

***Pyramiding wheat rust resistance genes
using marker-assisted selection***

By

SCOTT LLOYD SYDENHAM

*Submitted in the fulfilment of the requirements for the
degree*

Magister Scientiae Agriculturae

***Department of Plant Sciences (Plant Breeding)
Faculty of Natural and Agricultural Sciences
University of the Free State
Bloemfontein
Republic of South Africa***

November 2007

Supervisor: Dr. Liezel Herselman

Co-supervisor: Prof. ZA Pretorius

Declaration

"I, Scott Lloyd Sydenham, declare this dissertation hereby submitted by me for the degree Magister Scientae Agriculturae at the University of the Free State is my own independent work and has not previously been submitted by me to another University for any degree".

"I cede copyright of this dissertation in favour of the University of the Free State".

.....

Scott Lloyd Sydenham

.....

Date

*“Each of us has a fire in our heart for something.
It's our goal in life to find it and to keep it lit”.*

ML Retton

Acknowledgements

I would like to convey my sincere gratitude, appreciation and special thanks to various organisations, institutes and individuals who were instrumental during the course of my studies and research over the past two years.

- Dr. Liezel Herselman as my supervisor, without your continual support, encouragement and belief in me. Without your expertise, advice, amazing enthusiasm, long hours and dedication to the discipline, this study would not have been possible.
- To Professor ZA Pretorius my co-supervisor in helping me realise his vision, for his wealth of knowledge and experience.
- To my loving Mom and late Dad, for there unconditional love, continual support, motivation, belief in me and strength during hard times. Thank you for doing everything in your power to allow me to study and encourage me to pursue what I want in life. To Dad I know you are always there. To my mom a special thank you for your amazing support and assistance in anyway possible through this last year after the death of dad, you have done an incredible job to keep me heading on the right path during this hard and painful time. Without you this would not have been possible.
- To Cindy-Lee for being by my side at all times, for your support, love, encouragement, patience, understanding, always going beyond what is required to help me and easing my stress throughout my study. Especially for keeping me calm, high spirited and being there through my emotional and stressful times. My personal assistant.
- To Margie and Harry Guild, for their support of me, love, assistance where possible, enthusiasm and interest in the study.
- To Adré for your friendship, always willing to listen, teamwork and assistance beyond what was expected. And good times!
- To Sadie for your amazing handling of administrative affairs, care, support, encouragement and listening ear.
- To Rouxlene and Dr. Elizma, for your friendship, guidance and practical help.

- To Fred for his friendship, co-operation and assistance.
- To Oscar for his friendship, motivational talks and spiritual upliftment.
- To Prof Deventer for his assistance with the breeding aspects of this study.
- To the Winter Cereal Trust for financial support.

Dedication

This masters dissertation is dedicated
in loving memory of my Dad.
(10th May 1941- 29th Dec 2006)

*“Those who loved you and were helped by you will
remember you. You carved your name on their hearts
and not on marble”.*

CH Spurgeon

Table of contents

Declaration	i
Quotation	ii
Acknowledgements	iii
Dedication	v
Table of contents	vi
List of Figures	x
List of Tables	xi
List of abbreviations	xiii

Chapter 1

<i>General Introduction</i>	1
References	4

Chapter 2

<i>Wheat rusts: an ancient and continual threat</i>	6
2.1 Wheat	6
2.2 History of wheat production in South Africa	6
2.3 Global wheat production	7
2.4 Utilisation of wheat	8
2.5 Wheat taxonomy	8
2.6 Wheat genomics	8
2.7 History of wheat domestication	9
2.7.1 <i>Ancient wheat</i>	9
2.7.2 <i>Evolution of bread wheat</i>	9
2.7.3 <i>Wheat species</i>	10
2.8 Plant diseases	12
2.8.1 <i>Disease cycle</i>	13
2.9 Important diseases of wheat	14
2.9.1 <i>Karnal bunt</i>	14
2.9.2 <i>Loose smut</i>	14

2.9.3	Common bunt	15
2.9.4	Fusarium head blight	16
2.9.5	Black chaff	16
2.9.6	Mildew	17
2.9.7	Glume blotch	17
2.10	Rust Pathogens	17
2.10.1	Wheat rusts	18
2.10.2	Leaf rust	19
	<i>Characteristics</i>	19
	<i>Hosts</i>	20
	<i>Life cycle</i>	20
	<i>Economic importance</i>	21
	<i>Epidemics</i>	21
	<i>Virulence</i>	21
2.10.3	Stem rust	22
	<i>Characteristics</i>	22
	<i>Hosts</i>	23
	<i>Life cycle</i>	24
	<i>Economic importance</i>	24
	<i>Epidemics</i>	24
	<i>Ug99 (TTKS)</i>	25
	<i>Virulence</i>	26
2.10.4	Stripe rust	26
	<i>Characteristics</i>	27
	<i>Hosts</i>	28
	<i>Life cycle</i>	28
	<i>Epidemics</i>	28
	<i>Economic importance</i>	29
	<i>Virulence</i>	29
2.10.5	Managing rust	30
2.11	Resistance genes to be used in the current study	32
2.11.1	Lr19	32
2.11.2	Lr34	32
2.11.3	Sr2	33

2.11.4	Sr26	34
2.11.5	YrSp	34
2.11.6	Yr7D and Yr2B	35
2.12	Molecular plant breeding	35
2.12.1	Amplified fragment length polymorphism	36
2.12.2	Sequenced-tagged site (STS)	38
2.12.3	Microsatellites or simple sequence repeats	38
2.12.4	Application of SSR markers in wheat	39
	<i>SSR map of wheat</i>	40
2.12.5	Marker-assisted selection	41
2.12.6	Gene pyramiding	42
2.12.7	Gene pyramiding applications	44
2.13	References	45

Chapter 3

Rust resistance genotyping using linked molecular markers

		57
3.1	Introduction	57
3.2	Materials and methods	59
3.2.1	Plant material	59
3.2.2	Planting design	60
3.2.2.1	<i>Test planting</i>	60
3.2.3	Crossing programme design	60
3.2.4	Phenotypic screening	63
3.2.5	Sample collection	63
3.2.6	DNA Extraction	64
3.2.6.1	<i>Homogenising of leaf samples</i>	64
3.2.6.2	<i>DNA isolation</i>	64
3.2.7	SSR analysis	65
3.2.7.1	<i>PCR reactions</i>	65
3.2.7.2	<i>SSR-PCR cycling conditions</i>	65
3.2.8	Visualisation	68
3.2.8.1	<i>Polyacrylamide gel electrophoresis (PAGE)</i>	68

3.2.8.2	<i>Silver staining procedure</i>	68
3.2.8.3	<i>Agarose gel electrophoresis</i>	68
3.2.9	<i>AFLP analysis</i>	69
3.2.9.1	<i>DNA extraction for AFLP analysis</i>	69
3.2.9.2	<i>Restriction digestion</i>	69
3.2.9.3	<i>Adapter ligation</i>	70
3.2.9.4	<i>Pre-selective amplification</i>	70
3.2.9.5	<i>Selective amplification</i>	71
3.2.10	<i>Screening of parental lines, F₁ progeny and the double cross population</i>	71
3.2.10.1	<i>Data analysis</i>	71
3.3	Results	72
3.3.1	<i>Phenotypic screening</i>	72
3.3.1.1	<i>Leaf rust infection</i>	72
3.3.1.2	<i>Stem rust infection</i>	72
3.3.1.3	<i>Stripe rust infection</i>	73
3.3.2	<i>Genotyping</i>	75
3.3.2.1	<i>Parental screening</i>	75
3.3.2.2	<i>F₁ cross identification</i>	76
3.3.2.3	<i>Double cross population</i>	78
3.3.3	<i>Marker segregation</i>	81
3.3.4	<i>Genotypic frequencies in double cross population</i>	83
3.3.4.1	<i>Expected genotypic frequencies</i>	83
3.3.4.2	<i>Observed genotypic frequencies</i>	83
3.4	Discussion	88
3.5	References	94
Chapter 4		
	<i>General conclusions and perspectives</i>	98
	References	102
	Summary	105
	Opsomming	107

List of Figures

Figure 2.1	Diagrammatic representation of hybridisation events that occurred during the evolution of wheat.	10
Figure 2.2	Leaf rust (<i>P. triticina</i>) symptoms on a wheat leaf.	20
Figure 2.3	Stem rust (<i>P. graminis</i> f. sp. <i>tritici</i>) symptoms on the stem of a wheat plant.	23
Figure 2.4	Stripe rust symptoms (<i>P. striiformis</i> f. sp. <i>tritici</i>) on the leaf of a wheat cultivar.	27
Figure 3.1	Crossing scheme to combine resistance genes of the four wheat cultivars used in this study.	62
Figure 3.2	A silver stained polyacrylamide gel of Avocet YrSp/Kariega F ₁ individuals screened with marker Gwm148.	77
Figure 3.3	Comparing the cross success percentages of cross 1 and cross 2 within individual plantings confirmed by SSR and STS marker screening.	78
Figure 3.4	A silver stained polyacrylamide gel of marker Gwm111 indicating the allele sizes of Avocet, Blade, CsLr19-149-299 and Kariega segregating in the double cross population.	79
Figure 3.5	Frequency distribution showing the number of double cross F ₁ plants that tested positive for the presence of molecular markers.	80

List of Tables

Table 2.1	Diseases caused by <i>Puccinia</i> spp. on cereals	18
Table 2.2	Comparative characteristics of <i>Puccinia graminis</i> f. sp. <i>tritici</i> , <i>P. triticina</i> and <i>P. striiformis</i> f. sp. <i>tritici</i> of wheat	30
Table 3.1	Numerical characteristics of hybrids between parents differing in <i>n</i> allelic pairs	61
Table 3.2	Selected SSR markers, corresponding primer pair sequences, targeted genes or QTL, parental cultivar sources and references for the primer sets used in the study	66
Table 3.3	Optimal reaction and PCR cycling conditions for SSR markers used in the study	67
Table 3.4	<i>Mse</i> I- and <i>Eco</i> RI-adapter and primer sequences	70
Table 3.5	Primary leaf seedling infection types of parental and F ₁ genotypes to selected pathotypes of <i>Puccinia graminis</i> f. sp. <i>tritici</i> , <i>P. triticina</i> and <i>P. striiformis</i> f. sp. <i>tritici</i>	74
Table 3.6	Number and size of marker alleles identified during parental screening	75
Table 3.7	Segregation ratios of eight molecular markers tested on 900 individuals of the double cross population using Chi square analysis	82

Table 3.8	Expected genotypes, genotypic percentage, expected number of individuals (E) in the 900 individuals of the double cross population based on the combination of five rust resistant genes and two QTL	84
Table 3.9	Observed and expected genotypic frequencies of individuals of the double cross population individuals genotyped based on data from six molecular markers	87

List of Abbreviations

AFLP	Amplified fragment length polymorphism
APR	Adult plant resistance
ARC-SGI	Agricultural Research Council-Small Grain Institute
ATP	Adenosine 5'-triphosphate
Avr	Avirulence
BC	Before Christ
bp	Base pairs(s)
°C	Degrees Celsius
CAPS	Cleaved amplified polymorphic sites
cm	Centimetre(s)
cM	Centimorgan(s)
CTAB	Hexadecyltrimethylammonium bromide
DH	Double haploid
DNA	Deoxyribonucleic acid
dNTPs	2'-deoxynucleoside 5'-triphosphate
DTT	Dithiotreitol
E	Expected
EDTA	Ethylene-diaminetetraacetate
F₁	First generation
F₂	Second generation
FHB	Fusarium head blight
g	Gram(s)
<i>g</i>	Gravitational force
GH	Greenhouse
h	Hour(s)
km	Kilometre(s)
l	litre(s)
Lr	Leaf rust
Ltn	Leaf tip necrosis
M	Molar(s)
MAS	Marker-assisted selection

min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
ng	Nanogram(s)
NIL	Near-isogenic line
O	Observed
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PBC	Pseudo-black chaff
pH	Power of hydrogen
pmol	Picomole(s)
QTL	Quantitative trait loci
R	Rand(s)
R	Resistance
®	Reserved
r/s	Revolutions per second
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RIL	Recombinant inbred line
S	Susceptible
SA	South Africa
SAM	Selectively amplified microsatellite
SCAR	Sequence characterised amplified region
sec	Seconds
SNP	Single nucleotide polymorphism
Sr	Stem rust
SSR	Simple sequence repeat
STM	Sequenced-tagged microsatellite
STS	Sequenced-tagged site
TBE	Tris-borate/EDTA
TE	Tris-Cl/EDTA
Tris-Cl	Tris (hydroxymethyl) aminomethane
™	Trade Mark

U	Unit(s)
USA	United States of America
V	Volt(s)
v/v	Volume per volume
W	Watt(s)
w/v	Weight per volume
Yr	Yellow rust
µg	Microgram(s)
µl	Microlitre(s)
µm	Micrometre(s)
µM	Micromolar(s)

Chapter 1

General Introduction

Wheat is one of the most important cereal crops to the human race and rust diseases continually pose a threat to global wheat production (Khan *et al.*, 2005). Wheat is grown over large areas in South Africa and globally, primarily for human consumption (Bajaj, 1990; Curtis *et al.*, 2002).

Like all plants, wheat endures injury and are exposed to stress during all stages of development. This naturally will affect normal plant functioning and optimal development (Wiese, 1977). Wheat production globally and locally suffers large yield losses due to diseases (Scott, 1990). Fortunately man's battle against disease has been, for many years, fought relatively successfully through targeted wheat improvement.

There are numerous wheat diseases, caused by various pathogens. Of these, rust diseases have, for years, been a major concern and problem for breeders, farmers and commercial seed companies (Wiese, 1977; Marsalis and Goldberg, 2006). Rust diseases of wheat are the oldest known to man (Marsalis and Goldberg, 2006) and are important worldwide (Singh *et al.*, 2005; Kuraparthi *et al.*, 2007). Wheat rusts have been reported as devastating, having the ability to destroy entire susceptible wheat crops, in a matter of weeks, resulting in large economical losses (Haung and Röder, 2004; Marsalis and Goldberg, 2006).

Generally a resistance response against air-borne wheat pathogens could be caused by specific and non-specific resistance genes in the host plant (Khlestkina *et al.*, 2007). Fungal rusts are obligate parasites (Kolmer, 2005) that interact in a gene-for-gene relationship between the resistance gene(s) of the wheat plant and the virulence gene(s) of the pathogen (Kolmer, 2005; Khlestkina *et al.*, 2007; Kuraparthi *et al.*, 2007). Specific resistance genes within the host wheat plant triggers a protection mechanism against disease, normally with a hypersensitive response reaction and are usually expressed

during all plant developmental stages (Singh *et al.*, 2005; Khlestkina *et al.*, 2007; Kuraparthy *et al.*, 2007). Random mutational events and selection for virulence against rust resistance genes cause the development of new pathogen races (Kolmer, 2005). This ability of pathogens to mutate rapidly and multiply, and the use of air-borne dispersal mechanisms for long distance travel, pose a continual global threat (Singh *et al.*, 2005). A change in pathogen virulence results in previously developed resistant cultivars becoming ineffective and susceptible (Kolmer, 2005).

Breakdown of cultivar resistance is further complicated by the fact that rust spores can be spread thousands of kilometres by wind. Kolmer (2005) reported that pathogen races have been traced and followed across continents. Generally breeders try to develop resistant cultivars in their breeding programmes ahead of the potential of new pathogen races to ensure durable resistance (Singh *et al.*, 2005). Lately, epidemic losses due to rust diseases are rare, although diseases can occur at significant levels in particular production regions (Marsalis and Goldberg, 2006). In more recent years the spread of new rust races across continents has complicated the development of new resistant cultivars with durable rust resistance (Kolmer, 2005). These new virulent races, together with breeding objectives of high-yielding, pure and uniform varieties worldwide, have reduced the genetic base for disease resistance, affecting the number of potentially effective rust genes available for new cultivar development (Kolmer, 2005; Kuraparthy *et al.*, 2007).

Over the years, resistance genes have been incorporated throughout breeding programmes and depending on the gene, remained effective for a number of years. Experience has shown that some resistant cultivars containing single resistance genes were not effective for long periods; thus the need arose to pyramid genes against particular rusts into a single cultivar (Schnurbusch *et al.*, 2004).

Application of molecular techniques and marker-assisted selection (MAS) in a breeding programme can assist to reach a breeding objective in a shorter period of time. Extensive research has gone into many of the economically

important crops, including wheat. Results, developments and breakthroughs opened up new application frontiers for crops of interest (Röder *et al.*, 1998; Francia *et al.*, 2005).

New technologies can not replace the progress that traditional breeding programmes make, but MAS can help breeders to reach objectives more effectively and rapidly. With this merging of traditional breeding and new technologies in mind, the aim of the current study was conceptualised.

The main aim of this study was to pyramid several wheat rust resistance genes into a single genotype. The study focused on wheat genes and markers used and/or developed in South African breeding programmes. Gene pyramiding was accomplished by using four cultivars containing seven different rust resistance genes/quantitative trait loci (QTL) (five genes and two QTL) and selection was done using microsatellite or simple sequence repeat (SSR), sequence-tagged site (STS) and amplified fragment length polymorphism (AFLP) markers.

References

Bajaj YPS (ed) (1990) Biotechnology in Agriculture and Forestry 13 – Wheat. **Springer-Verlag pp 687.**

Curtis BC, Rajaram S and Macpherson HG (eds) (2002) Bread wheat improvement and production. **Food and Agriculture Organization of the United Nations, Rome Italy pp 554.**

Francia E, Tacconi C, Crosatti D, Barabaschi D, Dalli' Agilo E and Vale G (2005) Marker assisted selection in crop plants. **Plant Cell 82: 317-342.**

Haug XQ and Röder MS (2004) Molecular mapping of powdery mildew resistance genes in wheat. **Euphytica 137: 203-223.**

Khan RR, Bariana HS, Dholakia BB, Naik SV, Lagu MD, Rathjen AJ, Bhavani S and Gupta VS (2005) Molecular mapping of stem and leaf rust resistance in wheat. **Theoretical and Applied Genetics 111: 846-850.**

Khlestkina EK, Röder MS, Unger O, Meinel A and Börner A (2007) More precise map position and origin of a durable non-specific adult plant disease resistance against stripe rust (*Puccinia striiformis*) in wheat. **Euphytica 153: 1-10.**

Kolmer JA (2005) Tracking wheat rust on continental scale. **Current Opinion in Plant Biology 8: 441-449.**

Kuraparthi V, Chhuneja P, Dhaliwal HS, Kaur S, Bowden RL and Gill BS (2007) Characterization and mapping of cryptic alien introgression from *Aegilops geniculata* with new leaf rust and stripe rust resistance genes *Lr57* and *Yr40* in wheat. **Theoretical and Applied Genetics 114: 1379-1389.**

Marsalis MA and Goldberg NP (2006) Leaf, stem and stripe rust diseases of wheat. *New Mexico State University Guide A-415*. Available at (<http://www.cahe.nmsu.edu>). Cited July 2006.

Röder MS, Korzun V, Wendehake K, Plaschke J, Tixier M, Leroy P and Ganai MW (1998) A microsatellite map of wheat. *Genetics* **149**: 2007-2023.

Scott DB (1990) Wheat diseases in South Africa. *Pretoria: Department of Agricultural Development pp 62*.

Schnurbusch T, Paillard S, Schori A, Messmer M, Schachermyr G, Winzeler M and Keller B (2004) Dissection of quantitative and durable leaf rust resistance in Swiss winter wheat reveals a major resistance QTL in *Lr34* chromosomal region. *Theoretical and Applied Genetics* **108**: 477-484.

Singh RP, Huerta-Espino J and Williams HM (2005) Genetics and breeding for durable resistance to leaf and stripe rusts in wheat. *Turkish Journal of Agriculture* **29**: 121-127.

Wiese MV (1977) Compendium of wheat diseases. *St. Paul: The American Phytopathological Society pp 112*.

Chapter 2

Wheat rusts: an ancient and continual threat

2.4 Wheat

Wheat is a widely adapted crop, which is grown from temperate, irrigated dry, high rainfall, warm humid to dry cold climates. As a C₃ plant wheat is capable of thriving in cool environments. Optimal growth of wheat occurs at an average temperature of 25°C, with minimums at times as low as 3°C to 4°C and maximums of 30°C to 32°C. Cultivation of wheat in climatic regions where annual rainfall averages from 250 mm up to 1 750 mm have been reported (Curtis *et al.*, 2002).

2.5 History of wheat production in South Africa

Wheat was first planted in South Africa (SA) shortly after the arrival of Jan van Riebeeck in the Cape in 1652. One hundred years later wheat was propagated across other areas of South Africa (<http://www.Wintercrops.co.za>).

Wheat in SA is planted mainly between middle of April and middle of June in the winter rainfall areas (Western Cape) and between middle May and the end of July in the summer rainfall areas. Wheat is harvested in SA between November and December (USDA, 2006). Most of the wheat produced in South Africa is bread wheat, with a little durum wheat produced in certain areas. Geographically wheat is currently grown in the Western Cape, Northern Cape, Free State, North West, Mpumalanga, KwaZulu-Natal and Limpopo provinces. The two main regions responsible for three quarters of the production are in the Free State and Western Cape. Approximately 85% of the crop planted is under rainfall dependent climatic conditions while the rest is irrigated (http://www.fas.usda.gov/pecad/highlights/2004/10/RSA_wheat/index.htm, 2004).

Wheat production generally averages around 2 million tons (Curtis *et al.*, 2002). Some recent reports state production may have dropped below the 2 million ton mark. During the 2005 and 2006 wheat season the price of wheat fluctuated

between R1 600 per ton in June 2005 and R1 652 in June 2006, an increase of 3.1% for the period. This price increase of wheat contributed to a 38% increase in revenue generated by wheat farmers (Van Wyk, 2006). During 2007 the wheat price in South Africa fluctuated between R1 900 to over R2 000 per ton. The wheat price reached a high of R2 005 per ton in July 2007 and levelled to R1 900 at the end of the year (<http://www.sagis.org.za>, 2007).

2.3 Global wheat production

Wheat is widely cultivated over large areas and is an important food crop worldwide (Bajaj, 1990; Curtis *et al.*, 2002; Gupta *et al.*, 2002; Haung and Röder, 2004). Cultivation of wheat (*Triticum spp.*) dates back in history for 8 000 years. Wheat was one of the first domesticated food crops and has been a basic staple food for many civilisations (Curtis *et al.*, 2002). Harlan (1995) reported that wheat contributes 23.4% to the total world food production (based on dry matter yield). More recently Haung and Röder (2004) stated that wheat is a staple food for 35% of the human population. Wheat production worldwide increased exponentially during the period 1951-1990, accompanied by an expansion in the area used in wheat cultivation. Since 1986, when production reached 529 million tonnes, global wheat production has constantly been above the 500 million ton mark. The increase in global wheat production was attributed to an increase in yield per hectare as a result of variety improvement (Curtis *et al.*, 2002). Rajaram (2001) reported that global wheat production averaged around 600 million tonnes a year in 2001 and is expected to be one billion tonnes by the year 2020 in order to meet human consumption. Curtis *et al.* (2002) similarly reported that in future global wheat production is expected to reach 850 million tonnes a year by 2030 to keep up with human population growth.

Van Wyk (2006) reported that global wheat production declined by 1.1% in the 2005/2006 season and is expected to decline by 3.5% in the 2006/2007 season. In June 2005 the world wheat price was R807 per ton and by June 2006 it increased substantially to R1 020 per ton. The fact still remains that the world wheat price is much lower than the wheat price in South Africa, making it more cost-effective to import wheat from international markets (Van Wyk, 2006).

2.4 Utilisation of wheat

Worldwide wheat is used extensively during the production of many different types of foods. Approximately 90 to 95% of the globally grown wheat is common wheat (*Triticum aestivum* L.). It is the staple food of millions of people and forms an important part of many people's daily diet (Curtis *et al.*, 2002). Wheat is mainly utilised as flour for the production of products such as different types of bread, cakes and other baked products (Dendy and Dobraszczyk, 2001; Curtis *et al.*, 2002). Wheat is less extensively grown for and used as a source of animal feed (Dendy and Dobraszczyk, 2001). There are different wheat variety classes according to various grain characteristics such as hardness, protein content, starch content, etc. which are selected depending on the required end product and utilisation. The rest of the globally grown wheat is mainly durum wheat (*T. durum* Desf. Husn.), used in the production of semolina (coarse flour). Semolina is the main raw ingredient used to make biscuits and pasta products (noodles, spaghetti) (Curtis *et al.*, 2002).

2.5 Wheat taxonomy

Wheat is classified within the genus *Triticum* which is part the *Poaceae* family. The genus *Triticum* is further subdivided into a number of species which are classified according to the number of chromosome pairs they contain; diploid ($2n=2x=14$) (7 pairs e.g. einkorn wheat), tetraploid ($2n=4x=28$) (14 pairs e.g. durum wheat) and hexaploid ($2n=6x=42$) (21 pairs e.g. "common" bread wheat) (Dendy and Dobraszczyk, 2001; Curtis *et al.*, 2002).

2.6 Wheat genomics

Bread wheat or common wheat is an allohexaploid, with three closely related genomes (A, B and D), each consisting of seven chromosomes (Gupta *et al.*, 2002; Gill *et al.*, 2004; Dieguez *et al.*, 2006; Zaharieva and Monneveux, 2006). The three genomes of bread wheat originated from different species and combined during the evolution of wheat (Zaharieva and Monneveux, 2006). Wheat has a genome size of 16×10^9 bp, which is considered large (Curtis *et al.*, 2002; Gupta *et al.*, 2002). The bread wheat genome (AABBDD) is eight times larger than the maize genome and 40 times larger than the rice genome

(Gill *et al.*, 2004). Eighty percent of the genome consists of repetitive DNA sequences (Gupta *et al.*, 2002).

2.7 History of wheat domestication

2.7.1 Ancient wheat

Based on archaeological evidence, the history of wheat dates back to 17 000 BC, with the finding of emmer wheat seeds at a site on the shores of Israel. Carbonised evidence of thinner wild varieties of einkorn wheat in archaeological sites in Northern Syria dated back to around 10 000 BC, indicating that ancient man had been gathering and eating this wheat. Further archaeological evidence indicated that around 7 800 BC, near Damascus in Israel, hulled emmer wheat had gone through a domestication process through human intervention. Man probably selected plants with plumper grain that was non-brittle and stayed on the plant till harvest. The earliest evidence of ancient humans making use of bread wheat is dated back to 4 700 BC, in the region between the Black and Caspian seas. The wild grass *Aegilops squarrosa* grew in the same region, leading to hybridisation and creation of bread wheat. Soon after this, before 4 000 BC, free-threshing naked bread wheat was developed (Hopf and Zohary, 1993; Sauer, 1993).

2.7.2 Evolution of bread wheat

The three main cereal crops of today, wheat, maize and rice, all co-evolved from a single common grass ancestor some 40 million years ago (Gill *et al.*, 2004). Hybridisation and introgression of closely related species occur naturally. Furthermore, hybridisation between cultivated crops and their wild relatives has been well documented (Zaharieva and Monneveux, 2006). Gill *et al.* (2004) stated that “Humans and wheat have a remarkably parallel evolutionary history”. It is believed that the common grass ancestor of wheat that existed 3 million years ago diverged further into different diploid wheat species. Around 30 000 years ago two wild diploid wheat species hybridised to form a polyploid (tetraploid) wheat. Wheat was the first crop cultivated by man and is the youngest polyploid species compared to the other agriculturally important crops such as rice and maize (Gill *et al.*, 2004).

Figure 2.1 illustrates the hybridisation events between wild species in the past to produce new polyploid species during domestication and cultivation of wheat through the centuries by human civilisations. *Triticum urartu* (AA) hybridised with *Aegilops speltoides* (Tausch) Gren (BB) to create a new polyploid species, *T. turgidum* (AABB). *Triticum turgidum* (AABB) then crossed with *A. tauschii* Cross (DD) to form *T. aestivum* (AABBDD) or common wheat (Akhunov *et al.*, 2003; Gill *et al.*, 2004; Dieguez *et al.*, 2006; Zaharieva and Monneveux, 2006).

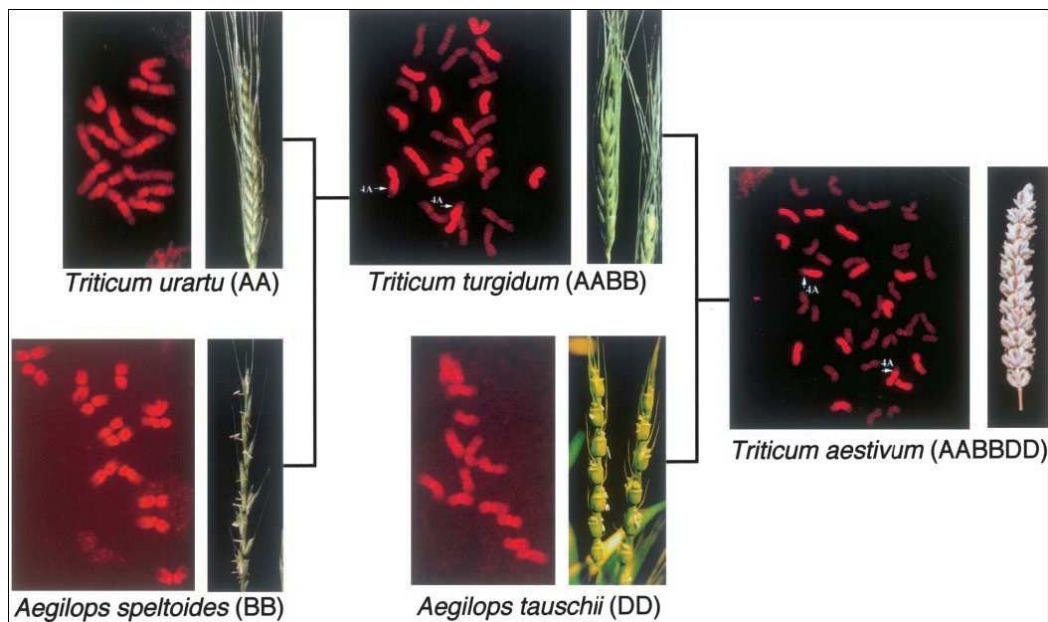


Figure 2.1 Diagrammatic representation of hybridisation events that occurred during the evolution of wheat (Gill *et al.*, 2004).

2.7.3 Wheat species

Triticum monococcum (Link) Thell. (einkorn) wheat is a diploid species with two sets of chromosomes ($2n=7x=14$) (Hopf and Zohary, 1993; Sauer, 1993). *Triticum monococcum* contains the A genome which consists of 5 billion base pairs (bp) grouped into seven pairs of chromosomes (Curtis *et al.*, 2002). Most varieties of this species produce one grain per spikelet, resulting in its common and Latin names. Einkorn wheat was initially domesticated from wild grass types 9 000 years ago and this resulted in plants that produced fuller grain that will remain on the plant till harvest. According to history, einkorn wheat was grown during the Neolithic period. Its use gradually dwindled as man moved into

the Bronze Age and other wheat species and varieties took preference (Hopf and Zohary, 1993; Sauer, 1993).

Triticum turgidum (Desf). Husn. (durum and emmer wheat) is a tetraploid ($2n=4x=28$) species (Hopf and Zohary, 1993). Durum wheat has a genome (AABB) size of roughly 10 billion bp organised into 14 pairs of chromosomes. The structure of the A and B chromosomes are essentially identical to the A and B genomes of common wheat (Curtis *et al.*, 2002). Wild durum wheat varieties' seeds are covered by a tusk. The tusk stays around the grain after threshing which requires a more labour intensive process of breaking and pounding the tusk in some manner to release the seed within. This tusk around the seeds is commonly referred to as the hulled variety. Emmer wheat is a hulled variety while durum wheat is a free-threshing grain. Durum wheat was selected from emmer wheat (Hopf and Zohary, 1993).

Triticum timopheevii (Zhuk). Zhuk is a tetraploid wheat with no great importance to world agriculture, due to it not being involved in polyploidisation events or further wheat cultivation. Timopheevi wheat was grown between the Black and Caspian seas (Sauer, 1993). However, *T. timopheevii* has contributed indirectly to the wheat industry by being the source of two stem rust resistance genes (*Sr36* and *Sr37*) which have been used extensively around the world in breeding programmes. Additionally *T. timopheevii* has contributed *Lr18* and *Sr40* resistance genes to the wheat breeding industry (Friebe *et al.*, 1996).

Bread wheat, *T. aestivum*, is a hexaploid ($2n=6x=42$) wheat. Today, there are a number of bread wheat types, classed according to growing period (winter, spring and intermediate), kernel colour (white and red) and end use in mind [bread (hard), biscuit (soft) and animal feed]. True winter wheat types need a cold period in order to produce a high yield. Winter wheat is normally planted during autumn in summer rainfall regions which tend to have a cold winter. Winter wheat grows slowly through winter and develops many tillers per plant (Trench *et al.*, 1992).

Intermediate wheat types tend to be grown in warmer parts of summer rainfall climates around the middle of winter. Most wheat grown under dryland conditions in summer rainfall climates are winter wheat types or intermediate types (Trench *et al.*, 1992).

Spring wheat types do not need a cold period in order to produce good grain. Spring wheat is planted under irrigation in summer rainfall climates, in the early spring or winter. Some of these varieties are planted before winter in winter rainfall regions of the Western Cape. This type of wheat grows faster than winter wheat and produces ears more quickly (Trench *et al.*, 1992).

2.8 Plant diseases

Plant diseases are as ancient as agriculture itself and are important to man due to damage caused to plants and products. Annually plant diseases cause huge economic losses and millions are spent in combating diseases on economically important crops (Jones and Clifford, 1978; Singh *et al.*, 1992). Losses caused by plant disease affect commercial farmers, subsistence farmers growing food for survival, food markets, wholesalers and the final consumer (Trench *et al.*, 1992). Plant disease is often differently interpreted, with many defining sentences or statements (Murray *et al.*, 1998). Murray *et al.* (1998) stated that “a plant disease is a harmful alteration of what is normally considered physiological and biological development of the plant concerned, resulting in abnormal morphological and physiological changes, displaying unique symptoms”. Trench *et al.* (1992) gave a similar definition. The plant disease definition by Murray *et al.* (1998) is vital in understanding why diseases of agriculturally important crops are such a concern to breeders and scientists. If no alteration and/or harm occurred during normal plant development it would be of no interest or importance (Murray *et al.*, 1998).

Plant diseases can be caused by a variety of biotic organisms including fungi, bacteria, phytoplasmas, viruses, viroids, nematodes and parasitic plants (Jones and Clifford, 1978; Trench *et al.*, 1992; Murray *et al.*, 1998). Furthermore, abiotic causes such as mineral deficiency can lead to substantial harmful changes in a plant's physiology. A key factor is that abiotic diseases do not

spread from an infected plant to a healthy plant, the same way biotic organisms can (Trench *et al.*, 1992; Murray *et al.*, 1998).

Trench *et al.* (1992) stated that an infectious disease results from a pathogen infecting a plant. The severity of a disease depends on three factors; the susceptibility of the plant or crop, the pathogen and the environment. The severity of the disease depends on the degree to which these factors overlap (Murray *et al.*, 1998).

2.8.1 Disease cycle

The understanding of a disease cycle or commonly referred to as life cycle, is necessary to effectively manage and control disease. A typical disease cycle caused by a transmissible pathogen is divided into several stages. Most plant diseases have the following stages: production of inoculum, dissemination, penetration, infection, colonisation and survival (Murray *et al.*, 1998).

The first stage of the disease cycle involves the production of inoculum. Inoculum is any part of a pathogen or the entire pathogen that is able to infect plants. There are different types of inoculum produced by specific pathogens, e.g. urediniospores and teliospores in rust fungi (Trench *et al.*, 1992; Murray *et al.*, 1998). The second stage is dissemination, which is the transportation or spread of inoculum from the location of production to the plant. Vectors that aid the spread of inoculum include wind, water, insects and human activities (Murray *et al.*, 1998). Due to modern agriculture and goals set for economic viability, farmers plant a single cultivar over a large area that reduces cultivar diversity and increases disease incidence. In SA, farmers employ poor crop rotation systems by planting the same crop year after year, which favours disease. Crops grown under irrigation, which is a necessity in certain areas, favour the spread of disease caused by bacteria and fungi due to constant free water flow (Trench *et al.*, 1992).

The next stage is penetration, which is the primary entry of the pathogen into the plant. Penetration is followed by infection when the pathogen contacts the internal tissues and creates a parasitic relationship with the host plant. Colonisation is the second last stage in the cycle and is the active growth and

development of the pathogen within the plant. The last stage of the disease cycle is survival, a mechanism of the pathogen to survive during unfavourable environmental conditions when susceptible host plants are unavailable (Murray *et al.*, 1998). Normally the pathogen will survive on a secondary wild relative of the host plant, or residue in the soil from the previous crop, until the next growth season (Trench *et al.*, 1992; Murray *et al.*, 1998; Eckardt, 2006).

2.9 Important diseases of wheat

2.9.1 Karnal bunt

Karnal bunt, alternatively known as partial bunt is caused by *Tilletia indica* Mitra (Singh *et al.*, 1992; Murray *et al.*, 1998). Karnal bunt is a floral infecting organism that infects seed of bread wheat, durum wheat and triticale (Singh *et al.*, 1992). It is stated by Singh *et al.* (1992) that Karnal bunt may have been sighted as early as 1909 by Howard at Faizalabad, Pakistan. Karnal bunt was first identified in 1930/1931 near the north Indian city of Karnal and named accordingly. Since its identification it has spread to northwest India, northern Pakistan, parts of Nepal, Iraq and Mexico. An epidemic in northern India in 1970 elevated the status of the disease from minor to noteworthy (Singh *et al.*, 1992; Murray *et al.*, 1998). During 1996, this disease was discovered in the southwestern parts of the United States (Murray *et al.*, 1998). Karnal bunt was identified in SA in 2000. Karnal bunt is important due to the strict international quarantine status of the disease (www.nda.agric.za/publications, 2001). Losses in terms of this disease are relatively minor regarding grain yield but significant in reduction of flower quality (Singh *et al.*, 1992; Murray *et al.*, 1998).

2.9.2 Loose smut

Loose smut is one of the most distinct and obvious wheat diseases. It occurs throughout areas of the world where wheat is grown. Loose smut is a seed borne fungal wheat disease caused by *Ustilago tritici* (Pers.) Rostr. This disease converts the flowering spikes of wheat into a dense black mass of spores. Yield losses from loose smut are normally proportional to incidence of infected spikes (Knox *et al.*, 2002). Complete yield loss does not occur and is commonly about 15%, although severe cases of yield loss of 27% have been reported. The loose smut fungus survives winters as a dormant fungal thread inside the embryo of

wheat seed. When infected seed germinate, the dormant pathogen is activated and extends toward the growing point of the plant (Trench *et al.*, 1992; Curtis *et al.*, 2002). Loose smut is visible from flowering onwards when the plant begins to form the head. The fungus infects all of the young spike tissue except for that of the rachis (backbone) (Trench *et al.*, 1992; Curtis *et al.*, 2002). The fungus produces plant growth hormones which results in infected plant heads flowering earlier than healthy heads. An infected head contains black spore masses in place of the seeds. The spores which are loosely held together are spread by wind onto neighbouring healthy plants. Due to infected heads flowering earlier than healthy heads, production and release of spores occur at the opportune moment when the rest of the crop is flowering. Spores are blown by wind into flowers of the healthy plants and enter the ovaries and become part of the developing grain. This is how a new cycle of infected seed is produced for the following year (Curtis *et al.*, 2002).

2.9.3 Common bunt

Common bunt is caused by the two closely related fungi namely *Tilletia tritici* (Berk.) Wint (Dromph and Borgen, 2001) and *Tilletia laevis* Kühn (Curtis *et al.*, 2002). Common bunt is alternatively known as stinking smut (Trench *et al.*, 1992; Curtis *et al.*, 2002) or hill bunt in different areas of the world. There are no obvious symptoms of infection until the grain heads fully emerge; a common symptom is stunted growth of an infected plant (Trench *et al.*, 1992; Curtis *et al.*, 2002). The kernels of the infected heads are replaced with smut balls filled with dark spores. At maturity, infected spikes may appear lighter in colour than normal. Glumes on the infected heads are spread wide which exposes the plump smut balls. When the smut ball is crushed in some manner, it has a distinct foul, fishy odour. Infection occurs in two ways, either from teliospores on the seed surface or from teliospores within the soil close to the vicinity of seed (Curtis *et al.*, 2002). Teliospores in the soil of the field remains viable for approximately two years but teliospores on or within infected seed can be viable for many years (Dromph and Borgen, 2001; Curtis *et al.*, 2002). Yield losses as a result of common bunt infection can be high under high inoculum pressure and ideal infection conditions. Reduction in grain quality results in high yield

losses, due to grain after infection not being of the correct standard for certain products (Curtis *et al.*, 2002).

2.9.4 *Fusarium head blight*

Fusarium head blight (FHB) is a major disease of wheat that is a deterrent to wheat production worldwide. FHB is caused mainly by *Fusarium graminearum* Schwabe. in North America and in cooler areas of Europe, by *F. culmorum* (Wm. G. Sm.) Sacc. (Murray *et al.*, 1998; Somers *et al.*, 2005).

FHB of wheat was first noted in SA in 1980. The main species that cause FHB in SA are *F. graminearum*, *F. culmorum* and *F. crookwellense*. *Fusarium graminearum* and *F. culmorum* are associated with warmer climates and *F. crookwellense* with cooler climates of the country. In SA, FHB spreads in localised specific regions, e.g. regular outbreaks occur on wheat grown under irrigation (Trench *et al.*, 1992; Kriel and Pretorius, 2006).

FHB occurs predominantly in the warm, humid conditions of KwaZulu-Natal (Trench *et al.*, 1992), especially under overhead irrigation (Trench *et al.*, 1992; Murray *et al.*, 1998). In the past some outbreaks of FHB have occurred in the southern parts of the Cape Province and eastern Free State (Trench *et al.*, 1992). As reported by Kriel and Pretorius (2006), regular epidemics of FHB have occurred during 1985, 1986, 1994 and 2000. FHB can cause yield loss up to 70% under favourable conditions and high inoculum pressure (Kriel and Pretorius, 2006).

2.9.5 *Black chaff*

Black chaff is caused by *Xanthomonas campestris* pv. *translucens* and is alternatively known as bacterial stripe or bacterial leaf streak. This bacterium is distributed worldwide in major cereal growing regions on all small grain crops with oats being the exception. This disease is most observed in sub-tropical and tropical climates that have a high rainfall or where overhead irrigation is used during the growing season. The primary source of inoculum of black chaff is infected seed. Economically, black chaff reduces grain yield up to 40%, as a result of smaller kernel size (Murray *et al.*, 1998).

2.9.6 Mildew

Powdery mildew is a common disease amongst cereals, occurring in all areas where important cereal crops are grown. Mildew is caused by the following fungi on the different crops: *Erysiphe (Blumeria) graminis* f. sp. *tritici* (wheat), *E. (Blumeria) graminis* f. sp. *hordei* (barley), *E. (Blumeria) graminis* f. sp. *avenae* (oats) and *E. (Blumeria) graminis* f. sp. *secalis* (rye). Yield losses due to mildew disease vary from 20% to 25%, depending on the region (Murray *et al.*, 1998).

2.9.7 Glume blotch

Glume blotch is caused by *Phaeosphaeria nodorum* and is a seed borne disease which can survive for up to 12 months in stubble. Spores are spread over short distances by splashing water. In SA it is considered a major disease with limited occurrence. The optimal conditions for this disease are moisture for 6 to 7 h and low temperatures (less than 7°C). Glume blotch occurs in the eastern and western Cape and occasionally in parts of KwaZulu-Natal and the Free State (Trench *et al.*, 1992).

2.10 Rust Pathogens

Rust fungi are obligate biotrophs that grow and reproduce on living plant tissue. There are around 5 000 species of rust fungi that cause diseases on many agriculturally important crops and other species of plants (Eckardt, 2006). The different species of rust and their relevant cereal host crop are listed in Table 2.1. The same rust species occasionally causes infection on more than one cereal crop.

The life cycles of rust fungi are extremely complex. Most life cycles involve up to five different spore producing stages. Rust fungi require two phylogenetically distinct hosts to complete their life cycles (Eckardt, 2006). Rust fungi are host specific and will develop compatible or incompatible interactions with their host plants in a gene-for-gene relationship (Eckardt, 2006; Khlestkina *et al.*, 2007). This relationship depends on whether avirulence (*Avr*) genes of the pathogen are present or not and on the corresponding resistance (*R*) genes in the host plant (Eckardt, 2006).

During infection of the host plant, fungi form specialised infection structures called haustoria. Haustoria penetrate the plant cell wall and create invaginations in the plasma membrane (Eckardt, 2006). This is the main source of nutrients for the fungus from the host cell. At this point of infection, a hypersensitive response within the host plant will normally be triggered (Eckardt, 2006; Khlestkina *et al.*, 2007). This leads to disease resistance in resistant hosts when the correct interaction between *Avr* factors of the pathogen and the *R* gene products of the host exists (Eckardt, 2006).

Table 2.1 Diseases caused by *Puccinia* spp. on cereals (Singh *et al.*, 1992)

Crop	Disease	Pathogen
Wheat (<i>Triticum</i> spp.)	Black (stem) rust	<i>Puccinia graminis</i> Pers. f. sp. <i>tritici</i> Eriks.
	Brown (leaf) rust	<i>P. triticina</i> Eriks.
	Yellow (stripe) rust	<i>P. striiformis</i> West. f. sp. <i>tritici</i> Eriks.
Barley (<i>Hordeum vulgare</i>)	Black rust	<i>P. graminis</i> Pers. f. sp. <i>tritici</i> Eriks.
	Leaf rust	<i>P. hordei</i> Otth
	Yellow rust	<i>P. striiformis</i> West. f. sp. <i>hordei</i>
Oat (<i>Avena sativa</i>)	Black rust	<i>P. graminis</i> Pers. f. sp. <i>avenae</i> Eriks.
	Crown rust	<i>P. coronata</i> Cda. f. sp. <i>avenae</i> Fraser & Ledingham
Rye (<i>Secale cereale</i>)	Stem rust	<i>P. graminis</i> Pers. f. sp. <i>secalis</i>
	Brown rust	<i>P. recondita</i> Rob. ex Desm. f. sp. <i>secalis</i>

2.10.1 Wheat rusts

Wheat rusts are important foliar diseases of wheat worldwide, causing extensive losses and damage to the wheat industry (Singh *et al.*, 1992). There are three types of wheat rusts, namely leaf, stripe and stem rust. Rust fungi all produce similar disease symptoms on their host plants and mostly have similar optimal conditions for infection (Marsalis and Goldberg, 2006).

Wheat rust pathogens belong to the genus *Puccinia*, family Pucciniaceae, order Uredinales and class Basidiomycetes. These rust fungi are specialised plant pathogens with narrow host ranges and are host specific (Curtis *et al.*, 2002; Singh *et al.*, 2002).

2.10.2 Leaf rust

Leaf rust (Lr) is the most common of the three wheat rust types. Leaf rust is additionally known as brown rust and is caused by *P. triticina* Eriks (Mesterházy *et al.*, 2000; Curtis *et al.*, 2002; Singh *et al.*, 2002; Singh *et al.*, 2005) and is an important disease worldwide (Mesterházy *et al.*, 2000; Singh *et al.*, 2005; Kuraparthi *et al.*, 2007). This leaf rust was first separated from similar rust on rye by Eriksson in 1894.

Characteristics

Leaf rust primarily occurs on wheat (Scott, 1990; Murray *et al.*, 1998). This pathogen produces both urediniospores and teliospores on the primary host (Scott, 1990). The pathogen survives on alternate hosts when conditions are not optimal (Singh *et al.*, 2002).

Leaf rust is characterised by orange-red pustules that develop on the upper surfaces of the leaves and even the leaf sheath (Figure 2.2). The urediniospores occur within the pustules. Leaf rust generally has a relatively low urediniospore output compared to stem rust (Scott, 1990; Singh *et al.*, 2002). Leaves of susceptible cultivars become brown and necrotic as the disease develops. On such leaves, many tiny black spots containing teliospores are visible on the abaxial surface (Scott, 1990).

Optimal conditions for leaf rust development are temperatures ranging from 10°C to 30°C, with at least 6 h of moisture, dew or soft rain (Scott, 1990; Curtis *et al.*, 2002; Singh *et al.*, 2002). Under ideal conditions, new generations of spores can be produced every 7 to 10 days. Under favourable conditions rust infection takes 6 to 8 h hours to reach completion (Marsalis and Goldberg, 2006).

Hosts

Puccinia triticina is mainly a pathogen of wheat (*T. aestivum*) and its immediate ancestors. Recent studies reported that the main alternate host of *P. triticina* is *T. speciosissimum* which appears to produce little direct inoculum, however may be a mechanism for genetic exchange between different races and populations in certain regions (Curtis *et al.*, 2002; Singh *et al.*, 2002).



Figure 2.2 Leaf rust (*P. triticina*) symptoms on a wheat leaf (ZA Pretorius).

Life cycle

Puccinia triticina survives between seasons (summer to winter to spring) and wheat crops via what is referred to as the green bridge, which normally is volunteer (self-sown) wheat or wild wheat relatives. Urediniospores of the leaf rust pathogen have the ability to travel long distances by wind, from one region to another. The formation of more and more urediniospores is the continual asexual cycle on the wheat crop. Shortly after development, teliospores can germinate in the presence of moisture to produce basidiospores which can infect the alternate hosts. After sexual recombination on the alternate host, aeciospores are produced that infect the wheat host plant. (Curtis *et al.*, 2002; Singh *et al.*, 2002).

Urediniospores initiate germination just 30 min after coming into contact with water (dew drops or rain), at an optimal temperature range of 15° to 25°C. A germ tube is formed which grows along the surface of the leaf in search of a stomata, initiating the internal infection (Curtis *et al.*, 2002; Singh *et al.*, 2002).

Economic importance

Leaf rust reduces grain yield and quality as a result of reduced floral set and grain shrivelling. In highly susceptible genotypes entire plants can be killed by early epidemics. Losses due to leaf rust damage are normally below 10% but can at times be as severe as 30% (Trench *et al.*, 1992; Boshoff *et al.*, 2002; Curtis *et al.*, 2002; Singh *et al.*, 2002).

Epidemics

In SA during the past years, leaf rust epidemics have occurred in the Swartland, eastern Cape areas and on wheat grown under irrigation in areas of KwaZulu-Natal (Trench *et al.*, 1992).

Virulence

Virulence is the ability of a pathogen to overcome a specific gene for resistance (Ezzahiri *et al.*, 1992; Singh *et al.*, 2002). As stated by Kuraparthi *et al.* (2007) there are more than 50 *Lr* genes documented. Virulence for a number of *Lr* genes singly and in combination exists. There is a continual battle between pathogen evolution and the wheat plant for survival (Ezzahiri *et al.*, 1992; Singh *et al.*, 2002). Mesterházy *et al.* (2000) reported that in Europe resistance genes *Lr9* and *Lr19* remained most effective, virulence for *Lr24*, *Lr25* and *Lr28* were rare and these genes were widely effective in most parts of Europe. *Lr24* is ineffective in SA, North and South America (Mesterházy *et al.*, 2000) and Australia while the *Lr19* gene remains effective in SA and China (Xing *et al.*, 2007). Virulence of rust races against resistance genes necessitates a continual search for new sources of resistance to be used in resistant cultivar development (Kuraparthi *et al.*, 2007). Each season it is vital to carry out pathogen surveys in specific wheat growing areas to be able to establish what pathogen races are present and what type of virulence exists. Genetic

recombination of a rust pathogen can occur on occasions during a single wheat season (Ezzahiri *et al.*, 1992; Singh *et al.*, 2002).

Historically there have been a few examples of durable resistant cultivars. These include Americano 25, Americano 44d, Surpreza, Frontana and Fronteira. Generally the agronomical life span of any resistant cultivar is five years or longer if a continual breeding programme exists (Curtis *et al.*, 2002). For more durable, long lasting resistance to leaf rust or any other rust, many effective resistance genes should be used in one cultivar. This is the goal of breeders globally and in SA (Scott, 1990).

2.10.3 Stem rust

Stem rust (Sr), also known as black rust, is caused by *P. graminis* Pers. f. sp. *tritici* Eriks. & Henn. (Curtis *et al.*, 2002; Singh *et al.*, 2002; 2006). Stem rust was first independently documented and reported by Italian scientists, Fontana and Tozzetti in 1767. In 1797 stem rust was officially named *P. graminis* by Persoon (Singh *et al.*, 2006). Stem rust is feared in most wheat growing regions due to its ability to turn a good healthy crop into nothing but black broken stems before harvest (Singh *et al.*, 2002; 2006). Historically stem rust has caused severe losses to wheat production globally (Singh *et al.*, 2006).

Characteristics

Stem rust is found mainly on the stems, but at times on leaves, sheaths, glumes and seeds (Marsalis and Goldberg, 2006). Raised, long and narrow, orange-red pustules occur in early stages of the disease on the stems and leaves of susceptible cultivars (Figure 2.3). With the termination of the disease, black sooty teliospores are formed and the bursting pustules take on a black colour (Scott, 1990; Marsalis and Goldberg, 2006).

Humid conditions and warmer temperatures of 15°C to 35°C are favoured. Stem rust requires a longer dew period of about 6 to 8 h, compared to leaf rust. Infection is completed in 8 to 12 h (Curtis *et al.*, 2002; Singh *et al.*, 2002).



Figure 2.3 Stem rust (*P. graminis* f. sp. *tritici*) symptoms on the stem of a wheat plant (ZA Pretorius).

Stem rust has a high output of urediniospores per day (Singh *et al.*, 2002). Urediniospores of stem rust can remain viable for long periods and are carried long distances by winds (Curtis *et al.*, 2002; Singh *et al.*, 2002). Wind is a great spreading agent of stem rust and causes concern as to how easy and far stem rust can spread. Long distance transport of urediniospores occurs annually and distances of 800 km across the North American Great Plains have been reported. Most years it has been found that stem rust spores can travel the 2 000 km from Australia to New Zealand. A few times in the last 75 to 100 years spores have travelled the 8 000 km from east Africa to Australia (Singh *et al.*, 2002; Kolmer, 2005).

Hosts

The primary host plants for stem rust are *Triticum aestivum*, *T. turgidum* and triticale. The main secondary host that occurs in nature throughout Europe is *Berberis vulgaris* L. (Curtis *et al.*, 2002).

Life cycle

The life cycle of *P. graminis* f. sp. *tritici* mostly consists of continual uredinial generations. Stem rust fungi spread via airborne spores in the form of urediniospores from one wheat plant to another and from field to field. Initial inoculum to start the infection process may be local from volunteer infected plants or an inoculum source from urediniospores that have travelled long distances (Curtis *et al.*, 2002; Singh *et al.*, 2002).

Uredinospore germination starts 1 to 3 h after contact with free water at optimal temperatures which is similar to leaf rust. In the case of stem rust moisture must last 6 to 8 h at favourable temperatures for the spores to germinate and produce a germtube. After germtube formation the development of an appressorium takes place and the host is penetrated (Curtis *et al.*, 2002; Singh *et al.*, 2002).

Economic importance

Stem rust is the most devastating of the rust diseases. It can cause losses of up to 50% on susceptible cultivars in a single season when conditions are favourable (Scott, 1990; Curtis *et al.*, 2002). Losses of 100% are possible on some susceptible cultivars (Curtis *et al.*, 2002).

Epidemics

In the past there have been a number of major stem rust epidemics in North America, namely in 1904, 1916 and the 1950's. These epidemics led to the understanding that there are different stem rust races that vary in the ability to infect different wheat varieties (Singh *et al.*, 2006). Other epidemics that occurred were in Australia in the 1940s on Eureka which contains *Sr6*, on Lee (*Sr9g*, *Sr11* and *Sr16*), Langdon (*Sr9e*, +) and Yuma (*Sr9e*, +) in the United States in the 1950s (Curtis *et al.*, 2002). Until recently stem rust was under control worldwide (Curtis *et al.*, 2002; Singh *et al.*, 2002). A new virulent stem rust race has recently been reported in central Africa (Kolmer, 2005). This particular race known as Ug99 (TTKS) could be a major threat to global wheat industries. Ug99 contains virulence for most resistance genes used extensively in breeding programmes and existing resistant cultivars around the world (Singh

et al., 2006). In the past 20 years there have been no major stem rust epidemics in SA. Breeders, researchers and pathologists should be aware of the potential danger and threat a new virulent race of stem rust could pose (Scott, 1990).

Ug99 (TTKS)

The new stem rust race Ug99 was first identified in Uganda during 1999 and named accordingly (Pretorius *et al.*, 2000). Since its first identification, Ug99 has been renamed to TTKS by Wanyera *et al.*, using the North American nomenclature system as reported by Singh *et al.* (2006). Ug99 is virulent for a number of key resistance genes used in breeding programmes around the world. Amongst others Ug99 shows virulence against *Sr31* and *Sr38 genes* (Singh *et al.*, 2006).

Ug99 migration is slowly taking place. In 2003 this new stem rust race was detected in Ethiopia. Recent reports suggest that Ug99 is well established and spreading in the eastern African highlands. This is a reason for concern when considering the fact that normally the east African highlands are considered a “hot spot” for evolution and formation of new rust races. Pathogen population build up is favoured in the east African highlands region due to optimal environmental conditions and the availability of potential host plants all year round (Singh *et al.*, 2006). Recent reports have stated that the Ug99 race has been detected in Yemen across the Red Sea. There is also some evidence that the Ug99 race has spread into Sudan (Anonymous, 2007).

The major concern of breeders, farmers and pathologists is that a significant quantity of world wheat germplasm is potentially at risk and susceptible to race Ug99. It has been predicted based on numerous models, climatic conditions and with large wheat production areas in mind, that Ug99 might follow a similar path to the progressive appearance of *Yr9* virulence during 1986 to 1998. Ug99 is expected to move through Africa across the Red Sea into Asia via step wise migration and aided by natural wind flow. If an epidemic does result from Ug99, losses will affect farming communities and food sources. Furthermore, agricultural markets and global wheat prices could be drastically affected. Stem

rust disease, which has been controlled for decades through successful genetic resistance, could once again become the reason for food shortages and famine in Africa, the Middle East and Asia, if Ug99 is left unchecked (Singh *et al.*, 2006).

Virulence

There are close to 50 different stem rust resistance genes catalogued and identified (Khan *et al.*, 2005; Singh *et al.*, 2006). Several of these resistance genes were derived from alien relatives of wheat. Except for one gene (*Sr2*), all of the 50 *Sr* genes are race-specific. *Sr2* provides a slow rusting resistance response to an adult plant (Singh *et al.*, 2006). Virulence exists in SA against *Sr24* and in Australia against *Sr27*. There is no record so far of virulence against *Sr26* even though it has been used extensively in Australian cultivar development (Pretorius *et al.*, 2000; Singh *et al.*, 2002). The following *Sr* genes are considered ineffective: 5, 6, 7a, 8a, 9a, 9b, 9d, 9e, 9f, 9g, 10, 11, 12, 15, 16, 17, 18, 19, 20, 21, 23, 30, 31, 34, 38, 41, 42 and *wld-1*. *Sr* genes that have remained effective against stem rust are: 2, 13, 14, 22, 24, 25, 26, 27, 28, 29, 32, 33, 35, 36, 37, 39, 40, 43, 44, 45 and 1A.1R (Singh *et al.*, 2006). Thatcher and Hope cultivars in the past have been successful sources of resistance against stem rust (Curtis *et al.*, 2002).

2.10.4 Stripe rust

Stripe rust, alternatively known as yellow rust (Yr), is caused by *P. striiformis* Westend. f. sp. *tritici* Eriks. (Ma *et al.*, 2001; Boshoff *et al.*, 2002; Curtis *et al.*, 2002; Singh *et al.*, 2002; Smith *et al.*, 2002; Lin and Chen, 2007). Stripe rust was first described by Gadd and Bjerkander in 1777 (Curtis *et al.*, 2002) and is considered a major foliar disease that causes considerable losses to wheat production worldwide (Ma *et al.*, 2001; Smith *et al.*, 2002; Lin and Chen, 2007). Stripe rust has a lower optimal temperature for development in comparison to the other two rust species, limiting it as major disease to specific localised regions. This rust is an important disease on wheat grown during winter or early spring or at high altitudes (Curtis *et al.*, 2002).

Characteristics

Stripe rust has a distinguishable characteristic of producing light yellow, straight-sided pustules that occur in stripes between the veins on the leaves and occasionally on the heads (Figure 2.4). As the pustules reach maturity, orange-yellow spores are produced. As the disease progresses the plant tissue around the infection area goes brown and looks scorched (Murray *et al.*, 1998; Marsalis and Goldberg, 2006). Towards the end of the season, black telia form within necrotic tissue that was killed by the orange-yellow rust pustules (Murray *et al.*, 1998). During telial development, narrow black stripes are formed on the surface of the leaf (Singh *et al.*, 2002).



Figure 2.4 Stripe rust symptoms (*P. striiformis*. f. sp. *tritici*) on the leaf of a wheat cultivar (ZA Pretorius).

Stripe rust has the potential to be as devastating as stem rust but is limited to cooler climates (Boukhatem *et al.*, 2002; Singh *et al.*, 2002). It usually occurs in the cold of winter or early spring, with temperatures ranging between 0°C and 15°C (Singh *et al.*, 2002). The temperature range for stripe rust infection is as follows; minimum of 0°C, optimal of 11°C and maximum of 23°C (Curtis *et al.*, 2002). Stripe rust occurs frequently in the northern parts of Europe, Mediterranean region, Middle East, western United States, Australia, east

African highlands, regions of China, India, New Zealand and SA as well as the Andean regions of South America. This rust type is considered important in tropical regions with higher altitudes such as countries of North Africa, the Indian Himalayan foothills, Pakistan and Mexico (Boshoff *et al.*, 2002). The urediniospores of stripe rust are sensitive to ultraviolet light, which renders them unsuitable for travel by wind over long distances (Curtis *et al.*, 2002; Singh *et al.*, 2002).

Hosts

Triticum spp. are the major hosts of stripe rust. Stripe rust is the only one of the three rusts of wheat to consistently spread past the initial infection point within the plant (Curtis *et al.*, 2002).

Life cycle

Puccinia striiformis is a pathogen with a life cycle that seems to only consist of the uredinial and telial stages. Urediniospores of stripe rust are the only known form of inoculum for wheat, which germinate and infect at a low temperature range. Pathogen populations of stripe rust can exist, undergo change in virulence, possibly mutate and result in epidemics, all independent of an alternate host (Curtis *et al.*, 2002; Singh *et al.*, 2002).

Epidemics

Epidemics occurred in Wales and England in 1978, 1981 and 1988 (Murray *et al.*, 1998). Only as recently as 11 years ago in 1996, stripe rust was first discovered in SA (Kolmer, 2005). Boshoff *et al.* (2002) reported that stripe rust was first observed in the Western Cape, near Moorreesburg, during August of 1996. After the first observation of stripe rust in 1996, it spread through most of the important wheat growing regions of SA (Boshoff *et al.*, 2002; Moldenhauer *et al.*, 2006). It is thought that the introduction of stripe rust into Australia was as a result of human air travel. It has been reported that some stripe rust airborne spores travelled the 2 000 km distance between New Zealand and Australia, although on average this is considered a rare event (Curtis *et al.*, 2002).

Economic importance

Losses due to stripe rust can be severe, 50% or more, due to shrivelled grain and damaged tillers (Boukhatem *et al.*, 2002; Singh *et al.*, 2002). Losses of up to 40% on susceptible cultivars have been reported at times during epidemic years (Murray *et al.*, 1998; Khan *et al.*, 2005). In Europe stripe rust is one of the most important and widely distributed rust pathogens on susceptible wheat crops. Severe outbreaks of the disease have resulted in 50% to 60% losses in the past (Moldenhauer *et al.*, 2006). Boshoff *et al.* (2002) stated that the stripe rust epidemic of 1996 in the Western Cape of SA resulted in producers spending R28 million on fungicides to treat the disease.

Virulence

To date there are 35 stripe rust resistance genes listed and designated (Kuraparthi *et al.*, 2007). In the global sense, there is a probability that virulence already exists for most of the *Yr* genes (Curtis *et al.*, 2002; Singh *et al.*, 2002). Virulence for the following *Yr* genes occurs in SA: *Yr2*, *Yr6*, *Yr7*, *Yr8*, *Yr17*, *Yr19* and *Yr25*, as reported by Boshoff *et al.* (2002). Later Kolmer (2005) reported an additional two genes, *Yr11* and *Yr14*, against which virulence exists. Boshoff *et al.* (2002) reported that although the adult resistance genes *Yr11* and *Yr14* offered relative resistance when compared to the susceptible cultivar Avocet S, these genes would not provide sufficient crop protection. Various resistance genes are used in different combinations where no virulence has been observed or if virulence is localised (Curtis *et al.*, 2002; Singh *et al.*, 2002). In fact, virulence to adult plant resistance (APR) genes is somewhat unknown due to most virulence surveys being done at the seedling stage (Singh *et al.*, 2002). The South African cultivar Kariega has an adult stripe rust resistance response as a result of containing two major QTL, designated as *QYr.sgi-7D* and *QYr.sgi-2B.1* (Ramburan *et al.*, 2004). Examples of historically successful resistant cultivars to stripe rust include Wilhelmina, Capelle-Desprez, Manella, Juliana and Carstens VI (Curtis *et al.*, 2002).

In summary, Table 2.2 gives an in depth comparison of the characteristics of stem, leaf and stripe rust.

Table 2.2 Comparative characteristics of *Puccinia graminis* f. sp. *tritici*, *P. triticina* and *P. striiformis* f. sp. *tritici* of wheat (Singh et al., 1992)

Differentiating characters	Stem rust (<i>Puccinia graminis</i> f. sp. <i>tritici</i>)	Leaf rust (<i>Puccinia triticina</i>)	Stripe rust (<i>Puccinia striiformis</i> f. sp. <i>tritici</i>)
Optimal temperature	25°C	20°C	10-15°C
Severity	More severe on stalks than on leaf sheaths, leaves and ears	Infects leaves exclusively, rare on leaf sheaths and stalks	Infects leaves and when severe, leaf sheaths, stalks, and ears
Uredinia	Large elongated, dark brown, bursting early, throwing epidermal fringes in the process	Small, oval, light brown, do not run together burst early with mild displacement of epidermis, found chiefly on upper surface of leaves	Arranged in rows of lemon yellow coloured sori, epidermal rupture not visible
Uredinospores	Brown, oval, thick wall, tiny spines, 25-30 x 17-20 µm having 4 germ pores at equatorial plane	Brown, spherical, minutely echinulated, 16-28 µm, 7-10 germ pores dispersed all over	Spherical to oval, spore wall colourless, contains yellow oil globule, minute echination 23-35 x 20-25 µm, 6-16 germ pores dispersed all over
Telia	Black, found on all aerial parts, mainly stem	Pustules do not burst epidermis, found chiefly on the leaf surface	Sori are flattened and dull black, chiefly on the lower surface and do not burst open easily
Teliospores	40-46 x 15-20 µm	35-63 x 12-20 µm	35-63 x 12-20 µm

2.10.5 Managing rust

Field scouting, or the continually monitoring of regions for infection, is vital to effective rust control (Kolmer, 2005; Marsalis and Goldberg, 2006). The monitoring process is important worldwide to allow wheat pathologists, breeders and scientists to prepare and anticipate the possible occurrence of new rust races (Kolmer, 2005). Preparations for the season ahead of time and based on historical data should help in rust control. Depending on temperatures for that

particular season, be it a mild winter perhaps, rust infection can persist into early spring. Bearing in mind, the worst infection time is when the flag leaf gets infected. This results in the highest damage if untreated. Flag leaf infection often results in the necessity for fungicide treatment; this is vital especially if warmer, wet weather is forecasted. A problem associated with chemical control is that it is expensive and carries environmental risks. There are several types of fungicides and depending on the chosen product, the price will differ. Every year millions of dollars are spent worldwide on fungicide treatment, not always with success (Marsalis and Goldberg, 2006).

According to Boshoff *et al.* (2002), R28 million was spent on fungicides to chemically control the stripe rust epidemic of 1996 in the Western Cape of SA. Spraying too early or too late will result in inadequate disease control, resulting in large losses in profits, increased costs and loss of time. Most of the time fungicides offer two to three weeks protection against infection. It is vital to remember that fungicides are not a cure for the problem but merely a preventive measure (Marsalis and Goldberg, 2006). Murray *et al.* (1998) reported that chemical control in Europe is generally practiced in areas where high-yielding susceptible wheat varieties are grown.

A cultural method of rust control involves the removal of volunteer wheat or cereals around fields, which tends to harbour inoculum during winter. Good disposal of crop debris after the harvesting period is important in dealing with inoculum build up (Murray *et al.*, 1998).

The main mechanism used to control wheat rusts or other cereal rust for that matter, is through the use of resistant cultivars. Resistance breeding has been around for years and there are a number of historically developed resistant cultivars (Singh *et al.*, 2002). Several strategies to improve resistant cultivars have been conceptualised, such as the incorporation of multiple race-specific resistance genes in the same cultivar (gene pyramiding or gene stacking) and making use of a different resistance gene carrying varieties in neighbouring wheat fields (Murray *et al.*, 1998).

2.11 Resistance genes to be used in the current study

2.11.1 *Lr19*

The *Lr19* gene occurs on an alien segment of DNA, transferred by Sharma and Knott (1966) (Prins *et al.*, 1997) from *Thinopyrum ponticum* (Prins and Marais, 1998; Prins *et al.*, 2001; Xing *et al.*, 2007) and was originally found on chromosome 7DL. *Lr19* is completely linked on the same translocation to *Sr25* (Prins *et al.*, 2001). *Lr19* is a seedling resistance gene and even though virulence to the *Lr19* gene is rare, it will likely have limited long term durability if introduced alone. Best possible durability will be obtained if it is combined with a slow rusting gene (Zhang *et al.*, 2005). Previously this gene has not been widely used in common wheat breeding programmes due to its linkage on the original translocation with the yellow pigment gene *Y* that tints flour yellow. In some countries yellow flour is not desired for bread making but the yellow pigment is desired in durum wheat for good pasta (Zhang *et al.*, 2005). In this study a CS *Lr19*-149-299 line with a modified form of the original *Lr19* translocation was used as a donor parent for *Lr19*. This line contains a shortened form of the original translocation which has been relocated to chromosome 7BL. *Sr25* and *Y* genes previously associated with *Lr19* have been removed. Recombinants of the shortened form are associated with white flour. Selection for the *Lr19* gene segment can be done using a dominant STS marker (STSLr19₁₃₀). Even though this marker is still distant from *Lr19* itself, the marker is useful in MAS programmes since the alien segment from which *Lr19* was transferred, does not normally recombine with its wheat homologue in the presence of homologous pairing gene *Ph1* (Prins *et al.*, 2001). *Lr19* still provides effective resistance against most, if not all, leaf rust races in SA and China (Xing *et al.*, 2007).

2.11.2 *Lr34*

The leaf rust resistance gene *Lr34* is non race-specific, effective at adult plant stages and has provided durable resistance to leaf rust (Schnurbusch *et al.*, 2004; Lagudah *et al.*, 2006). Lagudah *et al.* (2006) reported that *Lr34* confers APR and possibly even seedling resistance under appropriate conditions. *Lr34* is considered one of the most relevant genes in wheat rust disease resistance breeding programmes (Bossolini *et al.*, 2006). *Lr34*, known as a “slow rusting gene”, is located on chromosome 7DS and is associated with the stripe rust

gene *Yr18* and the morphological character leaf tip necrosis (Ltn) (Spielmeyer *et al.*, 2005; Bossolini *et al.*, 2006; Lagudah *et al.*, 2006). This *Lr34* locus provides durable resistance to both leaf rust and stripe rust (Bossolini *et al.*, 2006). Cultivars with *Lr34* display a longer latent period, have fewer uredina on the plant and the uredina are smaller in size (Schnurbusch *et al.*, 2004). Lagudah *et al.* (2006) reported that the STS co-dominant marker csLV34 can identify cultivars carrying the *Lr34* gene. Recently in 2007, STS marker csLV34 was used in a study to test and confirm a number of Australian cultivars containing the *Lr34* gene (Singh *et al.*, 2007).

2.11.3 *Sr2*

Sr2 is an important gene for stem rust resistance in modern wheat breeding (Spielmeyer *et al.*, 2003). At present there are several cultivars which contain *Sr2*. *Sr2* is a recessive gene, which confers a slow-rusting response, resulting in variable levels of disease development in adult plants (Sharp *et al.*, 2001; Kota *et al.*, 2006). This gene has offered durable, broad-spectrum resistance for some 50 years in the wheat breeding industry. The *Sr2* gene has been used extensively and is still being used in breeding programmes in Australia (Sharp *et al.*, 2001; Spielmeyer *et al.*, 2003; Hayden *et al.*, 2004). This gene was transferred from the tetraploid Yaroslav emmer wheat into hexaploid wheat in the 1920's (Sharp *et al.*, 2001).

The *Sr2* gene is located on chromosome arm 3BS (Hayden *et al.*, 2004; Kota *et al.*, 2006). According to Sharp *et al.* (2001) *Sr2* is linked to the leaf rust resistance gene *Lr27*. It is difficult to select for *Sr2* in breeding programmes as a result of it being a recessive gene (Sharp *et al.*, 2001; Hayden *et al.*, 2004; Kota *et al.*, 2006). The fact that *Sr2* resistance is only phenotypically visible in the adult plant stage furthermore delays progress in selection (Sharp *et al.*, 2001; Hayden *et al.*, 2004). However, two morphological markers, pseudo-black chaff (PBC) and seedling-leaf chlorosis are closely associated to the presence of *Sr2*. PBC is a pigmentation that develops on the glumes and under the stem nodes of the wheat plant, expressed by the partially dominant gene *Pbc*. The phenotype of PBC can assist in *Sr2* selection, although expression of PBC varies in different cultivar backgrounds and environments, making PBC an

unreliable marker across environments (Sharp *et al.*, 2001; Spielmeier *et al.*, 2003). Seedling-leaf chlorosis is conferred by a recessive gene (*sc*) which confers a distinct leaf chlorosis reaction to seedlings inoculated with virulent leaf or stem rust races, grown under high temperatures and in high light intensity conditions (Brown, 1997). This phenotypic marker is often used to select for the presence of *Sr2* in phenotypic screening experiments carried out in controlled greenhouse conditions (Sharp *et al.*, 2001). Hayden *et al.* (2004) reported the development of two sequence-tagged microsatellite (STM) markers (*stm559tgag* and *stm598tcac*) that were derived from microsatellite marker *Gwm533* to distinguish between *Sr2* and non-*Sr2* allelic presence in different cultivars.

2.11.4 *Sr26*

The stem rust resistance gene *Sr26* was introduced through translocation from the long arm of *Agropyron elongatum* chromosome 6A into chromosome 6A of wheat. At present there is no virulence toward *Sr26* worldwide. Eagle, an Australian cultivar, was the first cultivar to contain *Sr26*. Since then many varieties have been released containing this gene. Previously it was thought that the *Sr26* segment is associated with a yield penalty. This is no longer the case as a result of the reduction of the *Agropyron* chromosome segment (Mago *et al.*, 2005). Mago *et al.* (2005) reported the development of a STS marker *Sr26#43* to be used in MAS for the presence of *Sr26*.

2.11.5 *YrSp*

YrSp is a gene for seedling resistance to stripe rust, derived from the cultivar Spaldings Prolific. This is a dominant gene that confers an immune-type resistance, characterised by minute chlorotic patches on the leaf surface. The *YrSp* gene has been mapped to the short arm of chromosome 2B and was incorporated into Avocet S, an Australian wheat cultivar (Mathews, 2005). Two AFLP markers linked to the *YrSp* gene in Avocet S were identified by Mathews (2005).

2.11.6 Yr7D and Yr2B

Kariega, a South African cultivar, shows complete APR to stripe rust. *Yr* Kariega 7D (QYr.sgi-7D) and *Yr* Kariega 2B (QYr.sgi-2B) are the designated names given to the two major QTL found in the South African cultivar. It is thought that within these QTL there are other possible genes offering various forms of resistance. The two major QTL were mapped to chromosomes 7D and 2B, respectively. The 7D QTL represents potentially more durable resistance than the 2B QTL. It is believed that the *Lr34/Yr18* complex falls within the *Yr7D* QTL region as well (Ramburan *et al.*, 2004; Moldenhauer *et al.*, 2006).

2.12 Molecular plant breeding

In the last 50 years research has produced major advances in plant breeding, especially in the economically important crops such as maize, wheat, rice, sorghum and barley (Rajaram, 2001).

Genetic engineering and biotechnology hold great potential for plant breeding in future, as it promises to reduce the time taken to create varieties with desirable characters. With the use of molecular techniques it has become possible to accelerate the transfer of desirable traits among different cultivars and to introduce novel genes into a crop (Mohan *et al.*, 1997). Many agriculturally and economically important traits e.g. yield, quality and forms of resistance are controlled and influenced by many genes (Collard *et al.*, 2005; Francia *et al.*, 2005). These are known as quantitative traits or polygenic traits. Specific regions within the genome of an organism that harbours genes that affect a quantitative trait are referred to as QTL (Collard *et al.*, 2005).

Genetic or DNA markers generally detect a difference between individuals of a species or between different species at genomic level. These DNA markers may be used as signs or indicators of the presence of desired regions of DNA in MAS (Collard *et al.*, 2005). Marker types can be classified as co-dominant or dominant. A co-dominant marker can distinguish between homozygous and heterozygous individuals (Mohan *et al.*, 1997).

DNA markers have been used extensively in many crops to do genome mapping and to construct linkage maps. Linkage maps provide information on chromosomal regions that contain major genes and QTL (Collard *et al.*, 2005). Molecular markers have been used extensively in the development of detailed genetic and physical chromosome maps in a variety of organisms. Another important application of molecular markers in plant species has involved targeted improvement in efficiency of conventional breeding by indirect selection using markers linked to the desired traits (Gupta *et al.*, 1999).

The usefulness of a marker depends on the level of polymorphism (differences) detected between different individuals, its repeatability and how closely it is linked to a targeted gene. Molecular markers are mostly based on techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), AFLP, microsatellite or SSR, sequenced-tagged sites (STS), cleaved amplified polymorphic sites (CAPS) and single nucleotide polymorphisms (SNPs). All of these techniques are being used extensively to identify markers for specific traits and genes in many important crops (Mohan *et al.*, 1997; Gupta *et al.*, 1999; Haung and Röder, 2004; Francia *et al.*, 2005). RAPDs have been used in the past as a quick fingerprinting method and for diversity studies in most crops but its biggest disadvantage is a lack of repeatability. RAPDs have been put to limited use in bread wheat partly due to the low level of polymorphism (Gupta *et al.*, 1999). AFLPs are used for fingerprinting, fine mapping and marker identification in many crops. SSRs are extensively used together with AFLPs in mapping anchor markers to chromosomes and in many other approaches of MAS in a variety of crops. SNP detection is a relatively new technique that has found fairly limited application in plant genomics to date. SNPs seem to be used in diversity studies, mutational event studies and fingerprinting (Mohan *et al.*, 1997; Haung and Röder, 2004; Francia *et al.*, 2005).

2.12.1 Amplified fragment length polymorphism

The AFLP technique is a modified polymerase chain reaction (PCR)-based method whereby a specific set of restriction fragments are selectively amplified. The first step of this technique involves digestion of genomic DNA with two

different restriction enzymes, a frequent cutter and a rare cutter. A frequent cutter is considered a four base cutter and a rare cutter a six base cutter (Vos *et al.*, 1995; Blears *et al.*, 1998).

Enzyme digestion is followed by ligation of double stranded adapters. The adapter sequences correspond to the cut end of the double stranded DNA. Following ligation, a first PCR amplification is carried out. This first amplification step is often referred to as pre-selective amplification. Primers for the pre-selective amplification stage contain the known adapter-enzyme complex sequence and none or one randomly selected additional base at the 3' end. The randomly selected base reduces the number of restriction fragments amplified. A secondary amplification step is done with primers similar to the ones used in the pre-selective amplification, except that two to three additionally randomly chosen bases are included in the primer sequence. This step selectively amplifies specific fragments with the exact matching sequence, reducing the number of fragments 16 fold. Selective amplification helps with visualisation and result interpretation by reducing the thousands of fragments produced by the digestion to a workable number of selectively amplified fragments (Vos *et al.*, 1995; Blears *et al.*, 1998).

AFLP is a useful DNA fingerprinting technique that is capable of generating individual DNA profiles using different primer combinations. In combination with different targeting strategies, such as bulk segregant analysis or different populations (recombinant inbred lines (RILs), near-isogenic lines (NILs), F₂ and doubled haploid (DH) lines), AFLPs can be used to identify specific gene or trait markers. These AFLP markers can be sequenced and converted to simpler PCR markers, e.g. sequence characterised amplified region (SCAR) or STS markers (Vos *et al.*, 1995). Conversion of AFLP markers into SCAR or STS markers circumvents the disadvantages associated with AFLP analysis, namely being time-consuming, relatively difficult and expensive. It furthermore allows high-throughput analysis.

AFLP is a multi-locus technique, which makes it attractive for fingerprinting and mapping, due to large genome coverage and a high multi-plex ratio. It

furthermore produces many polymorphic fragments per primer due to its multi-locus nature (Bleas *et al.*, 1998).

2.12.2 Sequenced-tagged site

A STS is a short, unique sequence that identifies a specific target locus and is amplified by PCR. A STS marker is characterised by a set of PCR primers that are developed from the sequencing of a cloned RFLP, RAPD or AFLP fragment linked to a specific trait. STS primers amplify specific, targeted DNA areas and therefore have a locus specific nature. STS markers are useful for the detection of specific desired genes/QTL (Gupta *et al.*, 1999). In the last five years, a number of STS markers have been developed in wheat, including STS markers for the *Lr19* (Prins *et al.*, 2001), *Sr26* (Mago *et al.*, 2005) and *Lr34* (Lagudah *et al.*, 2006) resistance genes. These markers can be employed in MAS by researchers and breeders interested in transferring or selecting these genes.

2.12.3 Microsatellites or simple sequence repeats

Microsatellites or SSRs are a unique class of repetitive DNA sequences that are highly polymorphic and abundant throughout the genomes of eukaryotes (Powell *et al.*, 1996; Varshney *et al.*, 2000; Hayden and Sharp, 2001; Rakoczy-Trojanowska and Bolibok, 2004). Microsatellites have been used extensively in genome mapping of a number of animal, insect and plant species (Varshney *et al.*, 2000). A microsatellite region is generally a repeat motif of di-, tri-, tetra- or penta-nucleotides (Powell *et al.*, 1996) that occur randomly throughout the genome (Powell *et al.*, 1996; Fahima *et al.*, 1998; Röder *et al.*, 1998; Hayden and Sharp, 2001). A microsatellite region is normally flanked by highly conserved sequences of DNA (Powell *et al.*, 1996; Rakoczy-Trojanowska and Bolibok, 2004). The length of the microsatellite region is dependent on the number of motif repeats between the conserved flanking regions. These repeat units have high mutation rates, primarily due to DNA slippage within these repetitive regions which are highly polymorphic and can be used to distinguish between closely related individuals (Powell *et al.*, 1996; Fahima *et al.*, 1998). The flanking regions define the microsatellite region and primers are normally developed based on the sequence of the flanking regions (Powell *et al.*, 1996). Microsatellite regions are sources of ubiquitous informative genetic markers

throughout the genome. Hayden *et al.* (2001) reported that SSRs revealed a higher incidence of polymorphisms than other forms of genetic markers, which makes SSRs ideal for a self-pollinating crop such as wheat. SSR markers have become the DNA marker of choice for application in wheat research (Varshney *et al.*, 2000). Since SSR markers are multi-allelic and mostly co-dominant in nature, it makes them ideal for use in evolutionary studies, genetic relationship studies, DNA fingerprinting, genetic mapping and MAS. Most SSRs are locus specific and often chromosome specific (Powell *et al.*, 1996; Röder *et al.*, 1998; Hayden and Sharp, 2001; Hayden *et al.*, 2001; Gupta *et al.*, 2002) making SSRs ideal anchoring markers to specific chromosomes (Röder *et al.*, 1998).

Microsatellite analysis is a PCR-based technique (Röder *et al.*, 1998; Gupta *et al.*, 2002; Somers *et al.*, 2004) which makes it a rapid and reliable technique that can be used between laboratories and for high-throughput analysis (Röder *et al.*, 1998; Somers *et al.*, 2004). Development of primers for microsatellite analysis is initially complex, time-consuming, labour intensive and costly (Röder *et al.*, 1998; Hayden *et al.*, 2001). Conventional development of locus-specific microsatellite markers requires the isolation, identification and characterisation of individual loci within the genome. This process involves the construction and screening of DNA libraries with microsatellite specific targeting probes, followed by DNA sequencing of positively identified clones and subsequent PCR primer synthesis (Hayden and Sharp., 2001; Hayden *et al.*, 2001; Rakoczy-Trojanowska and Bolibok, 2004). Another approach used to generate SSR markers, namely selectively amplified microsatellite (SAM) analysis, has proven to be a different way to generate locus-specific SSR markers for genomic regions of interest (Hayden and Sharp, 2001; Hayden *et al.*, 2001). However, once microsatellite markers have been developed, their application in breeding programmes for MAS is effective (Röder *et al.*, 1998).

2.12.4 Application of SSR markers in wheat

Due to the hexaploid nature and relatively large genome size of wheat, development of SSR markers is time-consuming and expensive. The fact that the majority of SSR markers are co-dominant and detect high levels of

polymorphism, is an important advantage for crops with a large genome such as wheat (Röder *et al.*, 1998; Somers *et al.*, 2004; Sourdille *et al.*, 2004).

SSR map of wheat

The development of genetic maps of many crop species such as wheat has increased progressively since the 1980s (Somers *et al.*, 2004). Genetic maps of wheat were initially constructed using RFLP markers. Since the discovery of PCR by Mullis, PCR-based markers have become the markers of choice for genetic map construction (Somers *et al.*, 2004; Sourdille *et al.*, 2004). PCR markers that have been used for mapping include RAPDs, AFLPs and SSRs. The potential use of markers identified from mapping studies in plant breeding was one of the primary reasons to switch to PCR-based markers such as SSRs. Traditional plant breeding requires the analysis of thousands of plants in the shortest possible time and at the lowest cost. SSRs and high-throughput modern electrophoresis equipment give a good base for directed use of MAS within breeding programmes. However, the MAS process can only be as efficient as the availability and reliability of relevant markers. Molecular breeding therefore requires high density molecular maps of wheat to function optimally (Somers *et al.*, 2004). The first SSR map in wheat was constructed by Röder *et al.* (1998) using 279 SSRs. Since then a number of studies have been done using different targeting strategies and types of populations to acquire a higher density SSR map in wheat (Somers *et al.*, 2004; Sourdille *et al.*, 2004). In 2004 two research groups published SSR maps in wheat. Sourdille *et al.* (2004) used a deletion line strategy to construct a genetic-physical map using 725 SSRs. The other study by Somers *et al.* (2004) joined four independent genetic maps of bread wheat done on a series of populations. The later study used 1 235 SSR markers in order to construct a SSR consensus map in wheat. These two studies together with present and future studies will allow scientists a wider choice of specific markers for a targeted gene or region of a chromosome for use in a MAS breeding programme of wheat (Somers *et al.*, 2004).

2.12.5 Marker-assisted selection

MAS is an approach that involves the use of known molecular markers linked to traits of interest to aid and speed up the normal selection process in a breeding programme. Often MAS changes the selection criteria of a breeding programme as selection is no longer being based on phenotype but rather more directly towards selection of specific genes (Francia *et al.*, 2005). According to Mohan *et al.* (1997) progress in mapping and tagging a number of agriculturally important genes with molecular markers has been made, which forms the basis of MAS in crops. One of the strengths of MAS is the fact that markers are not environmentally influenced. With the increasing availability of a large number of different molecular markers, MAS can be used for selection of simple and quantitative traits (Mohan *et al.*, 1997; Röder *et al.*, 1998; Francia *et al.*, 2005). As stated by Witcombe and Hash (2000), MAS of resistance genes identified by molecular markers and QTL analysis is now considered possible in many important crops. According to Huang and Röder (2004) the application of tightly associated or co-segregating markers to specific resistance genes is important to perform MAS in wheat breeding programmes. It is possible, with the successful application of MAS, to pyramid several resistance genes into a single desired genotype (Witcombe and Hash, 2000).

MAS in a plant breeding programme ideally requires markers that co-segregate with or are closely linked (within 1 cM or less) to the gene or trait of interest. The technique used to visualise these markers should be quick, easy and economical for screening large populations. Generally PCR-based techniques are performed together with a good visualisation process (Mohan *et al.*, 1997). A careful selection process of weighing up all advantages and disadvantages of the molecular techniques, cost involved and breeding objectives of the MAS programme must be considered to select the correct technique (Gupta *et al.*, 1999).

MAS has a number of advantages for plant breeders (Collard *et al.*, 2005). With the aid of molecular markers it is now possible to select for agronomic traits of importance that previously was not possible (Mohan *et al.*, 1997). MAS could save time with the possibility of making many selections in one year or a single

growth season, depending on the crop. Plants can be analysed at the seedling stage rather than waiting until the plant is fully mature. MAS can save time for plant breeders by minimising complex field trials through substitution with the molecular marker screening tests. MAS applications can increase the reliability and efficiency compared to phenotypic screening of desired traits in field trials. MAS can furthermore be used for gene pyramiding of similar functioning target genes into new cultivars. The use of MAS in different breeding strategies, e.g. backcrossing, can help breeders prevent undesirable trait transfer to the target plant. Using the correct markers, MAS can aid breeders in transferring traits or genes that have low heritability. MAS, together with traditional breeding programmes and comprehensive crossing schemes, work hand in hand to make a normally labour intensive, time-consuming task easier and more efficient (Collard *et al.*, 2005).

2.12.6 Gene pyramiding

To fight rust disease, the use of different resistant cultivars has proven to be the most effective method of disease control (Liu *et al.*, 2000; Curtis *et al.*, 2002). A major task for modern plant breeders is to breed for durable resistance to pathogens and pests. Pyramiding of different resistance genes into one genotype is a good method to accomplish durable resistance (Liu *et al.*, 2000). Longer durability could be achieved with a combination of several partially resistant genes, or a series of singularly ineffective genes, transferred into one cultivar, to create superior resistance. This is preferred rather than using a single resistance gene (Chelkowski and Stepein, 2001). Even with that in mind, a new approach is gaining approval from breeders and scientists around the world: this approach focuses on using more slow rusting genes in breeding programmes to obtain longer durability in resistant cultivars. Markers linked to slow rusting QTL or regions known to confer a slow rusting response can be vital in the development and improvement of durability (Xu *et al.*, 2005).

Wang *et al.* (2007) reported that breeders are faced with many complex choices when designing an efficient crossing scheme and deciding on selection strategies aimed at combining a number of desired genes into a single target genotype or cultivar. Key factors that need careful consideration and planning

by a breeder when considering gene pyramiding are the number of genes being transferred, knowledge of the inheritance mechanism (dominant or recessive) of the genes to be transferred, the parental source of the desired genes, are genes in a homozygous or heterozygous state and population types and sizes to be used during each stage. All these factors will influence each other, depending on the crossing scheme and selection strategies used (Wang *et al.*, 2007; Ishii and Yonezawa, 2007a; 2007b). Gene pyramiding principals become even more complex when accumulating many genes from four or more donor parental lines (Ishii and Yonezawa, 2007a).

Ideally molecular markers tightly linked to the resistance genes of interest should be used. These markers have an important role to play during the selection process, especially in complex pyramiding schemes when more than two or three genes are involved in achieving durable and broad spectrum resistance (Liu *et al.*, 2000). Gene pyramiding for durable disease resistance without the desired markers in a traditional breeding programme would require special knowledge and availability of existing pathogen races. This would be a labour intensive, time-consuming and resource dependent approach. With the aid of MAS, this can be done without phenotypic screening (Smith *et al.*, 2002).

The development of cultivars with new and/or improved genetic resistances contributes to a reduction in economical losses, production costs and lowers environmental risk as a result of a reduction in fungicide usage. Since single gene resistant cultivars have seemed to promote the emergence of new pathogen races, pyramiding of multiple rust resistance genes should increase the viable commercial life of a cultivar (Khan *et al.*, 2005). The practical use of multigene pyramided lines as parents to develop new hybrid marketable cultivars with a number of desirable traits is potentially useful. As a larger variety of gene-pyramided lines become available, it would be potentially possible to breed selected and targeted superior market cultivars through MAS (Ishii and Yonezawa, 2007a).

2.12.7 Gene pyramiding applications

Gene pyramiding of different genes and traits using MAS is being done more and more frequently. In the last ten years, there are reported studies on successful marker-assisted pyramiding of disease resistance in wheat. Barloy *et al.* (2007) reported that in 1997 three leaf rust resistance genes, *Lr13*, *Lr34* and *37*, were successfully transferred by Kloppfers and Pretorius (1997). Liu *et al.* (2000) published the successful pyramiding of three powdery mildew resistance genes, *Pm3*, *Pm4a* and *Pm21* in wheat.

Barloy *et al.* (2007) recently reported on an attempt to pyramid two cyst nematode resistance genes that had been transferred from the wild grass species *Aegilops variabilis* into wheat. In this study the first step was the conversion of flanking RAPD markers into SCAR markers. These flanking SCAR markers for the two genes were used to carry out MAS during the study. Recombinant lines for the different genes were used as parental lines and subsequent F₁ lines and thereafter F₂ lines were screened for possible pyramiding. Gene pyramiding was confirmed in the F₃ population with desired resistance level reactions from the two transferred resistance genes.

In a recent study reported by Ashikari and Matsuoka (2006) on rice they aimed to firstly identify useful QTL, followed by gene pyramiding of a number of important QTL using MAS. All of the above examples are proof that gene pyramiding with the aid of MAS is possible and has been successful in the past. Therefore gene pyramiding can be used to pyramid several different selected target resistance genes in order to obtain durable resistance within wheat.

2.13 References

Akhunov ED, Goodyear AW, Geng S, Qi L-L, Echalier B, Gill BS, Miftahudin, Gustafson JP, Lazo G, Chao S, Anderson OD, Linkiewicz AM, Dubcovsky J, La Rota M, Sorrells ME, Zhang D, Nguyen HT, Kalavacharla V, Hossain K, Kianian SF, Peng P, Lapitan NLV, Gonzalez-Hernandez JL, Anderson JA, Choi D-W, Close TJ, Dilbirligi M, Gill KS, Walker-Simmons MK, Steber C, McGuire PE, Qualset CO and Dvorak J (2003) The organization and rate of evolution of wheat genomes are correlated with recombination rates along chromosome arms. ***Genome Research 13: 753-763.***

Anonymous (2007) Dangerous wheat disease jumps red sea. ***CIMMYT News.*** www.cimmyt.org. Cited May 2007.

Ashikari M and Matsuoka M (2006) Identification, isolation and pyramiding of quantitative trait loci for rice breeding. ***Trends in Plant Science 11: 344-350.***

Bajaj YPS (ed) (1990) Biotechnology in Agriculture and Forestry 13 – Wheat. ***Springer-Verlag pp 687.***

Barloy D, Lemoine J, Abelard P, Tanguy AM, Rivoal R and Jahier J (2007) Marker-assisted pyramiding of two cereal cyst nematode resistance genes from *Aegilops variabilis* in wheat. ***Molecular Breeding 20: 31-40.***

Blears MJ, De Grandis SA, Lee H and Trevors JT (1998) Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. ***Journal of Industrial Microbiology and Biotechnology 21: 99-114.***

Boshoff WHP, Pretorius ZA and van Niekerk BD (2002) Establishment, distribution and pathogenicity of *Puccinia striiformis* f. sp. *tritici* in South Africa. ***Plant Disease 86: 485-492.***

Bossolini E, Krattinger SG and Keller B (2006) Development of simple sequence repeat markers specific for *Lr34* resistance region of wheat using sequence information from rice and *Aegilops Tauschii*. ***Theoretical and Applied Genetics* 113: 1049-1062.**

Brown NM (1997) The inheritance and expression of leaf chlorosis associated with gene *Sr2* for adult plant resistance to wheat stem rust. ***Euphytica* 95: 67–71.**

Boukhatem N, Baret PV, Mingoet D and Jacquemin JM (2002) Quantitative trait loci for resistance against yellow rust in two wheat-derived recombinant inbred line populations. ***Theoretical and Applied Genetics* 104: 111-118.**

Chelkowski J and Stepein L (2001) Molecular markers for leaf rust resistance gene in wheat. ***Journal of Applied Genetics* 42: 117-126.**

Collard BCY, Jahufer MZZ, Brouwer JB and Pang ECK (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. ***Euphytica* 142: 169-196.**

Curtis BC, Rajaram S and Macpherson HG (eds) (2002) Bread wheat improvement and production No. 30. ***Food and Agriculture Organization of the United Nations, Rome Italy pp 554.***

Dendy DAV and Dobraszczyk BJ (eds) (2001) Cereals and Cereal Products – Chemistry and Technology. ***Aspen Publishers, Inc. pp 429.***

Dieguez MJ, Altieri E, Ingala LR, Perera E, Sacco F and Naranjo T (2006) Physical and genetic mapping of amplified fragment length polymorphisms and the leaf rust resistance *Lr3* gene on chromosome 6BL of wheat. ***Theoretical and Applied Genetics* 112: 251-257.**

Dromph KM and Borgen A (2001) Reduction of viability of soil borne inoculum of common bunt (*Tilletia tritici*) by collembolans. ***Soil Biology and Biochemistry* 33: 1791-1795.**

Eckardt NA (2006) Identification of rust fungi avirulence elicitors. ***The Plant Cell* 18: 1-3.**

Ezzahiri B, Diouri S and Roelfs AP (1992) The role of the alternate host, *Anchusa italica*, in the epidemiology of *Puccinia recondite* f. sp. *tritici* on durum wheats in Morocco. In: **Zeller FJ and Fischbeck G (eds)**. Proceedings of the 8th European and Mediterranean Cereal Rusts and Mildew conference. p. 69-70. ***Heft 24 Weihenstephan, Germany.***

Fahima T, Röder MS, Grama A and Nevo E (1998) Microsatellite DNA polymorphism divergence in *Triticum dicoccoides* accessions highly resistant to yellow rust. ***Theoretical and Applied Genetics* 96: 187-195.**

Friebe B, Jiang J, Raupp WJ, McIntosh RA and Gill BS (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. ***Euphytica* 91: 59-87.**

Francia E, Tacconi C, Crosatti D, Barabaschi D, Dall'Aglio E and Vale G (2005) Marker assisted selection in crop plants. ***Plant Cell* 82: 317-342.**

Gill BS, Appels R, Botha-Oberholster A-M, Buell CB, Bennetzen JL, Chalhoub B, Chumley F, Dvořák J, Iwanaga M, Keller B, Li W, McCombie WR, Ogihara Y, Quetier F and Sasaki T (2004) A workshop report on wheat genome sequencing: International genome research on wheat consortium. ***Genetics* 168: 1087- 1096.**

Gupta PK, Balyan HS, Edwards KJ, Isaac P, Korzun V, Röder M, Gautier MF, Joudrier P, Schlatter AR, Dubcovsky J, De la Pena RC, Khairallah M, Penner G, Hayden MJ, Sharp P, Keller B, Wang RCC, Hardouin JP, Jack P and Leroy P (2002) Genetic mapping of 66 new microsatellite (SSR) loci in bread wheat. *Theoretical and Applied Genetics* **105**: 413-422.

Gupta PK, Varshney RK, Sharma PC and Ramesh B (1999) Molecular markers and their applications in wheat breeding. *Plant Breeding* **118**: 369-390.

Harlan JR (1995) The living fields – our agricultural heritage. *Cambridge University Press, Cambridge pp 271*.

Hayden MJ and Sharp PJ (2001) Targeted development of informative microsatellite (SSR) markers. *Nucleic Acids Research* **29**: 1-6.

Hayden MJ, Khatkar S and Sharp PJ (2001) Targeting microsatellites (SSRs) in genetic linkage maps of bread wheat. *Australian Journal of Agricultural Research* **52**: 1143-1152.

Hayden MJ, Kuchel H and Chalmers KL (2004) Sequenced tagged microsatellites for *Xgwm533* locus provide new diagnostic markers to select for the presence of the stem rust resistance gene *Sr2* in bread wheat. *Theoretical and Applied Genetics* **109**: 1641-1647.

Hopf M and Zohary D (1993) Domestication of plants in the old World – The origin and spread of cultivated plants in West Asia, Europe and the Nile Valley. *Clarendon Press, Oxford pp 278*.

http://www.fas.usda.gov/pecad/highlights/2004/10/RSA_wheat/index.htm
(2004). Cited June 2006.

<http://www.nda.agric.za/publications> (2001). Cited May 2007.

<http://www.sagis.org.za> (2007) South African grain information services. Cited September 2007.

<http://www.Wintercrops.co.za> History of the wheat crop in SA. Cited July 2006.

Huang XQ and Röder MS (2004) Molecular mapping of powdery mildew resistance genes in wheat. *Euphytica* **137**: 203-223.

Ishii T and Yonezawa K (2007a) Optimization of the marker-based procedures for pyramiding genes from multiple donor lines: I. Schedule of crossing between the donor lines. *Crop Science* **47**: 537-546.

Ishii T and Yonezawa K (2007b) Optimization of the marker-based procedures for pyramiding genes from multiple donor lines: II. Strategies for selecting the objective homozygous plant. *Crop Science* **47**: 1878-1886.

Jones DG and Clifford BC (1978) Cereal diseases their pathology and control. *BASF pp* 279.

Khan RR, Bariana HS, Dholakia BB, Naik SV, Lagu MD, Rathjen AJ, Bhavani S and Gupta VS (2005) Molecular mapping of stem and leaf rust resistance in wheat. *Theoretical and Applied Genetics* **111**: 846-850.

Khlestkina EK, Röder MS, Unger O, Meinel A and Börner A (2007) More precise map position and origin of a durable non-specific adult plant disease resistance against stripe rust (*Puccinia striiformis*) in wheat. *Euphytica* **153**: 1-10.

Kloppers FJ and Pretorius ZA (1997) Effects of combinations amongst genes *Lr13*, *Lr34* and *Lr37* on components of resistance in wheat to leaf rust. *Plant Pathology* **46**: 737-750.

Knox RE, Menzies JG, Howes NK, Clarke JM, Aung T and Penner GA (2002) Genetic analysis of resistance to loose smut and an associated DNA marker in durum wheat doubled haploids. *Canadian Journal of Plant Pathology* **24**: 316-322.

Kolmer JA (2005) Tracking wheat rust on continental scale. *Current opinion in Plant Biology* **8**: 441-449.

Kota R, Speilmeyer W, McIntosh RA and Lagudah ES (2006) Fine genetic mapping fails to dissociate durable stem rust resistance gene *Sr2* from *pseudo-black chaff* in common wheat. *Theoretical and Applied Genetics* **112**: 492-499.

Kriel WM and Pretorius ZA (2006) Fusarium head blight: A summary of the South African situation. *Proceedings of the 2005 National Fusarium head blight Forum, Milwaukee, Wisconsin, USA*, p 243-245.

Kuraparthi V, Chhuneja P, Dhaliwal HS, Kaur S, Bowden RL and Gill BS (2007) Characterization and mapping of cryptic alien introgression from *Aegilops geniculata* with new leaf rust and stripe rust resistance genes *Lr57* and *Yr40* in wheat. *Theoretical and Applied Genetics* **114**: 1379-1389.

Lagudah ES, McFadden H, Singh RP, Huerta-Espino J, Bariana HS and Spielmeyer W (2006) Molecular genetic characterization of the *Lr34/Yr18* slow rusting resistance gene region in wheat. *Theoretical and Applied Genetics* **114**: 21-30.

Lin F and Chen XM (2007) Genetics and molecular mapping of genes for race-specific all-stage resistance and non-race-specific high-temperature adult-plant resistance to stripe rust in spring wheat cultivar Alpowa. *Theoretical and Applied Genetics* **114**: 1277-1287.

Liu J, Liu D, Tao W, Li W, Wang S, Chen P, Cheng S and Gao D (2000) Molecular marker-facilitated pyramiding of different genes for powdery mildew resistance in wheat. *Plant Breeding* **119**: 21-24.

Ma J, Zhou R, Dong Y, Wang L, Wang X and Jia J (2001) Molecular mapping and detection of the yellow rust resistance gene *Yr26* in wheat transferred from *Triticum turgidum* L. using microsatellite markers. *Euphytica* **120**: 219-226.

Mago R, Bariana HS, Dundas IS, Spielmeyer W, Lawrence GJ, Pryor AJ and Ellis JG (2005) Development of PCR markers for the selection of wheat stem rust resistance genes *Sr24* and *Sr26* in diverse wheat germplasm. *Theoretical and Applied Genetics* **111**: 496-504.

Marsalis MA and Goldberg NP (2006) Leaf, stem, and stripe rust diseases of Wheat. *New Mexico State University Guide A-415*. Available at (<http://www.cahe.nmsu.edu>). Cited July 2006.

Mathews LJ (2005) A host-pathogen study of stripe rust resistance in *Triticum aestivum*. *M.Sc. dissertation, University of the Free State, South Africa*.

Mesterházy A, Bartos P, Goyeau H, Niks RE, Czosza M, Andersen O, Casulli F, Ittu M, Jones E, Manisterski J, Manninger K, Pasquini M, Rubiales D, Schachermayr G, Strzembricka A, Szunics L, Todorova M, Unger O, Vanco B, Vida G and Walther U (2000) European virulence survey for leaf rust in wheat. *Agronomie* **20**: 793-804.

Mohan M, Nair S, Bhagwat A, Krishna TG, Yano M, Bhatia CR and Saski T (1997) Genome mapping, molecular markers and marker-assisted selection in crop plants. *Molecular Breeding* **3**: 87-103.

Moldenhauer J, Moerschbacher BM and van der Westhuizen AJ (2006) Histological investigation of stripe rust (*Puccinia striiformis* f. sp. *tritici*) development in resistant and susceptible wheat cultivars. *Plant Pathology* **55**: 469-474.

Murray TD, Parry DW and Cattlin ND (1998) A colour handbook of diseases of small grain cereal crops. *Manson Publishing Ltd. pp 142.*

Powell W, Machray GC and Provan J (1996) Polymorphism revealed by simple sequence repeats. *Trends in Plant Science 7: 215-222.*

Pretorius ZA, Singh RP, Wagiore WW and Payne TS (2000) Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis* f. sp. *tritici* in Uganda. *Phytopathology 84: 203.*

Prins R and Marais GF (1998) An extended deletion map of the *Lr19* translocation and modified forms. *Euphytica 103: 95-102.*

Prins R, Groenewald JZ, Marais GF, Snape JW and Koebner RMD (2001) AFLP and STS tagging of *Lr19*, a gene conferring resistance to leaf rust in wheat. *Theoretical and Applied Genetics 103: 618-624.*

Prins R, Marais GF, Pretorius ZA, Janse BJH and Marais AS (1997) A study of modified forms of the *Lr19* translocation of common wheat. *Theoretical and Applied Genetics 95: 424-430.*

Rajaram S (2001) Prospects and promise of wheat breeding in the 21st century. *Euphytica 119: 3-15.*

Rakoczy-Trojanowska M and Bolibok H (2004) Characteristics and a comparison of three classes of microsatellite-based markers and their application in plants. *Cellular and Molecular Biology Letters 9: 221-238.*

Ramburan VP, Pretorius ZA, Louw JH, Boyd LA, Smith PH, Boshof WHP and Prins R (2004) A genetic analysis of adult plant resistance to stripe rust in wheat cultivar Kariega. *Theoretical and Applied Genetics 108: 1426-1423.*

Röder MS, Korzun V, Wendehake K, Plaschke J, Tixier M, Leroy P and Ganai MW (1998) A microsatellite map of wheat. *Genetics 149: 2007-2023.*

Sauer JD (1993) Historical geography of crop plants – a select roster. **CRC Press, Boca Raton, Florida.**

Schnurbusch T, Paillard S, Schori A, Messmer M, Schachermyr G, Winzeler M and Keller B (2004) Dissection of quantitative and durable leaf rust resistance in Swiss winter wheat reveals a major resistance QTL in *Lr34* chromosomal region. ***Theoretical and Applied Genetics* 108: 477-484.**

Scott DB (1990) Wheat diseases in South Africa. **Pretoria: Department of Agricultural Development pp 62.**

Sharp PJ, Johnston S, Brown G, McIntosh RA, Pallotta M, Carter M, Bariana HS, Khatkar S, Lagudah ES, Singh RP, Khairallah M, Potter R and Jones MGK (2001) Validation of molecular markers for wheat breeding. ***Australian Journal of Agriculture* 52: 1357-1366.**

Singh D, Park RF and McIntosh RA (2007) Characterisation of wheat leaf rust resistance gene *Lr34* in Australian wheats using components of resistance and the linked molecular marker csLV34. ***Australian Journal of Agricultural Research* 58: 1106-1114.**

Singh RP, Hodson DP, Jin Y, Huerta-Espino J, Kinyua MG, Wanyera R, Njau P and Ward RW (2006) Current status, likely migration and strategies to migrate the threat to wheat production from race Ug99 (TTKS) of stem rust pathogen. Article available at <http://www.cababstractsplus.org/cabreviews>. *Cited August 2006.*

Singh RP, Huerta-Espino J and Roelfs AP (2002) The wheat rusts. FAO Plant production and Protection Series 30: BREAD WHEAT: Improvement and Production. www.wheat/FAOdocumentRepositoryfile/y4011eog.htm. *Cited March 2006.*

Singh RP, Huerta-Espino J and William HM (2005) Genetics and breeding for durable resistance to leaf and stripe rusts in wheat. *Turkish Journal of Agriculture* **29**: 121-127.

Singh US, Mukhopadhyay AN, Kumar J and Chaube HS (1992) Plant diseases of international importance- diseases of cereals and pulses. *Volume 1 Prentice-Hall, Inc.*

Smith PH, Koebner RMD and Boyd LA (2002) The development of a STS marker linked to yellow rust resistance derived from the wheat cultivar Moro. *Theoretical and Applied Genetics* **104**: 1278-1282.

Somers DJ, Isaac P and Edwards K (2004) A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* **109**: 1105-1114.

Somers DJ, Thomas J, DePauw R, Fox S, Humphreys G and Fedak G (2005) Assembling complex genotypes to resist *Fusarium* in wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* **111**: 1623-1631.

Sourdille P, Singh S, Cadalen T, Brown-Guedira GL, Gay G, Qi L, Gill BS, Dufour P, Murigneux A and Bernard M (2004) Microsatellite-based deletion bin system for the establishment of genetic-physical map relationships in wheat (*Triticum aestivum* L.). *Functional Integrated Genomics* **4**: 12-25.

Spielmeyer W, McIntosh RA, Kolmer J and Lagudah ES (2005) Powdery mildew resistance and *Lr34/Yr18* genes for durable resistance to leaf and stripe rust co-segregate at locus on the short arm of chromosome 7D of wheat. *Theoretical and Applied Genetics* **111**: 731-735.

Spielmeyer W, Sharp PJ and Lagudah ES (2003) Identification and validation of markers linked to broad-spectrum stem rust resistance gene *Sr2* in wheat. *Crop Science* **43**: 333-336.

Trench TN, Wilkinson DJ and Esterhuysen SP (1992) South African plant disease control handbook. *Farmer Support Group pp 553.*

USDA (2006) Commodity intelligence report. *United States Department of Agriculture:* http://www.pecad.fas.usda.gov/highlights/2006/10/saf_19oct06/. Cited July 2007.

Van Wyk BJ (2006) Quarterly agricultural economic sector review. *Compiled by Economic Research Unit:* <http://www.nda.agric.za>. Cited September 2007.

Varshney RK, Kumar A, Balyan HS, Roy JK, Prasad M and Gupta PK (2000) Characterization of microsatellites and development of chromosome specific STMS markers for bread wheat. *Plant Molecular Biology Reporter 18: 5-16.*

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research 23: 4407-4414.*

Wang J, Chapman SC, Bonnett DG, Rebetzke GJ and Crouch J (2007) Application of population genetic theory and simulation models to efficiently pyramid multiple genes via marker-assisted selection. *Crop Science 47: 582-588.*

Witcombe JR and Hash CT (2000) Resistance gene deployment strategies in cereal hybrids using marker-assisted selection: Gene pyramiding, three way hybrids, and synthetic parent populations. *Euphytica 112: 175-186.*

Xing L, Wen-xiang Y, Ya-ning L, Da-qun L, Hong-fei Y, Qing-fang M and Ting Z (2007) Identification of AFLP markers linked to *Lr19* resistance to wheat leaf rust. *Agricultural Sciences in China 6: 311-315.*

Xu X, Bai G, Carver BF, Shaner GE and Hunger RM (2005) Molecular characterization of slow leaf-rusting in wheat. *Crop Science 45: 758-765.*

Zaharieva M and Monneveux P (2006) Spontaneous hybridization between bread wheat (*Triticum aestivum* L.) and its wild relatives in Europe. **Crop Science 46: 512-527.**

Zhang W, Lukaszewski AJ, Kolmer J, Soria MA, Goyal S and Dubcovsky J (2005) Molecular characterization of durum and common wheat recombinant lines carrying leaf rust resistance (*Lr19*) and yellow pigment (*Y*) genes from *Lophopyrum ponticum*. **Theoretical and Applied Genetics 111: 573-582.**

Chapter 3

Rust resistance genotyping using linked molecular markers

3.1 Introduction

The three wheat rusts, leaf, stem and stripe rust, are foliar and stem diseases that cause significant reduction in yield and grain quality amounting to major losses to the wheat industry in different regions of the world (Bariana *et al.*, 2002; Singh *et al.*, 2006; Hiebert *et al.*, 2007; Kuraparthi *et al.*, 2007). Yearly millions of funds are spent around the world on fungicides in an attempt to control wheat rusts. Resistant cultivars have proven to be the most effective, economical and environmentally friendly form of rust disease control (Kuraparthi *et al.*, 2007; Lin and Chen, 2007). Although there have historically been many resistant cultivars, there exists a need for more durable rust resistant cultivars. The application of molecular markers and MAS strategies in breeding programmes can support plant breeders in accomplishing pyramiding of several rust resistant genes into new cultivars.

Many breeding programmes in a range of crops around the world are using different forms of molecular markers to screen for one to several alleles of economical and agricultural interest. There is an increasing number of useful molecular markers available, allowing for simultaneous and more accurate selection across a larger number of loci, more than previously thought possible (Mohan *et al.*, 1997; Wang *et al.*, 2007). Progress has been made in mapping and tagging numerous genes of interest with molecular markers and this forms the basis of MAS in crops (Mohan *et al.*, 1997; Somers *et al.*, 2004).

Two widely used molecular marker techniques in breeding programmes are SSR and AFLP analysis. SSR and AFLP analysis are useful forms of molecular markers for the application of genotype identification, paternity testing, linkage mapping, fine mapping, fingerprinting and MAS (Vos *et al.*, 1995; Hayden and Sharp, 2001; Somers *et al.*, 2004). The usefulness of SSR analysis in plant breeding arose from its high informative content and relative ease of

genotyping. The co-dominant nature of SSR markers makes it possible to distinguish between homozygous and heterozygous individuals, making SSRs attractive as diagnostic markers in hybridisation studies (Powell *et al.*, 1996). There are more than a 1 000 SSR markers available, covering the wheat genomes, a number of them are linked to various different traits of interest (Somers *et al.*, 2004). Although AFLP analysis is useful for adding many markers to linkage maps and detect high levels of polymorphism, its application in breeding programmes is limited because it is not suited for high-throughput analysis.

As stated by Knapp (1998), the use of MAS has emerged as an ideal strategy for increasing selection gains. A potential limitation of transferring a number of alleles while using MAS is population size. Large populations are required to ensure, with a reasonable amount of certainty, that the target genotype with all desired trait linked markers is present. The population size needed can be drastically affected by the different crossing and selection strategies chosen to develop the target genotype. It is imperative to select the most efficient method that would minimise the resources required to combine the targeted alleles into a new genotype (Wang *et al.*, 2007). Other factors to consider when applying MAS in a breeding programme is cost per marker assay, laboratory space, expertise needed and the overall cost of MAS. These factors must be weighed up against the added efficiency of MAS and possible saving of time compared to conventional phenotypic selection methods (Mohan *et al.*, 1997; Knapp, 1998; Ishii and Yonezawa, 2007a; 2007b).

A gene pyramiding or gene accumulation programme, when using many donor lines, can be divided into two simple steps. The first step is to acquire all markers linked to the traits of interest within a single genotype. Normally during the first step the target genes of interest are in a heterozygous condition, depending on gene sources. The second step involves a series of selections to identify individuals containing the desired genes in the homozygous state (Ishii and Yonezawa, 2007a; 2007b). It is now possible with MAS, using the desired linked markers, to produce multigene pyramided lines. These lines will have an

extremely high practical use throughout the breeding industry (Ishii and Yonezawa, 2007a).

The aim of this study was to pyramid seven rust resistance genes/QTL (leaf, stripe, stem rust) into a single genotype using five SSR, three STS and two AFLP markers. The study focussed on wheat genes applicable to the local wheat industry and markers used and/or developed in South African breeding programmes.

3.2 Materials and methods

3.2.1 Plant material

Four bread wheat lines or cultivars (spring types), namely *AvocetYrSp*, *Blade*, *CSLr19-149-299* (Chinese Spring containing *Lr19*) and *Kariega* were used as sources of donor material carrying the desired rust resistance genes. Seed of *AvocetYrSp* and *Blade* were obtained from the Plant Breeding Institute at the University of Sydney, Australia. *AvocetYrSp* is an Australian line carrying the *YrSp* and *Sr26* genes. *AvocetYrSp* was selected as female parent during the first and second series of crosses. *Blade* is an Australian variety carrying the *Sr2* and *Sr26* resistance genes to stem rust. The third parental line used was *CSLr19-149-299*, which was a source of *Lr19* and *Lr34*. The *CSLr19-149-299* line seed source was developed by and obtained from Prof GF Marais (University of Stellenbosch, South Africa). The fourth parental line used in the breeding programme is a South African cultivar, *Kariega*, [Agriculture Research Council – Small Grain Institute (ARC-SGI), South Africa] which is a source of two QTL, *QYr.sgi-7D* and *QYr.sgi-2B.1* and shows complete APR to stripe rust. These two QTL are thought to be within regions densely populated with resistance genes. The *QYr.sgi-7D* of *Kariega* contains the *Lr34* complex (Ramburan *et al.*, 2004). *Lr34* and *Sr2* are slow rusting genes while *Sr26*, *Lr19* and *YrSp* confer hypersensitive seedling resistance.

During the phenotypic screening, the following additional cultivars were used as controls: *ThatcherLr19* (University of the Free State germplasm bank), *Avocet S* and *Hartog* (University of Sydney, Australia) and *Morocco* (University of the Free State germplasm bank). *ThatcherLr19* was used as a control for the *Lr34*

and *Lr19* reaction. Avocet S was used to compare its stripe rust reaction to that of AvocetYrSp. Hartog was used as positive control for the *Sr2* reaction. Morocco was included due to its susceptibility to all three rusts.

3.2.2 Planting design

Parental lines and offspring were planted in a greenhouse at the University of the Free State. Plants were grown under standard greenhouse air-conditioner controlled conditions, with a temperature range from 10°C to 25°C and natural daylight length. Watering of the wheat plants was done daily. Plants were planted in 5 l pots, in natural top-soil mix, 3 to 5 plants per pot. The required mixture of 2:3:2 fertiliser (1 g/100 ml) and microelements (Chemicult®) were applied every two weeks or when needed. Wild garlic granules were applied directly to the soil to control aphids.

3.2.2.1 Test planting

Four rows of five pots per row were planted of the parental lines. Each row represented a different parental line and each pot contained five seeds. A total of 25 seeds each of AvocetYrSp, Blade and Kariega were planted and 15 seeds of CSLr19-149-299, due to a limited number of seeds. The purpose of the planting was to multiply seed, test seed viability, record flowering times and to obtain leaf material for marker optimisation.

3.2.3 Crossing programme design

Before attempting gene pyramiding, either through traditional breeding techniques or with the aid of MAS, the number of crosses required per generation and population size needs to be calculated. The population size necessary to transfer seven genes/QTL in the current study was determined from Table 3.1. An end population size of 1 000 was selected to ensure with reasonable certainty that a single genotype with seven rust resistance genes/QTL is identified.

Table 3.1 Numerical characteristics of hybrids between parents differing in n allelic pairs (Allard, 1960)

Number of allelic pairs	Kinds of gametes possible in F_1	Kinds of genotypes possible in F_2	Smallest perfect population in F_2
1	2	3	4
2	4	9	16
3	8	27	64
4	16	81	256
10	1024^a	59,049	1,084,576
21	2,097,152	10,460,353,203	4,398,046,511,104
n	2^n	3^n	4^n

^aTargeted double cross population size

Crosses during the programme were done using standard methods and specifically in one direction (one cultivar was always used as the female parent and another as the male parent). Parental lines were planted in three grouped plots. Each plot was planted two weeks apart to ensure synchronisation of flowering. Plots were divided into planting one, two and three respectively. Since Karioga flowered two weeks earlier than the other parental lines, planting of this line was delayed by two weeks. Each plot consisted of four rows, ten pots per row, with five plants per pot. Around 90 to 100 spikes of each cross were pollinated, with the aim of harvesting ± 10 seeds per cross.

The crossing design is given in Figure 3.1. For cross 1 AvocetYrSp was used as the female parent, to allow the maximum probability of transferring the YrSp gene to the offspring, due to reasons to be discussed later. Karioga was used as male parent. For cross 2, Blade was used as female and CSLr19-149-299 as male. CSLr19-149-299 was used as male parent due to a limited number of seeds and more crosses could be made using it as pollinator rather than female parent.

F₁ seed harvested from crosses 1 and 2 were planted (approximately 350 seeds per cross) in separate plantings as described earlier. Each planting was structured as follows: the two different F₁ crosses (cross 1 and cross 2) were alternated within each planting, two rows of ten pots per row of either cross 1 or 2.

The next set of crosses (cross 3, Figure 3.1) involved crossing of the F₁ offspring of cross 1 and cross 2. F₁ plants of cross 1 were used as female parent, to allow good transfer of *YrSp* from the *Avocet YrSp/Kariega* offspring and the F₁ plants from cross 2 as male parent. Approximately 220 spikes were pollinated during cross 3.

Half the harvested seed (1 200 seeds) of cross 3 (F₁ x F₁), were planted out in the greenhouse and the rest stored in a germplasm bank. Young leaf material was sampled for SSR analysis and plants left to self-pollinate.

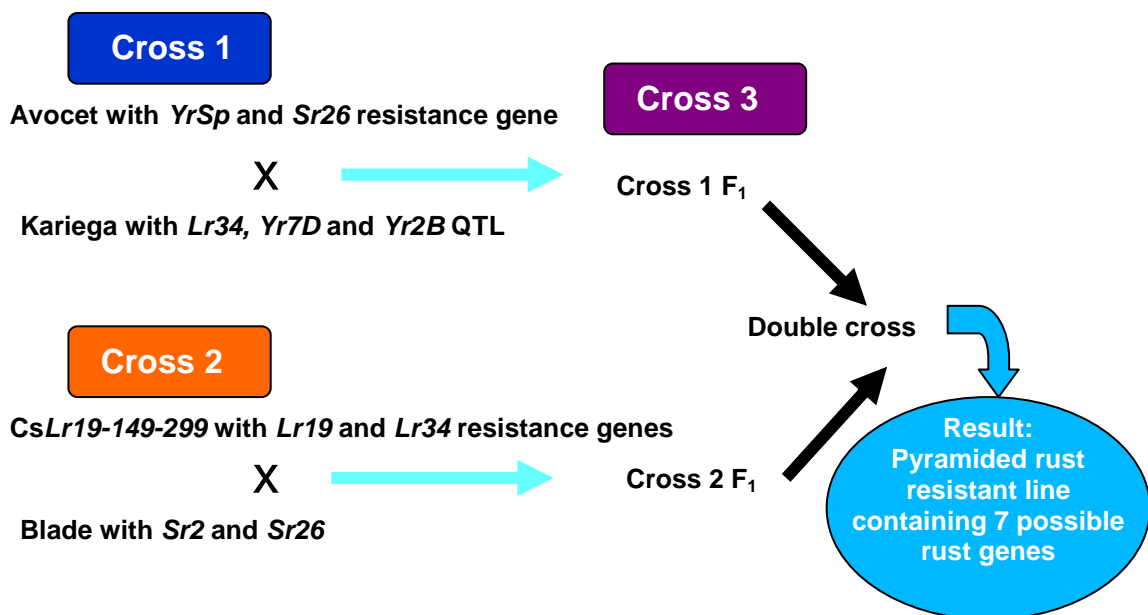


Figure 3.1 Crossing scheme to combine resistance genes of the four wheat cultivars used in this study.

3.2.4 Phenotypic screening

Five to ten seeds per cultivar/ F_1 lines were planted in a cluster in a total of 20 pots (9 cm in diameter) in a soil-peat mix. Six cultivars/ F_1 lines were planted per pot. Cultivar/ F_1 lines used for phenotypic screening were Blade, CSLr19-149-299, Kariega, Avocet YrSp, Blade/CSLr19-149-299 F_1 , Avocet YrSp/Kariega F_1 , ThatcherLr19, Avocet S, Hartog and Morocco. All cultivars/ F_1 lines were planted in duplicate, four pots per tray. Each tray represented a single experiment consisting of two pots per duplication, to be exposed to either a different rust pathogen and/or placed under different optimal growth conditions.

Inoculation was done on 8-day-old seedlings. Collected spores of leaf rust race UVPt18, stem rust race UVPgt55 and stripe rust race 6E22A+ were suspended in a light mineral oil before spraying lightly on the surface of leaves of seedlings in an inoculation chamber. Two trays were used for both UVPt18 and UVPgt55 inoculation and one tray for 6E22A+. After inoculation plants were allowed to dry at room temperature for 2 h. Leaf and stem rust infected plants were incubated overnight (17°C to 20°C) in a dew chamber. Stripe rust was incubated in a cold chamber at 10°C for 48 h.

Following overnight incubation, one tray of leaf rust infected plants were placed in the greenhouse under normal conditions and the other in a growth chamber at 12°C. For stem rust, one tray was placed under normal greenhouse conditions and the other in a greenhouse at 20°C to 28°C. The tray with stripe rust infected plants were placed in the greenhouse at 15°C to 18°C.

Phenotypic scoring of the different infection reactions was carried out 14 days after inoculation except for the leaf rust infected plants that were incubated in the growth chamber at 12°C, which were scored 19 days after inoculation. Plants were scored on a 0 to 4 scale.

3.2.5 Sample collection

Throughout the study, sample collection for DNA extraction was carried out as follows. Three to four young leaves of around 5 cm in length were sampled from four to six week old plants, under strict sterile conditions and placed in 5 ml

plastic sampling tubes. During sampling tubes with leaf material were placed on ice. Sampled leaf material was freeze-dried (Freeze mobile II) for two to three days and stored at -70°C.

3.2.6 DNA Extraction

3.2.6.1 Homogenising of leaf samples

Freeze-dried leaf material was homogenised using Qiagen's TissueLyser. Three to five, 1 to 2 cm pieces of freeze-dried leaf material from each wheat line were cut into smaller pieces into a 2 ml eppendorf tube. Two round stainless-steel ball bearings (5 mm in diameter) were added to the leaf material in each 2 ml eppendorf tube. Samples were homogenised for 1 min at 30 r/s.

3.2.6.2 DNA isolation

Total genomic DNA was isolated using a modified CTAB (hexadecyltrimethylammonium bromide) extraction method (Saghai-Marooft *et al.*, 1984). A volume of 750 µl CTAB buffer [100 mM Tris-Cl (tris (hydroxymethyl) aminomethane), pH 8.0, 20 mM EDTA (ethylene-diaminetetraacetate), pH 8.0, 1.4 M NaCl, 2% (w/v) CTAB, 0.2% (v/v) β-mercaptoethanol] was added to approximately 250 µl of fine leaf powder in a 2 ml eppendorf tube and incubated for 1 h at 65°C. The suspension was extracted with 500 µl chloroform: isoamylalcohol [24:1 (v/v)]. DNA was precipitated from the aqueous phase with 500 µl isopropanol at room temperature for 20 min and centrifuged at 12 000 *g* for 5 min. The precipitate was washed at room temperature for 20 min with 500 µl 70% (v/v) ethanol followed by centrifugation at 12 000 *g* for 5 min. The DNA pellet was air-dried for 1 h and resuspended in 100 µl TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) and treated with DNase-free RNase to a final concentration of 100 µg/ml through incubation at 37°C for 2 h. DNA quantity and quality were estimated from a 0.8% (w/v) agarose gel. Electrophoresis was carried out at 80 V for 1 h in 1x UNTAN (40 mM Tris-Cl, 2 mM EDTA, pH adjusted to 7.4 with acetic acid) buffer and DNA visualised using ethidium bromide staining. Based on these results DNA was further diluted one to four fold for SSR analysis.

3.2.7 SSR analysis

Reaction conditions for each SSR primer set were optimised following several methods and conditions from published articles. All SSR-PCR reactions were performed using a DYAD™ (DNA Engine) Peltier Thermal Cycler. Based on the presence of the specific resistance genes in the four parental lines, SSR markers were selected to target the specific gene or QTL regions within the wheat genome. Table 3.2 lists the selected SSR markers, primer sequences, gene or QTL targeted by each primer set, parental cultivar source and authors who published the SSR primer data.

3.2.7.1 PCR reactions

The SSR-PCR reactions were set up in one of two final volumes, 10 µl or 20 µl. Gwm501 was set up in a final volume of 20 µl, while the rest of the marker reactions were set up in 10 µl. PCR reactions for the 10 µl final volume contained 4 µl genomic DNA, 2 mM MgCl₂, 1x polymerase buffer (Promega), 200 µM 2'-deoxynucleotide 5'-triphosphates (dNTPs), 25 ng each of the forward and reverse primer and 0.25 U GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA).

The MgCl₂, dNTPs, primer and *Taq* polymerase concentrations were altered according to optimised conditions and are given in Table 3.3, together with optimised cycling conditions of the markers. SSR-PCR reactions for Gwm501 was setup in a 20 µl final volume containing 4 µl genomic DNA, 2 mM MgCl₂, 1x polymerase buffer (Promega), 400 µM dNTPs, 50 ng of each of the forward and reverse primer and 1 U GoTaq® Flexi DNA polymerase (Promega).

3.2.7.2 SSR-PCR cycling conditions

All SSR-PCR reactions were optimised and modified based on conditions from published data. Optimal reaction conditions for primer sets Gwm501, Gwm111 and stm559tgag were determined using the modified method of Taguchi (1986) as described by Cobb and Clarkson (1994). The detailed optimised PCR cycling conditions are given in Table 3.3. Cycling conditions of the remaining SSR markers were similar to the published methods.

Table 3.2 Selected SSR markers, corresponding primer pair sequences, targeted genes or QTL, parental cultivar sources and references for the primer sets used in the study

Marker	Forward primer sequence (5'- 3')	Reverse primer sequence (5'- 3')	Targeted gene or QTL	Parental cultivar source	Reference
Gwm111	TCTGTAGGCTCTCTCTCCGACTG	ACCTGATCAGATCCCACTCG	<i>QYr.sgi-7D</i>	Kariega	Röder <i>et al.</i> , 1998; Ramburan <i>et al.</i> , 2004
Gwm295	GTGAAGCAGACCCACAACAC	GACGCGTGCGACGTAGAG	<i>QYr.sgi-7D</i>	Kariega	Röder <i>et al.</i> , 1998; Ramburan <i>et al.</i> , 2004
Sr26#43	AATCGTCCACATTGGCTTCT	CGCAACAAAATCATGCACTA	<i>Sr26</i>	Avocet, Blade	Mago <i>et al.</i> , 2005
STSLr19 ₁₃₀	CATCCTTGGGGACCTC	CCAGCTCGCATACATCCA	<i>Lr19</i>	CsLr19-149-299	Prins <i>et al.</i> , 2001
Gwm148	GTGAGGCAGCAAGAGAGAAA	CAAAGCTTGACTCAGACAAA	<i>QYr.sgi-2B.1</i>	Kariega	Röder <i>et al.</i> , 1998; Ramburan <i>et al.</i> , 2004
Gwm501	GGCTATCTCTGGCGCTAAAA	TCCACAAACAAGTAGGCGCC	<i>QYr.sgi-2B.1</i>	Kariega	Röder <i>et al.</i> , 1998; Ramburan <i>et al.</i> , 2004
stm559tgag	AAGGCGAATCAAACGGAATA	TGTGTGTGTGTGTGAGAGAGAG	<i>Sr2</i>	Blade	Hayden <i>et al.</i> , 2004
csLV34	GTTGGTTAAGACTGGTGATGG	TGCTTGCTATTGCTGAATAGT	<i>Lr34</i>	In all except Blade	Lagudah <i>et al.</i> , 2006

Table 3.3 Optimal reaction and PCR cycling conditions for SSR markers used in the study

Marker	PCR Cycling conditions	Adjusted reaction concentrations
Gwm111	95°C 2 min; 1 cycle 95°C 1 min, 64.7°C 1 min, 72°C 1 min; 44 cycles 72°C 5 min; 1 cycle	300 µM dNTPs and 37.5 ng primer
Gwm295	95°C 2 min; 1 cycle 95°C 1 min, 64°C 1 min, 72°C 1 min; 44 cycles 72°C 5 min; 1 cycle	Same as 10 µl final volume ¹
Sr26#43	94°C 3 min; 1 cycle 94°C 45 sec, 55°C 45 sec, 72°C 1:15 min; 44 cycles 72°C 10 min; 1 cycle	Same as 10 µl final volume ¹
STSLr19 ₁₃₀ Gwm148	94°C 3 min; 1 cycle 94°C 1 min, 60°C 1 min, 72°C 2 min; 44 cycles 72°C 10 min; 1 cycle	STSLr19 ₁₃₀ 0.5 U GoTaq®
Stm559tgag	95°C 2 min; 1 cycle 95°C 30 sec, 64°C 30 sec, 72°C 30 sec; 44 cycles 72°C 10 min; 1 cycle	1.5 mM MgCl ₂ ; 37.5 ng primer
CsLV34	94°C 5 min; 1 cycle 94°C 1 min, 55°C 1 min, 72°C 2 min; 4 cycles 94°C 30 sec, 55°C 30 sec, 72°C 30 sec; 29 cycles 94°C 30 sec, 55°C 30 sec, 72°C 5 min; 1 cycle	Same as 10 µl final volume ¹
Gwm501	94°C 3 min; 1 cycle 94°C 1 min, 60.7°C 1 min, 72°C 2 min; 44 cycles 72°C 10 min; 1 cycle	Same as 20 µl final volume ²
Gradient (Taguchi)	95°C 30 sec; 1 cycle 95°C 30 sec, 56°C - 70°C 30 sec, 72°C 30 sec; 44 cycles 72°C 10 min; 1 cycle	As described by Cobb and Clarkson (1994)

¹10 µl final volume: 4 µl genomic DNA, 2mM MgCl₂, 1 x *Taq* Buffer, 200µM dNTPs, 25ng of each primer, 0.25 U GoTaq® Flexi DNA Polymerase

²20 µl final volume: 4 µl genomic DNA, 2mM MgCl₂, 1 x *Taq* Buffer, 400µM dNTPs, 50ng of each primer, 1 U GoTaq® Flexi DNA Polymerase

3.2.8 Visualisation

3.2.8.1 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels were run for all markers with csLV34 being the exception, which was run on a 2% (w/v) agarose gel. For PAGE gels, SSR-PCR products were mixed with either 5 μ l (for 10 μ l PCR reaction volumes) or 10 μ l (for 20 μ l PCR reaction volumes) formamide loading dye [98% (v/v) de-ionized formamide, 10 mM EDTA, pH 8.0, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol]. Reactions were denatured by incubation for 5 min at 95°C. Mixtures were immediately placed on ice prior to loading. SSR-PCR products (4.5 to 5.0 μ l) were separated on a 5% (w/v) denaturing polyacrylamide gel [19:1 acrylamide:bis-acrylamide; 7 M urea; 1x TBE Buffer (89 mM Tris-Borate; 2.0 mM EDTA)]. Electrophoresis was performed at constant power of 80 W for 1 to 2 h.

3.2.8.2 Silver staining procedure

SSR-PCR products separated using PAGE were visualised using the protocol described by the Silver Sequence™ DNA Sequencing System manual supplied by Promega. Stained gels were left standing in an upright position overnight to air-dry and be photographed. Photographs were done by exposing photographic paper (Ilford Multigrade IV RC) directly under the gel to a dim white light for approximately 20 s. The procedure above produced a negative image of exactly the same size of the SSR gel. SSR fragment lengths were determined by comparison with a 25 bp DNA ladder (Promega).

3.2.8.3 Agarose gel electrophoresis

SSR-PCR products of primer set csLV34 were mixed with 5 μ l non-denaturing condition loading dye (15% (w/v) Ficoll, 0.24% (w/v) bromophenol blue). PCR products (5 μ l) were separated on a 2% (w/v) agarose gel and visualised using ethidium bromide staining. Electrophoresis was performed at 100 V for 1 h in 1x UNTAN (section 3.2.6.2). SSR fragment lengths were determined by comparison with a 25 bp DNA ladder (Promega).

3.2.9 AFLP analysis

AFLP analysis was performed using primer pair combinations *EcoRI*-ACC/*MseI*-CAT and *EcoRI*-ACC/*MseI*-CTG. These two primer combinations amplify two markers linked to *YrSp* resistance in Karioga (Mathews, 2005). Primers and adapters were synthesised by Integrated DNA Technologies Inc, USA. Oligonucleotides used for adapters were PAGE purified. Adapters were prepared by mixing equimolar amounts of both strands, heating for 10 min 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). Sequences of adapters and primers are given in Table 3.4

3.2.9.1 DNA extraction for AFLP analysis

DNA extraction was carried out as described in section 3.3.5.2. DNA was further purified as follows. After the RNaseA step, DNA was extracted with 0.75 M ammonium acetate and an equal volume chloroform: isoamylalcohol [24:1 (v/v)] and centrifuged at 12 000 *g* for 5 min. DNA was precipitated by adding 500 µl ice-cold 100% (v/v) ethanol followed by overnight incubation at 4°C. Samples were centrifuged at 12 000 *g* for 15 min and the pelleted DNA was washed twice with 500 µl 70% (v/v) through centrifugation for 10 min at 12 000 *g*. The supernatant was discarded and the DNA pellet was air-dried, followed by overnight re-suspension in 50 µl TE buffer, pH 8.0 at 4°C. DNA concentration and purity was determined as described earlier.

3.2.9.2 Restriction digestion

Approximately 1 µg of extracted genomic DNA from the four parental lines were digested for 5 h at 37°C using 4 U *MseI* (New England Biolabs) and 1x *MseI*-buffer [(50 mM NaCl, 10 mM Tris-Cl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT) pH 7.9)] in a final volume of 50 µl. Following *MseI* digestion, DNA was further digested overnight at 37°C with 5 U *EcoRI* (Roche Diagnostics) and NaCl to a final concentration of 100 mM.

Table 3.4 *Mse*I- and *Eco*RI-adapter and primer sequences

Adapter/Primer	Sequence (5' - 3')
<i>Mse</i> I adapter-F	GACGATGAGTCCTGAG
<i>Mse</i> I adapter-R	TACTCAGGACTCAT
<i>Eco</i> RI adapter-F	CTCGTAGACTGCGTACC
<i>Eco</i> RI adapter-R	AATTGGTACGCAGTCTAC
<i>Mse</i> I+ C primer	GATGAGTCCTGAGTAAC
<i>Mse</i> I+ 3 primer	GATGAGTCCTGAGTAAC CNN CNN = CAT, CTG
<i>Eco</i> RI+ A primer	GACTGCGTACCAATTCA
<i>Eco</i> RI+ 3 primer	GACTGCGTACCAATTC ANN ANN = ACC

3.2.9.3 Adapter ligation

Adapters were ligated by adding a 10 µl reaction mixture containing 50 pmol *Mse*I-adapter, 5 pmol *Eco*RI-adapter, 1 U T4 DNA Ligase (USB Corporation), 0.4 mM adenosine triphosphate (ATP) and 1x T4 DNA Ligase buffer (66 mM Tris-Cl pH 7.6, 6.6 mM MgCl₂, 10 mM DTT, 66 mM ATP) to the 50 µl restriction digest solution, followed by overnight incubation at 16°C.

3.2.9.4 Pre-selective amplification

Pre-selective amplification was performed in a 50 µl final volume reaction mixture consisting of 5.0 µl of the undiluted digested/ligated solution, 1x polymerase buffer (Promega), 2 mM MgCl₂, 200 µM dNTPs, 30 ng of primer *Mse*I+C, 30 ng of primer *Eco*RI+A and 0.02 U GoTaq® Flexi DNA polymerase (Promega). Thermal cycling of the reactions was performed using a DYAD™ (DNA Engine) Peltier Thermal Cycler. Pre-selective cycling conditions were as follows: 94°C for 5 min, 1 cycle, 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min for 30 cycles and a final single cycle of 72°C for 10 min. Pre-selective fragments were separated on a 1.5% (w/v) agarose gel (ran at 80 V for an hour) using ethidium bromide to visualise the fragments (section 3.2.6.2). DNA purity

and quantity were determined and DNA dilutions made accordingly (1:15 to 1:50 using 0.1x TE buffer).

3.2.9.5 Selective amplification

Selective PCR was done in a total of 20 µl containing 5 µl diluted pre-selective DNA, 1x polymerase buffer, 2 mM MgCl₂, 200 µM dNTPs, 100 µg/ml Bovine serum albumin, 30 ng *Mse*I+3 primer, 30 ng *Eco*RI+3 primer and 0.75 U GoTaq® Flexi DNA polymerase. Primer combinations used were as follows: E-ACC with M-CAT and E-ACC with M-CTG. Selective amplification was performed under the following cycling conditions: 1 cycle at 94°C for 5 min, 94°C for 30 sec, 65°C for 30 sec, decreasing by 1°C every cycle and 72°C for 1 min for 9 cycles, 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min for 25 cycles and 1 cycle of 72°C for 2 min.

Visualisation of selective AFLP products were performed as described in sections 3.3.8.1 and 3.3.8.2. AFLP fragments sizes were compared to a 100 bp DNA ladder (Promega).

3.2.10 Screening of parental lines, F₁ progeny and the double cross population

All four parental lines (*Avocet YrSp*, *Blade*, *CSLr19-149-299* and *Kariega*) were screened with five SSR and three STS primer sets and two AFLP primer pairs to confirm the presence of the expected marker alleles. *Avocet YrSp/Kariega* (cross 1) F₁ hybrids were screened with SSR marker *Gwm148* and *Blade/CSLr19-149-299* (cross 2) F₁ hybrids with SSR marker *STSLr19₁₃₀* to confirm whether it was successfully crossed and not self-pollinated. Individuals from the double cross population were screened with all eight marker primer sets.

3.2.10.1 Data analysis

The number of alleles and allele sizes per SSR marker set were determined during the parental screening and compared to expected allele sizes of published data. SSR-PCR products were scored for the presence “1” or absence “0” of expected parental alleles. Within the double cross population,

the number of resistance genes and/or QTL in each individual genotype was determined and statistical analysis was carried out on the entire population. Chi square analysis was done using online GraphPad Software and Punnett square calculations were done using an online Punnett square calculator that is JAVA script encoded, created by Change Bio Science (<http://www.changbioscience.com/genetics/punnett.html>).

3.3 Results

3.3.1 Phenotypic screening

Leaf, stripe and stem rust infection types (0 to 4 scale) are given in Table 3.5. These ranged from 0 (representing a highly resistant reaction), through 1 (high resistance) and 2++ (low resistance-intermediate) to 3++4 (highly susceptible) reactions.

3.3.1.1 Leaf rust infection

Cultivars and F_1 lines expected to contain the *Lr19* gene (CS*Lr19*-149-299, Blade/CS*Lr19*-149-299 F_1 and Thatcher*Lr19*) displayed highly resistant reactions to leaf rust pathotype UVPt18 under normal greenhouse (GH) conditions. Kariega, Avocet*YrSp*, CS*Lr19*-149-299, Blade/CS*Lr19*-149-299 F_1 , Avocet*YrSp*/Kariega F_1 , expected to contain the slow rusting *Lr34* gene, displayed low to highly resistant reactions to leaf rust under the 12°C incubation. The positive control for leaf rust resistance, Thatcher*Lr19*, displayed a highly resistant reaction to leaf rust infection while Morocco, the susceptible control, displayed a highly susceptible reaction. Hartog developed a low resistance-intermediate reaction under the 12°C incubation and moderately to highly susceptible reaction under normal GH conditions. Overall, leaf rust reactions were as expected for all cultivars.

3.3.1.2 Stem rust infection

Cultivars and F_1 lines expected to contain *Sr26* (Avocet*YrSp*, Blade, Avocet*YrSp*/Kariega F_1 and Blade/CS*Lr19*-149-299 F_1) and *Sr2* (Blade and Blade/CS*Lr19*-149-299 F_1) displayed resistant reactions. Blade displayed indifferent results between duplication under both normal and warm GH conditions of 20°C to 28°C. This indifferent expression of resistance of Blade to

stem rust infection will be discussed later. The following cultivars/lines had a highly susceptible reaction: CSLr19-149-299, Kariega and Morocco. The Avocet S control developed a resistant reaction under both incubation conditions. Hartog under the high temperature incubation conditions displayed strong systemic seedling chlorosis as expected for *Sr2*. Reactions of all cultivars were as expected for stem rust infection with the exception of inconsistency in Blade regarding *Sr2*.

3.3.1.3 Stripe rust infection

Cultivars and F₁ lines expected to contain the seedling resistance *YrSp* gene (Avocet *YrSp* and Avocet *YrSp*/Kariega F₁) displayed a highly resistant reaction to stripe rust. The remaining cultivars all displayed highly susceptible reactions to stripe rust. All reactions to stripe rust were as expected.

Table 3.5 Primary leaf seedling infection types of parental and F₁ genotypes to selected pathotypes of *Puccinia graminis* f. sp. *tritici*, *P. triticina* and *P. striiformis* f. sp. *tritici*

Entry	Source	Seedling infection type				
		UVPt18 (GH ^a)	UVPt18(12°C ^b)	UVPgt55 (Gh ^a)	UVPgt55 (GH high ^c)	6E22A+ ^d
Blade	XII-11 BV1998	2+3	2cn	;1=	::1	3++
CSLr19-149-299	US (GFM)	0;	0;	3=	3-	3++
Kariega	III-51 GH2003	2+	;1	3	3+	3++
Avocet YrSp	X-16	3++	2=	1=	1	0;
Blade/Lr19 ^e	F ₁	0;	0;	;1	;1	3++
Avocet YrSp/Kariega	F ₁	3+	;1	;1	;1	0;
ThatcherLr19	I-19 1998	0;	0;	;1	1	3++
Avocet S	CMB GH2006	3	2cn	;1	;1	3+
Hartog	XII-43	2-	3++4	2	2	3+
Morocco	Control	3++	3++4	3++	4	3++

^aLeaf rust (UVPt) and stem rust (UVPgt) responses tested under normal greenhouse conditions (18°C-24°C)

^bTested in a growth chamber at continuous 12°C to allow detection of *Lr34*

^cTested in greenhouse at 20°C-28°C to allow systemic expression of seedling chlorosis, linked to *Sr2*, on 2nd and 3rd leaves

^dStripe rust responses tested at 15°C-18°C in greenhouse

^eBlade/CSLr19-149-299

3.3.2 Genotyping

3.3.2.1 Parental screening

The four parental lines, Avocet YrSp, Blade, CSLr19-149-299 and Kariega were screened with five SSR, three STS and two AFLP markers. Table 3.6 shows the relative allele sizes of the SSR and STS markers detected for the specific parental cultivar.

Table 3.6 Number and size of marker alleles identified during parental screening

Marker	Targeted gene/QTL	Number of alleles	Avocet YrSp allele size (bp)	Blade allele size (bp)	CSLr19-149-299 allele size (bp)	Kariega allele size (bp)
Gwm111	<i>QYr.sgi-7D</i>	4	214	182	210	204
Gwm295	<i>QYr.sgi-7D</i>	4	251	248	252	250
Gwm501	<i>QYr.sgi-2B.1</i>	4	168	165	174	177
Gwm148	<i>QYr.sgi-2B.1</i>	4	145	142	162	165
STSLr19 ₁₃₀	<i>Lr19</i>	2	Null allele	Null allele	100	Null allele
Sr26#43	<i>Sr26</i>	2	190	190	Null allele	Null allele
stm559gtag	<i>Sr2</i>	4	249	237	252	Null allele
csLV34	<i>Lr34</i>	2	150	229	150	150

*Allele sizes indicated in bold are linked to the resistant genes or QTL

Markers Gwm111, Gwm295, Gwm501, Gwm148 and stm559gtag each amplified four different allele sizes in the four parental lines. STS markers csLV34, STSLr19₁₃₀ and Sr26#43 amplified two alleles in the four parental lines. Markers Gwm111 and Gwm295, flanking the *QYr.sgi-7D* QTL in Kariega, amplified the expected 204 and 250 bp allele in Kariega, respectively. However, marker Gwm295 amplified a 251 bp allele in Avocet YrSp, making discrimination between the 250 bp allele of Kariega and the 251 bp allele of Avocet YrSp difficult. The other two parental lines did not contain the 204 and 250 bp alleles. Markers Gwm148 and Gwm501, flanking the *QYr.sgi-2B.1* QTL region in Kariega, amplified the expected 177 and 165 bp alleles in Kariega, respectively. These allele sizes were absent in the other three parental lines. Marker STSLr19₁₃₀ associated with the *Lr19* gene in CSLr19-149-299 amplified the expected 100 bp allele in CSLr19-149-299 and a null-allele in the other three parental lines. STS marker Sr26#43 associated with the *Sr26* gene in Avocet YrSp and Blade, amplified the expected 190 bp in these two cultivars

and a null-allele in the other two parental lines. Marker *stm559gtag*, linked to the *Sr2* gene in Blade, amplified the expected 237 bp allele in Blade, a null-allele in Kariega and a 249 and 252 bp allele in *AvocetYrSp* and *CSLr19-149-299*, respectively. STS marker *csLV34* associated with the *Lr34* gene in *CSLr19-149-299* and Kariega, amplified the expected 150 bp allele in these parental lines and a bigger 229 bp allele in Blade. This marker unexpectedly amplified the 150 bp allele, associated with the *Lr34* gene, in *AvocetYrSp*. To confirm this result, marker *csLV34* was tested on both *Avocet S* and *AvocetYrSp*. Marker *csLV34* amplified a 229 bp allele in *Avocet S* and again a 150 bp allele in *AvocetYrSp*, confirming the presence of the marker allele for *Lr34* in *AvocetYrSp*.

During the parental screening on different individuals of the parental lines, all expected SSR and STS markers, with the exception of *stm559gtag*, were observed in all individuals (30) screened. Marker *stm559gtag*, linked to *Sr2* resistance in Blade, did not amplify the expected 237 bp allele in all 30 initially tested individuals, even after three replications. After further testing on a second set of 30 Blade individuals, the expected 237 bp allele was amplified in 43 of the 60 tested Blade individuals, while the rest amplified a null-allele. Only Blade lines containing the expected 237 bp allele were used in subsequent crosses.

AFLP marker *EcoRI-ACC/MseI-CAT* did not amplify the expected 290 bp polymorphic fragment in *AvocetYrSp* and marker *EcoRI-ACC/MseI-CTG* the expected 59 bp fragment in Kariega. There was no detectable difference for those respective fragment sizes between the four parental lines, even after three replications. These two AFLP markers, linked to the *YrSp* gene, were not used further in the study.

3.3.2.2 *F*₁ cross identification

Two molecular markers were selected to identify successful crosses in the *F*₁ populations. SSR marker *Gwm148* was used to screen approximately 200 *F*₁ individuals of the *AvocetYrSp*/*Kariega* (cross 1, Figure 3.1). STS marker *STSLr19₁₃₀* was used to screen approximately 150 *F*₁ individuals of the *Blade*/*CSLr19-149-299* cross (cross 2, Figure 3.1). Marker *Gwm148* amplified

the expected 145 bp allele of Avocet *YrSp* as well as the expected 165 bp allele of Kariega. The presence of both the 145 bp allele of Avocet *YrSp* (female) and the 165 bp allele of Kariega (male parent) in the F₁ hybrids of cross 1 confirmed a cross (Figure 3.2). Marker STSLr19₁₃₀ amplified the expected 100 bp allele of *CSLr19-149-299*. The presence of the 100 bp allele of the male parent *CSLr19-149-299* in the F₁ hybrids of cross 2 confirmed a cross. Only F₁ hybrids that were confirmed as crosses were used for the production of the double cross.

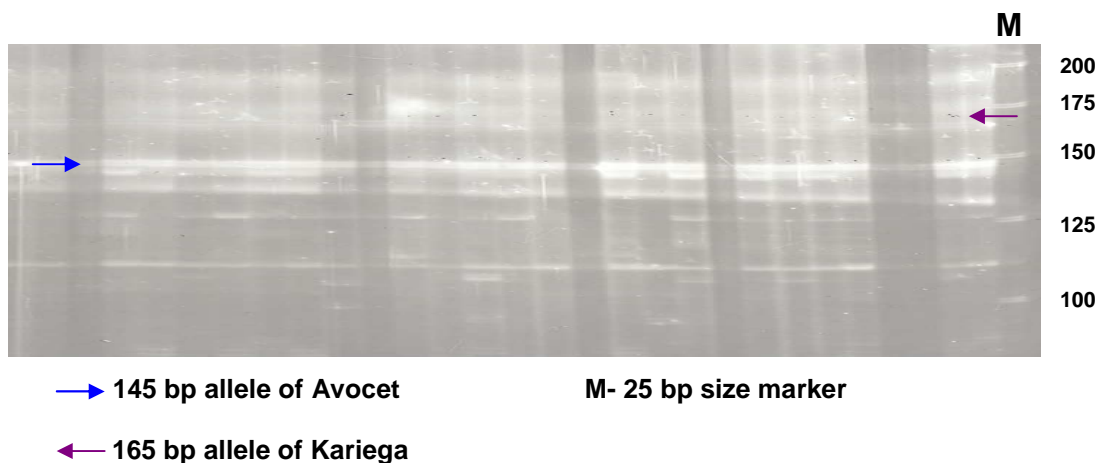


Figure 3.2 A silver stained polyacrylamide gel of Avocet *YrSp*/Kariega F₁ individuals screened with marker Gwm148.

Screening of F₁ hybrids with markers Gwm148 and STSLr19₁₃₀ confirmed that on average 83.45% of the Avocet *YrSp*/Kariega F₁ hybrids of cross 1 were true hybrids and that on average 87.46% of the Blade/*CSLr19-149-299* F₁ hybrids of cross 2 were true hybrids. Figure 3.3 displays the percentage of successful crosses within each planting.

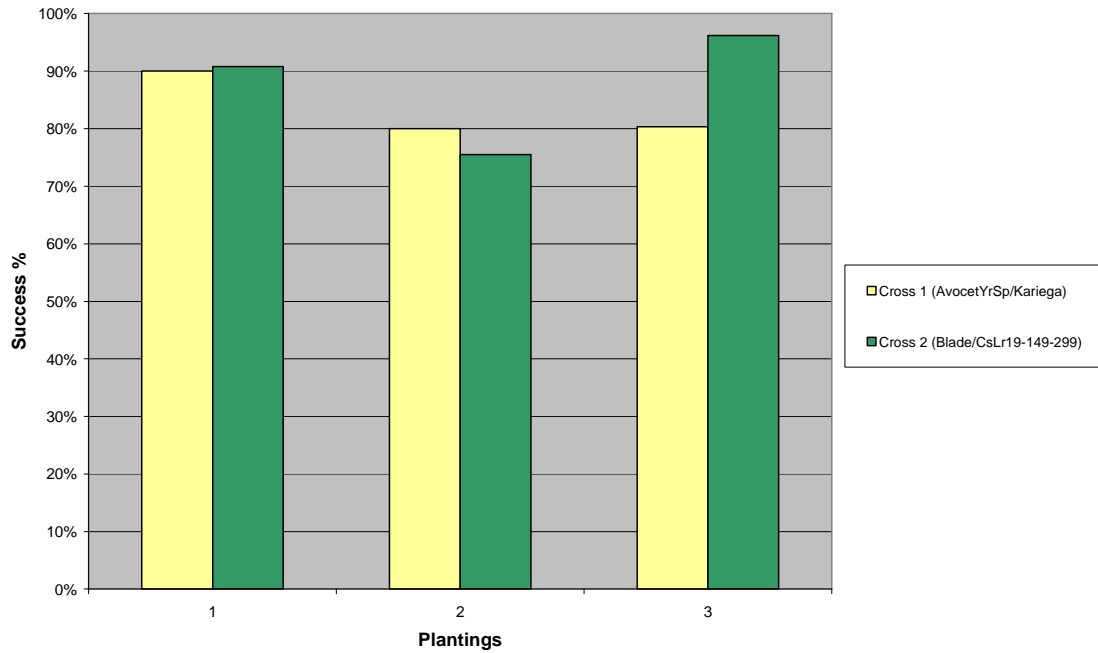


Figure 3.3 Comparing the cross success percentages of cross 1 and cross 2 within individual plantings confirmed by SSR and STS marker screening.

3.3.2.3 Double cross population

Five SSR markers and three STS markers were screened on 900 F_1 individuals of the double cross population. An example of segregation within the double cross population for marker Gwm111 is given in Figure 3.4. Since the two AFLP markers linked to the *YrSp* gene in *AvocetYrSp* could not discriminate between the four parental lines these AFLP markers were not screened on the double cross population. Single gene allele frequencies for the segregating population were determined. The number of individual plants of the double cross population containing markers linked to the desired resistance gene(s)/QTL ranged from two individuals containing none of the markers to three individuals containing all eight markers (Figure 3.5). Due to the high number of individuals being screened with eight different markers, not all reactions could be repeated and some markers were screened as missing values. Individuals containing no markers could be due to some markers scored as missing values for that individual. The three individuals containing eight markers confirmed the presence of markers associated to the presence of the four single genes (*Sr2*, *Sr26*, *Lr19* and *Lr34*) and two QTL (*QYr.sgi-7D* and *QYr.sgi-2B.1*). Due to the

inefficiency of the AFLP markers, the presence of the seventh gene (*YrSp*) could not be confirmed on genotypic level. The majority of the population contained either three or four markers, with 231 or 235 individuals respectively. Twenty-one of the individuals contained seven of the eight markers. Of those 21 individuals, seven might possibly contain all six genes, as a result of missing only one of the markers flanking either of the two QTL.

Based on these results, a normal distribution curve is observed for the presence of the eight possible markers in the 900 individuals of the double cross population (Figure 3.5).

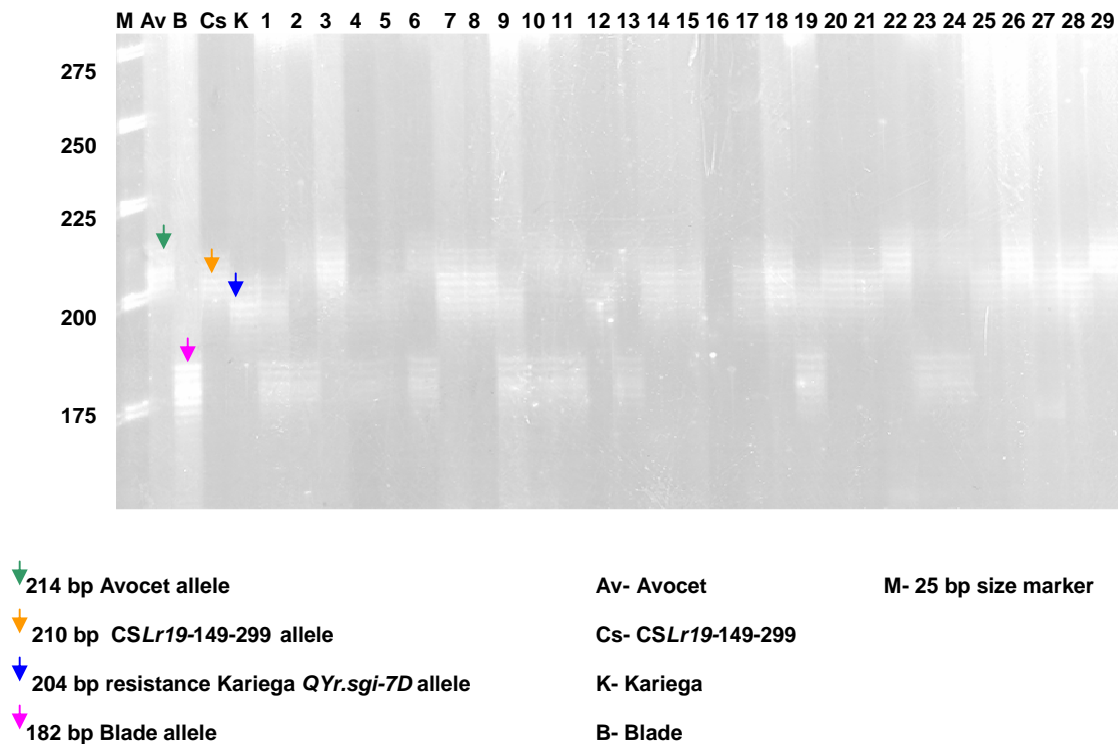


Figure 3.4 A silver stained polyacrylamide gel of marker Gwm111 indicating the allele sizes of Avocet, *Blade*, *CSLr19-149-299* and Kariega segregating in the double cross population.

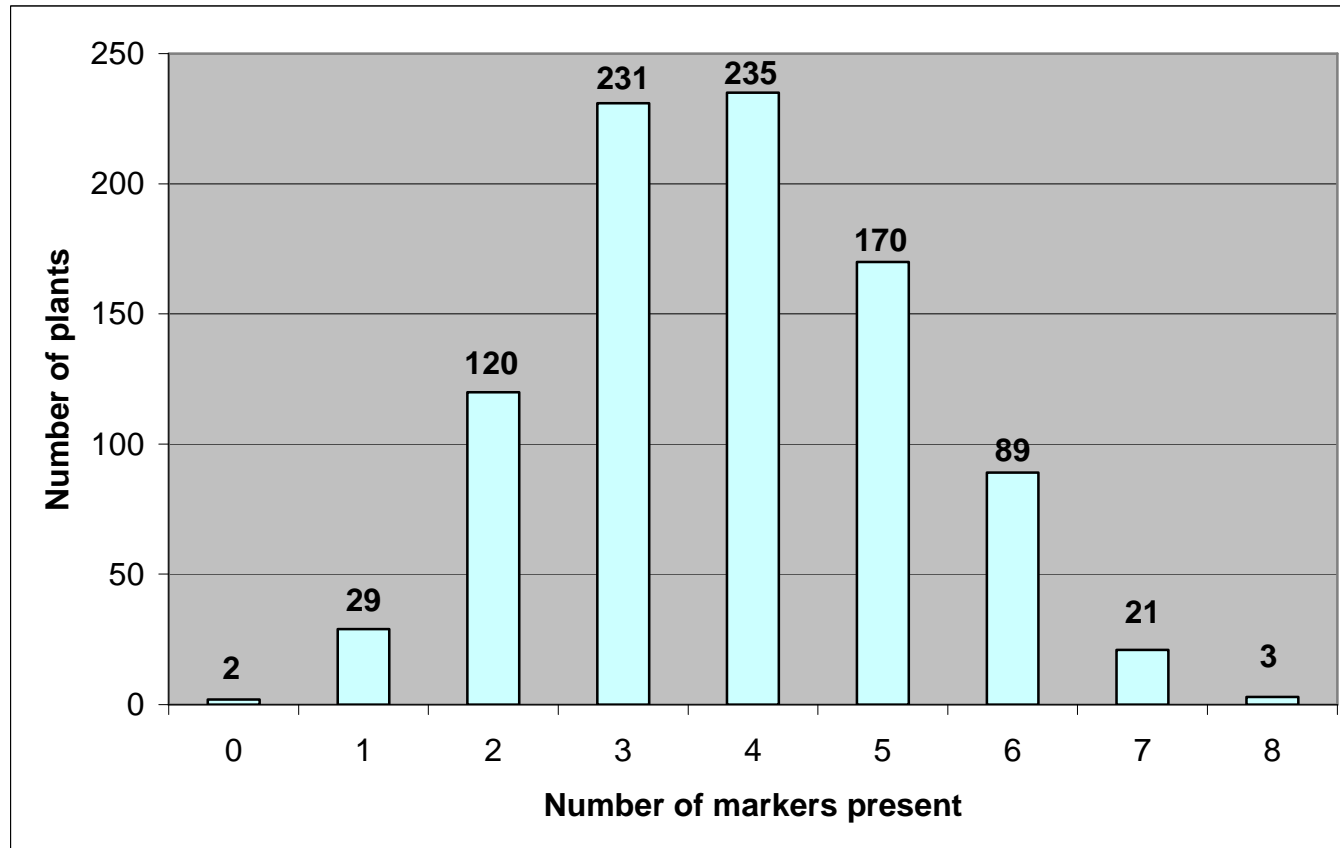


Figure 3.5 Frequency distribution showing the number of double cross F₁ plants that tested positive for the presence of molecular markers.

3.3.3 Marker segregation

Expected segregation ratios for the six single genes were calculated by single gene Punnett square analysis. Parental and F₁ marker data were used to determine the genotypes (homozygous or heterozygous) of the single genes used in the Punnett square calculations. Each single gene was given a theoretical code (A to G) in order to calculate the expected single gene ratios for the double cross population. The expected segregation ratios are given in Table 3.7. All genes in the double cross population, with the exception of *Sr26*, were initially expected to segregate in a 1:1 ratio (heterozygous to homozygous). The *Sr26* gene (expected to be present in the homozygous resistant state in two of the four parental lines) was expected to be present in a 3:1 ratio [homozygous and heterozygous resistant (R) to homozygous susceptible (S)]. All markers segregated according to the expected ratios except for *Sr26* that segregated closer to a 1:1 ratio in contrast to the expected 3:1 ratio. On a statistical level based on Chi square analysis all markers deviated significantly from their relevant expected ratios.

Table 3.7 Segregation ratios of eight molecular markers tested on 900 individuals of the double cross population using Chi square analysis

Marker	Heterozygote R ¹	Heterozygote S ²	Homozygous R	Homozygous S	Ratio E ³	Ratio O ⁴	Chi ²	P value	Total	Missing values
STSLr19₁₃₀	263			454	1:1	1:1.7	50.9	0.0001	717	183
csLV34	289		480		1:1	1:1.7	47.4	0.0001	769	131
Sr26#43	401			358	3:1	1:1.1	199.1	0.0001	759	141
stm559gtag		283		427	1:1	1:1.5	29.2	0.0001	710	190
Gwm148	352			464	1:1	1:1.3	15.4	0.0001	816	84
Gwm111	272			462	1:1	1:1.7	49.2	0.0001	734	166
Gwm501	338			490	1:1	1:1.4	27.9	0.0001	828	72
Gwm295	784	784/14*			1:1				798	102

* For marker Gwm295, 14 individuals were confirmed heterozygous S, while the 784 could be either heterozygous R or heterozygous S

¹Resistant

²Susceptible

³Expected ratio

⁴Observed ratio

3.3.4 Genotypic frequencies in double cross population

3.3.4.1 Expected genotypic frequencies

Each single gene/QTL was coded (A to G) as follows, A: *Lr19*, B: *QYr.sgi-7D*, C: *QYr.sgi-2B.1*, D: *Sr26*, E: *YrSp*, f: *Sr2* (recessive gene) and G: *Lr34*. Based on the cross design used in the study (Figure 3.1) and marker data generated during parental line screening, two coded F₁ genotypes were generated. F₁ from cross 1 (*AvocetYrSp/Kariega*) was coded as aaBbCcDdEeFFGG and F₁ from cross 2 (*Blade/CSLr19-149-299*) as AabbccDdeeFfGg. These two F₁ genotypes were used in the Punnett square analysis to give all possible genotypes expected for all five genes and two QTL used in this study. Results from the Punnett square generated are represented in Table 3.8. The resulting Punnett square gave 192 different possible genotypes. Within Table 3.8 the genotypic frequency percentage is given and this was used to calculate the expected number of individuals carrying the specific genotype out of the 900 individuals of the double cross screened. Depending on the genotype, either 3.5 or 7 individuals are expected to contain a particular genotype. Table 3.8 was used as basis to obtain a list of the possible genotypes observed and scored accordingly from the generated marker data.

3.3.4.2 Observed genotypic frequencies

In order to obtain the observed genotypic frequencies for the tested individuals of the double cross population, marker data for the respective genes were analysed. Since data for the two *Kariega* QTL (*QYr.sgi-7D* and *QYr.sgi-2B.1*) was generated using two flanking SSR markers each, one marker per QTL was selected to represent each QTL. Due to the difficulty observed during scoring of marker data generated by Gwm295, data from marker Gwm111 was used to detect the presence of the *QYr.sgi-7D* QTL. Marker data for both flanking markers for the *QYr.sgi-2B.1* QTL could be accurately scored, but since marker Gwm148 is more closely linked to the QTL, marker data from this marker was used to indicate the presence of the *QYr.sgi-2B.1* QTL.

In order to relate the observed genotypic frequencies with the expected frequencies (Table 3.9), certain adjustments had to be made to correlate the data. Firstly, due to the dominant nature of the STS marker Sr26#43, linked to the *Sr26* gene, homozygous (DD) and heterozygous (Dd) individuals could not be distinguished from each other based on marker data only. Expected genotypes in Table 3.8 that were identical at all the other loci but differed in being either Dd or DD were pooled. For example, aabbccDdEeFFGG and aabbccDDEeFFGG were combined into one genotype indicated as aabbccD-EeFFGG. The dash represents either the D or d allele it replaced. Although the STS marker linked to the *Lr19* allele was also a dominant marker and could thus not distinguish between homozygous (AA) or heterozygous (Aa) resistant individuals, Punnett square analysis indicated that only Aa or aa genotypes for the *Lr19* gene were possible in the double cross population. The presence of the marker allele was thus an indication of the heterozygous resistant individuals only. The other markers were all co-dominant and could distinguish between homozygous and heterozygous individuals.

Due to the inability of the two AFLP makers to distinguish between the parental lines, the presence of the *YrSp* gene could not be confirmed on the genotypic level. Punnett square analysis indicated that two genotypes for the *YrSp* gene were possible, namely heterozygous resistant (Ee) or homozygous susceptible (ee). Genotypic frequencies in Table 3.8 were further reduced by combining all similar genotypes with Ee or ee, e.g. aabbccD-EeFFGG and aabbccD-eeFFGG. Combining some of the expected genotypic frequencies resulted in 64 possible observed genotypic frequencies (Table 3.9). In order to obtain accurate observed genotypic frequencies, individuals containing one or more missing values were omitted from further analysis. A total of 464 individuals contained no missing values and were used in further analysis.

The expected number of individuals per possible observed genotype was calculated from the percentages of the original table and adjusted to a population of 512 to obtain whole number expectancies. All 64 expected genotypes were observed at least once. Thirty individuals contained only the *Lr34* gene and 23 individuals contained both the *Sr26* and *Lr34* genes, which is higher than the expected values (Table 3.9). The eight individuals indicated in bold in Table 3.9 contained all six scoreable genes/QTL in either a heterozygous or homozygous state. Because marker data from only six markers were taken into consideration, the number of individuals containing six genes/QTL is higher than the three individuals in Table 3.7 that were detected containing all eight marker alleles. The six individuals with the AaBbCcD-FfGG genotype (Table 3.9) contained *Lr19*, *QYr.sgi-7D*, *QYr.sgi-2B.1* in a heterozygous resistant state, *Sr26* in a homozygous or heterozygous resistant state, *Sr2* in a heterozygous susceptible state, *Lr34* in a homozygous resistant state and *YrSp* in either a heterozygous resistant or homozygous susceptible state. The genotypic data presented in Table 3.9 furthermore confirmed the pyramiding of the five rust genes and two QTLs in different possible gene combinations.

Table 3.9 Observed and expected genotypic frequencies of individuals of the double cross population genotyped based on data from six molecular markers

Genotype	Observed	Expected	Genotype	Observed	Expected
AaBbCcddFFGG	4	4	AaBbCcD-FFGG	4	12
AaBbCcddFFGg	3	4	AaBbCcD-FFGg	3	12
AaBbCcddFfGG	4	4	AaBbCcD-FfGG	6*	12
AaBbCcddFfGg	2	4	AaBbCcD-FfGg	2*	12
AaBbccddFFGG	2	4	AaBbccD-FFGG	5	12
AaBbccddFFGg	2	4	AaBbccD-FFGg	4	12
AaBbccddFfGG	1	4	AaBbccD-FfGG	7	12
AaBbccddFfGg	2	4	AaBbccD-FfGg	3	12
AabbCcddFFGG	7	4	AabbCcD-FFGG	10	12
AabbCcddFFGg	3	4	AabbCcD-FFGg	4	12
AabbCcddFfGG	3	4	AabbCcD-FfGG	3	12
AabbCcddFfGg	4	4	AabbCcD-FfGg	4	12
AabbccddFFGG	9	4	AabbccD-FFGG	8	12
AabbccddFFGg	6	4	AabbccD-FFGg	15	12
AabbccddFfGG	2	4	AabbccD-FfGG	3	12
AabbccddFfGg	2	4	AabbccD-FfGg	3	12
aaBbCcddFFGG	7	4	aaBbCcD-FFGG	7	12
aaBbCcddFFGg	5	4	aaBbCcD-FFGg	9	12
aaBbCcddFfGG	11	4	aaBbCcD-FfGG	4	12
aaBbCcddFfGg	2	4	aaBbCcD-FfGg	3	12
aaBbccddFFGG	11	4	aaBbccD-FFGG	12	12
aaBbccddFFGg	10	4	aaBbccD-FFGg	4	12
aaBbccddFfGG	5	4	aaBbccD-FfGG	7	12
aaBbccddFfGg	8	4	aaBbccD-FfGg	11	12
aabbCcddFFGG	14	4	aabbCcD-FFGG	13	12
aabbCcddFFGg	7	4	aabbCcD-FFGg	9	12
aabbCcddFfGG	6	4	aabbCcD-FfGG	9	12
aabbCcddFfGg	11	4	aabbCcD-FfGg	8	12
aabbccddFFGG	30	4	aabbccD-FFGG	23	12
aabbccddFFGg	11	4	aabbccD-FFGg	14	12
aabbccddFfGG	11	4	aabbccD-FfGG	18	12
aabbccddFfGg	15	4	aabbccD-FfGg	9	12

A: *Lr19*

B: *QYr.sgi-7D*

C: *QYr.sgi-2B.1*

D: *Sr26*

f: *Sr2*

G: *Lr34*

- either homozygous or heterozygous for allele

* numbers indicated in bold represents individuals containing six genes/QTL

3.4 Discussion

In this study both SSR and STS markers were successfully used in a breeding programme aiming at pyramiding, within a two year period, five different rust genes and two QTL from four parental donor lines. The purity and identity (with regard to the potential presence of the expected resistance genes) of the parental lines were confirmed during the parental screening using SSR and STS markers. Results confirmed the expected allelic sizes for the specific parents with all gene/QTL targeting markers with the exception of marker *stm559gtag*. The 237 bp allele amplified by marker *stm559gtag* and associated with the presence of the recessive gene *Sr2* from Blade, gave diagnostic difficulties. Results indicated that the diagnostic 237 bp allele, specifically related to the *Sr2* gene in Blade, was present in some of the individual Blade plants that were tested, while a null allele was amplified in the rest. Phenotypic screening confirmed the inconsistent expression of *Sr2* resistance in Blade. The inconsistent genotypic and phenotypic results might be attributed to either recombination between the marker allele and the *Sr2* gene or to the Blade seed source retaining a level of heterogeneity (if this trait was not specifically been selected for during the development of Blade). It is known that transfer of a recessive trait such as *Sr2* in a traditional breeding programme can be more difficult compared to a dominant trait (Spielmeyer *et al.*, 2003). However, a recessive trait is normally easy to fix in a homozygous state. Data of this study suggests that Blade might be heterogeneous for the presence of *Sr2*. This however should be confirmed in future by genotyping Blade individuals or derived individuals that express the *Sr2* gene using marker *stm559gtag*. For the purpose of this study, only Blade individuals containing the SSR marker linked to the *Sr2* gene was used in the breeding scheme to rule out genotyping errors.

Parental screening furthermore unexpectedly indicated the presence of the marker allele linked to the *Lr34* gene in Avocet *YrSp*. Marker *csVL34* has been successfully used to screen 84 Australian breeding lines for the presence of the *Lr34* gene (Singh *et al.*, 2007). The presence of *Lr34* within Avocet *YrSp* was unexpected since the line was developed from Avocet S, known not to contain the *Lr34* gene. It is possible, however, that *Lr34* was transferred from Spaldings Prolific, an old United Kingdom variety and source of the *YrSp* gene. Another

explanation lies in the fact that the marker locus and *Lr34* are not absolutely linked. Since *Lr34* is of common wheat origin the possibility exists that in some genotypes the marker allele might not be linked to *Lr34* and *vice versa*. Marker csLV34 was used to screen both Avocet S and AvocetYrSp.. Results indicated the presence of the 229 allele associated with absence of *Lr34* in Avocet S and the 150 bp allele associated with *Lr34* in AvocetYrSp. This result was the basis for stating that the marker allele associated with *Lr34* was present in three of the parents and for calculation of the expected single gene ratios and genotypic frequencies. Based on the single gene calculations and the *Lr34* resistant allele being possibly present in three of the four donor parents, a possible 100% *Lr34* resistance transfer was expected. It is suggested that in the development of the AvocetYrSp line, it potentially inherited *Lr34* from some source in its background. However, it should be noted that the possibility exists that the 150 bp fragment present in AvocetYrSp is in fact not associated with the *Lr34* gene. Another unexpected result with regard to AvocetYrSp was obtained with SSR marker Gwm295. This marker amplified a 242 bp allele in Avocet S (R Prins, personal communication) compared to a 251 bp fragment in AvocetYrSp.

The efficiency of the selected SSR and STS markers for high-throughput analysis using PAGE and silver staining was evaluated. Due to the large number of individuals of the double cross population (900) that needed to be screened and the cost involved, PAGE and silver staining were selected as the screening system of choice. Although PAGE and silver staining are more cost effective compared to analysis using fluorescently labelled primers, it is not as sensitive. SSR and STS markers STSLr19₁₃₀, csLV34, Sr26#43, stm559gtag and Gwm148 all produced clear dark banding patterns that were easy to analyse. In contrast, some difficulty was experienced in scoring markers Gwm111 and Gwm501 due to light staining intensity of these markers. Visualisation of these marker patterns should be more effective using fluorescently labelled primers and separation using an automated sequencer.

Although marker Gwm295 displayed good, clear, high intensity banding patterns after silver staining, the 1 bp difference between the Kariiega allele (250 bp) and the AvocetYrSp allele (251 bp) complicated scoring. Even though the

electrophoresis time was increased to get better separation of the alleles differing by 1 bp, it was impossible to identify these two alleles from each other in individuals of the double cross population. As a consequence, individuals were scored as having either of the two (*Kariega* and/or *AvocetYrSp*) alleles. Segregation ratios for this marker allele could thus not be determined. This marker was furthermore excluded when the observed genotypic frequencies for the *QYr.sgi-7D* QTL were determined even though this marker was more ideal being closer to the 7D QTL compared to the other flanking marker Gwm111. It is suggested that marker Gwm295 should in future be run on an automated sequencer in order to be able to more clearly distinguish between *Kariega* and *AvocetYrSp* individuals.

Screening of individuals of the two different F_1 crosses confirmed the advantages of application of MAS in a traditional breeding programme. Markers Gwm148 and STSLr19₁₃₀ were successfully used to distinguish true crosses from individuals that resulted from self-pollination. Only individuals confirmed to result from a cross were selected and used further in the breeding scheme. Marker Gwm148 was selected for cross 1 because it could clearly and efficiently distinguish between *Kariega* and *AvocetYrSp*. The 20 bp difference between the *AvocetYrSp* allele (145 bp) and the *Kariega* allele (165 bp) made marker Gwm148 ideal for identification of true hybrids. In the second set of F_1 offspring from cross 2, involving *Blade* and *CSLr19-149-299* as donor parents, marker STSLr19₁₃₀ was used for cross confirmation. STSLr19₁₃₀ was used to identify the presence of the expected 100 bp allele associated with *Lr19* gene of *CSLr19-149-299* which was used as male parent. This marker was particularly useful, as it produces an additional 130 bp monomorphic fragment present in all parental cultivars. The presence of the monomorphic fragment served as positive control that PCR reactions worked, especially in lines where a null allele, associated with the absence of *Lr19*, was amplified. Although the dominant nature of the marker made it impossible to distinguish between heterozygotes and homozygotes, only heterozygous resistant individuals were expected in the F_1 offspring.

The phenotypic evaluations helped to confirm what was seen at molecular level, confirming the transfer of the desired genes. The *YrSp* positive, resistant reaction observed was important for confirmation of *YrSp* transfer to the offspring since genotypic parental screening revealed that the AFLP markers linked to *YrSp* was not useful. In Mathews (2005), these AFLP markers were scored on an automated sequencer compared to silver staining used in the current study. As mentioned before, silver staining is not as sensitive as fluorescently labelled reactions run on an automated sequencer. This might explain the inability to detect the polymorphic fragments linked to *YrSp* resistance rendering the AFLP markers not fit for use for *YrSp* MAS in this study. On a molecular level it was no longer possible to select for *YrSp* transfer. Since such a possibility was envisaged, Avocet *YrSp* and derived F_1 individuals of Avocet *YrSp* were used as the female parent throughout the breeding programme to ensure the maximum probability of transferring *YrSp* to the offspring.

Genotyping of 900 individuals of the double cross population revealed that all markers segregated as expected. To correlate the expected and observed genotypic frequencies, certain assumptions were made for the 900 genotyped individuals. Since the two markers for the *Lr19* and *Sr26* genes were dominant STS markers, the homozygous nature of the parental lines containing these genes could not be confirmed. In order to calculate the expected segregation ratios and patterns, it was hypothesised that the parental lines were in a homozygous state for *Lr19* and *Sr26*. Since the marker linked to the *Lr19* gene was used to select individuals that resulted from a true cross in the F_1 individuals, it is possible that individuals from a homozygous or heterozygous parent for *Lr19* could have been selected. However, due to the use of the *Lr19* linked marker, only F_1 individuals heterozygous for *Lr19* were selected. The calculation of the segregation ratio for *Lr19* would not have been affected due to the ratios being calculated from what was expected in the F_1 individuals, based on marker data. The deviation of the *Lr19* segregation ratio from a 1:1 ratio is thought to be attributed to the complexity of translocation segment segregation and the lack of chromosome pairing genes. However, it was a different case for the two parental lines (Avocet *YrSp* or Blade) containing the *Sr26* gene. From

the resulting segregation ratio (closer to 1:1), it was hypothesised that one of the original parental lines were not homogenous for *Sr26* gene. If both parents contained the *Sr26* gene in a homozygous state, the marker allele linked to the *Sr26* gene would have segregated in a 3:1 ratio. Since a ratio closer to 1:1 was observed it confirmed that one of the parental lines (*Avocet YrSp* or *Blade*) was heterogeneous for the *Sr26* allele. STS marker *Sr26#43* was not a feasible marker to be used in cross identification during screening of the F_1 individuals since *Sr26* was present in both female parents (*Blade* and *Avocet YrSp*) and amplified a null allele in both male parents used in the initial crosses. Therefore, for the *Sr26* gene, no F_1 marker data was available to confirm on a genetic level whether F_1 individuals were homo- or heterozygous for the *Sr26* marker. As mentioned before, the original expected 3:1 ratio calculation was based on the theoretical assumption that both parents (*Blade* and *Avocet YrSp*) were homogeneous for *Sr26*. For the *Sr2* gene, based on single gene ratio calculations and the Punnett square, it became apparent that only homozygous susceptible (FF) and heterozygous susceptible (Ff) individuals were possible. Therefore it was assumed that in all individuals where only the *Sr2* allele was amplified, these individuals in fact were heterogeneous, containing the null allele of *Kariega*. This further confirmed that it would not be possible to get *Sr2* resistance expressed until further self pollination of the selected seed with all markers present and thereafter phenotypic testing for the presence of *Sr2*. The presence of the null allele in *Kariega* will complicate MAS selection for the *Sr2* gene in future studies. With the above mentioned gene complications for *Lr19*, *Sr26* and *Sr2* in mind, it is suggested that progeny tests should be done on the original parents, to confirm if the parents were pure breeding for the genes.

Marker data and genotypic frequency results of this study confirmed on marker level the possible successful pyramiding of six of the seven desired genes into a single genotype. However, the presence of the gene associated with the detected marker alleles need to be confirmed. Based on published results only, only three rust genes have been successfully pyramided (*Barloy et al.*, 2007). Potentially more genes might have been pyramided in unpublished reports. In this study, with the aid of MAS it was possible to pyramid four genes, *Sr26*, *Lr34*, *Lr19* and *Sr2* and two QTL, *QYr.sgi-7D* and *QYr.sgi-2B.1* within a two

year period. There is a 50% chance that the *YrSp* gene could be present in individuals containing all eight molecular markers. The presence of the *YrSp* gene will need to be confirmed through phenotypic screening in future. The potential use of these lines and molecular markers include the fact that both seedling resistance to leaf rust (*Lr19*), stem rust (*Sr26*) and stripe rust (*YrSp*) and adult stage resistance to leaf rust (*Lr34*), stem rust (*Sr2*) and stripe rust (*QYr.sgi-7D* and *QYr.sgi-2B.1*) could be confirmed. As these developed lines form the initial stage of developing pyramided derived lines, there is great potential for the future use of these lines in development of a number of pyramided lines with various rust resistance gene sources. It should be noted here that the presence of the all the associated trait makers does not necessarily mean with a 100% chance that the genes/QTL are in fact present.

Lines containing all eight markers and some of the lines containing seven of the eight markers should all be planted out and selfed for a few generations in an attempt to acquire all genes in a homozygous state. The SSR and STS markers could be used throughout the selfing process to detect homozygous individuals. MAS would have to be accompanied by phenotypic screening for *Sr2* and *YrSp* resistance. This would form part of the second stage of a complex pyramiding scheme (Ishii and Yonezawa, 2007a; 2007b). Once in a fixed state, the use of these lines in future breeding programmes harnesses great potential in developing durable rust resistance within a marketable cultivar. The presence of the desired rust resistance genes in lines identified using MAS should be confirmed using phenotypic screening and subsequent field trials.

This study was successful in using the selected SSR and STS markers associated to the desired rust genes in a MAS breeding programme designed to pyramid five different rust resistance genes and two QTL. This proved that in South Africa a study of this nature is possible. The future uses of the developed rust resistance gene pyramided lines of this study are countless. The use of these lines in combating the continual threat of wheat rusts in some manner should be helpful in future.

3.5 References

Allard RW (1960) Principles of plant breeding. **New York: Wiley pp 485.**

Bariana HS, Brown GN, Ahmed NU, Khatkar S, Conner RL, Wellings CR, Haley S, Sharp PJ and Laroche A (2002) Characterisation of *Triticum vavilovii*-derived stripe rust resistance using genetic, cytogenetics and molecular analysis and its marker-assisted selection. ***Theoretical and Applied Genetics* 104: 315-320.**

Barloy D, Lemoine J, Abelard P, Tanguy AM, Rivoal R and Jahier J (2007) Marker-assisted pyramiding of two cereal cyst nematode resistance genes from *Aegilops variabilis* in wheat. ***Molecular Breeding* 20: 31-40.**

Cobb BD and Clarkson JM (1994) A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. ***Nucleic Acid Research* 22: 3801-3805.**

Hayden MJ and Sharp PJ (2001) Targeted development of informative microsatellite (SSR) markers. ***Nucleic Acid Research* 29: 1-6.**

Hayden MJ, Kuchel H and Chalmers KL (2004) Sequenced tagged microsatellites for Xgwm533 locus provide new diagnostic markers to select for the presence of the stem rust resistance gene *Sr2* in bread wheat. ***Theoretical and Applied Genetics* 109: 1641-1647.**

Herselman L (2003) Genetic variation among Southern African cultivated peanut (*Arachis hypogaea* L.) genotypes as revealed by AFLP analysis. ***Euphytica* 133: 319–327.**

Hiebert CW, Thomas JB, Somers DJ, McCallum BD and Fox SL (2007) Microsatellite mapping of adult-plant leaf rust resistance gene *Lr22a* in wheat. ***Theoretical and Applied Genetics* 115: 877-884.**

<http://www.changbioscience.com/genetics/punnett.html>. Cited October 2007.

Ishii T and Yonezawa K (2007a) Optimization of marker-based procedures for pyramiding genes from multiple donor lines: I. Schedule of crossing between the donor lines. *Crop Science* **47**: 537-546.

Ishii T and Yonezawa K (2007b) Optimization of marker-based procedures for pyramiding genes from multiple donor lines: II. Strategies for selecting the objective homozygous plant. *Crop Science* **47**: 1878-1886.

Knapp SJ (1998) Marker-assisted selection as a strategy for increasing the probability of selecting superior genotypes. *Crop Science* **38**: 1164-1174.

Kuraparthi V, Chhuneja P, Dhaliwal HS, Kaur S, Bowden RL and Gill BS (2007) Characterization and mapping of cryptic alien introgression from *Aegilops geniculata* with new leaf rust and stripe rust resistance genes *Lr57* and *Yr40* in wheat. *Theoretical and Applied Genetics* **114**: 1379-1389.

Lagudah ES, McFadden H, Singh RP, Huerta-Espino J, Bariana HS and Spielmeier W (2006) Molecular genetic characterization of the *Lr34/Yr18* slow rusting resistance gene region in wheat. *Theoretical and Applied Genetics* **114**: 21-30.

Lin F and Chen XM (2007) Genetics and molecular mapping of genes for race-specific all-stage resistance and non-race-specific high-temperature adult-plant resistance to stripe rust in spring wheat cultivar *Alpowa*. *Theoretical and Applied Genetics* **114**: 1277-1287.

Mago R, Bariana HS, Dundas IS, Spielmeier W, Lawrence GJ, Pryor AJ and Ellis JG (2005) Development of PCR markers for the selection of wheat stem rust resistance genes *Sr24* and *Sr26* in diverse wheat germplasm. *Theoretical and Applied Genetics* **111**: 496-504.

Mathews LJ (2005) A host-pathogen study of stripe rust resistance in *Triticum aestivum*. *M.Sc dissertation, University of the Free State, South Africa*.

Mohan M, Nair S, Bhagwat A, Krishna TG, Yano M, Bhatia CR and Sasaki T (1997) Genome mapping, molecular markers and marker-assisted selection in crop plants. *Molecular Breeding* **3**: 87-103.

Powell W, Machray GC and Provan J (1996) Polymorphism revealed by simple sequence repeats. *Trends in Plant Science* **1**: 215-222.

Prins R, Groenewald JZ, Marais GF, Snape JW and Koeber RMD (2001) AFLP and STS tagging of *Lr19*, a gene conferring resistance to leaf rust in wheat. *Theoretical and Applied Genetics* **103**: 618-624.

Ramburan VP, Pretorius ZA, Louw JH, Boyd LA, Smith PH, Boshof WHP and Prins R (2004) A genetic analysis of adult plant resistance to stripe rust in wheat cultivar Kariega. *Theoretical and Applied Genetics* **108**: 1426-1423.

Röder MS, Korzun V, Wendehake K, Plaschke J, Tixier M, Leroy P and Ganal MW (1998) A microsatellite map of wheat. *Genetics* **149**: 2007-2023.

Saghai-Maroo MA, Soliman KM, Jorgensen RA and Allard RW (1984) Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceeding of the National Academy of Sciences USA* **81**: 8014–8018.

Singh D, Park RF and McIntosh RA (2007) Characterisation of wheat leaf rust resistance gene *Lr34* in Australian wheats using components of resistance and the linked molecular marker csLV34. *Australian Journal of Agricultural Research* **58**: 1106-1114.

Singh RP, Hodson DP, Jin Y, Huerta-Espino J, Kinyua MG, Wanyera R, Njau P and Ward RW (2006) Current status, likely migration and strategies to migrate the threat to wheat production from race Ug99 (TTKS) of stem rust pathogen. <http://www.cababstractsplus.org/cabreviews>. Cited July 2006.

Somers DJ, Isaac P and Edwards K (2004) A high-density microsatellite consensus map for bread wheat. *Theoretical and Applied Genetics* **109**: 1105-1114.

Spielmeyer W, Sharp PJ and Lagudah ES (2003) Identification and validation of markers linked to broad-spectrum stem rust resistance gene *Sr2* in wheat. *Crop Science* **43**: 333-336.

Taguchi G (1986) Introduction to quality engineering: Designing quality into products and processes. *Japan, Asian productivity organization pp 191*.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.

Wang J, Chapman SC, Bonnett DG, Rebetzke GJ and Crouch J (2007) Application of population genetic theory and simulation models to efficiently pyramid multiple genes via marker-assisted selection. *Crop Science* **47**: 582-590.

Chapter 4

General conclusions and perspectives

Wheat is one of the most important economical food crops in South Africa and the world (Bajaj, 1990; Curtis *et al.*, 2002; Khan *et al.*, 2005). As any crop, wheat has many diseases that reduce grain quality and yield loss (Scott, 1990). Wheat rusts (leaf, stem and stripe rust) are a group of important diseases that pose a major threat to wheat production locally and worldwide in all areas where wheat is grown (Boukhatem *et al.*, 2002; Khan *et al.*, 2005; Singh *et al.*, 2005; Kuraparthi *et al.*, 2007).

Over the years it has become known that the most efficient and environmentally sustainable manner for breeders and scientists to reduce losses due to rust pathogens is the development of resistant cultivars (Fahima *et al.*, 1998; Boukhatem *et al.*, 2002; Kuraparthi *et al.*, 2007). In the past there have been well documented resistant wheat cultivars. However, with the ongoing mutational events and genetic recombination of rust pathogens, long distance spore travel and new race virulence, the need for improved cultivars with durable rust resistance exists (Kolmer, 2005). Introgression of resistance genes from different related wild or cultivated species has provided an opportunity for new sources of genetic diversity for rust resistance in wheat (Khan *et al.*, 2005). In search of improved durable rust resistance it is widely thought that different gene combinations or gene pyramids would confer more durable resistance than the same genes deployed singularly (Liu *et al.*, 2000; Hiebert *et al.*, 2007).

Molecular marker approaches have improved the possible tracking of species-specific alleles in interspecific hybrid backgrounds and the investigation of recombination of chromosomal segments between parents. Recombination is crucial in the transfer of targeted genes/alleles from wild species and different cultivar sources into a single background (Edmé *et al.*, 2006). Edmé *et al.* (2006) reported on the importance and value that marker segregation analysis studies can play in the assessment of developed hybrids. During the present study intensive marker data was generated and based on segregation analysis

it was possible to trace and determine whether the desired recombination had in fact taken place. Based on the marker segregation patterns it was possible to conclude that the crossing scheme of the project was successful and originally assumed theoretical parental genotypes were accurately interpreted.

Wang *et al.* (2007) reported that many breeding programmes around the world for a range of crops, are using selected molecular markers to screen for one to several genes of interest. However, the true practical application of these markers will be extended further once accumulated into a single genotype to construct high-degree gene-pyramided lines (Ishii and Yonezawa, 2007b). The development of such lines would have high practical use as parents for a new inbred as well as hybrid market cultivars (Ishii and Yonezawa, 2007a). Marker-based gene pyramiding should be rather straight-forward when two or three donor lines are involved. However, pyramiding of many genes, such as five genes and two QTLs during this study from four donor lines, becomes far more complex and intensive as reported by Ishii and Yonezawa, (2007a). During the present study step one of a gene-pyramiding scheme, namely obtaining all markers linked to the target genes in a heterozygote state with in complex pyramiding programme (Ishii and Yonezawa 2007b) was achieved. This was evident from the genotypic fingerprinting results which confirmed genes present in a heterozygous/heterogeneous (*Lr19*, *Sr2*, *Sr26*, *QYr.sgi-7D* and *QYr.sgi-2B.1*) or homozygous/homogeneous (*Sr26* and *Lr34*) state. In future step two of this pyramiding scheme would need to be carried out in terms of careful selection using the pyramided markers and phenotypic selection of individuals that are homozygous for the targeted genes, in order to fix the genes (Ishii and Yonezawa, 2007a; 2007b).

Between two and three resistance genes for different diseases have over the years been successfully pyramided into single or desired genotypes (Barloy *et al.*, 2007). Three leaf rust genes were pyramided into a single wheat cultivar in 1997 through traditional breeding methods (Barloy *et al.*, 2007). In 2001 a report was released on the successful pyramiding of three powdery mildew resistance genes (Liu *et al.*, 2000). Barloy *et al.* (2007) reported the successful transfer of two nematode resistance genes into a number of lines. Based on limited

documentation it seems that a small number of genes have been successfully pyramided simultaneously. Past studies have targeted a single wheat rust and used related resistance genes in gene pyramiding attempts. However, in this study pyramiding several genes against leaf, stem and stripe rust into a single line was confirmed.

This study confirmed pyramiding of different rust genes and QTL (*Sr2*, *Sr26*, *Lr19*, *Lr34*, *QYr.sgi-7D* and *QYr.sgi-2B.1*) into a single genotype from four donor lines. This combination of slow rusting, non-host specific APR genes, host-specific seedling resistance genes and QTL regions go a long way towards achieving the desired goal of durable resistance to rusts in wheat. The potential use of the pyramided lines containing four genes and two QTL are limitless. A higher number of resistance genes are furthermore possibly present in the pyramided lines as some of these pyramided genes are linked to other resistance genes at the same loci. It has been well documented that *Lr34* is tightly linked to the slow rusting stripe rust resistance gene *Yr18* on chromosome 7DS (Spielmeyer *et al.*, 2005; Lagudah *et al.*, 2006). Spielmeyer *et al.* (2005) reported that *Lr34* and *Yr18* have provided durable resistance to leaf and stripe rust offering a partial level of resistance in a number of cultivars grown throughout the world. Sharp *et al.* (2001) furthermore reported that the slow rusting adult resistance gene *Sr2* is linked to leaf rust resistance gene *Lr27*. However, *Lr27* alone is an ineffective gene, it will only confer resistance in the presence of the complementary gene *Lr31*. The *Sr25* gene might also be present in the pyramided lines. The chromosome segment from *Thinopyrum ponticum* transferred into common wheat carrying leaf rust resistance gene *Lr19* is thought to contain the stem rust resistance gene *Sr25* (Prins *et al.*, 1997; Prins and Marais, 1998). It is reported that this *Lr19* translocated segment does not pair with the homoeologous chromosome 7DL arm of common wheat during meiosis in heterozygotes, resulting in the transmission of the entire translocated segment as a single, large linkage of genes (Prins *et al.*, 1997). However, it must be noted that the *CSLr19-149-299* line used in this study, developed by Prof GF Marais, contained a reduced translocated segment. Therefore, *Sr25* although closely associated on the *Lr19* translocated segment could possibly be missing from the new reduced segment. It can be assumed that the developed

pyramided lines from this study contain *Sr2*, *Sr26*, *Lr19*, *Lr34*, *QYr.sgi-7D* and *QYr.sgi-2B.1*, with a 50% chance of containing *YrSp* and possibly also the linked *Lr27*, *Sr25* and *Yr18* genes. These identified pyramided lines should in the future be screened using molecular and phenotypic markers to confirm the potential presence of these genes.

It is vital that these identified pyramided lines are self pollinated for a number of generations in an attempt to obtain all genes in a homozygous/homogenises state. This is especially necessary for *Sr2* resistance gene expression, due to the recessive nature of this gene. Homozygosity will prevent the loss of some of the pyramided resistance genes and enable the use of these pyramided lines in future breeding programmes involving backcrosses to a local South African commercial cultivar. The performance of these lines in normal field conditions combined with phenotypic screening to confirm the presence of the genes/QTL will offer interesting information regarding the success of the gene-pyramiding scheme employed in this study.

Results of this study are particularly applicable with the current state of world affairs with the initiation of the Global Rust Initiative to combat the potential threat that the stem rust race Ug99 poses to a large percentage of world wheat germplasm. With low numbers of reported resistant cultivars with resistance to Ug99, a source of rust pyramided lines as developed in this study could aid in combating local and global threats from Ug99 and any new virulent rust races.

Ultimately this study proved that, within a two year period, gene-pyramiding of many targeted rust resistance genes from four donor lines, against the three wheat rusts, using specifically selected SSR and STS markers, is possible.

References

Bajaj YPS (ed) (1990) *Biotechnology in Agriculture and Forestry 13 – Wheat*. Springer-Verlag pp 687.

Barloy D, Lemoine J, Abelard P, Tanguy AM, Rivoal R and Jahier J (2007) Marker-assisted pyramiding of two cereal cyst nematode resistance genes from *Aegilops variabilis* in wheat. *Molecular Breeding* 20: 31-40.

Boukhatem N, Baret PV, Mingoet D and Jacquemin JM (2002) Quantitative trait loci for resistance against yellow rust in two wheat-derived recombinant inbred line populations. *Theoretical and Applied Genetics* 104: 111-118.

Curtis BC, Rajaram S and Macpherson HG (eds) (2002) Bread wheat improvement and production. *Food and Agriculture Organization of the United Nations, Rome Italy* pp 554.

Edmé SJ, Glynn NG and Comstock JC (2006) Genetic segregation of microsatellite markers in *Saccharum officinarum* and *S. spontaneum*. *Heredity* 97: 366-375.

Fahima T, Röder MS, Grama A and Nevo E (1998) Microsatellite DNA polymorphism divergence in *Triticum dicoccoides* accessions highly resistant to yellow rust. *Theoretical and Applied Genetics* 96: 187-195.

Hiebert CW, Thomas JB, Somers DJ, McCallum BD and Fox SL (2007) Microsatellite mapping of adult-plant leaf rust resistance gene *Lr22a* in wheat. *Theoretical and Applied Genetics* 115: 877-884.

Ishii T and Yonezawa K (2007a) Optimization of the marker-based procedures for pyramiding genes from multiple donor lines: I. Schedule of crossing between the donor lines. *Crop Science* 47: 537-546.

Ishii T and Yonezawa K (2007b) Optimization of the marker-based procedures for pyramiding genes from multiple donor lines: II. Strategies for selecting the objective homozygous plant. *Crop Science* **47**: 1878-1886.

Khan RR, Bariana HS, Dholakia BB, Naik SV, Lagu MD, Rathjen AJ, Bhavani S and Gupta VS (2005) Molecular mapping of stem and leaf rust resistance in wheat. *Theoretical and Applied Genetics* **111**: 846-850.

Kolmer JA (2005) Tracking wheat rust on continental scale. *Current opinion in Plant Biology* **8**: 441-449.

Kuraparthi V, Chhuneja P, Dhaliwal HS, Kaur S, Bowden RL and Gill BS (2007) Characterization and mapping of cryptic alien introgression from *Aegilops geniculata* with new leaf rust and stripe rust resistance genes *Lr57* and *Yr40* in wheat. *Theoretical and Applied Genetics* **114**: 1379-1389.

Lagudah ES, McFadden H, Singh RP, Huerta-Espino J, Bariana HS and Spielmeier W (2006) Molecular genetic characterization of the *Lr34/Yr18* slow rusting resistance gene region in wheat. *Theoretical and Applied Genetics* **114**: 21-30.

Liu J, Liu D, Tao W, Li W, Wang S, Chen P, Cheng S and Gao D (2000) Molecular marker-facilitated pyramiding of different genes for powder mildew resistance in wheat. *Plant Breeding* **119**: 21-24.

Prins R and Marais GF (1998) An extended deletion map of the *Lr19* translocation and modified forms. *Euphytica* **103**: 95-102.

Prins R, Marais GF, Pretorius ZA, Janse BJH and Marais AS (1997) A study of modified forms of the *Lr19* translocation of common wheat. *Theoretical and Applied Genetics* **95**: 424-430.

Scott DB (1990) Wheat diseases in South Africa. *Pretoria: Department of Agricultural Development pp 62.*

Sharp PJ, Johnston S, Brown G, McIntosh RA, Pallotta M, Carter M, Bariana HS, Khatkar S, Lagudah ES, Singh RP, Khairallah M, Potter R and Jones MGK (2001) Validation of molecular markers for wheat breeding. *Australian Journal of Agriculture* **52**: 1357-1366.

Singh RP, Huerta-Espino J and William HM (2005) Genetics and breeding for durable resistance to leaf and stripe rusts in wheat. *Turkish Journal of Agriculture* **29**: 121-127.

Spielmeyer W, McIntosh RA, Kolmer J and Lagudah ES (2005) Powdery mildew resistance and *Lr34/Yr18* genes for durable resistance to leaf and stripe rust co-segregate at locus on the short arm of chromosome 7D of wheat. *Theoretical and Applied Genetics* **111**: 731-735.

Wang J, Chapman SC, Bonnett DG, Rebetzke GJ and Crouch J (2007) Application of population genetic theory and simulation models to efficiently pyramid multiple genes via marker-assisted selection. *Crop Science* **47**: 582-588.

Summary

Wheat (*Triticum aestivum* L.) is widely cultivated over large areas and is an important food crop worldwide. Wheat is extensively used during the production of many different types of foods. Wheat rusts (leaf, stem and stripe rust) are important foliar diseases of wheat worldwide, causing large losses and damage to the wheat industry. The ability of these rust pathogens to change and be dispersed over long distances pose a continual global threat. Annually millions around the world are spent on fungicides in an attempt to control wheat rusts. Resistant cultivars have proven to be the most effective, economical and environmentally friendly form of rust control. Although many resistant cultivars have been developed historically, a need for more durable resistance exists. The application of molecular markers and marker-assisted selection (MAS) strategies in breeding programmes can support plant breeders in accomplishing pyramiding of several rust resistant genes into new cultivars.

The aim of this study was to pyramid seven rust resistant genes/QTL (leaf, stripe and stem rust) into a single genotype using five SSR, three STS and two AFLP markers. The study focused on wheat genes applicable to the local wheat industry and markers used and/or developed in South African breeding programmes. In this study four bread wheat cultivars or lines (*AvocetYrsp*, *Blade*, *CSLr19-149-299* and *Kariega*) were used as parental sources of five resistance genes (*Sr2*, *Sr26*, *YrSp*, *Lr19* and *Lr34*) and two QTL (*QYr.sgi-7D* and *QYr.sgi-2B*). Selection after each cross was done using a MAS approach with SSR and STS markers linked to the different resistant genes/QTL. The study was conducted over a two year period, involving the development of two different sets of F₁ offspring and one double cross generation from a series of directional crosses.

Before crosses were made, the presence of the expected rust resistance genes was confirmed in the parental lines using specific SSR, STS and AFLP markers. The SSR and STS markers amplified the expected allele sizes in the parental lines, except for the unexpected detection of the *Lr34* gene in *AvocetYrsp*.

Results indicated that the *Sr2* marker was not consistently present in Blade, suggesting the Blade cultivar was heterogeneous for *Sr2*. The AFLP markers linked to the *YrSp* resistance gene did not detect differences between the parental lines and were excluded from further experiments. The F₁ generations were screened with one SSR or STS marker each to identify successful crosses. Genotyping of the F₁ generations indicated that on average, 85.5% of the tested F₁'s were true hybrids. Phenotypic screening was done on the parental lines and F₁ individuals for the three rust types and confirmed the presence of the expected genes in the parental lines as well as selected F₁ individuals.

A total of 900 individuals of the double cross generation were screened with five SSR and three STS markers associated with resistance genes and QTL to identify whether gene pyramiding within a single genotype was successful. The number of individual plants of the double cross population containing markers linked to the desired resistance gene(s)/QTL ranged from two individuals containing none of the markers to three individuals containing all eight markers. The three individuals containing eight markers confirmed the presence of markers associated to the presence of the four single genes (*Sr2*, *Sr26*, *Lr19* and *Lr34*) and two QTL (*QYr.sgi-7D* and *QYr.sgi-2B.1*). Due to the inefficiency of the AFLP markers, the presence of the seventh gene (*YrSp*) could not be confirmed on genotypic level. The future uses of the developed rust resistance gene pyramided lines of this study are countless. The use of these lines in combating the continual threat of wheat rusts in some manner should be helpful in future.

Keywords: amplified fragment length polymorphism (AFLP), DNA, diseases, durable resistance, genotyping, phenotypic screening, sequence-tagged site (STS), simple sequence repeats (SSR), virulence.

Opsomming

Koring (*Triticum aestivum* L.) word algemeen oor groot gebiede verbou en is 'n belangrike voedselgewas wêreldwyd. Koring word op groot skaal vir die produksie van baie verskillende tipes kossoorte gebruik. Een van die belangrikste koringblaar siektes wêreldwyd is roes (blaar-, stam- en streeproes) wat groot verliese en skade in die koringbedryf veroorsaak. Die vermoë van hierdie roespatogene om te verander en oor lang afstande te verprei hou wêreldwyd 'n deurlopende gevaar in. In 'n poging om roes op koring te beheer word daar wêreldwyd miljoene jaarliks aan swamdoders spandeer. Daar is gevind dat weerstandbiedende kultivars die mees effektiewe, ekonomiese en omgewingsvriendelike vorm van roes beheer is. Alhoewel daar in die verlede reeds baie weerstandbiedende kultivars ontwikkel is, bestaan daar steeds 'n behoefte vir verbeterde standhoudende weerstand. Die gebruik van molekulêre merkers en merker-ondersteunde seleksie (MAS) strategieë in teelprogramme kan plantetelers help om verskeie roes weerstandsgene in nuwe kultivars te stapel.

Die doel van hierdie studie was om sewe roes weerstandsgene/kwantitatiewe eienskap lokusse (QTL) (blaar-, streep- en stamroes) in 'n enkele genotipe te stapel deur van vyf mikrosatellietmerkers (SSR), drie volgorde-geteikende gebiede (STS) en twee geamplifiseerde fragment lengte polimorfisme (AFLP) merkers gebruik te maak. Die studie het op roesgene wat toepasbaar in die plaaslike koringindustrie, asook merkers wat in Suid-Afrikaanse teelprogramme gebruik en/of ontwikkel is, gefokus. In hierdie studie is vier broodkoring kultivars of lyne (*Avocet Yrsp*, *Blade*, *CSLr19-194-299* en *Kariega*) as ouer bronne vir vyf weerstandsgene (*Sr2*, *Sr26*, *YrSp*, *Lr19* en *Lr34*) en twee QTL (*QYr.sgi-7D* en *QYr.sgi-2B*) gebruik. Seleksies is na elke kruising gedoen deur van 'n MAS benadering gebruik te maak waar SSR en STS merkers gekoppel aan die verskillende weerstandsgene/QTL gebruik is. Die studie is oor 'n twee jaar periode uitgevoer en het die ontwikkeling van twee verskillende F₁ nageslagte en een dubbel-kruis generasie, vanaf 'n reeks spesifiek gerigte kruisings, behels.

Voordat kruisings gedoen is, is die teenwoordigheid van die verwagte roesgene in die ouerlyne bevestig deur van spesifieke SSR, STS en AFLP merkers gebruik te maak. Die SSR en STS merkers het die verwagte alleel groottes in die ouerlyne geamplifiseer, behalwe vir die onverwagte opsporing van die *Lr34* geen in *Avocet YrSp*. Resultate het aangetoon dat die *Sr2* merker nie deurgaans in Blade teenwoordig was nie. Die AFLP merkers gekoppel aan die *YrSp* weerstandsgene het nie verskille tussen die ouerlyne aangetoon nie en is nie in verdere eksperimente gebruik nie. Die F_1 generasies is afsonderlik met een SSR of STS merker elk getoets om suksesvolle kruisings te identifiseer. Genotipering van die F_1 generasies het aangetoon dat gemiddeld 85.5% van die getoetste F_1 individue wel ware hibriede was. Fenotipiese toetse vir al drie roestipes is op die ouerlyne en F_1 individue gedoen en het die teenwoordigheid van die verwagte gene in die ouerlyne, asook geselekteerde F_1 individue, bevestig.

'n Totaal van 900 individue van die dubbel-kruis generasie is met vyf SSR en drie STS merkers wat met die weerstandsgene en QTL geassosieer is, getoets om te bepaal of geenstapeling binne 'n enkele individu wel suksesvol was. Die aantal individuele plante van die dubbel-kruis populasie wat merkers gekoppel aan die verlangde gene/QTL bevat het, het gewissel van twee individue wat geen merkers bevat het nie tot drie individue wat al agt merkers bevat het. Die drie individue wat al agt merkers bevat het, het die teenwoordigheid van merkers geassosieer met die vier enkel gene (*Sr2*, *Sr26*, *Lr19* en *Lr34*) en twee QTL (*QYr.sgi-7D* en *QYr.sgi-2B.1*) bevestig. As gevolg van die ondoeltreffendheid van die AFLP merkers kon die teenwoordigheid van die sewende geen (*YrSp*) nie op genotipiese vlak bevestig word nie. Die toekomstige gebruik van die lyne met die gestapelde roes weerstandbiedende gene wat tydens die studie ontwikkel is, is ontelbaar. Hierdie lyne kan in die toekoms gebruik word om die deurlopende bedreiging van roes op koring in 'n mate te bestry.

Sleutelwoorde: DNA, fenotipiese toetse, geamplifiseerde fragment lengte polimorfisme, genotipering, mikrosatelliet merkers, siektes, standhoudende weerstand, virulensie, volgorde-geteikende gebiede.