A novel marker technique: Using miniature inverted-repeat transposable elements (MITEs) in combination with resistant gene analogues (RGAs)

by

Carol-Ann Lambert

Dissertation presented for the Degree of Doctor of Philosophy at the University of Stellenbosch

December 2001

Supervisor: Professor AE Retief

Stellenbosch University http://scholar.sun.ac.za

Declaration

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

Signature: Date:

Stellenbosch University http://scholar.sun.ac.za

SUMMARY

Given the organisation of the maize genome as well as demands placed on the saturation of molecular linkage maps it would be desirable to identify informative molecular markers that is located or linked to genic rich areas.

Sequences of gene products from different gene classes were investigated. Proteins containing a nucleotide binding site (NBS) and leucine-rich repeat (LRR) region comprise the largest class of disease resistance proteins. Resistant gene analogue (RGA) primers belonging to this specific class were derived from previous published literature studies. By means of similarity studies of short stretches of conserved amino acid and DNA sequences, primers were developed that belonged to the peroxidase and reductase gene classes. A novel class of transposable element was identified, that occurred in the gene rich areas of a diverse range of grass genomes. Of all the MITE families described so far, the *Heartbreaker* (*Hbr*) and *Hb*2 family elements were of particular interest.

The unique properties of MITEs, especially their high copy number, polymorphism, stability and preference for genic areas together with the RGA primers, were exploited to develop a new marker technique for the isolation of a class of molecular marker with a strong preference for genic areas.

Using the publicly available recombinant inbred population, Tx303 x CO159, 196 MITE/RGA markers were added to the existing recombinant inbred linkage map consisting of ±1033 already established markers. It became apparent that just like loci for disease resistance, the 196 MITE/RGA fragments were not randomly distributed across the maize genome but occurred in clusters spread across the ten maize chromosomes. Ninety-two (92) of the MITE/RGA fragments showed significant correlation to previously mapped maize resistance genes. To establish the conservation and specificity of both the *Hbr* and *Hb*2 elements, sequences of 19 MITE/RGA fragments were ascertained. When comparing the partial MITE element sequences from these fragments, a high degree of element conservation was

Stellenbosch University http://scholar.sun.ac.za

observed. One fragment showed good sequence correlation to a NADPH HC Toxin reductase protein product and mapped to the same chromosomal location as the *hm*1 gene locus in maize. This fragment can be considered a candidate gene for resistance against the pathogen, *Helminthosporium carbonum*. The *Hbr* primer used proved to be very specific for the *Heartbreaker* MITE element, this was in contrast to the non-specificity of the *Hb*2 primer.

The applicability of this technique was tested on two maize diseases that cause immense damage in the maize production industries in South Africa. Fourteen MITE/RGA markers were used to fine map the putative chromosomal locations for the *HtN*1, *Ht*1, *Ht*2 and *Ht*3 genes that confer resistance against *Setosphaeria turcica*, the northern corn leaf blight (NCLB) pathogen in maize. Three MITE/RGA fragments were identified that aided in the saturation of the linkage map for quantitative trait resistance (QTL) against gray leaf spot (GLS) in maize.

This novel MITE/RGA technique presented a unique opportunity to search for additional candidate genes by using polymerase chain reaction (PCR) analysis. When compared to the conventional amplified fragment length polymorphism (AFLP) technique, the MITE/RGA technique proved to be just as efficient but was more cost effective and less time consuming.

Stellenbosch University http://scholar.sun.ac.za

OPSOMMING

Die organisasie van die mielie genoom as ook die vereistes wat daar geplaas word op die versadiging van koppelingskaarte, vereis dat daar meer klem geplaas word op die ontwikkeling van molekulêre tegnieke wat merkers in geenryke areas identifiseer.

Die volgordes van geenprodukte, wat behoort tot verskillende geenklasse, is deeglik bestudeer. Proteïenprodukte wat bestaan uit 'n nukleotiedbindingsarea (NBA) en 'n leusienryke herhalende (LRH) area is een van die grootste klasse waaronder siekteweerstandsproteïene sorteer. Polimerase kettingreaksie (PKR) inleiers wat behoort tot hierdie spesifieke klas, is verkry vanuit vorige publikasies. Deur kort gekonserveerde aminosuur en DNS volgordes te vergelyk is inleiers ontwikkel wat behoort tot die peroksidase en reduktase gene klasse. 'n Nuwe klas transponeerbare elemente wat voorkom in die geenryke areas van diverse gras genome, is geïdentifiseer. Van al die miniatuur inversie herhalende transponeerbare elemente (MITE) wat al geïdentifiseer is, is die twee elemente, *Heartbreaker* (*Hbr*) en *Hb*2, van groot belang.

Unieke eienskappe van die MITEs, veral hul hoë kopie aantal, polimorfiese-indeks, stabiliteit asook voorkeur vir geenryke areas, tesame met die weerstandsgeen analoë (WGA) inleiers, is gebruik om 'n nuwe merker tegniek te ontwikkel. Hierdie nuwe tegniek identifiseer 'n klas merker wat 'n sterk voorkeur het vir geenryke areas.

Deur gebruik te maak van die openbare beskikbare rekombinante ingeteelde (RI) populasie, Tx303 x CO159, is 196 MITE/WGA-merkers gekarteer op die bestaande RIL koppelingskaart, wat alreeds bestaan uit ±1033 gevestigde merkers. Net soos die lokusse vir siekteweerstand het dit geblyk dat hierdie 196 merkers in groepe voorkom wat verspreid is oor die tien mielie chromosome. Twee-en-negentig (92) van die 196 gekarteerde MITE/WGA-merkers het betekenisvolle korrelasie gewys met reeds gekarteerde mielie weerstandsgene. Die volgordes van 19 MITE/WGA-fragmente is bepaal om sodoende die spesifisiteit en mate van konservering van die *Hbr* and *Hb*2 elemente te bereken. 'n Hoë mate van element konservering is

Stellenbosch University http://scholar.sun.ac.za

waargeneem. Een fragment het 'n baie goeie volgorde korrelasie gewys met 'n NADPH HC toksien reduktase proteïen produk en karteer op dieselfde chromosomale posisie as die *hm1* geen lokus. Hierdie fragment kan gesien word as 'n kandidaatgeen vir weerstand teen die mielie patogeen, *Helminthosporium carbonum*.

Die toepasbaarheid van hierdie tegniek is getoets op twee siekte toestande, wat lei tot groot verliese in die mielie industrie, in Suid-Afrika. Veertien van die MITE/WGA-merkers is gebruik om die waarskynlike chromosomale posisies van die *Ht*N1, *Ht*1, *Ht*2 en *Ht*3 gene, wat weerstand bied teen *Setosphaeria turcica*, die noordelike mielie blaarvlek (NMBV) patogeen, fyner te karteer. Drie MITE/WGA fragmente is geïdentifiseer wat gehelp het in die versadiging van die koppelingskaart vir die kwantitatiewe kenmerk weerstandbiedenheid (KKW) teen grys blaarvlek (GBV) in mielies.

Deur gebruik te maak van polimerase kettingreaksie (PKR) analise, verskaf hierdie tegniek die moontlikheid om te soek vir addisionele kandidaatgene. Hierdie tegniek is ook vergelyk met die konvensionele geamplifiseerde fragment lengte polimorfisme (AFLP) tegniek. Daar is gevind dat die nuwe tegniek net so informatief is, maar wel meer koste effektief en tyd besparend.

Stellenbosch University http://scholar.sun.ac.za

Acknowledgements

I would like to acknowledge:

- My supervisor, Prof AE Retief for his continuous support, assistance and encouragement.
- My companion and friend, Derick van Staden, for always being there for me and believing in me.
- Sensako (Pty) LTD, the Harry Crossley Trust and the NRF/THRIP funding program for their financial support.
- Sensako (Pty) LTD. for providing some plant material.
- Dr B Burr, Brookhaven National laboratory, New York, for providing the mapping populations.
- My colleagues and friends for their support
- The University of Stellenbosch for supplying the facilities
- May the glory go to our almighty God

Stellenbosch University http://scholar.sun.ac.za

TABLE OF CONTENTS

Stellenbosch University http://scholar.sun.ac.za

TABLE OF CONTENTS (cont.)

Stellenbosch University http://scholar.sun.ac.za

TABLE OF CONTENTS (cont.)

Stellenbosch University http://scholar.sun.ac.za

TABLE OF CONTENTS (cont.)

Stellenbosch University http://scholar.sun.ac.za

List of tables

Stellenbosch University http://scholar.sun.ac.za

List of figures

Stellenbosch University http://scholar.sun.ac.za

ABBREVIATIONS

γ	Gamma
°C	Degrees Celsius
µl	Microliter
Ac	Activator
AFLP	Amplified fragment length polymorphisms
AP-PCR	Arbitrary primed polymerase chain reaction
ASAP	Allele specific associated primers
BC	Backcross
bp	Base pair
CG	Candidate genes
cM	CentiMorgan
CTAB	Cetyltrimethylammonium bromide
DAF	DNA amplification fingerprints
DH	Double haploid
DIR	Direct repeat
DNA	Deoxyribonucleic acid
Ds	Dissociation
EDTA	Ethylenediaminetetra-acetic acid
F_1	First filial generation
F_2	Second filial generation
GLS	Gray leaf spot
Hbr-hm1	*Heartbreaker-hm1*
Ht	*Helminthosporium turcicum*
IRAP	Inter-retrotransposon amplified polymorphism
KCl	Potassium Chloride
LINE	Long interspersed nuclear elements
LRR	Leucine-rich repeat
LTR	Long terminal repeats
MAAP	Multiple arbitrary amplicon profiling
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
MITE	Miniature inverted-repeat transposable element
ml	Millilitre
mM	Millimolar
Mu	Mutator
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS	Nucleotide binding site
NCLB	Northern corn leaf blight
ng	Nanogram
NIL	Near isogenic line
PCR	Polymerase chain reaction
Per	Peroxidase
QTL	Quantitative trait loci

Stellenbosch University http://scholar.sun.ac.za

RAMPS	Random amplified microsatellite polymorphism
RAMS	Random amplified microsatellites
RAPD	Random amplified polymorphic DNA
Red	Reductase
REMAP	Retrotransposon-microsatellite amplified polymorphism
RGC	Resistant gene candidates
RFLP	Restriction fragment length polymorphism
RGA	Resistant gene analogue
R-genes	Resistance genes
RIL	Recombinant inbred line
SCAR	Sequenced characterised amplified region
sec	Second
SINE	Small interspersed nuclear elements
SPAR	Single primer amplification reaction
S-SAP	Sequence-specific amplification polymorphism
TD	Transposable-display
TE	Transposable element
Tm	Annealing temperature
Tris	Tris (hydroxymethyl) aminomethane
UPGMA	Unweighted pair-group arithmetic average
US	University of Stellenbosch
V	Voltage

Stellenbosch University http://scholar.sun.ac.za

INTRODUCTION
CHAPTER ONE

Cultivated cereal grains have helped to sustain an ever-growing world population, providing a valuable source of nutrition in the form of wheat, maize, barley and rice. Maize is undoubtedly one of South Africa's most important field crops. Over 10 million hectare of maize is planted annually in South Africa, presenting an average of 40% covering total land under cultivation.

Maize genome organisation

Intensive studies confirmed that the origin and genetic structure of *Zea mays* L. evolved as a hybrid between a wild teosinte, most likely *Z.mays* subsp. *parviglumis* and an unknown grass in the tribe Andropogoneae, more than 7000 years ago (Takahashi et al. 1999). The maize genome consists of approximately 3 x 10^9 base pairs of DNA per haploid genome (2n = 2x = 20). The ploidy level of the maize genome is a much-debated topic, it has always been widely regarded as a diploid organism (Mangelsdorf 1974). Recent genetic and cytological studies strongly suggested that maize is a segmental allotetraploid, consisting of two complete diploid genomes (Helentjaris et al. 1988; Moore et al. 1995; Gaunt and Doebley 1997). Genetic studies have shown that as many as 40% of RFLP and cDNA molecular probes commonly detect two or more unlinked loci in the maize genome (Helentjaris et al. 1988). The maize genome contains 60–80% repetitive DNA sequences interspersed within unrelated repeated DNA or unique sequences (Hake and Walbot 1980; Michaelson et al. 1991). White and Doebley (1998) speculated that the size of the maize genome is a result of amplification and reduction in the copy number of long terminal repeat (LTR)-retrotransposons in the intergenic regions of the genome.

1.1 Transposable elements

In the early 1950's Barbara McClintock, working on maize at the Cold Spring Harbor Laboratory, called the attention of researchers to the behaviour of genetic elements she called "controlling elements". These elements were first noticed because they inhibited the normal expression of the genes that they came in close contact with and

Stellenbosch University http://scholar.sun.ac.za

they did not have any fixed chromosomal location. Instead they seemed to move about in the genome, depending on their host for propagation and survival. These "controlling elements" could be inserted and later excised, after which the putative function of the effected gene would often return. The genes that could so easily be switched on and off were rendered unstable. With recombinant DNA technology studies flowed a wealth of data regarding the organisation and means of transposition of these "elements", now called transposable elements (Capy et al. 1994). Characteristically, active transposons are only found in a few populations within species (Capy et al. 1994) and randomly insert into a specific target sequence at a frequency of 10^{-6} – 10^{-5} (Chang and Peterson 1994). Just like all other expressed sequences, transposable elements are differentially active in different tissue, at different times of development and under different induction systems (Bennetzen 2000). Viewed by many as selfish or parasitic DNA, it was clear that a high level of transposable element activity could be deleterious to individuals.

According to their method and mechanism of transposition transposable elements were classified into two classes (Lai 1994). Class I elements, transpose directly from DNA to DNA e.g. *Ac/Ds, Spm/dspm (En/I)* and *Mutator* systems in maize and the *P* element of *Drosophila* (Lai 1994.) Class II elements, called the retrotransposons, "move" by reverse transcription of an RNA-intermediate e.g. *Ty* elements in yeast, *Cin4* elements in maize and the *copia*-like elements in *Drosophila*. Then there is the recently discovered group of transposable elements, a novel class III element, the miniature inverted-repeat transposable elements (MITE) (Wessler 1999).

1.1.1 Class I elements:

This family of DNA transposable elements are defined by the fact that they all share the same terminal inverted repeats (TIRs), ranging in size from 11bp (*Ac/Ds)* to a few hundred bases *(Mutator)* (Bennetzen 2000).

***Ac/Ds* and *Spm/dspm* transposable elements of maize**

McClintock (1950) identified two types of transposable elements in maize. The *Ac (Activator)* element cause mutations that reverts at high frequencies, for example the *Ac* mutations in the genes for kernel colour produce mottled kernels with patches of

Stellenbosch University http://scholar.sun.ac.za

normal colour (were the mutation has reverted back). *Ds (Dissociation)* elements cause stable mutations, kernels with these mutations stays colourless unless these *Ds* plants are crossed to a inbred containing an *Ac* mutation. *Ac* and *Ds* elements are derivates of the same transposable element (Bennetzen 2000). The tendency of the *Ac* and *Ds* elements to have a preference for genic rich sites, guided researchers to target insertion sites that are linked to the *Ac* or *Ds* elements (Casa et al. 2000). Studies linked to this type of research led the way to the isolation of numerous mutant alleles to be isolated for genetic (Kermicle 1980) and molecular studies (Athma et al. 1992; Weil et al. 1992). *Spm* is the autonomous member of the *Spm/dspm* family. In most cases the non-autonomous element of a family, such as the *Ds* and the *dspm* elements, contains deletions or are derivates of the autonomous element and is able to transpose by transactivation of the active element in the family (Bennetzen 2000).

Mutator (Mu) transposable elements of maize

The mutator elements in maize are one of the most active transposable element families described in any organism. Robertson (1978) described a mutator (*Mu)* transposable element in maize that increases the forward mutational rate by approximately 30-fold. He characterised the mutator quality of these maize stocks and named it "mutator lines". The mutagenic activities, defining the mutator lines are transmitted as a dominant phenotype when crossed with non-Mutator stocks (Robertson 1978). *Mu* elements are composed of at least six distinct subfamilies, sharing a conserved ~75% of a 210-215bp sequence, the terminal inverted repeat (TIR) (Bennetzen 1996). The majority of *Mu* elements are non-autonomous; their transposition requires the presence of at least one regulatory element referred to as *MuDR* (*MuR* or *MuA)* (Chomet et al. 1991; Green et al. 1994; Lisch et al. 1995). Mutator maize lines contain 10-50 mobile copies of the *Mu* element, whereas most maize strains as well as other plants have none (Alleman and Freeling 1986). Unlike the well-defined controlling *Ac/Ds* elements, *Mu* elements appear to segregate in a non-Mendelic or multigenic manner (Robertson 1978) and there are a myriad of ways that these class I elements can alter and modify normal gene expression: (i) they can masquerade as introns (Purugganan and Wessler 1992), (ii) induce alternative

Stellenbosch University http://scholar.sun.ac.za

splicing sites (Varagona et al. 1992) and (iii) change a gene's tissue-specific expression (Marillonnet and Wessler 1997).

Analysis of data suggested that the contribution of *Ac/Ds*, *Spm/dspm* and *Mu* to normal gene expression and evolution are minimal and sequences from these elements are rarely found near wild-type genes in the maize genome (Wessler 1998). Studies showed that DNA surrounding the *Ac/Ds, Spm/dspm* and *Mu* elements were primarily of a low copy number (Cresse et al. 1995). The copy number and activity of these elements are strongly controlled by the host plants, thus elements like these with a haploid copy number lower than 100 are likely to have no significant impact on the genome and phenotypic expression.

1.1.2 Class II elements: Retrotransposons

Retrotransposons or RNA transposable elements are members of the class II retroelements. The dispersion (Katsiotis et al. 1996), ubiquity (Voytas et al. 1992) and prevalence of retrotransposons in plant genomes provide an excellent basis for the development of an effective marker system. Retrotransposons constitute over 70% of the nuclear DNA in the maize genome and are even more numerous in plant species with larger and more complex genomes (Bennetzen 2000). Retrotransposons replicate by successive transcription, reverse transcription and insertion of the new cDNA copies back into the genome of its host, hence these elements do not excise when they transpose. The first mobile retrotransposon to be isolated in plants was the *Bs1*-element in maize, it was identified having transposed into the *adh1*-gene (Jin and Bennetzen 1989).

There are five types of retrotransposons and in each plant genome the retroelements are represented by at least four types. Long interspersed nuclear elements (LINEs), are presumably the most ancient type of element (Bennetzen 2000). The most numerous and largest type of retroelement are those flanked by long terminal repeats (LTR), coding for the proteins necessary for their movement/transposition (Grandbastien 1992). LTR-retrotransposons vary in size from several hundred base pairs to over 10kb, with the LTR regions varying from a few hundred to several thousand base pairs. These long defined conserved sequences can be used for

Stellenbosch University http://scholar.sun.ac.za

cloning of specific markers and flanking sequences (Kalender et al. 1999). The most abundant LTR-retrotransposon found in the maize genome is associated with the methylated, presumably heterochromatic DNA regions. Depending on the order of the coding domains, retrotransposons can be classified into two subclasses, they can either be *copia*-like or *gypsy*-like (Wessler 1998). The *Gypsy*-like retrotransposons acquire a gene that allows itself to be included in the cell membrane, leading to intercellular infections, hence they are called retroviruses and are believed to be found only in animals (Bennetzen 2000). The last class of retrotransposons is the small interspersed nuclear elements (SINEs) and are usually 100 to 300bp in size. In humans the *Alu*-SINE sequences are abundant (Schmid 1996), but in plant genomes SINE-elements are somewhat rare (Bennetzen 2000).

Intensive studies revealed that there are two classes of transposable elements that proof to be important components of especially maize genes (i) retrotransposons and (ii) MITE.

1.1.3 Class III: MITEs: A novel Class of transposable element in plant genes

Miniature inverted-repeat transposable elements (MITEs) are non-autonomous DNA elements that are most prevalent in the grass genomes, but are also wide-spread in different plants, nematodes, vertebrates and insects (Wang and Wessler 1999; Magbanua et al. 2001; Zhang et al. 2001). Rather than containing large numbers of LINEs and SINEs, as in mammalian genomes, many genes in flowering plants harbour MITEs. In the maize genome most MITE elements were found to be members of the *Tourist*- (Bureau and Wessler 1992; Bureau and Wessler 1994a) of which *Heartbreaker* (2000-4000 copies; Zhang et al. 2000) and *Hb2* (12 000 copies; Spell et al. 1988; Magbanua et al. 2001) are two subfamilies (Zhang et al. 1998), *Stowaway*- (80-323bp; Bureau and Wessler 1994b) and *mPIF*- (over 5000 copies; Zhang et al. 2001) MITE families.

A 314bp element called *Heartbreaker-hm1* (*Hbr-hm1*) was identified in the 3' untranslated region of a mutant allele in the maize disease resistance gene *hm1* (Casa et al. 2000Preliminary studies have shown that these non-autonomous DNA

Stellenbosch University http://scholar.sun.ac.za

elements are small (~100 to 500bp), have ~2000-4000 copies per haploid genome (Zhang et al. 1998; Casa et al. 2000) with a 90% sequence similarity (Wang and Wessler 1999) and a preference for insertion into 2-3bp A and T rich target sites (Casa et al. 2000) (*Tourist:* TAA-preference; *Stowaway:* TA-preference: Pozueta-Romero et al. 1996; Wessler 1998). Plant introns, like most MITE families, have a high A/T content whereas A/T richness have been shown to be a cis-requirement for the efficient splicing of introns in plant genomes (Goodall and Filipowicz 1989). Since most plant coding sequences (exons) are much more G/C rich than non-coding sequences (introns), MITEs will likely be inserted into regions of the plant genome were they will cause least "damage" to their host (Wessler 1998).

There are sequence similarities within MITE families but little, if any, sequence similarities between families. MITEs do share structural features, they have short stretches (125-500bp) of non-coding sequence, conserved terminal inverted repeats (TIR; 10-15bp) and they have the potential to form secondary DNA structures (Wessler 1998; Casa et al. 2000; Zhang et al. 2000). These structural similarities suggested that in the beginning MITE families transposed by a common mechanism. MITEs exhibit non-coding features and no element has yet been observed to excise, meaning that they exhibit a very low transposition activity or are no longer active (Wessler 1998; Casa et al. 2000; Zhang et al. 2000). The *Hbr* and *Hb2* elements are highly polymorphic between different maize inbred lines and yet they are stable from generation to generation (Wang and Wessler 1999). Just recently new classes of MITE families were identified in the genes of potato and bell pepper, namely *Alien* (Pozueta-Romero et al. 1995; Pozueta-Romero et al. 1996), *Emigrant* from *Arabidopsis* (Casacuberta et al. 1998) and *Bigfoot* from *Medicago* (Charrier et al. 1999). Nagel et al. (2001) identified 14 distinct MITE families that composed an estimated 7% of the *Oryza sativa* genome.

Attractive characteristics such as high copy number, sequence homology, polymorphisms and preference for genic rich areas make MITEs highly desirable attribute to be used in molecular marker techniques.

Stellenbosch University http://scholar.sun.ac.za

1.2 Resistant gene analogues (RGAs)

Agricultural crops are constantly challenged by pathogens and insects, biological control of host plant resistance remains the most efficient and environment friendly means of reducing parasitic losses. The specificity of pathogen recognition is often determined by a pathogen avirulence (*avr*) gene and a corresponding plant resistance (*R*) gene (Hammond-Kosack and Jones 1997). Biochemical studies have shown that two classes of host gene contribute to the specific host resistance reaction: (i) resistance genes involved in the recognition process and, (ii) resistance genes involved in the defence response (Li et al. 1998).

Many R-genes contain similar sequence motifs, although they determine resistance to very different pathogens. Isolation of resistance genes revealed five main classes of *R*-gene sequences, whose products appear to activate a similar range of defence mechanisms (table 1.1) (Hammond-Kosack and Jones 1997). Very recently, the cloning of resistant genes from different plant species revealed a large degree of conservation amongst some protein domains at the amino acid level for different classes of *R*-genes. One of the first resistant genes to be cloned and isolated was for *Helminthosporium carbonum* susceptibility1 (*hm*1) of maize, which confers resistance to the leaf spot fungus *Helminthosporium maydis.* The *hm*1 gene was mapped to maize chromosome 1 and codes for the enzyme, NADPH-dependent HC toxin reductase (Johal and Briggs 1992).

Molecular characterised disease resistance genes can be categorised in several classes based upon the function or amino acid sequence of the proteins they encode. The majority of resistance genes belongs to a class consisting either of an N-terminal nucleotide binding site (NBS) and a C-terminal stretch of leucine-rich repeats (LRR) (e.g., *RPS2-Arabidopsis* gene; Bent et al. 1994) and the *N*-gene of tobacco (Whitham et al. 1994), or to a class that consists only of a LRR-domain and no nucleotide binding site (Collins et al. 1998). The largest class of R genes, the nucleotide binding site (NBS)-leucine rich repeat (LRR) class (NBS-LRR) (Hammond-Kosack and Jones 1997) is also referred to as the superfamily of genes (Gedil et al. 2001). NBS-LRR proteins are numerous; the *Arabidopsis* genome is estimated to

Stellenbosch University http://scholar.sun.ac.za

have ~200 members in this family (Meyers et al. 1999) and they confer resistance against a wide variety of pathogens and pests, including nematodes, viruses, bacteria, fungi and insects (Hammond-Kosack and Jones 1997; Milligan et al. 1998; Rossi et al. 1998). Sequence comparison and hybridization experiments involving different resistance genes of different plant species, revealed structural similarities and short stretches of amino acid sequences conserved among the NBS-LRR regions, which suggested a common putative functional role of defence response for the encoded protein (Aarts et al. 1998). LRR proteins are involved in specific protein-protein interactions and are confined predominantly to eukaryotes. Resistance genes with putative NBS- and LRR- domains have been cloned from a number of dicot plant species, and confers resistance to bacteria, fungi and a virus (Collins et al. 1998).

Stellenbosch University http://scholar.sun.ac.za

Table 1.1 Five classes of cloned plant disease resistance genes as defined by Hammond-Kosack and Jones (1997).

Class	Gene	Plant	Pathogen	Infection type/organ attack	Predicted features of R protein
1	*Hm*1	Maize	*Helminthosporium maydis* (race 1)	Fungal necrotroph/leaf	Detoxifying enzyme HC-toxin reductase
2	*Pto*	Tomato	*Pseudimonas syringe* p.v. tomato (AvrPto)	Extracellular bacteria/leaf	Intracellular serine/ threonine protein kinase
3a	*RPS*2	Arabidopsis	*Pseudimonas syringe* p.v. tomato (AvrPpt2)	Extracellular bacteria/leaf	L.Zip/NBS/LRR Intracellular protein with amino terminal leucine zipper domain, and nucleotide binding site (NBS) and leucine rich repeat (LRR) domains
	*RPM*1	Arabidopsis	*Pseudimonas syringe* p.v. maculicola (AvrRPM1/avrB)	Extracellular bacteria/leaf	
	I_2	Tomato	*Fusarium oxysporium* f.sp. lycopersicon	Necrotrophic fungus/root and vascular tissue	
3b	*N*	Tobacco	*Mosaic virus*	Intracellular virus/leaf and phloem	Toll/NBS/LRR Intracellular protein with amino terminal domain homology with Drosophila Toll protein, and NBS and LRR domains.
	*L*6	Flax	*Melampsora llnl* (AL6, AM)	Biotrophic fungal rust with haustoria/leaf	
	M	Flax			
	*RPP*5	Arabidopsis	*Peronaspora parasitica*	Biotrophic downy mildew fungus with haustoria/leaf	

UNIVERSITEIT STELLENBOSCH BIBLIOTEEK

Stellenbosch University http://scholar.sun.ac.za

Table 1.1 (cont.)

Class	Gene	Plant	Pathogen	Infection type/organ attack	Predicted features of R protein
4	*Cf*-9 *Cf*-2 *Cf*-4 *Cf*-5	Tomato	*Cladosporium fulvum* (Avr9, Avr2, Avr4, Avr5)	Biotrophic extracellular fungus without haustoria/leaf	Extracellular LRR protein with single membrane spanning region and short cytoplasmic carboxyl terminus
5	*Xa*-21	Rice	*Xanthomonas oryzae* p.v. oryzae (all races)	Extracellular bacteria/leaf	Extracellular LRR protein with single membrane spanning region and cytoplasmic kinase domain

Stellenbosch University http://scholar.sun.ac.za

Generally, only the effect of the resistance gene on the phenotype can be recognized, and little if any information exists regarding the resistance gene product. Two strategies for the molecular cloning of resistance genes are usually: (i) transposon tagging and, (ii) map-based cloning. For transposon tagging the presence of a functional transposon system in the plant species of interest is required. Map-based cloning can be applied in any plant species with a sexual reproduction system (Schwarz et al. 1999). Using a map-based cloning strategy, several race-specific resistance genes have been isolated. Schwarz et al. (1999) identified an AFLP marker flanking the locus for powdery mildew resistance in barley.

Designing of NBS-LRR degenerate primers led to the rapid and efficient isolation of resistant gene candidates (RGC) in several plant species; i.e. potato, (Leister et al. 1996), soybean (Yu et al. 1996), wheat (Feulliet et al. 1997), *Arabidopsis* (Aarts et al. 1998; Speulman et al. 1998), lettuce (Shen et al. 1998) and barley (Schwarz et al. 1999). Close linkage as well as co-segregation, of the RGC with known resistance genes were well documented in these investigations.

1.2.1 Resistance to *Setosphaeria turcica*

Northern corn leaf blight (NCLB) in maize, is caused by the ascomycete *Setosphaeria turcica* (Lutrell) Leonard & Suggs, in its anamorphic stage *Exserohilum turcicum* (Pass.) Leonard & Suggs [syn. *Helminthosporium turcicum* (pass.)]. The disease occurs sporadically in humid areas where maize is grown and results in grain yield losses of 30% or more (Lim et al. 1974). Resistance to NCLB in maize is generally classified as either (i) monogenic resistance, which is race specific, or (ii) polygenic resistance which is not race specific. Monogenic resistance is controlled by five dominant genes (*Ht*M, *Ht*N1, *Ht*1, *Ht*2 and *Ht*3) and one recessive gene (*Ht*4) (Ullstrup 1970; Gevers 1975; Hooker 1977; Hooker 1981). The *Ht*1, *Ht*2, *Ht*3 and *Ht*4 genes form a chlorotic lesion type of resistance whereas the *Ht*M and *Ht*N1 genes cause a delay in appearance of necrosis and a reduction in sporulation (Gevers 1975; Robbins and Warren 1993).

Stellenbosch University http://scholar.sun.ac.za

The existence of a number of NCLB races and other maize crippling diseases make *Ht* scoring very difficult. The loci of the single genes on the maize chromosomal maps are well-defined, but to date only a few PCR markers, developed by Van Staden et al. (2001), are available to apply in marker-assisted selection.

1.2.2 Resistance to gray leaf spot (GLS)

Gray leaf spot of maize, caused by the fungus *Cercospora zeae-maydis* (Tehon and Daniels 1925), can reduce grain yields by up to 60% and is now recognized as one of the most significant yield-limiting diseases of maize in many parts of the world. In South Africa, the disease was first observed in KwaZulu-Natal in 1988 and has since spread rapidly to neighbouring provinces and countries. GLS development is highly dependent on environmental conditions and because of low resistance heritability field assessment of this disease is problematic. Symptoms of GLS are normally first observed as rectangular to irregular lesions on the lower leaves (Ward et al. 1999). Saghai Maroof et al. (1996) identified five independent quantitative trait loci (QTL) involved in GLS resistance.

Resistance to GLS is an essential trait in most maize improvement programs (Schechert et al. 1999). The most sustainable and long-term management strategy for GLS will rely heavily on the development of high yielding, locally adapted GLS resistant hybrids. Because of the number of genes involved in conferring GLS resistance and its low heritability it is difficult to transfer gray leaf spot resistance to susceptible elite lines. Thus, marker-assisted selection programs may proof to be very useful to plant breeders to indirectly select for genes affecting GLS resistance.

1.3 Marker technologies

Mapping genetic traits can be accomplished using a wide repertoire of genetic markers, many of which are based on DNA amplification techniques. Various techniques have been developed over the past few years to facilitate in the organisation of genotypes and detection of DNA polymorphisms; they are based on an array of morphological, biochemical and molecular descriptors. Prior to the development of molecular markers, mapping of disease resistant genes in maize

Stellenbosch University http://scholar.sun.ac.za

involved the development and use of endosperm marker-linked translocations and chromosome tester stocks (Burnham 1982). Unfortunately, they represented inappropriate genetic backgrounds for the mapping of disease and insect resistance genes. Plant scientists relied on the phenotype, pigmentation, quality, or other characteristics to classify and distinguish plant genotypes, but the efficiency of selection for these traits is a function of the heritability of the trait. For most morphological trait markers, genotypes generally can be ascertained only at the whole plant level, and frequently the mature plant is needed for references (Weising et al. 1995). In contrast to molecular markers, morphological characteristics are often strongly influenced by environmental factors and consequently special breeding programs and experimental designs are needed to distinguish phenotypic from genotypic variants.

Since 1959, isozyme studies have been successfully applied to many different organisms varying from bacteria to plant and animal species. Isozyme markers are based on protein polymorphisms (Weising et al. 1995). Isozymes can distinguish between homozygous and heterozygous individuals making them co-dominant markers and thus very effective in population genetic studies (Weising et al. 1995). Disadvantages in isozyme analysis are that polymorphisms will only be detected if the nucleotide base change leads to an amino acid substitution (Weising et al. 1995).

For effective and widespread application in plant genetics and breeding, a molecular marker should possess the following properties (Weising et al. 1995): (i) co-dominant Mendelian inheritance, (ii) high polymorphic behaviour, (iii) high copy number in the genome of interest, (iv) evenly distributed throughout the genome, (v) high reproducibility, (vi) no pleiotropic effects, (vii) easy and rapid access (for example amenable to automation) and (viii) easy exchange of data between laboratories. Molecular markers have become an indispensable tool in complex genome analysis, giving information regarding the coding and non-coding regions of any organism. Studies with molecular markers encompass various fields (e.g., genetics, biochemistry, breeding applications) and purposes (e.g., population structure, mating systems, genetic variation).

Stellenbosch University http://scholar.sun.ac.za

All DNA marker techniques can conveniently be categorised into two main groups (i) DNA hybridisation based techniques and (ii) PCR based marker techniques. The ease of obtaining and screening for DNA markers are essential to the efficiency in marker-assisted breeding programs.

1.3.1 DNA hybridisation based techniques

Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism has been the dominant DNA marker type since the 1980s (Soller and Beckman 1983). RFLP markers were first used for the detection of human disease alleles at closely linked loci, such as the sickle-cell anaemia allele (Goodburn et al. 1983).

The term RFLP was invented to denote the difference in molecular weight of homologous fragments of restriction enzyme digested genomic DNA observed in two genetically distinct individuals. The technique involves the restriction digest of total genomic DNA yielding reproducible sets of fragments of well-defined lengths after gel electrophoresis, thus reducing complex DNA to a population of fragments with discrete sizes. This is followed by Southern blot membrane transfer of the range of fragments, where specific DNA sequence(s) are revealed through the use of a well-defined, radioactively labelled multi- or single- locus probe. Point mutations within the recognition sequence, as well as insertions and deletions, will result in altered patterns of restriction fragments making it possible to detect polymorphisms between the different samples (Weising et al. 1995). Allelic restriction fragments are co-dominantly inherited, easily detectable, and independent of tissue of origin, age, and gene expression (Weising et al. 1995). These polymorphisms allow precise measurements of genetic similarity of the genotypes. However, due to the events by which polymorphisms are generated, the number of alleles at a given locus are limited (Weising et al. 1995).

In contrast to PCR derived markers, the automation of RFLP marker analysis is difficult and impractical (Rafalski and Tingey 1993). The technology has certain drawbacks: (i) the need for DNA sequence information and, (ii) large amounts of

Stellenbosch University http://scholar.sun.ac.za

intact DNA, (iii) it is time consuming and (iv) it requires use of short lived radioisotopes implying that the routine application of the method is undesirable and impractical in plant breeding projects where large outputs of samples are required (Waugh and Powell 1992).

The construction of genetic linkage maps based on RFLP analysis has been accomplished in many crop species (Shin et al 1990; Heun et al. 1991; Mukai et al. 1995) and since 1993 a well-characterised core RFLP map was available for the maize genome (Gardiner et al. 1993). In different plant species extensive genome mapping using the RFLP technique has been employed to: (i) identify markers linked to disease resistance genes (Paran et al. 1991; Schüller et al. 1992; Paran and Michelmore 1993; Hittalmani et al. 2000), (ii) mapping of agronomically important quantitative and qualitative genes (Kleinhofs et al. 1988; Barone et al. 1990; Graner and Bauer 1993; Laurie et al. 1993; Laurie et al. 1994; Yu et al. 1994; Ribaut et al. 1997; Agrama et al. 1999; Pernet et al. 1999; Sibov et al. 1999) and (iii) gene tagging (Yu et al. 1991). In addition RFLP markers have been used to map a myriad of single genes and quantitative trait loci (QTLs) for example mapping of the maize mosaic virus resistance (Ming et al. 1997), maize streak virus resistance (Pernet et al. 1999) and aluminium tolerance in maize (Sibov et al. 1999). Extensive RFLP linkage maps have been constructed for many crop species and agronomically important traits have been mapped by applying this RFLP technique (Tanksley et al. 1989; Lee 1995). In crops such as maize, tomato and wheat a large number of RFLP markers are available and extensive RFLP profiling is thus possible. However, in some common horticultural crops such as *Citrus* only a small number of polymorphic RFLP probes are available which limits the use of the method for analysis of these genomes.

1.3.2 PCR based molecular markers

The development of molecular marker technologies has enhanced our ability to map disease and insect resistance loci in maize and other plant species. Prior to the development of DNA markers certain maize resistance genes were mapped using

Stellenbosch University http://scholar.sun.ac.za

endosperm marker-linked translocations and marker arm tester stocks (Louie et al. 1991). These stocks only provided partial coverage of the maize genome.

PCR based markers can be classified into two separate groups: (i) Techniques that target multiple loci and (ii) techniques that target single loci. PCR-based methods often enable the analysis of large numbers of progeny in a relatively short time, yielding polymorphic markers more rapidly.

1.3.2.1 Multiple loci PCR based DNA marker techniques

Multiple arbitrary amplicon profiling (MAAP)

Multiple arbitrary amplicon profiling uses one or more oligonucleotide primers (≥5 base pairs) of arbitrary sequence to initiate DNA amplification creating characteristic profiles from anonymous genomes or DNA templates (Caetano-Anollés 1994). The generation of these profiles is easily accomplished and their application is versatile and universal (Caetano-Anollés 1994). However, the resolving power is limited since they do not provide direct access to allelic frequencies (Ender et al. 1996) as well as their information content is relatively lower than other assay methods (Caetano-Anollés 1994). The complexity of the profiles distinguishes the different MAAP techniques. Factors such as (i) primer-template mismatching, (ii) primer design, (iii) tailoring profile complexity, (iv) polymorphic DNA and (v) primer length can explain the differing complexity in profiles.

Different applications can be classified as a MAAP technique e.g., random amplified polymorphic DNA (RAPD-Williams et al. 1990), arbitrary primed polymerase chain reaction (AP-PCR-Welsh and McClelland 1990), DNA amplification fingerprints (DAF-Caetano-Anollés et al. 1991), random amplified microsatellite polymorphism (RAMP-Wu et al. 1994), tandom amplified microsatellites (RAMS-Hantula et al. 1996), single primer amplification reaction (SPAR-Gupta et al. 1994), inter simple sequence repeat-PCR (ISSA-Zietkiewicz et al. 1994) and the retrotransposon based MAAP techniques. Some of these MAAP applications will be discussed briefly.

Stellenbosch University http://scholar.sun.ac.za

(i) Random amplified polymorphic DNA (RAPD)

The random amplified polymorphic DNA assay is a modification of the PCR technique, where no prior knowledge of template DNA sequence is needed (Williams et al. 1990). The method is also referred to as the *blind analysis* of DNA (Haymer 1994). One short (9-10bp) oligonucleotide of a randomly chosen sequence, mixed with template genomic DNA and thermostable polymerase, is subjected to PCR temperature cycles under relaxed annealing conditions. Amplification is constrained to occur only in regions of the genome where the particular DNA sequence (complementary to the primer sequence) and the primer inverse is found within a size range that can successfully be amplified by PCR, thus generating a range of DNA fragments which can be separated by gel electrophoresis (Williams et al. 1990). Different primers generate different amplified bands, randomly distributed throughout the genome. It appears that the outcome of the reaction is partly determined by competition for priming sites.

RAPDs have been used as marker system in many different applications such as variety identification (Demeke et al. 1993), pedigree analysis, isolation of markers tightly linked to a trait or gene of importance (Michelmore et al. 1991; Paran et al. 1991; Paran and Michelmore 1993; Yang and Krüger 1994) and mapping in different populations (Quiros et al. 1993). In recent studies co-dominant RAPD markers linked to resistance genes were identified in the tomato genome (Kawchuk et al. 1994) and *Brassica nigra* (Quiros et al. 1995).

(ii) *Copia*-SSR, retrotransposon-microsatellite amplified polymorphism (REMAP) and inter-retrotransposon amplified polymorphism (IRAP)

These techniques are PCR-based assays and exploit the repetitive and dispersed nature of LTR-containing retrotransposons families for the visualisation of genomic polymorphisms. The techniques offer a simple and efficient method of generating both dominant and co-dominant genetic markers in virtually any eukaryote species for mapping studies. The LTR regions tend to be highly conserved (Kalender et al. 1999).

Stellenbosch University http://scholar.sun.ac.za

Copia-SSR, as described by Provan et al. (1999), and retrotransposon-microsatellite amplified polymorphism (REMAP), as described by Kalender et al. (1999), are two PCR-based assays, which make use of a combination of two classes of repetitive elements found widely distributed in eukaryotic genomes, namely the *Ty1-Copia* retrotransposons and simple sequence repeats (SSR). The intergenic region between the retrotransposon and SSR is amplified using an outward facing LTR-specific polymerase chain reaction (PCR) primer and 3' anchored simple-sequence repeat primers (Kalender et al. 1999) amplifying multiple polymorphic products from total genomic DNA. Using *Copia*-SSR, seven markers were mapped to four barley chromosome arms (Provan et al. 1999).

IRAP examines the polymorphisms between two retrotransposons insertion sites, using two outward facing LTR sequence specific primers (Kalender et al. 1999).

Amplified fragment length polymorphisms (AFLP)

A PCR-based DNA profiling technique, based upon selective restriction fragment amplification, namely amplified fragment length polymorphisms, was developed and described by Zabeau and Vos in 1993. The term amplified fragment length polymorphism was also used by Caetano-Anollés (1991) but refers to the amplified products of the technique known as DNA amplified fingerprints (DAF).

AFLP overcomes the problems experienced with RFLPs and combines the reliability of the RFLP- and power of the PCR-technique. Two approaches for AFLP analysis can be used (Zabeau and Vos 1993; Vos et al. 1995). The restriction fragments can be pre-selected using: (i) paramagnetic dynabeads or (ii) a pre-selective and selective round of PCR. Both approaches make use of a selective round of PCR amplification using oligonucleotides with 2-3 selective nucleotides before visualizing the products using radioactive labelling, fluorescent labelling or silver staining. Both protocols have been shown to be highly repeatable (Vos et al. 1995; Lin et al. 1996; Jones et al. 1997) except when more than 3 selective nucleotides are used, under these circumstances non-specific fragments will be amplified (Vos et al. 1995).

Stellenbosch University http://scholar.sun.ac.za

The genomic DNA of interest is digested with two restriction enzymes, a rare and frequent cutter. The rationale for using a rare and frequent cutter is that only those fragments flanked by the two restriction sites will be amplified, thus limiting the total number of fragments to be analysed. The use of two enzymes also makes it possible to label one strand of the double stranded PCR product using either radioactive- or fluorescent labelled primers (Hartl and Seefelder 1998). Rare cutter enzymes used can be *EcoR*I (Vos et al. 1995), *Hind*III (Meksem et al. 1995), *Pst*I (Milbourne et al. 1997; Castiglioni et al. 1999), *Sse*I (Donini et al. 1997) or *Mlu*I (Van Staden 2001) while the frequent cutter is *Mse*I (Hartl and Seefelder 1998). Double stranded adapters of a defined sequence are ligated to both ends of the DNA restriction fragments, thus generating primer binding sites for the subsequent DNA amplification reactions (Vos et al. 1995). The 5' termini of the primers are made complementary to the adaptors, where the 3' termini extend for a few arbitrary chosen nucleotides into the restriction fragments. Amplification products are resolved on poly-acrylamide gels and visualised using either radioisotopes, fluorochromes or silver staining. The complexity of the banding patterns can easily be tailored by varying the number of selective base pairs at the 3' termini. This technique is capable of detecting more than 50 independent loci in a single PCR reaction (Maughan et al. 1996). Due to occasional length polymorphisms, some AFLP markers cab be described as being co-dominant markers. The latter AFLP fragments have to meet the following criteria (i) they must originate from the two different parents using the same primer combination and (ii) they must map to the same locus (Vuylsteke et al. 1999).

Distribution of AFLP markers

Different restriction enzyme combinations can affect the number of polymorphisms detected. Generally the idea is that AFLP markers produced by different restriction enzymes should be distributed randomly throughout the genome (Qi et al. 1998). However, a tendency for the clustering of AFLP markers at centromeric regions have been observed when using the *EcoR*I/*Mse*I primer combination (Vuylsteke et al. 1999). In cytogenetic studies performed in the *Arabidopsis* genome it was shown that the pericentromeric heterochromatin regions fluoresces brightly when stained with the fluorochrome DAPI (Ross et al. 1996), which is known to show preference for AT-rich DNA regions. This can be the plausible explanation why AFLP fragments

Stellenbosch University http://scholar.sun.ac.za

amplified from restriction enzymes *EcoR*I and *Mse*I have centromeric preference since both the enzymes recognise AT-rich recognition target sequences (Vuylsteke et al. 1999). The clustering of AFLP fragments, generated by the *EcoR*I/*Mse*I restriction enzyme combination, in specific chromosomal regions was also reported in other plant AFLP linkage maps, such as potato (van Eck et al. 1995), barley (Powel et al. 1997) and soybean (Keim et al. 1997). Like the genomes of other higher plants, maize nuclear DNA is extensively (>50%) methylated at the 5' position of cytosine residues in the sequence islands of 5'-CG-3' and the 5'-CNG-3' nucleotide motifs (Bennetzen 1987). Vuylsteke and co-workers (1999) established that the AFLP fragments generated by the enzyme combination of *Pst*I/*Mse*I showed prevalence for the distal hypomethylated non-centromeric regions of the chromosome, which are associated with genes. Castiglioni et al. (1999) and Ridout and Donini (1999) concluded that the specific primer combination (*Pst*I/*Mse*I), or any other methylation sensitive restriction enzyme, provides better genome coverage with more random distribution of AFLP markers.

AFLP maps

Until very recently, genetic linkage maps of many plant species were primarily based on segregating restriction length polymorphism (RFLP) markers (Helentjaris et al. 1986; Burr et al. 1988; Beavis and Grant 1991; Shoemaker et al. 1992; Coe et al. 1995). Vuylsteke et al. (1999) used the high level of polymorphisms in the maize genome in combination with the high multiplex ratio of the AFLP technique to generate two high-density molecular AFLP linkage maps for maize. The maps were based on the (i) B73 x Mo17 recombinant inbred population and (ii) on a D32 x D145 immortalised F_2 population. A total of 1539 and 1355 AFLP markers were mapped in these two populations, respectively. The AFLP approach has recently been used to rapidly create linkage maps in a variety of different plant species, rice (Maheswaran et al. 1997), maize (Castiglioni et al. 1999; Vuylsteke et al. 1999), A*rabidopsis thaliana* (Alonso-Blanco et al. 1998) peach (Lu et al. 1998), barley (Becker et al. 1995; Castiglioni et al. 1998), *Eucalyptus* (Marques et al. 1998) and pine (Remington et al. 1999).

Stellenbosch University http://scholar.sun.ac.za

AFLP technology has been successfully used in the identification of markers tightly linked to disease resistance loci in potato (Meksem et al. 1995; Li et al. 1998), tomato (Thomas et al. 1995), barley (Paltridge et al. 1998), carrot (Bradeen and Simon 1998), wheat (Hartl et al. 1999) and maize (Lehmensiek 2000; Van Staden 2001). This technology has also been applied to determine the genetic relationship of different germplasm pools in rice (Zhu et al. 1998), potato (Milbourne et al. 1997; McGregor et al. 2000), barley (Russel et al. 1997a), coconut (Perera et al. 1998), soybean (Maughan et al. 1996), wheat (Barett and Kidwell 1998), grapevine (Cervera et al. 1998) and maize (Ajmone Marsan et al. 1998; Pejic et al. 1998).

Modified AFLP techniques

Waugh et al. (1997) reported the development of a modified AFLP technique known as sequence-specific amplification polymorphism (S-SAP). This technique involves the substitution of one of the AFLP primers with a sequence specific primer derived from the long terminal repeat region (LTR) of the Bare-1 *Ty1-Copia* retrotransposable element in the barley genome. These transposable elements are found dispersed throughout the euchromatic regions of the genome and as a result the S-SAP technique gives a wide-spread genome coverage (Provan et al. 1999). Another efficient and highly reproducible modified AFLP approach, called MuAFLP (Mutator AFLP), was used by Edwards et al. (2001). Their results demonstrated the suitability of this technique to identify insertion events within large numbers of genes expressed during specific stages of maize development.

Wessler (1999) and Casa et al. (2000) described the development of a modified AFLP technique called transposon-display (TD). Substituting one AFLP primer with a primer developed from the sequence from one of the MITE elements, a reproducible AFLP like display is generated expectantly in genic regions. When Wang and Wessler (1999) recovered some of the bands they got an indication that the bands were amplified from element-containing loci. Using MITEs, this technique provides researchers with a useful and informative tool to generate "smart" markers, preferentially anchored in genic regions. The TD approach has been successfully applied in the rice (Nagel et al. 2001) and maize genome (Casa et al. 2000; Magbanua et al. 2001; Zhang et al. 2001). Nagel et al. (2001) used the TD approach

Stellenbosch University http://scholar.sun.ac.za

on nine *Oryza* species providing enough tools to address the evolutionary influence of MITEs on the genus *Oryza*.

1.3.2.2 Single loci PCR based DNA marker techniques

Sequence characterised amplified region (SCAR)

The first sequence characterised amplified region marker to be derived from a polymorphic RAPD fragment was described by Paran and Michelmore in 1993. SCAR markers are generated by the cloning and sequencing of a specific polymorphic amplified fragment of interest followed by the designing of oligonucleotide primers that are complementary to the ends of the original fragment, they retain a co-dominant segregation behaviour. When these primers are used in a subsequent PCR reaction on the original template DNA, a single locus is amplified, hence the name sequence characterised amplified regions or SCAR. SCARs were derived from RAPD and AFLP markers linked to disease resistance and agronomically important traits in lettuce (Paran and Michelmore 1993), apples (Yang et al. 1997a; Yang et al. 1997b), tomatoes (Sobir et al. 2000), *Brassica* (Negi et al. 2000), rice (Gu Cho et al. 1996) and wheat (Myburg et al. 1998).

Allele-specific associated primers (ASAPs)

Allele-specific associated primers are dominant markers and they are a variation on the SCAR technique. The appropriate fragments can be observed through direct staining with ethidium bromide in micro-titre plates and no electrophoretical methods are therefore needed. These specific primers only generate a single DNA fragment in those individuals that posses the appropriate allele (Gu et al. 1995). ASAPs can also be derived from RFLPs and other DNA sequence variations identified near or within a certain gene of interest (Gu et al. 1995).

SCAR and ASAP markers have certain advantages: (i) they can behave as co-dominant (SCAR) or dominant (ASAP) markers and are thus much more informative, (ii) SCAR primers can be used as probes to screen genomic libraries, (iii) stringent PCR conditions are applied making SCARs and ASAPs more reproducible and favourable than RAPDs, (iv) they are locus specific and (v) they can be used as physical landmarks in the genome (Paran and Michelmore 1993; Weising et al.

Stellenbosch University http://scholar.sun.ac.za

1995). The generation of SCAR and ASAP markers are more laborious and expensive than other molecular marker techniques.

Sequence-tagged-site (STS)

A sequence-tagged-site is a short region (200–300 base pairs) of DNA sequence, for which a set of PCR primers are designed from a low copy-number DNA sequence (Olsen et al. 1989). PCR primers are then used to amplify a locus specific fragment. Any sequence can be converted to an STS provided that it contains a unique sequence occurring only once in the genome (Primrose 1995). When these primer sets are tested on several lines, polymorphisms may be found due to the size differences of the amplified fragment.

Cleaved amplified polymorphic sequence (CAPS)

SCAR or STS amplified products can be digested with a panel of restriction enzymes to identify RFLPs amongst individuals. The assay is referred to as cleaved amplified polymorphic sequences. With this technique many restriction enzymes can easily be tested on the amplified DNA enabling the generation of more polymorphisms (Rafalski and Tingey 1993; Jarvis et al. 1994). Eighteen co-dominant CAPS markers, evenly distributed throughout the genome, have been developed for the *Arabidopsis* genome (Jarvis et al. 1994). CAPS derived from sequenced cDNA clones were combined with the AFLP marker system to construct a genetic linkage map of the *Cryptomeria japonica* genome (Nikaido et al. 2000).

Repetitive DNA sequences

The term describes a class of DNA sequences found in multiple copies in any given genome (Haymer 1994). They represent a vast reservoir of DNA variation regardless of where it is situated in the genome of interest. Polymorphisms created by tandem repetitive DNA sequences result from variation in the number of repeat units of the core sequence (Nakamura et al. 1987). Depending on the sizes of the core sequences they are described as minisatellites (Jeffreys et al. 1985) or microsatellites (Litt and Luty 1989). Minisatellite loci are mainly concentrated at centromeric and telomeric regions of a chromosome in contrast to microsatellite loci, which are apparently randomly distributed throughout the genome (Jeffreys et al. 1985; 1988; 1990). In spite of its limitations for use in plant genomes, minisatellites

Stellenbosch University http://scholar.sun.ac.za

was used to produce fingerprinting profiles of rice cultivars (Dallas 1988) as well as tomato cultivars (Sharon et al. 1995).

As an example of a highly informative single locus PCR based marker technique the application of microsatellites will be discussed.

(i) Microsatellites

Microsatellites, have been referred to by a number of acronyms including simple-sequence length polymorphisms (SSLP; Tautz 1989), sequence-tagged microsatellite sites (STMS; Beckmann and Soller, 1990), simple-sequence repeats (SSR; Litt and Luty 1989) and short tandem repeats (STR; Edwards et al. 1991). STRs are defined as relatively short runs of tandemly repeated DNA sequences with a core sequence of 1 to 6 base pairs in length (Litt and Luty 1989). Microsatellites exhibit co-dominant Mendelian inheritance and can be classified as perfect (no interruptions in the run of repeat), imperfect (one or more interruptions in the run of repeat) or compound (a run of perfect or imperfect repeats adjacent to a run of another simple sequence repeat) (Weber and May 1989).

Different number of repeats in a microsatellite core sequence causing allelic variation, can easily and efficiently be detected by PCR amplification. A proposed hypothesis for the observed high levels of polymorphisms of microsatellite sequences is slipped-strand mispairing (SSM) of the two strands of the DNA double helix (Levinson and Gutman 1987). Earlier investigations into plant STRs proved that these markers are polymorphic and informative in barley (Weising et al. 1989), *Vitis vinifera* (Thomas and Scott 1993), soybean (Morgante et al. 1994), rice (Zhao and Kochert 1992), *Arabidopsis thaliana* (Bell and Ecker 1994) and maize (Chin et al. 1996). The utility of STR markers were illustrated in many plant genetic studies, which includes finding markers linked to a virus resistance gene in soybean (Yu et al. 1994), identification of chromosomal regions with significant effects on yield in rice (Zhang et al. 1994), germplasm assessment of rice, barley and soybean (Yang et al. 1994; Rongwen et al. 1995; Russel et al. 1997b) as well as the inclusion of

Stellenbosch University http://scholar.sun.ac.za

microsatellite loci in molecular linkage maps in several plant species (Wu and Tanksley 1993; Bell and Ecker 1994; Akagi et al. 1996).

1.4 Molecular marker applications

High-density molecular linkage maps and associated technologies have been used and applied successfully for a number of different purposes in genetic research and breeding. Among their applications are (i) identifying and detection of genes/traits of interest (Tanksley et al. 1992) or gene-tagging (Castiglioni et al. 1999), (ii) evolutionary studies (Castiglioni et al. 1999) (iii) facilitating marker-assisted selection (Tanksley et al. 1992; Castiglioni et al. 1999), (iv) chromosome walking and thus mapped-based cloning (Tanksley et al. 1992) and (v) constructing high-density molecular linkage maps ensure that the entire genome is covered with molecular markers, which is especially important when using linkage maps to detect and characterise QTL loci (Tanksley et al. 1992; Castiglioni et al. 1999).

1.4.1 Marker-assisted selection (MAS)

Many researchers have demonstrated the value of molecular marker technologies for identifying and mapping qualitative and quantitative traits in maize and several other crop species. Markers can increase the precision and efficiency of plant breeding, and also advance the acquisition of important genes from exotic populations or from wild species. One of the main objectives in plant breeding is the integration of one or more desirable traits/genes from a suitable donor parent in to the background of an elite variety or inbred line. In this pathway other excellent qualities of the elite variety is retained, while adding traits such as disease resistance, resistance to other environmental factors or nutritional qualities (Humberto Reyes-Valdés 2000). Using marker-assisted selection (MAS), linkage is sought between a specific DNA marker(s) and agronomically important loci. Thus, instead of selecting for the trait, the breeder can select for a marker, which can be detected very early in the selection scheme, shortening the breeding programs. Tight linkage between molecular markers and genes of interest are necessary for effective use in plant breeding. Yamamoto et al. (2000) proposed, that by developing a breeding program using MAS for mapped QTLs they will be able to perform QTL pyramiding, i.e. combining QTLs having different gene actions into one variety.

Stellenbosch University http://scholar.sun.ac.za

1.4.2 DNA profiling

DNA fingerprinting, DNA profiling or DNA typing involves the display of a set of DNA fragments from a specific DNA sample. It refers to the characterisation of either similarities or one or more distinctive features in the genetic make-up of an individual, a variety, race or a species. Eukaryotic and prokaryotic organisms can be distinguished at the DNA level by detecting variations in DNA sequence through molecular hybridisation or DNA amplification. Knowledge of genetic variation and the genetic relationship between genotypes are important considerations for the efficient rationalisation, utilisation and management of germplasm resources (Morell et al. 1995; McGregor et al. 2000). All fingerprinting methods are based on the principle that only a small fraction of the digested fragments are visualised as a simple banding pattern, which then constitutes the DNA fingerprint of that specific organism (Morell et al. 1995).

The genetic variability amongst new cultivars, within specific plant species, is most likely to diminish and this complicates the identification of specific cultivars (Rongwen et al. 1995). The general rule is that the closer the genetic make-up and genetic relationships, the greater similarity between the genomes and consequently the distinctive features in the genomes will be less (Rongwen et al. 1995). DNA based markers provide a solution to this problem as the detection of genetic variation is not restricted to the expressed gene level, thus providing unique profiles to identify and assess genetic diversity in plant cultivars (Morell et al. 1995).

1.5 Mapping strategies

1.5.1 Transposon tagging

Transposable elements (TE) have many potential applications in plant genetic research such as insertional mutagenesis (transposon tagging), gene mapping, gene cloning, gene transfer as well as the identification of tissue specific genes at certain given times (Lai 1994). Transposon tagging is a specialised application of mutagenesis in which a cloned transposable element (located near the gene of interest) is immobilised to create an insertion at a gene locus of interest (Lai 1994). The inserted transposon is then used as a molecular tag (Lai 1994). The principle and technique of transposon tagging was first utilized in *Drosophila melanogaster* by

Stellenbosch University http://scholar.sun.ac.za

Bingham et al. (1981). It relies on the phenotypic recognition of a mutable allele caused by the insertion of a known transposon (Lai 1994). Excision of the transposon, leading to the restoration of the gene function, generally proves that the element integrated into the gene of interest. The element sequence can be used as a probe to clone the mutable allele, which will include DNA sequences from the gene adjacent to the inserted element. Transposon tagging lends itself to be a resourceful approach by which the mutant phenotype of interest can be screened and the gene of interest can be identified and cloned (Lai 1994).

This method has a major advantage over traditional mutagenesis: (i) the gene can be cloned without any knowledge of the gene product and (ii) transposon tagging can also play an important role in "reverse" genetics by determining the gene's function after it has been cloned. A disadvantage however is the fact that there are numerous ways in which transposons can induce mutations and that these mutations are often unstable (Lai 1994). *Mu* mutagenesis have been established as the method for cloning unknown genes responsible for a certain phenotype. To understand the diverse and unique phenotypic expression of recessive lojap (*ij*) mutants in maize, a transposon tagging experiment was conducted using Robertson's Mutator (*Mu*) (Han et al. 1992; Byrne and Taylor 1996.). A new ij mutant was obtained from crosses of the reference allele of (*ij*-ref) to Mu lines (Han et al. 1992). Gray et al. (1998) isolated the lethal leaf-spot 1 gene (*lls*1) from maize via transposon tagging with Mutator. The sequence indicates that the *lls*1 gene encodes a novel function conserved in plants. ZmDB, maize genome collaboration, is currently using engineered *Mu* (*RescueMu*) to discover and investigate maize genes (http://www.zmdb.iastate.edu/site_map.html.) Unfortunately, this approach requires time and is very costly.

1.5.2 Comparative mapping

The use of common sets of DNA probes to detect and map homologous sequences across different isolated species has surprisingly revealed a high degree of conservation in terms of copy number and homology of low copy probes and locus order. This comparative approach was pioneered by Tanksley and co-workers (1989); they mapped tomato RFLP probes on the map of the tomato, chilli peppe0-

Stellenbosch University http://scholar.sun.ac.za

and potato genomes. Comparative mapping displays a broad range of co-linearity among related species. Ahn and Tanksley (1993) noted that a certain amount of conservation exists between the genomes of plant species belonging to the different Poacceae tribes of wheat, maize and rice, especially within short fragments of chromosomes. Tanksley et al. (1992) revealed, using comparative genetic maps, based on common RFLPs, that the genomes of tomato and potato are nearly identical in overall gene content and gene order. Whitkus et al. (1992) presented a comparative genome mapping study analysing the genomes of sorghum and maize, using RFLP probes of maize and isozymes. Comparative studies such as these offer researchers new insight into chromosomal evolution as well as chromosomal organisation, but it also provide a stern basis for interpreting genetic information among distantly related genomes which are not amenable to analysis by traditional cytogenic techniques.

1.5.3 Near-isogenic lines (NILs)

Near-isogenic lines, are the natural result of an introgressive backcross breeding program aimed at transferring a trait of interest into elite cultivars. The donor of the specific trait of interest is first crossed to the recurrent parent. The resulting F_1 hybrids are then crossed back to the recurrent parent after which the whole process of backcrossing is repeated numerous times until the unwanted portion (from the donor parent due to linkage drag) is largely eliminated and the desired trait or gene is introduced into the resulting isogenic lines (Muehlbauer et al. 1988), ideally into backcross (BC) 7. After each cross the progeny is selected on the basis of the phenotype in regards to the target gene. The BC7 genome is practically homozygous for the recurrent genotype except for the area of interest (gene for resistance or trait of interest) gained from the donor parent. One of the homologous chromosomes will always be contributed by the recurrent parent (Tanksley et al. 1995). The BC7s' target area must be completely homozygous to the donor parent and the surrounding genome completely homozygous to the recurrent parent; ideally BC7 is selfed for approximate two generations (Tanksley et al. 1995).

Stellenbosch University http://scholar.sun.ac.za

Near-isogenic lines carrying a disease resistance gene are theoretically identical to their recurrent parent, except for the presence of the resistance gene from the donor parent. Kaeppler and co-workers (1993) used NILs to detect linkage between molecular markers and introgressed regions. The probability that a certain marker, with a specified distance from the introgressed gene, will have a donor parent allele in a NIL, is a function of the distance between the marker and the gene as well as the number of backcrosses or selfs used in obtaining the NIL. Muehlbauer and co-workers (1988) suggested that NILs could be used to identify presumptive linkages of molecular and conventional genetic markers.

Due to the availability of well-defined NILs for many plant species they could be used very successfully in the identification of markers linked to agronomically important traits (Young et al. 1988; Paran et al. 1991; Mackil and Bonman 1992; Schüller et al. 1992).

1.5.4 Bulk segregant analysis (BSA)

Michelmore et al. (1991) developed bulk segregant analysis as a method for the rapid identification of markers linked to any specific gene or genomic region. BSA is a pooled sample approach to the construction of high resolution genetic maps. The method involves the comparison of two separate pooled DNA samples of individuals from a segregating population, originating from one single cross. This strategy depends on the existence of an easily selectable target locus and the ability to produce large segregating populations. Within each bulk or pool the individuals are identical for a selected trait or gene of interest, but they are arbitrary for all other genes. Molecular marker screening of all of the individuals in the two bulks from the segregating population is only necessary when a polymorphism, linked to the gene or trait of interest, is detected between the two bulks. Bulked segregant analysis provide researchers with a efficient molecular tool to focus on regions of interest or areas that are sparsely populated with markers (Michelmore et al. 1991).

BSA has been used successfully in the detection of markers linked to many traits, such as fungal resistance (Penner et al. 1993; Poulsen et al. 1995), presence of rye

Stellenbosch University http://scholar.sun.ac.za

chromatin in wheat (Francis et al 1995), daylight insensitivity in oat (Wight and Penner 1994), the *Rfp1* restorer gene for *pol* cytoplasmic male sterility in canola (Jean et al. 1998), identifcation of AFLP markers linked to the *R1*- and *R2* alleles in potato (Meksem et al. 1995; Li et al. 1998) and the identification of two loci linked to aluminium tolerance in maize (Sibov et al. 1999).

1.5.5 Mapped-based cloning/positional cloning

Plant species with well defined linkage maps and DNA libraries are well suited for mapped based cloning. Fine scale mapping relies on the density of known markers to localize the mutant locus to a small genomic region. The original concept behind mapped-based cloning was to find a marker linked to the gene of interest, creating a physical map, and then to "walk" to the gene via overlapping artificial chromosomes (bacterial, yeast or mammalian) or cosmids (Springer et al. 1994; Tanksley et al. 1995). Map-based cloning has three requirements: (i) the individuals within the population must be genetically different concerning the trait of interest and (ii) the gene(s) of interest can be mapped to a chromosomal position(s) adjacent to previously mapped DNA markers and (iii) a high density map of the DNA markers closely linked to the gene of interest is needed. Chromosome walking is hampered by the size of the genome together with the amount of repetitive DNA sequences present in the genome. Combining AFLP technology with the process of mapped-based cloning, Cnops and co-workers (1996) isolated the *tornado 1 (trn1)* mutant from the *Arabidopsis* genome. Schwarz and co-workers (1999) "landed" on the *Mla* locus in barley by identifying an AFLP marker flanking the locus.

1.5.6 Candidate genes (CG)

Pflieger et al. (1999) used consensus primers corresponding with conserved domains, (NBS-LRR and the kinase domains), in a PCR-based approach to isolate resistant gene analogues. They used these resistant gene analogues as candidate genes (CG) for quantitative trait loci (QTLs) involved in pepper-pathogen interactions. Gedil et al. (2001) identified one candidate gene, belonging to the NBS resistant gene family, showing linkage to downy mildew resistance in sunflower. The selection and use of candidate genes is becoming a wide-spread method and rely on two non-

Stellenbosch University http://scholar.sun.ac.za

exclusive approaches. The first one is named the "functional" candidate gene approach, based on a choice of gene(s), which may be functionally related to the trait of interest (Prioul et al. 1999). This approach has been used successfully to show the role of the polymorphisms of *p1* gene loci in maize (Byrne et al. 1996). The second approach is the "positional" candidate gene approach that relies on QTL mapping and on the identification and examining of known functional genes or mutations which map in the same chromosomal region (Prioul et al. 1999). Prioul et al. (1999) used RIL populations to identify candidate genes linked to biochemical traits in maize. Faris et al. (1999) described the use of candidate genes involved in defence response for the QTL analysis of resistance to several diseases in wheat.

To clone quantitative trait loci, the use of transposon tagging and mapped-based cloning is a option, but their application is not optimal because of the imprecise map location of the QTL as well as the partial effects of the QTL. The use of candidate genes provides an alternative method.

1.6 Mapping populations

Historically, maize has been used as the model plant organism in the development and evaluation of molecular markers mapping and manipulation of major genes. The choice of the population used for mapping purposes will have important repercussions on the efficiency and accessibility of mapping information.

1.6.1 Immortalised populations

An immortalised mapping population signifies a set of mapping strains that has been derived by a procedure that maintains the heterozygous alleles in each individual F_2 plant by random mating in the F_3 and subsequent populations, thus the genotype of each F_2 individual is maintained (Gardiner et al. 1993). These populations have aided in the development of core RFLP linkage maps that are relatively polymorphic and show an even distribution of markers throughout the genomes. These RFLP molecular markers allows for the minimum genome coverage needed to map any quantitative or qualitative trait.

Stellenbosch University http://scholar.sun.ac.za

Recombinant inbred lines (RILs) and double-haploid (DH) mapping populations are true breeding and immortal mapping populations. Immortalised mapping populations constitute a permanent population in which segregation is complete, or nearly complete, and therefore they can be used indefinitely for mapping purposes, this poses as a huge advantage over mortalised mapping populations. In comparison to F_2 or backcross segregating populations derived from a single meiosis, recombinant inbred individuals undergo multiple rounds of meiosis before homozygosity is reached.

Mapping genes with recombinant inbred lines (RILs)

Recombinant inbred lines are produced by continually selfing or sib-mating the progeny of individual members of an F_2 population, they undergo multiple rounds of meiosis until homozygosity is reached. Burr et al. (1988) developed an RFLP map of the maize genome based on segregation data using two publicly available recombinant inbred populations (Tx303 x CO159 and T232 x CM37). RILs are derivates of an F_2 population obtained from two pure breeding inbred lines, ensuring there will only be two alleles for any given locus segregating in the F_2 population. The advantages of using these two populations of B.Burr, are that it would be less difficult to find polymorphism or linkage and the indication of marker linkage to a gene order can be verified and checked against results obtained from the second family (Burr et al. 1988). Differences in linkage estimates will vary somewhat between the two populations, but without any meaningful discrepancies. Each RIL can now be visualised as having a set of chromosomes where the parental alleles appear as linked blocks, which are now essentially fixed. Any trait, morphological-, protein- or molecular marker can be mapped, as long as the parental genotypes can be distinguished. This pose as a limitation, a trait or marker cannot be mapped if the parental lines cannot be differentiated. Defining the recombinant inbred maps a number of isozymes and cloned genes with known map positions were used, as well as B-A translocations assigning markers to chromosome arms (Burr and Burr 1991).

The database for the two RI-families contains 334 mapped loci and covers approximately 1460cM. Two hundred and twenty of these loci were mapped using

Stellenbosch University http://scholar.sun.ac.za

both families, of the remainder, 57 were mapped to the T232 x CM37 family and 57 mapped to the Tx303 x CO159 family (Burr and Burr 1991). The designation of a set of core markers, evenly spaced and relatively polymorphic, on the linkage maps of the two RI populations, has facilitated in the dissection and definition of the ten maize chromosomes. Thus constructing computer-sortable chromosomal bins that serve as collection points for the mapping and characterising of other genetic loci. These defined bins with their core markers form the genomic backbone that is necessary for any integrating mapping project. In a RI mapping population, a genotype is represented by an inbred line, rather than by an individual, thus a more accurate assessment of the genetic component of variance can be made in studying quantitative traits. Shown in table 1.2 is only an indication of some of the RIL families available for maize genetic studies.

Table 1.2 Some available publicly recombinant inbred populations for mapping trait loci in the maize genome. Number of individuals per population as well as generations of inbreeding is indicated.

Parents	***No: of individuals***	***Generations of inbreeding***	***Source***
Hi31 x Ki14	91	?	J. Brewbaker (MaizeDB)
T232 x CM37	48	8	Burr et al.1988
Tx303 x CO159	41	8	Burr et al.1988
Tx303 x CO159	160	5	C. Stuber (MaizeDB)
Mo17 x B73	44	7	M. Albertsen (MaizeDB)
Mo17 x B73	350	7	Lee et al. 1998
PA326 x ND300	74	5	D. West (MaizeDB)
CK52 x A671	162	5	D. West (MaizeDB)
CG16 x A671	172	5	D. West (MaizeDB)
CH593-9 x CH606-11	101	5	D. West (MaizeDB)
CO220 x N28	173	5	D. West (MaizeDB)

Stellenbosch University http://scholar.sun.ac.za

1.6.2 Mortalised populations

A classical F_2 mapping population can be derived from selfing of F_1-hybrids or backcrossing of the F_1 individuals to one of the parents. In contrast to immortalised mapping populations, F_2 and backcrossing populations, unless they can be asexually propagated, can be grown only once and material derived from them will eventually get depleted. Often markers mapped in one segregating population have to be remapped in a second population in order to realign different maps using the same reference points. In contrast to an immortalised mapping population, an F_2 segregating population can be constructed for basically any trait of interest, given that the parents differ in the phenotypical expression of that trait. Thus they are essential for the mapping of traits that are not present in an immortalised population. The construction of an immortalised population is much more laborious and time consuming.

Stellenbosch University http://scholar.sun.ac.za

AIMS OF STUDY

Given the organisation of the maize genome as well as demands placed on the saturation of molecular linkage maps it would be desirable to identify informative molecular markers that is located or linked to genic rich areas. In the past isolation of resistance genes required labour intensive, time consuming and complex procedures of map-based cloning and/or transposon tagging. The development of a numerous myriad of different marker technologies provided researchers with the means to isolate, with greater efficiency, markers located in or near genic rich areas thus isolating and identifying possible candidate genes (CG).

Short stretches of conserved areas in different disease or insect resistance genes presented the opportunity to search for additional candidate genes in diverse species by polymerase chain reaction (PCR)-analysis, using degenerate oligonucleotides, homologous to the conserved areas. This approach has been used with success and indicated that it is possible to amplify gene analogues from diverse plant species (e.g., *Arabidopsis*, Aarts et al. 1998; maize, Collins et al. 1998 and rice, Mago et al. 1999). Using highly conserved sequence motifs, disease resistant candidate genes belonging to the NBS-LRR gene superfamily have already been cloned from numerous crop species.

In this study the unique properties, of the transposon group, miniature inverted-repeat transposable elements (MITEs), especially their high copy number, polymorphism, stability and preference for genic areas, were exploited to develop a new marker technique with a strong preference for genic areas. Bureau and Wessler (1994b) found a number of MITE-Stowaway elements that showed very close association with more than 40 monocotyledonous and dicotyledonous plant genes listed in the GenBank and EMBL nucleic acid databases. Wessler (1999) and Casa et al. (2000) used transposon-display (MITE-TD) to generate markers that were preferentially anchored in genic regions. The TD approach has been successfully applied in the other plant species (Casa et al. 2000; Magbanua et al. 2001; Nagel et al. 2001; Zhang et al. 2001).

Stellenbosch University http://scholar.sun.ac.za

Combining two different genetic elements (MITEs) and resistant gene analogues (RGA), molecular markers will be identified and mapped to supplement in the saturation of maize linkage maps. Markers that mapped close to, or shows promising linkage to known insect or disease resistant genes or agronomically important traits can be used in future experimental analysis to identify genetic stock harbouring specific resistant alleles.

The objectives of this study were to:

(i) use sequences from peroxidase and reductase gene products as a search query on data bases. By aligning the amino acid sequences identifying conserved areas, suitable primer pairs would be identified and used for analysis.

(ii) use combinations of *Hbr/Hb2* MITE elements and RGAs in a modified transposon-like display to amplify suitable profiles.

(iii) determine and examine the degree of MITE element conservation and primer specificity by comparing the *Heartbreake*r (*Hbr)* and *Hb*2 element sequences with data base sequences as well as by comparing the sequences of various amplified MITE/RGA fragments.

(iv) use the sequences of various excised MITE/RGA fragments as BLAST search queries to identify any similarities with mapped maize resistance genes.

(v) study linkage and chromosomal distribution of polymorphic MITE/RGA fragments using the existing linkage map of the commercially available RIL population (Burr et al. 1988).

(vi) compare mapping results and linkage analysis with other published studies.

(vii) apply this technique to fine map gene loci and quantitative trait loci (QTL) for *Setosphaeria turcica* resistance and gray leaf spot (GLS) resistance in maize respectively.

(viii) identify possible candidate genes linked to the resistance genes, *HtN*, *Ht*1, *Ht*2 and *Ht*3 as well as to any of the major or minor QTLs for GLS.

(ix) use elite maize lines to analyse the distribution, genome coverage and efficiency of the MITE/RGA display in comparison to conservative AFLP profiling.

Stellenbosch University http://scholar.sun.ac.za

MATERIAL AND METHODS

CHAPTER TWO

2.1 Plant Material

Recombinant inbred lines (RILs) are derivates of an F_2 population in which linked blocks of progenitor alleles are now fixed. Limited amounts of RI seeds from the second RI mapping population (Tx303 x CO159) (2 parents and 40 individuals) are being distributed by B. Burr, Brookhaven National laboratory, New York to laboratories ensuring that different research groups will be working on the same individuals in a given generation.

Gray leaf spot (GLS) plant material

The F_1 single cross between a GLS resistant male parent (Seed Co LTD, Zimbabwe) and a susceptible female parent (Sensako, South Africa) was selfed to produce a segregating F_2 generation which was planted at Hillcrest in 1998 and scored for GLS resistance on a rating scale of 1-9, where 1 is most resistant and 9 is highly susceptible. Of the F_2 population, 230 scored plants were used in linkage analysis and QTL mapping.

Elite maize lines

Seeds of 40 elite white (19) and yellow (21) maize inbred lines were received from Sensako/DeKalb. These inbred lines were used for AFLP and MITE/RGA fingerprinting, to make a comparison on efficiency, as well as the functional application of the two PCR based marker techniques.

2.2 Plant genomic DNA extraction

Most genomic DNA extraction protocols rely on cetyltrimethylammonium bromide (CTAB) as detergent to disrupt cell membranes. The DNA extraction protocol used for this study is described in the CIMMYT applied molecular genetics laboratory manual, which is largely based on the CTAB method by Saghai-Maroof et al. (1984). In total 40 RIL individuals plus two RIL parents of the second RIL (Tx303 x CO159) family under greenhouse conditions were planted. Leaves from three-week old seedlings were harvested, and stored (until lypholisation) in 50ml sterilin plastic tubes. The leaves were then frozen with liquid nitrogen and the frozen leaves were then transferred to a lypholiser for 3-5 days (depending on amount of leaves and

Stellenbosch University http://scholar.sun.ac.za

moisture in leaves). The dried leaves were ground to a fine powder and 300-500mg were used for genomic DNA extraction. Nine (9) ml of warm (65°C) pre-prepared CTAB extraction buffer [1% CTAB; 100mM Tris (pH7.5); 700mM NaCl; 50mM EDTA-(pH8.0); 140mM β-mercapto-ethanol] were added to the lypholised tissue. Plant tissue was then incubated for 60-90min in a 65°C water bath with gentle rocking. Tubes were removed from the water bath and 4.5ml of chloroform/octanol [24:1] were added to cooled tubes with gentle rocking at room temperature for 5min. Centrifugation for 10min at 1400 x g^{-1} at room temperature was followed by a second chloroform/octanol [24:1] extraction and centrifugation. The top aqueous layer was removed and 30µl of preboiled 10mg/ml RnaseA (Roche) were added and incubated at room temperature for 30min. After the incubation 6.0ml of isopropanol (2-propanol) were added by gentle inversion. Precipitated genomic DNA were removed by an inoculation needle and placed in a 2.5ml eppindorf tube containing 1ml of TE buffer [10mM Tris-HCl (pH7.5); 0.1mM EDTA (pH8.0)]. Phenol /chloroform extractions were performed on the dissolved genomic DNA. The genomic DNA was precipitated with 100% Ethanol and 50µl of 5mM NaCl. DNA was purified, firstly using 3ml of WASH-buffer I (100% EtOH; 2.5M Na-acetate; ddH_2O) followed by a quick wash using WASH-buffer II (100% EtOH; 1M NH_4-acetate; ddH_2O). The DNA was dissolved overnight in 200-250µl of ultra-high-quality water (SABAX).

Quantification of genomic DNA samples was determined on agarose gel (Whitehead Scientific) using six DNA quantification standards supplied by Life Technologies, GIBCO BRL, U.K.

2.3 Designing of primers

Primers were designed according to standard PCR guidelines using the Primer Design Version 0.5 computer programme (Scientific & Educational Software). The primers were specific for the target sequence, free of internal structures avoiding complementation at the 3'-ends within each primer or primer pair. Lyophilised primers were order from Life Technologies, GIBCO BRL, U.K. in a 50nmol concentration with some internal modifications for the RGA primers (table 2.2).

Stellenbosch University http://scholar.sun.ac.za

MITE primers

The availability of the *Hbr-* (Zhang et al. 2000) and *Hb2-* (Spell et al. 1988) element sequences as well as flanking regions of these transposable element loci facilitated the design of appropriate PCR primers. Table 2.1 shows the sequences of the primers used in this study.

Table 2.1 Sequences of the two MITE primers

Primer	***Source***	***Sequence 5'-3'***
Hbr-F	Casa et al. (1999)	GAT TCT CCC CAC AGC CAG ATT C
Hb2-F	Casa et al. (1999)	TCA CAG GCA CAG CTC AAC

RGA primers

Sequence similarities between translated nucleotide sequences and known biological proteins (in this case peroxidase and reductase proteins) provide strong evidence for the presence of homologous coding regions in different species and distantly related species.

Computer aided similarity searches of DNA and amino acid sequences with the EPLN and SwissProt sequence database were carried out using the FASTA version 3.3t07 algorithm (http://www.ebi.ac.uk/servicestmp/533115.372271-235644.htm1), (Genetics Computer Group- Pearson and Lipman 1988). Similarity searches were done for a maize resistance gene with a peroxidase- (ZmDB text search- accession TUC 12-280-5500.2) and a reductase- (*Helminthosporium carbonum* susceptibility1) gene product. Based on similarities between the gene product and sequence of different plant species, primers were developed with low degeneracy on the 3' end. Shown in Table 2.2 are the 16 primers used in this study. All ten primers grouped under NBS, originated from conserved motifs in genes with a putative NBS and LRR region, according to articles published by Collins et al. (1998) and Aarts et al. (1998).

Stellenbosch University http://scholar.sun.ac.za

Table 2.2 Sequences of the 16 resistant gene analogue primers with their annealing temperatures (Tm) in brackets

Primer	*Source*	*Sequence 5'-3'*
Group name: Per		
Per1-F (57°C)	ZmDB (Fasta3_t)	CAC CCM AAG GTC AGC CAI GAG C
Per1-R (57°C)	ZmDB (Fasta3_t)	GCT CIT GGC TGA CCT TKG GGT G
Group name: Red		
Red(C)1-F (54°C)	ZmDB (Fasta3_t)	TCA CCG GNG SIG CCG GGT WCA TCG
Red(C)1-R (54°C)	ZmDB (Fasta3_t)	CGA TGS ACC CGG CIW CKC CGG TGA
Red(D)1-F (54°C)	ZmDB (Fasta3_t)	TTC ATC GGC TCC TGG CTC GT
Red(D)1-R (54°C)	ZmDB (Fasta3_t)	ACG AGC CAG GAG CCG ATG AA
Group name: NBS		
RG_1-F (54°C)	Aarts et al. (1998)	GGI ATG GGI GGI GTI GGI AAR CAN ACN
RG_1-R (54°C)	Aarts et al. (1998)	NGT NGT YTT ICC IAC ICC ICC CAT ICC
PL1-5-F (51°C)	Collins et al. (1998)	AAG AAT TCG GNG TNG GNA AAA CAN C
PL1-5-R (51°C)	Collins et al. (1998)	GTN GTT TTN CCN CAN CCG AAT TCT T
Kin-F (51°C)	Collins et al. (1998)	CTA CTG NTN CTN GAY GAT GT
Kin-R (51°C)	Collins et al. (1998)	ACR TCR TCN AGN ANC AGT AG
GLPL-F (51°C)	Collins et al. (1998)	AAC TCG AGA ANG CCA ANG GCA AWC C
GLPL-R (51°C)	Collins et al. (1998)	GGS TTG CCN TTG GCN TTC TCG AGT T
WMA1-2-F (51°C)	Collins et al. (1998)	AYR AAN CCN TNW GCC ATC CA
WMA1-2-R (51°C)	Collins et al. (1998)	TGG ATG GCS NAN GGN TTY RT

CODES FOR NON-STANDARD OR MIXED BASES

I = deoxyinosine
N = A + C + G + T
R = A +G
Y = C +T
M = A +C
D = A + T + G
K = T + G
S = C + G
W = A +T
H = A +T + C
B = T + C + G
V = A + C + G

To simplify further analysis the primers were named according to their respective groups.

Stellenbosch University http://scholar.sun.ac.za

AFLP primers

Two AFLP primer combinations were applied in this study.

Table 2.3 Sequences of the AFLP adaptors and primers used

	Selective nucleotides	***Sequence***
Adaptor sequences		
***Mse*I-adaptor**		5' GAC GAT GAG TCC TGA G 3'
		3' TA CTC AGG ACT CAT 5'
***Mlu*I-adaptor**		5'biotin-CTC GTA GAC TGC GTA AC 3'
		3' CTG ACG CAT TGG CGC 5'
Primers used		
***Mse*I primer 5**	5' **GGC** 3'	5' GAT GAG TCC TGA GTA A-**GGC** 3'
***Mse*I primer 7**	5' **TAC** 3'	5' GAT GAG TCC TGA GTA A-**TAC** 3'
***Mlu*I primer 5**	5' **TGC** 3'	5' GAC TGC GTA ACC GCG-**TGC** 3'

2.4 Labelling and amplification conditions

2.4.1 AFLP analysis

AFLPs were performed as described by KeyGene, Inc. (Zabeau and Vos 1993; Vos et al. 1995) with minor modifications. The adaptors and primers, with selective 3' nucleotide sequences, used in this study are listed in Table 2.3.

Digestion, ligation and selection

Genomic DNA (200ng) was digested with 10units of each of the restriction enzyme *Mlu*I (Life Technologies, GIBCO BRL, U.K.) and *Mse*I (Life Technologies, GIBCO BRL, U.K.) using 1 x One-Phor-All Buffer PLUS [100 mM Tris-acetate (pH7.5); 100mM Mg-acetate; 500mM K-acetate] (USB Corporation, Pharmacia Biotech) in a total volume of 50µl for 1-2 hours at 37ºC.

The 50µl digested DNA mixture was supplemented with 10µl adapter/ligation solution, containing 50pmol *Mse*I adapter and 5pmol 5'-biotinylated *Mlu*I adapter, 1.2µl 10 mM ATP, 1X One-Phor-All Buffer PLUS [100mM Tris-acetate (pH7.5); 100mM Mg-acetate; 500mM K-acetate] (USB Corporation, Pharmacia Biotech) and 1 unit of T4 DNA ligase, (USB Corporation, Pharmacia Biotech) and incubated overnight at 37°C. The biotinylated fragments (*Mlu/Mlu* fragments and *Mlu/Mse*

Stellenbosch University http://scholar.sun.ac.za

fragments) were sifted and separated from the non-biotinylated fragments (*Mse*I/*Mse*I fragments) by binding to paramagnetic streptavidine coated beads (Dynal, Oslo, Norway). For each restricted sample, 20µl beads were washed three times with 20µl STEX-buffer [100mM NaCl; 10 mM Tris.HCl (pH7.5); 0.1mM EDTA (pH8.0)] and then resuspended in 20µl STEX-buffer [100mM NaCl; 10mM Tris.HCl (pH7.5); 0.1mM EDTA (pH8.0)]. The beads were subsequently added to the ligation mixture and incubated for 30 minutes on ice with gentle agitation every 10 minutes. The dynabead bounded fragments were collected using a magnetic particle separator. The beads were washed three times with 200µl of TE-buffer [(100mM NaCl; 10mM Tris.HCl (pH 7.5); 0.1mM EDTA (pH8.0)]. Finally the beads were resuspended in 100µl of TE buffer [(10mMTris.HCl (pH7.5); 0.1mM EDTA (pH8.0)].

Primer labelling

Only the *Mse*I primers were labelled. For ten PCR reactions, one µl (300ng) of the *Mse*I primer was added to 1µl 1 x One-Phor-All Buffer PLUS [100mM Tris-acetate (pH7.5); 100mM Mg-acetate; 500 mM K-acetate] (USB Corporation, Pharmacia Biotech), 10units [γ-^{33}P] ATP (25µCi) and 5units of T4 polynucleotide kinase (USB Corporation, Pharmacia Biotech), in a total volume of 10µl. Labelling was performed in heating blocks at 37° for 1 hour followed by a heat inactivation step at 65°C for 15 min.

AFLP fingerprinting amplification conditions

One µl of the biotinylated template DNA fragments was added to 30ng labelled *Mse*I primer, 30ng *Mlu*I primer, 100µM of each dNTP, 0.5units *AmpliTaq*® DNA polymerase (Perkin Elmer Biosystems), 1.5 mM $MgCl_2$ and 1/10 PCR Buffer II [100mM Tris-HCl (pH8.3) (at 25ºC); 500mM KCl] (Perkin Elmer Biosystems) in a total volume of 20µl. All amplification reactions were performed on a 9700 Perkin-Elmer GeneAmp PCR system thermocycler (Perkin Elmer-Biosystems). The cycle profile used for amplification was as follows: one cycle 72°C for 1min, one cycle of 94°C for 2min, followed by 12 cycles of 94°C for 20sec, 65°C for 30sec, 72°C for 2min, followed by 25 cycles of 94°C for 20sec, 56°C for 30sec, 72°C for 2min and one cycle at 72°C for 30min. The PCR conditions for the two *Mse/Mlu* AFLP primer combinations were consistent.

Stellenbosch University http://scholar.sun.ac.za

2.4.2 MITE/RGA analysis

MITE/RGA fingerprinting

Marker analysis was performed by end-labelling 30ng of the MITE primer (*Hbr*-F or *Hb2*-F) with 10units of [γ^{33}P] ATP (25μCi) using 5units of T4 polynucleotide kinase (USB Corporation, Pharmacia Biotech), 1 x One-Phor-All Buffer PLUS [100mM Tris-acetate (pH7.5); 100mM Mg-acetate; 500mM K-acetate] (USB Corporation, Pharmacia Biotech) to a total volume of 10μl. Labelling was performed in heating blocks at 37° for 1 hour followed by a heat inactivation step at 65°C for 15min.

Optimised PCR reactions were performed in a reaction mixture of 20μl containing: 50ng of genomic DNA (RIL individuals and both parents), 1μl of labelled MITE primer, 90ng of degenerate RGA primer, 100μM of each dNTP (Life Technologies, GIBCO BRL, U.K.) 0.5units of *AmpliTaq*® DNA polymerase (Perkin Elmer Biosystems), 2mM $MgCl_2$ and 1/10 PCR Buffer II [100mM Tris-HCl, (pH8.3) (at 25ºC); 500mM KCl] (Perkin Elmer Biosystems). By gently pipetting, the components were mixed. The cycling profile started with a denaturing step at 94ºC for 5min followed by 35 cycles [30 sec at 94ºC, 30 sec at 51°C (*Hbr*-F/RGA primer combinations) and 50°C (*Hb2*-F/RGA primer combinations) and 2min at 72ºC] followed by a final elongation step of 10min at 72ºC. All amplifications were performed on a 9700 Perkin-Elmer GeneAmp PCR system thermocycler (Perkin Elmer-Biosystems).

Thirty-two primer combinations were employed to detect polymorphisms between the recombinant parents corresponding to the same polymorphisms segregating in the 40 inbred individuals. These polymorphic fragments will be used for future documentation and mapping purposes. Eight MITE/RGA primer combinations were used on the 40 elite maize lines: i.e. four *Hbr*-F/RGA primer combinations *Hbr*-F/Red(D)1-R, *Hbr*-F/PL1-5-F, *Hbr*-F/Kin-F, *Hbr*-F/WMA1-2-R and four *Hb2*-F/RGA primer combinations; *Hb2*-F/Red(C)1-F, *Hb2*-F/PL1-5-F, *Hb2*-F/Kin2-R, *Hb2*-F/WMA1-2-F.

Stellenbosch University http://scholar.sun.ac.za

2.5 Polyacrylamide gel electrophoresis (PAGE)

The PCR samples were mixed with half the volume of loading dye (98% formamide; 10mM EDTA; 0.025% bromophenol blue and 0.025% xylene cyanol). Amplification products produced by the 32 different primer combinations were denatured by incubation at 90°C for 4 min, chilled on ice and run on a denaturing 4% acrylamide/bis-acrylamide (19:1), 7.5M urea and 1 X TBE gel for 90min to 2 hours at 40mA using a Model S2001 Sequencing Gel Electrophoresis Apparatus (Life Technologies, GIBCO BRL, U.K.). The gel was then transferred to 3MM Whatmans chromatography paper and dried on a gel dryer for 90min at 80°C. Gels were exposed overnight to Kodak BioMax MR film (Separations Scientific).

2.6 Gel analysis

For the recombinant inbred family only fragments polymorphic between the two parents were visually scored as present (1) and absent (0) and then subsequently scored separately for the 40 individuals of the inbred family.

For the two *Mse/Mlu* AFLP primer combinations and eight MITE/RGA primer combinations, both monomorphic and polymorphic fragments were scored. For each separate fragment amplified using the different primer combinations, each fragment were treated as a separate character and scored as either present (1) or absent (0) across all the 40 elite genotypes.

2.7 Statistical analysis

Genetic similarity and clustering analyses were performed using the unweighed pair group method arithmetic average (UPGMA) function of GENSTAT Version 531 software package (Numerical Algorithm Group, Oxford). Dendograms were constructed comparing the two sets of data generated from each individual fingerprinting method performed on the 40 elite maize lines.

2.8 Linkage analysis and mapping

The second recombinant inbred population of Burr et al. (1988) (Brookhaven National Laboratory, New York) was used to map 196 MITE/RGA fragments. Data of

Stellenbosch University http://scholar.sun.ac.za

approximately 1033 mapped markers for this second family of inbred lines were obtained from the databases at the National Agricultural Library (http://probe.nalusda.gov:8300). Linkage analysis was assessed using the software package MAPMAKER/EXP version 3.0b (Lander et al. 1987; Lincoln et al. 1992a). To identify linkage groups, pairwise comparisons and grouping of markers were carried out at a LOD score of 3.0 with no set maximum distances between the markers. Afterwards marker order was confirmed. Lod scores lower than 3 were disregarded where LOD scores higher than 3 were taken as significant values.

Setosphaeria turcica mapping

Linkage analysis was performed with the software package MAPMAKER/EXP version 3.0b (Lander et al. 1987; Lincoln et al. 1992a) using mapping data obtained with us-markers, identified by Van Staden (2001).

QTL mapping

Linkage analysis was performed with the software package MAPMAKER/EXP version 3.0b (Lander et al. 1987; Lincoln et al. 1992a) using the markers identified by Lehmensiek (2000). To include a locus in a linkage group a minimum LOD threshold of 3.0 was used. The chromosomal location of the QTL was determined by interval mapping (Lander and Botstein 1989) using MAPMAKER/QTL version 1.1b (Paterson et al. 1988; Lincoln et al. 1992b) at a LOD threshold of 2.0 and by composite interval mapping (Zeng 1994) using the program QTL Cartographer version 1.13 (Baston et al. 1994; Baston et al. 1997).

2.9 Isolation and cloning of AFLP fragments

After localisation by autoradiography of the significant AFLP fragments (identified from mapping positions on the RI family maps) on the acrylamide gels, the area containing the fragment was excised from the gel, by orientating the X-ray film with the original gel using autoradiography GLO stickers (Bel-Art products, Penquannock, NJ) as fluorescent markers. In table 2.4 the 26 MITE/RGA fragments identified for excision are shown, ten fragments for the *Hbr*-F primer combinations and 16 for the *Hb2*-F primer combinations.

Stellenbosch University http://scholar.sun.ac.za

Table 2.4 The 26 *Hbr-* and *Hb2* fragments excised from the gels. Also indicated are the chromosomes to which each of these 26 fragments map to.

Primer Combination	***Chromosome***	***Primer Combination***	***Chromosome***
Hbr-F/Perox-R (2)	1	*Hb2-F*/Perox-R (2)	7
Hbr-F/Red(C)-F (5)	2	*Hb2-F*/Red(C)-F (5)	2
Hbr-F/Red(D)-R (4)	3	*Hb2-F*/Red(C)-F (6)	4
Hbr-F/RG_1-F (2)	4	*Hb2-F*/Red(C)-F (7)	5
Hbr-F/PL1-5-F (1)	7	*Hb2-F*/Red(C)-R (7)	7
Hbr-F/PL1-5-F (9)	8	*Hb2-F*/RG_1-R (8)	8
Hbr-F/GLPL-F (1)	2	*Hb2-F*/RG_1-R (9)	6
Hbr-F/GLPL-F (3)	2	*Hb2-F*/PL1-5-F (7)	2
Hbr-F/WMA-R (2)	2	*Hb2-F*/Kin2-R (11)	9
Hbr-F/WMA-R (3)	3	*Hb2-F*/WMA-F (1)	7
		Hb2-F/WMA-F (13)	1
		Hb2-F/WMA-F (15)	1
		Hb2-F/WMA-F (17)	2
		Hb2-F/WMA-R (10)	5
		Hb2-F/WMA-R (11)	8
		Hb2-F/WMA-R (12)	8

Fragments were eluted in 60μl of TE-buffer [10mM Tris-HCl; (pH7.5); 0.1mM EDTA; (pH8.0)]. Five PCR reactions, each using 2μl of the eluted fragment were reamplified using the same MITE/RGA primer pair combination and under the same conditions as before, this time using only 30ng of each primer. Products were separated on a 2% low melting agarose gel (Whitehead Scientific) and sized using the 1Kb Plus DNA Ladder (Life Technologies, GIBCO BRL, U.K.). Reamplified fragments were excised from the gel and dissolved at 65°C for 10 min. Gel volume was made up to 500μl with ddH_2O (SABAX) followed by equal volumes of phenol and chloroform extraction. The fragment was precipitated with 20μl 0.5M NaCl and absolute (100%) ethanol. Washing was done with 500μl of 70% ethanol.

Stellenbosch University http://scholar.sun.ac.za

Quantification of the fragments was performed on a 1.5 % agarose gel (Whitehead Scientific) using DNA Quantitation Standards 15ng, 31ng, 65ng, 125ng, 250ng, 500ng (Life Technologies, GIBCO BRL, U.K.)

Purified DNA fragments were cloned into a bacterial plasmid using the pGEM-T Easy Vector System (Promega, Madison Wisconsin), ligated overnight at 4°C and transformed into 50µl of JM109 bacterial strain according to the manufacturer's instruction manual (Promega, Madison Wisconsin). Agar grids were prepared for a few selected colonies for each of the transformed ligation products. To verify the origin and authenticity of the fragments, 30ng of the MITE primer was end-labelled using [γ^{33}P] ATP. Radioactive PCRs on selected colonies, as well as on the recombinant inbred parents, were performed to discard fragments of incorrect size.

Overnight 5ml LB-medium (10g bacto®-tryptone, 5g bacto®- yeast extract and 10g NaCl per 1 litre) cultures were extracted using the PE-MiniPrep plasmid extraction kit (Perkin Elmer-Biosystems).

2.10 Fragment sequencing

Plasmid DNA was sequenced with the original MITE and RGA primer, using an ABI Prism BigDye Terminator Cycle Sequencing ready reaction kit (Perkin Elmer Biosystems) following instructions in the manufacturers manual. The PCR reactions were subjected to temperature profiles on a Perkin-Elmer GeneAmp PCR system 2400 thermocycler and electrophoresed on a 36cm denaturing 5% Long Ranger poly-acrylamide gel (FMC BioProducts, Rockland, ME). The samples were electrophoresed for ±3.5 hours at a limiting parameter of 3000V on an ABI 377 automated DNA sequencer (Perkin Elmer Biosystems). Data analysis was performed using the Sequencing Analysis version 3.3 software package.

2.11 DNA sequencing analysis

Computer aided similarity of sequence searches were used as queries on the EPLN plants library nucleic acid sequence database employing the FASTA algorithm version 3.3t07 (http://www.ebi.ac.uk/servicestmp/533115.372271-235644.htm1), (Pearson and Lipman 1988).

Stellenbosch University http://scholar.sun.ac.za

CHAPTER THREE

RESULTS

3.1 Designing of unique primer sets

Two miniature inverted-repeat transposable subfamily element (MITE) primers, *Heartbreaker* (*Hbr*) and *Hb2* were used in combination with different resistant gene analogue (RGA) primers to determine by linkage analysis and mapping the occurrence and distribution of MITE/RGA fragments in genic rich areas of the maize genome. Using the Fasta version 3.3t07 algorithm on the EPLN and SwissProt sequence database (http://www.ebi.ac.uk/servicestmp/ 533115.372271-235644.htm1) (Pearson and Lipman 1988), similarity searches were carried out for maize genes with a peroxidase and reductase protein product. Peroxidase was used as a query because it is supposed that the protein products of some of the *Ht* genes (discussed in later sections in more detail) are of a peroxidase nature. One of the first resistant genes to be cloned and isolated was for *Helminthosporium carbonum* susceptibility1 (*hm*1) of maize, which confers resistance to the leaf spot fungus *Helminthosporium maydis.* Because of the availability of this specific protein sequence, the reductase primer sets were designed. This sequence was either used as a search query or used directly to design suitable primers from. These primer sets were designated Per and Red primers. Primer sets developed based on the homology searches and sequence data are shown in Table 3.1.

Two different sets of reductase primers were designed using the EPLN and SwissProt sequence database. Collins et al. (1998) and Aarts et al. (1996), designed a subset of oligonucleotides based solely on conserved amino acid motifs among known nucleotide binding sites (NBS)-leucine rich repeats (LRR) resistance genes (PL1-5, GLPL, Kin, WMA1-2 and RG_1), of diverse plant genomes (flax, *Arabidopsis*, tomato and tobacco). Primers published in these two studies were used for analysis in this study. Collectively, the five primer sets were named: NBS (Table 2.2 Materials and Methods, Chapter 2). The availability of the *Hbr* - (Zhang et al. 2000) and *Hb2*- (Spell et al. 1988) original element sequences facilitated the designing of the forward *Hbr*- and *Hb2* primers.

Stellenbosch University http://scholar.sun.ac.za

Table 3.1 Primer sequence derived after similarity searches were conducted for maize genes with a peroxidase and reductase protein product using the Fasta3_t algorithm.

Primer	**Source**	***Sequence 5'-3'***
Group name: Per		
Per1-F	ZmDB (Fasta3_t)	CAC CCM AAG GTC AGC CAI GAG C
Per1-R	ZmDB (Fasta3_t)	GCT CIT GGC TGA CCT TKG GGT G
Group name: Red		
Red(C)1-F	ZmDB (Fasta3_t) (***Helminthosporium carbonum)***	TCA CCG GNG SIG CCG GGT WCA TCG
Red(C)1-R	ZmDB (Fasta3_t) (***Helminthosporium carbonum)***	CGA TGS ACC CGG CIW CKC CGG TGA
Red(D)1-F	ZmDB (Fasta3_t)	TTC ATC GGC TCC TGG CTC GT
Red(D)1-R	ZmDB (Fasta3_t)	ACG AGC CAG GAG CCG ATG AA

3.2 PCR profile amplification

The distribution and chromosomal organisation of the MITE/RGA fragments were determined in the parents and across 40 individuals of the publicly available recombinant inbred population (RIL) (Tx303 and CO159), produced by B. Burr, Brookhaven National Laboratory, (Burr et al. 1988). Figures 3.1 (a–b) and 3.2 (a-b) are examples and illustrates the partial amplification profiles displays obtained using different RGA-primers in combination with the *Hbr-F* and *Hb2-F* primer.

Stellenbosch University http://scholar.sun.ac.za

Figure 3.1 (a) A partial amplification profile using RGA-primer PL1-5-F together with MITE primer *Hbr-F* (group NBS). P_1 = Tx303 and P_2 = CO159. Some of the fragments scored, are indicated.

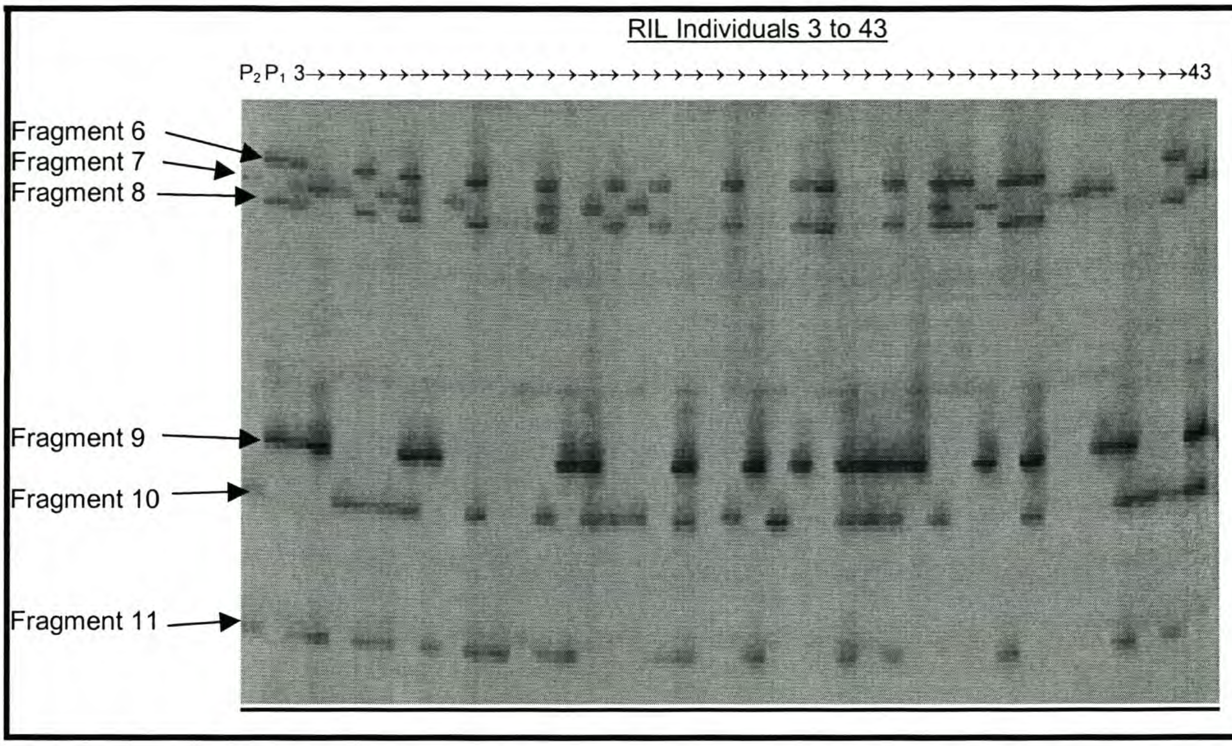
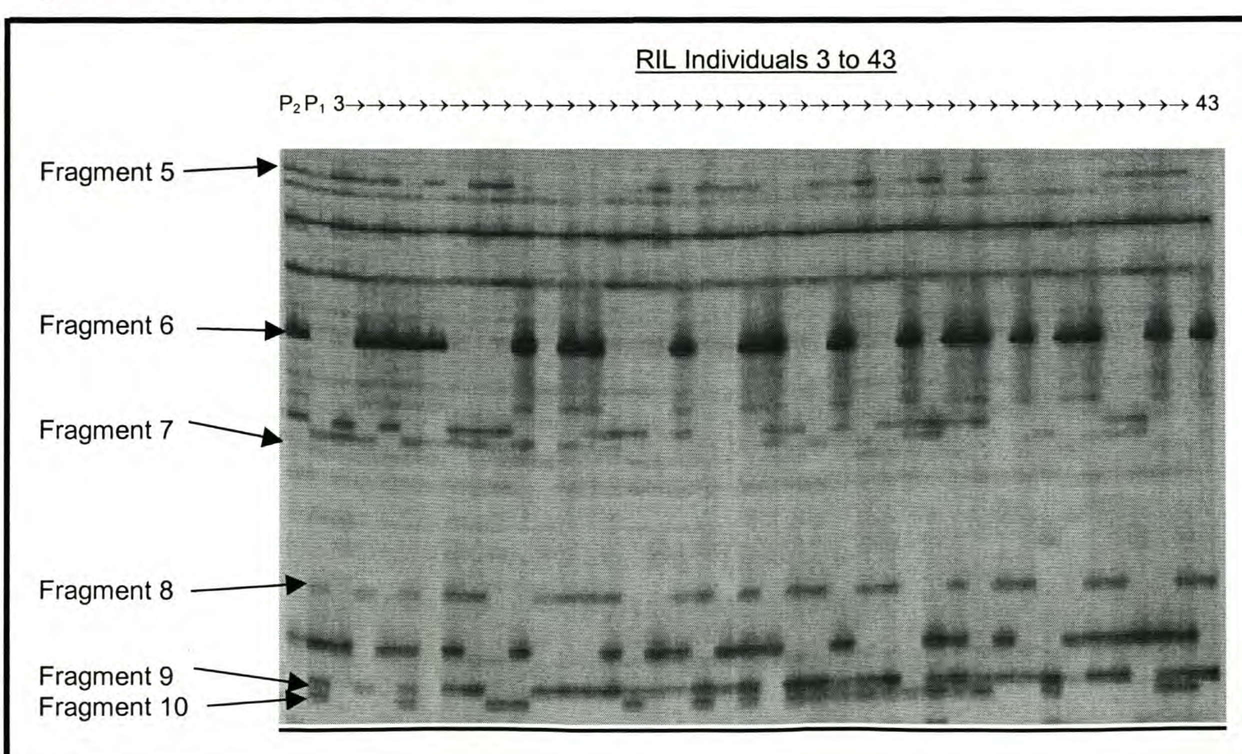


Figure 3.1 (b) A partial amplification profile using RGA primer Kin-F together with MITE primer *Hbr-F* (group NBS). P_1 = Tx303 and P_2 = CO159. Some of the fragments scored are indicated.



Stellenbosch University http://scholar.sun.ac.za

Figure 3.2 (a) A partial amplification profile using RGA-primer WMA-F together with MITE primer *Hb2-F* (group NBS). P_1 = Tx303 and P_2 = CO159. Some of the fragments scored are indicated.

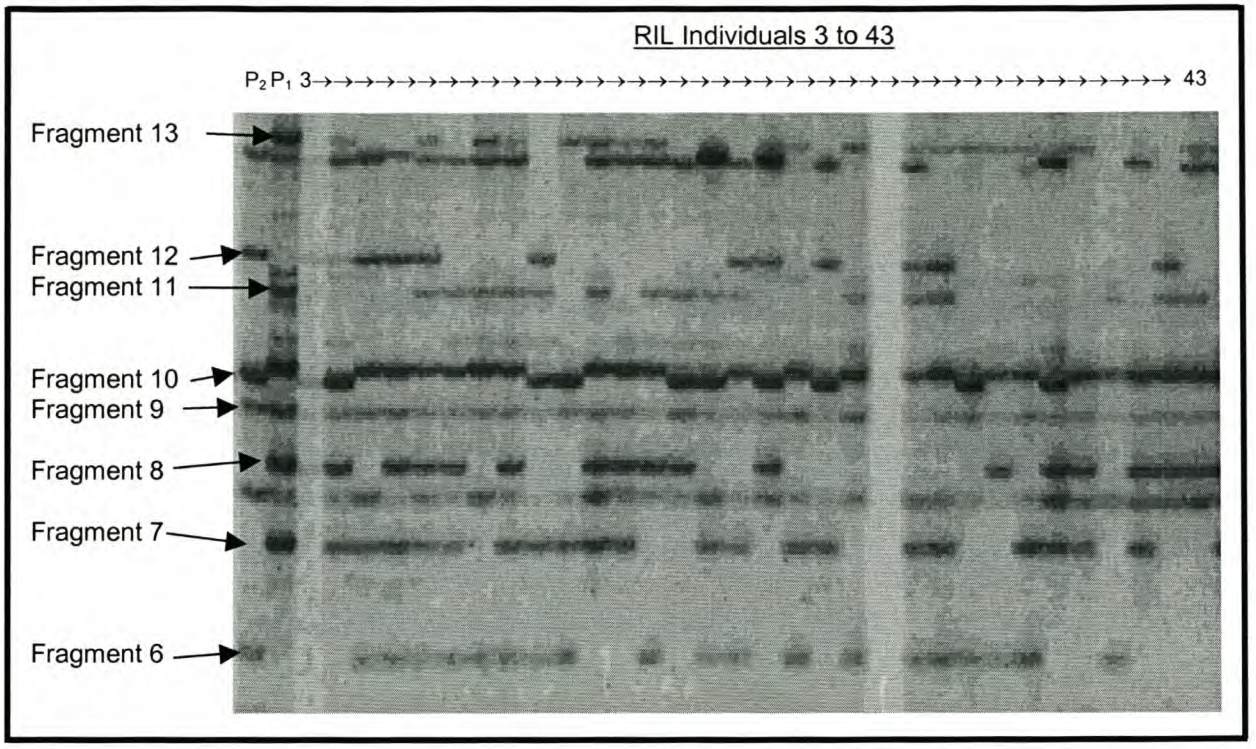
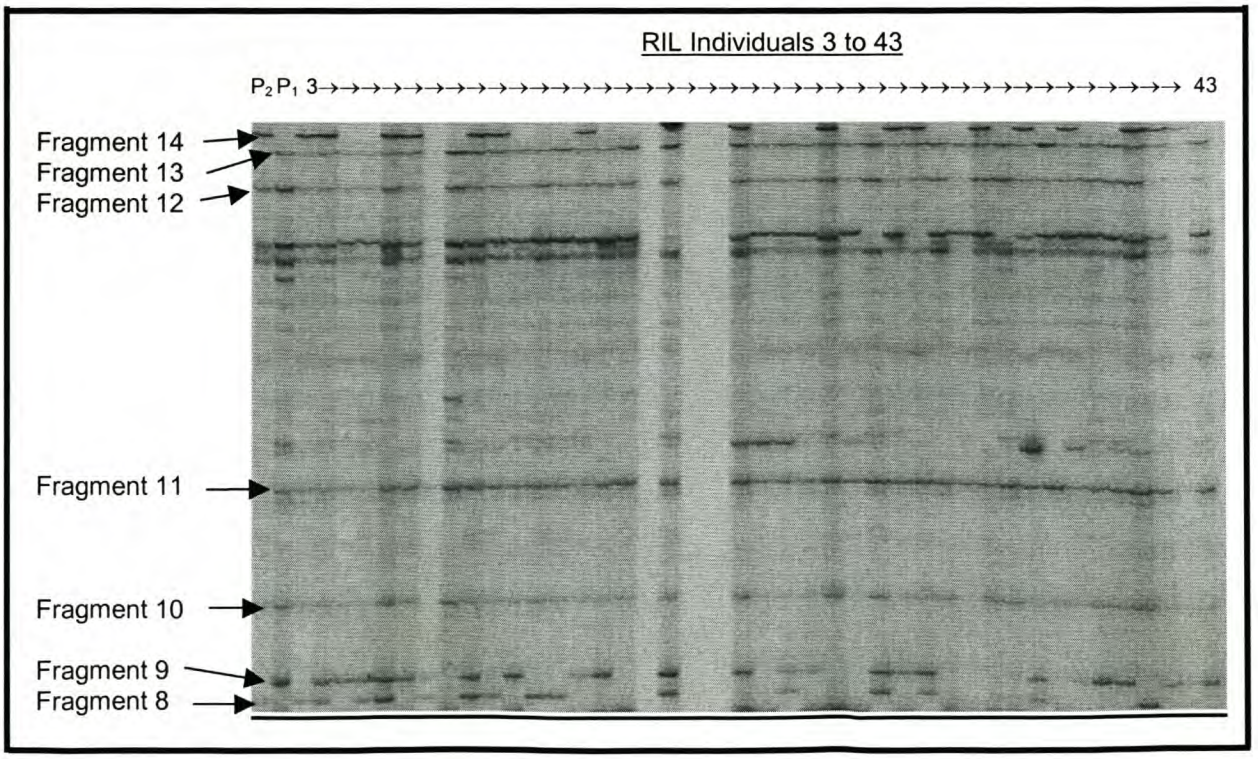


Figure 3.2 (b) A partial amplification profile using RGA-primer Red(C)-F together with MITE primer *Hb2-F* (group Red). P_1 = Tx303 and P_2 = CO159. Some of the fragments scored are indicated.



Stellenbosch University http://scholar.sun.ac.za

3.3 Primer specificity and sequence analysis

3.3.1 Element conservation

To determine the degree of conservation amongst the MITE element subfamilies, ten *Hbr*- and 16 *Hb2* fragments were non-randomly selected, excised from the gels and subsequently sequenced (Table 3.2). These partial *Hbr*/RGA- and *Hb2*/RGA-fragment sequences were compared to the original *Hbr*-element and *Hb2*-element sequences, to determine the extent of element sequence conservation.

Table 3.2 Twenty-six MITE/RGA fragments were isolated and sequenced

Primer Combination (no of fragments)	***Primer Combination (no of fragments)***
Hbr-F/Per (1)	*Hb2-F*/Per (1)
Hbr-F/Red (2)	*Hb2-F*/Red (4)
Hbr-F/NBS (7)	*Hb2-F*/NBS (11)

Figure 3.3 shows alignments of the sequences of the original *Hbr-hm*1 element sequence and the ten *Hbr*-fragments containing a partial sequence of the *Hbr*-element. All the fragments sequenced showed the *Hbr*-element, this confirms the specificity of the *Hbr* primer. The 14bp of terminal inverted-repeat (TIR) differed for all of the ten *Hbr* elements. They varied with as little as 1bp point mutation (93% similarity) for fragments PL1-5-F(9) and GLPL-F(3) to as much as 3 base pair point mutations (79% similarity) for fragments Perox-R(5) and GLPL-F(1). When analysing the internal *Hbr*-element sequence of 44bp, differences were found from as little as 1 base pair for fragments RG_1-F(2) and PL1-5-F(9) (98% similarity) to as much as 7 base pairs for fragments Perox-R(5) and WMA-R(3) (84% similarity). Fragments PL1-5-F(1) revealed an 18 base pair deletion.

Stellenbosch University http://scholar.sun.ac.za

Figure 3.3 Sequence alignment of the ten *Hbr*-elements with the *Hbr-hm1*-element (Zhang et al. 2000). Point mutations are indicated in blue, deletions indicated as •, primer sequence of *Hbr-F* indicated in green, the bold red indicated regions represents the 3bp host sequence (DIR) that is duplicated upon insertion and the Terminal inverted-repeat (TIR) of each *Hbr*-element is underlined.

Hbr-hm1 5'...GATTCTCCCC ACAGCCAGAT TC*TCTCCACA ACCAGATTTT CAGAAAAGCT GGTCAGAAAA AAGCTG*AACC AAACAGACCC TAA...3'

Red(C)-R (5) 5'...GATTCTCCCC ACAGCCAGAT TC*TCCTCACA GCCAGATTTT CAGAAAAGCT AGTCAGAAAA AAGCTG*AACC AAACAGCCAC TTA...3'

Red(D)-R (4) 5'...GATTCTCCCC ACAGCCAGAT TC*TCCCTACA GCCAGATTTT CAGAAAAGCT GGTCAGAAAA AAGCTG*AACC AAACATGCCC TGT...3'

RG_1-F (2) 5'...GATTCTCCCC ACAGCCAGAT TC*TCTCCACA GCCAGATTTT CAGAAAAGCT GGTCAGAAAA AAGCTG*AACC AAACAGGCGC ATT...3'

Per-R (5) 5'...GATTCTCCCC ACAGCCAGAT TC*TCTCCACA ACCAGATTGT CAGAAAAGCT TATTAGAAAA AAGACG*AAAC GAACATACCA TAG...3'

GLPL-F (1) 5'...GATTCTCCCC ACAGCCAGAT TC*TTCCTACA GCCAAATTTT TAGAAAAGCT GGTCAGAAAA AAGCTG*AACC AAATATGCCC GAA...3'

GLPL-F (3) 5'...GATTCTCCCC ACAGCCAGAT TC*TCCCAACA GCCAGATTTT TAGAAAAGCT GGTCAGAAAA AAGCTG*AACC AAACAGGCCC ACA...3'

PL1-5-F (1) 5'...GATTCTCCCC ACAGCCAGAT TC*T•••••••••••••••••••••••••••••• CAGAAAAGCT GGTCAGAAAA A •GCTG*AACC AAACAGGCCC TAT ...3'

PL1-5-F (9) 5'...GATTCTCCCC ACAGCCAGAT TC*TCTCCACA GCCAGATTTT CAGAAAAGCT GGTCAGAAAA AAGCTG*AACC AAACAGGCGC ATT...3'

WMA-R (2) 5'...GATTCTCCCC ACAGCCAGAT TC*TCCTCACA GCCAGATTTT CAGAAAAGCT AGTCAGAAAA AAGCTG*AACC AAACAGCCAC TTA...3'

WMA-R (3) 5'...GATTCTCCCC ACAGCCAGAT TC*TCCGCACA GCTAGATTCT TAGAAAAGCT GGTCAGAAAA AAGCTA*AACC AAACAGGTCC ATT...3'

Only nine of 16 *Hb2* fragment sequences, analysed showed a partial sequence that matched the original *Hb2*-element, sequence of an element insertion into maize W23 (waxy) allele (original *Hb2*-element; Spell et al. 1988). None of the remaining seven fragment sequences showed any similarity to each other or to the original *Hb2*-element sequence. Figure 3.4 are alignments of the nine partial *Hb2* fragment sequences with the original *Hb2*-element sequence, illustrating the extent and degree of conservation of the *Hb2*-element. The primer selected and designed for this study amplified a very small part of the *Hb2*-element. Part of the imperfect TIR (10 of 15bp) and 5bp directional repeat (DIR), from the *Hb2*-element were amplified. The 15bp terminal inverted-repeat of the original *Hb2*-element showed a 100% similarity to fragments WMA-R(10), WMA-R(12), WMA-F(13), WMA-F(17) and Red(C)-F(7). Fragment RG_1-R(9) showed a 1bp deletion in the TIR where fragments WMA-F(1), Perox-R(2) and WMA-F(1) showed 1 to 2bp point mutation. The directional repeats of fragments WMA-R(10), WMA-F(15) and Red(C)-F(7) showed a 100% similarity to the DIR sequence of the original sequence.

Stellenbosch University http://scholar.sun.ac.za

Figure 3.4 Sequence alignment of the nine 33bp *Hb2*-elements to the sequence of the DNA insertion in the W23 maize (waxy) W23 allele. (Spell et al. 1988). Primer sequence indicated in green, point mutations are indicated in blue, deletions are indicated as •, bold red indicated regions represents the 5bp (DIR) host sequence that is duplicated upon insertion and the 15bp Terminal inverted-repeat (TIR) region is underlined.

Sequence of insertion into maize W23 allele	5'...TCACAGGCAC AGCTCAACCA AACAGACCCT AAG...3'
WMA-R (10)	5'...TCACAGGCAC AGCTCAACCA AACAGACCCT AAG...3'
WMA-R (12)	5'...TCACAGGCAC AGCTCAACCA AACAGACCCT AAA...3'
WMA-F (1)	5'...TCACAGGCAC AGCTCAACCA AATAGACCCT TAA...3'
WMA-F (13)	5'...TCACAGGCAC AGCTCAACCA AACAGACCCT TAG...3'
WMA-F (15)	5'...TCACAGGCAC AGCTCAACCA AACAAACCCT AAG...3'
WMA-F (17)	5'...TCACAGGCAC AGCTCAACCA AACAGACCCT CAG...3'
Per-R (2)	5'...TCACAGGCAC AGCTCAACCA AACATAACCT AAA...3'
RG_1-R (9)	5'...TCACAGGCAC AGCTCAACCA AACAGA •CCT TAA...3'
Red(C)-F (7)	5'...TCACAGGCAC AGCTCAACCA AACAGACCCT AAG...3'

3.3.2 Specificity of the two MITE primers

To show the specificity of both the *Hbr*-F primer and the *Hb2*-F primers, partial sequences (Figures 3.3 and 3.4-including the forward primer sequences) of both of the MITE elements were used as a search query (83bp query search for *Hbr*-F primer and 33bp for the *Hb2*-F primer) on the Fasta DNA sequence data bank (http://www.ebi.ac.uk/servicestmp/533115.372271-235644. htm1). The queries were compared to 68 956 possible gene or gene product sequences from the Fasta EPLN plants library nucleic acid sequence database (Pearson and Lipman 1988), results from this search is shown in Table 3.3.

The partial sequence of the original *Hbr*-element, *Hbr-hm1,* showed nearly perfect fits (93-95% in an 80bp overlap) with five accessions of *Zea mays* L. miniature inverted-repeat transposable elements (MITEs). In contrast the *Hb2*-element gave hits (79-94% in a 23-34bp overlap) to numerous *Zea mays* L. and rice accessions, including the 316bp insert in the maize waxy gene (hit no:3*-Table 3.3).

Stellenbosch University http://scholar.sun.ac.za

Table 3.3 Database search results using the original *Hbr*- and *Hb2*- sequences as queries

Query	***Fragment size (bp)***	***Match***	***Overlapping Sequence (bp)***	***% Identity***
***Hbr*-F**	83	**AF203732**: *Zea mays* Miniature inverted repeat element	80	95%
		AF203730: *Zea mays* Miniature inverted repeat element	80	93.75%
		AF203733: *Zea mays* Miniature inverted repeat element	80	93.75%
		AF203729: *Zea mays* Miniature inverted repeat element	80	95%
Hb2-F	33	**AF205807**: *Zea mays* subsp. Huehuetangen	33	93.94%
		Z29642: *Zea mays* U3SNRNA Psuedogene	33	87.88%
		***X06934:** Maize 316bp insertion sequence	28	92.86%
		AF172282: *Oryza Sativa* ADH1-ADH2 region	31	87.1%

3.3.3 MITE/RGA DNA sequence analysis

Using the sequences of the 19 MITE/RGA fragments, computer aided similarity searches were conducted on the EPLN plants library nucleic acid sequence database employing the Fasta algorithm version 3.3t07 (http://www.ebi.ac.uk/servicestmp/533115.372271-235644.htm1) (Pearson and Lipman 1988). These queries excluded the MITE primer sequence but included the RGA primer sequence. Based on similarities between the queries and data base sequence, gene analogues were identified. Tables 3.4 (a) and 3.4 (b) summarizes the top two hits and percentage similarity scores for each of the *Hbr*- and *Hb2*/RGA fragments are shown.

Similarities were found with chromosomal areas corresponding to the maize-, rice-, tomato-, *Arabidopsis*-, petunia- and sorghum genomes. A number of hits were found with maize household genes e.g., alcohol dehydrogenase gene, accase gene (accA), glyceraldehyde-3-phosphate dehydrogenase (GAPC) genes and the *Mus*2 (mismatch

Stellenbosch University http://scholar.sun.ac.za

repair) gene as well as a stress induced gene, the NIK heat shock protein gene. Fragment *Hb2*/WMA-F(1) showed hits with two genes, one from the rice genome and the other was shown to be part of a tomato resistant gene. Fragment *Hb2*/Per-R(9) showed a high degree of similarity (78.36% of 305bp overlapping sequence) to the maize lethal leaf-spot 1 gene (*lls*1), this gene aids in programmed cell death suppression. Sequence *Hb2*/WMA-R(13) showed similarity to two *Zea mays* L. miniature inverted-repeat transposable element sequences (MITE). One fragment *Hb2*/WMA-F(15) showed, with a 80bp overlap, a 67.5% sequence similarity to a certain *Zea mays* L. NADPH HC toxin reductase. One specific fragment, *Hb2*/WMA-F(15), map to chromosome 1, bin1.07, which is also the chromosomal location for the *hm*1 gene (*Helminthosporium carbonum*) which has a NADPH HC Toxin reductase protein product. This fragment can be considered as a candidate gene for *hm1*. Only one fragment, *Hbr*/Per-R(5), did not show similarity to any known sequence in the database.

Stellenbosch University http://scholar.sun.ac.za

Table 3.4 (a) Hits accomplished with sequences from the ten *Hbr*/RGA fragments.

Query	*Fragment size (bp)*	*Match*	*Overlapping Sequence (bp)*	*% identity*
Red(C)-R (4)	246	**AP001111:** Genomic sequence for *Oryza sativa*	240	64.58%
		AC027661: Genomic sequence for *Oryza sativa*, subspecie Japonica	225	64.44%
Red(D)-R (5)	368	**AF105716:** Genomic sequence for *Zea mays*	95	76.84%
		AF090447: *Zea mays* cosmid II.2E10	118	71.19%
RG_1-F (2)	250	**AC069556:** Genomic sequence for *Arabidopsis* thaliana	63	69.84%
Per-R (5)	130	No Match found	-	-
GLPL-F (1)	401	**AP001859:** Genomic sequence for *Oryza sativa*	165	58.79%
		AF203033: Chladymonas Reinhardtii Nitrater reductase (NIT1) gene	165	58.79%
GLPL-F (3)	188	**AP002747:** Genomic sequence for *Oryza sativa*	138	68.12%
		AF031569: *Zea mays* 22-KDA Alpha zein gene cluster	135	68.15%
PL1-5-F (1)	545	**M99431:** Pharbitis NIK heat shock protein gene	260	55.39%
		AF061282: Sorghum bicolor 22 KDA kafirin cluster	261	60.65%
PL1-5-F (9)	247	**AP002071**: Genomic sequence for *Oryza sativa*	123	60.98%
WMA-R (2)	517	**AC027659:** Genomic sequence for *Oryza sativa*, subspecie Japonica	520	65.19%
		AC068654: Genomic sequence for *Oryza sativa*	507	64.90%
WMA-R (3)	545	**X66422:** *Zea mays* PG gene for polygalacturonase	231	84.85%
		U03682: *Zea mays* W-22 clone PREM-1B retroelement	231	83.11%

Stellenbosch University http://scholar.sun.ac.za

Table 3.4 (b) Hits accomplished with sequences from the nine *Hb2*/RGA fragments.

Query	*Fragment size (bp)*	*Match*	*Overlapping sequence (bp)*	*% identity*
WMA-R(10)	230	**AF123535:** *Zea mays* alcohol dehydrogenase	134	67.91%
		ZU90128: *Zea mays* Accase gene, introns continue	140	62.14%
WMA-R(12)	196	**AF090446**: *Zea mays* cosmid IV.1E1	194	72.68%
		AF123535: *Zea mays* alcohol dehydrogenase	188	73.40%
WMA-F(1)	487	**AB022172**: Part of *Oryza sativa* T580-9 Gene	174	60.92%
		AF004879: Lycopersicon esculentum resistance gene	222	56.76%
WMA-F(13)	214	**AF205891**: *Zea mays* Miniature inverted repeat element	85	77.65%
		AF205925: *Zea mays* Miniature inverted repeat element	87	74.71%
WMA-F(15)	178	**AF041047**: *Zea mays* NADPH HC Toxin Reductase	80	67.5%
		AL138649: Genomic sequence for *Arabidopsis* thaliana	102	61.77%
WMA-F(17)	88	**X73152:** *Zea mays* GAPC4 Gene	87	96.55%
		AJ238786: *Zea mays* Mus2 gene, Exons1-1 (DNA mismatch repair)	86	91.86%
Per-R(2)	590	**U77346:** *Zea mays* Lethal Leaf Spot1 (lls1) (novel cell death repressing function)	305	78.36%
		AJ251018: *Zea mays* PAO Gene	354	73.73%
RG_1-R(9)	179	**AJ271065:** Petunia hybrid partial mRNA	79	68.35
		AB012248: Genomic sequence for *Arabidopsis* thaliana	75	66.67%
Red(C)-F(7)	211	**L19495**: *Zea mays* transcriptional	145	68.28%
		AC0007069: *Arabidopsis* thaliana chromosome	138	65.21%

Stellenbosch University http://scholar.sun.ac.za

3.4 Polymorphic fragments

Applying the *Hbr*-element primer (Table 2.1-Materials and Methods) together with the 16 different gene analogue primers (Table 2.2-Materials and Methods) a total of 172 prominent fragments were scored on amplification profiles (Table 3.5). One hundred and six (61.6%) of these fragments scored showed to be polymorphic between parental lines Tx303 and CO159. Using the *Hb2*-element primer (Table 2.1-Materials and Methods) for amplification, the 16 different primer combinations (Table 2.2-Materials and Methods, Chapter 2) amplified a total of 208 scorable fragments (Table 3.5). One hundred and four fragments (50%) were polymorphic between parents Tx303 and CO159. The 210 polymorphic fragments were subsequently scored on the 40 individuals recombinant inbred family.

Table 3.5 Total number of fragments amplified for both the MITE elements. Shown in brackets are the numbers of primers used.

	Hbr-element		***Hb2-element***		***TOTAL***	
	F	**P**	**F**	**P**	**TF**	**TP**
Per (2)	12	8	16	7	28	15
Red (4)	44	28	44	20	88	48
NBS (10)	116	70	148	77	264	147
Total:	172	106	208	104	380	210

F = Total number of fragments scored P = Fragment polymorphic between the two RIL parents

TF = Total number of fragments scored for both MITE elements for the respective RGA group

TP = Total number of fragments for the two MITE elements polymorphic in the two RIL parents

3.5 Mapping and distribution of the MITE/RGA markers

The 40 recombinant inbred individuals were used to do linkage analysis and determine the marker distribution of the MITE/RGA fragments across the ten maize chromosomes, using the MapMaker/EXP software PC version 3.0b computer statistical package (Lander et al. 1987; Lincoln et al. 1992a). The main marker framework was built using a significant LOD score higher than 3.0 to infer the most probable marker order along each of the ten chromosomes.

Of the 210 polymorphic fragments, 196 were mapped applying multipoint linkage test analysis using as a framework, the approximately 1033 previously mapped conventional markers from the publicly available RIL population (Tx303 x CO159)

Stellenbosch University http://scholar.sun.ac.za

map, obtained from National Agriculture Library (http://probe.nalusda.gov:8300). For the *Hbr*/RGA primer combinations four fragments were found to be polymorphic between the parental lines but could not be placed on the linkage map, this was also the case for ten *Hb2*/RGA fragments. Table 3.6 summarises the chromosomal distribution of the 196 fragments across the 10 maize chromosomes.

Table 3.6 shows that no peroxidase fragments mapped to chromosomes 4, 6, 8 and 9, whereas all of the ten chromosomes hold at least two mapped reductase fragments and at least six mapped NBS fragments. When assessing the distribution of the 196 fragments across the ten chromosomes it *were* found that a total of 39 MITE/RGA markers mapped to chromosome 2 and only 9 fragments mapped to chromosome 9. Chromosome 9 contained the least amount of mapped *Hbr* fragments (33.3%) and the most mapped *Hb2* fragments (66.7%) whereas chromosomes 3 and 7 contained the most mapped *Hbr* fragments (65.2%) but the least amount of mapped *Hb2* fragments (34.8%). Chromosome 8 had an equal numbers of mapped *Hbr* and *Hb2* fragments (50%).

Table 3.6 Chromosomal distribution of the 196 mapped fragments across the maize genome.

	Chromosome									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Group class:										
Per	2	3	1	0	2	0	1	0	0	1
Red	5	7	3	2	7	5	6	6	2	2
NBS	16	29	19	13	6	9	16	14	7	12
TOTAL:	23	39	23	15	15	14	23	20	9	15
% *Hbr* fragments	56.5	48.7	65.2	46.7	46.7	42.9	65.2	50.0	33.3	60.0
% *Hb2* fragments	43.5	51.3	34.8	53.3	53.3	57.1	34.8	50.0	66.7	40.0

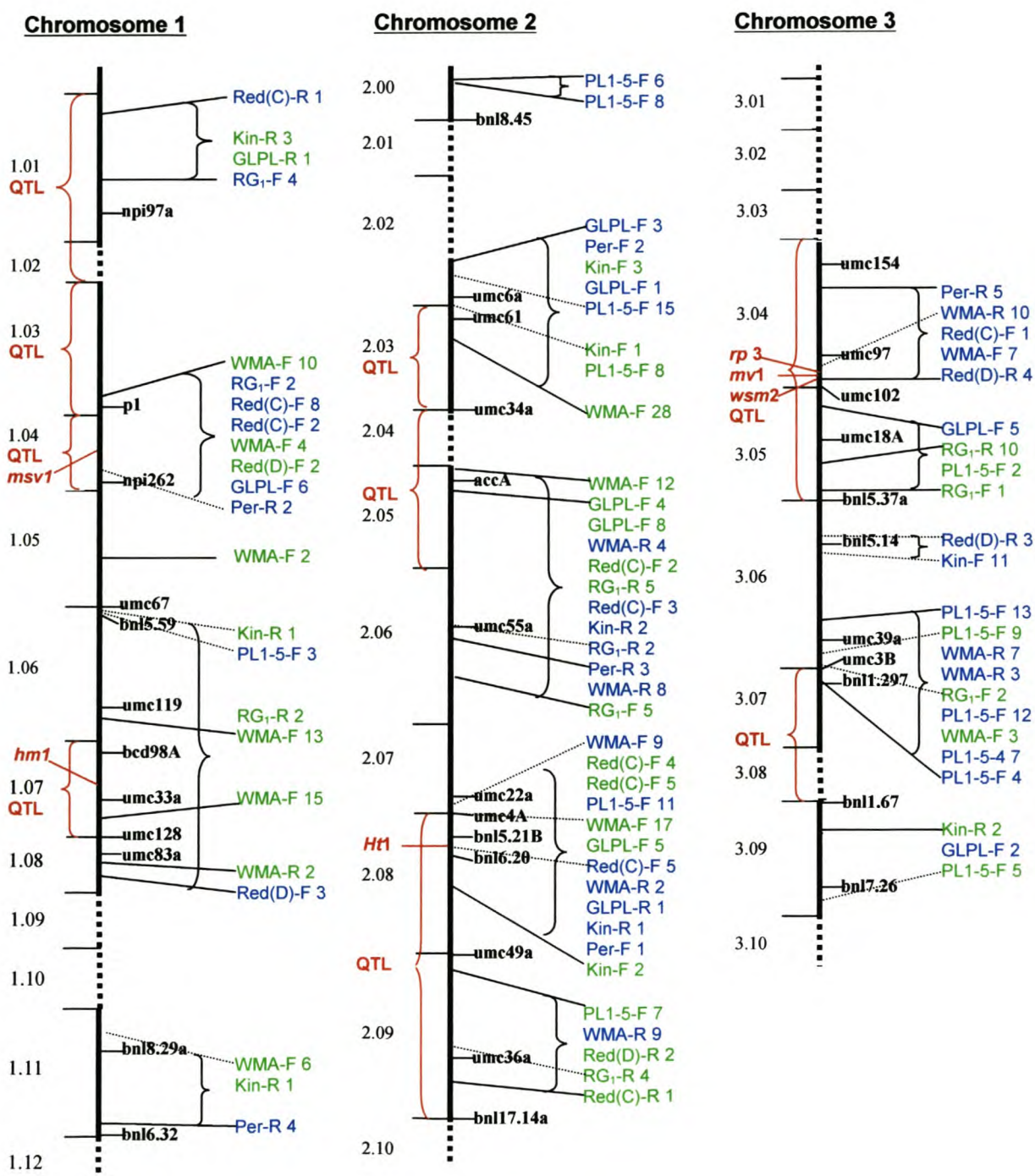
The 196 fragments were named after the RGA primer set used to amplify a specific fragment. A graphical representation of the data in Table 3.6 are given in Figures 3.5 (a-d), showing detailed chromosomal maps, indicating the distribution of the 196 MITE/RGA fragments in linkage groups spread across the ten maize chromosomes.

Stellenbosch University http://scholar.sun.ac.za

When a chromosomal bin was assigned to each of the 196 fragments, flanking (where possible)- and core markers from the RIL-map (Tx303 x CO159) were taken into consideration to fix the order of the mapped fragments. Linkage groups were assigned to each of the ten chromosomes by including the segregation data of the mapped loci obtained from the recombinant inbred lines. Linkage analysis of the 196 MITE/RGA fragments and mapping data is given in Addendum A. In addendum A, each linkage group of a chromosome is in order of appearance on that specific chromosome.

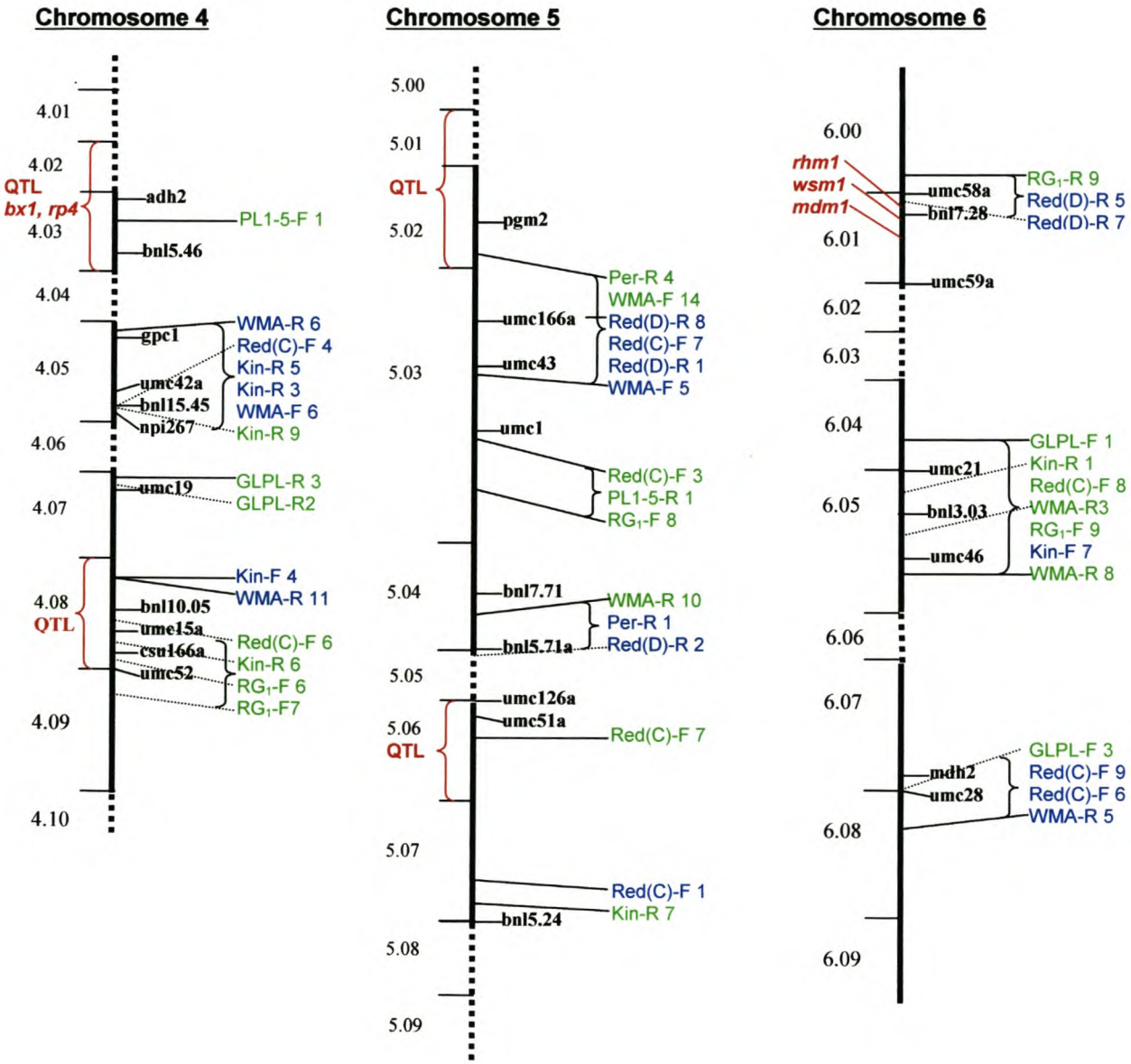
Stellenbosch University http://scholar.sun.ac.za

Figure 3.5 (a) Schematic presentation of maize chromosomes 1-3 showing the distribution of the MITE/RGA fragments in comparison to anchored markers on the map. Chromosomal bins containing no mapped MITE/RGA fragments are indicated as truncated (----) lines. *Hbr*-fragments are indicated in blue and the *Hb2*-fragments are indicated in green. Chromosomal bin locations of disease and insect resistance and Quantitative trait loci (QTL), corresponding to MITE/RGA fragments are indicated in red.



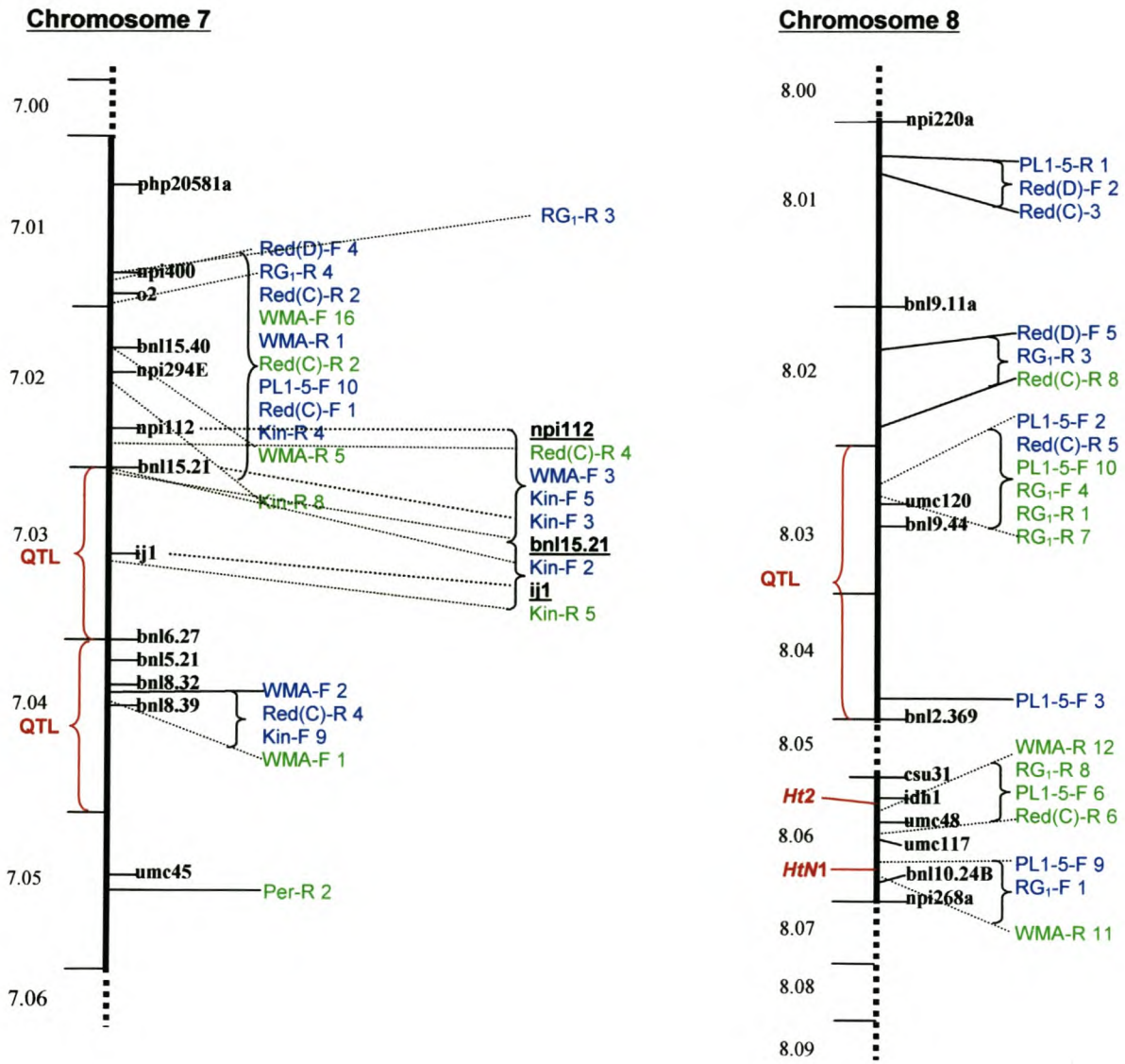
Stellenbosch University http://scholar.sun.ac.za

Figure 3.5 (b) Schematic presentation of maize chromosomes 4-6 showing the distribution of the MITE/RGA fragments in comparison to anchored markers on the map. Chromosomal bins containing no mapped MITE/RGA fragments are indicated as truncated (----) lines. *Hbr*-fragments are indicated in blue and the *Hb2*-fragments are indicated in green. Chromosomal bin locations of disease and insect resistance and Quantitative trait loci (QTL), corresponding to MITE/RGA fragments are indicated in red.



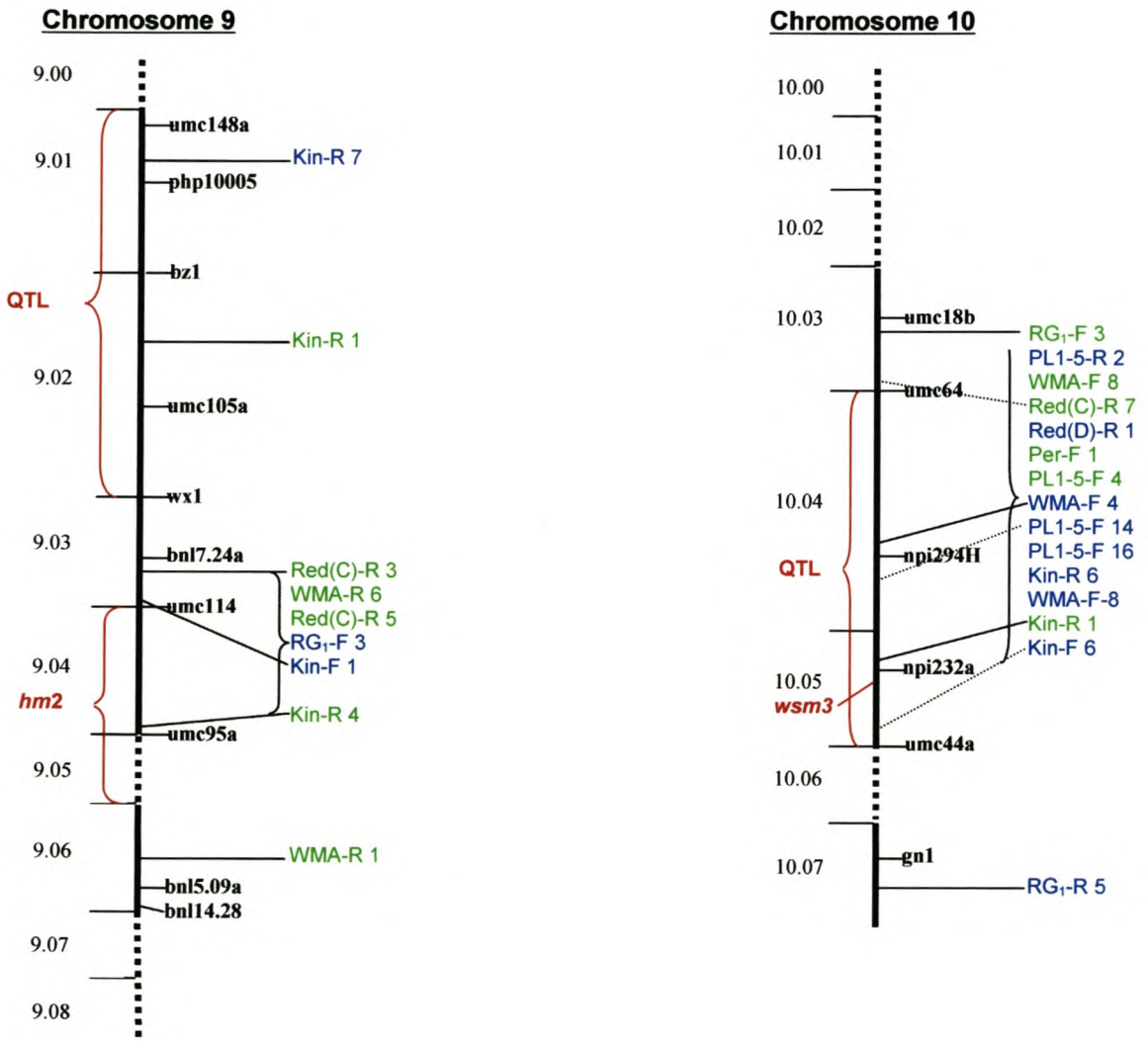
Stellenbosch University http://scholar.sun.ac.za

Figure 3.5 (c) Schematic presentation of maize chromosomes 7 and 8 showing the distribution of the MITE/RGA fragments in comparison to anchored markers on the map. Chromosomal bins containing no mapped MITE/RGA fragments are indicated as truncated (----) lines. *Hbr*-fragments are indicated in blue and the *Hb2*-fragments are indicated in green. Chromosomal bin locations of disease and insect resistance and Quantitative trait loci (QTL), corresponding to MITE/RGA fragments are indicated in red.



Stellenbosch University http://scholar.sun.ac.za

Figure 3.5 (d) Schematic presentation of maize chromosomes 9 and 10 showing the distribution of the MITE/RGA fragments in comparison to anchored markers on the map. Chromosomal bins containing no mapped MITE/RGA fragments are indicated as truncated (----) lines. *Hbr*-fragments are indicated in blue and the *Hb2*-fragments are indicated in green. Chromosomal bin locations of disease and insect resistance and Quantitative trait loci (QTL), corresponding to MITE/RGA fragments are indicated in red.



Stellenbosch University http://scholar.sun.ac.za

3.6 Clustering of previously mapped resistance genes compared to the MITE/RGA fragments

According to McMullen and Simcox (1995a; 1995b) all of the ten chromosomes contain at least one resistance gene. Table 3.7 (a) gives a detailed summary of some agronomically important genes of the maize genome, together with their chromosomal locations. Table 3.7 (b) compares the approximate positions of the mapped MITE/RGA fragments (this study) with those of mapped resistance loci. The schematic presentation of the 10 chromosomes given in Table 3.7 (b) is not according to scale, it merely shows the distribution of significant MITE/RGA markers across the genome. In total, 92 of the 196 MITE/RGA markers mapped to chromosomal positions of known resistance gene loci. All of the maize chromosomes showed a MITE/RGA linkage group or at least a single MITE/RGA fragment that mapped to the same approximate chromosomal location of certain resistance loci.

From Table 3.7 (b) it is apparent that each of the ten chromosomes has at least one mapped NBS fragment. Chromosomes 1, 2, 5 and 10 yielded mapped fragments of all three RGA (Per, Red and NBS) classes.

Stellenbosch University http://scholar.sun.ac.za

Table 3.7 (a) Chromosomal bin locations of disease/insect resistance genes and quantitative trait loci (QTL) (from McMullen and Simcox (1995a; 1995b)).

Disease/insect resistant trait	*Locus*	*Chromosome*									
		1	2	3	4	5	6	7	8	9	10
[1]Northern corn leaf blight (NCLB)	*Ht1*		2.08								
	Ht2								8.05/6		
	[1]Ht3							7.04			
	HtN1								8.06		
	QTL	1.01/2		3.07/8	4.02/3	5.01/2		7.03	8.03/4		
DIMBOA	*bx1*				4.02/3	5.06					
European corn borer (ECB)	QTL	1.01 1.07	2.03 2.08/9	3.04/5	4.02/3			7.04			10.04/5
Corn earworm (CEW)	QTL	1.03/4								9.01/2	10.06
[2]Gray leaf Spot (GLS)	QTL	1.05/6				5.03/4/5/6					
Anthrose stalk rot (ASR)	QTL				4.08						
Maize streak virusc (MSV)	*msv1*	1.04									
Stewart's wilt (SW)	QTL	1.05									
Carbonum leaf spot (CLS)	*hm1*	1.07									
	hm2									9.04/5	
Fusarium stalk rot (FSR)	QTL	1.07	2.04	3.04/5	4.04	5.02 5.04					10.06
Common rust	*rp1*										10.01
	rp3			3.04							
	rp4				4.02/3						
	rp5										10.01
	rp1-G										10.01
Southern rust	*rpp9*										10.01
Maize mosaic virus	*mv1*			3.04							
Wheat streak mosaic	*wsm1*						6.01				
	wsm2			3.04							
	wsm3										10.05
Maize dwarf mosaic	*mdm1*						6.01				
Southern corn leaf blight	*rhm1*						6.01				

[1] = Putative chromosomal location of the *Ht3* gene was given by Van Staden (2001)

[2] = Lehmensiek (2000) determined the chromosomal location of a major and some minor QTLs for GLS resistance

Stellenbosch University http://scholar.sun.ac.za

Table 3.7 (b) Chromosomal diagram comparing the location of mapped MITE/RGA fragments (Per, Red and NBS) with regards to mapped mono- and polygenic disease resistance genes.

Bin	Chromosome 1		2		3		4		5		6		7		8		9		10	
	Trait	Marker	Trait	Marker	Trait	Marker	Trait	Marker	Trait	Marker	Trait	Marker	Trait	Marker	Trait	Marker	Trait	Marker	Trait	Marker
0.1	QTL for ECB and NCLB	Red=1 NBS=3							QTL for NCLB	Per=1	*rhm1* *wsm1* *mdm1*	Red=2 NBS=1					QTL for CEW	NBS=2		
0.2							QTL for ECB NCLB *bx1* *rp4*	NBS=1												
0.3	QTL for CEW	NBS=2	QTL for ECB	NBS=3						Per=1			QTL for NCLB	NBS=2	QTL for NCLB	Red=1 NBS=5				
0.4	*msv1*	Per=1 Red=3 NBS=2			QTL for ECB FSR *rp3* *mv1* *wsm2*	Red=2 NBS=7			QTL for GLS	Red=5 NBS=5			QTL for ECB	Red=1 NBS=3			*hm2*	NBS=1	QTL for ECB *wsm3*	Per=1 Red=1 NBS=8
0.5																				
0.6									QTL for NCLB	Red=1					*Ht2* *HtN1*	Red=1 NBS=6				
0.7	*hm1*	NBS=3			QTL for NCLB	NBS=4														
0.8			*Ht1* QTL for ECB	Per=1 Red=3 NBS=8																
0.9																				

Stellenbosch University http://scholar.sun.ac.za

Non-parental fragments

Amplified fragments present in one or more of the progeny and absent in both of the parental lines, together with fragments present in the parental lines and absent in the progeny, were defined as being non-parental fragments. The observed percentage of non-parental fragments were low for the MITE/RGA primer combinations used in this study, *Hb2*/RGA primer combinations showed the highest percentage of non-parental fragments, 4.8%, with *Hbr*/RGA having a lower percentage of 2.3%.

3.7 Applying mapped MITE/RGA markers to fine map specific examples of resistance gene loci

Clusters of MITE/RGA markers, mapped to chromosomal bins where the putative positions of the *HtN*1, *Ht*1, *Ht*2 and *Ht*3 resistance genes are located. MITE/RGA fragments located near chromosomal positions for a major and some minor QTLs for resistance against gray leaf spot in maize were also identified.

Setosphaeria turcica

Multipoint linkage analysis *were* performed using the software package MAPMAKER/EXP version 3.0b (Lander et al. 1987; Lincoln et al. 1992a). The RIL population partial linkage maps for genes *HtN*1, *Ht*1, *Ht*2, and *Ht*3 are given in Table 3.8. Fourteen MITE/RGA markers, mapping to chromosomal bins where the putative positions of the *HtN*1, *Ht*1, *Ht*2 and *Ht*3 resistance genes are located, were analysed.

Shown in Table 3.8 are six MITE/RGA fragments that mapped to chromosomal bin 2.07. This was previously identified by Van Staden et al. (2001) as the chromosomal location of the *Ht*1 gene. The six markers mapped in close proximity with high LOD scores to important established and core markers. Core marker umc98a mapped 0.0cM to marker *Hbr*/WMA-F(9) with a LOD score of 11.41, marker us30 (University of Stellenbosch) mapped 0.0cM to marker *Hbr*/PI1-5-F(11) with a LOD score of 10.81 and us-marker 35 mapped 0.0cM to marker *Hbr*/GLPL-R(1) with a LOD score of 12.01. Also shown in Table 3.8 are four MITE/RGA markers that mapped to chromosomal bins 8.05/8.06, previously identified as the putative map positions of the *Ht*2 and *HtN*1 genes. These markers showed relatively high LOD scores with close proximity mapping to established markers. Marker *Hb2*/WMA-R(12) mapped 2.8cM

Stellenbosch University http://scholar.sun.ac.za

from us-marker 60 with a LOD score of 8.3, the core RFLP marker umc48 map 2.9cM from *Hb2*/RG_1-R(2) with a LOD score of 7.74.

The precise chromosomal location of the *Ht*3 gene is yet unknown, but It was putatively assigned to chromosome 7 (Van Staden et al. 2001). According to Table 3.8, four MITE/RGA fragments were identified in chromosomal bin 7.04. Flanking core markers, bln6.27 and bnl5.21a (Table 3.8), were used as anchors to map the cluster of MITE/RGA fragments to chromosome 7.

Table 3.8 Two point linkage scores with the putative positions of the *HtN*1, *Ht*1, *Ht*2 and *Ht*3 genes showing linkage to the SCAR markers, RFLP core markers (indicated in bold) and MITE/RGA markers. Map distances are indicated in cM followed by LOD scores in brackets.

Resistance gene: Ht1		***Resistance gene: Ht2/HtN1***		***Resistance gene: Ht3***	
Chromosome 2.07		**Chromosome 8.05/8.06**		**Chromosome 7.04**	
umc98a	0.0cM(11.41)	**bnl12.30a**	5.7cM(5.04)	**bnl6.27**	4.9cM(6.08)
Hbr/WMA-F(9)	8.5cM(4.76)	*Hb2*/WMA-R(12)	2.8cM(8.30)	*Hb2*/WMA-F(2)	0.0cM(11.41)
umc22	0.0cM(10.21)	us60	6.3cM(5.87)	*Hbr*/Kin-F(9)	0.0cM(9.91)
us30	0.0cM(10.81)	**csu31**	4.4cM(7.13)	*Hbr*/Red(C)-R(4)	3.2cM(6.92)
Hbr/PL1-5-F(11)	1.4cM(9.13)	**idh1**	1.3cM(9.99)	*Hbr*/WMA-F(2)	2.7cM(8.58)
umc125a	0.0cM(11.11)	us56	8.5cM(4.76)	**bnl5.21a**	
us24	1.3cM(9.99)	**umc48**	2.9cM(7.74)		
Hbr/Red(C)-F(5)	0.0cM(11.71)	*Hb2*/RG_1-R(2)	11.9cM(3.97)		
Hbr/WMA-R(2)	2.9cM(7.74)	*Hb2*/PL1-5-F(6)	2.7cM(8.58)		
umc122	0.0cM(11.41)	*Hb2*/Red(C)-R(6)			
us35	0.0cM(12.01)				
Hbr/GLPL-R(1)	0.0cM(12.01)				
Hb2/Kin-R(1)	1.4cM(9.13)				
bnl6.20					

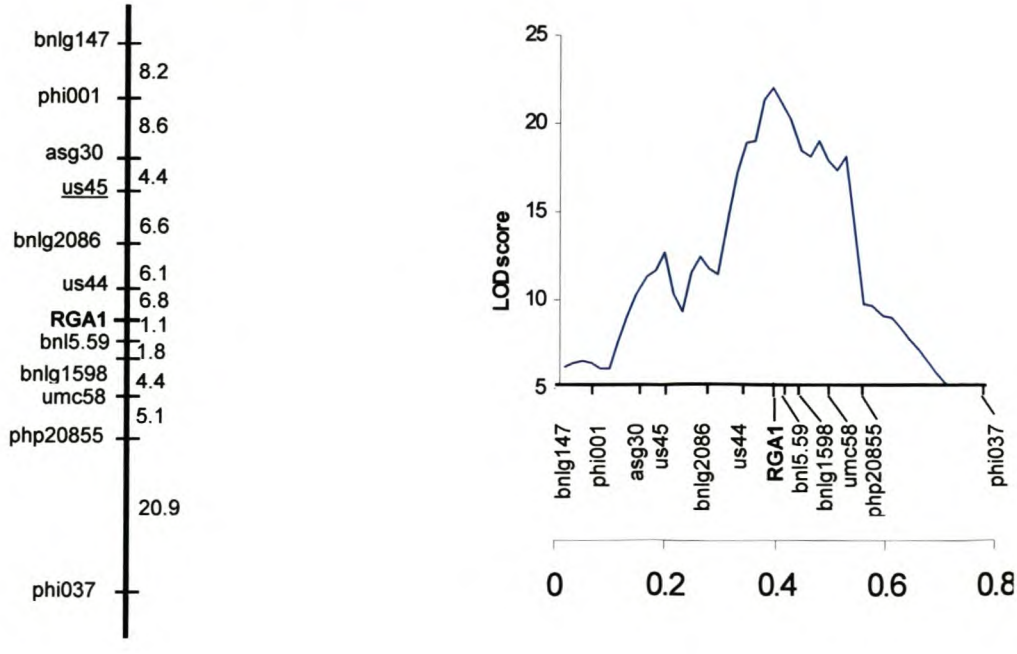
Stellenbosch University http://scholar.sun.ac.za

Gray leaf spot (GLS)

Twenty-eight MITE/RGA primer combinations were tested on available gray leaf spot (GLS) resistance and susceptible genetic plant material. Two bulks, a resistant and susceptible as well as the two parents (P_1 and P_2) were tested using 16 *Hb2*/RGA primer combinations and 12 *Hbr*/RGA primer combinations. Only those primer combinations that gave the best results and fragment distribution on the Tx303 x CO159 RIL population were selected for analysis on the GLS plant material.

One primer combination (*Hb2*/Kin-R) amplified a fragment which was present in the susceptible parental line and bulk and absent in the resistant parental line and bulk. This fragment, named RGA1, was amplified on the 230 F_2 plants and linkage analysis was performed using the markers previously identified by Lehmensiek (2000). Using segregating GLS data, marker RGA1 was localized on chromosome 1 between markers us44 and bnl5.59, with a multi-point distance of 6.8cM between markers us44 and RGA1 and 1.1cM between markers RGA1 and bnl5.59 (Figure 3.6). Interval mapping indicated that the marker RGA1 was situated 2.8cM distal to the QTL peak (Figure 3.6).

Figure 3.6 Linkage group on chromosome 1 analysed by linkage analysis of 230 F_2 plants using MAPMAKER/EXP. Map distances are given in cM.



Stellenbosch University http://scholar.sun.ac.za

Two other MITE/RGA fragments proved to be valuable, they were marker *Hb2*/Kin-R(1) and *Hbr*/PL1-5-F(3), both of which showed linkage to the core marker, bnl5.59. These markers mapped 1.5 cM from each other with a significant LOD score of 8.55. MITE/RGA marker *Hbr*/PL1-5-F(3) showed tight linkage to core marker, bnl5.59, with a LOD score of 5.56. Unfortunately linkage to us44 could not be determined, because different mapping populations were used.

3.8 MITE/RGA DISPLAY VERSUS CONVENTIONAL AFLP PROFILING

Resistant gene analogue primers were used in combination with the *Hbr-F* and *Hb2-F* primers, together with *Mse/Mlu* AFLP primer combinations to amplify fingerprinting profiles of the 40 elite maize lines.

3.8.1 Scoring of fragments for the AFLP- and MITE/RGA display profiles

When working with a relatively small number of samples the scoring of the fragments are often done manually direct from gel photographs or autoradiographs. For the 40 elite inbred lines all banding profiles were scored manually as a series of 0's and 1's, representing the absence or presence of a given band (e.g., 1001110101). The array of 0's and 1's is referred to as a data vector and was used to calculate genetic distance to construct the dendrograms. Band sharing is the tool used to estimate the level of similarity between multi-band patterns. Pairwise genetic distance matrices were produced by comparing the vector of a given sample (individual) with that of every other individual in the study.

Eight MITE/RGA primers combinations, those that showed the best amplification and fragment distribution among the 40 RIL individuals, were chosen for this profiling study. For the transposon like display profiles a total of 81 fragments were scored. Figure 3.7 shows the partial fingerprint display of one MITE/RGA primer combination, *Hb2*/Kin-R.

Figure 3.7 A MITE/RGA amplification profile, using RGA primer Kin-R together with MITE primer *Hb2*, on the 40 elite maize lines.

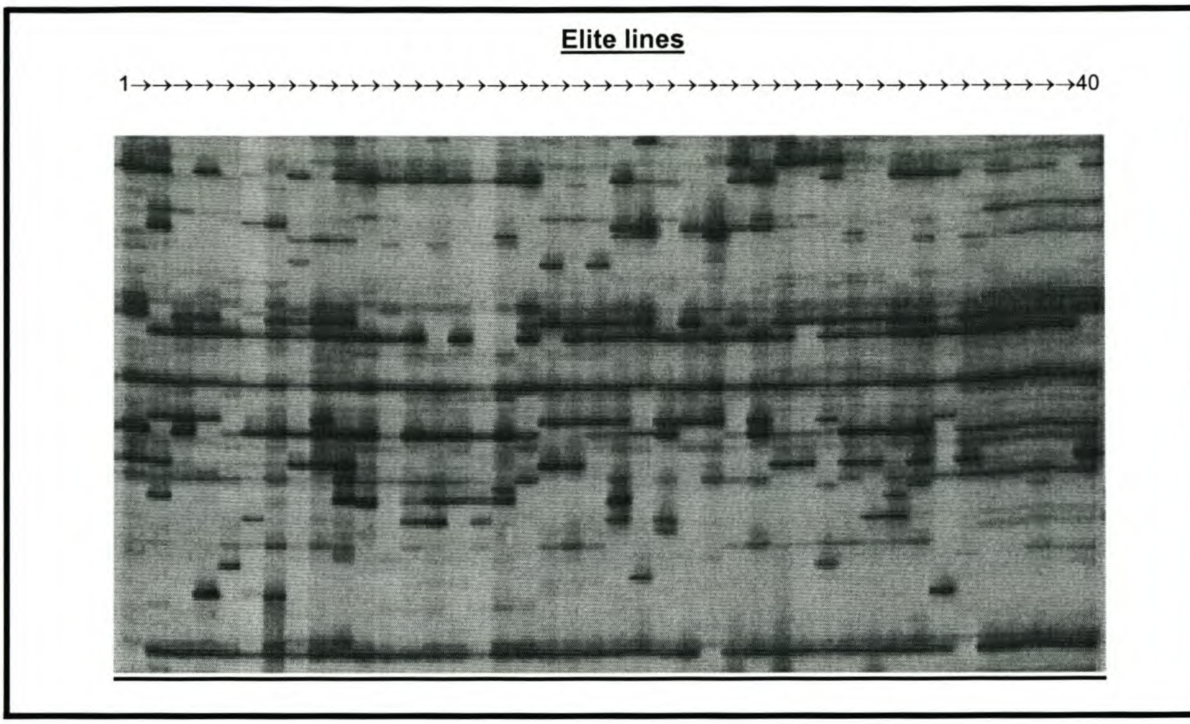
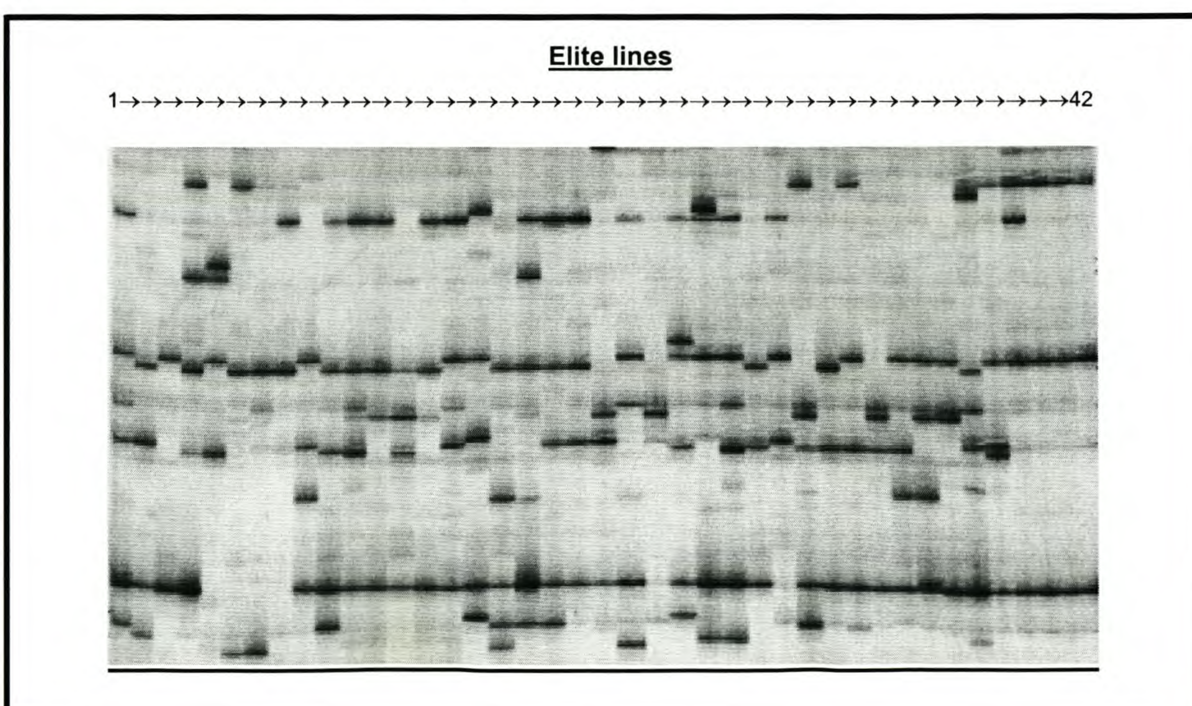


Figure 3.8 AFLP amplification profile, primer combination *Mlu*-GGC/*Mse*-TGC. (individuals 10 and 12 were not used in the MITE/RGA transposable like display.)



Stellenbosch University http://scholar.sun.ac.za

Stellenbosch University http://scholar.sun.ac.za

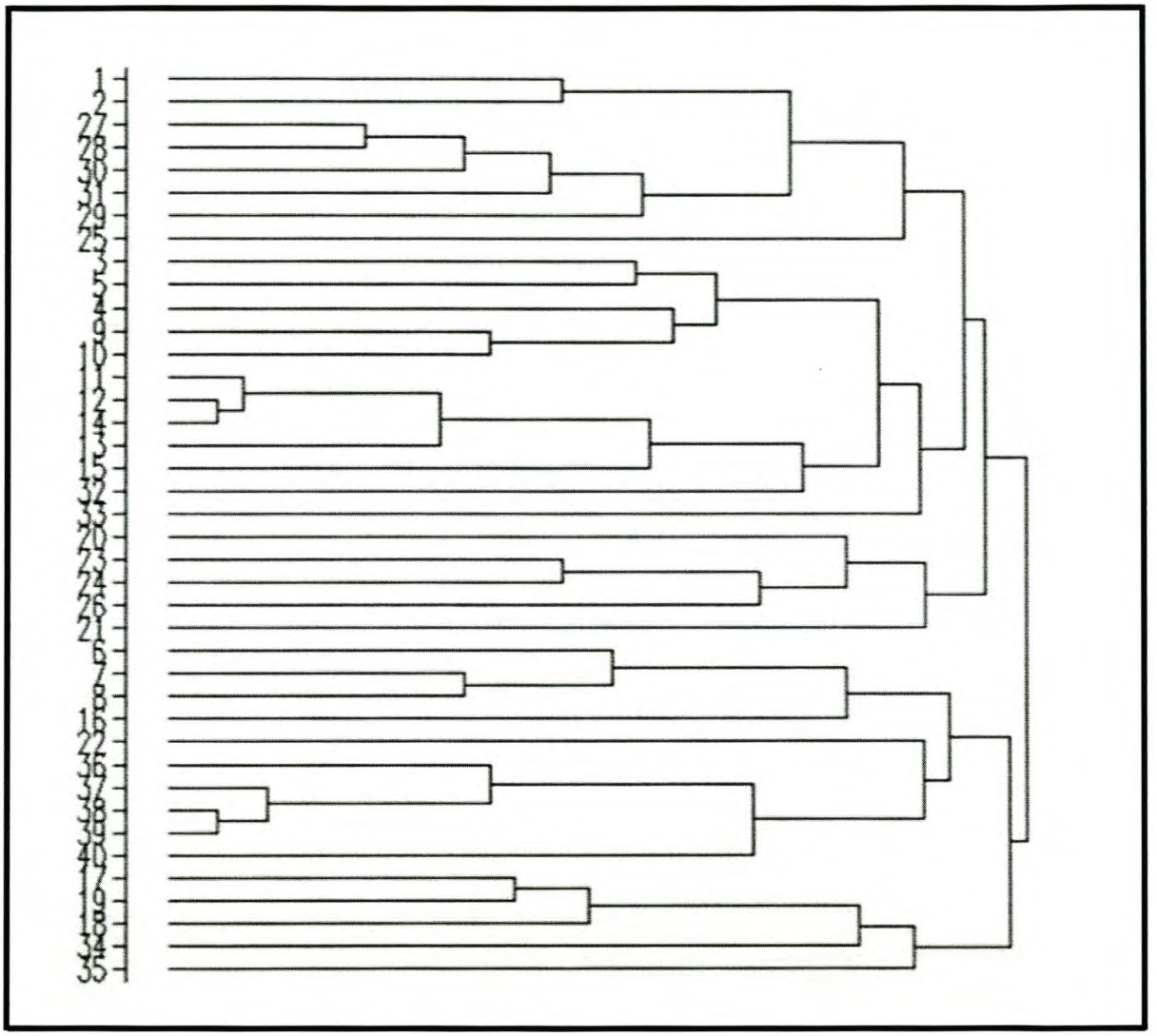
For AFLP analysis on the 40 elite inbred lines, a total of 100 fragments were scored using two primer combinations (*Mlu*-GGC/*Mse*-TGC and *Mlu*-GGC/*Mse*-TAC). Figure 3.8 shows the partial fingerprint display of one AFLP primer combination.

3.8.2 Statistical analysis

Numerous computer software packages are available to perform cluster analysis, the multivariate data analysis in this study was accomplished using the group average agglomerative clustering function of GENSTAT Version 531 software package (Numerical algorithm group, Oxford). Various algorithms for data clustering such as SINGLE, COMPLETE, WPGMA, and WPGMC are available, but the unweighted pair-group, using an arithmetic average (UPGMA) method, is the most commonly used and was therefore applied in this study. Dendrograms consisting of the clustering analysis of inbred varieties often complete the data analysis. In a study by Powell et al. (1996) and Maughan et al. (1996), UPGMA clustering was applied to different accessions of wild and cultivated soybean cultivars to show relationships between and within these two groups. UPGMA clustering was used to construct dendrograms, based on elite lines banding profiles, demonstrating clustering of the transposable like-display using a MITE–primer as anchor in conjunction with an RGA-primer. Figure 3.9 (a–b) shows the dendrograms determined for (a) the MITE/RGA profiling on the 40 elite maize lines and (b) the AFLP profiling on the 40 elite maize lines.

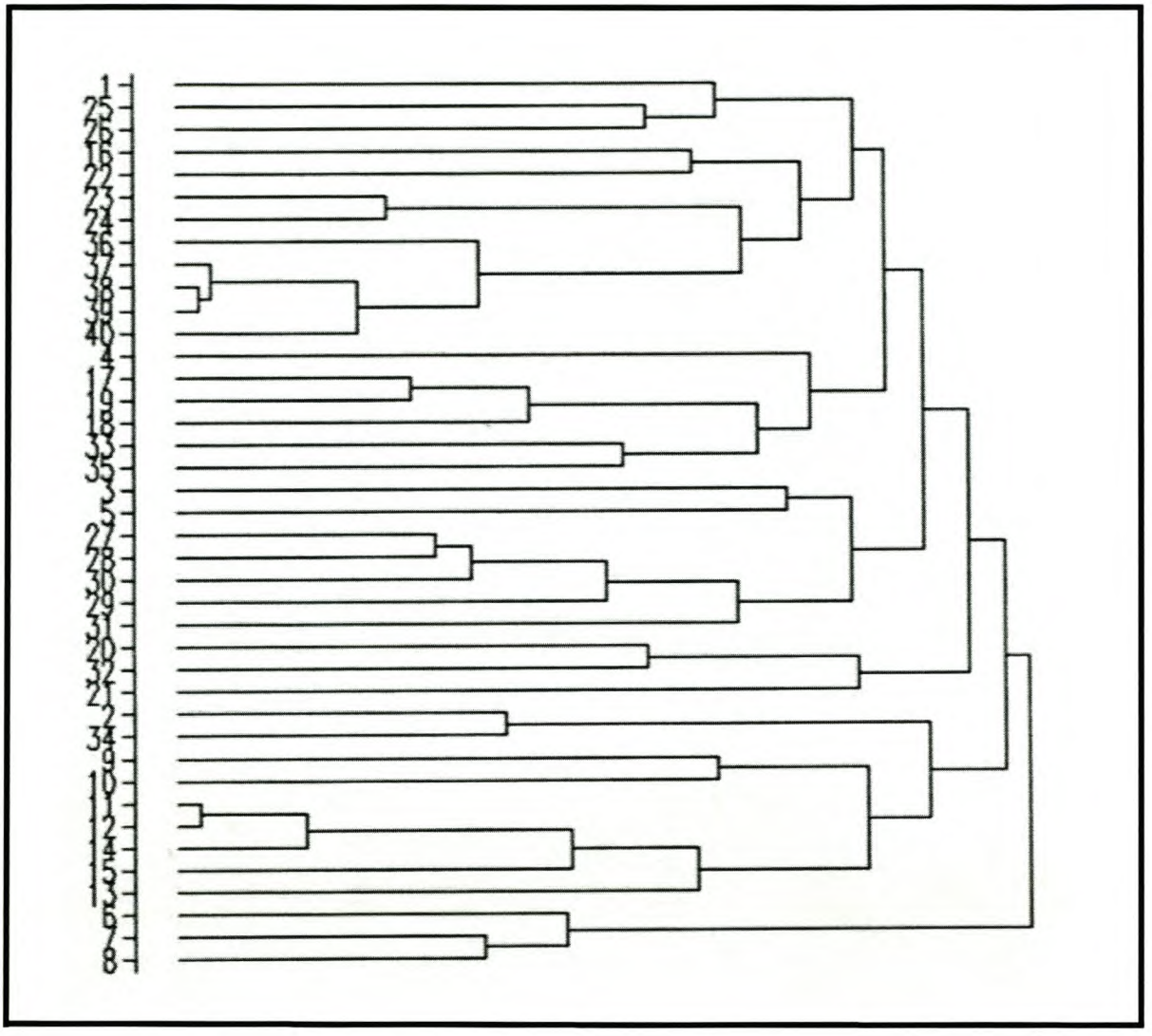
Stellenbosch University http://scholar.sun.ac.za

Figure 3.9 (a) MITE/RGA dendrogram displaying the UPGMA clustering analysis of the 81 MITE/RGA markers across the 40 elite maize lines.



Stellenbosch University http://scholar.sun.ac.za

Figure 3.9 (b) AFLP dendrogram displaying the UPGMA clustering analysis of the 100 AFLP markers across the 40 elite maize lines



It was noticeable that some of the 40 elite lines grouped in the same clusters, regardless of the profiling technique applied, for example; (i) lines 36, 37, 38, 39 and 40; (ii) lines 27, 28, 30, 29 and 31; (iii) lines 23 and 24 (iv) lines 17, 19, 18 and 35; (v) lines 6, 7 and 8; (vi) lines 11, 12, 14, 15 and 13; (vii) lines 3 and 5; (viii) lines 9 and 10.

Stellenbosch University http://scholar.sun.ac.za

CHAPTER FOUR
DISCUSSION

Maize (*Zea mays* L.) has been used effectively as a plant model species in for development and evaluation of different molecular markers for the identification, mapping and manipulation of genes effecting important agricultural traits. A significant attribute of any molecular marker assay system, affecting its general application in genetic studies, should be in the overall distribution of markers across the genome.

Two miniature inverted-repeat transposable subfamily elements (MITE), *Heartbreaker* (*Hbr*) and *Hb2* were used in combination with three different resistant gene analogues (RGA) to ascertain the distribution of these genic originating fragments across the ten chromosomes of the maize genome. Forty RI individuals of the public available recombinant inbred population (RIL) (Tx303 and CO159), produced by B. Burr, (Burr et al. 1988) were used for this purpose. Casa and co-workers (2000) analysed a MITE subfamily (*Heartbreaker*) using a modified AFLP approach, namely transposon-display (TD). Casa chose to work with a large RIL population (100 RI individuals), derived from a cross between B73 x Mo17. Two reasons for choosing to work with a recombinant inbred population, were given; (i) the transposon-display (TD) technique could show high frequencies of non-parental bands in the individuals which might indicate new transpositions or (ii) if these non-parental bands are very rare, then all polymorphic parental bands could be mapped after segregation analysis.

It would be preferable to develop a marker technique identifying markers located in genic regions, which are stable, highly polymorphic, and carries a high information index. The discovery of MITEs, with their high copy number, stability and genic preference, fulfil most of these criteria. Casa et al. (1999; 2000) and Zhang et al. (2000) discovered and reported that the *Heartbreaker (Hbr)* fragment in maize is highly polymorphic and is believed to originate in genic rich regions. If a primer, originating from the original *Hbr-* or *Hb2* element, was used in combination with

Stellenbosch University http://scholar.sun.ac.za

resistance gene analogue (RGA) primers, would the amplified fragments, preferentially originate from genic regions?

4.1 Unique primer sets

The availability of the original *Hbr* - (Zhang et al. 2000) and *Hb2*- (Spell et al. 1988) element sequences, facilitated in the design of the *Hbr*- and *Hb2* forward primers. Properties of MITE elements, such as their high copy number, polymorphism and preference for genic areas make them useful sequences for the identification and cloning of disease resistance genes. Gene analogue primers used in this study were derived from three main groups; (i) genes with a peroxidase protein product, (ii) genes with a reductase enzyme product (*hm1)* and (iii) genes consisting of partially conserved motifs within their nucleotide binding sites (NBS) and leucine-rich repeat (LRR) units. Genes from the NBS-LRR class are widely distributed among flowering plants and provide resistance to a variety of plant pathogens. The gene analogue primers from the NBS group (with partial conserved NBS and LRR motifs) were derived from the sequences of known genes and gene products, from diverse plant genomes (flax, A*rabidopsis*, tomato and tobacco) as published by Collins et al. (1998) and Aarts et al. (1996).

Using the term peroxidase as a query on the maize database (ZmDB) a particular accession, TUC 12-280-5500.2, was identified. Using the sequence data of this accession as a query on the database, the primer sequences were designed from matching nucleotide sequences. One reductase primer set, Red(D), was designed using the sequence of the *Helminthosporium carbonum* gene product as a search query on Fasta, followed by the designing of the primers from the sequence matches with other reductase gene products. The remaining reductase primer set, Red(C), was designed using the DNA sequence of the reductase *Helminthosporium carbonum* gene product.

Stellenbosch University http://scholar.sun.ac.za

4.2 Amplification of the MITE/RGA profiles

The PCR based method used in this study made use of a combination of two anchored primers, both believed to originate from genic areas; (i) a resistant gene analogue (RGA) primer used in combination with, (ii) a MITE element derived primer, generating a modified MITE transposon-like display (TD). Modified AFLP transposon-display (TD) protocols have been used to analyse plant transposable DNA elements, for example van der Broeck (1998) used this modified approach to study the *dTph*1 family in petunia whereas Kalender (1999) used it to study the *BARE*-1 element in barley.

This MITE/RGA technique was shown to be repeatable, with the only major differences detected in the fragment intensity of some amplification profiles.

4.3 Primer specificity and sequence analysis

4.3.1 Element conservation

The first *Hbr*-element to be identified was discovered as a 314bp insert into the *hm1* gene of maize. This element was designated *Hbr-hm1* and is flanked by 14bp terminal inverted-repeats (TIR), residing adjacent to the TIRs are 3bp directional repeats (DIR) which were duplicated upon insertion. The internal sequence of the *Hbr-hm1* element was found to be unlike any other transposable element. When compared to the original *Hbr*-element, *Hbr-hm1*, polymorphisms (either point mutations or deletions) were detected, within and between all of the ten *Hbr*-element sequences. Similar results were reported in a study by Zhang et al. (2000), where 27 inserts were compared with the original *Hbr-hm1* element and showed a >82% identity.

By sequencing analysis of non-mutant *waxy (Wx)* genes in two maize inbred lines, Spell et al. (1988) identified a DNA insertion with structural features similar to that of a transposable element. The insertion was 316bp in length, had a 15bp imperfect terminal inverted repeat (TIR) and was flanked by a 5bp direct repeat (DIR) generated, which is duplicated upon insertion. Spell and co-workers found that this insertion element was not homologous with any previously described maize

Stellenbosch University http://scholar.sun.ac.za

transposable element and was present in a very high copy number, approximately 12 000 copies per haploid genome. In later studies by Wessler (1999), the element was concluded to be a member of the MITE *Tourist* family and designated as *Hb2*. Only nine of the 16 *Hb*2-fragments, from the fragments studied, showed to have a partial sequence of the *Hb2* element so, the primer selected from the terminal inverted repeat (TIR) of the *Hb2* element, must prime to other sites in the maize genome. In those fragments that did show the element sequence, the selected primer amplified only 10bp of the original repeat internal sequence and 5bp of the directional repeat. Hence the nine fragments show a very high sequence similarity of 80-100%.

Either the presence or absence of the MITE element or differences in deletions, point mutations and insertions in the DNA recognition site sequence of any of the gene analogue primers will create the observed polymorphisms. The occurrence of the imperfect TIR indicates that the element can no longer transpose, for the simple reason that the TIR would not be recognised by any transposase enzyme. It can also be argued that the element transpose at an extremely low frequency (Spell et al. 1988). Although some degree of sequence difference for the *Hbr*- and *Hb2*-elements was found, the length of the two elements were highly conserved (except for the occasional deletions in two of the sequences), 90% for the *Hbr*-element (one out of ten) and 89% for the *Hb2*-element (one out of nine). These results suggest that most of the ±4000 *Hbr*-elements and ±12000 *Hb2*-elements in the maize genome are highly conserved in both their length and sequence, and that the two MITE subfamily elements might have spread very recently throughout the maize genome.

4.3.2 Specificity of the MITE element primers

To show that the MITE primers used for this study will amplify fragments originating partly from MITE elements as well as to authenticate the specificity of the *Hbr* and *Hb2*-primers, partial sequences of the two MITE elements (including primer sequences) (*Heartbreaker* and *Hb2*) were used as a search query on the EPLN DNA sequence database.

Stellenbosch University http://scholar.sun.ac.za

The query results achieved with the partial sequence of the original *Hbr*-element, confirmed the specificity of the *Hbr*-element forward primer it also explains why the ten sequenced fragments all contained a partial sequence of the original *Hbr*-element. In contrast the results achieved by the *Hb2*-element sequence just strengthens the suspicion regarding the specificity of the *Hb2* primer. The *Hb2* primer is not selective enough and does not detect only *Hb2* MITE elements. For purpose in this study it explains why only nine of the 16 fragments sequenced contained a portion of the *Hb2*-element sequence, even though the *Hb2*-F primer sequence was present in all of the 16 fragments.

4.3.3 MITE/RGA DNA sequence analysis

In total 19 different MITE/RGA fragments (including both *Hbr* and *Hb2* fragments) were isolated and sequenced. Sequencing data were used as queries on the EPLN DNA sequence database to determine if any of these fragments show some significant correlation to known mapped maize genes.

One fragment, *Hb2*/WMA-F(15), showed promising results. It mapped to the same chromosomal location as that of the *hm1* gene. However, it is expected that reductase fragment(s) would map to the chromosomal location of *hm1*, because the Red(D) primers were derived directly from the *hm1* gene's protein product. This specific fragment was detected using one of the NBS class primers and not a reductase primer. Still this fragment can be considered as a candidate gene for *hm1*. Poor results attained with the similarity searches can probably be ascribed to the RGA primers used in this study. Although developed and designed from the products of known resistance genes, they seem to have a higher sequence similarity with mapped maize household genes. It is also a known fact that the maize linkage map is not densely populated with resistance genes. If in the future more resistance genes are placed on the maize linkage map, it will be highly likely that a higher percentage of the MITE/RGA fragments will map in their proximity.

Stellenbosch University http://scholar.sun.ac.za

4.4 Polymorphic fragments

Through hybridisation experiments, Wessler and co-workers (1999) established that the haploid copy number of *Hbr*-elements in the maize genome is ±4000 in comparison with the ±12000 for the *Hb2*-element (Wessler, 1999 Plenary Talk 41st Annual Maize Genetics Conference).

In this study a total number of 380 MITE/RGA fragments were scored, of these 55.26% were found to be polymorphic between the parental lines Tx303 and CO159. In a TD-display Casa et al. (2000) mapped ±60% of their *Hbr*-anchored fragments, this results compared favourable, to the, 59.3% (102 out of 170), *Hbr*-fragments mapped in this study. In microsatellite studies the polymorphic index observed was between 53-69% (Senior et al. 1996), for RFLP studies a percentage of was 50-80% observed (Gardiner et al.1993) and for different AFLP marker assays a percentage of between 26-41% (Castiglioni et al. 1999; Vuylsteke et al. 1999) were observed. Therefore a polymorphic index of 55.26% proof to be in concurrence with other marker analysis systems.

The level of polymorphism detected by the MITE/RGA marker analysis technique is a possible reflection on when each of the MITE families might have spread throughout the specific population. The higher the polymorphic index, the more recent their transposition activity (Casa et al. 2000). Sequence similarity is also an indication of recent amplification activities, thus MITE families with a high sequence similarity will exhibit a high polymorphic index in a given mapping population. A polymorphic index of 55.26% and a very high sequence similarity of >84%, (see previous section 4.3) suggest that these two MITE subfamily elements most probably transposed very recently in the current population (Tx303 and CO159) used.

4.5 Mapping and distribution of the MITE/RGA fragments

On average, Casa and co-workers (2000) scored 52 fragments for each of their AFLP/*Hbr* primer combinations. In comparison, an average of 11.8 fragments were scored for each of the 32 primer combinations used in this study. A combination of AFLP and MITE primers will produce fragments wherever there is a restriction site in

Stellenbosch University http://scholar.sun.ac.za

close proximity to a binding site for the MITE element primer, as well as AFLP/AFLP fragments. However, using MITE and resistance gene analogue primers as anchors, fewer fragments (originating especially from genic rich areas) will be selectively amplified.

Applying the TD technique, Casa and co-workers (2000) scored and mapped 213 *Hbr* fragments using the 100 recombinant individuals from a B73 x Mo17 inbred mapping population (M. Lee). In this study, thirty-two primer combinations were used to map a total of 196 MITE/RGA markers, these markers were mostly found in clusters distributed across the ten maize chromosomes. Magbanua et al. (2001) mapped 565 polymorphic *Hb*2 bands and also found that the fragments were not evenly distributed across the ten maize chromosomes, but occurred as clusters. Casa and co-workers (2000) successfully applied the TD-display, using seven other MITE families, three families from the maize genome (*Hb*2, Magbanua et al. 2001; *Tourist*, unpublished data and *mPIF*; unpublished data) and four from the rice genome (*Gaijin, Tourist, Olo* and *Ditto*-unpublished data).

Figures 3.5 (a-d) shows the ten chromosome maps with the distribution of the 196 MITE/RGA fragments. For some household genes, significant linkage data based on the linkage map of the Tx303 x CO159 recombinant inbred family, (as illustrated in Addendum A) will be discussed briefly.

a) Maize chromosome 1

Three MITE/RGA markers (two NBS and one Red) mapped in close proximity (0.7-0.6cM) with high LOD scores (11.71) to the *p*1 locus in maize. Dominant *p*1 is involved in the synthesis of a red flavonoid pigment and regulates red to red-brown pigmentation in the pericarp as well as the soft floral parts of the cob. The tissue-specific pattern of expression of certain *p* alleles suggests that *p* may be a complex locus, with more than one functional unit (Lechelt et al. 1989).

b) Maize chromosome 2

Three MITE/NBS markers mapped in close proximity (1.4-6.3cM), with relative high LOD scores (9.42 to 5.87), to the *acc*A locus in maize. Maize acetyl-CoA carboxylase

Stellenbosch University http://scholar.sun.ac.za

(ACCase) is encoded by a small gene family, of which four genes have been characterized: A1, A2, B1, and B2 (Lutz and Gengenbach 1996). *Acc1* is a semi-dominant nuclear gene, mutant forms of which confer tolerance to certain herbicides (Egli et al. 1994). Caffrey et al. (1995) mapped the accA gene near the centromeric region of chromosome 2, in the interval defined by the RFLP markers umc131 and uox.

c) Maize chromosome 4

One MITE/NBS marker mapped in close proximity (1.3cM) with a high LOD score (9.7) to the *gpc*1 maize locus. The small maize multi-gene family consisting of *gpc*1, *gpc*2, *gpc*3 and *gpc*4, encodes for cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) (Manjunath and Sachs 1997). Based on the deduced amino acid sequence identity GAPC1 and GAPC2 were designated as group I (97% identical) and GAPC3 and GAPC4 as group II (99.4% identical) (Manjunath et al. 1997).

One MITE/NBS marker mapped in close proximity (4.2cM) with a high LOD score (7.40) to the *adh*2 locus in maize. The alcohol dehydrogenase2 (*adh*2) gene of maize has a nucleotide sequence closely related to that of the maize *adh*1 gene, indicating that the two genes arose from a progenitor gene by a duplication event (Dennis et al. 1985). The coding regions are 82% conserved at the nucleotide level and 87% conserved at the amino acid level. The *adh*2 gene together with the adh1 gene is induced by anaerobic conditions and under these conditions produces an increased level of mRNA (Dennis et al. 1985).

d) Maize chromosome 6

One MITE/NBS marker mapped 4.2cM from the gene *mdh*2 with a LOD score of 7.40. The malate dehydrogenase2 (*mdh*2) gene mapped to chromosome 6, bin6.07 (maizeDB).

e) Maize chromosome 7

Two MITE/RGA (one Red and one NBS) markers mapped in close proximity (7.7-5.9cM) with relative high LOD scores (5.48-6.38) to the *o*2 locus in maize. The *Opaque2* (*o*2) gene mutations cause an increase in the lysine content of the grain.

Stellenbosch University http://scholar.sun.ac.za

Lopes et al. (1995) used bulked segregant analysis to identify the genetic map location *o*2. Two *o*2 modifier loci were mapped to the telomere and centromere of chromosome 7L.

Two MITE/NBS markers were linked (4.6-1.5cM) with relative high LOD scores (6.60-8.55) to the *ij*1 locus in maize. The recessive nuclear mutation iojap (*ij*) in maize produces striped plants with normal chloroplasts in green sectors and poorly developed chloroplasts in the white sectors. The *ij* gene encodes a 24.8kDa protein that showed no significant sequence similarity with proteins listed in databases (Han et al. 1992).

g) Maize chromosome 9

The maize waxy (*wx*) locus map to chromosome 9, bin9.02-9.03 MITE/RGA marker showed significant linkage to this specific locus. *Wx* encodes an enzyme responsible for the synthesis of amylose in endosperm tissue (Wessler et al. 1986).

Segregation distortion

Fourteen fragments, showing a polymorphism between the parental lines, could not be placed on any of the chromosomal maps. A possible explanation for the inability to map the 14 fragments can be that some areas (chromosomal bins) on the ten maize chromosomes are less populated (less dense) with well-defined polymorphic markers. In all probability these 14 fragments do map to a specific chromosomal location, but linkage could not be established. Segregation distortion (deviation from 1:1 Mendelian inheritance) is commonly observed when mapping molecular markers in plant genomes (Casa et al. 2000). In a study by Casa et al. (2000) the percentage of segregation distortion for the *Hbr*-element markers were found to add up to 19.5%. Calculation of these values was possible since a large mapping population of 100 individuals were used. Only one of the 14 unlinked fragments showed clear signs of segregation distortion, 37 individuals exhibited the Tx303 parental allele were as only three individuals had the CO159 parental allele. Accurate segregation distortion values cannot be calculated for the 14 fragments in this study, mostly because a limited amount of inbred individuals (40) were available.

Stellenbosch University http://scholar.sun.ac.za

4.6.1 Clustering of previously mapped resistance genes compared with the MITE/RGA fragments

McMullen and Simcox (1995a; 1995b) gave chromosomal locations on the chromosomal positions of different important resistance genes. According the mapping data from McMullen and Simcox, this study demonstrated that each of the ten maize chromosomes contained a linkage group of MITE/RGA markers or at least one MITE/RGA marker that showed to map in the proximity of a resistance gene, either monogenic or polygenic loci.

Combining the *Hbr*- and the *Hb2*-element RGA fragments, it became apparent that just like loci for disease- and insect resistance, these fragments were not randomly distributed across the maize genome but occurred in clusters. McMullen and Simcox (1995) reflected, that it became apparent as more genes for disease response traits are placed on the maize linkage maps, that these loci are not randomly distributed but clustered. These clusters of disease resistance loci include not only genes conferring resistance to a single pathogen or pest, but also genes for resistance to unrelated organisms (McMullen and Simcox 1995a; 1995b). Yu et al. (1996) and Gedil et al. (2001) respectively reported that NBS-containing sequences appear to be clustered in several lettuce and soybean chromosomal regions that also correspond to the positions of some resistance genes. This supports the hypothesis; that a correlation exists between homologous resistance gene like fragments and known resistance loci (Aarts et al. 1996; Leister et al. 1996; Mago et al. 1999). Chromosomal regions containing clusters of resistance genes in maize could be more efficiently targeted for transposon tagging, by pre-selecting element populations linked to the genic region of interest.

The clustering of resistance genes in the maize genome suggests the possibility of constructing high resolution genetic maps in the regions of the gene clusters, which can then be used in the positional cloning of a number disease and insect resistance genes. McMullen and Simcox (1995a; 1995b) speculated about the functional significance of disease resistance gene clustering. They concluded that evolution as well as genomic organisation played a big part in disease gene clustering.

Stellenbosch University http://scholar.sun.ac.za

To determine whether the maize resistant gene analogue clones hybridise to resistance genes, Collins and co-workers (1998) used RGA clones as probes to map RFLP loci in maize. The mapping of these RGA probes were performed using the 54 individuals from the Tx303 x Co159, University of Missouri-Columbia, immortalised F_2 mapping population (Gardiner et al. 1993) and the 71 individuals from the H99 x Mo17 mapping population developed by, Mike Lee, Iowa State University. Eleven of the RGA probes plus one wheat RGA probe mapped to seven regions of the maize genome and showed close proximity and linkage to known disease resistance genes in maize. In this study, 92 MITE/RGA fragments were identified that showed linkage to some of the known disease and insect resistance genes as well as possible linkage to some QTL traits as defined by McMullen and Simcox (1995a; 1995b) and Lehmensiek (2000).

Collins et al. (1998) detected RGA clones that mapped close to; (i) chromosome 1- *msv*1, resistance to maize streak virus. Maize streak, incited by maize streak geminivirus (MSV), is an economically important disease of maize. Previous genetic studies have reported monogenic or multigenic inheritance of resistance to infection by MSV (Kyetere et al. 1995), (ii) chromosome 1- *hm*1 gene for resistance to *corbonum* leaf spot. Dominant hm1 keeps maize resistance to the maize leaf spot/ear mold disease (yellow flecks), caused by the ascomycetous fungus Cochliobolus carbonum, race 1 (Multani et al. 1996), (iii) chromosome 2- *Ht*1, resistance *to Setosphaeria turcica* (see later), (iv) chromosome 3- a complex that includes *rp*3, resistance to common rust. *Rp* genes confer resistance to *Puccinia sorghi*, the causal agent of maize common rust. Most known *Rp* genes map to the Rp1 complex near the end of the short arm of chromosome 10 (Hulbert et al. 2001) Resistance to wheat streak mosaic virus (*wsm2)*. WSMV is an economically important pathogen of wheat, maize acts as an over-summering host and map to the short arm of chromosome 6 (Simcox et al. 1993). Resistance to maize mosaic virus (*mv*1). Maize mosaic virus (MMV), transmitted by the leafhopper, causes a common disease of maize in many tropical and subtropical countries. The *mv* loci map to chromosome 3 near the centromere (Ming et al. 1997), (v) chromosome 4- *rp*4, resistance to common rust, (vi) chromosome 6- a complex that includes *rhm*1,

Stellenbosch University http://scholar.sun.ac.za

resistance to southern corn leaf blight. A recessive chlorotic-lesion type resistance to *C. heterostrophus*, causal agent of the southern corn leaf blight the *rhm1* locus map to chromosome 6 (Simcox et al. 1993). Resistance to wheat streak mosaic virus (*wsm*1) and *mdm*1, resistance against maize dwarf mosaic virus. Several unlinked loci are involved in resistance in maize to MDMV. Presently, all characterized sources of resistance to MDMV have involved the *mdm1* allele on the short arm of chromosome 6 (Simcox et al. 1993)] and finally, (vii) chromosome10- a complex that includes *rp*1, *rp5, rp*6, resistance to common rust and *rpp*9, resistance to maize southern rust.

Except for a few discrepancies, results from this study corresponded to what was found in the study conducted by Collins et al. (1998). Collins showed linkage of two RGA clones to the *rp*1 *rp*5 *rp*6 and *rrp*9 complex on the short arm of chromosome 10, no linkage of any of the 92 MITE/RGA fragments could be found to this specific complex.

Collins et al. (1998) did not find linkage to *Ht*2 and *HtN*1 or *hm*2, whereas in this study six MITE/RGA fragments were linked to *Ht*2 and *HtN*1 and one MITE/RGA fragment co-segregated with *hm*2. Collins et al. (1998) did identify a RGA clone that showed close linkage to the *hm*1 gene, although the original primers were not derived from a reductase protein product. Supporting these results, three MITE/NBS and one MITE/NBS mapped close to the *hm*1 and *hm*2 genes respectively. Unfortunately, no MITE/Red fragments showed linkage to either the *hm*1 or *hm*2 genes. This was rather disappointing since the reductase primers used, were originally derived from the sequence of the reductase protein product of the *hm*1 gene. A possible explanation for this can be that the specific MITE/Red primer combinations did not detect polymorphisms between the two recombinant inbred parental lines. So with no polymorphism detected between the two parents it was not possible to map any reductase fragments that could show linkage to the reductase genes (*hm1* and *hm2*). In a study by Bar-Zur et al. (1998), an association was made between the *HtN*1 gene and different isoperoxidases, unfortunately no MITE/Per fragments was mapped in the immediate vicinity of the *HtN*1 gene. One MITE/Per fragment did, however, map

Stellenbosch University http://scholar.sun.ac.za

close to the *Ht*1 gene, but since *Ht*- and *HtN*1 resistance do not have the same chlorotic lesion response to the pathogen, it cannot be assumed that the *Ht*1 gene is linked to a locus coding for a specific isoperoxidase.

McMullen and Simcox (1995a; 1995b) identified more than 20 quantitative trait loci (QTLs) influencing the resistance to known pathogens, but since the chromosomal positions of most QTLs are so poorly defined, Collins and co-workers (1998) did not attempt to show linkage of any of the RGA clones to QTLs. In this study probable linkage to numerous QTLs were shown, although as mentioned earlier, the exact positions of QTLs are in many instances undecided. However, extensive research has been done by Lehmensiek (2000) on QTL resistance to Gray leaf spot (GLS) and according to Table 3.7(b) five MITE/NBS fragments mapped in the proximity of a major QTL conferring resistance to GLS resistance.

Collins admitted failure in identifying candidate RGA loci for many of the known disease resistance loci in maize. This may in part be due to the fact that not all of the disease resistance genes in maize belongs to the NBS-LRR class, for example, *hm*1 and *hm*2 resistance genes encodes a reductase enzyme product that does not have a NBS region (Johal and Briggs 1992). Some of the RGA probes used by Collins et al. (1998) did not show any hybridisation in some of the maize lines, suggesting that different genotypes may have different sets of NBS-LRR gene classes (they identified one wheat RGA probe). To optimise the detection and mapping of candidate gene loci in a given genome it would be advisable to; (i) use a number of different mapping populations, with adequate population size, thus getting more accurate chromosomal mapping data and (ii) use more diverse primer sets derived from a wider repertoire of gene analogues, being more representative of a wider population of the different resistant genes in plant genomes.

4.6.2 MITE family elements inserts preferentially into genic areas

Schwarz et al. (1999) derived a candidate gene via a PCR based approach using degenerate oligonucleotide designed from the conserved areas and motifs from different plant resistant genes from the nucleotide-binding site (NBS) and the leucine-

Stellenbosch University http://scholar.sun.ac.za

rich repeat (LRR) motif. The sequenced AFLP fragment was identified near to the *Mla* locus that confers resistance to powdery mildew in barley. The sequence was compared using the BLAST algorithm. The amino acid deducted sequence showed 63% identity and 76% similarity to a putative resistance gene protein from potato. Through PCR analysis, applying analogue primers from conserved protein domains, on different plant species such as potato (Leister et al. 1996), rice (Mago et al. 1999), soybean (Yu et al. 1996), *Arabidopsis (*Aarts et al., 1998), researchers found that the PCR amplification products they obtained were homologous to known plant resistance genes.

Heartbreaker insertion sites, like those of the *Ac* and *Mutator* elements, were found to be preferentially to the low copy number regions of the maize genome (Zhang et al. 2000). This indicates their preference for genic rich areas since low copy number genomic regions may account for less than 20% of the maize genome (Casa et al. 2000). In a study by Zhang et al. (2000) they found that 20 out of 37 *Hbr* fragments tested and analysed have inserted in or near sequences that showed significant similarity to known genes or expressed regions in the maize genome. Unlike most transposable elements, the *Hbr*-element shows no clustering at centromeric areas. Jiang and Wessler (2001) explained that a bulk of MITE amplifications might have preceded LTR retrotransposition in maize, this provided researchers further evidence on the genic preferences of MITE elements. Tikhonov et al. (1999) found 33 MITEs in a 225kb region in the maize genome flanking the *adh1*-gene. None of these MITEs were found to be within a 166kb genomic region occupied by any LTR-retrotransposon.

Even though only a very small percentage of the total number of mapped fragments were sequenced it can be concluded that by taking in consideration the nature of the RGA primers as well as the genic preference of the MITE elements, that most of the amplified fragments derived from this altered MITE transposable-like display (TD), will have a genic origin.

Stellenbosch University http://scholar.sun.ac.za

Occurrence of non-parental fragments

It is not known with certainty whether MITEs are DNA or RNA mediated transposable elements, all scientific evidence favours the view that MITEs are non-autonomous DNA elements (Wessler 1998; Casa 1999). Supporting this view is the fact that in general *Hbr*-elements prefers genic regions. In this study, four and ten of the fragments scored for the *Hbr*- and *Hb2*-elements, respectively, appeared to be of non-parental inheritance. The low percentage of non-parental MITE/RGA fragments can and can most probably be due to; (i) mutation(s) in the recognition site(s) of the respective RGA primer in one or in both of the parental lines, (ii) new transpositions (which are very unlikely), (iii) pollen contamination due to outcrossing and (iv) residual heterozygosity, that is commonly observed in maize inbred lines (Casa et al. 2000).

Casa and co-workers (2000) calculated that the percentage of non-parental fragments for the *Hbr*-element markers varied from 0.2% to 2.5%, depending on the primer combination and mapping population they used. The incidence of non-parental bands can be compared to those observed in other molecular marker studies done in maize, for example; (i) non-parental band frequencies of 2-5% were observed in microsatellite studies; (ii) a frequency of 3% was observed for RFLP studies (Chin et al. 1996); (iii) van Staden (unpublished data) detected a low percentage (3%) of non-parental bands in an AFLP analysis. Thus the, non-parental band percentages, observed in the different studies, using a wide array of different marker techniques correlate, suggesting that the chances are extremely slim that any of the *Hbr*- or *Hb2*-elements are still active in the inbred background.

4.7 Applying the MITE/RGA markers to fine map examples of resistance genes.

Van Staden (2001) and Lehmensiek (2000), respectively, identified PCR-based molecular markers (the us-markers) linked to; (i) single gene resistance against the pathogen, *Setosphaeria turcica,* in maize and (ii) QTL resistance to gray leave spot (GLS) in maize. Mapping results, using the linkage maps of the two available RI mapping populations (B.Burr), mapping the *HtN*1, *Ht*1, *Ht*2 and *Ht*3 and QTL resistance for gray leaf spot (GLS) loci in respect to new molecular markers and established markers, proofed to be extremely valuable to this study.

Stellenbosch University http://scholar.sun.ac.za

Setosphaeria turcica

There are presently four dominant or partially dominant genes known that individually condition resistance in maize to *Setosphaeria turcica*. These genes, known as *HtN*1, *Ht1, Ht2,* and *Ht3* can be readily differentiated from one another by the reaction they confer in maize to the known physiologic races of *E. turcicum (*Zaitlin et al. 1992*)*. Clusters of MITE/RGA markers, mapping to chromosomal bins where the putative positions of the *HtN*1, *Ht*1, *Ht*2 and *Ht*3-resistance genes are located, were analysed.

*Ht*1 gene chromosomal location

Six additional MITE/RGA markers showed close linkage to the polymorphic us-markers, RFLP and SSR core markers, which map to chromosome 2, bin2.07. Previous research by Patterson et al. (1965), Bentolila et al. (1991) and Freymark et al. (1993) positioned the *Ht*1 gene in the region of this chromosomal location. For *Ht*1, van Staden (2001) mapped three SCAR markers (us24, us30, and us35) to chromosome 2 (bin 2.07), with flanking markers umc98a (proximal) and bnl6.50 (distal). Linkage data of 0.0cM with accompanying high LOD scores (10.81-12.01) are a strong indication that these MITE/RGA markers can be considered as possible candidate gene loci for the resistance gene locus, *Ht*1. Should a proper F_2 segregating mapping population be available for *Ht*1 resistance, the six candidate gene markers can be used together with the currently available us-markers to map the exact position of the *Ht*1 gene.

*Ht*2 and *HtN*1 gene chromosomal location

Van Staden (2001) mapped two SCAR markers (us56 and us60) to maize chromosome 8 (bins 8.05-8.06). Zaitlin et al. (1992) mapped the *Ht*2 gene to the same chromosomal region between two RFLP flanking markers, umc89a (proximal) and umc48a (distal). Van Staden (2001) produced a more defined map showing the putative position of the *Ht*2 gene. A putative chromosomal location for the *HtN*1 resistance gene was given by Paterson et al. (1965) and van Staden (2001). They mapped the *HtN*1 gene between the two anchor markers, bnl12.30a (proximal) and umc48a (distal).

Stellenbosch University http://scholar.sun.ac.za

One MITE/RGA marker, *Hb2*/WMA-R(12), shows very close linkage (2.8cM) with a high LOD (8.3) score to marker us60. The remaining three markers showed close linkage to the core anchor marker, umc48. *Ht*2 and *HtN*1, map in close proximity to each other but they are two different resistant genes exhibiting different phenotypical characteristics.

*Ht*3 gene chromosomal location

The location of the *Ht*3 gene is still unknown, but van Staden (2001) putatively mapped this gene, using well-defined *Ht*3 near isogenic stocks (NILs) and the T232 x CM37 public available RIL mapping population (Burr et al. 1988). Using RFLP markers bnl6.27 (proximal) and bnl5.21a (distal) as anchor- and core markers, the marker (us62) was mapped with a high LOD score of 9.41 to chromosome 7 (bin 7.04). Marker, us62 mapped 14.4cM from bnl6.27 and ~5.0cM from bnl5.21a. However, marker us62 was tested on RIL mapping population Tx303 x CO159, but detected no polymorphism between the parental lines and thus could not be mapped. As a result the MITE/RGA and us-marker could not be tested for the linkage as a different mapping population was used in the analysis. Fortunately, both flanking core markers, bln6.27 and bnl5.21a, are mapped on both of Burr's publicly available RIL populations. Using RIL population (Tx303 x CO159), the two core markers were used as anchors to map the cluster of MITE/RGA fragments to chromosome 7, bin 7.04. Marker *Hbr*/WMA-F(2) mapped 2.7cM from core marker bnl5.21a with a high LOD score of 8.58, marker *Hb2*/WMA-F(2) mapped 4.9cM from core marker bnl6.27 with a LOD score of 6.08. All four of the MITE/RGA markers mapped between core markers bnl6.27 and bnl5.21a. To further define the chromosomal location of the *Ht*3 gene, the four MITE/RGA markers must be placed on the linkage map of the first recombinant population together with the us-markers.

The 14 MITE/RGA markers that mapped in chromosomal locations for *Ht* resistance are of great potential value and aid in the saturation of the maize genome linkage map. Van Staden (2001) could not confirm the exact locations of the *Ht* resistance genes. Mapping of van Staden's us-markers together with the novel MITE/RGA markers on a well-defined F_2 segregating population will clarify to what extent the the MITE/RGA fragments can be considered as candidate genes for *Ht* resistance.

Stellenbosch University http://scholar.sun.ac.za

Gray leaf spot (GLS)

Gray leaf spot (GLS) in maize, caused by the fungus C*ercospora zeae-maydis* (Tehon and Daniels 1925), has become a major threat throughout maize growing regions. Because of the yield losses experienced by GLS infections there is a demand for maize inbred lines with GLS resistance. A number of candidate gene primers were used together with MITE primers to determine whether candidate gene markers linked to GLS resistance could be identified. The F_2 population (230 individuals) used in the study by Lehmensiek (2000), was used in linkage analysis and interval mapping. Resistant gene analogue primers were amplified together with MITE primers on the resistant and the susceptible parental line as well as the resistant and susceptible bulks. Equal volumes of DNA from 20 resistant plants with a GLS score of 1 and 16 susceptible plants with a GLS score of 9 derived from a 1998 F_2 mapping population planted were pooled in two contrasting bulks.

Mapping results of marker, us44, were obtained from Lehmensiek (2000), giving information regarding the chromosomal location of one major QTL for GLS resistance in maize, on chromosome 1, bin 1.05/6. Fragment RGA1 was identified on the 230 individuals of an F_2 GLS segregating mapping population. Although marker RGA1 did not map directly to the interval harbouring of the assumed position of the QTL, it is still an informative marker. Using the segregating GLS data from the 230 F_2 plants, Lehmensiek (2000) identified a marker us44, on chromosome 1, bin 1.05/6. QTL mapping confirmed the presence of a major QTL at this chromosomal location. Interval mapping indicated that the marker RGA1 was situated distally to the QTL peak. Using the marker, RGA1, the LOD value of the peak of the QTL was increased from 20.7 (Lehmensiek 2000) to 21.9 and the phenotypic variance explained by the QTL was increased from 36.7% to 38.9%.

Markers *Hb2*/Kin-R(1) and *Hbr*/PL1-5-F(3) were identified and mapped to chromosomal position 1.05/6. Comparing with the results of Lehmensiek (2000) suggested that the two fragments mapped in the proximity of us44. Because marker us44 was mapped using the T232 x CM37 RIL mapping population, linkage of the two markers to us 44 could not be tested, but they did show linkage to the same core

Stellenbosch University http://scholar.sun.ac.za

marker bnl5.59. Marker us44 was placed on the RIL linkage maps using RI population (T232 x CM37), this us-marker showed close linkage to a RFLP core marker, bnl5.59. In order to test for linkage of these two markers with the QTL for GLS resistance it would be advisable to use the same mapping population. Ultimately it would be preferable to map these two MITE/RGA markers on the 230 individuals of the F_2 GLS segregating mapping population.

Stellenbosch University http://scholar.sun.ac.za

4.8 MITE/RGA DISPLAY VERSUS CONVENTIONAL AFLP PROFILING

AFLP and modified AFLP technique are highly robust and reliable marker techniques but are expensive because of the patent rights and the numerous manipulations necessary to handle the genomic DNA. The DNA firstly needs to be digested with restriction enzymes, adapters needs to be ligated and primers need to be specially purified. Since no restriction digestion or adaptor ligation is necessary for MITE/RGA analysis, the application of MITE primers as anchors in combination with degenerate resistance gene analogue (RGA)-primers are especially useful. The MITE/RGA approach is less expensive and laborious, but will this application be able to compete with the efficiency of the AFLP technique? Will it be more feasible with more practical applications in comparison to conventional AFLP fingerprint analysis?

4.8.1 Profile amplification

MITE/RGA analysis

Using eight different MITE/RGA primer combinations, a total of 81 fragments were scored with an average of 40.5 fragments per primer combination. As shown before the 81 fragments occurred in clusters throughout the maize genome. The fuzziness seen on the x-ray photos can be ascribed to quality of the RGA primers. Mostly degenerate RGA primers were used, meaning that the primers are not specific and will form some sort of non-specific banding patterns.

AFLP analysis

Using two *Mse*I/*Mlu*I AFLP primer combinations, a total 100 fragments were scored with an average of 50 fragments per primer combination. The data of Castiglioni et al. (1999) and Vuylsteke et al. (1999) showed that different restriction enzyme combinations detect different numbers of polymorphisms. The use of different rare cutter restriction enzymes (*EcoR*I, *Mlu*I, *Pst*I) in AFLP analysis significantly affects the distribution of AFLP amplification sites across the genome. They established that AFLP fragments generated by the enzyme combination *Pst*I/*Mse*I showed prevalence for genomic single copy sequences. Numerous researchers reported that, the use of a methylation sensitive restriction enzyme, in an AFLP application, be that for identification of markers or for fingerprinting of individuals, such as *Mlu*I (Van Staden

Stellenbosch University http://scholar.sun.ac.za

2001), *Pst*I (Castiglioni et al. 1999; Van Staden 2001) and *Sse*I (Donini et al. 1997) would amplify AFLP markers that are more randomly distributed across the genome. Methylation sensitive restriction enzymes will give equal genome coverage. By using the methylation sensitive restriction enzyme, *Mlu*I, the tendency for clustering of AFLP markers at centromeric regions will be drastically reduced.

4.8.2 Statistical analysis

Statistical tools to analyse molecular genetic data are becoming increasingly powerful and very sophisticated. A polymorphism shows the existence of variants among a given set of tested individuals and must be quantified using the appropriate statistical procedures. These variants can be identified genetically at different levels. Most laboratory procedures provide the means to identify genetic differences between cultivars. Genetic diversity studies have two main objectives; (i) to analyse the level of polymorphism at a given hierarchical level and, (ii) to study the distribution of the polymorphisms among the different hierarchical levels (Kremer et al. 1998).

Based on the banding profiles of the 40 elite maize lines, the UPGMA clustering analysis was used to construct dendrograms depicting the genetic distances among the 40 elite lines. Most of the observed discrepancies (within the subgroups) can be explained by the fact that the two types of markers assays target different parts of the maize genome as well as the fact that the assays are based on different molecular applications. Thus, the informative nature of the two techniques differs considerably. The number of primer combination necessary to give an accurate account of the genetic distance between different lines depends on the genetic diversity of the specific germplasm. For example, if two maize lines are very closely related, numerous different primer combinations are needed to effectively distinguish among them. If two maize lines are distantly related with very dissimilar genetic backgrounds fewer primer combinations will be required. Pejic et al. (1998) did comparative analysis studies of genetic similarities among 33 maize inbred lines, using four different profiling techniques. They found that the average number of assays they needed to attain a precise estimate were 30-40 clone-enzyme combinations for

Stellenbosch University http://scholar.sun.ac.za

RFLPs, 40-50 primers for RAPDs, 20-30 primers for SSRs and 4-5 AFLP enzyme primer combinations.

4.8.3 Dendrogram analysis

When comparing clusters from the two dendrograms, generated with the two profiling techniques, clustering of some of the maize lines did not match. However some closely related lines did appear to stay clustered. Possible explanations for these discrepancies can be given; (i) the underlying assumptions made in calculating pedigree data, (ii) genome and pedigree sampling and (iii) the number of markers or probes employed in a specific study. Taking the few discrepancies between the dendrograms into account, the results in this study showed that by using the *Hbr*- or *Hb2*-transposable elements in combination with gene analogue primers, a functional and efficient fingerprint is generated.

MITE/RGA is a simple marker technique that can be applied without restriction digestion of genomic DNA and without the need to ligate any adapters. This marker technique approach could be used to develop a functional linkage map for maize where markers are not merely markers but also candidate genes. There are approximately 4000 *Hbr* elements, 12000 *Hb2* elements and a number of other MITEs in the maize- and other plant genomes. All of the MITE families can be used in combinations with basically any gene sequences or gene product sequence found in other plants such as rice, *Arabidopsis*, wheat, barley, flax and tomato, to identify and map genes in maize as well as other important crops.

Stellenbosch University http://scholar.sun.ac.za

CHAPTER FIVE
CONCLUSION

Plant genomes are populated with transposable elements. These transposable elements exhibit unique features that can be applied in modern day molecular analytical techniques. The cloning of resistance genes from diverse plant species revealed a degree of conservation amongst protein domains at the amino acid level. The unique properties of a novel transposable element, miniature inverted-repeat transposable elements (MITEs), together with resistant gene analogues (RGA) have been exploited to develop a new molecular marker technique, a modified transposable-display (TD) that can be applied very successfully in maize genetics.

The new technique, using the two types of maize "elements", MITEs and RGAs, aided in the expansion of the maize genetic linkage map, adding in total, 196, new markers to the existing Tx303 x CO159 linkage map. Just as for resistance genes, these new MITE/RGA markers were found to occur in clusters spread across the maize genome, mapping to all of the ten maize chromosomes. Evenly distributed molecular markers are a desired attribute of linkage maps as they ensure excellent genome coverage. The ability to map the markers with confidence on the ten maize chromosomes depicts on the reproducibility of the technique and the fact that it was possible to unambiguously score most of the fragments.

Ninety-two (92) new MITE/RGA markers mapped in the vicinity of chromosomal positions of well-characterised mono- and polygenic resistance genes in the maize genome. Possible candidate genes were identified through fine mapping of the chromosomal regions of the *HtN*1, *Ht*1, *Ht*2 and *Ht*3 gene loci, conferring resistance to *Setosphaeria turcica*, and QTL resistance against gray leaf spot (GLS) in maize. The candidate gene approach has proved successful in the cloning and characterization of resistance genes (Faris et al. 1999). The discovery and cloning of numerous disease resistance genes, belonging to the different gene classes, will assist in the understanding of the molecular genetic mechanisms underlying disease resistance in all crop species.

Stellenbosch University http://scholar.sun.ac.za

Comparing conventional AFLP with the modified transposon-display technique proved that: (i) the MITE/RGA technique is more cost effective and less time consuming as it does not require restriction digestion of genomic DNA and ligation of primer adaptors, (ii) fragments from the MITE/RGA fingerprint display originate essentially from genic rich areas and (iii) just as for the AFLP approach, numerous loci are amplified in one reaction applying the MITE/RGA technique. Unfortunately the applicability of this technique is limited to species that have identifiable MITE elements. A general assumption of the presence of MITEs cannot be made e.g. the same MITE element are not necessarily present in closely related. The primers used in this study is very specific for that particular MITE element and is of little use for other MITE families. Conversely, the different restriction enzyme recognition sites and the choice of AFLP adaptors also limit the application of the AFLP technique.

Stellenbosch University http://scholar.sun.ac.za

REFERENCES

Aarts, M.G.M., te Lintel Hekkert, B., Holub, E.B., Beynon, J.L., Stiekema, W.J., and Pereira, A. (1998). Identification of R-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. Mol. Plant Micr. Inter 11, 251-258

Agrama, H.A., Moussa, M.E., Naser, M.E., Tarek, M.A., and Ibrahim, A.H. (1999). Mapping of QTL for downy mildew resistance in maize. Theor. Appl. Genet 99, 519-523

Ahn, S., and Tanksley, S.D. (1993). Comparative linkage maps of the rice and maize genomes. Proc. Natl. Acad. Sci. USA 90, 7980-7984

Ajmone Marsan, P., Castiglioni, P., Fusari, F., Kuiper, M., and Motto, M. (1998). Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. Theor. Appl. Genet 96, 219-227

Akagi, H., Yokozeki, Y., Inagaki, A., and Fujimura, T. (1996). Microsattellite DNA markers for rice chromosomes. Theor. Appl. Genet 93, 1071-1077

Alleman, M., and Freeling, M. (1986). The *Mu* transposable elements of maize: Evidence for transposition and copy number regulation during development. Genetics 112, 107-119.

Alonso-Blanco, C., Peeters, A.J., Fusari, F., and Kuiper, M.M. (1998). Development of an AFLP-based linkage map of Ler, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of a *Ler/Cvi* recombinant inbred population. Plant J 14, 259-271

Athma, P., Grotewold, E., and Peterson, T. (1992). Insertional mutagenesis of the maize *P* gene by intragenic transposition of *Ac*. Genetics. 131,199-209

Barett, B.A., and Kidwell, K.K. (1998). AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. Crop Sci 38, 1261-1271

Barone, A., Ritter, E., Schachtschabel, U., Debener, T., Salamini, F., and Gebhardt, C. (1990). Localisation by restriction fragment length polymorphism mapping in potato of a major dominant gene conferring resistance to the potato cyst nematode *Clobodera rostochiensis*. Mol. Gen. Genet 244, 177-182

Stellenbosch University http://scholar.sun.ac.za

Bar-Zur, A., Tadmor, Y., Juvik, J.A., Shimoni, M., and Reuveni, R. (1998). Resistance to northern corn leaf blight in maize (*Zea mays*) conditioned by the *HtN* gene and the association with isoperoxidases. Can. J. Plant Path 20, 28-34

Baston CJ, Weir BS, Zeng Z-B. (1994). Zmap-a QTL cartographer. In Proceedings of the 5th World Congress on Genetic Applied to Livestock Production: Computing Strategies and Software, edited by C Smith, JS Gavora, B Benkel, J Chesnais, W Fairfull, JP Gibson, BW Kennedy and EB Burnside. Published by the organizing committee, 5th World Congress on Genetics Applied to Livestock Production, Guelph, Ontario, Canada 22, 65-66

Baston CJ, Weir BS, Zeng Z-B. (1997). QTL Cartographer: A reference manual and tutorial for QTL mapping. Department of Statistics, North Carolina State University, Raleigh, NC

Beavis , W.D., and Grant, D. (1991). A linkage map based on information from four F_2 populations of maize (*Zea mays* L.). Theor. Appl. Genet 82, 636-644

Becker, J., Vos, P., Kuiper, M., Salamini, F., and Heun, M. (1995). Combined mapping of AFLP and RFLP markers in barley. Mol. Gen. Genet 249, 65-73

Beckman, J.S., and Soller, M. (1990). Towards unified approach to the genetic mapping of eukaryotes based on sequence-tagged microsatellite sites. Bio/Technology 8, 930-932

Bell, C.J., and Ecker, J.R. (1994). Assignment of 30 Microsatellite loci to the linkage map of *Arabidopsis*. Genomics 19, 137-144

Bennetzen, J.L. (1987). Covalent DNA modification and the regulation of *Mutator* element in transposition in maize. Mol. Gen. Genet 208, 45-51

Bennetzen, J.L. (1996). The contributions of retroelements to plant genome organization. Trends Microbiol 4, 347-353

Bennetzen, J.L. (2000). Transposable element contribution to plant gene and genome evolution. Plant Mol. Biol 42, 251-269

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leun, J., and Staskawicz, B.J. (1994). *RPS*2 of *Arabidopsis thaliana*: A Leucine-Rich Repeat class of plant disease resistance gene. Science 265, 1856-1860

Bentolila, S., Guitton, C., Bouvet, N., Sailand, A., Nykaza, S., and Freyssinet, G. (1991). Identification of an RFLP marker tightly linked to the *Ht*1 gene in maize. Theor. Appl. Genet 82, 393-398

Stellenbosch University http://scholar.sun.ac.za

Bingham, P.M., Levis, R., and Rubi, G.M. (1981). The cloning of DNA sequences from the white locus of *Drosophila melanogaster* using a novel and general method. Cell 25, 693-704.

Bradeen, J.M and Simon, P.W. (1998). Conversion of an AFLP linked fragment to the carrot Y_2 locus to a simple co-dominant, PCR-based marker form. Theor. Appl. Genet 97, 960-967

Byrne, M and Taylor, WC. (1996). Analysis of Mutator-induced mutations in the lojap gene of maize. Mol. Gen. Genet 252, 216-220

Byrne, R.F., McMullen, M.D, Snook, M.E., Musket, T.A., Theur, J.M., Widstrom, N.W., Wiseman, B.R., and Coe, E.H. (1996). Quantitative trait loci and metabolic pathways: genetic control of the concentration of maysin, a corn ear worm resistance factor, in maize silks. Proc. Natl. Acad. Sci. USA 93,8820-8825

Bureau, T.E., and Wessler, S.R. (1992). *Tourist*: A large family of small inverted repeat elements frequently associated with maize genes. Plant Cell 4, 1283-1294

Bureau, T.E., and Wessler, S.R. (1994a). Mobile inverted-repeat elements of the *Tourist* family are associated with the genes of many cereal grasses. Proc. Natl. Acad. Sci. USA 91, 1411-1415

Bureau, T.E., and Wessler, S.R. (1994b). *Stowaway*: A new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants. Plant Cell 6, 907-916

Burnham, C.R. (1982). The location of genes to chromosome by the use of chromosomal interchanges. In: Maize for Biological Research. Plant Molecular Biology Association, Charlottesville, VA, pp 65-70

Burr, B., and Burr, F.A. (1991). Recombinant inbreds for molecular mapping in maize: theoretical and practical considerations. Trends Genet 7, 55-60

Burr, B., Burr, F.A., Thompson, K.H., Albertson, M.C., and Stuber, W.C. (1988). Gene mapping with Recombinant Inbreds in maize. Genetics 118, 519-526

Caetano-Anollés, G. (1994). MAAP: A versatile and universal tool for genome analysis. Plant Mol. Biol 25, 1011-1026

Stellenbosch University http://scholar.sun.ac.za

Caetano-Anollés, G., Bassam, B.J., and Gresshoff, P.M. (1991). DNA amplification fingerprinting: A Strategy for Genome Analysis. Plant Mol. Biol. Rpt, 9, 292-305

Caffrey, J.J., Wurtele, E.S., and Nikolau, B.J. (1995). Genetic mapping of two acetyl-CoA carboxylase genes. MNL 69, 3-4

Capy, P., Anxolabehere, D., and Langin, T. (1994). The strange phylogenies of transposable elements: are horizontal transfers the only explanation? Trends Genet 10, 7-12

Casa, A.M., Brouwer, C., Nagel, A., Wang, L., Zhang, Q., Kresovich, S., and Wessler, S.R. (2000). The MITE family *Heartbreaker* (*Hbre*): Molecular markers in maize. Proc. Natl. Acad. Sci. USA 97, 10083-10089

Casa, A.M., Kresovich, S., Wang, L., Nagel, A., Zhang, Q., and Wessler, S.R. (1999). MITEs, a novel class of "smart" molecular marker located preferentially in genic regions. Poster. 41st Annual Maize Genetic Conference.

Casacuberta, E., Casacuberta, J.M., Puigdomènech, P., and Mondorf, A. (1998). Presence of miniature inverted-repeat transposable elements (MITEs) in the genome of *Arabidopsis thaliana:* characterisation of the Emigrant family of elements. Plant J 19, 79-85

Castaglioni, P., Pozzi, C., Heun, M., Terzi, V., Muller, K.J., Rohde, W., and Salamini, F. (1998). An AFLP-based procedure for the efficient mapping of mutations and DNA probes in barley. Genetics 149, 2039-2056

Castaglioni, P., Ajmone-Marsan, P., van Wijk., and Motto, M. (1999). AFLP markers in a molecular linkage map of maize: codominant scoring and linkage group distribution. Theor. Appl. Genet 99, 425-431

Cervera., M.T., Cabezas, J.A., Sancha, J.C., Martínez de Toda, F., and Martínez-Zapatar, J.M. (1998). Application of AFLPs to the charactarization of grapevine (*Vitis vinifera* L.) genetic resources. A case study with accessions from Rioja (Spain). Theor. Appl. Genet 97, 51-59

Chang, R.Y., and Peterson, P.A. (1994). Chromosome labelling with transposable elements in maize. Theor. Appl. Genet 87, 650-656

Charrier, B., Foucher, F., Kondorosi, E., d'Aubenton-Carafa, Y., Thermes, C., Kondorosi, A., and Ratet, P. (1999). Bigfoot-A new family of Mite elements characterized from the Medicago genus. Plant J 18, 431-441

Stellenbosch University http://scholar.sun.ac.za

Chin, E.C.L., Senior, M.L., Shu, H., and Smith, J.S.C. (1996). Maize simple repetitive DNA sequences: Abundance and allele variation. Genome 39, 866-873

Chomet, P., Lisch, D., Hardeman, K.J., Chandler, V.L., and Freeling, M. (1991). Identification of a regulatory transposon that controls the *Mutator* transposable element system in maize. Genetics 129, 261-270

Cnops, G., den Boer, B., Gerats, A., Van Montagu, M., and Van Lijsebettens, M. (1996). Chromosome landing at the *Arabidopsis TORNADO1* locus using and AFLP-based strategy. Mol. Gen. Genet 253, 32-41

Coe, E., Hancock, D., Kowaleski, S., and Polacco, M. (1995). Gene list and working maps. MNL 69, 191-267

Collins, N.C., Webb, C.A., Ellis, J.G., Hulbert, S.H., and Pryor, A. (1998). The isolation and mapping of disease resistance gene analogs in maize. Mol. Plant Micr. Inter 11, 968-978

Cresse, A.D., Hulbert, S.H., Brown, W.E., Lucas, J.R., and Bennetzen, J.L. (1995). *Mu1*-related transposable elements of maize preferentially insert into low copy number DNA. Genetics 140, 315-324

Dallas, J.F. (1988). Detection of DNA "fingerprints" of cultivated rice by hybridization with a human minisatellite probe. Pro.c Natl. Acad. Sci. USA 85, 6831-6835

Demeke, T., Kawchuk, L.M., and Lynch, D.R. (1993). Identification of potato cultivars and clonal variants by random amplified polymorphic DNA analysis. Am. Pot. J 70, 561-570

Dennis, E.S., Sachs, M., Gerlach, W.L., Finnegan, E.J., and Peacock. (1985). Molecular analysis of the alcohol dehydrogenase-2 (Adh2) gene of maize. Nucl. Acid. Res 13, 727-743

Donini, P., Elias, M.L., Bougaard, S.M., and Koebner, R.M.D. (1997). AFLP fingerprinting reveals pattern differences between template DNA extracted from different plant organs. Genome 40, 521-526

Edwards, A., Civitello, A., Hammond, H.A., and Caskey, T.C. (1991). DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am. J. Hum. Genet 49, 746-756

Stellenbosch University http://scholar.sun.ac.za

Edwards, D., Jane C., Stevenson, D., Hegarty, M., Batley., Holdswoth, M., and Edwards, K. (2001). High throughput transposon mutagenesis in maize. Poster. 43rd Annual Maize Genetic Conference.

Egli, M., Lutz, S., Somers, D., and Gengenbach, B. (1994). Identification and mapping of maize acetyl-CoA carboxylase genes. MNL, 68, 92-93

Ender, A., Schwenk, K., Städler, T., Streit, B., and Schierwater, B. (1996). RAPD identification of microsatellites in *Daphnia*. Mol. Ecol 5, 437-441

Faris, J.D., Li, W.L., Liu, D.J., Chen, P.D., and Gill, B.S. (1999). Candidate gene analysis of quantitative disease resistance in wheat. Theor. Appl. Genet 98, 219-225

Feuillet, C., Schachermayr, G., and Keller, B. (1997). Molecular cloning of a new receptor-like kinase gene encoded at the *Lr10* disease resistance locus of wheat. Plant J 11, 45-52

Francis, H.A., Leitch, A.R., and Koebner, P.M.D. (1995). Conversion of a RAPD-generated PCR product, containing a novel dispersed repetitive element, into a fast and robust assay for the presence of rye chromatin in wheat. Theor. Appl. Genet 90, 636-642

Freymark, P.J., Lee, M., Woodman, W.L., and Martinson, C.A. (1993). Quantitative and qualitative trait loci affecting host-plant response *to Exserohilium turcicum* in maize (*Zea mays* L.). Theor. Appl. Genet 87, 537-544

Gardiner, J.M., Coe, E.H., Melia-Hancock, S., Hoisington, D.A., and Chao, S. (1993). Development of a core RFLP map in maize using an immortalized F_2 population. Genetics 134, 917-930

Gaunt, B.S and Doebley, J.F. (1997). DNA sequence evidence for the segmental allotetraploid origin of maize. Proc. Natl. Acad. Sci. USA 94, 6809-6814

Gedil, M.A., Slabaugh, M.B., Berry, S., Johnson, R., Michelmore, R., Miller, J., Gulya, T., and Knapp, S.J. (2001). Candidate disease resistance genes in sunflower cloned using conserved nucleotide-binding site motifs: Genetic mapping and linkage to the downy mildew resistance gene Pl1. Genome 44, 205-212

Gevers, H.O. (1975). A new major gene for resistance to *Helminthosporium turcicum* leaf blight of maize. Plant Dis. Rep 59, 296-299

Goodall, G.J., and Filipowicz, W. (1989). The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. Cell 58, 473-483

Stellenbosch University http://scholar.sun.ac.za

Goodburn, S.E.Y., Higgs, D.R., Clegg, J.B., and Wheatherall, D.J. (1983). Molecular basis of length polymorphism in the human beta-globin gene complex. Proc. Natl. Acad. Sci. USA 80, 5022-5026

Grandbastien, M-A. (1992). Retroelements in higher plants. Trends Genet 8, 103-108

Graner , A., and Bauer, E. (1993). RFLP mapping of the *ym4* virus resistance gene in barley. Theor. Appl. Genet 83, 689-693

Gray, J., Close, P., Greenberg, J.M., McElver, J., Hantke, J., Briggs, S., and Johal, G. (1998). Isolation of lls1, a novel suppressor of cell death in plants. Maize Genetics Conference Abstracts, 40

Green, B., Walko, R., and Hake, S. (1994). *Mutator* insertions in an intron of the maize *knotted-1* gene result in dominant suppressible mutations. Genetics 138:1275-1285

Gu, W.K., Weeden, N.F., Yu, J., and Wallace, D.H. (1995). Large-scale, cost effective screening of PCR products in marker-assisted selection applications. Theor. Appl. Genet 91, 465-470

Gu Cho, Y., Blair, M.W, Panaud, O., and McCouch, S.R. (1996). Cloning of variety-specific rice genomic DNA sequences: amplified fragment length polymorphisms (AFLP) from silver-stained polyacrylamide gels. Genome 39, 373-378

Gupta, M., Chyi, Y.S., Romero-Severson, J., and Owen, J.L. (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor. Appl. Genet 89, 998-1006

Hake, S., and Walbot, V. (1980). The genome of *Zea mays*, its organization and homology to related grasses. Chromosoma 79, 251-270

Hammond-Kosack, K.E., and Lones, J.D.G. (1997). Plant disease resistance genes. Annu Rev Plant Physiol Plant Mol. Biol 48, 575-607

Han, C., Coe, E Jr., and Martienssen, R.A. (1992). Molecular cloning and characterization of iojap (ij), a pattern striping gene of maize. EMBO J 11, 4037-4046

Hantula, J., Dusabenyagasani, M., and Hamelin, R.C. (1996). Random amplified microsatellites (RAMS)-a novel method for characterizing genetic variation within fungi. Eur. J. For. Path 26, 159-166

Stellenbosch University http://scholar.sun.ac.za

Hartl, L., Mohler, V., Zeller, F.J., Hsam, S.L.K., and Schweizer, G. (1999). Identification of AFLP markers closely linked to the powdery mildew resistance genes *Pm1c* and *Pm4a* in common wheat (*Triticum aestivum* L.) Genome 42, 322-329

Hartl, L., and Seefelder, S. (1998). Diversity of selected hop cultivars detected by fluorescent AFLPs. Theor. Appl. Genet 96, 112-116

Haymer, D.S. (1994). Random Amplified Polymorphic DNAs and Microsatellites: What are they, and can they tell us anything we don't know already know? Ann Entomol. Soc. Am 87, 717-722

Helentjaris, T., Slocum, M., Wright, S., Schaefer, A., and Nienhuis, J. (1986). Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor. Appl. Genet 73, 761-769

Helentjaris, T., Weber, D., and Wright, S. (1988). Identification of the genomic locations of duplicated nucleotide sequences in maize by analysis of restriction fragment length polymorphisms. Genetics 118, 353-363

Heun, M., Kennedy, A.E., Anderson, J.A., Lapitan, N.L.V., Sorrells, M.E., and Tanksley, S.D. (1991). Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). Genome 34, 437-447

Hittalmani, S., Parco, A., Mew, T.V., Zeigler, R.S., and Huang, N. (2000). Fine mapping and DNA marker-assisted pyramiding of the three major genes for blast resistance in rice. Theor. Appl. Genet 100, 1121-1128

Hooker, A.L. (1977). A second major gene locus in corn for chlorotic-lesion resistance to *Helminthosporium turcicum*. Crop. Sci 17, 132-135

Hooker, A.L. (1981). Resistance to *Helminthosporium turcicum* from *Tripsacum floridanum* incorporated into corn. MNL 55, 87-88

Hulbert, S.H., Webb, C.A., and Smith, S.M. (2001). A modified set of *Rp* differential lines. MNL 75, 40-41

Humbert Reyes-Valdés, M. (2000). A model for Marker-based selection in gene introgression breeding programs. Crop Sci 40:91-98

Jarvis, P., Lister, C., Szabo, V., and Dean, C. (1994). Integration of CAPS markers into the RFLP map generated using recombinant inbred lines of *Arabidopsis thaliana*. Plant. Mol. Biol 24, 685-687

Stellenbosch University http://scholar.sun.ac.za

Jean, M., Brown, G.G., and Landry, B.S. (1998). Targeted mapping approaches to identify DNA markers linked to the *Rfp1* restorer gene for the 'Polima' CMS of Canola (*Brassica napus* L.). Theor. Appl. Genet 97, 431-438

Jeffreys, A.J., Neumann, R., and Wilson, V. (1990). Repeat unit sequence variation in minisatellites: A novel source of DNA polymorphisms for studying variation and mutation by molecule analysis. Cell 60, 473-485

Jeffreys, A.J., Wilson, V., Neumann, R., and Keyte, J. (1988). Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucl. Acid. Res 19, 10953-10971

Jeffreys, A.J., Wilson, V., and Thein, S.L. (1985). Hypervariable 'minisatellite' regions in human DNA. Nature 314, 76-79

Jiang, N., and Wessler, S.R. (2001). A genome-wide analysis of MITE multimers in rice (*O.Sativa* cv *Nipponbare*). Poster. 43rd Annual Maize Genetic Conference.

Jin, Y-K., and Bennetzen, J.L. (1989). Structure and coding properties of *Bs1*, a maize retrovirus-like transposon. Proc. Natl. Acad. Sci. USA 86, 6235-6239

Johal, G.S., and Briggs, S.P. (1992). Reductase actvity encoded by the *HM*1 disease resistance gene in maize. Science 258, 985-987

Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, .F, Van de Wiel, C., Bredemeijer, G., Vosman, B., Mattches, M., Daly, A., Brettschneider, R., Bettini ,P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazques, A., and Karp, A. (1997). Reproducibility testing of RAPD, RFLPs and SSR markers in plants by a network of European laboratories. Mol. Breeding 3, 381-390

Kalender, R., Grob, T., Regina, M., Suoniemi, A., and Schulman, A. (1999). IRAP and REMAP: two new retrotransposons-based DNA fingerprinting techniques. Theor. Appl. Genet 98, 704-711

Katsiotis, A., Schmidt, T., and Heslop-Harris, J.S. (1996). Chromosomal and genomic organization of *Ty1-copia* retrotransposon sequence in the genus *Avena*. Genome 39, 410-417

Kawchuk, L.M., Lynch, D.R., Hachey, J., Bains, P.S., and Kulcsar, F. (1994). Identification of a codominant amplified polymorphic DNA marker linked to the verticillum wilt resistance gene in tomato. Theor. Appl. Genet 89, 661-664

Stellenbosch University http://scholar.sun.ac.za

Keappler, S.M., Phillips, R.L., and Kim, T.S. (1993). Use of near-isogenic lines derived by backcrossing or selfing to map qualitative traits. Theor. Appl. Genet 87, 233-237

Keim, P., Schupp, J.M., Travis, S.E., Clayton, K., Zhu. T., Shi, L., Ferreira, A., and Webb, D.M. (1997). A high-density soybean genetic map based on AFLP markers. Crop. Sci 37, 537-543

Kermicle, J.L. (1980). Probing the component structure of a maize gene with transposable elements. Science 208, 1457

Kleinhofs, A., Chao, S., Sharp, P.J. (1988). Mapping of nitrate reductase genes in barley and wheat. In: Miller, T.E., and Koebner, R.M.D (eds) Proc. 7th Int Wheat Symposium, bath Press, Bath, USA: pp 541-546

Kyetere, D., Ming, R., McMullen, M., Pratt, R., Brewbaker, J., Musket, T., Pixley, K., and Moon, H. (1995). Monogenic tolerance to maize streak virus maps to the short arm of chromosome 1. MNL 69, 137-136

Lai, C. (1994). Genetic application of transposable elements in eukaryotes. Genome 37, 519-525

Lander, E.S., and Botstein, D. (1989). Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121, 185-199

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E., and Newburg, L. (1987). MAPMAKER: and interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1, 174-181

Laurie, D.A., Pratchet, N., Bezant, J.H., and Snape, J.W. (1994). Genetic analysis of a photoperiod response gene on the short arm of chromosome 2 (2H) of *Hordeum vulgare* (barley). Heredity 72, 619-627

Laurie, D.A., Pratchet, N., Romer, C., Simpson, E., and Snape, J.W. (1993). Assignment of the *denso* dwarfing gene to the long arm of chromosome 3 (3H) of barley by the use of RFLP markers. Plant Breeding 111, 198-203

Lechelt, C., Peterson, T.A., Laird, A., Chen, J., Dellaport, S.L., Dennis, E.S., Starlinger, P., and Peacock, W.J. (1989). Isolation and molecular analysis of the maize P locus. Mol Gen Genet 219, 225-234

Lee, M. (1995). DNA markers and plant breeding programs. Adv. Agron 55, 265-344

Stellenbosch University http://scholar.sun.ac.za

Lee, M., Beavis, W.D., Tingey, S.V., Vogel, J.M., Woodman, W.L., Long, M.J., Cardinal, A.J., Krakowsky, M., Hallauer, A.R., Austin, D and Ritland, D. (1998). The intermated B73 x MO17 maize population-A progress report. Maize Genetics Conference Abstract, 40

Lehmensiek A. (2000). Genetic mapping of grey leaf spot resistance genes in maize. Ph.D. University of Stellenbosch. Stellenbosch, South Africa

Leister, D., Ballvaro, A., Salamini, F., and Gebhardt, C. (1996). A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nat. Genet 14, 421-429

Levinson, G., and Gutman, G.A. (1987). Slipped-Strand Mispairing: A major mechanism for DNA sequence evolution. Mol. Biol. Evol 4, 203-221

Li, X., van Eck, H.J., Rouppe van der Voort, J.N.A.M., Huigen, D-J., Stam, P., and Jacobsen, E. (1998). Autotetraploids and genetic mapping using common AFLP markers: the *R2* allele conferring resistance to *Phytophthora infestans* mapped on potato chromosome 2. Theor. Appl. Genet 96, 1121-1128

Lim, S.M., Kinsey, J.G., and Hooker, A.L. (1974). Inheritence of virulence in Helminthosporium turcicum to monogenic resistant corn. PhytoPath 64, 1150-1151

Lin, J-J., Kuo, J., Ma J., Saunders, J.A., Beard, H.S., Macdonald, M.H., Kenworthy, W., Ude, N., and Matthews, F. (1996). Identification of molecular in soybean comparing RFLP, RAPD and AFLP DNA mapping techniques. Plant. Mol. Biol. Rep 14, 156-159

Lincoln, S., Daley, M., and Lander, E. (1992a). Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical report. 3rd edition.

Lincoln, S., Daley, M., and Lander, E. (1992b). Mapping genes controlling quantitative traits with MAPMAKER/QTL1.1. Whitehead Institute Technical report. 2nd edition.

Lisch, D., Chomet, P., and Freeling, M. (1995). Genetic characterization of the *Mutator* System in Maize: Behavior and regulation of *Mu* transposons in a minimal line. Genetics 139, 1777-1796

Litt, M., and Luty, F.A. (1989). A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum .Genet 44:397-401

Stellenbosch University http://scholar.sun.ac.za

Lopes, M.A., Glóverson, L.M., and Larkins, B. (1995). Genetic mapping of *opaque2* modifier genes. MNL 69, 125

Louie, R., Findley, W.R., Knoke, J.K., and McMullen, M.D. (1991). Genetic basis of resistance in maize to five maize dwarf mosaic virus strains. Crop Sci 31, 14-18

Lu, Z.X., Sosinski, D., Reighard, G.L., Baird, W.V., and Abbott, A.G. (1998). Construction of a genetic linkage map and identification of AFLP markers for resistance to root-knot nematodes in peach rootstocks. Genomics 14, 604-610

Lutz, S., and Gengenbach, B.G. (1996). Characterization of two unique Long Interspersed Nuclear Elements (LINEs), colonist1 and colonist2. MNL 70:59

Mackill, D.J and Bonman, J.M. (1992). Inheritance of blast resistance in near isogenic lines of rice. PhytoPath 82, 746-749

Magbanua, Z.V., Zhang, Q., Wang, L., and Wessler, S.R. (2001). Transposon display and mapping reveal clusters of the MITE Hb2 in the maize genome. Poster. 43rd Annual Maize Genetic Conference.

Mago, R., Nair, S., and Mohan, M. (1999). Resistance gene analogues from rice: cloning, sequencing and mapping. Theor. Appl. Genet 99, 50-57

Maheswaran, M., Subudhi, P.K., Nandi, S., Xu, J.C., Parco, A., Yang, D.C., and Huang, N. (1997). Polymorphism, distribution, and segregation of AFLP markers in a doubled-haploid rice population. Theor. Appl. Genet 94, 39-45

Mangelsdorf, P.C. (1974). Corn: its origin, evolution, and improvement. Harvard, Cambridge, Mass

Manjunath, S., and Sachs, M.M. (1997). Molecular characterization and promoter analysis of the maize cytosolic glyceraldehyde 3-phosphate dehydrogenase gene family and its expression during anoxia. Plant Mol. Biol 33, 97-112

Marillonet, S., and Wessler, S.R. (1997). Retrotransposons insertion into the maize *waxy* gene results in tissue specific RNA processing. Plant Cell 9, 967-978

Marques, C.M., Araujo, J.A., Ferreira, J.G., Whetten, R., O'Malley, D.M., Liu, B-H., and Sederoff, R. (1998). AFLP genetic maps of *Eucalyptus globules* and *E.tereticornis.* Theor. Appl. Genet 96, 727-737

Stellenbosch University http://scholar.sun.ac.za

Maughan, P.J., Saghai-Maroof, M.A., Buss, G.R., and Huestis, G.M. (1996). Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. Theor. Appl. Genet 93, 392-401

McClintock, B. (1950). The origin and behaviour of mutable loci in maize. Proc. Natl. Acad. Sci. USA 36, 344-355

McGregor, C.E., Lambert, C.A., Greyling, M.M., Louw, J.H., and Warnich, L. (2000). A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tertraploid potato (*Solanum tuberosum* L.) germplasm. Euphytica 133, 135-144

McMullen, M.D., and Simcox, K.D. (1995a). Genomic organization of disease and insect resistance genes in maize. Mol. Plant Micr. Inter 8, 811-815

McMullen, M.D., and Simcox, K.D. (1995b). Clustering of disease resistance loci in the maize genome. MNL 69, 52-53

Meksem, K., Leister, D., Peleman, J., Zabeau, M., Salamini, F., and Gebhardt, C. (1995). A high-resolution map of the vicinity of the *R1* locus on chromosome V of potato based on RFLP and AFLP markers. Mol. Gen. Genet 249, 74-81

Meyers, B.C., Dickerman, A.W., Michelmore, R.W., Sivaramakrishnan, S., Sobral, B.W., and Young, N.D. (1999). Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J 20, 317-332

Michaelson, M.J., Price, H.J., Ellison, J.R., and Johnston, J.S. (1991). Comparison of plant DNA contents determined by feulgen microspectrophotometry and laser flow cytometry. Am. J. Bot 78, 183-188

Michelmore, R.W., Paran, I., and Kesseli, R.V. (1991). Identification of markers linked to disease resistance-genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88, 9828-9832

Milbourne, D., Russel, J., and Waugh, R. (1997). Comparison of molecular marker assay in inbreeding (barley) and outbreeding (potato) species. Pages 371-381 in: Karp, A., Isaac, P.G., and Ingram, D.S, eds. Molecular tools for screening biodiversity. Chapman and Hall GmbH, Germany.

Stellenbosch University http://scholar.sun.ac.za

Milligan, S.B., Bodeau, J., Yaghoobi, J., Kaloshian, i., Zabel, P., and Williamson, V.M. (1998). The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat of plant genes. Plant Cell 10, 1307-1319

Ming, R., Brewbaker, J.L., Pratt, R.C., Musket, T.A., and McMullen, M.D. (1997). Molecular mapping of a major gene conferring resistance to maize mosaic virus. Theor. Appl. Genet 95, 271-275

Moore, G., Devos, K.M., Wang, Z., and Gale, M.D. (1995). Grasses, line up and form a circle. Curr. Biol 5, 737-739

Morell, M.K., Peakall, R., Appels, R., Preston, L.R., and Lloyd, H.L. (1995). DNA profiling techniques for plant variety identification. Austr. J. Exp. Agri 35, 807-819

Morgante, M., Rafalski, A., Biddle P., Tingey, S., and Olivieri, A.M. (1994). Genetic mapping and the variability of seven soybean repeat loci. Genome 37, 763-769

Muehlbauer, G.J., Specht, J.E., Thomas-Compton, M.A., Staswick, P.E., and Bernard, R.L. (1988). Near-Isogenic Lines-A potential resource in the integration of conventional and molecular marker linkage maps. Crop Sci 28, 729-735

Mukai, Y., Syama, Y., Tsumura, Y., Kawahara, T., Yoshimaru, H., Kondo, T., Tomaru, N., Kuramoto, N., and Murai, M. (1995). A linkage map for sugi (*Cryptomeria japonica*) based on RFLP, RAPD and isozyme loci. Theor. Appl. Genet 90, 835-840

Multani, D., Johal, G., Gray, J., Meeley, R., and Briggs, S. (1996). Plant-pathogen micro evolution: molecular basis for the origin of a fungal disease in maize. Maize Genetics Conference Abstracts 38

Myburg, A.A., Cawood, M., Wingfield, B.D., and Botha, A-M. (1998). Development of RAPD and SCAR markers linked to the Russian wheat aphid resistance gene *Dn*2 in wheat. Theor. Appl. Genet 6, 1162-1169

Nagel, A., Hooligen, D., Temnykh, S., McCouch, S., and Wessler, S.R. (2001). Development and application of Transposable display (TD) for several families from rice. Poster. 43rd Annual Maize Genetic Conference.

Stellenbosch University http://scholar.sun.ac.za

Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E., and White, R. (1987). Variable number of tandem repeats (VNTR) markers for human gene mapping. Science 235, 1616-1622.

Negi, M.S., Devic, M., Delseny, M., and Lakshmikumaran, M. (2000). Identification of AFLP fragments linked to seed coat colour in *Brassica juncea* and conversion to a SCAR marker for rapid selection. Theor. Appl. Genet 101, 146-152

Nikaido, A.M., Ujino, T., Iwata, H., Yoshimura, K., Yoshimura, H., Suyama, Y., Murai, M., Nagasaka, K., and Tsumura, Y. (2000). AFLP and CAPS linkage maps of *Cryptomeria japonica*. Theor. Appl. Genet 100, 825-831

Olsen, M., Hood, L., Cantor, C., and Botstein, D. (1989). A common language for physical mapping of the human genome. Science 245, 1434-1435

Paltridge, N.G., Collins, N.C., Bendahmane, A., and Symons, R.H. (1998). Development of YLM, a codominant PCR marker closely linked to the *Yd2* gene for resistance to barley yellow dwarf disease. Theor. Appl. Genet 96, 1170-1177

Paran, I., and Michelmore, R.W. (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet 85, 985-993

Paran, I., Kesseli, R., and Michelmore, R.W. (1991). Identification of restriction fragment length polymorphisms and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce using near-isogenic lines. Genome 34, 1021-1027

Paterson, A.H., Lander, E.S., Hewitt, J.D., Peterson, S., Lincoln, S.E., and Tanksley, S.D. (1988). Resolution of quantitative traits into Mendelian factors by using a complete RFLP linkage map. Nature 355, 721-726

Patterson, E.B., Hooker, A.L., and Yates, D.E. (1965). Location of *Ht* in the long arm of chromosome 2. MNL 39, 86-87

Pearson, W.R., and Lipman, D.J. (1988). Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. USA 85, 2444-2448

Stellenbosch University http://scholar.sun.ac.za

Pejic, J., Ajimone-Marsan, P., Morgante, M., Kozumplick, V., Castiglioni, P., Taramino, G., and Motto, M. (1998). Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs and AFLPs. Theor. Appl. Genet 97, 1248-1255

Penner, G.A., Chong, J., Wight, C.P., Molnar, S.J., and Fedsak, G. (1993). Identification of and RAPD marker for the crown rust resistance gene Pc68 in oats. Genome 36, 818-820

Perera, L., Russel, J.R., Provan, J., McNicol, J.W., and Powell, W. (1998). Evaluating genetic relationships between indigenous coconut (*Cocos Nucifera* L.) accessions from Sri Lanka by means of AFLP profiling. Theor. Appl. Genet 96, 545-550

Pernet, A., Hoisington, D., Franco, J., Isnard, M., Jewell, D., Jiang, C., Marchand, J.L., Reynaud, B., Glaszmann, J.C., and González de loon, D. (1999). Genetic mapping of maize streak virus resistance from the Mascarene source. I. Resistance in line D211 and stability against different virus clones. Theor. Appl. Genet 99, 524-539

Pflieger, S., Lefebvre, V., Caranta, C., Blattes, A., Goffinet, B., and Palloix, A. (1999). Disease resistance gene analogs as candidates for QTLs involved in pepper-pathogen interactions. Genome 42,1100-1110

Poulsen, D.M.E., Henry, R.J. Johnston, R.P., Irwin, J.A.G., and Rees, R.G. (1995). The use of bulk segregant analysis to identify a RAPD marker linked to leaf rust resistance in barley. Theor. Appl. Genet 91, 270-273

Powell. W., Thomas, W.T.B., Baird, E., Lawrence, P., Booth, A., Harrower, B., McNicol, J.W., and Waugh, T. (1997). Analysis of quantitative traits in barley by the use of amplified fragment length polymorphisms. Heredity, 79:4859

Pozueta-Romero, J., Houlne, G., and Schantz, R. (1996). Non-autonomous inverted repeat *Alien* transposable elements are associated with genes of both monocotyledonous and dicotyledonous plants. Gene 171, 147-153

Pozueta-Romero, J., Klerin, M., Houlne, G., Schantz, M-L., Meyer, B., and Schantz, R. (1995). Characterization of a family of genes encoding a fruit-specific wound-stimulated protein of bell pepper (*Capsicum annuum)*: Identification of a new family of transposable elements. Plant Mol. Biol 28, 1011-1025

Primrose, S.B. (1995). Principles of genome analysis. Blackwell Science Ltd.

Stellenbosch University http://scholar.sun.ac.za

Prioul, J-L., Pelleschi, S., Séne, M., Thévenot, C., Causse, M., de Vienne, D., and Leonardi, A. (1999). From QTLs for enzyme activity to candidate genes in maize. J Exp. Bot 50, 1281-1288

Provan, J., Thomas, W.T.B., Forster, B.P., and Powel, W. (1999). *Copia-SSR*: A simple marker technique which can be used on total genomic DNA. Genome 42, 363-366

Purugganan, M., and Wessler, S.R. (1992). The splicing of transposable elements and its role in intron evolution. Genetica 86, 295-303

Qi, X., Stam, P., and Lindhout, P. (1998). Use of locus-specific markers to construct a high density map in barley. Theor. Appl. Genet 96, 376-384

Quiros, C.F., Ceada, A., Georgescu, A., and Hu, J. (1993). Use of RAPD markers in potato genetics: segregation in diploid and tetraploid families. Am. Pot. J 70, 35-42

Quiros, C.F., Truco, M.J., and Hu, J. (1995). Sequence comparison of two codominant RAPD markers in *Brassica nigra*: deletions, substitutions and microsatellites. Plant Cell Rep 15, 268-270

Rafalski, J.A., and Tingey, S.V. (1993). Genetic Diagnostics in plant breeding: RAPDs, Microsatellites and machines. Trends Genet 9, 275-279.

Remington, D.L., Whetten, R.W., Liu, B-H., and O'Malley, D.M. (1999). Construction of an AFLP linkage map with nearly complete genome coverage in *Pinus taeda*. Theor. Appl. Genet 98, 1279-1292

Ribaut, J.M., Jiang, C., Gonzales-de-Leon, D., Edmeads, G.O., and Hoisington, D.A. (1997). Identification of quantitative trait loci under drought conditions in tropical maize 2. Yield components and marker assisted selection strategies. Theor. Appl. Genet 94, 887-896

Ridout, C.J., and Donini, P. (1999). Use of AFLP in cereals research. Trends Plant Sci :76-79

Robbins, W.A., and Warren, H.L. (1993). Inheritance of resistance to *Exserohilum turcicum* in PI209135, 'Mayorbela' variety of maize. Maydica 38, 209-213

Robertson, D.S. (1978). Characterization of a mutator system in maize. Mutat. Res 51, 21-28

Stellenbosch University http://scholar.sun.ac.za

Rongwen, J., Akkaya, M.S., Bhagwat, A.A., Lavi, U., and Cregan, P.B. (1995). The use of microsatellite DNA markers for soybean genotype identification. Theor. Appl. Genet 90, 43-48

Ross, K., Fransz, P.F., and Jones, G.H. (1996). A light microscopic atlas of meiosis in *Arabidopsis thaliana.* Chromos. Res 4, 507-516

Rossi, M., Goggin, F.L., Milligan, S.B., Kaloshian, I., Ullman, D.E., and Williamson, V.M. (1998). The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. Proc. Natl. Acad. Sci. USA 95, 9750-9754

Russel, J., Fuller, J.D., Macaulay, M., Hatz, B.G., Jahoor, A., Powell, W. and Waugh, R. (1997a). Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. Theor. Appl. Genet 95, 714-722

Russel, J., Fuller, J.D., Young, G., Thomas, B., Taramino, G., Macaulay, M., Waugh, R., and Powell, W. (1997b). Discriminating between barley genotypes using microsatellite markers. Genome 40, 442-450

Saghai Maroof, M.A., Yue, Y.G., Xiang, Z.X., Stromberg, E.L., and Rufener, G.K. (1996). Identification of quantitative trait loci controlling resistance to gray leaf spot disease in maize. Theor. Appl. Genet 93, 539-546

Schechert, A.W., Welz, H.G., and Geiger, H.H. (1999). QTL for resistance to *Setosphaeria turcica* in tropical maize. Crop Sci 39, 514-523

Schmid, C.W. (1996). Alu: Structure, origin, evolution, significance and function of one tenth of human DNA. Prog. Natl. Acad. Res. Mol. Biol 53, 283-319

Schüller, M., Backes, G., Fduschbeck, G., and Jahoor, A. (1992). RFLP markers to identify the allelles on the *Mla* locus conferring powdery mildew resistance in barley. Theor. Appl. Genet 84, 330-338

Schwarz, G., Michalek, W., Mohler, V., Wenzel, G., and Jahoor, A. (1999). Chromosome landing at the *Mla* locus in barley (*Hordeum vulgare* L.) by means of high-resolution mapping with AFLP markers. Theor. Appl. Genet 98, 521-530

Senior, M.L., Chin, E.C.L., Lee, M., Smith, J.S.C., and Stuber, C.W. (1996). Simple sequence repeat markers developed from maize sequences found in the GenBank database: map construction. Crop Sci 36, 1676-1683

Stellenbosch University http://scholar.sun.ac.za

Shagai-Moroof, M.A., Soliman, K.A., Jorgenson, R.A., and Allard, R.W. (1984). Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81, 8014-8018

Sharon, D., Adato, A., Mhameed, S., Lavi, U., Hillel, J., Gomolka, M., Epplen, C., and Epplen, J.T. (1995). DNA fingerprints in plants using simple-sequence repeat and minisatellite probes. HortScience 30, 109-112

Shin, J.S., Chao, S., Corpuz, L., and Blake, T.K. (1990). A partial map of the barley genome incorporating restriction fragment length polymorphism, polymerase chain reaction, isozyme, and morphological marker loci. Genome 33, 803-810

Shen, K.A., Meyers, B.C., Islam-Faridi, M.N., Chin, D.B., Stelly, D.M., and Michelmore, R.W. (1998). Resistance gene candidates identified by PCR with degenerate oilgonucleotide primers map to clusters of resistance genes in lettuce. Mol. Plant Micr. Inter 11, 815-823

Shoemaker, J., Zaitlin, D., Horn, J., De Mars, S., Kirshman, J., and Pitas, J. (1992). A comparison of three agrigenetics maize RFLP linkage maps. MNL 66, 65-69

Sibov, S.T., Gasper, M., Silva, M.J., Ottoboni, L.M.M., Arruda, P., and Souza, A.P. (1999). Two genes control aluminium tolerance in maize: Genetic and molecular mapping analyses. Genome 42, 475-482

Simcox, K.D., McMullen, M.D., and Louie, R. (1993). Mapping of multiple disease resistance genes on the short arm of chromosome six. MNL 67, 117

Sobir, T., Ohmori, T., Murata, M., and Motoyoshi, F. (2000). Molecular characterization of the SCAR markers tightly linked to the *Tm*-2 locus of the genus *Lycopersicon.* Theor. Appl. Genet 101, 65-69

Soller, M., and Beckmann, J.S. (1983). Genetic polymorphisms in variety identification and genetic improvement. Theor. Appl. Genet 67, 25-33

Spell, M.L., Baran, G., and Wessler, S.R. (1988). An RFLP adjacent to the maize *waxy* gene has the structure of a transposable element. Mol. Gen. Genet 211, 364-366

Speulman, E., Bouchez, D., Holub, E., and Beynon, J.L. (1998). Disease resistance gene homologs correlate with disease resistance loci of *Arabidopsis thaliana*. Plant J 14, 467-474

Stellenbosch University http://scholar.sun.ac.za

Springer, P.S., Edwards, K.J., and Bennetzen, J.L. (1994). DNA class organization on maize *Adh1* yeast artificial chromosomes. Proc. Natl. Acad. Sci. USA 91, 863-867

Takahashi, C., Marshall, J.A., Bennett, M.D., and Leitch, I.J. (1999). Genomic relationships between maize and its wild relatives. Genome 42, 1201-1207

Tanksley, S.D., Young , N.D., Paterson, A.H., and Bonierbale, M.W. (1989). RFLP mapping in plant breeding new tools for an old science. Biotechnology 7, 257-264

Tanksley, S.D., Ganal, M.W., Prince, J.P., de Vincente, M.C., Bonierbale, M.W., Broun, P., Fulton, T.M., Giovannoni, J.J., Grandillo, S., Martin, G.B., Messeguer, R., Miller, J.C., Miller, L., Paterson, A.H., Pineda, O., Röder, .S., Wing, R.A., Wu, W., and Young, N.D. (1992). High density molecular linkage maps of the tomato and potato genomes. Genetics 132, 1141-1160.

Tanksley, S.D., Ganal, M.W., and Martin, G.B. (1995). Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet 11, 63-68

Tautz, D. (1989). Hypervariability of simple sequences as a general source of polymorphic DNA markers. Nucl. Acid. Res 17, 6463-6471

Tehon, L.R., and Daniels, E. (1925). Notes on parasitic fungi of Illinois. Mycologia 17, 240-249

Thomas, M.R., and Scott, N.S. (1993). Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). Theor. Appl. Genet 86, 985-990

Thomas, C.M., Vos, P., Zabeau, M., Jones, D.A., Norcott, K.A., Chadwick, B.P., and Jones, J.D.G. (1995). Identification of amplified restriction fragment polymorphism (AFLP) markers tightly linked to the tomato length *Cf-9* gene for resistance to *Cladosporium-Fulvum.* Plant J 8, 785-794

Tikhonov, A.P., SanMiguel, P.J., Nakajima, Y., Gorenstein, N.D., Bennetzen, J.L., and Avramova, Z. (1999). Collinearity and its exceptions in orthologous *adh* regions of maize and sorghum. Proc. Natl. Acad. Sci. USA 96, 7409-7414

Ullstrup, A.J. (1970). A comparison of monogenic and polygenic resistance to Helminthosporium turcicum in corn. PhytoPath 60, 1597-1599

Stellenbosch University http://scholar.sun.ac.za

Van der Broeck, D., Maes, T., Sauer, M., De Keukeleire, P., D'Hauw, M., van Montagu, M., and Gerats, T. (1998). Transposon display identifies individual transposable lines in high copy number lines. Plant L 13, 121-129

Van Eck H.J., Van Der Voort, J.R., Draaistra, J.R., van Zandvoort, P., van Enckevort, E., Segers, B., Peleman, J., Jacobsen, E., Helder, J., and Bakker. (1995). The inheritance of chromosomal localization of AFLP markers in a non-inbred potato offspring. Mol. Breeding 1, 397-410

Van Staden, D. (2001). AFLP and PCR markers for the *Ht*1, *Ht*2, *Ht*3 and *Htn*1 resistance genes in maize. Ph.D. University of Stellenbosch. Stellenbosch, South Africa

Varagona, M.J., Purugganan, M., and Wessler, S.R. (1992). Alternative splicing induced by insertion of retrotransposons into maize *waxy* gene. Plant Cell 4, 811-820

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. Nucl. Acid. Res 23, 4407-4414

Voytas, D.F., Cummings, M.P., Konieczny, A.K., Ausubel, F.M., and Rodermel, S.R. (1992). *Copia*-like retrotransposons are ubiquitous among plants. Proc. Natl. Acad. Sci. USA 89, 7124-7128

Vuylsteke, M., Mank, R., Antonise, R., Bastiaans, E., Senior, M.L., Stuber, C.W., Melchinger, A.E., Lübberstedt, T., Xia, X.C., Stam, P., Zabeau, M., and Kuiper, M. (1999). Two high-density AFLP® linkage maps of *Zea mays* L.: analysis of distribution of AFLP markers. Theor. Appl. Genet 99, 921-935

Wang, L., and Wessler, S.R. (1999). Display of Miniature Inverted Repeat Transposable Elements in maize. Poster. 41st Annual Maize Genetic Congress.

Ward, J.M.J., Stromberg, E.L., Nowell, D.C., and Nutter, F.W. (1999). Gray leaf spot: A disease of global importance in maize production. Plant Dis 83, 884-895

Waugh, R., and Powell, W. (1992). Using RAPD markers for crop improvement. Trends Biotech 10, 186-191

Stellenbosch University http://scholar.sun.ac.za

Waugh, R., McLean, K., Flavell, A.J., Pearce, S.R., Kumar, A., Thomas, W.T.B., and Powell, W. (1997). Genetic distribution of Bare-1-like retrotransposable elements in he barley genome revealed by sequence-specific amplification polymorphisms (S-SAP). Mol. Gen. Genet 253, 687-694

Weber, J.L., and May, P.E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Gen 44, 388-396

Weil, C.F., Marillonnet, S., Burr, B., and Wessler, S.R. (1992). Changes in state of the *Wx-m5* allele of maize are due to intragenic transposition of *Ds*. Genetics 130, 175-185

Weising, K., Nybom, H., Wolf, K., and Meyer, W. (1995). DNA fingerprinting in plants and fungi. CRC press, Inc.

Weising, K., Weigand, F., Driesel, A.J., Kahl, G., Zischler, H., and Epplen, J.T. (1989). Polymorphic simple GATA/GACA repeats in plant genomes. Nucl. Acid. Res 17, 10128

Welsh, J., and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acid. Res 18, 7213-7218

Wessler, S.R. (1998). Transposable elements associated with normal plant genes. Physiol. Plantarum 103, 581-586

Wessler, S.R. (1999). MITEs: Transposable elements that create allelic diversity and serve to anchor a novel class of "smart" markers. Plenary Talk, Abstract. 41st Annual Maize Genetic Congress.

Wessler, S.R., Baran, G., Varagona, M.J., and Dellaporta, S.L. (1986). Excision of Ds produces waxy proteins with a range of enzymatic activities. EMBO J 5, 2427-2432

White, S., and Doebley, J. (1998). Of genes and genomes and the origin of maize. Theor. Appl. Genet 14, 327-332

Whitham,S., Dineshkumar,S.P., Choi,D., Hehl,R., Corr,C., and Baker,B. (1994). The Product of the Tobacco Mosaic-Virus Resistance Gene-N-Similarity to Toll and the Interleukin-1 Receptor. Cell 78, 1101-1115

Whitkus, R., Doebley, J., and Lee, M. (1992). Comparative genome mapping of sorghum and maize. Genetics 132, 1119-1130

Stellenbosch University http://scholar.sun.ac.za

Wight , C.P., and Penner, G.A. (1994). The identification of random amplified polymorphic DNA markers for daylength insensitivity in oat. Genome 37, 910-914

Williams, J,G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acid. Res 18, 6531-6535

Wu, K.S., Jones, R., Danneberger, L., and Scolnik, P.A. (1994). Detection of microsatellite polymorphisms without cloning. Nucl. Acid. Res 22, 3257-3258

Wu, K.S., and Tanksley, S.D. (1993). Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol. Gen. Genet 241, 225-235

Yamamoto, T., Kuboki, Y., Lin, S.Y., Sasaki, T., and Yano, M. (2000). Fine mapping of quantitative trait loci *Hd-1, Hd-2* and *Hd-3* controlling heading date of rice, as single Medelian factors. Theor. Appl. Genet 97, 37-44

Yang, H., and Krüger, J. (1994). Identification of a RAPD marker linked to the *Vf* gene for scab resistance in apples. Euphatica 77, 83-87

Yang, H.Y., Schuyler, S.K., Krüger, J., and Schmidt, H. (1997). The use of a modified bulk segregant analysis to identify a molecular marker linked to a scab resistance gene in apple. Euphytica 94, 175-182

Yang, H.Y., Schuyler, S.K., Krüger, J., and Schmidt, H. (1997). A randomly amplified DNA (RAPD) marker tightly linked to the scab-resistance gene V_f in apple. J. Am. Soc. Hort. Sci 122, 47-52

Young, N.D., Zamir, D., Ganal, M.W., and Tanksley, S.D. (1988). Use of Isogenic lines and simultaneous probing to identify DNA markers tightly linked to the *Tm-2a* gene in tomato. Genetics 120, 579-585

Yu, Z.H., Makill, D.J., Bonman, J.M., and Tanksley, S.D. (1991). Tagging genes for blast resistance in rice via linkage to RFLP markers. Theor. Appl. Genet 81, 471-476

Yu, Y.G., Saghai Maroof, M.A., Buss, G.R., Maughan, P.J., and Tolin, S.A. (1994). RFLP and Microsatellite mapping of a gene for soybean mosaic virus resistance. Genetics 84, 60-64

Yu, Y.G., Buss, G.R., and Saghai Maroof, M.A. (1996). Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc. Natl. Acad. Sci. USA 93, 11751-11756

Stellenbosch University http://scholar.sun.ac.za

Zabeau, M., and Vos, P. (1993). Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application number 92402629.7, Publication number 0 534 858 A1

Zaitlin, D., DeMars, S.J., and Gupta, M. (1992). Linkage of a second gene for NCLB resistance to molecular markers in maize. MNL 66, 69-70

Zeng Z-B. (1994). Precision mapping of quantitative trait loci. Genetics 136, 1457-1468

Zhang, Q., Arbuckle, J., and Wessler, S.R. (2000). Recent, extensive, and preferential insertion of members of the miniature inverted-repeat transposable element family *Heartbreaker* into genic regions of maize. Proc. Natl. Acad. Sci. USA 97, 1160-1165

Zhang, Q., Gao, Y.J., Yang, S.H., Ragab, R.A., Shagai Maroof, M.A., and Li, Z.B. (1994). A diallele analysis of heterosis in elite hybrid rice based on RFLPs and microsatellites. Theor. Appl. Genet 89, 185-192

Zhang, Q., Mrozek, R., and Wessler, S.R. (1998). The maize MITE subfamilies tourist-*Hbr* and *Hb*2 may posses a recent and massive amplification history. Talk. 40th Annual Maize Genetic Conference.

Zhang, X., Jiang, N., Zhang, Q., Eggleston, W., Feschotte, C., and Wessler, S.R. (2001). Isolation of a novel class 2 (DNA) element with strong target site preference and MITE family members. Talk. 43rd Annual Maize Genetic Conference.

Zhao, X., and Kochert, G. (1992). Characterization and genetic mapping of a short, highly repeated, interspersed DNA sequence from rice *(Oryza sativa L.)*. Mol. Gen. Genet 231, 353-359

Zhu, J., Gale, M.D., Quarrie, S., Jackson, M.T., and Bryan, G.J. (1998). AFLP markers for the study of rice biodiversity. Theor. Appl. Genet 96, 602-611

Zietkiewicz, E., Rafalski, A., and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20, 176-183

Stellenbosch University http://scholar.sun.ac.za

ADDENDUM A

Linkage analysis of the 196 MITE/RGA fragments on the 10 maize chromosomes

Linkage groups on Chromosome 1

Group 1.1

	Markers	Distance
1	H*br*/Red(C)-R(1)	9.4 cM
3	*Hb2*/Kin-R-F(3)	0.0 cM
4	H*b2*/GLPL-R(1)	4.2 cM
2	*Hbr*/RG_1-F(4)	10.0 cM
10	npi97A	----------
		23.7 cM 5 markers log-likelihood=-30.12

	1	3	4	2
3	7.7			
	4.39			
4	9.5	0.0		
	4.06	11.11		
2	12.1	4.6	4.2	
	3.34	6.60	7.40	
10	15.0	11.2	12.3	10.0
	2.72	3.78	3.76	4.46

Group 1.2

	Markers	Distance
117	*Hb2*/WMA-F(10)	0.7 cM
266	p1	0.6 cM
105	*Hbr*/RG_1-F(2)	0.0 cM
40	*Hbr*/Red(C)-F(8)	0.0 cM
34	*Hbr*/Red(C)-F(2)	9.6 cM
112	*Hb2*/WMA-F(4)	7.6 cM
186	*Hb2*/Red(D)-F(2)	5.8 cM
96	*Hbr*/GLPL-F(6)	4.2 cM
67	*Hbr*/Per-R(2)	4.2 cM
276	npi262	15.0 cM
286	umc67	----------
		47.7 cM 11 markers log-likelihood=-51.35

Stellenbosch University http://scholar.sun.ac.za

	117	266	105	40	34	112	186	96	67	276
266	0.0									
	11.71									
105	1.3	0.0								
	9.99	11.71								
40	1.3	0.0	0.0							
	9.99	11.71	12.01							
34	1.3	0.0	0.0	0.0						
	9.99	11.71	12.01	12.01						
112	12.3	10.4	10.0	10.0	10.0					
	3.76	4.23	4.46	4.46	4.46					
186	28.0	24.9	23.8	23.8	23.8	8.0				
	1.42	1.66	1.82	1.82	1.82	5.24				
96	28.0	24.9	23.8	23.8	23.8	8.0	5.9			
	1.42	1.66	1.82	1.82	1.82	5.24	6.38			
67	32.7	29.0	27.5	27.5	27.5	13.8	10.8	2.9		
	1.01	1.21	1.35	1.35	1.35	3.13	4.00	7.74		
276	29.4	24.9	24.9	24.9	24.9	26.2	21.1	10.0	3.0	
	1.28	1.66	1.66	1.66	1.66	1.50	2.09	4.46	7.47	
286	43.8	43.8	52.8	52.8	52.8	46.9	52.8	26.2	16.8	15.5
	0.57	0.57	0.37	0.37	0.37	0.48	0.37	1.50	2.55	2.94

Group 1.3

	Markers	**Distance**
292	bnl5.59	5.0 cM
297	umc119	7.9 cM
286	umc67	13.1 cM
110	*Hb2*/WMA-F(2)	14.8 cM
276	npi262	----------
		40.9 cM 5 markers log-likelihood= -39.80

	292	297	286	110
297	3.7			
	5.83			
286	10.4	5.9		
	4.23	4.79		
110	27.5	24.0	13.8	
	1.35	1.31	3.13	
276	24.9	28.0	15.5	16.1
	1.66	1.07	2.94	2.74

Stellenbosch University http://scholar.sun.ac.za

Group 1.4

	Markers	Distance
286	umc67	1.3 cM
145	*Hb2*/Kin-R(1)	1.3 cM
73	*Hbr*/PL1-5-F(3)	5.8 cM
292	bnl5.59	3.3 cM
297	umc119	5.5 cM
178	*Hb2*/RG_1-R(2)	10.1 cM
120	*Hb2*/WMA-F(13)	4.9 cM
302	bcd98A	12.0 cM
306	umc33	3.9 cM
122	*Hb2*/WMA-F(15)	6.2 cM
312	umc128	1.3 cM
315	umc37	1.3 cM
323	umc83	4.3 cM
127	*Hb2*/WMA-R(2)	10.1 cM
22	*Hbr*/Red(D)-F(3)	----------
		71.1 cM 15 markers log-likelihood=-76.59

	286	145	73	292	297	178	120	302	306	122
145	1.5 8.55									
73	3.0 7.47	1.5 8.55								
292	10.4 4.23	8.8 4.53	6.5 5.62							
297	5.9 4.79	4.0 5.29	3.8 5.56	3.7 5.83						
178	15.5 2.94	13.3 3.33	10.4 4.23	6.1 6.13	5.9 4.79					
120	31.0 1.14	27.5 1.35	22.1 1.92	21.1 2.09	18.1 1.95	9.7 4.69				
302	44.7 0.50	39.4 0.64	30.7 1.07	27.5 1.35	27.3 1.00	19.4 2.22	4.6 6.60			
306	-	-	-	-	72.3 0.11	-	19.1 1.76	10.0 2.97		
122	57.3 0.29	41.6 0.61	32.7 1.01	52.8 0.37	54.9 0.26	41.0 0.68	14.9 3.14	16.8 2.55	3.8 5.56	
312	43.8 0.57	44.7 0.50	34.7 0.88	-	61.2 0.19	-	26.2 1.50	21.2 1.86	6.4 4.28	6.5 5.62
315	32.7 1.01	30.7 1.07	24.3 1.59	43.8 0.57	29.9 0.92	52.8 0.37	22.1 1.92	21.2 1.86	9.1 3.43	4.6 6.60

Stellenbosch University http://scholar.sun.ac.za

	286	**145**	**73**	**292**	**297**	**178**	**120**	**302**	**306**	**122**
323	43.8	41.6	32.7	59.5	43.2	74.1	24.9	16.8	8.7	6.3
	0.57	0.61	1.01	0.27	0.47	0.14	1.66	2.55	3.67	5.87
127	-	62.6	46.9	-	-	-	34.7	20.3	12.3	10.4
		0.22	0.48				0.95	2.04	2.72	4.23
22	39.0	29.0	23.1	43.8	29.9	49.0	24.9	27.5	20.3	10.4
	0.72	1.21	1.75	0.57	0.92	0.45	1.66	1.35	1.58	4.23

	312	**315**	**323**	**127**
315	1.4			
	9.13			
323	2.9	1.3		
	8.02	9.70		
127	6.5	6.5	4.5	
	5.62	5.62	6.87	
22	10.8	6.5	10.4	10.4
	4.00	5.62	4.23	4.23

Group 1.5

	Markers	**Distance**
2	*Hb2*/WMA-F(6)	4.4 cM
140	bnl8.29A	5.3 cM
3	*Hb2*/Kin-R(13)	16.8 cM
1	*Hbr*/Per-R(4)	5.0 cM
146	bnl6.32	----------
		31.6 cM 5 markers log-likelihood= -35.56

	2	**140**	**3**	**1**
140	4.4			
	7.13			
3	6.7	4.6		
	5.37	6.60		
1	36.7	29.4	13.8	
	0.83	1.28	3.13	
146	36.9	32.7	15.0	4.9
	0.76	1.01	2.72	6.08

Stellenbosch University http://scholar.sun.ac.za

Linkage groups on Chromosome 2

Group 2.1

	Markers	Distance
53	*Hbr*/PL1-5-F(6)	0.0 cM
55	*Hbr*/PL1-5-F(8)	6.1 cM
222	bnl8.45	15.1 cM
226	umc53A	----------
		21.3 cM 4 markers log-likelihood= -25.78

	53	55	222
55	0.0		
	12.01		
222	5.9	5.9	
	4.79	4.79	
226	29.4	29.4	14.4
	1.28	1.28	2.50

Group 2.2

	Markers	Distance
94	*Hbr*/GLPL-F(3)	0.0 cM
72	*Hbr*/Per-F(2)	1.8 cM
144	Hb2/Kin-F(3)	3.7 cM
92	*Hbr*/GLPL-F(1)	0.0 cM
62	H*br*/PL1-5-F(15)	5.9 cM
232	umc6	6.2 cM
142	*Hb2*/Kin-F(1)	3.8 cM
164	*Hb2*/PL1-5-F(8)	4.1 cM
236	umc61	2.7 cM
125	*Hb2*/WMA-F(28)	6.3 cM
244	umc34	----------
		34.6 cM 11 markers log-likelihood= -47.92

	94	72	144	92	62	232	142	164	236	125
72	0.0									
	11.41									
144	1.4	1.5								
	9.13	8.55								
92	5.9	4.5	2.9							
	6.38	6.87	7.74							
62	5.9	4.5	2.9	0.0						
	6.38	6.87	7.74	12.01						
232	10.4	8.8	6.9	6.3	6.3					
	4.23	4.53	5.12	5.87	5.87					

Stellenbosch University http://scholar.sun.ac.za

	94	**72**	**144**	**92**	**62**	**232**	**142**	**164**	**236**	**125**
142	16.8	15.0	13.8	13.8	13.8	5.1				
	2.55	2.72	3.13	3.13	3.13	5.82				
164	11.9	10.4	10.8	11.9	11.9	4.5	3.0			
	3.97	4.23	4.00	3.97	3.97	6.87	7.47			
236	10.0	10.8	8.8	14.9	14.9	10.4	9.1	4.4		
	4.46	4.00	4.53	3.14	3.14	4.23	4.29	7.13		
125	10.0	10.8	8.8	14.9	14.9	16.1	14.4	8.0	2.9	
	4.46	4.00	4.53	3.14	3.14	2.74	2.92	5.24	8.02	
244	19.4	21.2	18.4	27.5	27.5	26.2	34.7	19.4	10.8	6.7
	2.22	1.86	2.17	1.35	1.35	1.50	0.81	2.22	4.00	5.37

Group 2.3

	Markers	**Distance**
119	*Hb2*/WMA-F(12)	1.3 cM
253	accA	4.2 cM
208	*Hb2*/GLPL-F(4)	1.3 cM
210	*Hb2*/GLPL-F(8)	4.2 cM
4	*Hbr*/WMA-R(4)	0.0 cM
199	*Hb2*/Red(C)-F(2)	11.9 cM
181	*Hb2*/RG_1-R(5)	0.0 cM
35	*Hbr*/Red(C)-F(3)	0.0 cM
85	*Hbr*/Kin-R(2)	0.0 cM
97	*Hbr*/RG_1-R(2)	0.0 cM
271	umc55A	4.4 cM
68	*Hbr*/Per-R(3)	14.4 cM
8	*Hbr*/WMA-R(8)	1.5 cM
172	*Hb2*/RG_1-F(5)	----------
		43.5 cM 14 markers log-likelihood= -48.72

	119	**253**	**208**	**210**	**4**	**199**	**181**	**35**	**85**	**97**
253	1.4									
	9.42									
208	5.9	4.5								
	6.38	6.87								
210	7.7	6.3	1.3							
	5.48	5.87	9.99							
4	10.0	13.3	6.1	4.4						
	4.46	3.33	6.13	7.13						
199	10.4	13.3	6.3	4.5	0.0					
	4.23	3.33	5.87	6.87	11.11					
181	23.8	22.1	17.1	14.4	10.0	10.4				
	1.82	1.92	2.77	3.34	4.46	4.23				

Stellenbosch University http://scholar.sun.ac.za

	94	**72**	**144**	**92**	**62**	**232**	**142**	**164**	**236**	**125**
35	23.8	22.1	17.1	14.4	10.0	10.4	0.0			
	1.82	1.92	2.77	3.34	4.46	4.23	12.01			
85	23.8	22.1	17.1	14.4	10.0	10.4	0.0	0.0		
	1.82	1.92	2.77	3.34	4.46	4.23	12.01	12.01		
97	24.9	23.1	17.8	14.9	10.4	10.8	0.0	0.0	0.0	
	1.66	1.75	2.58	3.14	4.23	4.00	11.71	11.71	11.71	
271	24.9	23.1	17.8	14.9	10.4	10.8	0.0	0.0	0.0	0.0
	1.66	1.75	2.58	3.14	4.23	4.00	11.71	11.71	11.71	11.41
68	18.4	19.3	18.4	15.0	12.6	15.7	5.1	5.1	5.1	5.3
	2.17	1.99	2.17	2.72	3.13	2.52	5.82	5.82	5.82	5.56
8	41.0	39.0	29.4	34.7	29.4	23.1	14.9	14.9	14.9	12.8
	0.68	0.72	1.28	0.95	1.28	1.75	3.14	3.14	3.14	3.54
172	49.0	46.9	34.7	41.0	36.7	27.5	21.1	21.1	21.1	18.6
	0.45	0.48	0.95	0.68	0.83	1.35	2.09	2.09	2.09	2.40

	271	**68**	**8**
68	5.3		
	5.56		
8	15.5	15.7	
	2.94	2.52	
172	22.1	15.7	1.4
	1.92	2.52	9.42

Group 2.4.1

	Markers	**Distance**
287	umc22	9.1 cM
20	*Hbr*/WMA-F(9)	8.2 cM
201	*Hb2*/Red(C)-F(4)	0.0 cM
202	*Hb2*/Red(C)-F(5)	0.0 cM
58	*Hbr*/PL1-5-F(11)	0.0 cM
124	*Hb2*/WMAF-F(17)	0.0 cM
292	umc4A	3.9 cM
294	bnl5.21B	1.7 cM
37	*Hbr*/Red(C)-F(5)	0.0 cM
2	*Hbr*/WMA-R(2)	2.7 cM
91	*Hbr*/GLPL-R(1)	0.0 cM
84	*Hbr*/Kin-R(1)	1.6 cM
306	bnl6.20	7.4 cM
71	*Hbr*/Per-F(1)	4.8 cM
143	*Hb2*/Kin-F(2)	----------
		39.7 cM 15 markers log-likelihood=-48.82

Stellenbosch University http://scholar.sun.ac.za

	287	**20**	**201**	**202**	**58**	**124**	**292**	**294**	**37**	**2**
20	8.5									
	4.76									
201	0.0	8.2								
	10.51	5.00								
202	0.0	8.0	0.0							
	10.81	5.24	11.41							
58	0.0	7.7	0.0	0.0						
	11.11	5.48	11.41	11.71						
124	0.0	8.2	0.0	0.0	0.0					
	10.81	5.00	10.81	11.11	11.41					
292	0.0	9.1	0.0	0.0	0.0	0.0				
	10.51	4.29	9.91	10.21	10.51	10.21				
294	2.9	12.3	2.9	2.9	2.8	2.9	1.5			
	8.02	3.76	7.74	8.02	8.30	8.02	8.55			
37	2.9	17.1	6.3	6.1	5.9	4.5	3.1	1.3		
	7.74	2.77	5.87	6.13	6.38	6.87	7.19	9.70		
2	3.0	17.8	6.5	6.3	6.1	4.6	3.2	1.4	0.0	
	7.47	2.58	5.62	5.87	6.13	6.60	6.92	9.42	11.71	
91	2.9	11.9	6.3	6.1	5.9	4.5	3.1	1.3	2.7	2.8
	7.74	3.97	5.87	6.13	6.38	6.87	7.19	9.70	8.58	8.30
84	2.9	11.9	6.3	6.1	5.9	4.5	3.1	1.3	2.7	2.8
	7.74	3.97	5.87	6.13	6.38	6.87	7.19	9.70	8.58	8.30
306	5.1	16.1	9.1	8.8	8.5	6.9	5.5	3.0	1.4	1.4
	5.82	2.74	4.29	4.53	4.76	5.12	5.30	7.47	9.13	8.84
71	11.6	36.7	13.8	16.1	15.5	13.8	12.6	8.5	6.3	6.5
	3.56	0.83	3.13	2.74	2.94	3.13	3.13	4.76	5.87	5.62
143	19.3	34.7	25.5	29.0	29.0	26.9	21.4	17.6	13.8	11.6
	1.99	0.88	1.44	1.21	1.21	1.28	1.64	2.36	3.13	3.56

	91	**84**	**306**	**71**
84	0.0			
	12.01			
306	1.4	1.4		
	9.13	9.13		
71	10.4	10.4	6.7	
	4.23	4.23	5.37	
143	16.8	16.8	12.6	5.1
	2.55	2.55	3.13	5.82

Stellenbosch University http://scholar.sun.ac.za

Group 2.4.2

	Markers	Distance
209	*Hb2*/GLPL-F(5)	8.2 cM
294	bnl5.21B	2.8 cM
296	umc122	----------
		11.0 cM 3 markers log-likelihood= -21.89

	209	294
294	8.2	
	5.00	
296	6.5	2.8
	5.62	8.30

Group 2.5

	Markers	Distance
309	umc49	1.7 cM
163	*Hb2*/PL1-5-F(7)	7.5 cM
9	*Hbr*/WMA-R(9)	0.0 cM
188	*Hb2*/Red(D)-R(2)	2.6 cM
180	*Hb2*/RG_1-R(4)	0.0 cM
313	umc36A	4.1 cM
190	*Hb2*/Red(C)-R(1)	5.7 cM
321	bnl17.14	----------
		21.8 cM 8 markers log-likelihood= -34.71

	309	163	9	188	180	313	190
163	1.8						
	7.11						
9	8.4	8.0					
	3.91	5.24					
188	8.4	7.7	0.0				
	3.91	5.48	11.71				
180	14.4	11.9	2.8	2.7			
	2.50	3.97	8.30	8.58			
313	11.2	12.8	2.9	2.9	0.0		
	3.15	3.54	7.74	8.02	11.41		
190	18.1	14.4	8.0	7.7	4.2	4.5	
	1.95	3.34	5.24	5.48	7.40	6.87	
321	39.9	28.0	12.3	11.9	7.7	8.0	5.9
	0.57	1.42	3.76	3.97	5.48	5.24	6.38

Stellenbosch University http://scholar.sun.ac.za

Linkage groups on Chromosome 3

Group 3.1

	Markers	Distance
230	umc154	5.1 cM
70	*Hbr*/Per-R(5)	4.4 cM
246	umc97	1.7 cM
10	*Hbr*/WMA-R(10)	0.0 cM
42	*Hbr*/Red(C)-F(1)	0.0 cM
18	*Hbr*/WMA-F(7)	0.0 cM
28	*Hbr*/Red(D)-R(4)	5.9 cM
252	umc102	----------
		17.2 cM 8 markers log-likelihood= -29.99

	230	70	246	10	42	18	28
70	4.5						
	6.87						
246	5.3	3.4					
	5.56	6.37					
10	2.9	2.9	1.6				
	8.02	8.02	7.69				
42	4.4	4.4	1.6	0.0			
	7.13	7.13	7.97	11.71			
18	4.4	4.4	1.6	0.0	0.0		
	7.13	7.13	7.97	11.71	12.01		
28	4.4	4.4	1.6	0.0	0.0	0.0	
	7.13	7.13	7.97	11.71	12.01	12.01	
252	14.9	8.2	7.2	6.3	6.1	6.1	6.1
	3.14	5.00	4.88	5.87	6.13	6.13	6.13

Group 3.2

	Markers	Distance
252	umc102	2.7 cM
95	*Hbr*/GLPL-F(5)	1.3 cM
253	umc18A	5.0 cM
185	*Hb2*/RG_1-R	0.0 cM
159	*Hb2*/PL1-5-F(2)	0.0 cM
168	*Hb2*/RG_1-F(1)	3.8 cM
264	bnl5.37	----------
		12.8 cM 7 markers log-likelihood= -26.69

Stellenbosch University http://scholar.sun.ac.za

	252	95	253	185	159	168
95	2.8 8.30					
253	2.9 7.74	1.4 9.13				
185	10.0 4.46	5.9 6.38	4.6 6.60			
159	10.0 4.46	5.9 6.38	4.6 6.60	0.0 12.01		
168	10.4 4.23	6.1 6.13	4.8 6.34	0.0 11.71	0.0 11.71	
264	17.6 2.36	11.6 3.56	11.6 3.56	3.1 7.19	3.1 7.19	3.2 6.92

Group 3.3

	Markers	Distance
83	*Hbr*/Kin-F(11)	4.4 cM
269	bnl8.01	1.3 cM
27	*Hbr*/Red(D)-R(3)	1.4 cM
272	bnl5.14	----------
		7.0 cM 4 markers log-likelihood=-20.98

	83	269	27
269	4.6 6.60		
27	6.1 6.13	1.4 9.42	
272	3.0 7.47	0.0 11.11	1.4 9.13

Group 3.4

	Markers	Distance
60	*Hbr*/PL1-5-F(13)	5.5 cM
280	umc39A	1.6 cM
165	*Hb2*/PL1-5-F(9)	0.0 cM
7	*Hbr*/WMA-R(7)	1.3 cM
3	*Hbr*/WMA-R(3)	0.0 cM
169	*Hb2*/RG_1-F(2)	0.0 cM
282	umc3B	2.9 cM
59	*Hbr*/PL1-5-F(12)	1.4 cM
111	*Hb2*/WMA-F(3)	1.4 cM
54	*Hbr*/PL1-5-F(7)	1.4 cM
52	*Hbr*/PL1-5-F(4)	9.1 cM
284	bnl1.297	----------
		24.7 cM 12 markers log-likelihood=-38.62

Stellenbosch University http://scholar.sun.ac.za

	60	**280**	**165**	**7**	**3**	**169**	**282**	**59**	**111**	**54**	
280	5.7										
	5.04										
165	7.7	1.7									
	5.48	7.40									
7	8.0	1.7	0.0								
	5.24	7.40	11.71								
3	10.0	1.7	1.3	1.3							
	4.46	7.40	9.70	9.70							
169	10.0	1.8	1.3	1.4	0.0						
	4.46	7.11	9.70	9.42	11.41						
282	10.0	1.7	1.3	1.4	0.0	0.0					
	4.46	7.40	9.70	9.42	11.41	11.41					
59	5.9	5.7	4.2	4.4	2.8	2.8	2.8				
	6.38	5.04	7.40	7.13	8.30	8.30	8.30				
111	8.0	8.0	6.1	6.3	4.5	4.5	2.9	1.3			
	5.24	4.14	6.13	5.87	6.87	6.87	8.02	9.70			
54	9.7	10.7	7.7	8.0	6.1	6.1	4.4	2.7	1.3		
	4.69	3.37	5.48	5.24	6.13	6.13	7.13	8.58	9.70		
52	11.9	13.7	9.7	10.0	8.0	8.0	6.1	4.2	2.8	1.3	
	3.97	2.71	4.69	4.46	5.24	5.24	6.13	7.40	8.30	9.99	
284	2.9	5.9	4.5	4.6	6.5	6.5	6.3	6.3	4.6	6.3	8.2
	8.02	4.79	6.87	6.60	5.62	5.62	5.87	5.87	6.60	5.87	5.00

Group 3.5

	Markers	**Distance**
302	bnl1.67	8.9 cM
146	*Hb2*/Kin-R(2)	8.6 cM
310	bnl7.26	0.0 cM
93	*Hbr*/GLPL-F(2)	2.7 cM
161	*Hb2*/PL1-5-F(5)	----------
		20.2 cM 5 markers log-likelihood= -29.44

	302	**146**	**310**	**93**
146	9.1			
	4.29			
310	14.4	8.8		
	3.34	4.53		
93	15.5	9.1	0.0	
	2.94	4.29	11.71	
161	21.1	13.8	2.7	2.8
	2.09	3.13	8.58	8.30

Stellenbosch University http://scholar.sun.ac.za

Linkage groups on Chromosome 4

Group 4.1

	Markers	Distance
248	adh2	5.0 cM
158	*Hb2*/PL1-5-F(1)	3.5 cM
250	bnl5.46	----------
		8.6 cM 3 markers log-likelihood= -21.23

	248	158
158	4.2	
	7.40	
250	6.3	2.9
	5.87	7.74

Group 4.2

	Markers	Distance
6	*Hbr*/WMA-R(6)	1.3 cM
264	gpc1	2.6 cM
268	umc42A	2.6 cM
274	bnl15.45	0.0 cM
36	*Hbr*/Red(C)-F(4)	15.3 cM
88	*Hbr*/Kin-R(5)	4.4 cM
86	*Hbr*/Kin-R(3)	4.4 cM
17	*Hbr*/WMA-F(6)	1.3 cM
153	*Hb2*/Kin-R(9)	1.5 cM
285	npi267	----------
		33.5 cM 10 markers log-likelihood= -43.79

	6	264	268	274	36	88	86	17	153
264	1.3								
	9.70								
268	2.8	2.6							
	8.30	8.86							
274	3.1	4.6	1.4						
	7.19	6.60	9.13						
36	6.1	4.2	2.7	0.0					
	6.13	7.40	8.58	10.81					
88	21.1	17.1	20.3	13.8	14.4				
	2.09	2.77	2.26	3.13	3.34				
86	17.8	14.4	17.1	13.8	17.1	4.2			
	2.58	3.34	2.77	3.13	2.77	7.40			
17	29.4	23.8	20.3	16.8	20.3	9.7	4.2		
	1.28	1.82	2.26	2.55	2.26	4.69	7.40		

Stellenbosch University http://scholar.sun.ac.za

	6	264	268	274	36	88	86	17	153
153	23.1	19.4	16.1	12.6	16.1	8.5	2.9	1.4	
	1.75	2.22	2.74	3.13	2.74	4.76	7.74	9.13	
285	29.0	22.1	22.1	16.8	16.1	8.5	4.6	2.9	1.5
	1.21	1.92	1.92	2.55	2.74	4.76	6.60	7.74	8.26

Group 4.3

	Markers	Distance
213	*Hb2*/GLPL-R(3)	2.7 cM
212	*Hb2*/GLPL-R(2)	14.4 cM
294	umc19	----------
		17.1 cM 3 markers log-likelihood= -24.18

	213	212
212	2.7	
	8.58	
294	14.4	14.4
	3.34	3.34

Group 4.4

	Markers	Distance
77	*Hbr*/Kin-F(4)	0.0 cM
11	*Hbr*/WMA-R(11)	5.1 cM
307	bnl10.05	----------
		5.1 cM 3 markers log-likelihood= -15.86

	77	11
11	0.0	
	11.11	
307	5.3	5.1
	5.56	5.82

Group 4.5

	Markers	Distance
203	*Hb2*/Red(C)-(6)	5.0 cM
318	umc15A	1.4 cM
150	*Hb2*/Kin-R(6)	1.4 cM
320	csu166A	4.7 cM
173	*Hb2*/RG_1-F(6)	3.0 cM
321	umc52	9.2 cM
174	*Hb2*/RG_1-F(7)	----------
		24.8 cM 7 markers log-likelihood= -37.01

Stellenbosch University http://scholar.sun.ac.za

	203	318	150	320	173	321
318	4.5					
	6.87					
150	3.0	1.4				
	7.47	9.13				
320	4.8	2.9	1.5			
	6.34	8.02	8.55			
173	8.2	8.2	6.7	4.8		
	5.00	5.00	5.37	6.34		
32	8.2	8.0	6.5	6.5	2.9	
	5.00	5.24	5.62	5.62	8.02	
174	24.9	24.9	19.4	13.3	10.0	8.0
	1.66	1.66	2.22	3.33	4.46	5.24

Linkage groups on Chromosome 5

Group 5.1

	Markers	Distance
236	pgm2	9.5 cM
141	*Hb2*/Per-R(4)	4.2 cM
121	*Hb2*/WMA-F(14)	5.8 cM
252	umc166A	0.0 cM
31	*Hbr*/Red(D)-R(8)	0.0 cM
39	*Hbr*/Red(C)-F(7)	0.0 cM
32	*Hbr*/Red(D)-R(1)	3.6 cM
254	umc43	1.8 cM
16	*Hbr*/WMA-F(5)	----------
		24.9 cM 9 markers log-likelihood= -36.68

	236	141	121	252	31	39	2	254
141	9.7							
	4.69							
121	12.3	4.4						
	3.76	7.13						
252	20.3	10.0	6.3					
	2.26	4.46	5.87					
31	23.8	11.9	6.1	0.0				
	1.82	3.97	6.13	11.71				
39	23.8	11.9	6.1	0.0	0.0			
	1.82	3.97	6.13	11.71	12.01			
32	23.8	11.9	6.1	0.0	0.0	0.0		
	1.82	3.97	6.13	11.71	12.01	12.01		

Stellenbosch University http://scholar.sun.ac.za

	236	**141**	**121**	**252**	**31**	**39**	**2**	**254**
254	28.0	14.9	10.4	2.7	2.8	2.8	2.8	
	1.42	3.14	4.23	8.58	8.30	8.30	8.30	
16	23.8	11.9	10.0	1.3	2.7	2.7	2.7	1.3
	1.82	3.97	4.46	9.70	8.58	8.58	8.58	9.70

Group 5.2

	Markers	**Distance**
256	umc1	6.5 cM
200	*Hb2*/Red(C)-F(3)	2.7 cM
167	*Hb2*/PL1-5-R(1)	6.1 cM
175	*Hb2*/RG_1-F(8)	----------
		15.3 cM 4 markers log-likelihood= -26.59

	256	**200**	**167**
200	6.7		
	5.37		
167	4.8	2.9	
	6.34	8.02	
175	13.8	10.4	6.3
	3.13	4.23	5.87

Group 5.3

	Markers	**Distance**
281	bnl7.71	9.2 cM
132	*Hb2*/WMA-R(10)	7.6 cM
66	*Hbr*/Per-R(1)	1.2 cM
290	bnl5.71	1.4 cM
26	*Hbr*/Red(D)-R(2)	----------
		19.4 cM 5 markers log-likelihood= -28.62

	281	**132**	**66**	**290**
132	9.8			
	3.83			
66	7.4	8.5		
	4.63	4.76		
290	7.7	8.8	0.0	
	4.39	4.53	10.81	
26	15.0	12.3	2.9	0.0
	2.72	3.76	8.02	11.11

Stellenbosch University http://scholar.sun.ac.za

Group 5.4

	Markers	Distance
294	umc126A	7.9 cM
298	umc51	4.4 cM
204	*Hb2*/Red(C)-F(7)	----------
		12.3 cM 3 markers log-likelihood= -23.42

	294	298
298	8.0	
	5.24	
204	10.8	4.4
	4.00	7.13

Group 5.5

	Markers	Distance
43	*Hbr*/Red(C)-F(1)	8.9 cM
151	*Hb2*/Kin-R(7)	5.8 cM
310	bnl5.24	----------
		14.7 cM 3 markers log-likelihood= -23.61

	43	151
151	8.5	
	4.76	
310	14.4	5.5
	2.92	5.30

Linkage groups on Chromosome 6

Group 6.1

	Markers	Distance
184	*Hb2*/RG_1-R(9)	5.9 cM
223	umc85	4.1 cM
29	*Hbr*/Red(D)-R(5)	1.3 cM
30	*Hbr*/Red(D)-R(7)	3.0 cM
230	bnl7.28	----------
		25.4 cM 6 markers log-likelihood= -36.18

	184	223	29	30
223	5.9			
	6.38			
29	7.7	4.2		
	5.48	7.40		
30	9.7	5.9	1.3	
	4.69	6.38	9.99	
230	17.1	11.5	5.9	7.7
	2.77	4.19	6.38	5.48

Stellenbosch University http://scholar.sun.ac.za

Group 6.2

	Markers	Distance
206	*Hb2*/GLPL-F(1)	7.8 cM
256	umc21	7.7 cM
156	*Hb2*/Kin-R(1)	5.8 cM
205	*Hb2*/Red(C)-F(8)	3.1 cM
258	bnl3.03	8.9 cM
128	*Hb2*/WMA-R(3)	0.0 cM
176	*Hb2*/RG_1-F(9)	2.7 cM
80	*Hbr*/Kin-F(7)	2.7 cM
266	umc46	6.1 cM
131	*Hb2*/WMA-R(8)	----------
		44.7 cM 10 markers log-likelihood= -53.82

	206	256	156	205	258	128	176	80	266
256	6.7								
	5.37								
156	10.8	7.4							
	4.00	4.63							
205	14.9	6.9	6.7						
	3.14	5.12	5.37						
258	13.3	9.1	5.1	3.0					
	3.33	4.29	5.82	7.47					
128	34.7	30.7	20.3	12.8	6.7				
	0.95	1.07	2.04	3.54	5.37				
176	41.0	36.9	24.3	15.5	8.8	0.0			
	0.68	0.76	1.59	2.94	4.53	11.41			
80	34.7	36.9	20.3	18.6	8.8	2.9	2.9		
	0.95	0.76	2.04	2.40	4.53	8.02	8.02		
266	54.9	32.7	32.7	29.4	12.8	6.1	6.1	2.8	
	0.35	1.01	1.01	1.28	3.54	6.13	6.13	8.30	
131	34.7	36.9	29.0	26.2	13.8	6.1	6.3	2.9	6.1
	0.95	0.76	1.21	1.50	3.13	6.13	5.87	8.02	6.13

Group 6.3

	Markers	Distance
5	*Hbr*/WMA-R(5)	8.0 cM
38	*Hbr*/Red(C)-F(6)	0.0 cM
41	*Hbr*/Red(C)-F(9)	14.5 cM
298	umc28	0.0 cM
207	*Hb2*/GLPL-F(3)	4.1 cM
292	mdh2	----------
		26.7 cM 6 markers log-likelihood= -32.19

Stellenbosch University http://scholar.sun.ac.za

	5	38	41	298	207
38	8.2				
	5.00				
41	8.5	0.0			
	4.76	11.41			
298	23.1	16.1	13.8		
	1.75	2.74	3.13		
207	24.9	14.9	12.8	0.0	
	1.66	3.14	3.54	11.41	
292	29.4	24.9	22.1	4.4	4.2
	1.28	1.66	1.92	7.13	7.40

Linkage groups on Chromosome 7

Group 7.1.1

	Markers	Distance
6	*Hbr*/Red(D)-F(4)	7.7 cM
19	o2	6.1 cM
2	*Hbr*/RG_1-R(4)	6.3 cM
1	*Hbr*/Red(C)-R(2)	8.2 cM
4	*Hb2*/WMA-F(16)	0.0 cM
3	*Hbr*/WMA-R(1)	2.8 cM
8	*Hb2*/Red(C)-R(2)	0.0 cM
9	*Hbr*/PL1-5-F(10)	1.3 cM
10	*Hbr*/Red(C)-F(1)	2.8 cM
11	*Hbr*/Kin-R(4)	0.0 cM
12	*Hb2*/WMA-R(5)	0.0 cM
48	bnl15.40	4.1 cM
39	npi294E	2.9 cM
13	*Hb2*/Kin-R(8)	----------
		42.4 cM 14 markers log-likelihood=-54.20

	6	19	2	1	4	3	8	9	10	11
19	7.7									
	5.48									
2	17.1	5.9								
	2.77	6.38								
1	21.2	11.6	6.9							
	1.86	3.56	5.12							
4	21.1	8.0	4.4	9.5						
	2.09	5.24	7.13	4.06						
3	21.1	8.0	4.4	9.5	0.0					
	2.09	5.24	7.13	4.06	11.41					

Stellenbosch University http://scholar.sun.ac.za

	6	**19**	**2**	**1**	**4**	**3**	**8**	**9**	**10**	**11**
8	31.0	12.8	8.2	12.6	2.9	2.9				
	1.14	3.54	5.00	3.13	7.74	7.74				
9	28.0	11.9	7.7	14.4	2.8	2.8	0.0			
	1.42	3.97	5.48	2.92	8.30	8.30	11.41			
10	32.8	14.4	9.7	17.6	4.4	4.4	1.4	1.3		
	1.08	3.34	4.69	2.36	7.13	7.13	9.42	9.99		
11	34.7	13.8	6.7	10.7	3.1	3.1	3.2	3.0	1.4	
	0.88	3.13	5.37	3.37	7.19	7.19	6.92	7.47	8.84	
12	34.7	14.9	6.1	18.4	4.5	4.5	4.6	4.4	2.8	0.0
	0.95	3.14	6.13	2.17	6.87	6.87	6.60	7.13	8.30	10.51
48	32.7	18.6	6.5	20.3	4.8	4.8	3.1	2.9	1.4	0.0
	1.01	2.40	5.62	1.81	6.34	6.34	7.19	7.74	9.13	9.91
39	28.0	13.9	7.7	14.4	2.8	2.8	0.0	0.0	1.3	3.0
	1.42	3.54	5.48	2.92	8.30	8.30	11.41	12.01	9.99	7.47
13	23.1	10.8	8.5	16.4	3.0	2.9	3.1	2.9	4.6	7.4
	1.75	4.00	4.76	2.32	7.47	7.74	7.19	7.74	6.60	4.63

	12	**48**	**39**
48	0.0		
	10.81		
39	4.4	2.9	
	7.13	8.02	
13	8.8	6.9	2.9
	4.53	5.12	7.74

Group 7.1.2

	Markers	**Distance**
98	*Hbr*/RG_1-R(3)	10.0 cM
99	*Hbr*/RG_1-R(4)	5.9 cM
224	o2	----------
		15.9 cM 3 markers log-likelihood= -24.96

	98	**99**
99	10.0	
	4.46	
224	21.1	5.9
	2.09	6.38

Stellenbosch University http://scholar.sun.ac.za

Group 7.2

	Markers	Distance
260	npi112	1.5 cM
193	*Hb2*/Red(C)-R(4)	1.4 cM
14	*Hbr*/WMA-F(3)	0.0 cM
78	*Hbr*/Kin-F(5)	0.0 cM
76	*Hbr*/Kin-F(3)	14.4 cM
268	ijl	----------
		17.3 cM 6 markers log-likelihood= -24.94

	260	193	14	78	76
193	1.4				
	9.42				
14	2.9	1.3			
	8.02	9.99			
78	2.9	1.3	0.0		
	7.74	9.70	11.71		
76	3.0	1.4	0.0	0.0	
	7.47	9.42	11.41	11.41	
268	19.4	15.5	12.8	13.3	13.8
	2.22	2.94	3.54	3.33	3.13

Group 7.3

	Markers	Distance
264	bnl15.21	0.0 cM
75	*Hbr*/Kin-F(2)	4.1 cM
268	ijl	1.4 cM
149	*Hb2*/Kin-R(5)	----------
		5.6 cM 4 markers log-likelihood= -19.01

	264	75	268
75	0.0		
	10.51		
268	2.9	4.6	
	7.4	6.60	
149	5.1	4.8	1.5
	5.82	6.34	8.55

Stellenbosch University http://scholar.sun.ac.za

Group 7.4

	Markers	Distance
76	bnl8.32	2.6 cM
69	bnl5.21A	2.6 cM
1	*Hbr*/WMA-F(2)	4.1 cM
2	*Hbr*/Red(C)-R(4)	0.0 cM
3	*Hbr*/Kin-F(9)	0.0 cM
4	*Hb2*/WMA-F(1)	4.5 cM
60	bnl6.27	1.6 cM
87	bnl8.39	----------
		15.6 cM 8 markers log-likelihood= -30.45

	76	69	1	2	3	4	60
69	2.6						
	8.86						
1	2.7	2.7					
	8.58	8.58					
2	7.2	5.1	3.2				
	4.88	5.82	6.92				
3	8.0	8.0	4.4	0.0			
	5.24	5.24	7.13	9.91			
4	8.0	8.0	4.4	0.0	0.0		
	5.24	5.24	7.13	9.91	11.41		
60	10.8	10.8	8.8	5.7	4.9	4.9	
	4.00	4.00	4.53	5.04	6.08	6.08	
87	13.1	16.4	13.7	9.6	8.4	8.4	1.7
	2.92	2.32	2.71	3.20	3.91	3.91	7.40

Group 7.5

	Markers	Distance
307	umc151	8.9 cM
313	*umc45*	3.1 cM
140	*Hb2*/Per-R(2)	1.4 cM
318	umc168	----------
		29.2 cM 4 markers log-likelihood= -32.35

	307	313	140
313	8.2		
	5.00		
140	14.4	2.9	
	3.34	7.74	
318	23.8	16.1	17.8
	1.82	2.74	2.58

Stellenbosch University http://scholar.sun.ac.za

Linkage groups on Chromosome 8

Group 8.1

	Markers	Distance
223	npi220A	14.7 cM
64	*Hbr*/PL1-5-R(1)	0.0 cM
21	*Hbr*/Red(D)-F(2)	1.4 cM
46	*Hbr*/Red(C)-R(3)	27.9 cM
228	bnl911	----------
		44.1 cM 5 markers log-likelihood= -32.77

	223	64	21	46
64	16.1			
	2.74			
21	14.9	0.0		
	3.14	11.41		
46	22.3	1.6	1.5	
	1.69	7.97	8.55	
228	41.6	26.9	24.3	32.3
	0.61	1.28	1.59	0.86

Group 8.2

	Markers	Distance
228	bnl911	10.9 cM
24	*Hbr*/Red(D)-F(5)	1.3 cM
179	*Hb2*/RG_1-R(3)	9.5 cM
197	*Hb2*/Red(C)-R(8)	7.6 cM
242	umc120	0.0 cM
235	bnl944	----------
		29.3 cM 6markers log-likelihood=-35.49

	228	24	179	197	242
24	11.2				
	3.78				
179	11.2	1.3			
	3.78	9.99			
197	34.7	11.9	9.7		
	0.88	3.97	4.69		
242	34.7	21.1	17.8	8.0	
	0.88	2.09	2.58	5.24	
235	36.9	22.1	18.6	8.2	0.0
	0.76	1.92	2.40	5.00	11.41

Stellenbosch University http://scholar.sun.ac.za

Group 8.3

	Markers	Distance
50	*Hbr*/PL1-5-F(2)	0.0 cM
48	*Hbr*/Red(C)-R(5)	0.0 cM
166	*Hb2*/PL1-5-F(10)	0.0 cM
171	*Hb2*/RG_1-F(4)	0.0 cM
177	*Hb2*/RG_1-R(1)	0.0 cM
182	*Hb2*/RG_1-R(7)	0.5 cM
242	umc120	0.7 cM
235	bnl944	----------
		1.2 cM 8 markers log-likelihood= -13.54

	50	48	166	171	177	182	242
48	0.0 10.21						
166	0.0 12.01	0.0 10.21					
171	0.0 11.71	0.0 9.91	0.0 11.71				
177	0.0 12.01	0.0 10.21	0.0 12.01	0.0 11.71			
182	0.0 12.01	0.0 10.21	0.0 12.01	0.0 11.71	0.0 12.01		
242	0.0 10.71	0.0 9.91	0.0 11.71	0.0 11.41	0.0 11.71	0.0 11.71	
235	1.4 9.42	1.6 7.69	1.4 9.42	1.4 9.13	1.4 9.42	1.4 9.42	0.0 11.41

Group 8.4

	Markers	Distance
265	bnl2369	3.2 cM
51	*Hbr*/PL1-5-F(3)	----------
		3.2 cM 2 markers log-likelihood= -13.54

Group 8.5

	Markers	Distance
134	*Hb2*/WMA-R(12)	10.0 cM
183	*Hb2*/RG_1-R(8)	2.8 cM
284	umc48	11.7 cM
162	*Hb2*/PL1-5-F(6)	2.7 cM
195	*Hb2*/Red(C)-R(6)	6.1 cM
292	umc117	----------
		33.2 cM 6 markers log-likelihood= -40.24

Stellenbosch University http://scholar.sun.ac.za

	134	183	284	162	195
183	10.0				
	4.46				
284	11.2	2.9			
	3.78	7.74			
162	24.9	11.9	13.3		
	1.66	3.97	3.33		
195	24.9	17.1	19.4	2.7	
	1.66	2.77	2.22	8.58	
292	19.4	22.1	11.2	8.2	6.3
	2.22	1.92	3.78	5.00	5.87

Group 8.6

	Markers	Distance
292	umc117	29.7 cM
56	*Hbr*/PL1-5-F(9)	0.0 cM
104	*Hbr*/RG_1-F(8)	0.0 cM
133	*Hb2*/WMA-R(11)	4.2 cM
296	bnl1024B	0.0 cM
297	npi268	----------
		36.6 cM 6 markers log-likelihood= -30.94

	292	56	104	133	296
56	31.0				
	1.14				
104	31.0	0.0			
	1.14	12.01			
133	32.7	2.8	2.8		
	1.01	8.30	8.30		
296	18.6	6.1	6.10	2.9	
	2.40	6.13	6.13	8.08	
297	17.8	7.7	7.7	4.4	0.0
	2.58	5.48	5.48	7.13	12.01

Stellenbosch University http://scholar.sun.ac.za

Linkage groups on Chromosome 9

Group 9.1

	Markers	Distance
219	bnl9.07A	12.4 cM
221	umc148	8.1 cM
90	Hbr/Kin-R(7)	5.7 cM
221	php10005	----------
		26.3 cM 4 markers log-likelihood= -31.64

	219	221	90
221	10.2		
	3.60		
90	16.1	7.2	
	2.74	4.88	
222	15.5	4.9	5.9
	2.94	6.08	6.38

Group 9.2

	Markers	Distance
155	*Hb2*/Kin-R(1)	11.2 cM
230	umc105	----------
		11.2 cM 2 markers log-likelihood= -17.88

Group 9.3

	Markers	Distance
232	wx1	3.4 cM
237	bnl7.24A	9.6 cM
192	*Hb2*/Red(C)-R(3)	4.4 cM
130	*Hb2*/WMA-R(6)	1.3 cM
194	*Hb2*/Red(C)-R(5)	1.3 cM
106	*Hbr*/RG_1-F(3)	6.9 cM
74	*Hbr*/Kin-F(1)	2.3 cM
252	umc114	7.3 cM
148	*Hb2*/Kin-R(4)	0.0 cM
260	umc95	----------
		36.7 cM 10 markers log-likelihood=-46.78

	232	237	192	130	194	106	74	252	148
237	3.0								
	7.47								
192	13.8	8.0							
	3.13	5.24							
130	6.9	2.9	4.4						
	5.12	8.02	7.13						

Stellenbosch University http://scholar.sun.ac.za

	232	**237**	**192**	**130**	**194**	**106**	**74**	**252**	**148**
194	8.8	4.4	5.9	1.3					
	4.53	7.13	6.38	9.70					
106	8.8	2.8	7.7	2.8	1.3				
	4.53	8.30	5.48	8.30	9.99				
74	17.6	10.8	15.5	8.5	6.3	6.3			
	2.36	4.00	2.94	4.76	5.87	5.87			
252	15.0	8.8	13.8	6.9	4.8	4.8	1.5		
	2.72	4.53	3.13	5.12	6.34	6.34	8.55		
148	19.3	23.1	27.5	16.8	13.33	16.1	9.1	3.3	
	1.99	1.75	1.35	2.55	3.33	2.74	4.29	6.64	
260	24.3	22.1	26.2	16.1	12.8	15.5	8.8	4.9	0.0
	1.59	1.92	1.50	2.74	3.54	2.94	4.53	6.08	10.51

Group 9.4

	Markers	**Distance**
126	*Hb2*/WMA-R(1)	14.1 cM
274	bnl5.09	0.0 cM
275	bnl14.28	8.3 cM
281	bngl128	5.9 cM
280	npi97B	----------
		28.3 cM 5 markers log-likelihood= -32.97

	126	**274**	**275**	**281**
274	13.8			
	3.13			
275	14.4	0.0		
	2.92	11.11		
281	24.9	8.2	8.5	
	1.66	5.00	4.76	
280	26.2	13.3	11.2	5.9
	1.50	3.33	3.78	6.38

Stellenbosch University http://scholar.sun.ac.za

Linkage groups on Chromosome 10

Group 10.1

	Markers	Distance
227	umc18B	1.3 cM
170	*Hb2*/RG$_1$-F(3)	2.6 cM
65	*Hbr*/PL1-5-R(2)	0.0 cM
115	*Hb2*/WMA-F(8)	0.0 cM
196	*Hb2*/Red(C)-R(7)	5.7 cM
241	umc64	5.8 cM
25	Hbr/Red(D)-R(1)	5.8 cM
139	*Hb2*/Per-R(1)	2.7 cM
160	*Hb2*/PL1-5-F(4)	0.0 cM
15	*Hbr*/WMA-F(4)	5.8 cM
246	npi294H	4.1 cM
61	*Hbr*/PL1-5-F(14)	0.0 cM
63	*Hbr*/PL1-5-F(16)	0.0 cM
89	*Hbr*/Kin-R(6)	2.6 cM
19	*Hbr*/WMA-F(8)	5.8 cM
154	*Hb2*/Kin-R(1)	1.3 cM
251	npi232A	4.8 cM
79	*Hbr*/Kin2-F(6)	6.7 cM
253	umc44A	----------
		55.3 cM 19 markers log-likelihood=-71.02

	227	170	65	115	196	241	25	139	160	15
170	1.4 9.42									
65	4.4 7.13	2.8 8.30								
115	4.5 6.87	2.9 8.02	0.0 11.71							
196	4.4 7.13	2.8 8.30	0.0 12.01	0.0 11.71						
241	4.2 7.40	6.1 6.13	5.9 6.38	6.1 6.13	5.9 6.38					
25	12.3 3.76	10.0 4.46	5.9 6.38	4.4 7.13	5.9 6.38	5.9 6.38				
139	12.3 3.76	10.0 4.46	9.7 4.69	8.0 5.24	9.7 4.69	5.9 6.38	5.9 6.38			
160	12.3 3.76	10.0 4.46	9.7 4.69	8.0 5.24	9.7 4.69	5.9 6.38	5.9 6.38	2.7 8.58		
15	12.3 3.76	10.0 4.46	9.7 4.69	8.0 5.24	9.7 4.69	5.9 6.38	5.9 6.38	2.7 8.58	0.0 12.01	

Stellenbosch University http://scholar.sun.ac.za

	227	**170**	**65**	**115**	**196**	**241**	**25**	**139**	**160**	**15**
246	11.9	14.9	14.4	12.3	14.4	9.4	14.4	9.7	5.9	5.9
	3.97	3.14	3.34	3.76	3.34	4.92	3.34	4.69	6.38	6.38
61	10.0	8.0	11.9	10.0	11.9	11.9	11.9	7.7	4.2	4.2
	4.46	5.24	3.97	4.46	3.97	3.97	3.97	5.48	7.4	7.4
63	10.0	8.0	11.9	10.0	11.9	11.9	11.9	7.7	4.2	4.2
	4.46	5.24	3.97	4.46	3.97	3.97	3.97	5.48	7.4	7.4
89	10.0	8.0	11.9	10.0	11.9	11.9	11.9	7.7	4.2	4.2
	4.46	5.24	3.97	4.46	3.97	3.97	3.97	5.48	7.4	7.4
19	14.9	12.3	11.9	10.0	11.9	17.1	11.9	11.9	7.7	7.7
	3.14	3.76	3.97	4.46	3.97	2.77	3.97	3.97	5.48	5.48
154	29.0	29.0	27.5	24.3	27.5	32.7	23.1	23.1	16.1	16.1
	1.21	1.21	1.35	1.59	1.35	1.01	1.75	1.75	2.74	2.74
251	23.1	29.0	19.4	16.8	19.4	18.6	16.1	16.1	10.8	10.8
	1.75	1.21	2.22	2.55	2.22	2.40	2.74	2.74	4.00	4.00
79	36.7	36.7	24.9	22.1	24.9	34.7	24.9	24.9	17.8	17.8
	0.83	0.83	1.66	1.92	1.66	0.95	1.66	1.66	2.58	2.58
253	28.0	34.7	23.8	21.1	23.8	26.7	23.8	23.8	17.1	17.1
	1.42	0.95	1.82	2.09	1.82	1.57	1.82	1.82	2.77	2.77

	246	**61**	**63**	**89**	**19**	**154**	**251**	**79**
61	4.2							
	7.40							
63	4.2	0.00						
	7.40	12.01						
89	4.2	0.00	0.00					
	7.40	12.01	12.01					
19	7.7	2.7	2.7	2.7				
	5.48	8.58	8.58	8.58				
154	19.4	10.8	10.8	10.8	6.5			
	2.22	4.00	4.00	4.00	5.62			
251	10.4	10.8	10.84	10.8	8.5	1.5		
	4.23	4.00	4.00	4.00	4.76	8.55		
79	12.3	14.9	14.9	14.9	10.0	6.7	4.8	
	3.76	3.14	3.14	3.14	4.46	5.37	6.34	
253	9.4	14.4	14.4	14.4	14.4	6.5	2.9	6.1
	4.92	3.34	3.34	3.34	3.3	5.62	8.02	6.13

Stellenbosch University http://scholar.sun.ac.za

Group 10.2

	Markers	Distance
270	gln1	13.8 cM
172	*Hbr*/RG_1-R(5)	----------
		13.8 cM 2 markers log-likelihood= -18.54