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IDENTIFICATION AND CHARACTERISATION OF MOLECULAR MARKERS LINKED TO LEAF RUST RESISTANCE GENE *Lr41*

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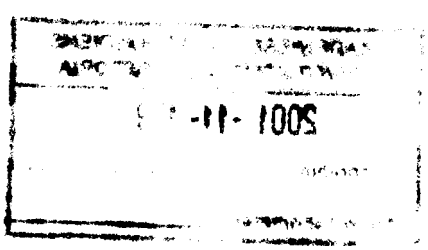

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PREFACE

The results presented in this thesis follow from a study which was carried out at the Department of Genetics, University of Pretoria, Pretoria, and the Department of Plant Pathology, University of the Orange Free State, Bloemfontein, under the supervision of Prof. A-M. Oberholster (Botha) and co-supervision of Dr. F.J. Kloppers.

The results presented here are original and have not been submitted in any form to another University.

The following manuscripts and abstracts have been published or submitted for publication from the results of the study.

1. **Botha A-M. & Lottering J-M. 2000.** Linked markers to leaf rust resistance gene *Lr41*. *South African Plant Breeders Association. Harare, Zimbabwe.* **1:** 13-16.

2. **Loots A., Botha A-M., Lottering J-M & Troskie C. 2000.** Comparative study between the genetic analysis of wheat cultivars using RAPDs, AFLPs and microsatellites. *South African Plant Breeders Association. Harare, Zimbabwe.* **1:** 13-16.

3. **Lottering J-M., Kloppers F.J. & Botha A-M. 1998.** Molecular marker linked to leaf rust resistance gene *Lr41*. *South African Genetic Society, 16th Cocress, South Africa.* **1.**

4. **Lottering J-M., Kloppers F.J. & Botha A-M. 1998.** DNA markers linked to leaf rust resistance gene *Lr41*. *Proceedings of the 9th assembly of the wheat breeding society, Australia.* **1:** 176-177.

5. **Lottering J-M., Kloppers F.J. & Botha A-M. 2000.** AFLP and RAPD markers linked to leaf rust resistance gene *Lr41* in wheat. *Theor. Appl. Gene.* (submitted)

6. **Lottering J-M., Troskie C, Kloppers F.J. & Botha A-M. 1998.** Markers linked to leaf rust resistance genes *Lr37* and *Lr41*. *South African Association of Bothany, South Africa.*

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OUTLINE

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ATP	Adenosin 5'-triphosphate
bp	Base-pairs
BSA	Bulked segregant analysis
cM	Centimorgan
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
EDTA	Ethylenediaminetetraacetic acid
kb	Kilobases (1kb = 10^3 base-pairs)
L	Liter
MAS	Marker-assisted selection
Mb	Megabases (1Mb = 10^6 base-pairs)
m/v	Mass per volume
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SCAR	Sequence characterised amplified region
SSR	Simple sequence repeats
TAE	Tris-acetate-EDTA buffer
T_m	Annealing temperature
Tris	Tris(hydroxymethyl)-aminomethane
U	One unit of *Taq* DNA polymerase enzyme is defined as the amount of enzyme that will incorporate 10 nmoles of dNTPs into acid insoluble material per 30 minutes at 74 °C under standard analysis conditions.
UPGMA	Unweighted pair-group mean arithmetic
UV	Ultraviolet

V	Volts
v/v	Volume per volume

CHAPTER 1

INTRODUCTION

From the earliest times world food security has depended on the production of cereal crops. The first cereals were probably cultivated about 9000 years ago by Neolithic man. He recognised that the seeds of certain grasses, when separated from the chaff were a rich source of food. All true cereals are from the family Gramineae, the most important being wheat, rice, maize, barley, oats, rye, sorghum and millet (Jones & Clifford, 1983).

Wheat is the number one food grain consumed directly by humans (Briggle & Curtis, 1987) and provides more nourishment for people than any other food source (Reitz, 1967; Johnson *et al.*, 1978). It is the major source of carbohydrates in the majority of countries all over the world (Leonard & Martin, 1963) and a significant amount is also used for animal feed. Today the common wheat (*Triticum aestivum* L.) is grown across a wide range of environments. In comparison with other crops, more land is devoted to the production of wheat than to any other agronomical commercial crop (Briggle & Curtis, 1987).

One significant implication in the cultivation of wheat is the reduction of genetic variation. This reduction makes this crop more vulnerable to disease and adverse climatic changes (Beharav *et al.*, 1997). The local primitive landraces are of great importance for broadening the narrow genetic basis of new agricultural cultivars. Their value lies in their wide range of variation, specific adaptation to different conditions and resistance or tolerance to various plant diseases and pests. The genetic significance of landraces is particularly high in Israel, which lies at the centre of wheat origin. The area consist of the natural distribution of wild relatives of wheat and barley (Harlan & Zohary, 1981).

Wheat leaf rust, caused by *Puccinia recondita* Rob. Ex Desm. *tritici* (Fig. 1.1), is one of the most important diseases in wheat (Schachermayr *et al.*, 1997). The fungus is adapted to a range of different climates, and the disease can be found in diverse wheat growing areas throughout the world (Kolmer, 1996). Wheat cultivars that are susceptible to leaf rust regularly suffered yield reduction of 5-15% (Samborski, 1985) or greater, depending on the stage of crop development when the initial rust infections occur (Chester, 1949).

Genetic resistance is the most economical and preferable method of reducing yield losses due to leaf rust. To date, more than 47 genes for resistance against wheat leaf rust (*Lr*) have been described and used in wheat breeding programs (McIntosh *et al.*, 1995). Resistance gene expression is affected by both genetic background, gene complementation and suppression and by environmental factors, including temperature and light (Kolmer, 1996). The resistance gene, *Lr41* is one of six resistance genes transferred from a wild relative, *Triticum tauschii* (Cox *et al.*, 1994). The *Lr41* gene is inherited in a single, dominant Mendelian fashion. Although genetic studies on the inheritance of *Lr* genes have been carried out for several years, information of the precise positions of *Lr* genes is relatively recent (Schachermayr *et al.*, 1997).

Various wheat breeding programs throughout the world have had mixed results in producing cultivars with durable resistance to leaf rust. Spring wheat breeding programs in North America (Kolmer *et al.*, 1991), Mexico (Rajaram, 1988) and Australia (McIntosh, 1992) have generally been very successful in producing cultivars that have high levels of durable and effective resistance (Kolmer, 1996). In contrast, the winter wheat grown in the southern plains of the United States often loses effective resistance after only a few years of cultivation (Marshall, 1989; Long *et al.*, 1992) due to the ability of the leaf rust pathogen to mutate and form new virulent races (Roelfs *et al.*, 1992).

The breeders aim is to combine genes, originating from wild relatives, into a commercially important cultivar. This process will ensure more durable resistance to the crops. One way of incorporating these genes is by using molecular markers as selection tools. Closely linked molecular markers for the individual *Lr* genes will allow plant breeders to accurately select for complex genetic backgrounds containing more than one *Lr* gene.

Fig. 1.1 Wheat flag leaves infected by the leaf rust pathogen *Puccinia recondita* f. sp. *tritici*.

The three molecular techniques used in this study were random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), amplified fragment length polymorphism (AFLP) (Zabeau *et al.*, 1993; Vos *et al.*, 1995) and simple sequence repeats. Both these techniques have been used successfully to produce molecular markers. The RAPD technique has been used to produce molecular markers linked to several important leaf rust resistance genes in wheat (Dedryver *et al.*, 1996; William *et al.*, 1997; Troskie *et al.*, 1999). The AFLP technique is a more resent innovation in genetic marker technology, but has shown great potential in molecular marker technology (Zabeau *et al.*, 1993; Vos *et al.*, 1995).

The aim of this study was two fold. Firstly, three different molecular techniques (random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellite were used in the identification and analysis of the level of DNA variation in a collection of wheat cultivars, hexaploid x-*Tritosecale* and rye. Due to the reduction of genetic variation, analysis of this kind will be of great importance in broadening the genetic basis of new cultivars (Chapter 3). Secondly, two molecular techniques, RAPD and AFLP, were used to generate closely linked molecular markers for the leaf rust resistance gene *Lr41*. These markers were converted into more specific, PCR-based, markers that can be used for marker assisted selection (Chapter 4).

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Plant breeding generally aims to improve agronomically relevant, or otherwise important traits, by combining characters present in different parental lines of cultivated species or their wild relatives. Through the centuries many plants have been domesticated. Characteristics like yield and quality of fruits were improved through a long process of artificial selection. This was, however, a long, time-consuming and costly process due to backcrossing, selfing and testing. During the selecting process, the breeder depends on accurate screening methods and the availability of lines with clear-cut phenotypical characters (Winter & Kahl, 1995). Breeders were therefore in need of a system to simplify the selecting process. With the discovery of the genetic basis of inheritance by Gregor Mendel in 1865, it became possible to start systematically selecting traits in progeny. These selecting of traits can now be made easier with the help of molecular techniques. It was, however, only after the demonstration of linkage between morphological and genetic markers by Morgan 1911 that indirect selection of plant traits was introduced as an efficient breeding tool (Chrispeels & Sadava, 1994).

At the beginning of modern genetics it was already obvious that phenotypic differences (polymorphisms) between parents were often co-inherited but this linkage was not always complete (Winter & Kahl, 1995). This segregation was the result of the occasional split of genetic material between parental chromosomes and is called recombination. Morgan (1911) defined the genetic distance between linked markers as the frequency of recombination between any two markers. The unit centimorgan (cM) was assigned to a distance that resulted in a rate of one recombination event in every 100 meiosis. The first genetic maps were based on recombination frequencies between morphological markers. These maps allowed the breeder to estimate the offspring's genotype without, or prior to, testing (Winter & Kahl, 1995).

The first map of the human genome based on molecular markers (Botstein *et al.*, 1980) fuelled the development of genomic maps in other organisms. The polymorphisms detected in restricted genomic DNA of plants have accelerated the development of molecular markers for plant breeding. Today, various new marker techniques and breeding strategies, which include a collection of molecular markers,

have been designed. The result of these efforts is an ever increasing number of markers for agronomically important traits, available for nearly all crops (Winter & Kahl, 1995).

The ideal molecular marker is a probe that targets only a single homologous locus in the genomes that are sampled (Devos & Gale, 1993). The specific targeting will reduce the time necessary to differentiate between cultivars by sampling the genome directly (Demeke *et al.*, 1992). One of the most important advantages of molecular markers is the usefulness for the manipulation of disease resistance genes in plants (Kelly, 1995). This manipulation has been aided by the fact that many disease resistance traits are determined by single, dominant genes. The ease of screening segregating populations for the phenotypic expression of these genes has facilitated the identification and characterisation of disease resistance markers. The economic importance of disease resistance genes has contributed to the world-wide focus on the use of molecular markers in plant breeding programs (Michelmore, 1995).

The characterisation of disease resistance genes, their function and how we can manipulate them to increase the levels of disease resistance in crops. Furthermore, identification of molecular markers for more than one resistance gene to the same pathogen allows the breeders to combine these resistance genes into one commercial genotype. This will allow the production of new breeding lines and cultivars that will hopefully display durable resistance to the pathogen (Kelly, 1995).

This review focuses on the wheat leaf rust disease caused by the fungus *P. recondita* f. sp *tritici* and the importance of DNA molecular marker technologies to resistance breeding. Special attention is given to the three molecular techniques; random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats, which were used during this study.

2.2 PLANT DISEASES CAUSED BY FUNGI

Fossil records of parasitic fungi date back to the Devonian period, which suggests that plant diseases originated along with the plants (Agrios, 1969). Fungi constitute a highly versatile group of eukaryotic carbonheterotrophic organisms that have successfully occupied most natural habitats (Knogge, 1996). Fungi are small, generally microscopic organisms that lack chlorophyll and conductive tissues. All plants are attacked by some kind of fungi, and each of the parasitic fungi can attack one or many kinds of plants (Agrios, 1969).

Cereals are subjected to attack by many groups of fungi. Some organisms are pathogenic on a broad host range, whereas others are limited to certain species (Tepper & Anderson, 1984). The damage they cause may result in a considerable reduction in grain yield and quality. The extent of the problem varies with the nature of the pathogen and the severity of the attack, but it has been estimated that there is an annual reduction in yield of about 12% on a world basis (Jones & Clifford, 1983).

2.2.1 Rust

Rust diseases have attracted considerable attention from plant pathologists because of their destructiveness and vexed biology. Their 'peaceful coexistence' with the 'special' hosts makes them elite among plant pathogens (Bilgrami & Dube, 1976). According to Chester (1949), the first person to recognise that rust was caused by a fungal parasite, was Felice Fontana in 1767. However, it was not until well into the 19th century that a distinction among the rust diseases was made (McIntosh *et al.*, 1995).

All the rust fungi are grouped together under the order Uredinales of the class Basidiomycetes. They are mainly identified on the basis of their teliospore morphology (Agrios, 1969). Rust diseases occur on a wide variety of economically important crops, as well as on ornamental plants (Bilgrami & Dube, 1976).

Table 2.1 The rust diseases of wheat, their primary hosts and symptoms (Roelfs *et al.*, 1992).

Disease	Pathogen	Primary hosts	Symptoms
Leaf rust	*Puccinia recondita* f.sp. *tritici*	Bread & durum wheat and triticale	Isolated uredinia on upper leaf surface and occasionally on leaf sheaths
Stem rust	*Puccinia graminis* f.sp. *tritici*	Bread & durum wheat, barley, and triticale	Isolated uredinia on upper and lower leaf surfaces, stem, and spikes
Stripe rust	*Puccinia striiformis* f.sp. *tritici*	Bread & durum wheat, triticale, and a few barley	Systemic uredinia on leaves and spikes but rarely on leaf sheathes

Of the three rust diseases of wheat, the most common is called leaf or brown rust (*Puccinia recondita* Rob. ex Desm. f.sp. *tritici*). It is probably the most important wheat disease on a world-wide basis (Samborski, 1985). Table 2.1 summarises primary hosts and symptoms cause by the three rust diseases.

2.2.2 Leaf Rust

In 1815 de Candolle showed that wheat leaf rust was caused by a distinct fungus and described it as *Uredo rubigo-vera*. The pathogen underwent a number of name change until 1956 when Cummins and Caldwell suggested *Puccinia recondita* (Roelfs *et al.*, 1992). Many researchers now accept *P. triticina* as the name of wheat attacking forms of leaf rust (Anikster *et al.*, 1997).

Leaf rust occurs to some extent wherever wheat grows. It can, however, effect other plants such as rye and some grasses. The formae specialis on a particular crop or grass is usually confined to that crop. Leaf rust is a common disease on wheat, but it became a severe problem in the mid-1970s (Knott, 1989). This fungus is known to have several alternative hosts (species of *Thalictrum*, *Anchusa* and *Isopyrum*) on which the sexual stage occurs. The uredinia (brown rust pustules) have a scattered distribution and this, together with their brown colour, enable it to be distinguished

from yellow rust. The leaf rust disease develops slowly in the spring. However, it is rarely noticed until more rapid spread occurs at the time of ear emergence or more usually later when environmental conditions tend to be more favourable. Development is most rapid at temperatures of 15-22 °C. Free water is essential for spore germination and high humidity for infection (Gair *et al.*, 1987).

Losses in grain yield are primarily attributed to reduced floret set. In severe epidemics with moisture stress, shrivelling of the grain occurs. Florets, tillers, and whole plants can be killed by early (pre-heading) epidemics. Losses due to leaf rust are usually small (<10%), but can be more severe (30% or more) (Roelfs *et al.*, 1992). In most years the disease only becomes severe too late in the season to be very damaging and more resistant varieties can escape disease altogether (Gair *et al.*, 1987).

2.3 PLANT RESISTANCE TO PATHOGENS

The phenomenon of plant resistance to fungal pathogens was first described by Farrer according to De Wit (1995). He showed that certain wheat cultivars were resistant to the stem rust pathogen, *P. graminis* (De Wit, 1995). The most common form of resistance found in higher plants is nonhost resistance. This resistance is exhibited by each plant against all potential pathogens (Heath, 1996). Therefore, every plant is resistant to the majority of fungal pathogens that may attempt to attack it (Heath, 1991). If a pathogen can overcome this non-host barrier, forming a basic compatible association, the plant species becomes a host. Alternative resistance systems then come into operation.

Host-specific resistance is only expressed against certain pathotypes of a pathogen. Such specificity of resistance must depend on the host-pathogen recognition processes, which involves a gene-for-gene relationship between avirulence genes in the parasite and resistance genes in the plant (Heath, 1996).

The current view is that race specific resistance is generally associated with the so-called hypersensitive response (HR), which is triggered by specific recognition of the

pathogen by the host (Atkinson, 1993). The HR can be considered as a rapid, programmed cell death (Greenberg *et al.*, 1994) which involves severe metabolic disturbance of cells in the zone of contact with the challenge (Tepper & Anderson, 1984).

2.3.1 Genetic Basis of Resistance

The use of inherited host resistance is commonly the preferred method of controlling cereal pathogens (Jones & Clifford, 1983). There are many advantages to the use of genetic resistance to control diseases. One of which is the low cost which at present relates only to the cost of varietal development (Jones & Clifford, 1983). Resistance is found to be controlled by one or more genes and inherited most frequently as a dominant character, although cases of recessive inheritance are found (Troskie *et al.*, 1999). Monogenic resistance is usually easy to detect, even at a seedling stage and is normally very specific, each resistance gene acting against one or a few specific races of the pathogen. This specificity gives the advantage of a high level of resistance against the designated races but, if following mutations, hybridisation, heterokaryosis or parasexuality new races develop, than resistance can be overcome (Jones & Clifford, 1983).

The specificity of most types of monogenic resistance suggests that there is some relationship between the physiologic races of the pathogen and the host varieties. A hypothesis suggested that for every gene determining resistance in the host, there is a corresponding gene conditioning avirulence in the pathogen (Jones & Clifford, 1983; De Wit, 1995). This genetic interrelationship between host and pathogen is known as the gene-for-gene hypothesis (De Wit, 1995).

Gene postulation Gene postulation applies the principles of gene-for-gene specificity to hypothesise which *Lr* genes may be present in hosts. This method uses the avirulent isolate/resistant host combination, as the definitive combination. Low or incompatible infection types are expressed only when hosts with a specific resistance gene are challenged with a pathogen isolate avirulent to the gene. All other combinations result in high or compatible infection types (Kolmer, 1996).

Genetic analysis The number and identity of leaf rust resistance genes in wheat cultivars can be conclusively determined only by genetic analysis. In this method the cultivar being studied is crossed with a susceptible parent and the F_1 plants are selfed to obtain F_2 populations, or are backrossed to the susceptible parent to obtain BCF_1 plants. This population is selfed to obtain the F_3 or BCF_2. The number of segregating resistance genes can then be determined by inoculating the F_3 and BCF_2 families, with specific rust races. (Kolmer, 1996).

Cytogenetic analysis This involves using monosomic (one missing chromosome) and telosomic (one missing chromosome arm) plants. Studies can be made of segregation generations from crosses of the selected resistant cultivars with standard susceptible monosomics for the 21 chromosomes to identify a gene's location on the chromosome (Sears, 1953).

2.4 GENOME STRUCTURE OF WHEAT

Wheat has an extremely large genome (1.7×10^{10} bp per haploid nucleus) with more than 60% repetitive DNA. The *Triticum* species has three genomes A, B and D, and is thought to be the result of an accidental hybridisation between the diploid einkorn *Triticum urartu* species (donating the A-genome) with an unidentified grass species (donating the B-genome). Research is beginning to show, that a wild grass-like *Aegilops* species (*A. speltoides*) is the mysterious donor of the B-genome (Jones & Clifford, 1983; Badaeva *et al.*, 1998). The hybridisation must have led to some tetraploid progeny. The resulting tetraploid underwent a chance interaction with *T. tauschii* adding the D-genome (Devos & Gale, 1993). These crosses led to the hexaploid wheat, which include the bread wheat, *T. aestivum* (Jones & Clifford, 1983).

The *Triticum* species behave like a typical allopolyploids in that, its chromosomes pair in a diploid-like fashion and the mode of inheritance is disomic. Each of the *Triticum* genomes from a polyploid series based on x = 7, which adds up to a 42 chromosome hexaploid species. The species can consist of three different ploidy levels: diploids (2n=2x=14), tetraploids (2n=4x=28) and hexaploids (2n=6x=42)

(Jones & Clifford, 1983). The tripling of the genetic content of the *Triticum* species makes it an extremely complex organism to work with (Devos & Gale, 1993).

2.4.1 Genetic Resistance against Wheat Leaf Rust

The leaf rust pathogen *P. recondita* f.sp. *tritici*, is the most widespread and regularly occurring rust on wheat. Genetic resistance is the most economical method of reducing yield losses due to leaf rust (Kolmer, 1996). However, resistance is not always durable and the genetics of durable resistance is poorly defined (Parlevliet, 1988).

Wheat rust fungi, like most organisms, are prone to mutation and recombination. This has led to new pathogen races that can overcome host plant resistance. Scientists are continually searching for new resistance genes in order to increase the genetic diversity of wheat. To date, 47 leaf rust resistance genes (*Lr*) have been designated and 46 genes have been mapped in wheat (McIntosh *et al.*, 1989). Although genetic studies on the inheritance of *Lr* genes have been carried out for several decades, information on the precise positions of *Lr* genes is relatively recent (Feuillet *et al.*, 1995).

Resistance gene expression is dependent on the genetics of host-parasite interaction, temperature conditions, plant developmental stage and interaction between resistance genes with suppressors or other resistance genes in the wheat genome (Kolmer, 1996). The resistant phenotype may vary from an immune type response or flecking to small or intermediately sized pustules, which could be associated with chlorosis or necrosis of host tissue (McIntosh *et al.*, 1995).

Genes expressed in seedling plants have not provided long-lasting effective leaf rust resistance. Adult-plant resistance genes *Lr13* and *Lr34* are single genes, and together, provided the most durable resistance to leaf rust in wheat throughout the world. Continued efforts to isolate, characterise, and map leaf rust resistance genes are essential given the ability of the leaf rust fungus to overcome deployed resistance genes (Kolmer, 1996).

2.4.2 Leaf rust resistance gene *Lr41*

The Asian goatgrass *Triticum tauschii* (syn. *Aegilops squarrosa* L; genomes DD, 2n=14) is proving to be a valuable source of genes for resistance to leaf rust. Resistance to leaf rust is common among goatgrass accession held in the Wheat Genetics Resource Center (WGRC) collection (Gill *et al.*, 1986; Cox *et al.*, 1993). Goatgrass is one of the wild ancestors of common wheat (AABBDD, 2n=42). Six genes have been transferred from *T. tauschii* (accession 'TA 2460') to produce stable, hexaploid wheat lines resistant to leaf rust. The line 'KS90WGRC10' has the leaf rust resistant gene *Lr41*. The location of the gene is on the chromosome 1D (Cox *et al.*, 1994). The gene, *Lr41* segregates independently of any other *T. tauschii*-derived leaf rust resistance genes (Kolmer, 1996).

2.4.3 Strategies for Using Resistance

The discovery of genes in the host plant that control resistance led to the initiation of many plant breeding programmes. These programs are designed to incorporate resistance genes into varieties of cereals, which also have desirable agronomic qualities. The incorporation of resistance genes into new varieties seemed quite sound but in most cases led to the "boom and bust" cycle (Kolmer, 1996). Due to the high degree of selection pressure placed on the pathogen population, mutant races capable of overcoming the resistance increased in the population at the expense of the previous pathogen races, which could not infect the new host plants. Thus, in breeding for resistance, an element of genetic vulnerability was also introduced which commonly led to the withdrawal of the new variety. Crop vulnerability to disease may be artificially induced, as in the case of breeding for resistance, or it may be natural where the host and the parasite, have evolved separately, and eventually brought together (Jones, 1983).

Resistance has been shown to be controlled by single genes, in many instances and was the exciting discovery by Biffen according to Jones & Clifford, (1983). The discovery made the transferring of single resistance genes from related species to

cultivated lines possible and is usually done through backcrosssing. Gene pyramiding is another method use by breeders (see 2.4.3.b) (Roelfs *et al.*, 1992).

2.4.3.a Resistance genes from related species

The reduction of variation in wheat is of great importance to wheat breeders. One method of broadening the narrow genetic basis of wheat is the transference of resistance genes from related species of lower-ploidy into species with higher-ploidy. For instance species of lower-ploidy (*T. tauschii,* AABB) into hexaploid (AABBDD) bread wheat. However, numerous studies in *Triticum* have revealed that the expression of resistance is reduced when the genes are transferred (Hanusovà *et al.*, 1996). The transfer is being complicated by interactions between resistance genes and suppressor genes in the different genomes. Bai & Knott, (1963) crossed ten leaf rust resistant accessions of *T. turgidum* var. *dicoccoides* (AABB) with susceptible bread wheat (AABBDD) and durum wheat (AABB). The F_1 plants from crosses with the durum wheat expressed leaf rust resistance, while the F_1 plants from crosses with the hexaploid wheat were susceptible. In the F_2 progenies from the hexaploid crosses, resistant plants had fewer D chromosome segments (average 3.2) compared to susceptible plants (average 11.5). Chromosomes 2B and 4B carried genes for leaf rust resistance, and 1D chromosome and 3D carried suppressors of resistance. Suppressors of leaf rust resistance have also been located to the A and B genomes (Kolmer, 1996). In spite of the existence of suppressors of resistance that may limit the use of transferred alien genes, several studies have shown that alien genetic variation could be successfully exploited (Hanusovà *et al.*, 1996).

Innes *et al.* (1994) investigated three methods for transferring genes from *T. tauschii* to common wheat: (1) The production of synthetic hexaploids (Kerber *et al.*, 1969; Dyck *et al.*, 1970; Joppa *et al.*, 1980) in a manner analogous to the evolution of hexaploid wheat; (2) A modification of the latter method in which undoubled inflorescence of triploid hybrids are pollinated by a hexaploid wheat to yield a derived hexaploid heterozygous for the target gene(s); and (3) direct introgression, in which the target gene is backcrossed directly into the recurrent hexaploid parent (Gill *et al.*, 1986). Of the three methods compared, direct introgression of gene transfer is

considerably more laborious, involving three successive generations of controlled crosses. These methods require a large number of pollinations to recover hybrid progeny. Of the three methods used the direct introgression have several advantages and thus having major use in transferring genes from diploid genotypes that cannot be readily hybridised with the tetraploid genotypes available (Innes & Kerber, 1994).

2.4.3.b Gene pyramids

The plant breeder aims to introduce into cultivars disease resistance that will give useful disease control for long enough to ensure that the commercial life of a cultivar is not curtailed by a breakdown in disease resistance. Prolonged resistance may result if a pathogen is unable to respond to host resistance with matching cultivar-specific pathogenicity as apparently, with some biotrophic parasites (Jenkyn & Plumb, 1981).

A gene pyramid involves the use of several genes in a single cultivar to provide a wider base of disease resistance. Most breeders world-wide use this approach for the three wheat rusts. Many gene pyramids have been successful, although some have quickly been rendered ineffective (Roelfs *et al.*, 1992).

The construction of *Lr* gene pyramids in a single wheat (*T. aestivum*) genotype is an important strategy in breeding for resistance to *P. recondita.* Pretorius *et al.* (1992) investigated the development of two-gene combinations among *Lr13, Lr34* and *Lr37.* Considerable progress has been made, but interaction among pathotype, the *Lr* gene, temperature and the wheat growth stage is important when confirming the presence of a gene (Kloppers & Pretorius, 1997).

If the objective is to develop pyramids of resistance genes, their accumulation and combination with other agronomic and yield characters is difficult, but not impossible to achieve (Roelfs *et al.*, 1992). Parlevliet (1988) has reviewed strategies for the control of cereal rusts. Resistance is often vulnerable to environmental factors, including temperature and light (Kolmer, 1996). In a severe epidemics, partial resistance seems to be less effective and hence selecting for this character by creating severe epidemics a doubtful procedure. The best breeding strategy to combine

polygenes for partial resistance is recurrent selection in which improvement of resistance is a continuous process. When partial resistance is controlled by a few genes with additive effects, accumulation of such genes can be achieved by carefully choosing the parents for crossing and through any breeding methodology (Roelfs *et al.*, 1992).

The breeding methodology for developing gene pyramids involves the identification of genetically different sources of resistance followed by the incorporation of this resistance into a high yielding and adapted background. The incorporation can be accomplished by any selection methodology (pedigree or bulk breeding) (Roelfs *et al.*, 1992). Conventional gene pyramiding techniques rely on field and glasshouse screening with differential rust races. They may also require the use of controlled environment facilities if the resistance genes are temperature-sensitive. Consequently the processes is complex and time-consuming (Poulsen *et al.*, 1995). However, the development of molecular marker technology has expanded the potential for pyramiding disease resistance genes. For the efficient application of the pyramiding strategy, the development of molecular markers, that are closely linked to the respective resistance genes, is essential (Feuillet *et al.*, 1995).

2.5 DNA BASED MOLECULAR MARKERS

Various molecular approaches are greatly increasing our ability to characterise and manipulate disease resistance genes in plants. Molecular techniques are providing the tools to characterise all stages of the interaction between plants and potential pathogens, from the earlier recognition events to the changes that occur during resistant and susceptible responses. Genetic molecular markers are DNA segments that behave as landmarks for genome analysis. The DNA segments usually represent variant or polymorphic traits that can be identified using general strategies such as molecular hybridisation or enzymatic amplification of DNA (Caetano-Anollès, 1993).

Molecular markers are useful for fingerprinting varieties, establishing phylogenies, tagging desirable genes (to assist their introgression into new varieties), determining similarities among inbreds (to maximise heterosis in hybrids), and mapping plant

genomes (Yu *et al.*, 1993). Furthermore, molecular markers are useful to localise monogenic and polygenic traits which allows the efficient introgression and selection of individuals with specific characteristics (Van Eck *et al.*, 1995). The most commonly used molecular marker systems are restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites. In order for the technique to become an effective tool in DNA molecular marker technology, it has to be simple and cost-effective, but it must maintain a high efficiency. For markers to have value in fingerprinting they must be reliable, practical, and validated in independent laboratories (Guidet, 1994).

Molecular markers are allowing the dissection of monogenic and polygenetic resistance, and will soon enhance the effective deployment of resistance genes to provide more stable resistance (Michelmore, 1995). In a breeding program, the applicability of molecular markers depends on a fast detection method, and on the specificity of the marker for the gene of interest in genetically diverse breeding material (Schachermayr *et al.*, 1997).

Several resistance genes have been characterised at the molecular level. The characterisation has led to the identification of several mechanistic classes and provides the opportunity for transgenic disease control strategies (Michelmore, 1995). However, the development of molecular markers that are specific for one particular gene appears to be difficult for a gene derived from the wheat gene pool, whereas it is easier to find specific markers if the gene originates from a wild relative of wheat (Schachermayr *et al.*, 1997). Table 2.2 presents a list of markers linked to leaf rust resistance in wheat.

Information on the chromosomal and genetic map location of a gene provides an alternative route for molecular markers (Rommens *et al.*, 1989). The development of detailed genetic linkage maps based on molecular polymorphic assay procedures will facilitate the identification of markers linked to important disease resistance genes (Tanksley *et al.*, 1989).

One of the most time-consuming requirements of DNA marker development is the need to screen the entire mapping population with every probe or primer. This problem has been solved by the development of bulked segregant analysis described by Michelmore *et al.* (1991).

Table 2.2 List of published DNA and other markers linked to leaf resistance genes in wheat.

Lr gene	Source	Marker type	Location	Reference
Lr1	*Triticum aestivum*	RFLP	5DL	Feuilet et al., 1995
Lr2	*T. aestivum*	RFLP	6BL	Parker et al., 1998
Lr9	*Ae. umbellulata*	RFLP	6BL	Schachermayr et al., 1997
Lr10	*T. aestivum*	RFLP-STS	1AS	Schachermayr et al., 1997
		RFLP		Nelson et al., 1997
Lr13	*T. aestivum*	RFLP	2B	Seyfarth et al., 1998
Lr18	*T. timopheevi*	N-band	5BL	Yamamori, 1994
Lr19	*Thinopyrum*	RFLP	7DL	Autrique et al., 1995
Lr20	*T. aestivum*	RFLP	5AL	Parker et al., 1998
Lr23	*T. turgidum*	RFLP	2BS	Nelson et al., 1997
Lr24	*A. elongatum*	RAPD-SCAR	2DL	Dedryver et al., 1996
		RFLP/RAPD		Schachermayr et al., 1997
Lr25	*Secale cereale*	RFLP	4BL	Procunier et al., 1995
Lr27	*T. aestivum*	RFLP	3BS	Nelson et al., 1997
Lr28	*T. speltoides*	RAPD/STS	2DS	Niak et al., 1998
Lr29	*A. elongatum*	RFLP	7DS	Procunier et al., 1995
Lr31		RFLP	4BL	Nelson et al., 1997
Lr32	*T. tauschii*	RFLP	3DS	Autrique et al., 1995
		RAPD/QTL	7BL	William et al., 1997
Lr34	*T. aestivum*	RFLP	7DS	Nelson et al., 1997
Lr35	*T. speltoides*	RFLP	2B	Seyfarth et al., 1998
Lr37	*T. ventricosum*	RAPD	2AS	Troskie et al., 1999
Lr41	*T. tauschii*	AFLP	1D	Lottering et al., 1999

2.5.1 The use of bulked segregant analysis (BSA) to identify markers

The most common way of mapping resistance genes is to screen near-isogenic lines (NILs) (Hartl *et al.*, 1995). The use of NILs is an ideal genetic system, but a time consuming process, labour-intensive and almost impossible in a number of vegetatively reproducing species. Michelmore *et al.* (1991) designed a 'in vitro near-isogenic lines' system, the system is called bulked segregant analysis (BSA). BSA consists of a comparison of two bulked DNA samples from a segregating population derived from a single cross. Each individual in the same bulk is identical for the trait

of interest (e.g. resistance to a disease) but arbitrary for another. Markers linked to the loci determining the trait used to construct the pools will be polymorphic between the two bulks (Page *et al.*, 1997).

BSA offer a rapid method for the identification of markers linked to any specific gene or genomic region and only small amounts of DNA are required, which need not be of high quality. The success of the approach will depend on the genetic divergence between the parents in the target region. The minimum size of the bulk will be determined by the frequency with which unlinked loci might be detected as polymorphic between the bulked samples (Michelmore *et al.*, 1991).

BSA has been developed mainly to find markers associated with monogenic traits using a segregating population (mainly F_2 population) resulting from the cross of two inbred lines. In such a case, the distribution of the alleles between the bulks is known for the gene involved in the expression of the trait (Page *et al.*, 1997).

2.5.2 DNA Based Molecular Marker Systems

2.5.2.a Random amplified polymorphic DNA (RAPD) markers

Random amplified polymorphic DNA (RAPD) was developed by two research groups. A group at Du Pont Co. (Wilmington, USA) called the new method RAPD (Williams *et al.*, 1990) and described its genetic mapping applications. Another group at the California Institute of Biological Research, USA (Welsh & McClelland, 1990) focused on genome fingerprinting and called their assay arbitrarily primed polymerase chain reaction (AP-PCR) (Innis *et al.*, 1990). Today the most commonly used name is RAPD. RAPDs are random amplifications of DNA fragments by polymerase chain reaction (PCR) using short 9-10 random primers (Welsh *et al.*, 1990, Williams *et al.*, 1990).

The RAPD technique has many advantages, one of which is that several loci may be detected per primer (Xu *et al.*, 1995) and in contrast with the conventional PCR,

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knowledge of the DNA sequence of the target locus is not required (Williams *et al.*, 1990; Waugh *et al.*, 1992). RAPDs can reveal polymorphisms even within genetically close groups (He & Quiros, 1991) and have been developed as a way to generate a great number of DNA markers in many plant species (Williams, 1993). These DNA markers are useful for genetic and breeding studies because they are not influenced by environmental conditions. The markers allow early screening of the plants since their detection requires very little plant material (Haley *et al.*, 1994). The RAPD technique is convenient, simple and fast (Barua *et al.*, 1992), therefore the markers represent a powerful tool for genome characterisation and open new possibilities for marker-assisted selection. However, the ordering of markers within short map distances requires the screening of large segregating populations to detect rare recombinant individuals. Large-scale testing is also necessary when markers linked to single-gene traits or to quantitative trait loci are used for MAS. In both cases, plants with a unique combination of marker alleles may need to be identified in a large population, thus rapidity, simplicity, and cost are important considerations (Aitken *et al.*, 1994).

Polymorphic RAPD markers are inherited in a Mendelian fashion, generally like that of a dominant trait. The dominant nature of RAPD markers would generally not allow differentiation of intra-locus homozygosity and heterozygosity (Haley *et al.*, 1994). However, pairs of RAPD markers tightly linked in repulsion phase would allow the identification of heterozygotes (Williams *et al.*, 1990).

The linkage between a RAPD fragment and the trait is revealed by a complete polymorphism (presence in one bulk and absence in the other), except for some recombinations that may occur between the marker and the gene considered. The distribution of the RAPD fragment in the plants of bulks allows an estimation of the recombination rate, which represents the tightness of the linkage between the fragment and the gene (Page *et al.*, 1997). RAPD markers have been found to be of limited use in the construction of linkage maps in wheat (Devos & Gale, 1992). However, it has been proposed that the latter may be useful in the characterisation of introgressed single chromosome segments (Schachermayr *et al.*, 1994).

Plants may have the same level of resistance with different associations of alleles at the various loci determining this trait. Thus, each genotype of the resistant bulk does not necessarily have all the resistance alleles. Moreover, some certainly have susceptibility alleles. In such a situation, even in the absence of any recombination event, a RAPD marker can be linked to resistance without being present within all the genotypes of the resistant bulk. RAPD marker may also be exhibited by some genotypes of the susceptible bulk. Therefore, in the case of a polygenic trait, the RAPD fragment ratios within the bulks reflect the tightness of the linkage between the marker and the trait. However, it depends on two factors, the genetic distance between the marker and the gene involved in the expression of the trait, and the importance of the effect of this gene (Page *et al.*, 1997).

The disadvantage of RAPD markers is that they behave as dominant markers and have the problem of reproducibility (Devos & Gale, 1992). Failure of RAPD bands to follow expected inheritance patterns, as well as, an excess of nonparental RAPD bands in offspring of known pedigree has also been reported (Riedy *et al.*, 1992). The reason that is suggested is competition among RAPD fragments to be a major source of genotyping error in RAPD analysis (Demeke *et al.*, 1997).

Principle of the RAPD technique

RAPDs are the amplification of DNA fragments by PCR reaction. The assay is based on the observation that a single short oligodeoxynucleotide of a randomly chosen sequence, when mixed with genomic DNA, dNTPs, buffer, and thermostable DNA polymerase, and subjected to temperature cycling amplified several DNA fragments (Innis *et al.*, 1990).

The RAPD assay is a modification of the basic PCR technique (Mullis *et al.*, 1986) but unlike the PCR, no knowledge of the target DNA sequence is required. Usually random oligonucleotides (9 or 10 bases) are used as primers to amplify discrete fragments of genomic DNA, and containing at least 50% G (guanine) and C (cytosine) and without internal inverted repeats. The complementary between the primer and the template need not be perfect (Williams *et al.*, 1991). The products are

easily separated by a standard electrophoretic technique and visualised under ultraviolet (UV) illumination of ethiduim bromide stained agarose gels (Demeke *et al.*, 1997). The conditions of the PCR reaction limit the band size to 100-3000 bp (Yu *et al.*, 1993). RAPD can reveal polymorphisms in genetically close groups and these polymorphisms may arise form single-base mutations or from insertion and deletion events (Aitken *et al.*, 1994).

Events favouring one or another product in early cycles of PCR may have a far greater effect on the nature and quality of the final products than under standard conditions (Xu *et al.*, 1995). The competitive aspect of RAPD analysis may explain in part why minor changes in almost any aspect of the amplification reaction have been reported to affect the outcome: DNA quality and quantity (Williams, 1993), choice of DNA polymerase (Schierwater & Ender, 1993), $MgCl_2$ concentration (Park *et al.*, 1994; Williams, 1993), choice of thermal cycler (Penner *et al.* 1993), primer concentration (Williams, 1993), use of ethiduim bromide vs. silver for detection of products (Caetano-Annollès *et al.*, 1992), and presence of RNA (Yoon & Glawe, 1993). The inconsistencies in RAPD reactions make the RAPD markers less useful (Xu *et al.*, 1995). Micheli *et al.* (1994), however, found that ethanol precipitation during DNA extraction contaminates the DNA, and is therefore the major (if not sole) source of variability in the RAPD reaction.

2.5.2.b Amplified fragment length polymorphic (AFLP) markers

With the discovery of the restriction enzymes in the late 1960s, a new tool became available to molecular biology (Appelhans, 1991). AFLP relies on the restriction of genomic DNA and subsequent PCR amplification of a defined subset of the restriction fragments generated. The AFLP technique finds numerous applications in plant and animal genetics (Domini *et al.*, 1997).

AFLP systems is one of the most recent innovations in genetic marker technology (Zabeau & Vos, 1993; Vos *et al.*, 1995). Amplified fragment length polymorphisms (AFLPs) can be used easily as markers for genetic typing and mapping applications (Caetano-Anollès *et al.*, 1992; Tingey *et al.*, 1993). The AFLP technique has been

used to identify markers linked to disease resistance (Thomas *et al.*, 1995) and to fingerprint DNA (Vos *et al.*, 1995). The technique is a reliable and reproducible molecular marker assay. The number of polymorphisms per reaction determined by AFLP is much higher than those performed by RFLP or RAPD (Lin & Kuo, 1995). AFLP markers do not require sequence information and a large number of markers can be assayed on a single gel (Vos *et al.*, 1995). The AFLP assay is a multi-locus marker system that enables the simultaneous investigation of many loci (Van Eck *et al.*, 1995). The large number of bands increases the difficulty in scoring (Mackill *et al.*, 1996). AFLPs behave as dominant markers, similar to RAPDs, although they can be scored as codominant markers with the appropriate software. AFLP procedure is more time consuming and expensive than RAPD. Almost every primer combination is usable and the total number of loci scored per primer is much higher than for RAPDs (Mackill *et al.*, 1996). The use of AFLPs avoids problems that may be encountered with reproducibility and optimisation of reaction conditions when using RAPDs. Furthermore are the primer sets of AFLPs readily available (Life technologies and Perkin Elmer) (Money *et al.*, 1996).

Principle of the AFLP technique

AFLP is a PCR-based technique for detecting DNA polymorphism in which small genomic restriction fragments are selectively amplified. The technique involves three steps: (1) restriction of the DNA and ligation of oligonucleotide adapters; (2) selective amplification of sets of restriction fragments; and (3) gel analysis of the amplified fragments (Vos *et al.*, 1995).

The technique involves digestion of genomic DNA with a rare and frequent cutter restriction enzymes (commonly *EcoR1* and *Mse1*) (Appelhans, 1991). High-quality genomic DNA is necessary for AFLP to ensure complete digestion by the restriction endonucleases (Lin & Kuo, 1995). The restriction fragments are ligated to double-stranded adapters, to the ends of the DNA-fragments to generate template DNA for amplification (Vos *et al.*, 1995).

PCR primers for AFLPs are designed in such a way that the 3' nucleotides of the primers extend to the unique sequences of each restriction fragment. These nucleotides serve as selective bases to amplify only those restriction fragments with complementary sequences (Appelhans, 1991). The basic principle of a PCR cycle is: (1) denaturation of the target DNA at 95°C; (2) annealing of the primer molecules to opposite DNA strands at 50°C flanking the target DNA; and (3) extension of the primers across the template by a thermostable DNA polymerase at 72°C. The newly synthesised DNA can then serve as a template in the following rounds of amplification. After 30-40 cycles of heat denaturation, annealing and primer extension, the target DNA has been amplified 10^9 fold. This exponential amplification makes it possible to amplify a specific DNA region out of chromosomal DNA (Appelhans, 1991).

Up to 100-150 single stranded amplification products can be detected using denaturing polyacryamide gels. The gel separates these amplicons and allows the identification of length differences as small as one base pair. The actual number of informative loci detected per primer combination is also determined by the polymorphism frequency within a species, but many plants are highly diverse, offering the potential to evaluate many polymorphic loci with a single primer combination. The 50-100 loci per electrophorectic lane detected by AFLP markers greatly improve the prospects for a high-density genetic map (Keim *et al.*, 1997).

2.5.2.c Microsatellite/Simple sequence repeats

Microsatellite markers (or simple sequence repeats, SSR) are genetic markers derived from tandemly oligonucleotide repeats such as $(GA)_n$,$(TG)_n$, $(AAT)_n$ or $(GT)_n$ (Röder *et al.*, 1998). The oligonucleotide repeats have basic motifs of <6 bp and are widely dispersed throughout eukaryotic genomes (Botha & Venter, 2000). SSRs have emerged as an important source of ubiquitous genetic markers due to the high number of polymorphisms obtained (Wang *et al.*, 1994). In general, only a few markers are sufficient to discriminate among even closely related species (Röder *et al.*, 1995; Bryan *et al.*, 1997).

In plants it has been demonstrated that SSRs are highly informative, locus-specific markers in many species (Bennett & Smith, 1976; Condit & Hubbell, 1991). These advantages are because SSRs are multiallelic and can therefore be use in evolutionary studies and phylogenetic studies among species (Buchanan *et al.*, 1994). One advantage of microsatellites is that primers developed for particular species have been shown to be applicable across a wide range of taxa (Schloettered *et al.*, 1991) and detect a much higher level of genetic variation than any other class of marker (Röder *et al.*, 1998). Such markers are especially crucial for genetic analysis in organisms with a narrow genetic base. The majority of the primer sets developed are genome-specific and detect only a single locus in one of the three genomes of wheat (A, B or D). The primer sets are all randomly distributed along the linkage map, with clustering in several centromeric regions. The apparent random distribution indicates that SSRs provide excellent coverage of the wheat genome (Botha & Venter, 2000).

2.5.2.d Sequence characterised amplified region (SCAR) markers

One of the big disadvantages of RAPD markers are the variation that occurs among laboratories. To circumvent this problem, Paran & Michelmore (1993) developed a dependable PCR-based technique called sequence characterised amplified region (SCAR). In this procedure, the polymorphic DNA fragment is cloned and sequenced. Forward and reverse primers are designed based on the sequence information for stable amplification of DNA. This marker technique has many advantages over RAPD markers, one of which is the use of longer and specific SCAR primers allows a more robust PCR reaction. The technique also eliminates the multiple banding pattern (Procunier *et al.*, 1997). The identification of RAPD/AFLP markers linked to the resistance gene and conversion of these markers into SCARs will allow routine marker-assisted selection (MAS) for the resistance gene in wheat breeding programs. Identification of SCARs for a resistant gene will also allow breeders to select for the gene in genetic backgrounds containing other resistance genes (Myburg *et al.*, 1998).

Some of the SCAR primers developed from RAPD have turned out to be codominant (Paran & Michelmore, 1993) and thus more informative. The combined use of different molecular markers will be beneficial for mapping studies in cereals (Demeke *et al.*, 1997).

PCR amplification of low-copy-number targets is, however, vulnerable to interference by the amplified extension of primer pairs, and annealing to non-target nucleic acid sequences in the sample ('mispriming') is possible (Chou *et al.*, 1992). Polymorphisms may also not occur if there is a point mutation, small deletion or addition of a DNA fragment in the PCR product amplified by SCAR primers. In this case the PCR amplified DNA products, have to be restricted with several enzymes in order to generate polymorphisms (Demeke *et al.*, 1997; Venter & Botha, 2000).

2.6 MOLECULAR MARKERS AND THEIR ROLE IN PLANT BREEDING

Genetic variability is the basis of all plant breeding. Conventional plant breeding focuses on selection of superior progeny from segregating populations, and selection is mostly based on phenotypic characteristics (Demeke *et al.*, 1997). During the selecting process the breeder depends on accurate screening methods and the availability of lines with clear-cut phenotypical characters (Winter & Kahl, 1995). Molecular techniques are increasingly being used by plant breeders to develope and improve cultivars (Demeke *et al.*, 1997). With the development of molecular markers, genetic maps of the plants can be constructed. These genetic maps lessen the chance of introgressing a gene while eliminating or inactivating another (Maisonneuve *et al.*, 1994). The use of markers and maps will accelerate the study and manipulation of genes in breeding programs (He *et al.*, 1992). By tagging economically important genes it is possible to identify individuals that carry these genes and to isolate and introgress genes based on map-based cloning (Devos & Gale, 1993).

Efficient management of crop diseases require an understanding of the functioning of resistance genes (Nelson *et al.*, 1997). For plant breeders, the linkage of markers with

morphological, physiological, disease resistant and biochemical characters is useful (Voorrips *et al.*, 1997). The closeness associated with the marker and the gene, must be 1 centiMorgan (cM) which equals 3 Mb in wheat. This distance will, however, not ensure close physical proximity of the marker to the gene, and will therefore hamper the use of such a marker in map based cloning and breeding programs (Feuillet *et al.*, 1995).

Molecular markers have the advantage of selecting genes without using any pathogenic tests. The selection of the resistance genes can therefore be carried out in areas where the pathogen don't occur naturally which makes it impossible to inoculate plants with the pathogen artificially (Kelly, 1995).

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The identification of a suitable source of disease resistance genes and their incorporation into adapted germplasm is a major challenge for plant breeders. An easily scorable marker linked to the gene conferring a particular resistance phenotype would therefore represent an important tool for plant breeders (Barua *et al.*, 1992). Once markers for an interesting trait have been established, these should allow the prediction of the resistance offspring derived from a cross, solely by the markers distribution pattern in the offspring's genome (Winter & Kahl, 1995). Closely linked markers will not only accelerate the process of breeding but will also help protect a breeder's rights, as these markers can also be used as an identification method (Nkongolo *et al.*, 1991).

2.7 FUTURE PROSPECTS - mapping of wheat genome

The plant breeder's aim is to improve agricultural crops by developing new crops with more durable resistance to diseases (Winter & Kahl, 1995). However, one of the greatest constraints in wheat genetics is the reduction of the genetic variation of cultivated wheat. This reduction makes wheat more vulnerable to disease and adverse climatic changes (Beharav *et al.*, 1997). The local primitive landraces are of great importance for broadening the narrow genetic basis of new agricultural cultivars (Harlan & Zohary, 1981). The genes of interest are usually inherited in a simple Mendelian fashion and are transferred to new cultivars through backcrossing. This

inheritance enables the breeders to combine genes of interest and transfer them into other cultivars (gene pyramiding). The combination of genes will broaden the genetic basis of the wheat plant, unfortunately epistasis and the need for six to seven backcrosses are currently impeding the pyramiding of genes (Dong & Quick, 1995).

In recent years the mapping of the wheat genome has become a primary focus of wheat genetics. However, due to the complexity of wheat's large genome it is lagging behind other cereal crops in marker development and genome mapping (Lanridge & Chalmers, 1998). Due to this lagging and the importance of knowledge about this crop, an incentive was launched to start an international genome mapping project (ITEC: International Triticeae Expressed Sequence Tags Cooperative)(9th International Wheat Genetics Symposium, Canada). This project will result in the identification and sequencing of several genes, by the end of this century and will advance our knowledge of the Triticeae genome (Schachermayr *et al.*, 1994). The knowledge would enable breeders to utilise a wider array of defence mechanisms against most known pathogens attacking wheat (Saidi & Quick, 1996).

The usage of molecular markers has moved over the past few years, from identification of trait linked markers to a broader concept of the mapping of the wheat genome. This will improve our knowledge and understanding of this complex genome. In future, more markers will be available to the breeders to utilise and manipulate the genome to our advantage.

CHAPTER 3

IDENTIFICATION AND ANALYSIS OF WHEAT, RYE AND TRITICALE GENOTYPES USING RAPD, AFLP AND MICROSATELLITE FINGERPRINTING

3.1 INTRODUCTION

The Gramineae family contains approximately 10 000 species, including one of the most important food crops in the world, wheat (*Triticum aestivum*). Other economically important members of this family include rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.) and rye (*Secale cereale* L.) (Van Deynze *et al.*, 1995). Wheat has an extremely large genome with more than 60% repetitive DNA and is a very complex organism to work with (Devos & Gale, 1993). Broadening of the narrow genetic basis of genotypes is one of the main aims of breeders (Beharav *et al.*, 1997). Physical - and genetic maps have given important insights into the relationship between physical and genetic distances in wheat (Van Deynze *et al.*, 1995). The identification of genotypes, estimation of genetic distance, and genome mapping have benefited from the application of DNA fingerprinting techniques (Yu *et al.*, 1993; Nybom, 1994).

The RAPD technique uses short, single random primers for the PCR and polymorphisms are monitored by the presence or absence of an amplified DNA fragment. While this technique is fast and convenient, its main drawback is, that the reproducibility between laboratories is relatively low due to the sensitivity of the random amplification step (Schachermayr *et al.*, 1994). RAPD analysis has, however, successfully been used to establish phylogenetic relationships among species, subspecies and genotypes (Landry *et al.*, 1994). The accurate identification of plant breeding material is extremely important, not only for the protection of breeder's rights, but also to accelerate plant breeding programs (Morell *et al.*, 1995).

Problems arising from the large wheat genome have limited the extensive use of RAPD analysis for the identification of wheat genotypes and varieties (Demeke & Adams, 1994). Accurate genotype specific markers can be generated where high levels of genetic variation exist between genotypes (Nybom, 1994). However, low levels of genetic variation are commonly observed among wheat genotypes (Gale *et al.*, 1990; Devos *et al.*, 1992). The lack of variation has necessitated the use of relatively high numbers of RAPD primers to distinguish closely related wheat genotypes (He *et al.*, 1992).

By using different molecular techniques, problems that may arise from one technique may be overcome by another. AFLP seem to overcome the major pitfalls present in RAPD analysis and appears to be as reproducible, heritable and intraspecific as RFLP (Paran & Michelmore, 1993; Mackill *et al.*, 1996). AFLPs have a large genome coverage (average 50-100 bands) and appear therefore, particularly good for fingerprinting and can be used to assay diversity within species and pedigrees (Barrett Kidwell, 1998; Barret *et al.*, 1998b; Liu *et al.*, 1998). One of the major drawbacks for AFLPs is that they are more expensive than RAPDs (Botha & Venter, 2000).

The third technique used in the fingerprinting of the cereal genotypes, was microsatellites or simple sequence repeats (SSRs). SSRs are genetic markers derived from tandemly repeated basic motifs of <6 bp. These sequence repeats are widely dispersed throughout eukaryotic genomes and are often highly polymorphic. The SSR molecular technique has become a very important source of genetic markers (Wang *et al.*, 1994). SSRs detect a much higher level of genetic variation, then any other marker. It is therefore, crucial in the genetic analysis of organisms with a narrow genetic base (Kam-Morgan *et al.*, 1989).

The aim of this study was to use different molecular techniques (random amplified polymorphic DNA, RAPD; amplified fragment length polymorphism, AFLP and microsatellites, SSRs) in the identification and genetic analysis of a collection of cereal genotypes. This study forms an important basis in the broadening of the reduced wheat genetic variation in wheat.

3.2 MATERIALS AND METHODS

Plant Materials

The germplasm used was obtained from the Small Grain Institute Bethlehem, South Africa and the *T. tauschii* material ('TAM107', 'TA2460' and 'KS90WGRC10') was obtained from TS Cox, USDA-ARS, Department of Agronomy, Kansas, USA. The cereal genotypes included in the study are summarised in Table 3.1. A bulk leaf sample, of all the genotypes, which consists of an equal sized, leaf segment of each plant, was

used to prepare representative genomic DNA extracts for each of the genotypes. The two genotypes 'Kiewiet', an AABBRR hexaploid tritcale (x Tritocoxecale Wittmack) and rye were include to provide a broader genetic background or 'outgroup' for genetic analysis.

DNA extraction

Representative DNA samples were prepared from the bulked leaf samples using a DNA isolation method based on that of Edwards and co-workers (1991). The equal concentration leaf samples were ground in liquid nitrogen into a fine powder in 1.5 ml Eppendorf tubes using glass pestles. A volume of 600 µL warm (65 °C) extraction buffer (100 mM Tris-HCl pH8.0, 500 mM NaCl, 50 mM EDTA, 1.25% (m/v) SDS, 20 mM $Na_2S_2O_5$) was subsequently added to the frozen tissue and incubate at 65 °C for 30 min. One volume of chloroform/isoamyl alcohol (24:1) was added to the mixture and this was vigorously vortexed to a white foam. The emulsion was centrifuged at 12000 x g for 15 min and the aqueous phase removed to a new 1.5 ml tube containing 900 µL ice-cold 100% ethanol. After 5 min the DNA was spooled out (Micheli *et al.*, 1994) using a sterile pipette tip and washed in 70% ethanol for 5 min. The DNA was air-dried and dissolved in 200 µL sterile water (Myburg *et al.*, 1998). The DNA concentration of each sample was determined with a fluorometer. The samples were diluted to 3.75 ng/µL for use in RAPD analysis, 100 ng/µl for the AFLP analysis and 10 ng/µl for the SSRs.

Table 3.1 Cereal genotypes.

No.	Genotype	Genus/Species	Description	Genome composition	Origin
1.	'Palmiet'	*T. aestivum*	Spring wheat	AABBDD	South Africa
2.	'Tugela'	*T. aestivum*	Winter wheat	AABBDD	South Africa
3.	'Molopo'	*T. aestivum*	Winter wheat	AABBDD	South Africa
4.	'SA1684/*5 Tugela'	*T. aestivum*	Winter wheat	AABBDD	South Africa
5.	'SA463/* Gamtoos'	*T. aestivum*	Spring wheat	AABBDD	South Africa
6.	'Betta'	*T. aestivum*	Winter wheat	AABBDD	South Africa
7.	'SA463/* Palmiet'	*T. aestivum*	Spring wheat	AABBDD	South Africa
8.	'SA2199/ Letaba'	*T. aestivum*	Winter wheat	AABBDD	South Africa
9.	'SA1684/*4 Molopo'	*T. aestivum*	Winter wheat	AABBDD	South Africa
10.	'Kiewiet'	x *Triticosecale* - outgroup		AABBRR	South Africa
11.	Rye	*Secale cereale* - outgroup		RR	South Africa
12.	'SST65'	*T. aestivum*	Winter wheat	AABBDD	South Africa
13.	'SST86'	*T. aestivum*	Winter wheat	AABBDD	South Africa
14.	'SST825'	*T. aestivum*	Winter wheat	AABBDD	South Africa
15.	'Inia'	*T. aestivum*	Spring wheat	AABBDD	South Africa
16.	'Corwa'	*T. aestivum*	Winter wheat	AABBDD	USA
17.	'SA1684'	*T. aestivum*	Winter wheat	AABBDD	Iran
18.	'SA2199'	*T. aestivum*	Winter wheat	AABBDD	former USSR
19.	'TA2460'	*T. tauschii*	Winter wheat	DD	USA
20.	'TAM107'	*T. aestivum*	Winter wheat	AABBDD	USA
21.	'KS90WGRC10'	x *T. tauschii*	Winter wheat	AABBDD	USA

RAPD primers and PCR conditions

Four 10-mer primers (Operon Technologies Inc. kits OPA_{19}; OPB_{13}; OPG_{13} and OPG_{16}) were used for RAPD analysis of the wheat genotypes and the two related cereal genotypes (Table 3.2). These primers were selected from previously obtained amplification profiles (Botha *et al.*, 1995; Myburg *et al.*, 1997) and was chosen for their ability to generate high number of unambiguously scorable bands.

The optimal conditions for the RAPD-PCR reaction were determined using modified Taquchi optimisation methods (Cobb & Clarkson, 1994). The RAPD-PCR reaction was performed in 25 µL reaction mixtures under oil overlay in 96 well microtiter plates

(BIOzym) as described (Myburg *et al.*, 1998). Amplification products were analysed by electrophoresis on 2% agarose gels (Seakern LE, FMC) at 3V/cm in 1 x TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA). Amplification profiles were visualised under UV light and detected by staining with ethidium bromide (0.5 µg/ml).

AFLP primers and PCR conditions

Three AFLP primer combinations (E-ACA:M-CTC; E-AAG:M-CAG; E-AGC:M-CTC) were selected for AFLP analysis of the cereal genotypes (Table 3.2). These primer combinations were selected on their ability of the primer to generate a high number of bands.

The AFLP analysis was done according to the manufacturer's instructions (Applied Biosystems, 1997). Three primer combinations were used and evaluated by using GeneScan software. The samples were loaded on a 5% polyacrylamide gel and run on the ABI Prism 377 DNA Sequencer.

SSR primers and PCR conditions

The SSR analysis was performed according to the technique described by Lee *et al.* (1995). The two primers used in this study were XGWM122 and XGWM337. The primers are chromosome-arm specific and amplified regions from chromosome 2AS. The 25 µL reaction contained; 20 pM primer, 20 ng DNA, 800 µM dNTPs, 1 U *Taq* polymerase, 1.5 mM $MgCl_2$ and 1 x *Taq* buffer (Promega, USA) (20mM $(NH_4)_2SO_4$, 75 Mm tris-HCL, pH 9.0 and 0.1% (v/v) Tween). The following temperature cycle was used; 40 cycles at 94 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min, and the final cycle at 72 °C for 10 min. The SSR amplification products were analysed on a 6% polyacrylamide gel and silver stained as described by Bassam and co-workers (1990).

Calculation of genetic distances

Each of the amplification products obtained from the RAPD, AFLP and SSR primers was considered to be an independent locus. The locus was named according to the

primer used and its size in base pairs. Presence (1) and absence (0) were scored and summarised in an input matrix (see appendix 7.1). The matrix was used to generate a pairwise distances matix between the different taxa, with the aid of PAUP 4.0 (Swofford, 1991).

UPGMA (Unweighted pair-group mean arithmetic) analysis (Sneath & Sokal, 1973) was used for genetic analysis of the pairwise distance matrix which generated a dendogram (Fig. 3.1) representing the character differences between the genotypes. All the calculations were done with the aid of PAUP 4.0 (Swofford, 1991).

3.3 RESULTS

RAPD, AFLP and SSR profiles

The profiles used in this study were only those products that could be unambiguously scored in all the genotypes. Bands that did not qualify were too faint to score accurately. A total of 55 scorable RAPD loci, amplified by four oligonucleotide primers (Fig. 3.2 and Fig. 3.3), were studied (Table 3.2). There were 379 AFLP loci scored, from only three AFLP primers. The two SSR primers used, identified 12 loci (Fig. 3.1). This means that a total of 446 loci were scored, with the techniques used. Of these, three (0.67%) were monomorphic for all the genotypes and 443 (99.33%) displayed polymorphisms in this study. The polymorpic loci were used to identify polymorphisms among the wheat genotypes and the two 'outgroups' ('Kiewiet' and rye). Of the 443 polymorphic loci, 215 (48.5%) were polymorphic among the wheat genotypes and the remaining 228 (51.4%) loci distinguished the rye and 'Kiewiet' genotypes.

None of the RAPD loci scored were unique markers that could be used for identification of wheat genotypes. More loci were scored with the AFLP primers. Markers $OPA19_X$, E-ACA:M-CTC_{176}, E-ACA:M-CTC_{182}, E-ACA:M-CTC_{208}, E-ACA:M-CTC_{320}, E-ACA:M-CTC_{352} are R-genome specific markers shared by 'Kiewiet' (AABBRR) and rye (RR), which distinguished them from the wheat genotypes.

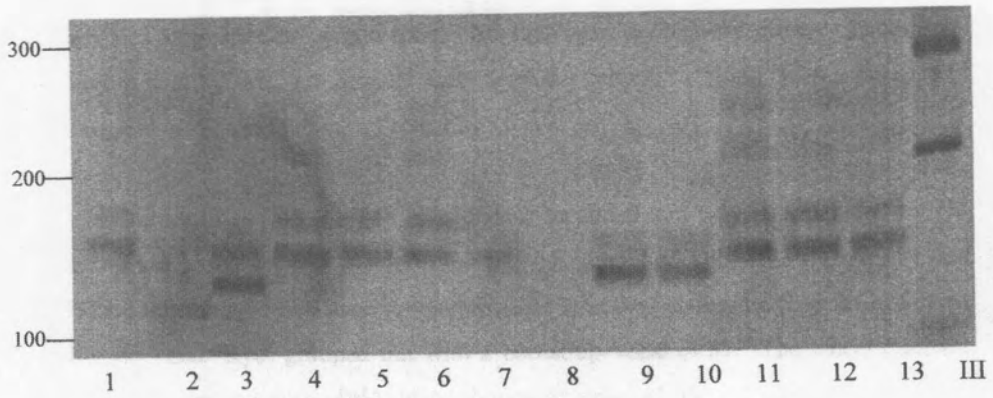

Fig 3.1 SSR profiles obtained from SSR primer pair XGWM122. Lane 1 = 'SA1684/*5 Tugela', 2 = 'SA463/*4 Gamtoos', 3 = 'Betta', 4 = 'SA463/*4 Palmiet', 5 = 'SA2199/ Letaba', 6 = 'SA1684/* Molopo', 7 = 'Kiewiet', 8 = Rye, 9 = 'SST65', 10 = 'SST86', 11 = 'SST825', 12 = 'Inia', 13 = 'Corwa'. The last lane contains a 100 bp molecular size marker.

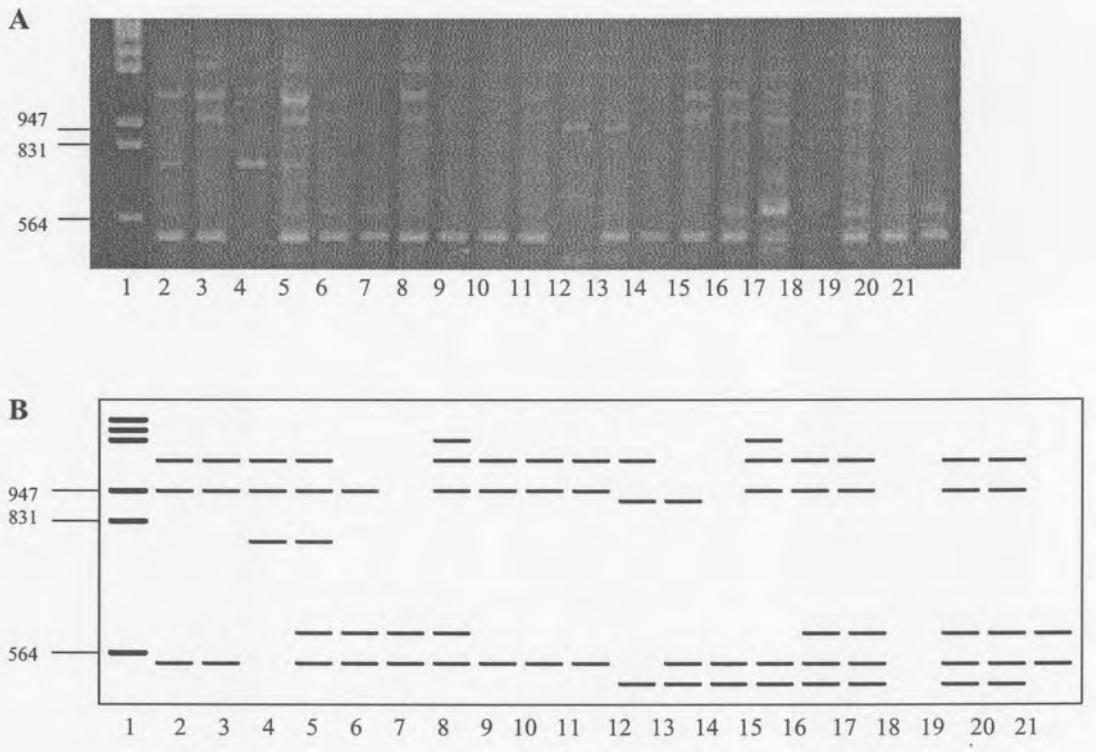

Fig. 3.2 (a) RAPD profiles obtained from RAPD primer OPG13. (b) Fingerprinting profile for the identification of the cereal cultivars with RAPD primer OPB13. Lane 1 is the molecular size marker III (lamda DNA digested with *Eco*RI and *Hind*III). Lane 2 = 'KS90WGRC10', 3 = 'TAM107', 4 = 'TA2460', 5 = 'SA2199', 6 = 'SA1684', 7 = 'Corwa', 8 = 'Inia', 9 = 'SST825', 10 = 'SST86', 11 = 'SST65', 12 = Rye, 13 = 'Kiewiet', 14 = 'SA1648/*4 Molopo', 15 = 'SA2199/*1 Letaba', 16 = 'SA463/*4 Palmiet', 17 = 'Betta', 18 = 'SA463/*4 Gamtoos', 19 = 'SA1684/*5 Tugela', 20 = 'Molopo, 21 = 'Tugela', 22 = 'Palmiet'.

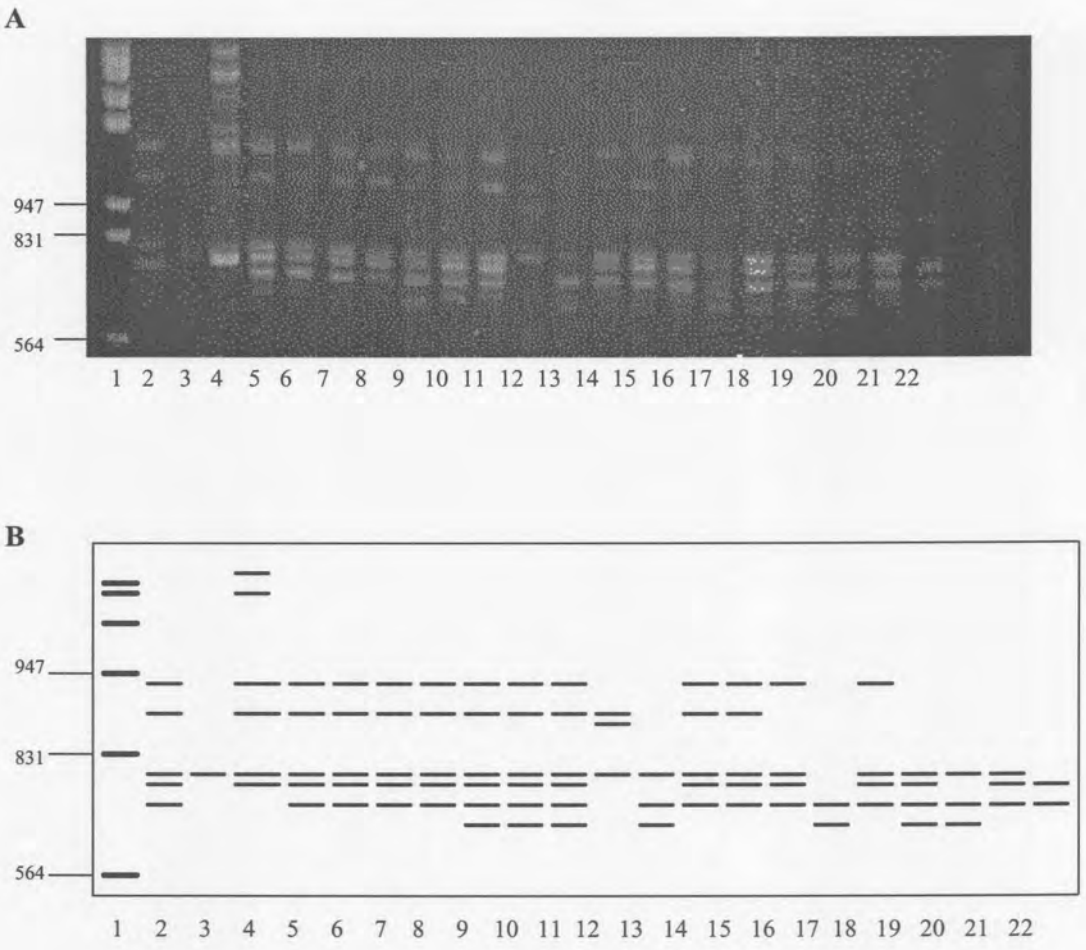

Fig. 3.3 (a) RAPD profiles obtained from RAPD primer OPB13. (b) Fingerprinting profile for the identification of the cereal cultivars with RAPD primer OPB13. Lane 1 is the molecular size marker III (lamda DNA digested with *Eco*RI and *Hind*III). Lane 2 = 'KS90WGRC10', 3 = 'TAM107', 4 = 'TA2460', 5 = 'SA2199', 6 = 'SA1684', 7 = 'Corwa', 8 = 'Inia', 9 = 'SST825', 10 = 'SST86', 11 = 'SST65', 12 = Rye,13 = 'Kiewiet', 14 = 'SA1648/*4 Molopo', 15 = 'SA2199/*1 Letaba', 16 = 'SA463/*4 Palmiet', 17 = 'Betta', 18 = 'SA463/*4 Gamtoos', 19 = 'SA1684/*5 Tugela', 20 = 'Molopo, 21 = 'Tugela', 22 = 'Palmiet'.

Table 3.2 List of RAPD, AFLP and SSR primers used in the analysis of the different cereal genotypes.

No.	Primer	Sequence	Monomorphic loci		Polymorphic loci		Total number of loci
			#	%	#	%	
1.	OPA19	CAACGTCGG	1	7.7	12	92.3	13
2.	OPB13	TTCCCCCGCT	0	0	15	100	15
3.	OPG13	CTCTCCGCCA	0	0	11	100	11
4.	OPG16	AGCGTCCTCC	0	0	16	100	16
5.	E-ACA:M-CTC	AATTCACA:CTCAAT	1	0.82	121	99.18	122
6.	E-AAG:M-CAG	AATTCAAG:CAGAAT	1	0.47	212	99.53	213
7.	E-AGC:M-CTC	AATTCAGC:CTCAAT	0	0	44	100	44
8.	XGWM122	^{F}GGGTGGGAGAAAGGAGATG ^{R}AAACCATCCTCCATCCTGG	0	0	6	100	6
9.	XGWM337	^{F}CCTCTTCCTCCCTACTTAGC ^{R}TGCTAACTGGCCTTTGCC	0	0	6	100	6
	Total		**3**		**443**		**446**
	Average		**0.67**		**99.33**		

^{F} = forward

^{R} = reverse

Distance analysis of wheat genotypes

The pairwise distances, which were generated with the aid of PAUP 4.0 (Swofford, 1991), were used in this study (Table 3.3). An average distance of 0.28 was obtained, among the South African wheat genotypes, while the distance obtained among the foreign and backcrossed wheat genotypes was 0.33 and 0.24, respectively. The genetic distances between the wheat genotypes range from 0.17 ('SA1684/*4Molopo' and 'Betta') to 0.44 ('Betta' and 'TAM107'). 'Kiewiet' and rye were used as the outgroup. The average distance between the wheat genotypes, 'Kiewiet' and rye were 0.38 and 0.41, respectively. The smaller genetic distance (approximately 0.96 simmilarity) between 'Kiewiet' and the wheat genotypes correlates with the fact that the genotypes share the two genomes - AABB. 'Kiewiet' and rye share the RR - genome, and this acounts for the 0.97 similarity between the genotypes.

Table 3.3 Pairwise distance matrix between the different genotypes, according to PAUP 4.0 (Swofford, 1991).

1.	**'Kiewiet'**	0																				
2.	**'KS90WGRC10'**	0,41	0																			
3.	**'TAM107'**	0,41	0,29	0																		
4.	**'TA2460'**	0,44	0,26	0,33	0																	
5.	**'SA2199'**	0,33	0,32	0,37	0,35	0																
6.	**'SA1684'**	0,34	0,33	0,34	0,39	0,21	0															
7.	**'Corwa'**	0,35	0,38	0,39	0,39	0,25	0,25	0														
8.	**'Inia'**	0,39	0,35	0,38	0,4	0,19	0,29	0,27	0													
9.	**'SST825'**	0,34	0,35	0,37	0,38	0,22	0,22	0,23	0,26	0												
10.	**'SST86'**	0,37	0,36	0,39	0,39	0,21	0,26	0,21	0,22	0,24	0											
11.	**'SST65'**	0,38	0,38	0,38	0,4	0,25	0,26	0,3	0,29	0,27	0,28	0										
12.	**Rye**	0,3	0,35	0,41	0,41	0,37	0,41	0,41	0,41	0,43	0,39	0,45	0									
13.	**'SA1684/*4Molopo'**	0,39	0,39	0,42	0,43	0,23	0,27	0,25	0,28	0,28	0,21	0,34	0,4	0								
14.	**'SA2199/Letaba'**	0,39	0,39	0,39	0,41	0,26	0,27	0,25	0,27	0,28	0,22	0,33	0,4	0,22	0							
15.	**'SA463/*4Palmiet'**	0,39	0,35	0,38	0,41	0,23	0,27	0,26	0,23	0,25	0,22	0,3	0,42	0,18	0,23	0						
16.	**'Betta'**	0,38	0,39	0,44	0,41	0,25	0,25	0,25	0,27	0,26	0,22	0,33	0,4	0,17	0,22	0,19	0					
17.	**'SA463/*4Gamtoos'**	0,42	0,37	0,43	0,43	0,25	0,28	0,3	0,28	0,26	0,27	0,32	0,44	0,28	0,26	0,26	0,24	0				
18.	**'SA1684/*5Tugela'**	0,38	0,37	0,38	0,39	0,27	0,25	0,28	0,29	0,24	0,23	0,33	0,41	0,26	0,22	0,22	0,22	0,28	0			
19.	**'Molopo'**	0,36	0,37	0,4	0,43	0,22	0,24	0,24	0,25	0,24	0,24	0,26	0,39	0,22	0,23	0,21	0,2	0,24	0,25	0		
20.	**'Tugela'**	0,42	0,4	0,42	0,43	0,28	0,31	0,28	0,33	0,29	0,24	0,37	0,44	0,24	0,24	0,23	0,2	0,26	0,2	0,29	0	
21.	**'Palmiet'**	0,41	0,36	0,39	0,42	0,28	0,27	0,28	0,3	0,29	0,26	0,32	0,4	0,26	0,24	0,23	0,24	0,28	0,26	0,22	0,27	0
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**

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UPGMA analysis of the similarity matrix yielded a dendogram (Fig. 3.3) that reflected some of the known relationships and origin of these genotypes included in the study. 'Tugela' and 'SA1684/*5 Tugela', and 'Betta' and 'SA1684/*4 Molopo' are grouped together. 'SA463/*4 Palmiet', 'Molopo', 'SA2199/*1 Letaba' are further related. 'SST 86' and 'Corwa' are grouped together. 'Palmiet' is the furthest related from the wheat genotypes. All the genotypes mentioned above are grouped together. 'SST825' and 'SA1684' grouped together, as well as 'Inia' and 'SA2199'. 'SA463/*4 Gamtoos' and 'SST65' are the furthest related to all the wheat genotypes mentioned above. 'TA2460', 'KGS10' and 'TAM07' are grouped together. Rye and 'Kiewiet' are correctly grouped, as an outgroup.

To test the significance of the data, a cladogram (Fig. 3.4) was also created with the 446 independent loci. The bootstrap value of 98 confirmed that 'Kiewiet' and rye belonged together and was correctly group as the outgroup. The *T. tauschii* genotypes formed a clade independently from *T. aestivum* genotypes, with a bootstrap value of 94. All the wheat genotypes are closely related and did not group in a clade, except for the 'Tugela' and the 'Tugela' backcross was the only wheat genotypes grouped together in a clade, with a bootstrap value of 77.

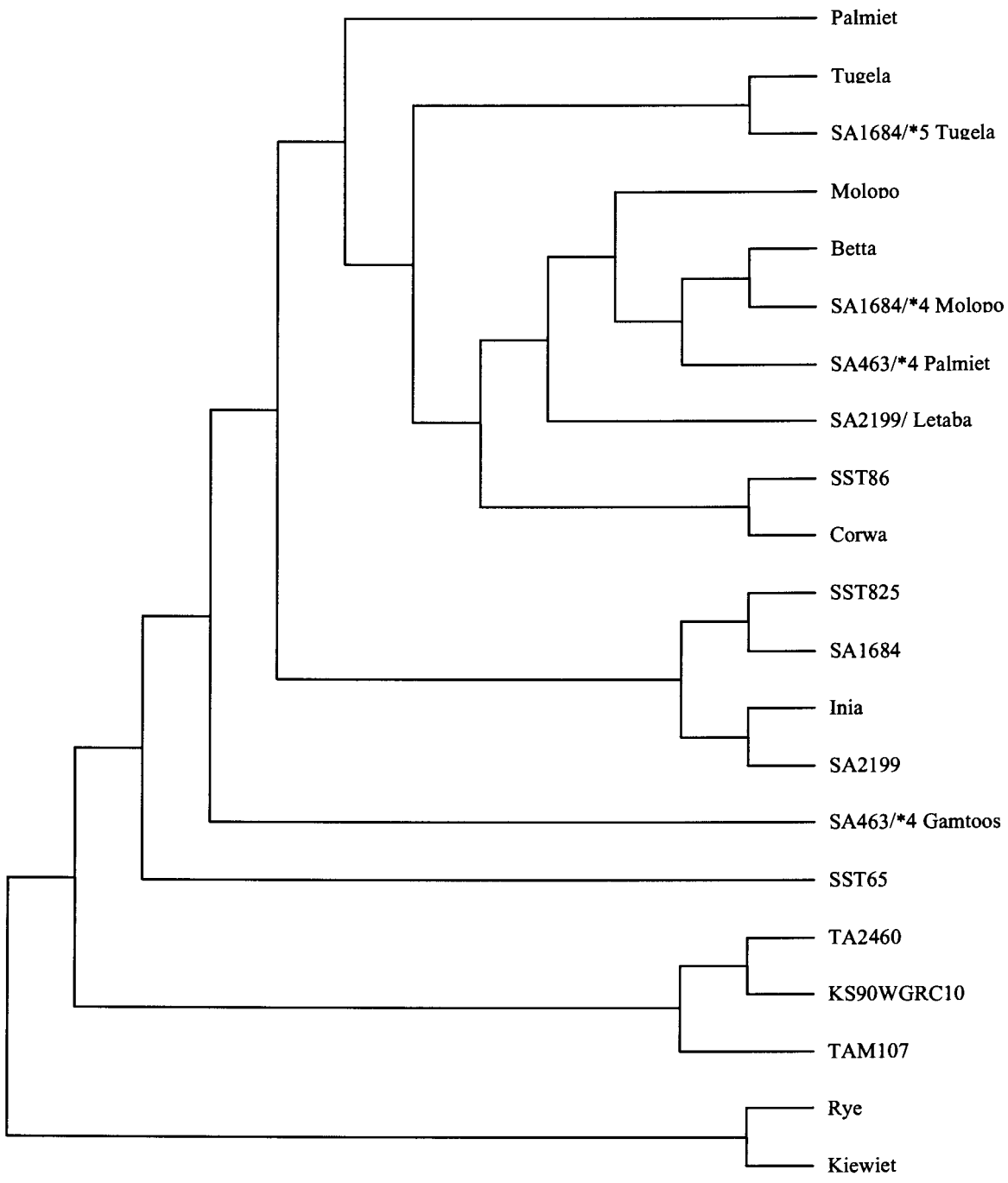

Fig. 3.4 UPGMA (Unweighted pair-group mean arithmetic) analysis of the pairwise distance matrix, generated a dendrogram representing the distances between the cereal genotypes.

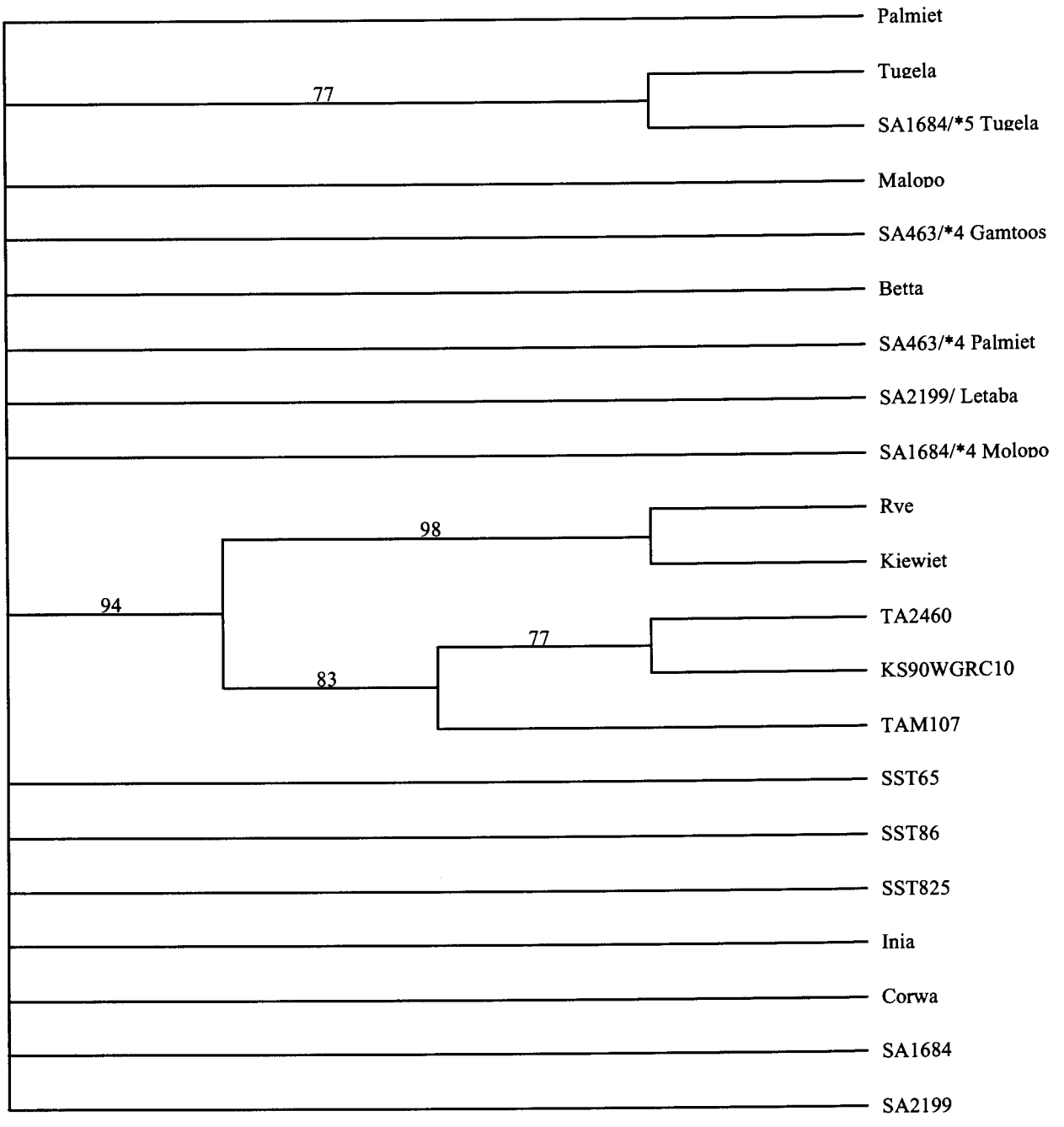

Fig 3.5 A cladogram of the different genotypes used in the study, of the wheat genotypes.

3.4 DISCUSSION

The identification and distance analysis of wheat genotypes will greatly improve our ability to broaden the genetic basis of new genotypes. The molecular methods used in this study, were RAPDs, AFLPs and SSRs. RAPD analysis has accelerated the use of DNA-based fingerprinting data for the identification and analysis of genotypes (Nybom, 1994). This is in part, due to the fact, that the technique does not require any previously obtained sequence data. The technique is able to distinguish between a large number of individuals and genetic loci. RAPDs are therefore, appropiated for use in taxonomically studies (Demeke *et al.*, 1992; Demeke *et al.* 1994). RAPDs can, however, be unsuccessful when working with geneticaly very close genotypes, as in the case of wheat (Devos & Gale, 1992). The four RAPD primers that were used in the study, failed to identify unique molecular markers for the wheat genotypes. The amount of primers tested will determine the success rate of RAPD as a marker detection system (Myburg *et al.*, 1997, Myburg *et al.*, 1998). Demeke *et al.* (1996) suggested that up to 965 RAPD primers are needed to identify molecular markers linked to a specific gene in wheat.

The AFLP technique overcomes the major pitfalls present in RAPD analysis (Mackill *et al.*, 1996) and offered a high level of utility when compared with other marker systems. One of the main advantages of AFLPs is the large genome coverage, and is therefore particularly good for fingerprinting (Powell *et al.*, 1996). We found that this was particularly true in this study. The average polymorphisms obtained from RAPDs were 11-16 bands (Table 3.2), while an average of more than 100 polymorphisms per AFLP primer was obtained. The main reason for this genome coverage by AFLPs, is the restriction digestion step (Botha *et al.*, 2000).

The RAPD and AFLP analysis of the wheat, x-*Tritosecale* and rye DNA yielded species specific amplification profiles. The AFLP primers were able to distinguished among the 'Kiewiet', rye, 'SA463/*4Palmiet, 'SST65', 'SA684', 'TA2460' and 'KS90WGRC10'. The rye and 'Kiewiet' could be distinguished by 228 loci (41 loci by RAPD and 187 loci by AFLP) and were correctly grouped as outgroups by the UPGMA analysis.

SSRs detects a much higher level of genetic variation, than any other marker technique (Kam-Morgan *et al.*, 1989). The majority of SSR primers are genome specific, and detects only a single locus (Rödel *et al.*, 1998). This is especially important in wheat, because of the three genomes present (A, B or D). The two SSR primers used, distinguished one of the three wheat genomes. Primer pair $XGWM122_{150}$ amplified a fragment, which was not identified in the rye genome. This fragment is probably amplified from the A or B genome, which occur in all the genotypes tested except for rye.

Calculation of pairwise distances between taxa (Table 3.3) revealed an average distance value of 0.29 between the cereal genotypes. The UPGMA analysis grouped the South African genotypes together, except for 'SST825', 'Inia' and 'SST65'. These two revealed an average distance value of 0.28. The backcross genotypes are grouped with the South African genotypes, with a distance value of 0.25. Only 'SA463/*4 Gamtoos' did not group with the South African genotypes. 'Corwa', which is a foreign wheat cultivar (USA), was closely grouped with 'SST86', and grouped with all the South African wheats. The distance between these was 0.21 and suggested that they are closely related.

The foreign genotypes are generally grouped together, with an average distance value of 0.33. South African genotypes 'SST825', 'Inia' grouped with foreign genotypes 'SA1684' and 'SA2199', respectively. 'TA2460', 'KS90WGRC10' and 'TAM107' are grouped together. The 'TA2460' and 'KS90WGRC10' grouping was expected, because these genotypes are from the wild relative, *T. tauschii*. 'KS90WGRC10' is the offspring of 'TAM107' and 'TA2460' and this explain the grouping.

In the process of boardening the genetic diversity of wheat genotypes, crossbreeding among genotypes that are to closely related will be a solution to the problem. The crossing of a South African wheat line with a foreign line will introduce new variation and in the process boarden the genetic diversity. To supply the breeder with this information, dendograms of different wheat genotypes are important. It is, however, crucial to use the best molecular technique for an accurate analysis of the data. In this study it was found that by using different techniques, most of the pitfalls of one technique can be overcome by the other. To conclude, the analysis of different wheat

genotypes worldwide, will provide breeders with information on their genetic relationship and will enable calculated crossings. That will result in improvement of agronomicaly important traits with a broad genetic base.

CHAPTER 4

IDENTIFICATION AND CHARACTERISATION OF RAPD AND AFLP MARKERS LINKED TO LEAF RUST RESISTANCE GENE *Lr41*

Submitted for publication in:

THEORETICAL APPLIED GENETICS

4.1 INTRODUCTION

The global human population is ever increasing and will lead to an increase per capita demand for food. It is predicted that there will be a net doubling of imports of grains with wheat being more than 50% of this increased requirement (Eastwood, 1999). One significant constraint to increased wheat production is the variety of diseases attacking the crop. The reduction of the genetic variation of cultivated wheat makes it more vulnerable to diseases, with consequent limiting effects on food production (McIntosh *et al.*, 1995).

Wheat rusts have been noted as diseases of great importance. Leaf rust caused by *Puccinia recondita* Rob. Ex Desm. *tritici* is one of the most common rust diseases and occurs almost wherever wheat is grown (Schachermayr *et al.*, 1997). One of the most cost-effective ways of controlling rust is breeding for resistance (Kolmer, 1996). To date, more than 47 leaf rust resistance genes (*Lr*) have been designated and mapped in wheat (Feuillet *et al.*, 1995). Most of these resistance genes are inherited in a dominant Mendelian fashion. Although genetic studies on the inheritance of leaf rust resistance genes have been carried out for several decades, information on the specific locations of these genes are relatively recent (Schachermayr *et al.*, 1997).

Various molecular approaches have increased our ability to characterise and manipulate disease resistant genes in plants. These molecular techniques are important tools in the characterisation of the interaction between plants and pathogens (Michelmore, 1995). DNA-based molecular markers can also be of great use in marker assisted selection (MAS) (Schachermayr *et al.*, 1994). DNA markers linked to leaf rust resistance genes *Lr1*, *Lr2*, *Lr9*, *Lr10*, *Lr13*, *Lr18-Lr20*, *Lr23-25*, *Lr27*, *Lr29*, *Lr31*, *Lr32*, *Lr34*, *Lr35* and *Lr37* have previously been reported (for review see Botha and Venter, 2000). These will allow for the detection of the specific genes in a complex genetic background of wheat and make gene pyramiding possible. Molecular makers such as random amplified polymorphic DNA (RAPD) (Welsh *et al.*, 1990; Williams *et al.*, 1990) and amplified fragment length polymorphism (AFLP) (Zabeau *et al.*, 1993; Vos *et al.*, 1995) can be used. RAPDs are convenient, simple and fast to run (Barua *et al.*, 1992), and therefore represent a powerful tool for

genome characterisation (Aitkent *et al.*, 1994). AFLP is a recent innovation in genetic marker technology (Zabeau *et al.*, 1993; Vos *et al.*, 1995). The AFLP assay is a multi-locus marker system that usually behave as a dominant marker, similar to RAPDs, although co-dominant behaviour has also been reported (Botha & Venter, 2000). The use of AFLPs alleviates problems with reproducibility and optimisation of reaction conditions that may be encountered when using RAPDs.

Lr41 is a recently designated gene from *Triticum tauschii*, a diploid ancester of common bread wheat. *Lr41* confers high levels of resistance to South African leaf rust pathotypes. However, one of the disadvantages of such race-specific resistance is that it often becomes ineffective (Pretorius, 1997). Such genes are especially vulnerable where they are used as monogenic resistance in cultivars. Identifying markers linked to such a gene will enable breeders to combine them with other effective genes in the same genotype. Thus, the aim of this study was to identify markers linked to the leaf rust resistance gene *Lr41* by using molecular techniques such as RAPDs and AFLPs. We also investigated the ease whereby AFLP markers can be converted into cheaper and easier to use sequence characterised amplified region (SCAR) markers.

4.2 MATERIALS AND METHODS

Wheat material

Lr41 resides in the wheat line 'KS90WGRC10' . Seeds of 'KS90WGRC10', 'TAM107' and 'TA2460' were obtained from T.S. Cox, USDA-ARS, Department of Agronomy, Kansas State University, Kansas, U.S.A. The pedigree of 'KS90WGRC10' is 'TAM107*3/TA2490. A direct 'TAM107/TA2460' hybrid was backcrossed as a female to 'TAM107', 'TAM107' was pollinated by a resulting BC_1F_1 plant. 'KS90WGRC10' is a BC_2F_2-derived F_5 line produced by self-pollinating a single BC_2F_3 family that was homogeneous for immune reaction to PRTUS25 (Cox *et al.*, 1992). A BC_2F_2 'Karee/KS90WGRC10' population developed by Z.A. Pretorius, Department of Plant Pathology, University of the Orange Free State, Bloemfontein, South Africa was used as segregating population to locate markers linked to *Lr41*.

The control genotypes, 'Karee', 'KS90WGRC10', 'TAM107' and 'TA2460' and the 34 individual BC_2F_2 'Karee/KS90WGRC10' plants were grown in a glasshouse in 1-l capacity plastic pots, containing a sterilised soil:peatmoss mixture (1:1 v/v). The glasshouse cubicle was set to maintain a temperature of approximately 20 °C, and natural daylight was supplemented with 120 mE m-2s-1 photosynthetically active radiation, emitted by cool white fluorescent tubes, arranged directly above plants, for 14h each day. Four week old plants of all individuals were tagged, numbered and harvested for DNA isolation. Subsequently, these plants were inoculated with pathotype UVPrt9 of *P. recondita* f.sp. *tritici*, a pathotype virulent to 'Karee', for phenotypic expression. Plants were placed in the dark in a dew-simulation chamber at 18-20 °C for 16h, and transferred to an air-conditioned glasshouse cubicle where they were incubated at 20 °C and a 14h daylight period. Twelve days post-inoculation, plants were evaluated for leaf rust infection types using a 0 (immunity) to 4 (susceptibility) cereal rust infection type scale (Roelfs *et al.* 1992).

DNA extraction

DNA samples were prepared from 0.3 g of uninfected leaf samples using a modified DNA isolation method (Edwards *et al.,* 1991). After precipitation of the DNA in ice-cold 100% ethanol for 5 min, the DNA was spooled out (Micheli *et al.,* 1994) using a sterile pipette tip and washed in 70% ethanol for 5 min. The DNA was air-dried and dissolved in 200 µl sterile water (Myburg *et al.,* 1998). The DNA concentration of each sample was determined fluorometerically and the samples were diluted to 2.5 ng/µl for use in RAPD analysis or 500 ng/µl for the AFLP analysis.

A bulked DNA sample, consisting of equal constentration of DNA from six of the susceptible and resistant individual plants respectively, was used as a representative DNA sample for each wheat line.

RAPD-PCR conditions

The 380 oligonucleotide primers used in the study were obtained from Operon Technologies, Alameda, Calif. (kits A—S). The optimal conditions for the RAPD-

PCR reaction were determined using modified Taguchi optimisation methods (Cobb and Clarkson, 1994). The RAPD-PCR reaction was performed in 25 µl reaction mixtures under oil overlay in 96 well microtiter plates (BIOzym) as described (Myburg *et al.,* 1998). All the amplification reactions were performed in duplicate and each set repeated at least once over time and material. Amplification products were analysed by electrophoresis on 2% agarose gels (Seakem LE, FMC) at 3V/cm in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Amplification profiles were visualised under UV light and detected by staining with ethidium bromide (0.5 µg/ml).

AFLP-PCR conditions

The AFLP analysis was done according to the manufacturer's instructions (Applied Biosystems, 1997). Sixty four primer combinations were used and evaluated using GeneScan software. The samples were loaded on a 5% polyacrylamide gel and run on an ABI Prism 377 DNA Sequencer. AFLP fragments that were polymorphic between the bulks and parent lines were loaded on a manual gel. The manual gels were run on a 6% denaturing polyacrylamide gel and silver stained as described (Bassam *et al.,* 1990).

RAPD and AFLP analysis

RAPD fragments that were polymorphic between the bulks, as well as the parental lines ('TAM2460', 'TA107', 'KS90WGRC10' and 'Karee'), were further analysed in a 12-plant subset of the BC_2F_2 population. RAPD markers that displayed recombination fractions in these plant populations were then analysed in all 34 individuals of the BC_2F_2 population. AFLP fragments that were polymorphic between the susceptible and resistant bulks, as well as the parent lines, and a 12 set of the BC_2F_2 population were removed from the manual gel and cloned. The AFLP markers were cloned into pGEM-T plasmid vectors (Promega) and transformed into JM109 high efficiency competent (>108 cfu/µg DNA) cells (Promega) according to the manufacturer's instructions. These cloned fragments were sequenced and used to design SCAR primers.

Development of SCAR markers

One of the five putative AFLP markers were selected for the development of a sequence characterised amplified region (SCAR) marker (Kesseli *et al.,* 1992) to verify the ease whereby these fragments can be converted into SCARs. The cloned fragments were sequenced with the ABI PRISM^{TM} dye terminator cycle sequencing ready reaction kit (Perkin Elmer, 1995) using the T7 forward and Sp6 reverse primers. The sequence data was used to design a 19 bp SCAR primer set containing the original 9 bases of the AFLP primer and the next 10 internal bases on the ends of the AFLP fragment. The primers were synthesised by Roche (UK).

SCAR amplification of genomic DNA was performed in 25 µl reaction mixtures, containing 5 ng template DNA, 10 x amplification buffer (20 mM $(NA_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.1% (v/v) Tween), 300 µM dNTPs, 0.001% (m/v) gelatine, 3.0 mM $MgCl_2$, 7.5 pmol 19-mer primer and 0.6 U Taq DNA polymerase (Advanced Biotechnologies) (Venter & Botha, 2000). The temperature cycle was performed using a thermal cycler (Perkin Elmer) programmed for an initial step of 1 min at 94 °C, followed by 30 cycles of: 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and there after the final step 10 min at 72 °C. Agarose electrophoresis was performed as described for RAPD analysis.

Linkage analysis

The linear order of the RAPD and SCAR markers was determined using MAPMAKER (Lander *et al.,* 1987). A logarithm of odds ratio (LOD) score of 3.0 was established for the linkage. Recombination fractions were transformed to linkage distances in centiMorgans (cM) according to Kosambi (1944).

Dot blot analysis

To determine the feasibility of converting the AFLP marker to a SCAR, a dot blot hybridisation of the AFLP marker E-AAG:M-CTA was performed according to the

technique described by Sambrook *et al.* (1989). The AFLP amplification products of wheat genotypes 'TA2460', 'TAM107', 'KS90WGRC10' and the susceptible and resistant BC_2F_2 bulks, were denatured with the addition of 0.1 volume of 3M NaOH followed by incubation at 60 °C for one hour. The sample was subsequently cooled and 1 volume of 5 x SSC buffer (0.9M NaCl, 0.09M $C_6H_5Na_3O_7.2H_2O$, pH7) was added. A positively charge nylon (Boeringer Mannheim) membrane was wetted with UHQ water and then soaked in 5 x SSC solution. Of each sample 2µl was applied to the membrane and the applied sample was immobilised by baking the membrane at 120 °C of 20 minutes.

Colony PCR products were labelled radioactively with a nick translation system (Promega). A 25 µl nick translation mixture contained: 3 uM dNTP mix (dCTP, dGTP, dTTP), 1 x nick translation buffer (50 mM Tris-HCL pH7.2, 10 mM $MgSO_4$ and 1 mM DTT), 1 µg sample DNA, 1 µM [α-32P]-dATP (70 µCi at 400 Ci/mmole and 10 mCi/ml) and 0.2 µl DNA polymerase I/ DNAse I mix (1 U/µl DNA polymerase I and 0.2 ng/µl DNase I in a solution containing 50 % glycerol, 50 mM Tris-HCL ph 7.2, 10 mM $MgSO_4$, 0.1 mM DTT and o.5 mg/ml nuclease-free BSA). The nick translation reaction was incubated at 15 °C for 1.5 hours. This was followed by purification of the probes from unincorporated radioactive dNTPs using a Sephadex G-50 column calibrated with a 1 x TEN buffer (0.1 m NaCl, 10 mM TrisHCL pH 8.0, 1 mM EDTA).

The membrane was pre-hybridised in a solution (5 x SSC, 1 % blocking reagent (Roche)) for 2 h at 60 °C. After incubation the pre-hybridisation solution was removed and a hybridisation solution (5 x SSC, 0.5 % SDS) was added to the membrane. The probe was denatured at 95 °C for five minutes and placed on ice, before the denatured probe was added to the hybridisation solution. The hybridisation step was performed for 16 hours at 52 °C. Post hybridisation procedures involved two washing steps of 30 minutes in 2 x SSC and 0.5 % SDS at 52 °C and a final wash step at 52 °C in 2 x SSC. Results were visualised using autoradiography (Sambrook *et al.*, 1989).

4.3 RESULTS

Phenotypic expression

Resistance was expressed by *Lr41* in 'KS90WGRC10', its donor source 'TA2460' and the resistant BC_2F_2 individuals as a very low fleck (;) infection type (Table 4.1), to pathotype UVPrt9 of *P. recondita* f.sp. *tritici*. 'Karee', 'TAM107' and susceptible BC_2F_2 individuals displayed clear susceptible infection types of 3 and 3^+, respectively (Table 4.1).

Table 4.1 Infection types produced by pathotype UVPrt9 of *Puccinia recondita* f. sp. *tritici* 12 days post-inoculation on individuals of a BC_2F_2 population of 'Karee/KS90WGRC10' (*Lr41*), and the control genotypes.

Genotypes	Infection types[a]
'Karee'	3^+
'TA2460'	;
'TAM107'	3
'KS90WGRC10'	;cn
30 Resistant Karee/KS90WGRC10	;
9 Susceptible Karee/KS90WGRC10	3^+

[a] Infection types (0-4 scale) (Roelf et al., 1992)

RAPD/AFLP analysis

RAPD analysis of the bulks and parental lines with 380 oligonucleotide 10-mer primers yielded on average of seven scorable bands per primer. These bands ranged in size from 300-3000 bp. Approximately 3190 RAPD loci were evaluated for potential linkage to the *Lr41* resistant gene. A total of 14 polymorphic bands were identified as potential markers. Only primers that could be reproduced umambigously were selected. This yielded a total of seven linked RAPD markers (Table 4.2) that yielded an average of 0.22 polymorphic bands per loci.

The 64 AFLP primer combinations were automatically scored on the ABI Prism 377 DNA Sequencer and yielded an average of 29 bands per primer ranging in size from 37-1500 bp. The low amplification results were due to some AFLP primer

combinations that did not amplified. Approximately 1859 AFLP loci were evaluated. Primer combinations with a high yield (bands per primer) and more than five polymorphisms were manually scored on a 5% polyacrylamide gel. A total of five putatively linked AFLP markers were identified (Table 4.2) and removed from the gels, these fragments were then cloned.

Table 4.2 RAPD and AFLP primers linked to leaf rust resistance gene *Lr41*.

Marker	Linkage phase	Recombination fraction (%)	Linkage distance (cM)[b]
$OPA12_{750}$	coupling	2.8	2.8
$OPA12_{1200}$	coupling	2.8	2.8
$OPK10_{1050}$	coupling	18	18.8
$OPK18_{880}$	coupling	24	26
$OPL1_{650}$	coupling	29	33
$OPM19_{830}$	coupling	15	15
$OPN1_{720}$	coupling	26	28.8
E-AAG:M-CTA_{100}	repulsion	nv	nv
E-AAG:M-CTA_{200}	repulsion	nv	nv
E-AAG:M-CTA_{300}	coupling	15	15
E-AAG:M-CTA_{1000}	coupling	nv	nv
E-AGG:M-CAA_{1300}	repulsion	nv	nv

[a] nv = not verified

[b] Estimated from recombination fractions according to Kosambi (1944)

Linkage analysis of RAPD and AFLP markers

The *Lr41* gene segregated in the BC_2F_2 population of 'Karee/KS90WGRC10' in a dominant monogenic, 3:1 Mendelian fashion at a significant level of 0.02 (Table 4.3). The RAPD and AFLP markers segregated with the gene of interest. Analyses of DNA polymorphisms revealed seven putative RAPD markers and five AFLP markers linked to the *Lr41* resistant gene. The seven RAPD markers are $OPA12_{1200c}$, $OPA12_{750c}$, $OPK10_{1050c}$, $OPK18_{880c}$, $OPL1_{650c}$, $OPM19_{830c}$ and $OPN1_{720c}$ (see appendix 7.2 for primer sequences) their linkage distances ranged from 2.8-33 cM (Table 4.2, Figure 4.1). Three of the AFLP markers, E-AAG:M-CTA_{100r}, AAG:M-CTA_{200r} and AGG:M-CAA_{1300r} (see appendix 7.3 for primer sequences) were linked to the repulsion phase of the *Lr41* gene and two markers, AAG:M-CTA_{300c} (Fig. 4.2) and AAG:M-CTA_{1000c} (see Table 7.2 for primer sequences) were linked to the coupling phase of the gene. Linkage was also verified through Southern analysis of the fragment, and the fragment segregated in accordance with the AFLP fragment.

Table 4.3 Segregation analysis of the BC_2F_2 population.

	Resistant	Susceptible	Sum of X^2
Expected value	25.5	8.5	
Observed value	25	9	
X^2	0.0098	0.0294	0.0392
Segregation	3	1	

MAPMAKER (Lander *et al.*, 1987) generated a linkage map with map size ranging from 2.8-19.6 cM. $OPA12_{1200c}$ and $OPA12_{750c}$ mapped to the one side of the resistance gene, while OPK_{1050c} and E-AAG:M-CTA_{300c} mapped to the other side of the gene (Fig. 4.1).

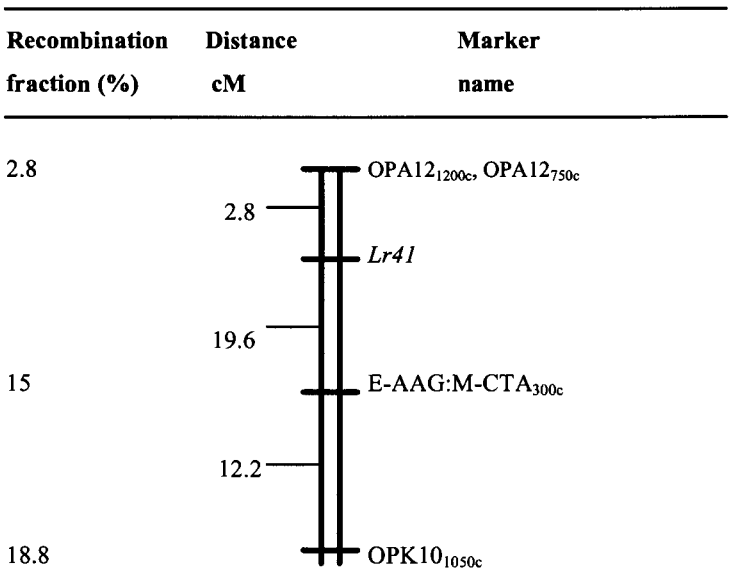

Fig. 4.1 Linear order of the molecular markers segregating in the BC_2F_2 population on a linkage map generated by MAPMAKER. Linkage distances were calculated according to Kosambi (1944). c = coupling phase marker.

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SCAR analysis of the AFLP markers

The one AFLP marker was selected for further analysis. The marker was cloned and five clones were sequenced. The sequences were used directly for the synthesis of SCAR primers (Table 4.4). Amplification with SCAR primer SCAR-AGCT$_{300}$ yielded a 300 bp product (Fig 4.2) in the resistant population. A small amount of non-specific product was detected in some of the BC_2F_2 individuals, but higher stringency annealing temperatures rectified this (Venter & Botha, 2000).

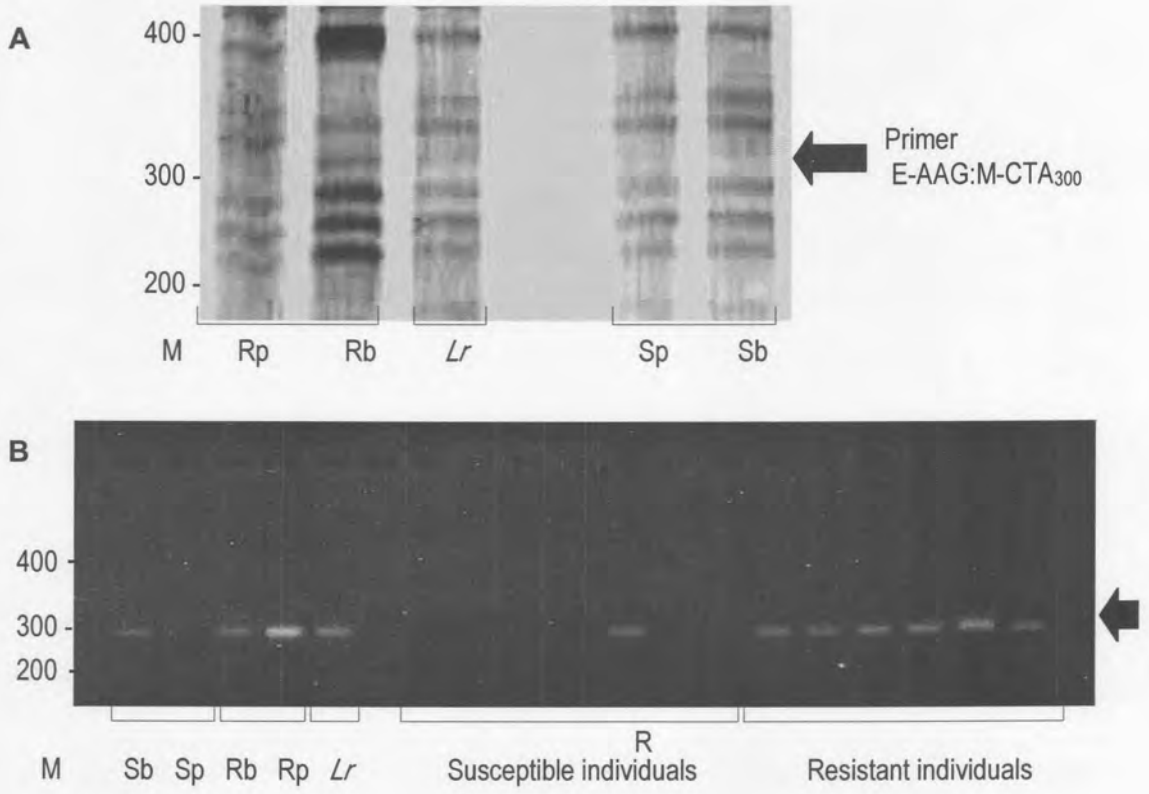

Fig. 4.2 (a) Coupling phase AFLP marker E-AAG:M-CTA linked to the *Lr41* resistance gene. (b) SCAR analysis of the original AFLP marker in a subset (12) of the 39 BC_2F_2 individuals. R = recombinant BC_2F_2 individual; Sb = susceptible bulk; Sp = susceptible parent (TAM107); Rb = resistant bulk; Rp = resistant parent (TA2460); *Lr* = KS90WGRC10. M is 100 bp molecular weight marker.

Table 4.4 Summary of SCAR primers derived from one AFLP marker linked to *Lr41* resistance gene

SCAR primer	AFLP marker	Sequence[a]
SCAR-AGCT$_{A\text{-}F}$	E-AAG:M-CTA	GTAACTAATAAAGCACC
SCAR-AGCT$_{A\text{-}R}$	E-AAG:M-CTA	AGTCCAGATCGAACTTAAC
SCAR-AGCT$_{B\text{-}F}$	E-AAG:M-CTA	CAATTCAAGACAGGATAAT
SCAR-AGCT$_{B\text{-}R}$	E-AAG:M-CTA	ATCATCCAGGATCAATG
SCAR-AGCT$_{300\text{-}F}$	E-AAG:M-CTA$_{300c}$	GTAACTAGCCCATTGCT
SCAR-AGCT$_{300\text{-}R}$	E-AAG:M-CTA$_{300c}$	GATTTAGGTGGAACTTAAC
SCAR-AGCT$_{C\text{-}F}$	E-AAG:M-CTA	GTAACTATATCTTTTAT
SCAR-AGCT$_{C\text{-}R}$	E-AAG:M-CTA	AAACTTAAGCGAACTTAAC
SCAR-AGCT$_{D\text{-}F}$	E-AAG:M-CTA	CAATTCAAGGATACCATGA
SCAR-AGCT$_{D\text{-}R}$	E-AAG:M-CTA	TGTATGATCAATACCATG

[a] The original AFLP primer sequence is underlined.

4.4 DISCUSSION

Various molecular approaches increased our ability to characterise and manipulate disease resistance genes in wheat. In this study RAPDs and AFLPs proved effective in the identification of markers linked to leaf rust resistance gene *Lr41*. Out of the 380 RAPD primers that were tested, only seven differentiated between the resistant and susceptible lines. As each primer amplified about seven fragments per primer on average and seven out of 3190 loci were different, which yield an average of 0.22% polymorphic bands per loci. The AFLP analysis was done with 64 AFLP primer combinations. Approximately 1859 AFLP loci were evaluated and five (0.27% polymorphic bands per loci) primers were linked to the resistance loci. Thus, the number of polymorphisms determined by AFLP proved significantly higher (30%) than those performed by RAPD (Lin *et al.*, 1995, Botha & Venter, 2000).

AFLP is a time consuming and expensive technique (Mackill *et al.,* 1996), it is also technically difficult and not optimal for high though-put screening associated with breeding programs. Another difficulty is that single bands on a gel can sometimes be compromised of several co-migrating amplification products making accurate analysis difficult (for review see Botha & Venter, 2000). To overcome these problems, an attempt was made to convert one of the AFLP markers into a SCAR marker. SCARs are sequence characterised amplified fragments and based on a longer and more specific primer that allows for a more robust PCR reaction which, eliminate multiple banding patterns (Procunier *et al.,* 1997) that occur during AFLP analysis.

The five AFLP markers that consisted out of three repulsion phase markers (E-AAG:M-CTA_{100}, E-AAG:M-CTA_{200} and E-AGG:M-CAA_{1300}) and two coupling phase markers (E-AAG:M-CTA_{300} and E-AAG:M-CTA_{1000}) were cloned and sequenced characterised. One AFLP marker, E-AGG:M-CTA_{300} was selected to test the ease whereby AFLP fragments can be converted into SCAR markers, as it proved more difficult with RAPD markers as initially anticipated. Several reports have shown that although it was theoretically easy to convert RAPD markers into SCAR markers, only a small subset of RAPD markers were converted into SCAR markers (Adam-Blondon *et al.,* 1994; Borovkova *et al.,* 1997; Blake *et al.,* 1996; Myburg *et al.,* 1998; Venter and Botha, 2000).

Primer-set SCAR-$AGCT_{300}$ produced single amplification products with the same molecular size and segregation phase as the original AFLP marker. Southern analysis of the fragment was carried out and the fragment segregated in accordance with the AFLP fragment. Thus, the developed SCAR primer-set was successful in detecting the polymorphism generated by the AFLP marker it derived from. A small amount of non-specific product was detected in some of the BC_2F_2 individuals, but this was rectified after reaction stringency was improved using higher annealing temperatures.

The seven RAPD primers and the SCAR primer were used to generate a linkage map. The size range from 2.8-19.6 cM with fragments mapping on both sides of the resistance gene *Lr41*. Even though the markers are distantly linked to the resistance gene, it is a starting point for a more detailed characterisation of the chromosomal region containing the *Lr41* gene. The combination of RAPD and AFLP techniques

proved to be an efficient way to define markers. The construction of a map based on RAPDs is probably not worthwhile (Devos & Gale, 1992) for wheat, but in combination with AFLPs valuable information can been gained.

The identification of the AFLP markers and the conversion of these markers into SCARs makes routine marker-assisted selection of leaf rust resistance in wheat a achievable goal, as the process seemed, unlike with RAPDs, relatively straight forward. By identifying and characterising SCAR markers linked to leaf rust resistance gene *Lr41*, breeders should in the near future be able to use this gene in combination with other genes, in a gene pyramiding approach to increase resistance in commercially important wheat cultivars.

CHAPTER 5

CONCLUSIONS

Wheat is one of the world's major food crops and is grown across a wide range of environments (Briggle & Curtis, 1987). One of the major problems in the production of wheat is the reduction of genetic variation (Beharav *et al.*, 1997). To address the problem we identified and determined the distances between cereal genotypes. Various molecular techniques can be applied to accomplish this. The molecular technique selected needs to be reliable and easy to perform in a relatively short time (Johnson *et al.*, 1995). The techniques used in this study, were RAPDs, AFLPs and SSRs. It was found that by combining the different methods, most of the pitfalls of one technique can be overcome by the other. RAPDs can be used in taxonomical studies (Demeke *et al.*, 1992; Demeke *et al.*, 1994). It was, however, found that the four RAPD primers, used in this study failed to identify unique molecular markers for the wheat genotypes. The AFLP technique offers a high level of utility (Powell *et al.*, 1996) and the average number of polymorphisms obtained were 100 polymorphisms per AFLP primer. The AFLP primers were able to distinguish among the genotypes, 'SA463/*Palmiet', 'SST65' 'SA684', 'TA2460', 'KS90WGRC10', 'Kiewiet' and rye. One of the major advantages of SSR is that the primers are genome specific, and can therefore only detect one of the three genomes of wheat. Primer pair $XGWM122_{150}$ amplified a fragment that was not identified in the rye and is probably specific for the A or B genome of *Triticum*.

The pairwise distance analysis between the genotypes revealed that the South African wheat genotypes are genetically very close, with an average distance value of 0.29. These results were confirmed by the cladogram. The results emphasised the reduction of the genetic variation. The genotypes, 'TA2460', 'TAM107' and 'KS90WGRC10' were not closely related to the wheat genotypes, used in the study. To broaden the genetic basics of these genotypes, that are not closely related genotypes need to be introduced into the South African genome. The *T. taushii* genotypes is not closely related to the South African genotypes and introduction of these genotypes will broaden the South African wheat diversity. This new variation will also additionally see the introduction of a leaf rust resistance gene, *Lr41*.

Leaf rust, is one of the most important diseases in wheat (Schachermayr *et al.*, 1997). The most economical method of reducing yield losses, caused by leaf rust, is breeding

for genetic resistance (Kolmer, 1996). A total of 47 leaf rust resistance genes have been identified (McIntosh *et al.*, 1998). Leaf rust gene *Lr41*, is a gene transferred from the wild relative *T. tauschii* (Cox *et al.*, 1994). Various molecular techniques are used to increase our ability to characterise resistance genes in wheat. RAPDs and AFLPs proved effective in the identification of molecular markers (Dedryver *et al.*, 1996; Williams *et al.*, 1997; Hartl *et al.*, 1999). It was found that the number of polymorphisms determined by AFLP was significantly higher than those performed by RAPDs. One of the objectives in the study was to provide breeders with an easy and fast characterisation tool for breeding for *Lr41* resistance.

AFLP marker, E-AAG:M-CTA$_{300}$, was converted into a SCAR marker. Primer set SCAR-AGCT$_{300}$ produced the same, single molecular size and segregation pattern as the original AFLP marker and was therefore, successful in detecting the polymorphism. The seven RAPD primers and the SCAR primer were used to generate a linkage map, with the size differ from 2.8-19.6 cM. Even though the markers are distantly linked to the resistance gene, *Lr41*, it is the starting point for more detailed characterisation of the chromosomal region 1D (Cox *et al.*, 1994).

In this study it was found that by combining different molecular techniques the best results were obtained. The knowledge obtained from the distance analysis of different cereal genotypes, and the characterisation and identification of molecular markers linked to the leaf rust resistance gene, *Lr41* expands our knowledge about the wheat genome. This in turn will improve breeder's ability to exploit and manipulate the wheat genome to our advantage.

CHAPTER 6

SUMMARY/OPSOMMING

SUMMARY

Wheat is one of the major food crops consumed by man and is grown in a wide range of environments. One constraint in the production of wheat is the different diseases attack the crop. One such a diseases is leaf rust. Leaf rust is caused by *Puccinia recondita* f.sp. *tritici* and is the most widespread and regularly occurring rust on wheat. Researchers are continually searching for new resistance genes and to date 47 leaf rust resistance genes (*Lr*) have been designated in wheat.

Three molecular techniques RAPDs (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphism) and SSRs (microsatellites) were used to identify and analyse the distances between different cereal genotypes. Four RAPD primers, three AFLP primers and two SSR primers were used to analyse the wheat genotypes, x-*tritosecale* and rye. The average polymorphisms obtained from the AFLPs were 100 polymorphisms per AFLP primer combination, and is much higher than with the RAPD primers (11-16 polymorphic loci). The four RAPD primers failed to identify unique molecular markers for the wheat genotypes. The AFLP primers were able to distinguished between the 'Kiewiet', rye, 'SA463/*4Palmiet', 'SA684', TA2460' and 'KG90WGRC10' genotypes. The SSR primer pair $XGWM122_{150}$ was able to amplify a fragment from the A or B genome. The pairwise distances between the genotypes revealed that the wheat genotypes were genetically very close, with an average distance value of 0.29 between the genotypes. The genotypes, 'TA2460', 'TAM107' and 'KS90WGRC10', were not closely related to the South African wheat genotypes and were genetically very close to each other. The rye and 'Kiewiet' genotypes were grouped as an outgroup by the UPGMA analysis and were the least closely related to the cereal genotypes.

RAPD (Random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) analysis were used to identify molecular markers linked to leaf rust resistance gene *Lr41*. Bulk and parental lines were screened with 380 RAPD primers and 64 AFLP primer combinations. A total of 3190 RAPD and 1859 AFLP loci, respectively were screened for linkage to the resistance gene. Twelve fragments

(seven RAPD and five AFLP), nine in coupling phase and three in repulsion phase, were identified as putative markers for the leaf rust resistance gene *Lr41*. Segregation analysis of these markers in a BC_2F_2 population revealed varying linkage distances that ranged from 2.8 to 33 cM. The coupling phase AFLP marker E-AAG:M-CTA_{300} was converted into a sequence characterised amplified region (SCAR) marker. AFLP markers have potential for marker assisted selection breeding programs, provided it can be converted into SCARs or equivalent marker typed systems.

OPSOMMING

Koring is een van die mees belangrikste graangewasse en groei in 'n verskeidenheid van omgewings. Een struikelblok in die produksie van koring, is die verskillende siektes wat die gewas infekteer. Een so 'n siekte is blaarroes. Blaarroes word deur *Puccinia recondita* f.sp. *tritici* veroorsaak en is die mees algemeenste en wydverspreidste roes op koring. Wetenskaplikes soek gedurig na nuwe weerstandsgene en 47 blaarroesgene (*Lr*) is tot op hede geïdentifiseer.

Drie molekulêre tegnieke RAPDs ('random amplified polymorphic DNA'), AFLPs ('amplified fragment length polimorphism') en SSRs (microsateliete) is vir die identifisering en analise van die afstande tussen die kleingraangewasse gebruik. Vier RAPD-inleiers, drie AFLP-inleiers en twee SSR-inleiers is vir die analise van koring, rog en korog gebruik. Die gemiddelde polimorfismes wat deur middel van die AFLPs verkry is, was 100 en is baie hoër as by RAPDs (11-16 polimorfismes). Die vier RAPD-inleiers was nie instaat om tussen die koring genotipes te onderskei nie. Die AFLP-inleiers het tussen 'Kiewiet', rog, 'SA463/*4Palmiet', 'SA684', TA2460' en 'KG90WGRC10', onderskei. Die SSR-inleier-paar, $XGWM122_{150}$ het 'n fragment geamplifiseer wat deel is van die D-genoom. Die afstande tussen die genotipes het gewys dat die Suid-Afrikaanse koring geneties baie na verwant is, met 'n gemiddelde afstandswaarde van 0.29. 'TA2460', 'TAM107' en 'KS90WGRC10', is geneties naby aan mekaar verwant en verder van die koringgenotipes verwant. Al koringgenotipes is geneties baie naby verwant aan mekaar.

RAPD- en AFLP-analise is vir die identifisering van molekulêre merkers, wat aan die weerstandbiedende blaarroesgeen *Lr41* gekoppel is, gebruik. Bulk en ouerlyne is met 380 RAPD-inleiers en 64 AFLP-kombinasie-inleiers geanaliseer. 'n Totaal van 3190 RAPD- en 1859 AFLP-loci is onderskeidelik vir moontlike koppeling aan die weerstandsgeen geanaliseer. Twaalf fragmente (sewe RAPD en vyf AFLP) is as moontlike merkers vir die geen *Lr41* geïdentifiseer. Nege van die moontlike merkers was aan weerstandbiedenheid en drie aan vatbaarheid, gekoppel. Segregasie-analise van die TK_2F_2-populasie het koppelingsafstande van 2.8 tot 29 cM geïdentifiseer. Die weerstand-gekoppelde AFLP-merker, E-AAG:M-CTA_{300c}, met 'n molekulêre afstand

van 300, is as SCAR ('sequence characterized amplified region')-merker ontwikkel. Die inleierpaar-SCAR-$AGCT_{300}$ het 'n enkele amplifikasieproduk, met dieselfde molekulêre grootte en segregasiepatroon as die oorspronklike AFLP-merker, geamplifiseer. AFLP-merkers het die potentiaal om in MAS ('marker-assisted selection') telingsprogramme gebruik te word, indien dit na SCAR-merkers omgeskakel word.

CHAPTER 7

LITERATURE CITED

AND

APPENDIX

LITERATURE CITED

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APPENDIX

Appendix 7.1 Input data matrix used for UPGMA analysis of RAPD, AFLP and microsatellite markers[a].

'Palmiet'
010011111110011010110001000001101111001110000001101011000110010011001100000000010100
010010000000000010101000001000010000011110011011000000010101100000000000000000000000
00000100000001000010001011010110111011001101001000010110100100001000110010011000100
01111011011000101011001010101001001100100011001001100001000100111000011100010010111
010000110101110010000100000010100001011000010000010100010110000000000000000000000101
0001001011010011010

'Tugela'
010001111110011000110001000001101101001010010001101011010001100100001101000000000101
00101100001010000110110001101000001110111000100100000100000100000000001001000001000
10100110011100100001001011110101110010110101001100110110100010001100001010010110010
11101111011000100010101000101001001100100001000101000010100100101010011100010010011
01000111010110001100010100000010001100001001000001001001010010001100001010010000000
0000000011010000110

'Molopo'
010101111110011010110001111101111111000101001000110001100000000001000010000000100100
010000000101011010111000000000000000000000000000000000000000000000000000000000000000
000001000000001000000010011100010101011011010010001100100000010001000010010101001100
011110010101001010110011001010010100001000110010011000011011001000000011100010010101
000000110101110011010101010100100001001010010000010101010110010010100000100000000101
0101001001000001010

'SA1684/*5 Tugela'
01010111111001101011000100100110111000101001100110101101000000010000110110101010100
001001010000000001110000111000000000010101001011000001000001000000000000001000100000
00001100000100100001001011110101110101001101001100110010100010001010011010010100010
00101011010100100010101000101001010100100010000101000011001001011110011100010010001
010001101101100011000100000010100011000010010000011010010101000001000000000000000101
0101001010111001011

'SA463/*4 Gamtoos'
000001110000011000110001001100000011000010010001000010000100000001011110110010000101
001011000010000000110110110010100000010111000101100000100000110000000001001000000000
100001000000000100001001011101000110110101011001100010110110010001000010011101001100
01111011010100100010101000101001010000101011000010100001101100110100011101000010001
00100011010110001100010101110010100100101001000001001000011000110110000110000000101
0001001001010010110

'Betta'
110001111110011000110001001001101010001010010101110011110011100000001101000101001001
0101100000000001010100001010000000000111000101100000000010100000000000000000000001000
0000010000010010000100101111000110101011010100100011011000001000100001101001010010001
0110100101100010001010110010100100100010001010100101000110101010000001110001001100101
0010010110101100011000101010100100101000010010000010011010110000101100100100100000101
0101001000110001010

'SA463/*4 Palmiet'
0101011111100110101100010010011011110011111011011010110010101000000011010000000001000
0101010010000000101010001101100000000101110001000000000100000010000000000000000000000
0000010000000100001000100101010111001110110100100011001000001000110001101001010001000
0111111010100100010101000101001110000100000101010100001101100100010011100000001000100
0000011010110011100010101001010000100001000000000101010101101001011001100100000001010
101001010110001010

'SA2199/ Letaba'
0101011101100110101100010000011111110010100110011000110100010001010011010001100010100
1000000000100011011000100010001000101111000101100000000101010000000000100000000000000
0000100000010010000000010011101001001111001010101000100101000100010000110100101010100
1101011011000100010101000101001010100100011001001100001101100100010011101010110101
0100001011011000110001010101001011010000100000000101001001010000010010000100100010101
1000100101001001 0

'SA1684/*4 Molopo'
1101011101100110101100010000011011110011101110011000110010001000010011010100000001010
0101100000001000101011001011101100001011100010011000100000001000000000010010000010000
0000100001001000000000100111000110100110010100100001011000001000110001101001010101000
1010010110001000101011001001010100001100110010011000011010101000100111000101010011
1001011010110001101010101010010010100101001000000111010101100001101001000101000010101
01001001010001000

'Kiewiet'
1011100011111111101111011111001000111010101100001100110100001000010011010010000010010
1001010001000010010000011010001100101100001111000000000000100010000000000000001010001
0000010001101001000000110000100001000110110001000001001000001000101101001001100110001
0101001011000000011010000000100100001010010001001010101001010101001011010101010000
1010010011000000010101100010000100101010100110100001001000010000000010001000000000000
1100010100000000 00

'Rye'
0011100011000011111111001101001011100010001000001111001010100000100101010000000110000
1110010111000010011000111000001000101110000111001010000000100010100000011001001001
0000010001100010000000110000100001001010100010100110010100110010010100000000010101001
1010000010101001001101100010000110001000001000100101010100000101010001100011011010
1010010100100000000001010001000100000101101001100000100000010000000001000000000000000
100000100000000000 0

'SST65'
1010111101100111101110011111011011110010010010011010110000000011000011100000001001100
1010101100000000100000111000000000100000000001001000000010000000100000010010010000010
1001100000000100100000000101001000011100100011001000010110000010001100010010101000100
0011001101010001001010100010100101010010000100001001100110110010001001111001001010100
0100001101011001110001001100101000110010100100000101000110010001101100001000000010101
0101011001010011010

'SST86'
10101111011001101011000100000110111110100100100110101101000000000000011010100001 0101
00101100001100001010100011010000100011110100101100001000001010000000000001000000100
10000100001000100000000100101000110010110010100100001001000001000100101001001010101 0
01111011011000100010111000101001010100101001000001010001101100100010011110010011001
01000011010100010100010010010010000100001001001001101011011001001001010010000000101
010101101101000101 0

'SST825'
10101111011001101011000110100100111110111111000010001101000000000000011110000000010 1
00100100000000000111000000000000000000011100001100000000000110000000001000000000100 0
00001100001010010000000101001001011101000011001000110010000010001000010010101010100
00111011010100010010101000100101010000101000100011100001000100101000011000010010001
00100010110110011100010000110010000100001000000000100110101010000010000000100100001 01
010101101101100101 1

'Inia'
10101111011001101011000110100100111100100000100110101100101000000100010101010000001
01000011000000000000101010010000000001010110101100100100011101010000000000000101000 00
01000010000000100000001001010001110101100101001000010010000010001000011010101010001 00
01101011010100100010101000101001010000100001000001100000101100100000011010000001000 0
01000000101010000010001000111001000010000100000000100101000101001010001010000000010 1
011000100010000101 0

'Corwa'
10101111011001101011000110000100111111111111000010000101000110010000110101000000010 1
00101010000000000101100001010000010000111100011100000000100100000000001000000010000 00
00000100001100100000000100001000111000010010100100011001000001000100001001001100101 0
01101011011000100010101000101011000100101001000010101001101010101010011100010010101
01000011010111001101010010010010000100001011001001010100010101100010000110111110010 1
010101100000000101 0

'TAM107'
0000110000000001101110011111111001111001001001001111011010000000001001011100000101010010001000000001100001010010010000001000110101000010010010101000010001010000000000000000100010000100011001100000101010000101001100100100010100000001000101000000101010101000000010011000001001001000010100000010100011000001100000000111000010100000101000100000100000000000000010000000100000010101101001010100111000001100100101010

'KS90WGRC10'
1010101000000001111110011111111011111001001001001111011000100011010010100100000001010010100010000010101100010100101000101010011001000000000001100000010000010010000010000011000000000000010000000110000101001001001000110110110001001010001000010000001001100110001000010101010011110010010000000000100111100001010101000000101000110100100100000100101000000000000001100010000011000000000111100000000100000100000010011101110111010011101010010100

[a] Each text string shows the name of the genotype or line and the presence (1) and absence (0) of the markers.

Appendix 7.2 Listing of RAPD primer sequences.

Nr	Primer	Sequence 5' to 3'
1.	OPA12	TCGGCGATAG
2.	OPK10	GTGCAACGTG
3.	OPK18	CCTAGTCGAG
4.	OPL1	GGCATGACCT
5.	OPM19	CCTTCAGGCA
6.	OPN1	CTCACGTTGG

Appendix 7.3 Listing of AFLP primer sequences.

Nr	Primer	Sequence	
		5' to 3'	**3' to 5'**
		***Eco*RI**	***Mse*I**
1.	E-AAG;M-CTA	AATTCAAG	ATCAATT
2.	E-AGG:M-CAA	AATTCAGG	AACAATT