AFLP and PCR markers for the *Ht1*, *Ht2*, *Ht3* and *Htn1* resistance genes in maize.

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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 part submitted it at any university for a degree.

SUMMARY

Maize is undoubtedly South Africa's most important field crop. The identification of markers and genes for traits of interest is important to sustain the improvement of maize cultivation. Northern corn leaf blight (NCLB) is a disease that occurs world-wide and can dramatically reduce yield. A number of single dominant resistance genes have been identified for NCLB and some have been mapped. Currently there are no simple PCR markers for any of these resistance genes, making marker-assisted selection (MAS) difficult.

The aim of this study was to develop PCR markers for the NCLB resistance genes *Ht1*, *Ht2*, *Ht3* and *Htn1* in maize. To accomplish this, the AFLP (amplified fragment length polymorphism) technique was first optimised. The results indicated that the *MluI*/*MseI* restriction enzyme combination produces a higher percentage of polymorphisms when compared to the *PstI*/*MseI* enzyme combination. It was also shown that the enzyme combination plays an important role in the percentage of polymorphic fragments observed, whereas the number of restriction enzymes used in AFLP analysis only significantly affects the total number of fragments scored.

Populations segregating for the different resistance genes were not available for this study. Nearly-isogenic lines (NILs) were used in combination with AFLP technology to identify markers that map close to the genes. AFLP markers common in at least two resistant or susceptible lines were cloned and converted to PCR markers. Two commercially available recombinant inbred line (RIL) populations were then used to map the identified markers.

For *Htn1* fifteen polymorphic fragments were present in both resistant lines. They were selected for sequence specific marker conversion. Seven of the fifteen sequence characterized amplified region (SCAR) markers were polymorphic on the NIL pairs and five mapped to one region of maize chromosome 8.05/06. Twenty-one AFLP markers were identified for *Ht1* and four SCAR markers were polymorphic In the *Ht1* NILs. Three of these were mapped to chromosome 2.07. Three AFLP markers were identified for *Ht2* of which two were converted to SCAR markers. Both SCAR markers were polymorphic on the *Ht2* NILs and mapped to chromosome

8.05/06. On the *Ht3* NILs, four AFLP markers were identified and two converted SCAR markers and one microsatellite marker (bnlg1666) were polymorphic. One of the SCAR markers and the microsatellite marker were mapped to chromosome 7.04 using a RIL population. This reports the first tentative mapping position for the *Ht3* locus.

The next step was to determine if a set of marker alleles could be used in a number of *Htn1* resistance lines to identify a common donor region selected by the breeders. Nine markers consisting of five SCAR markers, three converted RFLP markers and one microsatellite marker were used on 16 *Htn1* resistant lines. The marker allele of us3 was in 12 of the 16 lines in coupling with *Htn1* resistance. Second was the marker us5 in 11 of the 16 lines. Using this data 14 of the 16 lines shared a common introgressed region between the markers us3 and us5. A further common introgressed region between 11 of the inbred lines was found between the markers us14 and asg17.

The last aim of this study was to propose a new marker technique that might be more successful than the AFLP technique in the identification of markers closely linked to genes. A new marker approach was identified where a MITE (Hbr) primer was used as an anchor primer in combination with resistance gene analog primers. This was found to be a highly polymorphic marker technique that could be used to identify markers and possibly candidate genes. It is a robust technique, which is affordable since amplifications occur from undigested genomic DNA and the primers mainly amplify fragments from genic regions.

OPSOMMING

Mielies (*Zea mays*) is ongetwyfeld Suid Afrika se belangrikste lanbou gewas. Vir volgehoue opbrengs verbetering is die identifisering van merkers en gene vir belangrike eienskappe noodsaaklik. Noordelike blaarskroei (NBS) kan opbrengs wesenlik kan beïnvloed. Tans is daar reeds 'n aantal enkel weerstandsgene geïdentifiseer, maar geen PKR-merkers is beskikbaar vir merker gebaseerde seleksie nie.

Die doelwit van hierdie studie was om PKR-merkers te ontwikkel vir vier enkel weerstands gene (*Ht1, Ht2, Ht3* en *Htn1*) teen NBS in mielies. Om die doelstelling te bereik is die AFLP-tegniek eers geoptimiseer. Op grond van waargenome aantal polimorfismes, was *MluI/MseI* 'n beter restriksie ensiem kombinasie as *PstI/MseI*. In die studie is ook bewys dat die aantal (meer as twee) restriksie ensieme wat gebruik word slegs die aantal fragmente, en nie die persentasie polimorfismes, wesenlik beïnvloed nie.

Geen segregerende populasie was vir die verskillende gene beskikbaar nie. Naby isogeniese lyne (NILe) is daarom in kombinasie met die AFLP-tegniek gebruik om merkers te identifiseer wat naby die gene karteer. Alleenlik polimorfiese merkers wat in ten minste twee weerstand biedende of vatbare lyne voorgekom het, is gekloneer en omgeskakel na PKR-merkers. Daarna is twee kommersiële rekombinante ingeteelde lyn populasies gebruik om die gene te karteer.

Vyftien fragmente is gevind wat gekoppel was met die *Htn1* weerstand. Sewe van hierdie merkers is omgeskakel in polimorfiese SCAR-merkers waarvan vyf gekarteer is in een gebied op chromosoom 8.05/06. Een-en-twintig AFLP-merkers is geïndentifiseer vir *Ht1* en vier is omgeskakel na polimorfiese SCAR-merkers. Drie hiervan is gekarteer op chromosoom 2.07. Drie AFLP-merkers is geïndetifiseer vir *Ht2* waarvan 2 omgeskakel is na polimorfiese SCAR-merkers. Altwee hierdie merkers is gekarteer op chromosoom 8.05/06. Op die *Ht3* lyne is vier AFLP-merkers geïdentifiseer waarvan twee omgeskakel is na polimorfiese SCAR-merkers. Een mikrosatelliet merker (bnlg1666) is ook gevind wat die selfde polimorfiese patroon wys op die *Ht3* lyne. Die mikrosateliet en een van die SCAR-merkers het gekarteer

op chromosomale posisie 7.04. Hierdie is die eerste tentatiewe posisie vir die *Ht3* lokus.

Die volgende stap was om te bepaal of 'n stel polimorfiese merker-allele gebruik kan word om die donor DNA-segment te identifiseer wat die planttelers geselekteer het. Nege PKR-merkers wat bestaan het uit vyf SCAR-merkers, 3 omgeskakelde RFLP merkers en een mikrosateliet is gebruik op 16 *Hnt1* weerstandslyne. Us3 was die merker alleel wat in die meeste gevalle gekoppel was met die *Htn1* weerstandslyne (12/16). Tweede was die merker us5 (in 11 van die 16 lyne). Uit die data blyk dit dat 14 van die 16 lyne 'n donor segment het wat beide merkers us3 en us5 bevat. Merkers us14 en asg17 het in 11 van die 16 bestande lyne saam voorgekom.

Die laaste doelstelling van hierdie studie was om 'n nuwe tegniek te ontwikkel wat dalk meer suksesvol as AFLPs kan wees om merkers te identifiseer naby aan gene. 'n Nuwe tegniek word voorgestel waar 'n MITE (*Hbr*) inleier gebruik kan word in kombinasie met weerstandgeen-analoog inleiers. Dit is gevind dat hierdie kombinasie van inleiers 'n hoogs polimorfiese bandpatroon gee en dat die merkers ook dalk kandidaat-gene kan wees. Die tegniek is maklik uitvoerbaar, relatief goedkoop en maak gebruik van onverteerde genomiese DNA. Die fragmente wat geamplifiseer word is hoofsaaklik vanaf geenryke areas.

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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

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

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LITERATURE REVIEW

1 Introduction

Cereal grains, such as wheat, rice, maize and millet, were amongst the first plants to be domesticated nearly 10 000 years ago (Vasil 1994). They have helped to sustain the development of human civilization by providing valuable sources of food and nutrition. Cereals form the centrepiece of world agriculture by providing more than half of all food consumed by man. The increase in cereal production has prevented widespread hunger and famine by keeping pace with the ever-growing human population. The world population has risen from 2.8 to 5.8 billion people in the past 40 years, and is expected to double in the next 40-50 years, with the greatest increase occurring in the crowded areas of Asia and Africa (Kasha 1999). In the same time span the production of the dominant cereals, such as rice, wheat and maize, has increased, on average, 2.5 times leading to fewer hungry people than in 1960 (Anonymous 1996). Maize is South Africa's most important field crop. On average, 40 per cent of South Africa's total land under cultivation is annually planted with maize, i.e. approximately one rugby field of maize for every family consisting of 5 people. The question now is how to achieve at least the doubling of crop production during the next 40 years? Surely, this can only be achieved by understanding and manipulating the plant genomes that produce our food.

2 Resistance to *Setosphaeria turcica*

Northern corn leaf blight (NCLB) is caused by the ascomycete *Setosphaeria turcica* (Luttrell) Leonard & Suggs, in its anamorphic stage *Exserohilum turcicum* (Pass.) Leonard & Suggs [syn. *Helminthosporium turcicum* (Pass.)]. The disease occurs sporadically in most temperate and humid areas where maize is grown and results in grain yield losses of 30% or more when susceptible hybrids are grown (Lim et al. 1974, Raymundo and Hooker 1981). Symptoms of NCLB are wilting local lesions that turn necrotic at a later stage thereby destroying large portions of the leaf area in susceptible plants (Dingerdissen et al. 1996).

Resistance to NCLB in maize is generally classified as one of two types: monogenic resistance which is race specific and polygenic resistance which is not race specific. Monogenic resistance to NCLB is controlled by five dominant genes (*Ht1*, *Ht2*, *Ht3*, *Htm* and *Htn1*) and one recessive gene (*Ht4*) (Ullstrup 1970, Hooker 1977, Hooker 1981, Robbins and Warren 1993, Gevers 1975, Carson 1995). The *Ht1*, *Ht2*, *Ht3* and *Ht4* genes form a chlorotic lesion type of resistance whereas the *Htm* and *Htn1* genes cause a delay in appearance of necrosis and a reduction in sporulation (Gevers 1975, Raymundo and Hooker 1981, Robbins and Warren 1993).

In 1963 Hooker reported the first identification of the single dominant gene *Ht1* in the inbred line GE440 and in the popcorn variety Ladyfinger. According to Hooker (1963) the genes in the two corn types are identical, allelic or very closely linked. *Ht1* was the first monogenic trait locus mapped. It was mapped to the central region of the long arm of chromosome 2 (Patterson et al. 1965). More recently restriction fragment length polymorphisms (RFLPs) have been used to verify the position of the *Ht1* gene(s) on the long arm of chromosome 2 and to map the genes more precisely (Bentollila et al. 1991, Freymark et al. 1993).

Hooker (1977) found that the inbred line NN14 from Australia has two dominant resistance genes for chlorotic-lesion resistance. From NN14 the two inbred lines, NN14A and NN14B were developed, each containing one of the resistance genes. The inbred line NN14A contains a single dominant gene that mapped to the locus of *Ht1* and NN14B contains an independent locus that Hooker (1977) designated as *Ht2*. The *Ht2* resistance gene is characterised by elongated chlorotic lesions which occasionally extend over the entire leaf surface in seedlings, while no lesions develop on the upper foliage of mature plants after silking stage. Zaitlin et al. (1992) mapped the *Ht2* resistance gene to the long of chromosome 8. This was confirmed by Freymark et al. (1993). Simcox and Bennetzen (1993) found tight linkage between *Ht2* and *Htn1* and estimated that *Ht2* maps approximately 10 cM proximal to *Htn1*.

Ht3 is a dominant resistance gene for *Setosphaeria turcica* that was most probably derived from *Tripsacum floridanum* (Hooker 1981). Resistance is expressed in

greater magnitude in the homozygous than in the heterozygous individuals. Analysis of F_2 data from Simcox and Benetzen (1993) and preliminary data of Hooker (1981) indicate that *Ht3* is not linked to *Ht1*, *Ht2* or *Htn1*. According to Simcox and Bennetzen (1993) *Ht2* and *Ht3* have a very similar chlorotic-necrotic lesion phenotype and this can unfortunately lead to source contamination.

The *Htn1* gene was identified in the inbred line 11Mex44, which was developed from the Mexican variety Pepitilla (Gevers 1975). This line was subsequently used as a donor parent to introgress *Htn1* resistance into a number of highly susceptible North American inbred lines by recurrent backcrossing and selection (Gevers 1975). In 1993, Simcox and Bennetzen mapped the *Htn1* locus on chromosome 8 (0.8 cM distal to the RFLP marker umc117).

Due to low levels of naturally occurring NCLB disease pressure in South Africa, segregation studies are problematic. Furthermore, the existing number of NCLB races and diseases such as grey leaf spot make disease scoring even more difficult (Adipala et al. 1993, Gevers et al. 1994). NCLB occurs sporadic in African countries like; Cameroen, Uganda, Tanzania, Zimbabwe, South Africa and Kenya, especially on high altitudes areas where maize is grown under cool wet conditions (M. Barrow, personal communication, PANNAR Pty). Therefore marker-assisted selection is a necessity if resistance is an objective in inbred development. To date no PCR markers are available for any of the *Ht* resistance genes, thus making marker assisted selection (MAS) less feasible.

3 DNA marker technologies and applications

The aim of this study was to tag the *Ht* genes with PCR markers. A DNA marker can be classified as a segment of DNA with an identifiable physical location on a chromosome whose inheritance can be followed. The marker can be a gene, or it can be some section of DNA with no known function. Because DNA segments that lie near each other on a chromosome tend to be inherited together, markers are often used as indirect ways of tracking the inheritance pattern of genes that have not yet been identified, but whose approximate locations are known. Currently two

methods can be used to identify DNA markers for a gene of interest: 1) available markers from a high-density molecular linkage map can be tested on the parents for polymorphisms and used in a genome screening or 2) markers can be developed by using a high-volume marker technique together with special plant material to target the specific chromosome region(s) of interest. Thousands of DNA markers have been mapped for the maize genome. However if the approximate position of a gene is not known it could be costly and time consuming to screen existing markers for polymorphisms and linkage to the gene of interest.

The development of DNA marker technologies is an ongoing process. In this literature review the most commonly used markers are highlighted and their applications in the saturation of the maize genome is discussed.

3.1 Marker techniques based on DNA hybridisation.

3.1.1 Restriction fragment length polymorphisms (RFLPs)

RFLPs are manifested as variations in the size of DNA fragments complementary to a given probe in a Southern filter hybridisation. These polymorphisms are the consequence of heritable changes in the DNA: point mutations create or abolish restriction endonuclease sites while DNA rearrangements, insertions and deletions, alter the fragment size (Helentjaris 1987). The methodology entails the digestion of purified genomic DNA with restriction endonucleases that recognize and cleave at specific four to seven base pair sequences in DNA. The digested DNA is electrophoresed in agarose gels to separate the DNA fragments according to size. The DNA fragments are usually transferred to nitrocellulose sheets using the Southern blotting procedure and are then hybridised with a radioactively labelled DNA probe. The non-specific hybridisation products are washed off and the filter is exposed to X-ray film. Only fragments complementary to the labelled probe will be visible on the film. In order to detect defined loci within the genome, one needs to construct a set of clones, containing single copy genomic sequences (Helentjaris 1987). One such source is a cDNA library. Introns in genomic coding regions can, however, cause weak signals. Alternatively, low copy number genomic sequences can be used as probes.

The preparations for a maize genetic linkage map based on RFLPs were started as early as 1985. By 1987 a linkage group for each of the 10 chromosomes had been identified (Helentjaris et al. 1985, Helentjaris et al. 1986, Evola et al. 1986, Helentjaris 1987). While preparing the linkage map it was found that some of the low copy number probes detected more than one locus and that they mapped to different chromosomes (Helentjaris et al. 1988). This was the first evidence that common DNA sequences exist at different chromosomal locations and that maize is not a diploid but a segmental allotetraploid.

A well-characterized, dispersed core RFLP linkage map of maize was established in 1993 and in 1996 a composite map was available (Gardiner et al. 1993, Causse et al. 1996). The linkage maps of maize currently contain more than a thousand RFLP markers. These markers have been used to map a large number of single genes and quantitative trait loci (QTL), e.g. adaptation differences (Jiang et al. 1999), aluminium tolerance (Sibov et al. 1999), development functions (Khavkin and Coe 1997) and resistance QTL to downy mildew (Agrama et al. 1999), maize streak virus (Pernet et al. 1999) and maize mosaic virus (Ming et al. 1997) to name a few.

Another major application of DNA markers is for the fingerprinting of elite inbred lines. The fingerprint data can be used for purity tests, patent rights and to determine the genetic distance between lines. Maize breeders need to identify inbred lines that in combination produce heterosis and to combine this hybrid performance with specific traits of importance such as disease resistance. A number of studies have shown that RFLPs can reveal the genetic diversity between inbred lines to group them into heterotic groups and to predict hybrid performance (Livini et al. 1992; Ajmone Marsan et al. 1998; Dubreuil and Charcosset 1999). The limitation of RFLPs is the time consuming nature of the technique that limits the number of probes and individuals that can be screened. The technique is not cost effective for marker-assisted selection (MAS).

In summary, the RFLP technology is a very powerful molecular marker technique, because it provides a large amount of information for linkage maps. It forms the backbone of maize DNA markers. However, (1) large quantities of DNA are

required, (2) analysis of large populations are costly and (3) the technique is difficult to automate for applications in MAS programs.

3.1.2 DNA micro array technology

The DNA micro array technology developed at Stanford enables simultaneous monitoring of the expression patterns of many genes (reviewed in Schena 1996). Using an automated workstation thousands of DNA samples are immobilised at high density on a solid support, such as glass slides. These slides are then hybridised with fluorescent labelled probes developed from mRNA, which was obtained from a particular tissue or organ of interest. Direct comparison of gene expression in two tissues or inbreds can be accomplished by simultaneously hybridising with mRNA probes labelled with different flourochromes.

The ZmDB (http://www.zmdb.iastate.edu/) is a maize genome database and its goals are to discover maize genes, to sequence them and to analyse their phenotypic function. The ZmDB is in the process of arraying a full suite of maize genes that will be applicable in basic research and in the commercial arena.

In summary, DNA micro array technology is the most exiting technique currently available. It will change the current way of focussing on single or a few genes to seeing the bigger picture (thousands of genes).

3.2 PCR-based marker techniques and applications.

The introduction of PCR revolutionized the methodology of molecular biology (Mullis and Faloona 1987, Saiki et al. 1988). It is a powerful and sensitive technique with applications in almost every biological field.

The basic PCR methodology utilises two synthetic oligonucleotide primers (normally 10-30 bp long), which are complementary to the 5'-ends of a double-stranded DNA fragment with a length of between 80-10 000 bp. The fragments are amplified with a thermo stable DNA polymerase using different temperature cycles for denaturing, annealing and amplifying. Fragments are separated on an agarose gel and length

differences between samples can be detected. These differences can be due to insertion-deletion mutations between the primers or at the 5'-ends were the primers should anneal. If the annealing site of a primer does not have enough complementary binding sites, amplification will not occur.

In this literature review PCR based DNA marker techniques are divided into two groups: PCR based marker techniques that target multiple loci or that target a single locus.

3.2.1 Multiple loci PCR based marker techniques

High volume marker techniques became available in the early 1990's when researchers started to use arbitrary sequence primers to generate characteristic fingerprints from DNA templates. Multiple arbitrary amplicon profiling (MAAP) is the collective name given by Caetano-Anollés (1994) to techniques that use one or more oligonucleotide primer (5 bp or more) of arbitrary sequences to initiate DNA amplification and to generate fingerprints. The following methodologies can be grouped together under MAAP:

(a) AP-PCR (arbitrarily primed PCR: Welsh and McClelland 1990).
(b) RAPD (random amplified polymorphic DNA: Williams et al. 1990).
(c) DAF (DNA amplification fingerprinting: Caetano-Anolles et al. 1991).
(d) *Copia*-SSR (Copia-specific primer with anchored simple-sequence repeat primers (Provan et al. 1999).
(e) IRAP and REMAP (Retrotransposon-based DNA fingerprinting: Kalender et al. 1999).

In 1993 the AFLP technique was developed (Zabeau and Vos 1993). This is a very powerful technique and is based on the selective amplification of genomic restriction fragments. In 1996 researchers started to focus on gene sequences that were isolated from genes conferring gene-for-gene disease resistance in plants. These genes can be categorized into four classes based on their predicted protein products. Recently PCR primers, based on short stretches of amino acids conserved in these classes of resistance proteins, were used to amplify resistance

gene-like sequences called RGAs (resistance gene analogs).

RAPDs, AFLPs, Modified AFLPs and RGAs will be discussed in more detail.

3.2.1.1 Random amplified polymorphic DNA (RAPD)

The RAPD amplification reaction (Williams et al. 1990) is performed with genomic DNA as template using an arbitrary oligonucleotide primer. This results in the amplification of several discrete DNA products. Each amplification product is derived from a region of the genome that contains two short DNA segments which have homology to the primer. These segments must be on opposite DNA strands and must be sufficiently close to each other to allow DNA amplification to occur. The usage of short primers and low annealing temperatures ensures that several randomly distributed sites in the genome are amplified (reviewed by Rafalski and Tingey 1993).

The RAPD technology has provided researchers with a quick and efficient method to screen polymorphisms based on sequence differences at a large number of loci. Its technical simplicity is its main advantage over RFLPs. A disadvantage of RAPDs is that they are dominant as opposed to RFLPs which are co-dominant markers. RAPDs have been extensively used in different plant species for DNA fingerprinting, to generate linkage maps and for the identification of markers linked to traits of interest (Lanza et al. 1997, Lin and Ritland 1996, Michelmore et al. 1991, Stevens et al. 1995). This has indicated that RAPDs can efficiently generate randomly dispersed markers as well as markers linked to genes.

Despite obvious advantages some problems have also been encountered with the RAPD technique. Riedy et al. (1992) observed a fragment in the F_1 hybrid that could not be detected in either of its parental inbreds. Others have reported that 10-40% of RAPD fragments show non-Mendelian inheritance (Reiter et al. 1992 and Heun and Helentjaris 1993). The RAPD technique has been used successfully together with bulks to target particular areas of the genome. Nevertheless the use of RAPDs to define 'fingerprints' of individual genotypes should be viewed with some caution as it seems that the presence of some fragments can interfere with the amplification of

others (Heun and Helentjaris 1993).

For a genetic marker to be useful, its detection must be predictable. The RAPD technique should therefore be used with caution when applied to gene tagging and fingerprinting.

3.2.1.2 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphisms (AFLP) analysis was developed by Zabeau and Vos (1993) and selectively amplifies digested DNA fragments. Genomic DNA is digested with two restriction endonucleases and site-specific adapters are ligated to the DNA fragments. Primers complementary to the adapters and the restriction sites, are designed with selective nucleotides at the 3' ends of the primers. Only DNA fragments with nucleotides that match the selective nucleotides of the primer are amplified during PCR. The resolution of the DNA fragments on standard sequencing gels allows for the detection of amplified fragment length polymorphisms (AFLPs). As with the RAPD analysis, the AFLP assay does not require prior sequence knowledge. The AFLP analysis however detects a 10-fold greater number of loci (20-100) than the RAPD analysis. The AFLP analysis has the capacity to rapidly screen thousands of independent genetic loci.

The major advantages of AFLPs are (1) a high multiplex ratio, (2) a limited set of generic primers used, and (3) no sequence information is needed (Vuylsteke et al. 1999). The AFLP technique has been applied successfully to identify markers linked to disease resistance loci (Meksem et al. 1995; Thomas et al. 1995; Cervera et al. 1996; Sharma et al. 1996; Brigneti et al. 1997; Simons et al. 1997; Vos et al. 1998). It has also been used in maize germplasm analysis (Smith et al. 1993; Ajmone Marsan et al. 1998; Pejic et al. 1998) and the development of linkage maps (Castiglioni et al. 1999; Vuylsteke et al. 1999).

A major concern about the AFLP analysis is the clustering of fragments at the centromeres with the use of specific restriction enzymes. The clustering of *Eco*RI/*Mse*I markers in specific chromosome regions (centromeres) has been shown in linkage maps of maize (Castiglioni et al. 1999; Vuysteke et al. 1999), potato (van

Eck et al. 1995), barley (Becker at al. 1995) and *Arabidopsis* (Alonso-Blanco et al. 1998). An explanation for this phenomenon was given by Vuylsteke et al. (1999): It was shown that the pericentromeric heterochromatin fluoresces brightly in *Arabidopsis* when stained with the fluorochrome DAPI (Ross et al. 1996), which is known to show preference for AT-rich DNA. Vuylsteke et al. (1999) stated that this could explain the enrichment of *EcoRI/MseI* AFLP markers in the *Arabidopsis* centromeres and possibly in other plant genome centromeres, since the restriction enzymes *Eco*RI and *Mse*I have AT-rich target sequences (*Mse*I recognises 5'-TTAA-3', while *Eco*RI recognises 5'-GAATTC-3').

In plant genomes, cytosine (C) methylation of CpG and CpNpG nucleotides varies in frequency along a chromosome and acts to regulate gene expression (reviewed by Kass et al. 1997). In RFLP analysis it has been shown that methylation sensitive restriction enzymes such as *PstI* can be used to clone single-copy sequences in order to avoid repetitive DNA sequences (Burr et al. 1988). A similar approach has been used in AFLPs where the methylation-sensitive restriction enzyme *PstI* was used to increase the frequency of markers in genetically active euchromatic regions. Castiglioni et al. (1999), Vuylsteke et al. (1999) and Young et al. (1999) detected a more random distribution of *PstI* AFLP markers on linkage maps with a preferential localisation of markers in the hypomethylated telomeric regions of the chromosomes.

Theoretically thousands of rare and frequent cutter combinations can be used in AFLP analysis. The most commonly used is *Eco*RI/*Mse*I and *Pst*I/*Mse*I. Ridout and Donini (1999) observed that *Pst*I/*Mse*I primer combinations produce more polymorphisms than *Eco*RI/*Mse*I combinations in barley. Keygene has developed software which measures AFLP bands in a pixel image on a Fuji BAS 2000 thus determining the intensities of a band and enabling the distinction between homozygosity or heterozygosity. Simons et al. (1997) used three restriction enzyme combinations in barley to improve the AFLP analysis. They used *Pst*I or *EcoRI* in combination with *Taq*I and *Mse*I. Standard AFLP adapters, for each restriction site, were ligated to the restriction fragments but AFLP primers for *Taq*I were not added in the subsequent AFLP amplification reactions. As the fragments with a *Taq*I restriction site were not amplified, the number of fragments amplified with the

*Pst*I/*Mse*I and *Eco*RI/*Mse*I primer combinations were reduced.

In summary, AFLP analysis has the capacity to rapidly screen thousands of independent genetic loci and markers targeting methylation sensitive and insensitive areas can be identified thereby detecting gene islands in the genome.

3.2.1.3 Modified AFLP technology

A characteristic of AFLPs is the use of two restriction enzymes (a rare and a frequent cutter) for the digestion of the genomic DNA. Modified AFLP approaches normally only use one restriction enzyme, either a frequent or rare cutter, together with a primer complementary to unique sequence. Waugh et al. (1997) used the long terminal repeat (LTR) region of the Bare-1 Ty1-Copia retrotransposon in barley and Van den Broeck et al. (1998) and Casa et al. (2000) used transposon elements as anchors in a modified AFLP procedure named transposon display (TD).

In summary, instead of two restriction enzymes used for AFLPs, only one restriction enzyme is used in combination with a unique primer. The more loci the unique primer recognise the more fragments will be amplified.

3.2.1.4 Resistance gene analogs (RGAs)

Almost all resistance genes that have been isolated from plants can be placed in three classes based on the regions they share. These classes are involved in nucleotide binding (nucleotide binding site, NBS), protein-protein interaction (leucine rich repeat, LRR), or intracellular signalling (kinase domain). The NBS-LRR group, which contains both a leucine rich repeat and a nucleotide binding site, includes the *Arabidopsis RPS2* and *RPM1* and *RPP5* genes (Bent et al. 1994; Mindrinos et al. 1994; Grant et al. 1995; Parker et al. 1997), the tobacco *N* gene (Whitman et al. 1994), the flax *L6* and *M* genes (Lawrence et al. 1995; Anderson et al. 1997) and the tomato *Prf* gene (Salmeron et al. 1996). The LRR group which contains only a LRR domain but no NBS includes the tomato *Cf* genes (Jones et al. 1994; Dixon et al. 1996). The kinase group either contains a serine-theonine kinase (STK) domain, as

in the tomato *Pto* gene (Martin et al. 1993) or it is associated with a receptor domain resulting in a receptor-like kinase structure, as in the rice Xa21 gene (Song et al. 1995).

In recent studies, PCR primers based on short stretches of amino acids conserved among NBS-LRR resistance proteins, have been used to amplify resistance gene-like sequences in maize (Collins et al. 1998), potato (Leister et al. 1996), pepper (Pflieger et al. 1999), rice, barley and wheat (Chen et al. 1998). RGA fingerprinting only distinguishes 1-5 fragments with agarose-gel electrophoresis (Leister et al. 1996; Plieger et al. 1999) but numerous PCR-amplified products can be detected with high-resolution electrophoresis (Chen et al. (1998).

RGA fingerprinting has advantages over existing molecular markers in the evaluation of the diversity of host resistance. The RGA markers serve both as candidate genes and as informative markers. RGA fingerprints can be used in the identification of candidate resistance genes and to determine genetic relationships between germplasms. This data can then be used to select lines in resistance breeding programs (Chen et al. 1998). Disadvantages of RGA fingerprinting are the low polymorphic index and that it only focuses on resistance genes. Another problem is that not all resistance genes fall into one of the three groups mentioned earlier, e.g. *Hm1* and barley *Mlo* genes (Johal and Briggs 1992; Büschges et al. 1997)

RGA fingerprinting is one of a few techniques that actually focus on candidate genes as markers. The technique is limited due to the lack of conserved sequence of other groups of genes, other than the resistance genes.

3.2.2 Single locus PCR based markers

In contrast to PCR based markers that have arbitrary primers and that amplify fragments from more than one locus, single locus PCR based markers have sequence specific, unique primers. These unique primers can be derived from any low copy number fragment: (1) it can be single copy DNA from a genomic library, cDNA library or a RFLP probe that has been mapped. These sequence specific PCR-based markers have been called sequence tagged site (STSs) markers (Olson

et al. 1989, Adams et al. 1991, Erpelding et al. 1996), (2) if the unique primers flank a fragment that contains a simple sequence repeat (SSR) they are called microsatellite markers (Davies 1993, Senior and Heun 1993), 3) sequence specific primers can also be developed from a single RAPD or AFLP fragment that was cloned and sequenced. These sequence specific markers are designated as sequence characterized amplified region (SCAR) markers by some researchers (Paran and Michelmore 1993; Chalhoub et al. 1997) and as STS markers by others (Shan et al. 1999).

Almost all fragments of a multiple loci PCR marker technique can be converted to a single locus PCR based marker if the fragment is of low copy number and causes an ASLP (amplified sequence length polymorphism) between for example maize inbred lines. If the converted sequence specific PCR markers do not produce any polymorphisms between lines, the fragments can be digested with restriction enzymes. If a polymorphism is observed with a restriction enzyme, the marker is normally called a cleaved amplified polymorphic sequence (CAPS) marker (Konieczny and Ausubel 1993). In some cases the sequence specific primers derived from a fragment can be very specific and only amplify a DNA fragment in a specific DNA background. The marker is then called an allele specific associated primer (ASAP) marker (Gu et al. 1995). An ASAP marker can be used in large-scale, cost effective screening in a marker-assisted selection program since agarose gel electrophoresis is unnecessary.

Single nucleotide polymorphisms (SNPs) are another class of molecular markers and are the most common form of DNA polymorphisms. In a crop like maize, SNPs can be used in germplasm finger printing, marker assisted backcross conversion and marker assisted breeding. SNPs are highly amenable to automation and can be used to create a high-density genetic map. In general, primers are made from an EST database and are used to amplify fragments from different genotypes. The amplified fragments are sequenced to determine whether any SNPs are present. In a maize study 700 genes/ESTs of interest were analysed and variants were detected in 311 loci: insertions/deletions accounted for 27% of the total number of variations observed and the rest were comprised of transitions and transversions (SNPs) (Bhattramakki et al. 2000). These variations can be used to develop ASAP markers

for a specific genotype.

The advantages of converting an AFLP or RAPD fragment to a simple PCR based fragment are evident. It is less expensive and easy to score large population. However, few AFLP markers are successfully converted to polymorphic SCAR/STS markers. Normally only 25%-50% of the fragments converted to PCR based markers show the same polymorphism detected in the original AFLP marker (Shan et al. 1999). The reason for this is probably that AFLP polymorphisms are caused by restriction digestions and that the converted PCR primers are generated from sequences internal to the AFLP restriction sites.

In principle, all sequence specific PCR based marker techniques are the same: a single fragment is amplified from a predetermined locus by using sequence specific primers. Of all DNA marker techniques available, microsatellites or SSRs are the most polymorphic marker technique for maize (Pejic et al. 1998).

3.2.2.1 Microsatellite markers

Microsatellite or simple sequence repeats (SSRs) are short nucleotide sequences, usually from 2 to 6 bases (b) in length that are repeated in tandem arrays. Amplifiable polymorphisms are revealed because of differences in the numbers of tandem repeats that lie between sequences that are otherwise conserved for each locus. From these conserved sequences specific primers are generated for microsatellite markers. Microsatellite loci are highly polymorphic and useful as genetic markers in maize (Senior and Heun 1993; Chin et al. 1996; Taramino and Tingey 1996; Smith et al. 1997; Pejic et al. 1998) as well as in other plant species.

To date hundreds of mapped sequence specific primers for microsatellites are publically available from the Maize Database (http://www.agron.missouri.edu/) and they are updated on a regular basis. Microsatellite markers are ideal DNA markers for genetic mapping of important traits and for germplasm analysis because they are:
(1) Highly abundant and evenly distributed,
(2) Co-dominant,
(3) Highly polymorphic,

(4) Easily assayed by PCR and
(5) Accessible to other laboratories via published primer sequences

SSRs have been compared to RFLPs, RAPDs and AFLPs to determine which of these marker techniques is the most suitable for germplasm analysis in maize (Taramino and Tingey 1996; Smith et al. 1997; Pejic et al. 1998). In general, genetic similarity trees from RFLP, SSR and AFLP data correlate strongly with pedigree data. RAPDs showed low correlation probably due to a lack of reproducibility caused by mismatch annealing. SSR and AFLP technologies can replace RFLP markers in genetic similarity studies because of their comparable accuracy as well as easier standardisation and cost effectiveness over RFLPs. AFLPs are regarded as the most effective marker system because of their capacity to reveal several bands in a single amplification reaction. AFLPs have a higher assay efficiency index compared to any other DNA marker method (Pejic et al. 1998).

SSRs with their multi-allelism and co-dominance are well suited for marker-assisted selection once a gene has been mapped. Whereas AFLPs with their high multiplex ratio offer a distinctive advantage over SSRs when regional targeting using NILs or bulks or fingerprinting of inbred lines needs to be performed.

4 Transposon Tagging

At the forefront of maize genetics is the exiting research of transposon tagging. Transposon tagging is an efficient means of gene identification and analysis of the phenotypic consequences of altered gene expression. It was the striking and beautiful spotted kernel phenotypes that led to the discovery of transposable elements in maize by Barbara McClintock over 50 years ago. These mutations are disguised by their high frequency of somatic and germinal instability and are caused by the action of DNA or "class 2" elements (Wessler 1998). Elements in this group are characterized by short inverted repeats at their termini and, most important, transposition via a DNA intermediate.

Active Mutator lines have between 10-50 multiple mobile elements. These active lines have at least one regulatory *Mu* element (MuDR), which is needed for Mu

elements to be mobile (Lisch et al. 1995). Mu elements are efficient mutagens because the transposons insert preferentially into genes and the elements move throughout the genome (reviewed by Walbot 1991; Chandler and Hardeman 1992; Bennetzen 1996). Forward mutation is so high that populations as low as 1000 and up to 50 000 individuals (kernels or plants) are sufficient to recover mutants in all targets tested (Walbot 1991).

A number of techniques have been developed to identify the genes and to recover Mu element contiguous DNA. This can be done by AIMS (amplification of insertion mutagenized sites) markers where PCR is used to generate very short products (<250 bp) next to each Mu insertion (Frey et al. 1997; 1998). "Gene machines" such as the TUSC system of Pioneer HiBred (Benson et al. 1995) uses a primer from the Mu element in combination with primers from known gene sequences or gene motifs. ZnDB, a maize genome collaboration, is currently using engineered *Mu* (*RescueMu*) tagging to discover maize genes and to phenotypically analyse their functions (http://www.zmdb.iastate.edu/).

The major disadvantage of Mu gene tagging is the large amount of work involved in correlating a particular Mu element with a segregating mutant phenotype.

5 Mapping populations and strategies

A prerequisite for the construction of a mapping population is that the parents have sufficient DNA sequence polymorphisms. This is not a problem in maize, as it is a naturally outcrossing crop and has high levels of DNA polymorphisms between inbred lines (Helentjaris 1987). In the construction of a linkage map one should, however, consider whether a mortal or immortal mapping population should be used.

Often a geneticist is not interested in developing a molecular map but only wishes to find markers that are closely linked to a specific trait. This leads to the development of special mapping material that targets specific chromosome regions. Two strategies, called near-isogenic lines (NILs) and bulked segregant analysis (BSA), have been developed. These make the identification of markers for a specific trait possible without the use of a segregating population or genome screening (Young et

al. 1988; Michelmore et al. 1991).

5.1 Mortal or immortal mapping population

The major use of mapping populations is in the construction of comprehensive genetic linkage maps. Linkage maps are essential for effective marker-assisted breeding, QTL mapping and gene characterisation. The major disadvantage of a mortalised mapping population (classical F_2 population, or 1 backcross) is that the genotypes die and the DNA eventually becomes exhausted. Mapping populations can also not be exchanged between different groups. Often markers that have been mapped in one population need to be remapped in a new population to provide reference points for alignment in different maps.

In contrast, immortalised mapping populations, recombinant inbreds (RI) or immortalised F_2's' constitute a permanent population (Burr and Burr 1988; Gardiner et al. 1993). Recombinant inbred (RI) lines are produced by continually selfing or sib-mating the progeny of individual members of a F_2 population until homozygosity is achieved (Burr and Burr 1991). In such a RI mapping population, segregation is complete, or nearly complete and can be used indefinitely for mapping. New data is continuously added to the pre-existing map. Immortal populations also allow for the detection of QTLs as they can be evaluated in replicated trials over locations and years.

Mortilized mapping populations are, however, essential in mapping specific traits that are not present in immortalized mapping populations. It also takes longer to construct an immortalized population. A new type of mortilized mapping population is where a specific cross underwent random matings for 4 generations (Davis et al. 2000). The random mating process increases the average number of recombination events per individual by 3-fold compared with F_2 or RI derived mapping lines.

In the mapping of the maize genome real progress was made when a subset of RFLP core markers, that are relatively polymorphic and evenly spaced throughout the genome, was constructed by Gardiner et al. (1993) using an immortalized F_2 population. This was important as the core marker set allowed for the minimal

genome coverage needed to map any quantitave or qualitative trait. The designation of this set of core markers has facilitated the dissection of the maize genome into a series of computer-storable and sub dividable bins that serve as collection points for mapped genetic loci.

5.2 Bulked segregant analysis (BSA) and nearly isogenic lines (NILs)

Finding markers linked to a particular locus or trait can be laborious if markers that cover all chromosomes are tested. Two targeted mapping approaches have been described to ease this process: comparison of nearly isogenic lines (NILs) (Muehlbauer et al. 1988) and bulked segregant analysis (Michelmore et al. 1991).

NILs are the natural result of backcross-breeding programs aimed at transferring important agronomic characteristics into elite cultivars. The donor of the trait is first crossed to the recipient line. The generated F_1 hybrid is crossed back to the recipient line (designated as the recurrent parent), and the process is repeated several times until the wanted portion of the genome from the recurrent parent is retained and the desired trait is introduced. This new breeding line is then nearly isogenic to the recurrent parent (the original elite line) but still contains a limited amount of DNA from the donor, some of it flanking the introgressed gene, and some scattered at random sites in the genome. NILs have been successfully used in combination with marker techniques like RFLPs (Young et al. 1988, Paran et al. 1991, Messeguer et al. 1991, Diers et al. 1992, Schüller et al. 1992) RAPDs (Martin et al. 1991, Barua et al. 1993) and AFLPs (Maughan et al. 1996, Jin et al. 1998) to identify DNA markers linked to plant genes.

With the AFLP technique hundreds or even thousands of potentially polymorphic fragments can be screened between NILs to identify fragments derived from the donor parent. The limitation to this approach is the occurrence of donor parent DNA at scattered sites throughout the genome of the NILs that is not linked to the target trait (Jean et al. 1998). When using only one NIL pair the targeting power of the NILs is limited by the occurrence of the residual DNA from the donor line (Jean et al. 1998). Muehlbauer et al. (1988) calculated that 50-75% of the fragments retained from a donor parent in a BC_5S_1 NIL could be expected to be located on the marker

chromosome. Kaeppler et al. (1993) prososed that sets of NILs should be used to detect linkages between molecular markers and introgressed loci. Unfortunately, NILs are often not available for important agronomic traits and the production of NILs is a lengthy process.

Bulked segregant analysis (BSA) allows marker targeting using any population segregating for a given characteristic. This approach involves comparing two pooled DNA samples from individuals of segregating populations originating from a single cross. Within each pool, or bulk, the individuals are identical for a trait, gene or genomic interval of interest but are arbitrary for all other genes. Two contrasting pools for a trait are analysed to identify markers that distinguish them. Markers that are polymorphic between the pools will be genetically linked to the loci determining the trait used to construct the pools. A number of publications have reported success by using bulked segregant analysis to identify particular genes or genomic intervals (Giovannoni et al. 1991; Reiter et al. 1992; Barua et al. 1993; Chalmers et al. 1993; Kesseli et al. 1993; Williams et al. 1993; Delourme et al. 1994). The limitation to bulked segregant analysis is the chance of shared homozygosity at specific unlinked chromosomal regions in the bulks that might occur (Jean et al. 1998). With a segregating population derived only one generation after the original cross (F_2 and BC_1 population), it is very likely that some genomic regions will be uncovered where the markers will not yet have been randomised through meiosis and recombination. According to Jean et al. (1998) increasing the size of the bulks would not eliminate all false positives.

6 Marker-assisted breeding

Seed companies are currently using marker-assisted breeding in two major ways (1) for DNA fingerprinting and (2) using markers to introgress one or more genes from donor parents into elite inbred lines.

Maize seed companies fingerprint DNA of elite inbred maize lines to reveal pedigree relationships among inbreds, to predict hybrid performance, to determine maximum similarity to the recipient line and minimum similarity to the donor line in backcross breeding, to protect intellectual property rights, to assess genetic purity and identify

contaminants in hybrids. The characterization of inbreds lines by molecular markers and their subsequent use in predicting hybrid performance has been the major focus in recent research studies. One strategy that has been considered is based on the assumption that the specific combining ability (SCA) expressed by a hybrid is related to the genetic distances between its parental lines (Lee et al. 1989). The condition under which this "distance model" is efficient has been documented on an experimental (Melchinger et al. 1992, Burstin et al. 1995, Ajmone Marsan et al. 1997) and theoretical (Charcosset and Essioux 1994) level. Both types of studies have led to the conclusions that the "distance model" is efficient for (1) sets of hybrids between related inbreds, as is mostly the case within a heterotic group, and (2) sets of hybrids between related and unrelated inbreds. However, prediction efficiency with this approach is expected to be extremely low for sets of hybrids between unrelated inbreds limiting this approach for commercial use in applied programs (Charcosset et al. 1998).

Marker-assisted selection (MAS) has been advocated as a useful tool for rapid genetic advances in breeding of quantitative traits, traits that are difficult to select for (resistance genes) and for the selection of many traits simultaneously. Tight linkage between markers and genes of interest is necessary for effective use in plant breeding. Such linkage permits indirect selection for the presence of a desirable gene by assaying for the molecular marker (Tanksley et al. 1989). Its simplicity and reproducibility could shorten the breeding procedure significantly. The use of genetic markers for improving selection efficiency has been proposed using two approaches (Charmet et al. 1999). In the first approach a molecular score is added into the selection index in addition to the phenotypic one (Lande and Thompson 1990; Moreau et al.1998). This approach focuses primarily on population improvement rather than the fixation of the extreme genotypes. The second approach, known as genotype construction, simply considers and handles QTLs as Mendelian factors. This approach has mostly been used in backcross breeding programs to reduce linkage drag and to optimize population size (Hospital and Charcosset 1997).

MAS has been used successfully together with the "advanced backcross QTL analysis" to introduce QTLs from the wild species *Lycopersicon hirsutum* Hub. & Bonpl. into cultivated tomato ,*Lycopersicon esculentum,* lines (Tanksley and Nelson

1996). Selections out-performed the original elite variety in yield, soluble solids content, and fruit colour (Tanksley and McCouch 1997). Hospital et al. (1997) showed with simulations that for a few generations of recurrent selection, selection based on marker-phenotype is more efficient than pure phenotypic selection, however, the progress with marker-assisted selection declines rapidly. Moreover, they observed that marker-assisted selection might become less efficient than phenotypic selection because the rate of fixation of unfavourable alleles at QTLs with small effects is higher under marker-assisted selection than under phenotypic selection. This problem is more acute when the effects associated with markers are not re-evaluated at each generation.

The main conclusion is that MAS could be more efficient than pure phenotype selection in quite large populations and for traits showing relatively low heritability (Hospital et al. 1997). Furthermore, MAS should be most effective in early generations of selection among progeny from crosses between inbred lines (Lande 1992; Stromberg et al. 1994). Heritability is usually lowest (because replications are limited and experimental units tend to be small) and linkage disequilibruim is greatest in these generations (Falconer 1981). The paradox is that the power of mapping QTLs decreases as heritability decreases and is lowest for traits where MAS has the greatest theoretical impact (Lande and Thompson 1990; Lande 1992).

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AIM OF STUDY

The aim of this study was to develop PCR markers for the *Ht1*, *Ht2*, *Ht3* and *Htn1* resistance genes in maize. To accomplish this, a marker technique was chosen following the literature study. The AFLP technique proved to be the best high volume marker technique available. Different restriction enzyme combinations can be used in AFLP analysis and more than two restriction enzymes can be used simultaneously (Simons et al. 1997).

1) Therefore enzyme combinations and the effect of using more than two restriction enzymes in AFLP analysis were first evaluated.

It was clear from the start of this study that field populations segregating for the different resistance genes would be unavailable. The reasons for this are the overshadowing effect of gray leaf spot that make *turcicum* screening difficult and the fact that different races were needed to differentiate between the different *Ht* resistance genes. Bulks from segregating material were not available. Therefore nearly-isogenic lines were used in the development of markers close to the genes and commercially available recombinant inbred line (RIL) populations for mapping. From the literature study it was evident that between 25%-50% of the fragments identified using this approach would map to unlinked loci. The reason for this is that fragments of DNA from the donor parent are retained in the recurrent parent at scattered sites in the genome, that are not linked to the target trait.

2) Consequently, AFLP analysis was used on two NIL pairs to identify common markers in resistant or susceptible lines. These markers should map mostly to one area selected by the breeders and should correspond to previous mapping data of the genes.

If this approach worked, it could be used to map the *Ht3* resistance gene that has not been mapped.

The next step was to determine if a set of markers could be used in a number of *Htn1* resistance lines to determine a more precise gene position.

3) The last aim of this study was to propose a new marker technique that could be used, instead of AFLPs, on this model of two *Ht* NIL pairs to identify candidate genes.

The results of this study are arranged in publication form. Each of the six chapters consist of a full length publication with references:

Chapter 1
A comparison of AFLP enzyme combinations, for the identification of polymorphic markers in maize NIL pairs.

Chapter 2
The effect of using two, three and four restriction enzyme in AFLP analysis.

Chapter 3
Identification of AFLP and SCAR markers for the *Htn1* resistance gene in maize using two pairs of nearly isogenic lines.

Chapter 4
Identification of AFLP and SCAR markers for the *Ht1, Ht2 and Ht3* genes in maize.

Chapter 5
Using 9 PCR markers to identify common introgressed regions for 16 lines with *Htn1* resistance.

Chapter 6
Developing a new simple PCR marker technique that could be used for gene tagging and fingerprinting in maize.

CHAPTER 1

A comparison of two AFLP enzyme combinations for the identification of polymorphic markers in maize NIL pairs.

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With 2 tables

Abstract

The percentage of polymorphic markers on maize near isogenic lines (NILs) was compared using two amplified fragment length polymorphism (AFLP) restriction enzyme combinations *PstI/MseI* and *MluI/Mse*I. Sixteen primer combinations of each enzyme combination were used on three NIL pairs (K64R, K64R*3/*Htn1*; B73, B73*5/*Ht1* and B73, B73*8/*Htn1*). The total number of fragments observed with *MluI/MseI* was 1369 of which 110 were polymorphic (8%), compared to 2931 fragments observed with *PstI/MseI* of which 112 were polymorphic (3.8%). The percentage of observed polymorphisms between NIL pairs decreased with an increase in the number of backcrosses. This study has indicated that *MluI* is better than *PstI* when used in marker identification.

Key words: AFLPs-NILs-Enzyme combinations

Introduction

Theoretically, thousands of rare and frequent cutter restriction enzyme combinations can be used in AFLP analysis. The most commonly used combinations are *Eco*RI/*Mse*I and *Pst*I/*Mse*I. In linkage maps of maize (Castiglioni et al. 1999; Vuysteke et al. 1999), potato (van Eck et al. 1995), barley (Becker at al. 1995) and *Arabidopsis* (Alonso-Blanco et al. 1998) it was found that *EcoRI/MseI* fragments mainly cluster in specific chromosome regions (centromeres).

An explanation for this phenomenon was given by Vuylsteke et al. 1999: In *Arabidopsis* it was shown that the pericentromeric heterochromatin fluoresces brightly when stained with the fluorochrome DAPI (Ross et al. 1996), which is known to show preference for AT-rich DNA. The restriction enzymes *Eco*RI and *Mse*I have AT-rich target sequences (MseI recognizes 5'-TTAA-3', while EcoRI recognizes 5'-GAATTC-3').and Vuylsteke et al. (1999) argued that this is a plausible explanation for the clustering of *Eco*RI/*Mse*I AFLP markers in the *Arabidopsis* centromeres (Alonso-Blanco et al. 1998) and possibly in other plant genome centromeres.

In plant genomes, cytosine (C) methylation of CpG and CpNpG nucleotides varies in frequency along a chromosome and acts to regulate gene expression either at the gene level or regionally, thereby influencing entire regions of chromosomes (review Kass et al. 1997). In recent studies by Castiglioni et al. (1999), Vuylsteke et al. (1999) and Young et al. (1999) a random distribution of *PstI* AFLP markers were found in linkage maps, with a predominant localisation of markers in the hypomethylated telomeric regions of the chromosomes. The methylation-sensitive restriction enzyme *PstI* can therefore be used to increase the frequency of markers in genetically active euchromatic regions. Ridout and Donini (1999) also observed that the *PstI*/*Mse*I primer combinations produce more polymorphisms than *Eco*RI/*Mse*I combinations in barley.

An ideal enzyme combination is one that produces a high percentage of polymorphisms, which are essential in the scoring and isolation of markers. In this study a number of different methylated sensitive restriction enzyme were tested. Only the primers of the enzyme combinations *MluI*/*Mse*I and *PstI*/*Mse*I produced clear fingerprints and were compared using NIL pairs. The results showed that *MluI*/*Mse*I produce consistently higher percentages of polymorphic markers than *PstI*/*Mse*I using 3 NIL pairs.

Materials and Methods

Plant material and DNA Isolation

Seed of three pairs of NILs (K64R, K64R*3/*Htn1*; B73, B73*5/*Ht1* and B73, B73*7/*Htn1*) were obtained from Sensako (Delmas, South Africa). Seedlings were grown under greenhouse conditions and leaves of 6 week old plants were harvested and freeze-dried. The protocol described in the CIMMYT Applied Molecular Genetics Laboratory Manual, based on the method of Saghai-Maroof et al. (1984), was used in genomic DNA extractions.

AFLP analysis

The AFLP analysis was performed as described by Zabeau and Vos (1993) and Vos et al. (1995) with minor modifications. Total genomic DNA of maize (0.2-3 μg) was digested with one of the following restriction enzyme combinations: *Mlu*I/*Mse*I and *Pst*I/*Mse*I. The DNA was digested with 10 U of each restriction enzyme and 1X One-Phor-All Buffer Plus [100 mM Tris-acetate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech] in a total volume of 50 μl. The reactions were incubated in a 37°C water bath for 1 h.

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

The biotinylated fragments were separated from the non-biotinylated fragments (*Mse*I/*Mse*I fragments) by binding them to paramagnetic streptavidine beads (Dynal, Oslo, Norway) and selecting them with a paramagnetic particle separator. For each samples 20 μl beads were used. The beads were washed three times with 20 μl 1 STEX (100 mM NaCl/ 10 mM Tris.HCL pH 7.5/ 0.1 mM EDTA ph 8.0) and resuspended in 20 μl 1 STEX. The beads were subsequently added to the ligation mixture and incubated for 30 minutes on ice with gentle agitation every 10 min. The beads were washed 3 times with 200 μl 1 STEX and then resuspended in 100 μl TE

buffer (10mM Tris.HCL pH 7.5/ 0.1mM EDTA ph8.0).

Only the *Mse*I primer was labeled. For 10 PCR reactions, 1 μl (300ng) *Mse*I primer was added to 1 μl 1X One-Phor-All Buffer PLUS [100mM Tris-acetate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech], 1 μl [γ-^{33}P]ATP (25μCi) and 5 U T4 Polynucleotide Kinase in a total volume of 10 μl. The reaction was incubated at 37°C for 1 h and was terminated by placing it in a heating block at 68°C for 10 min.

One μl of the biotinylated template DNA fragments was added to 100 μM of each dNTP, 1.5 mM $MgCl_2$, 1 X buffer, 0.5 U Taq DNA polymerase (AmpliTag, Perkin Elmer), 30 ng labeled *MseI* primer and 30 ng *Mlu*I or *Pst*I primer in a total volume of 20 μl. PCR amplification was performed in a PCR Express thermal cycler from Hybaid. The cycle profile used for amplification was as follows: one cycle of 72°C for 1 min, one cycle of 94°C for 2 min, followed by 12 cycles of 94°C for 20 sec, 65°C for 30 sec, 72°C for 2 min, followed by 25 cycles of 94°C for 20 sec, 56°C for 30 sec, 72°C for 2 min and one cycle at 72°C for 30 min. In this study 32 AFLP primer combinations were used consisting of 16 *Mse*I/*Mlu*I and 16 *Mse*I/*Pst*I primer combinations.

Formamide loading buffer (10 μl) was added to each amplified sample. The reactions were denatured at 90°C for 5 minutes in a heating block and quickly chilled on ice. Four μl of each sample was loaded on 4% crylamide/bisacrylamide (19:1), 7.5 M urea and 1 X TBE gels and run at 60 Watts for approximately 2 h. The gels were dried on 3MM Whatman chromatographic paper using a gel drier and exposed to x-ray film (Biomax MR, Kodak) overnight.

Results and Discussion

The total number of AFLP fragments and polymorphisms observed in the 3 NIL pairs using *Mlu*I and *Pst*I in combination with *Mse*I are given in Table 1 A and B. The total number of fragments observed in the 3 NIL pairs with *Mlu*I was 1351 with 110 fragments polymorphic (8%), compared to 2886 fragments observed with *Pst*I of

which 112 were polymorphic (3.8%).

The percentages of observed polymorphisms between the NIL pairs with *Mlu*I/*Mse*I were 12, 7.9 and 4.2% and for and *Pst*I/*Mse*I 6.9, 3.4 and 1.3%, respectively. The differences in polymorphisms between NIL pairs can be appointed to the number of backcrosses used to develop the NIL.

Ridout and Donini (1999) observed that the *Pst*I/*Mse*I primer combinations produce more polymorphisms than the *Eco*RI/*Mse*I combinations in barley. The results of this study in maize indicate that *MluI/MseI* is a better choice than *PstI/MseI* for use in marker identification. Since both *PstI* and *MluI* are 6 base restriction enzymes with high GC content it is possible that the differences in total numbers of fragments observed could be due to methylation sensitivity.

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Table 1 (A): AFLP fragments and polymorphisms observed in NIL pairs using 16 primer combinations (PC) with the *MluI/MseI* enzyme combination. (n=total number of fragments scored and n_p=polymorphisms observed between NILs.)

	NIL pair K64R and K64R*3/Htn1		NIL pair B73 and B73*5/Ht1		NIL pair B73 and B73*8/Htn1	
PC	n	n_p	n	n_p	'n	n_p
Mlu-ac/Mse-aca	29	3	26	1	25	2
Mlu-ac/Mse-acc	24	8	28	2	26	1
Mlu-ac/Mse-ccg	22	3	20	3	18	1
Mlu-ac/Mse-ggc	18	1	21	2	20	1
Mlu-ac/Mse-ttg	56	7	46	3	46	1
Mlu-ac/Mse-gag	23	4	20	1	19	1
Mlu-ac/Mse-cat	19	2	21	2	19	1
Mlu-ac/Mse-cac	16	2	29	3	27	1
Mlu-gc/Mse-gaa	25	4	28	7	23	1
Mlu gc/Mse aca	34	0	39	0	39	0
Mlu gc/Mse-acc	34	3	33	3	30	0
Mlu gc/Mse-ccg	26	3	23	0	25	2
Mlu gc/Mse-ggc	29	3	28	5	24	1
Mlu gc/Mse-ttg	51	5	49	1	48	2
Mlu gc/Mse-tac	31	3	32	2	32	2
Mlu gc/Mse-gag	23	4	23	2	22	1
Total/Percentages	460	55 (12%)	466	37 (7.9%)	425	18 (4.2%)

Table 1 (B): AFLP fragments and polymorphisms observed in NIL pairs using 16 primer combinations (PC) with the *PstI/MseI* enzyme combination. (n=total number of fragments scored and n_p=polymorphisms observed between NILs.)

	NIL pair K64R and K64R*3/Htn1		NIL pair B73 and B73*5/Ht1		NIL pair B73 and B73*8/Htn1	
PC	n	n_p	'n	n_p	n	n_p
Pst-cc/Mse aca	88	6	97	4	92	1
Pst-cc/Mse-acc	75	6	90	3	85	2
Pst-cc/Mse-ccg	41	4	34	1	34	0
Pst-cc/Mse-ggc	31	2	34	0	33	0
Pst-cc/Mse-ttg	86	5	87	1	86	1
Pst-cc/Mse-tac	57	3	55	1	54	0
Pst-cc/Mse-gag	55	2	53	3	52	0
Pst-cc/Mse-cat	71	4	64	1	63	1
Pst-cc/Mse-cac	67	0	65	2	62	1
Pst-ag/Mse-acc	76	2	69	3	67	0
Pst-ag/Mse-ccg	40	5	37	1	37	0
Pst-ag/Mse-ggc	46	3	45	1	44	1
Pst-ag/Mse-tac	75	12	75	5	63	2
Pst-ag/Mse-gag	50	3	54	5	49	0
Pst-ag/Mse-cat	65	6	76	1	75	1
Pst-ag/Mse-cac	54	4	57	1	54	2
Total	977	67 (6.9%)	959	33 (3.4%)	950	12 (1.3%)

CHAPTER 2

The effect of using two, three and four restriction enzymes in AFLP analysis

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Abstract

The effect that two, three and four restriction enzyme digestions have on the percentage of polymorphic fragments in amplified fragment length polymorphisms (AFLP) analysis, was evaluated. Two primer combinations of each of the 4 different restriction enzyme combinations, were used on DNA templates digested with two, three and four enzymes. The results indicated that the enzyme combination used determined the percentage of polymorphic fragments amplified, whereas the number of restriction enzymes used only affected the total number of fragments scored.

Introduction

With amplified fragment length polymorphism (AFLP) analysis large numbers of fragments can be amplified with a single primer combination (Vuylsteke et al. 1999). The number of fragments amplified can be increased or reduced by the number of selective nucleotides used (Vos et al. 1995). However, in AFLP fingerprinting, (Smith et al. 1993; Ajmone Marsan et al. 1998; Pejic et al. 1998), linkage analysis (Castiglioni et al. 1999; Vuylsteke et al. 1999) and the identification of markers for genes (Meksem et al. 1995; Thomas et al. 1995; Cervera et al. 1996; Sharma et al. 1996; Brigneti et al. 1997; Simons et al. 1997; Vos et al. 1998), the percentage of polymorphic fragments scored is more important than the total number of fragments scored.

In barley it was shown that different enzyme combinations and the use of more than two enzymes at a time could optimise AFLP analysis. Ridout and Donini (1999) observed that the *Pst*I/*Mse*I primer combinations produce more polymorphisms than

the *Eco*RI/*Mse*I combinations. Simons et al. (1997) used three restriction enzyme combinations in barley to improve the AFLP analysis. They used *Pst*I or *EcoRI* in combination with *Taq*I and *Mse*I. Standard AFLP adapters for each restriction site were ligated to the restriction fragments but AFLP primers for *Taq*I were not added in the subsequent AFLP amplification reactions. This was done to reduce the number of fragments amplified as the fragments with a *Taq*I restriction site were not amplified.

Van Staden and Retief (Chapter 1) reported that enzyme combinations play an important role in the percentage of polymorphisms observed in maize AFLP analysis. They found that the enzyme combination *MluI/MseI* produces more polymorphisms per total number of fragments scored than *PstI/MseI*. The aim of this study was to determine if more than two restriction enzymes could increase the percentage of polymorphisms. This study confirmed that the primer combinations MluI/* produce a higher ratio of polymorphisms than PstI/* and that an extra rare or frequent cutter only decreases the total number of fragments dramatically but has very little effect on the ratio of polymorphisms to total number of fragments scored.

Material and Methods

Plant material & DNA Isolation

Seed of two pairs of NILs (B73/B73-Htn1, K64R/K64R-Htn1) were obtained from Sensako (Delmas, South Africa). Seedlings were grown under greenhouse conditions and leaves of 6 week old plants were harvested and freeze dried. The protocol described in the CIMMYT Applied Molecular Genetics Laboratory Manual based on the method of Saghai-Maroof et al. (1984), was used in genomic DNA extractions.

AFLP analysis

The AFLP analysis was performed essentially as described by Zabeau and Vos (1993) and Vos et al. (1995), using the two restriction enzyme (*Mlu*I/*Mse*I, *Mlu*I/*Msp*I,

*Pst*I/*Mse*I, *Pst*I/*Msp*I), 3 restriction enzyme (*Mlu*I/*Pst*I/*Mse*I, *Mlu*I/*Pst*I/*Msp*I) and 4 restriction enzyme (*Mlu*I/*Pst*I/*Mse*I/*Msp*I) combinations. The following adapter sequences were synthesized, PAGE purified, and made.

*Pst*I-adapter: 5'-Biotin-CTCGTAGACTGCGTACATGCA-3'
3'-CATCTGACGCATGT-5'

*Mlu*I-adapter: 5'-Biotin-CTCGTAGACTGCGTAAC-3'
3'-CTGACGCATTGGCGC-5'

*Mse*I-adapter: 5'-GACGATGAGTCCTGAG-3'
3'-TACTCAGGACTCAT-5'

*Msp*I-adapter: 5'-GACGATGAGTCCTGAT-3'
3'-TACTCAGGACTAGC-5'

Restriction enzyme digestions:

Total genomic maize DNA (0.2-3 μg) was digested with two restriction enzymes (*Mlu*I/*Mse*I), (*Pst*I*Mse*I), (*Mlu*I/*Msp*I) or (*Pst*I/*Msp*I), three restriction enzymes (*Mlu*I/*Pst*I/*Mse*I) or *Mlu*I/*Pst*I/*Msp*I), and four restriction enzymes (*Mlu*I/*Pst*I/*Mse*I/*Msp*I). The DNA was digested with 10 U of each restriction enzyme and 1X One-Phor-All Buffer Plus [100 mM Tris-acetate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech] in a total volume of 50 μl. The reactions were incubated in a 37°C water bath for 1-3 h.

Two restriction enzymes

The 50 μl digested DNA mixture of the two restriction enzyme combinations was supplemented with 10 μl adapter/ligation solution, containing 50 pmol *MseI* or MspI adapter and 5 pmol 5'-biotinylated *MluI* or *PstI* adapter, 1.2 μl 10 mM ATP, 1X One-Phor-All Buffer PLUS [100 mM Tris-aceate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech] and 1 U T4 DNA ligase, and incubated overnight at 37°C. To the ligation mixture 20 μl of washed Dyna Beads was added, the solution was mixed and incubated for 30 minutes on ice, with gentle agitation every 10 min.

The fragments linked to the biotin labelled beads were collected using a paramagnetic particle separator. The beads were washed three times with 200 µl of 1 STEX (100 mM NaCl/ 10 mM Tris.HCL pH 7.5/ 0.1 mM EDTA ph 8.0). Finally the beads with the fragments linked were resuspended in 100 µl TE buffer (10mM Tris.HCL pH 7.5/ 0.1mM EDTA ph 8.0).

Three restriction enzymes

The 50 µl DNA mixture digested with the three restriction enzyme combinations was supplemented with 10 µl adapter/ligation solution, containing 50 pmol *MseI* or *MspI* adapter and 5 pmol 5'-biotinylated *MluI* and *PstI* adapter, 1.4 µl 10 mM ATP, 1X One-Phor-All Buffer PLUS [100 mM Tris-aceate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech] and 2 U T4 DNA ligase, and incubated overnight at 37°C. To the ligation mixture 25 µl of washed Dyna beads was added, the solution was mixed and incubated for 30 minutes on ice with gentle agitation every 10 min. The protocol was continued as for the two-enzyme combination.

Four restriction enzymes

The 50 µl DNA mixture digested with the four restriction enzyme combinations was supplemented with 10 µl adapter/ligation solution, containing 50 pmol *MseI* and *MspI* adapter and 5 pmol 5'-biotinylated *MluI* and *PstI* adapter, 1.6 µl 10 mM ATP, 1X One-Phor-All Buffer PLUS [100 mM Tris-aceate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech] and 2 U T4 DNA ligase, and incubated overnight at 37°C. To the ligation mixture 25 µl of washed Dyna beads was added, the solution was mixed and the solution was incubated for 30 minutes on ice with gentle agitation every 10 min. The protocol was continued as for the two-enzyme combination.

<u>Labelling:</u>

Only the *MseI* and *Msp*I primers were labelled. For 10 PCR reactions, 1 µl (300 ng) *MseI* or *MspI* primer was added to 1 µl 1X One-Phor-All Buffer PLUS [100mM Tris-aceate (pH7.5), 100mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech], 1 µl [γ-^{33}P]ATP (25µCi) and 5 U T4 Polynucleotide Kinase in a total volume of 10 µl and incubated at 37°C for 1 h. The reaction was terminated by heat inactivation at 68°C

for 10 min.

Amplification:

One μl of the biotinylated template DNA fragments was added to 100 μM of each dNTP, 1.5 mM $MgCl_2$, 1 X buffer, 0.5 U Taq DNA polymerase (AmpliTag, Perkin Elmer), and 30 ng labelled *MseI* or MspI primer in combinations with 30 ng *MluI* or *PstI* primer in total volume of 20 μl. PCR amplification was performed in a PCR Express thermal cycler from Hybaid. The cycle profile used for amplification was as follows: one cycle 72°C for 1 min, one cycle of 94°C for 2 min, followed by 12 cycles of 94°C for 20 sec, 65°C for 30 sec, 72°C for 2 min, followed by 25 cycles of 94°C for 20 sec, 56-60°C for 30 sec, 72°C for 2 min and one cycle at 72°C for 30 min. Eight AFLP primer combinations were used: 2 *Mse*I/*Mlu*I, 2 *Mse*I/*Pst*I, 2 *Msp*I/*Mlu*I and 2 *Msp*I/*Pst*I.

Formamide loading buffer (10 μl) was added to each amplified sample. The reactions were denatured at 90°C for 5 minutes in a heating block and quickly chilled on ice. Four μl of each sample was loaded on 4% acrylamide/bisacrylamide (19:1), 7.5 M urea and 1 X TBE gels and run at 60 Watts for approximately 2 h. The gels were dried on 3MM Whatman chromatographic paper using a gel drier and exposed to x-ray film (Biomax MR, Kodak) overnight.

Results

A summary of the AFLP analysis observed with 8 primer combinations is given in Table 1. Two primers of each of the four different enzyme combinations were amplified on DNA templates digested with two, three and four restrictions enzymes. Table 1 indicates the number of polymorphic fragments, non-polymorphic fragments, total number of fragments and ratios observed between the two maize inbred lines B73 and K64R. Results observed on the two resistant NILs (B73-*Htn1* and K64R-*Htn1*) were consistent with the susceptible lines B73 and K64R and are not shown. No new polymorphisms were observed in the NIL pairs using three and four restriction enzymes.

The *Pst*I/* primer combinations produced a larger number of fragments but had a lower percentage of polymorphisms than the *Mlu*I/* primer combinations. By including an extra rare cutter in the DNA template digestion, the total number of fragments was reduced by 6%. The ratio of polymorphisms to total number of fragments was on average the same (57%). If an extra frequent cutter was added to the DNA template digestion the total number of fragments was reduced by 40%. The percentage of polymorphisms observed was, however, only 4% lower (from 57% to 53%).

In Figure 1 a x-ray of an AFLP gel is given to show the effect an extra rare and a frequent restriction enzyme digestion has on the AFLP profile. An extra rare cutter has a minimal effect on the total number of fragments scored. However, one can clearly see that the total number of fragments is dramatically affected by the inclusion of an extra frequent cutter.

Discussion

The results of this study support the previous data of van Staden and Retief (Chapter 1), that *Mlu*I/* enzyme combinations produce a higher percentage of polymorphic markers than *Pst*I/* enzyme combinations. As expected the total number of fragments is reduced with the inclusion of an extra rare or frequent cutter in maize AFLP analysis. The percentage polymorphic fragments are, however, not meaningfully increased or reduced.

In this study, the influence that extra restriction enzyme digestions have on DNA templates was evaluated. This allows selective amplification of a number of enzyme combinations from a single DNA template. It was evident that the procedure does not increase but decrease the number of polymorphic markers. The number of polymorphic fragments could probably be increased by digestion with restriction enzymes after PCR amplification in a cleaved amplified polymorphic sequence (CAPS) AFLP approach.

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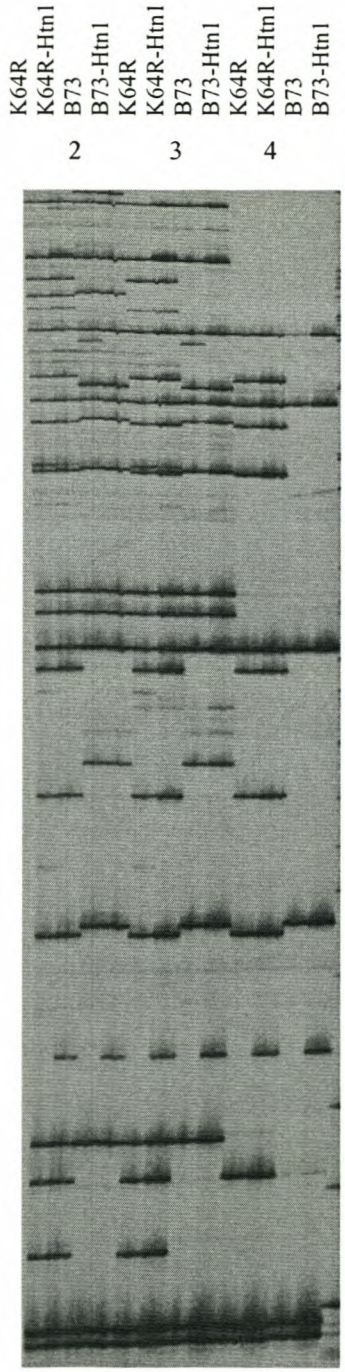
Table 1: Summary of fragments scored using B73 and K64R inbred lines with 8 primer combinations. The DNA templates were digested with two, three and four restriction enzymes (n_p=polymorphic fragments and n_{np}=non polymorphic).

Template DNA digestions/Primer combination	n_p	n_{np}	Total	Ratio
MluI/MseI (MluI-gc/MseI-ggc, MluI-gc/MseI-tac)	47	30	77	0.61
MluI/MseI + extra PstI	38	28	66	0.58
MluI//MseI + extra PstI and MspI	17	16	33	0.52
MluI/MspI (MluI-gc/MspI-cat, MluI-gc/MspI-ctc)	58	31	89	0.65
MluI/MspI + extra PstI	53	28	81	0.65
MluI/MspI + extra PstI and MseI	32	20	52	0.62
PstI/MspI (PstI-ag/MspI-cat, PstI-ag/MspI-ctc)	74	79	153	0.48
PstI/MspI + extra MluI	76	77	153	0.49
PstI/MspI + extra MluI and MseI	44	46	90	0.48
PstI/MseI (PstI-ag/MseI-ttg, PstI-ag/MseI-tac)	91	66	157	0.58
PstI/MseI + extra MluI	91	66	157	0.58
PstI/MseI + extra MluI and MspI	43	37	80	0.53
Summary of two enzymes	270	206	476	0.57
Summary of three enzymes	258	199	457	0.57
Summary of four enzymes	136	119	255	0.53

Figure 1: X-ray of an AFLP gel using the primer combination *Mlu*l-gc/*Mse*l-ggc on DNA templates digested with 2 (*Mlu*l/*Mse*l), 3 (*Mlu*l/*Mse*l + *Pst*l) and 4 enzymes (*Mlu*l/*Mse*l + *Pst*l and *Msp*l).



CHAPTER 3

Identification of AFLP and SCAR markers for the *Htn1* resistance gene in maize using two pairs of nearly isogenic lines.

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With 2 tables and 2 figures

Abstract

Two NIL pairs were used to identify markers for the *Htn1* resistance gene in maize. The amplified fragment length polymorphism (AFLP) marker technique was applied to DNA samples of B73, B73*7/*Htn1* and K64R, K64R*3/*Htn1*. Fifteen polymorphic fragments common in both resistant lines were selected for sequence specific marker conversion. Five sequence characterized amplified region (SCAR) markers mapped to one region of maize chromosome 8 using two recombinant inbred populations. Using more than one NIL pair and selecting for polymorphic fragments common to the resistant lines, eliminates polymorphic fragments that might be scattered throughout the genome.

Key words: AFLP – SCAR – NILs - *Htn1* - *Sethosphaeria turcicum*

Introduction

Northern corn leaf blight (NCLB) is a major foliar disease of maize (*Zea mays* L.) caused by the ascomycete *Setosphaeria turcicum*. The symptoms of NCLB are wilting local lesions which turn necrotic at a later stage thus causing the destruction of large portions of the leaf area in susceptible plants (Dingerdissen et al. 1996).

Five dominant genes (*Ht1*, *Ht2*, *Ht3*, *Htm* and *Htn1*) and one recessive gene (*Ht4*) control monogenic resistance to NCLB in maize (Ullstrup 1970, Hooker 1977, Hooker 1981, Robbins and Warren 1993, Gevers 1975, Carson 1995). *Ht1*, *Ht2*, *Ht3* and *Ht4* resistance is of a chlorotic lesion type while *Htm* and *Htn1* delays necrosis and reduces sporulation (Hooker 1977, Hooker 1981, Carson 1995, Robbins and Warren 1993, Gevers 1975). The single dominant resistance gene, *Htn1*, was identified in a Mexican race, Pepitilla (Gevers 1975). In 1993, Simcox and Bennetzen mapped *Htn1* to chromosome 8 (0.8 cM distal to the RFLP marker *umc117*).

Muehlbauer et al. (1988) proposed that nearly isogenic lines (NILs) could be used to identify markers linked to targeted traits. The use of NILs to identify markers close to a target gene relies on the use of high-volume marker techniques such as AFLPs (Tanksley et al. 1995). Hundreds or even thousands of potentially polymorphic fragments can be screened with the AFLP technique to identify only those fragments derived from a region adjacent to the targeted gene. The NIL approach has been used successfully in combination with the AFLP technique to identify DNA markers linked to plant genes (Maughan et al.1996, Jin et al. 1998).

The limitation of this approach is the occurrence of donor parent DNA that is not linked to the target trait but scattered throughout the genome (Jean et al. 1998). Muehlbauer et al. (1988) calculated that 50-75% of the fragments from a donor parent retained in a BC_5S_1 NIL can be expected to be located on the marker chromosome. Kaeppler et al. (1993) proposed using sets of NILs to detect linkages between molecular markers and introgressed loci.

In theory, AFLP fragments common in at least two resistant or susceptible lines from different NILs, should predominantly originate from the region targeted by selection during backcrossing. This applies if the donor source is the same and the selection pressure during backcrossing was for one trait only. In this study we used two NIL pairs and mapped 5 markers to a targeted introgressed region of *Htn1* using recombinant inbred populations of Burr et al. (1988).

Materials and methods

Plant material and DNA isolation

Seeds of the two NILs pairs (B73, B73*7/*Htn1* and K64R, K64R*3/*Htn1*) originating from the donor line, 11Mex44, were used. Dr. Benjamin Burr (Brookhaven National Laboratory, Upton, NY) kindly provided seeds of the recombinant inbred populations, CM37/T232 and CO159/TX303.

Leaves of six week old plants were harvested and lyophilised. The protocol described in the CIMMYT Applied Molecular Genetics Laboratory Manual, which is based on the method by Saghai-Maroof et al. (1984), was used for the DNA extractions.

AFLP analysis

AFLP analysis was performed as described by Zabeau and Vos (1993) with minor modifications. Total genomic DNA (0.2-3 μg) of the NIL pairs was digested with one of the following restriction enzyme combinations: *MluI/MseI*, *HindIII/MseI* or *PstI/MseI*. The cycle profile used for amplification was as follows: one cycle 72°C for 1 min, one cycle of 94°C for 2 min, followed by 12 cycles of 94°C for 20 sec, 65°C for 30 sec, 72°C for 2 min, followed by 25 cycles of 94°C for 20 sec, 56°C for 30 sec, 72°C for 2 min and one cycle at 72°C for 30 min. In this study 37 AFLP primer combinations were used consisting of: 18 *MseI/MluI*, 16 *MseI/PstI* and 3 *MseI/HindIII* primer combinations.

Cloning of AFLP fragments

AFLP fragments were visualised by autoradiography. Autoradiography glo-stickers (Bel-Art products, Penquannock, NJ) were used to orientate the exposed radiograph on the dried gel. Polymorphic fragments common in the resistant lines were identified and excised from the Whatman paper. The fragment was eluted in 50 μl of TE buffer and used as PCR template together with the relevant AFLP primers. The amplification products were run on a 2% low melting agarose gel and cleaned by phenol/chloroform extraction. Fragments were cloned using the pGEM®-T easy

vector system (Promega, Madison, WI). Target AFLP fragments are usually contaminated with slightly larger or smaller fragments and a method was therefore developed to confirm that the correct insert was cloned. Five white colonies were selected of each cloned fragment and used as template DNA in AFLP analysis. The amplification products of the colonies were run on a polyacrylamide gel alongside the original AFLP fingerprint. Two colonies containing fragments of the correct size were cultured overnight at 37°C in 3 ml LB medium containing ampicillin. Plasmids were extracted using the Perkin Elmer Miniprep kit and sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The Primer Designer, version 1.01, software program was used to design unique primers from the sequences obtained.

To confirm that the primers were developed from the correct insert they were labelled radioactively and used (separately) in combination with the opposite AFLP primers on the AFLP template DNA. This was done to determine if the original AFLP polymorphisms could be seen on the DNA of the NIL pairs. With this approach the nature of the original AFLP polymorphism in a NIL pair could be explained. If no polymorphism was detected in the NIL pairs using this procedure the fragment and primers were not used further.

Sequence characterized amplified region (SCAR) markers

The primer sets were used on genomic DNA of the NIL pairs to determine their use as sequence characterized amplified region (SCAR) markers. All PCR amplifications were performed in a PCR Express thermal cycler (Hybaid) using 25-50 ng genomic DNA, 0.5 U of AmpliTaq or AmpliTaq Gold^{TM} DNA polymerase (Perkin Elmer) with a final concentration of 1.5 mM $MgCl_2$. The PCR profile used for amplification was as follows: one cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 20 sec, annealing temp.(54°C-60°C) for 30 sec, 72°C for 2 min and one cycle at 72°C for 10 min.

Mapping of SCAR markers

The two RIL populations of Burr et al. (1988) were used to map the new SCAR markers. Data files of more than a thousand mapped markers of the two populations were obtained from the National Agricultural Library (http://probe.nalusda.gov:8300) and linkage analysis was performed using the software package MAPMAKER/EXP version 3.0b (Lincoln et al. 1992).

Results

AFLP analysis on two pairs of NILs

With the 37 AFLP primer combinations used, approximately 1800 fragments were scored per NIL pair. Two percent of the total number of fragments scored were polymorphic in the B73 NIL pair, developed from seven backcrosses, and 8% were polymorphic in the K64R NIL pair, developed from only three backcrosses. Twenty-six polymorphic fragments were identified in B73-*Htn1* and 65 fragments in K64R-*Htn1*. Of these, 15 were found to be common across resistant lines. The AFLP primer combinations used to identify the polymorphic fragments are listed in table 1. Figure 1 is an example of an AFLP marker common in the resistant lines K64-*Htn1* and B73-*Htn1*.

Linkage analyses of SCAR markers

The 15 AFLP markers were converted to SCAR markers. Of these, 7 produced polymorphisms with genomic DNA of the B73 and K64 NIL pairs. These markers were tested on the two RIL populations of Burr et al. (1988). Five markers were polymorphic in the parents and were mapped. Table 2 summarises the methodology used to visualise these markers. The markers *us1*, *us3*, *us5* and *us6* were polymorphic for RIL population CM37/T232 and *us6* and *us14* were polymorphic for RIL population CO159/TX303.

Linkage analysis was performed using the software package MAPMAKER/EXP version 3.0b (Lincoln et al. 1992). The four markers *us1*, *us3*, *us5* and *us6* were mapped to maize chromosome 8 in bin 8.05/6. Figure 2(A) illustrates the order of the new markers in relation to previously mapped RFLP markers with population CM37/T232. Mapping distances were calculated using multi-point linkage analysis. Of the four new markers mapped to chromosome 8, the marker *us1* was most proximal and *us6* was most distal. Using two-point linkage analysis *us1* was placed 8 cM (LOD score 5.24) distal to the RFLP marker bnl12.30a and the marker *us6* mapped 2.7 cM proximal to the RFLP marker umc48 (LOD score of 8.58). A two-point linkage distance of 5.9 cM and a LOD score of 6.38 was calculated between markers *us1* and *us6.*

Results of the multi-point linkage analysis using the RIL population CO159/TX303 are summarised in Figure 2(B). Two-point linkage analysis of markers *us6* and *umc48* resulted in a linkage distance of 6.3 cM (LOD score 5.37). The SCAR marker *us14* mapped 6.3 cM proximal to umc117 (LOD score 5.87). Both Figures 2(A) and 2(B) were aligned with the UMC 1998 chromosome 8 map in Figure 2(C).

Conclusion

The aim of this study was to map all AFLP markers common in the resistant lines of the two *Htn1* NIL pairs. Only five of the 15 markers identified mapped to chromosome 8.05/06. The inefficient conversion of AFLPs to polymorphic sequence specific PCR markers has also been reported by Shan et al. (1999). This restricts the development of simple PCR markers for genes in maize.

Using AFLPs on two NIL pairs, the *Htn1* introgressed region mapped to the same region reported by Simcox and Bennetzen (1993) who used a segregating population. The approach used in this study to map a single gene is an alternative to the classical F_2 population segregating for the trait (*Htn1*).

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Table 1: List of primer combinations with which polymorphic AFLP fragments, that were common between the resistant lines of the NILs pairs (B73, B73*7/*Htn1* and K64R, K64R*3/*Htn1*).

Marker code	Enzyme and primer combination with 3' selective nucleotides
us1	MseI-GAA/MluI-GC
us3	MseI-CCG/MluI-GC
us4	MseI-GGC/MluI-GC
us5	MseI-TTG/MluI-GC
us6	MseI-TAC/MluI-GC
us7	MseI-CAT/MluI-GC
us8	MseI-GGC/MluI-AC
us9	MseI-TTG/MluI-AC
us10	MseI-ACA/PstI-CC
us11	MseI-TTG/PstI-CC
us12	MseI-TAC/PstI-AG
us14	MseI-GAA/HindIII-GC
us15	MseI-ACC/ HindIII-GC
us16	MseI-CCG/ HindIII-GC
us17	MseI-CCG/ HindIII-GC

Table 2: Primer sequences, annealing temperatures and type of polymorphic SCAR markers.

Marker Code	Primers developed	Annealing Temperature	Type marker/ Visualising (gel)	Polymorphic on RIL population
us1	F-5'gcc agc cac cac tac ata cg-3' R-5'aat gag gct gac cga agt tg-3'	58°C	Co-dominant/ acrylamide	CM37/T232
us3	F-5'gtg cca tcc gat gcc g-3' R-5'ggg aaa aca aac ggt cga ac-3'	54°C	Dominant/ agarose	CM37/T232
us4	F-5'cac atg ggt gag gtt tga tg-3' R-5'cgt tga agg tgc ttt tgg ta-3'	57°C	Dominant/ agarose	No
us5	F-5'tcg tct ggt gtt cgg ttc ag-3' R-5'cag aac act aac cat gcg ag-3'	52°C	Dominant/ agarose	CM37/T232
us6	F-5'tac acc ggc tag gaa acg ag-3' R-5'cgt gaa agg cgt gtc tgc tt-3'	57°C	Dominant/ acrylamide	CM37/T232 and C0159/T303
us7	F-5'aca ttt agg cta tcg cac tca-3' R-5'gcc gta ggg gcc aaa aat aa-3'	57°C	Dominant/ agarose	No
us14	F-5'gtc ctc ccc gct gtt gta-3' R-5'tgt gtt tac tag cct cct gg-3'	60°C	Dominant/ agarose	C0159/T303

Figure 1: AFLP gel indicating the marker us4 identified with the primer combination MluI-GC/MseI-GGC on the resistant lines K64-*Htn1* and B73-*Htn1*.

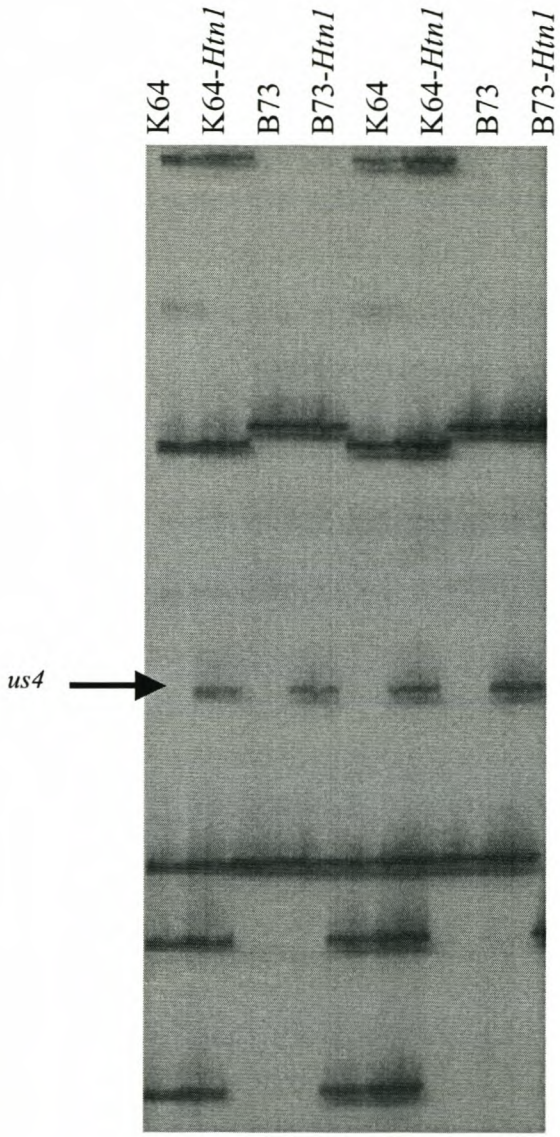
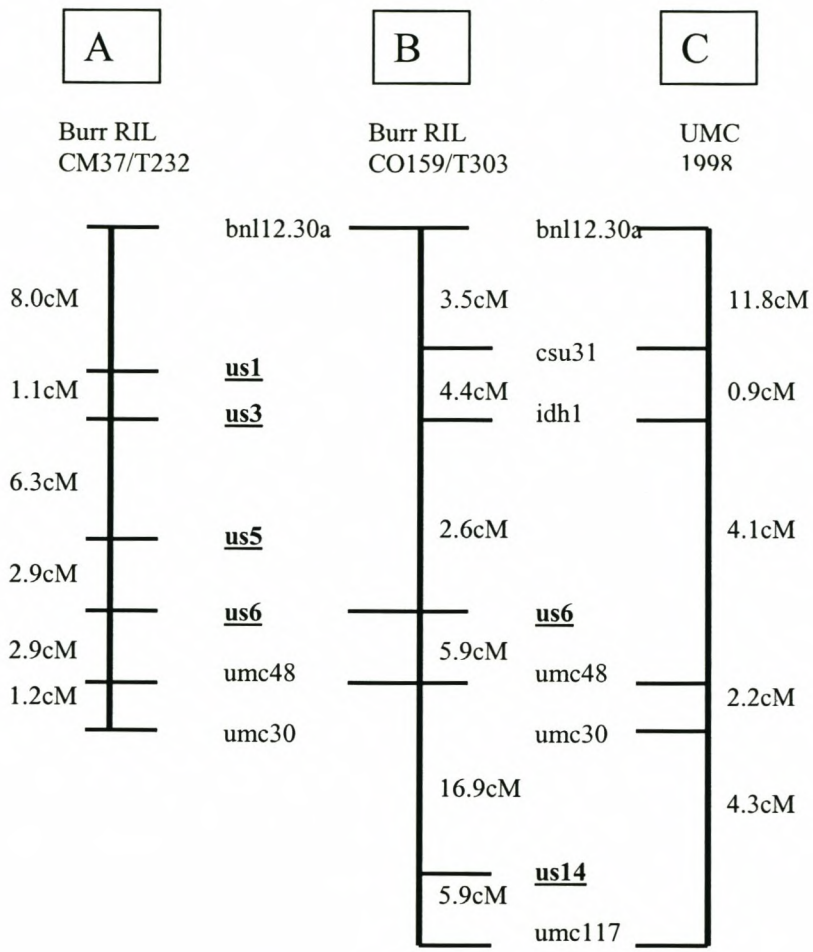


Figure 2: Multipoint linkage analysis of chromosome 8 using **(A)** the RIL population CM37/T232 and **(B)** the RIL population CO159/T303. In **(C)** the alignment of these maps with the UMC 1998 map is given. The SCAR markers are underlined and in bold.



CHAPTER 4

Identification of AFLP and SCAR markers for the *Ht1, Ht2 and Ht3* genes in maize.

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With 2 tables

Abstract

Amplified fragment length polymorphisms (AFLPs) were used on NIL pairs to identify markers for *Ht1*, *Ht2* and *Ht3*. These markers were converted to sequence characterized amplified region (SCAR) markers and were mapped using two commercially available recombinant inbred line (RIL) populations. Four SCAR markers were polymorphic in the *Ht1* NILs and three of these were mapped to chromosome 2.07. Two SCAR markers were polymorphic in the *Ht2* NILs and mapped to chromosome 8.05/06. In the *Ht3* NILs, two converted SCAR markers and one microsatellite marker (bnlg1666) were polymorphic. One of the SCAR markers and the microsatellite marker were mapped to chromosome 7.04. This reports the first tentative map position of the *Ht3* locus. The simple PCR markers, which were developed for *Ht1*, *Ht2* and *Ht3* can be used in fine mapping of the genes in segregation studies.

Introduction

The fungus *Setosphaeria turcica* (Luttrell) Leonard & Suggs, anamorphic stage *Exserohilum turcicum* (Pass.) Leonard & Suggs [syn. *Helminthosporium turcicum* (Pass.)] causes a foliar disease in maize called northern corn leaf blight (NCLB). NCLB occurs world-wide but is most prevalent and damaging when cool to moderate temperatures and moist conditions prevail during the growing season (Hooker 1975). The disease can cause extensive defoliation during grain-filling periods, resulting in yield losses of 30% or more (Perkins and Pedersen 1987).

Resistance to NCLB in maize is generally classified as one of two types: monogenic resistance which is race specific and polygenic resistance which is not race specific. Monogenic resistance to NCLB is controlled by five dominant (*Ht1*, *Ht2*, *Ht3*, *Htm* and *Htn1*) and one recessive gene (*Ht4*) (Ullstrup 1970, Hooker 1977, Hooker 1981, Robbins and Warren 1993, Gevers 1975, Carson 1995). *Ht1*, *Ht2*, *Ht3* and *Ht4* form a chlorotic lesion type of resistance whereas *Htm* and *Htn1* cause a delay in appearance of necrosis and a reduction in sporulation (Gevers 1975, Raymundo and Hooker 1981, Robbins and Warren 1993).

In 1963 Hooker reported the first identification of the single dominant gene *Ht1* in inbred line GE440 and in the popcorn variety Ladyfinger. According to Hooker (1963) the genes in the two corn types are identical, allelic or very closely linked. *Ht1* was the first monogenic trait locus for resistance to NCLB mapped. It was mapped to the central region of the long arm of chromosome 2 (Patterson et al. 1965). More recently restriction fragment length polymorphisms (RFLPs) have been used to verify the position of *Ht1* on the long arm of chromosome 2 and to map the gene more precisely (Bentollila et al. 1991, Freymark et al. 1993).

Hooker (1977) found that the inbred line NN14 from Australia has two dominant resistance genes for the chlorotic-lesion resistance. From NN14 the two inbred lines, NN14A and NN14B were developed, each containing one of the resistance genes. The inbred line NN14A contains a single dominant gene that mapped to the locus of *Ht1* and NN14B contains an independent locus that Hooker (1977) designated as *Ht2*. The *Ht2* resistance gene is characterised by elongated chlorotic lesions which occasionally extend over the entire length of leaves in seedlings, while no lesions develop on the upper foliage of mature plants after the silking stage. Zaitlin et al. (1992) first mapped the *Ht2* resistance gene to the long of chromosome 8. This was supported by data from Freymark et al. (1993). Simcox and Bennetzen (1993) found tight linkage between *Ht2* and *Htn1* and estimated that *Ht2* maps approximately 10 cM proximal to *Htn1*.

Ht3 is a dominant resistance gene for *Setosphaeria turcica* that was most probably derived from *Tripsacum floridanum* (Hooker 1981). The resistance shows incomplete dominance. Analysis of F_2 data from Simcox and Benetzen (1993) and preliminary

data from Hooker (1981) indicate in that *Ht3* is not linked to *Ht1*, *Ht2* or *Htn1*. According to Simcox and Bennetzen (1993) *Ht2* and *Ht3* have a very similar chlorotic-necrotic lesion phenotype and this can unfortunately lead to source contamination.

Due to the low natural incidence of NCLB disease in South Africa, segregation studies are problematic. Furthermore, the existence of different pathotypes of NCLB makes field scoring for the different single genes resistance difficult (Adipala et al. 1993, Gevers et al. 1994). Identifying markers for *Ht1*, *Ht2* and *Ht3* using pairs of NILs and mapping the introgressed regions using RIL populations was an attractive alternative to a classical F_2 mapping population. In this study, 4 PCR markers were identified for *Ht1*, 2 for *Ht2* and 3 for *Ht3*. These markers confirmed the mapping positions of *Ht1* and *Ht2* and a tentative mapping position for the *Ht3* locus was identified (chromosome 7.04).

Materials and Methods

Plant material and DNA extractions

Leaf samples of six NIL pairs (B73/B73-*Ht1*, B73/B73-*Ht1*, A619/A619-Ht2, H4460/H4460-Ht2, A619/A619-Ht3 and H4460/H4460-Ht3) were harvested from field trials of the South African seed company, Sensako (Delmas, South Africa). Dr. Ben Burr (Brookhaven National Laboratory, Upton, NY) provided seed of the recombinant inbred families, CM37/T232 and CO159/TX303, consisting of 48 and 41 inbred lines, respectively. The protocol described in the CIMMYT Applied Molecular Genetics Laboratory Manual, based on the method by Saghai-Maroof et al. (1984), was used for DNA extractions.

AFLP analysis

AFLP analysis was performed using the Dyna-bead bound template DNA approach originally described by Zabeau and Vos (1993) with minor modifications. Total genomic DNA of maize (0.2-3 μg) was digested with one of the following restriction enzyme combinations: *Mlu*I/*Mse*I or *Pst*I/*Mse*I. The cycle profile used for amplification was as follows: one cycle of 72°C for 1 min, one cycle of 94°C for 2 min,

followed by 12 cycles of 94°C for 20 sec, 65°C for 30 sec, 72°C for 2 min, followed by 25 cycles of 94°C for 20 sec, 56°C for 30 sec, 72°C for 2 min and one cycle at 72°C for 30 min.

Conversion of AFLPs to SCAR markers

Autoradiography glo-stickers (Bel-Art products) were used to mark the dried gel for orientation purposes. A specific AFLP fragment was exised with a scalpel and incubated overnight at 37ºC in 50µl TE buffer. One µl of the TE buffer containing the excised DNA fragment was amplified with the same set of AFLP primers. Amplification products were electrophoresed at 80V in a 1.5% low melting point agarose gel. The desired fragments were excised from the gel. The DNA was extracted by phenol/chloroform extraction and ethanol precipitated. A pGem®-T Easy Vector System II (Promega) was used to clone the fragments. The plasmids were extracted using a Perkin Elmer Miniprep kit and sequenced with an ABI PRISM 377 automatic sequencer. Primers were commercially synthesized. All PCR amplifications were performed in a PCR Express thermal cycler (Hybaid) using 25-50 ng genomic DNA, 0.5 U Amplitaq Gold^{TM} DNA polymerase (Perkin Elmer), 100 µM of each dNTP, 1.5 mM $MgCl_2$ and 30ng of each primer in a 20µl PCR reaction volume. The PCR profiles used for amplification were as follows: one cycle of 95°C for 10 min, followed by 35 cycles of 95°C for 20 sec, annealing temperature of 50-60ºC (depending on primer pair) for 30 sec, 72°C for 2 min and one cycle at 72°C for 10min. The amplification products were electrophoresed either in 1.5-2% agarose gels with 1X TBE buffer containing ethidium bromide (1/10 000 dilution of a 50 mg/ml solution) or were loaded on 4% acrylamide/bisacrylamide (19:1), 7.5 M urea and 1X TBE gels and run at 60 Watts for approximately 1 h.

Results

SCAR markers for *Ht1*

With 18 *MluI/Mse*I and 16 *PstI/Mse*I primer combinations, 21 AFLP markers were identified which were common in both resistant lines. These markers were converted to SCAR markers. Four of the SCAR markers were polymorphic in the two NIL pairs. These markers were further tested on the two RIL populations of Burr et al. (1988).

Three of these markers were polymorphic and could be mapped. In Table 1 (A) the fragment sizes, primer pairs and chromosome mapping results are given. Linkage analysis results indicated that the three markers (us24, us30, us35) map to maize chromosome 2.07. Multipoint linkage analysis was used with population CO159/T303 to determine the order of the new markers to previously mapped markers (Table 2). Genetic distances (cM) and LOD scores using two-point linkage analysis are also indicated in Table 2. The three SCAR markers mapped to a region of approximately 14 cM, flanked by the markers umc98 and bnl6.20.

SCAR markers for *Ht2*

Ten AFLP primer combinations (*MluI/Mse*I) were used to identify fragments common in either the susceptible (repulsion) or resistant (coupling) lines. Three AFLP markers, 1 repulsion and two in coupling were identified and converted to SCAR markers. Two of these (us56 and us60) were mapped, using the RIL population CO159/T303. The fragment size, primer pairs and chromosome mapping results are summarised in Table 1 (B). Linkage results indicated that the two markers (us56, us60) map to maize chromosome 8.05/06. The order of these markers relative to those of the RIL population, analysed by multi-point linkage analysis, is given in Table 2 together with two-point linkage data. Both markers mapped proximal to the RFLP marker umc48 and distal to bnl12.30a.

SCAR marker for *Ht3*

To identify markers for *Ht3*, 35 AFLP primer combinations (*MluI/MseI*) were used. Four AFLP markers were common in either the susceptible or resistant lines. Two of these (us62 and us64) were converted to polymorphic SCAR markers and one (us62) was mapped to chromosome 7.04 using RIL population CM37/T232.

To substantiate that this marker identifies an introgressed region, more mapped markers on chromosome 7.04 were used to screen the NIL pairs. Four microsatellite markers were selected from the maize database. One marker, bnlg1666, amplified the same polymorphic allele in both resistant lines. This marker was mapped on the same RIL population as marker us62. The mapping results are indicated in Table 2.

Discussion

AFLP analysis was applied on two NIL pairs each of the genes *Ht1*, *Ht2*, and *Ht3* to identify markers common in either the susceptible or the resistant lines. By using the RIL populations of Burr et al. (1988), the SCAR markers were mapped to small regions of specific chromosomes.

For *Ht1*, 3 SCAR markers were mapped to chromosome 2.07 having flanking RFLP markers umc98a (proximal) and bnl6.20 (distal). This data correlates with Paterson et al. (1965), Bentolila et al. (1991) and Freymark et al. (1993). The best estimate for the placement of the *Ht1* locus is given in the integrated maps of Freymark et al. (1993), who also indicated markers umc98a and bnl6.20 flank *Ht1*.

Two SCAR markers for *Ht2* mapped to chromosome 8.05/06 between flanking markers bnl12.30a (proximal) and umc48a (distal). Zaitlin et al. (1992) and Freymark et al. (1993) mapped the *Ht2* gene to the same region on chromosome 8.05/06. The superimposed data of Freymark et al. (1993) suggested the position of the *Ht2* locus to be between umc89a (proximal) and umc48a (distal). In this study the SCAR markers mapped distal to bnl12.30a and proximal to umc48a.

The locus for *Ht3* has not been mapped. A SCAR marker was identified using AFLP analysis on two *Ht3* NIL pairs. Using the RIL populations CM37/T232 of Burr et al. (1988) the SCAR marker (us62) was mapped with high LOD scores on chromosome 7.04. To confirm this mapping data, additional microsatellite markers from 7.04 were tested on the *Ht3* NIL pairs. The alleles of one marker, bnlg1666, were common in the resistant lines and differed from those detected in the susceptible lines. This marker was mapped using the same RIL population. SCAR marker us62 and microsatellite marker bnlg1666 mapped within a interval of 2.5 cM (LOD score of 9.41) on chromosome 7.04. Simcox and Benetzen (1993) supported preliminary data of Hooker (1981), who stated that *Ht3* is not linked to *Ht1*, *Ht2* or *Htn1*. This study reports the first tentative allocation of the *Ht3* locus to chromosome 7.04.

In this study, PCR markers were developed for the introgressed regions of the genes *Ht1*, *Ht2* and *Ht3*, using AFLPs on two NIL pairs each. Using this approach, the introgressed regions for *Ht1*, *Ht2* and *Ht3* were mapped and the data correspond to

known map positions of these genes. Furthermore, a tentative position was found for the resistance gene *Ht3*. The developed PCR markers can be used in fine mapping of the *Ht1*, *Ht2* and *Ht3* genes in segregation studies.

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Table 1: SCAR markers identified using NIL pairs for *Ht1*, *Ht2*, *Ht3*. The fragment sizes, primer pairs and chromosome mapping data are indicated.

SCAR	Fragment Size	SCAR Primers	Mapping population 1=CM37XT232 2=CO159XT303	Chromosome Bin
(A) *Ht1*				
us23	700bp	F-5'-gca tga cgg tag gat ggg ct-3' R-5'-atc aca tcg tca tca tcg gc-3'	Not polymorphic	
us24	360bp	F-5'ccg cca cca ttc tat tca gc-3' R-5'ggt acg acg aag gct ggc tc-3'	1 and 2	2.07
us30	200bp	F-5' ccc tgc tga gca aca aac tt-3' R-5'-ggt gga gaa gat tgg aga gg-3'	2	2.07
us35	250bp	F-5'-gtg tcg tat cgt gtt tgc-3' R-5'gat aaa cgt acg gtc tcc-3'	2	2.07
Ht2				
us56	200bp	F-5'-gtgctgtgatcatgcggcta-3' R-5'-tgacacacgaggggaagtat-3'	1 and 2	8.06
us60	800bp	F-5'-ctctcgttgatgatgatggatgg-3' R-5'atgctcagtgtcaggacgaa-3'	2	8.05
Ht3				
us62	280bp	F-5'-tgacggcgaatcgaggatgg-3' R-5'-cttcagcacaagcaggaatc-3'	1	7.04
us64	300bp	F-5'-agaggaaggtggttatggag-3' R-5'-gcataaagagagcacacc-3'	Not polymorphic	

Table 2: RIL population partial linkage maps for SCAR markers identified, using NIL pairs for *Ht1*, *Ht2* and *Ht3* (new SCAR markers are indicated in bold, two point linkage data are indicated in cM and LOD scores in brackets)

Ht1 **Chromosome 2.07**		***Ht2*** **Chromosome 8.05/06**		***Ht3*** **Chromosome 7.04**	
umc98a		bnl12.30a		bnl6.27	
	6.5 cM (5.62)		5.5 cM (5.30)		14.4 cM (3.34)
umc22		**us60**		**us62**	
	0.0 cM (10.21)		6.3 cM (5.87)		2.5 cM (9.41)
us30		csu31		bnlg1666	
	2.9 cM (8.02)		4.4 cM (7.13)		2.4 cM (9.69)
umc125a		idh1		bnl5.21a	
	0.0 cM (11.11)		1.3 cM (9.99)		1.1 cM (12.03)
us24		**us56**		bnl8.02	
	1.4 cM (9.42)		8.5 cM (4.76)		
umc122		umc48a			
	0.0 cM (11.41)				
us35					
	1.5 cM (8.55)				
bnl6.20					

CHAPTER 5

Using 9 PCR markers to identify common introgressed regions for 16 lines with *Htn1* resistance.

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With 1 table and 3 figures

Abstract

Nine PCR markers were used to identify a common introgressed region in 16 *Htn1* resistant lines. The nine markers consisted of 5 SCAR markers, 3 converted RFLP markers and 1 microsatellite marker. Not one of the markers was present in all the resistant lines. Fourteen of the 16 lines did, however, share a common introgressed region between the markers us3 and us5. A further common introgressed region between 11 of the inbred lines was found between the markers us14 and asg17. This could indicate the existence of two *Htn1* genes in close proximity or that some lines have lost the *Htn1* gene.

Introduction

Northern corn leaf blight (NCLB) caused by *Setosphaeria turcica* is a major disease of maize and occurs in Africa in most regions where cool to moderate temperatures and moist conditions prevail during the growing season (Carson 1995, Adipala et al. 1993). Gevers (1975) identified *Htn1*, a single dominant gene resistance to NCLB, which was derived from the Mexican maize race Pepitilla. It differs from the typical chlorotic-necrotic lesion resistance genes *Ht1*, *Ht2* and *Ht3* by extending the period between infection and disease symptom expression and by delaying the onset of sporulation (Gevers 1975, Raymundo and Hooker 1981). In 1993, Simcox and Bennetzen mapped the *Htn1* locus to chromosome 8 (0.8 cM distal to the RFLP marker *umc*117).

In the past, South African plant breeders have often used *Htn1* resistance, because it was effective and easy to incorporate into new lines. The donor parent 11Mex44 was used as a source for *Htn1* resistance and introgressed into a number of susceptible inbred lines by backcrossing (Gevers 1975).

When genes are introgressed from a donor parent, linkage drag should be limited during the introgression phase and the gene should not be lost due to environmental conditions, pathogen availability or epistatic gene effects. Genetic markers could be used in two ways in introgression programs: (1) to select for using markers for the gene that is to be introgressed and (2) to select for (or against) a particular background genotype (Visscher et al. 1996). This enables the breeder to introgress the gene and recover the background genome of the recurrent lines with the minimum number of backcrosses. Visscher et al. (1996) showed through simulation studies that markers or marker haplotypes should cover ~10-20 cM surrounding the estimated gene position, to ensure that the target allele frequency does not decrease in later backcross generations.

Nine PCR markers, covering approximately 34 cM of chromosome 8.05/06, were used in retrospect to determine if a common introgressed region in 16 *Htn1* resistant lines could be identified. Not one marker was common in all the resistant lines. However, fourteen lines did contain a common introgressed region between the markers us3 and us5.

Material and Methods

Plant material and DNA isolation

Seed of two pairs of NILs (B73/B73^{7}.*Htn1*, K64R/K64R^{3}.*Htn1*) and the donor line 11Mex 44 were obtained from seed company Sensako (Delmas, South Africa). Six publicly available *Htn1* resistant lines were obtained from the ARC-Grain Crop Institute (Potchefstroom, South Africa) and six *Htn1* inbreds from Quality Seed (Scottsvile, South Africa). As control for *Htn1* resistance the chromosome marker 808C (W22*Htn1*/*Htn1*) was obtained from the Maize Genetics Cooperation-Stock Center.

Seedlings were grown under greenhouse conditions and leaves of 6 week old plants were harvested and freeze dried. The protocol described in the CIMMYT Applied Molecular Genetics Laboratory Manual based on the method by Saghai-Maroof et al. (1984), was used in DNA extractions.

Nine PCR markers

The nine markers consisted of 5 SCAR markers, 3 converted RFLP markers and 1 microsatellite. The 5 SCAR markers identified by van Staden et al. (Chapter 3) were used. RFLP probes received from the University of Missouri, Columbia, Mo., USA were sequenced and two 20 bp primers were commercially synthesized for each probe and microsatellite primer sequences were obtained from the Maize Database website (http://www.agron.missouri.edu).

PCR amplifications were carried out in a PCR Express thermal cycler (Hybaid) using 25-50 ng genomic DNA, 0.5 U Amplitaq or Amplitaq Gold™ DNA polymerase (Perkin Elmer) 100 µM of each dNTP, 1.5 mM $MgCl_2$ and 30 ng of each primer in a 20 µl PCR reaction volume. The forward primers of the three markers us1, us6 and bnlg666 were radioactively labelled at the 5'-end using 1 µCi [γ-^{33}P]ATP and 1 U FPLCpure™ T4 Polynucleotide Kinase (Pharmacia) in 1X One-Phor All PLUS buffer at 37°C for 1h. The labelling reaction was terminated by heat inactivation at 68°C for 10 min. The PCR profile used for amplification was as follows: one cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 52°C-60°C for 20 sec, 72°C for 2 min and one cycle at 72°C for 10 min. All amplification products that were not radioactively labelled were electrophoresed at 80V for 1-2 h in a 1.5-2.5% agarose gel prepared with 1X TBE buffer containing ethidium bromide (1/10 000 dilution of a 50mg/ml solution). The products were visualised by illumination with ultraviolet light. Radioactively labelled amplification products were loaded on a 4% acrylamide/bisacrylamide 19:1, 7.5 M urea and 1X TBE gels and run at 60 Watts for approximately 1 h. The gels were dried on 3MM Whatman chromatographic paper using a gel drier and exposed to x-ray film overnight.

Linkage analysis

Linkage analysis was performed with the software package MAPMAKER/EXP version 3.0b (Lander et al. 1987). The data files used in linkage analysis with the RIL populations were obtained from the Maize Database website

Results

Nine PCR markers that cover an area of approximate 34 cM on maize chromosome 8.05/06, amplified polymorphic fragments associated with *Htn1* resistance. These markers are listed in Table 1 with their primers, annealing temperatures and type. The nine markers included 5 SCAR markers (us1, us3, us5, us6, us14) developed by van Staden et al. (Chapter 3), 3 converted RFLP markers and 1 microsatellite from the maize database.

The 9 markers were tested on 18 maize inbred lines of which 16 possessed *Htn1* resistance. This was done to determine if a common introgressed region could be identified. Figure 1A-C shows a schematic presentation of the introgressed regions identified in these lines. The nine PCR markers are given in linear map order determined by multipoint linkage analysis (van Staden et al. Chapter 3).. Two-point linkage distances between markers are indicated. Markers in brackets are closely linked to the primary markers and were used alternatively for two-point linkage analysis.

The two NIL pairs used by van Staden et al. (Chapter3), the original donor line 11Mex44 and the chromosome marker from the maize stock center are shown in Figure 1(A). The markers indicate a crossover position in the nearly isogenic line B73-*Htn1* on the proximal end of the introgressed region between the markers us1 and bnlg666 and distally between us14 and asg17. The donor line, 11Mex44, the chromosome marker 808C and the nearly isogenic line K64R*3/*Htn1* had introgressed regions that range beyond the region spanned by the markers. Not one of the markers associated with *Htn1* resistance were found on the two susceptible lines (B73 and K64R).

All six inbred lines from the ARC, indicated in Figure 1(B) had introgressed regions

distal to the microsatellite marker bnlg666. Although a number of cross-over events have taken place in these lines, all six inbred lines had a common introgressed area between us3 and us5

The six lines from Quality seed, shown in Figure 1(C), had introgressed regions distal to us1. Four of the inbred lines had a common introgressed region between the markers us3 and us5. One of the inbreds QMI8 505-6 did not show any introgressed region with the markers.

Discussion

The donor line 11Mex44, the chromosome marker 808C and the nearly-isogenic line K64R*3/*Htn1* had the largest introgressed region of the lines tested and ranged beyond the span of the markers. The other inbred lines had introgressed regions distal to the microsatellite marker bnlg666 indicating that the *Htn*1 gene must be distal to this marker. Eight of the inbreds had introgressed regions that stretched beyond the marker asg17. More markers are needed distal to asg17 to identify all cross-over events in these inbred lines.

One of the inbred lines, QMI8 505-6, did not reveal any marker associated with resistance. This line has a Mo17 background, which is known to contibute quantitative trait loci, involved in resistance to *Setosphaeria turcica* (Adipala et al. 1993, Carson 1995, Dingerdissen et al. 1996). This could explain why the line has no introgressed region for *Htn1*.

Of the sixteen *Htn1* resistant inbred lines used, 14 had common introgresed regions between us3 and us5. The second most common introgressed region (11 of 16 inbreds) was between us14 and asg17. Simcox and Benetzen (1993) mapped the *Htn1* locus to this area (0.8 cM distal to umc117). This suggests that there are either two similar genes in close proximity or that some of the resistant lines tested have lost the *Htn1* gene. However, in this study, not a single marker was identified that was present in all of the resistant lines. The markers will have to be mapped in conjunction with a *Htn1* segregating population to determine the position of the locus.

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Table1: The nine PCR based markers used in this study, primers, type of marker and annealing temperature are indicated.

Marker	Primers	Marker type/ visualise (gel)	Annealing temperature
us1	F-5'gcc agc cac cac tac ata cg-3' R-5'aat gag gct gac cga agt tg-3'	Co-dominant/ acrylamide	58°C
us3	F-5'gtg cca tcc gat gcc g-3' R-5'ggg aaa aca aac ggt cga ac-3'	Dominant/ Agarose	54°C
us5	F-5'tcg tct ggt gtt cgg ttc ag-3' R-5'cag aac act aac cat gcg ag-3'	Dominant/ Agarose	52°C
us6	F-5'tac acc ggc tag gaa acg ag-3' R-5'cgt gaa agg cgt gtc tgc tt-3'	Dominant/ acrylamide	57°C
us14	F-5'gtc ctc ccc gct gtt gta-3' R-5'tgt gtt tac tag cct cct gg-3'	Dominant Agarose	60°C
asg17	F-5'-tgt gag gcc aac tac att gc-3' R-5'-tcc agt gct ata cct gct ga-3'	Co-dominant Agarose	60°C
umc30	F-5'-ctc gcg tga cat ggc tat gg-3' R-5'-acg acg aga gag tga cca cc-3'	Repulsion phase Agarose	57°C
umc48	F-5'-cca acc tct cct ctc tcc ag-3' R-5'-ctt agc ttg ttg cca agt gc-3'	Repulsion phase Agarose	59°C
bnlg666	F-5'-aaa agg caa gta gc tag cat gca ttt gca g-3' R-5'-ggc tca cgt ccg tat cc aaa cca aca-3'	Co-dominant Acrylamide	57°C

Figure 1 (A): Schematic presentation of 9 PCR markers (in linear order with two-point LOD scores) on two NIL pairs (K64R/K64R-*Htn1*, B73/B73-*Htn1*), the donor line 11Mex 44 and chromosome marker 808C. Introgressed regions identified by markers are indicated in grey.

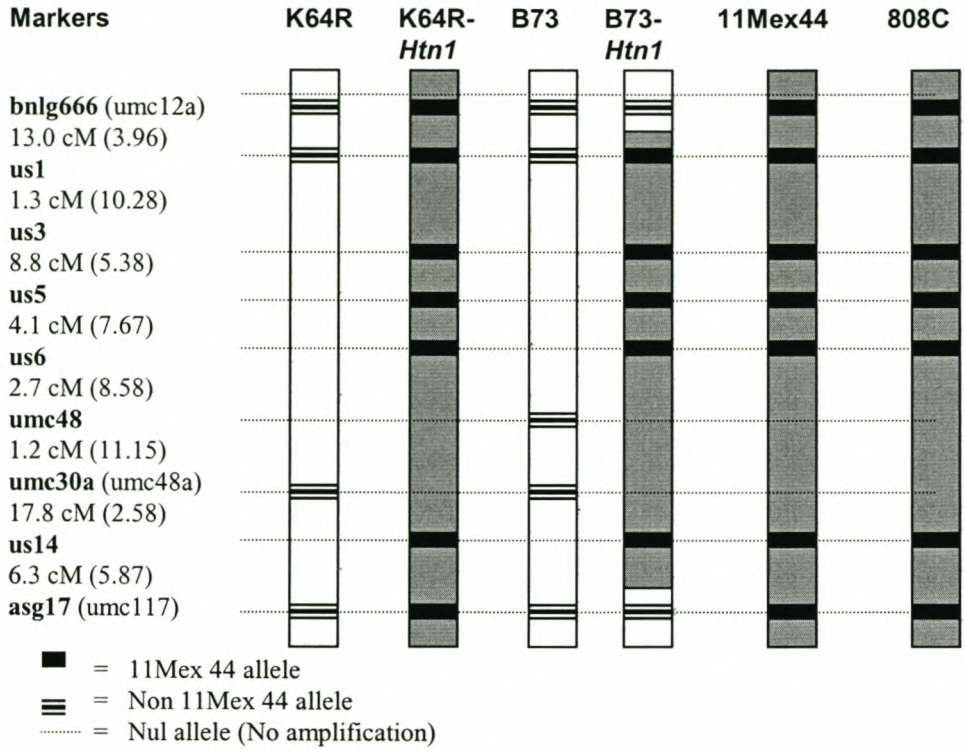


Figure 1 (B): Schematic representation of 9 PCR markers on 6 *Htn1* resistant inbred lines from the ARC-Grain Crop Institute (Potchefstroom, South Africa).

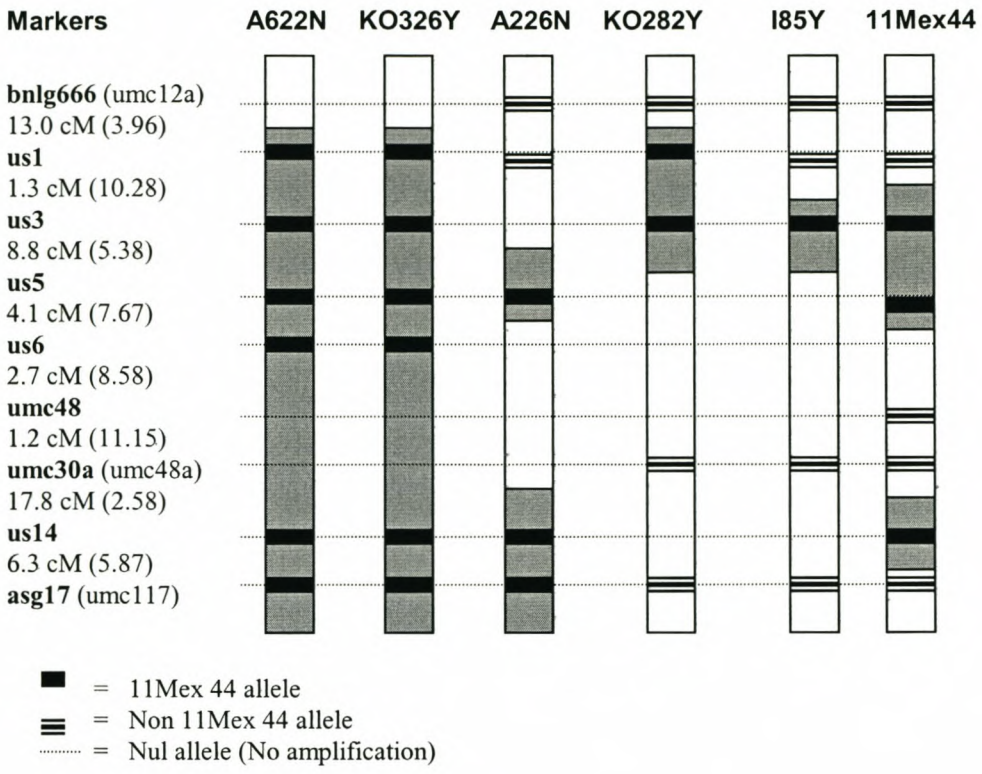
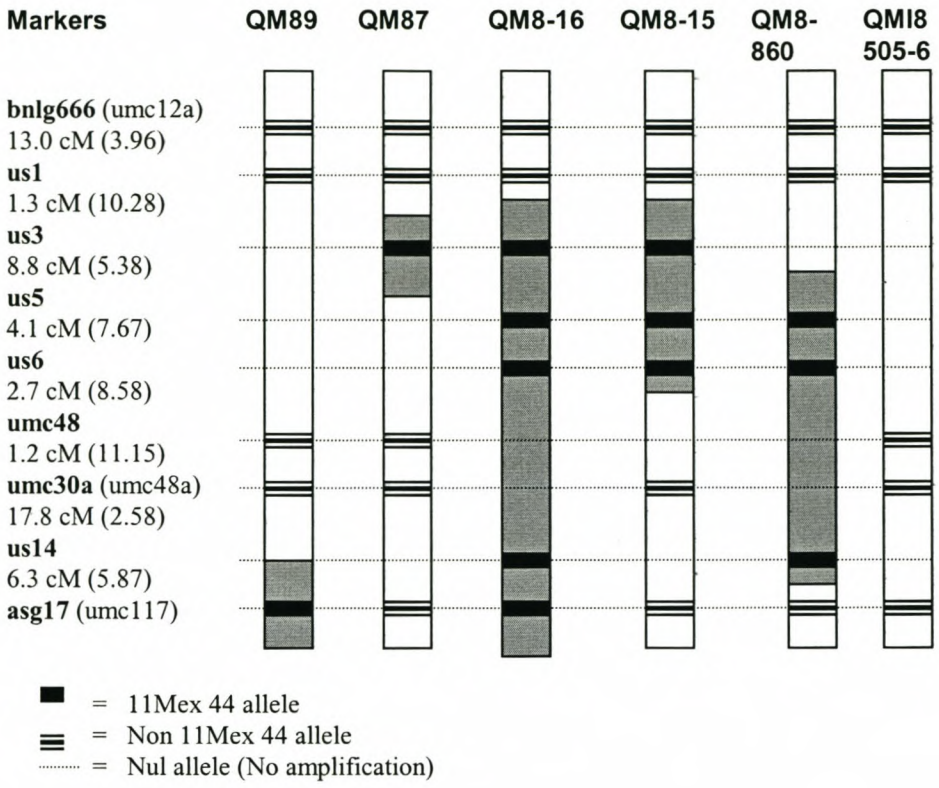


Figure 1 (C): Schematic representation of 9 PCR markers on 6 *Htn1* resistant inbred lines from Quality Seed (Scottsvile, South Africa).



CHAPTER 6

Developing a new simple PCR marker technique that could be used for gene tagging and fingerprinting in maize.

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With 4 tables and 1 figure

Abstract

Different primer combinations were used to develop a new type of marker technique that is easy, repeatable, highly polymorphic and targets genic regions. First, a modified AFLP approach was investigated using a rare cutter (*HindIII*) in combination with a MITE (Hbr element) primer or in combination with a gene analog primer. The second approach was to use a MITE (Hbr) primer as an anchor in combination with gene analog primers. This was found to be a highly polymorphic marker technique that should be useful for fingerprinting and gene tagging. Amplifications occur from undigested genomic DNA and the primers mainly amplify fragments from genic regions. This technique produced a highly polymorphic, repeatable banding pattern.

Introduction

In recent years a number of DNA "fingerprinting" techniques have been developed that can be used to identify markers linked to important genes such as disease resistance loci. The most commonly used high volume marker techniques currently available are the random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and the amplified fragment length polymorphism (AFLP) techniques (Vos et al. 1995). A number of modifications of the AFLP technique have been developed such as sequence-specific amplified polymorphism (S-SAP) where one of the standard AFLP primers are substituted by a primer complementary to a specific sequence. Waugh et al. (1997) used the long terminal repeat (LTR) region of the Bare-1 Ty1-Copia retrotransposon in barley and Van den Broeck et al. (1998) and Casa et al. (2000) used transposon elements as anchors in a modified AFLP procedure called

transposon display (TD). Efforts are directed towards the development of a marker technique that amplifies fragments preferentially in genic regions. Two classes of DNA sequences that appear to be associated with genes in maize are gene analogs and MITEs.

Almost all resistance genes, isolated from plants, can be placed to three classes based on the regions they share, such as those involved in nucleotide binding (nucleotide binding site, NBS), protein-protein interaction (leucine rich repeat, LRR), or intracellular signalling (kinase domain). The NBS-LRR group contains both leucine rich repeats and a nucleotide binding site and includes *Arabidopsis RPS2*, *RPM1* and *RPP5* genes (Bent et al. 1994; Mindrinos et al. 1994; Grant et al. 1995; Parker et al. 1997), the tobacco *N* gene (Whitman et al. 1994), the flax *L6* and *M* genes (Lawrence et al. 1995; Anderson et al. 1997) and the tomato *Prf* gene (Salmeron et al. 1996). The LRR group contains a LRR domain but no NBS and includes the tomato *Cf* genes (Jones et al. 1994; Dixon et al. 1996). The kinase group contains a serine-theonine kinase (STK) domain, such as in the tomato *Pto* gene (Martin et al. 1993) or is associated with a receptor domain resulting in a receptor-like kinase structure, such as in the rice *Xa21* gene (Song et al. 1995).

In recent studies, PCR primers based on short stretches of amino acids conserved among NBS-LRR resistance proteins were used to amplify resistance gene-like sequences in maize (Collins et al. 1998), potato (Leister et al. 1996), pepper (Pflieger et al. 1999), rice, barley and wheat (Chen et al. 1998). Resistance gene analogs (RGA) fingerprinting only produces (1 to 5) fragments on agarose-gel electrophoresis (Leister et al. 1996; Pflieger et al. 1999), whereas numerous PCR-amplified products could be detected by high-resolution electrophoresis (Chen et al. 1998). Problems associated with RGA's are the high degree of degeneracy of the primers and the fact that at least two conserved areas are necessary for amplification in an unknown gene.

Many genes of flowering plants and especially grasses harbour miniature inverted repeat transposable elements (MITEs). With plant database searches, (nucleic- and amino-acid-level searches of Gen Bank and EMBL databases) a number of MITE families have been found: Tourist (Bureau and Wessler 1992; Bureau and Wessler

1994a), Stowaway (Bureau and Wessler 1994b), Alien (Pozueta-Romero et al. 1996), Emigrant (Casacuberta et al. 1998), Bigfoot (Charrier et al. 1999) Hb2 (Spell et al. 1988) and Hbr (Zhang et al. 2000). These elements have structural but not sequence similarities, they are relatively short sequences (125-500 bp), have terminal inverted repeats, and have target site preference and high copy numbers. Their target-site preference and high copy number serve to distinguish MITEs as a unique group from other elements such as Ds1 (Zang et al. 2000). Zhang et al. (2000) found that the Hbr-element is a highly conserved element, that the loci are highly polymorphic between maize inbreds and that the polymorphisms are preferentially associated with maize genic regions. So far no direct evidence has been found for transposition of the Hbr family. Casa et al. (2000) proposed that MITEs are ideal anchors in genes for a new class of markers.

The aim of this study was to propose a new marker technique that could be used in gene tagging and fingerprinting in maize. The Hbr element was used as a anchor in combination with AFLP primers as described by Casa et al. (2000) to amplify fragments from maize DNA. Furthermore, gene analogue primers were used in combination with AFLP primers and finally MITE primers were used as anchores in combination with gene analog primers. The latter was found to produce the best fragments for a new type of marker technique.

Material and Methods

Plant material and DNA Isolation

Seed of the following maize near isogenic lines B73, B73*7-*Ht1*, B73*6-*Ht1* and A619, A619-*Ht1* was obtained from Sensako (Delmas, South Africa). Leaves of 6 week old plants were harvested and freeze dried. The protocol described in the CIMMYT Applied Molecular Genetics Laboratory Manual, based on the method by Saghai-Maroof et al. (1984), was used for DNA extractions.

MITE primer

The MITE primer that was used for analysis in this study came from the Hbr MITE sequence and was only used in the forward direction. The sequences were the same as those used by Casa et al. (2000) : 5'- GAT TCT CCC CAC AGC CAG ATT C-3".

Gene Analog Primers

Six resistance gene analog primers described by Collins et al. (1998) and two of Aarts et al. (1998) were used. These primers are listed in Table 1.

Modified AFLP technique

AFLP analysis was performed as described by Zabeau and Vos (1993) with some modifications. Total genomic DNA of maize (0.2-3 μg) was digested with only one restriction enzyme *Hind III*. Digestions were carried out with 10 U of each restriction enzyme and 1X One-Phor-All Buffer Plus [100 mM Tris-acetate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech] in a total volume of 50 μl. The reactions were incubated in a 37°C water bath for 1 h.

The 50 μl digested DNA mixture was supplemented with 10 μl adapter/ligation solution, containing 5 pmol *HindIII* adapter, 1.2 μl 10 mM ATP, 1X One-Phor-All Buffer PLUS [100 mM Tris-acetate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech] and 1 U T4 DNA Ligase, and incubated overnight at 37°C.

Only the MITE primer or gene analog primers were labelled. For 10 PCR reactions, 1 μl (300ng) of the primer was added to 1 μl 1X One-Phor-All Buffer PLUS [100mM Tris-acetate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech], 1 μl [γ-^{33}P]ATP (25 μCi) and 5 U T4 Polynucleotide Kinase in a total volume of 10 μl and incubated at 37°C for 1 h. The reaction was terminated by placing it in a heating block at 68°C for 10 min.

One μl of the *HindIII* template DNA fragments was added to 100 μM of each dNTP, 1.5 mM $MgCl_2$, 1 X buffer, 0.5 U Taq DNA polymerase (AmpliTaq, Perkin Elmer), 30 ng labelled primer and 60 ng *HindIII* primer with one selective nucleotide A, C, G or

T in total volume of 20 μl. Amplification was performed in a PCR Express thermal cycler from Hybaid. The cycle profile used for amplification was as follows: one cycle of 72°C for 1 min, one cycle of 94°C for 2 min, followed by 12 cycles of 94°C for 20 sec, 60°C for 30 sec, 72°C for 2 min, followed by 25 cycles of 94°C for 20 sec, Tm of gene analog primer for 30 sec, 72°C for 2 min and one cycle at 72°C for 30 min.

Formamide loading buffer (10 μl) was added to each amplified sample. The reactions were denatured at 90°C for 5 minutes in a heating block and quickly chilled on ice. Four μl of each sample was loaded on 4% acrylamide/bisacrylamide (19:1), 7.5 M urea and 1 X TBE gels and run at 60 Watts for approximately 2 h. The gels were dried on 3MM Whatman chromatographic paper using a gel drier and exposed overnight to x-ray film (Biomax MR, Kodak).

MITEs in combination with gene analog primers

Marker analysis was performed by end-labelling 30 ng of the MITE primer (Hbr-F) with 10 U of [γ^{33}P] ATP (25μCi) using 5 U of T_4 Polynucleotide Kinase (USB Corporation, Pharmacia Biotech), 1x of One-Phor-All Buffer [100mM Tris-aceate (pH7.5), 100 mM Mg-acetate, 500 mM K-acetate] (USB Corporation, Pharmacia Biotech) in a total volume of 10μl. Labelling was performed in a heating block at 37° for 1 h followed by a heat inactivation step at 65°C for 15 min.

RGA primers, which were used in the amplification reactions, are listed in Table 1. Optimised PCR reactions were performed in a reaction mixture of 20 μl containing: 50 ng of genomic DNA (RIL individuals and parents), 1 μl of labelled MITE primer, 90 ng of degenerate RGA primer, 100 μM of each dNTP (Gibco, BRL, Life Technologies) 0.5 U of *AmpliTaq*® DNA polymerase (Perkin Elmer), 2 mM $MgCl_2$ and 1/10 PCR Buffer II [100 mM Tris-HCl, pH8.3 (at 25ºC); 500 mM KCl] (Perkin Elmer). By gently pipetting, the components were mixed. The cycling profile started with a denaturing step at 94ºC for 5 min followed by 35 cycles [30 sec at 94ºC, 30 sec at Tm (see Table 2) and 2 min at 72ºC] followed by a final elongation step of 10 min at 72ºC. All amplification reactions were performed in a 9700 Perkin-Elmer GeneAmp

PCR system thermocycler. Eight primer combinations were employed to detect polymorphisms between the nearly-isogenic lines and the B73 and A619 genotypes.

Electrophoresis

The PCR samples were mixed with half the volume of loading dye (98% Formamide, 10 mM EDTA, 0.025% Bromophenol blue and 0.025% Xylene cyanol). Amplification products produced by the 8 different primer combinations were denatured by incubation at 90ºC 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 90 min to 2 hours at 60W using a Model S2 Sequencing Gel Electrophoresis Apparatus (Life Technologies, GIBCO BRL). The gel was transferred to 3MM Whatmann chromatography paper and dried on a gel drier for 90 min at 80ºC. Gels were exposed to Kodak BioMax MR film for 1-2 days.

Results

Hbr primer in combination with AFLP primers

Casa et al. (2000) used two Hbr primers in a nested primer approach for their transposon display. This minimizes the fuzzy bands caused by the AFLP-AFLP primer amplifications when using a frequent cutter like *MseI* for modified AFLPs. In this study it was found that one Hbr primer could be used in combination with a rare cutter like *HindIII* for a modified transposon display. Using a rare cutter instead of a frequent cutter creates larger fragments that cause less competition between the AFLP-AFLP primers, which in turn reduces most of the fussy bands.

A summary of the fragments amplified on the near isogenic lines using the Hbr MITE primer in combination with the 4 HindIII primers is given in Table 3. In total 122 fragments were amplified, of which only two were polymorphic in the *Ht1* resistant lines. Between the B73 and A619 genotypes, 23 and 34 polymorphic fragments were found, respectively. This gave a percentage of 47% (57/122) polymorphic fragments.

Resistance Gene Analog (RGA) primers in combination with AFLP primers

Gene analog primers have a number of degenerate nucleotides in the primer to account for the possibilities of the codon translations. When these are used as anchors in combination with AFLP primers fussy bands are produced (Data not presented). This is therefore not a good marker system. If sequence specific primers from known genes are used this problem is overcome.

Hbr primer in combination with gene analog primers

Eight RGA primers were used in combination with the Hbr anchor primer to determine if these primer combinations could be used as a marker technique in maize. Table 4 is a summary of the fragments that were amplified on the genomic DNA of the NIL pairs. The Hbr primer in combination with the gene analogs gave highly repeatable banding patterns containing between 10 and 30 amplified fragments. In total 154 fragments were scored of which 51 were polymorphic (33%) between the inbred genotypes B73 and A619. Six polymorphic fragments were found in the resistant *Ht1* lines and one polymorphism was common in two of the *Ht1* resistance lines. This marker, identified with the primer combination Hbr/WMA1-2-R, was sequenced and converted into a simple PCR marker for *Ht1*. When the Hbr-F primer was used in combination with the reverse primer, developed from the sequenced fragment, a co-dominant marker was detected. The sequenced fragment was compared to the sequence of the original Hbr-hm1-element to determine whether the part of the Hbr element that was amplified was conserved. Fig 1 shows an alignment of the fragment sequence, cloned from the Hbr/WMA1-2-R amplification, with the original Hbr element that was found in *Hm1* by Zhang et al. (2000). Between the sequence alignments of the original Hbr element from *Hm1* and the fragment Hbr/WMA1-2-R a number of deletions and insertions were found.

Discussion

The use of the Hbr element in combination with gene analog primers produced the best profiles for a new marker technique. The fingerprints could be assayed with great ease, the method was inexpensive and the amplified polymorphic fragments were reliable and repeatable. This is a simple marker technique where DNA does not need to be digested or adapters ligated. The fragments do not only serve as

markers but can also be candidate genes, because of the genic areas that these primer sets target. Using the pairs of *Ht1* NILs, a marker/candidate gene was identified for the *Ht1* locus.

The presence or absence of the MITE element and deletions and insertions in the DNA sequence cause the observed polymorphisms. This marker technique approach could be used to develop a functional linkage map of maize, where the markers are not just markers but also functional candidate genes. Primers from gene sequences found in other plants such as rice and *Arabidopsis* can be used to identify similar genes in maize. MITEs are more stable and more abundant than other transposable elements. Although they do not alter the gene function, their sequences can be used in a number of ways to identify genes not previously mapped in the maize genome.

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Table 1: Resistant gene analog primers used.

Primer	*Source*	*Sequence 5'-3'*
RG1-F	Aarts et al. (1998)	GGI ATG GGI GGI GTI GGI AAR CAN ACN
RG1-R	Aarts et al. (1998)	NGT NGT YTT ICC IAC ICC ICC CAT ICC
PL1-5-F	Collins et al. (1998)	AAG AAT TCG GNG TNG GNA AAA CAN C
PL1-5-R	Collins et al. (1998)	GTN GTT TTN CCN CAN CCG AAT TCT T
GLPL-F	Collins et al. (1998)	AAC TCG AGA ANG CCA ANG GCA AWC C
GLPL-R	Collins et al. (1998)	GGS TTG CCN TTG GCN TTC TCG AGT T
WMA1-2-F	Collins et al. (1998)	AYR AAN CCN TNW GCC ATC CA
WMA1-2-R	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

Table 2: Annealing temperatures for primers used.

Primer	*Annealing temperature*
RG1-F	54°C
RG1-R	54°C
PL1-5-F	51°C
PL1-5-R	51°C
GLPL-F	51°C
GLPL-R	51°C
WMA1-2-F	51°C
WMA1-2-R	51°C

Table 3: Summary of the fragments that were amplified on the NIL pairs with the 4 *HindIII* primers in combination with the specific Hbr-F primer.

	Total	**Polymorphic fragments on Ht1 lines**	**B73 specific fragments**	**A619 specific fragments**
Hbr/Hind-PA	36	1	5	9
Hbr/Hind-PC	20	0	5	9
Hbr/Hind-PG	28	0	7	5
Hbr/Hind-PT	38	1	6	11
	122	2	23	34

Table 4: Summary of the fragments that were amplified from genomic DNA of the NIL pairs using the Hbr F primer in combination with the gene analogs primers.

	Total	**Polymorphisms on Ht1 NILs**	**B73 specific fragments**	**A619 specific fragments**
Hbr-F/RG1-F	28	3	7	3
Hbr-F/RG1-R	13	1	2	4
Hbr-F/PL1-5-F	15	0	3	2
Hbr-F/PL1-5-R	17	0	3	4
Hbr-F/GLPL-F	11	0	3	3
Hbr-F/GLPL-R	13	0	4	3
Hbr-F/WMA1-2-F	29	0	2	1
Hbr/WMA1-2-R	28	2	3	4
	154	6	27	24

Fig1: Sequence alignment of the Hbr-F/WMA1-2-R fragment with the original Hbr-element found in hm1 by Zhang et al. (2000).

Hbr-F

Fragment cloned 5'-*GATTCTCCCCACAGCCAGATTC* **TTCCCACAGC**

Zhang et al. (2000). 5'-GATTCTCCCCACAGCCAGATTC

CAGATTCT**TC** CCACA**G**CCAG ATT**C**TTAGAA AAG**T**TGGTCA GAAAAAAGCT

TCT CCACAACCAG ATTTTCAGAA AAGCTGGTCA GAAAAAAGCT

GAACCAAACAG**G**CCCAATATCTTTTTCACATCCGAAATCTCCTTACATATGCA

GAACCAAACAGACCCTAA

GGCAAACCCGTATAAATCTTTCGCAGCATCCACTTCCACCGGACAGAGCATC

AACGGCAAATCCGAGAAATGCATAAATAAACAATTACAATCGAGAAAACAA

GATTTAGAAAACTGC*ACGAATCCTTAGGCCATCCA*-3'

WMA1-2-R

CONCLUSION

In this study a number of contributions were made towards maize research. The main aim of the study was to develop PCR markers for the 4 resistance genes *Ht1*, *Ht2*, *Ht3* and *Htn1*. The AFLP technique was chosen as a high volume marker technique in combination with nearly isogenic lines to identify markers.

It has been reported that enzyme combinations play an important role in the number of polymorphisms observed in AFLP analysis (Ridout and Donini 1999). The results of this study indicated that the enzyme combination *MluI/MseI* is a better choice than *PstI/MseI*, which is normally used in maize. Both *PstI* and *MluI* are 6 base restriction enzymes with high GC content and the differences detected in the total number of fragments and percentages of polymorphisms could be due to methylation sensitivity. Furthermore, it was found that when more than two restriction enzymes were used in AFLP analysis, the total number of fragments were reduced as expected. No differences could, however, be observed in the percentage of polymorphisms. The number of polymorphic fragments could probably be increased by digestion with restriction enzymes after PCR amplification in a cleaved-amplified polymorphic sequence (CAPS) AFLP approach.

No segregating population was available for this study, hence nearly isogenic lines were used in combination with the AFLP technology to identify markers. It was anticipated that polymorphic markers common in resistant or susceptible lines of two NIL pairs would be from the target area selected by the breeders. The polymorphic fragments from a single NIL pair have a 25-50% chance of mapping to unlinked regions. In this study all markers that were common between two resistant or susceptible lines from a given gene mapped to a single region in the genome using commercially available RIL mapping populations. This shows that two NIL pairs can be used instead of segregating populations to map single genes.

Using the *Htn1* NIL pairs, 15 AFLP markers were identified of which 7 were converted to polymorphic SCAR markers. Only 5 SCAR markers could be mapped. All of the markers mapped to chromosome 8.05/06. The inefficient conversion of AFLPs to polymorphic sequence specific PCR markers has also been reported by

Shan et al. (1999). This restricts the development of simple PCR markers for genes in maize. Using AFLPs with two NIL pairs, the *Htn1* introgressed region was mapped to the same region reported by Simcox and Bennetzen (1993) who used a segregating population. Sixteen *Htn1* resistant inbred lines were evaluated with 9 PCR markers to determine if they had common introgressed regions. Fourteen had a common introgressed region between the markers us3 and us5. The second most common introgressed region (11 of 16 inbreds) was between the markers us14 and asg17. Simcox and Benetzen (1993) mapped the *Htn1* locus to this area (0.8 cM distal to umc117). This indicates that there are either two similar genes in close proximity or that some of the lines tested have lost the *Htn1* gene. The markers will have to be mapped using a *Htn1* segregating population to determine the gene(s) position.

For *Ht1*, 3 SCAR markers were mapped to chromosome 2.07. This data correlate with Paterson et al. (1965), Bentolila et al. (1991) and Freymark et al. (1993). Two SCAR markers for *Ht2* mapped to chromosome 8.05/06 between flanking markers bnl12.30a (proximal) and umc48a (distal). Zaitlin et al. (1992) and Freymark et al. (1993) mapped the *Ht2* gene to the same region on chromosome 8.05/06. The locus for *Ht3* has not been mapped previously. A SCAR marker was identified using AFLP analysis on DNA from two *Ht3* NIL pairs. Using the RIL populations CM37/T232 of Burr et al. (1988) the SCAR marker (us62) was mapped with high LOD scores on chromosome 7.04. To confirm this mapping data, additional microsatellite markers from bin 7.04 were tested on the *Ht3* NIL pairs. The alleles of one marker, bnlg1666, were common in the resistant lines and differed from those detected in the susceptible lines. This marker was mapped using the same RIL population. SCAR marker us62 and microsatellite marker bnlg1666 mapped within an interval of 2.5cM (LOD score of 9.41) on chromosome 7.04.

This study indicated that introgressed regions for genes can be mapped using two NIL pairs and a commercially available mapping population. The limiting factor for this type of study is that the same donor source should be used. The donor parent should also have enough polymorphic sites compared to the recipient line in the area that one targets. The two NIL pairs approach also only detects fragments in an area were the introgressed regions of different resistant lines overlap. As a result, two

resistant lines with large introgressed regions could overlap only in a small area of the gene making the identification of common markers difficult. The PCR markers developed in this study can be used in the future for fine mapping of the *Ht* genes using segregating populations. Most of the stocks of the different genes have been contaminated and these PCR markers can be used to identify which of the Ht genes are present. Breeding companies that want to pyramid the single dominant genes can effectively use these PCR markers for marker-assisted selection.

A number of strategies were evaluated in order to develop a new marker technique that can be used to identify marker/candidate genes for *Ht1*, *Ht2*, *Ht3* and *Htn1*. The results showed that one needs at least one specific primer that anchors the DNA amplification. MITEs are a new class of transposable elements that have not been shown to transpose and could serve as such an anchor for DNA amplification close to genes. By using resistance gene analog primers in combination with a MITE primer the chances are very good that the fragments detected are from genic regions. The markers that are developed from MITEs in combination with gene analogs can serve as markers and candidate genes. Currently this marker technique is used on the NIL pairs to identify candidate genes for *Htn1*, *Ht1*, *Ht2* and *Ht3*.