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ABSTRACT

The objective of this study was to develop and apply closely linked molecular markers and information from maize genetic maps for marker assisted selection (MAS) of gray leaf spot (GLS) in the maize breeding programme. Application of biotechnology techniques, especially molecular markers linked to resistance genes would facilitate the rapid identification of resistance to diseases in pyramided lines. This will compliment the conventional breeding programme and can add to the competitiveness of the maize industry. Molecular markers linked to the resistance genes may be useful to plant breeders to support the introgression of the resistance alleles into elite inbred lines. Furthermore, this can be done without inoculation and at an early stage of plant development. Bulked segregant analysis was used to identify microsatellite (SSR) and amplified fragment length polymorphism (AFLP) markers linked to quantitative trait loci (QTL's) involved in resistance to grey leaf spot in maize. Two markers were identified linked to the resistance trait in one segregating population. SSR marker bnlg1057 (on chromosome 1) explained 25 % and marker us44 (in the same chromosomal region) explained 27 % of the variation in the GLS disease ratings from population CML7 x I137TNW.

INTRODUCTION

Maize is the major food staple for most indigenous, rural populations in Africa. African countries consume nearly one-fourth of the total maize produced in Africa and for many countries, the per capita consumption of maize may be as high as 100 kg per year. Farming systems in South Africa are quite diverse. At the one end of the spectrum are approximately 55 000 commercial farmers producing 4 to 9 million metric tons of grain annually. GLS management for these farmers are varied but vital to the continued economic production of maize. In contrast, there are more than a million small-scale subsistence farmers in South Africa, many of whom are women and children. The impact of GLS on these small-scale farmers could be devastating, as the disease management options available to them are very limited and maize accounts for as much as 70 % of their food production. The competitiveness of the maize industry could benefit immensely if effective resistance contributes just 1 % to increased yield and reduced risk. Household food security benefits for millions of resource-poor and rural populations inside and outside this country are impossible to calculate but it is the most important outcome of projects of this nature.

Grey leaf spot (GLS) of maize, caused by the fungus *Cercospora zeae-maydis*, 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. In South Africa the disease was first observed in KwaZulu-Natal in 1988 and has since rapidly spread to neighbouring provinces and countries. It can reduce grain yields by 30 - 60 %, depending on hybrid susceptibility and favourable weather conditions. It is now recognised as one of the most significant yield-limiting diseases of maize worldwide. GLS is an extremely environmentally sensitive disease requiring high humidity and extended leaf wetness. Symptoms of GLS are normally first observed on the lower leaves. Typical mature GLS lesions are gray to tan in colour, sharply rectangular, long and narrow, and run parallel to the leaf veins. Methods to control GLS include the discontinuation of conservation tillage, the use of crop rotation, the application of costly foliar fungicides and growing resistant cultivars. Cultivar resistance is the most cost-effective, efficient, acceptable and sustainable option, however. 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. Recovery through conventional breeding is therefore difficult and to-date only a few high-yielding maize hybrids resistant to GLS are available in South Africa. Molecular markers linked to the resistance genes may be useful to plant breeders to support the introgression of the resistance alleles into elite inbred lines. Lehmensik *et al*. (2001) located QTL's for GLS resistance on chromosomes 1, 3 and 5 utilizing a resistant inbred line from Zimbabwe.

MATERIALS AND METHODS

Plant material and disease evaluations

Four different populations were developed and used for marker development: i) S196YxV613Y (resistant) and R2565Y (susceptible), ii) CML7 x I137TNW, iii) CML8 x I137TNW and iv) A1.P100 x I137TNW. The plant material used in the study originated from CIMMYT as well as ARC-GCI inbred lines. Crosses were advanced to F_2 and 250 plants from each population were evaluated for disease resistance under natural disease infestation in a hot spot area for GLS. The resistant and susceptible parents were planted to determine the disease infestation and progress. Disease ratings were recorded three times during the growing season, using the susceptible parents as indicators. Three of the populations were advanced to the F_3 generation and the GLS ratings confirmed in F_3 families. Leaf tissue samples were taken from 250 plants of each F_2 population for DNA analysis.

AFLP analysis

DNA was extracted from leaf samples using a modified CTAB (cetieltrimethyl-ammoniumbromide) procedure. Leaf material was ground to a fine powder with silica gel and suspended in CTAB extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% (m/v) CTAB, and 0.2% (v/v) ß-Mercaptoethanol) and incubated for 1 h at 65°C. The suspension was extracted with chloroform : isoamylalcohol (24:1 (v/v)) and the phases separated by centrifugation at 10 000 x g for 10 min. The DNA was precipitated from the aqueous phase with 0.66 volume isopropanol at room temperature, and centrifuged for 15 min at 12 000 x g. The precipitate was washed with 70% (v/v) ethanol and air dried. The pellet was re-suspended in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and precipitated with 0.75 M ammonium acetate and 2 volumes absolute ethanol after a chloroform : isoamylalcohol (24:1 (v/v)) extraction. After an overnight incubation at -20°C, the DNA was recovered by centrifugation at 12 000 x g for 15 min and washed twice with 70% (v/v) ethanol for 5 min. The ethanol was removed and the pellet air-dried. The DNA was re-suspended in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and treated with 0.1 $\mu g.\mu l^{-1}$ DNase-free RNase, prepared by heating a stock solution of 10 $mg.ml^{-1}$ RNase (Roche Boehringer Mannheim, Randburg, South Africa) for 10 min at 94°C. The concentration of the DNA was determined spectrophotometrically at 260 nm.

Selective genotyping and bulked segregant analysis (BSA) was used to identify amplified fragment length polymorphism markers (AFLPs) and/or microsatellites (SSR) linked to QTL's involved in GLS resistance. The bulk samples were constructed by mixing equal amounts (m/m) of DNA from 5 - 8 plants from each of the two extreme ends of the disease spectrum, i.e. resistant and susceptible plants according to disease ratings. The AFLP methodology as described by Vos *et al*. (1995) was followed with minor modifications. Approximately 1 µg of genomic DNA was digested with either *Eco*RI or *Mlu*I

(rare cutter) for 5 hours, followed by digestion with *Mse*I (frequent cutter) overnight at 37°C. The digested DNA was incubated overnight at 16°C with 5 pmol *Eco*RI and 50 pmol *Mse*I adapters (Vos *et al*., 1995) and 1U T4 DNA ligase.

Pre-selective PCR was carried out with primers+1 (Vos *et al*., 1995) in a 50 µl volume containing 5 µl of the ligated DNA, pre-amp primer, Taq polymerase buffer, 2 mM $MgCl_2$ and Taq DNA polymerase (Promega). Samples were amplified in a Thermo Thermal Cycler for 30 cycles of 30 sec at 94°C, 1 min at 56°C and 1 min at 72°C. Quality and quantity of pre-amplification products were determined with electrophoresis in a 1.5% (m/v) agarose gel.

For selective PCR the pre-amplification products were diluted 1:10. PCR was conducted in a 20 µl reaction mixture containing 5 µl pre-amplification product, 0.25 ng.µl^{-1} *Eco*RI+3 primer (Vos *et al*., 1995), 1.5 ng.µl^{-1} *Mse*I +3 primer, 2 mM $MgCl_2$, Taq polymerase buffer and 0.02 U.µl^{-1} Taq DNA polymerase (Promega). The samples were amplified for one cycle at 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min, after which the annealing temperature was lowered 1°C for each of 9 cycles, followed by 24 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min. After amplification, reactions were stopped with an equal volume of loading buffer (95% (v/v) formamide, 20 mM EDTA, 0.025% (m/v) bromophenol blue, 0.025% (m/v) xylene cyanol) and denatured at 94°C for 3 min, followed immediately by chilling on ice. A 5% (m/v) denaturing polyacrylamide (19 acrylamide : 1 N,N'-methylene-bis-acrylamide ratio) gel was prepared with 7 M urea and 1 x TBE buffer (89 mM Tris-borate, 2.5 mM EDTA, pH 8.3). Two glass plates were prepared before casting the gel. One plate was treated with Sigmacote (Sigma-Aldrich) and the other with bind silane (950 µl absolute ethanol, 5 µl acetic acid (absolute), 3 µl bind silane (Promega)). The gel was pre-run at constant 80 W for 30 min. PCR products (5 µl) were separated on the prepared gel at 80 W constant power for approximately 2 h using a standard DNA sequencing unit (C.B.S. Scientific Company, California, USA).

The separated amplified DNA fragments were visualized with a silver staining kit (Promega) according to the manufacturer's instructions. The gel was left upright overnight to air dry and photographed by exposing photographic paper (Kodak Polymax II RC) directly under the gel to about 20 sec of dim light. This produced a negative image of exactly the same size as the gel.

Microsatellite (SSR) analysis

The microsatellite primer sequences were obtained from the Maize Genome Database website (http://www.maizegdb.org). Additional markers as developed by Stellenbosch University (Lehmensik *et al*., 2001), namely us40, us42, us44 and us45 were tested. Primers were used for the amplification of 25 ng genomic DNA in a total reaction volume of 25 µl. Reactions consisted of 30 ng of each of forward and reverse primers, 200 µM dNTP, 2 mM $MgCl_2$, 1 x Buffer (supplied by manufacturer), 0.5 U

Super-Therm polymerase (JMR-Holdings, London, UK). The amplification protocol consists of one cycle at 94°C for 1 min, followed by 10 cycles at 94°C for 1 min, 65°C to 55°C decreasing 1°C each cycle and 72°C for 1.5 min. This is followed by 30 cycles at 94°C 1 min, 55°C 1 min and 72°C for 1.5 min. A final extension at 72°C for 5 min is followed by a hold step at 4°C. Amplification products were electrophoresed in 5% denaturing polyacrylamide or 2% agarose gels.

Linear regression analysis

Linkage of molecular markers with GLS resistance was investigated with standard analysis of variance (ANOVA) for linear regression of GLS disease scores on marker genotypes. This analysis was used to calculate the proportion of the total phenotypic variance explained by each marker.

Linkage analysis and QTL mapping

Linkage analysis was performed with the software package MAPMAKER/EXP version 3.0b (Lander *et al*. 1987). To include a locus in a linkage group a minimum LOD threshold of 3.0 was used. The location of QTL's was determined by interval mapping (Lander and Botstein, 1989) using MAPMAKER/QTL version 1.1b (Patterson *et al.* 1988).

RESULTS

Population S196Y x V613Y (resistant) and R2565Y (susceptible)

AFLP and microsatellite analysis revealed 101 putative markers that were used for the construction of a putative linkage map. These markers mapped to 12 putative linkage groups covering a map distance of 1 681.4 cM (Figure 1). QTL analysis revealed two major (QTL1 and QTL2) and two minor (QTL3 and QTL4) QTL's on four different linkage groups. The two major QTL's each explained 100 % of the phenotypic variation. QTL1 mapped 19.1 cM from marker M451 and QTL2 mapped 19.4 cM from marker M498. Minor QTL3 explained 56 % of the variation and mapped 4.4 cM from marker M4511 while QTL4 explained 32 % of the variation and mapped 0 cM from marker M432.

Populations CML7 x I137TNW and CML8 x I137TNW

Preliminary screening of the ten selected plants from each population with SSR markers indicated that the two populations (CML 7 and CML 8 as resistant parents) had major putative QTL's linked to marker bnlg1057 on chromosome 1. Both populations also had QTL's on chromosome 8. Marker bnlg1057 was tested for linkage in 250 plants from population CML 7 x I137TNW and turned out to explain 25.2 % of the variation in the disease ratings. Marker us44 identified by Lehmensik *et al*. (2001) (also on chromosome 1) displayed 26.7% linkage to GLS resistance. Markers us40 and us42 on chromosome 5 were not significantly linked in any of the populations. Eight AFLP fragments showed putative linkage on the 10 plants of the bulks, but were not linked when tested in the larger populations.

Population A1.P100 x I137TNW

The resistant parent displayed possible additive disease resistance (results not published – J. Lake). The parents and bulks from this population were screened with 144 *Mlu*I/*Mse*I primer pair, as well as 144 *Eco*RI/*Mse*I combinations. Several putative markers were identified, but unfortunately no linked markers could be identified when the larger population was tested. The explanation for the lack of linkage could be that the disease ratings were not accurate due to inconsistent infestation of the pathogen. It is also possible that many QTL's are adding to resistance and these are too small to detect with a method using low density markers.

DISCUSSION

Bulked segregant analysis was used in a number of successful marker development programmes for different crops, including soybean (Mienie *et al*., 2002) and dry bean (Mienie *et al*., 2005). Markers linked to GLS resistance in maize were developed by Lehmensik et al. (2001) utilizing the same approach. The AFLP technique can be used to increase the density of markers on the plant genome, but these markers are anonymous and tend to bind to certain regions of the chromosome.

In the current study microsatellite (SSR) and AFLP techniques were used in combination with a BSA approach. Two major and two minor QTL's were identified to be linked to the resistance found in population (S196Y x V613Y) x R2565Y. These were AFLP markers and would be difficult to use for MAS. The GLS resistance trait in population CML7 x I137TNW had one major QTL on chromosome 1 linked to SSR marker bnlg1057, explaining 25.2% of the variation found in the segregating population. This could be the same resistance gene found in the population of Lehmensik *et al*. (2001) as the marker developed by this author, us44, displayed linkage to the same QTL on chromosome 1. The QTL identified by Lehmensik and co-workers on chromosome 5 could not be located in our population. This marker can thus be used in MAS utilizing CML7 as the source of resistance. No significant markers could be found in the other two populations used in this study that linked to resistance QTL. From field observations (unpublished data) it was clear that these resistance sources displayed additive effects, probably from several minor QTL's. When markers need to be found for relatively small QTL's adding to a specific trait, a marker technique must be used that covers the genome at high density, at most 10 cM apart. This can be achieved using techniques like microarrays, DArTs (Diversity Array Technology) and SNPs (single nucleotide polymorphism).

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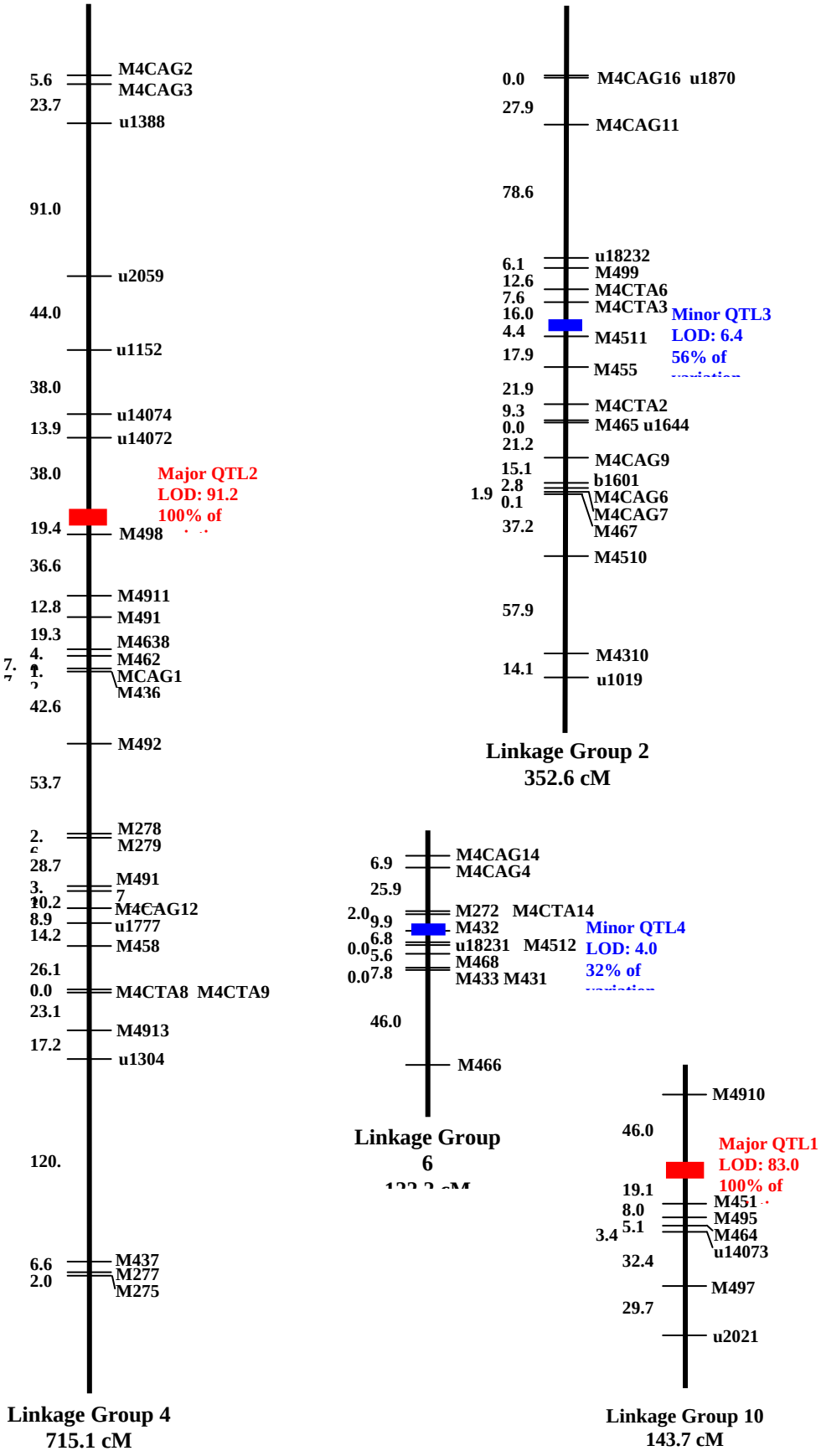

Figure 1. Genetic linkage map of population (S196Y x V613Y) x R2565Y indicating location of GLS QTL