease scores. The Lrstat value and % variance explained by each marker interval is given for the growth chamber study and the two field classification times. Scores with intervals not significant (NS) are also indicated.

QTL INTERVAL AND LOCATION		Growth chamber		Transformed reaction type				Transformed percentage LAI			
				Early score		Final score		Early score		Final score	
		[a]IM	[b]mIM	IM	mIM	IM	mIM	IM	mIM	IM	mIM
QTL-1 (7D)	LrStat	22.1	13.0	51	78.5	45.5	55.9	51.8	83.8	78.9	106.3
Xgwm295 – Ltn	% Var	16	8	34	25	31	16	35	26	48	29
QTL-2 (2B)	LrStat	NS[e]	NS	60.1	69.8	93.6	79.5	28.6	42.1	55.2	70.4
Xgwm148 – s12m60a	% Var			33	21	46	25	17	11	30	16
QTL-3 (1A)	LrStat[c]	NS	18.2	NS	NS	10.4	9.0	NS	10.2	NS	NS
s15m19D – s23m18E	% Var[d]		10			6	2		2		
QTL-4 (4A)	LrStat	38.5	16.8	NS	NS	NS	NS	NS	NS	NS	NS
s21m40A – s22m85A	% Var	23	11								
QTL-5 (4A)	LrStat	37	17.8	12.2	47.3	NS	NS	25.9	72.0	7.9	42.7
Xwmc219 – s12m34A	% Var	22	11	7	11			15	22	4	9
QTL-6 (7A)	LrStat	17.2	11.7	NS	NS	NS	NS	NS	NS	NS	NS
s19m86C – s18m47B	% Var	11	7								

[a]Indicates Interval Mapping
[b]Indicates modified Interval Mapping using a selected set of marker-co-factors
[c]Indicates the maximum likelihood ratio statistic score obtained on the chromosome interval in question. Permutation tests were done to determine the likelihood-ratio statistic (Lrstat) threshold values for each disease score used and correspond to genome-wide α levels of: 0.63(*Suggestive), 0.05(**Significant) and 0.001 (***Highly significant).
[d] Percentage phenotypic variance explained

An examination of the pedigrees of the cultivars used to map QTL reveals a common donor of *Yr18*. The *Yr18* gene is present in 'Opata 85' (ITMI; Nelson *et al.*, 1995b) and 'Frontana' is recognised as the common ancestor and donor of *Yr18*. 'Anza' which is present in the pedigree of 'Kariega', and 'CD87' (via 'Condor'), is a derivative of 'Frontana'. QTL-1 can most probably be ascribed to the presence of *Yr18* also linked to *Lr34* (Singh, 1992b). This common germplasm may also explain the consistent detection of QTL on chromosome 7D by Boukhatem *et al.* (2002), Bariana *et al.* (2001), Börner *et al.* (2002) (*Lr34* detected, but linked to *Yr18*) and this study and QTL on 2B by Boukhatem *et al.* (2002), Börner *et al.* (2002) and this study.

5.4.2 Other QTL detected

Some QTL were not consistently detected using the different disease scores (Table 5.5), with additional minor QTL being detected, a trend also found by Boukhatem *et al.* (2002). This suggests the occurrence of different QTL for the different disease components involved in the expression of APR to stripe rust (Table 5.5). Alternatively, this may be due to non-synchronised environmental testing conditions, i.e., growth chamber conditions.

QTL-3 on 1A, not detected at a significant level with single marker regression, was detected in the field using transformed reaction type and transformed percentage LAI (Fig. 5.2; Table 5.5) as well as in growth chamber studies. The QTL accounted for little variation in the field (2-6%) and in the growth chamber (10%) and no trend was evident in relation to the time of scoring (Table 5.5). Börner *et al.* (2002) detected QTL for leaf rust on 1A, and the leaf rust resistance gene *Lr10* is also located on 1A. It is worth mentioning that none of the catalogued stripe rust resistance genes are located on 1A, and this is the first report of QTL for stripe rust resistance, although of small effect, on this chromosome.

When considering the early and final field scores for percentage leaf area infected and host reaction type, it is clear that QTL-5 on 4A (accounting for between 4-22% of the variation) is generally not detected as time progressed (Figure 5.3; Table 5.5). Increased leaf damage due to pathogen infection at the final scoring date precludes the detection of small differences in scores and, as a result, minor QTLs may go undetected. Alternatively, these QTL may be expressed at an earlier stage in the plants response to infection and an earlier scoring time

may have shown this more accurately. Börner *et al.* (2002) also mapped QTL for stripe rust resistance in adult plants on 4A in the ITMI population.

5.4.3 Growth chamber tests

In addition to the low correlation between the field host reaction type (early/final) and the growth chamber reaction type (Table 5.1), the QTL analysis (Table 5.5) clearly indicates that the present growth chamber test results are not in agreement with the results obtained with the field data. Although at least one major QTL (QTL-1) was detected, QTL-2 that is consistently detected in the field trail was not detectable with the growth chamber results. In addition, another QTL was detected (QTL-6) which was not detectable in the field results. The results obtained indicate that a different set of QTL are detected in the expression of resistance in the growth chamber, suggesting that the growth chamber environment may still need adjustment to accurately reflect trial conditions in Kwa-Zulu Natal.

It is interesting to note that the susceptible 'Avocet S' parent contributed QTL-4 under growth chamber conditions (Table 5.5). Several cases have been reported where components of APR to powdery mildew in wheat have been contributed by the susceptible parent due to the effect of 'defeated seedling resistance genes' (Nelson 1978; Keller *et al.*, 1999; Chantret *et al.*, 1999; Paillard *et al.*, 2000). 'Avocet S' is a susceptible selection from 'Avocet', which was heterogenous for the seedling resistance gene *YrA* as well as adult plant stripe rust reaction (Wellings *et al.*, 1988; McIntosh *et al.*, 1995) and it has been shown that a component of the *YrA* resistance is located on chromosome 3D, and another component on an unknown chromosome (Wellings *et al.*, 1988). This unidentified component of the defeated *YrA* seedling gene may therefore be responsible for the detection of QTL-4 in the growth chamber. However, it is also possible that the adult plant stripe rust resistance observed in 'Avocet' might have been retained under the conditions in which the susceptible selection of 'Avocet S' was made. In studying *Yr18* resistance to different pathotypes in various environments, Johnson *et al.* (2000) reported that 'Avocet S', one of the control genotypes in that particular study, had significantly lower stripe rust scores than 'Avocet R' in four out of five comparisons in New Zealand and the UK. This indicates that some form of reduced susceptibility is detectable in 'Avocet S' under certain conditions as was evident in this study under growth chamber conditions. In Mexico, the two 'Avocet' selections have been found to be equally susceptible (Johnson *et al.*, 2000).

In general, the major QTL (QTL-1 and 2) account for some 55% of the explained variation in the field (an approximate value, averaged over disease scores, scoring times, and QTL mapping techniques) while the minor QTL (QTL-3, 4 and 5) account for some 14% (calculated as described). With approximately 69% of the variation accounted for, the study can be regarded successful in generating a partial linkage map providing good genome coverage. It also illustrates the usefulness of partial maps in QTL analysis. However, the results also highlight the need for linkage maps with comprehensive genome coverage, and software packages capable of detecting variation ascribable to epistatic, and QTL x environment interactions in order to account for all the variation in the trait.

5.5 QTL interaction

Epistasis is the interaction of alleles at different loci, where the allelic effects of one locus is dependent on the genotype at another locus, often referred to as non-additive gene action. Additve gene action, as defined by Falconer and Mackay (1996), implies an absence of dominance with reference to a single locus, or the absence of epistasis with two or more loci. In the absence of epistasis, the total genetic value of an individual is the sum of the individual locus values, as loci are independent, but this is not always the case.

Using single markers and regression analysis (Zeng, 1993; 1994) an epistatic interaction term was calculated by Map Manager QTX for various combinations of markers (Table 5.6). A high significance threshold (P=0.000001) is applied to avoid false positives as suggested by the developers of Map Manager QTX. A number of marker combinations gave significant interaction terms (Table 5.6) with certain chromosome combinations repeatedly being detected, although with different marker combinations.

Markers on chromosomes 2B and 7D produced significant interaction terms in most marker combinations (Table 5.6). Chromosome 2B markers consistently produced significant interaction terms in combination with markers on 7D, 2D and 1B, while chromosome 7D markers in combination with markers on chromosome 2B, 1A and 4A were consistently significant (Table 5.6). The highest interaction terms (34.7, 38.5, and 53.6) were obtained for 2B-7D chromosome combinations, again highlighting the importance of these regions in APR to stripe rust (Table 5.6) consistent with the classification of these QTL as major QTL.

Table 5.6. Markers showing significant interaction statistics for the growth chamber data, transformed reaction type and transformed % LAI. Chromosomes consistently showing significant interaction are highlighted.

Chromsome/ Marker (1)	Chromosome/ Marker (2)	Growth Chamber		Transformed reaction type				Transformed % LAI			
				Early score		Final score		Early score		Final score	
		Lrstat[a]	IX[b]	Lrstat	IX	Lrstat	IX	Lrstat	IX	Lrstat	IX
Xgwm148 -2B	*Ltn - 7D*							94.2	17.3		
S12m24D - 2B	*Ltn - 7D*			135.4	34.7	141.2	38.5			177.3	53.6
S12m24D - 2B	*Xwmc167 – 2D*			60	8	77.8	9.3				
S12m24D - 2B	*SRAP6 - 3B*									49.3	7.6
S12m24D - 2B	*s19m39A - 1B*									54.5	7.3
Xgwm682 - 2B	*Xwmc219 -4A*					93	7.4				
Xgwm682 - 2B	*Xgwm332 - 7A*					96.4	11.8				
Xgwm682 - 2B	*s22m88B - 6A*							33.5	7.1		
S23m12C - 2B	*Xwmc167 - 2D*									42.6	10.7
S23m12C - 2B	*s16m46A - 1B*					54.8	6.9				
S16m40A - 2B	*s18m93B - 5A*					72.3	11.5				
S16m40A - 2B	*s12m60A - 1B*							34.6	9.4		
Xpsp3030 - 2B	*s13m85B - 5A*									46.8	10.5
Xgwm372 – 2B	*SRAP6 - 3B*							37.2	9.8		
S16m51A – 2B	*s12m34A -4A*	38.7	9.2								
S15m17B – 2B	*s22m85A - 4A*			39.8	14.9						
Xgwm111.2-7D	*s15m19D 1A*			33.6	12.5						
Ltn - 7D	*s23m18E - 1A*					69.8	11.6	69.1	7.3	11.6	10.3
Ltn - 7D	*Xgwm186 - 5A*									95.4	11
Ltn - 7D	*Xwmc167 - 2D*					61.1	12.3				
Ltn - 7D	*Xwmc219 -4A*					67.9	15.2	116.2	12.6	119	11.5
Xgwm295 – 7D	*s25m48B – 4A*	44.4	11.7								
Xwmc219 –4A	*Xpsp3200 - 6D*							34	8.5		
s23m12A 4B	*Xgwm169 - 6A*	33.9	24.1								

[a]Likelihood ratio statistic as described previously

[b]Interaction statistic calculated by Map Manager QTX. A significance level of P=0.000001 was applied

The percentage of trait variation accounted for by interaction effects is not known. The detection of significant 2B-7D interaction effects suggests that breeding programmes should select for both regions to obtain adequate APR to stripe rust.

A major limitation of quantitative genetics has been that statistically detected genetic effects are generally not reliable indicators of underlying gene action, whereas QTL mapping methods provide a more direct route to understanding gene action (Holland, 2001). As a result of QTL experiments, more evidence of epistasis has been obtained, largely because (i) the estimation of effects in specific chromosomal regions provides considerable power over biometric methods that test average or total gene effects of the entire genome, and (ii) QTL studies can estimate gene action effects (e.g. the effect of the QTL, *a*) and not average allelic and genotypic effects (Holland, 2001). Furthermore, statistical methods for estimating epistasis in QTL experiments are improving, as illustrated by the maximim likelihood procedures developed by Wang *et al.* (1999) and Kao *et al.* (1999).

Since additive x additive forms of epistasis are a form of genic interaction for which favourable alleles can be 'fixed' and exploited in homozygous crops, selfing species such as wheat might tend to exhibit strong epistatic interactions and outcrossing species strong dominance interactions (Holland, 2001). Implications of epistasis for plant breeding will depend on the breeding system of the crop and on methods for breeding and seed production as these factors will determine the type of epistatic effects that can be propagated reliably (Hollland, 2001). Nevertheless, it is clear that epistasis is important and marker-assisted selection strategies should be designed to exploit it.

5.6 Targeting important chromosomes

Chromosomes harbouring QTL were identified early on in this study and these chromosomes were then targeted with more SSR markers in order to increase the number of markers and the density of marker linkage maps. Both major QTL were detected with approximately 70 markers, but the chromosomal location of QTL-2 was confirmed only after a total of 160 markers were mapped (data not shown). The approach of targeting chromosome 7D was not successful due to the low polymorphism level generally associated with the D genome (see Chapter 3). Although 22 7D SSRs were tested, the low polymorphism obtained for this

chromosome (36%, Table 3.3), resulted in very few markers being mapped and a sparse linkage map (Fig. 4.4).

The approach of targeting identified regions with additional SSRs proved to be successful in the cases of chromosomes 2B and 4A where an increase in marker density improved the QTL profile as illustrated for 4A in Fig. 5.4. QTL-5 was identified early in the study at a low significance level (data not shown) and a total of 160 mapped markers, but an increase in the number of markers mapped to this chromosome improved the QTL profile and distinct peaks were detected close to mapped markers. The QTL on 1A was detected later in the study, but a similar approach should be just as effective for improving the QTL resolution on this chromosome, as there are additional SSR loci mapped to 1A in other studies that were not tested.

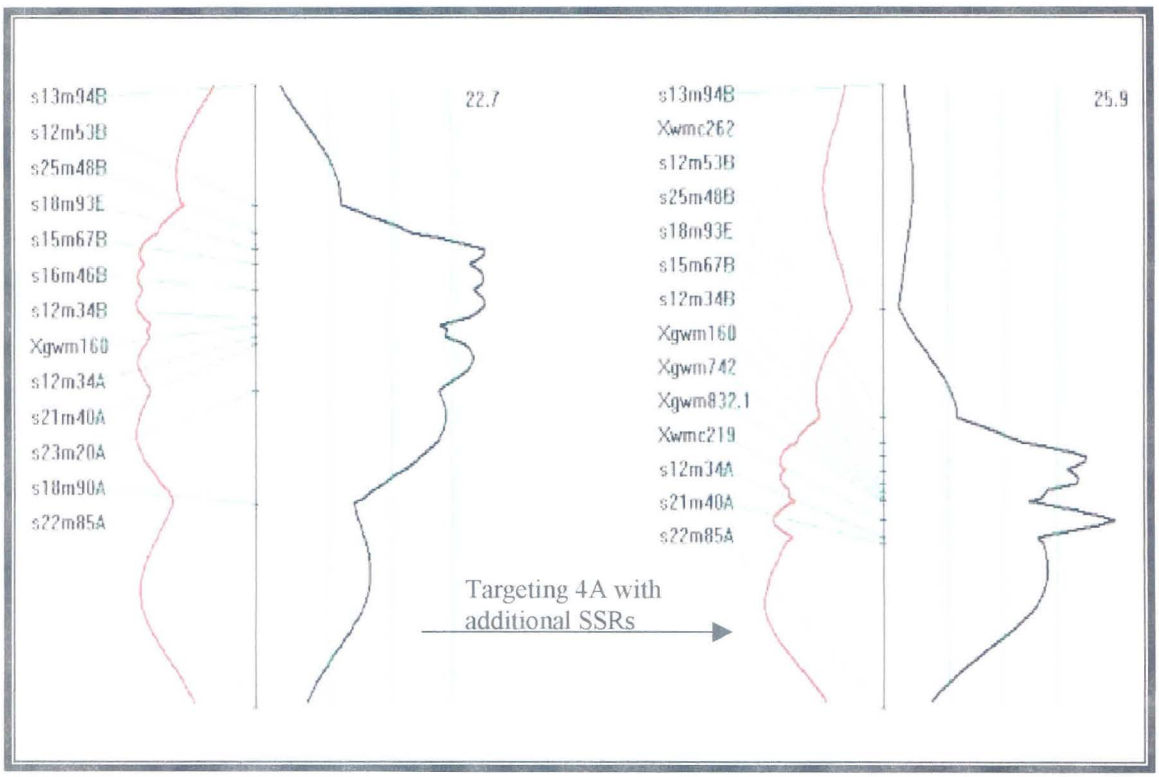


Figure 5.4. The results of interval mapping on 4A using transformed LAI (Early score) phenotypic data, illustrating the improvement in QTL resolution obtained after the chromosome was targeted with additional SSR markers. Marker positions are indicated on the left. Lines corresponding to suggestive, significant and highly significant Lrstat thresholds are indicated on the right, together with the maximum Lrstat value obtained for the interval.

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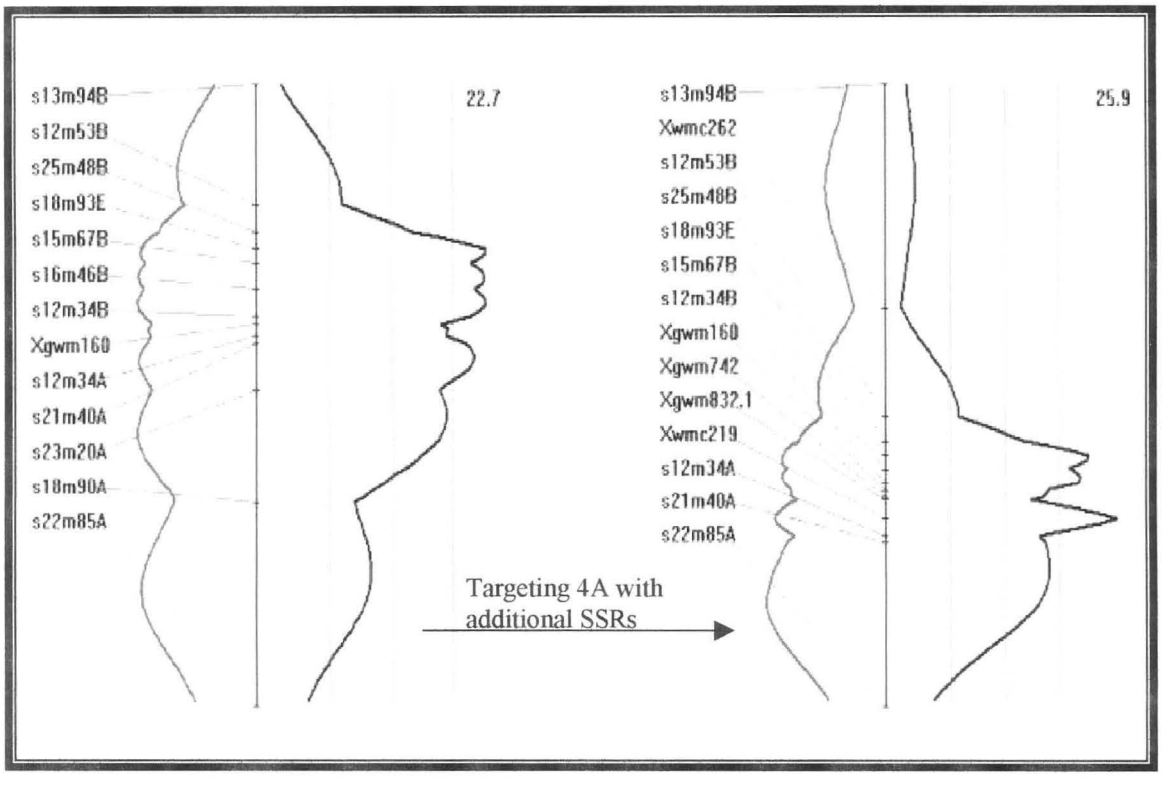

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The SRAP and RGA techniques were optimized, exhibit polymorphism and show Mendelian segregation, and thus have application in the development of linkage maps (Fig 4.4) with SRAP10 mapping in the region of QTL-2 (Fig. 5.3). None of the four RGAs mapped to QTL regions (Fig. 4.4). As both techniques are based upon degenerate PCR, it is important to ensure that markers mapped are highly reproducible, and easy to score to avoid any errors that may affect the quality of the linkage map produced.

5.7 Inferences about QTL and QTL position

It is important in any QTL study to recall that the approach does not pre-suppose any basis of inheritance in terms of the number of genes segregating in a segregating population, i.e., whether the trait is monogenic or multigenic. If a resistance trait is controlled by many genes, the identification of QTL does not indicate whether the genes are race specific or not (Young *et al.,* 1996). QTL represent segments of chromosome affecting a particular trait, and not necessarily single loci (Falconer and MacKay, 1996).

A QTL has been described as a statistical description of the phenotypic effects of a genetic locus or linked group of loci in defined sets of populations and environments (Paterson, 1998). QTL mapping relies on the frequency of recombination events, which beyond a given marker density, can only be increased by studying larger populations (Stam, 1994). Beavis (1994) reported that in simulated QTL mapping experiments involving 100-200 progeny individuals, only a fraction of the true QTL was detected, and suggested the use of at least 500 individuals in order to generate comprehensive and reliable data. Walling *et al.* (2002) recorded a higher proportion of putative QTL at marker positions compared to non-marker (between marker) positions for simulated data, with both linear regression and maximum likelihood interval mapping techniques, thus suggesting a bias toward marker positions when the QTL effect is small. Many of the software packages and models available, are still under development to optimise QTL detection, so the accuracy of experimental results are affected by the reproductive biology of the species, the availability of suitable data analysis methods and experimental resources.

The development of multiple marker QTL methods (Zeng 1993, 1994; Jansen and Stam, 1994) have produced better estimates of QTL positions but in order to improve QTL detection, the models upon which QTL software are based should focus on including terms

for QTL interactions, and QTL-environment interactions. If the proportion of the total variation due to these components can be accounted for, this will facilitate QTL studies. In order to improve QTL experiments, projects need to use better scoring methods, larger population sizes, multiple replications and environments, QTL verifications and appropriate quantitative genetic analysis (Young *et al.,* 1999).

The availability of a 'grass genome' map, prepared by Moore *et al.* (1995a), and extended by Gale and Devos (1998) details gene and DNA sequence similarity between genomes of the many species of the Gramineae which will enable studies in relatively small genomes such as rice to be applied to the larger wheat genome. The map of Gale and Devos (1998) describes 9 different grass genomes in terms of only 25 rice linkage blocks. The consensus regions of maps can be used to construct maps of other grass species rapidly, and to predict from one crop species to another, the location of key genes for adaptation (Gupta *et al.,* 1999). The extension of this synteny map with additional genes will allow for identification of these genes in other grasses and also offers the opportunity to detect QTL regions affecting similar traits that may overlap among members of the Gramineae.

The minor QTL (QTL-3, QTL-4, QTL-5, QTL-6 Table 5.5) identified in this study need verification but the chromosome 2B and 7D regions (Table 5.5) have been verified to be important in other independent studies i.e. Boukhatem *et al.* (2002), Bariana *et al.* (2001), Börner *et al.* (2002). The SSRs *Xgwm682-2B, Xgwm372-2B, Xgwm148-2B* and *Xpsp3030-2B* have been mapped to the region of QTL-2 (Fig. 5.3) and are candidates for marker validation on suitable genetic material, to test if they can be employed in breeding programmes. The AFLP markers mapped in the same region (Fig. 5.3) need conversion into a more suitable assay before marker validation. QTL-1 (Fig 5.3) can be targeted for more closely linked markers in order to generate a more dense linkage map for this region. The BSA approach is effective to study a particular genomic region against a randomized genetic background and is useful for identifying markers in regions that lack markers, such as gaps in the genetic maps. It has been successfully applied to develop markers linked to agronomically important traits in wheat (Eastwood *et al.,* 1994; Bai *et al.,* 1999; Parker *et al.,* 1999) and to fill gaps in linkage maps (Campbell *et al.,* 2001).

This study has demonstrated that a partial linkage map can be utilised to detect loose linkage to QTL for APR. In addition to major QTL, minor QTL were detected which may not have

been possible if a BSA strategy had been adopted. Targeting the chromosomes harboring QTL with additional SSR markers proved to be a successful strategy to increase the density of the linkage map of the chromosome, thus improving QTL resolution and, at the same time mapping markers that may be validated and used in MAS. Now that the QTL regions have been identified, these regions may be targeted for the mapping of more markers with the aim of ultimately identifying the gene or genes involved in APR to stripe rust.

CHAPTER 6: CONCLUSION

The most important findings of this study can be summarised as follows:

(i) A partial linkage map of the 'Kariega X Avocet S' DH mapping was constructed with 212 markers. The study has demonstrated that anchor SSRs, augmented with AFLP markers can provide good genome coverage, and can be used to generate linkage maps in a relatively short time and at a reduced cost compared to complete maps.

- The 'Kariega X Avocet S' linkage map can be used for the mapping of other genes of interest, for the facilitation of marker-assisted breeding, for the development of a high-resolution map to facilitate map-based cloning, for the mapping of additional traits for which the DH population is segregating and for providing a framework for understanding the genetic basis of complex traits.

- A feature of the linkage map is the low level of polymorphism observed in the D genome, more especially chromosome 7D, a result confirmed by other studies. Marker techniques such as SRAP and RGA can be used to target this region that is known to carry economically important genes.

- The use of the SRAP marker technique in detecting polymorphism and developing wheat linkage maps was demonstrated. A reproducible protocol was developed and 6% polymorphism was estimated for this population. The RGA technique was optimised for radioisotope detection and the polymorphism estimated (9.5%) shows much promise for future work. These marker techniques may be used to extend the existing linkage map.

(ii) QTL for APR to stripe rust were mapped and important chromosomal regions were identified. Two major QTL, explaining about 55% of the variation in APR in the population and two minor QTL explaining about 14% of the variation were detected which may not have been detected with BSA. This study has demonstrated that a partial linkage map can detect linkage to QTL and this can be verified by targeting the region with more markers.

- The program Map Manager QTX and the modified interval mapping (mIM) technique of Wang *et al.* (1999) were successfully used to detect major and minor QTL, and confirm their chromosomal location. Evidence for QTL interaction was found, chromosomes 2B and 7D consistently displaying significant interaction terms in the analysis, highlighting their importance in APR to stripe rust. The Map Manager QTX software package is easy to use and suited to linkage and QTL analysis.

- QTL were detected on chromosomes 2B, 7D (major) 1A and 4A (minor) using the early and final field disease scores, with the QTL on 4A more prominent at the early disease scoring. These regions may now be further characterised.

- QTL detected under growth chamber conditions did not agree well with those detected using field disease scores. However, the identification of some of the QTL detected in the field is promising and it is evident that further optimization is required before growth chamber data can be used to supplement field disease scoring.

- The QTL on 7D appears to correspond to *Yr18,* a gene exhibiting APR to stripe rust (Singh, 1992a).

- Targeting identified QTL regions with additional SSR markers mapped previously in the same region, is an efficient strategy and results in a dense map for the chromosome region involved.

Additional uses of the 'Kariega X Avocet S' linkage map was illustrated by mapping two important genes, that were segregating in the population. These genes may be adopted in South African breeding programmes.

- *Sr26,* the seedling resistance gene for stem rust, for which the DH population was segregating, was mapped to chromosome 6A, and confirmed in 'Avocet S'. Markers linked to *Sr26*, may aid the introgression of this gene into other cultivars by adopting MAS.

- The map position of *Ltn*, the gene controlling the development of leaf tip necrosis, was confirmed as 7DS. The gene is linked to the *Yr18* complex on this chromosome, which is desirable in breeding programmes (Singh and Rajaram, 1994).

The study has highlighted major QTL that are important in the expression of APR to stripe rust and other minor QTL that need to be distinguished as either new or existing loci. The aim of the study, i.e., an understanding of the resistance mechanisms involved in the APR of 'Kariega' was therefore achieved, and the results should serve as a basis for further work involving characterizing of the regions identified.

After this work was initiated, a further pathotype (7E22A-) of PST was identified in 2002 (Boshoff, unpublished) bringing the total number of pathotypes present in South Africa to three. The DH population available, and the linkage map generated in this study, will allow this new pathotype to be investigated as was done for pathotype 6E22A- in this study. Recent indications are that resistance to stripe rust in many South African cultivars is monogenic (Bender and Pretorius, 2001) and may therefore have little potential for durability. Boshoff *et al.* (2002) report that 73% of commercial South African cultivars tested were either susceptible or heterogenous for their response to the two stripe rust pathotypes tested (6E16A- and 6E22A-). These findings highlight the importance of the disease and the potential losses that could be incurred by South African wheat farmers.

Based on the development of PST in South Africa, and the ability of the disease to cause large-scale losses as was observed in other areas of the world, the results of this study becomes more significant. It is important to identify new sources of stripe rust resistance in existing breeding lines so that the different sources of resistance can be pyramided in suitably selected cultivars. Gene pyramiding is one of the options available to plant breeders to produce cultivars that are more stable to pathogen infection, and markers closely linked to resistance genes can then aid gene pyramiding through MAS. Studies similar to this aid in the identification of genes and closely linked markers for gene pyramiding.

CHAPTER 7: FUTURE PROSPECTS

The linkage maps generated in this study and the phenotypic data available provide the opportunity to initiate a directed BSA approach. The BSA technique is, however, not as effective as the use of complete linkage maps for complex traits that are controlled by many loci (Langridge and Chalmers, 1998), and was therefore not adopted in this study. Studies have indicated that BSA is more useful in tagging QTL of large effect and that some QTL may be missed (Grattapaglia *et al.,* 1996; Miklas *et al.,* 1996; William *et al.,* 1997). Campbell *et al.* (2001) recently used a BSA approach where progenies were bulked on the basis of flanking markers rather than phenotypic scores to map markers closer to regions of interest and to combine different linkage groups of the same chromosomes.

A set of 'targeted bulks' aimed at targeting QTL regions can be constructed utilizing phenotypic trait information and information from markers closely linked to QTL, thus combining the methods used by Michelmore *et al.* (1991) and Campbell *et al.* (2001). This may reduce some of the false positives associated with bulking samples based entirely on phenotypic data. The SRAP, RGA and additional AFLP markers, together with the 'targeted bulks' should facilitate the identification of markers more tightly linked to the targeted QTL. Sparse linkage maps have been generated for 7DS (Bariana *et al.,* 2001; Boukhatem *et al.,* 2002) so it is important to target this region. In addition, chromosomal location and marker linkage information for other genes identified via BSA, can be obtained by placing the markers on the 'Kariega X Avocet S' linkage map, i.e., comparative mapping techniques can be used together with BSA and the linkage map developed to reduce some of the costs involved with marker characterization

Markers developed using the SRAP technique showed Mendelian segregation and were used to generate the linkage maps described. This study can be extended to map additional SRAP markers. The PCR products mapped can then be sequenced, and the sequences aligned with the recently sequenced rice genome. It will be interesting to see if the technique actually targets expressed sequences in wheat, as was demonstrated in *Brassica* species (Li *et al.,* 2001).

The linkage map can be saturated with additional markers, to produce dense maps for the different regions identified. Marker techniques such as IRAP and REMAP (Kalendar *et al.,*

1999) have been shown to have application in the development of wheat linkage maps (Boyko *et al.,* 2002) and these can be mapped in the 'Kariega X Avocet S' population. It is known that wheat genes are present in clusters (Boyko *et al.,* 2002) so it is likely that the QTL regions identified harbour additional genes e.g. QTL for stripe rust resistance, powdery mildew resistance and leaf rust resistance have been identified on 7DS (Börner *et al.* 2002). Map-based cloning then becomes a possibility, with the opportunity of identifying and cloning many different genes from the same chromosomal region. Once isolated, these genes will have application in MAS programmes, and the development of genetically modified wheat cultivars.

APR is generally recognised as a more durable form of resistance (McIntosh *et al.,* 1995; Chen and Line, 1995). This work can serve as a basis for understanding the molecular basis of this durability by examining the growth of the fungus on the DH lines with different levels of resistance. Insight into the molecular mechanisms occurring between the host and pathogen that govern durability can then be acquired, resulting in a better understanding of resistance mechanisms in plants.

QTL regions identified in this study can be compared with those identified in other cereals utilising the 'grass genome' maps developed by Gale and Devos (1998). This synteny mapping may be used to reduce some of the complexity involved with working with the wheat genome, by comparing it to the smaller, less complex rice genome. Important regions, common among the cereals may be identified and targeted in this manner. The less diverse D genome of wheat may also be targeted in a similar manner by using the maps developed for the D genome donor of bread wheat, *Aegilops tauschii* (Boyko *et al.,* 2002).

The strict quality requirements imposed on wheat cultivars are controlled largely by protein quality and quantity where wheat grain and flour assessments are usually done by measuring milling characteristics such as grain protein, hectoliter mass, etc. 'Kariega' is currently the South African spring wheat quality standard. The linkage map developed offers the opportunity to map the various components controlling wheat quality, provided that the parental lines show differences for these traits. Mapping of the quality components of 'Kariega' offers the prospect of targeting the more important components required by millers and bakers and selecting for these in breeding programmes. Furthermore, 'Kariega' has been used in many of the breeding programmes of the Small Grain Institute and this study can be

extended to map some of the favourable agronomic characteristics of this cultivar and identify markers suitable for MAS.

The identification of some of the QTL detected in the field, under growth chamber conditions (QTL-1) is promising and highlights that further optimization is required before growth chamber data can be used to supplement field disease scores. This study material can be used to refine growth chamber disease scoring of APR by testing the DH lines at different scoring times and under varying environmental conditions, to attempt to synchronize results with those obtained in the field. Growth chamber testing for APR can obviate some of the time, cost and labour required for field testing.

A number of linkage maps for agronomically important, environmentally adapted cultivars have been developed. These include three linkage maps that relate to germplasm used in Australian wheat breeding (Chalmers *et al.,* 2001), a cross involving the French cultivar 'Courtot' (Cadalen *et al.,* 1997) and maps developed by crossing the Swiss varieties 'Forno' and 'Oberkulmer' (Messmer *et al.,* 1999). These linkage studies have proved to be valuable resources to the groups involved, as will the 'Kariega X Avocet S' linkage map, generated in this study.

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Appendix 1: Solutions used for the various techniques in this study

Solutions used for seed germination:

70 % ethanol (w/v)
70 ml ethanol
30 ml distilled water
store at 4 ˚C

30 % JIK(w/v)
30 ml JIK(household bleach)
70 ml distilled water

Solutions used for DNA isolations:

2 % CTAB isolation buffer(w/v)
20 g CTAB (2 %)
280 ml 5M NaCl (1.4 M)
40 ml 0.5 M EDTA pH 8.0 (20 mM)
100 ml 1M Tris-Cl pH 8.0 (100mM)
dissolve on a hot plate. Adjust to pH 8.0
distilled water to 1000ml
Autoclave

0.5 M EDTA
186.12 g $Na_2EDTA.2H_20$
add about 750 ml distilled water
adjust to pH 8.0 with NaOH pellets
until solution becomes clear
distilled water to 1000 ml
Autoclave

Wash buffer

76 ml absolute ethanol
0.1 ml 10 M NH_4OAc
distilled water to 100 ml

24:1 Chloroform isoamlyalcohol

20 ml isoamylalcohol
480 ml chloroform
store covered in foil at 4 ˚C

5 M NaCl

292.2 g NaCl
distilled water to 1000 ml
Autoclave

10 M NH_4OAC

38.5 g NH_4OAc
distilled water to 50 ml

7.5 M NH_4OAc

150 ml 10 M NH_4OAc
distilled water to 1000 ml
Filter sterilize

Solutions required for PAGE:

6 % (w/v) Sequencing gel mix (7M Urea)
37.5 ml 40 % acrylamide stock solution (Promega)
105.105 g Urea (Promega)
25 ml 10X TBE
distilled water to 250 ml
store at 4 ˚C, cover in foil

10X TBE

108 g Tris
55 g Boric Acid
20 ml 0.5 M EDTA pH 8.0
distilled water to 1000 ml – autoclave

10 % Ammonium persulphate (w/v)

1 g ammonium persulphate
distilled water to 10 ml
aliquot and store at –20 ˚C

Solutions used for probe transformations:

100 mg/ml Ampicillin

100 mg ampicillin (USB)
1 ml distilled water
filter sterilize (0.22 μm)

1M $CaCl_2$

54 g $CaCl_2.6H_2O$
200 ml distilled water
filter sterilize (0.22 μm)
store in 1 ml aliquots at –20 ˚C

LB Media

10 g Bacto-tryptone
5 g Bacto-yeast extract
10 g NaCl
pH 7.0 with NaOH
distilled water to 1000 ml
For LB agar add 2g bacto-agar per 100 ml LB

50 mM $CaCl_2$

1 ml 1M $CaCl_2.6H20$
distilled water to 100ml
filter sterilize (0.45 μm)
keep chilled until use

50 % Glycerol (v/v)

5 ml glycerol
5 ml distilled water
store in 800 μl aliquots
Autoclave

Solutions used for RFLP:

20X SSC

175.3 g NaCl
88.2 g sodium citrate
distilled water to 1000 ml pH 7.0

10 % SDS (w/v)

100 g SDS
distilled water to 1000 ml

0.4 N NaOH

16 g NaOH
distilled water to 1000ml

0.2 N HCl (add acid to water)

20 ml concentrated HCl
distilled water to 1000 ml

1.5 M NaCl; 0.5 N NaOH

87.66 g NaCl
20 g NaOH
distilled water to 1000 ml

5X HSB

175.3 g NaCl
30.3 g PIPES
7.45 g $Na_2EDTA.2H_20$
pH to 6.8 with 4 M NaOH
distilled water to 1000 ml

Prehybridisation solution

6 ml distilled water
2 ml 5X HSB
1 ml Denhardts III
1 ml carrier DNA (boil and add last)

Wash buffer 2

890 ml distilled water
10 ml 20X SSC
100 ml 10 % SDS

Carrier DNA

5 g Salmon testes DNA
distilled water to 1000 ml
dispense into 50 ml aliquots and freeze

Denhardts III

2 g gelatin
2 g Ficoll-400
2 g PVP-360
10 g SDS
5 g $Na_4P_2O.10H_20$
distilled water to 100ml – store at 65 ˚C

Wash buffer 1

800 ml distilled water
100 ml 20X SSC
100 ml 10 % SDS (w/v)

Solutions used for AFLP:

1X $TE_{0.1}$ buffer

1 ml 50X TE buffer
0.545 g Tris
set pH to 8.0 with conc. HCl
distilled water to 500ml

5X RL Buffer

50 mM TrisHAc pH 7.5
50mM MgAc
250 mM KAc
25 mM DTT
250 ng/μl BSA

AFLP loading dye

39.2 ml formamide
0.8 ml EDTA (from a 0.5 M stock)
0.02 g bromophenol blue
0.02 g xylene cyanol FF
dissolve, aliquot and store at –20 ˚C

Appendix 2: A list of SSR primers tested, the suggested annealing temperature and chromosomal location.

SSR	Annealing[1] temp.(°C)	CHROM.	SSR	Annealing temp.(°C)	CHROM.
GDM 6	55	2DL	GWM 666	60	3A; 5A; 6B
GDM 33	60	1DS; 1A	GWM 674	60	3A
GDM 62	55	3DS	WMC 149	61	2B
GDM 87	60	2D; 2B	WMC 245	61	2D
GWM 55	60	2B; 2D	GWM 682	55	2B
GWM 68	60	7B; 5B	GWM 702	60	2D
GWM 156	60	5A	GWM 720	60	3A
GWM 162	60	3AL	GWM 726	50	2A
GWM 192	60	5	GWM 735	50	7A; 7D
GWM 194	50	4DL	GWM 742	55	4A
GWM 251	55	4B	GWM 751	50	3A
GWM 260	55	7AS	GWM 757	60	3A
GWM 265	55	2A	GWM 780	50	7D
GWM 320	55	2D	GWM 781	55	4A
GWM 397	55	4A	GWM 783	50	7B
GWM 408	55	5B	GWM 789	60	1D
GWM 415	55	5A	GWM 815	55	2D
GWM 494	60	6A	GWM 820	60	1D
GWM 608	60	2D; 4D	GWM 832	55	4A
GWM 611	55	7BL	GWM 848	55	1D
GWM 613	60	6B	GWM 855	50	4A
GWM 437	50	7DL	GWM 885	60	7D
GWM 148	60	2B	GWM 886	60	2D
GWM 301	55	2D	GWM 903	60	1B; 1D
GWM 484	55	2D	GWM 928	55	7D
GWM 251	55	4B	GWM 937	60	4A
GWM 112	55	3BL; 7BL	GWM 959	50	4A
GWM 106	60	1D	GWM 972	50	2B
GWM 47	60	2A; 2B	GWM 974	50	7D; 7A; 7B
GWM 191	60	2BL; 5B; 6B	GWM 1000	50	7D; 3D
GWM 210	60	2BS; 2DS	GWM 1002	60	7D
GWM 302	60	7B	GWM 1007	60	7D
GWM 311	60	2DL	GWM 1014	55	7D
GWM 344	55	7BL	GWM 1025	55	7B
GWM 349	55	2DL	GWM 1038	55	3A
GWM 356	55	2AL	GWM 1044	60	7D
GWM 388	60	2BL	GWM 1049	55	1D
GWM 400	60	7B	GWM 1071	55	3A
GWM 495	60	4BL	GWM 1081	60	4A
GWM 497	55	2AS	GWM 1093	60	4A
GWM 515	60	2A; 2D	GWM 1110	55	3A
GWM 538	60	4BL	GWM 1121	50	3A
GWM 558	55	2A	GWM 1123	60	7D
GWM 595	60	5AL	GWM 1128	55	2B

GWM 1144	60	7B	CFA 2135	60	1A
GWM 1151	60	2A	CFA 2153	60	1A
CFD 11	60	2D; 2B	CFA 2158	60	1A; 1B; 1D
CFD 14	60	7D	CFA 2174	60	7A; 3D; 7D
CFD 15	60	1A; 1D	CFA 2179	60	4A
CFD 21	60	7D; 1D	CFA 2191	60	3B
CFD 31	60	4A; 7D	CFA 2226	60	3B; 1A
CFD 41	60	7D	WMC 43	60	3D
CFD 46	60	7D	WMC 94	60	7D
CFD 53	60	2D	WMC 154	60	2B
CFD 66	60	7D	WMC 166	60	2D; 7B
CFD 68	60	7D	WMC 213	60	2B
CFD 79	60	5D	WMC 219	60	4A
CFD 143	60	6D; 3B	WMC 232	60	4A
CFA 2040	60	7A; 7D	WMC 262	60	4A
CFA 2099	60	2A; 7D	WMC 265	60	2B
CFA 2114	60	6A	WMC 272	60	2B

[1]Indicates annealing temperature suggested in publication or via personal communication.

Appendix 3: Sequences of the *Sse*I and *Mse*I primers with one and two selective nucleotides only.

Primer	M primer sequence	S primer sequence
00	5' AGACTGCGTACATGCAGG 3'	5' GATGAGTCCTGAGTAA 3'
01	5' GACTGCGTACATGCAGGA 3'	
02	5' GACTGCGTACATGCAGGC 3'	
03	5' GACTGCGTACATGCAGGG 3'	
04	5' GACTGCGTACATGCAGGT 3'	
11	5' GACTGCGTACATGCAGGAA 3'	5' GATGAGTCCTGAGTAAAA 3'
12	5' GACTGCGTACATGCAGGAC 3'	5' GATGAGTCCTGAGTAAAC 3'
13	5' GACTGCGTACATGCAGGAG 3'	5' GATGAGTCCTGAGTAAAG 3'
14	5' GACTGCGTACATGCAGGAT 3'	5' GATGAGTCCTGAGTAAAT 3'
15	5' GACTGCGTACATGCAGGCA 3'	5' GATGAGTCCTGAGTAACA 3'
16	5' GACTGCGTACATGCAGGCC 3'	5' GATGAGTCCTGAGTAACC 3'
17	5' GACTGCGTACATGCAGGCG 3'	5' GATGAGTCCTGAGTAACG 3'
18	5' GACTGCGTACATGCAGGCT 3'	5' GATGAGTCCTGAGTAACT 3'
19	5' GACTGCGTACATGCAGGGA 3'	5' GATGAGTCCTGAGTAAGA 3'
20	5' GACTGCGTACATGCAGGGC 3'	5' GATGAGTCCTGAGTAAGC 3'
21	5' GACTGCGTACATGCAGGGG 3'	5' GATGAGTCCTGAGTAAGG 3'
22	5' GACTGCGTACATGCAGGGT 3'	5' GATGAGTCCTGAGTAAGT 3'
23	5' GACTGCGTACATGCAGGTA 3'	5' GATGAGTCCTGAGTAATA 3'
24	5' GACTGCGTACATGCAGGTC 3'	5' GATGAGTCCTGAGTAATC 3'
25	5' GACTGCGTACATGCAGGTG 3'	5' GATGAGTCCTGAGTAATG 3'
26	5' GACTGCGTACATGCAGGTT 3'	5' GATGAGTCCTGAGTAATT 3'

Appendix 4: +3 M primer pairs. The core sequence remains the same and only the selective nucleotides differ. For example the selective nucleotide sequence for M53 is CCG.

		First selective nucleotide			
Second	Third	A	C	G	T
A	A	31	47	63	79
	C	32	48	64	80
	G	33	49	65	81
	T	34	50	66	82
C	A	35	51	67	83
	C	36	52	68	84
	G	37	53	69	85
	T	38	54	70	86
G	A	39	55	71	87
	C	40	56	72	88
	G	41	57	73	89
	T	42	58	74	90
T	A	43	59	75	91
	C	44	60	76	92
	G	45	61	77	93
	T	46	62	78	94

Appendix 5: List of RGA primer combinations tested and mapped in this study

	NLRRfor	RLRRfor	Ptokin1	Ptokin2	Ptokin3	Ptokni4	Ptokin1IN	Ptokin2IN	CLRRINV1	XLRRINV1	Xa1NBSfor	Xa1LRfor	RLKfor	AS3
NLRRrev	X[1]	X			X									X
RLRRrev	X	X			X									
Ptokin1				X	X	X	X	X						X
Ptokin2			X		X	X	X	X						X
Ptokin3			X	X		X	X	X						
Ptokin4			X	X	X		X	X						X
Ptokin1IN					X									X
CLRRINV1											X	X		
CLRRINV2									X		X	X		
XLRRINV1											X	X		
XLRRINV2										X	X	X		
Xa1NBSrev											X	X		
Xa1Lrrev											X	X		
RLKrev							X	X					X	
AS3							X	X						

[X]Indicates primer combination was tested.

Marker	Primer combination
RGA1	Pto kin 3/Pto kin 2_1
RGA2	Pto kin 3/Pto kin 2_2
RGA3	Pto kin 3/Pto kin 2_3
RGA4	Pto kin 2/Pto kin 4_1
RGA5	Pto kin 2/Pto kin 4_2
RGA6	Pto kin 2/Pto kin 4_3
RGA7	Pto kin 1IN/Pto kin 4_1
RGA8	Pto kin 1IN/Pto kin 4_2

Appendix 6: SRAP primer sequences and markers

Primer	Sequence (5' to 3')	Base pairs
me1	5'- TGAGTCCAAACCGGATA - 3'	17
me2	5'- TGAGTCCAAACCGGAGC - 3'	17
me3	5'- TGAGTCCAAACCGGAAT - 3'	17
me4	5'- TGAGTCCAAACCGGACC - 3'	17
me5	5'- TGAGTCCAAACCGGAAG - 3'	17
em1	5'- GACTGCGTACGAATTAAT – 3'	18
em2	5'- GACTGCGTACGAATTTGC – 3'	18
em3	5'- GACTGCGTACGAATTGAC – 3'	18
em4	5'- GACTGCGTACGAATTTGA – 3'	18
em5	5'- GACTGCGTACGAATTAAC – 3'	18
em6	5'- GACTGCGTACGAATTGCA – 3'	18

Marker	Primer combination
SRAP1	em1me1_1
SRAP2	em1me1_2
SRAP3	em2me2_1
SRAP4	em1me2_1
SRAP5	em1me2_2
SRAP6	em2me3_1
SRAP7	em2me3_2
SRAP8	em2me3_3
SRAP9	em2me3_4
SRAP10	em1me5_1
SRAP11	em1me5_2
SRAP12	em1me5_3
SRAP13	em1me4_1
SRAP14	em1me4_2
SRAP15	em1me4_3
SRAP16	em6me4_1
SRAP17	em6me4_2
SRAP18	em6me4_3

Appendix 7: List of the main effect and interaction effect markers used for modified interval mapping. A significance threshold of P=0.000001 was used to choose interaction effect markers, in order to avoid false positives.

	Growth chamber	Transformed reaction type		Tranformed % LAI	
		Early score	Final score	Early score	Final score
Main Effect Markers	*s23m53B-4A*	*Xgwm372-2B*	*Xgwm372-2B*	*Xgwm372-2B*	*Xgwm372-2B*
	Xgwm160-4A	*Xgwm148-2B*	*Xgwm148-2B*	*Xgwm148-2B*	*Xgwm148-2B*
	s19m86C-4A	*Ltn-7D*	*Ltn-7D*	*Ltn-7D*	*Ltn-7D*
	s18m47B-7A	*Xgwm295-7D*	*Xgwm295-7D*	*Xgwm295-7D*	*Xgwm295-7D*
	Ltn-7D	*Xwmc219-4A*	*s15m19D-1A*	*Xwmc219-4A*	*s15m19D-1A*
				s18m93E-4A	*Xcfd31-3D*
				Xcfd31-3D	
Interaction Effect Markers	*s16m51A-2B*	*s15m19D-1A*	*Xwmc219-4A*	*Xpsp3200-6D*	*Xwmc219-4A*
	s11m61C-5D	*s23m18E-1A*	*s23m18E-1A*	*SRAP6-3B*	*s23m18E-1A*
	Xgwm169-6A	*s22m85A-4A*	*Xgwm349-2D*		
	s11m61D-5D	*Xgwm349-2D*	*Xgwm186-5A*		
			Xgwm332-7A		
			s21m40A-4A		