

# **Targeting quantitative trait loci for adult plant stripe rust resistance in wheat**

**by**

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## Declaration

“I hereby declare that the dissertation submitted by me in fulfilment of the requirements for the degree of *Magister Scientiae Agriculturae* in the Faculty of Natural and Agricultural Sciences, Department of Plant Sciences (Plant Breeding), University of the Free State, Bloemfontein is my own independent work and has not previously been submitted by me at another University/Faculty. I furthermore cede copyright of the thesis in favour of the University of the Free State.”

.....  
Onoufrios Agathoclis Philippou

.....  
Date

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## **Dedication**

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## Quote

“To know the cause of a disease and to understand the use of the various methods by which the disease may be prevented amounts to the same thing in effect as being able to cure the malady.”

**Hippocrates** (460 - 377 BC)

## List of abbreviations

<b>AFLP</b>	Amplified fragment length polymorphism
<b>APR</b>	Adult plant resistance
<b>ARC-SGI</b>	Agricultural Research Council - Small Grain Institute
<b>ATP</b>	Adenosine triphosphate
<b>bp</b>	base pair(s)
<b>BSA</b>	Bulk segregant analysis
<b>CAPS</b>	Cleaved amplified polymorphic sequences
<b>cDNA</b>	complementary DNA
<b>CTAB</b>	Hexadecyltrimethylammonium bromide
<b>DArT™</b>	Diversity arrays technology
<b>DH</b>	Doubled haploid
<b>DHPLC</b>	Denaturing high pressure liquid chromatography
<b>DIG</b>	Digoxigenin
<b>DNA</b>	Deoxyribonucleic acid
<b>dNTP</b>	2'-deoxynucleoside 5'-triphosphate
<b>DTT</b>	1,4 dithiothreitol
<b>EDTA</b>	Ethylenediaminetetraacetate
<b>gDNA</b>	genomic DNA
<b>IM</b>	Interval mapping
<b>ITMI</b>	International Triticeae Mapping Initiative
<b>kb</b>	kilobase(s)
<b>LAI</b>	Leaf area infected
<b>LOD</b>	logarithm of odds
<b>LRS</b>	likelihood ratio statistics
<b>MAS</b>	Marker-assisted selection
<b>PAGE</b>	polyacrylamide gel electrophoresis
<b>PCR</b>	Polymerase chain reaction
<b>PIC</b>	Polymorphic information content
<b>QTL</b>	Quantitative trait loci



<b>RAPD</b>	Random amplified polymorphic DNA
<b>RFLP</b>	Restriction fragment length polymorphism
<b>SCAR</b>	Sequence characterised amplified region
<b><i>Scmv</i></b>	Sugarcane mosaic virus
<b>SNP</b>	Single nucleotide polymorphism
<b>SSR</b>	Simple sequence repeat
<b>STR</b>	Simple tandem repeat
<b>STS</b>	Sequence-tagged site
<b><i>Taq</i></b>	<i>Thermus aquaticus</i>
<b>TBE</b>	Tris-borate/EDTA
<b>tBSA</b>	targeted bulk segregant analysis
<b>TE</b>	Tris-Cl/EDTA
<b>Tris-HCl</b>	Tris (hydroxymethyl) aminomethane hydrochloride

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## **List of presentations delivered from thesis project**

### **Poster**

Herselman, L., O.A. Philippou, Z.A. Pretorius, R. Prins, 2007. The effectiveness of targeted bulk segregant analysis to identify AFLP markers closely linked to QTL for adult plant stripe rust resistance in wheat. The 2<sup>nd</sup> International Conference on Plant Molecular Breeding (ICPMB). Sanya, Hainan, China, March 23-27, 2007.

# *Chapter 1*

## **General Introduction**

Bread wheat (*Triticum aestivum* L.) is one of the most important and widely grown cereal crops in the world. World trade for wheat is greater than for all other crops combined (Curtis, 2002). Wheat originated in the Middle East from where it spread to North Africa, Eurasia, western Europe, the Americas and the Southern hemisphere (Pagesse, 2001). Cultivated wheats are divided into three main groups: diploids ( $2n=2x=14$ ; einkorn), tetraploids ( $2n=4x=28$ ; emmer, durum, Polish and Persian wheat) and hexaploids ( $2n=6x=42$ ; spelt, bread, club and Indian shot wheat). Hexaploid bread wheat presumably originated in northwestern Iran or northeastern Turkey as a result of hybridisation between tetraploid wheat and diploid *Aegilops tauschii* >8 000 years BC (Feldman, 2000). Due to the polyploid genetic structure of *Triticum* species and the associated genetic diversity, these plants have successfully adapted in a variety of environments throughout the world (Maratheé and Gómez-Macpherson, 2001).

Abiotic and biotic stress factors hamper high yields of this crop leading to great economical losses. Biotic stress factors such as stripe rust (*Puccinia striiformis* f. sp. *tritici*) disease is a challenge to traditional plant breeding programmes because of the ability to cause high yield losses if the disease is not controlled (Tolmay, 2006). Hence, the cultivation of traditional wheat crops in areas which are suitable for the disease to form and spread has proven to be devastating to wheat producers globally. However, productivity in these areas can be increased by cultivation of durable adult plant resistant (APR) wheat cultivars which is the most useful, cost-effective and environmentally friendly way to control stripe rust disease in wheat (Lin and Chen, 2007). APR for stripe rust in wheat cultivars such as the South African cultivar Kariega can endure infection of stripe rust unlike other susceptible wheat cultivars such as Avocet S (Prins *et al.*, 2005). Wheat cultivars that have APR against stripe rust assist breeders in generating durable resistance. In contrast, monogenic resistance is frequently overcome by the disease which produces new virulent pathotypes able to attack previously

monogenic resistant plants. Thus, breeding programmes preferably breed for durability instead of specific resistance as a counter attack to stripe rust infection (Tian *et al.*, 2002).

Stripe rust was first reported in South Africa in 1996 in the Western Cape and has since then spread to all wheat producing areas, causing considerable damage and it has cost wheat farmers millions of rands to control the disease (Boshoff *et al.*, 2002). Presently, the major wheat producing areas around South Africa are the mediterranean, winter rainfall region in the Western Cape and the central irrigation areas including the Northern Cape (Tolmay, 2006). Since many records of durable rust resistant cultivars have been published, it might hopefully in future aid traditional and molecular wheat breeders using marker-assisted selection (MAS) for quantitative traits (Bariana and McIntosh, 1995). William *et al.* (2007) stated that the development of technologies will increase the amount of available markers, improve the efficiency, throughput, and cost effectiveness of MAS, thereby making it more productive and affordable to many breeding programmes. Although markers are currently available for relatively few traits, MAS should be incorporated with traditional breeding to exploit its impact in breeding programmes. When used together with phenotypic selection, MAS will improve response to selection for certain traits, thereby increasing rates of genetic progress (William *et al.*, 2007).

The undertaking of MAS has been welcomed with enthusiasm and expectation in wheat breeding programmes, generating remarkable investments in the development of molecular linkage maps and research to detect associations between phenotypes and markers. Molecular linkage maps have been constructed for a wide range of crop species. There is a substantial difference of opinion with respect to the applications of MAS among different cereal crop species (Ruane and Sonnino, 2007). For example, Koebner (2003) observed a greater interest for MAS in maize (*Zea mays* L.) compared to wheat, due to the breeding structure and programmes in place.

Given the abovementioned factors confronting wheat crops in South Africa, a study was undertaken to investigate the specific goals presented in this dissertation. The aim of this study was to determine how effective the targeted bulk segregant analysis (tBSA) approach

was in uncovering markers for the stripe rust resistance on chromosomes 2B and 7D governing APR quantitative trait loci (QTL) previously reported by Ramburan *et al.* (2004).

The aims of this project would be achieved by:

- i) selecting specific double haploid (DH) lines from the original 150 F<sub>1</sub> Karioga x Avocet S DH population based on QTL marker intervals as identified by Ramburan *et al.* (2004),
- ii) pooling these into five bulks and analysing these bulks and controls with amplified fragment length polymorphism (AFLP), a multi-loci detection technique for which no sequence information of the target region is needed,
- iii) validation on the individuals constituting these bulks,
- iv) addition of putative markers to the existing linkage map and QTL analysis using the 150 individuals of the Karioga x Avocet S DH population.



## Chapter 2

# Application of traditional and molecular plant breeding for achieving durable rust resistance in wheat

### 2.1 Introduction

Wheat is one of the most abundantly produced and edible (Röder *et al.*, 1998) cereal crops in the world. It is a member of the family Gramineae (Poaceae), genus *Triticum* (Zhang *et al.*, 2006) and has been studied intensively for a variety of agronomic traits found within the genome (Lagudah *et al.*, 2001). Wheat, in the form of wild einkorn (*Triticum monococcum* ssp. *aegilopoides* (Link) Thell.), had been harvested as early as the late Paleolithic and early Mesolithic Ages, 16 000 - 15 000 BC (Stallknecht *et al.*, 1996). Cultivated wild wheat remains have been dated back to the early Neolithic Age 10 000 BC (Zohary and Hopf, 1993). Cultivated wild einkorn continued to be harvested in the upper area of the Tigris-Euphrates regions during the Neolithic and early Bronze Age, 10 000 - 4 000 BC, giving way to emmer wheat by the mid-Bronze Age (Stallknecht *et al.*, 1996).

Wild species of wheat (*T. urartu* Tumanian ex Gandilyan), is a diploid ( $2n = 2x = 14$ ) with one genome (AA), crossed with *Aegilops speltoides* L., a diploid ( $2n = 2x = 14$ ) with one genome (BB). The fertile hybrid offspring produced from this cross had two genomes comprising the tetraploid (AABB). Wild emmer (*T. turgidum* ssp. *dicoccoides* (Körn. ex. Asch & Graebn.) Thell.) is a fertile tetraploid ( $2n = 4x = 28$ ) with two genomes (AABB). Bread wheat (*T. aestivum* L.), an allohexaploid ( $2n = 6x = 42$ ) originated from a cross between this tetraploid and a wild emmer relative *T. tauschii* (Cosson) Schmalh., with one genome (DD) (Gill *et al.*, 2004). Bread wheat has an extremely large genome of  $16 \times 10^9$  bp, consisting of seven groups of chromosomes, each group containing a set of three homologous chromosomes belonging to the AABBDD genomes (Lagudah *et al.*, 2001), with more than 80% repetitive DNA (Röder *et al.*, 1998, Francki and Appels, 2002). Wheat is described to be one of the most complex crop species for genetic analysis, due to the structure and size of its genome (Langridge *et al.*, 2001, Francki and Appels, 2002).

## 2.2 Economic importance of wheat

Wheat was the first cultivated crop (Gill *et al.*, 2004) and is still one of the most important food crops in the world (Röder *et al.*, 1998). Wheat plays an important role in every day life during human consumption, since it provides an immense source of proteins and calories. Wheat is mainly grown in temperate regions and occupies approximately 17% of all global agricultural areas. To meet the demands of human wheat consumption by 2050, production of wheat must increase at a rate of 2% annually on land that will not increase much beyond the present level (Gill *et al.*, 2004). Wheat is the second largest agricultural crop planted in South Africa (FAOstat database, 2007). During the 2006/2007 season, 764 000 ha of wheat was planted in South Africa and the projected planting for wheat during 2007/2008 season is 655 000 ha, which is 14.36% less than the previous season. The increasing wheat prices are due to supply and demand constraints in South Africa (Anonymous, 2007a). In 2007 wheat was South Africa's second highest agricultural import (FAOstat database, 2007), in order to meet local requirements. Import prices on 17 July 2007 in Durban harbour was R2 394 per ton and for Cape Town harbour R2 364 per ton (Anonymous, 2007a).

One of the main economic importances of bread wheat production is grain yield (Kuchel *et al.*, 2007). Major advances in understanding the wheat plant must be achieved to increase yields and protect crops from abiotic (heat, frost, drought and salinity) and biotic stresses (pests, viruses, bacteria and fungi) which accounts for an estimated average loss of 25% (Gill *et al.*, 2004).

## 2.3 Diseases of wheat

There are many different types of biotic stresses that affect wheat globally (Murray *et al.*, 1998). The Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), is a serious economic pest of wheat in South Africa (Liu *et al.*, 2001). Powdery mildew is a fungal disease in wheat growing areas worldwide caused by *Blumeria graminis* DC f. sp. *tritici* Em. Marchalof (Miranda *et al.*, 2006). Wheat diseases that are considered to be of global importance include fusarium head blight caused by *Fusarium graminearum* Schwabe [telomorph *Gibberella zeae*

(Schwein.) Petch] (William *et al.*, 2005), stem rust (black rust) caused by *Puccinia graminis* Pers. f. sp. *tritici*, leaf rust (brown rust) caused by *P. triticina* (Eriks.) and stripe rust (yellow rust) caused by *P. striiformis* Westend. f. sp. *tritici* Eriks (Marsalis and Goldberg, 2006).

## **2.4 Rusts of wheat**

Wheat rusts (*Puccinia* spp.) are destructive diseases that can be found in most wheat producing areas of the world (Kolmer, 2004). In South Africa wheat rusts have played an important role in wheat production in the past (Pretorius *et al.*, 2007). Wheat rust fungi are dispersed in the form of dikaryotic urediniospores, which can be carried for thousands of kilometres from primary infection locations by wind, causing worldwide epidemics. Wheat rust fungi are virulent obligate parasites with a high degree of specificity that interact in a gene-for-gene association with wheat. The development of many different pathogenic races makes durable rust resistance complicated to achieve. New virulent rust races, specific against resistance genes on recently infected wheat, can quickly increase in occurrence over large areas, consequently making existing resistance genes useless (Kolmer, 2005). There are three rust diseases that occur on wheat caused by a particular species of the rust fungus *Puccinia*, namely stem, leaf and stripe rust (Marsalis and Goldberg, 2006). Despite the fact that these three wheat rusts originated from the same genus, they differ in morphology, life cycle and develop at dissimilar environmental growth conditions (Pienaar, 2004).

### **2.4.1 Stem rust (black rust)**

*Puccinia graminis* f. sp. *tritici* that causes wheat stem rust, is an obligate parasite (Kolmer, 2005) and one of the most important and widespread diseases of cereals, infecting wheat, barley and triticale (Murray *et al.*, 1998). Other alternative host plants stem rust survives on include *Agropyron distichum* L., *Hordeum murinum* L., *H. vulgare* L., *Lolium italicum* A. Braun non Courtois, *Bromus maximus* Roth and *Dactylis glomerata* L. (Pretorius *et al.*, 2007). Stem rust develops in warm temperatures, optimally between 18°C and 29°C, but can occur at temperatures between 15°C and 40°C. Stem rust occurs mainly on stems, however it can be found on leaves, sheaths, glumes, awns and even seed. Symptoms begin as oblong

lesions that are usually reddish-brown in colour. In the late stages of the disease, pustules erupt to produce numerous black spores. Severe infections with numerous stem lesions may weaken plant stems (Marsalis and Goldberg, 2006). Stem rust is a macrocyclic, heteroecious rust and needs both cereals and barberry (*Berberis vulgaris* L.) to complete its life cycle (Murray *et al.*, 1998). Stem rust infection of barberry produces round, yellow-to-red coloured pustules underneath the leaves. Spores (aeciospores) produced on barberry plants infect wheat and a different type of spore (basidiospores) produced on wheat infects barberry plants. Although both hosts are needed, epidemics on wheat can develop rapidly as spores (urediniospores) can infect the same plants on which they were produced (Marsalis and Goldberg, 2006). In temperate regions teliospores can survive in crop debris from previous infected plants. However, it can survive as dormant mycelium during the winter season (Murray *et al.*, 1998).

#### **2.4.2 Leaf rust (brown rust)**

Wheat leaf rust, also known as brown rust, is the most common and widespread of the three wheat rusts (Kolmer, 2005). Alternative hosts for leaf rust include meadow rue (*Thalictrum* spp.), rue anemone (*Isopyrum* spp.) and clematis (*Clematis* spp.) (Marsalis and Goldberg, 2006). Leaf rust is an important disease of wheat especially in temperate climates and occurs regularly where wheat is grown. Leaf rust develops optimally at 100% humidity together with temperatures ranging between 15°C and 22°C (Murray *et al.*, 1998) and the disease will progress up to temperatures of 27°C. Leaf rust is normally found on leaves although it can infect glumes and awns. Symptoms on infected tissue start as tiny, circular to oval yellow spots on the upper leaf surface. The spots later develop into orange coloured pustules surrounded by a chlorotic halo. The pustules produce numerous spores that are easily dispersed, producing an orange dust on the leaf surface. Black spores may be produced later in the development of the disease, resulting in orange and black lesions on the same leaf. Small orange lesions are present on seed heads, however, lesions do not develop into dispersing pustules. This helps to differentiate leaf rust from stem rust (Marsalis and Goldberg, 2006).

### 2.4.3 *Stripe rust (yellow rust)*

Stripe rust disease in wheat is caused by the biotrophic fungus *P. striiformis* (Yan *et al.* 2003; Lin and Chen, 2007). Of the three rust diseases in wheat, stripe rust is the most damaging to grain yields in cool, moist environments (Singh *et al.*, 2000). The worldwide distribution of stripe rust, also known as yellow rust, is more restricted in comparison with leaf and stem rust (Boshoff *et al.*, 2002). Stripe rust develops at lower temperatures, optimally between 10°C and 15°C and normally occurs in regions that have predominantly cooler climates and are near the coast (Zadoks, 1961; Murray *et al.*, 1998). Optimal disease progression tends to cease at temperatures above 21°C (Marsalis and Goldberg, 2006). Only very susceptible varieties are at risk of infection from stripe rust during wet periods in semi-arid regions (Murray *et al.*, 1998).

#### 2.4.3.1 *Economic importance of stripe rust*

Stripe rust is an important disease obstructing wheat production worldwide (McIntosh *et al.*, 1995), including South Africa. The disease's quick dispersal and ability to survive in both summer and winter rainfall regions gives it the ability to attack susceptible high yielding cultivars during optimal climatic growth conditions. Stripe rust has a powerful impact on the economy as a result of yield losses and costs of chemicals used to control the disease (Pretorius *et al.*, 2001).

Yield losses due to stripe rust vary considerably on a yearly basis due to growth conditions, such as inoculum pressure, weather conditions and cultivar susceptibility. In 1994 reports throughout the world showed 40 - 84% yield losses (McIntosh *et al.*, 1995). Control of the disease on susceptible wheat cultivars affected by rusts has cost wheat farmers in South Africa millions of rands. In 1996 Western Cape wheat farmers spent R28 million on fungicides, nevertheless 5 - 50% yield losses still occurred. In 1997 farmers in the eastern Free State spent R18 million on fungicides. In 1998 costs to control the disease, excluding yield loss and quality, on approximately 42 000 ha was estimated to be R6 million (Boshoff, 2000; Pienaar, 2004). In South Africa, wheat grain yield losses as high as 65% were recorded

on fungicide trials for both spring and winter wheat infected with stripe rust (Pretorius *et al.*, 2007).

#### 2.4.3.2 Symptoms

Stripe rust disease gets its name from the typical symptoms on mature wheat leaves consisting of yellow-orange straight-sided pustules that occur in stripes on leaves arranged between the veins and heads. Pustules on young leaves are scattered and difficult to differentiate from those of leaf rust. Stripe rust occurs on heads, resulting in the arrangement of masses of spores lodged between the glume and the lemma (Murray *et al.*, 1998). The elongated pustules are thin and differ in length. Mature pustules produce yellow-orange spores. As the disease develops, tissues around the pustules turn brown and dry, producing necrotic tissue. Chlorosis or yellowing of leaves may be observed. Stripe rust infected wheat fields displaying severe symptoms are easily noticeable from a distance (Marsalis and Goldberg, 2006). At the end of the season, black telia form in necrotic tissue killed by yellow rust pustules (Murray *et al.*, 1998).

#### 2.4.3.3 Life cycle

Stripe rust disease is hemicyclic with only binucleate urediniospores (Line, 2002) and teliospores (Steele *et al.*, 2001). At the end of the season telia sometimes form lesions, germinate and produce basidiospores (Murray *et al.*, 1998), but no alternate host has been identified (Zadoks, 1961). Stripe rust survives in wheat as mycelium during intercrop periods or during the asexual stage as uredinia (Line, 2002). Stripe rust can endure cold temperatures but if temperatures drop below  $-5^{\circ}\text{C}$  the pathogen can be killed. Disease epidemics occur during periods of high humidity with temperatures between  $10^{\circ}\text{C}$  to  $15^{\circ}\text{C}$ . The disease tends to be inhibited during warmer conditions ( $21^{\circ}\text{C}$ ) and infections mainly occur during late winter, spring and early summer by wind dispersal or other vectors (Marsalis and Goldberg, 2006). Wind disperses urediniospores over long distances. Urediniospores which have not infected the wheat can remain in a dormant stage for at least seven days. The initial wheat plant infected by the urediniospores on the initial point of infection by the disease on a crop

is known as a hot spot. A hot spot is a regular pattern observed in relation to the spread of the disease to the prevailing wind direction. Stripe rust forms urediniospores in large quantities (Murray *et al.*, 1998).

#### 2.4.3.4 *Stripe rust in the world*

Stripe rust in the past was more significant in regions with cool and wet environments and is frequently observed in northern Europe, the Mediterranean region, Middle East, western United States, Australia, east African highlands, China, the Indian subcontinent, New Zealand and the Andean regions of South America. Stripe rust is more significant in higher altitude and tropical regions, such as northern African countries, the Himalayan foothills of India, Pakistan and Mexico (Boshoff *et al.*, 2002). Stripe rust is destructive in the western United States and has become increasingly more important in the south-central states of the United States (Yan *et al.*, 2003). It is one of the most important and prevalent rust pathogens in Europe and losses of 50 - 60% on susceptible varieties were observed during severe disease outbreaks (Moldenhauer *et al.*, 2006). Stripe rust did not occur in environments such as Australia until 1979 (Steele *et al.*, 2001) and was detected in New Zealand in 1980. Although stripe rust was detected in eastern Australia since 1979, it was only reported in Western Australia in 2002. The failure to move westwards was probably due to quarantine precautions, weather patterns and geographic barriers (Wellings *et al.*, 2003). Stripe rust was first reported in central Africa in northern Zambia in 1958 (Boshoff *et al.*, 2002).

#### 2.4.3.5 *Stripe rust in South Africa*

Stripe rust was first observed in South Africa in Moorreesburg, Western Cape, during August 1996 (Boshoff *et al.*, 2002) and later spread to all wheat production areas of the country (Ramburan *et al.*, 2004) e.g. Western Cape, eastern Free State and KwaZulu-Natal. Stripe rust caused considerable damage during the 1996/1997 season (Pretorius *et al.*, 1997). A decade later, stripe rust annually infects wheat producing regions. Disease severity depends on favourable moisture and temperature growth conditions (Pretorius *et al.*, 2007). Chemical control can be effective in some stripe rust infected areas and was the preferred approach in

controlling the disease in South Africa in the past (McIntosh *et al.*, 1995). It is estimated that more than R100 million has been spent from 1996 to 1999 on fungicides to control stripe rust (Boshoff *et al.*, 2002).

#### 2.4.3.6 Disease control

Wheat producing countries around the world affected by stripe rust have important wheat breeding programmes in place to produce wheat varieties resistant to stripe rust (Murray *et al.*, 1998). Although chemical control is used to control stripe rust, it is expensive and harmful to the environment (Singh *et al.*, 2000). Plant breeders have developed genetic resistance within wheat cultivars to a variety of rust races. Some resistant cultivars are highly effective with the result that many races common in the past are almost non-existent today. In many situations varieties remained resistant for three to four years before showing signs of susceptibility. However, mutation in stripe rust allow for development of new races. Therefore, wheat breeders are constantly monitoring old cultivars for sustained tolerance to the pathogen, in addition to developing new cultivars with improved resistance to new and old stripe rust races (Marsalis and Goldberg, 2006).

#### 2.4.3.7 Genetic resistance to stripe rust

There are numerous types of resistances described for cereals, with two major resistance types, specific resistance and nonspecific resistance. Specific resistance is often effective and is generally under monogenic control. However, pathogens can easily overcome specific resistance, leading to yield losses (Tian *et al.*, 2002). Nonspecific resistance is polygenically controlled and is often less effective. However, nonspecific resistance is considered to be durable (Boukhatem *et al.*, 2002). A particular form of polygenic resistance is APR, giving a high level of protection that is expressed only in adult plants but not during seedling stage. In some instances high yields appear to be incompatible with APR (Boshoff *et al.*, 2002). Stripe rust resistance can also be grouped into seedling and APR. Seedling resistance is effective throughout the growth cycle of the plant and follows a gene-for-gene relationship (Imtiaz *et al.*, 2003) but expresses low infection types in seedling tests. This type of resistance is short



lived in the field since stripe rust has the ability to mutate and infect plants with new virulent strains (Singh *et al.*, 2001). Plants that are susceptible during seedling stage but show resistance during the adult plant stage are described as having durable APR which is effective in the control of stripe rust disease (Imtiaz *et al.*, 2003).

Development of resistant cultivars to control stripe rust disease is the preferred method of control (Lin and Chen, 2007) and is an economical and environmentally safe method to reduce crop losses. The development of durable resistant cultivars to stripe rust requires the availability of the desired genetic resources, an understanding of the genetics of the host-pathogen interaction and suitable methods to utilise the desired genes (Imtiaz *et al.*, 2003). Plant breeders are interested in APR as it is often regarded to have the potential for durability (Johnson, 1988; Chen and Line, 1993), particularly those APR genes that have been characterised to be slow rusting genes (Singh *et al.*, 2000). Understanding the genetics of resistance to disease is important for planning crosses in breeding programmes, identifying resistance genes, understanding genetic diversity and developing genetic markers to assist in selection (Imtiaz *et al.*, 2003).

## **2.5 Breeding strategies**

### **2.5.1 Traditional plant breeding**

Traditional plant breeding programmes use phenotypic characteristics for the identification of genetic variation (Langridge and Chalmers, 2004). Global demand for wheat production is increasing, placing pressure on breeding programmes to develop elite cultivars which are adaptable to different environments without compromising grain quality, agronomic performance or resistance to pathogens causing diseases (Francki and Appels, 2002). Global wheat breeding programmes over the last forty years made major progress in improving yield potential, stability and development of durable resistant cultivars for many different types of pathogens (Francki and Appels, 2002; William *et al.*, 2005).

### 2.5.2 *Molecular plant breeding*

Although traditional plant breeding has made considerable contributions during the last few decades, major progress has been seen in biotechnology and molecular biology. This scientific progress has shown an increase in understanding and characterising traits important to plant breeders at molecular level (William *et al.*, 2005). One of the benefits of molecular breeding programmes compared to traditional breeding programmes is that selection based on phenotype has moved to selection based on genotype (Ruane and Sonnino, 2007). Molecular plant breeding programmes have assisted in effectively developing improved wheat varieties, using biotechnology for genome wide expression studies and comprehensive mapping initiatives (Francki and Appels, 2002).

A major objective of wheat breeding programmes is rust resistance (Bariana *et al.*, 2001), especially stripe rust, as it is the most damaging to grain yields in cool, moist wheat planting regions (Singh *et al.*, 2000). Therefore the development of stripe rust resistant cultivars is the most effective and economic method of managing stripe rust (Yan *et al.*, 2003; Lin and Chen, 2007). Several APR genes to stripe rust have been reported (Bariana *et al.*, 2001). There are 40 genes which have been identified as conferring stripe rust resistance at 37 loci in wheat and catalogued as *Yr1* through *Yr37* and 23 temporarily designated genes were identified (McIntosh *et al.*, 2005; Li *et al.*, 2006) and integrated into commercial cultivars (McIntosh *et al.*, 1995). For rust resistance, selection in the past was based on seedling and/or field responses of breeding populations (Bariana *et al.*, 2001). Presently, breeding programmes base selection on durable APR (Lin and Chen, 2007). Although breeding programmes prefer durable resistance, breeding for APR is complex and involves the late developmental stage of expression, potential multigenic control and high environmental variation (Varshney *et al.*, 2004). Molecular breeding programmes attempt to utilise molecular markers which are not influenced by the environment to tag genes responsible for APR against stripe rust and develop molecular markers as simple molecular tools for breeders to use in the introduction of APR into new wheat cultivars (Boyd, 2001).

## 2.6 History of wheat genomics

Since man started cultivating wheat as a domestic crop, they have tried to improve wheat using visual phenotypic outcomes to determine required results. Centuries later we are still trying to improve wheat with more tools that aid in observations of phenotypes and genotypes (Gill and Friebe, 2002). In the early 1920s, the nuclear genome of wheat was analysed based on chromosome pairing behaviour in interspecific hybrids, which provided information on genome constitution, phylogeny and evolution of *Triticum* and *Aegilops* species (Sax, 1922). Wheat aneuploids were used in the 1930s to initiate the cytogenetic analysis and gene mapping of individual chromosome arms of wheat (Sears, 1954). Later in history, in the 1970s, the substructures of wheat chromosomes were analysed using modern staining methods to construct karyotypes of wheat (Rayburn and Gill, 1986). In the 1980s, restriction fragment length polymorphism (RFLP) analysis was used for genetic linkage mapping in wheat (Chao *et al.*, 1989). By the end of the century, production of deletion stocks were used to develop physical maps based on molecular markers for all 21 chromosome pairs (Gill *et al.*, 1996). Molecular markers are heritable units linked with economically important traits that molecular plant breeders use as selection tools (Varshney *et al.*, 2004). These selection tools improve the understanding of the wheat genome (Röder *et al.*, 1998; Lin and Chen, 2007). Since wheat has a large genome, genetic analysis of quantitative disease resistance using closely linked markers provide an alternative means for the selection of APR in molecular breeding programmes and can be done in the absence of pathogens (Bariana *et al.*, 2001).

### 2.6.1 Molecular marker system technologies

Molecular markers are polymorphic when there is variation between the DNA sequences of individuals under study and are merely an indicator of sequence polymorphism. Sequence polymorphism between individuals is attributable to the insertion or deletion of multiple bases, or single nucleotide polymorphisms (SNPs) (Varshney *et al.*, 2004). The most powerful analytical tools to detect DNA polymorphism both at specific loci and whole genome level are molecular markers (Somers, 2004). A number of molecular markers are

being used in breeding programmes, nevertheless the costs associated with MAS are the main restriction to their widespread use by plant breeders (William *et al.*, 2007). Molecular markers have been used for many applications in plant breeding, such as estimating genetic diversity, fingerprinting, MAS and genetic linkage mapping (Gupta *et al.*, 1999) to produce advanced lines from desirable crosses (Somers, 2004). Molecular markers have been made available by numerous researchers globally and begun to significantly contribute to further improve plant breeding (William *et al.*, 2005).

## **2.6.2 Low-throughput molecular markers**

### *2.6.2.1 Restriction fragment length polymorphism*

RFLP are co-dominant markers capable of identifying variable DNA fragment sizes (Varshney *et al.*, 2004). RFLPs have been used for genetic fingerprinting, cultivar identification (Wünsch and Hormaza, 2002), germplasm evaluation, as an indirect selection criteria and genetic mapping (Poehlman and Sleper, 1995). RFLPs use endonuclease restriction enzymes that cleave genomic DNA at specific nucleotide sequences called restriction sites to produce restriction fragments (Varshney *et al.*, 2004). Restriction fragments are identified by Southern blotting, a technique by which fragments are separated by agarose gel electrophoresis according to size and transferred to a membrane (Southern, 1975; Varshney *et al.*, 2004). The membrane is made either from nitrocellulose or nylon and contains the immobilised DNA that hybridises with a radioactive labelled DNA probe (Varshney *et al.*, 2004). Alternatively, fragments can be visualised either by pre-treatment or post-treatment of the agarose gel, using staining methods such as ethidium bromide staining, silver staining (Kochert, 1994) or using digoxigenin (DIG), a labelled DNA probe (Morioka *et al.*, 1999). The restriction sites vary between individuals due to deletions, insertions, mutations and methylation modification between or within restriction sites (Appels *et al.*, 1986).

RFLPs are time-consuming and automation is difficult, resulting in the method not being used regularly within plant breeding programmes. A large quantity of DNA is used in

comparison to the polymerase chain reaction (PCR)-based techniques (Weising and Kahl, 1997). RFLPs, in comparison with some of the PCR-based marker techniques, obtained lower levels of polymorphism (Williams *et al.*, 1990) and are limited to low copy sequences (William *et al.*, 1997) which is not sufficient in wheat (Gupta *et al.*, 1999). Usually less than 10% of RFLP loci are polymorphic in wheat limiting their use for molecular analysis of agronomic traits (Ma *et al.*, 2001). Several RFLP-based maps of bread wheat, either for groups of homoeologous or for the entire chromosome groups, have been published (Peng *et al.*, 2000).

#### 2.6.2.2 Random amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) is a PCR-based marker technique (Varshney *et al.*, 2004), where random segments of genomic DNA are amplified with a single decamer (10-mer) primer of arbitrary nucleotide sequence. Incompatible pairing between primer and template, deletions of primer sites and deletions and insertions between primer sites are considered to be accountable for polymorphism (Welsh and McClelland, 1990; Williams *et al.*, 1990). Since RAPD analysis does not need information of the targeted DNA sequence (Williams *et al.*, 1990) it is mostly used to compare DNA of biological systems that has not been tested before. A major disadvantage of using RAPDs is non-reproducibility due to a low annealing temperature, causing unspecific binding of primers (Varshney *et al.*, 2004). Most RAPD markers are dominant although some rare co-dominant RAPD markers have been observed (Roy, 2000).

Advantages of RAPD technique include that it is rapid and requires small amounts of DNA with no radioactivity (Botha and Venter, 2000). RAPDs have been successfully used to map leaf rust resistance genes *Lr9*, *Lr24*, *Lr28*, *Lr29* and *Lr34* (Schachermayr *et al.*, 1994; William *et al.*, 1997). However, as with RFLPs, RAPDs are not as useful in wheat as with other crops, due to low levels of polymorphism within wheat (Gupta *et al.*, 1999).

### 2.6.2.3 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) technology was originally developed and described in the early 1990's by Zabeau and Vos (1993), working at Keygene, as a genetic fingerprinting technique. AFLP analysis is a PCR-based DNA marker analysis system and is a highly sensitive method able to detect polymorphisms in DNA throughout the whole genome (Vos *et al.*, 1995). AFLP analysis is based on the amplification of subsets of genomic restriction fragments using PCR (Savelkoul *et al.*, 1999). Genomic DNA is digested by two restriction enzymes, one rare cutter (e.g. *EcoRI*) and one frequent cutter (e.g. *MseI*) producing DNA fragments of different lengths (Vos *et al.*, 1995).

Vos *et al.* (1995) stated the principle for using two restriction enzymes is the following:

- (i) The frequent cutter will produce DNA fragments which amplify well and are in the most favourable size range for separation on denaturing gels.
- (ii) By using the rare cutter the amount of fragments amplified is reduced, since only the rare cutter and frequent cutter fragments are amplified, limiting the amount of selective nucleotides needed for selective amplification.
- (iii) The use of two restriction enzymes makes it possible to label one strand of the double stranded PCR products.
- (iv) Using two different restriction enzymes gives the greatest flexibility in manipulating the number of fragments to be amplified.
- (v) Large numbers of different fingerprints can be generated by the various combinations of a low number of primers.

Adapters are ligated to the ends of the DNA fragments to create a template DNA for amplification. The sequence of the adapters and the adjoining restriction site function as primer binding sites for subsequent amplification of the aliquot of genomic restriction fragments (Savelkoul *et al.*, 1999). Adapter-specific primers have extensions on their 3' ends of selective nucleotides which can only prime DNA synthesis from a subset of the restriction sites (Varshney *et al.*, 2004). Only restriction fragments in which nucleotides flanking the restriction sites match the selective nucleotides will be amplified. Selective amplification

using one nucleotide extension amplifies one out of four of the adapter-ligated fragments, whereas selective amplification using three nucleotide extensions in both primers amplify one out of 4 096 of the adapter-ligated fragments. Polymorphisms obtained from AFLP analysis are due to (i) mutations in restriction sites, (ii) mutations in sequences adjacent to the restriction sites and complementary to the selective primer extensions and (iii) insertions or deletions within the amplified fragments (Savelkoul *et al.*, 1999). Sequencing gels or even ordinary denaturing polyacrylamide gels (PAGE) are used to separate PCR products, which are visualised by silver staining. Alternatively, primers are labelled either by radioisotope ( $P^{33}$ ) or fluorescent dye so that the AFLP can be visualised by autoradiography or fluorescent imaging methodologies, respectively (Mueller and Wolfenbarger, 1999).

The AFLP technique, like most other techniques, has disadvantages. AFLP analysis amplifies dominant markers and is time consuming compared to simple sequence repeat (SSR) analysis, laborious and more expensive compared to other PCR-based techniques. It requires good quality DNA to guarantee complete enzymatic digestions because non-reproducible variation in DNA profiles occurs when DNA is partially digested (Varshney *et al.*, 2004). The advantages compared to other marker technologies are that it is highly efficient in revealing polymorphisms (Shan *et al.*, 1999) and has higher reproducibility, resolution and sensitivity on the whole genome level than other techniques (Wolfe and Liston, 1998). AFLP analysis obtains a higher number of amplified products (50-100), increasing the probability of detecting polymorphisms as compared to other marker systems (Varshney *et al.*, 2004). AFLP analysis utilises infinite amounts of loci which can be analysed with different primer combinations. The AFLP analysis is able to differentiate amongst genotypes which is not obstructed as a result of their bi-allelic nature (presence or absence) (Mackill *et al.*, 1996) and shows intra-specific homology (Powell *et al.*, 1996). Compared with other DNA marker systems, the AFLP technique requires no sequence information (Ma and Lapitan, 1998).

AFLP analysis simultaneously detects a large number of amplification fragments which is useful for mapping, especially in regions containing genes of interest (Peng *et al.*, 2000). Due to high levels of polymorphisms AFLP analysis has become predominantly beneficial in the study of genetic variation in strains or closely related species of plants and population

genetics to determine differences within populations and in linkage studies to generate maps for QTL analysis (Wolfe and Liston, 1998). Wheat has a large and complex genome as a result of both 90% repetitive and highly methylated DNA sequences and having a polyploidy ancestry. *SseI*, a methylation sensitive restriction enzyme is preferred for AFLP analysis in wheat compared to *EcoRI*, a methylation insensitive restriction enzyme. By avoiding fragments that terminate in methylated sites, highly methylated repetitive DNA is avoided and while the number of fragments amplified is reduced, the number of polymorphisms identified is increased (Donini *et al.*, 1997).

#### 2.6.2.4 Microsatellites

Microsatellites or SSRs or simple tandem repeats (STRs) (Archibald, 1991) is a PCR-based marker system (Jacob *et al.*, 1991). SSRs are polymorphic loci present in genomic DNA of one to four base pairs (bp) tandem repeats (e.g. ATATAT) (Turnpenny and Ellard, 2005). It has been estimated that repeats longer than 20 bp occur every 33 kb in plants (Varshney *et al.*, 2004). Loci are amplified by PCR using primers (18 - 25 bp long) which are specific for conserved nucleotide sequences flanking regions of repeats of two to four bp (Manifesto *et al.*, 2001). SSRs are highly abundant and evenly distributed, highly polymorphic, co-dominant, easily assayed by PCR and accessible due to published primer sequences (Saghai-Marooif *et al.*, 1984; Varshney *et al.*, 2004) and has made significant contributions to plant genetic studies. Microsatellites are restricted to intraspecific and intragenomic analysis and are therefore suitable for comparative analysis or for introgression studies involving wild species related to wheat (Gupta *et al.*, 1999). SSRs are locus-specific in most species (Taramingo and Tingey, 1996) and an extensive effort is needed to screen the whole genome with SSR markers in attempts to identify markers for a gene with an unknown chromosomal location. The research effort and cost therefore involved restrict their use in many laboratories (Brown *et al.*, 1996). SSR primers are useful for rapid and accurate detection of polymorphic loci and information could be used for developing a physical map based on these sequence tags (Varshney *et al.*, 2004).



Compared with RFLPs, SSRs are easier to handle, cost effective, appropriate for automation and can be used on a larger scale. SSRs have already been developed in various crop species. SSRs are abundantly found in eukaryotic genomes and express a greater level of polymorphism in hexaploid bread wheat than any other marker system (Ma *et al.*, 2001). One of the first SSR maps for wheat was developed by Röder *et al.* (1998) which consisted of 279 microsatellite loci amplified by 230 primer sets. Seventy nine of these primer sets were integrated and efficiently analysed for mapping purposes on genomic DNA of wild emmer wheat, *T. dicoccoides* [*Triticum turgidum* (L.) Thell. *ssp. dicoccoides* (Koern)Thell.] (Peng *et al.*, 2000). This map played a role in tagging and mapping genes for a variety of agronomic traits such as the yellow rust resistance gene *YrH52* in wild emmer wheat (Peng *et al.*, 1999) and *Yr15* transferred into wheat from *T. dicoccoides* (Ma *et al.*, 2001). Approximately 570 SSR primer sequences were available in 2005 for wheat, which is a small number relative to the genome size of wheat (Song *et al.*, 2005). Although the International Triticeae Mapping Initiative (ITMI) was the first organisation to publically fund research on the linkage map of bread wheat and more than 1 000 markers have been mapped, significant gaps remain in some linkage groups (Somers *et al.*, 2004; Anonymous, 2007b).

#### 2.6.2.5 Sequence specific PCR markers

The conversion of multi-locus marker types such as AFLPs and RAPDs through cloning, sequencing and primer design to sequence specific markers successfully deal with problems of reproducibility, high-throughput and co-migrating amplification products (Lottering *et al.*, 2002). Cleaved amplified polymorphic sequences (CAPS) are based on the restriction enzyme site variation in DNA fragments produced by PCR and are co-dominant markers. Sequence information for the primers can come from a gene bank, genomic or cDNA clones, or cloned AFLP/RAPD fragments (Varshney *et al.*, 2004). Paran and Michelmore (1993) resolved the problem of RAPD reproducibility by deriving sequence characterised amplified region (SCAR) markers from the initial RAPD markers for the *Dm* resistance genes in lettuce. They defined SCARs as “a genomic DNA fragment at a single genetically defined locus that is identifiable by PCR amplification using a pair of specific oligonucleotide primers”. The two ends of the RAPD amplified product are cloned and sequenced and used

as primers for the amplification of the single fragments or SCARs. SCAR markers can be dominant or co-dominant. SCAR markers are more reproducible than RAPDs, can be developed into plus/minus arrays where electrophoresis is not required and show less variability among different thermocyclers and when different DNA polymerases are used (Paran and Michelmore, 1993; Schachermayr *et al.*, 1994; Roy, 2000). The inability to convert RAPD markers to SCARs have however been reported (Venter and Botha, 2000). This is probably due to the fact that annealing of a short primer (10 bp) to a long template results in the middle base pairs annealing more tightly than those on the 3' and 5' ends (Chen and Wu, 1997). This results in a specific primer that contains two bases that are not complementary to the template. The incidence of repetitive sequences might also play an important role, as it causes the specific primer to amplify homologous loci located on another chromosome which gives rise to a similar banding pattern (Penner, 1996).

Success has however been reported with the development of SCAR markers for wheat. Dedryver *et al.* (1996) reported a SCAR-marker developed from a RAPD marker for *Lr24*, whilst a RAPD-SCAR marker was developed for the wheat aphid resistance gene *Dn2* (Myburg *et al.*, 1998). The construction of a SCAR is however not limited to the use of RAPD technology. Xu *et al.* (2001) reported on the conversion of AFLP markers linked to the *Vf* gene in apples to a SCAR marker. As is the case with RAPDs, the internal sequences from both ends of the AFLP marker were used to design the 25 bp SCAR primers.

A sequence-tagged site (STS) is a short unique sequence (200 - 500 bases long) amplified by PCR that identifies a known location on a chromosome. Its sequence does therefore not occur anywhere else in the genome. STSs can be amplified by PCR from a genomic library or genomic DNA (gDNA) using specific oligonucleotide primers (Olson *et al.*, 1989). As a result, a single band will be obtained with electrophoresis, corresponding to the size of the target region. Once constructed, STS primer sets offer advantages of safety (no radioisotopes), relative ease and greater throughput while incorporating the advantages of PCR (Martin *et al.*, 1995). STS primers developed in cereals are potentially transferable between related species, as is the case for RFLPs (Talbert *et al.*, 1994).

Schachermayr *et al.* (1997) developed a RFLP-STS marker for *Lr10*, whilst Hu *et al.* (1997) was successful in developing a RAPD-STS marker for the powdery mildew resistance gene *Pm1* in wheat. Shan *et al.* (1998) managed to develop chromosome specific STS primer pairs from polymorphic AFLP fragments. Prins *et al.* (2001) succeeded in the conversion of a fragment associated with *Lr19* to a dominant STS marker. From their studies it became clear that not all AFLP products will be suited for conversion. One of the disadvantages of this technique is the requirement to isolate and develop markers for each new crop (Brady *et al.*, 1996).

Another sequence specific marker is single nucleotide polymorphism (SNP) which is an abundant source of sequence variation between individuals that can be targeted for molecular marker development. SNPs are single base nucleotide substitutions (A, C, G or T) and are abundant in plant genomes. SNPs have the advantage of automation and can be screened in a digital format analysis, enabling high-throughput analysis (Wang *et al.*, 1998). It is expensive to develop SNPs, since each DNA locus has to be sequenced and suitable PCR primers need to be designed. Primers are used to amplify a corresponding fragment from all other possible genotypes. Fragments produced from possible genotypes are sequenced and compared with one another to determine the SNP for each haplotype (Varshney *et al.*, 2004). Of all the molecular marker technologies available, SNP produces the greatest marker density (Edwards and McCouch, 2007).

Different methods are used to identify SNPs within a genetic locus, such as direct sequencing, single-strand conformation polymorphism, chemical cleavage of mismatches and enzyme mismatch cleavage (Varshney *et al.*, 2004). SNPs are frequently used for finding markers close to or within a gene of interest and can be used to detect functional nucleotide polymorphism. Development of SNP markers requires knowledge of DNA sequence in an individual followed by re-sequencing in other varieties to find variable base pairs. Alternatively, direct sequencing of SNPs can be developed through ecotilling with the CEL I enzyme (Comai *et al.*, 2004) or by denaturing high pressure liquid chromatography (DHPLC) measuring small rare differences from PCR amplified sequences which are hybridised to a reference sequence (Kwok, 2001). In addition to SNP development, both DHPLC and

ecotilling are viable technologies for SNP detection. There is a large number of other SNP assay technologies being developed and no single method is superior to the others.

The benefits of SNP assays include increased speed of genotyping, lower cost and the parallel assay of multiple SNPs (Edwards and McCouch, 2007). The development of newer markers such as SNPs and the availability of newer technologies such as DNA chips and microarrays will accelerate genome mapping and tagging of genes in this polyploid crop for efficient wheat breeding programmes (Gupta *et al.*, 1999). A novel approach was used to develop primers to amplify and sequence gene fragments for fine mapping and targeted SNP analysis using rice-wheat gene colinearity in the region especially from the *Bo1* boron toxicity tolerance locus of the hexaploid wheat genome. SNPs identified were used to generate markers closely linked to *Bo1* on the distal end of chromosome 7BL. SNPs were observed between the two cultivars Cranbrook and Halberd, at a low frequency (one every 613 bp). Furthermore, SNPs were distributed unevenly and were limited to only two genes (Schnurbusch *et al.*, 2007).

### **2.6.3 High-throughput molecular markers**

DNA-based marker methods such as AFLPs, RAPDs and RFLPs are dependent on gel electrophoresis and associated with difficulties in correlating fragments on gels with allelic variants (Jaccoud *et al.*, 2001) and are thus characterised as low-throughput. As a result high-throughput (not dependent on gel electrophoresis) hybridisation methods of nucleic acids immobilised on solid states (DNA chips) were developed to replace gel-based analysis systems. High-throughput genotyping such as DNA microarrays (Lipshutz *et al.*, 1999) allow simultaneous analysis of hundreds of thousands of oligonucleotides attached to a solid silicon surface in an ordered array to create a microarray. The DNA or RNA sample is amplified using PCR to allow the attachment of fluorescently labelled nucleotides and then hybridised to the array. Each oligonucleotide or cDNA on the array performs as an allele specific probe. Ideal matching sequences hybridise more efficiently, emanating a stronger fluorescent signal than mismatched combinations. The fluorescent signals are measured by high resolution fluorescent scanning and analysed electronically, thus allowing the identification of

heterozygous base pair mutations, insertions and deletions (Chee *et al.*, 1996). Microarrays were developed to genotype SNPs (Wang *et al.*, 1998). Initially DNA chips that analyse SNPs required prior DNA sequencing (Jaccoud *et al.*, 2001).

To avoid sequencing, Diversity arrays technology (DArT™) were developed for detection of specific DNA fragments derived from the total genomic DNA of an organism or a population of organisms (Jaccoud *et al.*, 2001). The advantages and disadvantages of the DArT marker analysis as a alternative array-based method is a cost effective marker technology which produces extremely high quality data needed for high-density mapping. In addition, the ease in which sequences of the markers are obtained, allow large-scale alignments between genetic and physical maps. This makes DArT the method of choice especially for species with high levels of genetic variation, when limited financial resources are available or for complex polyploid genomes that may not be amenable to the whole-genome sequence approach (Wittenberg, 2007). DArT analysis was used to type hundreds to thousands of genomic loci in parallel within the hexaploid genome of bread wheat. DArT generated a large number of high-quality markers in wheat (99.8% allele-calling concordance and approximately 95% call rate). The genetic relationships among bread wheat cultivars revealed by DArT coincided with knowledge generated with other methods (SSR, RFLP and AFLP) and even closely related cultivars could be distinguished (Akbari *et al.*, 2006). A comparison between the different low and high-throughput molecular marker systems discussed in sections 2.6.2 and 2.6.3 is given in Table 2.1.

#### ***2.6.4 Molecular markers in plant breeding***

MAS in the developed world has not yet produced desired results for crops in commercial breeding programmes (Somers *et al.*, 1999). When applying MAS as a tool for genetic improvement in developing countries certain criteria are taken into consideration, namely financial costs, benefits compared with traditional breeding or other biotechnology applications, development costs of MAS and application of MAS taking into consideration intellectual property rights (Ruane and Sonnino, 2007).

**Table 2.1 Comparison between different molecular marker technologies (Edwards and McCouch, 2007)**

Marker Type	PCR-based	Uses restriction enzymes	Level of Polymorphism	Abundance	Co-dominant	Automation	Loci per assay	Specialised equipment
RFLP	No	Yes	Moderate	Moderate	Yes	No	1 to few	Radioactive isotope
AFLP	Yes	Yes	Moderate	Moderate	No	Yes	Many	Polyacrylamide gels/capillary
SSR	Yes	No	Low	Moderate	Yes	Yes	1 to about 20	Polyacrylamide gels/capillary
SNP	Yes	No	Variable	Highest	Yes	Yes	1 to thousands	Variable
DArT™	Yes	Yes	High	Moderate	No	Yes	Many	Microarray
CAPS	Yes	Yes	Variable	Moderate	Yes	Yes	Single	Agarose gels
SCAR	Yes	No	Low	Moderate	Yes/No	Yes	Single	Agarose gels

The main reason to move toward PCR-based markers and mainly SSR marker maps is the potential to use the maps in plant breeding programmes (Gupta *et al.*, 1999; Ruane and Sonnino, 2007).

Gene pyramiding for a single important trait through MAS allows breeders to identify the presence of multiple genes/alleles related to a single trait, particularly when alleles do not individually exert detectable effects on the expression of the trait (Koudandé *et al.*, 2000). However, if several genes are closely linked to molecular markers, molecular breeding strategies can be used to produce lines with gene combinations. Detection of recessive genes in traditional breeding programmes is done through selfing to detect individuals with recessive alleles for the target trait. Markers linked to recessive alleles allow breeders to avoid the selfing step and progeny testing in backcross approaches (William *et al.*, 2007).

The intended application, ease and cost involved are the main factors that influence the choice of marker system (Gupta *et al.*, 1999). In areas where seasonal and geographical

considerations are taken into account, markers provide an advantage in identifying traits which are only screened in certain seasons or geographical regions due to the presence of biotic or abiotic stresses. Difficulties in QTL detection and interactions between QTL and the environment may make markers unreliable for traits below a certain threshold heritability estimate (William *et al.*, 2007). The advantage of molecular marker technologies is that (some techniques) small amounts of DNA are required allowing high-throughput analysis for early selection (Roy, 2000). Analysis during early generations to test quality parameters requires a large number of seed where MAS can be used based on protein profiles or DNA based markers. Early detection using genetic analysis can be done using seeds or young plant tissue, which improves selection efficiency (William *et al.*, 2007).

In general, the development of molecular markers linked to one particular gene appeared to be more challenging for a gene derived from the wheat gene pool, compared to markers linked to the genes that originated from a wild relative of wheat (Schachermayr *et al.*, 1997). This is due to the large genome size of bread wheat, the low levels of molecular polymorphisms within the species and the overwhelming presence of repetitive sequences (William *et al.*, 1997). A comparative genetic study among some *Triticeae* species by Peng *et al.* (2000), using molecular markers, detected slight similarities among certain genes, indicating possible transfer of genes from wild wheat into bread wheat.

There exist many examples of the application of molecular markers in wheat breeding. Garg *et al.* (2001) found that AFLPs delivered the highest number of polymorphic fragments per assay, followed by RAPDs and SSRs, but that SSRs delivered the highest polymorphic information content (PIC) in wheat. In contrast, Bohn *et al.* (1999) found that the PIC values were similar for RFLPs, AFLPs and SSRs in wheat. Powell *et al.* (1996) indicated that the marker index, which is a product of the number of polymorphic loci in the analysed cultivars and the average PIC values were low for RFLPs and SSRs but high for AFLPs.

Even though polymorphic SSR markers detected 22% variation within the wheat genome (Ma *et al.*, 1996), it was speculated that SSR markers can detect more polymorphic alleles per marker than RFLP (Ma and Lapitan, 1998). However, SSR markers are the only markers

available in wheat that are amenable to large scale applications, which is a requirement in all molecular breeding programmes. Since it is vital for a marker to be co-dominant and robust SSR analysis is useful in MAS (William *et al.*, 2007). However, AFLP markers do not require DNA sequence information, as is the case with STS-PCR and SSR markers and the developmental costs required for marker development are therefore much lower. Compared to RFLP or other PCR-based marker systems, AFLP is relatively fast, reliable and cost-effective (Ma and Lapitan, 1998). The different molecular marker techniques contributed significantly to the development of plant genetic maps (Gupta *et al.*, 1999) and the identification of DNA markers for useful genes such as *Lr41* (Lottering *et al.*, 2002) and *Lr19* (Prins *et al.*, 2001) for leaf rust and *Pm1c* for powdery mildew resistance (Hartl *et al.*, 1999).

#### **2.6.5 Doubled haploids**

Plants produced by natural or artificial doubling of chromosomes of haploid plants are called doubled haploids (DH) (Prem *et al.*, 2004). DH offer a major advantage by its ability to acquire homozygosity, reducing the time needed for developing homozygous lines in cross-pollinated crops and can be used to produce hybrid lines (Rajhathy, 1976). Thus, the use of DH populations reduces the breeding cycle by accelerating the development of homozygous lines (Kott, 1997), as compared to other breeding populations (Prem *et al.*, 2004). DH populations can be continuously propagated, which allows different research groups to work with the same genetic material and all generated data contribute to a common database (Burr *et al.*, 1994). The development of DH populations depends on the availability of haploid plants, since it is a prerequisite for DH plant production. In the 1920s naturally occurring haploid plants were discovered. Apart from naturally produced plants which are rare and confined to a few species the use of haploid plants were not a practical technique until methods were developed for controlled production of haploid plants (Kimber and Riley, 1983). Haploid plants are produced either by interspecific or intergeneric crossing followed by the elimination of selective chromosomes, for example, crossing barley or wheat with maize (Zhu and Wu, 1979). In the early 1960s the development of tissue culture techniques gave way to haploid plant production by *in vitro* culturing of unfertilised ovules (gynogenesis) or



from mature or immature pollen grains (androgenesis) (Bossoutrot and Hosemans, 1983). However, anther cultures produce low frequencies of haploids, making it difficult to differentiate diploids from DH which regenerate from the same somatic tissue of anther walls. The production of DH populations using anther cultures is time consuming and laborious for use in breeding programmes (Prem *et al.*, 2004).

In traditional breeding programmes homozygosity is accomplished after selectively selfing for several generations, taking up to 10 - 12 years depending on the crop (Allard, 1960). Homozygosity for qualitative and recessive traits is even more difficult due to many loci and masking of recessive alleles in heterozygous plants (Prem *et al.*, 2004). Considerations should be taken into account for selection in the case of mutations, normally recessive in nature, or qualitative traits controlled by recessive alleles (Maluszynski *et al.*, 1995). Since haploids express recessive genes, transgressive segregants for recessive traits are recovered by doubling the chromosomes and making them diploid.

DH have been used for many different approaches such as mutation breeding, disease resistance studies, biotechnological gene transfer, molecular breeding etc. DH populations in combination with molecular breeding using DNA-based methods such as RFLP and AFLP analysis are being used in plant breeding programmes as a result of the large range of applications (Prem *et al.*, 2004). DH are being used in studies for gene linkages and interactions (Ma *et al.*, 2007) and provide the plant breeder with a highly efficient marker aided selection tool. Since DH are homozygous for all loci, they are frequently used for genetic mapping (Prem *et al.*, 2004).

#### **2.6.6 Bulk segregant analysis and targeted bulk segregant analysis**

In order to use high-density molecular maps for studies on genome organisation, the conversion of linkage maps to physical maps needs to be done. This has become feasible due to the development of techniques for manipulation of large DNA fragments. Physical maps can confirm the order of DNA markers in the target region and by determining the relationship between genetic and physical distances, thus influencing the choice of strategy to

be used (Grandillo and Fulton, 2002). The importance of targeting strategies such as bulk segregant analysis (BSA) described by Michelmore *et al.* (1991), tBSA and fine mapping is indicated by the vast amount of techniques which have assisted in the development of these targeting processes. Different types of molecular markers have been developed, varying in their cost, ease of generating markers, dependability and long term usefulness in assisting these targeting strategies. Many mapping populations have been developed, to take advantage of different molecular marker approaches and to increase the subsequent power of the maps generated (Bennetzen, 2000).

BSA was introduced in an effort to simplify the identification of markers in the absence of near-isogenic lines (Melchinger, 1990). BSA was initially proposed for screening qualitative traits known to express variation at a single locus of large effect (Giovannoni *et al.*, 1991). The method involves grouping of individuals (called bulks) from two phenotypic extremes from a segregating population. Due to the simplicity and low cost of the BSA technique, it was initially proposed for screening more complex traits and is often restricted to segregating generations which are simpler and cheaper to produce, such as F<sub>2</sub>, DH, backcross or similar populations (Giovannoni *et al.*, 1991; Mackay and Caligari, 2000). DNA isolated from the parental lines and two bulks is screened with DNA markers. Polymorphisms between the two bulks are derived from regions of the genome that do not share similarity between individuals that made up the bulks. According to Michelmore (1994), the arbitrary nature of the pooled segregants will ensure that the chance of identifying a molecular marker that differentiates between the segregating offspring is heightened. Therefore, the rest of the genome is randomly contributed by the parents and should show no polymorphisms between the bulks. The method is important due to its ability to identify markers associated with the trait of interest without the need for full map construction (Langridge and Chalmers, 2004).

However, selected markers used in BSA should be repeatable when screening with PCR-based marker assays. AFLP markers in particular have proved to be suitable for these assays. A disadvantage posed by BSA compared to genetic map analysis, is that genetic distance between the marker and trait is not provided. It is always necessary to check the marker/trait associations by screening the individuals from the population to confirm that the marker is

reliable for the trait of interest. Screening of individuals is usually done to validate the expected marker pattern. BSA has been widely used and has become an important method in marker development programmes (Langridge and Chalmers, 2004).

In several studies, a BSA approach led to the identification of DNA markers linked to useful genes in wheat, apparently irrespective of the type of marker system. Hartl *et al.* (1999) were able to link an AFLP marker to powdery mildew resistance. William *et al.* (1997) used BSA for the detection of QTL associated with leaf rust resistance in wheat. Kölliker *et al.* (2001) reported on the use of bulked leaf samples (opposed to later bulking of DNA) from individual white clover plants for the assessment of genetic diversity with the use of AFLP analysis (Gustine and Huff, 1997). The bulking of plant material rather than bulking of DNA has been successfully used in various studies (Golembiewski *et al.*, 1997). BSA is a fast method to identify molecular markers closely linked to genes of interest (Lin and Chen, 2007).

Targeted BSA is a modification of BSA which was developed as a tool for marker enrichment in the target regions of selected chromosomes (Lübberstedt *et al.*, 2002). This method minimises the experimental effort in analysing large numbers of putatively linked markers and enables the selection of closely linked markers without analysing all individuals of the mapping population. Targeted BSA in comparison to conventional BSA is more effective as a result of narrowing the target regions by enriching markers and reducing the amount of work in future mapping (Dußle *et al.*, 2000). Dußle *et al.* (2000) stated using tBSA on QTL analysis could be effective for identifying closely linked markers in targeted regions, especially if combined with AFLPs. They reported that the saturation of two chromosome regions conferring resistance genes linked to sugarcane mosaic virus (*SCMV1* and *SCMV2*) was successful using SSR and AFLP markers by saturating regions using tBSA in European maize (*Zea mays* L.).

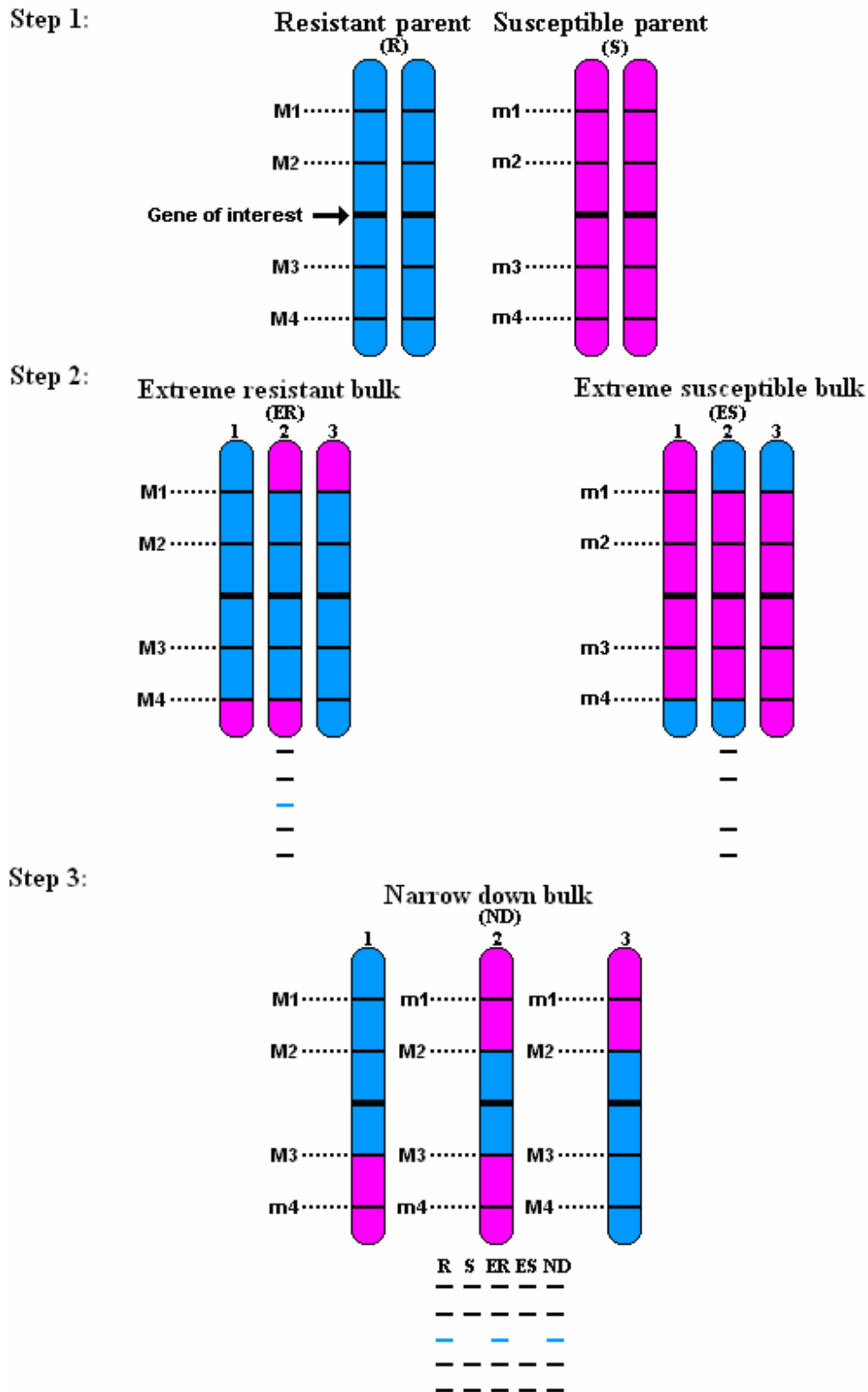


Figure 2.1 A graphical representation of a hypothetical tBSA approach to target the region between markers M2 and M3.

BSA can be used in combination with different targeting strategies in order to target specific regions of a chromosome around a gene of interest. One application is the use of existing marker data, as depicted in Figure 2.1. In step 1, marker data (M1 to M4 for the resistant parent and m1 to m4 for the susceptible parent) for the two parental lines (for e.g. a disease resistant and disease susceptible parent) are known. Furthermore, both phenotypic and marker data for the offspring of the segregating population is known. During step 2, individuals are selected to represent the extreme resistant and extreme susceptible bulks. Individuals are selected based on phenotypic data (similar to the traditional BSA approach) as well as known marker data. Individuals in the extreme resistant bulk contain all four markers flanking the gene of interest. These markers are associated with the resistant parent (M1 to M4). Individuals of the extreme susceptible bulk contain the four markers around the gene associated with the susceptible parent (m1 to m4). During step 3, in an attempt to target the region between the two flanking markers closest to the gene of interest (M2 and M3), a narrow down bulk is constructed for which individuals were chosen that had recombinations within the M1 and M2 region, as well as the M3 and M4 region.

In order to prevent detection of new markers within the M1 and M2 as well as M3 and M4 intervals, individuals are selected to have both M1 and m1 markers for the M1 and M2 interval and both M4 and m4 markers for the M3 and M4 interval. The presence of both parental alleles in these regions will theoretically eliminate detection of differences in these regions. Any markers that are only amplified in the resistant parent, extreme resistant bulk and narrow down bulk should theoretically be targeting the region closest to the gene of interest.

### ***2.6.7 Quantitative Trait Loci Analysis***

A QTL is a location on a chromosome which is considered to regulate an organism's phenotype for a quantitative trait (Collard *et al.*, 2005). Quantitatively inherited traits have a strong genetic component but which, under normal conditions of measurement, cannot be shown to be controlled by individually recognised loci. Expressions of these traits are usually influenced by the environment. QTL mapping makes the detection, localisation and

characterisation of genetic factors contributing to the variation of polygenically inherited traits feasible (Young, 1996). In order to perform QTL analysis, a plant breeder needs two critical sets of information. Namely, a linkage map of the genome of the species and a good method available to measure (such as leaf infection scores) how the trait of interest varies among plants (Ramburan *et al.*, 2004).

Individuals in a segregating population whose trait has been measured and which have been genotyped are crossed. The trait and genotypes of their produced offspring are determined as well. There are other experimental approaches which will give the same result, but all QTL analyses rely on a linkage map and reliable trait measures (Guzman-Novoa *et al.*, 2002).

To visualise how the data is analysed, researchers use genetic markers dispersed along the chromosome at a given location. The DNA sequences of these markers vary between the two copies of the chromosome (Collard *et al.*, 2005). The chances of recombination occurring are high if the markers are far apart and low if the markers are close together (Boake *et al.*, 2002).

Using a computer programme, the variation in the trait is correlated with each marker. If the trait is always high with a certain sequence of a marker and always low with another sequence, then the gene for the trait is probably close to the marker on the chromosome. If the variation in the trait is more or less random in relation to the marker, then there is little or no linkage between the trait and that chromosomal location. The level of association between the trait and location is called a logarithm of odds (LOD) score (Manly *et al.*, 2001). Most quantitative traits have high LOD scores for two to four locations in an organism's genome. There are of course exceptions to this. The higher the LOD score, the more significant a chromosomal region may be in regulating the trait. These chromosomal locations probably do not code directly for a particular trait, but instead for factors which shape the trait (Gershenfeld *et al.*, 1997).

Knowing the positions of QTL would allow for the use of MAS for traits difficult to manage by a traditional breeding programme (Araus *et al.*, 2004). However, QTL analysis in wheat

has been limited as a result of the large genome size and abundant repeated sequences. The genetic dissection of complex traits is one of the most difficult and most important challenges facing science today (Lin and Chen, 2007). By knowing which chromosomal locations are important, researchers can conceivably track how their genetic manipulations are affecting the regulation of the trait (Snape *et al.*, 2001). Many QTL have been detected in the wheat genome, for example Fusarium head blight (Haberle *et al.*, 2007), APR for stripe rust (Ramburan *et al.*, 2004; Prins *et al.*, 2005), etc.

### **2.6.8 Genetic mapping**

One of the most important applications of molecular markers is mapping of the whole genome. Molecular genetic maps of whole genomes based on allelic variation at individual marker loci, have been constructed for all major crops (Somers, 2004). Before the use of PCR-based markers, the construction of wheat genetic maps had been slow as a result of the low level of polymorphism in wheat (Chao *et al.*, 1989). The importance of genetic maps for different crop species has gradually increased since they were first introduced in the 1980s. RFLP markers were first used to produce wheat genetic maps (Chao *et al.*, 1989; Devos and Gale, 1992) and soon after, PCR-based markers were used for the construction of genetic maps, including RAPDs (Williams *et al.*, 1990), AFLPs (Vos *et al.*, 1995) and SSRs (Röder *et al.*, 1998; Gupta *et al.*, 1999). Traditional plant breeding needs to analyse thousands of plants in a short period of time at low cost. SSR markers and high-throughput capillary electrophoresis are beneficial for use of MAS in breeding programmes (Somers *et al.*, 2004). The ITMI has supervised and managed various institutions around the world to provide genetic mapping information for wheat (Francki and Appels, 2002).

A genetic map is more effective in molecular breeding if it is densely populated with markers. This provides molecular breeding strategies with more choice in the quality of markers and more probability of polymorphic markers in an important chromosome interval. The first microsatellite map in wheat possessed 279 microsatellites (Röder *et al.*, 1998). This marker density is useful for QTL and gene mapping, but is limiting for the precise transfer of QTLs between different genetic backgrounds. Specifically, the limitation comes from the

lack of polymorphic markers immediately flanking QTLs. Research in wheat genomics has increased the use of genetic maps, especially in map-based gene cloning efforts. Map-based cloning requires an accurate, fine genetic map to correctly position a gene of interest between close flanking markers (Somers *et al.*, 2004).

## **2.7 Previous studies on a Karioga x Avocet S DH population**

A project was approved by the Agricultural Research Council - Small Grain Institute (ARC-SGI) in 1999 to use molecular methods to detect QTL responsible for stripe rust APR using DH populations (Pretorius *et al.*, 2007). Prins *et al.* (2005) developed a DH mapping population from a cross between Karioga and Avocet S. Avocet S (Figure 2.2) is an Australian stripe rust susceptible wheat cultivar and the South African cultivar Karioga is a hexaploid hard red spring wheat cultivar (Prins *et al.*, 2005). Karioga (Figure 2.3) has a high yield potential with high protein content, a high falling number and excellent baking quality and is ranked top of the class for milling and baking characteristics (Barnard *et al.*, 2002).

Karioga expresses complete APR to stripe rust without losing any yield (Ramburan *et al.* 2004; Prins *et al.*, 2005) and is therefore of particular interest to wheat breeders. APR in Karioga could assist in developing new approaches to control stripe rust and develop a linkage map for breeding programmes. Analyses using APR to stripe rust field data identified two major QTL (*QYr.sgi-7D* and *QYr.sgi-2B*) in Karioga. *QYr.sgi-7D* was located on chromosome 7D and assumed to be the APR gene *Yr18*. *QYr.sgi-2B* was located on chromosome 2B associated with a chlorotic and/or necrotic response. Two minor QTL (*QYr.sgi-1A* and *QYr.sgi-4A*) were additionally identified on chromosome 1A and 4A respectively in the resistant cultivar Karioga (Ramburan *et al.*, 2004; Prins *et al.*, 2005; Moldenhauer *et al.*, 2006; Pretorius *et al.*, 2007).

Mapping results indicated that the most closely linked marker to the 7D QTL (*QYr.sgi-7D*) was the SSR locus *gwm295-7D* located at ~10 cM. Due to the low level of polymorphism on the 7D chromosome the construction of a dense linkage map for this chromosome was prevented, resulting in markers flanking 34 cM away from each other for *QYr.sgi-7D*





**Figure 2.2** A leaf of Avocet S showing susceptible symptoms to stripe rust.



**Figure 2.3** A leaf of Kariega indicating adult plant resistance to stripe rust.

(Ramburan *et al.*, 2004). The *Lr34/Yr18* complex is present on chromosome 7D (Singh *et al.*, 2000) and is linked to the gene *Ltn*. In the study of Ramburan *et al.* (2004), the *Ltn* gene mapped 12 cM away from the SSR marker *Xgwm295-7D* closest to the (*QYr.sgi-7D*) QTL region. It was not achievable to map *Lr34* in the DH population due to the presence and perplexing effects of other *Lr* genes.

Due to Kariega's pedigree it is highly likely that the *Yr18* gene is linked to the *QYr.sgi-7D* QTL region. A second major QTL was located on chromosome 2B. It was postulated that stripe rust resistance genes on 2BS include *Yr27*, *Yr31* and *Yr32*. However, the relationship between these genes on *QYr.sgi-2B* QTL region, *Yr27*, *Yr31* and *Yr32* still need to be determined (Ramburan *et al.*, 2004).

## *Chapter 3*

# **Increased marker resolution for adult plant stripe rust resistance QTL regions in Kariega**

### **3.1 Introduction**

Stripe rust resistance is important in wheat breeding programmes since it is a major disease hampering production of wheat globally (Boshoff *et al.*, 2002). Adult plant resistance (APR) to stripe rust has been reported in many cultivars (Bariana and McIntosh, 1995) including the South African cultivar Kariega (Ramburan *et al.*, 2004). Since stripe rust develops rather late in the season (Zadoks, 1961), selection for stripe rust resistance has been based either on seedling tests in the greenhouse and/or field responses of breeding populations (Bariana *et al.*, 2001). Breeders observed differences in levels of resistance at seedling and adult plant growth stages. Most breeders select for resistance based on field tests, since the environment plays a major role on stripe rust pathogens of wheat (Line, 2002). The application of durable quantitative resistance requires extensive field testing in a breeding programme and this type of resistance is generally more difficult to breed for than specific qualitative resistance (Castro *et al.*, 2002). The methodology of testing different types of disease resistance may include pathological seedling or adult plant greenhouse tests, field tests and molecular DNA markers. Molecular markers can aid in pyramiding, selection and detection of resistance genes in breeding programmes through marker-assisted selection (MAS) (Hysing, 2007). Closely linked markers provide an alternative means for the selection of important APR genes for stripe rust in breeding programmes, making it possible to determine resistance in the absence of stripe rust pathogens (Boyd, 2001).

Molecular plant breeding advances have provided many tools over the past few years for genetic analysis. The use of these techniques in combination with each other can improve and accelerate the development of genetic analysis by providing valuable information in a shorter period of time (Somers *et al.*, 1999). Specific genes can be targeted through the use of

gene targeting strategies such as bulk segregant analysis (BSA) and targeted BSA (tBSA). BSA (Michelmore *et al.*, 1991) is a method which compares two pooled DNA samples of individuals from a segregating population originating from a single cross. Individuals within each pool share an identical trait of interest, but are random for all other genes (Duñle, 2002). The tBSA method is a tool for marker enrichment in the target regions and was first described by Lübberstedt *et al.* in 2002. The tBSA approach reduces the time necessary for analysing large numbers of putatively linked markers and allows the selection of closely linked markers without analysing all individuals of the mapping population. Different techniques can be used in combination with BSA/tBSA, e.g. amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR). The AFLP method is widely used in mapping of wheat as it produces high levels of polymorphism and the procedure is relatively simple. AFLP markers seem to be the most efficient way to find markers tightly linked to resistance genes (Campbell *et al.*, 2001). Yu *et al.* (2004) reported SSR markers are particularly useful for constructing comparative framework maps for wheat species, since they amplify closely related markers to provide anchoring of chromosomes.

In a previous study two major quantitative trait loci (QTL) (*QYr.sgi-2B* and *QYr.sgi-7D*) and two minor QTL (*QYr.sgi-1A* and *QYr.sgi-4A*) against APR for stripe rust in the South African cultivar Kariega were identified based on analysis of field data for APR to stripe rust (Ramburan *et al.*, 2004; Prins *et al.*, 2005). Ramburan *et al.* (2004) detected low levels of polymorphism on chromosome 7D, resulting in markers flanking a region of 34 cM around the *QYr.sgi-7D* QTL region and higher levels of polymorphism on chromosome 2B, resulting in markers flanking a region of 25 cM around the *QYr.sgi-2B* QTL region. Markers more closely linked to the 2B and 7D QTL regions are needed for the construction of a dense linkage map. Thus the aim of this study was to determine how effective the tBSA approach was in uncovering additional AFLP markers for the 2B and 7D QTL target regions. This was achieved by i) selecting specific double haploid (DH) lines from the original 150 F<sub>1</sub> Kariega x Avocet S DH population based on QTL marker intervals as identified by Ramburan *et al.* (2004), ii) pooling these into five bulks and analysing these bulks and controls with AFLP, a multi-loci detection technique for which no sequence information of the target region is needed, iii) validation on the individuals constituting these bulks, iv)

addition of putative markers to the existing linkage map and QTL analysis using the 150 individuals of the Kariega x Avocet S DH population.

## **3.2 Materials and methods**

### **3.2.1 Plant material**

Kariega [pedigree: SST44{CI13523(Agent)/3\*T4(Anza)//K4500.2/Sapsucker S] is a hard red spring wheat cultivar with APR to stripe rust (Pretorius *et al.*, 1997). Avocet S is a soft white spring wheat cultivar susceptible to stripe rust. These two parental lines were used to develop a DH population using the wheat x maize technique (Ramburan *et al.*, 2004) with a F<sub>1</sub> plant derived from Kariega as a female and Avocet S as a male parent. The DH population consisted of 150 lines and segregated for APR to stripe rust (Prins *et al.*, 2005). Seed of parental lines and the DH population was planted in pots in the greenhouse to generate leaf material for DNA extractions.

### **3.2.2 DNA isolation**

Fresh young leaf material was harvested in the greenhouse and ground using a mortar and pestle to a fine powder after adding liquid nitrogen. Total genomic DNA was isolated using the CTAB (hexadecyltrimethylammonium bromide) DNA isolation method (Saghai-Marooif *et al.*, 1984). Approximately 250 µl of fine powder and 750 µl CTAB buffer, pH 8.0 [100 mM Tris-HCl (tris (hydroxymethyl) aminomethane hydrochloride), 1.4 M NaCl, 20 mM EDTA (ethylenediaminetetraacetate), 2% (w/v) CTAB, 0.2% (v/v) β-mercapthoethanol] were incubated at 65°C for one hour. An additional 500 µl of chloroform:isoamylalcohol [24:1 (v/v)] was added followed by centrifugation at 12 000 g for five minutes. DNA from the aqueous phase was precipitated with 500 µl isopropanol and incubated at room temperature for 20 minutes followed by centrifugation at 12 000 g for five minutes. The supernatant was discarded and tubes drained upside down. The pelleted DNA was washed by adding 500 µl ice-cold 70% (v/v) ethanol, followed by incubation for 20 minutes at room temperature. Tubes were centrifuged at 12 000 g for five minutes and the supernatant discarded. Pellets

were air-dried for one hour and re-suspended overnight at 4°C in 200 µl TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA).

Following overnight re-suspension, 0.1 µg/µl DNase-free RNase was added to the DNA followed by incubation for two hours at 37°C. DNA was extracted from the solution by adding 0.75 M ammonium acetate and an equal volume of chloroform:isoamylalcohol [24:1 (v/v)]. After gentle mixing, tubes were centrifuged at 12 000 g for five minutes. DNA from the upper aqueous phase was precipitated overnight at -20°C with 500 µl ice-cold absolute ethanol. Following overnight incubation, tubes were centrifuged at 12 000 g for 15 minutes and the supernatant discarded. Pellets were washed twice with 500 µl ice-cold 70% (v/v) ethanol through centrifugation at 12 000 g for ten minutes. The supernatant was discarded and the pellet air-dried at room temperature and re-suspended in 50 µl TE buffer, pH 8.0. The quantity and quality of the DNA was estimated by measuring absorbencies at 260 nm and 280 nm using a spectrophotometer and by visual comparison with known concentrations of standard bacteriophage lambda DNA (100 - 400 ng/µl). Genomic DNA was separated through a 0.8% (w/v) agarose gel using 1 x UNTAN (40 mM Tris-HCl, 2 mM EDTA, pH adjusted to 7.4 with acetic acid) buffer at 60 V for 45 minutes. DNA samples were diluted to a final concentration of 200 ng/µl and stored at -4°C until further use.

### **3.2.3 Construction of bulks**

For the purpose of this study, five bulks were constructed, namely an extreme resistant, extreme susceptible, narrow down 7D (+7D±2B), 7D (+7D-2B) and 2B (-7D+2B) bulks. R Prins used stripe rust phenotypic data generated by Ramburan *et al.* (2004) in combination with molecular marker data generated by Prins *et al.* (2005) to identify DH lines to be used in each of the five bulks. Phenotypic trait test data scoring leaf infections from stripe rust were ranked from highest to lowest for the selected resistant trait (Ramburan *et al.*, 2004). Figures 3.1a-3 depicts which individuals were pooled in each bulk. The extreme resistant bulk was constructed from seven DH lines selected within the 10 most resistant lines based on all phenotypic scores. All of them had Karioga alleles for the closest markers to the 7D QTL (*Ltn* and *gwm295*, Figure 3.1a) and 2B QTL (*gwm148* and *s12m60a*, Figure 3.1a), except for



**Narrow down +7D±2B bulk**

DH	7D-QTL	2B-QTL	4A-QTL	1A-QTL
81	K	K	A	A
105	K	K	A	A
48	K	A	K	A
68	K	A	K	A
57	K	A	K	A
141	K	A	K	A
83	K	A	K	A
43	K	A	K	A
Ltn				
gwm295	K	K	A	A
gwm111	K	A	K	A
cdf 46	K	A	K	A
gwm885	K	A	K	A
gwm437	K	A	K	A
§18m90b	K	A	K	A
§23m12c	K	A	K	A
gwm682	K	A	K	A
gwm372	K	A	K	A
gwm148	K	A	K	A
§12m60a	K	A	K	A
§12m24d	K	A	K	A
psp3030	K	A	K	A
§16m40a	K	A	K	A
SRAP10	K	A	K	A
§26m92f	K	A	K	A
§15m56c	K	A	K	A
§26m92g	K	A	K	A
§12m34a	K	A	K	A
§15m56a	K	A	K	A
gwm501	K	A	K	A
§12m53b	K	A	K	A
§25m48b	K	A	K	A
§18m93e	K	A	K	A
§15m67b	K	A	K	A
§12m34b	K	A	K	A
gwm160	K	A	K	A
gwm742	K	A	K	A
gwm832	K	A	K	A
wmc219	K	A	K	A
§12m34a	K	A	K	A
§21m40a	K	A	K	A
§18m90a	K	A	K	A
§15m19d	K	A	K	A
§23m18e	K	A	K	A
§13m84a	K	A	K	A
§18m47c	K	A	K	A
psp3027	K	A	K	A
Glu-A1-1	K	A	K	A
§25m48a	K	A	K	A

**Figure 3.1c** Marker alleles (Kariaga =K, Avocet S =A) for QTL regions of selected DH lines constituting the narrow down

+7D±2B bulk.

**+7D-2B bulk**

DH	7D-QTL	2B-QTL	4A-QTL	1A-QTL
127	K	A	A	A
72	K	A	A	A
79	K	A	A	A
21	K	A	A	A
74	K	A	A	A
35	K	A	A	A
101	K	A	A	A
109	K	A	A	A
Ltn				
gwm295	K	A	A	A
gwm111	K	A	A	A
cdf 46	K	A	A	A
gwm885	K	A	A	A
gwm437	K	A	A	A
§18m90b	K	A	A	A
§23m12c	K	A	A	A
gwm682	K	A	A	A
gwm372	K	A	A	A
gwm148	K	A	A	A
§12m60a	K	A	A	A
§12m24d	K	A	A	A
psp3030	K	A	A	A
§16m40a	K	A	A	A
SRAP10	K	A	A	A
§26m92f	K	A	A	A
§15m56c	K	A	A	A
§26m92g	K	A	A	A
§12m34a	K	A	A	A
§15m56a	K	A	A	A
gwm501	K	A	A	A
§12m53b	K	A	A	A
§25m48b	K	A	A	A
§18m93e	K	A	A	A
§15m67b	K	A	A	A
§12m34b	K	A	A	A
gwm160	K	A	A	A
gwm742	K	A	A	A
gwm832	K	A	A	A
wmc219	K	A	A	A
§12m34a	K	A	A	A
§21m40a	K	A	A	A
§18m90a	K	A	A	A
§15m19d	K	A	A	A
§23m18e	K	A	A	A
§13m84a	K	A	A	A
§18m47c	K	A	A	A
psp3027	K	A	A	A
Glu-A1-1	K	A	A	A
§25m48a	K	A	A	A

**Figure 3.1d** Marker alleles (Kariaga =K, Avocet S =A) for QTL regions of selected DH lines constituting the +7D-2B bulk.



DH	7D GTL	2B GTL	4A GTL	1A GTL	Other
70	A	A	A	A	s25m48a
10	A	A	A	A	Glu-A1-1
136	A	A	A	A	psp3027
51	A	A	A	A	s18m47c
132	A	A	A	A	s13m84a
95	A	A	A	A	s23m18e
8	A	A	A	A	s15m19d
53	A	A	A	A	s18m90a
150	A	A	A	A	s21m40a
99	A	A	A	A	s12m34a
					wmc219
					gwm832
					gwm742
					gwm160
					s12m34b
					s15m67b
					s18m93e
					s25m48b
					s12m53b
					gwm501
					s15m56a
					s12m34a
					s26m92g
					s15m56c
					s26m92f
					SRAP10
					s16m40a
					psp3030
					s12m24d
					s12m60a
					gwm148
					gwm372
					gwm682
					s23m12c
					s18m90b
					gwm437
					gwm885
					cdf 46
					gwm111
					gwm295
					Ltn

**Figure 3.1e** Marker alleles (Kariaga =K, Avocet S =A) for QTL regions of selected DH lines constituting the narrow down -7D+2B bulk.

DH 73 for which the marker information of *gwm148* was lacking and *s12m60a* had an Avocet S allele. However, this individual met the traditional criterion for being in the most resistant bulk based on its phenotypic score. Care was taken to choose individuals that had both Kariega and Avocet S alleles for the minor QTL regions (4A and 1A) to avoid detecting these regions during the tBSA approach. However, it was not possible to obtain an equal number of individuals that met the specified criteria of the bulk with and without the minor QTL. This was a problem encountered throughout the selection of individuals for the various bulks.

The extreme susceptible bulk consisted of individuals chosen within the 17 most susceptible lines based on all phenotypic scores. All of these lines also had Avocet S alleles for the 7D and 2B QTL intervals and a mixture of parental alleles for the 4A and 1A regions (Figure 3.1b). Both the extreme resistant and susceptible bulks were chosen based on their phenotypes, but marker information was used to validate its integrity.

In an attempt to target the region in the 7D QTL interval (*Ltn-gwm295*), a narrow down +7D±2B bulk was constructed for which individuals were chosen that had recombinations within that region, with Avocet S alleles for markers neighbouring this region (i.e. *gwm111* and *cf446*, Figure 3.1c). The presence of both parental alleles in the 2B, 4A and 1A QTL regions theoretically eliminated their detection in this bulk. Individuals in this bulk had relatively high to medium phenotypic scores and it was therefore decided to create another bulk targeting 7D which had a relative high leaf area infected (LAI) phenotype with no medium scores.

Ramburan *et al.* (2004) previously showed that the 7D region is primarily explaining the variance in leaf area infected observed in the DH population, which indicated a partial resistance reaction in contrast to the hypersensitive response reaction of the 2B region. The +7D-2B bulk had individuals that had Kariega alleles for a larger region surrounding the 7D QTL (*Ltn-gwm295-gwm111*, Figure 3.1d). In this bulk care was also taken to exclude individuals carrying the 2B QTL and once again a mixture of 4A and 1A QTL-carrying and non-carrying regions were achieved.

In order to target the 2B QTL only, a -7D+2B bulk was constructed consisting of individuals with 2B QTL Kariega alleles (*gwm148-s12m60a*, Figure 3.1e), but lacking the 7D QTL. Both parental alleles for the 4A and 1A regions were present. Individuals present in this bulk in general had a high hypersensitive response action, typically as described by Ramburan *et al.* (2004) for the 2B QTL region. Equal proportions of genomic DNA from the selected individual DH lines were used to create the five bulks of a final concentration of 200 ng/μl each and stored at -4°C until further use.

### 3.2.4 AFLP analysis

*MseI*-primers were screened in combination with *SseI*-primers. Names were given beginning with m and s for *MseI*-primers and *SseI*-primers, respectively. Primer pair combinations (Tables 3.1 and 3.2) that have not been used to construct the existing Kariega x Avocet S linkage map (Ramburan *et al.*, 2004; Prins *et al.*, 2005) were selected to screen parental lines and bulk samples. Primers and adapters were synthesised by Integrated DNA Technologies, Inc, USA. Oligonucleotides used for adapters were polyacrylamide gel electrophoresis (PAGE) purified. Adapters were prepared by mixing equimolar amounts of both strands, heating for 10 minutes at 65°C in a waterbath and leaving the mixture to cool down to room temperature. AFLP analysis was performed according to Vos *et al.* (1995), with minor modifications (Herselman, 2003).

#### 3.2.4.1 Restriction digestion and ligation of genomic DNA

Approximately 1.0 μg of genomic DNA of each sample was digested with 4 U *MseI* (New England Biolabs) and 1 x *MseI* buffer [50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM 1,4 dithiothreitol (DTT), pH 7.9] at 37°C for five hours. After *MseI* digestion, restriction fragments were further digested with 5 U *SseI* (Amersham Bioscience), followed by overnight incubation at 37°C. Ligation of adapters was done by adding a solution containing 0.4 mM adenosine triphosphate (ATP), 50 pmol *MseI*-adapter, 5 pmol *SseI*-adapter, 1 x T4 DNA Ligase buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM MgCl<sub>2</sub>, 10 mM DTT, 66 mM ATP) and 1 U T4 DNA ligase (USB Corporation), followed by overnight incubation at 16°C.

**Table 3.1 *MseI* AFLP adapter and primer sequences used in this study**

<b>Adapter</b>	<b>Sequence</b>		
<i>MseI</i> -adapter	5'-GACGATGAGTCCTGAG 3'-TACTCAGGACTCAT-5'		
<b>Primer</b>	<b>Sequence (5' - 3')</b>	<b>Primer</b>	<b>Sequence (5' - 3')</b>
<i>MseI</i> +0-primer	5'-GATGAGTCCTGAGTAA-3'		
<i>MseI</i> +3-primers			
m31	GATGAGTCCTGAGTAAAAA	m55	GATGAGTCCTGAGTAAACGA
m32	GATGAGTCCTGAGTAAAAAC	m56	GATGAGTCCTGAGTAAACGC
m33	GATGAGTCCTGAGTAAAAG	m57	GATGAGTCCTGAGTAAACGG
m34	GATGAGTCCTGAGTAAAAT	m58	GATGAGTCCTGAGTAAACGT
m35	GATGAGTCCTGAGTAAACA	m59	GATGAGTCCTGAGTAAACTA
m36	GATGAGTCCTGAGTAAACC	m60	GATGAGTCCTGAGTAAACTC
m37	GATGAGTCCTGAGTAAACG	m61	GATGAGTCCTGAGTAAACTG
m38	GATGAGTCCTGAGTAAACT	m62	GATGAGTCCTGAGTAAACTT
m39	GATGAGTCCTGAGTAAAGA	m63	GATGAGTCCTGAGTAAAGAA
m40	GATGAGTCCTGAGTAAAGC	m64	GATGAGTCCTGAGTAAAGAC
m41	GATGAGTCCTGAGTAAAGG	m65	GATGAGTCCTGAGTAAAGAG
m42	GATGAGTCCTGAGTAAAGT	m66	GATGAGTCCTGAGTAAAGAT
m43	GATGAGTCCTGAGTAAATA	m67	GATGAGTCCTGAGTAAAGCA
m44	GATGAGTCCTGAGTAAATC	m68	GATGAGTCCTGAGTAAAGCC
m45	GATGAGTCCTGAGTAAATG	m69	GATGAGTCCTGAGTAAAGCG
m46	GATGAGTCCTGAGTAAATT	m70	GATGAGTCCTGAGTAAAGC T
m47	GATGAGTCCTGAGTAAACAA	m71	GATGAGTCCTGAGTAAAGGA
m48	GATGAGTCCTGAGTAAACAC	m72	GATGAGTCCTGAGTAAAGGC
m49	GATGAGTCCTGAGTAAACAG	m82	GATGAGTCCTGAGTAAATAT
m50	GATGAGTCCTGAGTAAACAT	m85	GATGAGTCCTGAGTAAATCG
m51	GATGAGTCCTGAGTAAACCA	m86	GATGAGTCCTGAGTAAATCT
m52	GATGAGTCCTGAGTAAACCC	m87	GATGAGTCCTGAGTAAATGA
m53	GATGAGTCCTGAGTAAACCG	m88	GATGAGTCCTGAGTAAATGC
m54	GATGAGTCCTGAGTAAACCT	m90	GATGAGTCCTGAGTAAATGT

**Table 3.2 *SseI* AFLP adapter and primer sequences used in this study**

<b>Adapter</b>	<b>Sequence</b>
<i>SseI</i> -adapter	5'-TCGTAGACTGCGTACATGCA-3' 3'-CATCTGACGCATGT-5'
<b>Primer</b>	<b>Sequence (5' - 3')</b>
<i>SseI</i> +0-primer	5'- GTAGACTGCGTACATGCAG-3'
<i>SseI</i> +2-primers	
s19	GTAGACTGCGTACATGCAGGA
s20	GTAGACTGCGTACATGCAGGC
s23	GTAGACTGCGTACATGCAGTA
s24	GTAGACTGCGTACATGCAGTC
s25	GTAGACTGCGTACATGCAGTG

#### 3.2.4.2 *Pre-amplification reactions*

Pre-amplification reactions were performed in 50 µl reaction mixtures containing 5 µl template DNA from undiluted restriction/ligation mixtures, 30 ng of each pre-amplification primer [*MseI*-primer+0 and *SseI*-primer+0 (Tables 3.1 and 3.2)], 1 x Promega polymerase buffer, 2 mM MgCl<sub>2</sub>, 200 µM 2'-deoxynucleoside 5'-triphosphates (dNTPs) and 0.02 U GoTaq<sup>®</sup> Flexi DNA Polymerase (Promega, Madison, WI, USA). All PCR-amplifications were performed using a PCR thermal cycler (DNA Engine DYAD<sup>™</sup>, BIO-RAD, USA) using the following cycling programme: an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 56°C for one minute and 72°C for one minute and a final elongation step at 72°C for five minutes. The quality and quantity of pre-amplification reactions were estimated by separation through a 1.5% (w/v) agarose gel in 1 x UNTAN buffer at 60 V for 45 minutes. Based on electrophoresis results, pre-amplification reactions were diluted accordingly (1:20 to 1:50) with 1 x TE buffer (pH 8.0) prior to selective amplification.

#### *3.2.4.3 Selective amplification reactions*

Selective amplification reactions were performed in a total of 20 µl reaction volumes containing 5 µl of diluted pre-amplification DNA, 1 x Promega polymerase buffer, 2 mM MgCl<sub>2</sub>, 200 µM dNTPs, 100 µg/ml bovine serum albumin, 30 ng *Mse*I-primer+3, 30 ng *Sse*I-primer+2 and 0.75 U GoTaq<sup>®</sup> Flexi DNA Polymerase. Selective amplification was initiated with denaturation at 94°C for five minutes, followed by one cycle of 94°C for 30 seconds, 65°C for 30 seconds and 72°C for one minute. The annealing temperature was lowered by 1°C per cycle during the next eight cycles after which 25 cycles were performed at 94°C for 30 seconds, 56°C for 30 seconds and 72°C for one minute, followed by one final elongation step at 72°C for two minutes.

#### *3.2.4.4 Denaturing polyacrylamide gel electrophoresis*

Selective amplification reactions were mixed with 20 µl of formamide loading dye [98% (v/v) de-ionised formamide, 10 mM EDTA pH 8.0, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] and denatured at 95°C for five minutes. Mixtures were immediately placed on ice prior to loading. PCR products (5 µl in total for each sample) were separated through a 5% denaturing polyacrylamide gel [19:1 acrylamide: bis-acrylamide, 7 M urea and 1 x TBE buffer (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA)]. Electrophoresis was performed at a constant power of 80 W for approximately two hours.

#### *3.2.4.5 Silver staining for DNA visualisation*

Polyacrylamide gels were silver stained according to the protocol of the Silver Sequence<sup>™</sup> DNA Sequencing System manual supplied by Promega. Stained gels were left upright to air dry overnight and photographed by exposing photographic paper (ILFORD MULTIGRADE IV RC DE LUXE) placed under the gel, to dim light for approximately 20 seconds. This produced a negative image of the same size and scale as the gel. AFLP fragment lengths were determined by comparison with a 100 bp DNA ladder (Promega).

**3.2.5 Targeted bulk segregant analysis to identify markers closely linked to quantitative trait loci**

The five bulks (extreme resistant, extreme susceptible, narrow down +7D±2B, +7D-2B and -7D+2B) were used together with the parental lines (Kariega and Avocet S) for screening with AFLP markers. The parental lines and bulk samples were screened using 184 selected primer pair combinations and ran in adjacent lanes on a polyacrylamide gel. The gel was initially scored for each of the primer pair combinations that produced a clear, intense band in the resistant parent (Kariega) and extreme resistant bulk but not in the susceptible parent (Avocet S) and extreme susceptible bulk. In addition, the tBSA approach was implemented to assist in targeting QTL regions, depending on whether the putative marker had the desired banding pattern which might be linked to either the 7D or 2B QTL regions. Markers that were possibly linked to the 7D QTL region were selected based on a pattern where bands were additionally to the resistant parent and extreme resistant bulk present in the narrow down +7D±2B and +7D-2B bulks and absent in the -7D+2B bulk, susceptible parent and extreme susceptible bulk. For the 2B QTL region, markers were selected that showed a pattern where bands were present in the resistant parent, extreme resistant bulk, the narrow down +7D±2B and -7D+2B bulks and absent in the +7D/-2B bulk, susceptible parent and extreme susceptible bulk (Figure 3.2). Primer pair combinations that met these criteria were then tested on all individuals constituting a specific bulk it was originally detected in and only those that amplified in all were used to score the rest of the 150 individuals of the DH population.

	Kariega (resistant parent)	Avocet S (susceptible parent)	Extreme resistant	Extreme susceptible	Narrow Down +7D±2B	+7D-2B	-7D+2B
7D Marker	■		■		■	■	
2B Marker	■		■		■		■

**Figure 3.2 Expected banding patterns in the two parental lines and five bulk samples for markers that were derived from the 7D and 2B YrQTL regions using tBSA.**

### 3.2.6 Genetic linkage mapping of putative AFLP markers

In the present study data obtained from the tBSA approach was added to the existing Kariega x Avocet S linkage data set produced on 150 DH lines (Ramburan *et al.*, 2004; Prins *et al.*, 2005), which was further curated in 2006 (R. Prins personal communication). The absence of a band in one parent was treated as the presence of the other parental allele at that locus. Data was analysed using Map Manager QTX software version b20 (Manly *et al.*, 2001). Linkage at  $p < 0.05$  was considered significant using the Kosambi mapping function (Kosambi, 1944) in Map Manager. Markers were added to the existing linkage map using the “distribute” function of Map Manager. To avoid inflation of the map length (Castiglioni *et al.*, 1999) double crossovers were checked and removed where necessary. The best order and map position of the markers were confirmed using the software programme Record (Van Os *et al.*, 2005). Linkage maps were drawn using MapChart 2.2 (Voorrips, 2002).

### 3.2.7 QTL mapping

Ramburan *et al.* (2004) detected two major QTL for APR to stripe rust in the Kariega x Avocet S DH population on chromosomes 2B and 7D using different phenotypic values. In the present study, Map Manager QTX b20 (Manly *et al.*, 2001) was used to confirm the positions of the 2B and 7D QTL regions on the linkage map containing the newly mapped AFLP markers. Interval mapping (IM) was done using a single trait of the Ramburan *et al.* (2004) study i.e. mean host reaction type for final field data. A LOD threshold of 3.0 was chosen for declaring a putative QTL significant.

## 3.3 Results

A total of 184 AFLP primer pair combinations were tested on the two parental lines and five bulk samples. *SseI* primers s19, s20 and s23 were tested with all selected *MseI* primers (Table 3.1) while *SseI* primers s24 and s25 were tested with *MseI* primers in Table 3.3 previously not tested by Ramburan *et al.* (2004). A total of 79 of the 184 (42.9%) AFLP *SseI/MseI*-primer combinations tested on the parental lines and bulk samples detected 105



**Table 3.3 Primer pair combinations used to screen parental lines, five bulk samples, individuals comprising the bulks and the entire DH population**

<b>Number of putative markers</b>	<b>Marker ID</b>	<b>Possible chromosome identity</b>	<b>Individuals constituting bulks samples</b>	<b>Tested on 150 individuals</b>	<b>Most probable map position</b>
1	<i>s19m35a</i>	2B	+	+	2B
2	<i>s19m41a</i>	2B	-	-	/
3	<i>s19m56a</i>	2B	-	-	/
4	<i>s19m68a</i>	2B	-	-	/
5	<i>s20m34k</i>	7D	-	-	/
6	<i>s20m34l</i>	2B	-	-	/
7	<i>s20m34n</i>	2B	-	-	/
8	<i>s20m38a</i>	2B	+	+	2B
9	<i>s20m38b</i>	7D	+	+	7D
10	<i>s20m53g</i>	2B	+	+	2B
11	<i>s20m58a</i>	2B	-	-	/
12	<i>s20m66a</i>	2B	-	-	/
13	<i>s20m86g</i>	2B	-	-	/
14	<i>s20m88a</i>	7D	+	+	7D
15	<i>s20m88c</i>	2B	+	+	2B
16	<i>s23m32f</i>	2B	-	-	/
17	<i>s23m44a</i>	2B	-	-	/
18	<i>s23m49a</i>	2B	-	-	/
19	<i>s23m52a</i>	2B	+	+	2B
20	<i>s23m53d</i>	2B	+	+	2B
21	<i>s23m54a</i>	2B	+	+	2B
22	<i>s23m56a</i>	2B	-	-	/
23	<i>s23m70</i>	2B	-	-	/
24	<i>s24m64a</i>	2B	+	+	2B
25	<i>s24m88a</i>	2B	-	-	/
26	<i>s25m49</i>	2B	-	-	/
27	<i>s25m61a</i>	2B	-	-	/
28	<i>s25m61b</i>	2B	-	-	/
29	<i>s25m64b</i>	2B	-	-	/
30	<i>s25m82d</i>	2B	+	+	/
31	<i>s25m86h</i>	2B	-	-	/
32	<i>s25m86g</i>	2B	-	-	/

+ = markers showing expected segregation patterns  
 - = markers not showing expected segregation patterns  
 / = not tested

putative markers that could discriminate between the extreme resistant and susceptible bulks. Of these, 32 (Table 3.3) were identified as true candidates when considering the 2B and 7D specific bulks as well (Figure 3.2). These 32 putative markers were amplified by 27 (14.7% of the total AFLP markers tested) primer combinations. Twenty-nine of these markers were identified as possible candidates for mapping to chromosome 2B and three to chromosome 7D. After validation of these 32 markers on all individuals constituting the five bulk samples, 11 promising markers were identified (Table 3.3). These markers discriminated between all individuals in the extreme resistant and susceptible bulks. Nine of these markers met the criteria of all the bulks and its individuals (i.e. present in most of the individuals of the narrow down +7D±2B bulk, present in all individuals of the -7D+2B bulk and absent in all individuals of the +7D-2B bulk) for the *QYr.sgi-2B* QTL region. This suggested that the nine markers should map to the *QYr.sgi-2B* QTL region. Two of the 11 promising markers met the criteria for possible mapping to the *QYr.sgi-7D* QTL region. These two markers showed a banding pattern where fragments were present in most individuals of the narrow down +7D±2B bulk, present in all individuals of the +7D-2B bulk and absent in all individuals of the -7D+2B bulk. This suggested that these two markers should map to the *QYr.sgi-7D* QTL region.

The 11 putative markers, amplified using nine (4.9% of the total AFLP primers tested) AFLP primer combinations, were further validated on the rest of the individuals of the F<sub>1</sub> DH population consisting of 150 individuals. Mapping data for the 150 individuals of the DH population was scored and placed in a binary system where the presence of a Kariëga or Avocet S allele was denoted as A for Kariëga and B for Avocet S, respectively. Data was placed in Map Manager QTX b20 and analysed using the curated Kariëga x Avocet S linkage map of Prins *et al.* (2005). Ten of the 11 putative markers mapped to either chromosomes 2B or 7D. The one marker that was not mapped (*s25m82d*) showed linkage to the distal end of the short arm of chromosome 2B but could not be placed on the map with accuracy.

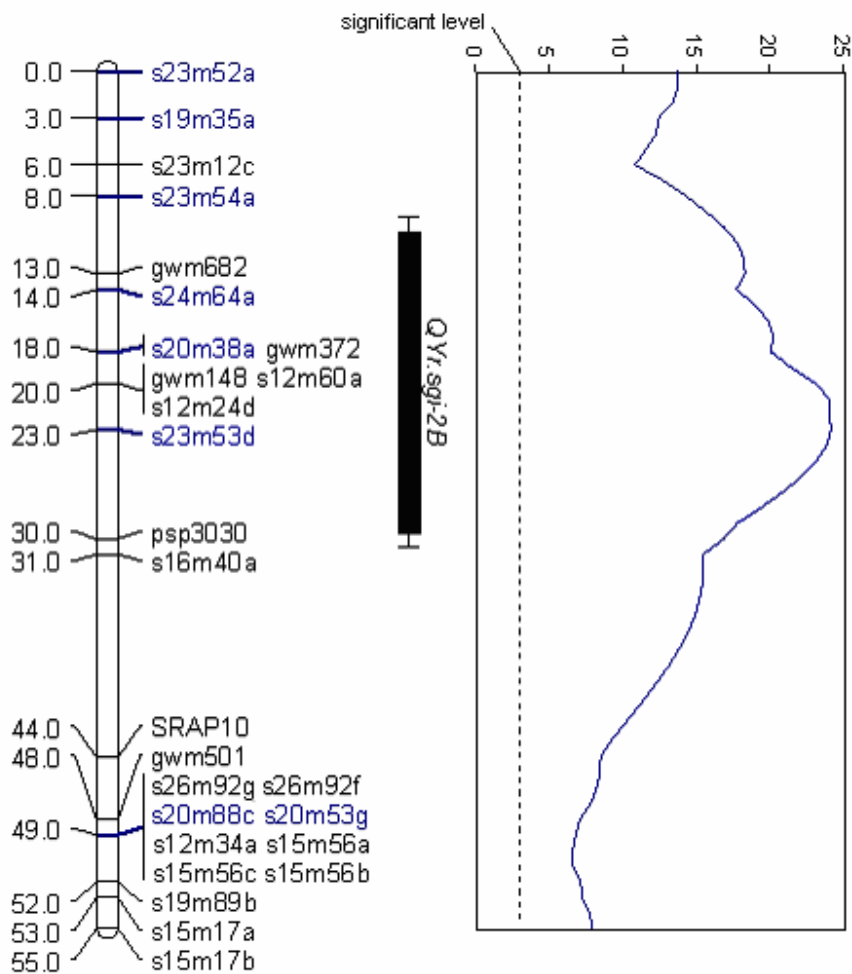
Eight of the eleven putatively identified AFLP markers mapped to chromosome 2B of which five mapped within the previously identified *QYr.sgi-2B* QTL region. Six markers mapped within a 23 cM interval on the distal end of the short arm of chromosome 2B. Markers *s20m38a* and *s23m53d* mapped to opposite sides of marker *gwm148* (Figure 3.3), previously shown to be significantly associated with mean host reaction type for final field data as well as leaf area infected of the *QYr.sgi-2B* QTL region.

Marker *s20m38a* mapped 2 cM and *s23m53d* 3 cM from marker *gwm148*. Another two markers (*s20m88c* and *s20m53g*) mapped to the long arm of chromosome 2B, outside the previously indicated *QYr.sgi-2B* QTL region.

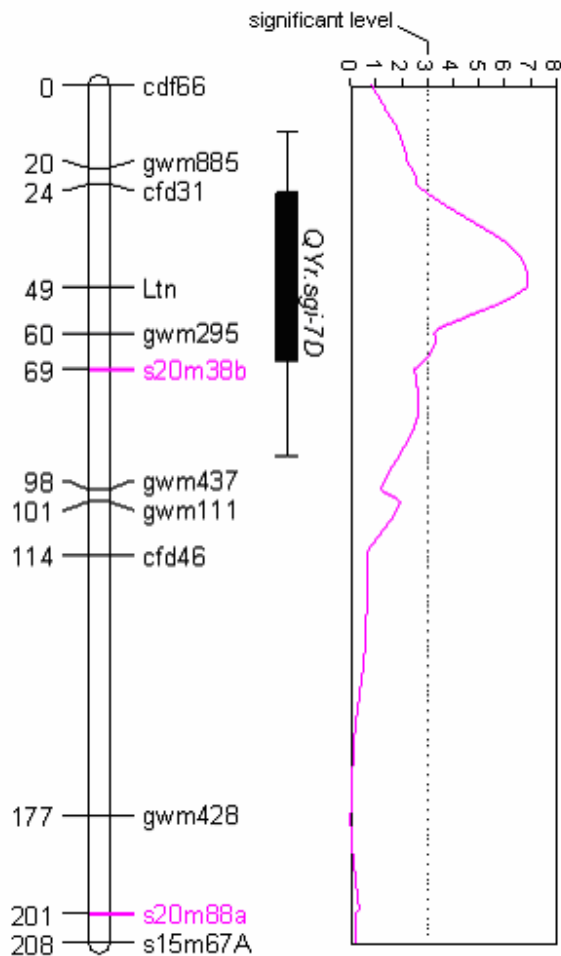
Two of the AFLP markers mapped to chromosome 7D, of which one mapped within the previously identified *QYr.sgi-7D* QTL region. Marker *s20m38b* mapped 9 cM from the SSR marker *gwm295* and 20 cM from the *Ltn* gene on chromosome 7D (Figure 3.4). The other 7D marker, *s20m88a*, mapped to the distal end of the long arm of chromosome 7D.

QTL analysis was conducted using the newly constructed 2B and 7D chromosome maps. The mean host reaction type for final field data trait was used for QTL analysis. The positions of the *QYr.sgi-2B* and *QYr.sgi-7D* QTL on chromosomes 2B and 7D were confirmed using interval mapping with additional genotype data derived from eight AFLP markers on chromosome 2B and two AFLP markers on chromosome 7D.

Interval mapping for chromosome 2B indicated a peak LOD score of 23.9 linked to marker *s23m53d*. This position explained 52% of the phenotypic variation. Marker *s23m53d* (Figure 3.5) mapped 3 cM away from the SSR marker *gwm148*, previously indicated to be the closest marker to the *QYr.sgi-2B* QTL region spanning 25 cM on chromosome 2B. Results from the present study indicated that the *QYr.sgi-2B* QTL region spanned a 21 cM region between markers *s23m54a* and *psp3030* on chromosome 2B.



**Figure 3.3** Left: A linkage map of chromosome 2B indicating previously mapped (black) and newly mapped (blue) markers using a targeted BSA approach. Marker names and their genetic distances (cM) are indicated at the right and left sides of the linkage map, respectively. Right: The QTL likelihood-profile for the mean host reaction type for final field data trait. LOD score and map distance are indicated on the x and y axes, respectively. The bar graph in the middle of the figure represents the standard 1 LOD (shaded region) and/or 2 LOD support intervals for the *QYr.sgi-2B* QTL region.



**Figure 3.4** Left: A linkage map of chromosome 7D indicating previously mapped (black) and newly mapped (pink) markers using a targeted BSA approach. Marker names and their genetic distances (cM) are indicated at the right and left sides of the linkage map, respectively. Right: The QTL likelihood-profile for the mean host reaction type for final field data trait. LOD score and map distance are indicated on the x and y axes, respectively. The bar graph in the middle of the figure represents the standard 1 LOD (shaded region) and/or 2 LOD support intervals for the *QYr.sgi-7D* QTL region.

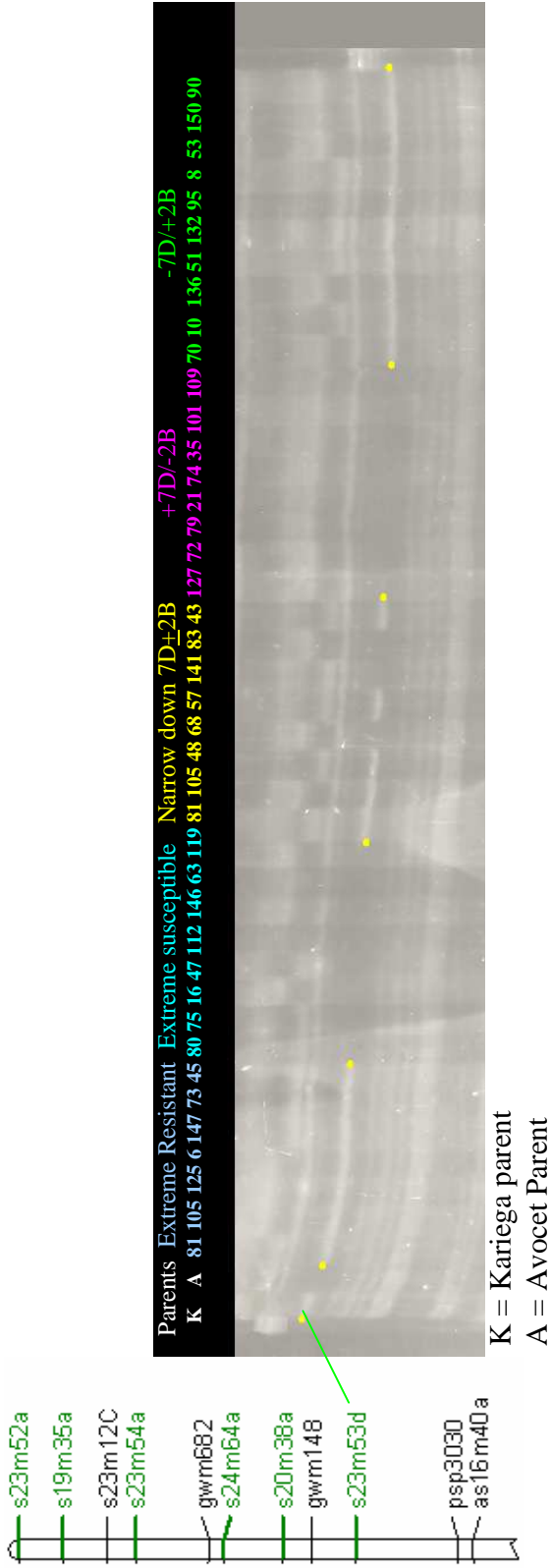


Figure 3.5 A section of the 2B chromosome depicting the position of the marker corresponding to the presence/absence of the marker in the different bulks on a silver stained polyacrylamide gel.

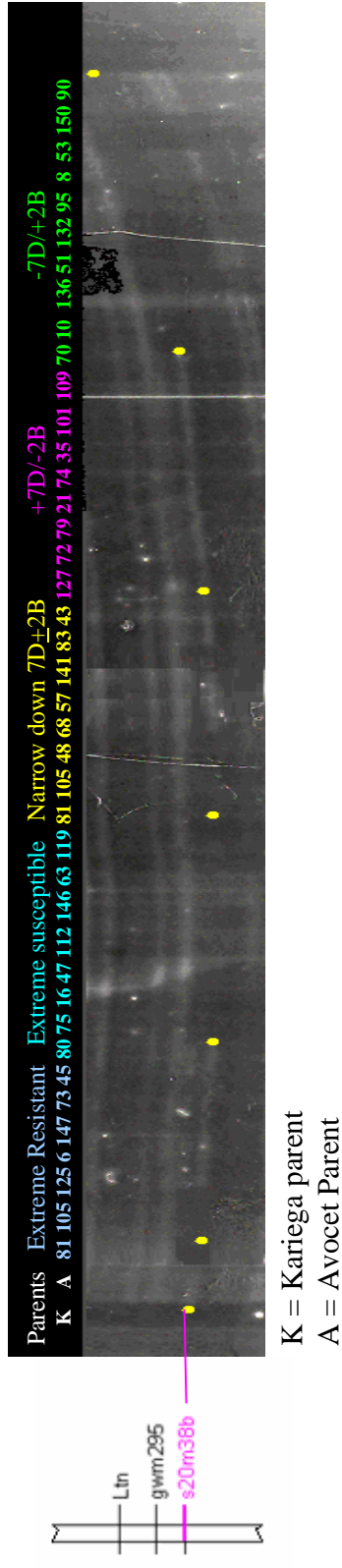


Figure 3.6 A section of the 7D chromosome depicting the position of the marker corresponding to the presence/absence of the marker in the different bulks on a silver stained polyacrylamide gel.

Interval mapping for chromosome 7D indicated a peak LOD score of 6.84 for the gene *Ltn*. This position explained 19% of the phenotypic variation. The newly mapped marker *s20m38b* (Figure 3.6) mapped 20 cM away from the *Ltn* gene with a LOD score of 2.65, explaining 8% of the phenotypic variation. The *QYr.sgi-7D* QTL region mapped in the same region as previously reported by Ramburan *et al.* (2004). In the present study the *QYr.sgi-7D* QTL region spanned a 40 cM region between markers *cf31* and *s20m38b* on chromosome 7D, with the latter mapping just outside the QTL region.

### 3.4 Discussion

Successful application of MAS in traditional wheat breeding programmes requires the identification of molecular markers tightly linked to the gene of interest. Furthermore, since selection for APR to stripe rust is difficult, closely linked markers provide an alternative means for the selection of resistant gene(s)/QTL in breeding programmes in the absence of pathogens. In the present study, AFLP technology was used in combination with tBSA to identify markers more closely linked to two APR QTL, *QYr.sgi-2B* and *QYr.sgi-7D* in the Kariega x Avocet S DH mapping population. The tBSA approach involved the selection and bulking of DH individuals based on existing marker data of flanking markers as well as phenotypic trait data in order to enrich the 2B and 7D QTL regions.

The AFLP marker system proved to be effective in revealing polymorphisms and was able to add marker information to the existing Kariega x Avocet S linkage map. In general, the AFLP marker results of the present study have confirmed the value of AFLP analysis in combination with tBSA as a reproducible, fast (less time needed to screen as compared with BSA) and cost effective approach. Should a traditional BSA approach of screening parental lines against the extreme resistant and susceptible bulks, have been followed in the present study, 105 putative markers would have been identified (amplified by 79 primer combinations). Therefore, based on screening of extreme bulks only, 42.9% of the AFLP primer combinations tested would have looked promising and would have been tested on the individuals comprising the bulks samples. However, when the narrow down +7D±2B, +7D-2B and -7D+2B bulks were taken into consideration, only 14.7% of the initially tested

primers combinations, looked promising. The number of putative markers was reduced from 105 to 32 and only 27 AFLP primer combinations had to be tested on the individuals comprising the bulk samples. This reduction made the approach more time and cost-effective. These results confirmed results obtained by Dußle *et al.* (2000) who reported that the advantage of tBSA approach compared to BSA approach is that the method minimises the experimental effort in analysing large numbers of putatively linked markers and enables the selection of closely linked markers without analysing all individuals of the mapping population.

The tBSA approach seems to be effective even though difficulties were experienced during construction of the bulk samples. Since the original mapping population consisted of only 150 individuals it was difficult to select DH lines with the correct marker profile for the targeted region (e.g. 7D or 2B) that also had an equal number of Kariega and Avocet S background alleles in the non-target region of that specific bulk. One such example is the narrow down  $+7D\pm 2B$  YrQTL bulk in which the area between *Ltn* and *gwm295* is specifically targeted. Seven of the eight recombinant individuals with a resistance phenotypic score had the Kariega 4AYrQTL region and only one individual the Avocet S alleles. This might explain why only two markers were mapped to the 7D chromosome. This emphasised the need to screen the individuals comprising a bulk separately, once putative markers have been identified. Successful application of the tBSA approach requires experience and the development of the correct population type which can be laborious and time consuming from both the breeding and molecular perspective (Semagn *et al.*, 2006).

Validation of the 32 markers identified during the screening of the parental lines and bulk samples on all individuals constituting the five bulk samples, identified 11 promising markers of which 10 mapped to either chromosome 2B or 7D. The tBSA approach was efficient since 10 of the 11 markers (91%) putatively identified after screening of the individuals constituting the bulk samples mapped to either chromosome 2B or 7D. Even the one marker that was not placed onto the map showed some linkage to chromosome 2B. Since 91% of the AFLP markers identified by tBSA mapped within the pre-selected 2B and 7D chromosome regions, tBSA seems to enrich markers in target regions while substantially



reducing the subsequent mapping effort. Similar results were obtained by Dußle *et al.* (2000) who used the tBSA approach to saturate two chromosome regions in maize. Two major genes conferring resistance to sugarcane mosaic virus were previously identified on chromosomes 3 and 6 using a combined QTL and BSA approach. Both these chromosome regions were further enriched using SSR and AFLP markers in combination of tBSA.

The efficiency of the strategy in the current study was furthermore confirmed after five markers mapped within the previously identified *QYr.sgi-2B* and one within the previously identified *QYr.sgi-7D* QTL regions. Even though only two markers were added to chromosome 7D, this result was not surprising. It is known from the literature (Desai *et al.*, 2006) that the D chromosomes of wheat show low levels of polymorphisms leading to low marker density on these chromosomes. The tBSA approach can be used to add closely linked markers to target chromosomes, especially low level polymorphic chromosomes such as the 7D chromosome with areas low in marker numbers. A complicating factor in genetic mapping using a random markers approach is that certain chromosomes are more polymorphic than others. Once an initial map is in place, it often produces results in unnecessary detail in highly polymorphic regions and continues to result in gaps in other chromosome regions (Campbell *et al.*, 2001). In the current study this was also the case, since more markers were added to the more polymorphic 2B chromosome compared to the 7D chromosome. Even though a low number of markers were added to the existing map, the use of AFLP analysis in combination with tBSA in this dissertation has been proven useful in closing gaps on the 2B and 7D chromosome regions.

Following the tBSA approach, marker *s23m53d* mapped 3 cM from marker *gwm148* previously shown to be significantly associated with mean host reaction type for final field data as well as leaf area infected of the *QYr.sgi-2B* QTL region. The LOD score for *gwm148* was 20.1 and 25.1 for *s23m53d* indicating that the application of *s23m53d* in a MAS programme would be expected to give improved results. Markers linked more closely to a gene of interest are useful in increasing the efficiency of gene isolation by reducing the region of interest on a specific chromosome and this can be an important consideration for attempts to clone and sequence genes. Even though two markers were added to the 7D

chromosome, both mapped outside the *QYr.sgi-7D* QTL region. Marker *s20m38b* mapped 9 cM from the SSR marker *gwm295* and 20 cM from the *Ltn* gene previously shown to be associated with the trait of interest on chromosome 7D. Although these markers will not be useful for MAS, the tBSA approach succeeded to add two additional markers to the low density marker region of the 7D chromosome.

AFLP primer combination *s20m38* was of particular interest since it amplified markers within or closely mapped to both the targeted 2B and 7D QTL regions. This primer combination could be useful in a MAS breeding programme to detect the presence or absence of either a 2B and/or 7D QTL regions. Since AFLP markers are not adapted for large-scale application in plant breeding, these markers should in future be converted to SCAR or STS markers which would help to reduce screening costs.

In summary, this dissertation has evaluated the use of AFLP analysis as markers for targeted mapping approaches. The combination of AFLPs and a tBSA approach has proved to be useful in the identification of a QTL, the placement of closely linked markers to known QTL and targeting chromosome areas with low marker numbers.

## *Chapter 4*

### **General conclusions and recommendations**

This study aimed at targeting QTL regions associated with APR to stripe rust identified in South African wheat cultivar Kariega. Results from this study indicated that the tBSA approach proved to be efficient in combination with the AFLP multi-locus profiling technique to uncover additional markers for the 2B and 7D QTL target regions. Although 184 AFLP markers were screened in the present study, the AFLP marker system proved to be effective in revealing polymorphisms and was able to add marker information to the existing Kariega x Avocet S linkage map. Despite the difficulties experienced in finding enough DH lines for a specific bulk that also had an equal number of Kariega and Avocet S background alleles in the non-target region of a specific bulk, tBSA yielded markers in the target region. AFLP analysis in combination with tBSA was shown to be reproducible, faster and a more cost effective approach compared to a traditional BSA approach. A reduction of 28.2% of AFLP primers that needed to be tested was achieved. Validation of the 32 markers identified after bulk screening on all individuals constituting the five bulk samples in which they were identified, pointed out 11 true candidates, emphasising the need to screen the individuals comprising a bulk separately, once putative markers have been identified in the bulks. The tBSA approach was efficient in enriching markers in the target region, while substantially reducing the mapping effort, since 10 of these 11 markers (91%) mapped to either chromosome 2B or 7D. Even the one marker that was not placed onto the map showed some linkage to chromosome 2B. Five markers mapped within the previously identified *QYr.sgi-2B* and one within the previously identified *QYr.sgi-7D* QTL regions. A complicating factor in genetic mapping using a random marker approach is that certain chromosomes may be more polymorphic which was also the case in this study, since more markers were added to the more polymorphic chromosome 2B compared to chromosome 7D.

Although a low number of markers were added to the existing map, these markers succeeded in increasing marker resolution on the 2B and 7D target regions. Marker *s23m53d* mapped 3 cM from marker *gwm148* previously shown to be significantly associated with the *QYr.sgi-*

2B QTL region. The LOD score for *gwm148* was 20.1 and 23.9 for *s23m53d* indicating that the application of *s23m53d* in a MAS programme may give improved results. Even though two markers were added to the 7D chromosome, both mapped outside the *QYr.sgi-7D* QTL region. Marker *s20m38b* mapped 9 cM from the SSR marker *gwm295* and 20 cM from the *Ltn* gene previously shown to be associated with the trait of interest on chromosome 7D. Although these markers will not be useful for MAS, the tBSA approach succeeded to add two additional markers to the low density marker region of the 7D chromosome. Several other traits have been shown to be associated with the *Ltn* gene, including the *Lr34/Yr18* slow rusting region (Lagudah *et al.*, 2006) and adult plant powdery mildew resistance (Spielmeyer *et al.*, 2005). Lagudah *et al.* (2006) developed a sequenced tagged site marker, *csLV34* and established that the genetic linkage between *csLV34* and *Lr34/Yr18* was estimated at 0.4 cM. Since they indicated that amplification products from the *csLV34* locus was “diagnostic” for *Lr34/Yr18*, also associated with *Ltn* and the *QYr.sgi-7D* QTL, this sequence tagged site marker should be added to the present linkage map and tested for its applicability as useful marker for the *QYr.sgi-7D* QTL.

AFLP primer combination *s20m38* was of particular interest since it amplified markers within or closely mapped to both the targeted 2B and 7D QTL regions. This primer combination could be useful in a MAS breeding programme to detect the presence or absence of either a 2B and/or 7D QTL region. Indications are that a large number of AFLP primer combinations need to be screened to successfully transfer a specific QTL interval.

This study underlined the value and integrity of the Kariega x Avocet S mapping population, its partial linkage map and the initial QTL analyses. The findings of Ramburan *et al.* (2004) were validated in this study as its applied knowledge led to an improvement of the maps in the major QTL regions.

In order to improve the mapping of new markers on the current linkage map using the tBSA approach, phenotypic and marker data for the 2B and 7D QTL regions are currently being extended from the initial 150 DH population to an extended 256 DH population. The extended population, in combination with the improved linkage maps will allow the

identification of additional lines (possibly also recombinants) that can be used to improve the ‘designer’ bulks for another round of tBSA. The most recent Kariega x Avocet S chart has already been improved with the addition of DArT<sup>TM</sup> markers (R. Prins, personal communication). The new bulks would consist of individuals with markers linked closer to the QTL regions of interest. An extended population should eliminate the problems associated with background markers due to the limited DH population size (150 individuals) used in this dissertation.

An additional outcome could be the development of AFLP-derived STS/SCAR markers that could lead to cheaper markers that are more suitable for MAS.

The validation of Ramburan et al’s (2004) work will also result in more confidence in expanding the set of individual DH lines that are currently being used in host-pathogen interaction studies which aim to unravel the resistance mechanisms of the individual QTL and its interactions.

In general we can conclude that this study has shown that tBSA of a complex trait can be successfully applied to identify markers in the target region.

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## Summary

Stripe rust, caused by *Puccinia striiformis* West. f. sp. *tritici* is one of the most damaging diseases of wheat (*Triticum aestivum* L.) globally. The South African wheat cultivar Kariega expresses APR and has retained yield levels acceptable for commercial production, which is of great importance to plant breeders. A Kariega x Avocet S partial linkage map has made a significant contribution to understanding the genetics underlying APR to stripe rust (*Yr*) in Kariega. Two major *Yr*QTL with indications of different resistance mechanisms were identified on chromosomes 2B and 7D.

In this study we investigated the effectiveness of identifying AFLP markers closely linked to the *Yr*QTL using a targeted bulk segregant analysis (tBSA) approach in F<sub>1</sub> doubled haploid (DH) individuals. Individual Kariega x Avocet S DH lines were characterised and bulked based on stripe rust phenotypes and DNA marker allele profiles. In agreement with standard BSA, an extreme resistant bulk (both QTL present: +7D+2B) and extreme susceptible bulk (both QTL absent: -7D-2B) were constructed based on phenotypic data and verified with marker allele data. Three additional bulks (+7D-2B; -7D+2B and narrow down +7D±2B with marker recombinations in 7D QTL interval) were constructed based on a combination of phenotypic and marker data, with a strong emphasis on the presence or absence of marker alleles representing a specific QTL interval as required by a specific bulk.

A total of 184 AFLP primer combinations (*Sse*I and *Mse*I) were tested on the two parental lines and five bulks. Thirty-one of these primer combinations detected 32 putative markers that could discriminate between the extreme resistant and susceptible bulks and that were putatively linked to either the 7D or 2B QTL regions. After validation of these markers on all individuals used in the extreme resistant and extreme susceptible bulks, nine markers were identified that were present in the extreme resistant and the specific -7D+2B bulk, but absent in the extreme susceptible bulk. Another two markers were identified that were present in the extreme resistant, +7D-2B and narrow down +7D±2B bulks, but absent in the extreme susceptible bulk. These markers were mapped onto the existing Kariega x Avocet S partial

linkage map using Map Manager QTXb20. Six AFLP markers mapped within or close to the *QYr.sgi.2B* and one close to the *QYr.sgi.7D* QTL regions.

The tBSA approach was efficient since 10 of the 11 markers (91%) putatively identified after screening of the individuals constituting the bulk samples mapped to either chromosome 2B or 7D. AFLP analysis in combination with tBSA was shown to be reproducible, faster and a more cost effective approach compared to a traditional BSA since tBSA lead to a reduction of 28.2% of primers that need to be tested. Following the tBSA approach, marker *s23m53d* mapped 3 cM from marker *gwm148* previously shown to be significantly associated with mean host reaction type for final field data as well as leaf area infected of the *QYr.sgi-2B* QTL region. This resulted in an increase in LOD score from 20.1 to 23.9 using interval mapping. Even though two markers were added to the 7D chromosome, both mapped outside the *QYr.sgi-7D* QTL region. Marker *s20m38b* mapped 9 cM from the SSR marker *gwm295* and 20 cM from the *Ltn* gene previously shown to be associated with the trait of interest on chromosome 7D.

In summary, the combination of AFLP analysis and a tBSA approach has proved to be useful in the identification of QTL, the placement of closely linked markers to known QTLs and targeting chromosome areas with low marker numbers. Indications are that a large number of AFLP primer combinations need to be screened to successfully target a specific QTL interval for increased map resolution.

**Keywords:** amplified fragment length polymorphism (AFLP), doubled haploids (DH), interval mapping (IM), linkage mapping, marker-assisted selection (MAS), targeted bulk segregant analysis (tBSA).

## Opsomming

Streeproes, wat deur *Puccinia striiformis* West. f. sp. *tritici* veroorsaak word, is een van die koringsiektes wat wêreldwyd die grootste skade op koring (*Triticum aestivum* L.) veroorsaak. Die Suid-Afrikaanse koring kultivar Kariëga bevat volwasse plant weerstand (APR) teen streeproes asook kommersieël aanvaarbare opbrengsvlakke wat baie belangrik vir plantetelers is. 'n Kariëga x Avocet S gedeeltelike koppelingskaart het 'n betekenisvolle bydrae tot kennis aangaande die onderliggende genetika van APR teen streeproes (*Yr*) in Kariëga gemaak. Twee hoof streeproes kwantitatiewe eienskap lokusse (*Yr*QTL), met aanduidings van verskillende weerstandsmeganismes, is op chromosome 2B en 7D geïdentifiseer.

Die doeltreffendheid om geamplifiseerde fragment lengte polimorfisme (AFLP) merkers wat naby aan die *Yr*QTL gekoppel is te identifiseer deur gebruik te maak van geteikende massa segregerende analise (tBSA) in  $F_1$  dubbel haploïde (DH) individue, is in hierdie studie ondersoek. Individuele Kariëga x Avocet S DH lyne is gekarakteriseer en op grond van streeproes fenotipes en DNA merker alleel profiele bymekaar gevoeg. Ooreenkomstig met standaard massa segregerende analise (BSA) is 'n uiters weerstandbiedende poel (beide QTL teenwoordig: +7D+2B) en 'n uiters vatbare poel (beide QTL afwesig: -7D-2B), gebaseer op fenotipiese data en bevestig met merker alleel data, saamgestel. Drie addisionele poele (+7D-2B; -7D+2B en 'n kleiner geteikende +7D±2B poel met merker rekombinasie van die 7D QTL interval) is op grond van 'n kombinasie van fenotipiese en merker data, met 'n sterk klem op die teenwoordigheid of afwesigheid van merker allele wat die spesifieke QTL intervale verteenwoordig, soos deur die spesifieke poel vereis, saamgestel.

'n Totaal van 184 AFLP voorvoerder kombinasies (*Sse*I and *Mse*I) is op die twee ouerlyne en vyf poele getoets. Een-en-dertig van hierdie voorvoerder kombinasies het 32 moontlike merkers wat tussen die uiters weerstandbiedende en vatbare poele kon onderskei en moontlik aan of die 7D of die 2B QTL gebiede gekoppel is, opgespoor. Nadat hierdie merkers op al die individue wat gebruik is om die uiters weerstandbiedende en vatbare poele saam te stel bevestig is, is nege merkers geïdentifiseer wat in die uiters weerstandbiedende poel en die

spesifieke -7D+2B poel teenwoordig was, maar afwesig was in die uiters vatbare poel. Die ander twee merkers wat geïdentifiseer is, was teenwoordig in die uiters weerstandbiedende poel, +7D-2B en kleiner geteikende +7D±2B poele, maar afwesig in die uiters vatbare poel. Hierdie merkers is op die bestaande Kariëga x Avocet S gedeeltelike koppelingskaart gekarteer deur van Map Manager QTXb20 gebruik te maak. Ses van die AFLP merkers het binne of naby die *QYr.sgi.2B* en een naby die *QYr.sgi.7D* QTL gebiede gekarteer.

Die tBSA benadering was effektief aangesien 10 van die 11 merkers (91%) wat aanvanklik, op grond van toetse op individue wat gebruik is om die poele saam te stel, as moontlike merkers geïdentifiseer is, of na chromosoom 2B of 7D gekarteer het. AFLP analise in kombinasie met tBSA was 'n meer herhaalbare, vinniger en koste-effektiewe benadering in vergelyking met tradisionele BSA aangesien 28.2% minder voorvoeders getoets is met tBSA in vergelyking met BSA. Gebaseer op tBSA resultate, het merker *s23m53d* 3 cM van merker *gwm148*, voorheen aangetoon as betekenisvol gekoppel aan die gemiddelde gasheerreaksie tipe vir die finale veld data asook blaaroppervlakte geïnfekteer van die *QYr.sgi-2B* QTL gebied, gekarteer. Dit het gebaseer op interval kartering tot 'n verhoging van die LOD telling vanaf 20.1 na 23.9, gelei. Alhoewel twee addisionele merkers op chromosoom 7D gekarteer is het beide buite die *QYr.sgi-7D* QTL gebied gekarteer. Merker *s20m88b* het 9 cM van die mikrosatelliet merker *gwm295* en 20 cM van die *Ltn* geen, voorheen aangetoon as geassosieer met die geteikende eienskap op chromosoom 7D, gekarteer.

Opsommend is gevind dat die kombinasie van AFLP analise en 'n tBSA benadering bruikbaar is vir die identifisering van QTL, die plasing van naby gekoppelde merkers aan bekende QTL en die tekening van chromosoom areas met lae merker getalle. Resultate dui daarop dat 'n groot aantal AFLP voorvoeder kombinasies getoets moet word om spesifieke QTL gebiede suksesvol te teiken vir verbeterde kaart resolusie.

**Sleutelwoorde:** geamplifiseerde fragment lengte polimorfisme, dubbel haploïede, geteikende massa segregerende analise, interval kartering, koppelingskartering, merker-ondersteunde seleksie,