Genetic mapping of gray leaf spot resistance genes in maize

by

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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.

Signature: Date:

Summary

Gray leaf spot (GLS) of maize, caused by the fungus *Cercospora zeae-maydis*, can reduce grain yields by up to 60% and it is now recognized as one of the most significant yield-limiting diseases of maize in many parts of the world. The most sustainable and long-term management strategy for GLS will rely heavily on the development of high-yielding, locally adapted GLS resistant hybrids.

Molecular markers could be useful to plant breeders to indirectly select for genes affecting GLS resistance and to identify resistance genes without inoculation and at an early stage of plant development. Only two studies in the USA have examined quantitative trait loci (QTL) association with GLS resistance.

The aim of this study was to map GLS resistance genes in a resistant Seed Co LTD, Zimbabwean inbred line. Molecular markers linked to the GLS resistance QTL were identified by using the amplified fragment length polymorphism (AFLP) technique together with bulked segregant analysis. Eleven polymorphic AFLP fragments were identified and converted to sequence-specific PCR (polymerase chain reaction) markers. Eight of the 11 converted AFLP markers were added to the maize marker database of the University of Stellenbosch.

Five of the 8 converted AFLP markers were polymorphic between the resistant and the susceptible parent. They were amplified on the DNA of 230 plants of a segregating F_2 population and linkage analysis was performed with MAPMAKER/EXP. Two linkage groups consisting of two markers each, with a linkage distance of 10.4 cM (LOD 22.83) and 8.2 cM (LOD 55.41) between the two markers, were identified. QTL mapping with MAPMAKER/QTL confirmed the presence of QTL in both linkage groups.

Two publicly available recombinant inbred families (Burr *et al.*, 1988) were used to localize the converted AFLP markers on the genetic map of maize. The QTL, which were identified with the AFLP markers, were mapped to chromosomes 1 and 5. Another AFLP marker was mapped to chromosome 2 and a further to chromosome 3.

To obtain more precise localizations of the QTL on chromosomes 1 and 5, sequence-tagged site markers and microsatellite markers were used. The markers were amplified on the DNA of the 230 plants of the F_2 population and linkage analysis was performed with MAPMAKER/EXP. The order of the markers was in agreement with the UMC map of the Maize Genome Database. Interval mapping using MAPMAKER/QTL and composite interval mapping using QTL Cartographer were performed. The QTL on chromosome 1 had a LOD score of 21 and was localized in bin 1.05/06. A variance of 37% was explained by the QTL. Two peaks were visible for the QTL on chromosome 5, one was localized in bin 5.03/04 and the other in bin 5.05/06. Both peaks had a LOD score of 5 and 11% of the variance was explained by the QTL.

To test the consistency of the detected QTL, the markers flanking each QTL were amplified on selected plants of two F_2 populations planted in consecutive years and regression analysis was performed. Both the QTL on chromosome 1 and the QTL on chromosome 5 were detected in these populations. Furthermore, the presence of a QTL on chromosome 3 was confirmed with these populations. A variance of 8 -10% was explained by the QTL on chromosome 3.

In this study, a major GLS resistance QTL was thus mapped on chromosomes 1 and two minor GLS resistance QTL were mapped on chromosomes 3 and 5 using a resistant Seed Co LTD, Zimbabwean inbred line. Markers were identified which could be used in a marker-assisted selection program to select for the GLS resistance QTL.

Opsomming

Grys blaarvlek (GBV) van mielies, veroorsaak deur die swam *Cercospora zeae-maydis*, kan graanopbrengs met tot 60% verlaag en word beskou as een van die vernaamste opbrengs-beperkende siektes wêreldwyd. Die toepaslikste langtermyn stragtegie vir GBV beheer sal wees om plaaslike mieliebasters met hoë opbrengs en GBV weerstand te ontwikkel.

Molekulêre merkers kan nuttig deur plantetelers gebruik word om weerstandsgene te selekteer. Seleksie is moontlik in die afwesigheid van inokolum en op 'n vroeë stadium van plant ontwikkeling. Slegs twee vorige studies (in die VSA) het kwantitatiewe-kenmerk-lokusse (KKL), vir GBV-weerstand ondersoek.

Die doel van hierdie studie was om die GBV weerstandsgene in 'n weerstandbiedende ingeteelde lyn (Seed Co BPK, Zimbabwe) te karteer. Molekulêre merkers gekoppel aan die GBV weerstands KKL is geïdentifiseer deur gebruik te maak van die geamplifiseerde-fragmentlengte-polimorfisme-(AFLP-) tegniek en gebulkte-segregaat-analise. Elf polimorfiese merkers is geïdentifiseer en omgeskakel na volgorde-spesifieke PKR (polimerase kettingreaksie) merkers. Agt van die elf omgeskakelde AFLP-merkers is by die mieliemerker databasis van die Universiteit van Stellenbosch gevoeg.

Vyf van die 8 omgeskakelde AFLP-merkers was polimorfies tussen die bestande en vatbare ouers. Hulle is geamplifiseer op die DNA van 230 plante van 'n segregerende F_2-populasie en is gebruik in 'n koppelingstudie met MAPMAKER/EXP. Twee koppelingsgroepe, elk bestaande uit twee merkers, met onderskeidelik koppelingsafstande van 10.4 cM (LOD 22.83) en 8.2 cM (LOD 55.41) tussen die merkers, is geïdentifiseer. KKL-kartering het getoon dat KKL in albei koppelingsgroepe aanwesig is.

Twee kommersieël beskikbare, rekombinant-ingeteelde families (Burr *et al.*, 1988) is gebruik om die omgeskakelde AFLP-merkers op die mielie genetiese kaart te plaas. Die KKL wat met die AFLP-merkers geïdentifiseer is, is gekarteer op chromosome 1 en 5. 'n Verdere AFLP-merker is op chromosoom 2 gekarteer en 'n ander op chromosoom 3.

Ten einde die KKL op chromosome 1 en 5 meer akkuraat te karteer, is volgorde-ge-etikeerde en mikrosatelliet merkers gebruik. Die merkers is geamplifiseer op die DNA van die 230 plante van die F_2-populasie en koppelings-analises is uitgevoer. Die volgorde van die merkers was dieselfde as die van die UMC-kaart in die Mielie Genoom Databasis. Interval kartering met MAPMAKER/QTL en komposiet interval kartering met QTL Cartographer is uitgevoer. Die KKL op chromosoom 1 het 'n LOD-telling van 21 gehad en is in bin 1.05/06 geplaas. Die KKL was verantwoordelik vir 37% van die variansie. Twee pieke was onderskeibaar vir die KKL op chromosoom 5, een in bin 5.03/04 geleë en die ander in bin 5.05/06. Elke piek het 'n LOD-telling van 5 gehad en die twee KKL was verantwoordelik vir 11% van die variansie.

Om die herhaalbaarheid van die effek van die KKL te toets is die merkers naaste aan elke KKL geamplifiseer op geselekteerde plante van twee F_2-populasies wat in opeenvolgende jare geplant is. Regressie analise is op die data gedoen. Beide die KKL op chromosoom 1 en die KKL op chromosoom 5 kon in hierdie populasies geïdentifiseer word. Verder kon die aanwesigheid van 'n verdere KKL op chromosoom 3 in hierdie populasies bevestig word. Laasgenoemde KKL was verantwoordelik vir 8-10% van die totale variansie.

In hierdie studie is daar dus 'n hoof GBV-weerstands KKL gekarteer op chromosoom 1 en twee kleiner GBV-weerstands KKL gekarteer op chromosome 3 en 5. Merkers is geïdentifiseer wat moontlik in merker-gebaseerde-telingsprogramme gebruik kan word om plante te selekteer wat die GBV-weerstands KKL het.

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Abbreviations

β	beta
°C	degrees Centigrade
γ	gamma
μCi	microcurie
μg	microgram
μl	microliter
μM	micromolar
A	adenosine
AFLP	amplified fragment length polymorphism
ASAP	allele-specific associated primer
ATP	adenosine triphosphate
1997 bulk(s)	bulk(s) of the 1997 F_2 population
1998 bulk(s)	bulk(s) of the 1998 F_2 population
B97R	resistant bulk of the 1997 F_2 population
B97S	susceptible bulk of the 1997 F_2 population
B98R	resistant bulk of the 1998 F_2 population
B98S	susceptible bulk of the 1998 F_2 population
BC	backcross
bp(s)	base pair(s)
BSA	bulked segregant analysis
C	cytosine
CAPS	cleaved amplified polymorphic sequence
cM	centiMorgans
CIM	composite interval mapping
CTAB	N-cetyl-N, N, N-trimethyl ammonium bromide
ddH_2O	double distilled water
DH	double haploid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
et al.	et alibi
G	guanosine
GLS	gray leaf spot
h	hour(s)
HCl	hydrochloric acid
i.e.	that is
IM	interval mapping
K	Potassium

Abbreviations (cont.)

kb	kilobase
LB	"Luria Bertani"
LOD	log of the ratio of the likelihoods
M	molar
MAS	marker-assisted selection
mg	milligram
Mg	magnesium
$MgCl_2$	magnesium chloride
min	minute(s)
ml	milliliter
mM	millimolar
NaCl	sodium chloride
NaOAc	sodium acetate
ng	nanogram
NH_4	ammonium
NH_4OAc	ammonium acetate
$(NH_4)_2SO_4$	ammonium sulfate
NIL(s)	nearly isogenic line(s)
no.	number
P1	resistant parent
P2	susceptible parent
PCR	polymerase chain reaction
QTL	quantitaitve trait locus/loci
QTL1	QTL on chromosome 1
QTL3	QTL on chromosome 3
QTL5	QTL on chromosome 5
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RIL(s)	recombinant inbred line(s)
RNase	ribonuclease
SSR	simple sequence repeat polymorphism
STS	sequence-tagged site
T	thymidine
Taq	*Thermus aquaticus*
TBE	89 mM Tris-borate and 2.5 mM EDTA, pH 8.3
TE	10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	unit(s)
V	volts

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Chapter 1
Introduction

1.1 GLS (gray leaf spot) disease in maize

Gray leaf spot (GLS) of maize, caused by the fungus *Cercospora zeae-maydis* (Tehon and Daniels, 1925), has become a major threat throughout the maize growing regions of the United States during the past decade and appears to be increasing each year (Wang *et al.*, 1998 and Ward *et al.*, 1999). In South Africa, the disease was first observed in KwaZulu-Natal in 1988 and has since spread rapidly to neighbouring provinces and countries. During the 1990 to 1991 growing season the first economic losses caused by *C. zeae-maydis* were reported in South Africa (Ward *et al.*, 1999). GLS can reduce grain yields by 30 to 60%, depending on hybrid susceptibility and favorable weather conditions and it is now recognized as one of the most significant yield-limiting diseases of maize in many parts of the world (Ward *et al.*, 1999).

Symptoms of GLS are normally first observed on the lower leaves (Ward *et al.*, 1999). Lesions first appear as small tan spots that are rectangular to irregular in shape and have chlorotic borders that are more easily discernible when diseased leaves are viewed through transmitted light. Mature GLS lesions are gray to tan in color, sharply rectangular, long and narrow, and run parallel to the leaf veins (Latterell and Rossi, 1983) (Figure 1). The name gray leaf spot was derived from the grayish cast produced by sporulating lesions (Ward *et al.*, 1999).

Figure 1. Maize leaf with GLS lesions

GLS losses occur when photosynthetic tissue is rendered non-functional due to lesions and/or the blighting of entire leaves. The blighting and premature death of leaves limits radiation interception and the production and translocation of photosynthate to developing kernels (Ward *et al.*, 1999). The number of kernels per ear and the kernel size are the two components of yield most affected by GLS epidemics (Ward *et al.*, 1999). In severely infected fields, stalk lesions are common, resulting from the spread of the fungus through leaf sheaths (Latterell and Rossi, 1983). When the leaf lesions cover most of the photosynthetic surface and extreme water loss occurs, the stalks deteriorate and become so weak that lodging precludes mechanical harvesting (Latterell and Rossi, 1983). GLS normally does not begin to develop until several days after flowering and late maturing lines tend to be more resistant than early lines (Bubeck *et al.*, 1993).

If maize residue from a previous crop affected with gray leaf spot is left on the surface and minimum tillage is practiced, the disease is likely to be far more severe the following season, as the pathogen overwinters in infected maize debris (Latterell and Rossi, 1983, Thompson *et al.*, 1987, Elwinger *et al.*, 1990 and Ward *et al.*, 1997). GLS is an extremely environmentally sensitive disease

requiring high humidities and extended leaf wetness (Latterell and Rossi, 1983, Thompson *et al.*, 1987, Donahue *et al.*, 1991, Gevers *et al.*, 1994, Wang *et al.*, 1998 and Ward *et al.*, 1999).

The severity of GLS may be greater in the absence of other foliar pathogens as more tissue is available for the colonization of *C. zeae-maydis*, and the development of maize hybrids with resistance to other pathogens may thus have contributed to the increase in gray leaf spot (Wang *et al.*, 1998).

Wang *et al.* (1998) obtained 91 monoconidial isolates of *C. zeae-maydis* from diseased leaves collected throughout the United States and analyzed them for genetic variability at 111 amplified fragment length polymorphism (AFLP) loci. By using cluster analysis, two very distinct groups of *C. zeae-maydis* isolates were revealed. These results were confirmed by nucleotide sequence differences of the 5.8S ribosomal DNA (rDNA) and the internal transcribed spacer (ITS) regions in the two groups. They found that isolates from the one group, that was most prevalent, were generally distributed throughout the main maize-producing regions of the United States, whereas isolates of the other group were mainly found in the eastern third of the country. Both groups of isolates were present in the same fields at some locations.

Methods to control GLS include the discontinuation of conservation tillage, the use of crop rotation, the application of foliar fungicides, and the use of hybrids with resistance (Latterell and Rossi, 1983, Ward *et al.*, 1997 and Coates and White, 1998). As inoculum can spread between different fields, the effectiveness of crop rotation and conventional tillage for GLS control may depend on the number of growers in the region that utilize these controls (Coates and White, 1998). As it is also important to preserve the economic and environmental advantages of conservation tillage systems, tillage is not a viable control option and crop rotation may not be an effective control (Coates and White, 1998). Foliar applied fungicides provide an effective control (Ward *et al.*, 1997), but may

not be economical for grain production (Coates and White, 1998). Furthermore, the pathogen may develop resistance to the fungicides (Ward *et al.*, 1999). Host resistance is therefore expected to be the most cost-effective, efficient and acceptable control measure (Huff *et al.*, 1988, Ulrich *et al.,* 1990, Gevers and Lake, 1994, Saghai Maroof *et al.*, 1996, Coates and White, 1998 and Ward *et al.*, 1999).

1.2 GLS host resistance

Corn germplasm has been evaluated for the inheritance of resistance to GLS by using diallel analysis (Thompson *et a*l., 1987, Huff *et al.*, 1988, Ulrich *et al.,* 1990, Donahue *et al.*, 1991, Gevers *et al.*, 1994 and Hohls *et al.*, 1995), generation mean analysis (Thompson *et a*l., 1987 and Coates and White, 1998), and statistical modeling of resistance (Elwinger *et al.*, 1990), and by examination of quantitative trait loci (QTL) and restriction fragment polymorphisms (RFLPs) associated with resistance (Bubeck *et al.*, 1993 and Saghai Maroof *et al.*, 1996).

In the diallel analyses both the general combining ability (Huff *et al.*, 1988, Thompson *et al.*, 1987, Ulrich *et al.,* 1990, Donahue *et al.*, 1991, Gevers *et al.*, 1994) and the specific combining ability (Huff *et al.*, 1988, Donahue *et al.*, 1991 and Gevers *et al.*, 1994) were significant for the inbreds under study. Ulrich *et al.* (1990) and Donahue *et al.* (1991) found resistance to be highly heritable and controlled by additive gene action. Thompson *et a*l. (1987) and Huff *et al.* (1988) also concluded that additive gene action was more important than nonadditive gene action for their sets of inbred lines. Both additive and nonadditive genetic effects played a major role in the resistance mechanism in South African maize breeding material (Gevers *et al.*, 1994). Breeding material, presumably

originating from teosinte germplasm, which exhibited levels of resistance due to a major gene, *GLS1*, was also identified (Gevers and Lake, 1994). Hohls *et al.* (1995), using South African inbred lines, found that GLS in maize can be expressed in terms of an additive-dominance model, with dominance almost complete.

Generation mean analysis in the study by Thompson *et al.* (1987) indicated that resistance is not very complex and is mainly controlled by additive gene action. Coates and White (1998) found that a simple additive-dominance model was able to explain the inheritance of resistance for all populations. Dominance effects were detected in all populations, with dominance being significant at early ratings, but not at late ratings. In both studies, the expression of resistance was environmentally dependent.

Statistical modeling of resistance indicated that dominance was important and that a model more complex than simple additivity was required to fully explain inheritance of resistance to gray leaf spot (Elwinger *et al.*, 1990). It was also found that few, rather than many genes controlled the inheritance of resistance to GLS.

Bubeck *et al.* (1993) studied QTL associated with GLS resistance in three populations and by means of 109 RFLP marker loci identified QTL associated with resistance on all maize chromosomes. Only one region on chromosome 2 was associated with GLS resistance in all three populations under study. Some marker-associated effects showed dominance, but most indicated additive gene action. Some of the favorable factors observed originated from the susceptible parent. The QTL effect on GLS resistance was found to be inconsistent over environments. Individual markers in their study accounted for 4-26% of the phenotypic variation.

Saghai Maroof *et al.* (1996) identified five independent QTL. They crossed the inbred Va14 (resistant) with the inbred B73 (susceptible) to obtain a large F_2 population. The plants were scored for GLS disease reaction at six different times throughout the disease season. Each F_2 was selfed to produce a F_2 derived F_3 line. F_3 lines were planted in two separate GLS disease blocks. Seventy-one RFLP markers were used to screen 239 F_2 individuals including those with extreme GLS disease phenotypes. The QTL located on chromosomes 1, 4 and 8 had large effects on GLS resistance and were remarkably consistent across three disease evaluations over 2 years and 2 generations. The QTL on chromosome 1 explained 35-56% of the variance and the QTL on chromosome 4 and 8 explained 8.8-14.3% and 7.7-11%, respectively. Cumulatively, the QTL on chromosome 1, 4 and 8 explained 44-68% of the variance in the different populations. Smaller QTL effects were found on chromosomes 2 and 5 which explained 4.8-7.7% and 5.7% of the variance, respectively. As the QTL on chromosome 5 was not reproducible over replications it was assumed to be a false positive. The resistance QTL were derived from parent Va14, except for the QTL located on chromosome 4, which was from the susceptible parent B73. The QTL on chromosome 1 and 2 appeared to have additive effects, whereas those on chromosome 4 seemed to be dominant and those on chromosome 8 recessive.

Resistant inbred lines are being used in breeding programs to transfer gray leaf spot resistance to susceptible elite lines which are widely used as parents in commercial hybrids. Attempts to transfer resistance from one source to the other have not always been successful (Coates and White, 1998). Difficulties in transferring resistance may be due to the number of genes involved, difficulty in selecting the best genotypes, the evaluation of an insufficient number of families, or a combination of these factors. Furthermore, the development of the disease is highly dependent on environmental effects and it is therefore very difficult to make assessments of the disease for inheritance studies and resistance breeding (Saghai Maroof *et al.*, 1996). Marker-assisted selection programs may

thus be useful to plant breeders to indirectly select for genes affecting GLS resistance, thus selecting desirable individuals on their genotype rather than their phenotype, independent of environmental influence (Berloo and Stam, 1998 and Toojinda *et al.*, 1998).

1.3 MAS (marker-assisted selection)

Marker-assisted selection (MAS) is the method whereby molecular markers enable plant breeders to select indirectly for genes affecting quantitative traits by selecting for molecular markers closely linked to these genes (Berloo and Stam, 1998).

The ability to select desirable individuals in a breeding program based on genotypic configuration is an extremely powerful application of DNA markers and QTL mapping (Young, 1996). Partial resistance loci can be treated as Mendelian factors and manipulated just like any other major gene (Young, 1996 and Yamamoto *et al.*, 1998) and several resistance genes can therefore be pyramided into a valuable genetic background by using marker-assisted selection (Melchinger, 1990, Young, 1996, Qi *et al.*, 1998a, Yamamoto *et al.*, 1998). QTL from diverse donors can be rapidly introduced into a desirable background or deployed in a set of cultivars, resulting in a higher level of resistance (Ragot *et al.*, 1995, Young, 1996 and Qi *et al.*, 1998a). MAS is also useful to check for the resistance genes without inoculation (Melchinger, 1990, Kelly, 1995, Pelsy and Merdinoglu, 1996 and Lübberstedt *et al.*, 1998a) and at an early stage of plant development (Melchinger, 1990, Lübberstedt *et al.*, 1998a,b and Qi *et al.*, 1998b).

By using markers, lines with cross-overs very near to a gene of interest can be selected, thereby reducing "linkage drag" from the donor parent. In a backcross program, markers can also be used to select for lines with minimal donor germplasm in regions unlinked to an introgressed segment, since such chromosomal segments are sometimes associated with undesirable traits (Melchinger, 1990, Tanksley and Nelson, 1996, Young, 1996, Toojinda *et al.*, 1998 and Yamamoto *et al.*, 1998).

Furthermore, desirable alleles from the parent with an otherwise less desirable phenotype can be selected to create new cultivars with phenotypes superior to the parents (Bubeck *et al.*, 1993, Saghai Maroof *et al.*, 1996, Toojinda *et al.*, 1998 and Pernet *et al.*, 1999a). Bernacchi *et al.* (1998) applied an advanced backcross QTL strategy to cultivated tomato using a wild species as the donor parent, and found that a significant portion of the QTL had allelic effects opposite to those of the parents. These agronomically useful and novel alleles would have been overlooked in phenotypic evaluations of exotic germplasm, but could be detected and transferred with a MAS approach.

A prerequisite for MAS is that the initial population is polymorphic for the marker and the gene of interest, and that both are in extreme linkage disequilibrium (Melchinger, 1990). Lande and Thompson (1990) and Berloo and Stam (1998) have investigated the efficiency of MAS compared to conventional phenotypic selection. Both found that the relative efficiency of MAS is greatest for characters with low heritability, if a moderate or large fraction of the additive genetic variance is significantly associated with the marker loci. Lande and Thompson (1990) also found that MAS is very useful to select traits on the basis of their molecular markers when individuals do not express the phenotypic traits of interest, such as before development of the adult phase.

Miklas *et al.* (1996) suggested that a backcrossing program, that utilizes coupling-phase linkages (marker and resistant allele on the same homologous

chromosome or chromatid), may be the most effective way to transfer resistance traits via indirect selection. The utility of the marker-QTL associations for indirect selection would depend on recombination frequency between the loci and the unwanted occurrence or absence of the informative markers in germplasm to which the resistance is to be introgressed. Marker-assisted backcrossing has been applied in barley to introgress stripe rust resistance QTL into a genetic background unrelated to the mapping population (Toojinda *et al.*, 1998).

The efficiency of MAS depends on the consistency of the estimated QTL position and effects across populations (Bohn *et al.*, 1997). Because of the poor consistency of QTL across populations, QTL mapping must be performed in each population separately as a pre-requisite for MAS (Lübberstedt *et al.*, 1998a).

In recent years a number of molecular marker techniques have been developed, making the selection of plants based on molecular markers more efficient. These include, amplified fragment length polymorphism (AFLP; Zabeau and Vos, 1993 and Vos *et al.*, 1995), restriction fragment length polymorphism (RFLP; Botstein *et al.*, 1980), microsatellite or simple sequence repeat polymorphism (SSR; Tautz, 1989) and random amplified polymorphic DNA (RAPD; Williams *et al.*, 1990).

1.4 AFLPs (amplified fragment length polymorphism markers)

The AFLP technique developed by Zabeau and Vos (1993) is based on the selective amplification of genomic restriction fragments using the PCR (polymerase chain reaction, Saiki *et al.*, 1988). DNA is digested with a frequent (e.g. *Mse*I or *Taq*I) and a rare cutter restriction enzyme (e.g. *Eco*RI, *Mlu*I or *Pst*I)

and double-stranded DNA adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. Primers complementary to the adapters and to the restriction sites are designed with two or three selective nucleotides added at the 3' ends of the primers. Restriction fragments will only be amplified if the nucleotides flanking the restriction site correlate with the selective nucleotides. Only one of the primers used in the selective amplification is labeled and therefore, only the restriction fragments containing the primer site of the labeled primer are detected. The amplified fragments are analyzed by denaturing gel electrophoresis. To visualize the fragments, the primers can either be labeled with radioactivity (Vos *et al.*, 1995) or fluorescence (Hartl and Seefelder, 1998), or the fragments can be detected by silver staining the polyacrylamide gel (Cho *et al.*, 1996). Alternatively, AFLP products can be blotted onto a nylon membrane and subsequently hybridized with an alkaline phosphotase-labeled AFLP probe, which hybridizes to the primer sequence (Lin *et al.*, 1999).

Polymorphisms such as the presence or absence of restriction enzyme sites, sequence polymorphisms adjacent to these sites, insertions, deletions and rearrangements are detected by the AFLP technique (Cervera *et al.*, 1996).

The following reasons are given by Vos *et al.* (1995) for using two restriction enzymes:

i. The frequent cutter will produce small DNA fragments easily amplified and separated on denaturing gels.
ii. Only the rare cutter/frequent cutter fragments are amplified, and therefore using the rare cutter reduces the number of fragments amplified. This in turn reduces the number of selective nucleotides needed for selective amplification.
iii. With the use of two restriction enzymes it is possible to label only one strand of the double stranded PCR products, thus preventing the

occurrence of doublets on the gels due to unequal mobility of the two strands of the amplified fragments.

iv. By using two restriction enzymes the flexibility in tuning the number of restriction fragments to be amplified is greatest.

v. By using various combinations of a low number of primers large numbers of different fingerprints can be generated.

A very important advantage of the AFLP technology is the high number of loci that can be analyzed per experiment (Vos *et al.*, 1995, Russell *et al.*, 1997, Vuylsteke *et al.*, 1999 and Zhu *et al.*, 1999a). The AFLP analysis detects a greater number of loci than RAPD or microsatellite analysis (Thomas *et al.*, 1995, Cervera *et al.*, 1996, Maughan *et al.*, 1996 and Sharma *et al.*, 1996) and is also more efficient at detecting informative markers than RFLP analysis (Sharma *et al.*, 1996, Walton *et al.*, 1996, Ajmone Marsan *et al.*, 1998 and Castiglioni *et al.*, 1999). Besides the ability to detect multiple discrete genetic loci, AFLP analysis is also fast, robust and reliable (Thomas *et al.*, 1995, Vos *et al.*, 1995, Cervera *et al.*, 1996, Maughan *et al.*, 1996, Sharma *et al.*, 1996 and Castiglioni *et al.*, 1999) and does not require prior sequence knowledge of the DNA (Vos *et al.*, 1995 and Vuylsteke *et al.*, 1999).

The disadvantage of this analysis is the inability to provide a known degree of genome coverage. It is therefore possible that some proportions of the polymorphic markers scored are from the same region of the genome, and that other regions are under-represented (Walton *et al.*, 1996, Rouppe van der Voort *et al.*, 1997 and Shan *et al.*, 1999). The clustering of AFLP bands has been noted around the centromeres of maize (Walton *et al.*, 1996, Castiglioni *et al.*, 1999 and Vuylsteke *et al.*, 1999) and barley (Qi *et al.*, 1998b). A difference in clustering was, however, observed when enzymes differing in methylation sensitivity were used to digest the genome. *Eco*RI/*Mse*I fragments were clustered in the centromeric regions, whereas methylation sensitive *Pst*I/*Mse*I fragments were randomly distributed (Castiglioni *et al.*, 1999 and Vuylsteke *et al.*, 1999). It is

believed that the centromeric suppression of recombination is the main reason for the clustering of markers (Castiglioni *et al.*, 1999). As recombination occurs primarily in genes, or perhaps unique sequences, and hypomethylated regions of the maize genome are associated with genes, the methylation sensitive *Pst*I/*Mse*I fragments were more randomly distributed than the *Eco*RI/*Mse*I fragments which may contain repetitive sequences and are therefore mainly clustered around the highly repetitive regions at the centromere (Castiglioni *et al.*, 1999 and Vuylsteke *et al.*, 1999).

Donini *et al.* (1997) found that partial restriction of wheat DNA occurred only in a small number of cases (1%). However, they did detect a variable number of differences within an accession between the amplification profiles of wheat DNA extracted from bulked seeds and those of wheat DNA extracted from leaves. These differences were shown to be neither due to genotypic mixtures nor to pathogen contamination, but were likely a result of differences in DNA methylation between organs. It was therefore suggested that DNA is extracted from physiologically uniform tissue in phylogenetic studies based on AFLP fingerprinting.

Rouppe van der Voort *et al.* (1997) investigated whether AFLP markers can be used to align genetic maps obtained from different potato genotypes. The ability to collate information from genetic maps obtained from different crosses is important in the application of molecular markers for genetic studies in crop plants. They showed that 89% of the AFLP markers, characterized by primer combination and mobility, are indeed allelic. Sequencing of the homologous AFLP markers confirmed that 19 out of 20 markers were identical. Qi *et al.* (1998b) and Zhu *et al.* (1999a) also indicated that AFLP fragments of similar size and intensity are homologous and are therefore transferable between populations.

AFLP markers can be converted into sequence-specific PCR markers (Bradeen and Simon, 1998 and Shan *et al.*, 1999). The AFLP fragments are isolated from the polyacrylamide gel, re-amplified, cloned and sequenced, and primers designed. A PCR assay, which is less expensive and less time and labour consuming (Qu *et al.*, 1998 and Shan *et al.*, 1999), can thus be used to screen the progeny (Cervera *et al.*, 1996). The isolation of AFLP fragments from YAC clones instead of plant DNA is easier, as yeast has a simpler genome structure and contamination with co-segregating bands is less likely (Cnops *et al.*, 1996).

The AFLP approach has been used:

i. in species diversity studies in maize (Walton *et al.*, 1996 and Ajmone Marsan *et al.*, 1998), soybean (Maughan, *et al.*, 1996), lens (Sharma *et al.*, 1996), wild barley (Pakniyat *et al.*, 1997), barley (Schut *et al.*, 1997) and hops (Hartl and Seefelder, 1998);
ii. to construct a high-density linkage map in maize (Castiglioni *et al.*, 1999 and Vuylsteke *et al.*, 1999), potato (Rouppe van der Voort *et al.*, 1997), rice (Zhu *et al.*, 1999a), barley (Lahaye *et al.* 1998, Qi *et al.*, 1998b and Richter *et al.*, 1998) and sugar beet (Schondelmaier *et al.*, 1996 and Nilsson *et al.*, 1999);
iii. to target markers linked to dominant genes of interest in *Populus* (Cervera *et al.*, 1996), soybean (Maughan, *et al.*, 1996), barley (Simons *et al.*, 1997), potato (Ballvora *et al.*, 1995), *Arabidopsis thaliana* (Cnops *et al.*, 1996) and tomato (Thomas *et al.*, 1995);
iv. to identify markers linked to QTL in barley (Powell *et al.*, 1997 and Qi *et al.*, 1998a) and *Brassica oleracea* (Voorrips *et al.*, 1997);
v. in selective amplified microsatellite polymorphic locus (SAMPL) analysis in lettuce (Witsenboer *et al.*, 1997);
vi. in the cDNA-AFLP method to detect differentially expressed transcripts in potato (Bachem *et al.*, 1998); and
vii. to generate mRNA fingerprints in wheat (Money *et al.*, 1996).

1.5 Converted RFLPs (restriction fragment length polymorphism markers)

RFLP technology is very reliable and provides a known degree of genome coverage based upon mapped locations of the RFLP probes (Walton *et al.*, 1996). However, large amounts of DNA are needed for the technique and it is time-consuming, tedious and expensive (Kelly, 1995, Beaumont *et al.*, 1996, Larson *et al.*, 1996, Schondelmaier *et al.*, 1996, Castiglioni *et al.*, 1999 and Vuylsteke *et al.*, 1999).

The conversion of RFLP markers to sequence-tagged site (STS) or cleaved amplified polymorphic sequence (CAPS) markers captures some of the advantages of the RFLP technique, while avoiding the disadvantages of Southern blot analysis (Tragoonrung *et al.*, 1992 and Rafalski and Tingey, 1993). Selection with these markers is thus faster and cheaper, and only a little DNA is needed in the amplification reaction. For STS markers to be useful they must co-segregate with the RFLP locus from which they derive and they should also provide co-dominant and reliable assays (Larson *et al.*, 1996). A number of studies have reported that STS markers co-segregate with RFLP markers (Tragoonrung *et al.*, 1992, Talbert *et al.*, 1994, Larson *et al.*, 1996 and Zaitlin *et al.*, 1993).

The sequence information for the locus of interest is used to create primer pairs, which are about 20 bases long, contain 50% GC nucleotides and harbor no inverted repeat sequences (Tragoonrung *et al.*, 1992). These primer sets are then used to amplify a segment of DNA at the locus. To identify RFLPs among individuals, which do not show size polymorphisms on an agarose gel, the amplified bands are digested with a number of restriction enzymes. These markers are termed CAPS (Rafalski and Tingey, 1993).

Insertion/deletion or point mutation polymorphisms can be distinguished with converted RFLPs (Tragoonrung *et al.*, 1992). RFLPs in the amplified bands may, however, be difficult to identify, because variation outside the STS region can not be detected as in RFLP analysis (Rafalski and Tingey, 1993 and Tragoonrung *et al.*, 1992).

1.6 Microsatellite markers

Microsatellites are stretches of tandemly arranged short sequence motifs that individually range from two to six nucleotides. Primers complementary to the conserved sequences flanking the repeat are used to amplify the intervening microsatellite. Polymorphisms are detected when the alleles differ in the number of tandem repeats in the amplified fragment (Chin *et al.*, 1996 and Smith *et al.*, 1997). Some amplified bands can be separated on agarose gels (Lübberstedt *et al.*, 1998c and Senior *et al.*, 1998). Others need to be separated on non-denaturing polyacrylamide gels stained with ethidium bromide (Brown *et al.*, 1996), or denaturing polyacrylamide gels (Saghai Maroof *et al.*, 1994 and Smith *et al.*, 1997). Primers for the amplification of microsatellites, which need to be separated on denaturing polyacrylamide gels, can either be labeled fluorescently (Smith *et al.*, 1997) or radioactively (Saghai Maroof *et al.*, 1994).

The development of microsatellite markers, also known as simple sequence repeat polymorphism (SSR) markers, is time consuming and expensive as genomic libraries have to be developed, clones have to be sequenced and primers synthesized before the markers can be amplified and run on a gel (Brown *et al.*, 1996, Maughan *et al.*, 1996 and Witsenboer *et al.*, 1997). However, once the primers have been developed, the primer sequences can be published

and used by different laboratories (Rafalski and Tingey, 1993). To date more than 1000 microsatellites have been published for maize. The primer sequences are listed in the Maize Genome Database and can be accessed via the World Wide Web at http://www.agron.missouri.edu/ssr.html.

Microsatellite markers are highly polymorphic, co-dominant, locus specific and can be analyzed quickly and simply with the inexpensive PCR-based assay. Furthermore, they require very little DNA and are usually fully transferable between crosses. Microsatellite markers are therefore very useful in studying genome regions of particular interest (Chin *et al.*, 1996, Brown *et al.*, 1999 and Zhu *et al.*, 1999a).

Microsatellite sequences have also been used to develop inter-simple-sequence-repeat (ISSR) markers (Ratnaparkhe *et al.*, 1998). As microsatellites are often clustered in a genome, they can be used to identify different markers in the same region. The technique uses the sequence of the microsatellite with variation at the 5' and 3' anchors.

1.7 BSA (bulked segregant analysis)

The identification of markers linked to desirable genes is facilitated by the availability of nearly isogenic lines (NILs). To develop NILs, a donor parent carrying a gene of interest is crossed to a recurrent parent with economically favorable properties. Progeny with the desirable gene are selected for backcrossing to the recurrent parent. Backcrossing is continued for a number of generations until the newly developed line is theoretically nearly isogenic with the recurrent parent, except for the segment containing the target gene (Melchinger,

1990). The development of these lines is, however, time consuming, costly and often unnecessary in breeding programs (Chagué *et al.*, 1996). Furthermore, the power to detect markers linked to the desired gene is limited by the occurrence of residual DNA from the donor cultivar at scattered sites in the genome of the NILs (Jean *et al.*, 1998).

Alternatively, bulked segregant analysis (BSA) can be used to rapidly identify, from a large pool of markers, those putatively linked to targeted genes (Michelmore *et al.*, 1991). By means of this approach polymorphic markers are evaluated across two DNA pools with the individuals for the trait or gene of interest being identical within each pool but arbitrary for all other genes. Thus, markers polymorphic between the two bulks are likely to be genetically linked to the loci determining the trait used to construct the bulks. The selected markers, which are polymorphic between the pools, can then be mapped across the entire population and analyzed for association with a specific trait (Miklas *et al.*, 1996).

The most important limitation to BSA is the chance occurrence of shared homozygosity at specific unlinked chromosomal regions in the bulks and it is very likely that with a segregating population derived only one generation after the initial intercross (e.g double haploid, F_2 and BC_1 populations), some genomic regions will not yet have been randomized through meiosis and recombination (Jean *et al.*, 1998).

As the phenotype in polygenic traits is influenced both by multiple genetic loci and the environment, individuals can have extreme phenotypes due to different sets of QTL or due to non-genetic factors. Therefore, the success of BSA for the identification of QTL is dependent on the magnitude of the phenotypic effect of individual QTL, the population size sampled and the influence of non-genetic factors on the phenotype (Wang and Paterson, 1994). A disadvantage of DNA pooling strategies is that it may not always detect QTL of smaller effects which

normally influence a complex trait (Wang and Paterson, 1994 and Grattapaglia *et al.*, 1996).

Wang and Paterson (1994) and Quarrie *et al.* (1999) have indicated that backcross populations are better than F_2s, but poorer than recombinant inbred or double haploid (DH) populations for identifying QTL using BSA. Recombinant inbred lines (RILs) or DH populations are useful in BSA, as polymorphisms with both dominant and co-dominant markers are informative.

BSA has been applied in a number of studies to identify markers linked to quantitative traits. Two DNA pools, one consisting of plants with generalized mosaic symptoms and another with dispersed, chlorotic spots and ring symptoms were used by McMullen *et al.* (1994) to identify 3 genes controlling resistance to wheat streak mosaic virus in maize. Each pool consisted of 25 plants. Quarrie *et al.* (1999) illustrated how BSA coupled with physiological studies can help to identify traits important in determining drought resistance in maize. Fifty plants were pooled in each bulk. Using BSA, 4 RAPD markers linked to a locus involved in quantitative resistance to tomato yellow leaf curl virus have been identified (Chagué *et al.*, 1997). Their resistant bulk consisted of 100 individuals and the susceptible bulk of 29 individuals. BSA was also used together with the RAPD analysis to identify 3 loci linked to QTL controlling leaf rust resistance in bread wheat (William *et al.*, 1997) and to identify 14 markers, distributed over 3 linkage groups, associated with low linolenic acid loci in canola (Somers *et al.*, 1998). Miklas *et al.* (1996) used selective mapping together with BSA and identified 14 RAPD markers linked to 7 QTL conditioning disease resistance in common bean. In these studies between 3 and 10 plants were used per bulk. Wang and Paterson (1994) suggested that 10 plants in a pool are sufficient to avoid detecting false positive markers, even with moderate deviations from Mendelian segregation.

1.8 Linkage analysis

The construction of genetic maps involves both the ordering of loci and the measurement of distance between them (Lynch and Walsh, 1998). Genetic maps are calculated from the recombination rates between loci as a result of chromosome crossovers at meiosis (Qi *et al.*, 1998b). The probability of a recombination event occurring between two loci is called the recombination fraction. The recombination fraction ranges from 0 for loci right next to each other through 0.5 for loci far apart or on different chromosomes, so that it can be taken as a measure of the map distance between gene loci (Terwilliger and Ott, 1994).

The recombination fraction is, however, underestimated for loci, which are further apart because of the occurrence of multiple crossovers. The recombination fraction must therefore be transformed by a map function into the map distance. Mapping functions such as Haldane or Kosambi were derived to predict the number of crossovers from the observed recombination frequency. Map distance is reported in Morgans or centiMorgans (cM), where 100 cM = 1 Morgan (Lynch and Walsh, 1998). One cM corresponds to a recombination fraction of 1% (Terwilliger and Ott, 1994). There is no universal relationship between the actual physical distance and the map distance between loci, as a cM can correspond to a span of between 10 kb (kilo bases) to 1000 kb, depending on the species (Lynch and Walsh, 1998). As the maize genome is 3000 Mb (mega bases) in size and the total map distance is about 1700 cM, 1 cM corresponds to approximately 1800 kb.

Environmental factors may influence recombination rates and therefore genetic distance may vary between different mapping populations (Powell and Nilan, 1963 and Qi *et* al., 1998b). However, in general, recombination rates are under genetic control and mainly depend on chromosome structure (Qi *et al.*, 1998b). A number of studies have indicated that genetic linkage maps are stable with the

orders of the anchor markers being identical and the distances between tightly linked markers being very similar (Burr *et al.*, 1988 and Qi *et al.*, 1998b).

With the computer program MAPMAKER/EXP linkage maps can be constructed from genotype data of F_2 or backcross populations by simultaneous multipoint analysis of any number of loci (Lander *et al.*, 1987 and Lincoln *et al.*, 1992a). The program uses the maximum LOD scores for a test of two-point linkage where the maximum LOD score is defined as the $\log_{10}$ of the ratio of the likelihoods obtained for the maximum likelihood estimate p and for $p = 0.5$ (Melchinger 1990).

1.9 QTL (quantitative trait locus/loci)

Most complex traits (e.g. resistance traits that cannot be fitted to Mendelian ratios) are controlled by multiple loci. Their phenotypes are measured quantitatively, so they are known as quantitative characters. The genetic loci associated with quantitative traits are called quantitative trait loci (Young, 1996). A QTL is a segment of chromosome affecting the trait, not necessarily a single locus (Falconer and Mackay, 1996).

Because all metabolic and developmental pathways are influenced to some degree by aspects of the environment, the expression of most quantitative traits is not completely under genetic control (Lynch and Walsh, 1998). A quantitative resistance trait can result from the expression of a unique gene and environmental factors (Pelsy and Merdinoglu, 1996).

The difference between qualitative and quantitative traits depends not so much on the magnitude of the effect of individual genes as on the relative importance of heredity and environment in producing the final phenotype (Lee, 1995). As environmental factors have a large effect on QTL, common QTL cannot always be detected across environments (Bubeck *et al.*, 1993, Lübberstedt *et al.*, 1998b, Tuberosa *et al.*, 1998 and Agrama *et al.*, 1999) and across populations (Bubeck *et al.*, 1993, Bohn *et al.*, 1997 and Lübberstedt *et al.*, 1998a, b). Some QTL are, however, stable across environments (Ragot *et al.*, 1995, Saghai Maroof *et al.*, 1996 and Pernet *et al.*, 1999a, b).

Earlier mapping investigations in maize showed that although QTL affecting a number of quantitative traits were distributed throughout the genome, certain chromosomal regions appeared to have larger effects than others (Stuber, 1995). To date, a number of different complexly inherited traits have been mapped in maize, such as QTL affecting grain yield (Edwards *et al.,* 1992, Beavis *et al.*, 1994 and Graham *et al.*, 1997), morphological traits (Beavis *et al.*, 1991, Edwards *et al.,* 1992 and Veldboom *et al.*, 1994), disease and insect resistance (Bubeck *et al.*, 1993, Freymark *et al.*, 1994, Jung *et al.*, 1994, Byrne *et al.*, 1996, Saghai Maroof *et al.*, 1996, Bohn *et al.*, 1997, Holland *et al.*, 1998, Lübberstedt *et al.*, 1998a, b, Agrama *et al.*, 1999, Pernet *et al.*, 1999a, b, and Welz *et al.*, 1999) and physiological traits such as abscisic acid concentration in leaves of drought-stressed maize (Tuberosa *et al.*, 1998 and Sanguinet *et al.*, 1999), low-phosphorous stress (Reiter *et al.*, 1991), thermotolerance (Ottaviano *et al.*, 1991) and protein and starch concentration (Goldman *et al.*, 1993).

Marker-based techniques together with segregating populations have made it possible to locate QTL to chromosomal regions and to estimate the effects of QTL (Quarrie *et al.*, 1997 and Tuberosa *et al.*, 1998). Molecular markers can also be used across related species and therefore QTL for a particular trait can be compared across species, to search for homoeologous genes (Quarrie *et al.*, 1997). This enables the comparison of related species to determine whether

complex traits have the same biochemical, physiological and developmental mechanisms. Furthermore, it is possible to compare the localization of QTL with that of mutants and/or cDNA and EST (expressed sequence tagged) clones of known function, thus obtaining important clues on possible candidate genes which are essential in the expression of the trait under investigation (Gilpin *et al.*, 1997 and Tuberosa *et al.*, 1998).

1.10 QTL mapping

QTL mapping is a highly effective approach for studying genetically complex forms of plant disease resistance as the roles of specific resistance loci can be described and race-specificity of partial resistance genes can be assessed. Furthermore, interactions between resistance genes, plant development, and the environment can be analyzed. QTL mapping also provides a framework for marker-assisted selection of complex disease resistance characters and the potential cloning of partial resistance genes (Young, 1996).

The basic methodology for mapping QTL involves making a cross between two inbred strains differing substantially in a quantitative trait. Segregating progeny are scored both for the trait and for a number of genetic markers (Lander and Botstein, 1989). Experimental designs for estimating effects and map positions of QTL are extensions of standard methods for mapping single genes, and are based on linkage disequilibrium between alleles at a marker and alleles at the linked QTL (Falconer and Mackay, 1996). A linkage map of polymorphic marker loci that adequately covers the whole genome, and variation for the quantitative trait within or between populations or strains, are needed to map QTL (Falconer and Mackay, 1996).

DNA-based markers are very useful to map QTL as they are (Falconer and Mackay, 1996):

i. highly polymorphic, so that pairs of individuals or lines are likely to carry different alleles at each locus;
ii. abundant, so comprehensive marker coverage of the genome is achieved;
iii. neutral, both with respect to the quantitative trait of interest and to reproductive fitness; and
iv. co-dominant, so that all possible genotypes at a maker locus can be identified.

To identify QTL by linkage to marker loci, individuals are scored for their genotype at the marker locus and the phenotype for the quantitative trait. If a difference in mean phenotype among marker genotype classes is detected, the presence of a QTL linked to the marker can be inferred (Falconer and Mackay, 1996).

If alleles that increase the value of the trait are homozygous in one parental line and the alleles that decrease the value of the trait fixed in the other parental line, the alleles are in association. The alleles are in dispersion if each line has some increasing and some decreasing alleles fixed. The number of QTL detected by linkage with markers is always an underestimate of the number of loci, because two closely linked QTL may appear as only one if in association, or may not be detected at all if in dispersion (Falconer and Mackay, 1996).

Darvasi and Soller (1994) found that fairly wide marker spacings of about 50 cM are optimum for initial studies of marker-QTL linkage. Once a QTL has been detected additional markers at chromosomal regions of interest should be used to provide a better estimation of the QTL map position (Darvasi, 1997). If too few markers are used, the loci may not be very closely linked to the QTL producing the effect and the total phenotypic variation explained by the marker locus may

be underestimated (Edwards *et al.*, 1992). Employing a larger number of linked markers may also be useful to determine whether different traits occurring in the same region are controlled by two or more linked genes or by pleiotropic effects of one gene (Veldboom *et al.*, 1994).

Large populations may not be necessary to characterize traits conditioned by few QTL of intermediate to major effect, as 40 RILs were as effective as 70 RILs for identifying QTL affecting greenhouse-leaf and field resistance to common bacterial blight (Miklas *et al.*, 1996). The larger population was, however, more effective in identifying and resolving QTL of relatively minor effect.

The F_2 design, derived from selfing of F_1s, has an advantage over designs which use backcross, RIL or DH populations, as it generates three genotypes at each marker locus, which allows the estimation of the degree of dominance associated with detected QTL (Lynch and Walsh, 1998). As F_2 intercrosses provide information about twice as many meioses as backcrosses of the same size, fewer progeny are required for detecting QTL having purely additive effects (Lander and Botstein, 1989 and Falconer and Mackay, 1996). In the case of dominant effects, one backcross will be more efficient than the F_2 and the other less efficient (Lander and Botstein, 1989 and Falconer and Mackay, 1996). To estimate homozygous effects, backcrosses to both parents are necessary, which are also less efficient than the F_2 population (Falconer and Mackay, 1996).

All estimates of the number of QTL are minimum estimates of the true number of loci affecting a trait, because (Falconer and Mackay, 1996 and Young, 1996):

i. experiments are limited in their power to separate closely linked loci;
ii. there must always be other loci with effects too small to be detected by an experiment of particular size;
iii. the loci found are those differentiating the two strains compared, other loci would probably be found in other strains;

iv. inappropriate choice of cut-off for declaring a QTL; and
v. inadequate disease scoring methods.

To increase the power of QTL mapping the environmental noise should be reduced by progeny testing and the genetic noise can be reduced by studying several genetic regions simultaneously (Lander and Botstein, 1989).

To introgress QTL by marker-assisted selection, the location of the QTL need not be known with great accuracy (Lee, 1995 and Kearsey and Farquhar, 1998). QTL mapping will thus be sufficient to identify useful QTL, which could have been missed by conventional mass selection, and incorporate them into elite lines (Veldboom *et al.*, 1994 and Kearsey and Farquhar, 1998).

The ultimate achievement of QTL mapping technology will be the molecular cloning of the underlying genes, including those that confer partial resistance (Young, 1996). Map based gene cloning of QTL and their detailed analysis, however, will require somewhat greater mapping precision than is currently available (Lee, 1995 and Kearsey and Farquhar, 1998).

The candidate gene approach has been employed as a method to combine QTL analysis with the extensive data available on the cloning and characterization of genes involved in plant defense (Faris *et al.*, 1999). This method involves the use of genes, potentially involved in the biochemical pathways leading to trait expression, as molecular markers for QTL analysis. Candidate genes that contribute to quantitative resistance provide breeders with a very useful molecular marker, which can be used to select desirable alleles at QTL and to make the most desirable combinations. Furthermore, the molecular cloning of the QTL is circumvented. Resistance QTL involving candidate genes, however, will have to be mapped at a much higher resolution to determine if they actually do coincide with the candidate gene of a distinct, but related, function (Faris *et al.*, 1999).

1.11 Interval mapping and composite interval mapping

The traditional approach to mapping QTL involves studying single genetic markers one-at-a-time. For example, if at any particular marker, M, in a F_2, the individuals which were homozygous M_1M_1 were significantly taller on average than those which were M_2M_2, then it could be deduced that there was a QTL affecting height linked to this marker (Kearsey and Farquhar, 1998). There are, however, a number of short-comings with this approach (Lander and Botstein, 1989):

i. if the QTL does not lie at the marker locus, its phenotypic effects may be seriously underestimated;
ii. the genetic locations of QTL are not well resolved because distant linkage cannot be distinguished from small phenotypic effect; and
iii. if the QTL does not lie at the marker locus, substantially more progeny may be required.

Interval mapping of QTL was introduced to remedy these problems. Intervals between adjacent pairs of markers along a chromosome are scanned and the likelihood profile of a QTL being at any particular point in each interval is determined, i.e. the log of the ratio of the likelihoods (LOD) of there being one vs. no QTL at a particular point (Lander and Botstein, 1989). This approach has the following advantages (Lander and Botstein, 1989):

i. QTL are efficiently detected, while the overall occurrence of false positives is limited;
ii. phenotypic effects are accurately estimated;
iii. the probable position of the QTL is given by support intervals;
iv. it requires fewer progeny; and

v. QTL likelihood maps can also be used to distinguish a pair of linked QTL from a single QTL, provided that they are not so close that recombination between them is very rare.

Although the interval mapping technique has become the standard method for mapping QTL, it has a problem in distinguishing multiple linked QTL effects (Zeng 1994). To overcome the problem of testing whether one or more than one QTL are present on the same chromosome, Zeng (1994) introduced the composite interval method. This method combines interval mapping with multiple regression and involves an interval test in which the test statistic on a marker interval is made to be unaffected by QTL located outside the defined interval. To achieve this other genetic markers are fitted in the statistical model as a control when performing interval mapping. This method has several advantages over the interval mapping method (Zeng, 1994):

i. it reduces a multiple dimensional search problem for multiple QTL to a one dimensional search problem by confining the test to one region at a time;
ii. the sensitivity of the test statistics to the position of the individual QTL is increased and the precision of QTL mapping improved by conditioning linked markers in the test; and
iii. the efficiency of QTL mapping is improved by selectively and simultaneously using other markers in the analysis.

Although statisticians have developed a number of methods of QTL analysis, all produce essentially similar QTL locations and gene effects, while there is only a slight variation in the confidence intervals. This is largely due to the low chiasma frequency per chromosome, around two on average, which limit recombination and hence QTL resolution. Because of the wide confidence interval, it is difficult to identify more than three QTL per chromosome (Kearsey and Farquhar, 1998). In order to reduce the confidence interval significantly, populations of several thousand individuals have to be scored (Kearsey and Pooni, 1996).

1.12 The utilization of RILs (recombinant inbred lines) to map new markers

RILs are produced by inbreeding the progeny of individual members of a F_2 population derived from two well-established progenitor inbreds. After sufficient generations of inbreeding to achieve homozygosity, each recombinant inbred line is fixed for a different combination of linked blocks of parental alleles (Burr and Burr, 1991).

RILs undergo multiple rounds of meiosis before homozygosity is reached and, therefore, the probability of recombination between very closely linked markers is high. As a result map distances can be more accurately estimated (Burr and Burr, 1991).

RILs represent a permanent population, because all alleles are fixed and can therefore be used indefinitely for mapping of new DNA probes and passed on to other research groups (Burr *et al.*, 1988). A number of publicly available RIL families have been produced in maize. These can be used to map new markers, as long as the parental genotypes can be distinguished. As maize contains a high degree of polymorphisms it is particularly well suited for mapping. To map a new marker one merely types each RIL for the parental allele it received and compares this data by computer with the existing RIL database containing more than 1000 markers (Burr and Burr, 1991). By using computer programs such as MAPMAKER/EXP linkage and map positions can thus be obtained.

Aim of study

In Southern Africa, gray leaf spot was first observed in KwaZulu-Natal in 1988 and the disease has since spread rapidly to neighbouring provinces and countries. GLS can reduce grain yields by up to 60% and it is now recognized as one of the most significant yield-limiting diseases of maize in many parts of the world. Methods to control GLS such as tillage and crop rotation may not be viable, as it is important to preserve the economic and environmental advantages of conservation tillage systems. Although foliar applied fungicides are an effective control, they may not be economical for grain production and the pathogen may develop resistance to these fungicides. The most sustainable and long-term management strategy for GLS will therefore rely heavily on the development of high-yielding, locally adapted GLS resistant hybrids.

The development of GLS is highly dependent on environmental effects, field assessment of the disease is problematic and heritability of resistance is relatively low. Recovery of resistance genes through conventional breeding is therefore difficult and to date only a few high-yielding maize hybrids resistant to GLS are available in South Africa. Maize inbred lines, which exhibit resistance to GLS and also maintain other agronomically important traits, are in demand.

Molecular markers could be useful to plant breeders to indirectly select for genes affecting GLS resistance and to check for the resistance genes without inoculation and at an early stage of plant development. Only two studies in the USA have examined quantitative trait loci association with GLS resistance (Bubeck *et al.*, 1993 and Saghai Maroof *et al.*, 1996).

The aim of this study was to map GLS resistance genes using a resistant Seed Co LTD, Zimbabwean inbred line.

To achieve this aim the objectives were:

- ❖ to identify molecular markers linked to the GLS resistance QTL by using the AFLP technique together with bulked segregant analysis,
- ❖ to amplify the polymorphic AFLP fragments on the DNA of the individual plants constituting the bulks to measure the frequency with which the particular fragments occur in each bulk,
- ❖ to convert the polymorphic AFLP fragments to sequence-specific PCR markers,
- ❖ to amplify the converted AFLP markers on the plants of a F_2 population and to then perform linkage analysis with MAPMAKER/EXP and to identify QTL with MAPMAKER/QTL,
- ❖ to map the converted AFLP markers to one of the maize chromosomes using existing linkage maps of two commercially available RIL populations (Burr *et al.* 1988),
- ❖ to obtain a more precise localization of the QTL by using converted RFLP and microsatellite markers,
- ❖ to produce linkage maps with MAPMAKER/EXP using the genotype data obtained with a F_2 population for all markers,
- ❖ to perform interval mapping and composite interval mapping with MAPMAKER/QTL and QTL Cartographer, respectively, using the genotype and phenotype data of each plant of a F_2 population,
- ❖ to test the consistency of the detected QTL by amplifying the markers flanking each QTL on selected plants of F_2 populations planted in different years and performing regression analysis.

Chapter 2
Materials and methods

2.1 Plant material

The plant material used in this study was obtained from the seed company Sensako. F_2 plants were visually assessed by the breeder and scored for resistance on a rating scale of 1-9, where 1 is most resistant and 9 is highly susceptible. Although the plants were grown in field plots with naturally infested corn debris, each plant was artificial inoculated to ensure high disease pressure. At least three rows of the susceptible parent were planted in each generation to determine the progress the disease has made. The first disease ratings were recorded when the susceptible parent had a GLS score of 7 and on average two to three ratings were taken.

The F_1 single cross between a GLS resistant male parent (P1, Seed Co LTD, Zimbabwe) and a susceptible female parent (P2, Sensako, South Africa) was backcrossed to P2 during the summer of 1995/6. During the winter of 1996 the backcross F_1 generation was selfed to produce a segregating F_2 generation, which was planted on Sensako's research farm at Hillcrest in the 1996/7 season (Table 1A). From the F_2 population ten resistant and ten susceptible plants with scores of 1 and 9, respectively, were chosen for bulk segregant analysis (BSA, Table 1A). These bulks will be referred to as B97R (resistant) and B97S (susceptible) in the text.

Table 1. List of F_2 populations used in this study. The number of plants scored for GLS resistance in each population and the number of plants used in DNA extractions are given. The type of analysis performed with each population is indicated.

	Population	No. of plants scored	No. of plants DNA extracted	Analysis
	1997			
A	P2*1 / P1 F_2	20	20	BSA (B97R, B97S)
	1998			
B	P2 / P1 F_2	230	230	BSA (B98R, B98S); QTL analysis
	1999			
C	P2 / P1 F_2	977	111	Regression analysis
	2000			
D	P2 / P1 F_2	1063	48	Regression analysis

Another F_1 single cross between the GLS resistant male parent (P1) and the susceptible female parent (P2) was selfed during the summer of 1995/6. This F_2 segregating population was planted on the research farm at Hillcrest in 1998 (Table 1B), 1999 (Table 1C) and 2000 (Table 1D). Two-hundred and thirty, 977 and 1063 plants from the 1998, 1999 and 2000 F_2 populations, respectively, were scored for resistance. The distributions of the GLS disease scores in the F_2 populations of 1998, 1999 and 2000 are given in Figure 2.

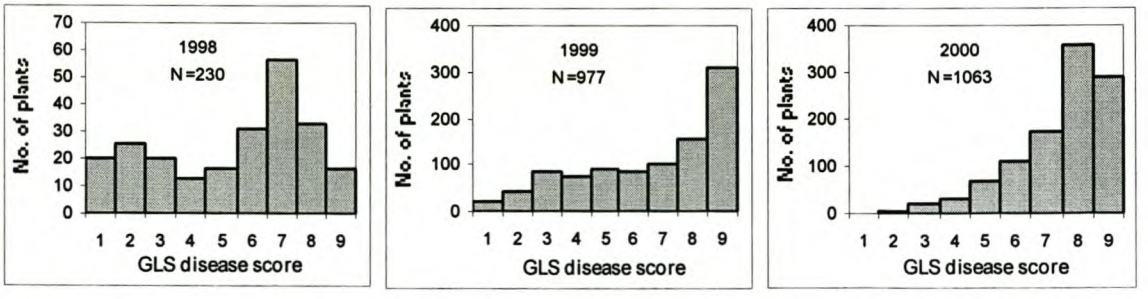

Figure 2. Distributions of the GLS disease scores in the 1998, 1999 and 2000 F_2 populations.

The 230 plants of the 1998 F_2 population were used in linkage analysis and QTL mapping. The 20 most resistant and 16 most susceptible plants of this population

were also used in bulk segregant analysis (Table 1B). In the text these bulks will be referred to as B98R (resistant) and B98S (susceptible).

Of the 1999 and 2000 F_2 populations 111 and 48 plants, respectively (Table 1C and 1D), were selected and used in the regression analysis to test the consistency of the QTL detected in the 1998 F_2 population.

Furthermore, two publicly available recombinant inbred families (Burr *et al.*, 1988) were used to map cloned AFLP fragments. Population I, T323 X CM37 and population II, CO159 X Tx303 consisted of 48 and 41 lines, respectively.

2.2 Genomic DNA extraction, quantification and pooling

For genomic DNA extraction the protocol described in the CIMMYT Applied Molecular Genetics Laboratory manual (Saghai Maroof *et al.*, 1984), based on the method used by Murray and Thompson (1980), was followed. Two to three leaves of a plant were sampled, rolled and placed in a 50 ml centrifuge tube, which was capped and placed on ice until it could be taken to the laboratory to be frozen at –80°C. The frozen leaves were dried in a lyophilizer for at least 3 days. After lyophilization the leaves were stored in a freezer at –20°C.

Prior to DNA extraction, lyophilized leaves were ground to a fine powder with a coffee grinder. Four hundred mg of this tissue was weighed into a 50 ml centrifuge tube. Nine ml prewarmed (65°C) CTAB extraction buffer (1% CTAB, 100 mM Tris-HCl pH 7.5, 700 mM NaCl, 50 mM EDTA pH 8.0 and 140 mM β-mercaptoethanol) was added and tubes were gently inverted for a number of times to mix. The mixture was incubated with continuous gentle rocking in a

water bath at 65°C for 1.5 h. After tubes had cooled down for 5 min at room temperature 4.5 ml of chloroform-octanol (24:1) was added and then mixed gently for 10 min. The suspension was centrifuged at 1500 x *g* for 10 min at room temperature and the top aqueous layer was pipetted into a 30 ml glass tube. The chloroform-octanol step was repeated once. After centrifugation, the top aqueous layer was pipetted into a 15 ml glass tube containing 40 µl of 10 mg/ml RNase A, gently inverted and incubated for 30 min at room temperature.

The DNA was precipitated with 6 ml isopropanol (2-propanol), mixed gently by inversion and removed by coiling it around a sterile, plastic inoculation needle. The inoculation needle with the DNA wound around it was placed in a 15 ml glass tube containing 1 ml TE-buffer and dissolved overnight at room temperature. The needle was removed once the DNA had dissolved off it.

The DNA was again precipitated by adding 50 µl 5 M NaCl and 2.5 ml absolute ethanol and mixed by gentle inversion. Another inoculation needle was used to remove the precipitated DNA and the needle was placed in a 5 ml plastic tube containing wash 1 (76% ethanol, 0.2 M NaOAc) for 10 min. Hereafter the DNA wound around the inoculation needle was briefly rinsed in wash 2 (76% ethanol, 10 mM NH_4OAc), removed from the needle into an Eppendorf, briefly dried and then dissolved overnight in 50-200 µl ddH_2O. Samples were stored at –20°C.

The DNA was quantified on a gel against a known concentration of lambda DNA and by spectrophotometry (Ultraspec III spectrophotometer). Equal volumes of standardized DNA were pooled in the two contrasting DNA bulks.

2.3 AFLP analysis

The AFLP methodology was based on the method used by Vos *et al.* (1995) and Zabeau and Vos (1993). Genomic DNA (150 ng) was digested with the two restriction enzymes *Mse*I (frequent-cutter) and *Mlu*I (rare-cutter). Digestions were carried out with 10 U *Mse*I, 10 U *Mlu*I and 1X One-Phor-All Buffer PLUS [100 mM Tris-acetate (pH 7.5), 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 waterbath for 1 h.

The 50 µl digested DNA mixture was supplemented with 10 µl adapter/ligation solution, containing 50 pmol Mse adapter and 5 pmol 5'-biotinylated Mlu adapter, 1.2 µl 10 mM ATP, 1X One-Phor-All Buffer PLUS [100 mM Tris- acetate (pH 7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech] and 1 U T4 DNA ligase, and incubated overnight at 37°C. The structure of the adapter sequences was:

Mse: 5'- GACGATGAGTCCTGAG
TACTCAGGACTCAT-5'

Mlu: 5'- biotin – CTCGTAGACTGCGTAAC
CTGACGCATTGGCGC-5'

The complexity of the DNA mixture was reduced by selecting the biotinylated Mlu fragments using streptavidine beads (Dynal). The beads were washed four times with 10 mM Tris-HCl, 0.1 mM EDTA, 100 mM NaCl in a volume equivalent to that of the beads (20 µl per reaction) and resuspended in the same volume. Twenty µl of beads was added to each 60 µl digested/ligated DNA sample and the mixture was incubated on ice for 30 min with gentle agitation every 5–10 min. Hereafter 120 µl wash solution (10 mM Tris-HCl, 0.1 mM EDTA, 100 mM NaCl) was added and the beads were collected with a magnet and washed 3 times with 200 µl wash solution. Finally, the remaining Mlu-Mse and Mlu-Mlu fragments were suspended in 100 µl of TE buffer and stored at -20°C.

Only the Mse primer was labeled. One μl (300 ng) of the Mse primer was added to 1X One-Phor-All Buffer PLUS [100 mM Tris- acetate (pH 7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech], 1 μl [γ-^{33}P]ATP (1 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 65°C for 10 min.

One μl of the biotinylated DNA fragments was added to 100 μM of each dNTP, 2.5 mM $MgCl_2$, 1X NH_4 buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20], 0.5 U *Taq* DNA polymerase (BIOTAQ™ polymerase, Bioline), 30 ng labeled Mse primer and 30 ng Mlu primer in a total volume of 20 μl. The Mse primers and the Mlu primers had 3 and 2 selective nucleotides, respectively. The PCR cycle profile was performed in a Hybaid PCR Express thermocycler. 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.

After amplification 10 μl of formamide loading buffer was added to each 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, 7.5 M urea and 1X 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 overnight. AFLP bands were identified as dominant markers, where a polymorphism is defined as the presence of a given band in one of the bulks and the corresponding parent and absent in the other bulk and corresponding parent.

2.4 Conversion of AFLPs to sequence-specific PCR markers

The gel was run as described above. Autoradiography glo-stickers (Bel-Art products, Penquannock, NJ) were used to mark the dried gel for orientation purposes. The gel was exposed to X-ray film for 1-2 days. To recover a specific AFLP fragment a small rectangle containing the autoradiographic image of the fragment was cut out from the X-ray film with a scalpel. The glo-stickers were then used to align the film over the gel in the exact orientation as during exposure. The segment of gel underneath the rectangular hole in the film was excised with a scalpel and transferred into a 1.5 ml microfuge tube containing 50 µl of TE buffer. It was incubated at 37°C overnight. The gel was re-autoradiographed to confirm that the correct band had been excised.

One to 3 µl of the TE buffer containing the excised DNA fragment was used in the amplification reaction with the same set of AFLP primers. The DNA was added to 50 µM of each dNTP, 2.5 mM $MgCl_2$, 1X NH_4 buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20], 0.5 U *Taq* DNA polymerase (BIOTAQ™ polymerase, Bioline), 30 ng Mse primer and 30 ng Mlu primer in a total volume of 20 µl. The PCR cycle profile was performed in a Hybaid PCR Express thermocycler. The cycle 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, 58°C for 30 sec, 72°C for 2 min and one cycle at 72°C for 10 min.

Amplification products were electrophoresed at 80 V for approximately 1 h in a 1.5% low melting point agarose gel. The desired fragments were excised from the gel and transferred to a 1.5 ml microfuge tube and heated in a heating block at 65°C. Once the agarose had melted, water was added up to the 500 µl mark indicated on the tube and the DNA was extracted by phenol/chloroform extraction. Five-hundred µl phenol was added to the tube and the samples were vortexed and centrifuged for 10 min at room temperature. The supernatant was transferred to a new tube and the samples were vortexed and centrifuged again.

The supernatant was then transferred to another tube and 500 µl chloroform was added and the samples were vortexed and centrifuged. After the supernatant had been transferred to a new tube 20 µl 5 M NaCl was added together with 2 volumes of 100% ethanol. The samples were centrifuged, left to dry for a few minutes and redissolved in 10 µl ddH_2O. One µl of each sample was loaded onto an agarose gel together with standard concentrations to determine the concentration of the fragments.

The fragments were cloned using the pGem®-T Easy Vector System II (Promega, Madison, WI). Instructions given by the supplier were followed for the ligation and transformation reactions. To make sure that the clones contained fragments of the correct size, 5 white colonies were selected of each cloned fragment, amplified with the same AFLP primer pairs and run on a polyacrylamide gel together with the AFLP fingerprints of the parents. One clone containing the correct size fragment was cultured overnight at 37°C in 3 ml LB medium.

Plasmids were extracted using the Perkin Elmer Miniprep kit and sequenced at the DNA Sequencing Facility of the University of Stellenbosch with an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) using the T7 and SP6 primer. Samples were loaded onto an ABI PRISM 377 automatic sequencer and run at 1.5 V for 8 h. The Primer Designer - version 1.01 software program (Copyright 1990, Scientific & Educational Software Serial number 50132) was used to design unique 20-bp primer pairs.

Thirty ng of each new primer was used in a 25 µl amplification reaction containing 20 ng genomic DNA as a template, 2 mM $MgCl_2$, 50 µM of each dNTP, 1X NH_4 buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20] and 0.5 U *Taq* DNA polymerase (BIOTAQ™ polymerase, Bioline). The PCR cycle profile was performed in a Hybaid PCR Express thermocycler. The cycle 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, 45-68°C (depending on the primer pair) for 30 sec, 72°C for 2 min and one cycle at 72°C for 10 min. The amplification products were electrophoresed at 100 V for 1-2 h in a 1.5-2 % agarose gel prepared with 1X TBE buffer containing ethidium bromide (6 µl of a 50 mg/ml solution). The 1-kb plus ladder (Gibco BRL) was used as a molecular weight marker. The products were visualized by illumination with ultraviolet light.

Primer pairs, which did not indicate a size difference between the DNA of the parents on an agarose gel, were run on a polyacrylamide gel. Either the forward or the reverse primer was labeled. One µl (300 ng) of the primer was added to 1X One-Phor-All Buffer PLUS [100 mM Tris- acetate (pH 7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech], 0.5 µl [γ-^{33}P]ATP (1 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 65°C for 10 min. Amplification was as above using 30 ng labeled primer per reaction. After amplification 10 µl of formamide loading buffer was added to each sample. The reactions were denatured at 90°C for 5 minutes in a heating block and quickly chilled on ice. Two µl of each sample was loaded on 4% acrylamide/bisacrylamide, 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.

Primer pairs, which did not indicate a size difference between the DNA of the parents on an agarose gel nor on a polyacrylamide gel, were digested with restriction enzymes (*Rsa*I, *Alu*I, *Cfo*I, *Tsp*509I, *Hpa*II, *Mn*lI, *Aci*I, *Msp*I, *Tru*91, *Hae*III, *Na*III, *Taq*I or *Hinf*I). Restriction enzyme digestions were carried out directly on 20 µl of the amplification products. Three units of the restriction endonuclease were added and the amplification products were incubated at 37°C for 1 h. Digested fragments were electrophoresed in a 2% agarose gel at 100 V for 1-2 h.

2.5 Conversion of RFLPs and analysis

Probes received from the University of Missouri, Columbia, Mo., USA were immediately streaked onto LB medium plates containing 100 μg/ml ampicillin and grown overnight at 37°C. Individual colonies were picked and cultured overnight at 37°C in 50-100 ml LB medium containing 100 μg/ml ampicillin. Plasmids were extracted using the Nucleobond® AX PC Kit 100 (Macherey-Nagel). Plasmid DNA concentrations were determined with a spectrophotometer (Ultraspec III spectrophotometer). All probes were amplified by the polymerase chain reaction to verify that the insert sizes were correct. Twenty ng of plasmid DNA was amplified in a 25 μl reaction containing 2 mM $MgCl_2$, 50 μM of each dNTP, 15 pmol each of the M13 forward and the M13 reverse primer or the T7 and SP6 primer, 1X NH_4 buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20] and 0.5 U *Taq* DNA polymerase (BIOTAQ™ polymerase, Bioline). The following PCR cycle profile was performed in a Hybaid PCR Express thermocycler: one cycle of 94°C for 7 min, followed by 35 cycles of 94°C for 45 sec, 60°C for 1 min, 72°C for 2 min and one cycle at 72°C for 2 min. The PCR products were loaded onto a 1% agarose gel in 1X TBE buffer containing ethidium bromide (6 μl of a 50 mg/ml solution) and run at 100 V for 2 h. The products were visualized under ultraviolet light.

The probes were sequenced at the DNA Sequencing Facility of the University of Stellenbosch with an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) using the M13 forward and M13 reverse primers. Samples were loaded onto an ABI PRISM 377 automatic sequencer and run at 1.5 V for 8 h. The Primer Designer - version 1.01 software program (Copyright 1990, Scientific & Educational Software Serial number 50132) was used to select unique primer pairs. Two 20-bp primers were selected for each probe.

To amplify the new primer pairs 30 ng of each primer was used in a 25 μl amplification reaction containing 20 ng genomic DNA as a template, 2.5 mM

$MgCl_2$, 50 μM of each dNTP, 1X NH_4 buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20] and 0.5 U Taq DNA polymerase (BIOTAQ™ polymerase, Bioline). The following cycle profile was performed in a Hybaid PCR Express thermocycler: one cycle of 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec - 1 min, 54-60°C (depending on the primer pair) for 30 sec - 1 min, 72°C for 2 min - 2.3 min and one cycle at 72°C for 10 min. The amplification products were electrophoresed at 100 V for 4 h in a 2% agarose gel prepared with 1X TBE buffer containing ethidium bromide (6 μl of a 50 mg/ml solution). A 1-kb plus ladder (Gibco BRL) was used as a molecular weight marker. The products were visualized by illumination with ultraviolet light and photographed.

Restriction enzyme digestions were carried out directly on 20 μl of the amplification products. Three units of the restriction endonuclease (*Rsa*I, *Alu*I, *Cfo*I, *Tsp*509I, *Hpa*II, *Mn*lI, *Aci*I, *Msp*I, *Tru*91, *Hae*III, *Na*III, *Taq*I or *Hinf*I) were added and the amplification products were incubated at 37°C for 1 h. Digested fragments were electrophoresed in a 1.5% agarose gel at 100 V for 1-2 h.

2.6 Microsatellite analysis

The microsatellite primer sequences were obtained from the Maize Genome Database website (http://www.agron.missouri.edu/ssr.html). Thirty ng of each microsatellite primer was used in a 20 μl amplification reaction containing 10 ng genomic DNA as a template, 2 mM $MgCl_2$, 50 μM of each dNTP, 1X NH_4 buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween 20] and 0.5 U Taq DNA polymerase (BIOTAQ™ polymerase, Bioline). The PCR cycle profile was performed in a Hybaid PCR Express thermocycler. The cycle 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, 45-70°C (depending on the primer pair) for 30 sec, 72°C for 30 sec and one cycle at 72°C for 30 min. The amplification products were electrophoresed at 80 V for 1-2 h in a 2% agarose gel prepared with 1X TBE buffer containing ethidium bromide (6 μl of a 50 mg/ml solution). The products were visualized by illumination with ultraviolet light.

Microsatellite primer pairs, which did not indicate a size difference between the DNA of the parents on an agarose gel, were run on a polyacrylamide gel. Either the forward or the reverse primer was labeled. One μl (300 ng) of the primer was added to 1X One-Phor-All Buffer PLUS [100 mM Tris- acetate (pH 7.5), 100 mM Mg-acetate, 500 mM K-acetate, Pharmacia Biotech], 0.5 μl [γ-^{33}P]ATP (0.5 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 65°C for 10 min. The microsatellites were amplified as above using 30 ng labeled primer per reaction. After amplification 10 μl of formamide loading buffer was added to each sample. The reactions were denatured at 90°C for 5 minutes in a heating block and quickly chilled on ice. Two to 4 μl of each sample was loaded on 4% acrylamide/bisacrylamide, 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.

2.7 QTL analysis

2.7.1 Chi-square analysis

Chi-square analysis was performed on each marker to detect deviations from the expected Mendelian segregation of a 1:2:1 and a 3:1 ratio for co-dominant and

dominant markers, respectively. All calculations were performed on a spreadsheet using the program Microsoft® Excel 97.

2.7.2 Linkage analysis

Linkage analysis and the order of the markers were determined for the F_2 population and the RIL populations by using the software package MAPMAKER/EXP version 3.0b (Lander *et al.*, 1987 and Lincoln *et al.*, 1992a).

The database used in the linkage analysis with the 1998 F_2 population is given in Addendum III (page 137). Only the recessive genotype data were used for the 2 dominant markers and the genotypes of the homozygous dominant and heterozygous plants were designated as missing data. The datafiles used in linkage analysis with the RIL populations were obtained from the Maize Genome Database website (http://www.agron.missouri.edu).

MAPMAKER's error function was on and the order function was used to determine the linear order of the markers. Multipoint analysis was used to determine the distances between the markers. To include a locus in a linkage group a minimum LOD threshold of 3.0 and a distance threshold of 50 Haldane cM were used.

2.7.3 QTL mapping

The chromosomal location of the QTL was determined by interval mapping (Lander and Botstein, 1989) using MAPMAKER/QTL version 1.1b (Paterson *et al.*, 1988 and Lincoln *et al.*, 1992b) at a LOD threshold of 2.0. The genotype and phenotype data used is given in Addendum III (page 137).

QTL mapping was also performed with the interval mapping and composite interval mapping method (Zeng, 1994) using the program QTL Cartographer

version 1.13 (Baston *et al.*, 1994 and Baston *et al.*, 1997). Models 3 (interval mapping) and 6 of QTL Cartographer were applied. For model 6, 5 markers were used to control the genetic background (n_p) and the window size (w_s) was 10.

2.8 Linear regression analysis

Linkage of molecular markers to genetic factors responsible for GLS resistance was investigated by standard ANOVA for linear regression of GLS scores on genotypes for each marker, scored as 1, 2 and 3 for the homozygous resistant, heterozygous and homozygous susceptible allele, respectively.

The regression of GLS score on marker genotype was used to calculate the proportion of the total phenotypic variance explained by each marker and uses the standard F-statistic. All calculations were performed on a spreadsheet using the program Microsoft® Excel 97.

Chapter 3
Results

3.1 AFLP analysis

The aim of this study was to map GLS resistance genes in a Seed Co LTD, Zimbabwean inbred line. To identify molecular markers linked to the GLS resistance QTL, bulked segregant analysis was used together with the AFLP technique.

Two pairs of bulks, one made from plants of the 1997 F_2 population (Table 1A, page 32) and the other made from plants of the 1998 F_2 population (Table 1B, page 32), were used to target the GLS resistance QTL. Equal volumes of standardized DNA of 10 plants with a GLS disease score of 1 and 10 plants with a GLS disease score of 9 from the 1997 population and 20 and 16 plants, respectively, of the 1998 population were pooled in the two contrasting bulks.

Ten AFLP primer combinations (Table 2) were used to screen the *Mse*I/*Mlu*I-digested parental and bulk DNA. Between 35 and 75 distinguishable bands were amplified with the different primer combinations with an average of 45 bands per primer combination. In total about 450 loci were screened. Approximately 50% of the fragments were polymorphic between the parents.

Table 2. Primer combinations used in AFLP analysis. The Mlu-5 primer was used in combination with the ten Mse primers.

Name of primer	*Sequence*	*Name of primer*	*Sequence*
Mlu-5	5'-gac tgc gta acc gcg tgc-3'		
Mse-1	5'-gat gag tcc tga gta aga a-3'	**Mse-6**	5'-gat gag tcc tga gta att g-3'
Mse-2	5'-gat gag tcc tga gta aac a-3'	**Mse-7**	5'-gat gag tcc tga gta ata c-3'
Mse-3	5'-gat gag tcc tga gta aac c-3'	**Mse-8**	5'-gat gag tcc tga gta aga g-3'
Mse-4	5'-gat gag tcc tga gta acc g-3'	**Mse-9**	5'-gat gag tcc tga gta aca t-3'
Mse-5	5'-gat gag tcc tga gta agg c-3'	**Mse-10**	5'-gat gag tcc tga gta aca c-3'

Of the 10 primer combinations tested, 7 showed polymorphic fragments with both the parent and the 1997 bulk DNA and one or two polymorphisms could be identified per primer combination. Eleven polymorphisms were detected in total (Figure 3). (The fragments were named after the Mse primer that was used to amplify them. If more than 1 polymorphic fragment was amplified with the same primer combination, the polymorphic fragments were indicated with a 1 or 2 after the primer number, e.g. AF2.1 was amplified with primer Mse-2 and it was one of 2 fragments polymorphic with the same primer combination.)

Of the 11 polymorphisms detected with the 1997 bulks, 6 could also be detected between the resistant and the susceptible 1998 bulk. No polymorphism could be detected with fragments AF2.1 and AF2.2 between the resistant and the susceptible 1998 bulk (Figure 3A). It was assumed that the intensity of the fragments AF2.1 and AF2.2 was the same in the 1998 bulks as the overall intensity of the fragments of lane B98S with the primer Mse-2 (Figure 3A) seems to be lower than the intensity of the other lanes.

The AFLP fragment AF5.1 was not amplified on either 1998 bulk DNA, while fragment AF5.2 was amplified on both the resistant and susceptible bulk DNA (Figure 3B). A difference in the intensity of the bands of the resistant and the susceptible 1998 bulk was visible with fragment AF6.1, whereas fragment AF6.2 was present in the 1998 susceptible but not the resistant bulk (Figure 3C).

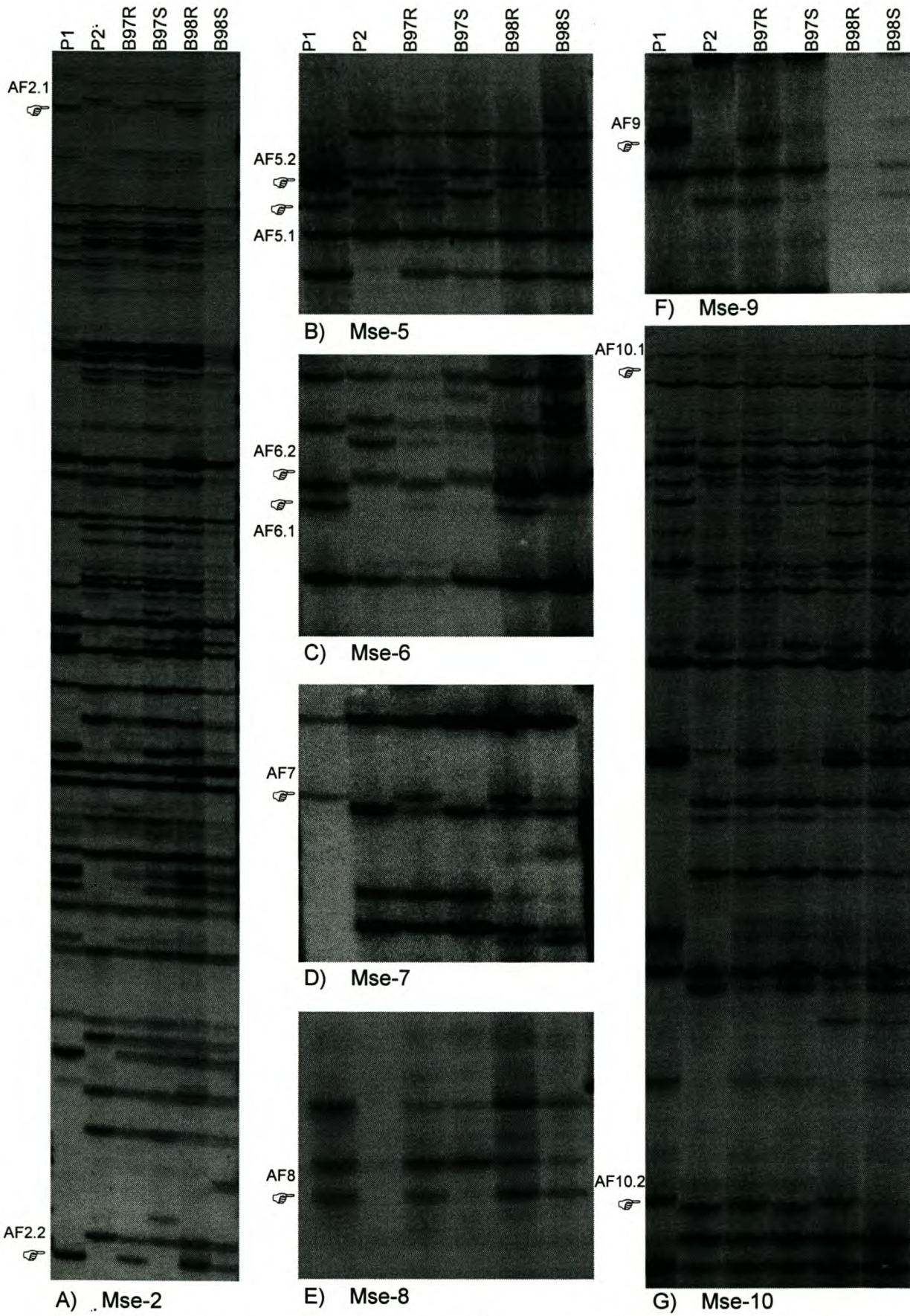

Figure 3. AFLP primer Mlu-5 amplified with primers A) Mse-2, B) Mse-5, C) Mse-6, D) Mse-7, E) Mse-8, F) Mse-9, and G) Mse-10 on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent) and the bulks (lane B97R = 1997 resistant bulk, lane B97S = 1997 susceptible bulk, lane B98R = 1998 resistant bulk, lane B98S = 1998 susceptible bulk). The polymorphic fragments are indicated.

With fragments AF7 (Figure 3D) and AF8 (Figure 3E) a difference in the intensity of the bands of the resistant and susceptible 1998 bulk could be detected. Fragment AF9 did not amplify successfully on the 1998 bulk DNA (Figure 3F). Fragments AF10.1 and AF10.2 were present in the resistant 1998 bulk and absent from the susceptible bulk (Figure 3G).

Once polymorphisms had been identified, the primers were amplified on the 20 individual plants making up the 1997 bulks to measure the frequency with which the particular alleles occur in each bulk. A summary of the polymorphisms detected and the frequency of a particular allele in each bulk is given in Table 3. The highest number of plants having the same polymorphic fragment in coupling with the resistance allele was 8, produced by the primer combination Mlu-5/Mse-6. Six polymorphic fragments (AF2.1, AF2.2, AF5.1, AF7, AF8 and AF9) were present in 7 individual plants of the resistant bulk, 1 fragment (AF10.2) was present in 6 and 2 fragments (AF5.2 and AF10.1) were present in 5 individual plants of the resistant bulk. Only 3 of the primer combinations amplified a fragment from one of the susceptible plants (fragments AF2.1, AF6.1 and AF10.2, Table 3). Only one fragment linked in repulsion phase with the resistance allele was identified (AF6.2, Table 3). This fragment was present in the 10 plants of the susceptible bulk and 2 plants of the resistant bulk.

Table 3. Summary of the 7 polymorphic Mlu/Mse primer combinations and the frequency of the alleles in the individual plants of the 1997 bulks.

Primer combinations	*Name of poly-morphic fragment*	*Frequency of the allele of the resistant parent in the resistant bulk*	*Frequency of the allele of the resistant parent in the susceptible bulk*
Mlu-5/Mse-2	AF2.1	7	1
	AF2.2	7	0
Mlu-5/Mse-5	AF5.1	7	0
	AF5.2	5	0
Mlu-5/Mse-6	AF6.1	8	1
	AF6.2	2	10
Mlu-5/Mse-7	AF7	7	0
Mlu-5/Mse-8	AF8	7	0
Mlu-5/Mse-9	AF9	7	0
Mlu-5/Mse-10	AF10.1	5	0
	AF10.2	6	1

The 11 polymorphic AFLP fragments were converted to sequence-specific PCR markers. They were excised from the polyacrylamide gel and cloned. To make sure that the clones contained fragments of the correct size, 5 white colonies were selected of each cloned fragment, amplified with the AFLP primer pairs and run on a polyacrylamide gel together with the AFLP fingerprints of the parents. Between 3 and 5 of the 5 clones had the correct size fragment. One of the clones with the correct size fragment was cultured, plasmids were extracted and the fragment was sequenced. The sequence of each fragment is given in Addendum I (page 129). All the sequences had the Mlu-5 primer sequence at the one end and a Mse primer sequence at the other end (note that reverse primer sequences are in reverse complement). The newly identified primer pairs, which were used to amplify the converted AFLP markers, are highlighted in Addendum I.

3.2 Converted AFLP markers

The new primer pairs were amplified on the resistant and the susceptible parental DNA. To detect size differences between the parents the amplified products were run on agarose gels. If no polymorphisms could be depicted on agarose gels the amplified products were separated on polyacrylamide gels to detect smaller size differences of one or more nucleotides. If no polymorphisms could be observed on polyacrylamide gels the amplified products were digested with 13 different restriction enzymes (*Rsa*I, *Alu*I, *Cfo*I, *Tsp*509I, *Hpa*II, *Mn*lI, *Aci*I, *Msp*I, *Tru*91, *Hae*III, *Na*III, *Taq*I or *Hinf*I) and run on agarose gels to search for point mutation polymorphisms within the amplified products.

The primer pairs for the marker obtained from fragment AF9 did not amplify a fragment with the expected size and was therefore discarded. Two other

markers obtained from the fragments AF2.2 and AF7 were also discarded as multiple bands were amplified with the primer pairs for these markers.

Even after restriction enzyme digestion with 13 different enzymes, no polymorphisms could be detected between the parents with the 3 markers obtained from the fragments AF2.1, AF6.2 and AF10.2. Five markers (obtained from the fragments AF5.1, AF5.2, AF6.1, AF8 and AF10.1) were polymorphic between the parents. The 3 non-polymorphic and 5 polymorphic markers were added to the maize marker database of the University of Stellenbosch (us). The marker obtained from the fragment AF2.1 was designated as us39, AF5.1 as us40, AF5.2 as us41, AF6.1 as us42, AF6.2 as us43, AF8 as us44, AF10.1 as us45 and AF10.2 as us46.

The 5 markers, which were polymorphic between the parents (us40, us41, us42, us44 and us45), were amplified on the DNA of the 20 individual plants of the 1997 bulks to determine if the same F_2 plants as in the AFLP analysis had the allele of the resistant and the susceptible parent. This was done to confirm that the correct fragment had been isolated and cloned. With all markers except for marker us45 the same plants, which had the allele of the resistant parent and the allele of the susceptible parent with the original AFLP primers, had the allele of the resistant and susceptible parent with the sequence-specific PCR primers. Marker us45 was amplified on the DNA of two plants on which the AFLP fragment was not amplified (discussed under section 3.2.5, page 53).

3.2.1 Marker us40

Marker us40 was amplified at an annealing temperature of 66°C on the parental DNA and the products were loaded onto a polyacrylamide gel. A fragment of about 160 bps was amplified with this marker. A size difference of 2 base pairs (bps) could be detected between the parents (lanes P1 and P2; Figure 4). The plants in lanes 2, 4, 5, 6, 8, 9 and 10 (Figure 4), making up the resistant bulk, had

the allele of the resistant parent. Six of these 7 plants (lanes 2, 4, 5, 6, 8 and 9; Figure 4) were heterozygous. The plants making up the susceptible bulk (lanes 11-20; Figure 4) had the allele of the susceptible parent.

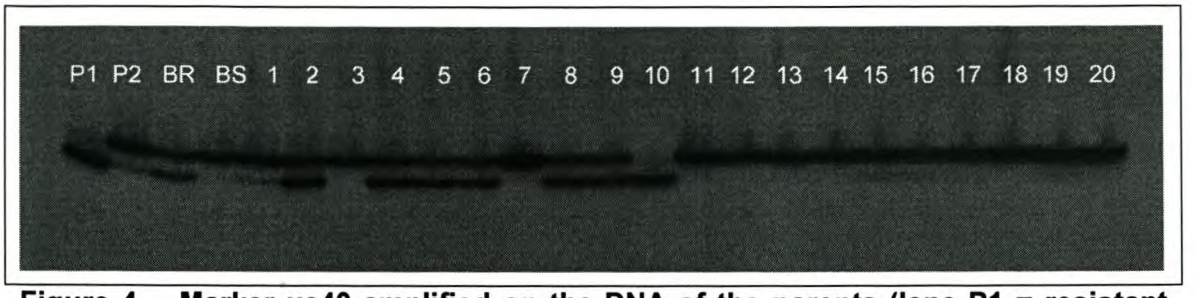

Figure 4. Marker us40 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent) and the bulks (lane BR = 1997 resistant bulk, lane BS = 1997 susceptible bulk) and the DNA of the 10 resistant (lanes 1-10) and 10 susceptible (lanes 11-20) plants making up the bulks.

3.2.2 Marker us41

Marker us41 amplified a fragment of about 240 bps on the resistant and a fragment, approximately 10 bps larger, on the susceptible parental DNA at an annealing temperature of 60°C. The polymorphism could be visualized on a polyacrylamide gel. Five of the ten plants (lanes 2, 4, 6, 8 and 9; Figure 5) of the resistant bulk had the allele of the resistant parent, of which 3 (lanes 6, 8 and 9; Figure 5) were heterozygous. None of the plants (lanes 11-20; Figure 5) of the susceptible bulk had the allele of the resistant parent.

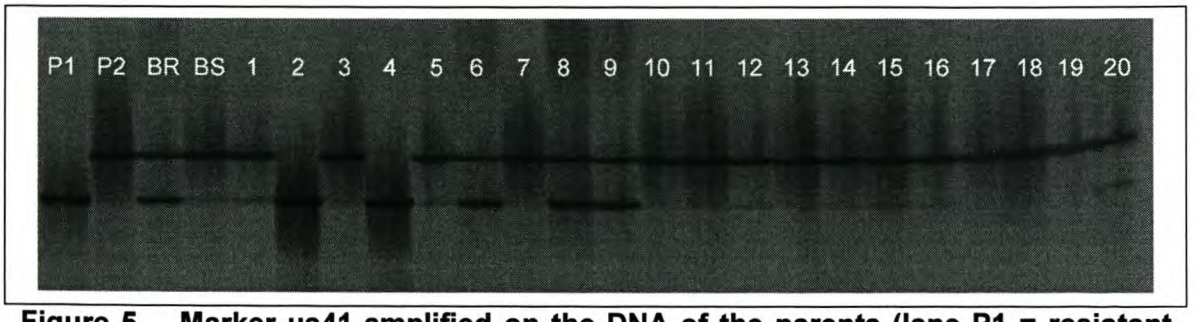

Figure 5. Marker us41 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent) and the bulks (lane BR = 1997 resistant bulk, lane BS = 1997 susceptible bulk) and the DNA of the 10 resistant (lanes 1-10) and 10 susceptible (lanes 11-20) plants making up the bulks.

3.2.3 Marker us42

Marker us42 amplified a fragment of about 160 bps on the DNA of the parents at an annealing temperature of 56°C. A 2 bps size difference between the parents was visible on a polyacrylamide gel. Eight of the ten plants (lanes 2, 4, 5, 6, 7, 8, 9 and 10; Figure 6) of the resistant bulk had the allele of the resistant parent, of which 5 (lanes 4, 6, 7, 8 and 9; Figure 6) were heterozygous. The ten plants (lanes 11-20; Figure 6) making up the susceptible bulk had the allele of the susceptible parent with one of the plants (lane 17; Figure 6) being heterozygous.

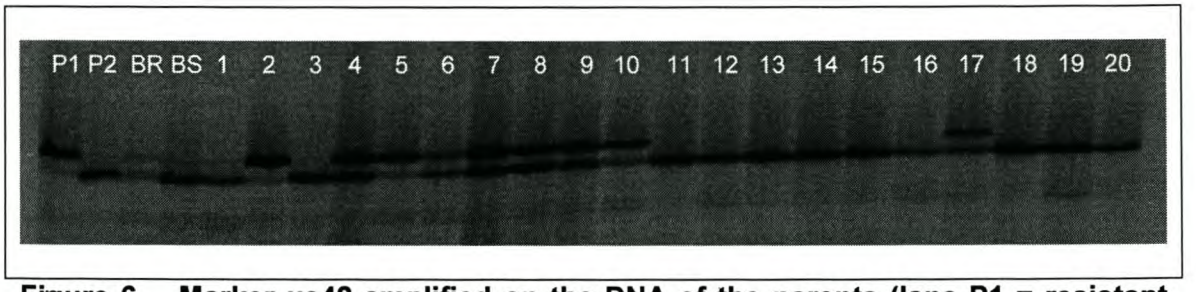

Figure 6. Marker us42 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent) and the bulks (lane BR = 1997 resistant bulk, lane BS = 1997 susceptible bulk) and the DNA of the 10 resistant (lanes 1-10) and 10 susceptible (lanes 11-20) plants making up the bulks.

3.2.4 Marker us44

A 270 bps fragment was amplified on both the resistant and the susceptible parental DNA with the marker us44 at an annealing temperature of 60°C. Marker us44 was polymorphic after digestion with restriction enzyme *Cfo*I and produced fragments of about 120 bps and 150 bps in the resistant and susceptible parent, respectively. Seven of the ten plants (lanes 1, 3, 4, 5, 6, 7 and 9; Figure 7) of the resistant bulk had the allele of the resistant parent of which two (lanes 6, 7; Figure 7) were heterozygous. The ten plants (lanes 11-20; Figure 7) of the susceptible bulk had the allele of the susceptible parent.

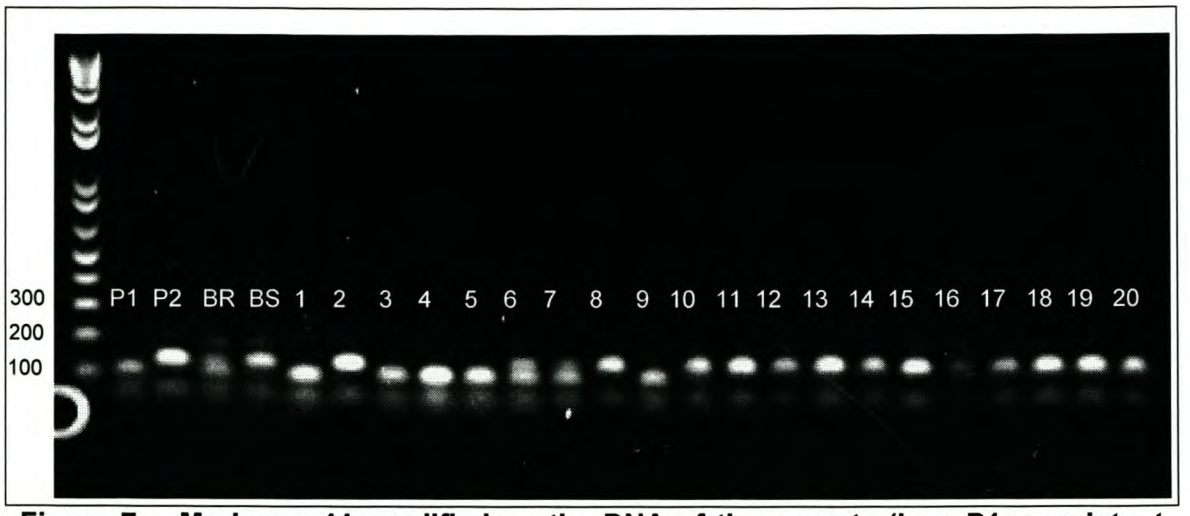

Figure 7. Marker us44 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent) and the bulks (lane BR = 1997 resistant bulk, lane BS = 1997 susceptible bulk) and the DNA of the 10 resistant (lanes 1-10) and 10 susceptible (lanes 11-20) plants making up the bulks and digested with *Cfo*I. The 1-kb plus ladder is the molecular mass marker.

3.2.5 Marker us45

At an annealing temperature of 66°C marker us45 amplified a fragment of approximately 600 bps on the resistant but not the susceptible parental DNA and was thus a dominant marker. This marker was present in 7 (lanes 1, 3, 4, 5, 6, 7 and 9; Figure 8) of the ten plants making up the resistant bulk and absent in the susceptible bulk (lanes 11-20; Figure 8). Except for the plants in lanes 1 and 3, the same F_2 plants as in the AFLP analysis had the allele of the resistant parent. The AFLP fragment was not amplified on the DNA of the plants of lanes 1 and 3 in the AFLP analysis.

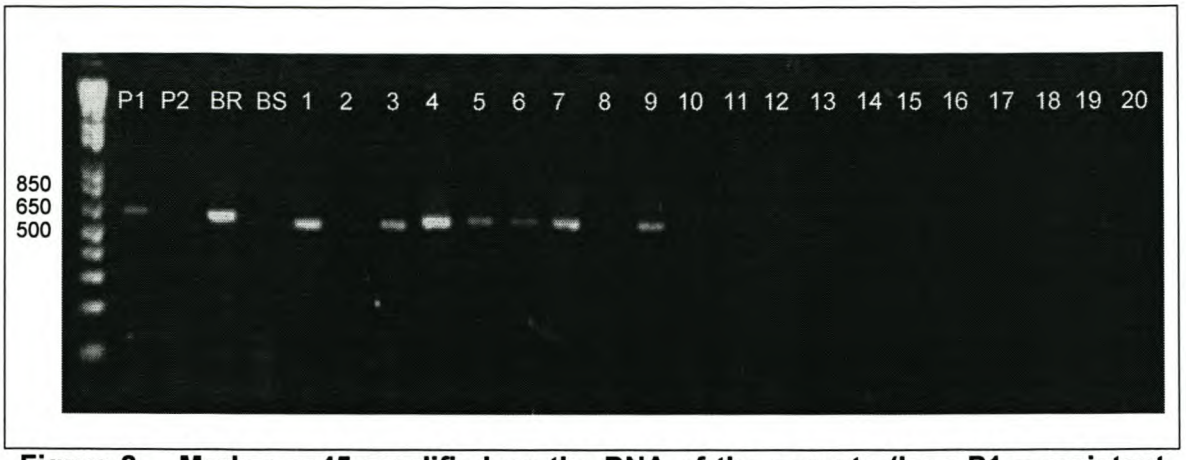

Figure 8. Marker us45 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent) and the bulks (lane BR = 1997 resistant bulk, lane BS = 1997 susceptible bulk) and the DNA of the 10 resistant (lanes 1-10) and 10 susceptible (lanes 11-20) plants making up the bulks. The 1-kb ladder plus is the molecular mass marker.

3.3 QTL analysis with the converted AFLP markers

The 5 polymorphic markers (us40, us41, us42, us44 and us45) were amplified on the 230 plants of the 1998 F_2 population (Table 1B, page 32) and linkage analysis was performed with MAPMAKER/EXP version 3.0b. Two linkage groups were identified, one group included the two markers us44 and us45 and the other included markers us40 and us42. The two-point linkage distance between the markers us44 and us45 was 10.4 cM (LOD 22.83) and between markers us40 and us42 was 8.2 cM (LOD 55.41). Marker us41 was unlinked.

Interval mapping with MAPMAKER/QTL using the two linkage groups and the genotype and phenotype data of the 230 F_2 plants confirmed the presence of QTL in both linkage groups. A LOD value of 18.12 and a variance contribution of 43% was calculated with the markers us44 and us45 and a LOD value of 4.85 and a variance contribution of 10% was calculated with the markers us40 and us42.

3.4 Mapping of the AFLP markers using RIL populations

Two publicly available recombinant inbred families (Burr *et al.*, 1988) already mapped for more than 1000 markers, were used to localize the converted AFLP markers on the genetic map of maize. RIL family I (T323 X CM37) consisted of 48 lines and RIL family II (CO159 X Tx303) consisted of 41 lines. Four of the five markers (us40, us41, us44 and us45), which were polymorphic between the parents of the F_2 population, were polymorphic between the parents of at least one of the RIL populations. Two other converted AFLP markers (us39 and us46), which were not polymorphic between the parents of the F_2 population, were polymorphic between the parents of at least one of the RIL populations. The 6 polymorphic markers were amplified on the DNA of the individual plants of the RIL populations. The genotype data were added to the existing datafiles of the RIL populations and linkage analysis was performed.

Markers us44 and us45 were mapped to chromosome 1 in bin 1.05 and 1.04, respectively (Figure 9A). Marker rz421 from the RIL database was the nearest marker to marker us44 at a distance of 1.2 cM (LOD value 11.15) and the nearest marker to marker us45 was rz672a at a distance of 5.3 cM (LOD value 7.41) (Figure 9B). With the RIL population a two-point linkage distance of 14.9 cM (LOD value 3.55) was calculated between markers us44 and us45.

Marker us40 was localized on chromosome 5 in bin 5.04 (Figure 10A), 1.3 cM (LOD value 10.28) distal to marker bnl5.71 from the RIL database (Figure 10B). As marker us42 showed linkage with marker us40 in the F_2 population (section 3.3), it can be inferred that this marker is also localized on chromosome 5.

Marker us41 was localized on chromosome 3 in bin 3.04 (Figure 11A), 1.3 cM (LOD value 9.7) distal to marker npi220b (Figure 11B) and marker us39 was localized on chromosome 2 in bin 2.02 (Figure 12A), 6.9 cM (LOD value 5.12) distal to marker umc6 (Figure 12B). No linkage could be detected between

marker us46 and the markers of the RIL population and this marker could therefore not be localized to a chromosome.

To determine whether the converted AFLP markers us41 on chromosome 3 and the us39 on chromosome 2 are associated with GLS resistance, a simple linear regression analysis was performed (section 3.5).

The QTL identified with the linkage group consisting of the markers us44 and us45 was mapped to chromosome 1 (QTL1) and the QTL identified with the linkage group consisting of the markers us40 and us42 was mapped to chromosome 5 (QTL5). To obtain a more precise localization of the QTL on chromosomes 1 and 5, converted RFLP and microsatellite markers were used (sections 3.6 - 3.9).

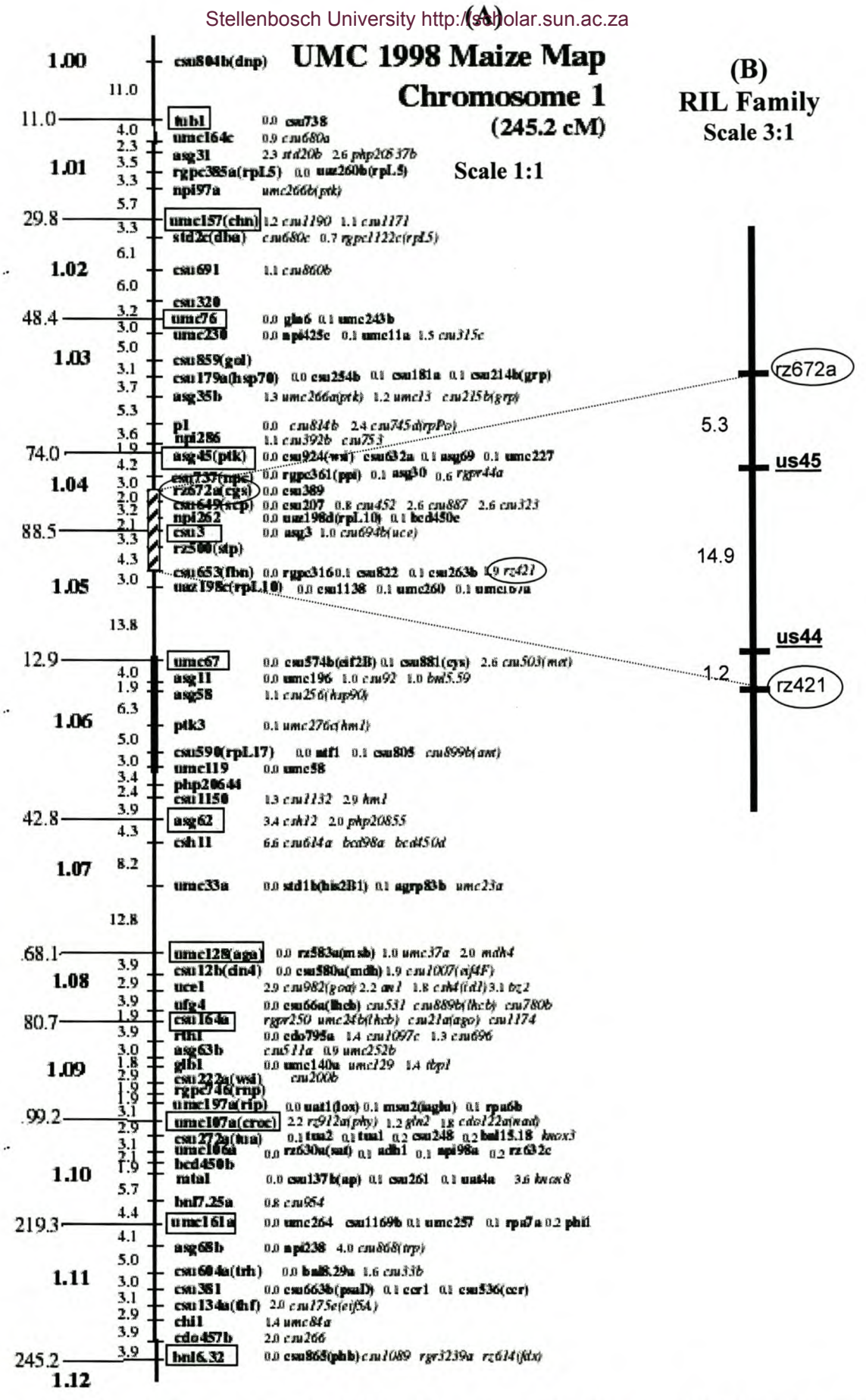

Figure 9. (A) Map of chromosome 1 obtained from the Maize Genome Database (http://www.agron.missouri.edu). (B) Linkage group obtained with the RIL family of Burr *et al.* (1988) using MAPMAKER/EXP. Markers which occur on both maps are circled. Map distances are given in centiMorgans.

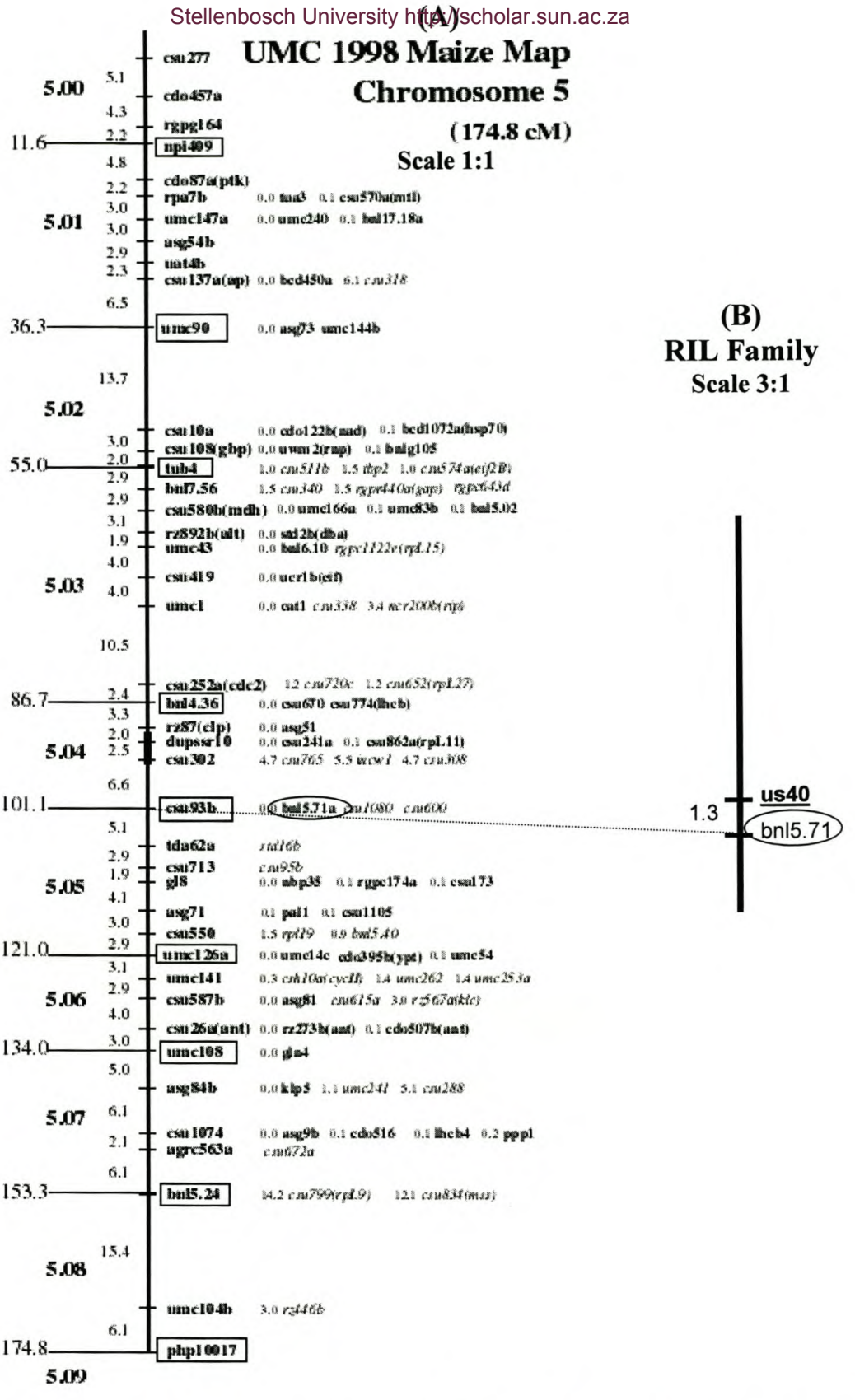

Figure 10. (A) Map of chromosome 5 obtained from the Maize Genome Database (http://www.agron.missouri.edu). (B) Linkage group obtained with the RIL family of Burr *et al.* (1988) using MAPMAKER/EXP. Markers which occur on both maps are circled. Map distances are given in centiMorgans.

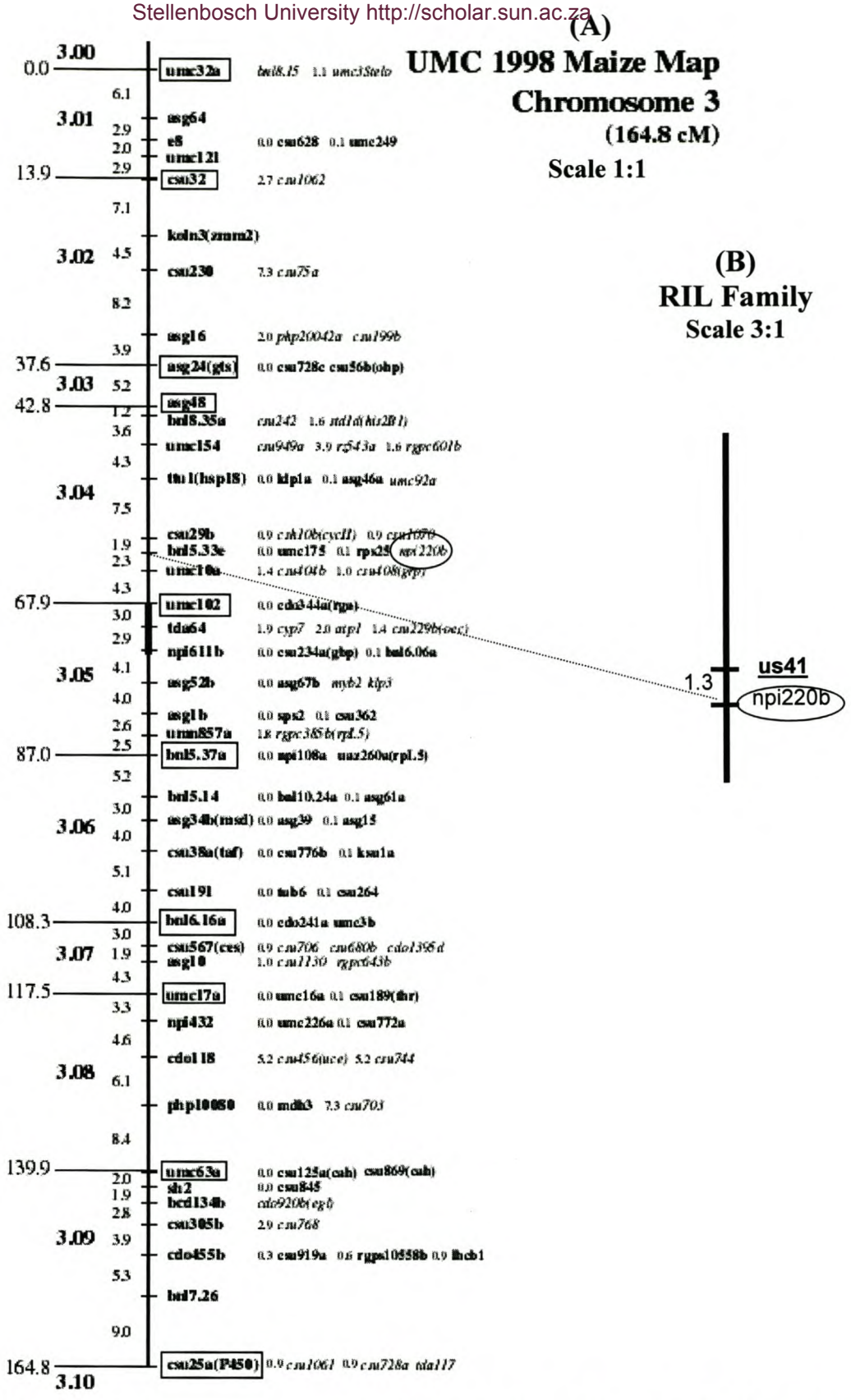

Figure 11. (A) Map of chromosome 3 obtained from the Maize Genome Database (http://www.agron.missouri.edu). (B) Linkage group obtained with the RIL family of Burr *et al.* (1988) using MAPMAKER/EXP. Markers which occur on both maps are circled. Map distances are given in centiMorgans.

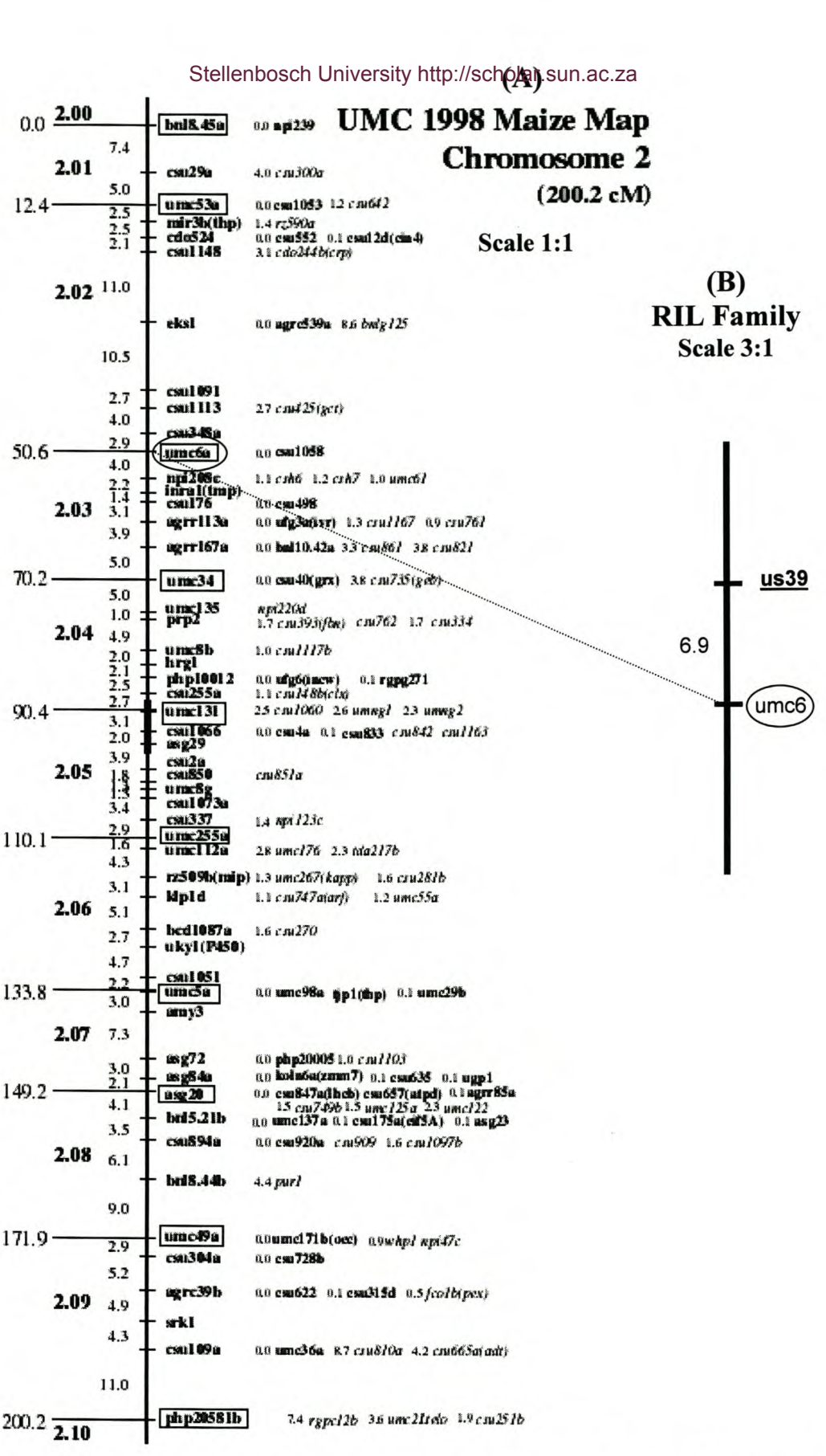

Figure 12. (A) Map of chromosome 2 obtained from the Maize Genome Database (http://www.agron.missouri.edu). (B) Linkage group obtained with the RIL family of Burr *et al.* (1988) using MAPMAKER/EXP. Markers which occur on both maps are circled. Map distances are given in centiMorgans.

3.5 Regression analysis

As marker us41 on chromosome 3 was not linked to any of the other converted AFLP markers, QTL mapping could not be performed with this marker. To determine whether it is associated with GLS resistance, a standard ANOVA for linear regression of GLS score on marker genotype was performed, using the 1998 F_2 population. This indicated no significant regression ($P = 0.204$), therefore no linkage for this marker.

Furthermore, the converted AFLP marker us39 on chromosome 2 was not polymorphic between the resistant and susceptible parent. To test for a link between GLS resistance and this marker, a microsatellite marker (bnlg125, Table 4) was obtained, occurring in the same bin (bin 2.02) as the non-polymorphic AFLP marker.

Table 4. Microsatellite marker bnlg125 with its bin position, primer sequences and annealing temperature.

Microsatellite	*Bin*	*Primer sequences*	*Annealing temperature*
bnlg125	2.02	gggacaaaagaagaagcagag// gaaatgggacagagacagacaat	48°C

Amplification of microsatellite marker bnlg125 on the parental DNA (annealing temperature of 48°C) resulted in a size difference which could be depicted on an agarose gel. The alleles produced with bnlg125 from the resistant and susceptible parental DNA had sizes of about 350 and 410 bps, respectively (lanes P1 and P2; Figure 13).

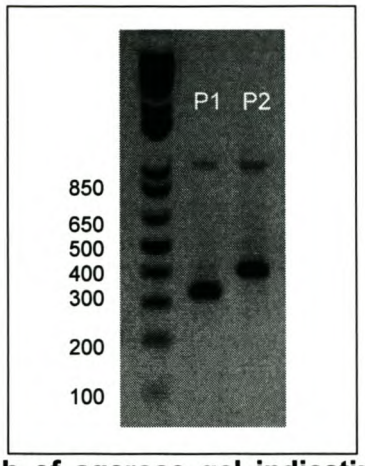

Figure 13. Photograph of agarose gel indicating microsatellite marker bnlg125 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent). The 1-kb plus ladder is the molecular mass marker.

The microsatellite marker was amplified on the DNA of the 230 plants of the 1998 F_2 population. A standard ANOVA for linear regression of GLS score on marker genotype indicated no significant regression (P = 0.757), therefore no linkage for this marker.

The AFLP markers on chromosomes 2 and 3 could thus be false positive markers (i.e., markers that appear polymorphic between bulks but are not linked to the trait expression (Grattapaglia *et al.*, 1996)). However, neither marker was polymorphic in the 1998 bulks, and it could thus be that the markers were associated with GLS resistance in the 1997 population, in which they were polymorphic, but not in the 1998 population.

3.6 Converted RFLPs on chromosome 1

Sixteen RFLP probes, spread over chromosome 1, were received from the University of Missouri (Table 5). To make screening of the progeny easier and faster, the RFLP probes were converted into STS (sequence-tagged site) markers. They were thus sequenced and primers identified. The sequences of the probes and the identified primers are given in Addendum II (page 131). No suitable primer pairs could be identified for probe asg75.

Table 5. List of RFLP probes obtained from the University of Missouri. The bin position of each probe is given together with the probe vector, selective agent, insert size and the enzyme. M13F/R = M13 forward and M13 reverse primers.

Probe/ locus name	*Bin position*	*Vector*	*Selective agent*	*Insert size*	*Enzyme*	*PCR primers*
npi286	1.03	pUC19	ampicillin	825	PstI	M13F/R
umc11	1.03	pUC19	ampicillin	890	PstI	M13F/R
umc76	1.03	pUC19	ampicillin	760	PstI	M13F/R
umc13	1.03	pUC19	ampicillin	640	PstI	M13F/R
umc8	1.03	pUC19	ampicillin	1180	PstI	M13F/R
asg30	1.04	pGEM3Zf	ampicillin	1700	PstI	T7, SP6
npi262	1.04	pUC19	ampicillin	490	PstI	M13F/R
asg75	1.04	pGEM3Zf	ampicillin	450	PstI	T7, SP6
umc227	1.04	pUC19	ampicillin	750	PstI	M13F/R
npi279	1.05	pUC19	ampicillin	875	PstI	M13F/R
npi598	1.05	pUC19	ampicillin	1100	PstI	M13F/R
bnl5.59	1.06	pUC12	ampicillin	2250	PstI	M13F/R
uaz249	1.06	pZL1	ampicillin	400	XbaI/SalI	M13F/R
umc58	1.06	pUC19	ampicillin	920	PstI	M13F/R
php20855	1.07	pUC19	ampicillin	1300	PstI	M13F/R
umc23	1.07	pUC19	ampicillin	670	PstI	M13F/R

Each STS primer set was amplified on the DNA of the resistant and susceptible parent. The following 6 converted RFLPs had to be discarded, because multiple fragments were amplified in both parents: umc11, umc13, npi262, umc227, uaz249 and umc23. One of the converted RFLPs (umc58) was polymorphic between the parents after agarose gel electrophoresis. To search for point mutation polymorphisms within the amplified products, the 9 remaining converted RFLPs were digested with the following 13 different restriction enzymes: *Rsa*I, *Alu*I, *Cfo*I, *Tsp*509I, *Hpa*II, *Mnl*I, *Aci*I, *Msp*I, *Tru*91, *Hae*III, *Na*III, *Taq*I and *Hinf*I.

A 700 bp fragment was amplified with the primer pairs for marker umc58 on the DNA of the resistant parent (lane P1; Figure 14) whereas a slightly smaller fragment of 670 bp was amplified on the susceptible parent (lane P2; Figure 14).

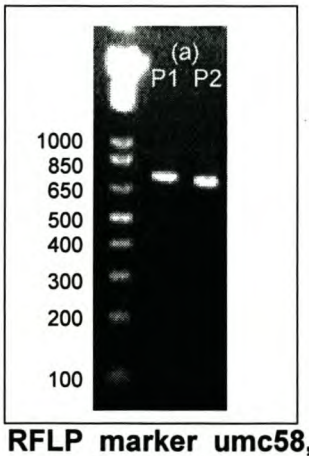

Figure 14. The converted RFLP marker umc58, amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent). The 1-kb plus ladder is the molecular mass marker.

After restriction enzyme digestion, 4 other converted RFLPs produced PCR products, which were polymorphic between the resistant and susceptible parent. PCR products amplified with markers npi286, asg30 and bnl5.59 and digested with restriction enzymes *Hpa*II, *Cfo*I, and *Tru*91, respectively, resulted in length polymorphisms that made the markers co-dominant. Marker php20855 was

polymorphic between the parents after restriction enzyme digestion with *Hpa*II. The plants having the allele of the resistant parent could, however, not be distinguished from the heterozygous plants, and the marker was thus recorded as a dominant marker.

With the primers for probe npi286 a fragment of approximately 400 bps was amplified at an annealing temperature of 60°C on the resistant and susceptible parent DNA. Upon digestion with the restriction enzyme *Hpa*II a fragment of 310 bps was produced in both parents together with a 280 bps fragment in the resistant parent (lane P1a; Figure 15) and a 380 bps fragment in the susceptible parent (lane P2a; Figure 15).

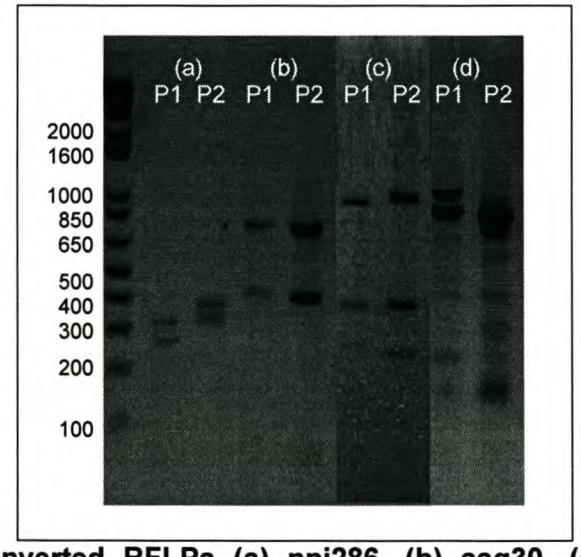

Figure 15. The converted RFLPs (a) npi286, (b) asg30, (c) bnl5.59, and (d) php20855 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent) and digested with *Hpa*II, *Cfo*I, *Tru*91 and *Hpa*II, respectively. The 1-kb plus ladder is the molecular mass marker.

The primers for probe asg30 amplified a fragment of approximately 1500 bps on the resistant and susceptible parent DNA at an annealing temperature of 55°C. Upon digestion with the restriction enzyme *Cfo*I a fragment of about 800 bps was produced in both parents and a fragment of 420 bps and 400 bps was produced

in the resistant parent (lane P1b; Figure 15) and the susceptible parent (lane P2b; Figure 15), respectively.

With the primers for probe bnl5.59 a fragment of approximately 2 kb was amplified on the parent DNA at an annealing temperature of 60°C. Upon digestion with the restriction enzyme *Tru*9I three distinguishable fragments were produced, including a 915 bp fragment in the resistant parent (lane P1c; Figure 15) clearly distinguishable from a slightly larger 980 bp fragment in the susceptible parent (lane P2c; Figure 15).

At an annealing temperature of 55°C marker php20855 amplified a fragment of about 1300 bps on both the resistant and the susceptible parent DNA. After restriction enzyme digestion with *Hpa*II, marker php20855 produced a fragment of approximately 900 bps in both the resistant and the susceptible parent (lane P1d and P2d, respectively; Figure 15) and a slightly larger fragment of approximately 1100 bps in the resistant parent (lane P1d; Figure 15). The absence of the larger fragment in the susceptible parent allowed for the distinction of the susceptible plants from the heterozygous plants and the plants dominant for the allele of the resistant parent.

As only five of the 16 converted RFLP markers were polymorphic, microsatellite markers were also chosen to saturate chromosome 1.

3.7 Microsatellite markers on chromosome 1

Seventeen microsatellite primer pair sequences were accessed via the Internet (Table 6). The primer pairs were tested for polymorphisms by amplification on the parental DNA.

Table 6. The microsatellites on chromosome 1 are listed with their bin positions, primer sequences and annealing temperatures. An indication of the polymorphism is given: (a) polymorphism can be detected on an agarose gel, (p) on a polyacrylamide gel and (n) no polymorphism could be detected.

Microsatellites	*Bins*	*Primer Sequences*	*Annealing temperature*	*Poly-morphism*
phi056	1.01	acgcccagactctgttccttctc// atggcggcaggccgattgtt	57°C	p
bnlg147	1.02	aggaagctttggtcaagtctta// gctcactcgatttgttgtgcta	56°C	p
phi001	1.03	tgacggacgtggatcgcttac// agcaggcagcaggtcagcagcg	58°C	a
bnlg652	1.05	cgcacgtcgggagagagggaga// gccgcaaacatagccgccaaaaat	59°C	a
bnlg1832	1.05	gcgcccacaacaagtaaatt// cctcattgtaaggggcagaa	48°C	a
bnlg1886	1.05	tctctctcacatgcacgcc// tttgatttggggaaccagag	45°C	n
bnlg2086	1.05	cggaacctgctgcagttaat// gagatgcaggaatgggaaaa	54°C	p
bnlg421	1.06	ggggcaaggacttgtcggt// agccagttgcccagcatct	69°C	n
bnlg1057	1.06	ttcaccgcctcacatgac// gcaacgctagctagctttg	45°C	n
bnlg1598	1.06	ggcaagattcggaccagg// cggttaggagcagtacgtca	50°C	p
bnlg257	1.07	tgcagagacgagcgtttgaatgct// gctctgaggttttcatacggggtt	54°C	n
bnlg615	1.07	cttccctctccccatctcctttccaa// gcaacctgtccattctcaccagcggatt	62°C	n
phi037	1.08	cccagctcctgttgtcggctcagac// tccagatccgccgcacctcagtca	70°C	p
phi094	1.09	aaagaggaggaacgcgaaggac// tcacatcctggcggtcacca	62°C	n
bnlg400	1.09	agctgtgactgtgaagggaaaa// cgtcacaccgctgtttcttg	59°C	n
phi120	1.11	gactctcacggcgaggtatga// tgatgtcccagctctgaactgac	49°C	n
bnlg504	1.11	cggcagctccagcaccggcat// agtgtccacataccgccacacacgttt	64°C	p

The annealing temperatures used to amplify each microsatellite marker are given in Table 6. Of the 17 microsatellite markers, the following 8 did not reveal a polymorphism between the resistant and susceptible parent DNA: bnlg1886, bnlg421, bnlg1057, bnlg257, bnlg615, phi094, bnlg400 and phi120. With 3 of the 17 microsatellite markers (phi001, bnlg652 and bnlg1832) a polymorphism between the parents could be seen on a 2% agarose gel. Microsatellite markers phi056, bnlg147, bnlg2086, bnlg1598, phi037 and bnlg504 produced polymorphic fragments, which could be depicted when the samples were loaded onto a 4% polyacrylamide gel (Table 6).

A size difference between the resistant and susceptible parent could be detected with microsatellite marker phi001 and it was thus a co-dominant marker. A band of about 120 bps was amplified from the resistant (lane P1a; Figure 16) and a smaller band of about 90 bps was amplified from the susceptible parent (lane P2a; Figure 16). Microsatellite markers bnlg652 and bnlg1832 were dominant. A fragment of about 100 bps was amplified from the resistant but not the susceptible parent DNA with the primer pairs for marker bnlg652 (lanes P1b and P2b, respectively; Figure 16). With the primer pairs for marker bnlg1832 a fragment of about 210 bps was amplified from the susceptible but not the resistant parental DNA (lanes P2c and P1c, respectively; Figure 16).

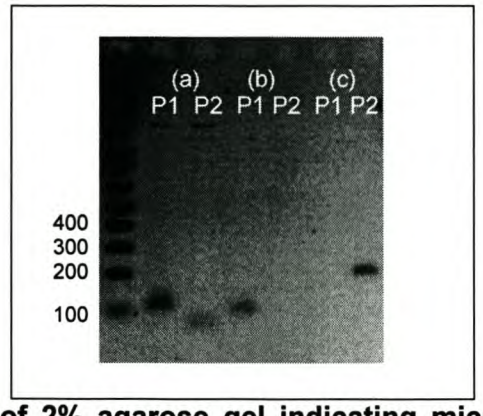

Figure 16. Photograph of 2% agarose gel indicating microsatellite markers (a) phi001, (b) bnlg652 and (c) bnlg1832 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent). The 1-kb plus ladder is the molecular mass marker.

The microsatellite markers, which produced polymorphism depicted by polyacrylamide gel electrophoresis, were co-dominant. The alleles produced by the primer pair for microsatellite marker phi056 from the resistant and susceptible parent DNA were 93 and 84 bps in size, respectively (lanes P1a and P2a; Figure 17). Microsatelite marker bnlg147 produced alleles of 118 and 114 bps (lanes P1b and P2b; Figure 17) and marker bnlg2086 produced alleles of 232 and 234 bps (lanes P1c and P2c; Figure 17) from the resistant and susceptible parental DNA, respectively.

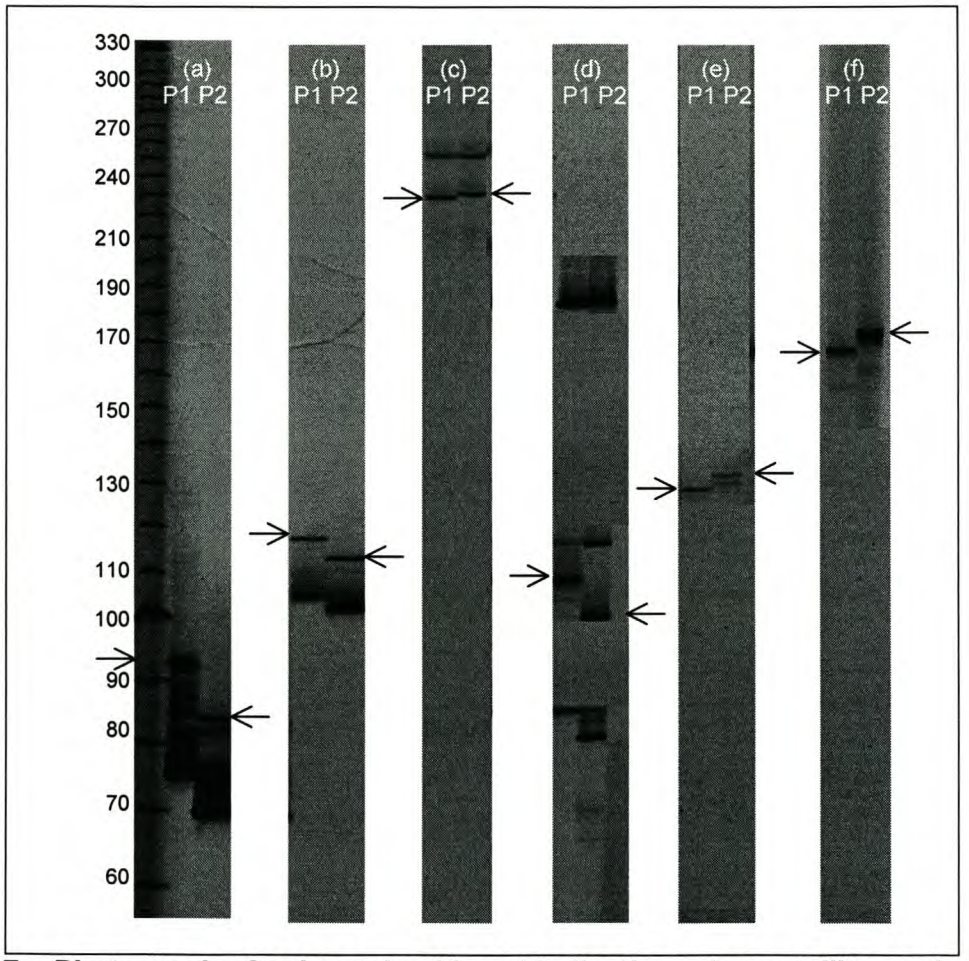

Figure 17. Photograph of polyacrylamide gel indicating microsatellite markers (a) phi056, (b) bnlg147, (c) bnlg2086, (d) bnlg1598, (e) phi037 and (f) bnlg504 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent). The allele of the resistant and the susceptible parent is indicated with an arrow on the left and the right side, respectively. The 30-330 ladder is the molecular mass marker.

Alleles of 110 and 100 bps were produced by marker bnlg1598 (lanes P1d and P2d; Figure 17) from the resistant and susceptible parent DNA, respectively. With marker phi037 alleles of 130 and 134 bps (lanes P1e and P2e; Figure 17) and with marker bnlg504 alleles of 169 and 175 bps (lanes P1f and P2f; Figure 17) were amplified on the resistant and susceptible parent DNA, respectively.

3.8 Microsatellite markers on chromosome 5

Sequences of nine microsatellite primer pairs on chromosome 5 were obtained from the Maize Genome Database website (Table 7). The primer pairs were tested for polymorphisms by amplification on the parental DNA.

Table 7. The microsatellites on chromosome 5 are listed with their bin positions, primer sequences and annealing temperatures. An indication of the polymorphism is given: (a) polymorphism can be detected on an agarose gel, (p) on a polyacrylamide gel and (n) no polymorphism could be detected.

Microsatellites	*Bins*	*Primer sequences*	*Annealing temperature*	*Poly-morphism*
bnlg143	5.01	gcactgccggagtgccttct// atgccgtgatctgtgacatctaacc	51°C	p
bnlg565	5.02	taagaacgacgaacggtaactg// gctcactgcacgccaacac	50°C	p
bnlg557	5.03	tcacgggcgtagagagaga// cgaagaaacagcaggagatgac	50°C	p
bnlg150	5.04	gaaaaacccccctccccatat// aatggccgaacacaattcaa	53°C	p
mmc0282	5.05	ctctttctttatttgttccgtt// ggactacacatcaccagca	48°C	a
umc1019	5.06	ccagccatgtcttctcgttctt// aaacaaagcaccatcaattcgg	50°C	n
bnlg1847	5.06	gacgctagagagaggcgaag// atgtaacaagaaggcccgtg	68°C	p
bnlg1306	5.07	caccttgaaagcatcctcgt// caaaaacaaatggcagctga	58°C	p
bnlg389	5.09	ggtcaccctccctttgcag// attgcctacacagtttgattgg	65°C	n

The annealing temperatures used to amplify each microsatellite marker are given in Table 7. Two of the 9 microsatellite markers on chromosome 5 (umc1019 and bnlg389) were not polymorphic between the parents of the F_2 population. The size difference produced by marker mmc0282 could be detected on a 2% agarose gel and the polymorphisms produced by the remaining microsatellite markers could be depicted on a 4% polyacrylamide gel (Table 7).

With the primer pairs for microsatellite marker mmc0282 an allele of 90 bps was amplified on the resistant and an allele of 170 bps on the susceptible parental DNA (lanes P1 and P2, respectively; Figure 18).

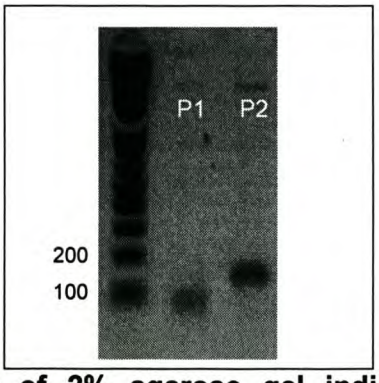

Figure 18. Photograph of 2% agarose gel indicating microsatellite marker mmc0282 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent). The 1-kb plus ladder is the molecular mass marker.

By running the amplified products on a polyacrylamide gel, the polymorphisms of markers bnlg143, bnlg565, bnlg557, bnlg150, bnlg1847 and bnlg1306 were detected. Microsatelite marker bnlg143 produced alleles of 224 and 232 bps (lanes P1a and P2a; Figure 19) from the resistant and susceptible parental DNA, respectively. Alleles of 77 and 131 bps (lanes P1b and P2b; Figure 19) were amplified with the primer pair for marker bnlg565 and alleles of 102 and 104 bps (lanes P1c and P2c; Figure 19) were amplified with the primers for marker bnlg557 on the resistant and susceptible parental DNA, respectively. Marker

bnlg150 produced alleles of 66 and 63 bps (lanes P1d and P2d; Figure 19), bnlg1487 produced alleles of 97 and 95 bps (lanes P1e and P2e; Figure 19) and bnlg1306 produced alleles of 162 and 184 bps (lanes P1f and P2f; Figure 19) from the resistant and susceptible parental DNA, respectively.

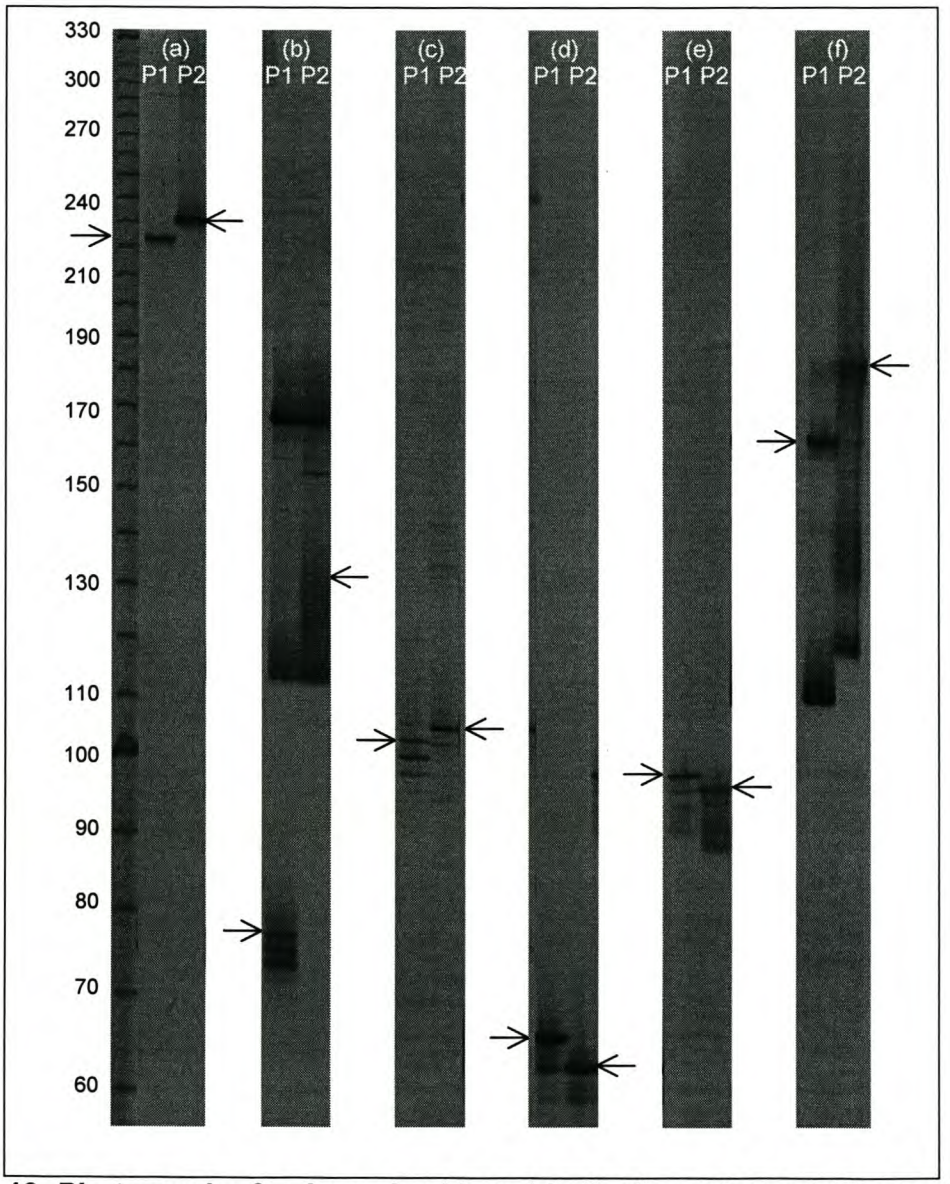

Figure 19. Photograph of polyacrylamide gel indicating microsatellite markers (a) bnlg143, (b) bnlg565, (c) bnlg557, (d) bnlg150, (e) bnlg1847, and (f) bnlg1306 amplified on the DNA of the parents (lane P1 = resistant parent, lane P2 = susceptible parent). The allele of the resistant and the susceptible parent is indicated with an arrow on the left and the right side, respectively. The 30-330 bps ladder is the molecular mass marker.

3.9 QTL analysis

3.9.1 Chi-square analysis

The converted RFLP markers (npi286, asg30, bnl5.59, umc58 and php20855) on chromosome 1, and the co-dominant microsatellite markers bnlg147, phi001, bnlg2086, bnlg1598 and phi037 on chromosome 1 were amplified on the 230 plants of the 1998 F_2 population. The microsatellite markers on chromosome 5 (bnlg143, bnlg565, bnlg557, bnlg150, mmc0282, bnlg1847 and bnlg1306) were also amplified on the 230 plants of the 1998 F_2 population.

These markers and the converted AFLP markers (us44, us45, us40 and us42) were tested for segregation according to the 1:2:1 and 3:1 expected Mendelian ratio for co-dominant and dominant markers, respectively, using the chi-square test. One converted RFLP marker, npi286, one converted AFLP marker, us45 and one microsatellite marker, bnlg143 showed distorted segregation at the 5% significance level (Table 8). Markers npi286 and us45 were skewed towards the allele of the susceptible parent, whereas marker bnlg143 was skewed towards the heterozygous genotype. When the significance level was increased to 1% only marker npi286 showed distorted segregation (Table 8). As marker npi286 showed distorted segregation with $P \leq 0.001$ it was not used in the construction of the linkage map.

Table 8. Number of observed and expected alleles (RR is homozygous for the allele of the resistant parent, SS is homozygous for the alleles of the susceptible parent and RS is heterozygous) for the markers on chromosome 1 and 5. Results of the chi-square test for distorted segregation are given.

		Observed				*Expected*			*Chi-square analysis*	*P(2df)*
Chromosome 1		RR	SS	RS	Total	RR	SS	RS		
Co-dominant markers:										
Bin	Marker									
1.02	bngl147	46	61	121	228	57	57	114	2.8333	0.2425
1.03	npi286	30	75	111	216	54	54	108	18.9166**	0.0001**
1.03	phi001	55	64	110	229	57	57	115	1.0611	0.5883
1.04	asg30	47	65	96	208	52	52	104	4.3462	0.1138
1.05	bnlg2086	47	53	130	230	58	58	115	4.2261	0.1209
1.05	us44	45	63	106	214	54	54	107	3.0467	0.2180
1.06	bnl5.59	48	63	104	215	54	54	108	2.3209	0.3133
1.06	bnlg1598	52	66	110	228	57	57	114	2.0000	0.3679
1.06	umc58	56	71	101	228	57	57	114	4.9386	0.0846
1.08	phi037	57	53	119	229	57	57	115	0.4934	0.7814
dominant markers:										
Bin	Marker									
1.04	us45	156	74		230	173	58		6.3130*	0.0426*
1.07	php20855	177	48		225	169	56		1.6133	0.4463
Chromosome 5										
Co-dominant markers:										
Bin	Marker									
5.01	bnlg143	42	54	130	226	57	57	113	6.3894*	0.0410*
5.02	bnlg565	46	49	128	223	56	56	112	4.96412	0.08357
5.03	bnlg557	42	64	124	230	58	58	115	5.6174	0.0603
5.04	bnlg150	53	56	120	229	57	57	115	0.6070	0.7382
5.04	us42	58	54	116	228	57	57	114	0.2105	0.9001
5.05	ua40	54	54	122	230	58	58	115	0.8522	0.6531
5.05	mmc0282	48	55	127	230	58	58	115	2.9304	0.2310
5.06	bnlg1847	46	57	123	226	57	57	113	2.8407	0.2416
5.07	bnlg1306	56	61	113	230	58	58	115	0.2870	0.8663

* Significantly different at $P \leq 0.05$

** Significantly different at $P \leq 0.01$

3.9.2 Linkage map construction

The datafile used in linkage analysis is given in Addendum III (page 137). The linkage analysis results produced by MAPMAKER/EXP version 3.0b are given in Addendum IV (page 140). In linkage analysis only the recessive genotype data were used for the dominant markers us45 and php20855 and the genotypes of the homozygous dominant and heterozygous plants were designated as missing

data. MAPMAKER's error detection function was used to eliminate mistakes due to mistypings of a locus in the raw data. The order command was used to determine the linear order of the markers. Multipoint analysis was used to determine the distances between the markers. Two linkage maps, consisting of the 11 markers on chromosome 1 and 9 markers on chromosome 5, were produced.

The order of the markers on chromosome 1 and the distance between the markers is indicated in Figure 20A. MAPMAKER's multipoint analysis indicated that the 11 markers on chromosome 1 spanned a total distance of 73.8 cM. The order of the markers was in agreement with the order of the markers on the UMC 1998 maize map (circled markers, Figure 20A and 20B) and the position of the markers us44 and us45, obtained with the RIL population, was thus confirmed.

In Figure 21A the order of the markers on chromosome 5 and the distance between the markers is indicated. A total distance of 104.6 cM was spanned with the 9 markers. The position of marker us40 in bin 5.04 obtained with the RIL population (section 3.4, page 55) is in agreement with the position obtained with the F_2 population, if compared to the bin positions of the microsatellite markers (Table 7, page 70).

3.9.3 QTL mapping

The linkage maps of chromosomes 1 and 5 produced by MAPMAKER/EXP were used together with the genotype and phenotype data (Addendum III, page 137) of each F_2 plant to localize the QTL with the programs MAPMAKER/QTL and QTL Cartographer. Models 3 (interval mapping) and 6 of QTL Cartographer were applied to the data. The interval mapping results for chromosomes 1 and 5 produced by MAPMAKER/QTL are given in Addendum IV (page 146).

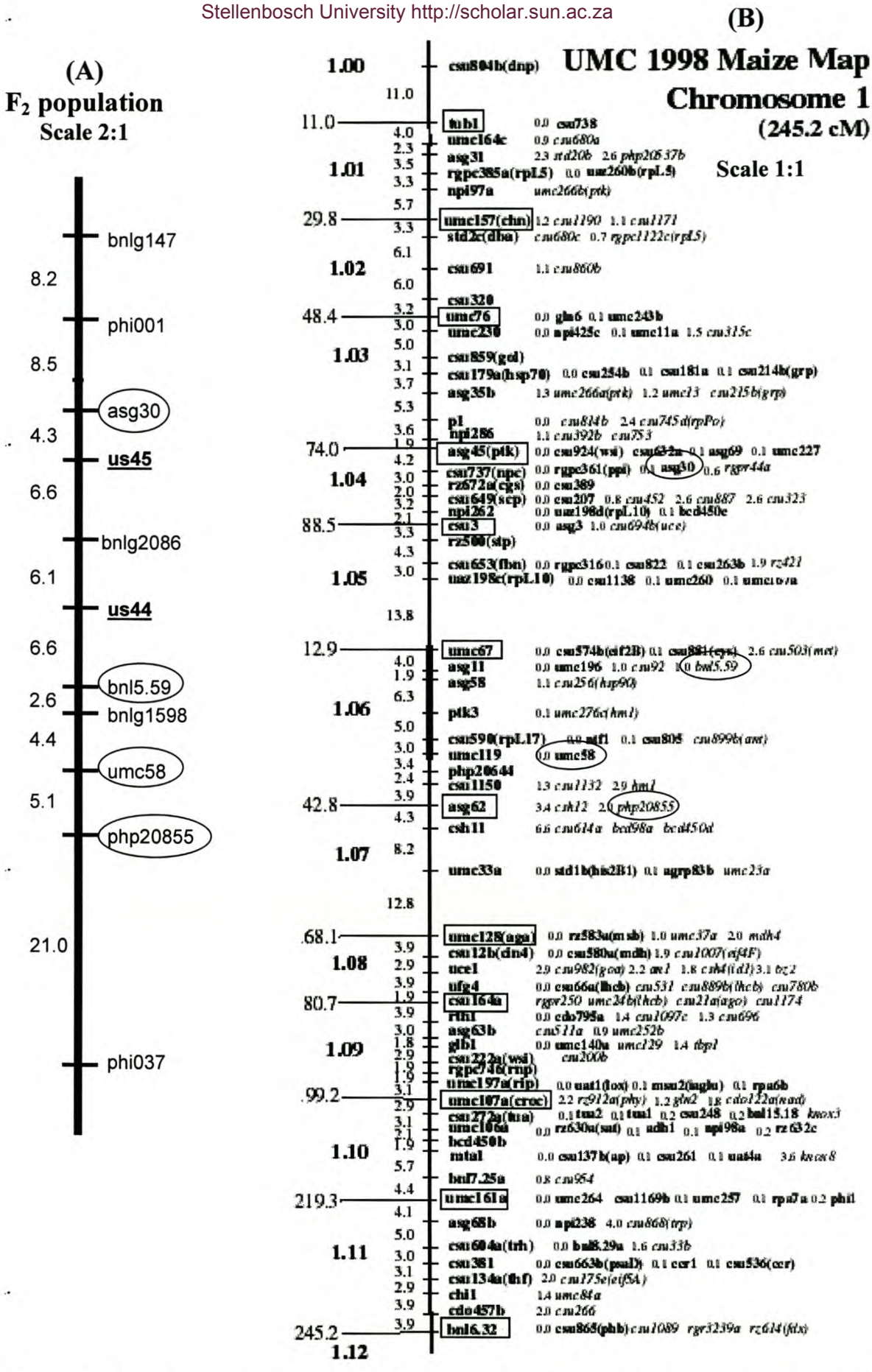

Figure 20. (A) Linkage group obtained with the 230 plants of the F_2 population using MAPMAKER/EXP. (B) Map of chromosome 1 obtained from the Maize Genome Database (http://www. agron.missouri.edu). Markers which occur on both maps are circled. Map distances are given in centiMorgans.

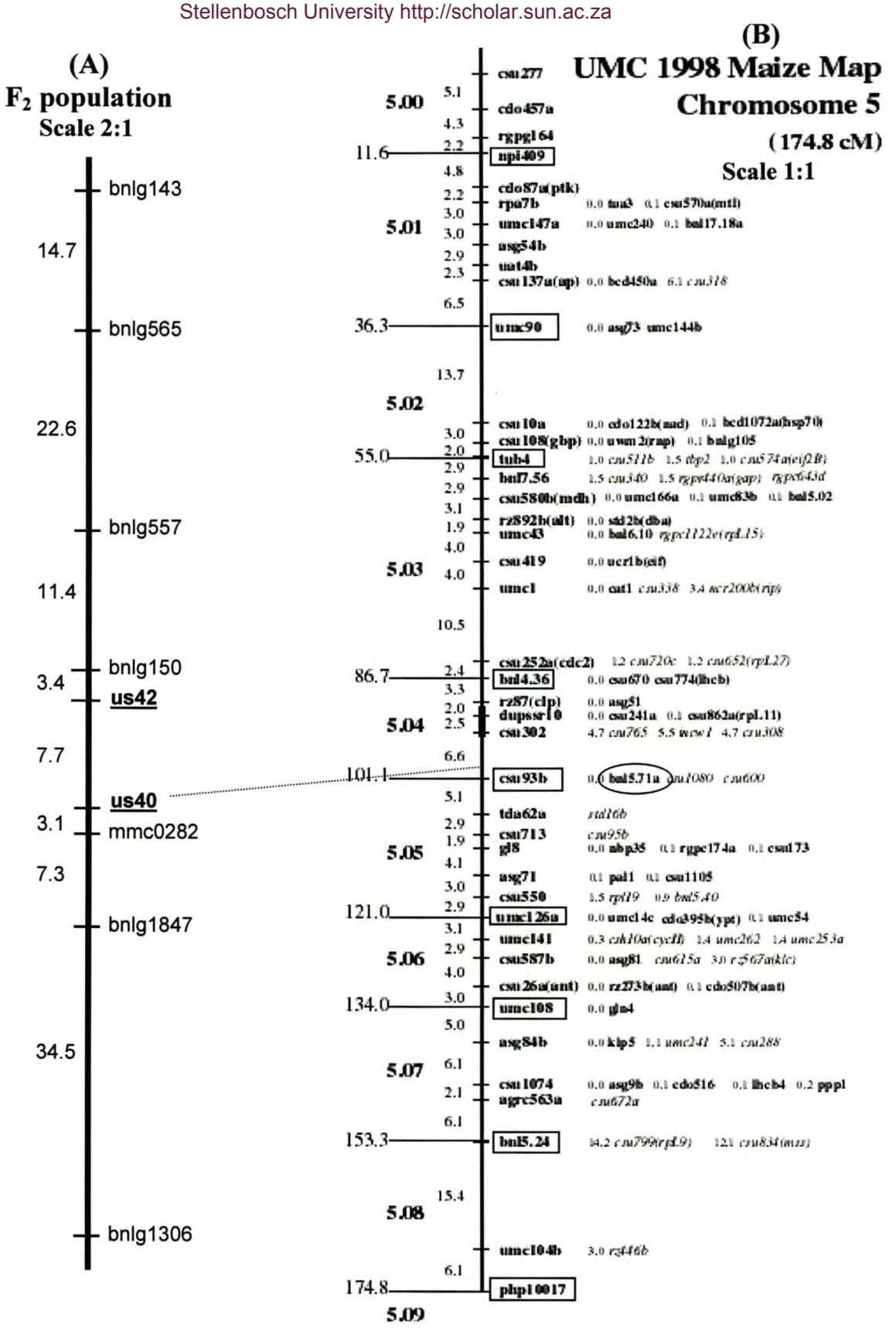

Figure 21. (A) Linkage group obtained with the 230 plants of the F_2 population using MAPMAKER/EXP. (B) Map of chromosome 5 obtained from the Maize Genome Database (http://www. agron.missouri.edu). Map distances are given in centiMorgans.

As the phenotypic GLS data showed deviations from the normal distribution (Figure 2, page 33), the data were transformed prior to analysis. The transformation of the score data to the $\log_{10}$ of the score data did not alter the QTL that was identified and the original data were therefore used in QTL analysis.

Using MAPMAKER/QTL, the highest peak with a LOD value of 20.7 was identified on chromosome 1 between markers us44 and bnl5.59, 3.1 cM proximal to marker us44 (Figure 22). The boundary of the confidence interval was 6 cM proximal to marker bnlg2086 and 6 cM proximal to marker us44. The phenotypic variance explained by QTL1 was 36.7%.

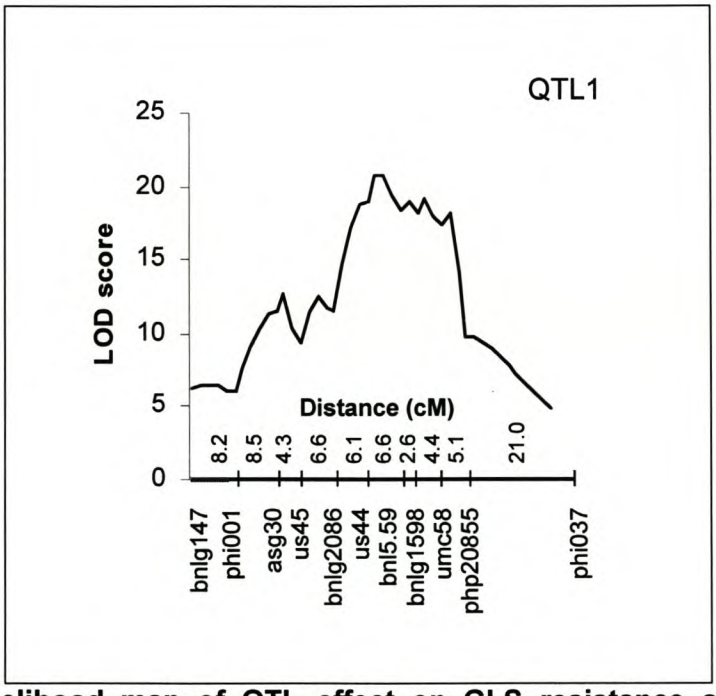

Figure 22. Likelihood map of QTL effect on GLS resistance as generated by MAPMAKER/QTL using the genotype and phenotype data of 230 F_2 plants with 11 markers on chromosome 1. The distances between the markers are given in cM.

Two peaks were visible on chromosome 5 (Figure 23). A LOD value of 5.2 was calculated for the highest peak between markers mmc0282 and bnlg1847, 3.6 cM proximal to marker mmc0282. The second peak was located between markers bnlg557 and bnlg150, 4.6 cM proximal to marker bnlg557 and the LOD value for the second peak was 4.82. The boundary of the confidence interval was 16 cM proximal to marker bnlg565 and 10 cM proximal to marker bnlg1847. 10.6% of the total phenotypic variance was explained by the highest peak and 10.5% was explained by the lower peak.

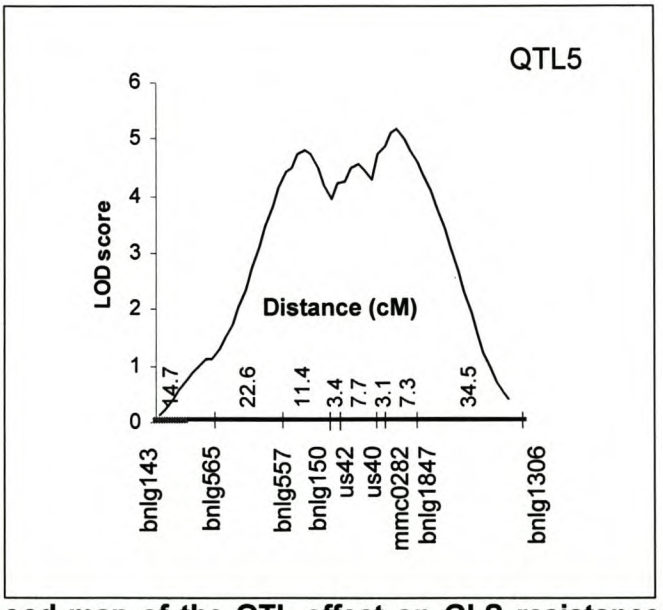

Figure 23. Likelihood map of the QTL effect on GLS resistance as generated by MAPMAKER/QTL using the genotype and phenotype data of 230 F_2 plants with 9 markers on chromosome 5. The distances between the markers are given in cM.

By examining QTL1 and QTL5 simultaneously, the cumulative variance explained was 46.6% (Addendum IV, no. 16). No substantial difference in cumulative variance was observed if one (45.9%) or both peaks (46.6%) were included for QTL5 (Addendum IV, no. 14 and 16).

Interval mapping with QTL Cartographer (model 3) produced the same results as MAPMAKER/QTL (dotted line, Figure 24). The LOD score for the highest peak of chromosome 1 was the same as that produced by MAPMAKER/QTL (20.7). The LOD values for the 2 peaks of chromosome 5 were slightly lower than those produced by MAPMAKER/QTL (4.3 for the highest peak and 4.08 for the lower peak).

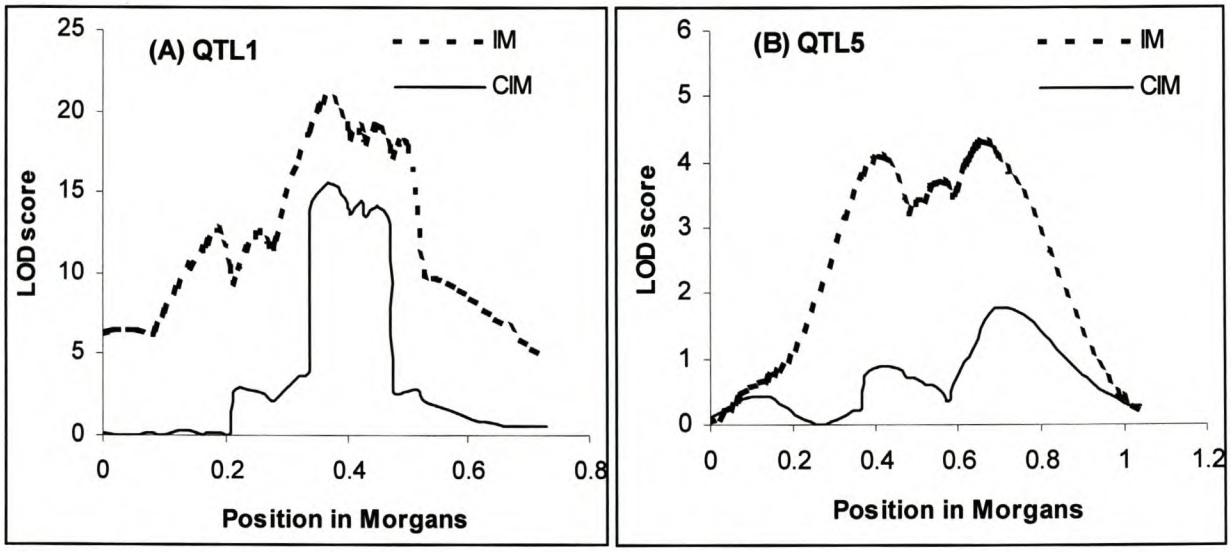

Figure 24. Interval mapping (IM - dotted line) and composite interval mapping (CIM- black line) results generated by QTL Cartographer on chromosome 1 (A) and chromosme 5 (B).

Further analysis using composite interval mapping (model 6) resulted in more prominent peaks (solid line, Figure 24), albeit localized between the same markers as those identified by interval mapping (dotted line, Figure 24). The LOD scores calculated using composite interval mapping were, however, lower (15.49 for the peak on chromosome 1 and 1.78 and 0.9 for the highest and lowest peaks on chromosome 5, respectively).

The gene action of the QTL was tested using MAPMAKER/QTL, to determine whether it was largely additive, dominant or recessive (Addendum IV, no. 7-12). None of the models could be deemed unlikely by 1 LOD (10-fold) or more for either QTL1 or QTL5. The free model, however, accounted for most of the variance with 37% (LOD 20.87) for QTL1 and 10.6% (LOD 5.19) and 10.6% (LOD 4.82) for the two peaks of QTL5, respectively. The additive model also accounted for a high percentage of the variance with 36.2% (LOD 20.44) for QTL1 and 10.5% (LOD 5.14) and 10.4% (LOD 4.77) for the two peaks of QTL5, respectively.

3.10 Consistency of the QTL

To determine the consistency of the QTL identified on chromosomes 1 and 5 using the 1998 F_2 population, the flanking markers for each QTL were tested on F_2 populations planted in 1999 (Table 1C, page 32) and 2000 (Table 1D, page 32). Furthermore, the markers on chromosomes 2 and 3 were also tested on the populations to determine whether an association between these markers and GLS resistance could be detected.

To limit the number of progeny to be genotyped, selective genotyping as introduced by Lander and Botstein (1989), was used. DNA was extracted from 111 plants of the 1999 F_2 population, including 19 plants with a GLS score of 1, 18 plants with a GLS score of 2 and 31 plants with a GLS score of 9. Of the F_2 population of 2000, DNA was extracted from 48 plants, including 5 plants with a GLS score of 2, 14 plants with a GLS score of 3 and 14 and 9 plants with a GLS score of 8 and 9, respectively. Figure 25 shows the distribution of the plants

scored for GLS resistance and the distribution of the plants which were genotyped (black) in the 1999 and 2000 F_2 populations.

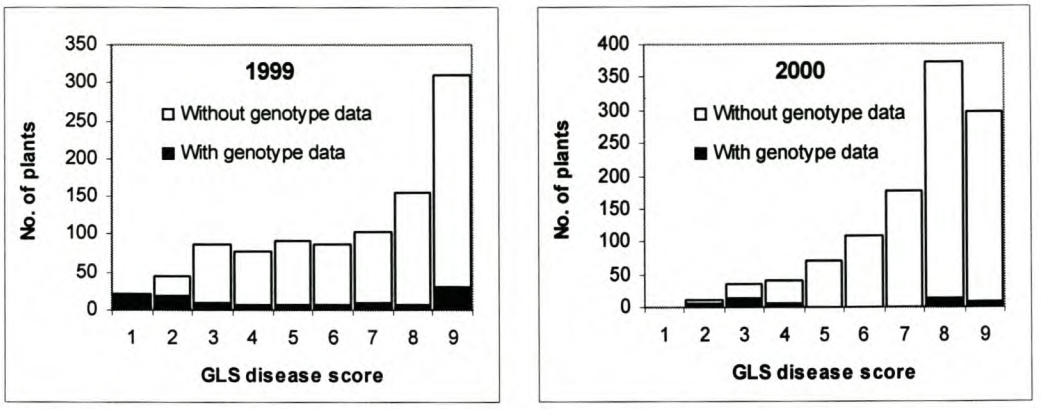

Figure 25. Distribution of GLS disease scores in the 977 plants of the 1999 F_2 population and the 1063 plants of the 2000 F_2 population. Marker genotypes were determined for the 111 and 48 plants shown in black for the 1999 and 2000 population, respectively.

The following flanking markers were amplified on the 111 and 48 F_2 plants of the 1999 and 2000 population, respectively: us44 and bnlg1598 on chromosome 1, and bnlg557, bnlg150, mmc0282 and bnlg1847 on chromosome 5. Markers us41 on chromosome 3 and bnlg125 on chromosome 2 were also amplified on the F_2 plants. Marker bnlg1598 was chosen as the right flanking marker for QTL1 instead of the actual flanking marker bnl5.59, as this marker was easier to amplify without the need for restriction enzyme digestions. The genotype data of the 1999 and 2000 population is given in Addenda V (page 153) and Addendum VI (page 156), respectively. A standard ANOVA for linear regression of GLS score on marker genotype was used to calculate the proportion of the total phenotypic variance explained by each marker (Table 9).

Table 9. Regression analysis results for the association between markers and GLS resistance in the 1998, 1999 and 2000 F_2 populations. R^2 = proportion of phenotypic variation explained by the markers, *F* = Fisher F-ratio, *P* = significance. Significant markers (P < 0.05) are highlighted.

	1998 F_2 population			1999 F_2 population			2000 F_2 population		
Bin Marker	R^2	*F*	*P*	R^2	*F*	*P*	R^2	*F*	*P*
Chromosome 1									
1.05 us44	0.306	93.263	<0.001	0.401	50.249	<0.001	0.262	15.971	<0.001
1.06 bnlg1598	0.274	85.301	<0.001	0.327	51.926	<0.001	0.315	21.105	<0.001
Chromosome 5									
5.03 bnlg557	0.093	23.304	<0.001	0.008	0.763	0.385	0.121	6.302	0.016
5.04 bnlg150	0.082	20.283	<0.001	0.075	8.810	0.004	0.205	11.849	0.001
5.05 mmc0282	0.099	24.968	<0.001	0.014	1.594	0.209	0.078	3.897	0.054
5.06 bnlg1847	0.101	25.255	<0.001	0.010	0.843	0.361	0.042	2.021	0.162
Chromosome 3									
3.04 us41	0.007	1.626	0.204	0.098	11.606	0.001	0.082	4.104	0.049
Chromosome 2									
2.02 bnlg125	0.000	0.096	0.757	0.018	1.586	0.211	0.009	0.413	0.524

The markers on chromosome 1 accounted for the highest proportion of the variance in both the 1999 and 2000 F_2 populations. Marker us44 explained 40% of the variation in the 1999 population ($P < 0.001$) whereas marker bnlg1598 explained 32% ($P < 0.001$) of the variance in the 2000 population. Of the chromosome 5 markers, bnlg150 explained the highest proportion of the variance in both the 1999 population (8%, $P = 0.004$) and the 2000 populations (21%, $P = 0.001$). Marker us41 on chromosome 3 accounted for 10% ($P = 0.002$) and 8% ($P = 0.049$) of the variance in the 1999 and 2000 populations, respectively, and could therefore be linked to GLS resistance in these populations. Marker bnlg125 on chromosome 2 explained an insignificant ($P > 0.05$) amount of the phenotypic variance and the marker could therefore not be associated with GLS resistance.

In comparison, the regression analysis results obtained with the 1998 F_2 population are also given in Table 9. QTL1 explained the highest amount of the variance (31-40%) in all three populations. Markers bnlg557 and bnlg1847

explained the highest proportion of the variance (9%, $P < 0.001$ and 10%, $P < 0.001$, respectively) for the two peaks of QTL5 in the 1998 population, whereas only marker bnlg150 explained a significant amount of the variance in the 1999 and 2000 population ($P = 0.004$ and 0.001, respectively). Although the QTL on chromosome 3 (QTL3) explained between 8 and 10% of the variance in the 1999 and 2000 population, no significant amount of the phenotypic variation was accounted for by this marker in the 1998 population. Marker bnlg125 on chromosome 2 explained an insignificant amount of the phenotypic variance in all three populations.

Chapter 4
Discussion

Resistance to GLS is an essential trait in most maize improvement programs (Schechert *et al.*, 1999), but only a few high-yielding maize hybrids resistant to GLS are available in South Africa. Maize inbred lines, which exhibit resistance to GLS and maintain other agronomically important traits are therefore in demand. Recovery through conventional breeding is difficult, because the development of GLS is highly dependent on environmental effects, field assessment of the disease is problematic and the heritability of resistance is relatively low. Molecular markers linked to the resistance genes may thus be useful to plant breeders to support the introgression of the resistance alleles into elite high-yielding inbred lines. Furthermore, this can be done without inoculation and at an early stage of plant development.

The main aim of this study was to map GLS resistance genes using a resistant Seed Co LTD, Zimbabwean inbred line. Markers closest to the QTL could be used to indirectly select for GLS resistance genes in breeding programs. As QTL are environmentally sensitive the usefulness of the selected markers across seasons was determined.

4.1 AFLP analysis

To detect GLS resistance QTL, bulked segregant analysis was used together with the AFLP technique. The AFLP technique was applied as a high number of loci can be analyzed per experiment, it is fast, robust and reliable, and it does not require prior sequence knowledge of the DNA.

4.1.1 The AFLP technique

Genomic DNA was digested with the enzyme combination *Mlu*I and *Mse*I to increase the likelihood of obtaining single copy regions. As *Mlu*I is a methylation sensitive enzyme it will only digest the non-methylated regions of the genome enriched for single copy sequences (McCouch *et al.*, 1988) and will thus recognize relatively few sites in maize DNA (Burr *et al.*, 1988).

Castiglioni *et al.* (1999) and Vuylsteke *et al.* (1999) used both the restriction enzyme *Eco*RI and the methylation sensitive enzyme *Pst*I in combination with *Mse*I to digest the maize genome. Their studies indicated that the *Pst*I/*Mse*I primer combinations amplified less AFLP bands than the *Eco*RI/*Mse*I primer combination and produced more randomly distributed AFLP markers across chromosomes and chromosome regions. It was believed that the more random distribution of the *Pst*I/*Mse*I AFLP bands on the genetic map was due to the preferential localization of the markers in the distal genomic regions of the genome associated with genes. As the restriction enzymes *Eco*RI and *Mse*I have AT-rich target sequences, the *Eco*RI/*Mse*I AFLP bands can contain repetitive sequences and the probability of identifying these markers in highly repetitive regions near centromeres is greater.

By using the *Mse*I-*Mlu*I restriction enzyme combination in this study, between 35 and 75 distinguishable bands were amplified with the ten different primer combinations with an average of 45 bands per primer combination. Thus in total

approximately 450 loci were screened. Approximately 50% of the fragments were polymorphic between the parents. In comparison, Ajmone Marsan *et al.* (1998) used the *Mse*I/*Eco*RI restriction enzyme combination to digest the maize genome and observed between 30 and 120 distinguishable fragments after amplification with six different primer combinations. An average of 34.8 of these amplified fragments was polymorphic among the maize lines.

Castiglioni *et al.* (1999), who used both the *Mse*I/*Eco*RI and the *Pst*I/*Mse*I restriction enzyme combinations to digest the maize genome, found that the *Eco*RI assay produced an average of 14.9 and the *Pst*I assay an average of 19.6 polymorphic fragments per primer combination. It was also observed that the profiles generated by the *Pst*I/*Mse*I primer combinations were clearer and easier to score than the profiles generated by the *Mse*I/*Eco*RI primer combinations due to a lower number of bands per gel and a reduced background. Vuylsteke *et al.* (1999), who generated two high-density linkage maps of maize using AFLP markers, also observed that the methylation sensitive enzyme *Pst*I produced more polymorphisms than the enzyme *Eco*RI. A greater number of polymorphisms were also detected with the *Pst*I/*Mse*I primers than the *Eco*RI/*Mse*I primers in barley (Powell *et al.*, 1997).

4.1.2 Bulked segregant analysis

Equal volumes of standardized DNA of ten resistant plants with a GLS score of 1 and ten susceptible plants with a GLS score of 9 of a F_2 population planted in 1997 were pooled in two contrasting bulks. AFLP analysis was used to identify from a large pool of markers, those putatively linked to the GLS resistance genes. Ten plants in a pool are sufficient to avoid detecting false positive markers, even with moderate deviations from Mendelian segregation (Wang and Paterson, 1994). Additionally, two contrasting bulks consisting of DNA of 20 resistant and 16 susceptible plants of a F_2 population planted in 1998 were

made. Miklas *et al.* (1996) suggested the use of separate contrasting bulks for each individual environment to identify QTL with minor effects.

With seven of the 10 AFLP primer combinations used, one or two polymorphic fragments per primer combination could be detected between both the parents and the 1997 bulks. Amplification of the AFLP markers on the individual plants constituting the bulks, indicated that most of the fragments, which were polymorphic between the bulks, were present in 7 of the individual plants constituting the resistant bulk and absent in the plants constituting the susceptible bulk.

Three of the 11 polymorphic fragments detected with the 1997 bulks could also be detected in the 1998 bulks by an absence/presence of band polymorphism. Three other fragments were distinguishable in the 1998 bulks by a difference in band intensity. The 5 fragments, which were polymorphic in the 1997 bulks but not in the 1998 bulks, were also cloned and sequenced, to determine whether they were indicative of a QTL in the 1997 population, which could not be detected in the 1998 population.

Fewer polymorphisms were thus observed between the 1998 bulks than the 1997 bulks. This could be, because the 1997 population had been backcrossed to the recurrent parent, whereas the 1998 had not, or due to the difference in the number of plants, which were pooled per bulk (10 in the 1997 bulks versus 20 and 16 in the resistant and susceptible bulk, respectively of 1998). Pooling a larger number of plants in each bulk would be a more stringent control and should thus reduce the number of false positive markers. This, however, would perhaps make the detection of minor QTL by using BSA more difficult. To solve this problem two or more comparative bulks consisting of fewer plants could be used from the same population, e.g. four bulk pairs of 10 individuals each have been used and it was found that a marker, which was polymorphic in three of the four bulks, was associated with a QTL (Grattapaglia *et al.*, 1996).

4.2 Converted AFLP markers

The eleven fragments, which were polymorphic in the 1997 bulks, were isolated from the gel, cloned and sequenced and sequence-specific primer pairs were identified. This was done to make the screening of the plants of the F_2 population easier, faster and less expensive and also to enable the mapping of the markers on the maize chromosomes using the publicly available RIL populations (Burr *et al.*, 1988). As the alleles produced by the converted AFLP markers were single bands, multiple loadings of amplified products could be made onto a single polyacrylamide gel and the running costs were thus reduced.

4.2.1 Conversion of AFLPs

A summary of the AFLP fragments, which were cloned, is given in Table 10. Of the 11 converted AFLP markers, 3 were discarded because multiple fragments were amplified on the DNA of the parents or the amplified products did not have the expected size. The cloned AFLP fragments for these three markers were very small (between 174 and 235 bps) and it was difficult to identify suitable primer pairs for them. It is therefore more useful to isolate and convert larger (>240 bps) fragments.

The 8 converted AFLP markers were added to the maize marker database of the University of Stellenbosch. As the positions of most of these markers are known, they may be useful in other projects. They could also be added to the existing datafiles of the RIL populations and to the UMC map of the Maize Genome Database and would thus contribute to the coverage of the maize genome maps.

Five of the eight remaining converted AFLP markers were polymorphic between the parents (markers designated us40, us41, us42, us44 and us45). Amplification of these markers on the individual plants constituting the 1997 bulks confirmed that the correct fragments had been isolated. The same plants having the original

AFLP fragments had the fragments produced with the primer pairs for the converted AFLP markers, with the exception of marker us45. Marker us45 was amplified on the DNA of two additional plants. The absence of this fragment in two plants in the AFLP analysis could be due to partial restriction during the *MseI*/*MluI* digestion of the genome, or due to a mutation at the restriction site. The latter is, however, more likely as a mutation was observed with the same two plants and a marker on chromosome 1 in a previous study (Lehmensiek, 1998).

Table 10. Summary of the AFLP markers. The name of the AFLP fragment and the sequence-specific PCR marker is given together with the original primer combination. An indication of the polymorphisms between the parents, the type of marker and the map positions are given.

Name of AFLP fragment	*Name of converted AFLP marker*	*Primer combination*	*Polymorphic between GLS parents*	*Type of marker*	*Map position*
AF2.1	us39	Mlu-5/Mse-2	No		bin 2.02
AF2.2		Mlu-5/Mse-2	Discarded		
AF5.1	us40	Mlu-5/Mse-5	Yes	Co-dominant	bin 5.04
AF5.2	us41	Mlu-5/Mse-5	Yes	Co-dominant	bin 3.04
AF6.1	us42	Mlu-5/Mse-6	Yes	Co-dominant	bin 5.04
AF6.2	us43	Mlu-5/Mse-6	No		
AF7		Mlu-5/Mse-7	Discarded		
AF8	us44	Mlu-5/Mse-8	Yes	Co-dominant	bin 1.05
AF9		Mlu-5/Mse-9	Discarded		
AF10.1	us45	Mlu-5/Mse-10	Yes	Dominant	bin 1.04
AF10.2	us46	Mlu-5/Mse-10	No		Unlinked

In this study 5 of the 11 AFLP markers were successfully converted into sequence-specific PCR markers. An experiment by Shan *et al.* (1999) indicated that only 6 out of 26 wheat or barley AFLP markers retained their specificity after they had been converted to sequence-specific PCR markers. Inefficient conversion of the AFLP markers may occur, as the primers are designed from sequences internal to the original AFLP primers and nucleotide and restriction site differences specific to the AFLP primers will thus not be reflected in the primers developed from an internal sequence (Shan *et al.*, 1999).

Different methods could be applied to obtain polymorphisms for the markers, which were not polymorphic between the parents. The inverse PCR procedure could be used to generate larger DNA fragments and to thereby improve the ability to design appropriate primers (Bradeen and Simon, 1998). Inverse PCR amplifies DNA sequences outside the region of the cloned AFLP fragment by using primers that point away from each other into the unknown DNA sequence. Alternatively, outwardly orientated locus-specific primers can be designed from the internal AFLP fragment sequence and used in conjunction with adapter primers to amplify from up to 22 different restriction-ligation reactions unknown regions that flank the internal sequence (Schupp *et al.*, 1999).

Conversion of the AFLP markers into sequence-specific PCR markers not only made the screening of the F_2 plants easier, faster and cheaper, but also resulted in the conversion of four of the dominant AFLP markers into co-dominant markers. It has been stated that the use of dominant markers in linkage analysis using an F_2 population can lead to errors, as the amount of information produced by each data-point is decreased in situations where heterozygous genotypes are found (Beaumont *et al.*, 1996, Schondelmaier *et al.*, 1996 and Jiang and Zeng, 1997). It is therefore important to combine dominant markers with co-dominant markers in a QTL mapping study. Alternatively, DH or RIL populations could be used to avoid the problems associated with dominant markers. These populations, however, are time-consuming and costly to develop.

4.2.2 Amplification with the converted AFLP markers

A large number of plants of the resistant bulk of 1997 were heterozygous when amplified with the sequence-specific PCR markers (6 and 5 plants with marker us40 and us42, respectively on chromosome 5; 3 with marker us41 on chromosome 3 and 2 with marker us44 on chromosome 1). Only 1 and 3 plants were homozygous for the allele of the resistant parent with markers us40 and us42, respectively and 2 plants with marker us41. A larger number of plants (5)

were homozygous for the allele of the resistant parent with marker us44. The difference in the number of homozygous resistant and heterozygous plants with a marker could be an indication of the effect of the QTL, as a greater number of homozygous resistant and less heterozygous plants were observed with the marker of the more significant QTL1 (us44) than with the markers of the less significant QTL3 (us41) and QTL5 (us40 and us 42).

The AFLP fragment AF5.1 was not present in the bulks of the 1998 F_2 population (Figure 3, page 47). Amplification of the co-dominant sequence-specific PCR marker, produced for this fragment (marker us40, page 50), on the DNA of the plants of the 1998 F_2 population, however, indicated that the allele of the resistant parent should have been represented in the resistant 1998 bulk. The absence of the original fragment in the 1998 F_2 population could, therefore have occurred due to incomplete digestion of the genome or a mutation at the restriction site. Partial restriction of DNA has only been observed in a small number of cases (1%) with bread wheat (Donini *et al.*, 1997) and is also unlikely in our study, as aberrant AFLP patterns resulting from partial restriction should be easily recognized (Vos *et al.*, 1995).

4.3 QTL analysis with the converted AFLP markers

The 5 sequence-specific PCR markers, which were polymorphic between the parents, were amplified on the 230 plants of the 1998 F_2 population. Linkage analysis was performed and 4 of the 5 markers were linked in two linkage groups. The two-point linkage distance between one group of markers was 10.4 cM (LOD 22.83) and between the other was 8.2 cM (LOD 55.41). As only two

flanking markers are needed with the program MAPMAKER/QTL to determine whether a QTL is present, QTL could be indicated at both linkage groups.

4.4 Mapping of the AFLP markers using RIL populations

RILs represent a permanent population, which can be used indefinitely to map new markers, as long as the parental genotypes can be distinguished. Two publicly available RIL populations (T323 X CM37 and CO159 X Tx303; Burr *et al.*, 1988) were used in this study. The datafiles of the RIL populations, which were used to map the AFLP markers, consisted of more than 1000 markers and they should thus present adequate coverage of the maize genome.

Four of the markers (us40, us41, us44 and us45), which were polymorphic between the GLS resistant and the susceptible parent, were also polymorphic between the parents of one of the RIL populations and could therefore be mapped. Both markers us44 and us45 were mapped on chromosome 1, whereas markers us40 and us41 were mapped on chromosomes 5 and 3, respectively (Table 10, page 90). As marker us42 showed linkage with marker us40 in the F_2 population, it could also be mapped to chromosome 5 (Table 10, page 90).

Marker us39 was not polymorphic between the parents of the F_2 population but was polymorphic between the parents of one of the RIL populations and could therefore be mapped to chromosome 2 (Table 10, page 90).

4.5 Regression analysis

A standard ANOVA for linear regression of GLS score on marker genotype indicated that the markers on chromosomes 2 and 3 were not linked to GLS resistance QTL in the 1998 F_2 population.

Interestingly, the original AFLP fragments AF5.2 on chromosome 3 and AF2.1 on chromosome 2 were not polymorphic in the 1998 bulks (Figure 3, page 47). This would thus indicate no association with GLS resistance using BSA. Possibly, these markers were associated with minor GLS resistance QTL that were present in the 1997 but not in the 1998 population. Regression analyses with two other populations, one planted in 1999 and the other in 2000, confirmed the presence of a QTL on chromosome 3. This therefore suggests that the expression of the QTL may be environment dependent and fortifies the use of separate bulks for each individual environment. Bubeck *et al.* (1993) planted the same population in the same location in two different years and also found that only one of the eight detected GLS resistance QTL was present in both years. Similarly, Agrama *et al.* (1999) found that two of the three QTL controlling the resistance to Sorghum downy mildew were significant in the one season but not the other.

As the marker on chromosome 2 was not associated with GLS resistance in any of the 3 F_2 populations, it is assumed that this is a false positive marker (i.e., a marker that appears polymorphic between bulks but is not linked to the trait expression (Grattapaglia *et al.*, 1996)). BSA analysis is limited by the chance occurrence of shared homozygosity at specific unlinked chromosomal regions in the bulks. In a segregating population derived only one cross after the original intercross (e.g. DH, F_2 and BC_1 populations), it is very likely that some genomic regions will be uncovered where the markers have not yet been randomized through meiosis and recombination (Jean *et al.*, 1998).

4.6 Converted RFLPs on chromosome 1

QTL analysis using the F_2 population and mapping of the AFLP markers using the RIL populations indicated the presence of QTL on chromosomes 1 (QTL1) and 5 (QTL5). To obtain a more accurate position for the QTL on chromosome 1, converted RFLP markers were used.

Sixteen RFLP probes were converted into STS markers to make the screening of the progeny easier. Only 5 of the 16 RFLPs could be successfully converted to STS markers. One marker showed a size difference whereas the other 4 markers showed restriction site differences. Tragoonrung et al. (1992) also observed more site than size polymorphisms in barley.

After restriction enzyme digestion, two of the converted RFLPs produced fragments, which were of equal size in each parent, together with the fragments, which were polymorphic between the parents (npi286 and php20855, Figure 15, page 65). The sum of the 2 fragments of each parent was larger than the size of the undigested fragment. It could thus be possible that the marker had been amplified on another segment of DNA homologous to the segment on chromosome 1. A duplication of chromosome segments has been reported in maize and it has been found that the bins 1.06 - 1.07, in which marker php20855 resides, are duplicated on chromosome 9 (McMullen and Simcox, 1995).

4.7 Microsatellite markers on chromosome 1

Nine out of 17 microsatellite markers on chromosome 1 were polymorphic between the resistant and susceptible parent (53%), compared to 5 out of 16

converted RFLPs (31%). The microsatellite markers were thus more polymorphic than the converted RFLP markers.

Three microsatellite markers amplified products, which could be visualized on a 2% agarose gel. This eliminated the need to label the primers with radioactivity and made microsatellite analysis faster than STS analysis, because restriction enzyme digestions were not necessary.

The number of publicly available microsatellite markers is increasing very rapidly. Over 1000 microsatellite primer pairs have already been published for maize and can be accessed via the Internet. The increasing number of available microsatellite primer pairs and the higher number of polymorphisms detected with these markers, make them more feasible to study genome regions of particular interest than converted RFLPs.

4.8 Microsatellite markers on chromosome 5

Both microsatellite markers and converted RFLP markers on chromosome 1 were used and it was found that microsatellite markers are more polymorphic than converted RFLP markers. Microsatellite markers were therefore used to obtain a more accurate position for the QTL on chromosome 5.

Seven of the 9 microsatellite markers obtained, were polymorphic between the resistant and the susceptible parent and one of the microsatellite markers amplified products, which could be visualized on a 2% agarose gel. Chromosome 5 seems to be more polymorphic between the resistant and susceptible parent than chromosome 1, as 78% (7 out of 9) of the microsatellite markers on

chromosome 5 were polymorphic opposed to 53% (9 out of 17) on chromosome 1.

A number of extra bands were visible with some of the microsatellite markers on both chromosomes 1 and 5 (Figures 17, page 69 and 19, page 72). These bands are probably PCR artefacts resulting from slippage during PCR amplification (smaller bands) or due to chance homologies of the primers at other sites (Tautz, 1989). As was stated previously, a duplication of chromosome segments has been reported in maize and one of the microsatellite markers, with which an extra band was amplified (bnlg1598) is localized in bin 1.06, one of the segments that has been found to be duplicated (McMullen and Simcox, 1995).

4.9 QTL analysis

To identify QTL by linkage to marker loci, individuals are scored for their genotype at the marker locus and the phenotype for the quantitative trait. If a difference in mean phenotype among marker genotype classes is detected, the presence of a QTL linked to the marker can be inferred (Falconer and Mackay, 1996). A linkage map of polymorphic marker loci that adequately covers the whole genome is needed to map QTL (Falconer and Mackay, 1996).

4.9.1 Chi-square analysis

The majority of the markers followed Mendelian segregation. Markers npi286 and us45 on chromosome 1 were both skewed towards the alleles of the susceptible parent, while marker bnlg143 on chromosome 5 was skewed towards the heterozygous genotype. As marker us45 is a dominant marker, no distinction

could be made between a susceptible plant and a faulty amplification and a few genotypes could thus have been recorded incorrectly. This is a disadvantage when using dominant markers. Only marker npi286 was eliminated from further analysis, because *P* was smaller than 0.001 for this marker.

A significant deviation of the segregation ratio from the expected 1:2:1 F_2 ratio for co-dominant and the 3:1 ratio for dominant markers has been recorded in a number of studies in maize (Veldboom *et al.*, 1994, Tuberosa *et al.*, 1998, Castiglioni *et al.*, 1999 and Vuylsteke *et al.*, 1999) and also in barley (Larson *et al.*, 1996 and Zhu *et al.*, 1999b) and rice (Xu *et al.*, 1997). Genetic, physiological and/or environmental factors are known to cause segregation distortion (Xu *et al.*, 1997).

4.9.2 Linkage map construction

Eleven markers on chromosome 1, and 9 markers on chromosome 5 were used to construct two linkage groups using MAPMAKER/EXP. As MAPMAKER/EXP could not place the 2 dominant markers into the linkage group, only the recessive genotype data were included for these 2 markers and the genotypes of the homozygous dominant and heterozygous plants were designated as missing data. MAPMAKER's error detection function was on to eliminate mistakes due to mistypings of a locus in the raw data. Genotyping errors can increase the genetic map length and it has been shown that a 3 per cent error rate in genotyping can double the genetic map length (Kearsey and Farquhar, 1998).

The linear order of the markers on the linkage map of chromosome 1 obtained with the F_2 population was in agreement with the order of the markers on the published map of the Maize Genome Database. Linkage analysis of the AFLP markers on chromosomes 1 and 5 with the locus-specific markers confirmed the localizations of the AFLP markers obtained with the RIL populations.

A two-point distance of 10.4 cM and a multipoint distance of 12.7 cM was calculated between the markers us45 and us44 on chromosome 1 and a two-point distance of 8.2 cM and a multipoint distance of 7.7 cM was calculated between the markers us40 and us42 on chromosome 5. The distances calculated between markers using multipoint analysis and two-point analysis may be considerably different, as multipoint analysis can take much more information, such as flanking marker genotypes and some amount of missing data, into account (Lander *et al.*, 1987).

A slight difference in the linkage distance between markers on chromosome 1 was observed between the maps of the F_2 population, the RIL population and the Maize Genome Database (Figure 9, page 57 and Figure 20, page 76). Slight differences in distance occur, when the number of markers used differ between maps, different inbred lines are used in each cross, and the cross-over frequencies between the lines vary (Chagué *et al.*, 1996 and Voorrips *et al.*, 1997).

4.9.3 QTL mapping

As a normal distribution of phenotypes is an inherent assumption for interval mapping (Lander and Botstein, 1989), the phenotypic GLS data, which showed deviations from normality (Figure 2, page 33), was transformed prior to analysis. Similarly to the results of Beavis *et al.* (1994) and Lübberstedt *et al.* (1998b), the transformation did not alter the QTL that was identified. Therefore, the original data were used in QTL analysis.

The results obtained by interval mapping with the programs MAPMAKER/QTL and QTL Cartographer were very similar. The peak of QTL1 was between markers us44 and bnl5.59 with a LOD score of 20.7. The confidence interval surrounding the QTL peak was 6 cM in length. Two peaks, 28 cM apart, were visible for QTL5. One peak was situated between markers mmc0282 and

bnlg1847 and the LOD score of the peak was 5.2 and 4.3 with MAPMAKER/QTL and QTL Cartographer, respectively. The other peak was situated between the markers bnlg557 and bnlg150 and had a LOD score of 4.8 and 4.1 with MAPMAKER/QTL and QTL Cartographer, respectively. The confidence interval surrounding the QTL peaks was 50 cM in length. By using interval mapping it was not clear whether one or two QTL are present on chromosome 5 as both peaks were prominent and the confidence interval was large.

Composite interval mapping using QTL Cartographer was performed, as this program is supposed to overcome the problem of testing whether one or more than one QTL is present on the same chromosome (Zeng, 1994). Composite interval mapping provides an interval test in which the test statistic on an interval is unaffected by all those QTL which are located outside the interval being tested and its adjacent two intervals (Zeng, 1994). Firstly a stepwise regression analysis is run. The stepwise regression analysis ranks the markers for their effect on the quantitative trait. A marker with the largest F-statistic is assigned rank 1. The test is repeated until all the remaining markers are ranked. Model 6 of QTL Cartographer chooses the most important markers from the prior run stepwise regression analysis to control for the genetic background. When testing at any point on the genome it will use the number of specified markers (in our case 5). A default value of 10 was used for the window size. The window size blocks out a region of the genome on either side of the markers flanking the test site. Since the flanking regions are tightly linked to the testing site, the signal from the flanking regions will be eliminated from the test site (Basten *et al.*, 1997).

Composite interval mapping resulted in more prominent peaks, localized between the same markers as those identified by interval mapping. The LOD scores calculated using composite interval mapping were, however, lower (15.49 for QTL1 compared to 20.7 using interval mapping and 1.78 and 0.9 for the highest and lowest peak of QTL 5, respectively, compared to 4.3 and 4.1 using interval mapping). The increase of precision of composite interval mapping is

gained by making the test conditional on nearby markers so that the sensitivity of the test statistic to the position of a QTL is emphasized in a short region of the interval conditioned. As the test under composite interval mapping is a conditional test, the test statistic on many intervals is smaller than those produced by interval mapping. This is a disadvantage of the program (Zeng, 1994).

From the mapping results, we can thus maintain that one QTL is present on chromosome 1 and at least one QTL, but probably two, are present on chromosome 5. QTL1 had the largest effect on GLS resistance and explained 37% of the variance. A smaller QTL effect was explained by QTL5 (11%). Cumulatively, the QTL explained 47% of the variance. Examining multiple QTL simultaneously can extend the sensitivity of QTL mapping by reducing the unexplained noise that must be accounted for and the estimates of QTL effects are thus considered to be more accurate (Paterson *et al.*, 1988).

The effect of different gene dosages on phenotype can be determined in a F_2 population, because all three possible gene dosages (homozygous and heterozygous) at a locus are represented. A 1-LOD (10-fold) reduction in likelihood was considered to mean that a type of gene action was unlikely. However, if a type of gene action is not rejected, it is still not sufficient evidence to assert that the relevant gene exhibits only that type of gene action (Paterson *et al.*, 1991). In our study the free model accounted for most of the variation of both QTL1 and QTL5.

It is worth mentioning that both QTL1 and the highest peak of QTL5 localized to the regions where the QTL for GLS resistance, introgressed from the inbred line Va14, were reported by Saghai Maroof *et al.* (1996). Interestingly, they assumed that the QTL on chromosome 5 was a false positive QTL, as it was not reproducible in their F_3 populations. QTL in common across different mapping populations have been reported by Bubeck *et al.* (1993), who detected one

region on chromosome 2 associated with GLS resistance in three different populations. The occurrence of QTL in the same chromosomal regions in different inbred lines could indicate that genetically similar sources of resistance were used in the parental lines.

4.10 Consistency of the QTL

It has been reported that the consistency of the identification of QTL in one population across seasons is low. Bubeck *et al.* (1993) for example planted the same population in the same location in two different years and found that only one of the eight detected GLS resistance QTL could be identified in both years. Tuberosa *et al.* (1998) identified QTL controlling leaf abscisic acid concentration in maize in field trails conducted over two years and found that of the 16 different QTL identified in at least one sampling, only 4 QTL were significant across samplings.

In our study, the consistency of QTL1 and QTL5 was tested on selected plants of F_2 populations planted in 1999 and 2000. The flanking markers of each QTL were amplified on the DNA of the selected plants. Furthermore, the markers on chromosome 2 and 3 were tested on the populations to determine whether an association between these markers and GLS resistance could be established. A standard ANOVA for linear regression of GLS score on marker genotype was employed to confirm an association between GLS resistance and the markers.

The results of the regression analysis indicated that the highest proportion of the variance (32-40%) was accounted for by the markers on chromosome 1 in both the 1999 and 2000 population. Only the flanking markers for the first peak of

QTL5 (Figure 23, page 79) explained a significant amount of the variance (7-20%) in the 1999 and 2000 population. This could confirm that two QTL are present on chromosome 5 and that only one of the two QTL was detectable in the 1999 and 2000 population.

Furthermore, regression analysis confirmed the presence of a QTL on chromosome 3. QTL3 was detectable in both the 1999 and 2000 population. The variance accounted for by the marker on chromosome 3 was 10% in the 1999 and 8% in the 2000 population. Interestingly, Bubeck *et al.* (1993) also identified a GLS resistance QTL where QTL3 was localized. The detection of QTL3 in the 1999 and the 2000 population but not the 1998 population could indicate that the environment has an effect on QTL3 and that this QTL will not always be detected across seasons.

If a large number of QTL are segregating for a given trait, only a fraction will be identified per experiment and therefore the chance that any two independent experiments will have the same set of QTL is very small (Beavis *et al.*, 1994). Beavis *et al.* (1994) also maintains that if the number of QTL is large and the power to identify QTL is small, it is possible that two independent experiments will not identify any QTL in common. This suggests that two breeders selecting for the same trait in the same environment on independent samples of progeny from the same cross will select for different arrays of QTL.

Since only one marker for the QTL on chromosome 3 was used in regression analysis, the precise localization of the QTL could not be determined. The distance between the marker and the GLS resistance QTL may still be great and therefore the QTL effects calculated could be under-estimated.

Although the genetic effects calculated by regression analysis were fairly consistent, the difference in the number of plants used per population in the regression analysis was large. The calculated genetic effects could, therefore, be

biased and should only be used as an indication of the presence or absence of a QTL.

4.11 Resistance genes in maize

Resistance genes in maize (McMullen and Simcox, 1995, Bohn *et al.*, 1997, Ming *et al.*, 1997 and Welz *et al.*, 1999) as well as in barley (Richter *et al.*, 1998) seemed to be clustered instead of being equally distributed among chromosomes. The clusters may be closely linked resistance genes or the gene action at the QTL may be pleiotropic, either through shared physiological pathways or through multi-functional gene products (McMullen and Simcox, 1995 and Welz *et al.*, 1999). Except for a few cases, the biochemical and physiological bases of resistance to pathogens and pests have yet to be elucidated in maize.

The GLS resistance QTL1, identified in this study, occurs in the same region as QTL for resistance to southwestern corn borer and sugar cane borer (Bohn *et al.*, 1997), northern corn leaf blight (Freymark *et al.*, 1994), maize streak virus (Pernet *et al.*, 1999a, b), common smut (Lübberstedt *et al.*, 1998a), common rust (Lübberstedt *et al.*, 1998b) and the corn earworm resistance factor maysin (Byrne *et al.*, 1996).

QTL for resistance to sugar cane borer (Bohn *et al.*, 1997), northern corn leaf blight (Schechert *et al.*, 1999 and Welz *et al.*, 1999 and 2000), *Fusarium* stalk rot (Welz *et al.*, 2000), common rust (Lübberstedt *et al.*, 1998b) and common smut (Lübberstedt *et al.*, 1998a) have been detected in the same region as the GLS resistance QTL5.

In the region of the QTL on chromosome 3, a major maize mosaic virus resistance gene (Ming *et al.*, 1997) and a wheat streak mosaic virus gene (McMullen *et al.*, 1994) have been identified and QTL for resistance to sugarcane mosaic virus (Xia *et al.*, 1999), sugar cane borer (Bohn *et al.*, 1997), *Fusarium* stalk rot (Welz *et al.*, 2000), and common rust (Lübberstedt *et al.*, 1998b).

It has been speculated that minor wild-type allelic variants at major qualitative mutant loci are responsible for substantial amounts of quantitative genetic variation, and therefore qualitative and quantitative loci should be localized on the same chromosomal region (Beavis *et al.*, 1991, Freymark *et al.*, 1993, Goldman *et al.*, 1993 and Richter *et al.*, 1998). Although net blotch resistance loci have been found in the neighbourhood of mapped QTL in barley (Richter *et al.*, 1998), QTL for resistance against common rust in maize were not preferentially located in map positions close to qualitative gene loci (Lübberstedt *et al.*, 1998b). In the latter study it was concluded that different biological mechanisms appear to be involved in quantitative versus qualitative resistance.

4.12 MAS using flanking markers for QTL1, QTL3 and QTL5

Kelly (1995) described the most desirable marker as one that retains linkage with the resistance gene and is clearly expressed and functional across a broad range of genetic backgrounds.

Flanking markers us44 and bnlg1598 on chromosome 1, markers bnlg557, bnlg150, mmc0282 and bnlg1487 on chromosome 5 and marker us41 on chromosome 3 could be used to support breeders in the introgression of the resistance QTL into high-yielding inbred lines through backcrossing. By selecting

for the presence of these markers, the need for subjective disease screening may be reduced and the speed and efficiency of backcrossing increased. By combining several QTL with different environmental specificities into a single genotype, one might be able to improve the phenotype which is somewhat shielded against the vagaries of environment.

With respect to the populations of 1998, 1999 and 2000 used in our study, only one plant of each population was homozygous for the allele of the resistant parent at all 7 loci. These 3 plants were resistant (GLS score of 1 in the 1998 and 1999 populations and a GLS score of 3 in the 2000 population), thus indicating that the flanking markers are useful in MAS. However, a high number of plants will have to be screened to obtain plants which are homozygous for the allele of the resistant parent at all loci. The usage of these markers in MAS in breeding programs will, therefore, have to be assessed.

Chapter 5
Conclusion

The AFLP technique together with bulked segregant analysis was useful to identify GLS resistance QTL on three chromosomes. It is, however, not known how many QTL were unidentified in the populations under study and whether the undetected QTL have larger or smaller effects on GLS resistance, than the ones identified. Other studies have indicated that BSA is more useful in tagging QTL of large effects and that some QTL might be missed (Grattapaglia *et al.*, 1996, Miklas *et al.*, 1996, Chagué *et al.*, 1997 and William *et al.*, 1997).

Alternatively, QTL could be identified by using the AFLP technique to construct a high-density linkage map and using this map together with the genotype and phenotype data of each marker in QTL mapping. This approach to identify QTL has been used in barley (Powell *et al.*, 1997 and Qi *et al.*, 1998a, b). By using 21 Pst/Mse primer combinations, 550 and 565 AFLP markers could be mapped using a maize RIL and F_2 population, respectively (Vuylsteke *et al.*, 1999). The markers were uniformly distributed and the average distance between the markers was 2 and 2.5 cM for the RIL and F_2 population, respectively. A good coverage of the genome was thus obtained with the 21 primer combinations used and over 5 times more markers could be used in QTL mapping than the average number of markers (about 100) which is normally used in maize QTL mapping studies.

Map based gene cloning of QTL is the ultimate achievement of the QTL mapping technology (Young, 1996). This, however, will require great mapping precision

and therefore a very large mapping population. Alternatively, different candidate genes could be used as molecular markers in QTL analysis. Although molecular cloning of the QTL is circumvented, resistance QTL involving candidate genes will have to be mapped at a high resolution to determine if they actually do coincide with the candidate gene of a distinct, but related, function (Faris *et al.*, 1999).

In this study, it was possible to identify marker loci associated with GLS resistance genes in maize by using BSA and the AFLP technique. Furthermore, markers were identified, which could be used in a MAS program to select for the GLS resistance QTL on chromosomes 1, 3 and 5. The main aim of this study, i.e. to map GLS resistance QTL, thereby identifying markers close to the QTL which could be used for MAS in breeding programs for GLS resistance, was therefore achieved.

Chapter 6
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Addendum I: Sequences of cloned AFLP fragments

The original primer combination used to amplify the AFLP fragment is given in brackets. The size of each fragment is given in base pairs (bps) and the newly identified primer pairs are highlighted.

AF2.1 (Mse-2/Mlu-5) 760 bps

GATGAGTCCTGAGTAAACAAGCGCAATAATGGCGCACCATCGGCCTTGTCCCTGCTTTCTCTGTGCAG
AGGTCAAGCTTAGCCTGCCCACGGGATAGGAGAGGGATGCCAGGATGGGATTGGGCGCCAGGGTCAA
AGGTGACGGGTGAGGGGTCGCTTTTCCCGGCTCGGTTGCCTGCACTGCACTGCAGCACcTGCACCCTC
TAGTTGACCGCAACCTGTTTCTcTCtGTCTCTCTCTCTCTCTCTCTCTTCGTGGCAGCAGGAGCGTTTTCT
CTACCTTTTTCCCAGCTTTACGTTCGGCCTCGCTTGAGTCTTTTGTTTTATTTTCAAACACCCCGGGCTG
CCCGCCCCGTCTGCGTCTTTTGTTTTTTTTTCCCCGGAGGTGGGACCTGGGCCGGGCCTCGCGCGTAC
CCATCCGTTGACCTAGCGAGCGTACCGAGCCGTGCGGAGCACAGGAGTCTTTGCTTGACACCCACGT
CTGTCTGATACTCTGATCTCTGATCTGCCCTCCGCTTTCCTCTGTTTGCCAGCCCAGGGACTGCTGTGA
CTAATGAGCTGAAGCGACACCTCCACAGCTCCACTAGACATTAGTCAACCGTCGGTGAATGGTCGTGG
TTGCAAGGCAGGTGTTGTTGCTAGTGGAGTACTAGCCCTTTATTTTTTTGTTTTTTTTTTGTTTTTGGCGG
GAAAAGCAAGCCCAACTAGGCAACTAGGAGCATTTTATGCCAATGTATGGTCCGTGCACGCGGTTACG
CAGTC

AF2.2 (Mse-2/Mlu-5) 174 bps

GACTGCGTACCGCGTGCCTTTTGCAATGCGGCGGGTGAGCTTGTGCTTTGGTTGATCCCCTAACACGT
GTGCAGAAGAAGGAAATCTTCCCGGGAGCTGTGCACCGAACAGCTTCCACGAATGCACTTCGGCCACA
GTTAGGGCATGTGTAGTGGTGTTTACTCAGGACTCATC

AF5.1 (Mse-5/Mlu-5) 298 bps

GACTGCGTAACCGCGTGCGGTACAGCGGCCTGACGTGATCGGCGATCTGATAGCCAACTTGGTGTCG
TCGTGACCGTGATGATGTGAGAGCCACAGCGCGGGCCTGAGCGTTTTGTGTGCAATGAGCTAGCAGC
ACTGCATCTGCATGCACCGCAGTCAAGGTTCACAGGCCGCTACAACTGTAGTAGTAGTAGCAGTGTGG
GTCAGAAAACCATTTTCTCGCTCATCCAGAAAGCCCCGCCGGTCCATTTATCCCACGCCAGCCTTGCCA
GGCCCAGGCGCCTTACTCAGGACTCAT

AF5.2 (Mse-5/Mlu-5) 303 bps

GACTGCGTAACGCGTGCACACGGCGCTTGGTTGCTTGTCCAAGATCTGGAATTCTTGATTAGTGCCGG
GGAAATATCTCTCGTGGGAGGGCATCGGCATCTGTTASGGCTTATACAACCTAGATAATATGTTACGAT
TTTCTAAAAACTAGATGCATCTAAGAAACTCCTTCATATAACCTAACATATCTAAGCCTTATATCTTATTCA
CTAGAATCCATCAAGATATGGACTGTATATGTGTGATATGGTAGCTCCAGTAGATTATGCGATATTCTGG
TCTAAGCCTTACTCAGGACTCAT

AF6.1 (Mse-6/Mlu-5) 251 bps

GATGAGTCCTGAGTAATTGTATATATCCATAGAGGGTAAACGGATTTACGTACATGAGTATAAAGATTTT
ATACCTACGAGA ACTCATCAAACACGTTCTTATATCTCTGTCTGTCACTACGGA ACATAGACACTACTA
ACGCTGTTGTATTACTGTCGCTCTCTTGCTACGCGCCCTCCCTCACTTGAAGATCTTCACCGAGAAAGG
AAGTCAAACATCAAGCCATCGTCAA GCACGCGGTTACGCAGTC

AF6.2 (Mse-6/Mlu-5) 258 bps

GATGAGTCCTGAGTAATTGGTAGCTCTGTGTTTCACGCAAAAGAGAAGAACTAACTCACGCAGACGCGA
TGATCCGAAGTTACGCGGGAACAAGCTAGCTAAGCGTTGGGGGGTAAAACACATGTTTGTGGTACGTA
GTACTGCTGCTAGACTAGCACGAGGATTTCACACAACAGTTCCAGTGGAAGGCCATGTACTGACGAAG
ATACTGCCGGTTTCGTTGAGGATCCCTCCGCCGCCGCACGCGGTTACGCAGTC

AF7 (Mse-7/Mlu-5) 224 bps

GACTGCGTAACCGCGTGCAGGCACCGGCTGAAGCTGTTCCGCGCCGACCCGTTCGACTACCACGCCA
TCgcCGATGCCGTGCGGGGCTGCgCCGGCGTCTTCTGCATGTTCAACACGCCCGACGACCAGGCCCAA
TGCGATGTGAGTACAAGACGGCAGCTTGCTTGCTTTCTTTCGCCTCTCTCTCTCTCTCCTCTGCATATTA
GTATTACTCAGGACTCATC

AF8 (Mse-8/Mlu-5) 271 bps

GATGAGTCCTGAGTAAGAGGGGAAGAAGGGCGCCGGACCTAGGCCCACTGCTTTATAGCCTCGGAAG
GTCGGTCCCGAGTGCTAGCAACGGATCCCGCATCCCGCGCTKGTGCTCCGAGCTCCGCGGAGTCGGA
CGCGCGTGGGAAGAGGATGGACTGGGATCGTGGGCCCGCGATTCAGAGACAGCGATCCGGCGAATG
CAGCATCAGTGGCTGACGGGCGGTCCCACACGTCAGCGGACTGTGGTGCAGTGGCACGCGGTTACGC
AGTC

AF9 (Mse-9/Mlu-5) 235 bps

GACTGCGTAACCGCGTGCTAATCCTAATTCCAAACCGTTCTCTGGCTGAGACGTTGAAATGCCACATTT
TATTCCATGAAAGTGATATATGATGTAAAGAATGAGTGAGTGGCTAGATGGTTTGTACACAGACCAGAC
CACCCTGTCAACATCACAAACCGTTCTCTGGCTGAGACGTTGAAATGCCACATCTTTATTCCATGAAAGT
GATATATGATGTTACTCAGGACTCATC

AF10.1 (Mse-10/Mlu-5) 771 bps

GATGAGTCCTGAGTAACACCTCACATGTCGACACGCCATGCATTAGGCACAGCTAGCTAGCTTACCTTG
TTCCCGGGCGACGACAGGCTCTTCTTCGCCCTGAAGGTCCTCTGGTGGTTCCTGCAAGCGCGGTTCGC
CGCCGGGGACAGGAACCATATCAGAACCGGTTCAGGCCGCCGGGAGAGGAAGAGTAGTAAACTGACT
AATAAGATCTCTGCTTGCGTACTTGCTGCCGCCGCCGGAGAAGAAGCTCATCTTGGTCCACACCTAGCT
AGCTCCGGCCGGAACGAACGAAAGACCGTACCTAACCTGACGATGAGCAGAAGCTGAGTAGCTAGCTA
GTCGTCGGCACTGCACACAGATCACTGCATACTGTGTGTTTACGTATATATAATAATACTATTACATACT
GCTGGCTCTGCGCTGCTGGGCCTTGCCTGGCAAAnCTGCGTGAGCGGTGCGTCTGGATTTCAGTTTCA
CCGGCCGCGCCGGCGCCGCTAGATCTTGCGTGTTGCGCTGCGTCGGGCTCGGGGCCAGCTGCCTGC
TGCATGTCATCATCATTCATCACCATGCATATTATTACGAGATCGTTCATCAGCCGCGCCCCTGAGTGC
ATAGATGTGGTGTAAAGAGATGGCGGAAATAGGGCGCAGAGGATCATACGCCACAACAAAATAGCTCA
AATAAAGACTAGCCTGGCTCGCACCACTGATCGAGCTAGCGATGCTCTTCATGGCCAGACGTACATGA
GGAGCACGCGGTTACGCAGTC

AF10.2 (Mse-10/Mlu-5) 247 bps

GACTGCGTAACCGCGTGCTCGTGCCTAGCGAGAAGCTTTTCGAATTTTGGTGCTATCCACCATCCACAT
CCATGGCCATCAGTATTGACCTAAGATGGTAGAGTGAGATGATGTTAGCGTAAAAAACAGAAACTACAC
ACTGATATATAGTACAATGCACATGAGTTGCGACCGCATACACATGAATAAATATTACATTTTATCCAGT
CAAAAGATCTAGAAAGTTATGTGTTACTCAGGACTCATC

Addendum II: Sequences of RFLP probes

The probes were obtained from the University of Missouri. The identified primer pairs are highlighted. Sequencing ambiguities are indicated with a 'N'.

npi286

GTTTGNAAANCGACGGCNNNGTTGAATTTCNAGCTCGGGTACCCGGGGNATCNTNTAGAGNCGACNTG
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GGGGTAGGCCTTGTGGCATTGGATTCCCGGCGTCGATCAGCGTCGTCGCGCAGATGTGGGCCTTGTG
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CGGCATGCACGCACGCACGCACACAGCGCTGCGCCCGTCCCTCGTCTCGTCTCTCATCTGCGCCAGG
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GAGGAAGAGGTCAACAAGAGCTACAGCTACCTATCTATAGGGTGTGGTGCGTGCCGTGCCGCGACCG
ACGTGCCGTGCGCCCGTGCCATTGGCGCTGGTACTGGCCGTCGTCCGTCGTCCAAGTAGCCTCTGAG
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NCGNGCCGNNCTNNANGCANGCAANNTTNGCGDNATCATNGNCNNNNNNGGTTCTGNGTGNAAATGT
ATCCGNTCACAATTCACACAACATNCAAGCCGGAANATAAAGTGNAAANNCTTGGGNNCCTAATGNGTG
NGCTTANCTNNNATTAATTGGG

umc11

GTTNCAAACGACGGCNAGNGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGCATA
AATGTATGGTGCATGGTAATGCCGGCCTACGTGTACCACACCANCACTNTNTTTGNGDTNAANATCGTT
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umc76

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umc13

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umc8

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asg30

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npi262

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asg75

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CNTATTGGGCGCT

umc227

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npi279

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CGNAATCATGGCATNCTGGTTCCTGGGNGAAATGGTANT

npi598

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bnl5.59 forward

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bnl5.59 reverse

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uaz249

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umc58

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php20855

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umc23

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ACCGGAAGAATCACCATGCAGGTCANNCGACGGAACCGTCGTCGTACTTCGTTCCACCTGGCTCTCNC
GAGGAGGAAGAACCGCTCGTTGGGCCCCACCTTCCTTTGGTCGCCCTCGCCCCCACCGGGGGACGCC
GGTGGCCCTTGGGCTCACCGGCCAGCCCCGGTACCC

Addendum III: Datafile used in QTL analysis

A = homozygous for the allele of the resistant parent
B = homozygous for the allele of the susceptible parent
H = heterozygous
C = homozygous for the allele of the resistant parent and heterozygous (dominant marker)
- = missing genotypes

```
data type f2 intercross

230 21 1

*bnlg147    HHHHABHHAHAHHHHHAAAAHHHAHAHHAAHAAAHAHBBHAHHAHHHHHHHBAHHHAAH
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*phi001     HHHHABHHAHAHAHHHAAAAHHHAHAHHAAHAAAHAHBBHAHHAHHHHHHHHHHAHAAH
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*asg30      HHBHAB--AHAHAAHHAAAAHHHAHAHHHA-AAAHAAHAAAHHAAHHHHHHH--AHA-H
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HA-HHHHHHHBHABBHHHBBHBBH-BBBHH-BBH-ABBBHHH-HHABB-HBBBHHHBBHBHBBBHBHHBB-
BBA-HBBHHHBBBHBHHBABAABBBBHBB

*us45       CCBBCBCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
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BBCBCBBCCBBBBCBCCCCBCCBBBBCBB

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*us44       AHBHABHHHAAAAAHHAAHAHHHAHAHHAAHAHAHAAAAAAHAAAHAHHHHHHHAHA-H
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*bnl5.59    AHBAABHAHAAAAAHHAAHAAHHAAHHHAAHAHAHAAAABAHAAAHAHAHHHHBAHAAH
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*umc58     AHBAHBHAHAAAAAHAAAHAAHHAAHHHAAHAHAHAAAAAAHAAAHAHAHHHHBAHAAH
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*php20855   CCBCBBCCCCCCCCCCCCCCCCCCCBCCCCCCCCCCCCCCCCCCCCCCCCCBCCCCC
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*bnlg565    HAAHHHHAAAAABHHHBHHHHHHHHABBBHHAAHHBHAAHHAHBHHHHHHHBHHHHHHH
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*us40 AHAAHAAAAAAHHHAHBHHAHHHBHAHHHAAHABHBAHABHAHBHBHAHHHHHAHHHHH
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*us41 HAHBAAHHBHAHABHBHHAHHAHHHHHBBBHAHHHHHHHHHHHHHHAHHAHBABAHBHHA
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8 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9

Addendum IV: QTL analysis results using MAPMAKER

```
*********************************************************************
* Output from:                                  Tue Jul 12 20:09:32 2000 *
*                                                                    *
*                            MAPMAKER/EXP                            *
*                           (version 3.0b)                           *
*                                                                    *
*********************************************************************

data from 'GLS.TXT' are loaded
  F2 intercross data  (230 individuals, 21 loci)
'photo' is on: file is 'GLS.OUT'

3> sequence all
sequence #1= all

4> group
Linkage Groups at min LOD 3.00, max Distance 50.0

group1= 1 2 3 4 5 6 7 8 9 10 11
-------
group2= 12 13 14 15 16 17 18 19 20
-------
unlinked= 21

5> error detection on
'error detection' is on.

6> order
Linkage Groups at min LOD 3.00, max Distance 50.0
Starting Orders: Size 5, Log-Likelihood 3.00, Searching up to 50 subsets
Informativeness: min #Individuals 1, min Distance 0.9
Placement Threshold-1 3.00, Threshold-2 2.00, Npt-Window 7
=====================================================================
Linkage group 1, 11 Markers:
   1 bnlg147       2 phi001        3 asg30         4 us45          5 bnlg2086
   6 us44          7 bnl5.59       8 bnlg1598      9 umc58        10 php20855
  11 phi037

Most informative subset: 1 2 3 11 5 6 7 8 9 10
Searching for a unique starting order containing 5 of 10 informative loci...
Got one at log-likelihood 3.84

Placing at log-likelihood threshold 3.00...
Start:   1 5 6 7 10
Npt-End: 1 5 6 7 10 (11)
Npt-Err: 1 (2) 5 6 7 10 11
Npt-Err: 1 2 5 6 7 (8) 10 11
Npt-Err: 1 2 5 6 7 8 (9) 10 11
Npt-Err: 1 2 (3) 5 6 7 8 9 10 11
Npt-Err: 1 2 3 (4) 5 6 7 8 9 10 11
Uniquely ordered all 11 markers
```

```
Map:                        Apriori
  Markers          Distance    Prob  Candidate Errors
    1  bnlg147       8.2 cM
    2  phi001        8.6 cM    1.0%  [#182 B-A-B 4.00] [#130 B-A-H 2.16] 7 more
    3  asg30         4.4 cM    1.0%  [#189 B-H-B 1.81] [#132 H-A-- 1.44] 1 more
    4  us45          6.6 cM    1.0%  [#94 --B-A 3.58]  [#191 H-B-H 1.73] 2 more
    5  bnlg2086      6.1 cM    1.0%  [#207 B-H-B 1.79] [#216 B-H-B 1.79] 9 more
    6  us44          6.6 cM    1.0%  -
    7  bnl5.59       2.6 cM    1.0%  [#40 A-B-A 5.21]  [#142 H-A-H 2.16]
    8  bnlg1598      4.4 cM    1.0%  [#218 B-H-B 2.33] [#19 H-B-H 2.32]  1 more
    9  umc58         5.1 cM    1.0%  [#80 H-B-- 1.84]  [#16 H-A-- 1.38]  6 more
   10  php20855     21.0 cM    1.0%  [#186 H-B-H 1.40] [#108 H-B-H 1.40]
   11  phi037      ----------
                    73.8 cM   11 markers   log-likelihood= -504.37

order1= 1 2 3 4 5 6 7 8 9 10 11
other1=
================================================================================
Linkage group 2, 9 Markers:
  12 bnlg143      13 bnlg565      14 bnlg557      15 bnlg150      16 us42
  17 us40         18 mmc0282      19 bnlg1847     20 bnlg1306

All markers are informative...
Searching for a starting order containing 5 of all 9 loci...
Got one at log-likelihood 5.64

Placing at log-likelihood threshold 3.00...
Start:   12 14 15 16 18
Npt-4:   12 (13) 14 15 16 18
Npt-End: 12 13 14 15 16 18 (20)
Npt-Err: 12 13 14 15 16 18 (19) 20
Npt-Err: 12 13 14 15 16 (17) 18 19 20
Uniquely ordered all 9 markers


Map:                        Apriori
  Markers          Distance    Prob  Candidate Errors
   12  bnlg143      14.7 cM
   13  bnlg565      22.6 cM    1.0%  [#133 H-A-B 1.33] [#151 H-A-B 1.33]
   14  bnlg557      11.4 cM    1.0%  [#156 H-B-A 1.78] [#152 A-B-H 1.48] 6 more
   15  bnlg150       3.4 cM    1.0%  [#173 H-A-H 1.81] [#209 H-B-H 1.81] 3 more
   16  us42          7.7 cM    1.0%  -
   17  us40          3.1 cM    1.0%  [#135 H-A-H 2.02] [#134 A-H-A 2.01] 2 more
   18  mmc0282       7.3 cM    1.0%  [#187 H-B-H 2.03] [#185 B-H-B 2.02] 6 more
   19  bnlg1847     34.5 cM    1.0%  [#126 H-B-A 1.48] [#163 H-B-A 1.40] 9 more
   20  bnlg1306    ----------
                   104.6 cM   9 markers   log-likelihood= -545.24

order2= 12 13 14 15 16 17 18 19 20
other2=
================================================================================
```

```
7> list loci

                                Error                Linkage   Haplotype
 Num  Name        Genotypes     Prob    Chrom        Group     Group      Class     New?

   1  bnlg147     228 codom   1.00%   -            group1    -          -         -
   2  phi001      229 codom   1.00%   -            group1    -          -         -
   3  asg30       208 codom   1.00%   -            group1    -          -         -
   4  us45         74 codom   1.00%   -            group1    -          -         -
   5  bnlg2086    230 codom   1.00%   -            group1    -          -         -
   6  us44        214 codom   1.00%   -            group1    -          -         -
   7  bnl5.59     214 codom   1.00%   -            group1    -          -         -
   8  bnlg1598    228 codom   1.00%   -            group1    -          -         -
   9  umc58       227 codom   1.00%   -            group1    -          -         -
  10  php20855     48 codom   1.00%   -            group1    -          -         -
  11  phi037      229 codom   1.00%   -            group1    -          -         -
  12  bnlg143     226 codom   1.00%   -            group2    -          -         -
  13  bnlg565     223 codom   1.00%   -            group2    -          -         -
  14  bnlg557     230 codom   1.00%   -            group2    -          -         -
  15  bnlg150     229 codom   1.00%   -            group2    -          -         -
  16  us42        228 codom   1.00%   -            group2    -          -         -
  17  us40        230 codom   1.00%   -            group2    -          -         -
  18  mmc0282     230 codom   1.00%   -            group2    -          -         -
  19  bnlg1847    226 codom   1.00%   -            group2    -          -         -
  20  bnlg1306    230 codom   1.00%   -            group2    -          -         -
  21  us41        220 codom   1.00%   -            unlinked  -          -         -

8> sequence 1-11
sequence #2= 1-11

9> make chromosome chr1
chromosomes defined: chr1

10> anchor chr1
1    - anchor locus on chr1
2    - anchor locus on chr1
3    - anchor locus on chr1
4    - anchor locus on chr1
5    - anchor locus on chr1
6    - anchor locus on chr1
7    - anchor locus on chr1
8    - anchor locus on chr1
9    - anchor locus on chr1
10   - anchor locus on chr1
11   - anchor locus on chr1
chromosome chr1 anchor(s): bnlg147 phi001 asg30 us45 bnlg2086 us44 bnl5.59
bnlg1598 umc58 php20855 phi037
```

```
11> frame chr1

setting framework for chromosome chr1...

================================================================================
chr1 framework:
            Apriori
  Markers          Distance   Prob  Candidate Errors
    1  bnlg147       8.2 cM
    2  phi001        8.6 cM   1.0%  [#182 B-A-B 4.00] [#130 B-A-H 2.16] 7 more
    3  asg30         4.4 cM   1.0%  [#189 B-H-B 1.81] [#132 H-A-- 1.44] 1 more
    4  us45          6.6 cM   1.0%  [#94 --B-A 3.58]  [#191 H-B-H 1.73] 2 more
    5  bnlg2086      6.1 cM   1.0%  [#207 B-H-B 1.79] [#216 B-H-B 1.79] 9 more
    6  us44          6.6 cM   1.0%  -
    7  bnl5.59       2.6 cM   1.0%  [#40 A-B-A 5.21]  [#142 H-A-H 2.16]
    8  bnlg1598      4.4 cM   1.0%  [#218 B-H-B 2.33] [#19 H-B-H 2.32]  1 more
    9  umc58         5.1 cM   1.0%  [#80 H-B-- 1.84]  [#16 H-A-- 1.38]  6 more
   10  php20855     21.0 cM   1.0%  [#186 H-B-H 1.40] [#108 H-B-H 1.40]
   11  phi037      ----------
                    73.8 cM   11 markers   log-likelihood= -504.37
================================================================================

12> lod
Bottom number is LOD score, top number is centimorgan distance:

      1     2     3     4     5     6     7     8     9     10

2      9.6
      50.81

3     15.2  10.7
      35.33 46.08

4     23.5  18.6   6.4
      13.17 16.79 27.18

5     27.1  20.3  12.3  13.1
      20.24 29.39 41.58 21.63

6     26.6  24.7  11.8  10.4   7.3
      19.00 22.03 40.78 22.83 55.84

7     35.5  31.9  16.9  14.4  15.4   6.9
      13.36 16.42 30.93 17.59 35.02 55.22

8     42.2  37.9  20.6  22.4  18.4  10.1   3.5
      10.47 13.38 26.79 13.82 32.25 47.87 72.07

9     43.7  43.6  25.4  23.5  24.5  14.1   8.2   5.3
      10.39 11.09 22.46 13.17 24.47 39.24 54.94 69.42

10    40.3  36.8  18.0   0.0  26.9  15.8   8.7   9.3   4.4
       4.22  4.99 10.03 20.47  7.56 12.37 15.88 16.41 21.68

11    65.6  79.4  49.3  60.0  47.3  36.4  34.1  28.8  25.8  15.8
      3.69  2.27  6.92  2.92  7.71 12.64 14.15 19.48 23.20 12.37
```

```
13> sequence 12-20
sequence #3= 12-20

14> make chromosome chr5
chromosomes defined: chr1 chr5

15> anchor chr5
12   - anchor locus on chr5
13   - anchor locus on chr5
14   - anchor locus on chr5
15   - anchor locus on chr5
16   - anchor locus on chr5
17   - anchor locus on chr5
18   - anchor locus on chr5
19   - anchor locus on chr5
20   - anchor locus on chr5
chromosome chr5 anchor(s): bnlg143 bnlg565 bnlg557 bnlg150 us42 us40 mmc0282
bnlg1847 bnlg1306

16> frame chr5
setting framework for chromosome chr5...
================================================================================
chr5 framework:
           Apriori
  Markers          Distance   Prob  Candidate Errors
   12  bnlg143      14.7 cM
   13  bnlg565      22.6 cM   1.0%  [#133 H-A-B 1.33] [#151 H-A-B 1.33]
   14  bnlg557      11.4 cM   1.0%  [#156 H-B-A 1.78] [#152 A-B-H 1.48] 6 more
   15  bnlg150       3.4 cM   1.0%  [#173 H-A-H 1.81] [#209 H-B-H 1.81] 3 more
   16  us42          7.7 cM   1.0%  -
   17  us40          3.1 cM   1.0%  [#135 H-A-H 2.02] [#134 A-H-A 2.01] 2 more
   18  mmc0282       7.3 cM   1.0%  [#187 H-B-H 2.03] [#185 B-H-B 2.02] 6 more
   19  bnlg1847     34.5 cM   1.0%  [#126 H-B-A 1.48] [#163 H-B-A 1.40] 9 more
   20  bnlg1306   ----------
                  104.6 cM   9 markers   log-likelihood= -545.24
================================================================================

17> lod
Bottom number is LOD score, top number is centimorgan distance:

      12    13    14    15    16    17    18    19

13    15.2
     31.88

14    40.8  24.3
      9.08 20.31

15    49.9  33.3  12.6
      5.96 13.41 41.96

16    60.9  35.3  15.0   4.0
      3.99 12.38 37.18 72.43

17    58.4  39.9  22.9  13.2   8.2
      4.38  9.68 24.09 41.06 55.41
```

```
        12     13     14     15     16     17     18     19
18     61.8   40.1   21.7   15.6   11.4    4.4
       3.54   9.44  24.95  35.44  45.15  69.58

19     64.0   44.3   26.0   17.8   17.5    9.7    8.6
       2.99   7.47  19.60  30.81  31.40  48.51  52.07

20    106.6   86.2   67.5   69.5   63.1   43.9   42.1   34.9
       0.68   1.43   3.42   3.05   4.03   9.34   9.81  13.18


19> quit
save data before quitting? [yes] y
saving map data in file 'GLS.MAP'... ok
saving two-point data in file 'GLS.2PT'... ok

        ...goodbye...
```

```
*************************************************************************
* Output from:                                  Tue Jul 12 20:26:28 2000 *
*                                                                        *
*                              MAPMAKER/QTL                              *
*                             (version 1.1b)                             *
*                                                                        *
*************************************************************************

'photo' is on: file is 'GLS.OUT'

3> show trait

Trait 1 (gls):

-------------------------------------------------------------------------
distribution:                        quartile |   fraction within n deviations:
mean   sigma   skewness  kurtosis   ratio    |   1/4    1/2   1     2     3
5.43   2.47    -0.45     -1.10      1.20     |   0.20   0.20  0.59  1.00  1.00
-------------------------------------------------------------------------

  0.48  |
  1.72  |*********************
  2.96  |*************************
  4.19  |***********************************
  5.43  |*****************
  6.66  |*********************************
  7.90  |************************************************************
  9.13  |**********************************************
 10.37  |
 11.60  |

4> sequence [all]
The sequence is now '[all]'

5> scan

QTL maps for trait 1 (gls):
Sequence: [all]
LOD threshold: 2.00  Scale: 0.25 per '*'

No fixed-QTLs.
Scanned QTL genetics are free.

POS     WEIGHT  DOM     %VAR  LOG-LIKE |
-----------------------------------------| 1-2 8.2 cM
0.0     1.231  -0.123  11.8%    6.244  | *****************
2.0     1.257  -0.160  12.7%    6.475  | ******************
4.0     1.249  -0.201  13.0%    6.526  | ******************
6.0     1.210  -0.252  12.6%    6.408  | ******************
8.0     1.140  -0.315  11.7%    6.152  | *****************
-----------------------------------------| 2-3 8.6 cM
0.0     1.131  -0.323  11.5%    6.119  | *****************
2.0     1.308  -0.333  15.3%    7.703  | ************************
4.0     1.429  -0.288  18.1%    9.135  | ****************************
6.0     1.509  -0.243  19.9%   10.364  | ********************************
8.0     1.550  -0.215  20.8%   11.356  | *********************************
-----------------------------------------| 3-4 4.4 cM
```

0.0	1.557	-0.209	20.9%	11.614	**********
2.0	2.196	0.404	28.8%	12.622	**********
4.0	2.461	0.362	21.7%	10.374	**********
					4-5 6.6 cM
0.0	2.369	0.199	17.5%	9.341	**********
2.0	2.342	0.569	26.2%	11.515	**********
4.0	2.136	0.696	28.0%	12.435	**********
6.0	1.760	0.627	22.9%	11.813	**********
					5-6 6.1 cM
0.0	1.621	0.562	20.5%	11.444	**********
2.0	1.874	0.392	27.6%	14.681	**********
4.0	1.970	0.255	30.8%	17.122	**********
6.0	2.003	0.170	31.6%	18.788	**********
					6-7 6.6 cM
0.0	2.005	0.166	31.6%	18.878	**********
2.0	2.139	0.372	36.7%	20.700	**********
4.0	2.134	0.391	36.7%	20.707	**********
6.0	2.030	0.359	33.5%	19.292	**********
					7-8 2.6 cM
0.0	1.937	0.340	30.9%	18.429	**********
2.0	2.000	0.372	32.9%	19.035	**********
					8-9 4.4 cM
0.0	1.902	0.378	30.4%	18.114	**********
2.0	1.973	0.321	33.5%	19.050	**********
4.0	1.866	0.202	30.9%	17.883	**********
					9-10 5.1 cM
0.0	1.808	0.145	29.4%	17.342	**********
2.0	2.417	0.692	39.9%	18.169	**********
4.0	2.632	0.555	33.3%	14.081	**********
					10-11 21.0 cM
0.0	1.541	-0.776	19.5%	9.763	**********
2.0	2.307	0.060	22.3%	9.675	**********
4.0	2.242	0.069	24.0%	9.422	**********
6.0	2.109	0.006	24.2%	8.988	**********
8.0	1.867	-0.191	22.3%	8.410	**********
10.0	1.613	-0.421	19.6%	7.792	**********
12.0	1.467	-0.501	17.6%	7.180	**********
14.0	1.362	-0.503	15.6%	6.565	**********
16.0	1.267	-0.465	13.6%	5.958	**********
18.0	1.179	-0.395	11.6%	5.375	**********
20.0	1.087	-0.318	9.7%	4.833	**********

					12-13 14.7 cM
0.0	0.053	-0.239	0.3%	0.130	
2.0	0.087	-0.349	0.6%	0.254	
4.0	0.123	-0.453	1.0%	0.410	
6.0	0.157	-0.546	1.4%	0.580	
8.0	0.195	-0.606	1.8%	0.746	
10.0	0.237	-0.633	2.1%	0.892	
12.0	0.282	-0.627	2.2%	1.012	
14.0	0.326	-0.603	2.3%	1.108	

```
---------------------------------------| 13-14 22.6 cM
0.0     0.341  -0.590   2.3%    1.137  |
2.0     0.438  -0.604   2.9%    1.300  |
4.0     0.540  -0.598   3.7%    1.504  |
6.0     0.644  -0.559   4.5%    1.747  |
8.0     0.750  -0.494   5.3%    2.031  | *
10.0    0.850  -0.400   6.2%    2.357  | **
12.0    0.938  -0.291   7.0%    2.717  | ***
14.0    1.011  -0.188   7.7%    3.097  | *****
16.0    1.063  -0.095   8.3%    3.474  | ******
18.0    1.093  -0.014   8.7%    3.828  | ********
20.0    1.100   0.054   8.8%    4.146  | *********
22.0    1.093   0.110   8.7%    4.420  | **********
---------------------------------------| 14-15 11.3 cM
0.0     1.089   0.124   8.6%    4.493  | **********
2.0     1.158   0.159   9.9%    4.737  | ***********
4.0     1.187   0.153  10.5%    4.823  | ************
6.0     1.173   0.106  10.4%    4.740  | ***********
8.0     1.122   0.027   9.7%    4.515  | ***********
10.0    1.043  -0.067   8.5%    4.201  | *********
---------------------------------------| 15-16 3.4 cM
0.0     0.982  -0.124   7.6%    3.968  | ********
2.0     1.007  -0.279   8.4%    4.234  | *********
---------------------------------------| 16-17 7.7 cM
0.0     0.984  -0.355   8.2%    4.253  | **********
2.0     1.049  -0.333   9.1%    4.488  | **********
4.0     1.075  -0.297   9.4%    4.567  | ***********
6.0     1.062  -0.267   9.0%    4.482  | **********
---------------------------------------| 17-18 3.1 cM
0.0     1.019  -0.244   8.2%    4.286  | **********
2.0     1.101  -0.189   9.3%    4.744  | ***********
---------------------------------------| 18-19 7.3 cM
0.0     1.113  -0.173   9.3%    4.868  | ************
2.0     1.170  -0.175  10.3%    5.126  | *************
4.0     1.187  -0.148  10.6%    5.184  | *************
6.0     1.162  -0.094  10.1%    5.027  | *************
---------------------------------------| 19-20 34.5 cM
0.0     1.119  -0.044   9.2%    4.818  | ************
2.0     1.136  -0.073   9.6%    4.616  | ***********
4.0     1.146  -0.103   9.9%    4.378  | **********
6.0     1.146  -0.133  10.0%    4.105  | *********
8.0     1.132  -0.157   9.9%    3.796  | ********
10.0    1.106  -0.183   9.6%    3.455  | ******
12.0    1.064  -0.200   9.0%    3.088  | *****
14.0    1.008  -0.211   8.1%    2.705  | ***
16.0    0.941  -0.224   7.2%    2.315  | **
18.0    0.859  -0.220   6.1%    1.933  |
20.0    0.766  -0.200   4.9%    1.572  |
22.0    0.670  -0.184   3.8%    1.243  |
24.0    0.571  -0.158   2.8%    0.954  |
26.0    0.479  -0.143   2.0%    0.711  |
28.0    0.390  -0.126   1.3%    0.513  |
30.0    0.310  -0.115   0.9%    0.358  |
32.0    0.240  -0.113   0.5%    0.240  |
34.0    0.177  -0.111   0.3%    0.156  |
---------------------------------------|
Results have been stored as scan number 1.
```

```
6> show peaks

LOD score peaks for scan 1.1 of trait 1 (gls).
Sequence: [all]
No fixed-QTLs.
Scanned QTL genetics are free.
Peak Threshold: 2.00  Falloff: -2.00

============================================================
QTL-Map for peak 1:
Confidence Interval:  Left Boundary= 2-3 + 8.0
                     Right Boundary= 2-3 + 0.0

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
3-4        4.4     2.0      free        2.1955  0.4037

chi^2= 58.128 (2  D.F.)        log-likelihood= 12.62
mean= 2.545   sigma^2= 4.348   variance-explained= 28.8%
============================================================
QTL-Map for peak 2:
Confidence Interval:  Left Boundary= 5-6 + 6.0
                     Right Boundary= 6-7 + 6.0

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
6-7        6.6     4.0      free        2.1338  0.3914

chi^2= 95.359 (2  D.F.)        log-likelihood= 20.71
mean= 2.948   sigma^2= 3.863   variance-explained= 36.7%
============================================================
QTL-Map for peak 3:
Confidence Interval:  Left Boundary= 13-14 + 16.0
                     Right Boundary= 19-20 + 10.0

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
18-19      7.3     4.0      free        1.1873 -0.1477

chi^2= 23.874 (2  D.F.)        log-likelihood= 5.18
mean= 4.268   sigma^2= 5.457   variance-explained= 10.6%
============================================================



7> sequence [6-7:try]
The sequence is now '[6-7:try]'

8> map

============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
6-7        6.6     3.1      free        2.1447  0.3898

chi^2= 96.108 (2  D.F.)        log-likelihood= 20.87
mean= 2.936   sigma^2= 3.843   variance-explained= 37.0%
============================================================
```

```
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
6-7        6.6     3.4      dominant    1.5938  1.5938

chi^2= 67.404 (2  D.F.)        log-likelihood= 14.64
mean= 2.936   sigma^2= 4.369   variance-explained= 28.4%
============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
6-7        6.6     2.1      recessive   1.2622 -1.2622

chi^2= 52.882 (2  D.F.)        log-likelihood= 11.48
mean= 4.698   sigma^2= 4.797   variance-explained= 21.4%
============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
6-7        6.6     3.0      additive    2.1127  0.0000

chi^2= 94.125 (2  D.F.)        log-likelihood= 20.44
mean= 3.164   sigma^2= 3.896   variance-explained= 36.2%
============================================================

9> sequence [18-19:try]
The sequence is now '[18-19:try]'


10> map

============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
18-19      7.3     3.6      free        1.1872 -0.1545

chi^2= 23.898 (2  D.F.)        log-likelihood= 5.19
mean= 4.273   sigma^2= 5.456   variance-explained= 10.6%
============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
18-19      7.3     4.3      dominant    0.7413  0.7413

chi^2= 12.931 (2  D.F.)        log-likelihood= 2.81
mean= 4.247   sigma^2= 5.748   variance-explained= 5.9 %
============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
18-19      7.3     3.4      recessive   0.8252 -0.8252

chi^2= 17.950 (2  D.F.)        log-likelihood= 3.90
mean= 5.016   sigma^2= 5.596   variance-explained= 8.3 %
============================================================
QTL map for trait 1 (gls):
```

```
INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
18-19      7.3     3.6      additive    1.1941  0.0000

chi^2= 23.677 (2  D.F.)        log-likelihood= 5.14
mean= 4.181   sigma^2= 5.464   variance-explained= 10.5%
============================================================

11> sequence [14-15:try]
The sequence is now '[14-15:try]'

12> map

============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
14-15      11.3    4.6      free        1.1883  0.1457

chi^2= 22.177 (2  D.F.)        log-likelihood= 4.82
mean= 4.083   sigma^2= 5.459   variance-explained= 10.6%
============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
14-15      11.3    4.1      dominant    0.8752  0.8752

chi^2= 15.398 (2  D.F.)        log-likelihood= 3.34
mean= 4.025   sigma^2= 5.615   variance-explained= 8.0 %
============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
14-15      11.3    5.8      recessive   0.7288 -0.7288

chi^2= 14.278 (2  D.F.)        log-likelihood= 3.10
mean= 5.041   sigma^2= 5.692   variance-explained= 6.8 %
============================================================
QTL map for trait 1 (gls):

INTERVAL   LENGTH  QTL-POS  GENETICS    WEIGHT  DOMINANCE
14-15      11.3    4.8      additive    1.1705  0.0000

chi^2= 21.986 (2  D.F.)        log-likelihood= 4.77
mean= 4.181   sigma^2= 5.470   variance-explained= 10.4%
============================================================
```

```
13> sequence [6+4][18+4]
The sequence is now '[6+4][18+4]'

14> map

============================================================
QTL map for trait 1 (gls):

INTERVALS  LENGTH  QTL-POS  WEIGHT  DOMINANCE
6-7        6.6     4.0      2.1050  0.2792
18-19      7.3     4.0      1.1340  0.0839

chi^2= 127.812 (4  D.F.)        log-likelihood= 27.75
mean= 1.804   sigma^2= 3.306   variance-explained= 45.9%
============================================================

15> sequence [6+4][18+4][14+5]
The sequence is now '[6+4][18+4][14+5]'

16> map

============================================================
QTL map for trait 1 (gls):

INTERVALS  LENGTH  QTL-POS  WEIGHT  DOMINANCE
6-7        6.6     4.0      2.0910  0.2923
18-19      7.3     4.0      0.7801 -0.0318
14-15      11.3    5.0      0.5160  0.2283

chi^2= 131.530 (6  D.F.)        log-likelihood= 28.56
mean= 1.576   sigma^2= 3.258   variance-explained= 46.6%
============================================================

17> sequence [18+4][14+5]
The sequence is now '[18+4][14+5]'

18> map

============================================================
QTL map for trait 1 (gls):

INTERVALS  LENGTH  QTL-POS  WEIGHT  DOMINANCE
18-19      7.3     4.0      0.7398 -0.2143
14-15      11.3    5.0      0.6559  0.1533

chi^2= 27.518 (4  D.F.)        log-likelihood= 5.98
mean= 3.995   sigma^2= 5.367   variance-explained= 12.1%
============================================================


19> quit
save data before quitting? [yes] n

        ...goodbye...
```

Addendum V: Phenotype and genotype data of the selected plants of the 1999 F2 population

Bin	1.05	1.06	5.03	5.04	5.05	5.06	3.04	2.02
Score	us44	bnlg1598	bnlg557	bnlg150	mmc0282	bnlg1847	us41	bnlg125
1	A	A	H	H	H	H	H	B
1	A	A	H	H	H	H	B	H
1	H	H	H	H	H	H	A	H
1	H	H	A	A	H	H	A	H
1	A	A	-	A	A	A	H	H
1	A	A	H	H	B	B	H	A
1	H	H	H	H	A	A	A	H
1	-	-	H	A	H	H	H	A
1	A	A	H	H	A	A	H	A
1	A	A	B	A	H	H	-	H
1	A	H	H	H	H	A	A	H
1	A	A	A	A	A	A	A	H
1	A	A	H	H	H	H	H	A
1	A	A	H	H	B	B	A	A
1	A	A	H	A	H	H	H	A
1	A	A	B	H	A	A	A	H
1	A	H	H	A	A	A	-	A
1	A	H	H	A	A	A	H	A
1	-	A	-	A	H	-	H	-
2	H	H	H	A	A	A	H	A
2	A	A	H	H	H	B	H	B
2	A	A	H	A	A	A	A	H
2	H	A	H	A	H	H	H	B
2	A	A	H	H	A	H	H	-
2	H	H	A	A	A	A	B	H
2	A	A	B	H	H	H	A	H
2	A	-	B	B	B	B	A	A
2	A	A	H	A	H	H	H	B
2	H	A	B	B	B	B	H	H
2	A	A	H	B	H	B	A	B
2	-	A	B	H	H	B	A	A
2	-	A	A	A	H	H	B	B
2	A	A	-	A	A	-	A	-
2	A	A	-	H	H	-	A	-
2	-	A	-	H	H	-	H	-
2	-	H	-	H	H	-	H	-
2	A	A	-	H	H	-	A	-
3	-	H	B	A	H	H	B	B
3	A	A	H	H	H	A	A	H
3	A	A	A	A	A	A	H	A
3	A	H	H	A	H	H	H	B
3	-	H	H	A	A	A	A	A
3	-	A	A	A	A	A	H	H
3	A	A	-	H	H	-	B	-
3	-	A	-	H	B	-	H	-

Bin	1.05	1.06	5.03	5.04	5.05	5.06	3.04	2.02
Score	us44	bnlg1598	bnlg557	bnlg150	mmc0282	bnlg1847	us41	bnlg125
3	-	A	-	A	A	-	H	-
4	H	H	H	H	H	H	H	H
4	A	A	A	H	A	A	H	H
4	H	A	B	A	A	A	H	A
4	H	H	A	A	H	H	H	B
4	H	H	H	H	A	A	B	H
4	A	H	H	A	A	A	A	A
5	A	H	H	A	A	A	H	B
5	H	H	A	A	A	-	B	H
5	H	H	B	B	B	-	A	H
5	A	H	H	H	A	A	A	A
5	A	H	B	B	H	H	H	H
5	A	H	H	H	H	H	H	H
6	H	H	H	H	H	H	A	H
6	B	H	A	A	A	A	A	H
6	A	A	H	H	A	A	H	A
6	H	B	B	B	B	B	H	B
6	A	A	B	B	B	B	B	H
6	-	H	H	H	H	H	B	A
7	-	H	A	A	A	H	A	H
7	-	H	A	A	H	A	B	A
7	-	A	B	A	A	A	H	A
7	-	B	H	A	H	B	A	B
7	-	A	A	H	A	A	H	H
7	-	H	H	H	H	H	H	A
7	H	A	-	H	H	-	H	-
7	H	H	-	H	A	-	B	-
7	H	H	-	H	H	-	H	-
7	-	H	-	A	H	-	A	-
8	-	B	A	A	A	A	H	A
8	-	H	H	H	H	H	B	H
8	-	H	H	H	B	B	H	H
8	-	A	H	H	H	H	A	H
8	-	H	B	B	H	H	B	H
8	-	H	H	H	H	A	A	H
9	-	H	B	B	H	H	H	H
9	-	H	H	H	H	B	H	H
9	-	H	H	H	H	H	A	H
9	-	H	H	H	A	A	B	H
9	H	H	B	H	H	H	B	B
9	H	H	H	H	H	H	A	H
9	H	H	H	H	H	H	B	H
9	B	B	H	H	B	B	B	B
9	H	H	H	H	H	H	B	A
9	-	H	H	A	A	A	B	A
9	H	H	H	H	H	H	A	H
9	H	A	B	B	H	B	H	B
9	H	B	B	H	H	H	H	A
9	B	B	A	A	A	A	A	H

Addendum VI: Phenotype and genotype data of the selected plants of the 2000 F2 population.

Bin	1.05	1.06	5.03	5.04	5.05	5.06	3.04	2.02
Score	us44	bnlg1598	bnlg557	bnlg150	mmc0282	bnlg1847	us41	bnlg125
2	H	H	A	H	A	H	A	H
2	A	H	H	H	A	H	A	H
2	H	H	A	A	A	A	H	A
2	H	A	H	H	B	B	A	A
2	A	A	H	H	H	H	H	H
3	A	A	H	H	H	A	B	A
3	A	A	A	A	A	H	H	B
3	H	H	H	H	H	H	A	B
3	A	A	H	H	H	H	A	H
3	A	A	A	A	A	A	A	A
3	A	A	B	H	B	B	A	H
3	H	A	A	A	A	H	H	H
3	H	H	A	A	H	H	H	A
3	A	A	H	H	H	H	H	B
3	A	A	A	H	H	B	A	A
3	A	A	A	A	A	A	H	H
3	A	A	B	H	H	A	H	H
3	A	A	H	H	B	B	A	H
3	H	B	A	A	A	A	H	A
4	A	A	H	A	A	A	H	B
4	A	A	H	H	H	H	A	A
4	H	H	H	A	H	H	H	A
4	H	H	A	A	A	A	H	H
4	A	A	A	A	A	A	B	H
4	A	A	H	H	H	H	H	B
8	A	A	H	H	H	H	H	H
8	H	H	H	H	H	H	A	H
8	A	H	H	H	H	H	A	B
8	A	A	H	A	A	H	H	H
8	A	A	H	H	H	H	H	A
8	B	B	H	H	B	B	B	H
8	B	H	B	B	B	B	H	A
8	H	H	H	H	H	H	B	H
8	H	A	B	B	H	H	H	A
8	H	H	A	A	A	A	H	H
8	H	B	A	A	H	H	A	H
8	A	H	A	A	A	H	A	H
8	H	H	A	B	B	B	B	H
8	H	H	H	H	H	H	A	A
9	H	H	B	H	A	A	B	A
9	H	H	H	H	H	H	H	H
9	B	B	B	B	B	B	H	B
9	B	B	H	H	H	H	A	A
9	H	H	B	B	H	H	B	B
9	B	B	H	B	B	H	B	B
9	B	B	B	B	B	B	H	H
9	-	B	A	A	A	A	H	H
9	B	B	B	B	B	B	H	H

Bin	1.05	1.06	5.03	5.04	5.05	5.06	3.04	2.02
Score	us44	bnlg1598	bnlg557	bnlg150	mmc0282	bnlg1847	us41	bnlg125
9	H	H	B	H	H	A	B	A
9	-	H	B	H	H	B	H	B
9	H	H	H	H	H	H	H	A
9	H	H	B	B	H	H	B	B
9	B	B	H	H	H	H	H	-
9	B	B	H	H	H	H	B	B
9	H	H	H	H	H	H	B	B
9	H	H	H	H	B	-	B	B
9	H	A	H	H	H	-	H	B
9	-	B	A	A	A	A	B	H
9	H	H	B	B	B	H	B	H
9	B	H	H	H	H	H	H	A
9	A	H	-	H	H	-	H	-
9	-	B	-	B	H	-	H	-
9	A	H	-	H	H	-	H	-
9	-	A	-	H	H	-	H	-
9	B	B	-	A	A	-	H	-

A = homozygous for the allele of the resistant parent
B = homozygous for the allele of the susceptible parent
H = heterozygous
- = missing data