IDENTIFICATION OF MOLECULAR MARKERS LINKED TO RESISTANCE QUANTITATIVE TRAIT LOCI (QTL) TO COMMON BACTERIAL BLIGHT IN DRY BEAN

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This dissertation is dedicated in loving memory to

Theveratnum (Thevers) Naidoo for always

believing and supporting me.

Rest in peace.

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DECLARATION

I hereby declare that this dissertation, unless otherwise acknowledged to the contrary in the text, is the result of my own investigation, under the supervision of Dr. C.M.S. Mienie, Plant Breeding and Biotechnology Department, Grain Crops Institute, Potchefstroom.

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We certify that the above statement is correct.

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ABSTRACT

Common bacterial blight (CBB) is a major disease limiting dry bean production in South Africa. All locally grown commercial cultivars are susceptible to the disease and improvement of the cultivars, by introducing stable resistance, is important. Two resistance sources, GN Nebr.#1 sel 27 and XAN 159 each contribute 2 independent quantitative trait loci (QTL) with major effects on CBB resistance. Sequence characterized amplified region (SCAR) markers linked to these four QTL are available for DNA marker-assisted breeding (Miklas *et al.*, 2000). Wilk 2 has shown better resistance in greenhouse trials when tested against the other available resistant cultivars. The aim of this study was to determine if there is an additional resistance QTL present in Wilk 2 and to develop flanking markers if possible. The AFLP technique was employed as a molecular tool to identify markers linked to CBB resistance. Twenty primer pair combinations of the *Eco*RI/*Mse*I-AFLP approach were screened. Seventy-nine putative markers were identified, tested on the segregating population and mapped using MAPMAKER-EXP along with the four available SCARs. One linkage group was obtained with two major and one minor QTL identified. The highest linkages to the resistance trait obtained through STATGRAPHICS were 81.17% and 76.16%. The available SCARs showed a lower linkage than expected (BC 420 58.44% to SAP 6 5.84%). Six markers were selected for further development of new SCARs. Polymorphic SCAR markers explained between 15.03 to 71.16% of the phenotypic variation for CBB resistance. The SCAR markers developed can be used for MAS, with SN 3-400 (E-AAC/M-CTC 2) being able to distinguish between the homozygotic and heterozygotic progeny, SN 2A-600 (E-AAC/M-CAT 3) and SN 3-300 (E-AAC/M-CTC 2) for selecting for the homozygous resistant plants, which could be highly efficient in a segregating population. The SCAR SN 1-1 (E-AAC/M-CAT 2) was unique to Wilk 2 and can be used in breeding programmes for pyramiding of CBB resistance genes.

OPSOMMING

Bakteriese skroei (CBB) is een van die belangrikste siektes wat droëboonproduksie in Suid-Afrika beïnvloed. Alle plaaslik verboude kommersiële kultivars is vatbaar vir die siekte. Dit is noodsaaklik om kultivars te verbeter deur die daarstelling van stabiele weerstand. Vier onafhanklike kwantitatiewe eienskap lokusse (QTL), afkomsitig van twee weerstandsbronne, GN Nebr.#1 sel 27 en XAN 159 (twee QTL elk), speel 'n balangrike rol in CBB weerstand. Volgorde gekarakteriseerde geamplifiseerde gebied (SCAR) merkers, gekoppel aan elk van die vier QTL, is beskikbaar vir DNA merker gesteunde teling (Miklas *et al.,* 2000). Glashuis proewe het aangetoon dat Wilk 2 beter weerstand vertoon in vergelyking met ander beskikbare weerstandbiedende kultivars. Die doel/fokus van hierdie studie was om te bepaal of Wilk 2 enige addisionele QTL besit, asook die ontwikkeling van merkers aan weerskante van die QTL, indien moontlik. Molekulêre merkers gekoppel aan CBB weerstand is geïdentifiseer deur gebruik te maak van die AFLP tegniek. Twintig voorvoerder kombinasies van die *Eco*RI/*Mse*I-AFLP benadering is gesif en 79 moontlike merkers is geïdentifiseer en op die segregerende populasie getoets. Hierdie moontlik merkers, asook die vier beskikbare SCARs, is gekarteer deur gebruik te maak van MAPMAKER-EXP. Een verbindingsgroep is verkry en twee hoof en een geringer QTL is geïdentifiseer. STATGRAPHICS het aangetoon dat 81.17% en 76.16% die beste koppeling aan die weerstandseienskap toon. Die beskikbare SCARs het swakker as die verwagte koppeling (BC 420 58.44% tot en met SAP 6 5.84%) getoon. Ses merkers is vir die ontwikkeling van nuwe SCARs geselekteer. Polimorfiese SCAR merkers het tussen 15.03 en 71.16% van die fenotipiese variasie vir CBB weerstand verklaar. Die ontwikkelde SCAR merkers kan vir merker ondersteunde seleksie gebruik word deurdat SN 3-400 (E-AAC/M-CTC 2) tussen homosigotiese en heterosigotiese nageslag onderskei terwyl SN 2A-600 (E-AAC/M-CAT 3) en SN 3-300 (E-AAC/M-CTC 2) homosigotiese weerstandbiedende plante selekteer, wat baie effektief kan wees vir 'n segregerende populasie. SCAR SN 1-1 (E-AAC/M-CAT 2) was uniek aan Wilk 2 en kan in teelprogramme gebruik word.

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LIST OF ABBREVIATIONS

A	Adenine
AFLP	Amplified fragment length polymorphism
AP-PCR	Arbitrarily primed polymerase chain reaction
bp	Base pairs
BSA	Bulk segregant analysis
C	Cytosine
$CaCl_2$	Calcium chloride
CAPS	Cleaved amplified polymorphic sequence
CBB	Common bacterial blight
cM	Centimorgan
CTAB	Cetryltrimethylammonium bromide
DAF	DNA amplification fingerprinting
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid disodium salt
G	Guanine
GN. Nebr. sel 27#1	Great Northern Nebraska selection 27 number 1
HCl	Hydrochloric acid
IPTG	Isopropyl-ß-D-thiogalactopyranoside
ISSRs	Inter-simple sequence repeats
K	Potassium
kb	kilobases
KCl	Potassium chloride
LB medium	Luria-Bertini medium
LOD score	$\log_{10}$ of the odds ratio
MAS	Marker assisted selection
Mg^{2+}	Magnesium ions
$MgCl_2$	Magnesium chloride
min	Minute
N	Nitrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
NIL	Near isogenic line
P	Phosphorus
PCR	Polymerase chain reaction

QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal deoxynucleic acid
RFLP	Restriction fragment length polymorphism
RIL	Recombinant inbred line
RGA	Resistance gene analogues
SCAR	Sequence characterized amplified region
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
sec	Second
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat
STS	Sequence tagged site
T	Thymine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Xap	*Xanthomonas axonopodis* pv. *phaseoli*
Xapf	*Xanthomonas axonopodis* pv. *phaseoli* var *fuscans*
X-Gal	5-bromo-4-chloro-3-indoyl-ß-D-galactoside

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CHAPTER 1

INTRODUCTION

Dry beans (*Phaseolus vulgaris* L.) are an important source of protein, B-complex vitamins and minerals (Paradez-Lopez *et al.*, 1986) and form the staple food in the basic diet of many Latin-American countries (De Leon *et al.*, 1992). In Africa, beans are the second most important source of protein after groundnuts (Technology Impact, 1997-1998) and production amounts to 2 049 000 tons, of which 373 000 t is produced in Uganda, 332 000 t in Ethiopia, 309 000 t in Angola and 217 500 t in Tanzania. The mean production in South Africa for the last nine years is given as 74 542 t (Technology Impact, 1997-1998).

In South Africa, beans are produced commercially in Mpumalanga (56%), Free State (28%), North West (7%), Kwa-Zulu Natal (5%) and Northern Cape (4%) provinces. The major areas for small-scale farmer bean production are Mpumalanga, Eastern Cape and Kwa-Zulu Natal (Fourie, 2002a).

Diseases are one of the most important factors associated with low bean yields in many bean-producing countries (Beebe and Pastor-Corrales, 1991). Some 200 pathogens are known to attack the common bean but fewer than a dozen cause substantial economic damage. Diseases like anthracnose, angular leaf spot, rust, common bacterial blight (CBB), bean common mosaic virus and bean golden mosaic virus are very widespread and can decrease seed yield considerably. One or more of these diseases is almost always associated with the bean crop wherever it is grown. Other bean diseases can also cause significant crop loss, but they tend to be confined to specific environments (Allen *et al.*, 1998).

Common bacterial blight (CBB) is caused by *Xanthomonas axonopodis* pv. *phaseoli* (Xap) and its fuscans variant *Xanthomonas axonopodis* pv. *phaseoli* var *fuscans* (Xapf). It is a major disease that limits dry bean production in South Africa (Technology Impact, 1997-1998) and ranks near the top of the list of bean production problems throughout most of the world (CIAT, 1985). Common bacterial blight was reported in South Africa prior to 1931 (Doidge and Bottomley, 1931) while fuscous blight was first reported in 1962 (Boelema, 1967).

The most reliable and effective control strategy for bacterial diseases is the use of resistant cultivars (Rands and Brotherton, 1925). High levels of cultivar resistance would minimise yield losses, reduce bactericide use and production costs, and facilitate integrated disease and crop management and the production and distribution of pathogen-free seed (Singh and Miklas, 2002). Molecular marker technology can be used to build capacity in finding and mapping marker genes for dry bean characteristics, which are of importance to dry bean production yields in Africa. Marker technology can complement conventional breeding programmes by confirming phenotypic evaluation on genetic level and is essential in gene pyramiding which can not be done on phenotypic level alone. It will aid in accelerated production of resistant cultivars, which can reach the producer much faster. This will be further elucidated in the following chapter.

All locally grown commercial cultivars are susceptible to CBB and thus improvement of cultivars by introducing stable resistance is important. Two resistance sources, GN Nebr.#1 sel 27 and XAN 159 each contribute 2 independent quantitative trait loci (QTL) with major effects on common blight resistance. Sequence characterized amplified region (SCAR) markers linked to these four QTL are available for DNA marker-assisted breeding (Miklas *et al.*, 2000). An additional resistance source, Wilk 2, has shown superior resistance to XAN 159 in greenhouse testing under South African conditions (Fourie, 2002). The aims of the study are to:

- Determine if Wilk 2 contributes any additional resistance QTL to CBB resistance and if so,
- Develop flanking markers for the QTL.

Development of suitable markers will assist in the improvement of resistance in Teebus, a commercially important local navy bean that is highly susceptible to CBB.

CHAPTER 2

LITERATURE REVIEW

The genus *Phaseolus* contains the common bean (*Phaselous vulgaris* L.). It is a staple food in many countries. Bacterial diseases are a serious concern as they cause a serious yield loss. CBB is found in 83% of seed production areas and 79% of the commercial areas of South Africa (Fourie, 2002b). The most effective to control diseases is the use of resistant cultivars. MAS help improve selection efficiency for resistant cultivars. There are various DNA technologies available for MAS. The use of PCR-based markers reduces time and labour costs by eliminating the need for phenotypic evaluations.

2.1 Common Bean (*Phaseolus vulgaris* L.)

The genus *Phaseolus* is made up of over 50 herbaceous species that ranges in type from annual to perennial, prostate to erect, climbing to bush. The four *Phaseolus* spp. cultivated in the New World are *P. vulgaris*, (common, field, green, snap and wax bean), *P. lunatus* L. (small- and large seeded lima bean), *P. coccineus* (scarlet runner bean), and *P. acutifolius* A. Gray (tepary bean) (Hall, 1991) of which *P. vulgaris, P. coccineus* and *P. acutifolius* are agronomically important in South Africa (Liebenberg and van Wyk, 1999). *P. vulgaris* is the most widely grown of the four species. It is cultivated extensively in south and central America and is also grown in north America, eastern Africa, eastern Asia, and western and southeastern Europe (Schwartz and Pastor-Corrales, 1989).

The common bean is a diploid organism (n=11) with relatively small chromosomes (Zheng *et al.*, 1991) and a small genome estimated to be 637 Mbp or 0.66 pg/1C (Arumuganathan and Earle, 1991). It has been estimated, via DNA reassociation kinetics, that 60% of the genome is comprised of single copy sequences (Talbot *et al.*, 1984). The chromosome number and genome size of *P.vulgaris* are very similar to those of *P. acutifolius* and *P. coccineus* (Arumuganathan and Earle, 1991), both of which are partially compatible with the common bean and represent an important source of germplasm for plant improvement (Hucl and Scoles, 1985). A rudimentary linkage map was developed through the years with mostly morphological markers and a few isozymes

(Basset, 1991; Vallejos and Chase, 1991a,b). Freyre *et al.* (1998) integrated three genetic linkage maps (Vallejos *et al.*, 1992; Nodari *et al.*, 1993a; Adam-Blondon *et al.*, 1994) resulting in a core map that has a higher density of markers and an expanded length. Electrophoretic analysis of the major seed storage protein (phaseolin) and a group of isozymes has led to the identification of a Mesoamerican and an Andean gene pool (Gepts *et al.*, 1986; Koenig and Gepts, 1989). DNA restriction fragment length polymorphisms (RFLP) shows that DNA probes can be used to differentiate between the two groups, but low levels of polymorphisms were detected within each gene pool, with moderate levels found between the gene pools (Chase *et al.*, 1991).

P. vulgaris is the most important food legume for direct human consumption in the world. It is one of the leading sources of protein and an important source of calories for many of the poorest continents (Schwartz and Pastor-Corrales, 1989). Research in the medical field has shown that a dry bean diet can counter and even control certain coronary diseases, colon cancer and diabetes (Liebenberg and van Wyk, 1999). Production occurs in a wide range of cropping systems and environments spanning regions as diverse as Latin America, Africa, the Middle East, China, Europe, the United States, and Canada. Latin America is the leading bean producer and consumer and beans are a traditional and very important food for the lower income strata. The highest per capita consumption in the world occurs in eastern Africa. Beans are an important source of dietary protein in Kenya, Tanzania, Malawi, Uganda and Zambia (Schwartz and Pastor-Corrales, 1989).

2.1.1 Common beans in Africa

P. vulgaris is an ancient New World domesticate. They reached Africa from Brazil with the slave trade. It became established as a food crop in Africa before the colonial era. The total annual production in Africa is estimated at two million tons of dry seed, which is about 25% of the world production (Schwartz and Pastor-Corrales, 1989).

Beans in Latin America and Africa are primarily a small farmer crop, grown with few purchased inputs, and besieged by an array of biological, edaphic, and climatic problems, making beans notoriously low in yield. In tropical bean production regions, diseases, insect pests and low soil fertility are the most important production constraints (Schwartz and Pastor-Corrales, 1989).

The common bean is adapted to temperate and cool tropical climates. In Africa, production is concentrated in the cool highlands of central and tropical eastern Africa where beans are the most important pulse crop. Production can be severely constrained by soil infertility, including acidity. Mean growth temperature ranges from 16 to 24°C. Annual precipitation is in the range of 500–2 000 mm. Average annual rainfall varies substantially with location and, in some places can be markedly variable from year to year. The wide variability of production environments results in a wealth of diversity in cropping systems as well as in agronomic constraints to bean production (Schwartz and Pastor-Corrales, 1989).

A continuous effort is being made to obtain higher productions per unit area in order to increase profitability and to meet the ever-increasing demand for food, and especially protein. The optimal profitability, production, marketing and consumption of dry beans can only be accomplished by means of proper and purposeful research. Proper planning and management and the application of the latest technology and aids can contribute further to promote opportunities in the dry bean industry (Liebenberg and van Wyk, 1999).

2.1.2 Crop Production Systems

Beans are produced in a wide range of production systems in Africa. Large-scale monoculture production of navy beans for canning and export still occurs in some areas, but this industry has collapsed in northern Tanzania, Uganda and Ethiopia (Schwartz and Pastor-Corrales, 1989).

In the Great Lakes Region of central Africa, beans are grown primarily for home consumption and usually in association with other crops. The crop most commonly associated with beans is maize, although the bean-banana-coffee association predominates in some areas. Other companion crops include sweet potatoes, peas, cassava, yams, cocoyams, potatoes, and peanuts (groundnuts). In the coffee-growing areas of north Kivu, Zaire, coffee is always associated with beans. In Rwanda, 60% of bean production is estimated as being in association with banana. The banana association plays an important role in reducing drought stress for the associated bean crop and thus improves the stability of the system. Associated cropping offers several

advantages to the small farmer: it enables greater productivity where land is restricted; it decreases the risk of complete crop failure, and often decreases disease severity. (Schwartz and Pastor-Corrales, 1989).

The main production constraints reported are poor agronomic practices, soil infertility, lack of improved cultivars, moisture stress, weed competition, and damage caused by insects and diseases (Schwartz and Pastor-Corrales, 1989). The poorer the farmer and the less fertile the soil, the more important yield stability becomes. His decision to grow beans in complex associations and often in varietal mixtures therefore stems from the need to maximize stability of performance rather than productivity per se. Significant production constraints that farmers consider include insect attack, drought, excess rain and associated diseases, low soil fertility and insufficient compost and manure, and lack of land. The two most limiting factors have been shown to be soil fertility and diseases. A clear negative reaction between soil fertility and disease is often found. Gains through increasing soil fertility are offset by losses from increasing disease pressure if diseases are not controlled. If a farmer is forced by economic or labor considerations to choose between increasing soil fertility or controlling diseases, the latter is more likely to bring about significant yield increases (Graf and Trutmann, 1987).

Successful crop production is ensured by the development of applicable technology and its effective transfer. The most important resource of agriculture is the successful implementation of developed technology. The development of new technology presupposes the availability of know-how, equipment, funds and above all a well-structured programme. The successful transfer of technology is assured if the producer is innovative and progressive. Most of the above elements are present in the South African dry bean industry, and production is therefore stable and the industry is growing. It is important to breed new cultivars that are agronomically more acceptable, offer greater resistance to diseases, can be cultivated successfully in different production areas and can produce a better yield (Liebenberg and van Wyk, 1999).

2.1.3 Disease as a production constraint

In most tropical bean production regions, diseases are often the most important constraint to bean production. More plant pathogens, greater pathogenic variation, and more virulent isolates of these pathogens are found attacking beans in Latin America and Africa than in temperate regions. The prevalence and importance of each disease varies considerably with locality, season, year and cultivar; however, some pathogens such as those that cause anthracnose, angular leaf spot, common bacterial blight, and bean common mosaic virus, are widespread and economically important (Schwartz and Pastor-Corrales, 1989).

A plant is diseased when it is not functioning normally. Disease is the result of an interaction among the plant, its environment, and one or more harmful factors in the environment. These harmful agents may be infectious organisms or infectious agents that can reproduce only in the living plant. Abiotic agents, such as toxic chemicals, nutrient deficiencies, drought, and heat, also cause plant diseases. Biotic and abiotic agents that cause disease are called pathogens. The visible indications of distress shown by diseased plants are called symptoms and may include yellowing (chlorosis) of leaves, discolorations, dead spots or patches, wilting, stunting, malformations, and numerous other irregularities. The abnormal functioning of the plant generally leads to reductions in quality and quantity of yield. Parts of the pathogen seen on diseased plants are called signs of the disease. Symptoms and signs are useful in determining the cause of a disease. Accurate disease diagnosis is critical to developing and recommending effective disease control procedures (Hall, 1991).

There are various fungal, viral and bacterial diseases of bean in Africa. The most important virus pathogen in Africa is the bean common mosaic virus (BCMV) and its necrotic strains are common and damaging. Among the bacterial diseases, bacterial wilt, bacterial brown spot, CBB and halo blight are widespread and important. The major fungal diseases of beans in Africa, as in Latin America, are angular leaf spot, anthracnose and rust (Schwartz and Pastor-Corrales, 1989).

Some diseases, such as anthracnose, bean common mosaic virus, CBB, and white mold, can cause extensive or complete crop failure and are important throughout the bean

production areas of the world. Annual production losses in world bean production as a result of diseases average about 10%. It is apparent that losses in bean production would be much higher in the absence of disease control practices (Hall, 1991).

2.2 Common Bacterial Blight (CBB)

2.2.1 Aetiology and Biology

The genus *Xanthomonas* is a phytopathogenic bacterium and consists of 5 species, each currently subdivided into a number of pathovars. CBB is caused by the bacterium Xap and its brown pigmented variant Xapf. The two bacteria cause similar symptoms and may occur together on the same plant (Allen *et al.*, 1998).

Both bacteria are typical xanthomonads. They are Gram-negative, do not form spores, are in the shape of rods measuring 0.4 x 1.0 μm and possess a single polar flagellum. Xap produces yellow-pigmented, smooth, convex and mucoid colonies on nutrient glucose agar (Dye *et al.*, 1980). The yellow pigment is called xanthomonadin and is due to an extracelluar polysaccharide slime called xanthan (Starr *et al.*, 1977). Xap and Xapf differ in genome size but both have the same host range and exhibit similar symptoms (Singh and Miklas, 2002). Xapf produces a diffusible brown pigment on media containing tyrosine (Dye *et al.,* 1980), which distinguishes it from Xap.

CBB enters the plant passively through wounds or natural openings such as stomata and hydathodes (Zaumeyer and Thomas, 1957) and multiply in intercellular spaces dissolving the middle lamella. Bacteria may block the vascular elements of the leaves and stems causing their disintegration and plant wilting (Santana, 1985) but do not infect the plant systemically (Haas, 1972). Macroscopically visible water-soaked spots become necrotic, enlarge and may coalesce. Bacteria exudates may appear on the surface of infected tissues and are disseminated to initiate secondary infection (Allen *et al.*, 1998).

The host range of Xap includes common bean (*Phaseolus vulgaris* L.), scarlet runner bean (*P. coccineus* L.), *P. lunatus*, urd bean (*Vigna mungo* (L.) Hepper), mung bean (*V. radiata* (L.) Wilczek var. *radiata*), tepary bean (*P. acutifolius* A. Gray var. *acutifolius*), *V. aconitifolia* (Jacq.) Marechal, *V. angularis* (Wild.) Ohwi *et* Ohasi, *V. umbellate*, *Lablab*

purpureus (L.) Sweet, *Strophostyles helvola* (L.) Elliot, soybean (*Glycine max* (L.) Merril, *Mucuna deeringiana* (Bort.) Merrill, *Lupinus polyphyllus* Lindl., cowpea (*V. unguiculata* (L.) Walp. ssp. *unguiculata*), *Macroptilum lathyroides*, *Pisum sativum*, *Strophostyles helvola* and *Mucuna deeringiana* (Seattler, 1989; Allen *et al.*, 1998).

Variation in pathogenicity of Xap isolates has been shown within and among geographic regions (Schuster *et al.*, 1973; Valladares-Sanchez *et al.*, 1979). There is evidence that the interaction between Xap and *P. vulgaris* is quantitative in nature (Allen *et al.*, 1998). Phage typing and plasmid profile analysis have proved reliable in distinguishing Xap isolates while bacteriocin typing and polyacrylamide gel electrophoresis appear unreliable (Sutton and Wallen, 1970; Fujimoto, 1985). Restriction fragment length polymorphism (RFLP) appears promising for the study of population structures and variability of Xap and Xapf. Initial studies indicate that isolates from different geographic regions form clonal groups (Gilbertson *et al.*, 1987; Lazo *et al.*, 1987). Xap and Xapf isolates of the same geographic origin were shown to show similar RFLP patterns but Andean and Mesoamerican groups do not occur (CIAT, 1992; Otoya and Pastor-Corrales, 1992; Otoya *et al.*, 1994).

Ten Xap and Xapf isolates were subjected to random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphic (AFLP) techniques to differentiate isolate variation at subgeneric level. It was found that a high degree of DNA polymorphism exists among isolates with a low presence of shared fragments between isolates. Similar results were obtained with the different DNA techniques, i.e. the Xap and Xapf isolates were clustered into 2 separate groups demonstrating that these are two distinct groups of bacteria (Fourie, 2002a).

2.2.2 Symptoms

CBB symptoms occur on leaves, pods and stems. The initial symptoms on leaves appear as water-soaked spots on the lower surface. As the spots enlarge the centers become necrotic. The lesions enlarge irregularly, coalesce and become surrounded by narrow chlorotic zones, which turn brown (Figure 2.1). On susceptible cultivars, necrosis may be extensive and wind and rain may cause premature defoliation (Zaumeyer and Thomas, 1957).

On pods, symptoms first appear as small water-soaked spots, which enlarge, turn dark reddish-brown and may become slightly sunken. Under humid conditions, a yellow slimy exudate may be produced, forming a yellow crust when dry. Heavy infection during seed and pod development may cause pods and seeds to shrivel. Bacteria occur on or inside seeds, on which they are sometimes symptomless (Weller and Seattler, 1980; Aggour, 1988; Seattler, 1989). Severely infected, pale-colored seeds bear yellow or butter-colored lesions. Seeds may be wrinkled and spotted when less severely infected (Allen *et al.*, 1998).

On the stem of seedlings, symptoms often arise from seedborne infection, resulting in the destruction of growing tips and primary leaves (Zaumeyer and Thomas, 1957). On older stems, lesions initially appear as water-soaked spots. They enlarge, become reddish-brown in color and may extend up the stem (Santana, 1985) or girdle it if infection is at a node (Burkholder, 1921; Zaumeyer and Thomas, 1957). The stem is weakened and may break in windy conditions (Allen *et al.*, 1998).

2.2.3 Epidemiology

Xap survives between seasons on contaminated seed (Weller and Seattler, 1980). The bacterium can be either borne externally or as an internal infection, retaining viability for as long as 36 years (Seattler *et al.*, 1986; Seattler, 1989). Under certain conditions, the pathogen can survive in infested soil and plant debris (Gilbertson *et al.*, 1990; Opio *et al.*, 1994), with periods of survival of 3 to 18 months depending on the environment.

The CBB pathogen can also survive on weeds and non-host plants (Cafati and Seattler, 1980; Ramos, 1988; Opio *et al.*, 1992a). Seedborne inoculum constitutes the means of international distribution of Xap (Schuster and Coyne, 1975) as well as the primary source of primary infection of susceptible common bean cultivars. The minimum population in seed that can initiate field infection is estimated at 10^2 colony-forming units; 0.2% seed infection can lead to epidemic development (Opio *et al.*, 1993).

The number of infection loci, the presence of vectors, the crop growth stage, environmental conditions and cultural practices influence disease development and

secondary spread. Plant susceptibility to infection generally increases from flowering to pod filling (Coyne and Schuster, 1974b), then decreases as the crop matures (Santana, 1985). Environmental conditions that favour disease spread include warm temperatures, high relative humidity, rainfall, wind and windborne soil and irrigation water (Sutton and Wallen, 1970; Clafin *et al.*, 1973; Steadman *et al.*, 1975; Seattler, 1989). Damage is greatest at temperatures of about 28°C and *in vitro* growth is most rapid in the range of 28-32°C (Patel and Walker, 1963; Mack and Wallen, 1974; Santana, 1985). Photoperiod also influences CBB severity, with damage being greater in short days; this may in part account for observed environmental effects on plant susceptibility (Coyne *et al.*, 1973; Santana, 1985). Cultural practices including intercropping with maize can also influence the rate of spread of CBB (van Rheenen *et al.*, 1981).

2.2.4 Crop loss

Xap and Xapf both occur together on the plant, therefore the plant damage and yield loss caused by either bacterium cannot be ascertained. Natural infection has been shown to cause yield losses of 38% in Canada (Sutton and Wallen, 1970). An outbreak in 1976 of common blight in Michigan affected 75% of the bean crop, causing an estimated yield loss of 10-20% (Seattler, 1989). Work in Uganda estimates that for every 1% increase in the incidence of CBB during reproductive growth there is a yield loss of 3.5-11.5% kg ha^{-1}, depending on the season (Opio *et al.*, 1992b).

Greater damage occurs with early plant infection due to premature defoliation, which reduces photosynthetic area, interferes with translocation of water and nutrients and reduces seed numbers and size. Lesions on pods and seeds reduce quality (Allen *et al.*, 1998). Figure 2.2 shows the different effects that CBB can have on a resistant and susceptible cultivar.

Fourie (2002b) conducted a disease survey in 1995-98 in seed production fields and commercial dry bean producing areas to determine incidence, severity and spread of bacterial diseases in South Africa. CBB was found to occur in 83% of seed production areas and 79% of commercial fields. Incidence of bacterial diseases was found to be high although the severity was generally low. Xapf was found to be more widespread than Xap in both seed production and commercial fields.

2.2.5 Disease Management

2.2.5.1 Current practices

Cultural methods are important in the control of CBB. Due to the importance of seedborne infection as a primary source of inoculum, use of clean seed is a potentially effective control measure where applicable (Weller and Seattler, 1980; Webster *et al.*, 1983). Chemical control of diseases in beans is by copper-based bactericides but is almost nonexistent because of the scarcity of agrochemicals, limited access to equipment with which to apply pesticides, and the meager capital available to smallholders for buying them. Chemical control is only feasible under special conditions such as seed multiplication or a component of integrated control strategy (Allen *et al.*, 1998). However, current cultural practices adopted by many bean farmers do seem to limit disease spread and severity. Traditional practices such as shifting cultivars, with its intervening periods of bush fallow, the burial of crop debris in mounds, and the cultivation of crop mixtures, provide some measurement of disease management. Studies by CIAT (1986 and 1987) have shown that rouging of diseased seedlings and removal of diseased basal leaves at weeding can decrease disease incidence. The chosen time of sowing and plant population may aid in escape from disease.

Various studies on the effect of crop association on disease severity have shown that diseases of beans are usually, but not invariably, less severe in a maize intercrop system (Msuku and Edje, 1982; van Rheenen *et al.*, 1981).

2.2.5.2 Host plant resistance

Particularly for small scale farmers, where other measures may have practical limitations, host plant resistance appears the most suitable control measure. Efforts have yielded only moderate levels of resistance with immunity to the disease having not yet been found in *P. vulgaris*. Most of the moderate resistance lines are of Andean origin. Resistant lines like Great Northern Nebraska No 1 selection 27 and PI 207262, bred in temperate regions (Coyne and Schuster, 1976), have been used to improve foliar resistance in the tropics. Poor adaptability and instability of seed color of these lines and their progenies, however have limited their use (Beebe and Pastor-Corrales, 1991). High

levels of resistance have been transferred from the tepary bean, *P. acutifolius*, (Schuster, 1955; Coyne and Schuster, 1974a) to *P. vulgaris* (Honma, 1956; McElroy, 1985). Near-immune self-fertile lines, compatible with *P. vulgaris* have been obtained from some crosses (McElroy, 1985) and resistant lines with different growth habits and grain types have been identified, such as XAN 159, XAN 160 AND XAN 161 (Beebe and Pastor-Corrales, 1991).

The ultimate aim is the development of safe, economic and durable disease control strategies for all farm situations. This will probably be achievable only through a combination of measures in an integrated control system including cultural practices, crop and varietal mixtures and chemicals as well as host-plant resistance (Allen *et al.*, 1998).

2.3 DNA based marker techniques

There are three marker types: morphological (traits), biochemical (seed storage proteins and isozymes), and molecular or DNA-based polymorphisms, that can be used to establish linkages with traits of economic importance. Polymerase chain reaction (PCR) based molecular markers have the greatest application for marker-assisted selection (MAS) (Kelly and Miklas, 1999).

The polymerase chain reaction is the basis for most DNA based marker technology (Stalker and Mozingo, 2001). PCR technologies are based on annealing two oligonucleotide primers that flank a target region to the duplex of genomic DNA (Erich *et al.*, 1991). The reaction is set up such that, as the DNA unwinds, each primer hybridizes to its complementary DNA sequence on each strand, and then the DNA polymerase is used to extend the primer on each strand. The cycle of denaturation, primer hybridization, and DNA synthesis is used to exponentially multiply the number of copies of the target sequence. The advantage of PCR is that it requires only very small amounts of DNA and allows the DNA from a single extraction to be screened for hundreds of marker loci (Stalker and Mozingo, 2001).

Many PCR-based genetic markers are available. These markers have been identified by either specific primers determined from known DNA sequences (e.g. allele-specific

primers and primers flanking minisatellites and microsatellites) or by arbitrary primers. The widespread use of these markers is limited as they rely on predetermined variation or on the genomic distribution and organization of tandem repeats. Microsatellites have been used successfully in groundnuts and beans to assess genetic diversity between genotypes (Hopkins *et al.*, 1999, Gaitán-Solís *et al.*, 2002).

Due to the advent of a wide range of analytical tools for DNA analysis, there are various types of molecular markers available to plant breeders, geneticists, and germplasm scientists. Co-dominant restriction fragment length polymorphisms (RFLPs) are abundant in most crops and valuable as genetic markers. Dominant RAPD markers proved easier to generate (Williams *et al.*, 1990) but were less reproducible across laboratories and were less informative as markers in segregating populations. Newer markers have been identified, such as AFLPs (amplified fragment length polymorphisms) that combine RFLP and RAPD technologies (Vos *et al.*, 1995), and microsatellite or simple sequence repeats (SSRs). These newer markers have gained popularity in higher plants through comparative mapping and DNA fingerprinting applications (Mohan *et al.*, 1997; Staub *et al.*, 1996). Scientists saw the potential for these highly abundant markers and used them extensively to expand the genetic linkage maps for most commodities. Many of these maps had previously been limited to either morphological traits or protein markers (Bassett, 1991; Gepts, 1988). Maps proved valuable in positioning traits in relation to each other but were limited in the traits segregating in the mapping population (Kelly and Miklas, 1999).

2.3.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP represents the first marker system that had a large number of polymorphisms and is widely used both to create linkage maps and to implement indirect selection strategies. They can be used to study both recessive genes and multiple alleles (Stalker and Mozingo, 2001).

RFLPs are produced by digesting DNA with restriction endonucleases that recognize a specific DNA sequence and then cleave the DNA strand in or near the sequence. Fragments thus produced can be separated on size differences by gel electrophoresis. Plants often produce so many fragments that the resulting gel is not interpretable. For

complex genomes, a probe is made from cloned DNA that is homologous to a specific DNA sequence in the species being investigated. Radioactivity is used to label probes and bands are visualized when the unhybridized radioactivity is washed away and then an autoradiograph is produced. Sequences as rare as one in a million can be detected and any unique DNA sequence can be used as a probe as long as it binds with some part of the digested DNA fragments. RFLPs can be used to tag genes, and can be used in the selection process in a plant-breeding programme, due to the reduced time needed to screen large populations in segregating generations (Stalker and Mozingo, 2001).

Since RFLPs were more informative due to the expression of co-dominance in the early generation populations, they became the marker of choice among geneticists interested in genetic mapping. The application to breeders was restricted by the costly and sophisticated techniques required in the generation of RFLPs. Germplasm specialists used RFLPs to assist in the classification of related and wild germplasm, but costs have limited their extensive deployment. In certain instances, these maps are being used as a basis for the cloning of specific genes into crops for which an efficient regeneration and transformation system is available (Kelly and Miklas, 1999).

RFLPs have been used to construct genetic maps in several crop species (O'Brien 1990). RFLPs can be used to initiate chromosome walks to clone genes in complex genomes (Gessler *et al.*, 1990; Rommens *et al.*, 1989). The use of RFLP analysis is restricted due to it being laborious and time-consuming but is a valuable method to detect and tag genes. Therefore methods are required for rapidly obtaining markers linked to resistance genes for genetic analysis and for physically characterizing the region (Paran and Michelmore, 1993).

2.3.2 Random Amplified Polymorphic DNA (RAPD)

Williams *et al.* (1990) described a DNA polymorphism assay based on the amplification of segments of genomic DNA using single primers of arbitrary sequence. PCR with arbitrary primers detects change in the DNA sequence at sites in the genome, which are defined by the primer used. Sequence variation is revealed by the number and length of amplified products, which may be phylogenetically conserved or individual-specific.

RAPD polymorphisms could be caused by differences in nucleotide sequence at the priming sites or by structural rearrangements within the amplified sequence. RAPD amplification can likely be initiated from genomic sites that do not perfectly match the primer sequence. Sequencing the products of RAPD analysis, however, does not provide information on mismatches within the RAPD primer sequence because, after a few cycles, the majority of templates will be identical in sequence to the primer (Paran and Michelmore, 1993).

RAPDs offer a fast, efficient method for generating scorable DNA polymorphisms from a wide range of species. It is useful in genotype identification, population and pedigree analyses, phylogenetic studies, and genetic mapping (Halward *et al.*, 1992). Using arbitrary primers in PCR has the following advantages for detecting polymorphism: (a) a universal set of primers can be used and applied to a wide range of species; (b) no extensive preparations are required, such as isolation of cloned DNA probes, Southern blots, or nucleotide sequencing and (c) the nature of primers allows for greater information transfer and collaboration among research laboratories. PCR methods also require significantly lower amounts of genomic DNA as compared to Southern blotting in conventional RFLP analysis (Williams *et al.,* 1990).

RAPD markers allow the quick construction of genetic maps for any plant species or the saturation of specific genomic regions with molecular markers (Martin *et al.*, 1991; Paran *et al.*, 1991; Michelmore *et al.*, 1991). Each amplification often detects five to ten RAPD loci. RAPDs are one of the most widespread uses of PCR. Procedures are technically easy and rapid to perform as compared to RFLP methods. RAPD analysis is simple, fast, does not involve the use of radioactive isotopes, and can be scaled up to analyze large numbers of samples. In order to utilize markers identified by RAPD analysis for map-based cloning, a single locus must be identified unequivocally. The method is very sensitive to DNA polymorphisms, but the disadvantage is that only dominant markers are detected (Stalker and Mozingo, 2001). RAPD amplified products often contain repetitive DNA sequences and can therefore not be used as hybridization probes. In addition, the RAPD technique is sensitive to changes in the reaction conditions (Paran and Michelmore, 1993).

RAPD markers proved to be cheaper but more variable technology with regard to the problems of reproducibility between laboratories (Jones *et al.*, 1997; Weeden *et al.*, 1992). Variability has been attributed to differences in PCR protocols, equipment, and the type of heat labile polymerase, all of which can be theoretically controlled. In practice, however, variability between laboratories cannot be controlled due to differences in equipment, which affects portability of RAPDs. Breeders saw the advantage of RAPD markers as a rapid, cost-effective tool for the indirect selection of traits once linkages between the marker and trait have been identified. As an extension of single gene tagging, linkages between RAPD markers and quantitative trait loci (QTL) controlling complex traits such as yield, stress tolerance (Schneider *et al.*, 1997), and canning quality (Walters *et al.*, 1997) have also been identified.

2.3.3 Amplified Fragment Length Polymorphism (AFLP)

AFLPs are based on PCR-based techniques that combine the benefits of RFLPs and RAPDs. The technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The principle of the AFLP technique is the amplification of subsets of genomic restriction fragments using PCR (Vos *et al.,* 1995). There are four steps in the AFLP technique: DNA digestion, ligation, amplification and gel analysis. Genomic DNA is first digested by two restriction enzymes. Double-stranded oligonucleotide adapters, homologous to either 5'- or 3'-end generated during restriction digestion, are ligated to the DNA fragments. The ligated DNA fragments are amplified by PCR using primers complementary to the adapter and restriction site sequence with additional selective nucleotides at their 3'-end. The use of selective primers reduces the complexity of the mixture. Only those fragments with complementary nucleotides extending beyond the restriction site will be amplified by the selective primers under the stringent conditions. Figure 2.3 shows the steps involved in the AFLP technique. Polymorphisms are revealed by analysis of amplified fragments on a denaturing polyacrylamide gel, and comparison of the patterns generated for each sample (Blears *et al.*, 1998).

The AFLP technique can be used for DNAs of any origin or complexity. Fingerprints are produced without any prior sequence knowledge using a limited set of generic primers. The number of fragments detected in a single reaction can be "tuned" by selection of specific primer sets. The AFLP technique is robust and reliable because stringent reaction conditions are used for primer annealing: the reliability of the RFLP technique is combined with the power of the PCR technique (Blears *et al.*, 1998).

The capacity to reveal many polymorphic bands in one reaction is a major advantage of AFLP markers. The numerous bands on a gel are analyzed simultaneously making AFLP an extremely efficient technique. AFLP has the capacity to screen a much greater number of loci for polymorphism than other available PCR-based techniques, such that the number of polymorphisms detected per reaction is much higher. AFLP is superior in terms of the number of sequences amplified per reaction and their reproducibility. The markers produced are reliable and reproducible within and between laboratories, and are relatively easy and inexpensive to generate. A virtually unlimited number of markers can be generated by simply varying the restriction enzymes, and the nature and number of selective nucleotides (Blears *et al.*, 1998).

AFLP is a DNA fingerprinting technique that detects genomic restriction fragments and resembles in that respect the RFLP technique, with the major difference that PCR amplification instead of Southern hybridization is used for detection of the restricted fragments (Vos *et al.*, 1995). Due to the nature of the RFLP technique, only the restriction site is scanned for differences in DNA sequence. The selective nucleotides included in AFLP provide additional possibilities for polymorphisms to be detected beyond the restriction site itself. AFLP has the capacity to detect more point mutations than RFLPs. In a single hybridization experiment, RFLP can detect, at most, a few genetic loci compared to the 100-200 loci detected by AFLP. In addition to a greater number of polymorphisms per reaction, AFLP is also superior in terms of efficiency, as it does not require template DNA sequencing. Fingerprints are produced without prior knowledge of DNA sequences (Blears *et al.*, 1998).

The resemblance with the RFLP technique was the basis to choose the name AFLP. The name AFLP should not be used as an acronym, because the technique displays the absence or presence of restriction fragments rather than length differences. Other fingerprinting techniques, e.g. RAPDs, DNA amplified fingerprinting (DAF) and Arbitrarily

primed PCR (AP-PCR) are very sensitive to reaction conditions, DNA quality and PCR temperature profiles and this therefore limits their application (Vos *et al.*, 1995).

RAPD is also a PCR-based technique similar to AFLP. However, AFLP uses primers specific to the adapter and restriction site sequence, whereas RAPD uses arbitrary primers. RAPD analysis is easier to perform than AFLP but the RAPD technique is very sensitive to reaction conditions, template DNA concentration and purity, and PCR temperature profiles, limiting its application. AFLP analysis uses stringent annealing conditions, which guarantee a better reproducibility (Folkertsma *et al.*, 1996).

The AFLP technique is not simply a fingerprinting technique but is an enabling technology in genome research, because it can bridge the gap between genetic and physical maps. The AFLP technique is a very effective tool to reveal restriction fragment polymorphisms. These fragment polymorphisms, i.e. AFLP markers can be used to construct high-density genetic maps of genomes or genome segments. In most organisms AFLP will prove to be the most effective way to construct genetic DNA marker maps compared to other existing marker technologies. AFLPs are amendable to high throughput marker generation and have gained popularity in mapping and germplasm comparison studies (Vos *et al.*, 1995).

The AFLP technique is powerful and reliable in identifying markers closely linked to genes of interest, but has some disadvantages for use in MAS and map based cloning. Limitations to the large scale, locus-specific application of AFLPs includes their dominant type of inheritance, the intensity of labor involved, and the high costs. Hence, conversion of AFLP markers into sequence specific PCR markers (eg. SCAR markers) is required for screening large breeding populations at low costs (Dussle *et al.,* 2002).

The AFLP technique was chosen for this study as it is reliable and is highly reproducible across laboratories. It also detects a higher number of polymorphisms in one reaction compared to the other DNA based techniques. No prior knowledge of sequence of the organism needs to be known before the technique is attempted.

2.3.4 Sequence Characterized Amplified Region (SCAR)

To add greater specificity to PCR techniques, Paran and Michelmore (1993) developed SCARs from PCR markers. SCARs are PCR-based markers that represent single, genetically defined loci that are identified by PCR amplification of genomic DNA with pairs of specific oligonucleotide primers; they may contain high-copy, dispersed genomic sequences within the amplified region. Polymorphism may be either retained as the presence or absence of amplification of the band or as length polymorphisms that converted dominant RAPD/AFLP loci into codominant SCAR markers. There is a gap between the ability to obtain linked markers to a gene of interest in a short time and the use of these markers for map-based cloning approaches and for routine screening procedures. To address these problems, SCARs were developed as PCR-based genetic markers (Paran and Michelmore, 1993).

SCARs are superior to RAPDs because they are less sensitive to reaction conditions. They detect only a single locus and can be potentially converted to co-dominant genetic markers. Like RAPDs, SCARs do not require radioactivity, and dominant SCARs may be used as a quick plus/minus assay for a particular product (Stalker and Mozingo, 2001).

SCARs are similar to sequence-tagged sites (STS) that are characterized by a short single-copy DNA sequence that can be amplified by PCR from a genomic library or genomic DNA using specific oligonucleotide primers. SCARs are primarily defined genetically; therefore, they can be used not only as physical landmarks in the genome but also as genetic markers. SCARs can contain repetitive DNA sequences within the amplified fragment as they are analyzed by PCR only; their uniqueness is determined by the sequence and spacing of the primer sequences, rather than by hybridization. Regardless of the genomic copy number within the amplified fragment, SCARs can be useful genetic markers as long as they originate from single loci, i.e., only one amplification site exists in the genome (Paran and Michelmore, 1993).

The use of longer oligonucleotide primers for SCARs allows a robust and more reproducible assay than could be obtained with the short primers used for RAPD analysis. It facilitates the use of molecular markers for other applications such as marker-assisted selection and fingerprinting. SCARs can also be useful in molecular taxonomy,

since they may detect single loci that can be examined across species (Paran and Michelmore, 1993).

In order for a SCAR to be useful as a genetic marker or a physical landmark, it must be demonstrated that the primers identify only a single locus. When the SCAR primers amplify the same sized fragment from both parents, various methods can be tried to identify polymorphism: 1) increasing the annealing temperature may reveal different amounts of mismatch within a primer sequence, 2) polymorphisms can be searched for between more genetically diverged lines from which mapping populations have been derived, 3) single base differences between the two alleles might be identified by various methods e.g. enzyme restriction. SCARs may identify polymorphisms that are less accessible by other techniques. The small fragments amplified allow the detection of chromosomal changes such as small insertions and deletions (Paran and Michelmore, 1993).

SCARs can be readily applied to commercial breeding programmes, as they do not require the use of radioactive isotopes. Although the codominant SCARs are the most useful for genetic studies, the dominant SCARs may be ultimately more useful in breeding applications if a quick plus/minus assay can be developed to detect the product. This would eliminate the need for electrophoresis to resolve products as well as decreasing the cost and increasing the speed of analysis (Paran and Michelmore, 1993).

Sequence-specific PCR markers have been successfully developed by conversion of different marker types such as RFLPs, RAPDs and SSRs (Bradshaw *et al.,* 1994; Cheung *et al.,* 1997; Jung *et al.,* 1999). The conversion of AFLP markers into PCR-based markers has been accomplished for several species such as carrot (Bradeen and Simon, 1998), brassica (Negi *et al.,* 2000), asparagus (Reamon-Büttner and Jung., 2000), soybean (Meksem *et al.,* 2001, Mienie *et al.*, 2002), apple (Xu *et al.,* 2001), barley and wheat (Shan *et al.,* 1999). However the conversion of AFLP markers seems to be more difficult than the conversion of other marker types due to the loss of their sequence specificity after amplification of the AFLP-derived internal primers (Shan *et al.,* 1999). Hence, AFLP polymorphisms related to *Eco*RI or *Mse*I restriction site differences will not be reflected in primers from an internal sequence (Shan *et al.* 1999).

With a size of 500-1500 bp, RAPD fragments are easier to convert to either SCARs or cleaved amplified polymorphic sequence (CAPS) markers than AFLP markers (Barret *et al.,* 1998). Dussle *et al.* (2002) was able to convert two short AFLP bands (150-300 bp) into PCR-based insertion/deletion and cleaved amplified polymorphic sequence (CAPS) markers without using methods like inverse PCR or chromosome walking. Mienie *et al.* (2002) was able to convert two AFLP fragments into SCAR markers in soybean for the nematode *Meloidogyne javanica.*

SCARs were derived from 8 RAPD markers linked to disease resistance genes to downy mildew in lettuce (Paran and Michelmore, 1993). All pairs of SCAR primers resulted in the amplification of single major bands of the same size as the RAPD fragment cloned. The amplified fragment contained no obvious repeated sequences beyond the primer sequence. Five out of the 8 pairs of SCAR primers amplified an alternate allele from both parents of the mapping population; therefore, the original RAPD polymorphism was likely due to mismatch at the primer sites (Paran and Michelmore, 1993).

Bulk segregant analysis (BSA) was used in a previous study with AFLP to identify several markers closely linked to the sugarcane mosaic virus resistance genes *Scmv1* and *Scmv2* (Dussle *et al.*, 2000). In this study the AFLP bands were sequenced to convert them into more simple and reliable PCR-based STS markers. One dominant AFLP marker was converted into an insertion/deletion marker and a second AFLP marker into a CAPS marker. Mapping of both converted PCR-based markers confirmed their localization to the same chromosome region as the original AFLP markers. Thus, these markers would be useful for MAS and facilitate map-based cloning of SCMV resistance genes (Dussle *et al.,* 2002).

In mapping BAC clones of lettuce in order to analyze resistance gene clusters, Meyers *et al.* (1998) identified duplicates of AFLP markers in the same chromosome region. Genes conferring resistance to different pathogens are often clustered in the same chromosome region in the maize genome (McMullen and Simcox, 1995). The closer a marker is linked to a specific resistance gene, the higher the probability of being duplicated with the resistance gene during evolution. Taking into account that only 4 out of the 8 converted AFLP markers showed polymorphism between inbred lines, it seems very likely that extending the fragment size by inverse PCR would increase the number of polymorphic STS primers (Dussle *et al.,* 2002).

The application of simple STS markers enables a faster DNA analysis for a high number of individuals required for fine mapping. An agarose gel could separate the polymorphisms revealed by STS markers, where no silver staining/radioactivity is required to visualize the results. Compared to the analyses of AFLP markers in which polyacrylamide gels and silver staining/radioactivity is required, the application of STS markers can reduce costs to about 20% (Dussle *et al.*, 2002).

2.3.5 Bulk segregant analysis (BSA)

To facilitate the identification of linked markers, mapping populations and methods have been developed which include near isogenic lines and BSA (Michelmore *et al.*, 1991). The BSA technique makes use of plants from the extremes of the phenotypic spectrum of a segregating population by pooling the DNA from these plants in two bulks. Each bulk contains individuals that are identical for a particular trait or genomic region, but seemingly heterozygous for all other regions (Michelmore *et al.* 1991). The principle of DNA pooling is the grouping together of informative individuals in order to study a selectable marker linked to a particular gene of interest in a randomized genetic background of unlinked loci (Wang and Paterson, 1994). BSA does not reveal novel types of variation, but allows the rapid screening of many loci and therefore the identification of polymorphisms. BSA can be used to obtain a high-resolution map around a quantitative trait locus (Michelmore *et al.*, 1991).

2.4 Marker-assisted selection (MAS)

Marker–assisted selection has been a plant-breeding tool since it was proposed by Sax in 1923 (Arus and Moreno-Gonalez, 1993). The theory behind this method is that plant breeders could observe easy-to-score phenotypes to select difficult-to-score or low heritability traits that are linked to them (Tanksley, 1983). A good marker should (a) allow the separation of homozygotes from heterozygotes, thus allowing more genetic gain per generation than is possible without using the marker; (b) have early expression in the plant, thus saving time waiting for the desired phenotype to develop; and (c) not have interactions with other markers (Arus and Moreno-Gonalez, 1993). Marker-assisted selection offers an additional tool for obtaining germplasm lines. Molecular markers allow

more efficient selection and offer a mechanism to eliminate undesirable traits associated with hybridizing diverse genotypes (Stalker and Mozingo, 2001).

Successful plant breeding requires selecting many traits with complex inheritance. Desirable quantitative traits usually have both genetic and environmental components (Dudley, 1993), and separation of these components to achieve maximum efficiency in breeding programmes is necessary (Gebhardt and Salamini, 1992). Breeders originally depended on markers that had a morphological effect on the plant because these were the only markers available. However, most morphological marker types do not fit the description of a "good" marker because they have either dominance effects, late expression, exist in epistatic relationships, or have deleterious effects on the plant (Tanksley, 1983).

AFLPs are amendable to high throughput marker generation and the PCR-based inter simple sequence repeats (ISSRs) have gained popularity in mapping and germplasm comparison studies. There are however, limited reports of their use for tagging genes for indirect selection. A relatively new class of markers known as resistance gene analogues (RGA), developed from sequences of cloned resistance genes may have potential for tagging resistance traits (Geoffroy *et al.*, 1998; Kanazin *et al.*, 1996). These markers might be less useful for MAS of specific resistance genes because most of them will arise from polymorphisms generated through accumulated mutations in silenced pseudogenes (Kanazin *et al.*, 1996). The most recent markers are the single nucleotide polymorphisms (SNPs), which appear useful for mapping, manipulation, and study of diseases in humans (Landegren *et al.*, 1998), and will likely have comparable application in plant genomics (Cregan *et al.*, 1998).

The development of molecular markers has great potential for increasing breeding efficiency due to many of the marker systems having large numbers of polymorphisms; alternate alleles rarely have deleterious effects at the molecular or whole plant level; they are often co-dominant, allowing all genotypes to be distinguished in each generation; and they rarely segregate in epistatic ratios. Scoring of molecular markers do not depend on gene expression and are not affected by the environment. The use of markers also reduces time and space necessary to evaluate plant populations. Molecular markers allow more efficient selection and offer a mechanism to eliminate undesirable traits

associated with hybridizing diverse genotypes. A linkage map with many markers, especially when the genome is saturated with markers, can be used to locate genes of interest (Stalker and Mozingo, 2001).

Quantitative traits appear to have the most potential for MAS, because environmental effects confound selection for them. Since the cost to evaluate these complex traits with low to moderate heritability is high, MAS would be more likely to show the greatest gain. Due to complex inheritance, low heritability, and confounding environmental effects, markers associated with QTL have been difficult to find and once found have exhibited limited usefulness across a range of genetic backgrounds or environments. Efforts to utilize MAS for the improvement of quantitative traits have been limited, but may improve as better quantitative data is generated and denser linkage maps become available from map co-integration across laboratories (Freyre *et al.*, 1998).

2.4.1 MAS in common bean

The quality of a marker used for MAS depends on its predictive and/or diagnostic value (Borchardt and Weissleder, 2000). Whereas the predictive value of a marker is determined by the inheritance of the marker and the linkage between marker and trait, the diagnostic value can be measured as the frequency of the desired linkage phase between marker and trait. Taking into account that resistant individuals of different populations harbor different resistance alleles of the same gene, cosegregation of these markers with the resistance trait in each population is not consequently preconditioned. In the case of a low diagnostic value, the allelic phase of a marker has to be checked in each cross before it can be used in MAS (Borchardt and Weissleder, 2000).

Breeders have used markers identified using marker techniques to incorporate new resistance genes or combine genes from different sources, especially in cases where normal screening methods are laborious and expensive. To improve the reproducibility of previously identified RAPD markers, allele specific associated primers (ASAP), and/or SCAR markers have become the basis for indirect selection of economically valuable traits in common bean (Kelly and Miklas, 1998; Melotto *et al.*, 1996).

Although indirect selection for qualitative traits appears promising (Kelly, 1995), the comparative efficiency of direct versus indirect selection for major gene traits comes into question. Often, such traits are easier, faster, and more cost effective to select for directly than indirectly. For agronomic traits such as plant growth habit, height, flowering, and maturity, indirect selection will not likely improve efficiency over routine field selection. Markers for genes conditioning photoperiodism and growth habit are possible exceptions. Improvement of other traits such as disease and insect resistance could be accelerated using indirect selection. Disease and insect resistance present a set of challenges different from those involved with agronomic traits. One major difference is that disease resistance is evasive and breeders must work with two biological organisms and have expertise in handling both. The limitation in disease screening methods becomes apparent when the breeder is challenged with mixed races of a pathogen or by the presence of many resistance genes in the host. When screening conditions are not ideal, escapes can occur and other disease causing organisms can and do interfere with the successful detection of resistant individuals. Indirect selection of race-specific genes offers breeders a viable alternative to ensure that favorable gene combinations are present in new breeding lines. RAPD Markers tightly linked to individual race-specific resistance genes form the basis for effective indirect selection of major gene resistance (Kelly and Miklas, 1999).

Breeding for durable resistance has challenged both plant breeders and pathologists. Non-specific resistance has been reported to offer more durable resistance but its deployment creates a major challenge for the breeder since epistatic major gene resistance often masks this type of resistance. Compounded by complex inheritance, non-specific resistance is not easily transmitted or identified in the progeny. Improving the durability of major gene resistance would be a more attractive alternative for plant breeders since genetic manipulations are easier to accomplish and outcomes are more predictable than the inconsistent expression of minor genes (Kelly 1995).

The application of dominant RAPD markers for indirect selection has been restricted to specific *Phaseolus* gene pools because of the presence in susceptible germplasm of DNA fragments of equal size (kb) to the markers linked to the resistance genes. To overcome gene pool specificity of a RAPD marker, Miklas *et al.* (1996a) showed that recombinant individuals, resistant but without the linked marker, could be crossed with

susceptible germplasm containing the marker. In such populations, resistant progeny are obtained by selecting against the marker. Conversely, resistant recombinants with susceptibility-linked markers can be used in crosses with susceptible lines lacking the susceptibility-linked marker to obtain resistant progeny by direct selection for the marker. This recombination-facilitated MAS strategy overcomes gene pool specificity of a dominant RAPD genetic marker by significantly broadening its range of application across gene pools. This strategy can also be used in the presence or absence of gene pool specificity to reverse the linkage orientation of a RAPD marker to fit a particular breeding program (Kelly and Miklas, 1999).

Duvick (1996) states that even though major resistance genes have only short useful lifetimes when used one at a time, the opportunity to pyramid genes (using MAS) with some knowledge of their complementarity will make major gene resistance more useful than at present. Bean breeders have a unique opportunity to utilize resistance genes from two major gene pools to develop complementary resistance to a wide range of bean pathogens. When several genes are to be pyramided using MAS, Beaver and Macchiavelli (1998) suggested waiting till the F_4 generation to screen lines for linked markers. Screening in the F_4 would improve the probability of identifying lines with the desired genotype and would drastically reduce the minimum of lines that need to be evaluated. Traditional gene pyramiding requires the incorporation of several different resistance genes into a single cultivar. Due to the race specificity of many of these genes, screening nurseries have to be systematically inoculated with different races of the pathogen in an attempt to ensure that gene combinations are being maintained.

2.4.2 Molecular markers for CBB resistance

Genetic studies have shown that resistance to CBB is quantitatively inherited, but involving a few major genes (Beebe, 1989). One, two or three genes appear to confer resistance depending on sources and methods of evaluation (McElroy, 1985; Drijfhout and Blok, 1987; Scott and Michaels, 1988; Silva, 1988).

The highest levels of CBB resistance have been reported in tepary bean (*P.acutifoilus*) and in runner bean (*P.coccineus*). McElroy (1985) studied CBB resistance in *P.acutifolius* and the XAN lines, XAN-159, XAN-160, and XAN-161. These lines were developed at

CIAT derived from interspecific crosses between *P. vulgaris* and *P. acutifolius* accession PI 319443. He also reported that *P. acutifolius* accession PI 319443 showed quantitative inheritance, predominantly additive gene effects, and partial dominance for CBB resistance. The data was found to be consistent with the hypothesis of one prominent major gene and two other genes of lesser effect controlling resistance. McElroy (1985) concluded that most genes controlling CBB resistance in *P. acutifolius* accession PI 319443 have been recovered in XAN-159.

Phenotypic observations of complex resistance have been supported by QTL data. Nodari *et al.* (1993a) identified the genomic locations associated with QTL for CBB resistance but there has not been a study to identify the genomic locations for genes controlling CBB resistance. This study was also able to explain 75% of the phenotypic variation for CBB resistance in leaves with four QTL in a F_2 segregating population. One major effect QTL explained 35% of the variation.

The presence of two resistance genes in OAC 88-1 is supported by the identification of two independent QTL that explained 80% of the variation for CBB resistance derived from the tepary line PI 449795 (Bai *et al.*, 1997). The potential commonality of the genes in OAC 88-1 to existing resistance sources in XAN159 and GN No.1 sel 27 is supported by the observation that one or more loci conditioning CBB resistance were in common across four different tepary lines (Urrea *et al.*, 1999). These observations support the presence of independent loci for CBB resistance in XAN 159 and OAC 88-1.

Three QTL explaining 77% of the variation was identified for the GN Nebr. #.1 sel 27 resistance source. One major effect QTL, defined by the RAPD marker $OAP6_{820}$ explained 60% of the variation (Miklas *et al.*, 1996b). A survey revealed that most lines with CBB resistance derived from GN No.1 sel 27 possessed $OAP6_{820}$ indicating this marker should be useful for MAS of CBB resistance. P.N. Miklas (unpublished data) has developed a SCAR for $OAP6_{820}$ and was found to explain the variation for CBB resistance in other mapping populations segregating for the GN No.1 sel 27 resistance source.

A SCAR marker, SU 91 that was derived from the XAN 159 resistance source segregating in a population explained 24% of the phenotypic variation in leaf reaction (Pedraza *et al.*, 1997). In addition, the marker SU 91 maps to a major QTL for CBB

resistance in another population in which another SCAR (Y5) has been developed targeting a second QTL (Jung *et al.*, 1998). Breeding lines with both SCARs were more resistant than lines with only the SU 91 SCAR marker. The marker SU 91 is derived from *P. acutifolius* and is likely to be located within the introgressed genomic fragment containing CBB resistance.

Three resistance sources, GN Nebr. #1 sel 27, XAN 159 and OAC 88-1 (dry bean genotypes) have each contributed two independent quantitative trait loci (QTL) with major effect for CBB resistance. SCARs linked with five of the six QTL are available for DNA marker-assisted selection. Three of the SCAR markers (SAP 6, SU 91 and BC 420) can be amplified in the same polymerase chain reaction at a common annealing temperature. These markers select for 3 independent QTL on linkage groups B6, B8 and B10, which contribute a substantial effect on CBB resistance. The marker SAP 6 is derived from GN Nebr. #1 sel 27 and BC 420 and SU 91 are from XAN 159. BC 420 is associated with the *V* locus and the purple colour of the flower (Miklas *et al.*, 2000). SCAR markers SAP 6 and BC 420 do not flank the resistance QTL. Resistant lines Wilk 2 and XAN 159 both show the markers BC 420 and SU 91, while Teebus shows the SAP 6 marker (Fourie, 2002b). SCAR markers are able to determine the presence of different CBB resistance QTL in pyramided lines.

Since linkages between traits are not common, both coupling and repulsion linkages have been reported between markers associated with disease resistance traits. A repulsion linkage between QTL affecting Bean Golden Mosaic and CBB resistance were observed on two separate linkage groups, which partially explained the low, but consistent, negative phenotypic correlation (Miklas *et al.*, 1996b). Jung *et al.* (1996) reported an association between web blight and CBB with the RAPD marker $BC409_{1250}$, but the marker was not mapped in that population. A QTL for CBB resistance has been located on linkage group 7 in a region that includes the phaseolin locus *Phs* (Nodari *et al.*, 1993b). Specific markers tagging these QTL need to be developed for use in MAS. These linkages may represent initial visualization of resistance gene clusters in common bean (Miklas *et al.*, 1998) as has been observed in other crops.

The combination of resistance QTL, as in the pyramiding of qualitative genes, will likely contribute to more effective and durable CBB resistance. Diverse sources of CBB resistance could be combined in an attempt to obtain superior resistant lines. Using

traditional breeding, the resistance of GN Nebr. #1 sel 27 and PI 207262 were combined in the highly resistant XAN 112 (Beebe and Pastor-Corrales, 1991) and these same resistance sources were likely combined in some of the Wilk and VAX lines (Zapata *et al.*, 1991; Singh and Muñoz, 1999).

The identification of RAPDs and development of SCARs linked with QTL from diverse sources with major to moderate influence on resistance to CBB, implies that MAS will be useful in combining resistance sources to CBB in common bean. The independence of the resistance genes to be combined must be closely monitored as many different lines and cultivars have common sources of CBB resistance. A potentially interesting use of markers would be in the introgression of resistance genes across species of *Phaseolus*. Markers linked to a block of monogenically inherited CBB resistance genes segregating in a tepary bean population have been identified (Park *et al.*, 1998) and may facilitate the recovery of CBB resistant plants from interspecific populations.

The failure to achieve better genome coverage suggests that researchers intending to do genome analysis should use wider crosses. In fact, most populations used are originally developed in the context of a dry bean-breeding programme and therefore was designed to achieve breeding objectives rather than the objectives of genomic research. Although this makes the detection of marker polymorphisms more difficult, the information obtained through the use of a typical breeding population may ultimately be more directly applicable to plant breeding problems (Jung *et al.*, 1996).

From a breeder's perspective, linkages among resistance loci are beneficial when they are derived from an individual parental line, because when crossed with susceptible parents these resistances will be inherited as a single unit and will likely remain intact in subsequent progeny (Geffroy *et al.*, 1998). When linked resistance genes are donated by different parents recombination between the loci is necessary to obtain both resistance sources in a single line. An appealing use of MAS would be the indirect selection of gene clusters using flanking markers.

2.4.3 Linkage map of common bean

A genetic linkage map is a tool with many applications in basic and applied genetic research. First, it provides information on the genetic control of traits, especially those with complex inheritance and their linkage relationships to other traits (Lander and Botstein, 1989). Molecular markers can also be used as indirect selection tools to simplify breeding or to provide information about genome evolution (Gepts *et al.*, 1993). Other markers with limited polymorphism, such as a small number of morphological traits or isozymes, may also provide additional information for linkage mapping in common bean (Tsai *et al.*, 1998).

Linkage maps have become widespread and essential tools for crop improvement and other biological studies (Gepts, 1999). Assembling a linkage map for species that do not have a long and intensive history of genetic research is possible. This is due to the development of molecular marker technologies that have led to an increase in linkage maps due to the increase in the number of polymorphic loci detected within any given segregating population. Phenotypic markers were used before molecular markers became available. The accumulation of phenotypic markers in the same population was time consuming and difficult due to the deleterious nature of these markers or epistatic interactions. Many RFLP maps have been produced but the overall trend has been toward an increased reliance on PCR-based markers due to their relative speed and reduced DNA requirements (Gepts, 1999).

A primary genetic linkage map, consisting of easily scored polymorphic marker loci spaced throughout a genome, is an essential prerequisite to detailed genetic studies in any organism (Lander *et al.*, 1987). Associations between the trait and the segregating markers can be done with statistical methods. Genetic mapping of QTLs is based on the idea that genetic markers that tend to be transmitted together with values of the trait are likely to be close to a gene affecting that trait. Thus an association is sought between marker variants and trait values, with higher levels of association suggesting closer genetic map distance (Doerge *et al.*, 1994). Linkage of a DNA marker to a QTL is done by making a cross between two plants differing for one or more characters. Segregating progeny (F_2, backcross or recombinant inbred lines) are obtained and evaluated for the

character of interest as well as their genotypes at DNA marker loci throughout the genome (Michelmore *et al.,* 1991).

Lander and Botstein (1989) described a set of analytical methods that modified and extended the classical theory for mapping QTLs. They adapted the method of likelihood of the odds (LOD) scores applied in human genetics to interval mapping of QTLs. The evidence for a QTL is given by the LOD score, which indicates the probability of the data to have arisen assuming the presence of a QTL than assuming its absence. If genetic markers have been scored throughout the genome, the method of maximum likelihood can estimate the phenotypic effect and the LOD score for a putative QTL at any given location. The LOD score is defined as the $\log_{10}$ of the odds ratio, and summarizes the strength of evidence in favour of the existence of a QTL with this effect at this position. If the LOD score exceeds a pre-determined threshold, the presence of a QTL is inferred. The threshold for the LOD score depends on the size of the genome and the density of the markers genotyped. Interval mapping allows interference about points throughout the genome and avoids confounding phenotypic effects with recombination, by using information from flanking genetic markers (Paterson *et al.,* 1991a,b). When the selective genotyping approach is followed, standard linear regression procedures cannot be followed as the biased selection of progeny would lead to a gross overestimation of phenotypic effects. Specially developed programmes like MAPMAKER/QTL (Lincoln *et al.,* 1992) are widely used as programmes that can analyze data with the genotypes of non-extreme individuals entered as missing data.

In recent years, molecular marker analyses have provided new insights into the common bean origin and diversification (Gepts, 1988), its process of domestication (Sonnate *et al.*, 1994) and the inheritance of disease resistances (Nodari *et al.*, 1993b; Adam-Blondon *et al.*, 1994; Yu *et al.*, 1998; Vallejos *et al.,* 2000). The first linkage maps of common bean were based on morphological traits (Basset, 1991) and isozyme and seed protein markers (Vallejos and Chase, 1991b) but provided limited genome coverage. A considerable improvement was obtained with the development of three genetic linkage maps with DNA markers (Vallejos *et al.*, 1992; Nodari *et al.*, 1993a; Adam-Blondon *et al.*, 1994). These three established maps have subsequently been integrated, resulting in a core map that has a higher density of markers and an expanded length (Freyre *et al.*,

1998). In spite of these advances, a connection between linkage groups and chromosomes has not been established.

A linkage map, based mainly on RFLP markers, has been constructed using a backcross progeny between a Mesoamerican breeding line and an Andean cultivar (Vallejos *et al.*, 1992). A genomic library of size-selected (500 – 4000 bp) *Pst*I fragments was the main source of probes (Chase *et al.*, 1991). Ninety-five percent of the 362 clones tested yielded hybridization patterns typical of single copy sequences. Sixty percent of the clones tested revealed polymorphisms between the parental genotypes with at least one of four restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, and *Hind*III). Twenty-eight of the clones that had not detected RFLP's with any of the previously used enzymes were tested with four new enzymes: *Bam*HI, *Bgl*II, *Kpn*I and *Xba*I. About 50% of these clones revealed polymorphisms with at least one of these enzymes. The estimated polymorphism between the parental genotypes is 80% with at least one of the 8 restriction enzymes. These enzymes differed in their ability to detect polymorphisms between the parental genotypes: *Kpn*I (40%); *Dra*I (42%); *Bam*HI, *Bgl*II and *Hind*III (53%); *Eco*RI (62%); and *Eco*RV (64%).

Segregation data have been used to assemble a linkage map for the common bean (Vallejos *et al.*, 1992) with the aid of the computer software MAPMAKER (Lander *et al.*, 1987). The map comprising of 227 RFLP loci, was constructed using stringent linkage criteria (LOD 3.0, 25 cM, and three point LOD exclusion threshold – 3.0). Eight of the 219 genomic clones hybridize to homologous sequences located in different linkage groups. The map also includes two loci of the chlorophyll a/b binding protein in groups *B* and *D*. Also included are: one phenotypically identified pigmentation locus (P), nine isozymes and nine seed proteins. The seed proteins are: phaseolin, the α-amylase inhibitor proteins, and other globulins (Vallejos and Chase, 1991b). Electrophoretic variation at the protein level could be due to either variation at the DNA level and/or variation in post-translational events – protease processing and/or glycosylation. Southern analysis of genomic blots with a phaseolin clone (Sun *et al.*, 1981) has shown perfect co-segregation between a restriction fragment identified by this clone and phaseolin protein bands identified by sodiumdodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). These results suggests that variation at the protein level in phaseolin is due to variation at the DNA level and that the locus mapped with the protein data corresponds to the

structural gene of phaseolin. RAPD markers have also been used and Williams *et al.*, (1990) have added *OA1a* in linkage group (LG) *D* and *OA10a* in LG *F* to the map. In summary, a linkage map of the common bean included 250 markers assigned to 150 loci/locus clusters and comprises 980 cM, or approximately 82% of the bean genome.

The common bean has the advantage of having a small genome, which is one of the smallest genomes in the legume family (Arumuganathan and Earle, 1991). There is also an advantage of the availability of large germplasm collections (e.g. at CIAT) and of breeding materials and near-isogenic lines. There is also a good understanding of the organization of genetic diversity in the primary gene pool of the species (Gepts, 1993a; 1998).

There have been several linkage maps developed for the common bean and can be distinguished by several characteristics, including the type of parents used, the segregating generation in which they were established, the traits segregating in each population, the type of markers, the number of markers and the total map length. A common additional characteristic of the linkage maps that have enough markers is that the total map length appears to be close to 1 200 cM (Gepts, 1999). The core linkage map contains 550 molecular markers, principally RFLP and RAPD markers. Sequences of known molecular and biochemical function have been mapped as RFLPs. Two hundred and forty markers are placed as framework markers and 329 in intervals. There are 46 markers that are unassigned. The core map consists of 11 linkage groups, numbered B1 to B11. The length of the map is 1226 cM or an average of two cM per interval.

Between gene pool polymorphism or variation reaches 80% for RFLPs and RAPDs, intra-gene-pool polymorphism reaches 30-40% for RFLPs (Nodari *et al.*, 1992) and 60% for RAPDs (Haley *et al.*, 1994). Future maps will be based more on crosses between parents belonging to the same gene pool, whether they belong to different races or the same race (Singh *et al.*, 1991).

RAPDs were used in two studies to construct linkage maps of common bean. Jung *et al.* (1996) used RAPD markers to construct a partial linkage map in a recombinant inbred population derived from the common bean. The linkage map spanned 545cM and

included 75 of 85 markers used in the study. The population was evaluated for resistance to common bacterial blight, foliar resistance to web blight and resistance to rust. Two to six markers accounted for 14% to 34% of the phenotypic variation for each trait. Significant marker locus-trait associations were found for 14 mapped loci and 7 of the 9 unmapped markers. Five markers were significantly associated with resistance to CBB of which three accounted for 21% of the phenotypic variation. The distribution of detected QTL appeared to be nonrandom with most significant markers associated with more than one trait or closely linked to markers significantly associated with variation for a different trait (Jung *et al.*, 1996).

RAPD markers were also used to construct a partial genetic linkage map for studying the genetics of bacterial disease resistance in common bean. The linkage map spanned 426cM and included 168 RAPD markers and two classical markers with 11 unassigned markers. One to four QTL accounted for 18% to 53% of the phenotypic variation for traits. The *V* locus was found linked to a QTL with a major effect on CBB resistance (Jung *et al.*, 1997).

The genetic linkage map and the mitotic chromosome map of the common bean was integrated using fluorescent in situ hybridization of pooled closely linked RFLP markers. Pooled RFLP probes showed clear and reproducible signals and allowed the assignment of all linkage groups to the chromosomes of the two *Phaseolus vulgaris* cultivars used. High variation in the number of 45S rDNA loci was observed among cultivars, suggesting that these terminal sites are highly recombinogenic in common bean (Pedrosa *et al.*, 2003).

In general, Pedrosa *et al.* (2003) found that there was no correlation between chromosome and linkage group sizes or between number of markers per linkage group and chromosome size. The 5S rDNA loci of *Phaseolus vulgaris* were conserved. In contrast, a high degree of variation in size and number of loci was observed for the 45S rDNA sequence within and between the cultivars, although in all cases individuals were structurally homozygous. It seems that a higher degree of amplification and dispersion of the 45S rDNA units has occurred among Andean cultivars. Variation in the size of the signal in a locus was interpreted as a difference in copy number between alleles. The detection of clustered markers dispersed on relatively small chromosome regions in common bean, however, suggested that no major chromosome rearrangement is present

within the species. This is consistent with the fact that genetic maps constructed from different intraspecific crosses revealed the same order of markers with minor exceptions (Freyre *et al.*, 1998; Jung *et al.*, 1999).

A RFLP linkage map was used to identify genetic elements affecting QTLs in two bean genotypes under two levels of mineral nitrogen i.e. low and high NH_4NO_3. QTLs affecting nodule number and response to Xap were identified and mapped. Under low N, three genomic regions influenced both traits, with seven linked markers. Four QTLs identified by interval mapping analysis accounted for 45% of the total variation of the nodule number and one QTL mapped to the same region as a QTL for CBB. Under high N, three additional regions were linked to nodule number. Four regions for CBB were mapped adjacent to or in the same region as a QTL for nodule number. Thus N showed dual and opposite effects on the expression of nodule number and CBB (Tsai *et al.*, 1998).

An increased susceptibility to one or more diseases was observed among the selected N_2-fixing plants. The selection process could be greatly enhanced by the use of genetic markers. RFLPs could be useful as markers to determine genetic relationships, to identify and map loci affecting quantitative traits, and to monitor these loci during introgression or crosses between two divergent parents (Nodari *et al.*, 1992, 1993a, b; Paterson *et al.*, 1991a, b).

With the identification of over 30 RAPD markers and five SCAR markers linked to 17 different resistance genes in *P. vulgaris*, the feasibility and potential for MAS in disease resistance breeding in common bean is clearly possible. When costs are balanced against applications, breeders may choose MAS as a practical alternative to direct selection for multiple traits. As additional markers are detected for other genes, the value of the technology will increase since MAS for more than one trait can be practiced in breeding populations. The value of MAS of quantitative traits will become more important and useful as phenotypic data and the technology for the detection of QTL improves. The direct incorporation of single gene resistance to the prevalent races of a pathogen within a particular area is currently the breeding method of choice, but this resistance is often short-lived, forcing breeders to continuously incorporate new resistance genes into their programs. Combining different genes with resistance to the prevalent races, although more difficult and expensive to achieve initially, should provide a more durable resistance

for long-term protection. Marker-assisted selection provides opportunities to pyramid genes not feasible with traditional breeding methods. Genetic markers will likely be used in the development of future bean cultivars possessing durable disease resistance (Kelly and Miklas, 1999).

2.5 Development of flanking resistance markers for CBB resistance in dry bean cultivar, Wilk 2

CBB occurs in temperate, subtropical and tropical regions (Singh, 1991) and can cause severe damage under favorable environmental conditions. In Eastern and Southern Africa common blight has been reported in 19 of the 20 bean producing countries (Allen, 1995) and is considered as one of the five most widespread biotic constraints of high importance across the sub-Saharan Africa (Gridley, 1994). The disease is widespread throughout all the production areas in South Africa and is favored by moderate temperatures and high relative humidity (Sutton and Wallen, 1970), which are also ideal conditions for dry bean production.

Common blight resistance is inherited quantitatively and the complex inheritance and low heritabilities make the transfer of resistance genes into local cultivars difficult. Lines developed through pyramiding are often not suitable as commercial seed type and resistance must be transferred to cultivars of different market classes (Singh and Munoz, 1999). Molecular markers linked to resistance have been developed and indirect selection using these markers has proven to be most applicable to breeding for resistance. It will further be desirable to pyramid the resistance genes to the same pathogen into a single cultivar of suitable market type in order to achieve more stable resistance, and the use of MAS can contribute considerably when pyramiding is attempted. In order to pyramid CBB resistance, SCAR markers linked to CBB resistance QTL should be developed. SCAR markers are the only way to determine the presence of different CBB resistance QTL in pyramided lines.

The availability of CBB resistant dry bean cultivars will have a big impact on both commercial and small holder farming local and internationally. The production of disease resistant dry bean cultivars will be the most cost effective way to reduce the excessive

input costs of commercial farmers and will contribute to a more stable provision of dry bean seeds for both commercial and small holder farmers.

Teebus is a small white bean that is used extensively in the canning industry. A study by Fourie (2002a) on 21 locally grown commercial dry bean cultivars showed Teebus as being the most susceptible cultivar to CBB. Teebus had a disease rating of 9 in a scale of 1 to 9. The improvement of CBB resistance in South African cultivars is necessary for yield stability as all local cultivars are susceptible to CBB. A yield loss study by Fourie (2002b) showed yield losses of 43.5% in Teebus diseased plots.

CBB was found to be the most important bean bacterial disease in South Africa when compared to halo blight and bacterial brown spot (Fourie, 2002a). In greenhouse trials, XAN 159 and Wilk 2 showed the best resistance to CBB, with XAN 159 having a mean disease rating of 2.3 and Wilk 2 a rating of 1.3. This implies that Wilk 2 possesses an additional resistance QTL that is not present in XAN 159 and therefore shows a lower disease rating than XAN 159.

XAN 159 was developed at CIAT from a *P. vulgaris* x *P. acutifolius* population and one major and a few minor genes control resistance in this line. Wilk 2 was developed at Cornell University by R.E. Wilkinson in the 1980's and although the exact pedigree and germplasm are not known, it seems to have combined CBB resistance genes from all three species: *P. vulgaris*, *P. coccineus* and *P. acutifolius*, including line XAN 159 or its sisters. Wilk 2 has shown the highest source of CBB resistance in large seeded beans. XAN 159 was used as a donor parent in a backcross programme to improve resistance of 2 local cultivars, Teebus (small white canning bean) and Kranskop (high yielding speckled sugar). Segregating populations were phenotypically evaluated under field and greenhouse conditions. In addition to phenotypic disease reaction, existing SCAR markers (Miklas *et al.*, 2000) were tested on identified resistant lines. Two separate near-isogenic lines (NILs) using Wilk 2 and XAN 159 as resistance donor parents and Teebus as a recurrent parent were developed.

PCR studies indicated that both SCAR markers BC 420 and SU 91 found in XAN 159 and Wilk 2 are present in these NILs as well as having the SCAR marker SAP 6 present in Teebus. The NILs show all three SCAR markers present. It is difficult to distinguish

between the 2 NILs as they show similar growth patterns and disease resistance in the field. Due to both XAN 159 and Wilk 2 showing the exact same SCARs, they cannot be differentiated on the basis of the available SCARs. An important objective is to determine whether genes, additional to the QTLs linked to the markers present in XAN 159, are present in Wilk 2 and to develop SCAR markers that are able to differentiate between these highly resistant lines.

The resistance source GN Nebr. #1 sel 27 is used extensively in breeding programmes around the world as a resistance source for CBB. The SCAR SAP 6 is derived from this resistance source and is selecting for an independent QTL on linkage group B10 (Miklas *et al.*, 2000). SAP 6 is present in the susceptible lines of local dry bean cultivars and is not useful as a marker for MAS. It does not flank the resistance QTL and it is therefore possible that the linkage between the SCAR and the QTL has been broken. The identification and development of a flanking SCAR marker for the QTL on linkage group B10 is needed.

A segregating population using Wilk 2 as the resistant parent and Teebus as the susceptible parent needs to be developed for linkage analysis of the phenotype with molecular markers. The AFLP technique using BSA could be used to evaluate and detect the CBB resistance QTL. The conversion of AFLP markers to SCAR markers is used to make the markers easier and faster to use as well as being more cost effective in the long term.

Figure 2.1. Symptoms of chlorosis caused by common bacterial blight on dry bean leaves

Figure 2.2. Effect of common bacterial blight on resistant and susceptible cultivars of dry beans under field conditions

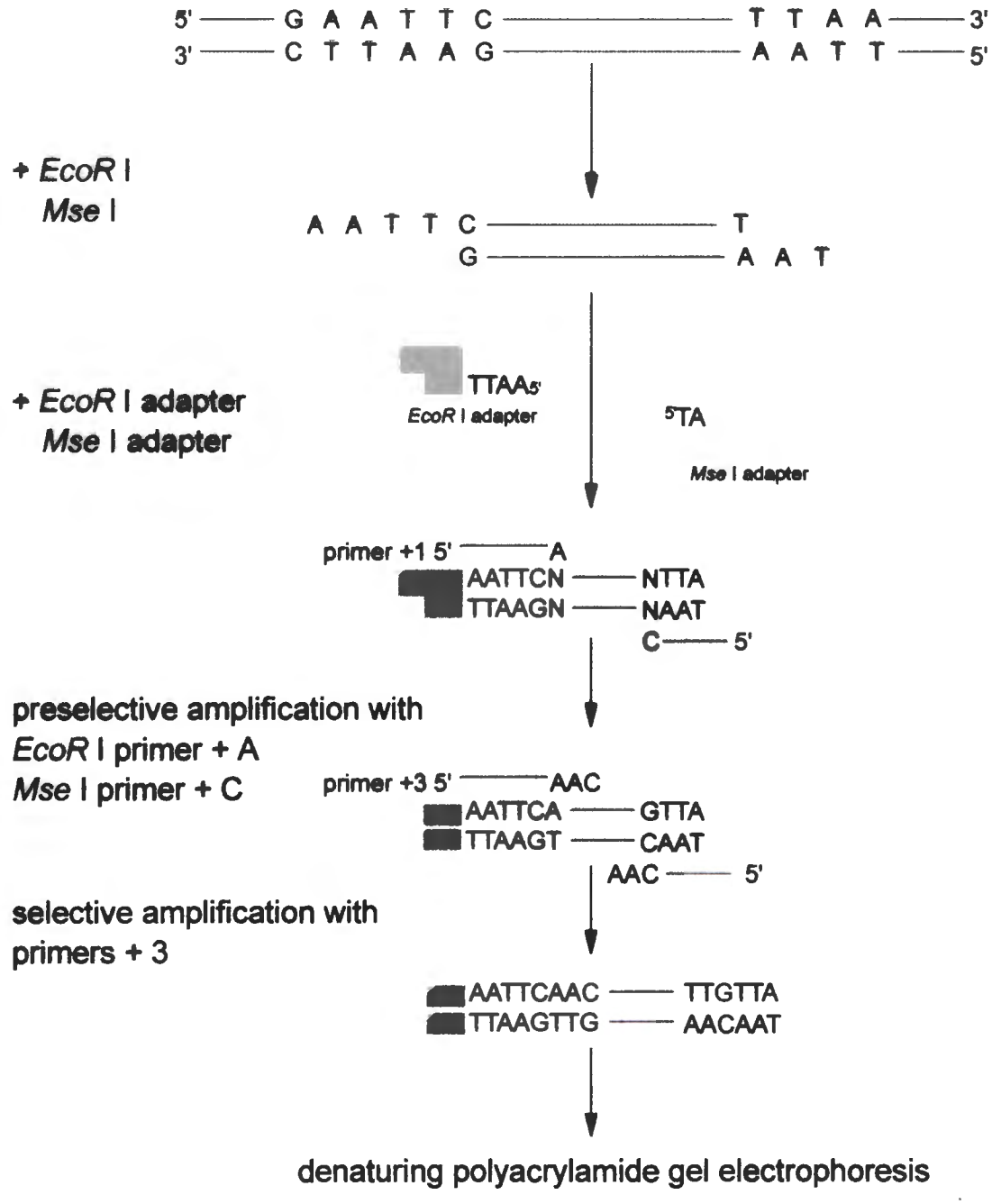

Figure 2.3. AFLP process showing the different steps in the technique (Instruction manual, AFLP™ Analysis System I, Life Technologies)

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant material

Wilk 2 is a large seeded (*Phaseolus vulgaris* L.) bean type and seed was obtained from CIAT (International Center for Agriculture in the Tropics, Cali, Columbia). It has shown better resistance to common bacterial blight (CBB) in the field and was therefore used as the resistant parent. Teebus is a commercial South African cultivar and is a small white canning bean type. It was used as the susceptible parent as it was shown to be susceptible to CBB in greenhouse trials. Seed was obtained from Agricultural Research Council-Grain Crops Institute, Agricultural Research Council, Potchefstroom, South Africa.

3.2 Plant breeding and phenotypic evaluations

A series of crosses were made between Wilk 2 and Teebus. The F_1 seeds resulting from the crosses were planted in the greenhouse and evaluated for disease resistance. The seeds were planted in 15 cm-diameter plastic pots in sterile soil and maintained in a greenhouse at 18°C night/28°C day. Inoculum was prepared by suspending 48- to 72 h-old *Xanthomonas axonopodis* pv. *phaseoli* cultures in sterile distilled water and adjusting it turbidimetrically to contain approximately 10^8 CFU ml^{-1}. Fourteen to 20 day-old plants with fully expanded first trifoliate leaves were inoculated, using the multiple-needle inoculation method (Andrus, 1948). Control plants were inoculated with sterile distilled water. Inoculated plants were kept in a greenhouse at 18°C night/28°C day. Plants were rated for infection 14 days after inoculation on a 1 to 9 scale with 1 being resistant and 9 susceptible. Only the resistant plants were allowed to self-pollinate and advance to a F_2 generation.

Seeds from the F_2 progeny were planted in a randomized pattern with Wilk 2 and Teebus plants as positive and negative controls. The F_2 population was phenotypically evaluated as for the F_1 population. The plant breeding and phenotypic evaluations were

performed by Dr. D Fourie from the Grain Crops Institute, Agricultural Research Council, Potchefstroom, South Africa.

3.3 DNA isolation

Leaf material was sampled from the F_2 population and freeze-dried. DNA was isolated from lyophilized leaf material by a modified CTAB (cetyltrimethylammonium bromide) extraction procedure (Dellaporta *et al.*, 1983). Silica gel was used to finely grind the leaf material, which was then resuspended in 2 x CTAB extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% (m/v) CTAB, 0.2% (v/v) β-mercaptoethanol) and incubated for 1 hour at 65°C. The suspension was extracted with an equal volume of chloroform: isoamylalcohol (24:1 (v/v)) and the phases separated by centrifugation at 10 000 x g for 10 mins. The DNA was precipitated from the aqueous phase with 0.66 x volume isopropanol at room temperature, centrifuged for 15 mins at 12 000 x g. The precipitate was washed with 70% (v/v) ethanol and air-dried. The pellet was resuspended in TE buffer (100 mM Tris-HCl (pH 8.0), 1 mM EDTA), 7.5 M ammonium acetate was added and the suspension subjected to a chloroform: isoamylalcohol (24:1 (v/v)) extraction after which the DNA was precipitated with 2.5 volumes of absolute ethanol. After an overnight incubation at -20°C, the DNA was recovered by centrifugation at 12 000 x g for 15 mins and washed twice with 70% (v/v) ethanol for 5 mins. The ethanol was removed and the pellet allowed to air dry. The DNA was resuspended in TE buffer (100 mM Tris-HCl (pH 8.0), 1 mM EDTA) and treated with 0.1 μg μl^{-1} DNase-free RNase A, prepared by heating a stock solution of 10 mg ml^{-1} RNase A (Roche Boehringer Mannheim, Randburg, South Africa) for 10 mins at 94°C. The concentration of the DNA was determined spectrophotometrically at 260 nm.

3.4 Evaluation of restriction enzymes

Wilk 2 genomic DNA was used to evaluate the cutting efficiency of different enzymes used in AFLP analysis. The enzymes used were *Mlu*I, *Eco*RI, *Pst*I, *Mse*I, *Hind*III and *Taq*I. Two μg of DNA was cut with between 2 U and 5 U of enzyme for 5-6 hours. The restricted DNA was run on a 1.5% (m/v) agarose gel. Based on the results of this test, *Eco*RI and *Mse*I were chosen as the enzymes for the AFLP analysis.

3.5 PCR of existing SCAR markers

Miklas *et al* (2000) published data on four SCAR markers that are linked to CBB resistance. The sequences of the existing SCAR markers can be found in Table 3.1. SCAR markers SAP 6, BC 420 and SU 91 were amplified in the same reaction at an annealing temperature of 58°C. SCAR marker BC 409 was amplified separately at an annealing temperature of 70°C. Each amplification reaction contained 50 ng of DNA, 1 x *Taq* buffer, 2 mM $MgCl_2$, 200 µM of dNTP's, 10 pmol of each primer and 1 U of *Taq* DNA polymerase (Promega) in a total reaction volume of 25 µl. Samples were overlaid with mineral oil and amplification was performed in the Hybaid Omnigene Cycler (Hybaid Limited, United Kingdom). The programme was 5 mins at 94°C for 1 cycle, 94°C for 1 min, X°C (annealing temperature) for 1 min, 72°C for 1 min 30 secs for 30 cycles, with a final elongation step of 72°C for 5 mins. Data was scored on the presence or absence of a band.

3.6 Bulk segregant analysis

Screening of AFLP selective primers for polymorphisms with putative linkage to CBB resistance was conducted with DNA samples from the two parents, Wilk 2 and Teebus, and two bulk samples. Bulk samples were constructed by mixing equal amounts (m/m) of DNA from ten F_2 plants, each of which was either resistant or susceptible to CBB according to the phenotypic evaluation.

3.7 AFLP analysis

3.7.1 Restriction endonuclease digestion of genomic DNA and ligation of adapters

A slightly modified protocol as developed by Zabeau and Vos (1993) and according to the manufacturers (Promega Corporation, Madison, WI) instructions was followed. Approximately 2 µg of genomic DNA was digested at 37°C with *Mse*I for five hours followed by overnight digestion with *Eco*RI. Fragments were then ligated to *Eco*RI and *Mse*I adapters overnight at 16°C. The sequences of the adapters and primers used for AFLP analysis are given in Table 3.2.

3.7.2 Pre-amplification reactions

Pre-selective PCR was carried out with 30 ng of primers+1 (Table 3.2) in a 50 µl reaction volume containing 5 µl of ligated DNA, *Taq* polymerase buffer, 2 mM $MgCl_2$, 200 µM dNTP's (Gibco) and Taq DNA polymerase (Promega). Samples were overlaid with mineral oil and amplified in an Omnigene Thermal Cycler for 30 cycles of 30 secs 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.

3.7.3 Selective AFLP amplification

For selective PCR the pre-amplification products were diluted 1:10 with 0.1 x TE buffer. PCR was conducted in a 20 µl reaction mixture containing 5 µl of pre-amplification product, 30 ng *Eco*RI+3 primer, 30 ng *Mse*I+3 primer, 2 mM $MgCl_2$, 200 µM dNTP's, 100 µg ml^{-1} BSA, *Taq* polymerase buffer and 0.75 U μl^{-1} *Taq* DNA polymerase (Promega). After overlaying the samples with mineral oil, the samples were amplified for one cycle at 94°C for 30 secs, 65°C for 30 secs and 72°C for 1 min, after which the annealing temperature was lowered by 1°C for each of the following nine cycles, followed by 30 cycles of 94°C for 30 secs, 56°C for 30 secs and 72°C for 1 min. Samples were stored at 4°C. After amplification the 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). A 100 bp ladder (0.5 µl/well) (Promega Corporation, Madison, WI) was used as a standard. The samples and ladder were denatured at 94°C for 3 mins, followed by immediate chilling on ice.

3.7.4 Preparation of polyacrylamide gel and glass plates

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 (8.9 mM Tris-base, 88.95 mM Boric acid and 2.85 mM EDTA, pH 8.3). Two glass plates were prepared before casting the gel. One plate was treated with approximately 2 ml of Wynn's C-Thru (WYNN Oil, Bramley, South Africa) and the other with bind silane (950 µl absolute ethanol, 5 µl acetic acid, 3 µl bind silane (Promega)). One times TBE was used as the electrophoresis buffer. The gel was pre-run at constant power (80 W) for 30 mins. PCR products (5 µl) were separated on the prepared gel at 80W constant power for approximately 2 hrs using a standard DNA sequencing unit (C.B.S. Scientific Company, California, USA).

3.7.5 Silver staining

The separated amplified DNA fragments were visualized with a silver staining kit from Promega according to the manufacturers instructions. The plates were separated after electrophoresis using a plastic wedge. The gel (short plate) was fixed with 10% (v/v) acetic acid for 30 mins. Milli-Q water was used to wash the gel three times for 10 mins each. The gel was stained in 0.1% (m/v) silver nitrate (Promega Corporation, Madison, WI) containing formaldehyde to a final concentration of 0.056% (v/v) for 30 mins. The gel was rinsed briefly before development in pre-chilled (10°C) developing solution (30 g l^{-1} sodium carbonate, 0.056% (v/v) formaldehyde and 2 mg l^{-1} sodium thiosulphate). Development of the silver stained fragments were terminated with 10% (v/v) acetic acid added directly to the developing solution. The gel was fixed for 2 mins and rinsed twice in water. The gel was left upright overnight to air dry and photographed by exposing photographic paper (Kodak Polymax II RC) directly under the gel to dim light for about 20 secs. This produced a negative image of exactly the same size as the gel.

3.8 Data analysis

The intense bands were manually scored and translated as follows: 1 for the presence of a band, 0 for the absence of a band and a blank space for missing or unclear values. The scored data was transferred to Microsoft Excel 2000 in columns according to the order in which the samples were loaded in the gel and the primer combinations used.

3.9 Statistical analysis

3.9.1 Regression analysis

Linkage to the resistance trait was determined with a general linear model of the STATGRAPHICS Plus computer program (Manugistics, Rockville, Maryland, USA, 1998), using genetic marker data as the independent variable and the phenotypic evaluation data as the dependent variable. The association between the trait and DNA marker was considered significant if the probability was < 0.05. The coefficient of determination (R^2) was used as a measure of the magnitude of association.

3.9.2 Mapping of markers

Interval mapping with Mapmaker-EXP (Lincoln *et al.*, 1992) was used to link the putative markers to the existing CBB markers. Linkage data was used to assign markers to linkage groups if the $\log_{10}$ of the odds ratio (LOD) was $\geq$ 3.0 and the distance were $\leq$ 37 cM with Kosambi mapping function. The scan command of MAPMAKER-QTL (Lincoln *et al.*, 1992) was used to identify the position of putative QTL's. The Haldane map function was used. The linkage map of the putative markers obtained from MAPMAKER-EXP was also put through Windows QTL Carthographer to identify the position and effects of the QTL's.

3.10 SCAR marker development

3.10.1 Isolation of AFLP fragments

The silver stained AFLP polyacrylamide gels containing the relevant fragments were air-dried. A scapel blade was used to remove the AFLP fragment and rehydrated in 10 µl of distilled water in a 0.5 ml microcentrifuge tube and stored at -20°C (Cho *et al.*, 1996). The original AFLP reaction conditions were used to reamplify the fragment directly from the piece of gel without any purification. One microlitre was electrophoresed on a sequencing gel for determination of purity, and subjected to a possible second and third round of isolation and amplification where necessary.

3.10.2 Cloning of AFLP fragments

The Wizard PCR Preps DNA Purification system (Promega Corporation) was used to purify the PCR products. The AFLP markers were cloned into pGEM-T plasmid vectors (Promega) and transformed into JM 109 high efficiency competent (>108 cfu mg^{-1} DNA) cells (Promega) according to the manufacturer's instructions.

3.10.3 Sequencing of fragments and design of primers

The cloned fragments were sent to the Central DNA Sequencer, University of Stellenbosch, Stellenbosch, South Africa to be sequenced and used to design SCAR primers. The T 7 forward and SP 6 reverse primers from the pGEM-T kit (Promega) were used in the sequencing reaction. The sequence data was used to design a SCAR primer set containing the original nine bases of the AFLP primer and the adjacent internal bases on the ends of the AFLP fragment. The primers were synthesized by Invitrogen (UK).

3.10.4 Optimisation and amplification of SCAR markers

A range of annealing temperatures with differing ramp rates was used for optimization along with DNA, $MgCl_2$, primer, and dNTP concentrations to test reaction conditions for the SCAR primers. The annealing temperatures were tested between 37°C and 60°C to determine the optimal temperature for each primer. DNA concentration was varied between 200 ng to 1µg. $MgCl_2$ concentration ranged between 2 to 5 mM, while primer concentration varied between 30 to 60 ng. Range of dNTP was between 100 to 200 µM per reaction.

SCAR amplification of genomic DNA was performed in 25 µl reaction mixtures, containing 200 ng template DNA, 1 x Taq buffer, 2 – 4 mM $MgCl_2$, 200 µM of dNTP's, 30 ng of each primer and 1 U of Taq DNA polymerase (Promega). Samples were overlaid with mineral oil and amplification was performed in the Hybaid Omnigene Cycler (Hybaid Limited, United Kingdom). The programme was 94°C for 3 mins for 1 cycle, 94°C for 1 min, 40°C for 1 min, 72°C for 1 min for 34 cycles, and 72°C for 5 mins. Data were scored on the presence or absence of a band. Amplification products were analyzed by electrophoresis on 2% (m/v) agarose gels in 1 x TAE buffer (40 mM Tris-acetate, 1 mM

EDTA, pH 8.0) or a 5% (m/v) denaturing polyacrylamide (19 acrylamide: 1 N,N'-methylene-bis-acrylamide ratio) gel in 1 x TBE buffer (8.9 mM Tris-base, 88.95 mM Boric acid and 2.85 mM EDTA, pH 8.3). Amplification profiles were visualized under UV light after staining with ethidium bromide (0.5 $\mu g\ ml^{-1}$).

3.11 Testing of developed SCARs on other CBB resistance sources

Genomic DNA was isolated from XAN 159, Wilk 6, Vax 3, Vax 4, Vax 5, Vax 6 and GN. Nebr. sel 27 #1 as per the method described in Chapter 3.3. PCR amplification of the SCARs was performed using the same reaction conditions as for the F_2 population. The products were screened on a 2% (m/v) agarose gel in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) or a 5% (m/v) denaturing polyacrylamide (19 acrylamide: 1 N,N'-methylene-bis-acrylamide ratio) gel in 1 x TBE buffer (8.9 mM Tris-base, 88.95 mM Boric acid and 2.85 mM EDTA, pH 8.3). Amplification profiles were visualized under UV light after staining with ethidium bromide (0.5 $\mu g\ ml^{-1}$).

Table 3.1 Sequence of existing SCAR markers (Miklas *et al.*, 2000).

SCAR	Primer Sequence (5'-3')	Size (bp)	Annealing Temperature
SAP 6	GTCACGTCTCCTTAATAGTA	820	58°C
	GTCACGTCTCAATAGGCAAA		
BC 420	GCAGCGTTCGAAGACACACTGG	900	58°C
	GCAGGGTTCGCCCAATAACG		
SU 91	CCACATCGGTTAACATGAGT	700	58°C
	CCACATCGGTGTCAACGTGA		
BC 409	TAGGCGGCGGCGCACGTTTTG	1250	70°C
	TAGGCGGCGGAAGTGGCGGTG		

Table 3.2 Sequences of AFLP adapters and primers used in the AFLP process.

Name	Type	Sequence (5'-3')
Eco-A	Primer+1	AGACTGCGTACCAATTCA
Mse-A	Primer+1	GACGATGAGTCCTGAGTAAA
Mse-C	Primer+1	GACGATGAGTCCTGAGTAAC
Mse-G	Primer+1	GACGATGAGTCCTGAGTAAG
Mse-T	Primer+1	GACGATGAGTCCTGAGTAAT
E-AAC	Primer+3	GACTGCGTACCAATTCAAC
E-AAG	Primer+3	GACTGCGTACCAATTCAAG
M-CAA	Primer+3	GATGAGTCCTGAGTAACAA
M-CAC	Primer+3	GATGAGTCCTGAGTAACAC
M-CAG	Primer+3	GATGAGTCCTGAGTAACAG
M-CAT	Primer+3	GATGAGTCCTGAGTAACAT
M-CTA	Primer+3	GATGAGTCCTGAGTAACTA
M-CTC	Primer+3	GATGAGTCCTGAGTAACTC
M-CTG	Primer+3	GATGAGTCCTGAGTAACTG
M-CTT	Primer+3	GATGAGTCCTGAGTAACTT
M-ACA	Primer+3	GATGAGTCCTGACTAAACA
M-ACC	Primer+3	GATGAGTCCTGACTAAACC
*Eco*RI	Adapter	5'-CTCGTAGACTGCGTACC
		CATCTGACGCATGGTTAA-5'
*Mse*I	Adapter	5'-GACGATGAGTCCTGAG
		TACTCAGGACTCAT-5'

CHAPTER 4

RESULTS

Common bacterial blight (CBB) resistance is inherited quantitatively; therefore it is difficult to transfer resistance into local cultivars. All local commercial cultivars are highly susceptible to CBB. This disease (CBB) can cause a serious loss of yield in dry bean production, therefore it is essential to develop cultivars that are both resistant and of marketable importance to the industry.

Wilk 2 was chosen as the resistant parent in this study as it has shown superior resistance phenotypically to CBB than XAN 159 in greenhouse testing under South African conditions (Fourie, 2002). XAN 159 was one of the resistant sources used in the development of the sequence characterized amplified regions (SCARs) available for CBB through random amplified polymorphic DNAs (RAPDs). Wilk 2 has the highest source of resistance in large seeded beans but it is not of a highly marketable type of bean. Teebus is extensively used in the industry as a canning bean but unfortunately is highly susceptible to CBB. It was therefore used in this study as the susceptible parent to improve resistance to CBB.

4.1 Efficiency of restriction enzymes

The genomic DNA of Wilk 2 was subjected to restriction digestion with six enzymes to determine the most effective frequent and rare cutter enzymes. The rare cutter enzymes used were *Mlu*I, *Eco*RI, *Hind*III and *Pst*I and the frequent cutter enzymes were *Mse*I and *Taq*I. *Eco*RI and *Hind*III were equally efficient. *Eco*RI was chosen as all the primers were available in the laboratory. *Mlu*I and *Pst*I only gave rise to large fragments (> 10 kb). Therefore the enzymes used in this study were *Eco*RI and *Mse*I for the amplified fragment length polymorphism (AFLP) analysis. Figure 4.1 shows the agarose gel electrophoresis of the products of the restriction digestion of the genomic DNA of Wilk 2 using the six different restriction enzymes.

4.2 AFLP analysis

The AFLP method is widely used for the identification of polymorphic bands. It was chosen as it generates a large number of restriction fragments and thereby facilitating the detection of more polymorphisms than the other molecular techniques. On average any given AFLP primer pair generates conservatively 10-20 variety specific bands. The AFLP technique was performed on the two parents, two bulks, 20 individuals of the bulks and 40 individuals of the F_2 segregating population.

Genomic DNA was isolated and restricted with *Eco*RI and *Mse*I enzymes overnight and then subjected to ligation with adapters. Preselective amplification was performed using primers with only one selective nucleotide. Figure 4.2 shows the preamplification products of the two parents and two bulks on a 1.5% (m/v) agarose gel. Pre-amplified products were visible as a white smear < 947 bp.

A total of twenty primer pair combinations were tested on the parents and bulks. All 20 primer combinations were informative, but only ten were further tested on the individuals of the bulks and population. This was based on the highest number of polymorphisms per primer combination. Bands were scored in the range of 600 to 100 bases. The frequency of polymorphism on the individuals of the bulks was found to be 36.8% with 30.7% found between the parents. An average of 58.4 bands per primer combination were amplified while the polymorphic bands per primer combination was between 16.8 in the parents and 2.72 for the individuals of the population.

Figure 4.3 shows the AFLP amplification using the primers E-AAC/M-CAT (E-Eco primer; M-Mse primer). The band E-AAC/M-CAT 2 was present in eight of the ten resistant individuals and none of the susceptible individuals. The AFLP fragment E-AAC/M-CAT 3 showed a band in nine of the ten resistant individuals and none of the susceptible individuals.

Selective amplification using E-AAC/M-CTC showed a band (E-AAC/M-CTC 2) present in nine of the resistant individuals and none of the susceptible individuals (Figure 4.4). It was a coupling phase marker, i.e. present in the resistant parent. Figure 4.5 shows the

coupling phase marker (E-AAG/M-CTT 6), which was amplified in eight of the ten resistant individuals of the bulks.

Table 4.1 shows the data obtained from the AFLP analysis on the F_2 population. The AFLP technique proved to be very efficient in detecting polymorphisms in this study. A large number of fragments were detected with 1097 fragments detected with only 20 primer combinations. A total of 337 polymorphic bands were detected between the parents of which 164 were linked to the bulks. In the individuals of the population there were 220 polymorphisms detected of which only 79 were informative. The highest polymorphism frequency (36.8%) was found in the individuals of the population. A total of 79 informative AFLP fragments were manually scored on the basis of one for the presence of a band and zero for the absence of a band and a blank space for missing or unclear values. The data was used for regression analysis to determine linkage to CBB resistance using the programme STATGRAPHICS.

4.3 Regression analysis

The programme STATGRAPHICS was used to determine the magnitiude of linkage of the possible AFLP markers and the existing SCAR markers to CBB resistance. Seventy-nine AFLP fragments were analysed and 58 fragments were found to be informative in the individuals of the population at the 99% confidence level. Linkages of the markers to CBB resistance ranged from 5.03% to 81.17%. The highest percentages of phenotypic variation to CBB resistance obtained were found in the AFLP markers E-AAC/M-CTC 2 (81.17%-Figure 4.4) and E-AAC/M-CAT 3 (76.16%-Figure 4.3). The existing SCAR markers explained the following percentages, BC 420 57.19%, SU 91 18.06%, BC 409 10.61% and SAP 6 only 5.73% of the phenotypic variation to CBB resistance. The low linkage found for SAP 6 was explained due to it being a false positive (Miklas, P.N., 2002, personal communication). SAP 6 was also present in the susceptible parent Teebus and is therefore not useful as a marker in MAS. Fifty-eight AFLP markers along with the four existing SCAR markers were used in the creation of a linkage map using MAPMAKER-EXP.

4.4 Development of a linkage map

Data was converted to MAPMAKER format from the raw one and zero format on the basis of Teebus being regarded as Parent B and Wilk 2 as Parent A. Teebus was considered as the homozygous parent and therefore became Parent B.
Using MAPMAKER-EXP only one linkage group was obtained meaning that the clustering of the scored markers was only on one group and three markers were unlinked. The linkage group spanned 621.3 cM and contained 63 markers. There were ten repulsion phase markers and 53 coupling phase markers in the linkage group. A coupling phase marker has a band present in the resistant parent and absent in the susceptible parent, while a repulsion phase has only a band present in the susceptible parent. It was found that other putative markers alongside the existing SCARs had a higher linkage to CBB resistance. Possible QTLs on other chromosomes could have been missed due to the selective procedure of identifying highly linked polymorphisms.

Six putative markers were selected for cloning and sequencing for further development of new SCARs on the basis of their percentage linkage explaining the phenotypic variation (R^2) to CBB resistance and their relative positions on the linkage map. These fragments were E-AAC/M-CTC 2 (81.17%), E-AAC/M-CAT 2 (76.16%), E-AAG/M-CAT 2 (76.16%), E-AAG/M-CTT 6 (61.55%), E-AAC/M-CAT 3 (66.48%) and E-AAG/M-ACC 2 (40.40%). All six fragments were coupling phase markers.

4.5 Sequences of cloned AFLP fragments

Six AFLP fragments identified in Section 4.4, which significantly explained the phenotypic variation to CBB resistance ($P \leq 0.001$), were cloned and sequenced. The sequences of the 5 fragments used for SCAR primer development are given in Figure 4.6. The sequence data of E-AAG/M-CAT 2 was not usable and therefore not used to develop SCAR primers for that fragment. Fragment 2 (E-AAC/M-CAT 3) showed two restriction fragments in tandem resulting in SN 2A and SN 2B. Three clones of fragment 1 (E-AAC/M-CAT 2) were sequenced, i.e. SN 1, SN 1-1 and SN 1-2, all of which differed in sequence. Similarily, 2 clones of fragment 3 (E-AAC/M-CTC 2) were selected and showed different sequences (i.e. SN 3 and SN 3-1) and therefore SCAR primers were developed for each clone. The primer length varied from 17 to 21 bases.

4.6 SCAR primer optimization and linkage

Genomic DNA of the two parents was used to test and optimize the reaction conditions for the designed SCAR primers. Polymorphisms found between the two parents were then tested on the F_2 segregating population and the linkage to CBB resistance was determined with regression analysis. Table 4.2 shows the sequences of the SCAR primers developed from the sequencing data of the AFLP fragments. The five AFLP fragments selected for sequencing were coupling phase markers.

SCAR primers designed from fragments SN 1-2 (E-AAC/M-CAT 2), SN 3-1 (E-AAC/M-CTC 2), SN 4 (E-AAG/M-CTT 6) and SN 6 (E-AAG/M-ACC 2) did not show any polymorphisms between the 2 parents and were not tested further. The optimal annealing temperature of SN 1 (E-AAC/M-CAT 2), SN 2A (E-AAC/M-CAT 3), SN 2B (E-AAC/M-CAT 3) and SN 3 (E-AAC/M-CTC 2) was found to be 40°C with the $MgCl_2$ concentration to be between 2 and 4mM. A two-step programme with annealing temperatures 45°C for 6 cycles and 40°C for 30 cycles was found to be optimal for SCAR SN 1-1 (E-AAC/M-CAT 2). The optimal dNTP concentration was 200 µM with 200 ng of genomic DNA. The optimal separation of the PCR products of SCAR primers SN 1-1 (E-AAC/M-CAT 2), SN 2B (E-AAC/M-CAT 3) and SN 3 (E-AAC/M-CTC 2) were found to be under denaturing PAGE conditions. The other SCAR markers could be separated on a 2% (m/v) agarose gel.

Amplification with SCAR primer SN 1 (E-AAC/M-CAT 2) (Figure 4.7) showed a polymorphic band present in the susceptible parent, Teebus at the annealing temperature of 40°C and a 2 mM $MgCl_2$ concentration. The band was in the repulsion phase. The SCAR marker only explained 15.02% of the phenotypic variation to CBB resistance as compared to the AFLP marker explaining 76.16%. SN 1 became an unlinked marker when subjected to MAPMAKER-EXP and clearly differed from the original fragment scored.

The SCAR marker SN 1-1 (E-AAC/M-CAT 2) (Figure 4.8) amplified two bands, one of which was polymorphic between the two parents. Regression analysis of the SCAR with the F_2 population was higher and more significantly explained the phenotypic variation to CBB resistance (64.71%) than SN 1 (15.02%). SN 1-1 unfortunately did not map close to

the original AFLP fragment. It was positioned between the existing SCAR marker BC 420 and the fragment E-AAC/M-CTC 2. The SCAR primer SN 1-2 did not show any polymorphism between the 2 parents and was not further tested (results not shown).

The SCAR marker SN 2A (E-AAC/M-CAT 3) showed two polymorphic bands, both of which were coupling phase markers (shown in Figure 4.9 as a (SN 2A-600) and b (SN 2A-300)) that were significantly explained the phenotypic variation to CBB resistance (57.78 and 54.81% ($P \leq 0.001$)) Amplification was optimal at an annealing temperature of 40°C at a 2 mM $MgCl_2$ concentration. Both bands were in the same phase as the AFLP fragment from which they were derived (coupling phase). The polymorphic band SN 2A-600 explained 57.78% of the phenotypic variation to CBB resistance and was found to be positioned 2.2 cM away from the original AFLP fragment on the linkage map (seen in Figure 4.12). The SCAR marker SN 2A-600 (57.78%) had a lower phenotypic explanation than the AFLP fragment from which it was developed (66.48%).

SCAR SN 2B (E-AAC/M-CAT 3) was amplified at a 2 mM $MgCl_2$ concentration. The amplification products were separated on a 5% polyacrylamide gel for optimal separation of the PCR products (Figure 4.10). The polymorphic band explained 40.40% of the phenotypic variation to CBB resistance. It was positioned 79.4 cM away from the original AFLP fragment. According to the linkage map, the SCAR SN 2B is positioned 5.6 cM away from the AFLP fragment E-AAG/M-ACC 2 used to design the SCAR primer SN 6. The primer SN 6 did not show any polymorphism when tested with the parents. Both the AFLP fragment and SN 2B explained 40.40% of the phenotypic variation to CBB resistance. It may be possible that SN 2B and E-AAG/M-ACC 2 are targeting the same part of the resistance QTL.

Three polymorphic bands (SN 3-300, SN 3-400 and SN 3-450) were observed for SN 3 (E-AAC/M-CTC 2) (Figure 4.11). All three bands linked in the coupling phase and explained between 71.16 to 54.03% of the phenotypic variation to CBB resistance. On the linkage map all three polymorphic bands mapped on the same linkage group with only SN 3-300 mapping close to the original AFLP fragment. The other fragments are most likely repeat sequences of the same fragments but found at different positions on the linkage map.

Table 4.3 shows the regression analysis linkage of the AFLP fragments, the developed and existing SCAR markers to CBB resistance and the relative positions as obtained with MAPMAKER-EXP. The R^2 value is the percentage that the marker explains the phenotypic variation to CBB resistance. The linkage group 1 contained 67 of the 71 markers with 4 markers being unlinked. A partial linkage map of the linkage group 1 can be seen in Figure 4.12. The linkage group was too large to be drawn as it contained 67 markers. Therefore a partial linkage map was drawn containing only the existing SCAR markers, the AFLP fragments used for cloning and the developed SCAR markers from this study.

Table 4.4 shows the linkage data of the AFLP fragments selected for cloning and the resulting SCAR markers from these fragments. SN 4 (E-AAG/M-CTT 6) and SN 6 (E-AAG/M-ACC 2) did not show any polymorphism between the two parents and therefore were not tested further.

4.7 Quantitative trait loci (QTL) map

By using the linkage map obtained through MAPMAKER-EXP, both programmes of Windows QTL Cartographer and MAPMAKER-QTL was used to determine the positions and effects of the QTLs. Three QTLs were identified with additive and dominant effects.

Two major additive and dominant QTLs were identified which had significant effects on CBB resistance. The first major QTL (1) was positioned 0.9 cM above the fragment E-AAC/M-CTC 2. The AFLP fragment explained 80.97% of the variation effect coded for CBB resistance of the first QTL. The SCAR marker SN 3-300 (E-AAC/M-CTC 2) is also closely linked to the QTL. The second major QTL (2) is found 0.2 cM below the AFLP fragment E-AAC/M-CAT 2. The AFLP fragment is able to explain 76.16% of the phenotypic variation effect coded for by the second QTL.

A third QTL was detected that was not significantly linked to CBB resistance. It also showed additive and dominant effects. It is possible that this QTL is a minor QTL that is not essential for CBB resistance but has an additive effect when linked to the major QTLs for CBB resistance. This QTL (3) was found to be positioned 3.2 cM above the

SCAR marker SN 1-1 (E-AAC/M-CAT 2). The SCAR marker explained 64.71% of the variation effect coded for by the third QTL.

4.8 Testing of additional resistance sources

Seven additional resistance sources (XAN 159, Wilk 6, Great Northern Nebraska sel 27 #1, Vax 3, Vax 4, Vax 5 and Vax 6) were tested to determine their linkage with the developed SCAR markers, and to determine their source of resistance.

The SCAR marker SN 1 (E-AAC/M-CAT 2) amplified a positive band in Teebus (susceptible parent) and all the other resistance sources tested except XAN 159 and Wilk 2. GN Nebr. sel 27 #1 is probably the source of resistance for the SCAR marker SN 1 as it is amplified in the cultivar and not in XAN 159 (Table 4.5). The Vax lines have been derived from GN Nebr. sel 27 #1 background. SN 1-1 (E-AAC/M-CAT 2) was amplified in the resistant parent used in the study (Wilk 2), Wilk 6 and Vax 3 (Table 4.5). SN 1-1 was not amplified in XAN 159 and can therefore be used to differentiate between XAN 159 and Wilk 2. The SCAR marker SN 1-1 seems to target an additional resistance QTL that has a minor but additive effect on CBB resistance. This SCAR marker (SN 1-1) developed in this study would be useful in differentiating the NILs derived from the Wilk 2 and XAN 159 lines.

Table 4.5 summarizes the presence of the developed SCAR markers in the additional resistance sources. XAN 159, Wilk 2 and Vax 3 were the only positive reactions for SCAR SN 2A-600 (E-AAC/M-CAT 3), while SN 2B (E-AAC/M-CAT 3) only had positive reactions for Wilk 2 and XAN 159. SCAR marker SN 2A-600 was the only developed SCAR that mapped close to the original AFLP fragment from which it was derived. Both SCAR markers SN 2A-600 and SN 2B amplify resistance QTLs from XAN 159 that have probably been transferred to Wilk 2 through breeding (Table 4.5). The SCAR marker SN 3-600 (E-AAC/M-CTC 2) was present in Wilk 2, Wilk 6 and XAN 159. It was not present in any of the other cultivars tested and the resistance QTL is probably derived from XAN 159.

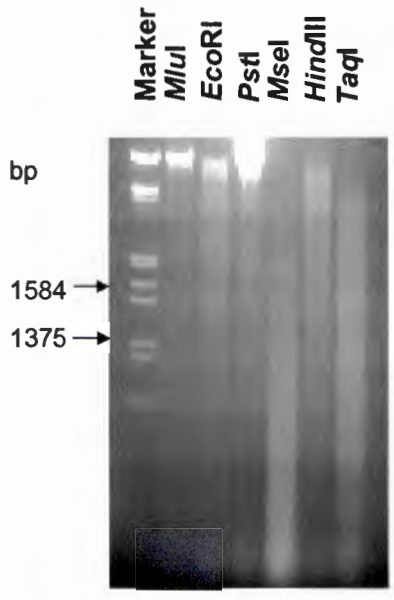

Figure 4.1. Agarose gel electrophoresis of restriction digestion of Wilk 2 genomic DNA using different restriction enzymes. Marker- Lambda DNA digested with *Eco*RI and *Hind*III.

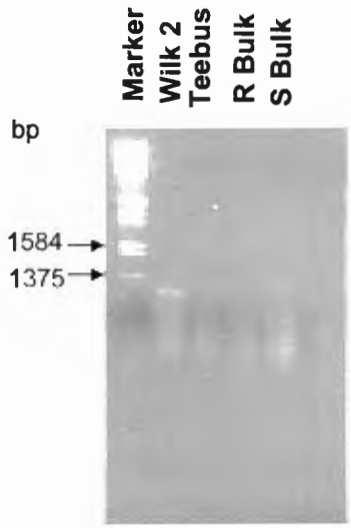

Figure 4.2. Agarose gel electrophoresis of pre-amplification products of genomic DNA after enzyme restriction and ligation with adapters. Lane 1:Marker-Lambda DNA digested with *Eco*RI and *Hind*III, Lane 2: Wilk 2, Lane 3: Teebus, Lane 4: Resistant bulk made up of 10 individuals, Lane 5: Susceptible bulk made up of 10 individuals.

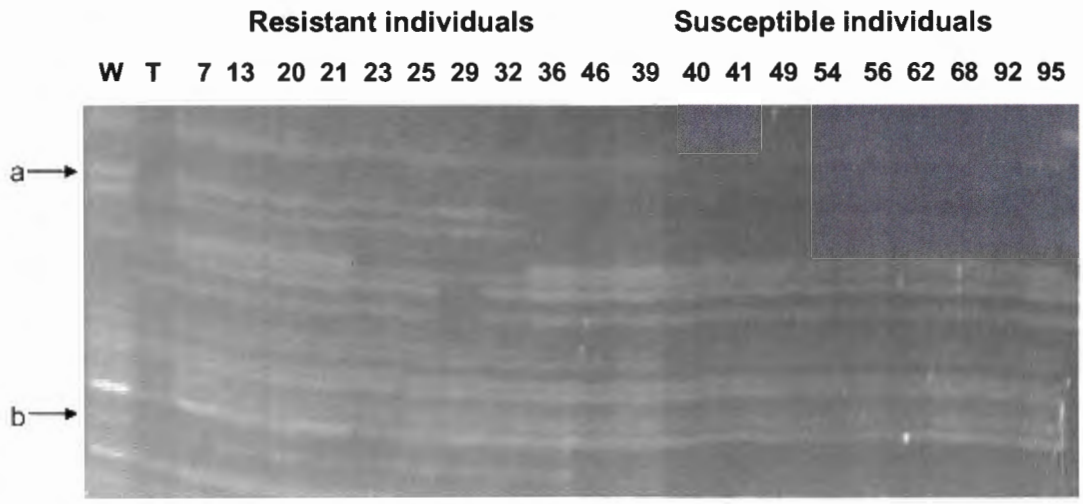

Figure 4.3. Selective amplification with Eco primer E-AAC and Mse primer M-CAT. Separation of amplified products under polyacrylamide gel electrophoresis conditions. Arrow (a) indicates the AFLP marker E-AAC/M-CAT 2 and arrow (b) indicates E-AAC/M-CAT 3. W: Wilk 2; T: Teebus; Nr. 7 to 95: Individuals of bulks.

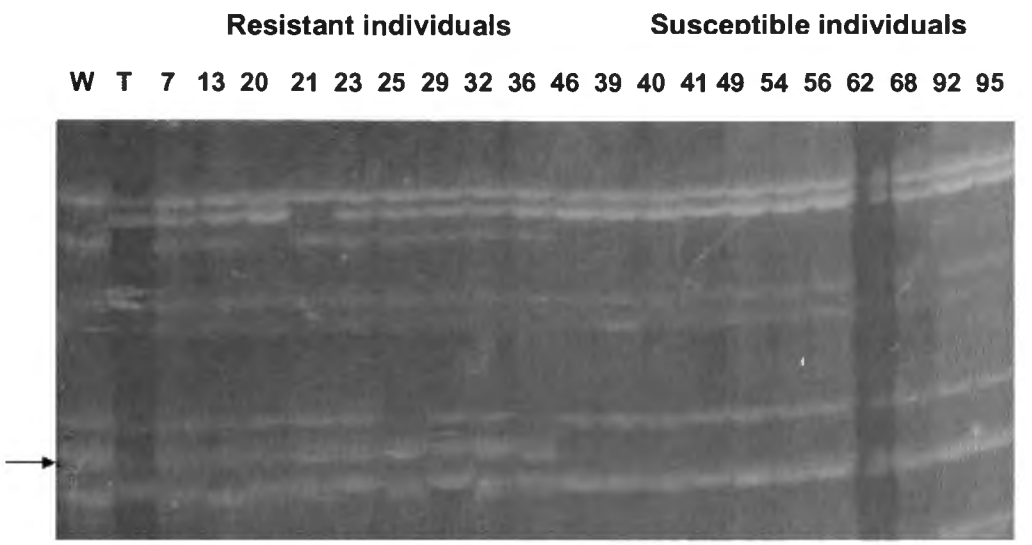

Figure 4.4. Selective amplification with Eco primer E-AAC and Mse primer M-CTC. Separation of amplification products under polyacrylamide gel electrophoresis conditions. The arrow indicates the AFLP marker E-AAC/M-CTC 2. W: Wilk 2; T: Teebus; Nr. 7 to 95: Individuals of bulks.

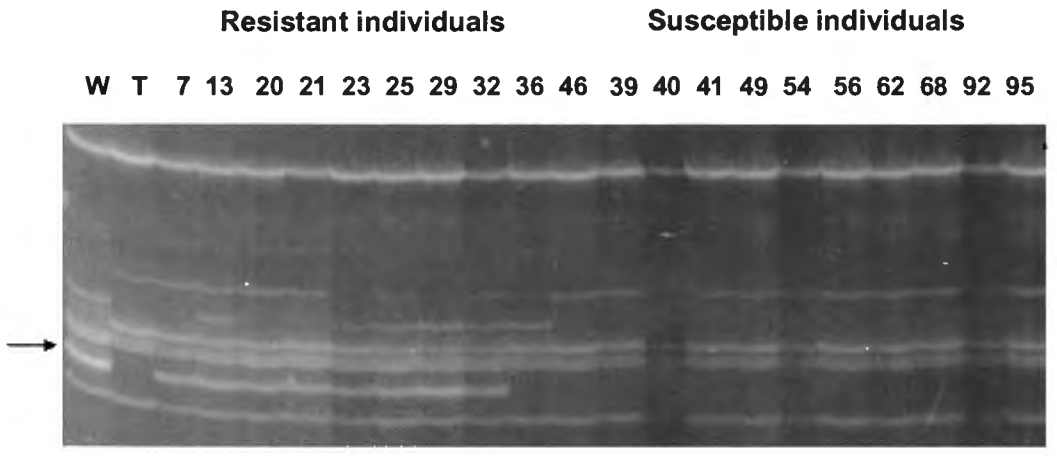

Figure 4.5. Selective amplification with Eco primer E-AAG and Mse primer M-CTT. Separation of amplification products under polyacrylamide gel electrophoresis conditions. Arrow indicates the AFLP marker E-AAG/ M-CTT 6. W: Wilk 2; T: Teebus; Nr. 7 to 95: Individuals of bulks.

SN 1: AAC-CAT2 (119bp)

GATGAGTCCTGAGTAACATATCAAAACACAATTCTAACACATGGAATTGGTTTTCGA
AGATATCATAAGTGTTTTCAATCAATTAACACATTTCTTTTTT**GTTGAATTGGTACGC**
AGTC

SN 1-1: AAC-CAT2 (119b)

GATGAGTCCTGAGTAACATCCTCTTGCCAGGGATTCATTTAGGTAAATTCTTCCTTT
GCCGAGAGGTTTCTTTCACACACTCTCGATTGTGATTCACTTA**GTTGAATTGGTACG**
CGGTC

SN 1-2: AAC-CAT2 (120bp)

GATGAGTCCTGAGTAACATTCCCAACTTTCCATGCACAATTATCCTCAAAGCTGCC
TCTCCACCCCTCAATTTAATCTAACTTTTTTATATTTCATCTGAG**GTTGAATTGGTAC**
GCAGTC

SN 2A: AAC-CAT3 (104bp)

GATTGTCCTGAGTAACATCACGGTGTACGACATCATTTGCATGTTGGTAGGCTAGA
GCCTCAGCTGCTTCCAAGCTATGTTGTG**GTTGAATTGGTACGCAGTC**

SN 2B: AAC-CAT2 (104bp)

GATGAGTCCTGAGTAACATCACAATAAGTAGGGCCGGTCGCCCGAATCAATTAATC
CTGAAAAATGTGTCTTTTCCATAGACCAT**GTTGAATTGGTACGCAGTC**

SN 3: AAC-CTC2 (188bp)

GACTGCGTACCAATTCAACATCCAAATCCGCATGCCTCCTCGAGGAATGCCTATCT
GCGCGGATGGCAAGGCCACCAATGGGGTGCCCTTCGTCTTCGTCTACTCCGCTAT
TTTCAAGAGGTTGAAGCTGCGCCTCCCTTTCACTTTCTTCGAAAAGGAGCTGAT**GAT**
GGAGTTACTCAGGACTCATC

SN 3-1: AAC-CTC2 (188bp)

GACTGCGTACCAATTCAACATCCAAATCCGCATTCCTCCTCGAGGAATGCCTATCT
GCGCGGATGGCAGGGCCACCAGTGGGGTGCCCTTCGTCTTCGTCTACTCCGCTAT
TTTCAAGAGGTTGAAGCTGCGCCTCCCTTTCACTCTCTTCGAAAAGGAGCTGATGA
TG**GAGTTACTCAGGACTCATC**

SN 4: AAG-CTT6 (121bp)

GATGAGTCCTGAGTAACTTCTGGTATACAGATGCACTGAATATATCATTGAGTCTG
CACTCGTTTCTCTAGACGACACAATACATGTTCTGTATTGATGGTA**CTTGAATTGGT**
ACGCAGTC

SN 6: AAG-ACC2 (161bp)

GATGAGTCCTGAGTAAACCAGCACAACAACATCAAGCAAAGCAGAGGCTTCAACAT
CCTTCAAAGGATTTGGATTCTTCAAAACATTGAACATCACAAGGTTCAACAATCTCC
CCCTATTTGATGAAGACAAATCCCTGATG**CTTGAATTGGAACGCAGTC**

Figure 4.6. Sequences of fragments. Bold indicates sequences of the AFLP primers and the underlined parts are the sequences of SCAR primers designed.

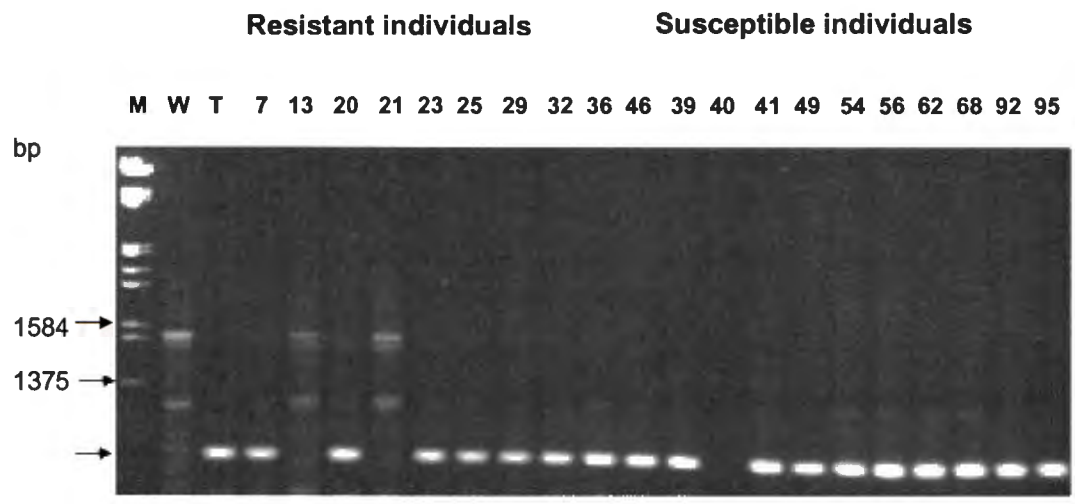

Figure 4.7. Agarose gel electrophoresis of amplification products of parents and individuals of bulks with SCAR marker SN 1 (E-AAC/M-CAT2). Arrow indicates the repulsion phase marker amplified by SN 1. Marker- Lambda DNA digested with *Eco*RI and *Hind*III. W: Wilk 2; T: Teebus; Nr. 7 to 95: Individuals of bulks.

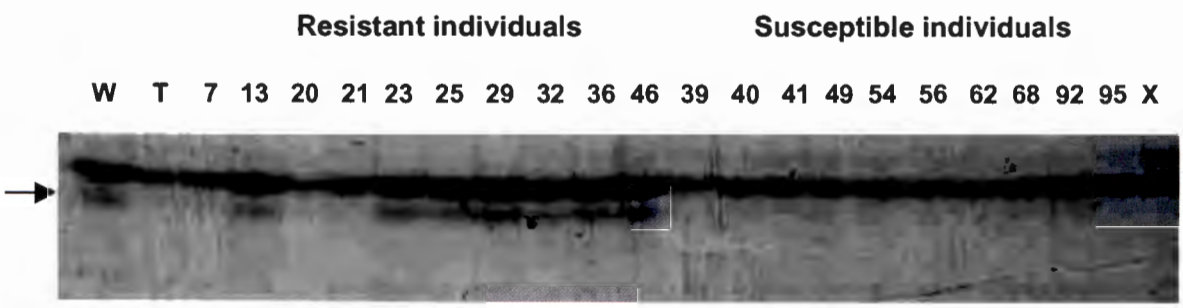

Figure 4.8. Amplification products of parents and individuals of bulks with SCAR marker SN 1-1 (E-AAC/M-CAT2). Separation of amplified products was under polyacrylamide gel electrophoresis conditions. Arrow indicates the coupling phase marker produced by SN 1-1. W: Wilk 2; T: Teebus; Nr. 7 to 95: Individuals of bulks; X: XAN 159.

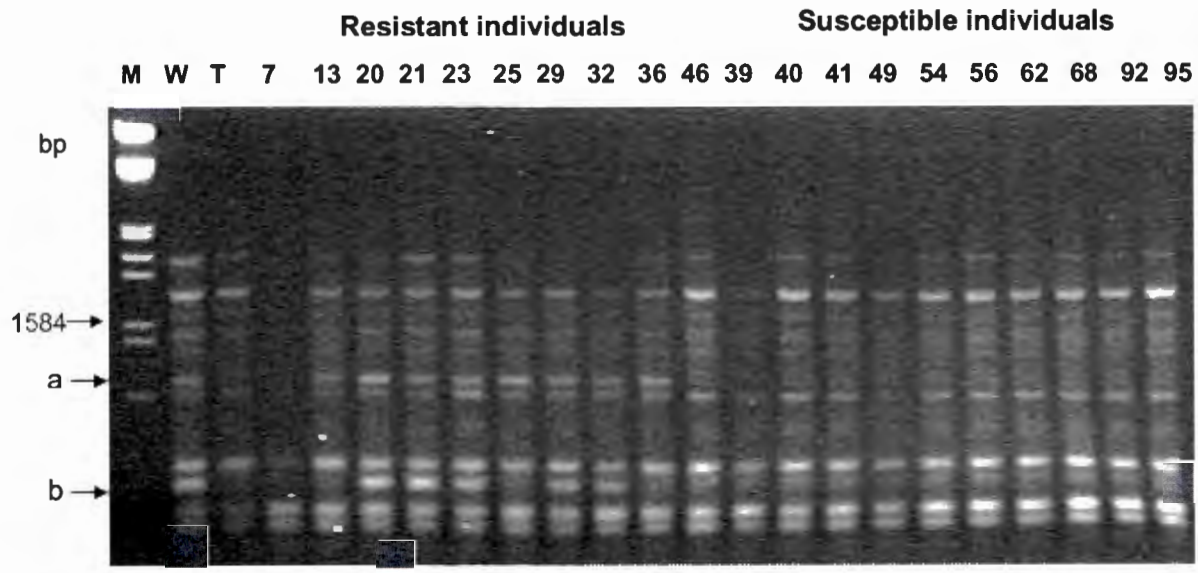

Figure 4.9. Amplification products of parents and individuals of bulks with SCAR marker SN 2A (E-AAC/M-CAT3). Separation of amplified products by agarose gel electrophoresis. The arrows indicate the two polymorphic bands amplified, with arrow (a) indicating SN 2A-600 and arrow (b) indicating SN 2A-300. Marker- Lambda DNA digested with *Eco*RI and *Hind*III. W: Wilk 2; T: Teebus; Nr. 7 to 95: Individuals of bulks.

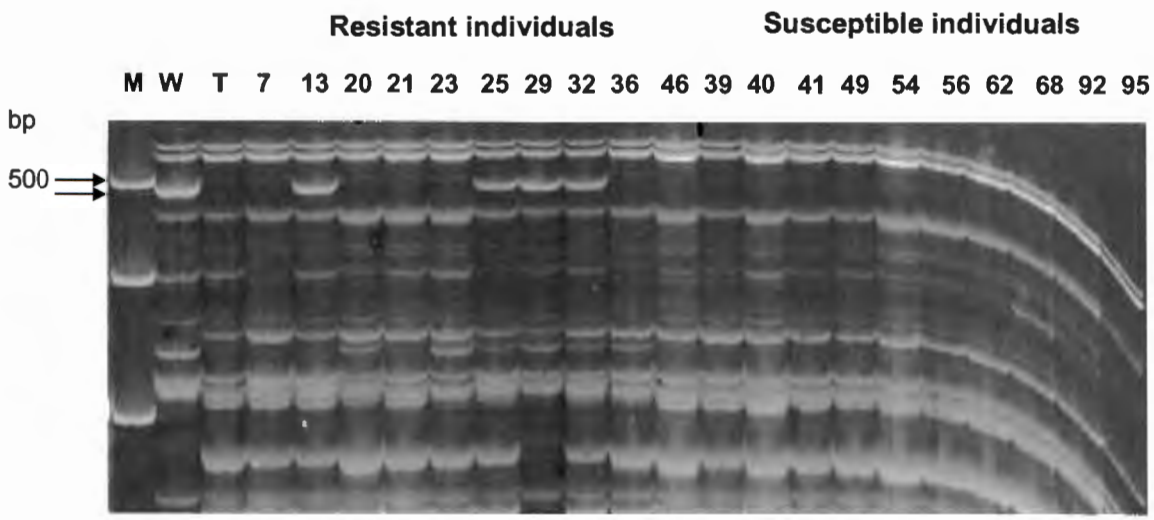

Figure 4.10. Amplification products of parents and individuals of bulks with SCAR marker SN 2B (E-AAC/M-CAT3). Separation of amplified products under polyacrylamide electrophoresis conditions. Arrow indicates the polymorphic band produced. Marker-100 bp ladder. W: Wilk 2; T: Teebus; Nr. 7 to 95: Individuals of bulks.

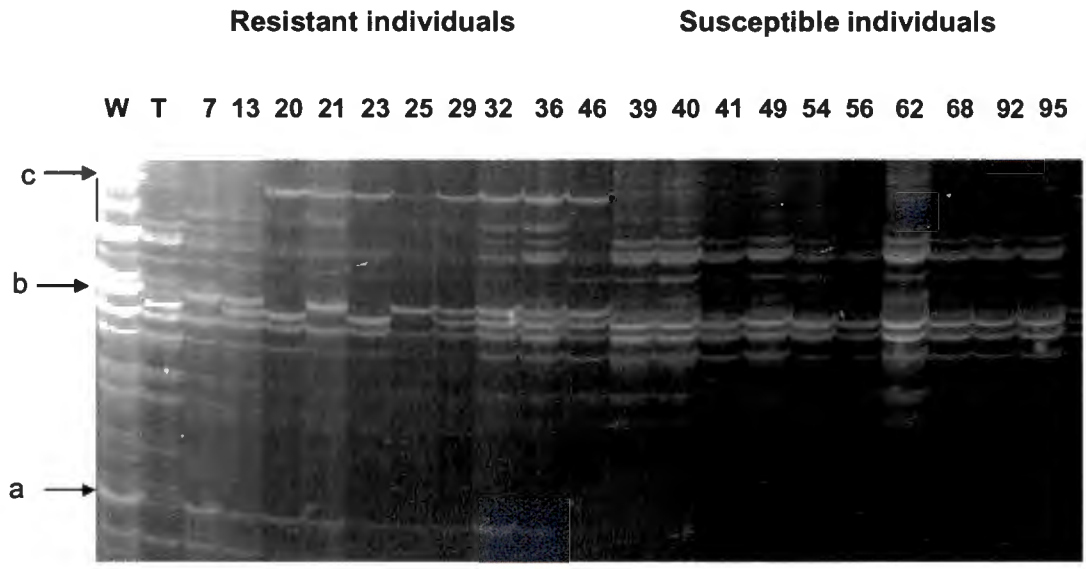

Figure 4.11. Amplification products of parents and individuals of bulks with SCAR marker SN 3 (E-AAC/M-CTC2) on a denaturing polyacrylamide gel. The arrows indicate the three polymorphic bands produced during amplification with (a) indicating SN 3-300, (b) indicating SN 3-400 and (c) indicating SN 3-450. W: Wilk 2; T: Teebus; Nr. 7 to 95: Individuals of bulks.

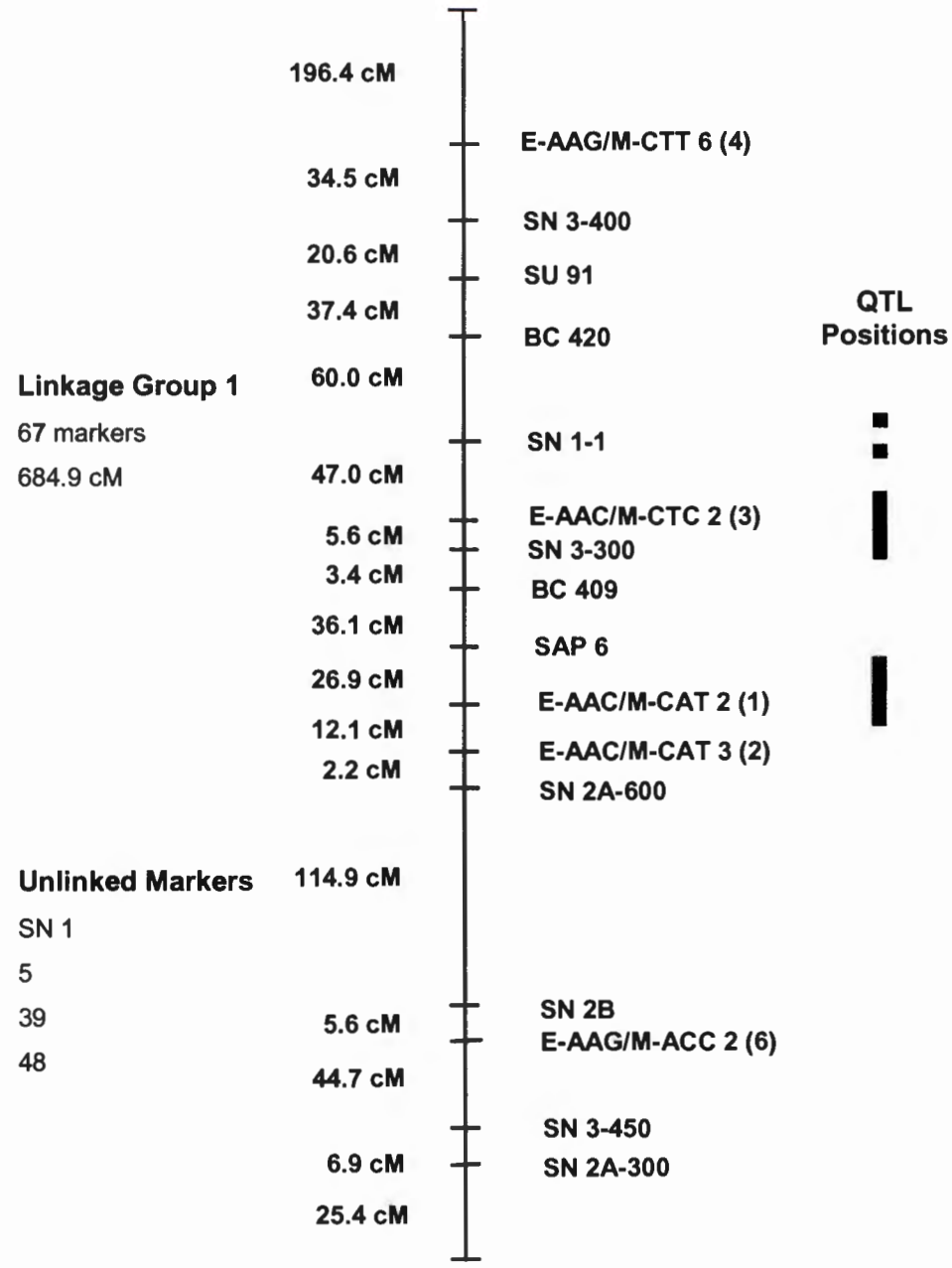

Figure 4.12 Partial linkage map with existing and developed SCAR markers. The numbers in brackets indicate the SCAR number to which the fragment was converted. The QTL positions are indicated by bars on the right side of the linkage group. The solid bars indicating the major QTLs and the dashed bar the minor QTL.

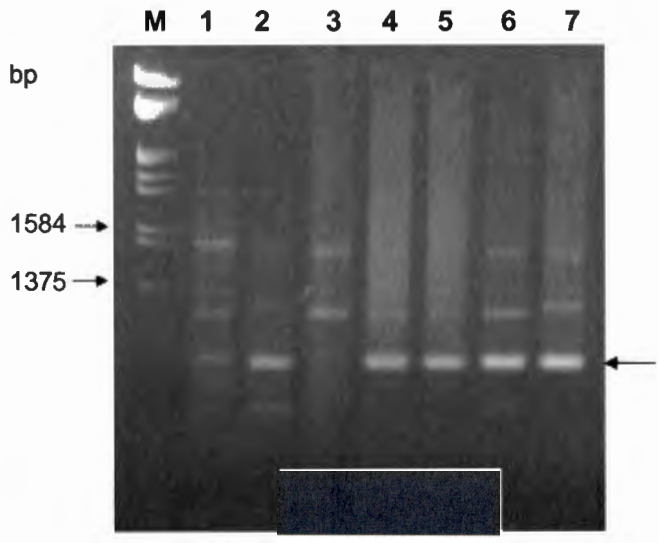

Figure 4.14. Agarose gel electrophoresis of amplification products of additional resistance sources with SCAR marker SN 1 (E-AAC/M-CAT2). Arrow indicates the polymorphic band produced. M: Lambda DNA digested with *Eco*RI and *Hind*III. Lane 1-Wilk 2; 2-Teebus, 3-XAN 159, 4-Vax 3, 5-Vax 4, 6 Vax 5 and 7-Vax 6.

Table 4.1 *Eco*RI/*Mse*I AFLP analysis data

	Parents	Bulks	Individuals of bulks (20)	Individuals of population (40)
Primers tested	20	20	10	10
Total fragments	1097	1097	666	598
Polymorphisms	337	164	228	220
Informative primers	20	20	10	10
Informative bands	-	119	86	79
Polymorphism rate (%)	30.7	14.9	34.2	36.8

Table 4.2 Sequences of SCAR primers developed

Primer	Sequence	No. of bases
SN 1F	TTAACATATCAAAACACAA	19
SN 1R	GAATTCAACAAAAAAGAA	18
SN 1-1F	TTAACATCCTCTTGCCAG	18
SN 1-1R	GAATTCTTCTAAGTGAATCA	20
SN 1-2F	TTAACATTCCCAACTTTC	18
SN 1-2R	GAATTCAACCTCAGATGA	18
SN 2AF	TTAACATCACGGTGTACG	18
SN 2AR	GAATTCAACCACAACATAGC	20
SN 2BF	TTAACATCACAATAAGTAGGG	21
SN 2BR	GAATTCAACATGGTCTATGG	20
SN 3F	GAATTCAACATCCAAATCC	19
SN 3R	TTAACTCCATCATCAGC	17
SN 3-1F	GAATTCAACATCCAAATCCG	20
SN 3-1R	TTAACTCCATCATCAGCTCC	20
SN 4F	TTAACTTCTGGTATACAGATG	21
SN 4R	GAATTCAAGTACCATCAA	18
SN 6F	TTAAACCAGCACAACAACAT	20
SN 6R	GAATTCAAGCATCAGGG	17

F: Forward primer

R: Reverse primer

Table 4.3 Linkage positions of AFLP markers and SCAR markers.

Marker Number	Primer combination	Distance (cM)	Linkage	R^2 (%) (P≤0.001)
51	E-AAG/M-ACA 6	33.8	R	31.41
54	E-AAG/M-ACA 12	56.0	R	39.87
1	E-AAC/M-CAA 1	27.5	R	21.89
18	E-AAC/M-CAT 4	0.0	R	7.29
19	E-AAC/M-CAT 5	10.5	R	7.29
49	E-AAG/M-ACA 4	11.3	R	7.15
25	E-AAC/M-CTG 1	4.5	C	53.48
35	E-AAG/M-CAT 1	3.3	C	62.08
21	E-AAC/M-CTC 1	10.6	C	49.78
24	E-AAC/M-CTC 5	4.4	C	53.48
42	E-AAG/M-CTT 6	2.1	C	61.55
4	E-AAC/M-CAA 4	6.7	C	61.55
57	E-AAG/M-ACC 3	4.4	C	62.08
34	E-AAG/M-CAG 8	9.1	C	57.35
20	E-AAC/M-CAT 6	10.2	C	43.54
72	SCAR SN 3-400	12.4	C	54.03
30	E-AAG/M-CAG 3	4.2	C	46.11
44	E-AAG/M-CTT 8	18.4	C	36.91
60	SU 91	20.1	C	18.06
40	E-AAG/M-CTT 4	14.1	C	36.91
50	E-AAG/M-ACA 5	3.2	C	46.30
61	BC 420	13.3	C	57.19
43	E-AAG/M-CTT 7	19.1	C	57.39
31	E-AAG/M-CAG 4	1.1	C	42.76
37	E-AAG/M-CTT 1	26.5	C	43.00
70	SN 1-1	7.0	C	64.71
47	E-AAG/M-ACA 3a	4.6	C	40.25
7	E-AAC/M-CAG 1	0.0	C	54.03
32	E-AAG/M-CAG 6	11.4	C	53.39

Marker Number	Primer combination	Distance (cM)	Linkage	R^2 (%) (P≤0.001)
8	E-AAC/M-CAG 2	11.3	C	57.93
14	E-AAC/M-CAG 8	8.7	C	54.03
10	E-AAC/M-CAG 4	4.0	C	66.48
22	E-AAC/M-CTC 2	6.8	C	81.17
71	SCAR SN 3-300	5.6	C	71.16
63	BC 409	2.1	R	10.61
36	E-AAG/M-CAT 2	5.7	C	71.16
13	E-AAC/M-CAG 7_1	10.2	C	71.16
2	E-AAC/M-CAA 2	18.1	C	64.12
62	SAP 6	0.8	R	5.73
23	E-AAC/M-CTC 3	7.1	C	57.39
12	E-AAC/M-CAG 6	10.1	C	66.48
46	E-AAG/M-ACA 2	8.9	C	75.76
16	E-AAC/M-CAT 2	1.2	C	76.16
33	E-AAG/M-CAG 7	0.0	C	70.77
29	E-AAG/M-CAG 2	2.3	C	70.77
41	E-AAG/M-CTT 5	8.6	C	70.71
17	E-AAC/M-CAT 3	1.1	C	66.48
11	E-AAC/M-CAG 5	1.1	C	71.16
65	SCAR SN 2A-600	8.8	C	54.78
6	E-AAC/M-CAA 6	11.1	C	49.21
3	E-AAC/M-CAA 3	16.3	C	28.44
15	E-AAC/M-CAT 1	15.0	C	46.85
38	E-AAG/M-CTT 2	19.0	C	46.30
26	E-AAC/M-CTG 2	18.7	C	43.00
58	E-AAG/M-ACC 4	12.9	C	46.85
45	E-AAG/M-ACA 1	13.1	C	34.09
69	SCAR SN 2B	5.6	C	40.40
56	E-AAG/M-ACC 2	0.0	C	40.40
9	E-AAC/M-CAG 3	1.1	C	40.40
55	E-AAG/M-ACC 1	15.9	C	43.54

Marker Number	Primer combination	Distance (Cm)	Linkage	R^2 (%) (P≤0.001)
27	E-AAC/M-CTG 5	15.6	C	49.78
52	E-AAG/M-ACA 7	6.7	C	43.64
73	SCAR SN 3-450	6.9	C	66.48
66	SCAR SN 2A-300	1.3	C	57.78
59	E-AAG/M-ACC 11	25.4	R	50.34
53	E-AAG/M-ACA 11	-	C	36.91
	684.9 cM	**67 markers**		

R^2: Coefficient of determination

P: Probability

C: Coupling phase

R: Repulsion phase

Table 4.4 Linkage data of AFLP fragments and developed SCAR markers.

AFLP marker	Linkage phase	R^2(%) (P≤0.001)	SCAR	Linkage phase	R^2(%) (P≤0.001)
E-AAC/M-CAT 2	C	76.16	SN 1	R	15.02
			SN 1-1	C	64.71
E-AAC/M-CAT 3	C	66.48	SN 2A-600	C	57.78
			SN 2A-300	C	54.81
			SN 2B	C	40.40
E-AAC/M-CTC 2	C	81.17	SN 3-300	C	71.16
			SN 3-400	C/D	54.03
			SN 3-450	C	66.48
E-AAG/M-CTT 6	C	61.55	SN 4	NP	NP
E-AAG/M-ACC 2	C	40.40	SN 6	NP	NP

R^2: Coefficient of determination

P: Probabilty

C: Coupling phase

R: Repulsion phase

C/D: Co-dominant

NP: Not polymorphic

Table 4.5 Developed SCAR markers in additional CBB resistance sources.

SCAR	SN 1	SN 1-1	SN 2A-600	SN 2B	SN 3-300
Resistance Source					
Wilk 2	-	+	+	+	+
Teebus	+	-	-	-	-
XAN 159	-	-	+	+	+
Wilk 6	+	+	-	-	+
GN Nebr. sel 27#1	+	-	-	-	-
Vax 3	+	+	+	-	-
Vax 4	+	+	-	-	-
Vax 5	+	+	-	-	-
Vax 6	+	+	-	-	-

+: Positive reaction

- : Negative reaction

CHAPTER 5

DISCUSSION

The phenotype of the plant can be divided into two types of variance, genetic and environmental. The genetic variance is made up of additive, dominance and epistatic effects. These effects are able to estimate the heritability of the trait and able to predict the response of the trait to selection. It is also able to estimate the minimum number of genes controlling the trait and the identification of molecular markers linking to the trait can be used to identify the genes (McClean, 1998).

A molecular marker is a readily detectable sequence of DNA or protein whose inheritance can be monitored. There are various desirable properties of molecular markers which include high numbers of polymorphisms, co-dominant inheritance, occurrence throughout the genome, easy, fast and inexpensive to generate and reproducibility within and between laboratories (Ford-Lloyd and Painting, 1996). The AFLP technique is highly sensitive, highly reproducible and is widely applicable.

In this study the existing sequence characterized amplified region (SCAR) markers, SU 91, BC 420, BC 409 and SAP 6 were tested for linkage to common bacterial blight (CBB) resistance genes and showed low linkages to CBB resistance quantitative trait loci (QTL). These markers are used in the breeding programme at ARC-GCI (Potchefstroom, South Africa) for the identification of CBB resistant cultivars, as they are able to select for three QTL for CBB resistance. SAP 6 showed a very low linkage to CBB resistance in the F_2 population used and is also not useful as a marker in marker assisted selection (MAS) as it is present in the susceptible cultivars and therefore not targeting a resistance QTL. SU 91 also showed a low linkage to CBB resistance (18.06%) with BC 420 (57.19%) being the most highly linked of the existing SCAR markers available for CBB resistance. The SCAR marker, BC 409 was tested but also showed a very low linkage of 10.61%. There is therefore a need for more tightly linked markers for the identification of CBB resistance QTL that are applicable in the breeding material used at the ARC-GCI. In this study, six amplified fragment length polymorphism (AFLP) fragments were used for SCAR primer development. Nine SCAR primer pairs were developed of which five primer pairs amplified eight polymorphic bands. The SCARs showed between 15.03% to

71.16% explanation of the phenotypic variation to CBB resistance. Only one SCAR marker was unlinked on the map (SN 1).

The F_2 population was used in this study for mapping and identifying QTL for CBB resistance. The advantage of using a F_2 population for mapping is that the additive and dominant gene actions can be measured at a specific locus. Recombinant inbred lines (RILs) can only measure the additive gene action, as they are essentially homozygous. The problem with using a F_2 population for mapping is that the population is not eternal and the source of tissue for isolation of DNA will be exhausted at some point in time, leading to the use of another population for mapping (McClean, 1998). In this study the F_2 population used was highly selective as the parents differed significantly with respect to CBB resistance and the resistant progeny could be easily selected on the phenotypic level during the breeding process (D. Fourie, 2003, personal communication).

It has also been recommended not to confine the selection of F_2 plants to marker genotypes that are completely homozygous for the desired allele or heterozygous for a few markers (Chao and Ukai, 2000). When several genes are to be pyramided using MAS, Beaver and Macchiavelli (1998) suggested waiting till the F_4 generation to screen lines for linked markers. Screening in the F_4 would improve the probability of identifying lines with the desired genotype and would drastically reduce the number of lines that need to be evaluated. Traditional gene pyramiding requires the incorporation of several different resistance genes into a single cultivar. Due to the race specificity of many of these genes, screening nurseries have to be systematically inoculated with different races of the pathogen in an attempt to ensure that gene combinations are being maintained.

Based on the criteria of enzyme cutting efficiency, availability of primers and optimization of technique, *Eco*RI and *Mse*I were chosen as the enzymes to be used for the AFLP technique in this study. Different regions of the plant genome can be targeted and using different enzyme combinations increases the possibility of finding a linked marker. In the plant genome the base sequence AT is more commonly followed by AG or TC (Powell *et al.*, 1996). Examples of rare-cutting enzymes used in AFLP analysis include *Eco*RI, *Mlu*I, *Ase*I, *Hind*III, *Apa*I and *Pst*I. The most commonly used frequent cutters are *Mse*I and

*Taq*I. However, the DNA of most eukaryotes is AT-rich making *Mse*I (/TTAA) the preferred frequent cutter for AFLP analysis (Blears *et al.*, 1998). A+T-rich recognition sequences such as those of *Eco*RI (G/AATTC) or *Mse*I are more frequent in G+C-poor genomes, and conversely G+C-rich recognition sequences, such as that of *Apa*I (GGGCC/C), are more frequent in G+C-rich genomes. *Hind*III (A/AGCTT) appears to be the most suited to digest genomic DNA with a G+C content of 40-50 mol% (Janssen *et al.*, 1996). In this study, the analysed sequences of the fragments showed that they were rich in adenine and thymine, explaining the successful digestion of the genomic DNA by the enzymes *Eco*RI, and *Mse*I.

The basis of the observed polymorphism in an AFLP reaction in terms of nucleotide variation may be single base mutation in restriction site sequences or in sequences complementary to the adaptor and selector nucleotides (Matthes *et al.*, 1998). AFLPs allows the specific co-amplification of high numbers of restriction fragments whose numbers may be controlled by the cleavage frequency of the rare cutter enzyme, the number and nature of selective bases. The number of fragments that can be analyzed simultaneously, however, is dependent on the resolution of the detection system. Most AFLP fragments correspond to unique positions on the genome and are characterized by its size and its primers required for amplification (Vos *et al., 1995*). The AFLP technique also permits detection of restriction fragments in any background or complexity, including pooled DNA samples and cloned (and pooled) DNA segments.

In this study AFLPs have been used in conjunction with bulk segregant analysis (BSA), which is able to target resistance genes more efficiently than the AFLP technique alone. This has also been applied in a wide variety of studies including soybeans (Lin *et al.*, 1996; Mienie *et al.*, 2002), roses (von Malek *et al.*, 2000), rice (Jia *et al.*, 2001), maize (Dussle *et al.*, 2000; Agrama *et al.*, 2002), brassica (Negi *et al.*, 2000) and sunflower (Horn *et al.*, 2003). Low levels of polymorphisms have been reported in various studies.

In this study although only ten primer combinations were tested on the individuals of the F_2 population, 220 polymorphisms were detected between the parents and individuals of the population of which 79 linked polymorphic AFLP markers to CBB resistance were obtained. von Malek *et al.* (2000) used 114 primer combinations that identified only

seven polymorphic AFLP markers. Eight AFLP loci were identified in rice of which only two were closely linked to the gene of interest (Jia *et al.*, 2001). Mapping genes for sorgum downy mildew in maize identified three AFLP markers that were closely linked to the trait of interest (Agrama *et al.*, 2002). Horn *et al.* (2003) used 48 primer combinations to identify 17 polymorphic AFLP markers of which only two were closely linked. Negi *et al.* (2000) used 16 primer combinations to identify seven polymorphic AFLP fragments. Compared to other studies using AFLP with BSA, very high levels of polymorphic fragments were detected in this study.

Meksem *et al.* (2001) found that AFLP markers contain ten to twenty times higher polymorphism rate than expected. AFLPs are efficient in detecting polymorphism but become costly and time laborious for large scale screening of large numbers of genotypes. Therefore, the conversion of AFLP markers closely linked to resistance genes is an important step to implement useful markers for MAS and map based cloning. It is so far unknown if resistance genes cluster due to linkage or whether some of them are identical and display pleiotropy. It is difficult to convert an AFLP fragment into a SCAR marker, as a polymorphism has to be obtained within the enzyme restriction site. These primers have to amplify a polymorphism within the original polymorphism detected by the AFLP primers.

The most common method of determining the association between any of the markers and the quantitative trait is by analyzing the phenotypic and genotypic data by a one-way analysis of variance and regression analysis (McClean, 1998). If the variance for the genotype class is significant ($P \leq 0.01$), then the molecular marker used to define the genotype class is considered to be associated with a QTL. The R^2 value is considered to be the amount of total genetic variation that is explained by the specific molecular marker. This study was able to identify markers explaining 81.17% to 5.73% of the phenotypic variation to CBB resistance. Based on these results 58 AFLP markers were selected for mapping onto a linkage map based on the probability values. The SCAR marker SAP 6 showed low linkage to CBB resistance (5.73%) in the mapping population used for this study. It has been suggested that SAP 6 is a false positive (Miklas, P.N., 2002, personal communication) and it does not seem to target the resistance QTL as reported.

To derive linkage relationships among markers the programme MAPMAKER-EXP is commonly used (McClean, 1998). This programme is based on the maximum likelihood method where the output is a linear relationship among markers and the distance between markers is measured in centimorgans (cM). The linkage map obtained in this study was 684.9 cM in length containing 67 markers comprising of the four existing SCAR markers, 56 AFLP markers and four developed SCAR markers. The four developed SCAR markers that were linked in this study produced seven polymorphic bands. The linkage map is not a true representative of the bean core linkage map as only one linkage group was obtained instead of 11 groups. The bean core map is 1226 cM in length and contains 550 molecular markers of which 46 are unassigned. There are 240 framework markers and 329 markers in intervals. Once markers have been detected that are associated with the trait of interest QTL selection can be performed on lines within the population. A QTL is basically the locus that controls the quantitative trait. Only those lines containing the alleles with positive effect on quantitative traits will be advanced. The breeder would like to identify as early as possible those lines containing QTL alleles that contribute to a high value of the trait under selection. Molecular markers could aid in a faster detection of the preferred individuals in the population, as no phenotypic evaluations are required.

The main reason for obtaining only one linkage group was due to BSA being used and the highly selective nature of markers chosen for mapping. BSA uses plants from the extremes of the phenotypic spectrum of a segregating population by pooling the DNA from these plants in two bulks. Each bulk contains individuals that are identical for a particular trait but seemingly heterozygous for all other regions and traits. BSA is used for rapid screening of many loci and therefore the identification of polymorphisms (Michelmore *et al.,* 1991). BSA in conjunction with the AFLP technique amplified a total of 220 polymorphic bands in the individuals of the population, of which only 79 were selected for regression analysis. After regression analysis 58 bands were selected for mapping with the probability of less than 0.01 showing that there is a significant relationship between CBB and the predictor variables at the 99% confidence level.

Another possible reason for one linkage group is that the markers were selected for high linkage to CBB resistance and therefore only targets the clustered resistance genes in a particular region of the genome. Meyers *et al.,* 1998 identified duplicates of AFLP

markers in the same chromosomal region. In the maize genome, genes conferring resistance to different pathogens are often clustered in the same chromosomal region (McMullen and Simcox, 1995). The disregarded polymorphic bands could have targeted other regions of the genome and therefore contributed to resolving the one linkage group into smaller more distinct linkage groups.

It is also possible that only a portion of the genome was being targeted with the primer combinations as the selective nucleotides of the primers only differed by three bases and therefore resulted in one linkage group. Furthermore another reason for obtaining only one linkage group would be that the individuals used in the F_2 population was not selected randomly but selected on the basis of their phenotypic evaluation. The entire F_2 population was also not used for mapping. Sixty individuals were chosen on the basis of the number of samples that could be analysed with relative ease simultaneously on the same gel. The markers were tested and found to be statistically skewed which could also explain the resulting linkage group. The F_3 phenotypic data was unavailable and therefore the heterozygotic and homozygotic individuals in the F_2 population could not be identified. It is possible that QTL on other chromosomes could have been missed with all the markers clustering on only one linkage group.

Common beans lend themselves as a good model system to study QTL that affect yield as it has a small genome of which 60% is single copy sequences (Talbot *et al.*, 1984) which is an advantage for MAS and linkage map analysis. During this study, two major QTL were detected on the linkage group having both dominant and additive effects. Another minor QTL was also detected linked to CBB resistance. The two AFLP fragments E-AAC/M-CAT 2 and E-AAC/M-CTC 2 were able to significantly explain the phenotypic variation of the QTL to which they were tightly linked. The minor QTL was additive and closely linked to the SCAR marker SN 1-1 explaining 64.71% of the phenotypic variation of the QTL. CBB is reported to have a few major QTLs and in this study three QTLs could be distinguished in the linkage group (Figure 4.12). This correlates with the findings of Beebe (1989) who suggested that CBB is a complex disease that is inherited quantitatively and involves a few major genes. One, two or three genes appear to confer resistance depending on sources and methods of evaluation (McElroy, 1985; Drijfhout and Blok, 1987; Scott and Michaels, 1988; Silva, 1988).

There have been a large number of studies to identify the number and to determine the phenotypic variation of CBB resistance QTLs. Nodari *et al.* (1993a) was able to explain 75% of the phenotypic variation by identifying four QTL with one major effect QTL explaining 35% of the variation. A QTL on linkage group B7 was found in a region that contains the phaseolin locus *Phs* (Nodari *et al.*, 1993b). Three QTL explaining 77% of the phenotypic variation was identified in the resistance source GN Nebr. #1 sel 27 (Miklas *et al.*, 1996b). Bai *et al.* (1997) identified two independent QTL that explained 80% of the variation of CBB resistance in the resistance source OAC 88-1 having two resistance genes. Jung *et al.* (1997) found four QTL accounting for 18% to 53% of the phenotypic variation of CBB resistance and the *V* locus was linked to a QTL with major effect. The three resistance sources, GN Nebr. #1 sel 27, XAN 159 and OAC 88-1 have each contributed two independent QTL with major effect for CBB resistance. There are SCARs linked with five of the six QTL available for MAS. The markers SAP 6, SU 91 and BC 420 select for three independent QTL on linkage groups B6, B8 and B10 which contribute a substantial effect on CBB resistance (Miklas *et al.*, 2000).

In this study it was therefore expected to detect at least three major QTL and a possible minor QTL with the mapping population used. Chao and Ukai (2000) reported that for detecting four QTL, the population size for a single marker is 67 plants in the F_2 and F_3 populations that are required for obtaining at least one plant with the desired trait genotype with 5 cM being the map distance between a QTL and its right or left marker. It was suggested that 39 plants of the F_2 population should be used. However, optimal sample size also depends on costs, time and also labour constraints. However, in this study 60 plants of the F_2 population were used for this study, which is greater than the suggested population size of 39 plants. MAPMAKER/QTL has also been used for fine-scale QTL analyses of several morphological and physiological traits of rice using the high-density linkage map and DNA markers (Mohan *et al.*, 1997). It is generally difficult to determine the precise location and gene action of individual QTL. To overcome these problems, construction of well characterized genetic stocks, such as near isogenic lines (NILs), carrying one or multiple chromosomal segments of one parental line in the genetic background of another parental line is needed.

The optimization of DNA template concentration is usually in the range of five to 500 ng. A higher concentration can lead to impurities problems (Ford-Lloyd and Painting, 1996).

It is therefore necessary to maintain a standard concentration to achieve consistent results. In this study the optimal DNA concentration for all SCAR primers was found to be 200 ng. The Mg^{2+} ion concentration may have a qualitative effect upon banding patterns and therefore needed standardization. The optimal Mg^{2+} ion concentration in this study was found to be between 2-4 mM depending on the SCAR primers. The higher the Mg^{2+} ion concentration, the more specific is the primer binding properties. The reasoning behind the two annealing temperatures in one PCR programme was to increase the intensity of the polymorphic band produced by the SCAR primer set of SN 1-1 (E-AAC/M-CAT 2). The first annealing temperature for a few cycles was higher to induce specific priming for the polymorphic sequence and to reduce the non-specific banding pattern. The second annealing temperature was essentially to produce a high number of copies of the selected sequence, thereby resulting in a brighter polymorphic band.

In this study it was found that from nine AFLP converted to SCAR primers, five were successful in detecting polymorphisms. Four SCARs were linked onto the linkage map. The SCAR SN 1 (E-AAC/M-CAT 2) became a repulsion phase marker from a coupling phase AFLP fragment and explained 15.03% of the phenotypic variation to CBB resistance. It was clearly not the same fragment that was isolated as it was in the wrong phase, had a lower explanation of the phenotypic variation than the AFLP fragment and did not map on the same linkage group. It was also much smaller than the AFLP fragment from which it was derived. A single band on a gel can be comprised of different amplification products. This can be due to the type of electrophoresis used. Both agarose and polyacrylamide gel electrophoresis separates fragments based on size. While being able to separate DNA quantitatively (i.e. according to size) it can't separate equal sized fragments qualitatively (i.e. according to base sequence). It is therefore possible to obtain co-migration of the same size fragments that differ in sequence occurring as a single band on a gel. This could lead to the wrong fragment being cloned and sequenced.

The SCAR SN 1-1 (E-AAC/M-CAT 2) was able to give a better explanation of the phenotypic variation (64.71%) than SN 1 to CBB resistance but did not map close to the AFLP fragment from which it was derived. This SCAR marker is positioned close to an additional minor QTL that is not significantly linked to CBB resistance but has an additive

effect when coupled with the major resistance QTLs of CBB leading to an increase in disease resistance for the plant. This explains the higher disease resistance rating that Wilk 2 obtains in greenhouse trials as compared to XAN 159 as this SCAR marker is only present in Wilk 2. It was however able to differentiate between the resistance cultivars Wilk 2 and XAN 159 by amplifying a fragment in Wilk 2 and not in XAN 159. This SCAR can now be used to differentiate between the two NILs developed, which used Wilk 2 and XAN 159 as donor parents and Teebus as the recurrent parent. This was previously not possible as both NILs show the same pattern on both the phenotypic and molecular levels. SN 1-1 can be used in the breeding programme to pyramid the resistance genes from Wilk 2 and XAN 159 into a single NIL with Teebus for an increased resistance to CBB. This NIL would then be able to replace the susceptible Teebus cultivar and would not show the serious yield losses that the cultivar incurs due to CBB susceptibility.

The two SCAR marker fragments SN 2A-600 (E-AAC/M-CAT 3) and SN 3-300 (E-AAC/M-CTC 2) both mapped relatively close to the original AFLP fragment from which it was derived (2.2 cM and 5.6 cM respectively). Mienie *et al.* (2002) identified two SCAR markers in soybean mapping 3.8 cM and 2.4 cM respectively from resistance QTL. SN 2A-600 explained 57.78% of the phenotypic variation to CBB resistance compared to the AFLP fragment (66.48%) showing that the SCAR marker explains less of the phenotypic variation of the QTL than the AFLP fragment. The SCAR SN 3 (E-AAC/M-CTC 2) showed three polymorphic bands, all of which significantly explained the phenotypic variation of CBB resistance. For optimal separation of the bands produced during PCR, the samples were run under denaturing PAGE conditions. The fragment SN 3-300 was the closest mapped polymorphic band amplified by SN 3 primers and showed the highest linkage from all three bands, i.e. was linked 71.16% to CBB resistance. This marker had the same linkage as the original AFLP fragment and was positioned between the AFLP fragment from which it was derived and the existing SCAR marker BC 409 which explained 10.61% of the phenotypic variation to CBB resistance QTL. The SCAR fragment explains more of the phenotypic variation to CBB resistance than the existing SCAR marker (BC 409) and could therefore be used as a closer and tighter linked marker of the major resistance QTL.

The fragment SN 3-400 is a co-dominant marker, which could be useful in detecting the heterozygote individuals of the population used for mapping. SN 3-400 and SN 3-450 seems to target additional minor QTLs positioned at the ends of the linkage group. These QTLs could be on other linkage groups of the bean genome that were not targeted in this study.

A large number of bands of which three were polymorphic were amplified with the SCAR primer set SN 3 (E-AAC/M-CTC 2). The designed sequence primers amplifying multiple fragments could be indicative of the primers not being specific for one locus. A possible solution would be the design of primer sequences that are longer in bases and thereby increasing the selectivity or selecting primers to amplify shorter fragments. The fragments being amplified are most likely repeat sequences that are similar or identical to the AFLP fragment chosen and therefore amplify in the same reaction. For SCAR marker SN 2A (E-AAC/M-CAT 3), although a single locus is not amplified, the 600 bp fragment cosegregated with CBB resistance variation. The same can be applied for the SN 3 (E-AAC/M-CTC 2) and the 300 bp fragment amplified.

The SCAR marker SN 2B was developed from a tandem sequence of the AFLP fragment E-AAC/M-CAT 3 and was not mapped close to the fragment from which it was derived. It is therefore not amplifying the original AFLP fragment. However, it did map close to the fragment E-AAG/M-ACC 6 (5.6 cM) from which the SCAR SN 6 was developed. The SCAR marker SN 2B explained the same percentage of the phenotypic variation to CBB resistance as the AFLP fragment (40.40%). SN 6 was not polymorphic when tested with the parents therefore it is possible to use the SCAR marker SN 2B instead of SN 6 to target the same part of the resistance QTL situated nearest.

On the linkage map two major QTL were identified (Figure 4.12) situated close to the AFLP fragments E-AAC/M-CTC 2 and E-AAC/M-CAT 2. The minor QTL was situated close to the developed SCAR marker SN 1-1 (E-AAC/M-CAT 2). BC 409 is situated close to the major resistance QTL but shows a very low linkage to the QTL. The developed SCAR marker SN 3-300 (E-AAC/M-CTC 2) is situated closer to the resistance QTL and has a much higher explanation of the phenotypic variation as caused by the

resistance QTL. SN 3-300 can therefore be used for screening cultivars instead of BC 409 to target the major QTL for CBB resistance.

The other major resistance QTL can be targeted by the developed SCAR marker SN 2A-600 (E-AAC/M-CAT 3) although it is positioned 14.3 cM away. The SCAR is able to explain 57.78% of the phenotypic variation caused by the QTL. SU 91 shows low linkage to CBB resistance (18.06%) in the population used for mapping. The SCAR marker, SN 3-400 (E-AAC/M-CTC 2) is positioned 20.6 cM away from SU 91 and shows a much higher linkage to CBB resistance (54.03%) as well as being a co-dominant marker. It could therefore be possible to replace SU 91 with a developed SCAR marker that is linked higher; able to detect heterozygotic individuals, can be amplified in the same reaction and screen in one gel as SN 3-300. The SCAR SN 1-1 (E-AAC/M-CAT 2) is able to detect a minor QTL responsible for the additional resistance that Wilk 2 shows that is absent in XAN 159. This minor QTL is responsible for a higher disease resistance rating when coupled with the major resistance QTLs and can therefore be used to differentiate between resistance QTLs derived from Wilk 2 and XAN 159.

This study was able to successfully convert five SCAR primers into polymorphic markers from nine SCARs. This was a high rate of conversion of SCAR primers from AFLP fragments into successful polymorphic markers. The conversion of AFLP markers into PCR-based markers has been attempted for several species but has not been successful in all studies. Techniques such as enzyme restriction and southern analysis have been used to obtain polymorphisms from non-polymorphic SCAR products. Shan *et al.* (1999) was not successful in obtaining polymorphisms from the SCAR primers even after restriction digestion by enzymes. Horn *et al.* (2003) was also unable to detect any polymorphisms from the converted SCAR markers. Meksem *et al.* (1995) found polymorphisms after restriction digestion of the PCR products of the SCAR primers. Cho *et al.* (1996) used Southern analysis to successfully convert the cloned AFLP fragments into single copy sequences. The successful conversion of a SCAR marker from the most closely linked AFLP fragment was accomplished by Jia *et al.* (2001) in rice and by von Malek *et al.* (2000) in roses. Negi *et al.* (2000) was able to convert an AFLP fragment into a simple codominant SCAR marker. Four AFLP markers were cloned and converted to SCAR markers for sorghum downy mildew in maize (Agrama *et al.*, 2002). From seven AFLP fragments two were successfully converted into SCAR markers explaining

41% and 42% of the gall index variation for *Meloidogyne javanica* in soybean (Mienie *et al.*, 2002). AFLP fragments were converted to co-dominant STS markers in wheat (Guo *et al.,* 2003). From ten AFLP bands six STS markers were able to be generated which identified eight insertion/deletion polymorphisms, two microsatellites and eight SNPs (Meksem *et al.*, 2001).

MAS for resistance to CBB using molecular markers can improve selection efficiency for resistance due to the low heritability of CBB and the independent QTL for resistance to different bacterial diseases. Molecular markers have several advantages over phenotypic markers, including carrying out selection not directly on the trait of interest but on molecular markers linked to that trait, thereby improving the efficiency of conventional breeding. The closer a marker is linked to a specific resistance gene, the higher the probability of it being duplicated with the resistance gene during evolution. A marker developed for a gene in one cross may not be useful in other crosses even though the same gene may be segregating in the second cross, unless the marker is from the gene itself. It would therefore be recommended to develop markers as close as possible to the resistance gene or to develop close flanking markers for the gene itself. Using markers makes it possible to concentrate the selecting effort on recessive alleles or some alleles that have small effects but have important economic value.

The use of SCAR markers instead of the AFLP technique is very useful in terms of time, labour and costs. SCARs are more user friendly, quicker to use and cost effective when screening large numbers of genotypes, as compared to the AFLP technique. These simple PCR based markers are invaluable in plant breeding programmes as they are able to reduce or eliminate the time required for phenotypic evaluations, are not affected by environmental conditions and allow more selections in a season of individuals than by conventional breeding. It is also not affected by the plant growth stage and pathogen infection and rating of plants is not required, thereby saving both time and labour costs.

The application of simple PCR markers enables a faster DNA isolation for a high number of individuals required for mapping. The polymorphisms revealed by simple PCR markers could be revealed on agarose gels, where no radioactivity or silver staining is required to visualize the results. It is possible to reduce costs by using simple PCR markers instead of AFLPs. Resistant individuals of different populations can harbour

different resistance alleles of the same gene, and cosegregation of these markers with the resistance trait in each population is preconditioned.

The deployment of resistant cultivars is the most effective and economical way of controlling diseases; so breeding for resistant cultivars is a priority for dry bean improvement. DNA markers linked to known resistance genes could be used as a tool for identifying and mapping new resistance genes. This study was part of a programme aimed at developing NILs for effective disease reduction in the field. The most effective way to do this would be to use DNA markers as a selection tool. The success of MAS depends ultimately on identifying marker(s) as close to the gene as possible for its utility across all populations.

CHAPTER 6

CONCLUSIONS

The common bean is one of the major sources of dietary protein in Latin America and Africa (Evans, 1986). Common bacterial blight (CBB) is a serious concern in South Africa as it causes a substantial yield loss on susceptible dry bean cultivars. The most effective control of this disease is the use of resistant cultivars. Unfortunately, all local commercial cultivars are highly susceptible to CBB. Marker assisted selection (MAS) enhances breeding efficiency by enabling plant breeders to select indirectly for genes affecting traits. This eliminates the time and space needed for phenotypic evaluations.

Therefore, one of the main aims of the breeding programme at the ARC-Grain Crops Institute (Potchefstroom, South Africa) is to improve the resistance of these local cultivars by using a backcross breeding programme. Wilk 2, a resistant cultivar shows a higher resistance to CBB than XAN 159 and it is therefore thought that Wilk 2 possesses an additional resistance quantitative trait loci (QTL) than XAN 159. Teebus is a local commercial cultivar that is highly susceptible to CBB, but is used extensively in the canning industry. The backcross programme produced near isogenic lines (NILs) with Teebus as the recurrent parent and Wilk 2 and XAN 159 separately as the donor resistance parent. These NILs have shown a vast improvement in resistance to CBB during field tests. Both Wilk 2 and XAN 159 have identical banding patterns when tested with the existing sequence characterized amplified region (SCAR) markers for CBB resistance. These NILs also can not be differentiated on the phenotypic level, as they look identical in the field. One of the aims of this study was to identify the additional QTL if present and develop a marker that could be used to pyramid the Wilk 2 and XAN 159 derived resistance QTLs.

A combination of amplified fragment length polymorphism (AFLP) and bulk segregant analysis (BSA) was used for the identification of markers closely linked to CBB resistance QTLs. A high number of polymorphisms were detected, making AFLP an efficient technique in targeting resistance genes for CBB. Two significant major QTLs were identified and one minor QTL linked to CBB resistance. The SCAR marker SN 1-1 (E-AAC/M-CAT 2) targets the minor additional resistance QTL only present in Wilk 2 and

is able to explain 64.71% of the phenotypic variation of the QTL. This SCAR marker can be used in future studies to differentiate between the Wilk 2 and XAN 159 derived NILs. The minor QTL has additive effects when coupled with the major QTL of CBB resistance, thereby explaining the higher disease rating of Wilk 2. The other polymorphic SCAR markers explained larger portions of phenotypic variations to CBB resistance and seem to target the QTLs for CBB resistance. The most significant phenotypic explanation by a SCAR marker is SN 3-300 (E-AAC/M-CTC 2) with 71.16% and is positioned 5.6 cM away from the original AFLP fragment. It is closely linked to the major resistance QTL. The other major resistance QTL is found near the fragment E-AAC/M-CAT 2 and is able to explain 76.16% of the phenotypic variation. The SCAR marker SN 2A-600 (E-AAC/M-CAT 3) is able to explain 57.78% of the phenotypic variation caused by the second major resistance QTL. Therefore, all three QTLs identified in this study can be targeted using the SCAR primers developed significantly explaining the phenotypic variations for CBB resistance.

More than 30 random amplified polymorphic DNA (RAPD) markers and five SCAR markers have been identified linked to 17 different disease resistance genes in the common bean. This makes the feasibility and potential for MAS in disease resistance breeding clearly possible. When selecting with regard to quantitative traits, using molecular markers can enhance selection efficiency. Since the selection of the marker type, unlike selection based on phenotype, can be performed in a laboratory without restrictions imposed by the growing season, it is free from the effects of environmental variation. In practice, it is recommended to use flanking markers for MAS unless the map distance between a QTL and its neighboring marker is very small.

Future work includes screening the BC_5F_5 populations of the crosses between Wilk 2 and XAN 159 as donor parents and Teebus as the recurrent parent with the SCAR marker SN 1-1 (E-AAC/M-CAT 2) developed in this study to determine the linkage of the marker in the populations. This SCAR can also be used to screen the crosses of the two NILs to ensure that the resistance genes from Wilk 2 as well as from XAN 159 have been stacked, thus aiding in gene pyramiding of the resistance genes of CBB. The marker could then be used in routine marker screening in the breeding programme.
(E-AAC/M-CAT 2)

The development of flanking markers can be accomplished by two options; the first being to identify closely situated and highly linked AFLP fragments for cloning and sequencing. These fragments could be converted into SCAR markers. The second being attempting cleaved amplified polymorphic sequence markers (CAPS) for the SCAR markers that did not show any polymorphism between the parents. This would entail identifying the restriction enzyme cutting sites within the sequences of these fragments and subjecting the amplified products to restriction digestion. Polymorphisms would be identified by size differences between the PCR products of the parents. These could then be mapped onto the linkage map and the linkage analysis to CBB resistance determined.

The developed SCARs could be mapped onto the bean core map using two populations obtained from Dr P.N. Miklas (USDA, United States of America). The mapping population is already available at the ARC-GCI (Potchefstroom, South Africa). The position of the SCAR markers could then be determined on the bean core map using MAPMAKER-EXP. This would aid in resolving the different linkage groups and the positions of the QTLs.

To obtain wider genome coverage and possibly more distinct linkage groups, more primers could be screened of the *Eco*RI and *Mse*I combinations, as well as the *Hind*III combinations. This would give a different variety of restriction fragments that could be targeting a wider region of the genome. NILs or RILs could be used to obtain a better representative of the linkage groups of the bean genome.

It would also be possible to develop SCAR primers from flanking AFLP fragments to obtain a more closely linked flanking marker for SAP 6. The AFLP markers situated around SAP 6 that are highly linked to the resistance QTL can be used to develop SCAR markers. This would help replace the existing SCAR marker with low linkage to CBB resistance, is present in the susceptible plants and is not useful in MAS. The *Eco*/*Mse* primer combinations could be used to screen Wilk 2, Teebus and XAN 159 to identify a co-dominant marker between XAN 159 and Wilk 2 to aid in gene pyramiding to ensure that both XAN 159 and Wilk 2 resistance can be identified in one reaction.

The use of markers in breeding programmes can lead to highly efficient selection of homozygous resistant individuals of the population. The SCAR markers developed can be used for MAS, with SN 3-400 (E-AAC/M-CTC 2) being able to distinguish between the homozygotic and heterozygotic progeny and able to replace the existing SCAR marker SU 91, SN 2A-600 (E-AAC/M-CAT 3) and SN 3-300 (E-AAC/M-CTC 2) targeting the major QTLs and selecting for the homozygous resistant plants, which could be highly efficient in a segregating population. SN 3-300 can be used to replace the existing SCAR marker BC 409. The SCAR SN 1-1 (E-AAC/M-CAT 2) can be used routinely in breeding programmes when the pyramiding of resistance genes from XAN 159 and Wilk 2 into a single NIL is attempted for improved resistance against CBB.

CHAPTER 7

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