Genetics of stem rust resistance in South African winter wheat varieties

by

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DECLARATION

I, Chemonges Martin, declare that the thesis that I herewith submit for the Doctoral Degree in Plant Breeding at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

…….. ……..

Chemonges Martin **Date**

DEDICATION

To my beloved wife Nancy Cherotich and children Kayla Martins Chelangat and Liana Martins Cheptoek.

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
APR	Adult plant resistance
ARC-SG	Agricultural Research Council, Small Grain
ASR	All stage resistance
Avr	Avirulence
BAC	Bacterial artificial chromosome
BC	Backcross
BIL	Backcross inbred line
bp	Base pair(s)
BSA	Bulked segregant analysis
CAPS	Cleaved amplified polymorphic sequence
CC	Coiled-coil
CDL	Cereal disease laboratory
CI	Coefficient of infection
CIMMYT	International Center for Maize and Wheat Improvement
cM	CentiMorgan(s)
CSSL	Chromosome segment substitution line
CTAB	Hexadecyltrimethylammonium bromide
DArT	Diversity array technology
DH	Doubled haploid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DS	Disease severity
EDTA	Ethylene-diaminetetraacetate
EST	Expressed sequence tag
EtBr	Ethidium bromide
F_1	First filial generation
F_2	Second filial generation
F_3	Third filial generation
FAM	6-Carboxylfluorescein
g	Gravitational force

GWAS	Genome wide association study
HEX	Phosphoramidite
ICIM	Inclusive composite internal mapping
Indel	Insertion or deletion
ISRTN07	2nd International stem rust trap nursery
IT	Infection type
K	Potasium
KASP	Kompetitive allele specific polymerase chain reaction
LD	Linkage disequilibrium
LOD	Logarithm of odds
LRGS	Low resolution wheat genome scan
MAS	Marker-assisted selection
$MgCl_2$	Magnesium chloride
ML	Maximum likelihood
MRMS	Moderately resistant and moderately susceptible
MRR	Moderately resistant and resistant
MSS	Moderately susceptible and susceptible
N	Nitrogen
NA	North American
NaCl	Sodium chloride
NGS	Next generation sequencing
NIL	Near isogenic line
NLR	Nucleotide-binding and leucine-rich repeats domains
P	Phosphorous
PCR	Polymerase chain reaction
Pgt	*Puccinia graminis* f. sp. *tritici*
PVE	Percentage of variation explained by phenotype
QTL	Quantitative trait loci
®	Registered
R	Resistant
RIL	Recombinant inbred line
RSB	Recurrent selection backcross
S	Susceptible

SA	South Africa
SCAR	Sequence characterised amplified region.
SNP	Single nucleotide polymorphism
spp.	Species
SSR	Simple sequence repeat
STARP	Semi-thermal asymmetric reverse polymerase chain reaction
STS	Sequence-tagged site
Ta	Annealing temperature
TACCA	Targeted chromosome-based cloning via long range assembly
Taq	*Thermus aquaticus*
TBE	Tris-HCl/Borate/EDTA
TE	Tris-HCl/EDTA
TEMED	N,N,N'N-tetramethylethylenediamine
Tris-HCl	Tris(hyroxymethyl) aminomethane hydrochloride
UFS	University of the Free State
UK	United Kingdom
TAE	Tris-HCl/Acetic acid/EDTA
URGI	Unité de Recherche Génomique Info
USA	United States of America
USDA-ARS	United States Department of Agriculture-Agricultural Research Services
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

LIST OF SI UNITS

%	Percentage
°C	Degrees Celsius
µl	Microlitre(s)
µM	Micromolar(s)
cm	Centimetre(s)
g	Gram(s)
h	Hour(s)
K	Kilo
ℓ	Litre(s)
M	Metre(s)
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar(s)
ng	Nanogram(s)
pH	Power of hydrogen
r/s	Revolutions per second
s	Second(s)
t/ha	Tonne(s) per hectare
U	Unit(s)
V	Volt(s)

ABSTRACT

Stem rust, caused by the fungus *Puccinia graminis* Pers. f. sp. *tritici* Eriks. and E. Henn (*Pgt*), is an important disease of wheat worldwide. Although resistant varieties have been utilised as an effective and efficient way of managing wheat stem rust, the emergence of new and virulent stem rust races threatens wheat cultivation. There is thus a continuous need to search for new sources of stem rust resistance. This study was conducted to elucidate the genetics of stem rust resistance in South African winter wheat varieties.

To understand the origin and inheritance of all stage resistance (ASR) to *Pgt* race PTKST, four resistant varieties Komati, Koonap, Limpopo and SST 387 were crossed with the stem rust susceptible wheat parent Line 37-07. Seedling phenotyping of the F_2 and F_3 offspring showed that a single dominant gene conferred stem rust resistance in each of the four populations. Allelism tests indicated that either the same gene or closely linked alleles confer resistance in the four wheat varieties. However, allelism tests with Norin 40 (*Sr42*) and CnsSrTmp (*SrTmp*) indicated that it is either a closely linked gene, *Sr42* or *SrTmp* that confers resistance to *Pgt* race PTKST. Multi-race phenotyping ruled out the involvement of *Sr42*, but suggested the likely presence of *SrTmp* among South African winter wheat varieties.

Simple sequence repeat (SSR) marker genotyping using bulk segregant analysis (BSA) in four F_2 mapping populations identified marker loci on chromosome 6DS linked to stem rust resistance in all four varieties. Linkage mapping identified two flanking SSR markers, and *barc183* and *wms4862* as closely linked [≤2.0 centiMorgan (cM)] to resistance gene(s) in two mapping populations (SST387/Line37-07 and Line37-07/Koonap). In the Line37-07/Komati mapping population, SSR markers *psp3200* and *barc183* were closely linked (≤2.9 cM). In the Line37-07/Limpopo mapping population, SSR markers *wms4528* and *barc183* were closely linked (≤0.9 cM). Two single nucleotide polymorphism (SNP) markers, *BS00085929* and *BS00085937*, detecting different alleles in the resistant parents, mapped distally to stem rust resistance gene(s) at an average of 8.4 and 9.2 cM, respectively. Quantitative trait loci (QTL) analysis indicated that *psp3200*, *wms4528*, *barc183* and *wms4862* flanked the stem rust resistance gene(s). Major QTL detected in Komati, SST 387, Koonap and Limpopo explained 73.0, 96.2, 71.4 and 85.2% of phenotypic variation for stem rust resistance to race PTKST, respectively. Flanking SSR markers, *wms4862* and *barc183*, were predictive of stem rust

resistance in advanced winter wheat lines, hence confirming the chromosome location and their usefulness in marker-assisted selection (MAS). This study represents the first report of markers developed for stem rust resistance genes in South African winter wheat varieties. Resistant genotypes tested negative for diagnostic SNP markers linked to *Sr42, SrTmp* and *SrCad* on chromosome 6DS. From the marker data it can be concluded that the mapped resistance gene(s) are possibly novel or allele(s) of *Sr42, SrTmp* and/or *SrCad*.

Identification and mapping of an adult plant resistance (APR) gene in the South African winter wheat variety PAN 3161 were conducted by field screening a F_2 population and F_3 families against *Pgt* race PTKST. SSR marker genotyping combined with BSA on 128 F_2 plants identified markers on the short arm of chromosome 4D as linked to a stem rust resistance gene. SSR marker *wmc720* flanked the APR gene *SrPan3161* distally at 1.8 cM. Another set of four co-segregating SSR markers *gpw7414, gpw8038, wmc52* and *cfd23* flanked the APR gene proximally at 1.8 cM. QTL analysis identified a single major QTL explaining 71.5% of the phenotypic variation for resistance to *Pgt* race PTKST. The flanking SSR markers *wmc52, cfd23* and *wmc720* were predictive of *SrPan3161* in F_3 families thus validating the chromosome location and their effectiveness in MAS.

Keywords: Allelism, Diagnostic, Inheritance, Markers, Marker-assisted selection, Resistance, Simple sequence repeat, Single nucleotide polymorphism, Stem rust, Wheat

CHAPTER 1

INTRODUCTION

Wheat is the second most important cereal crop in the world (FAOSTAT, 2017, 2018). It contributes 30% of the world's edible dry matter and 60% of the daily calorie intake in several developing countries (FAOSTAT, 2015). In 2017, wheat production in Africa accounted for only 3.5% of the world production, lacking behind other regions like Oceania (4.2%), the Americas (13.8%), Europe (35.0%) and Asia (43.5%) (FAOSTAT, 2017). In Africa, South Africa (SA) is the fifth largest wheat producer after Algeria (2.44 million tonnes), Ethiopia (4.83 million tonnes), Morocco (7.09 million tonnes) and Egypt (8.80 million tonnes). In 2017, SA (1.54 million tonnes) accounted for 5.7% of total wheat production in Africa but recorded a wheat negative trade deficit of 1.64 million tonnes (FAOSTAT, 2017). Although wheat production has been increasing, it is currently constrained by many abiotic and biotic stresses (Oerke, 2006; Keller et al., 2018). Generally, plant pathogens are estimated to reduce crop yields annually by 10-16% (Strange and Scott, 2005; Chakraborty and Newton, 2011). However, for wheat Savary et al. (2019) estimated the yield losses due to pathogens at 21.5%.

Stem (black) rust is among the major diseases of wheat and, historically, has severely affected wheat (Park, 2007; Singh et al., 2011; Khan et al., 2013). Yield losses caused by *Pgt* in the middle of the 20th century reached 20-30% in eastern and central Europe and many other countries including Australia, China and India (Leonard and Szabo, 2005). In Ethiopia, losses due to stem rust on susceptible wheat varieties were estimated as high as 70% (Bechere et al., 2000). Similar to other countries, wheat rusts are considered important biotic stress factors with the potential to cause serious economic losses in SA (Pretorius et al., 2007).

Hexaploid common bread wheat (*Triticum aestivum* L.), tetraploid durum wheat (*T. turgidum* spp. *durum* L.), barley (*Hordeum vulgare* L.), triticale (X *Triticosecale*) and wheat progenitors are primary hosts for the stem rust fungus (Roelfs et al., 1992; Mamo et al., 2015). In some areas where the alternate host (*Berberis vulgaris* L.) of *Pgt* exists, sexual recombination can give rise to more virulent races (Upadhyaya et al., 2015). The use of resistant varieties has been the most economical and environmentally friendly option of controlling stem rust (Gao et al., 2015; Kumssa et al., 2015; Olivera et al., 2018; Hatta et al., 2018).

Before 1999, stem rust outbreaks were rare except in Ethiopia where a major epidemic occurred on the widely grown wheat variety Enkoy in 1993 and 1994 (Singh et al., 2011). In 1999, a new stem rust race Ug99 was first discovered in Uganda (Pretorius et al., 2000). The discovery of Ug99 that is virulent to the commonly used *Sr31* resistance gene, has initiated global interest into combating the disease (Pretorius et al., 2012). At present, 13 variants of in the Ug99 race group have been described, differing in virulence for stem rust resistance genes *Sr9h*, *Sr21*, *Sr24*, *Sr31*, *Sr36*, and *SrTmp* (Singh et al., 2011, 2015; Pretorius et al., 2012; Fetch et al., 2016; Newcomb et al., 2016; Patpour et al., 2016a, 2016b, Bhavani et al., 2019). Variants in the Ug99 race group have been reported in 13 countries worldwide including Uganda, Egypt, Eritrea, Ethiopia, Iran, Kenya, Mozambique, Rwanda, SA, Sudan, Tanzania, Yemen and Zimbabwe (Singh et al., 2008; Nazari et al., 2009; Visser et al., 2011; Pretorius et al., 2012; Newcomb et al., 2016; http://rusttracker.cimmyt.org/?page_id=305). The stem rust race TTKSK is virulent to the commonly used stem rust resistance genes *Sr31* and *Sr38* and only 5-15% of wheat varieties were reported to be resistant against Ug99 (Jin and Singh, 2006; Singh et al., 2008, 2015; Bhavani et al., 2019).

It has been reported that many global wheat growing areas are environmentally conducive for the development of stem rust, and in many of these areas, susceptible varieties are being grown (Singh et al., 2011; Pardey et al., 2013). *Pgt* continues to evolve as was shown by emergence of a new virulent non-Ug99 race, TKTTF that was first detected in Turkey in 2007. During the 2013/2014 season in Ethiopia, this race TKTTF caused up to 100% yield loss on Digalu that was one of the most widely planted varieties (Olivera et al., 2012, 2015; Singh et al., 2015). *Pgt* is known to spread over long distances and wind trajectory studies have predicted the likely arrival of new, more virulent races in the bread baskets of the world (Hodson, 2011, Meyer et al., 2017a, 2017b, Allen-Sander et al., 2019; Visser et al., 2019). New *Pgt* races have recently appeared in Ethiopia (Olivera et al., 2015), Italy (Bhattacharya, 2017), Germany (Olivera Firpo et al., 2017) and United Kingdom (Lewis et al., 2018). Given the high virulence and geographical coverage of Ug99 and other *Pgt* races, they present a risk to wheat production in the major wheat growing regions. Although the damaging effects of stem rust can be mitigated by fungicide applications, the extra input costs and potential negative consequences of chemical treatments on the environment warrant the use of host resistance genes to control *Pgt* (Wanyera et al., 2009; Mamo et al., 2015; Soko et al., 2018).

Thus, discovery and deployment of new sources of resistance to stem rust should be explored, such as wild relatives (Bajgain et al., 2015, 2016; Guerrero-Chavez et al., 2015; Olivera et al., 2018). Presently, over 70 *Sr* genes have been characterised (Hatta et al., 2018; Saini et al., 2018; Aoun et al., 2019). Over 31 *Sr* genes are effective against at least one race of the Ug99 lineage (Singh et al., 2011, 2015; Rouse et al., 2014), of which the majority are from secondary and tertiary gene pools (Niu et al., 2011, 2014; Qi et al., 2011; Mago et al., 2013; Singh et al., 2015; Olivera et al., 2018), while only five *Sr* genes viz. *Sr28*, *Sr42*, *Sr57*, *SrTmp*, *SrCad* and *Sr9h* are derived from *T. aestivum* (Hiebert et al., 2011; Rouse et al., 2014). Currently, many designated and temporarily designated *Sr* genes namely *Sr13*, *Sr22*, *Sr23*, *Sr25*, *Sr26*, *Sr32*, *Sr33*, *Sr35*, *Sr38*, *Sr42*, *Sr47*, *Sr50*, *SrHuw234*, *SrND643*, *SrNing* and *SrYanac* are effective against Ug99 races and can be deployed in wheat using MAS (Bhavani et al., 2019). Because of limited genetic diversity for stem rust resistance in hexaploid wheat, many varieties remain susceptible to stem rust (Singh et al., 2011; Yu et al., 2015; Olivera et al., 2018).

Resistance genes deployed individually can be overcome by new virulent *Pgt* races (Pujol et al., 2015). Consequently, combining *Sr* genes into new wheat varieties is believed to result in increased durability of resistance (Singh et al., 2006, 2011, 2015). However, combining several alien genes into one variety will increase the total amount of alien chromatin that could lead to potential negative effects on yield stability and end-use quality (Liu et al., 2013; Yu et al., 2015). This can be mitigated by combining effective genes from the primary gene pool of wheat that are rarely associated with deleterious linkage drag (Bernardo et al., 2013; Bajgain et al., 2015; Guerrero-Chavez et al., 2015; Yu et al., 2015). Combining genes using conventional methods is difficult as it requires simultaneous testing of the same wheat breeding material with several different rust races before selection (Haile and Röder, 2013). It is furthermore difficult for breeding programmes to maintain all necessary rust races required for rust evaluations especially for quarantine races (Wu et al., 2009). Hence by using molecular markers it is possible to combine several resistance genes to achieve durable resistance (Zhang et al., 2019). Rust resistance genes can be tagged using tightly linked deoxyribonucleic acid (DNA) markers and selection based on these markers improves the efficiency of resistance breeding (Todorovska et al., 2009). Identifying molecular markers closely linked to resistance genes can result in rapid incorporation of multiple resistance genes into breeding lines (Lopez-Vera et al., 2014; Dunckel et al., 2015). These markers can be used to predict the presence of specific genes with high accuracy without the need for disease evaluation, thus helping with

the transfer of several genes into adapted germplasm (Tsilo et al., 2008, 2009; Bernardo et al., 2013).

In SA, a number of winter wheat varieties have been identified to possess resistance to stem rust. However, genes conferring resistance in these varieties are not well characterised (Figlan et al., 2014). Characterising previously identified and new sources of resistance will allow the development of varieties with effective gene combinations that are broadly resistant to stem rust (Hiebert et al., 2011; Bajgain et al., 2016). Race specific resistance is the most utilised source of resistance for stem rust (Haile and Röder, 2013; Bajgain et al., 2016). Several of the APR genes confer minor effects with 5-20% reduction in disease severity (Bajgain et al., 2016). Combination of APR and/or major genes should be a more attractive, both farmer- and environmentally-friendly, rust control strategy (Leonard and Szabo, 2005; Haile and Röder, 2013; Bajgain et al., 2016). Therefore, understanding the inheritance of stem rust resistance genes and developing markers linked to these genes will improve the efficiency of identifying and deploying these genes in South African wheat varieties.

This study was conducted to elucidate the genetics of stem rust resistance in South African winter wheat varieties and the key objectives were: (1) Determine the mode of inheritance of stem rust resistance genes in South African winter wheat varieties; (2) Evaluate seedling and adult plant stem rust resistance in South African winter wheat varieties; (3) Screen resistant varieties for known stem rust resistance genes using molecular markers; (4) Identify molecular markers closely linked to gene(s)/QTL conferring resistance to stem rust in South African winter wheat varieties and (5) Evaluate and identify new genes for resistance to stem rust in South African winter wheat varieties.

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CHAPTER 2

LITERATURE REVIEW

2.1 General introduction

Wheat is ranked the second most important cereal crop in the world (FAOSTAT, 2016). It contributes 20% of protein and 21% of food calories to over 4.5 billion people living in 94 developing countries (Braun et al., 2010), where it is used in making an array of food products such as bread, pastas, injera, cakes and cookies (Pena, 2002). It provides 60% of the daily calorie intake and 30% of the world's edible dry matter in many developing countries (FAOSTAT, 2015). SA has been a net importer of 1.3 million tonnes of wheat for the past decade (SAGL, 2018). As the world population continues to increases significantly, a 60% increase in wheat production will be needed to meet food demand in developing countries by 2050 (Singh and Trethowan, 2007; Singh et al., 2007). Although wheat production has been increasing, it is currently constrained by many factors like low soil fertility, droughts, rusts etc.

2.2 Wheat production and economic importance in South Africa

Wheat is produced in 32 of SA's 36 crop production areas and the main-wheat producing provinces are Free State (summer rainfall), Western Cape (winter rainfall) and Northern Cape (irrigation) (DAFF, 2016; Nhemachena and Kirsten, 2017). Other important wheat production areas are Mpumalanga and North West that are mainly under irrigation (SAGL, 2012). SA records an annual wheat production of between 1.5 to 3 million tonnes and productivity rates of 2.0-2.5 t/ha under dry land and over 5 t/ha under irrigation (DAFF, 2016). During the past decade, SA has had a total wheat requirement of 2.9 million tonnes against a production of 1.6 million tonnes, hence a net importer of about 1.3 million tonnes. For the 2017/2018 cropping season, wheat production was at 1.54 million tonnes that was 19.6% lower than the 2016/2017 season. The average wheat productivity in the 2017/2018 cropping season was 3.12 t/ha (SAGL, 2018). Van Lill and Purchase (1995) reviewed the breeding of winter wheat varieties between 1930 and 1990 in SA and reported that yield and baking quality had improved by 87% and 20%, respectively. Similarly, SAGIS (2015) has also noted a significant increase in productivity of dryland wheat from less than 0.5 t/ha in 1936 to 3.5 t/ha in 2015. Nhemachena and Kirsten (2017) summarised the total number of wheat varieties that have been released in SA from 1891 to 2013 based on growth type and pointed out that winter wheat varieties (36) ranked third behind spring wheat (89) and facultative wheat (51).

2.3 Taxonomy, origin and genome structure of wheat

Wheat belongs to the grass family Gramineae (Poaceae) and genus *Triticum* (Zhang et al., 2006). It originated from the fertile crescent region of south-western Asia (Kingfisher, 2004). Wheat comprises of two major species, bread wheat (*T. aestivum*) and durum wheat (*T. turgidum* var. *durum*). Bread wheat contributes towards 95% of total wheat grown in the world (Belderok et al., 2000; Shewry and Hey, 2015). Wheat species occur in three ploidy levels:diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) (Handcook, 2004). The tetraploid and hexaploid genomes are allopolyploids because they have dissimilar genomes. Tetraploid wheat arose from the natural hybridisation between *T. monococcum* L. var. *monococcum* (AA) and *Aegilops speltoides* L. (BB). Hexaploid wheat resulted from a cross between tetraploid wheat (AABB) and the wild relative *Ae. tauschii* ((Coss.) Schmalh.) followed by the doubling of the chromosome number (Belderok et al., 2000). During meiosis, hexaploid wheat behaves as a diploid resulting in pairing of homologous chromosomes. This is due to the presence of a gene on chromosome 5B called *Ph1* that causes homologous pairing within the same genome. Hexaploid wheat has a complex genome with a size of 16×10^9 base pairs (bp) (Arumuganathan and Earle, 1991), with seven groups of chromosomes, each with three homologous chromosome pairs (Lagudah et al., 2001), with at least 80% repetitive DNA (Röder et al., 1998; Francki and Appels, 2002). Hence, wheat is characterised as among the most complex crop species, due to the unique size and structure of its genome (Langridge et al., 2001; Francki and Appels, 2002).

2.4 Rust diseases of wheat

Rust pathogens can infect more than one host (Voegele et al., 2009). Stem rust, caused by *Puccinia graminis*, has been shown to infect at least 365 cereal and grass species (Anikster, 1984). Wheat brown or leaf rust, caused by *P. triticina* Erikss. is the most common among the three wheat rusts (Roelfs et al., 1992). Leaf rust prefers low temperatures of between 10 and 30^{o}C compared to the stem rust pathogen favouring 15-35^{o}C. Leaf rust causes less severe yield losses of often <10% but these losses can be as high as 30% or greater during epidemics (Roelfs et al., 1992). Stem rust is recorded as the most devastating of all wheat rusts that can cause up to 100% yield losses. Stripe or yellow rust, caused by *P. striiformis* Westend f. sp. *tritici* Erikss. is predominantly a disease of wheat grown in cool environments (2-15^{o}C) and can result in losses of 50-100% (Roelfs et al., 1992).

2.4.1 Wheat stem rust

2.4.1.1 Life cycle of wheat stem rust

The wheat stem rust pathogen reproduces by both sexual and asexual means. The wheat stem rust pathogen is a heteroecious fungus that requires two unrelated hosts to complete its lifecycle; the gramineous or primary host (wheat, barley, triticale) and the alternate host (*Berberis* spp.). *Pgt* produces thick-walled, two-celled teliospores towards the end of the wheat growing season. Initially each teliospore cell is dikaryotic but karyogamy happens as teliospores mature. Matured teliospores will not be dispersed immediately but remain dormant on infected wheat straw up to the onset of the spring season, where its germination coincides with new growth of the alternative host (Roelfs, 1985; Roelfs and Groth, 1988). Meiosis occurs after karyogamy; however, it is stopped during the dormancy period (Boehm et al., 1992). Each teliospore produces a basidium that undergoes meiosis to produce four haploid basidiospores that are dispersed by wind to infect barberry plants (Leonard and Szabo, 2005). Basidiospores usually infect the upper surface of barberry leaves producing flask-shaped pycnia. Pycnia then produces pcyniospores that are exuded in nectar and either dispersed by insects or rain. Pycniospores that usually consist of a single haploid nucleus with surrounding cytoplasm, serve as male gametes. While the hyphae at the top of pycnia serve as the female gametes. Two mating types; + and - with monogenic genetic control are believed to exist (Roelfs, 1985). Serving as male gametes, pycniospores are brought into contact with haploid females (n), flexuous hyphae of the opposite mating type that extrude from the top of the pycnium (Anikster et al., 1999). A dikaryon (n+n) consisting of two haploid nuclei is formed and the resulting hypae grows throughout the leaf mesophyll to produce an aecium on the abaxial leaf surface. From the aecium, single celled, dikaryotic (n+n) aeciospores are produced that can then infect the wheat host (Figure 2.1; Leonard and Szabo, 2005).

In the presence of alternate host, the primary infection of wheat is by aeciospores that infect and produce hyphae within the host (Kolmer et al., 2007). These hyphae then produce uredinia that yield dikaryotic urediniospores (n+n) that represent the asexual stage of the life cycle. In many wheat-growing regions, where the alternate host is not present, the primary source of infection are windblown urediniospores (Kolmer et al., 2007). Urediniospores re-infect the host during the growing season and these infections cause the principal damage to wheat plants resulting in yield losses. Upon maturity of the host, teliospores (n+n) are produced that will overwinter and begin the cycle the following growing season (Leonard and Szabo, 2005).

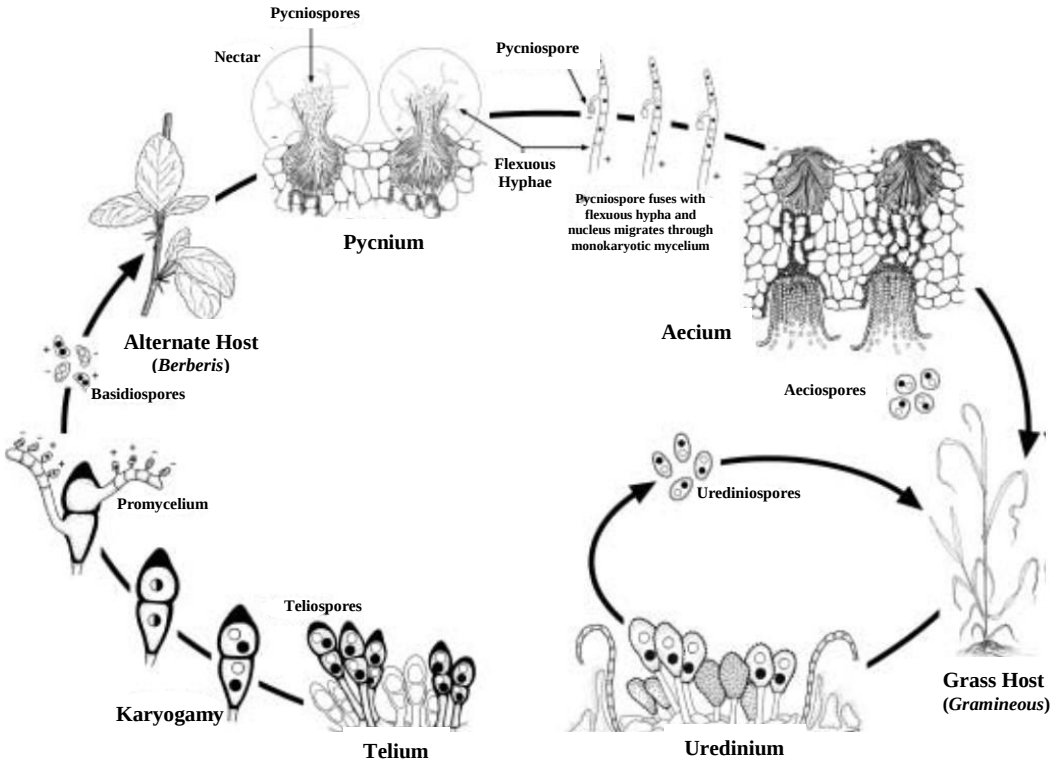

Figure 2.1 Life cycle of the stem rust fungus, *Puccinia graminis* (Source: Leonard and Szabo, 2005).

2.4.1.2 Significance of stem rust

Pgt infections are favoured by warmer weather conditions, however infections on susceptible wheat genotypes can happen over a wider range of geographic regions. As infection of stems become severe, this affects the flow of nutrients to wheat heads, hence resulting in formation of shrivelled grains (Leonard and Szabo, 2005). Roelfs et al. (1992) noted that *Pgt* infected stems become weakened and are hence prone to lodging, leading to further loss of grains.

Stem rust is an economically important disease of wheat globally, causing substantial yield losses up to 100% under prolonged severe epidemics (Admassu and Fekadu, 2005; Park, 2007). Yield losses caused by stem rust races in the mid of the 20th century reached 20-30% in eastern and central Europe and many other countries including Australia, China and India (Leonard and Szabo, 2005). The last stem rust epidemic in SA was recorded in 1984 in the Western Cape Province where a 60% susceptibility was observed on the *Sr24*-derived cultivars SST44 and Gamka (Le Roux and Rijkenberg, 1987b). Similarly, during the period of 1983 to 1985, stem rust caused yied losses of at least 40% in SA (Le Roux and Rijkenberg, 1987a, 1987b;

Pretorius, 1983). In 2004, the Agricultural Research Council, Small Grain (ARC-SG) of SA reported that 26% and 56% of winter and spring wheat types, respectively, expressed susceptibility to at least one of the tested stem rust races (Pretorius et al., 2007). Currently, Soko et al. (2018) observed a yield reduction of 47.9% due to *Pgt* on the susceptible parent Line 37-07. Better protection against *Pgt* was observed in cultivars containing ASR and APR with yield reductions of 6.4% and 19.5%, respectively (Soko et al., 2018).

Previously, stem rust control was believed to have been sustainably achieved globally (Singh et al., 2011a), but this scenario changed when a virulent *Pgt* race Ug99 (TTKSK) was detected in Uganda in 1998, that rendered the most widely used stem rust resistance gene (*Sr31*) ineffective (Pretorius et al., 2000). With emergence of this new stem rust race TTKSK, named following the North American (NA) stem rust differential set (Jin et al., 2007), studies have shown that only 5-15% of wheat breeding germplasm stocks worldwide are resistant (He et al., 2008; Singh et al., 2015). It was further reported that most of the global wheat growing areas are environmentally conducive for the development of stem rust, and in many of these areas, susceptible varieties are grown (Pardey et al., 2013), hence a great risk for wheat production.

2.4.2 Diversity and geographical spread of Ug99 and other important stem rust races

To date, 13 variants of the stem rust race Ug99 have been described in Africa and Asia (Bhavani et al., 2019). Historical data has shown that prevalent *Pgt* races change from year to year and geographical regions (Table 2.1).

2.4.3 Diversity within wheat stem rust races in South Africa

Since the early 1980s, over 30 wheat and triticale *Pgt* races have been documented in SA (Figlan et al., 2014; Terefe et al., 2016). Early *Pgt* pathotyping in SA in 1922 and 1939 led to the identification of two standard races, 34 and 21, respectively (Pretorius et al., 2007). Stem rust surveys conducted from 1981 to 1985 identified 38 *Pgt* races (Le Roux and Rijkenberg, 1987b).

Table 2.1 Identification of virulent stem rust races in different geographical regions

Virulent *Pgt* race	Country	Year	Reference
Ug99 (TTKSK)	Uganda	1998	Pretorius et al., 2000
TPMK or race 15B	USA	1953/54	Leonard, 2001
TPMK, RCRS, QFCS, QCCS, QTHJ, QFBS, RKMQ, RKQQ and RCMS	USA	1997/98	McVey et al., 2002
QFCS, TTTT, QFCN, QCCJ and MCCF	USA	2003	Jin, 2005
QFCS, MCCF, MCCD, TPMK, QCCN and TTTT	USA	2004	Kolmer et al., 2007
pt 34-1 and 2-7 +38	Australia	2001	Park, 2007, 2008
JRCQC, TRTTF and TTKSK	Ethiopia	2009	Olivera et al., 2012
PTKST and TTKST	Eritrea	2011	Walday et al., 2011
TTKSK, TTKST and TTTSK	Tanzania	2009	Hale et al., 2013
TTKST, TTKTK and TTKSK	Egypt	2014	Patpour et al., 2016a
TTKTK and TTKTT	Egypt, Kenya, Rwanda and Uganda	2014	Patpour et al., 2016b
TKTTF, TTKSK, RRTTF and JRCQC	Ethiopia	2013/14	Olivera et al., 2015
21, 94, 126 and 326	Australia	1925/54/64	Park, 2007, 2015
TTKSK, TKTTF, HKNTF, TTTTH and HKPPF	Ethiopia	2014	Hailu et al., 2015

Table 2.1 Continued

Virulent *Pgt* race	Country	Year	Reference
TTKSK, TTKST,TTKTK, TTKTT and TTHST	Kenya	2008-2014	Newcom et al., 2016
TTHSK and PTKTK	Kenya	2014	Fetch et al., 2016
34C0MRGQM, 34C3MTGQM, 34C3MKGQM, 34C3MKGSM, 34C6MTGSM and 34C6MRGQM	China	2013/14	Li et al., 2018
TTTTF	Italy	2016	Bhattacharya, 2017
40A and 40-1	India	2016	Kumar et al., 2016
TKTTF	Germany	2013	Olivera Firpo et al., 2017
UK-01	UK	2013	Lewis et al., 2018
QFCSC, QCCJB, QFCJC, RKQSC, RKQSF, QFCJC, RTHJF and TMRTF	Canada	2011/12	Fetch et al., 2017
QFCSC	USA	-	Jin et al., 2014

USA = United States of America, UK = United Kingdom.

Le Roux (1985) reported the first *Pgt* race, 2SA100, with virulence towards *Sr24* present in two South African varieties Gamka and SST44. Between 1986 and 1987 during the annual stem rust surveys eight races were reported namely 2SA4, 2SA100, 2SA101, 2SA6, 2SA32, 2SA2, 2SA43 and 2SA101. Stem rust pathotype 2SA4, with virulence towards *Sr9e*, was the most prevalent race accounting for 77% of isolates from field samples, while 2SA100 accounted for 17% (Le Roux, 1989). Results from stem rust surveys conducted between 1991 and 1997 showed the presence of five stem rust races; three on bread wheat 2SA4, 2SA36 and 2SA100 while two races, 2SA102 and 2SA103, were reported on triticale (Boshoff et al., 2000).

However, within the Ug99 race group five *Pgt* races TTKSF, TTKSP, PTKST, PTKSK and TTKSF+ have been reported in SA (Terefe et al., 2010, 2019). The first Ug99 variant TTKSF (isolate 2SA88), was detected during the 2000/2001 season in the Western Cape (Boshoff et al., 2002). Race TTKSF is virulent towards *Sr5*, *Sr6*, *Sr9e*, *Sr8b* and *Sr38* (Boshoff et al., 2002; Terefe et al., 2016). A characterisation study done by Visser et al. (2009) indicated strong genetic resemblance of race TTKSF to TTKSK, except that race TTKSF was avirulent on *Sr31* (Pretorius et al., 2007). This provided evidence that race TTKSF did not evolve from the older SA *Pgt* race 3SA43 as was previously believed (Pretorius et al., 2007). Terefe et al. (2010) identified race TTKSP (2SA106) in 2007 as the second South African Ug99 race, with virulence towards *Sr24*. Visser et al. (2011) reported that TTKSP has a similar phenotype compared to race PTKST except for its avirulence to *Sr31* and similar to race TTKSF except for virulence to *Sr24*.

The third Ug99 race PTKST (isolate 2SA107) was detected nearby Cedera and Greytown in 2009. Race PTKST possesses virulence to two important stem rust resistance genes *Sr24* and *Sr31* (Pretorius et al., 2010), but unlike race TTKSK, race PTKST is avirulent to *Sr21* (Terefe et al., 2010; Visser et al., 2011). The reported virulence of race TTKSP to *Sr24* was significant as 39.1% of the commercial South African varieties contained *Sr24* as a major resistance gene or in combination with other genes (Le Roux and Rijkenberg, 1987a). Current evidence suggests that *Sr24* is rare among modern South African wheat varieties (Pretorius et al., 2012). Race PTKST is avirulent towards *Sr13*, *Sr14*, *Sr21*, *Sr22*, *Sr25*, *Sr26*, *Sr27*, *Sr29*, *Sr32*, *Sr33*, *Sr35*, *Sr37*, *Sr39*, *Sr42*, *Sr43*, *Sr44*, *SrTmp*, *SrEm* and *SrSatu* (Pretorius et al., 2010). In Ethiopia and Kenya, other races with similar virulence phenotypes as 2SA107 were previously identified that suggest that race PTKST, just like race TTKSF might have spread to SA from East Africa (Terefe et al., 2010).

The fourth Ug99 lineage race reported from SA is 2SA88+ (TTKSF+) that was identified in 2010 (Pretorius et al., 2012). Race TTKSF+ is believed to be a local adaptation from race TTKSF with additional virulence on the winter wheat variety Matlabas. A recent study by Terefe et al. (2016) identified nine *Pgt* races from 521 isolates. The most prevalent stem rust races were TTKSF (Ug99 lineage) (39-85%) and BPGSC+*Sr27, Kiewiet, Satu* (10-20%). However, importantly, two new *Pgt* races BFBSC and TTKSF+*Sr9h* were detected in 2010 (Terefe et al., 2016). Terefe et al. (2016) showed that 14 stem rust genes *Sr13*, *Sr14*, *Sr22*, *Sr25*, *Sr26*, *Sr29*, *Sr32*, *Sr33*, *Sr35*, *Sr36*, *Sr37*, *Sr39*, *Sr42* and *Sr43* confer resistance to new and old South African *Pgt* races and can be used in wheat resistance breeding.

The fifth Ug99 lineage race reported from SA 2SA42 (PTKSK) was identified in the Free State near Bethlehem during the 2017 annual rust surveys. PTKSK is similar to PTKST, except for its avirulence for *Sr24* (Terefe et al., 2019). Although it is the first time race PTKSK is reported in SA, it is unlikely to cause significant damage to the South African breeding industry because race PTKSK is less virulent than race PTKST (Terefe et al., 2019).

Recently, Boshoff et al. (2018) have reported on phenotypic and genotypic variation within stem rust race 2SA55 that was initially described in 2000 (Boshoff et al., 2000). This race is reportedly related to non-Ug99 races occurring in SA.

2.4.4 Stem rust resistance genes in South African wheat and triticale varieties

According to stem rust inheritance studies by Le Roux and Rijkenberg (1988, 1989), resistance to stem rust in South African spring and winter wheat varieties in the late 1980s was conferred by *Sr5*, *Sr9e*, *Sr24*, *Sr27*, *Sr36* and possibly *Sr31*. This information was generated by analysing segregation of resistance among F_2 and F_3 generations, pedigree data and rust specificity. Since *Sr27* virulence was first detected in 1988, significant damage was observed on triticale varieties at that stage as 75% of varieties were found susceptible to *Sr27* virulent races (Smith and Le Roux, 1992). Pretorius and Brown (1999) performed seedling chlorosis, pseudo-black chaff and field stem rust analysis on 54 South African wheat varieties and postulated that the *Sr2* resistance gene was present in 10 of these entries. A comprehensive review of stem rust research in SA has indicated the vulnerability of wheat genotypes to stem rust as only a few resistance genes are being deployed in commercial varieties with a reported increase in the number of stem rust collections made annually (Pretorius et al. 2007). Evaluating 65 South African varieties with different stem rust races and molecular markers linked to some stem rust

resistance genes, Pretorius et al. (2012) reported that only three varieties; Inia, PAN 3377 and Steenbras carried *Sr*2 and concluded that 88% of the tested entries were susceptible to at least one of the stem rust races. The presence of *Sr24* was confirmed in 12 and *Sr31* in five wheat entries (Pretorius et al., 2012). Prins et al. (2001) developed a sequenced-tagged site (STS) marker for selection of the *Sr25*/*Lr19* gene. Stem rust resistance evaluation studies confirmed that some African (including South African) spring wheat varieties possess *Sr2*, *Sr36*, *Sr24*, *Sr31* and *Sr57* genes (Prins et al., 2016). The ARC-SG reported on the incorporation of several stem rust genes such as *Sr2*, *Sr25*, *Sr26* and *Sr39* into wheat breeding lines to improve their resistance to stem rust (Figlan, 2016). Recently, Wessels et al. (2019) mapped a gene in the bread wheat variety Matlabas and postulated that it is *Sr9h*.

2.4.5 Control of stem rust

2.4.5.1 Cultural control

Some cultural practices can be partially useful to control cereal rusts (Chaves et al., 2008). For instance, the use of early maturing varieties in Australia and early planting of wheat in Argentina was shown to be effective in controlling stem rust even before the use of resistant sources (Borloug, 1954; McIntosh, 1976). The following cultural practices are recommended for the management of wheat stem rust (https://www.agric.wa.gov.au):

a) Removal of volunteer plants

Self-sown volunteer plants such as wheat or barley, if present in fields, form green bridges for *Pgt* and should therefore be destroyed using herbicides or heavy grazing as soon as they appear or at least four months before the onset of the wheat growing season. This reduces the local carryover and minimises the risk of early infection.

b) Wheat field monitoring

Warm conditions favour the development of stem rust and the disease becomes more visible especially after the emergence of flag leaves. In areas where a green bridge exists or stem rust has been reported before, the following actions are recommended:

- Monitoring of susceptible wheat crops at intervals of 7-14 days post flag leaf emergence till early dough grain development.
- Careful examination of different parts of the crop specifically on the lower stems for early signs of stem rust infection.

- In situations where stem rust is observed in the field, move in the field following a 'W' pattern to sample up to 100 stems from 10 different locations, so as to help determine the number of stems infected with stem rust.

In some situations, fertilisation and irrigation planning in terms of amounts, timing and frequency can help to control diseases. Wheat cultivation on large farms can also be arranged such that plots with early maturing varieties are sown downstream to the late maturing varieties (Chaves et al., 2008). All these cultural control practices should be tested on a case to case basis in view of the anticipated epidemic in a particular area for it to be effective (Roelfs et al., 1992; Singh et al., 2002). Eradication of large areas infested with stem rust alternate hosts, for instance barberry in the USA and northern Europe, was also successful in the prevention of regular stem rust outbreaks (Roelfs, 1978; Peterson et al., 2005). Fortunately, on a global scale, it is only in North-western United States of America (USA) and eastern Europe regions where stem rust alternate hosts are instrumental in stem rust epidemiology (Chaves et al., 2008).

2.4.5.2 Chemical control

Many studies have reported on the effectiveness of fungicides in controlling stem rust outbreaks (Wanyera et al., 2009; Kang et al., 2010; Tadesse et al., 2010; Soko et al., 2018). Wanyera et al. (2009) revealed that different fungicides have varying efficacies in managing stem rust. Various fungicides such as propiconazole, tebuconazole, triadimefon, flutriafol, cyproconazole, azoxystrobin, tridimenol, prothioconazole and trifloxystrobin are effective in controlling stem rust (Loughman et al., 2005; Wanyera et al., 2009; Xue et al., 2012; Wanyera et al., 2016; Soko et al., 2018). These fungicides can be applied either singly or as combinations (Wanyera et al., 2009). However, the use of fungicides has negative consequences on the environment (Steffenson et al., 2007). The misuse of fungicides can also result in pathogen tolerance (Oliver, 2014). Given the above downsides, alternative and more sustainable stem rust control strategies such as gene discovery, gene mapping and deployment in adapted varieties should be explored.

2.4.5.3 Genetic resistance

Biffen (1905) showed that wheat stripe rust resistance was a simply inherited trait and this formed the basis for plant disease control and is now a major plant breeding objective (Byerlee, 1996). This form of disease control is cost-effective and more environmentally-friendly (Bockus et al., 2001). Growing of resistant varieties is highly encouraged worldwide in the

management of stem rust particularly in high risk environments prone to stem rust outbreaks. Partially or highly resistant varieties are useful in reducing disease development and preventing losses. In environments prone to rusts or during high-risk seasons, it is vital to avoid planting susceptible or highly susceptible varieties. Several studies have recommended that rust can effectively and efficiently managed using resistance genes (Bhavani et al., 2019; Jaleta et al., 2019).

2.5 Mechanisms of stem rust resistance

2.5.1 Race specific resistance

Race-specific resistance genes, also known as seedling resistance or all-stage resistance (ASR), are effective against some races of a pathogen and basically conform to the gene-for-gene model (Flor, 1956), where resistance is triggered by the interaction between host-resistance (R) genes and pathogen avirulence (*Avr*) genes (Periyannan et al., 2017). The use of ASR genes is a common practice in most wheat improvement programmes (Chaves et al., 2008). However, these ASR genes are often subject to rust pathogen breakdown especially when used as a sole source of resistance (Smale et al., 1998). ASR genes usually have an effective lifespan of up to five years (Singh and Huerta-Espino, 2001). The durability of ASR can be improved by combining several ASR genes into well-adapted varieties (Bernardo et al., 2013). Over the past number of years efforts have led to the identification and characterisation of several stem rust ASR genes such as *Sr32* (Mago et al., 2013), *Sr43* (Niu et al., 2014), *Sr44* (Liu et al., 2013), *Sr48* (Bansal et al., 2009), *Sr54* (Ghazvini et al., 2013), *SrTA10181* and *SrTA10171* (Olson et al., 2013a; Wiersma et al., 2016), *Sr9h* (Rouse et al., 2014), *SrWLR* (Zurn et al., 2014), *Sr45* (Periyannan et al., 2014), *Sr46* (Yu et al., 2015), *Sr49* (Bansal et al., 2015), *SrTm4* (Briggs et al., 2015), *SrND643* (Basnet et al., 2015), *Sr59* (Rahmatov et al., 2016), *Sr60* and *SrTm5* (Chen et al., 2018b).

2.5.2 Adult plant resistance

APR or non-race specific resistance often referred to as slow rusting (Vanderplank, 1963), is known to be effective to all races of a given pathogen species and to a limited extent more than one pathogen species (Ellis et al., 2014; Jones et al., 2016). APR is mainly effective at adult plant stage and is often detected during field evaluations (Roelfs et al., 1992; Ellis et al., 2014). Bajgain et al. (2016) noted that APR is often initiated at the booting stage and is effective especially during critical plant growth stages such as at flowering and grain filling. This form of resistance confers minor effects with an associated 5-20% reduction in rust severities (Singh

et al., 2000). Although APR genes singly may have small effects, combinations of 3-5 APR genes increase the host resistance response (Singh et al., 2000, 2011b). Similarly, Bansal et al., (2014) observed lower mean stem rust responses in recombinant inbred lines (RILs) containing both *Sr56* and *Sr57*, compared to those that carried these genes singly. APR is often polygenic and is considered a preferred mode of durable resistance as it confers less selection pressure on the pathogen (Yu et al., 2014; Riaz et al., 2017). To date, only a few stem rust APR genes have been identified including *Sr2* (McFadden, 1930), *Sr55* (Herrera-Foessel et al., 2014), *Sr56* (Bansal et al., 2014), *Sr57* (Lagudah et al., 2006, 2009; Krattinger et al., 2009) and *Sr58* (Singh et al., 2013b; McIntosh et al., 2014).

2.5.3 Progress in the fight against stem rust

Over 70 stem rust resistance genes have so far been characterised (McIntosh et al., 2017; Aoun et al., 2019). However, the majority of these originated from alien chromosome regions with only 12 *Sr* loci that have been derived from primary wheat gene pool accessions with resistance to *Pgt* Ug99 races (Jin et al., 2007; Randhawa et al., 2018). Several studies have indicated that the primary gene pool as a source of resistance against Ug99 *Pgt* races is limited. Only a few stem resistance genes such as *Sr2*, *Sr13a*, *Sr13b*, *Sr14*, *Sr22*, *Sr25*, *Sr26*, *Sr28*, *Sr33*, *Sr35*, *Sr42*, *Sr45*, *Sr57*, *Sr58*, *SrTmp*, *SrND643*, *SrIRS*^{Amigo} and *Sr8155B1* have been reported to confer resistance in adapted wheat varieties (Pretorius et al., 2000; Jin et al., 2007; Hiebert et al., 2011; Ghazvini et al., 2012; Randhawa et al., 2018). Only 10 ASR genes *Sr13*, *Sr21*, *Sr22*, *Sr33*, *Sr35*, *Sr45*, *Sr46*, *SrTA1662*, *Sr50* and *Sr60* have been cloned (Periyannan et al., 2013; Saintenac et al., 2013; Chen et al., 2015, 2018a; Mago et al., 2015; Steuernagel et al., 2016; Zhang et al., 2017; Arora et al., 2018; Chen et al., 2020). These cloned genes encode coiled-coil (CC) nucleotide-binding, leucine-rich repeats domains (NLR proteins) and kinase domains and confer stem rust resistance to the Ug99 race group (Hatta et al., 2018). Two wheat multi-pathogen genes *Lr34/Yr18/Sr57/Pm38/Ltn1* (Krattinger et al., 2009) and *Lr67/Yr46/Sr55/Pm46/Ltn3* (Moore et al., 2015) have also been cloned. Cloning of stem rust resistance genes has led to the development and deployment of diagnostic gene-based markers to combine various *Sr* genes in wheat varieties (Randhawa et al., 2018; Chen et al., 2020).

Some of these cloned stem rust resistance genes such as *Sr*22, *Sr33*, *Sr35* and *Sr45* have also been shown to confer resistance to Ug99 when transformed into the barley variety Golden Promise (Hatta et al., 2018). However, the transgenic barley lines remained susceptible to barley leaf rust *P. hordei* Otth, suggesting that the cloned genes confer specific resistance to

stem rust. Hence, the identification and cloning of wheat stem rust resistance genes will not only protect wheat but also barley against virulent stem rust races (Hatta et al., 2018).

It is anticipated that in the coming years, there will be a likely increase in the number of cloned R genes due to development of rapid gene cloning technologies such as AgRenSeq (Arora et al., 2018), targeted chromosome-based cloning via long range assembly (TACCA) (Thind et al., 2017), MutRenSeq (Steuernagel et al., 2016) and MutChromSeq (Sánchez-Martïn et al., 2016).

A major breakthrough in the fight against *Pgt* has led to the identification of fungal genes, *AvrSr35* and *AvrSr50* that are responsible for avirulence against *Sr35* and *Sr50*, respectively (Chen et al., 2017; Salcedo et al., 2017). This implies that researchers can now develop markers that will detect virulent *Pgt* strains early in the growing season, hence reducing losses that would be caused by these pathogens. These findings will complement the already cloned *Sr* genes in selecting gene combinations that will result in durable stem rust resistance. Stem rust resistance incorporated into varieties has been largely monogenic, posing a threat of being overcome by new virulent pathogen variants (Pujol et al., 2015). A classic example is when *Sr31* that was widely deployed in many wheat varieties was defeated by *Pgt* race TTKSK (Pretorius et al., 2000). It is now broadly believed that this risk can be minimised through the deployment of several resistance genes in a single genotype (Pujol et al., 2015; Zhang et al., 2019).

Recently, the wheat genome that is known to be complex in nature, has been sequenced (IWGSC, 2018) comprising of 107 891 high confidence genes. In addition, scientists have developed a comprehensive database with the bread wheat reference genome sequence together with other wheat databases (Alaux et al., 2018). This database is enriched with the latest genomic data useful for wheat improvement.

2.6 Characterisation and mapping of stem rust resistance genes and quantitative trait loci

2.6.1 Mapping populations

According to Singh et al. (2015), a mapping population is defined as a population that is suitable for linkage mapping of genetic markers. These populations are usually created by crossing two or more genetically diverse entries and managing the progeny in a systematic fashion. Ideally these parental entries must come from the same species, but in situations of

limited variation, related species may be used as one of the parents. Mapping populations are used to calculate the genetic distances and order between pairs of genes or loci and to map them on specific locations of the genome (Schneider, 2005; Semagn et al., 2006; Singh et al., 2015). This is also vital for the identification of markers closely linked to genes of interest that can be used in MAS of genes. There are two major mapping populations, namely primary and secondary populations. Primary mapping populations are created through hybridisation of homozygous lines usually with contrasting traits of interest and include F_2, F_2 derived F_3 ($F_{2:3}$), RILs, backcross (BC), backcross inbred lines (BILs), doubled haploids (DHs), immortalised F_2, chromosome segment substitution lines (CSSLs), advanced intercross lines, interconnected populations and recurrent selection backcross (RSB) populations. Secondary mapping populations are developed by crossing two lines or individuals from a mapping population; they are mainly useful for fine mapping of a genomic region of interest (Singh et al., 2015). It is a disadvantage that F_2 populations cannot be easily preserved, because F_2 plants are frequently not immortal and hence can only be phenotyped once, unlike immortal populations (RILs and DHs) that represent permanent resources that can be replicated indefinitely and shared among research groups (Schneider, 2005; Semagn et al., 2006). Immortal populations like RILs undergo several meiosis events before homozygosity is attained hence result in higher levels of recombination compared to F_2 or BC populations (Schneider, 2005). In terms of population sizes, between 50 to 250 individuals are needed for preliminary mapping, otherwise large population numbers are required for higher resolution fine mapping (Mohan et al., 1997).

2.6.2 Mapping and targeting strategies

There are two major types of gene mapping strategies namely linkage and association mapping. Singh et al. (2015) stated that linkage mapping is generally based on phenotypic and genotypic analyses of a mapping population developed by crossing two individuals with contrasting traits of interest. Linkage mapping has a limitation in that crosses cannot be made in all situations such as forest trees and mapping populations are at times too small with only a few alleles being studied (Gupta et al., 2005). This problem can be addressed by analysing a collection of germplasm lines or breeding lines and samples from natural populations to identify marker-trait associations, and this is referred to as association mapping. Once close linkage between the marker and the trait has been identified, markers can be used as basis for indirect selection of the gene/QTL, i.e. MAS.

The genome wide association study (GWAS) approach includes panels of genotypes that are not necessarily related to each other, e.g. wild relatives, landraces, and breeding germplasm. Due to the large number of genotypes that can be evaluated with GWAS, many novel QTL can be discovered (Saccomanno et al., 2018). GWAS refers to statistically significant associations identified between molecular markers and the phenotypic trait when the marker and the causal gene are in linkage disequilibrium (LD), by means of models that are selected depending on the population and the data under analysis. LD, defined as the non-random association of alleles at different loci, has to be carefully considered in association mapping. Patterns of LD vary depending on several factors: in general, LD is greater in self- than in out-crossing species and can vary with species, population structure and genomic region (Gupta et al., 2005). Within the same species, varieties have much higher LD extent than landraces (Saccomanno et al., 2018).

Targeting strategies are important as cost-effective ways of identifying regions of the genome with genes of interest (Singh et al., 2015). These targeting strategies include BSA and near isogenic lines (NILs) among others. In the absence of NILs, BSA can be an effective alternative for tagging of a given trait (Michelmore et al., 1991).

2.6.2.1 Bulked segregant analysis

BSA was developed as a short-cut for tagging QTL (Collard et al., 2005) and is used to locate markers in particular chromosome regions (Michelmore et al., 1991). Briefly, two pools or 'bulks' of DNA samples are combined from between 10 to 20 individual plants from a segregating population; these two bulks usually differ for a trait of interest (e.g. resistant vs. susceptible to a particular disease). In constituting DNA bulks, all loci are randomised, except for the region containing the gene of interest. Markers are screened across the two bulks. Polymorphic markers may represent markers that are linked to a gene or QTL of interest. The entire population is then genotyped with these informative polymorphic markers and a localised linkage map may be generated. This enables QTL analysis to be performed and the position of a QTL to be determined (Collard et al., 2005). This method has been successfully employed to identify molecular markers linked to several stem rust resistance genes or chromosome arms in wheat such as *Sr42/SrCad/SrTmp/SrTA10187* (Olson et al., 2013a; Lopez-Vera et al., 2014; Babiker et al., 2016; Kassa et al., 2016; Wiersma et al., 2016), *Sr6* (Tsilo et al., 2009), *Sr22* (Khan et al., 2005), gene/QTL on chromosome 4AL (Turner et al., 2016), *SrND643* (Basnet et al., 2015), gene/QTL on chromosome 7AL (Pujol et al., 2015), *SrWLR* (Zurn et al., 2014), *Sr28* (Rouse et al., 2012), *SrVL* (Bansal et al., 2012), *Sr40* (Wu et

al., 2009), *Sr56* (Bansal et al., 2014), *Sr46* (Yu et al., 2015), *Sr49* (Bansal et al., 2015), gene/QTL on chromosome 5DL (Gireesh et al., 2015), *SrTm4* (Briggs et al., 2015) and *SrPI410966* (Campbell et al., 2016). In *Aegilops tauschii* Coss. for *Sr46* (Yu et al., 2015), *SrTA1662* (Olson et al., 2013b) and *Sr55* (Forrest et al., 2014). In barley BSA was used to identify traits on chromosome 5HL (Mamo et al., 2015).

2.6.2.2 Quantitative trait loci analysis for stem rust resistance loci

Using 151 RILs, five significant QTL were identified on chromosomes 2B, 3B, 6A, 6D and 7A that are believed to correspond with *Sr16, Sr12, Sr8a, Sr5* and *Sr15,* respectively (Zurn et al., 2018). A genetic evaluation of a 159 global wheat landrace collection led to the identification of two QTL associated with adult plant stem rust resistance on chromosomes 1BL and 2B. Similarly, Kankwatsa et al. (2017) evaluated a panel of 159 wheat landraces and cultivars and identified four genes *Sr2*, *Sr55*, *Sr57* and *Sr58*. Prins et al. (2016) used QTL analysis to confirm the presence of the adult plant stem rust resistance locus *Lr34/Yr18/Sr57/Pm38/Ltn1* on chromosome 7DS and marker-trait associations on chromosome 6AS and 3BS in a diverse set of African wheat lines.

Three consistent QTL were detected on chromosome arms 5A, 3BS and 1BS that explained 5.9, 9.0 and 78.5%, respectively, of the stem rust phenotypic variation observed in European winter wheat genotypes (Getie et al., 2016). Furthermore, a joint mapping experiment conducted to test responses of wheat genotypes to NA stem rust races identified 59 small and medium effect QTL on 20 chromosomes, of which 15 QTL were detected in different environments (Bajgain et al., 2016).

Laidò et al. (2015) screened a collection of 230 tetraploid wheat genotypes with stem rust race TTKSK and identified 35 resistance QTL on all wheat chromosomes, of which 17 QTL were regarded as most important because they were selected based on multiple associations. Five chromosomes 1AL, 2AL, 4AL, 5BL and 7BS carried QTL proposed to be novel (Laidò et al., 2015). Babiker et al. (2015) also reported that a major QTL on chromosome 7AL in a spring wheat landrace conferred resistance to race TTKSK and suggested that this was either a new gene or an allele closely linked to *Sr15*. Similarly, Bajgain et al. (2015a) identified nine QTL effective against both African and NA stem rust races. A single QTL on chromosome 2B associated with APR was detected across all test environments (Bajgain et al., 2015a). In a study evaluating resistance among NA spring genotypes, seven QTL on chromosomes 3B, 4D,

5A, 5B, 6A, 7A and 7B were associated with APR and represented putative novel genes (Bajgain et al., 2015b). While analysing resistance against stem rust races TRTTF and QTHJC using a synthetic haploid W7984 x Opata reference population, Dunckel et al. (2015) observed major QTL on chromosomes 1AS, 2BS, 6AS and 6AL.

A review by Yu et al. (2014) revealed that 141 stem rust resistance loci distributed across the 21 chromosomes of wheat confer resistance to Ug99 (race TTKSK). Several resistance loci hotspots were detected for instance 19 QTL on chromosome 3BS, nine on 6BS, nine on 5BL and seven on 2BL. Specific stem rust resistance genes have been mapped to all these resistance hotspots, except chromosome arm 6BS (Yu et al., 2014).

2.7 Molecular markers

2.7.1 Microsatellites or simple sequence repeat markers

Microsatellites or SSRs are tandemly repeated DNA sequences of between 1 to 6 bp and are usually unevenly distributed in both prokaryote and eukaryote genomes (Asp et al., 2007). SSR markers are viewed as more efficient than SNP markers for diversity analysis (Singh et al., 2013a; Xu et al., 2013). This is attributable to higher mutation rates in SSRs compared to SNPs (Chao et al., 2009). In 2007, Ganal and Röder reported that about 2 000-2 500 SSR markers have been mapped (Ganal and Röder, 2007). By 2013, over 4 000 SSR markers had been developed and applied in numerous wheat genetic mapping studies (Ren et al., 2013). Indeed, currently the wheat Unité de Recherche Génomique Info/research unit in genomics and bioinformatics (URGI) database alone has 5 294 SSR markers sourced from various research groups worldwide (Alaux et al., 2018).

Previously SSR markers were developed from screening genomic DNA libraries for positive clones in a process that is rather difficult and laborious (Deng et al., 2016). Later, bacterial artificial chromosome (BAC) sequences (Waldbieser et al., 2003; Huo et al., 2008) and expressed sequence tags (ESTs) (Kantety et al., 2002; Varshney et al., 2002; Zhang et al., 2005) were widely used in identifying SSR markers. Though this was a reduced cost approach, it often produced multiple sets of markers for a single locus.

With great advances in sequencing technologies, numerous crop species' genome sequences are now available, so it is becoming common place to develop SSR markers from these sequences. For example, Han et al. (2015) searched a Chinese Spring wheat genome database

comprising of 10 603 760 sequences and identified 364 347 SSR markers. Similarly, Deng et al. (2016) conducted an *in silico* search of public databases and mined 20 666 SSR markers from genome sequences of nine model plants and eight *Triticeae* species.

2.7.1.1 Development of wheat microsatellite genetic linkage maps

In 1997, Bryan et al. reported that for hexapoloid wheat, 49 SSR primer pairs that amplified 76 loci were identified from over 200 sequenced clones. As a follow-up of work done by Bryan et al. (1997), 53 new microsatellites, designated as Psp were mapped in hexaploid wheat (Stephenson et al., 1998). Röder et al. (1998) developed a wheat linkage map using 279 SSR markers designated as Gwm that covered all 21 wheat chromosomes. Gupta et al. (2002) extended the wheat SSR marker pool by an additional 66 markers covering 20 of the 21 chromosomes in addition to the 384 SSRs already mapped. They abbreviated these markers Wmc from the wheat microsatellite consortium. A total of 65 SSR markers designated as Gdm were developed from *Ae. tauchii,* of which 55 were genetically mapped (Pestsova et al., 2000). An additional 100 SSR markers were isolated and developed from an *Ae. tauchii* library and showed a transferability of 92% to the D-genome of bread wheat (Guyomarc'h et al., 2002).

Sourdille et al. (2004) created a SSR-based deletion map of wheat using 725 SSR markers covering 21 wheat chromosomes using 84 deletion lines. During the same period, Somers et al. (2004) produced a SSR marker-dense consensus map for hexaploid wheat containing a total of 1 235 SSR markers from various research groups (Gwm, Wmc, Gdm, Cfa, Cfd, Gpw and Barc). Song et al. (2005) mapped a further 347 SSR markers in bread wheat extending the wheat linkage map by 80.7 cM. A high-density consensus durum wheat map containing SSR and diversity array technology (DArT) markers was developed by Marone et al. (2012) using 1 898 loci and an average marker density of 1.6 cM covering all 14 chromosomes. Both the hexaploid (Somers et al., 2004) and durum wheat (Marone et al., 2012) high-density consensus maps provide valuable markers to be utilised in mapping of stem rust resistance genes/QTL.

2.7.1.2 Application of microsatellites in wheat breeding for stem rust resistance

SSR markers can be used to map genes/QTL (Gao et al., 2018; Randhawa et al., 2018; Wessels et al., 2019). SSRs are being utilised as backbone markers for localisation of individual genes on all 21 chromosomes of wheat, including disease resistance genes and other important agricultural traits (Ganal and Röder, 2007). For these QTL, a larger number of SSRs are being applied in several marker-assisted projects that are in progress (Ganal and Röder, 2007). A

great number of SSR markers are currently available for MAS for stem rust resistance genes/QTL (https://maswheat.ucdavis.edu/). This means that wheat breeders can now use these resources to aid indirect selection for stem rust resistance genes/QTL.

By analysing online SSR marker resources, primers flanking these SSRs have been designed for wheat and used to assess relationships among wheat varieties and their relatives. Jaiswal et al. (2017) also utilised these vast sources of public genome sequences and developed a wheat microsatellite online database (TaSSRDb). This database contains the highest number of *in silico* microsatellites (476 169) and 268 SSR markers reported as closely linked to 11 important wheat traits. TaSSRDb is believed to be a useful wheat genomic resource with applications in diversity analysis, traceability and variety identification, linkage mapping and gene/QTL discovery (Jaiswal et al., 2017).

2.7.2 Single nucleotide polymorphism markers

SNPs refer to single-base differences among individuals (Barley and Edwards, 2007). They are sequenced-based molecular markers that are bi-allelic in nature and occur at a much higher frequency in genomes compared to other DNA markers (Lai et al., 2012; Ren et al., 2013). SNPs can be grouped as transitions (C-T or A-G) or transversions (C-G, G-T, A-C or A-T), hence they represent direct markers with exact forms of allelic variants (Edwards et al., 2007; Lai et al. 2012). C to T transitions are the most common form of non-random mutations (Edwards et al., 2007). Given their low mutation rates, SNPs are excellent markers for understanding complex traits and evolution of genomes (Syvanen, 2001). SNPs have lower expected heterozygosity compared to SSRs (Taramino and Tingey, 1996; Chao et al., 2009). Therefore, to effectively utilise SNP data, haplotype-based analysis is preferred to individual SNP analysis and is more powerful in detecting association with phenotypes (Rafalski, 2002; Brumfield et al., 2003). SNPs are useful in association mapping and in creating high density-linkage maps (Akhunov et al., 2009).

2.7.2.1 Development of wheat single nucleotide polymorphism genotyping platforms and high-density genetic maps

SNPs are the most abundant form of molecular markers and with the advent of new sequencing projects, a great number of wheat SNP markers were discovered (Berkman et al., 2012; Lai et al., 2012; Jia et al., 2013; Ling et al., 2013; Luo et al., 2013). Although many of these SNP

platforms had initially targeted diploid genotypes, to date, several studies have also reported on the discovery of SNPs from tetraploid and hexaploid wheat genotypes (Table 2.2).

Although a great number of wheat SNP markers have been discovered, there are still gaps in these SNP maps especially on the D-genome. Ishikawa et al. (2018) recently utilised a genome-specific amplicon sequencing approach and mapped 12 551 D-genome specific SNP markers in common wheat varieties. These D-genome specific SNP markers are therefore envisaged to improve the marker density on the previously sparsely covered D-genome of wheat. The D-genome specific SNP markers are also expected to be useful in many global wheat genotypes as they possessed a high polymorphic rate when tested on six Japanese wheat varieties (Ishikawa et al., 2018).

The first two genotyping arrays, Illumina iSelect 9K (Cavanagh et al., 2013) and Illumina iSelect 90K SNP array (Wang et al., 2014) detected low levels of polymorphic SNPs; however, this has been improved with the advent of modern arrays such as Affymetrix Axiom Wheat660K (Cui et al., 2017), Affymetrix Axiom 820K (Winfield et al., 2016), 35K wheat relative array (King et al., 2017) and TaBW280K (Rimbert et al., 2018). To facilitate the utility of these SNPs for practical wheat breeding, Allen et al. (2017) have formulated a wheat breeders' array with 35 143 polymorphic SNPs currently hosted as web resource "CerealDB".

Several SNP genotyping platforms were developed for identification and genotyping of multiple SNP markers, which is high throughput but quite costly. Therefore, cost-effective SNP genotyping platforms mendable to practical breeding systems have been developed such as Competitive allele specific polymerase chain reaction KASP (He et al., 2014; Semagn et al., 2014; Smith and Maughan, 2015) and semi-thermal asymmetric reverse polymerase chain reaction (PCR) (STARP) markers (Long et al., 2017). Unlike KASP markers, STARP markers use various reagents from different companies, hence is slightly cheaper for genotyping of individual SNPs and indel (insertion or deletion) markers (Long et al., 2017).

Table 2.2 Discovery of single nucleotide polymorphism (SNP) markers in tetraploid and hexaploid wheat genotypes

Crop	Source	Number of SNPs	Population	SNP discovery system	Reference
Tetraploid and hexaploid wheat	Intron sequences	96	53 Tetraploid and 38 hexaploid lines	Illumina BeadArray platform Golden Gate^{Tm} assay	Akhunov et al., 2009
Hexaploid wheat	Intron sequences	359	20 Wheat cultivars, 13 wheat accessions	Template-directed dye-terminator incorporation assay with fluorescence polarisation detection (FP-TDI)	Chao et al., 2009
Tetraploid durum wheat	Consensus sequences	2 659	4 Durum cultivars	Complexity reduction of polymorphic sequences (CRoPS) technology	Trebbi et al., 2011
Hexaploid wheat	ESTs and NGS databases	1 114	5 Wheat varieties	Competitive allele specific polymerase chain reaction (KASP) genotyping technology	Allen et al., 2011
Hexaploid wheat	Wheat exome	95 265	8 Wheat varieties	Sequence capture targeted re-sequencing approach	Allen et al., 2013
Durum wheat	Contigs	9 983	2 Durum varieties	Sequence-based genotyping (SBG)	Van Poecke et al., 2013
Hexaploid wheat	Wheat transcriptome	9 000	7 Mapping populations	Illumina iSelect SNP assay	Cavanagh et al., 2013
Hexaploid wheat	Wheat transcriptome	90 000	8 Mapping populations	Illumina iSelect SNP assay	Wang et al., 2014
Tetraploid wheat	Wheat transcriptome	26 626	13 Mapping populations	Illumina iSelect SNP assay	Maccaferri et al., 2015
Hexaploid, diploid and tetraploid accessions and wheat relatives	Exome-capture SNP sequences	819 571	43 Bread wheat accessions and wheat relatives	Affymetrix Axiom 820K SNP array	Winfield et al., 2016
Hexaploid wheat	Exome-capture SNP sequences	35 143	5 Mapping populations	Axiom 35K (Wheat breeders' array)	Allen et al., 2017
Hexaploid wheat	Genic and inter-genic regions of wheat genome	280 226	8 Wheat lines	TaBW280K genotyping array	Rimbert et al., 2018

ESTs = Expressed sequence tags, NGS = Next generation sequencing.

The STARP marker technology is highly flexible because either the gel-size or the gel-free fluorescence-based separations are possible for marker analysis (Long et al., 2017). In fact, a few STARP markers have already been developed for stem rust resistance genes (Klindworth et al., 2017; Saini et al., 2018). Similarly, Rasheed et al. (2016) validated the application of KASP assays for wheat genes such as stem rust (*Sr2* and *Sr36)*, leaf rust (*Lr21*, *Lr47* and *Lr68*), yellow rust (*Yr15* and *Yr36*) and other agronomically important wheat traits. This extensive KASP validation led to development of a robust SNP marker toolkit for numerous wheat functional genes that is 45 times more superior to DNA gel-based PCR platforms in generating marker data (Rasheed et al., 2016). Many studies have reported the use of SNP markers in construction of high-density wheat genetic (Wu et al., 2015; Zhai et al., 2015; Cui et al., 2017; Rimbert et al., 2018) and consensus maps (Li et al., 2015; Wen et al., 2017).

2.7.2.2 Identification of molecular markers linked to stem rust resistance loci

Several molecular markers that are diagnostic or tightly linked to wheat stem rust resistance genes/QTL have been developed and used for deploying these *Sr* genes/QTL in breeding programmes (Table 2.3).

2.8 Summary and motivation for this study

SA is one of the major producers of bread wheat in sub-Saharan Africa. However, the country remains a net importer of wheat. The productivity of wheat in SA is constrained by both biotic and abiotic factors. Among the biotic factors, stem rust is of great concern contributing towards risk and cost of production when susceptible varieties are planted. This has been compounded by the emergence of new virulent stem rust races such as Ug99 and its variants. A few stem rust resistance genes/QTL have been deployed in South African wheat varieties, hence evidence of their vulnerability to virulent stem rust races. Before 1999 stem rust control in SA was largely a success mainly due to the benefits that accrued from the annual stem rust surveys initiated in the early 1920s. From these annual rust survey new and virulent stem rust races have been detected in a timely manner hence enabling deployment of effective and efficient control strategies. The recent emergence of race Ug99 and other virulent stem rust races has re-emphasised the significance of stem rust surveys and the characterisation of wheat varieties and breeding lines to identify useful stem rust resistance genes.

Table 2.3 Single nucleotide polymorphism and other markers linked to various stem rust resistance genes/quantitative trait loci (QTL)

Gene/QTL	Chromosome	Marker	Reference
QTL	1B and 2B	SNP	Kankwatsa et al., 2017
QTL	7AL	SNP	Babiker et al., 2015; Pujol et al., 2015
QTL	2B	SNP	Bajgain et al., 2015a
QTL	1AS, 2BS, 6AS and 6AL	SNP	Dunckel et al., 2015
QTL	2BL and 6AL	SNP	Guerrero-Chavez et al., 2015
Sr2, *Sr8a* and *Sr11*	3B, 4D, 5A, 5B and 6A	SNP	Bajgain et al., 2015b
Sr2 and other genes	3BS	SNP	Kumssa et al., 2015
Sr2	3BS	SSR	Spielmeyer et al., 2003; Hayden et al., 2004; Mago et al., 2011
Sr6	2D	SNP	Mourad et al., 2018
Sr6	2D	SSR	Tsilo et al., 2009, 2010
Sr7a	4AL	SNP	Turner et al., 2016; Saini et al., 2018
Sr7a	4AL	SSR	Turner et al., 2016
Sr8a	6AS	SNP	Hiebert et al., 2017
Sr8a	6AS	SSR	Bhavani et al., 2008
Sr9a	2BL	SSR	Tsilo et al., 2007
Sr9h	2BL	SNP	Randhawa et al., 2018
Sr9h	2BL	SSR	Hiebert et al., 2010; Rouse et al., 2014

Table 2.3 Continued

Gene/QTL	Chromosome	Marker	Reference
Sr11	6BL	SNP	Nirmala et al., 2016
Sr12	3BL	SNP	Hiebert et al., 2016a
Sr13	6AL	SNP/Gene	Zhang et al., 2017; Saini et al., 2018
Sr13	6AL	SSR/STS	Admassu et al., 2011; Simons et al., 2011; Periyannan et al., 2014
Sr15	7AL	SNP	Babiker et al., 2015; Gao et al., 2018
Sr15	7AL	SSR	Neu et al., 2002; Jayatilake et al., 2013
Sr21	2AL	SSR/CAPS/Gene	Chen et al., 2015, 2018a
Sr22	7AL	SSR/STS/Gene	Khan et al., 2005; Periyannan et al., 2011; Steuernagel et al., 2016
Sr24	3DL	SSR/PCR	Mago et al., 2005
Sr25	7DL	SNP	Yu et al., 2017b
Sr25	7DL	SSR/STS	Prins et al., 2001; Gupta et al., 2006a, 2006b; Li et al., 2006; Liu et al., 2010
Sr26	6AL	SSR/PCR/SCAR	Mago et al., 2005; Liu et al., 2010; Rai et al., 2017
Sr26	6AL	SNP	Qureshi et al., 2018; Zhang et al., 2018
Sr28	2BL	SNP	Babiker et al., 2017
Sr28	2BL	SSR	Bansal et al., 2012; Rouse et al., 2012
Sr31	1BL/IRS	STS/SCAR	Mago et al., 2002; Das et al., 2006
Sr32	2DS	STS	Mago et al., 2013

Table 2.3 Continued

Gene/QTL	Chromosome	Marker	Reference
Sr33	1DS	Gene	Periyannan et al., 2013
Sr35	3AL	SSR/Gene	Babiker et al., 2009; Zhang et al., 2010; Saintenac et al., 2013
Sr36	2BS	SSR	Tsilo et al., 2008
Sr38	2AS	CAPS	Robert et al., 1999; Helguera et al., 2003
Sr39	2BS	SCAR/STS	Gold et al., 1999; Mago et al., 2009
Sr40	2BS	SSR	Wu et al., 2009
Sr42	6DS	SNP	Gao et al., 2015
Sr42	6DS	SSR	Ghavzini et al., 2012
Sr42/Tmp/Cad	6DS	SNP	Gao et al., 2015; Hiebert et al., 2016b; Kassa et al., 2016
Sr43	7DL	SSR	Niu et al., 2014
Sr44	7DS	STS	Liu et al., 2013
Sr45	1DS	Gene	Steuernagel et al., 2016
Sr46	2DS	SSR/STS	Yu et al., 2015
Sr46	2DS	Gene	Arora et al., 2018
Sr47	2BL	SSR	Klindworth et al., 2017
Sr47	2BL	SNP	Klindworth et al., 2017
Sr48	2AL	SSR/PCR	Bansal et al., 2008, 2009

Table 2.3 Continued

Gene/QTL	Chromosome	Marker	Reference
Sr49	5BL	SNP	Bansal et al., 2015
Sr50	IDL	PCR	Anugrahwati et al., 2008; Mago et al., 2015
Sr51	3DS	STS	Liu et al., 2011a
Sr52	6AL	STS	Qi et al., 2011
Sr53	5DL	SSR/STS	Liu et al., 2011b
Sr54	2DL	SSR	Ghazvini et al., 2013
Sr55	4DL	SSR	Herrera-Foessel et al., 2014
Sr55	4DL	SNP/Gene	Forrest et al., 2014; Moore et al., 2015
Sr56	5BL	SSR/STS	Bansal et al., 2014
Sr57	7DS	STS/Gene	Suenaga et al., 2003; Lagudah et al., 2006, 2009; Krattinger et al., 2009
Sr58	IBL	AFLP	William et al., 2003; Singh et al., 2013b
Sr59	2DS	SNP	Rahmatov et al., 2016
SrTmp	6DS	SSR	Hiebert et al., 2016b
SrTmp	6DS	SNP	Hiebert et al., 2016b
SrCad	6DS	SNP	Kassa et al., 2016
SrCad	6DS	SSR	Hiebert et al., 2011
Sr60 and *SrTm5*	5AS/7AL	SNP	Chen et al., 2018b

Table 2.3 Continued

Gene/QTL	Chromosome	Marker	Reference
Sr60	5AS	PCR/Gene	Chen et al., 2020
SrTm4	2AL	STS/SSR	Briggs et al., 2015
SrTA10187	6DS	SNP	Wiersma et al., 2016
SrTA10187	6DS	SSR	Olson et al., 2013a
SrWLR	2BL	SNP	Zurn et al., 2014
Sr8155B1	6AS	SNP	Nirmala et al., 2017; Saini et al., 2018
SrH and *SrY*	2BL	SNP	Randhawa et al.2018
SrND643	4AL	SNP	Basnet et al., 2015
Sr1662	IDS	PCR/Gene	Arora et al., 2018
Sr1662	IDS	SSR/STS	Olson et al., 2013b
Sr8155B1	6AS	SNP	Nirmala et al., 2017; Saini et al., 2018
SrPI410966	2BS	SSR	Campbell et al., 2016
Sr-1644-1Sh/5Sh	1Sh/5Sh	SNP	Yu et al., 2017a

SSR = Simple sequence repeat, Gene = Gene specific marker, STS = Sequence tagged site, CAPS = Cleaved amplified polymorphic sequence, PCR = Polymerase chain reaction, SCAR = Sequence characterised amplified region, AFLP = Amplified fragment length polymorphism.

Although some South African winter wheat varieties contain ASRgenes for stem rust resistance, the nature of their genetics are unknown. Therefore, it is important to characterise these existing sources of stem rust resistance to firstly determine the number of resistant genes involved, their potential vulnerability to new virulent races and lastly to develop molecular markers that can effectively and efficiently be used in MAS to facilitate future breeding for stem rust resistance.

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CHAPTER 3

INHERITANCE OF STEM RUST RESISTANCE IN SOUTH AFRICAN WINTER WHEAT VARIETIES

3.1 Abstract

A number of South African winter wheat varieties have been observed to express resistance to *Pgt*, but the basis of their stem rust resistance was unknown. To understand the origin and inheritance of ASR to *Pgt* race PTKST, four resistant varieties Komati, Koonap, Limpopo and SST 387 were crossed with the stem rust susceptible wheat parent Line 37-07. Results of seedling phenotyping of the F_2 and F_3 offspring showed that a single dominant gene conferred stem rust resistance in each of the four resistant parents. Allelism tests were conducted among these resistant varieties and the resulting progeny were all resistant to *Pgt* race PTKST suggesting that either the same gene or closely linked genes confer resistance in the four wheat varieties. However, an allelism test of resistant parents with Norin 40 (*Sr42*) and CnsSrTmp (*SrTmp*) were inconclusive and indicated that it is either a closely linked gene, *Sr42* or *SrTmp* that confers resistance to *Pgt* race PTKST. Multi-race phenotyping results ruled out the involvement of *Sr42* or *SrCad*, but suggest the likely presence of *SrTmp* in all four wheat varieties. The variety PAN 3161 that possesses APR was also crossed to the susceptible wheat parent Line 37-07 to study the nature of stem rust resistance inheritance. The F_2 segregation ratios from the Line37-07/PAN3161 population conformed to a 3:1 ratio indicating that a single dominant gene confers APR in this variety. However, F_3 family segregation ratios of Line37-07/PAN3161 conformed to a 7:8:1 ratio suggesting that PAN 3161 carries two dominant APR genes.

3.2 Introduction

Stem rust, caused by the obligate pathogen *Pgt* is an economically important disease of wheat globally (Singh et al., 2016) and causes substantial yield losses when epidemic outbreaks occur (Park, 2007; Soko et al., 2018). Globally, wheat production has been under threat by the emergence of a new *Pgt* race Ug99, virulent for *Sr31*, one of the most commonly deployed stem rust resistance genes in recent decades (Pretorius et al., 2000). At present, 13 variants of Ug99 are known to exist differing in virulence for *Sr9h, Sr21, Sr24, Sr31, Sr36* and *SrTmp* (Singh et al., 2011, 2015; Pretorius et al., 2012a; Fetch et al., 2016; Newcomb et al., 2016; Patpour et al., 2016a, 2016b). These *Pgt* races have been reported in at least 13 countries

including Egypt, Eritrea, Ethiopia, Iran, Kenya, Mozambique, Rwanda, SA, Sudan, Tanzania, Yemen and Zimbabwe (Singh et al., 2008; Nazari et al., 2009; Visser et al., 2011; Pretorius et al., 2012a; Terefe et al., 2019; http://rusttracker.cimmyt.org/?page_id=305). Previous estimates indicated that 5-15% of global wheat varieties are resistant to Ug99 (Jin and Singh, 2006; Singh et al., 2008; Mago et al., 2011). The stem rust pathogen continues to evolve as was shown recently by emergence of a new and virulent non-Ug99 race TKTTF, first identified in Turkey in 2007. During the 2013/2014 seoaon in Ethiopia, race TKTTF caused up to 100% yield losses in Digalu, that was one of the most widely planted varieties (Olivera et al., 2012, 2015; Singh et al., 2015). Digalu carries *SrTmp* (Olivera et al., 2015) that is postulated to be present in SA winter wheat varieties. Race TKTTF has spread towards many countries including Azerbaijan, Egypt, Iran, Iraq and Sweden and has acquired virulence for *Sr25* (Patpour et al., 2017). This illustrates the risk that *Pgt* poses to wheat production worldwide.

Five members of the Ug99 race group TTKSF, TTKSP, PTKST, PTKSK and TTKSF+ have been reported in SA (Terefe et al., 2010, 2019). Among these races, PTKST is potentially the most destructive with combined virulence to *Sr24*, *Sr31*and *Sr38*. PTKST is characterised by avirulence to *Sr9h*, *Sr21*, *Sr26*, *Sr27*, *Sr36*, *SrKiewiet*, *SrSatu* and *SrTmp* and virulence to *Sr5*, *Sr6*, *Sr7b*, *Sr8a*, *Sr8b*, *Sr9a*, *Sr9b*, *Sr9d*, *Sr9e*, *Sr9g*, *Sr10*, *Sr11*, *Sr17*, *Sr24*, *Sr30*, *Sr31*, *Sr38* and *SrMcN*) (Pretorius et al., 2010).

The use of resistant varieties remains the most economic and environmentally friendly way of controlling stem rust (Bajgain et al., 2016). In SA, a number of winter wheat varieties have been shown to possess uncharacterised sources of stem rust resistance against races of *Pgt*, including Ug99 races (Pretorius et al., 2012b). Characterising previously identified and new sources of resistance will contribute towards the development of varieties with effective gene combinations that are broadly resistant to stem rust (Hiebert et al., 2011; Bajgain et al., 2016). This can be achieved through a search for race-specific ASR representing the most utilised source of resistance to *Pgt* (Haile and Röder, 2013; Bajgain et al., 2016). Alternatively, APR can be exploited as several of these genes have been reported to confer minor reduction (5-20%) in disease severity (Bajgain et al., 2016). Combination of APR and/or ASR genes may result in high levels of protection as well as longer lasting resistance and is therefore considered a more attractive rust control strategy (Leonard and Szabo, 2005; Haile and Röder, 2013; Bajgain et al., 2016).

South African winter wheat varieties Komati, Koonap, Limpopo and SST 387 possess ASR (Pretorius et al., 2012b), while PAN 3161 possesses APR to *Pgt* race PTKST. However, the nature of inheritance of these resistances is unknown. The aims of this study were to (1) determine the origin and inheritance of resistance against race PTKST in these winter wheat varieties, (2) assess the allelic relationship between the resistance gene(s) among the four winter wheat varieties with ASR and (3) evaluate the relationship between the resistance gene(s) in the four winter wheat varieties showing ASR and other known resistance genes such as *Sr42* and *SrTmp*.

3.3 Materials and methods

3.3.1 Development of F_2 and F_3 mapping populations

The stem rust resistant winter wheat varieties Komati, Koonap, Limpopo, SST 387 and PAN 3161 were each crossed with the stem rust susceptible variety Line 37-07 (pedigree: Kasyob/Genaro-81//Cham4). The spring wheat variety Line 37-07 represents entry 37 of the 2nd International Stem Rust Trap Nursery (ISRTN07) and was selected based on its susceptibility to *Pgt* race PTKST in field trials in SA. Twelve F_1 seeds from each population, originating from a single spike, were raised in 2 ℓ pots filled with steam-sterilised soil in a greenhouse at 18-22°C. Pots were watered daily and fertilised weekly with 50 ml water-soluble fertiliser (Effekto® NPK 19:8:6 (43)). Spikes produced on 10 F_1 plants were covered individually with glassine crossing bags before flowering to prevent out-crossing and F_2 seeds harvested at maturity. F_2 seeds were bulked and used for seedling phenotyping.

In addition, one hundred seeds from each of the four F_2 populations were randomly selected and space planted in a field near Bethlehem in the eastern Free State to develop F_3 populations. The trial was planted and maintained under the supervision of wheat research staff at the seed company Sensako. Upon ripening, F_2 adult plants were pulled from the ground for each population and harvested (F_3 seeds) as single plants.

3.3.2 Development of wheat intercrosses

The winter wheat varieties Komati, Koonap, Limpopo and SST 387 were crossed to each other (intercrosses) to develop four F_1 populations. Ten seeds of each F_1 combination were vernalised for six weeks in a cold room at a temperature of ±5°C. Ten F_1 seedlings per intercross were transplanted into 2 ℓ pots filled with steam-sterilised soil. Spikes produced on the F_1 plants

were covered individually with glassine crossing bags before the onset of flowering to prevent out-crossing and F_2 seeds harvested at maturity.

The resistance gene identified in the South African winter wheat varieties mapped to the short arm of chromosome 6D, where *Sr42*, *SrTmp* and *SrCad* are all located (results are presented in Chapter 4). Unfortunately, no seed of the *SrCad* containing variety AC Cadillac (Hiebert et al., 2011) was available. To assess the relationship between the mapped resistance gene(s) and *Sr42* and *SrTmp*, crosses were made between Komati, Koonap, Limpopo and SST 387 with Norin 40 carrying *Sr42* (Ghazvini et al., 2012) and CnsSrTmp carrying *SrTmp* (Hiebert et al., 2016). The four South African winter wheat varieties were used as female parents in all crossing combinations while Norin 40 and CnsSrTmp were used as male parents. Seeds harvested from these crossing combinations were vernalised for six weeks through incubation in a cold room kept at ±5°C before seedlings were transplanted in 2 l pots and raised in a greenhouse as described above to develop F_2 populations. Molecular marker analysis was carried out using diagnostic SNP markers linked to *Sr42*, *SrTmp* and *SrCad* as described in Chapter 4.

3.3.3 Establishment of field trials

Field trials from 2017 to 2018 at Redgates Research Station, Pannar Seed, Greytown, SA were established by hand sowing entries in 1 m rows with 50 cm inter-row spacing. In 2017, 200 F_2 seeds of Line37-07/PAN3161were planted on May, 25th. Twenty seeds of each parents' F_2 (resistant x susceptible) progeny were hand sown in two, 1 m row plots with intra- and inter-row spacing of 10 x 50 cm. The F_3 families that originated from 56 individual F_2 plants were used to plant three rows with 10 seeds per row for each family on May 30th, 2018 at the Greytown field trial site. Trial planting dates allowed for sufficient vernalisation of longer growers, ruling out growth period as a major factor in final ratings.

3.3.4 Stem rust phenotyping

3.3.4.1 Greenhouse stem rust phenotyping

The parental lines, stem rust resistant control lines and F_2 progeny (±450 seeds planted per F_2 population) were screened in the greenhouse to determine their stem rust seedling infection types (ITs) using race PTKST. Seed was sown in a sterilised soil-peat moss (50:50) mixture in 10 cm diameter pots which were maintained in a growth chamber at 25°C until seedling emergence, before placement at 18-23°C under natural light in a greenhouse. Seedlings were

watered daily and fertilised with Multi-feed classic water-soluble fertiliser (Effekto® NPK 19:8:6 (43)) once before inoculation. Primary leaves of seedlings were inoculated eight days after planting with a 5 mg/ml suspension of freshly collected urediniospores of *Pgt* race PTKST suspended in Soltrol 130® isoparaffinic oil (Chevron Phillips Chemical Co., Borger, Texas, USA). After drying for 1 h in a growth cabinet set at 25°C seedlings were placed in a dark dew chamber for 16-18 h set at ±18°C. Upon removal seedlings were dried for 3 h under light in a growth chamber set at 25°C, and returned to a greenhouse cubicle with a mean night- and day temperature regime of 18-23°C. Seedlings with ITs of 0, fleck (;), 1 and 2 or combinations thereof were considered as resistant while seedlings with ITs of 3-4 were considered as expressing susceptible phenotypes. For QTL analysis the individual IT scores were converted to a linear scale of 0 to 9 following the method described by Zhang et al. (2014).

F_3 populations were evaluated following the same inoculation and incubation procedures described for the F_2 populations. From each F_3 population, 30 kernels were randomly selected per plant and planted in 10 cm diameter pots. F_3 seedlings were grown and managed as described for F_2 populations. Because of the need for vernalisation, F_3 families were advanced from F_2 plants that were not phenotyped. At F_3 level, families with at least 15 resistant seedlings and zero susceptible seedlings were classified as homozygous resistant, whereas families with at least 15 susceptible plants and zero resistant plants were classified as homozygous susceptible. Families with both resistant and susceptible seedlings were classified as segregating families.

For the resistant variety intercrosses, between 444 and 451 F_2 seedlings per combination were inoculated with freshly collected urediniospores from *Pgt* race PTKST. Standard seedling inoculation, incubation conditions and rating of seedlings were followed as described above. For the allelism tests between the resistant South African cultivars Komati, Koonap, Limpopo and SST 387 with the two known chromosome 6DS stem rust resistance genes Norin 40 (*Sr42*) and CnsSrTmp (*SrTmp*), between 372 and 737 seedlings were screened per combination with *Pgt* race PTKST.

3.3.4.2 Multi-race stem rust phenotyping

In an effort to assess the response of PAN 3161 as well as to resolve the relationship between the ASR mapped in the South African winter wheat varieties Komati, Koonap, Limpopo and SST 387 and other chromosome 6D genes *Sr42*, *SrTmp* and *SrCad*, the resistant parents,

susceptible parent and chromosome 6D control lines Norin 40, CnsSrTmp and AC Cadillac were tested against an array of *Pgt* races QCCJB, QTHJC, TRTTF, TTKSK, and TTKTT (Table 3.1) to differentiate between *Sr42*, *SrTmp* and *SrCad*. The *Pgt* races were used in replicated seedling trials to determine the seedling ITs of the South African winter wheat varieties SST 387, Komati, Koonap and Limpopo as well as the chromosome 6D *Sr* control entries Norin 40 (*Sr42*), CnsSrTmp (*SrTmp*) and AC Cadillac (*SrCad*). The multi-race screening was conducted at the United States Department of Agriculture-Agricultural Research services (USDA-ARS), Cereal Disease Laboratory (CDL), St. Paul, MN under supervision of Dr Matt Rouse during April 2019. Stem rust phenotyping was done following procedures described previously (Rouse et al., 2011).

Table 3.1 *Puccinia graminis* f. sp. *tritici* races used in seedling tests to determine the resistance gene(s) present in the South African winter wheat varieties SST 387, Komati, Koonap and Limpopo

Race	Isolate	Origin	Avirulence/Virulence formula
QCCJB	Unknown	USA	*Sr6, Sr7b, Sr8a, Sr9a, Sr9b, Sr9e, Sr11, Sr24, Sr30, Sr31,* Sr36, *Sr38, Sr42, SrCad, SrMcN, SrTmp / Sr5, Sr9d, Sr9g, Sr10, Sr17, Sr21*
QTHJC	75ND717C	USA	*Sr7b, Sr9a, Sr9e, Sr24, Sr30, Sr31, Sr36, Sr38, SrCad, SrTmp / Sr5, Sr6, Sr9b, Sr21, Sr8a, Sr9d, Sr9g, Sr10, Sr11, Sr17, Sr42, SrMcN*
TRTTF	06YEM34-1	Yemen	*Sr8a, Sr24, Sr31, SrCad / Sr5, Sr6, Sr7b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr17, Sr21, Sr30, Sr36, Sr38, Sr42, SrMcN, SrTmp*
TTKSK	04KEN156/04	Kenya	*Sr24, Sr36, Sr42, SrCad, SrTmp / Sr5, Sr6, Sr7b, Sr8a, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr17, Sr21, Sr30, Sr31, Sr38, SrMcN*
TTKTT	14KEN58-1	Kenya	*Sr36, Sr42 / Sr8a, Sr5, Sr6, Sr7b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr17, Sr21, Sr24, Sr30, Sr31, Sr38, SrCad, SrMcN, SrTmp*

3.3.4.3 Field stem rust phenotyping

The stem rust resistant parental lines Komati, Koonap, Limpopo, SST 387, PAN 3161 and susceptible parent Line 37-07 alongside stem rust resistant controls Norin 40 and CnsSrTmp were also assessed for their adult plant response by planting 20 seeds of each parent in 1 m row plots with inter spacing of 50 cm in field trials during the 2017 and 2018 seasons. Field trials were planted near Greytown, KwaZulu-Natal with regular irrigation and sufficient fertiliser applied to support optimum plant development. Spreader rows of the susceptible variety Line 37-07 were included and replicated within the trial. Rows planted with Line 37-07 were inoculated annually between six and eight weeks after planting, using an ultralow-volume sprayer (ULVA; Micron Group, Bromyard, England), with a suspension of freshly collected urediniospores in Soltrol® 130 isoparaffinic oil (± 3 mg/ml) to achieve early infection and consequent epidemic development. Urediniospores of *Pgt* race PTKST was increased annually in the greenhouse, prior to field inoculation on the variety Federation/Kavkaz (*Sr31*) and used for field inoculation. The stem rust susceptible wheat entries Line 37-07 and McNair were planted adjacent to every experimental row. Stem rust susceptible spreader blocks and head rows, planted with Line 37-07, were annually inoculated with race PTKST using fresh urediniospores and covered with a plastic tent overnight to facilitate dew formation.

The modified Cobb scale (Peterson et al., 1948) combined with a host infection response (Roelfs et al., 1992) was used to determine the field response of cultivars during grain fill. Scoring for stem rust host response was done twice during the growing season from end of flowering (growth stage 65) to just before the hard dough stage (growth stage 87) (Zadoks et al., 1974). F_2 plants were scored based on their host reaction type as resistant [R, moderately resistant and resistant (MRR) and moderately resistant and moderately susceptible (MRMS)] and susceptible [S and moderately susceptible and susceptible (MSS)]. The F_3 families were classified as homozygous resistant (combinations of R, MRR and MRMS), segregating (R, MRMS and S) and homozygous susceptible (S and MSS). Coefficients of infection (CI) = DS x IT were chosen for QTL analysis, where DS = disease severity, infection type immune = 0, R = 0.2, MR = 0.4, MRMS = 0.6, MS = 0.8 and S =1.0 (Stubbs et al., 1986; Yu et al., 2011). The Shapiro-Wilk statistical test was used to assess the normality of the linearised stem rust data.

3.4 Data analysis

Observed stem rust phenotypic segregation ratios of the F_2 populations and F_3 families were evaluated using the Chi-square (Cochran, 1952) test to evaluate the goodness of fit to known Mendelian segregation ratios. For the allelism experiment, Chi-square tests were done to determine the goodness of fit to the known segregation ratios. Besides, if no segregation for stem rust was observed in the F_2 progeny, it was believed that the respective parents carried the same gene(s) or very closely linked genes but when segregation in F_2 was observed, it suggested that more than one independent gene is involved.

3.5 Results

3.5.1 Evaluation of inheritance of stem rust resistance genes in South African winter wheat varieties

Komati, Koonap, Limpopo and SST 387 displayed resistant seedling ITs ranging mostly from a ;1^{-} to ;12 whereas the susceptible parent Line 37-07 produced a distinctively compatible seedling IT of 3++ (Table 3.2; Figure 3.1). Parent PAN 3161 expressed a susceptible seedling IT of 3 (Table 3.2). Varieties Komati, Koonap, SST 387 and PAN 3161 expressed resistant (R) to moderately resistant (MR) host reaction types; Limpopo, Norin 40 and CnsSrTmp were MR to moderately susceptible (MS) whereas Line 37-07 expressed a susceptible response (S) in field evaluations for the 2017 and 2018 seasons (Table 3.2).

Variation in seedling ITs observed in the respective F_2 populations and F_3 families are presented in Figures 3.2 and 3.3. The resistant control genotype Norin 40 (*Sr42*) and wheat lines carrying *SrTmp* including CnsSrTmp (*SrTmp*) expressed similar resistant seedling ITs (Table 3.2; Figure 3.4). The different wheat lines carrying *SrTmp* showed slight variation in their phenotypic responses to *Pgt* race PTKST with the lowest seedling IT recorded for Digalu (IT 2=) and the highest for McNSrTmp (IT 22+) (Figure 3.4).

To facilitate mapping of resistance gene(s) identified in South African winter wheat varieties, 200 seedlings were selected per F_2 combination represented by 150 resistant seedlings (ITs $\leq$ 2+) and 50 susceptible seedlings (ITs $\geq$3) per mapping population (Figure 3.5; Appendix A). The four resistant winter wheat varieties, F_2 populations and F_3 families, segregated for a single resistance gene to race PTKST (Tables 3.3, 3.4 and 3.5) as described below for each cross.

a) Line37-07/Komati

In the Line37-07/Komati F_2 population, there was a distribution of 433 resistant and 127 susceptible seedlings. The Chi-square ($\chi 2$) value of 1.610 (P = 0.205) showed that this distribution conformed to a segregation ratio of 3:1 expected for a single dominant resistance gene (Table 3.3). F_3 families segregated in a 14 homozygous resistant, 26 segregating and eight homozygous susceptible ratio ($\chi 2$ = 1.03, P = 0.596) that did not deviate significantly from the expected 1:2:1 ratio for a single dominant resistance gene (Table 3.4). Among the segregating F_3 seedlings, there was a distribution of 377 resistant and 121 susceptible seedlings with the Chi-square value of 0.07 (P = 0.797) confirming the F_2 distribution that conformed to a segregation of 3:1 expected for a single dominant resistance gene (Table 3.5).

b) Line37-07/Koonap

In the Line37-07/Koonap F_2 population, there was a distribution of 382 resistant and 124 susceptible seedlings (Table 3.3). The Chi-square value of 0.066 (P = 0.797) showed that this distribution conformed to a segregation ratio of 3:1 expected for a single dominant resistance gene (Table 3.3). F_3 families segregated in a 13 homozygous resistant, 36 segregating and 16 homozygous susceptible ratio ($\chi 2$ = 0.54, P = 0.763) that did not deviate significantly from the expected 1:2:1 ratio for a single dominant resistance gene (Table 3.4). Among the segregating F_3 seedlings, there was a distribution of 490 resistant and 157 susceptible seedlings with the Chi-square value of 0.09 (P = 0.759) confirming the F_2 distribution that conformed to a segregation of 3:1 expected for a single dominant resistance gene (Table 3.5).

c) Line37-07/Limpopo

In the Line37-07/Limpopo F_2 population, there was a distribution of 462 resistant and 143 susceptible seedlings (Table 3.3). The Chi-square value of 0.600 (P = 0.439) showed that this distribution conformed to a segregation ratio of 3:1 expected for a single dominant resistance gene (Tables 3.3 and 3.5). F_3 families segregated in a 11 homozygous resistant, 36 segregating and 14 homozygous susceptible ratio ($\chi 2$ = 1.20, P = 0.550) that did not deviate significantly from the expected 1:2:1 ratio for a single dominant resistance gene (Table 3.4). Among the segregating F_3 seedlings, there was a distribution of 525 resistant and 163 susceptible seedlings with the Chi-square value of 0.32 (P = 0.572) confirming the F_2 distribution that conformed to a segregation of 3:1 expected for a single dominant resistance gene (Table 3.5).

Table 3.2 Greenhouse seedling infection types (ITs) and adult plant field responses of parental lines as well as Norin 40 (*Sr42*) and CnsSrTmp (*SrTmp*) to *Puccinia graminis* f. sp. *tritici* race PTKST

Variety	Pedigree	Year released	IT[a]	Field response[b]
Komati	Molopo//P1-137729/5*Tugela-26	2002	;12 to 12c	10R-40MR
Koonap	Proprietary information	2010	;12	5R-50MR[c]
Limpopo	SA1684/4*Betta	1994	;1c	10MR-50MRMS
SST 387	Proprietary information	2008	;$1^{=}$ to ;$1^{=}$c	30-40MRR
PAN 3161	Proprietary information	2007	3	5R-20MR
Line 37-07	Kasyob/Genaro-81//Cham4	na[d]	3 to 3^{++}	90-100S
CnsSrTmp	na[d]	na	2	20MR-60MRMS
Norin 40	na	na	2	10MR-50MRMS

[a]Seedling infection types (IT) according to Stakman et al. (1962), [b]Range in adult plant stem rust host responses recorded for each variety. The first data point for each variety represents the highest early season host response recorded over two seasons and the second data point the follow-up rating ± 14 days later, [c]Field response according to Peterson et al. (1948) combined with a reaction type according to Roelfs et al. (1992), [d]Not applicable.

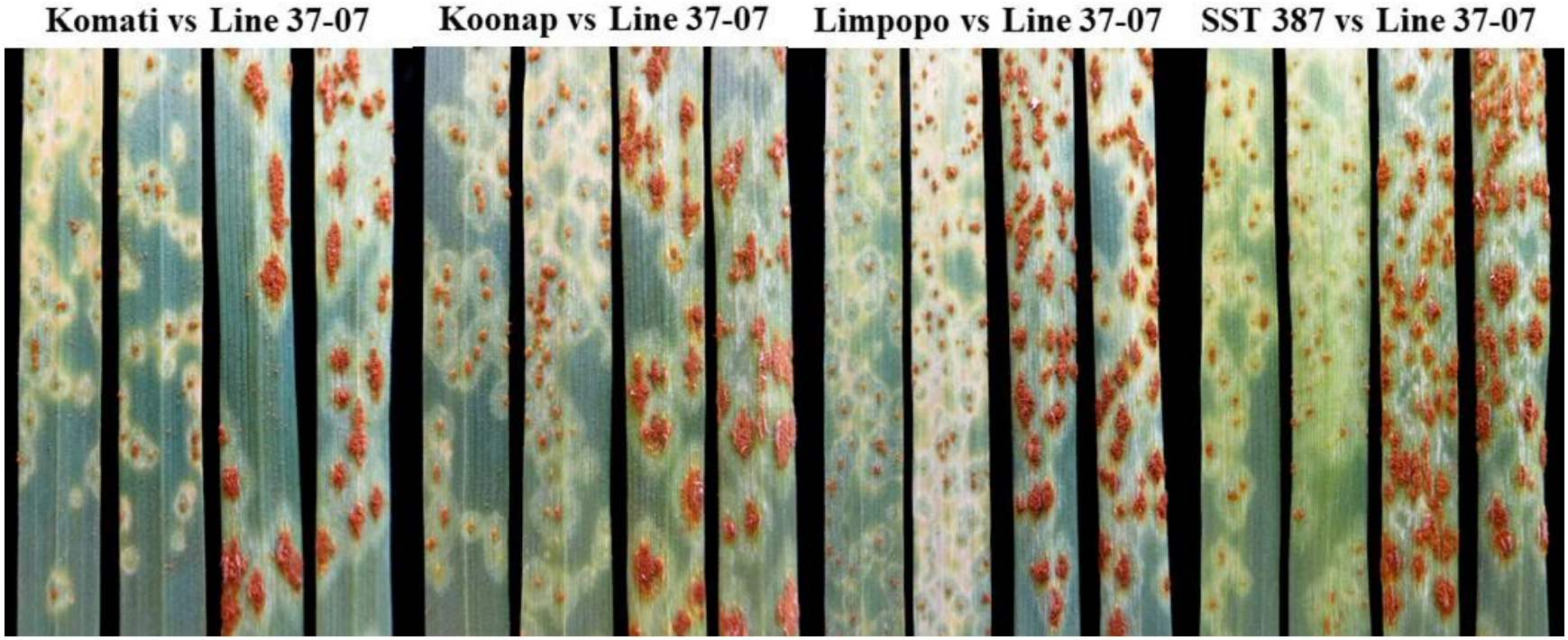

Figure 3.1 Seedling infection types produced by *Puccinia graminis* f. sp. *tritici* race PTKST on the resistant parents Komati, Koonap, Limpopo, and SST 387 (1st two leaves of each plate) and the susceptible parent Line 37-07 (3rd and 4th leaf of each plate)

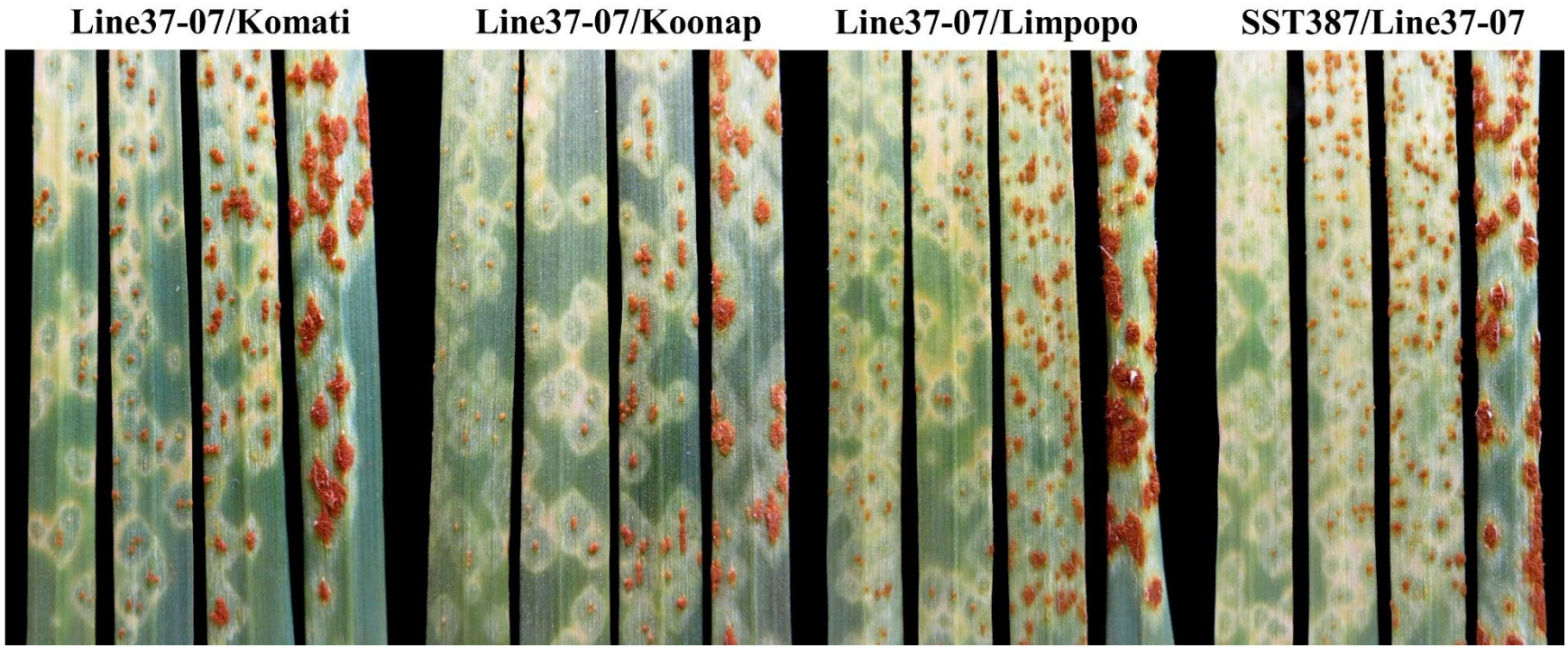

Figure 3.2 Variation in seedling infection types of F_2 populations considerd resistant (1st to 3rd leaf of each plate) and susceptible (4th leaf) to *Puccinia graminis* f. sp. *tritici* race PTKST

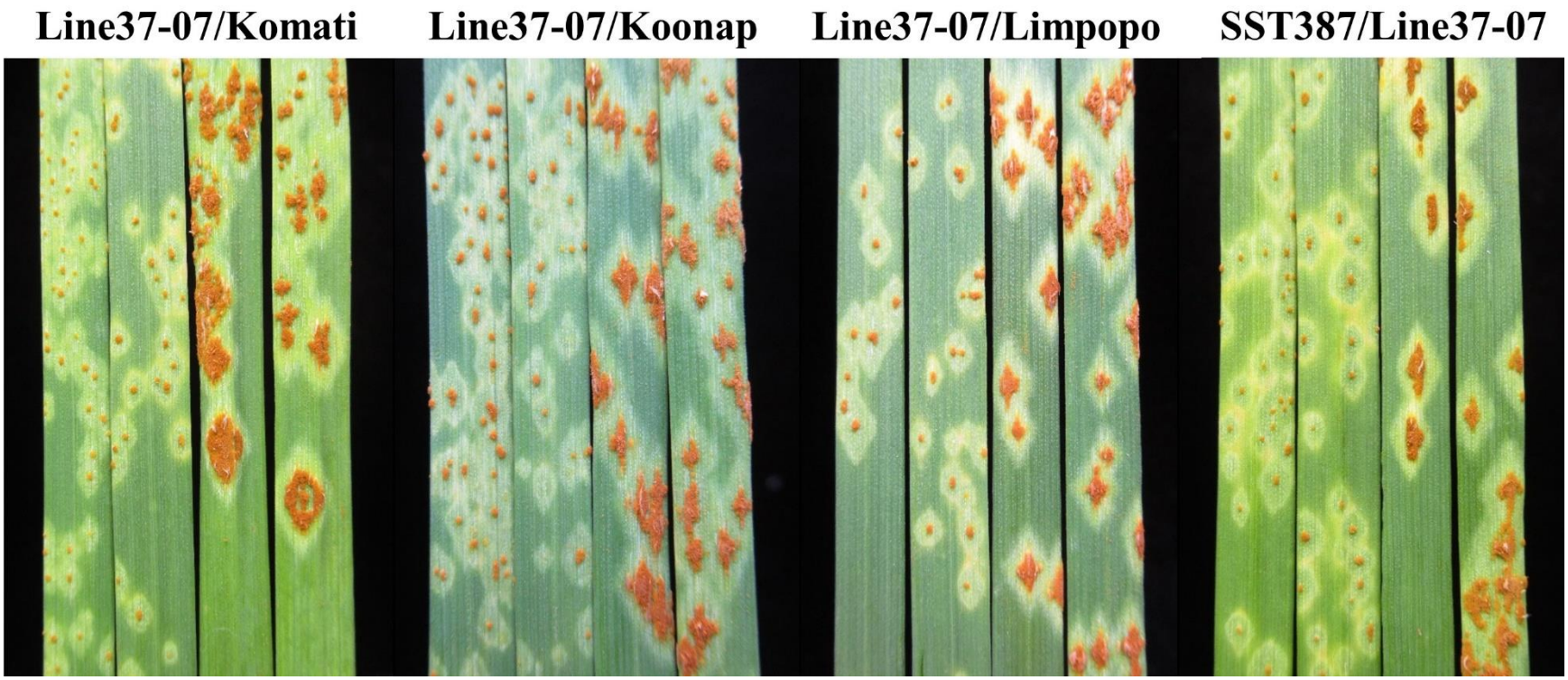

Figure 3.3 Variation in seedling infection types of F_3 families considered resistant (1st and 2nd leaf) and susceptible (3rd and 4th leaf of each plate) to *Puccinia graminis* f. sp. *tritici* race PTKST

Figure 3.4 Comparative seedling infection types (left to right) on the *Sr42* carrying line Norin 40 (2) and the *SrTmp* lines Digalu (2=), Triumph-64 (2-), CnsSrTmp (2), McNSrTmp (22+) and Federation*4/Kavkaz (3+) as a susceptible control *Sr31* line produced by *Puccinia graminis* f. sp. *tritici* race PTKST

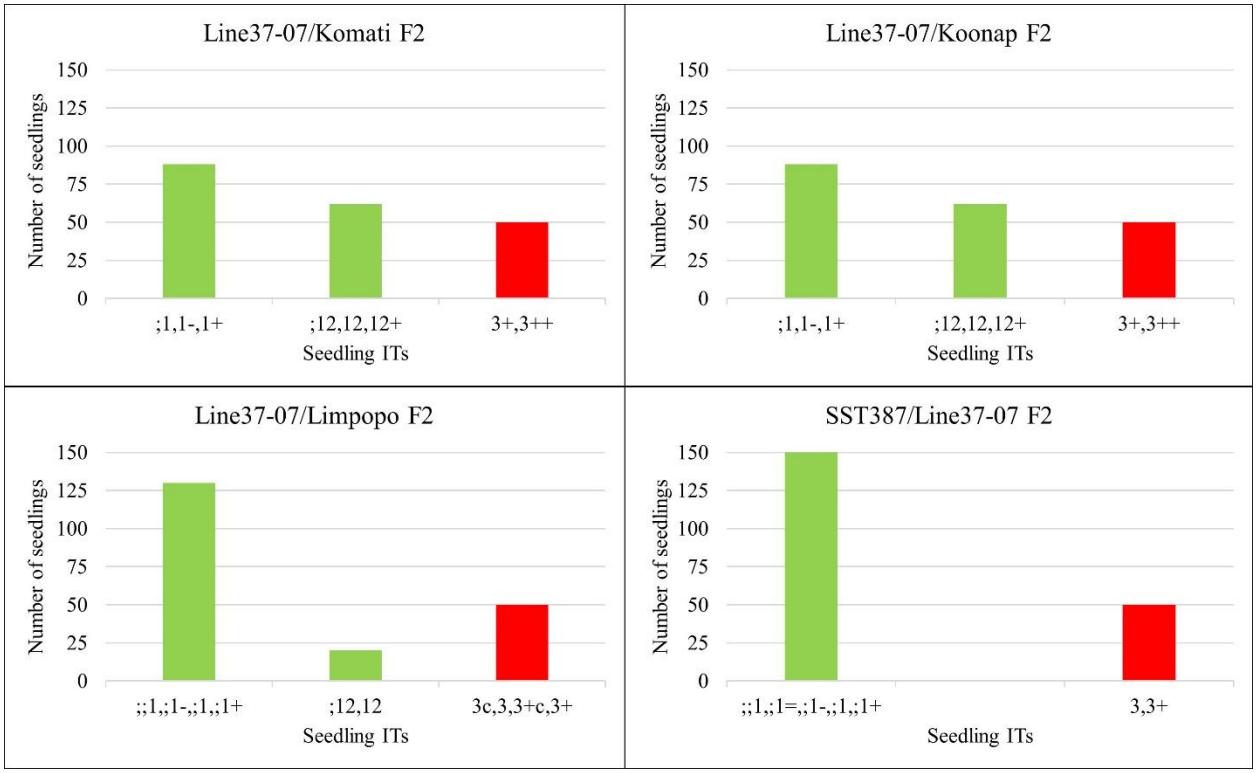

Figure 3.5 Distribution of seedling infection types (ITs) for *Puccinia graminis* f. sp. *tritici* race PTKST among different F_2 seedlings selected to represent the resistant (green bars) and susceptible (red) individuals for each F_2 combination

d) SST387/Line37-07

In the SST387/Line37-07 F_2 population, there was a distribution of 441 resistant and 132 susceptible seedlings (Table 3.3). The Chi-square value of 1.178 (P = 0.278) showed that this distribution conformed to a segregation ratio of 3:1 expected for a single dominant resistance gene (Table 3.3). F_3 families segregated in a 15 homozygous resistant, 40 segregating and 14 homozygous susceptible ratio ($\chi 2$ = 0.90, P = 0.637) that did not deviate significantly from the expected 1:2:1 ratio for a single dominant resistance gene (Table 3.4). Among the segregating F_3 seedlings, there was a distribution of 556 resistant and 197 susceptible seedlings with the Chi-square value of 0.27 (P = 0.607) confirming the F_2 distribution that conformed to a segregation of 3:1 expected for a single dominant resistance gene (Table 3.5).

Though 100 seeds per F_2 population were planted to raise F_3 populations, only 48, 65, 61 and 69 families of Komati, Koonap, Limpopo and SST 387, respectively, were obtained mainly due to seedling emergence challenges (Table 3.4). A total of 102 bulked F_3 homozygous lines consisting of 14, 13, 11 and 15 homozygous resistant and 8, 16, 11 and 14 homozygous susceptible F_3 lines for Komati, Koonap, Limpopo, and SST 387, respectively, were used to validate markers that flanked stem rust resistance gene(s) (Table 3.4 and Chapter 4).

Table 3.3 Greenhouse phenotypic evaluation of F_2 mapping populations based on seedling infection types to *Puccinia graminis* f. sp. *tritici* race PTKST

Crosses	Generation	Number of seedlings	Observed frequency	Expected ratios	$\chi 2$ (3:1)	P value
Line37-07/Komati	F_2	560	433:127 (3.4:1)	3:1	1.610	0.205
Line37-07/Koonap	F_2	506	382:124 (3.1:1)	3:1	0.066	0.797
Line37-07/Limpopo	F_2	605	462:143 (3.2:1)	3:1	0.600	0.439
SST387/Line37-07	F_2	573	441:132 (3.3:1)	3:1	1.178	0.278

Table 3.4 Validation of greenhouse phenotypic evaluation of F_3 families based on seedling infection types to *Puccinia graminis* f. sp. *tritici* race PTKST

Crosses	Generation	Number of families	Observed frequency	Expected ratios	$\chi 2$ (1:2:1)	P value
Line37-07/Komati	F_3	48	14:26:8 (1.8:3.2:1)	1:2:1	1.03	0.659
Line37-07/Koonap	F_3	65	13:36:16 (1:2.8:1.2)	1:2:1	0.54	0.763
Line37-07/Limpopo	F_3	61	11:36:11 (1:3.3:1)	1:2:1	1.20	0.550
SST387/Line37-07	F_3	69	15:40:14 (1.1:2.9:1)	1:2:1	0.90	0.637

Table 3.5 Greenhouse phenotypic evaluation of segregating F_3 families based on seedling infection types to *Puccinia graminis* f. sp. *tritici* race PTKST

Crosses	Generation	Number of seedlings	Observed frequency	Expected ratios	$\chi 2$ (3:1)	P value
Line37-07/Komati	F_3	498	377:121 (3.1:1)	3:1	0.07	0.797
Line37-07/Koonap	F_3	647	490:157 (3.1:1)	3:1	0.09	0.759
Line37-07/Limpopo	F_3	688	525:163 (3.2:1)	3:1	0.32	0.572
SST387/Line37-07	F_3	743	556:197 (2.8:1)	3:1	0.27	0.607

3.5.2 Allelic relationship of genes conferring resistance to stem rust in South African winter wheat varieties

All F_2 progeny of the four-winter wheat resistant variety intercrosses Komati/Limpopo (n = 444), Koonap/Komati (n = 444), Koonap/Limpopo (n = 447) and Limpopo/SST387 (n = 451) phenotyped with *Pgt* race PTKST were found resistant (Table 3.6 and Figure 3.6). The resistance was consistently expressed as a ;1^- to 1^+ which was clearly different from the susceptible control genotype Line 37-07 (Tables 3.1 and 3.6; Figure 3.6). Although it was not possible to observe any clear susceptible seedlings in crosses with *Sr42* or *SrTmp* (Table 3.7; Figures 3.6-3.8), it is worth noting that some F_2 seedlings showed intermediate range ITs that deviated from the more resistant ITs expressed by the resistant parents. This was more evident for F_2 offspring from the CnsSrTmp/Komati and Norin40/SST387 combinations (Figures 3.7 and 3.8).

Table 3.6 Results of greenhouse seedling screening with *Puccinia graminis* f. sp. *tritici* race PTKST to determine allelic relationship for gene(s) conferring resistance to stem rust in South African winter wheat varieties

Cross	Number of F_2 seedlings			
	Total	Resistant	Susceptible	Its
Komati/Limpopo	444	444	0	1 to 1+
Koonap/Komati	444	444	0	;1 to 1^+
Koonap/Limpopo	447	447	0	1 to 1+
Limpopo/SST387	451	451	0	;1 to 1^+

ITs = Infection types.

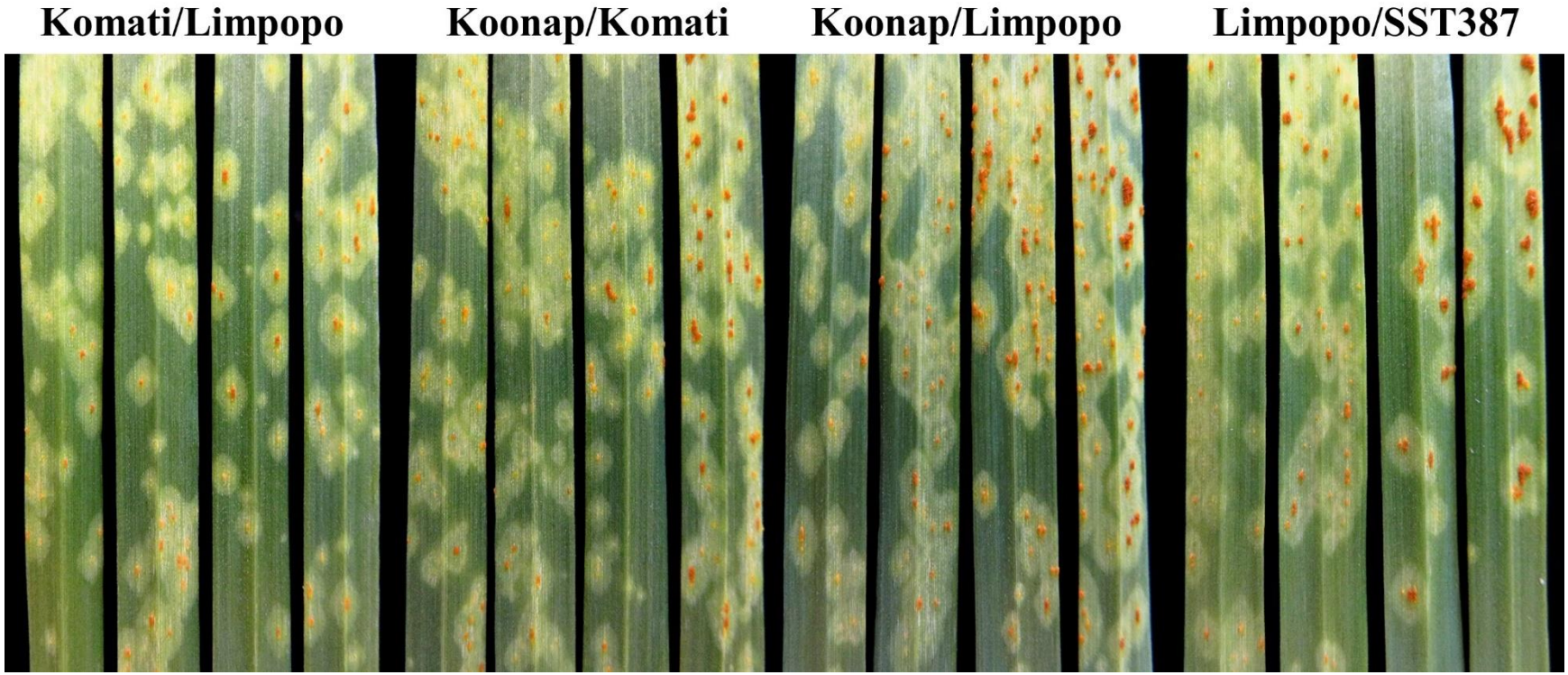

Figure 3.6 Resistant seedling infection types produced by *Puccinia graminis* f. sp. *tritici* race PTKST on F_2 seedlings derived from intercrosses among four resistant South African winter wheat varieties

Table 3.7 Greenhouse evaluation of allelic relationships for genes conferring resistance to *Puccinia graminis* f. sp. *tritici* race PTKST in South African winter wheat varieties

Cross	Genes involved	ITs	Total	Resistant	Susceptible
Komati/CnsSrTmp	*SrKm/SrTmp*	2^- to 2^+	585	585	0
Komati/Norin40	*SrKm/Sr42*	2^- to 2^+	372	372	0
Koonap/CnsSrTmp	*SrKn/SrTmp*	2^- to 2^+	642	642	0
Koonap/Norin40	*SrKn/Sr42*	2^- to 2^+	737	737	0
Limpopo/CnsSrTmp	*SrLm/SrTmp*	2 to 2^+	412	412	0
Limpopo/Norin40	*SrLm/Sr42*	2^- to 2^+	443	443	0
SST387/CnsSrTmp	*SrSt/SrTmp*	2^- to 2^+	489	489	0
SST387/Norin40	*SrSt/Sr42*	2^- to 2^+	447	447	0

ITs = Infection types.

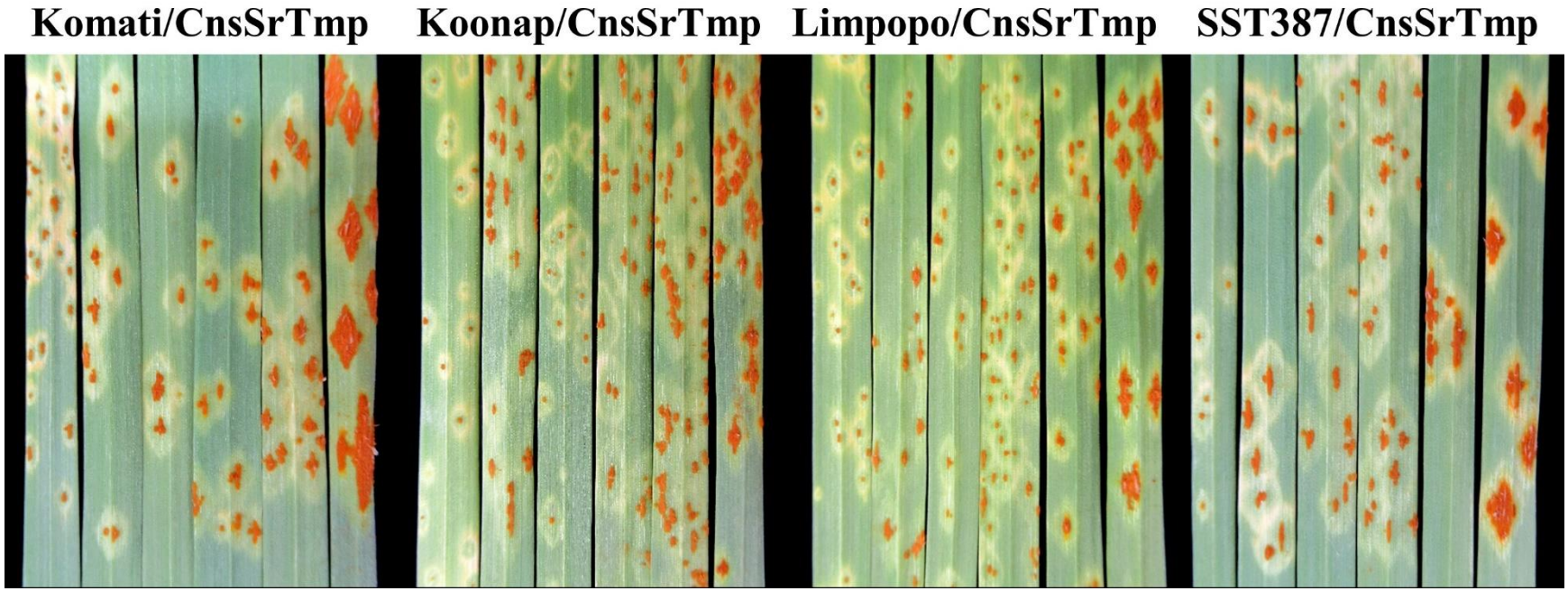

Figure 3.7 Seedling infection types (ITs) produced by *Puccinia graminis* f. sp. *tritici* race PTKST on F_2 intercrosses involving the resistant control CnsSrTmp. The 1st leaf on each photo represents the resistant parent's IT; 2nd to 5th leaves of each plate represent variation in resistant ITs and the 6th leaf the susceptible variety Line 37-07

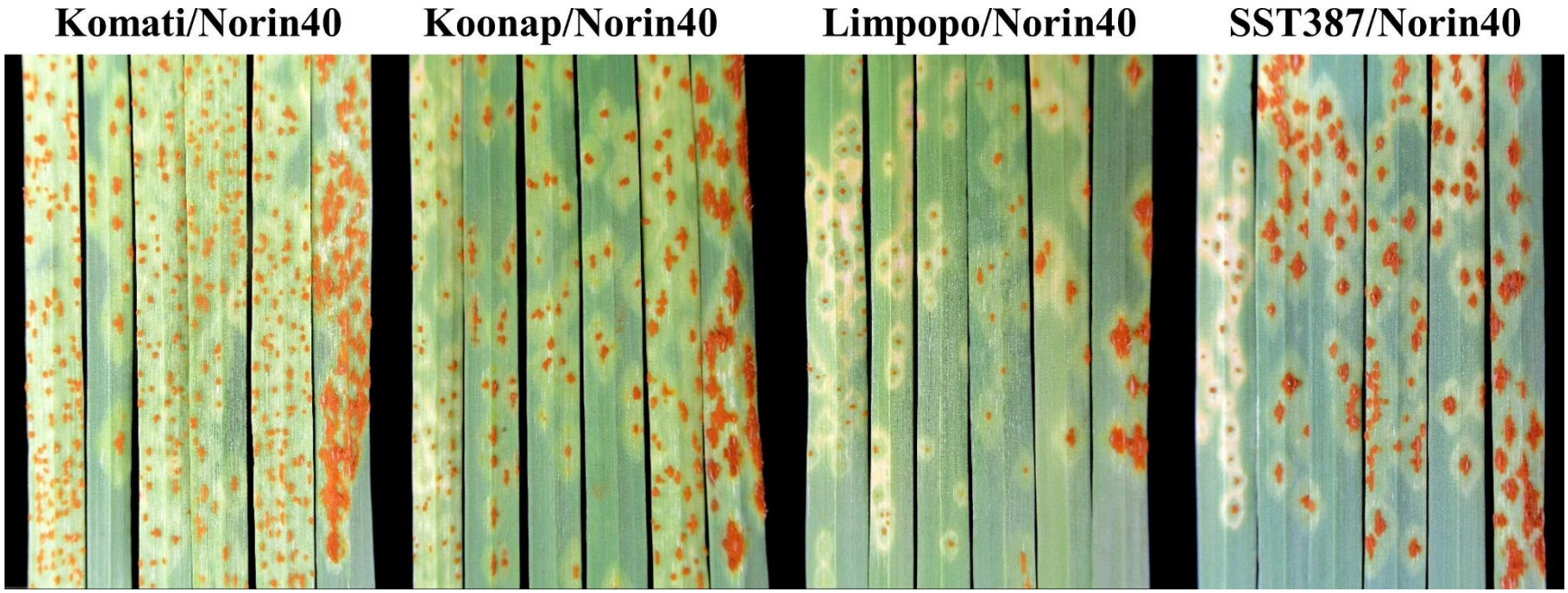

Figure 3.8 Seedling infection types (ITs) produced by *Puccinia graminis* f. sp. *tritici* race PTKST on F_2 intercrosses involving the resistant control Norin 40. The 1st leaf on each photo represents the resistant parent's IT; 2nd to 5th leaves of each plate represent variation observed in resistant ITs and the 6th leaf the susceptible variety Line 37-07

3.5.3 Multi-race seedling phenotyping of South African winter wheat varieties and chromosome 6D resistant control lines Norin 40, CnsSrTmp and AC Cadillac

Different ITs were expressed by the five *Pgt* races tested on both the South African winter wheat varieties and the resistant controls (Table 3.8). Two races QCCJB and TTKSK were avirulent on all tested *Sr* genes with ITs ranging from 0 to 2+. However, the other three races, QTHJC, TRTTF and TTKTT were either virulent or avirulent on the different *Sr* genes (Table 3.8). Race QTHJC was only virulent on Norin 40 (*Sr42*) (IT 33+) but avirulent on *SrSt*, *SrKm*, *SrKn*, *SrLm SrTmp* and *SrCad* (ITs 2 to 22+). Race TRTTF was virulent on *SrSt*, *Sr42* and *SrTmp* (ITs 3 to 3+) but intermediate on *SrKm* (IT 23) and avirulent on *SrKn* and *SrLm* (IT 2+) and *SrCad* (IT 2). Race TTKTT was avirulent on only *Sr42* (IT 2) but virulent on *SrSt* and *SrTmp* (IT 33+), *SrKn* (IT 3+), *SrKm* (IT 3) and *SrCad* (32+). However, the infection type response for *SrLm* was intermediate (2+3).

Table 3.8 *Puccinia graminis* f. sp. *tritici* (*Pgt*) seedling infection types produced with multi-race testing of South African winter wheat varieties and lines carrying known resistance genes in chromosome 6D (controls)

Entry	R gene	Infection types for *Pgt* races				
		QCCJB	QTHJC	TRTTF	TTKSK	TTKTT
PAN3161	*SrPan3161*	2+	3	3+	3+	3+
Line 37-07	-	2	3+	2+	33+	33+
SST 387	*SrSt*	2	22+	3+	2	33+
Komati	*SrKm*	2	2+	23	2	3
Koonap	*SrKn*	22-	2	2+	2	3+
Limpopo	*SrLm*	22-	22+	2+	22-	2+3
Norin 40	*Sr42*	2	33+	3	2-	2
CnsSrTmp	*SrTmp*	2+	2	3	2	33+
AC Cadillac	*SrCad*	0	2	2	2-	32+

[1]Seedling infection type data was confirmed in a replicated trial.

3.5.4 Assessment of adult plant stem rust resistance in the South African winter wheat variety PAN 3161

During the field evaluation PAN 3161 expressed a strong resistant host response to stem rust with severities from 5-20% and resistant reaction types of R to MR. The susceptible parent, Line 37-07 was severely affected in severity (80-100% infection) and showed a compatible (S) reaction type (Table 3.1; Figure 3.9). Severities and reaction types of the segregating F_2 populations ranged from highly resistant (0, TR), intermediate (10-20MRMS) to highly susceptible (100S) (Figure 3.9). The segregation in the F_2 population of Line37-07/PAN3161 fitted a 3:1 ratio, hence suggesting that a single dominant gene confers resistance to stem rust race PTKST (Table 3.9). From the 170 F_2 plants phenotyped, 128 plants consisting of 77 resistant plants (0, TR, 0-30), 35 intermediates (30-60MRMS) and 16 susceptible plants (70-100MS, S) were sampled for mapping of the APR gene. Segregation of the 56 F_3 families conformed to a 7:8:1 ratio for two dominant Mendelian genes (Table 3.9).

3.6 Discussion

3.6.1 Evaluation of inheritance of stem rust resistance genes in South African winter wheat varieties

The parents Komati, Koonap, Limpopo and SST 387 and the two chromosome 6D resistant controls Norin 40 and CnsSrTmp expressed low seedling ITs and R to MRMS host reaction types in field trials supporting the presence of ASR gene(s) in the South African winter wheat varieties. Line 37-07 expressed a susceptible IT in the seedling stage with no APR response in the field confirming the suitability of this variety for mapping ASR and APR genes against *Pgt* race PTKST. PAN 3161 expressed susceptible seedling responses against races TTKSF (3+), TTKSF+9h (3), TTKSP+24 (3+), PTKSK+31 (3+), PTKST+24+31 (3) and a strong APR response (5R-20R) against race PTKST confirming the presence of APR in this variety. Molecular analysis using markers linked to the APR locus *Lr34/Yr18/Sr57* indicated the presence of this gene complex in PAN 3161 (Chapter 5). However, the strong APR response expressed by PAN 3161 suggested the presence of additional APR gene(s) besides *Lr34/Yr18/Sr57,* hence the motivation for the APR inheritance study done in the current study.

Seedling ITs of the resistant parents Komati, Koonap, Limpopo and SST 387 ranged from ;1$^{=}$ to 12c and appeared lower when compared to the chromosome 6D resistant parents Norin 40 and CnsSrTmp (IT = 2). In addition, Komati, Koonap and SST 387 also expressed more resistant field responses when compared with Limpopo, Norin 40 and CnsSrTmp.

Table 3.9 Results obtained with the field evaluation of mapping populations based on host response to *Puccinia graminis* f. sp. *tritici* race PTKST

Season	Crosses	Generation	No. of plants/families	Observed frequency	Expected ratios	$\chi 2$	P value
2017	Line37-07/PAN3161	F_2	170	142:28 (5.1:1)	3:1	3.76	0.052
2018	Line37-07/PAN3161	F_3	56	21:30:5 (4.2:6:1)	7:8:1	0.29	0.593

$\chi 2$ = Chi-square value and $P \leq 0.05$.

Figure 3.9 Field infection types produced by *Puccinia graminis* f. sp. *tritici* race PTKST on stems of the resistant parent PAN 3161 and the susceptible parent Line 37-07 (left) as well as variation observed in host responses in the F_2 (middle) and F_3 (right) populations, respectively

The phenotypic results were not conclusive as to whether the same resistance gene(s) could be involved in the four South African varieties. Furthermore, phenotypic expression of resistance in these varieties were not identical to that observed for the two chromosome 6D control varieties. *Pgt* evaluations among F_2 populations and F_3 families fitted a 3:1 and 1:2:1 ratio, respectively, confirming that a single dominant gene conferred resistance to *Pgt* race PTKST at the seedling stage in each of Komati, Koonap, Limpopo and SST 387.

From the Line37-07/PAN3161 field stem rust evaluations, the difference between the number of genes segregating in the F_2 populations and F_3 families could have resulted from underestimation of resistant phenotypes at F_2 level as a result of lower expression of resistance genes (in heterozygous form) or a lack of complementary effects in the susceptible parent Line37-07. Besides, population sizes were small and segregation observed for growth period between plants in the field might also have had an effect on stem rust development. The most likely segregation was obtained with F_3 families as PAN 3161 tested positive for molecular markers linked to the APR locus *Lr34/Yr18/Sr57* located on chromosome 7D (Chapter 5), while the second putative APR gene was mapped to short arm of chromosome 4D (Chapter 5). Of the 56 F_3 families, 21 families consisting of 16 homozygous resistant (0R, 0-20MR and 0-20MS) and five homozygous susceptible plants (30-60S) were sampled for the validation of molecular markers identified and mapped in the F_2 population.

To confirm the true nature of the APR gene in PAN 3161, this variety should be tested at seedling stage against an array of stem rust races to uncover races that can help explain if some ASR could be present in PAN 3161. Once its APR nature is confirmed and also to minimise the confounding effects of *Lr34* in PAN 3161, selections of Line37-07/PAN 3161 F_2 lines lacking *Lr34* should be advanced to RILs, hence facilitating extensive stem rust field phenotyping in different locations and seasons. The use of RILs will improve the efficiency of phenotyping and mapping of the APR gene as well as the development of effective molecular markers for tagging the gene.

3.6.2 Allelic relationship for genes conferring resistance to stem rust in South African winter wheat varieties

All F_2 progeny of the four-winter wheat resistant variety intercrosses phenotyped with *Pgt* race PTKST had resistant ITs, hence suggesting that the resistant parents Komati, Koonap, Limpopo and SST 387 possess the same gene for resistance or closely linked gene(s). Since no clearly

susceptible F_2 progeny were observed in crosses involving varieties containing either *Sr42* or *SrTmp*, results suggest that Komati, Koonap, Limpopo and SST 387 possess either the same gene or an allele closely linked to *Sr42* or *SrTmp*.

Allelism tests have become useful in many studies. For instance, Olivera et al. (2008) evaluated the reaction of F_2 progeny of a cross involving two resistant *Ae. sharonensis* (Sharon goatgrass) accessions (1644 and 2229) with two stem rust races TTTT and TPMK. In their study only resistant progeny were produced, hence suggesting that either the same gene or two closely linked genes conferred resistance to stem rust in both resistant accessions. Similarly, Turner et al. (2016) have used allelism tests to suggest that the resistance observed in Jagger, a winter wheat variety, was actually *Sr7a* as all F_2 progeny were resistant to stem rust race TTTTF. Resistance to stem rust race TTKSK in different barley accessions was also demonstrated to be contributed by the known *rpg4/Rpg5* complex or by an allele very closely linked to it as no segregating plants were identified among F_2 progeny of crosses between resistant barley accessions and lines containing the *rpg4/Rpg5* complex (Mamo et al., 2015). Olson et al. (2013) used allelism tests to confirm that the gene transferred from an *Ae. tauschii* accession P1 603225 to hexaploid wheat was *Sr35*. Similarly, Rouse et al. (2012) indicated that resistance to stem rust race TTKSK in North Dokata line SD 1691 was conferred by *Sr28*. Allelism tests among two wheat cultivars Webster and Gabo 56 validated that the resistance in both cultivars was an allele at the *Sr9* locus and was eventually designated *Sr9h* (Rouse et al., 2014).

A study involving intercrosses between the South African winter wheat variety Matlabas and three wheat lines CItr 4311, RL 6071/Webster and Webster known to carry *Sr9h* produced only resistant F_2 progeny to *Pgt* race TTKSF suggesting that Matlabas also possesses *Sr9h* (Wessels et al., 2019). Recently, Gao et al. (2018) tested 120 $F_{2:3}$ families from crosses between MV Zelma with two *Sr15* carriers W2691 and Prelude, and found that the progeny were resistant to stem rust race TTKSK suggesting that MV Zelma also possesses *Sr15*. Similar applications of allelism tests in resolving gene identities have been reported for leaf rust (Loladze et al., 2014; Kthiri et al., 2018) and stripe rust resistance genes (Zhou et al., 2014).

Results of screening the F_2 populations and F_3 families suggest that a single gene confers resistance in each of the four stem rust resistant varieties Komati, Koonap, Limpopo and SST 387. Intercrosses among these four stem rust resistant varieties revealed that the same gene is likely to be involved in resistance to *Pgt* race PTKST. However, results from intercrosses

between the resistant parents with the chromosome 6D resistant carriers Norin 40 and CnsSrTmp were inconclusive as to whether *Sr42* and/or *SrTmp* is involved as no clear recombinants were identified.

3.6.3 Phenotypic relationship between the identified stem rust resistance gene(s) and known chromosome 6D genes *Sr42, SrTmp* and *SrCad*

Multi-race phenotyping was done to postulate the presence of *Sr42, SrTmp* and/or *SrCad* in South African winter wheat varieties Komati, Koonap, Limpopo and SST 387. Considering the seedling ITs produced by *Pgt* race QTHJC it is unlikely that the South African wheat varieties carry *Sr42* on its own. This race produced compatible seedling ITs of 3 to 3+ on Norin 40 whereas the seedling ITs for the South African varieties were clearly lower varying from 2 to 2+. Ghazvini et al. (2012) reported that race QTHJC is virulent on *Sr42* (IT 3+). The South African winter wheat varieties can thus only carry *Sr42* if a second R gene(s), effective against race QTHJC is present in each of them.

Pgt race TRTTF initially identified in Yemen is virulent on *Sr42* and *SrTmp* (Ghazvini et al., 2012; Olivera et al., 2012; Hiebert et al., 2016) and in accordance produced compatible seedling ITs on Norin 40 (IT = 3) and CnsSrTmp (IT = 3). In addition to these, race TRTTF is virulent to *Sr7b*, *Sr9a*, *Sr9e*, *Sr30* and *Sr36* whereas race QTHJC is avirulent. Olivera et al. (2012) reported that 19 *Sr* genes *Sr8a*, *Sr9e*, *Sr9e+Sr13*, *Sr22*, *Sr24*, *Sr25*, *Sr26*, *Sr27*, *Sr31*, *Sr32*, *Sr33*, *Sr35*, *Sr37*, *Sr39*, *Sr40*, *Sr46*, *Sr47*, *SrSatu* and *Sr50* confer resistance to race TRTTF. The higher seedling ITs produced by Komati (23) and SST 387 (3+) to race TRTTF suggest that they are likely to carry *SrTmp* or alternatively *Sr42* in combination with other R gene(s) against race QTHJC. However, both Koonap and Limpopo expressed more resistant seedling ITs (2+) indicating that they do not possess *Sr42* or *SrTmp* or alternatively that a second R gene(s) against race TRTTF is involved in these varieties in combination with *Sr42* or *SrTmp*. Race TRTTF is avirulent on *SrCad* (Hiebert et al., 2016, 2017). Similarly, in the present study race TRTTF was avirulent on *SrCad* (IT 2).

Hiebert et al. (2016) observed that race TTKSK is avirulent on *Sr42, SrTmp* and *SrCad.* Results for race TTKSK were similar to that of the more avirulent race QCCJB in that they produced clearly low seedling ITs to the four South African varieties and the chromosome 6D controls. Race TTKSK is similar to race QCCJB low on *Sr42, SrCad* and *SrTmp* but high on *Sr6, Sr7b, Sr9a*, *Sr9b*, *Sr9e*, *Sr11*, *Sr30* and *Sr36.*

Seedling ITs recorded for *Pgt* race TTKTT were high on CnsSrTmp (33+) and more compatible towards AC Cadillac (32+) and lacked virulence for Norin 40 (2). *Pgt* race TTKTT is virulent on both *Sr24* and *SrTmp* (Newcomb et al., 2016; Patpour et al., 2016b). Newcomb et al. (2016) reported that race TTKTT (IT 0) is avirulent on AC Cadillac. Considering the seedling ITs for varieties SST 387 (33+), Komati (3), Koonap (3+) and Limpopo (2+3) to race TTKTT in combination with results obtained with races QTHJC and TRTTF, the presence of *Sr42* in these varieties can be ruled out. Further differences in virulence between *Pgt* races TRTTF and TTKTT include avirulence for race TTKTT to *Sr8a*, *Sr24*, *Sr31* and *SrCad*. Limpopo reportedly do not to carry *Sr24* and *Sr31* (Pretorius et al., 2012b).

To assess whether Koonap possesses *Sr24* or *Sr31* the two dominant SSR markers *Sr24#12* and *iag91* were used, respectively. Genomic DNA was isolated from ten freeze-dried leaves as described in Chapter 4. Markers amplified the expected PCR products in the two positive controls LcSr24Ag (*Sr24*) and Federation/Kavkaz (*Sr31*) with no product generated in Koonap confirming the absence of both genes.

Pretorius et al. (2012b) showed that race TTTTF is virulent on Komati (IT 3+) and Limpopo (IT 2/3). These results could suggest that Komati and Limpopo likely carry *SrTmp*. North American race TTTTF has a virulence/avirulence formula of *Sr5*, *Sr6*, *Sr7b*, *Sr8a*, *Sr9a*, *Sr9b*, *Sr9d*, *Sr9e*, *Sr9g*, *Sr10*, *Sr11*, *Sr17*, *Sr21*, *Sr30*, *Sr36*, *Sr38*, *SrMcN*, *SrTmp*/*Sr22*, *Sr24*, *Sr31*, *Sr35* (Rouse et al., 2011).

Seedling ITs obtained with *Pgt* races TRTTF and TTKTT for the cultivars SST 387 and Komati exclude the role of *SrCad* which was clearly low (IT = 2) to race TRTTF and thus supports the involvement of *SrTmp*. Considering the narrow genetic base of South African winter wheats together with the F_2 intercross results in this Chapter, it is likely that *SrTmp* is the resistance gene involved in all the four South African winter wheat varieties resistance to race PTKST. The low seedling responses observed for Limpopo and Koonap to race TRTTF, virulent to *SrTmp*, can be explained by avirulence to *Sr8a*. In further support of this Lombard (1986) identified *Sr8a* in derivatives of the variety Betta appearing in the pedigree of Limpopo. Betta is further to this an indirect parent in Komati through Molopo (Betta//Monon/Arthur.Oh130) (Smit el al., 2010). The wheat variety Betta, commonly used in wheat improvement by South African breeding programmes, has previously been reported as the potential source of stem rust resistance in South African winter wheats as it commonly appears directly or indirectly in the pedigrees of cultivars released by ARC-SG and Sensako from 1983 to 2002 (Smit et al., 2010;

Pretorius et al., 2012b). This further supports the theory that the same ASR gene is likely to occur in SST 387, Komati, Koonap and Limpopo.

3.7 Conclusions and recommendations

A single dominant gene is postulated to confer ASR to *Pgt* race PTKST in each of the winter wheat varieties Komati, Koonap, Limpopo and SST 387. Allelism tests among these resistant parents indicated that it is the same gene or closely linked genes or alleles of the same gene in all four varieties. The test varieties are only protected by a single gene thus opening a risk for pathogen adaptation and resistance breakdown. Therefore, to broaden the resistance base in South African winter wheat varieties, new sources of resistance should be characterised and combined with identified resistance gene(s). Allelism tests between the four resistant varieties Komati, Koonap, Limpopo and SST 387 and Norin 40 (*Sr42*) and CnsSrTmp (*SrTmp*) indicated that the test varieties possess either novel gene(s) or very closely linked alleles to *Sr42* and *SrTmp*. Results from screening the Line37-07/PAN3161 F_2 population indicated the presence of a single APR gene which was contradicted by results obtained for the 56 F_3 families that indicated the presence of two APR genes. The population sizes for the field stem rust resistance study should also be increased to at least 100 F_3 families to improve the validity of the inheritance work. To further aid the characterisation of ASR and APR, the F_2 populations should be advanced to develop RIL populations that will result in effective and efficient inheritance studies across different seasons and locations. These stem rust inheritance studies thus formed a basis for the molecular mapping of ASR (Chapter 4) and APR (Chapter 5). Allelism test results between the identified stem rust resistance gene(s) and *Sr42* and *SrTmp* were inclusive. However, the allelism results taken together with multi-race phenotyping results rule out the involvement of either *Sr42* or *SrCad* in all tested South African winter wheat varieties and seem to suggest the likely presence of *SrTmp*.

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CHAPTER 4

IDENTIFICATION AND MOLECULAR MAPPING OF ALL STAGE STEM RUST RESISTANCE GENE(S) IN SOUTH AFRICAN WINTER WHEAT VARIETIES

4.1 Abstract

This study was conducted to identify and map stem rust resistance in four South African winter wheat varieties Komati, SST 387, Koonap and Limpopo. F_2 populations were developed by crossing four resistant parents and a single susceptible parent Line 37-07 and tested against *Pgt* race PTKST at seedling stage. SSR marker genotyping using BSA in F_2 populations identified marker loci on chromosome 6DS linked to stem rust resistance genes in all four varieties. Linkage mapping identified two SSR markers, *wms4862* and *barc183*, as closely linked to resistance gene(s) in two mapping populations of SST387/Line37-07 and Line37-07/Koonap. *Barc183* mapped distally to the resistance gene in SST 387 and Koonap at 2.0 and 1.4, respectively. *Barc183* mapped proximally to the stem rust resistance gene in Komati and Limpopo at 2.7 and 0.9 cM, respectively. *Wms4862* mapped proximally to the resistance gene in SST 387 and Koonap at 1.9 and 0.9 cM, respectively. In the Line37-07/Komati mapping population, SSR markers *psp3200* and *barc183* were closely linked and flanked the resistance gene. *Psp3200* mapped distally to stem rust resistance gene at 2.9 cM. In the Line37-07/Limpopo population, SSR markers, *wms4528 and barc183*, were closely linked and flanked the stem rust resistance gene(s). *Wms4528* mapped proximally at 0.6 cM to the stem rust resistance gene in Limpopo. This study reports two SNP markers, *BS00085929* and *BS00085937* that mapped distally to the stem rust resistance gene(s) at an average of 8.4 and 9.2 cM, respectively. These SNP markers detected different alleles in the resistant parents. *BS00085929* and *BS00085937* were closely linked to the resistance gene(s) and can be useful for MAS. QTL analysis showed that *psp3200, wms4528, barc183* and *wms4862* flanked the stem rust resistance gene(s) in the four tested populations. Additionally, major QTL was detected in Komati, SST 387, Koonap and Limpopo explaining 73.0, 96.2, 71.4 and 85.2% of the phenotypic variation for stem rust resistance to race PTKST, respectively. Flanking markers *wms4862* and *barc183* were predictive of stem rust resistance in 102 advanced winter wheat F_3 families, hence confirming the chromosome location and their usefulness in MAS. This study represents the first report of markers developed for stem rust resistance genes in South African winter wheat varieties. Resistant genotypes tested negative for diagnostic SNP markers

linked to *Sr42*, *SrTmp* and *SrCad* on chromosome 6DS. It is thus possible that the resistance mapped here is novel or allele(s) of *Sr42*, *SrTmp* and/or *SrCad*.

4.2 Introduction

Stem rust is an important disease of wheat worldwide and is capable of causing yield losses of up to 100% during an epidemic (Park, 2007). For decades, the use of resistant varieties has remained the most economic and environmentally friendly way of controlling stem rust (Bajgain et al., 2016; Olivera et al., 2018). However, the identification of the *Sr31* virulent *Pgt* race in Uganda, named Ug99 (Pretorius et al., 2000) and other virulent races coupled with their eventual spread to different parts of the world, indicates that *Pgt* is still a potential threat to world wheat production. With this eminent danger posed by *Pgt*, many scientific groups have reported on the identification and characterisation of over 70 *Sr* genes (Hatta et al., 2018; Saini et al., 2018; Aoun et al., 2019). Most of these genes have not been deployed in commercial wheat varieties either because they were derived from wheat relatives, possessing deleterious effects or that they have been defeated by new virulent *Pgt* races (Pretorius et al., 2000; Olivera et al., 2012; Guerrero-Chavez et al., 2015; Singh et al., 2015; Fetch et al., 2016; Patpour et al., 2016a). Hence, there exists a continuous need for identification and deployment of new stem rust resistant sources.

Combining of different *Sr* genes using conventional methods is difficult as it requires simultaneous testing of the same wheat breeding material using several different rust races before lines are selected (Haile and Röder, 2013). It is also often difficult for breeding programmes to maintain all necessary rust races required for rust evaluations (Wu et al., 2009) and quarantine races cannot be screened. Molecular markers enable plant breeders to combine several genes more efficiently to achieve durable resistance. Stem rust resistance genes can be tagged using tightly linked DNA markers and selection based on these markers improves the effectiveness and efficiency of breeding programmes (Todorovska et al., 2009).

One of the cost-effective approaches for identifying molecular markers is the use of BSA (Michelmore et al., 1991). Genotyping of bulks and parental lines can be used to search for associations between phenotypes and genotypes. BSA has been successfully employed to develop markers closely linked to several wheat stem rust resistance genes (Olson et al., 2013; Lopez-Vera et al., 2014; Babiker et al., 2016; Kassa et al., 2016; Wiersma et al., 2016). The

objective of this study was therefore to identify and map molecular markers linked to stem rust resistance in South African winter wheat varieties.

4.3 Materials and methods

4.3.1 Greenhouse trial

The parental seed increase trial was done at the University of the Free State (UFS) in 2016/2017. Four winter wheat varieties Komati, Koonap, Limpopo and SST 387 and stem rust resistant control lines (Table 4.1) were vernalised at 5-6°C for six weeks before being transplanted to the greenhouse. Ten seeds from each of the stem rust resistant control lines Thew, LC*Sr24*Ag and Agent, LC*Sr25*Ars and Agatha, Federation/4*Kavkaz, Norin 40, Triumph 64 and AC Cadillac were planted in the greenhouse alongside the vernalised stem rust resistant parents and susceptible parent Line 37-07. Greenhouse conditions were set at 22°C day and 18°C night temperatures using natural day/night lengths. Seedlings were planted in 2 ℓ pots with steam-sterilised soil. Pots were watered daily and fertilised weekly with 50 ml water-soluble fertiliser [Effekto® NPK 19:8:6 (43)]. Whenever plants showed signs of iron deficiency, 100 ml of iron solution per pot from a mixture of 2 g of Iron chelate in 10 ℓ water was applied. Plants were watered twice daily by hand.

Table 4.1 Stem rust resistant control lines and their associated stem rust resistance genes

Control line	***Sr* gene**
Thew	*Sr15*
LC*Sr24*Ag and Agent	*Sr24*
LC*Sr25*Ars and Agatha	*Sr25*
Federation/4*Kavkaz	*Sr31*
Norin 40	*Sr42*
Triumph 64	*SrTmp*
AC Cadillac	*SrCad*

4.3.2 Development and selection of individuals of mapping populations

The four mapping populations were developed as described in Chapter 3 (section 3.3.1). To facilitate genotyping about 500-600 F_2 plants of each of four populations were phenotyped as described in chapter 3 (Section 3.3.4.1) and based on phenotyping results, 150 resistant and 50 susceptible F_2 seedlings were selected for creating DNA bulks.

4.3.3 Marker-screening of parental lines

Leaf samples for DNA isolation were collected from four-week old seedlings. Four winter wheat stem rust resistant parental varieties as well as the susceptible spring wheat Line 37-07 were screened using 104 SSR markers to detect polymorphisms between parental lines.

4.3.4 DNA isolation

Leaf material used for DNA isolation was sampled from 5-10 four-week-old seedlings per parental line to obtain a representative sample using strict sterile conditions. Leaf material was collected from resistant parents, stem rust resistant control lines and susceptible parent Line 37-07. Sampled leaves were immediately placed on ice before being freeze-dried using the Alpha 1-2 LD plus freeze-drier (Martin Christ, Osterode am Hartz, Germany) for 72 h. Freeze-dried leaf material was homogenised using Qiagen's Tissue Lyser (Haan, Germany). Approximately 250 mg of freeze-dried leaf pieces of 1-2 cm in length was transferred to a 2 ml microcentrifuge tube together with two 5 mm stainless-steel ball bearings and homogenised for 1 min at 30 r/s.

DNA was isolated using a modified two-day CTAB (hexadecyltrimethylammonium bromide) extraction method (Saghai-Maroof et al., 1984). For each sample, 750 µl CTAB buffer [100 mM Tris-HCl (tris(hydroxymethyl) aminomethane hydrochloride), pH 8.0; 20 mM EDTA (ethylene-diaminetetraacetate), pH 8.0; 1.4 M NaCl (Sodium chloride); 2% (w/v) CTAB; 0.2% (v/v) β-mercaptoethanol] was added to 250 µg of fine leaf powder and incubated for 1 h at 65°C. The suspension was extracted with 500 µl chloroform: isoamyl alcohol [24:1 (v/v)] and centrifuged using a Sigma 2-16K centrifuge (Sigma, Shropshire, UK) at 12 000 *g* for 5 min at 4°C. DNA was precipitated with 500 µl (0.66 volumes) ice-cold 2-isopropanol at room temperature (20-25°C) for 20 min and centrifuged at 12 000 *g* for 5 min at 4°C. The DNA pellet was washed with 500 µl 70% (v/v) ice-cold ethanol and incubated for 20 min at room temperature before centrifuging at 12 000 *g* for 5 min at 4°C. The DNA pellet was air-dried for 1 h at room temperature. The dried DNA pellet was re-suspended in 200 µl 1x TE buffer (10 mM Tris-HCl, pH8.0; 1 mM EDTA, pH 8.0) and stored at 4°C till the next day. The re-suspended DNA pellet was treated with 100 mg/ml DNase-free RNase enzyme the next day and incubated at 37°C for 1 h. DNA quantity and quality were estimated on a 0.8% (w/v) agarose gel run at 100 V for 20 min in 1x UNTAN (40 mM Tris-HCl; 2 mM EDTA; pH adjusted to 7.4 with acetic acid) buffer and visualised using Ethidium bromide (EtBr). A digital gel image was recorded using ultraviolet (UV) light and the BIO-RAD Gel DOC^{Tm} EZ Imager

(Bio-Rad, CA, USA). DNA concentrations were determined with a Jenway 7315 spectrophotometer (Jenway, Staffordshire, UK) at A_{260} and A_{280} and the $A_{260:280}$ ratio was used to assess quality of extracted DNA and subsequently DNA samples were diluted using 0.1x TE buffer to working solutions of 20 ng/µl

4.3.5 Microsatellite analysis

PCR reactions were performed in a Bio-Rad T100^{Tm} thermal cycler (Bio-Rad, CA, USA). All PCR reactions were set up in a final volume of 10 µl. PCR reactions contained 80 ng genomic DNA, 2 mM $MgCl_2$, 1x GoTaq Flexi polymerase buffer (Promega Corporation, Madison, WI, USA), 200 µM of each dNTP (deoxynucleotide triphosphate), 25 ng of each primer and 0.25 U GoTaqR Flexi DNA *Taq* polymerase (Promega). Primers were sourced from Integrated DNA Technologies (Coralville, IA, USA). PCR programmes consisted of 5 min at 95^{o}C followed by 35-42 cycles of 30 s at 95^{o}C, 30-60 s at an appropriate annealing temperature and 30-60 s at 72^{o}C for the extension step. PCR reactions concluded with a further elongation step of 10 min at 72^{o}C. PCR products were screened using the Gel-Scan 3000 Real-Time DNA Fragment Analysis System (Corbett Research, Sydney, Australia). The 5% (w/v) non-denaturing gel with final volume of 25 ml consisted of 1x Tris-HCl/Borate/EDTA (TBE) buffer (2 mM EDTA; 89 mM Tris-HCl; 89 mM Boric acid), 5% (w/v) acrylamide:bis-acrylamide (19:1), 0.12% (v/v) N, N, N', N';-tetramethylethylenediamine (TEMED) and 0.08% (v/v) ammonium persulfate. Gels were poured and left to polymerise for 4-5 h or overnight. The upper buffer chamber (negative electrode) of the system contained 0.5x TBE buffer made up in deionised water. The bottom buffer chamber (positive electrode) contained 0.5x TBE buffer and 1% (v/v) EtBr in deionised water. PCR products were mixed with deionised formamide loading dye. A pre-run at 800 V for 45 min at 37°C was performed prior to sample loading. The final volume of the sample as well as loading dye depended on the intensity of PCR fragments. A 1-2 µl volume of diluted sample was loaded on the gel and a pulse-run initiated for 15 s then excess product was rinsed out. The actual run was at 1 200 V for 45 min at 37°C until all targeted fragments were visible and detected. A 25 bp ladder (HyperLadder V from Bioline, Taunton, MA, USA) was loaded for each gel run and used to determine relative amplified fragment sizes. PCR products were visualised and analysed using the Gel Scan 3000 Real-Time DNA Fragment Analysis System.

4.3.6 Molecular identification of F_1 hybrids

Forty-seven F_1 plants generated from crosses between the four resistant winter wheat varieties and the susceptible Line 37-07 were tested with four SSR markers *barc343*, *cfd2*6, *gwm312*

and *wmc285* to distinguish between true crosses and self-pollinated plants. These SSR markers were selected because they amplified DNA products with size differences of 15-45 bp between the male and female parents allowing accurate scoring.

4.3.7 Screening of parental lines for presence of known stem rust resistance genes

To confirm the presence of known stem rust resistance genes, the South African winter wheat parental varieties were tested alongside respective controls (Table 4.1) using different molecular markers (Table 4.2) closely linked to stem rust resistance genes namely *Sr15* (Neu et al., 2002), *Sr24* (Mago et al., 2005), *Sr25* (Prins et al., 2001; Zhang and Dubcovsky, 2008; Liu et al., 2010), *Sr31* (Mago et al., 2002), *Sr42* (Ghazvini et al., 2012), *SrCad* and *SrTmp* (Hiebert et al., 2011, 2016). The above *Sr* genes were selected based on previous infection types observed on most South African varieties and the fact that most of these genes have been involved in development of many South African wheat lines (Pretorius et al., 2012). Sequences of markers closely linked to various wheat stem rust resistance genes are shown in Table 4.3.

Table 4.2 Molecular markers linked to known stem rust resistance genes tested in the current study

Sr gene	Locus	Marker	Ta	Marker type	Source
Sr15	7AL	*STS638*	60	STS	Neu et al., 2002
Sr24	3D	*barc71*	55	SSR	Mago et al., 2005
Sr24	3D	*Sr24#12*	59	AFLP/STS	Mago et al., 2005
Sr25	7DL, 7AL	*BF145935*	50	STS	Liu et al., 2010
Sr25	7DL, 7AL	*GB*	50	STS	Prins et al., 2001
Sr25	7DL, 7AL	*PSY-D1*	58	STS	Zhang and Dubcovsky, 2008
Sr25	7DL, 7AL	*PSY-E1*	58	STS	Zhang and Dubcovsky, 2008
Sr31	1BL/IRS	*IB-267*	55	STS	Mago et al., 2002
Sr31	1BL/1RS	*Iag95*	55	SCAR	Mago et al., 2002
Sr42/SrCad	6D, 2B	*barc183*	58	SSR	Ghazvini et al., 2012
Sr42/SrCad	6D	*cfd49*	60	SSR	Ghazvini et al., 2012
SrCad	6DS	*FSD+RSA*	44	PCR	Hiebert et al., 2011
SrTmp	6DS	*gpw5182*	60	SSR	Hiebert et al., 2016

Ta = Annealing temperature, STS = Sequence tagged site, SSR = Simple sequence repeat, AFLP = Amplified fragment length polymorphism, PCR= Polymerase chain reaction, SCAR = Sequence characterised amplified region.

Table 4.3 Sequences of microsatellite markers linked to known wheat stem rust resistance genes used in current study

***Sr* gene**	**Locus**	**Marker**	**Ta**	**Forward primer (5'- 3')**	**Reverse primer (5'- 3')**
Sr2	3B	*csSr2*	60	CAAGGGTTGCTAGGATTGGAAAAC	AGATAACTCTTATGATCTTACATTTTTCTG
Sr15	7AL	*STS638*	60	GCGGTGACTACACAGCGATGAAGCAATGAAA	GCGGTGACTAGTCCAGTTGGTTGATGGAAT
Sr24	3D	*barc71*	55	GCGCTTGTTCCTCACCTGCTCATA	GCGTATATTCTCTCGTCTTCTTGTTGGTT
Sr24	3D	*Sr24#12*	60	CACCCGTGACATGCTCGTA	AACAGGAAATGAGCAACGATGT
Sr25	7DL, 7AL	*BF145935*	50	CTTCACCTCCAAGGAGTTCCAC	GCGTACCTGATCACCACCTTGAAGG
Sr25	7DL, 7AL	*GB*	50	CATCCTTGGGGACCTC	CCAGCTCGCATACATCCA
Sr25	7DL, 7AL	*PSY-D1*	58	TTGCAGTGCAATGGTTTTCCA	GACTCCTTTGACGATGTCTTC
Sr25	7DL, 7AL	*PSY-E1*	58	CTACGTTGCGGGCACCGTT	AGAGAAAACCATTGCATCTGTA
Sr31	1B/1RS	*IB-267*	55	GCAAGTAAGCAGCTTGATTTAGC	AATGGATGTCCCGGTGAGTGG
Sr31	1BL/1RS	*Iag95*	55	CTCTGTGGATAGTTACTTGATCGA	CCTAGAACATGCATGGCTGTTACA
Sr42/SrCad	6D, 2B	*barc183*	58	CCCGGGACCACCAGTAAGT	GGATGGGGAATTGGAGATACAGAG
Sr42/SrCad	6D	*cfd49*	60	TGAGTTCTTCTGGTGAGGCA	GAATCGGTTCACAAGGGAAA
SrCad	6D	*FSD+RSA*	44	GTTTTATCTTTTTATTTC	CTCCTCCCCCCA
SrTmp	6D	*gpw5182*	60	TCCACTTCACTAACAAACACGG	AAAAGCTGTATAGGCAGTTCGC

Ta = Annealing temperature.

4.3.8 Genome-wide screening using microsatellite markers to detect unknown resistance genes

4.3.8.1 Selection of microsatellite markers

The parental lines were initially screened using 104 low resolution wheat genome scan (LRGS) SSR markers (Wessels and Prins, 2016) to identify polymorphic markers. Each chromosome was represented by five SSR markers, except chromosome 4A that was represented by only four SSR markers spread across the length of its chromosome. Wessels and Prins (2016) reported that the LRGS set was 82% informative in both populations they tested. This high level of polymorphism increased the chances of these SSR markers being informative in screening of parental lines used in the current study.

4.3.8.2 Screening of parental lines to identify polymorphic markers using bulked segregant analysis

Genomic DNA was extracted from all parents used in the four mapping populations namely Komati, SST 387, Koonap, Limpopo and Line 37-07 as described in section 4.3.5. Parents were initially screened using 104 LRGS SSR markers (Wessels and Prins, 2016). An additional 31 SSR markers (Wmc, Gwm, Barc, Cfd and Cfa) covering the entire wheat genome were screened to obtain 4-10 polymorphic SSR markers per chromosome. Annealing temperatures for each SSR marker were obtained from the GrainGenes web database (http://wheat.pw.usda.gov).

The chromosome location associated with stem rust resistance in the four populations was determined using BSA (Michelmore et al., 1991). For each mapping population, two resistant and two susceptible DNA bulks were constituted. Genomic DNA (20 ng/µl per sample) from 20 resistant and 20 F_2 susceptible seedlings (Appendix B) were mixed in equivalent concentrations and volumes per bulk. SSR markers that were informative among the parents, and resistant and susceptible bulks (Table 4.4) were tested on all individuals constituting the bulks to test their consistent linkage and ultimately used to genotype all 200 individuals of each mapping population. Only SSR markers located on 6DS chromosome showed possible linkage to stem rust resistance.

4.3.9 Development of a high-density map for the identified chromosome region

4.3.9.1 Screening of additional 6DS microsatellite markers within the identified chromosome region

To improve the marker resolution around the region of interest in chromosome 6DS, which initially had only three SSR markers namely *cfd49*, *barc183* and *gwm469* (Table 4.4), a set of 37 additional SSR markers (Appendix C) were screened for polymorphisms among the parents of the four mapping populations. SSR markers that were polymorphic among the parents and between resistant and susceptible bulks were ultimately genotyped on all 200 F_2 seedlings per population.

Table 4.4 Sequences of microsatellite markers linked to stem rust resistance gene(s) on chromosome 6DS in South African winter wheat varieties

SSR name	Forward primer: 5'-3'	Reverse primer: 5' - 3'	Locus
cfd75	GCATAAACTTGGACCCTGGA	GCTAAGCCACGCTACCACTC	6D
gdm132	ACCGCTCGGAGAAAATCC	AGGGGGGCAGAGGTAGG	6D
cfd42	AGGTTCTAGGGGGCATGTCT	GCTCTCAATGACTGCACTGG	6D
psp3200	GTTCTGAAGACATTACGGATG	GAGAATAGCTGGTTTTGTGG	6D
cfd49	TGAGTTCTTCTGGTGAGGCA	GAATCGGTTCACAAGGGAAA	6D
barc183	CCCGGGACCACCAGTAAGT	GGATGGGGAATTGGAGATACAGAG	2B, 6D
gwm469	CAACTCAGTGCTCACACAACG	CGATAACCACTCATCCACACC	1B, 6D

SSR = Simple sequence repeat of microsatellite.

4.3.9.2 Screening of single nucleotide polymorphism markers located on the short arm of chromosome 6D

To improve the resolution of markers around the chromosome 6DS region, 200 F_2 seedlings from each of the four mapping populations were genotyped using KASP SNP markers. Twenty-two SNP markers (Table 4.5) previously mapped to the short arm of chromosome 6D were selected to genotype parents and all individuals of four mapping populations. The preliminary stem rust resistance mapping positioned the gene(s) between two SSR markers *psp3200* and *barc183*, located between deletion bins 6DS-6 and 6DS-4 (Sourdille et al., 2004). Therefor only SNP markers that map between *psp3200* and *barc183*, were targeted and selected. These 22 SNP markers were initially tested on the parents and the stem rust resistant control lines to identify polymorphic markers. Only polymorphic SNP markers were then ultimately screened on each of 200 individuals of the four mapping populations.

Table 4.5 Chromosome 6DS single nucleotide polymorphism markers tested on South African winter wheat varieties

SNP marker	Chromosome	Position (cM)	Avalon	Cadenza	Inheritance
BS00021867	6D	0	A:G	G:G	Dominant
BS00022481	6D	0	C:C	A:A	Co-dominant
BS00075409	6D	0	C:C	T:T	Co-dominant
BS00076638	6D	0	C:C	T:T	Co-dominant
BS00085929	6D	0	A:A	G:G	Co-dominant
BS00085937	6D	0	G:G	C:C	Co-dominant
BS00182757	6D	0	T:T	C:C	Co-dominant
BS00151031	6D	1.08	C:C	None	Co-dominant
BS00181153	6D	2.16	T:T	C:T	Dominant
BS00111704	6D	3.36	A:A	C:C	Co-dominant
BS00009806	6D	3.36	A:A	C:C	Co-dominant
BS00010742	6D	3.36	T:T	C:C	Co-dominant
BS00015915	6D	3.36	A:A	A:G	Co-dominant
BS00021983	6D	3.36	A:A	C:C	Partially co-dominant
BS00022795	6D	3.36	G:G	T:T	Co-dominant
BS00115727	6D	3.36	C:T	C:C	Partially co-dominant
BS00137632	6D	3.36	A:A	A:G	Partially co-dominant
BS00139001	6D	3.36	A:A	G:G	Co-dominant
BS00140915	6D	3.36	A:A	A:G	Dominant
BS00153610	6D	3.36	A:G	A:A	Partially co-dominant
BS00167764	6D	3.36	C:C	A:A	Co-dominant
BS00009514	6D	3.36	G:G	A:G	Partially co-dominant

Avalon and Cadenza are the two European wheat varieties in which the respective SNPs were identified. SNP markers located at the most distal end of chromosome 6DS were selected based on Allen et al. (2011, 2013, 2017).

For KASP SNP genotyping, three primers were used; two allele-specific forward primers that result in bi-allelic discrimination and a single common reverse primer. Oligonucleotides with standard FAM (6-Carboxylfluorescein) and HEX (Phosphoramidite) compatible tails (FAM tail: 5-ʾGAAGGTGACCAAGTTCATGCT-3ʾ and HEX tail: 5ʾ-GAAGGTCGGAGTCAACGGATT-3ʾ) were added to the forward primer sequences at the 3'-end.

Sample plate preparation

The 384-well PCR plates were bar-coded to ensure that the fluorescent plate reader captures the correct information about samples loaded. Two µl of DNA (50 ng) was pipetted with an electronic pipette (Max 10 µl) or fluidX plate loaders in to each well of 384-well PCR plates. PCR plates were centrifuged using an Eppendorf 5804 centrifuge (USA Scientific, Florida, CA, USA) at 2 250 *g* for 2-5 s. The DNA was then oven dried at 60^{o}C for 1 hr. Thereafter, PCR plates were placed on a bench top to normalise at room temperature (23-25 ^{o}C) before dispensing the KASP assay.

Dispensing the assay and reaction mix

The assay mix containing three primers and PCR grade water (Bioline) was vortexed before use for uniform concentrations and centrifuged for about 5 s. The KASP assay mix containing *Taq* polymerase, dNTPs, HEX and FAM was only vortexed. The final assay containing both primers and KASP mix were pipetted into 16-well plates and the solution thoroughly mixed before placing on the Meridian WWP (LGC Genomics, Middlesex, UK). A Meridian dispenser wash test step was performed before the actual run after which 3 µl of the assays was dispensed into each well of the 384-well PCR plate. PCR plates were centrifuged at 2 250 *g* for 5 s and thereafter sealed using the K-Seal (LGC Genomics, Middlesex, UK) at 175^{o}C for 4 s.

Polymerase chain reactions

PCR was carried out in a total volume of 5 µl. The PCR programme did not contain an extension step because very small PCR products were amplified. Amplification was performed in a hydrocycler (LGC, Middlesex, UK) starting at 94^{o}C for 15 min, followed by a touchdown phase of 10 cycles at 94^{o}C for 20 s and 65^{o}C for 60 s with a 1^{o}C decrease in temperature per cycle, followed by 35 cycles of 94^{o}C for 20 s and 65^{o}C for 60 s.

Data analysis

Following PCR reactions, plates were read using a FLUOstar omega SNP microplate reader (BMG LABTECH, Ortenberg, Germany) that detects fluorescence scores for HEX and FAM using Omega software. Raw data was imported into KlusterCaller software for final visualisation and exported to Excel as XX (Homozygote-parent 1), XY (Heterozygote) and YY (Homozygote-parent 2).

4.3.10 Genotyping of parents using single nucleotide polymorphism markers linked to *Sr42*, *SrTmp* and *SrCad*

Parents of the mapping populations and stem rust resistant control lines were genotyped with nine selected SNP markers (Table 4.6). These markers are reported to be diagnostic for *Sr42*, *SrTmp* and *SrCad* (Gao et al., 2015; Kassa et al., 2016) located on chromosome 6DS. This was done to test the relationship between these genes and the mapped resistance gene(s) and also attempt to map these polymorphic SNP markers in the four mapping populations.

4.3.11 Construction of linkage maps

Two-hundred individuals of each population were genotyped with eight SSR and five SNP markers indicated during BSA analysis to be linked to stem rust resistance. SNP markers that showed good quality for allele calling, based on scatter plot clutters and with less than 10% missing data, were used for linkage analysis. The susceptible parent's allele was coded as A, resistant parent's allele as B and heterozygotes containing both male and female alleles as H. The genotypic data were used to create a partial linkage map for chromosome 6DS. Dominant and phenotypic marker data were coded as A and C for susceptible and resistant alleles, respectively. Chi-squared ($\chi 2$) analysis was performed to test for segregation distortion of markers from the expected ratios of 1:2:1 and 3:1 for co-dominant and dominant markers, respectively. Linkage maps were created using JoinMap software version 4.1 (Van Ooijen, 2018) with a minimum logarithm of odds (LOD) score of 3.0, with independence LOD for grouping and maximum likelihood (ML) algorithm for mapping. Genetic distances were generated using Kosambi's mapping function (Kosambi, 1943). Linkage maps were drawn using Map Chart software 2.3 (Voorrips, 2002). Linkage maps of the Line37-07/Komati, SST387/Line37-07, Line37-07/Koonap and Line37-07/Limpopo mapping populations combined with stem rust phenotyping data were used to locate chromosome regions associated with race PTKST resistance.

Table 4.6 Sequences of single nucleotide polymorphism markers linked to stem rust resistance gene(s) located on chromosome 6DS

Marker ID	Forward primer (5'-3')	Alternative allele KASP primer (5'-3')	Reverse primer (5'-3')	*Sr gene*
kwm907	ATCTTGCTAGTTCATGAGCTACTACA**T**	ATCTTGCTAGTTCATGAGCTACTACA**A**	GTGAATTCGAGAAGGTTACAATTAAGCATA	*SrCad*
kwm997	CCTCATCGTAGTTTTCTTCTTCTATGT**A**	CTCATCGTAGTTTTCTTCTTCTATGT**C**	GCTTCCGGCACCCGTCCCAA	*SrCad*
kwm994	TAACTTGGATCTTGACGTCTTTGAT**G**	CTTTAACTTGGATCTTGACGTCTTTGAT**A**	TCACTAAGCTCAAGATTCGTCGCGA	*SrCad*
kwm987	TCAGGGATCTTGACCGCT**A**	CAGGGATCTTGACCGCT**G**	TGAGCACCTCGGTAAGTTGT	*SrCad*
IWB15852	GTATGTTTTGATCTGAAGCAAACTAATTCTA**T**	ATGTTTTGATCTGAAGCAAACTAATTCTA**C**	GTGTAAGATATGCAGAGGCGATGTTATTT	*Sr42/Tmp/Cad*
IWB31561	TCTTCCGTGAAGTGCTAATCTG**T**	CTTCCGTGAAGTGCTAATCTG**C**	CACAAGTTTGCGAGGACAACAAACAAATA	*Sr42/Tmp/Cad*
IWB36391	AAGATGATGAACAAGTGGCCCCT**T**	GATGATGAACAAGTGGCCCCT**C**	ACGCAAAACCGCATACATCATTGGATTT	*Sr42/Tmp/Cad*
TP43472	GTACCGCAGCGACGACGC**A**	ACCGCAGCGACGACGC**G**	CCCGTCTTCTTCACTCACGGCTT	*Sr42/Tmp/Cad*
TP93838	GCACGCCGTCGTCTCTGT**C**	GCACGCCGTCGTCTCTGT**A**	GCGGCGGCAGCGGAGACAA	*Sr42/Tmp/Cad*

Sr gene = Stem rust resistance gene to which respective SNP markers are linked.

4.3.12 Quantitative trait loci analysis

To eliminate the effect of a dominant trait in influencing the position of gene(s) in F_2 populations (Wessels et al., 2019), QTL analysis was done based on data from BSA for all four mapping populations. QTL analysis was done using inclusive composite internal mapping (ICIM) methods of QTL ICIMapping sofware version 4 (Meng et al., 2015). QTL ICIMapping uses a stepwise regression approach and evaluates information from all markers simultaneously. Stem rust phenotypic data was incorporated into the linkage maps. The ICIM for additive and dominance effects QTL (ICIM-ADD) method was used to search for QTL associated with stem rust resistance. Other parameters such as walking speed was set at 1 cM, while the probability used by stepwise regression was 0.001. Significant QTL were declared at a threshold LOD score of 3.0 after performing 1 000 permutations with type 1 error of 5%. Percentage of variation explained by phenotype (PVE) (%) was also calculated to ascertain the percentage of phenotypic variation for stem rust resistance explained by QTL. For QTL analysis the individual IT scores (Appendix A) were converted to a linear scale of 0-9 following the method described by Zhang et al. (2014).

4.3.13 Validation of identified stem rust resistance linked markers in F_3 populations

To assess the usefulness of SSR markers closely linked to stem rust resistance gene(s) identified in the current study for MAS, two SSR markers were tested on 102 F_3 families derived from four mapping populations. SSR markers were validated by testing their robustness in predicting phenotypes using homozygous resistant and homozygous susceptible F_3 plants. Leaf material used for DNA isolation was sampled from 10 individual plants per F_3 family to obtain a representative sample. Each family was genotyped as a bulk containing 10 individuals. So ideally for the 102 F_3 families, a total of 1 020 F_3 DNA samples were created and assessed. DNA isolation and SSR analysis were performed as described in sections 4.3.5 and 4.3.6, respectively.

4.4 Results

4.4.1 Screening of parental lines for presence of known stem rust resistance genes

All parental lines (resistant and susceptible) tested negative for *Sr2, Sr15, Sr24*, *Sr25, Sr31, Sr42/Tmp/Cad* and *Sr57*. All control lines tested positive for the respective stem rust resistance genes screened.

4.4.2 Identification of polymorphisms among parents

The four South African winter wheat stem rust resistant parents Komati, Koonap, Limpopo and SST 387 and susceptible parent Line 37-07 were screened with 104 LRGS SSR markers to identify polymorphic markers. From the initial screening 57, 62, 64 and 70 polymorphic markers were detected between Line 37-07 and Komati, Koonap, Limpopo and SST 387, respectively (Table 4.7). With this level of polymorphism, some chromosomes carried only one polymorphic marker. To increase the number of polymorphic markers, a further 31 SSR markers (wmc, gwm, barc, cfd and cfa) were screened on the parents of the mapping populations. This ensured that each chromosome had a minimum of four polymorphic SSR markers spread along its length. In total, 135 polymorphic SSR markers were identified and selected for further analyses. The levels of polymorphism between Line 37-07 and resistant parents varied with Komati, Koonap, Limpopo and SST 387 possessing 68, 71, 75 and 87 polymorphic markers, respectively.

Table 4.7 Polymorphic microsatellite marker coverage in four mapping populations

Locus	Koonap	Komati	Limpopo	SST 387	Average/chromosome
4B	4	5	4	5	4.50
5A	4	3	4	4	3.75
7A	3	4	4	4	3.75
4D	4	4	2	5	3.75
2A	4	2	3	5	3.50
6A	4	4	4	2	3.50
1B	3	3	4	4	3.50
3B	4	3	3	4	3.50
7B	4	4	3	3	3.50
1A	4	3	4	2	3.25
2D	1	2	5	5	3.25
5D	2	3	4	4	3.25
3D	4	2	2	4	3.00
4A	3	2	3	3	2.75
5B	2	3	3	3	2.75
6D	2	2	3	4	2.75
1D	3	2	3	2	2.50
3A	2	2	2	2	2.00
2B	2	2	2	1	1.75
7D	2	1	1	3	1.75
6B	1	1	1	1	1.00
Total	**62**	**57**	**64**	**70**	
Polymorphism rate (%)	**59.6**	**54.8**	**61.5**	**67.3**	

4.4.3 Molecular identification of F_1 hybrids

Forty-seven F_1 plants generated from crosses between the resistant winter wheat varieties and the susceptible parent were assessed for being true-crosses using four SSR markers and all 47 F_1 plants (100%) were confirmed as true crosses since all plants possessed two alleles, one from the male parent and the second from the female parent. These F_1 plants were used in generation of the F_2 populations.

4.4.4 Identification of the chromosome location linked to stem rust resistance using bulked segregant analysis

About 506-605 F_2 plants of each of the four populations were phenotyped with stem rust race PTKST (Chapter 3, Table 3.3). A total of 200 F_2 plants, 150 resistant and 50 susceptible plants, were sampled for genotyping. However, for BSA, two resistant and susceptible bulks for each population were formed. Each F_2 DNA bulk consisted of 10 individuals. In addition, two DNA bulks (consisting of 10 individual plants each) were also formed for each of the four resistant and one susceptible parent, resulting in 10 parental DNA bulks. For the Komati, Koonap, Limpopo and SST 387 populations, 68, 71, 75 and 87 previously identified polymorphic markers, respectively, were tested on resistant and susceptible bulks as well as the resistant and susceptible parents to detect linkage to resistance.

Only SSR markers known to map to the short arm of chromosome 6D showed linkage between parents and bulks, indicating possible association with stem rust resistance. To confirm this possible linkage, this chromosome region was screened with eight additional SSR markers. Nine SSR markers *cfd49*, *barc183*, *gwm469*, *psp3200*, *cfd75*, *gdm132*, *cfd42*, *wms4528* and *wms4862* showed linkage to resistance in all four mapping populations (Table 4.8). As expected for F_2 mapping populations segregating for a dominant resistant phenotype, the resistant and susceptible bulks contained both homozygous and heterozygous alleles. The possible resistance linkage on chromosome 6DS was confirmed by screening all individuals constituting the bulks with the nine SSR markers that showed possible linkage. All nine SSR markers showed consistent linkage to resistance when screened on all individuals constituting the bulks. Ultimately, seven SSR markers were genotyped on all 200 individuals per population. SSR marker *gwm469* that also showed consistent linkage between individuals of the bulks, was omitted from further screening because it was difficult to score. Only one 6DS linked marker, *psp3200,* showed dominant inheritance while the other markers expressed co-dominant inheritance. *Cfd49* amplified a 214 bp fragment in Koonap, Komati and Limpopo

and a 202 bp fragment in SST 387 compared to 158 bp and 160 bp fragments observed in the susceptible parent Line 37-07 and control Norin 40 (*Sr42*), respectively. In addition, *barc183* amplified fragments of 200-202 bp in length in the resistant parents compared to 169 bp in Norin 40. *Wms4862* amplified a 200 bp fragment in Norin 40 and a 210 bp fragment in the resistant parents. *Wms4528* produced a single allele of 207 bp in the resistant parents including Komati and two alleles of 205 and 207 bp in Norin 40. *Wms4528* showed linked polymorphism between the parents and bulks of the Komati population. However, when screened on individuals that constituted the bulks, scoring was not clear, hence this marker was ultimately not mapped in the Komati population.

Table 4.8 Microsatellite markers linked to stem rust resistance gene(s) on 6DS in South African winter wheat varieties

Marker	Range (bp)	Locus	Ta	Inheritance
barc183	179	2B, 6DS	58	Co-dominant
psp3200	171 (165-182)	6DS	58	Dominant
gdm132	(144) 144-164	6DS	60	Co-dominant
cfd49	155-220	6DS	60	Co-dominant
cfd42	17 (202-225)	6DS	60	Co-dominant
cfd75	297 (305-315)	6DS	64	Co-dominant
wms4862	210	6DS	60	Co-dominant
wms4825	207	6DS	60	Co-dominant

Ta = Annealing temperature.

4.4.5 Segregation patterns

All markers linked to stem rust resistance gene(s) were analysed using chi-square analysis to assess single locus segregation patterns before construction of linkage maps (Tables 4.9, 4.10 and 4.11). The five co-dominant SSR markers had a good fit to the expected 1:2:1 segregation ratio in the Line37-07/Koonap, Line37-07/Limpopo and SST387/Line37-07 mapping populations (Tables 4.9 and 4.10). The only significant deviation from the expected segregation ratios was observed in the Line37-07/Komati mapping population (Table 4.9). However, *psp3200*, the only dominantly inherited marker showed an expected segregation ratio of 3:1 in all populations (Tables 4.9 and 4.10). Segregation distortion was random and was skewed mainly towards the male parent allele B (Table 4.9).

Table 4.9 Segregation ratios for microsatellite markers in Line37-07/Koonap and Line37-07/Komati mapping populations

Population	Marker	No. of plants	AA	BB	AB/BB	AB	$\chi 2$
Line37-07/Koonap	*psp3200*	200	54	0	146	0	0.43
	barc183	200	49	49	0	102	0.08
	cfd75	200	53	40	0	107	2.67
	gdm132	200	50	43	0	107	1.47
	cfd42	200	55	36	0	109	5.23
	wms4862	200	51	44	0	105	0.99
	wms4528	200	53	45	0	102	0.72
Line37-07/Komati	*psp3200*	200	52	0	142	0	0.11
	barc183	200	50	67	0	83	8.67*
	cfd75	200	50	71	0	79	13.23**
	gdm132	200	47	71	0	82	12.24**
	cfd42	200	50	71	0	79	13.23**
	wms4862	200	48	68	0	84	9.12*

Co-dominant markers scored as AA, AB and BB, Dominant markers as AA and AB/BB. $\chi 2$ = Chi-square value, * = 0.05, ** = 0.01.

Table 4.10 Segregation ratios for microsatellite markers in Line37-07/Limpopo and SST387/Line37-07 mapping populations

Population	Marker	No. of plants	AA	BB	AB/BB	AB	$\chi 2$
Line37-07/Limpopo	*psp3200*	200	48	0	152	0	0.11
	barc183	200	47	63	0	90	4.56
	gdm132	200	47	54	0	99	0.51
	cfd42	200	44	52	0	104	0.96
	wms4862	200	50	61	0	89	3.63
	wms4528	200	47	60	0	93	2.67
SST387/Line37-07	*psp3200*	200	48	0	152	0	0.11
	barc183	200	51	58	0	91	2.11
	cfd75	200	62	50	0	88	4.32
	cfd42	200	47	56	0	97	0.99
	wms4862	200	52	57	0	91	1.87
	wms4528	200	48	59	0	93	2.19

Co-dominant markers scored as AA, AB and BB, Dominant markers as AA and AB/BB. $\chi 2$ = Chi-square value.

4.4.6 Construction of partial linkage maps for chromosome 6DS using microsatellite markers

BSA analysis identified 7-8 SSR markers as being linked to stem rust resistance on chromosome 6DS that were ultimately used for construction of partial linkage maps of the four populations (Figure 4.1). Total mapped distances for chromosome 6DS varied across different mapping populations with Line37-07/Komati, Line37-07/Limpopo, SST 387/Line37-07, and Line37-07/Koonap showing distances of 46.1, 49.5, 61.9 and 66.2 cM, respectively (Figure 4.1). In the Line37-07/Komati mapping population, *SrKm* was flanked by *psp3200* and *barc183*. In the Line37-07/Limpopo mapping population, *SrLm* was flanked by *wms4528* and *barc183*. SSR markers *barc183* and *wms4862* were closely linked and flanked stem rust resistance genes *SrSt* and *SrKn* (Figure 4.1). *Psp3200* mapped 2.4 cM distally from *SrKm* (Figure 4.1). *Barc18*3 mapped proximally to the stem rust resistance gene(s) *SrKm and SrLm* at 2.3 and 0.8 cM, respectively (Figure 4.1). *Barc18*3 mapped distally to the stem rust resistance gene(s) *SrSt and SrKn* at 2.0 and 1.4 cM, respectively (Figure 4.1). *Wms4862* mapped proximally to the stem rust resistance gene(s) *SrKm, SrLm, SrSt and SrKn* at 5.7, 3.2, 1.9 and 0.9 cM, respectively (Figure 4.1). *Cfd49* mapped distally to the stem rust resistance gene(s) *SrKm, SrLm, SrSt, SrKn* and at 6.1, 4.0, 13.0, 20.0 cM, respectively (Figure 4.1). To aid comparison, the linkage maps of the South African mapping populations were compared with the chromosome 6DS map indicating the position of *Sr42* in Norin 40 (Ghazvini et al., 2012).

4.4.7 Improvement of marker density of chromosome 6DS linkage maps using both microsatellite and single nucleotide polymorphism markers

Nine of the 22 (40.9%) chromosome 6DS SNP markers tested were polymorphic between the resistant and susceptible parents. Four SNP markers *BS00021867, BS000151031, BS00181153* and *BS0011704* were omitted from linkage mapping because these SNPs had more than 10% missing data. However, only five SNP markers had a good fit to the expected 1:2:1 segregation ratio for co-dominant markers in F_2 populations (Table 4.11). These five (22.7%) SNP markers with less than 10% missing data were successfully mapped to chromosome 6DS, thus confirming chromosome 6DS as the region containing the stem rust resistance gene(s). It also improved the marker density in this region. The sequences of these five SNP markers are shown in Table 4.12. Eight SSR markers *psp3200*, *cfd49*, *barc183*, *wms4528*, *wms4862*, *cfd75*, *gdm132* and *cfd4*2 (Table 4.8) together with five SNP markers *BS00085937, BS00085929, BS00076638, BS00075409, BS00022481* (Table 4.12) were used to create the final chromosome 6DS linkage maps for the four populations (Figure 4.2).

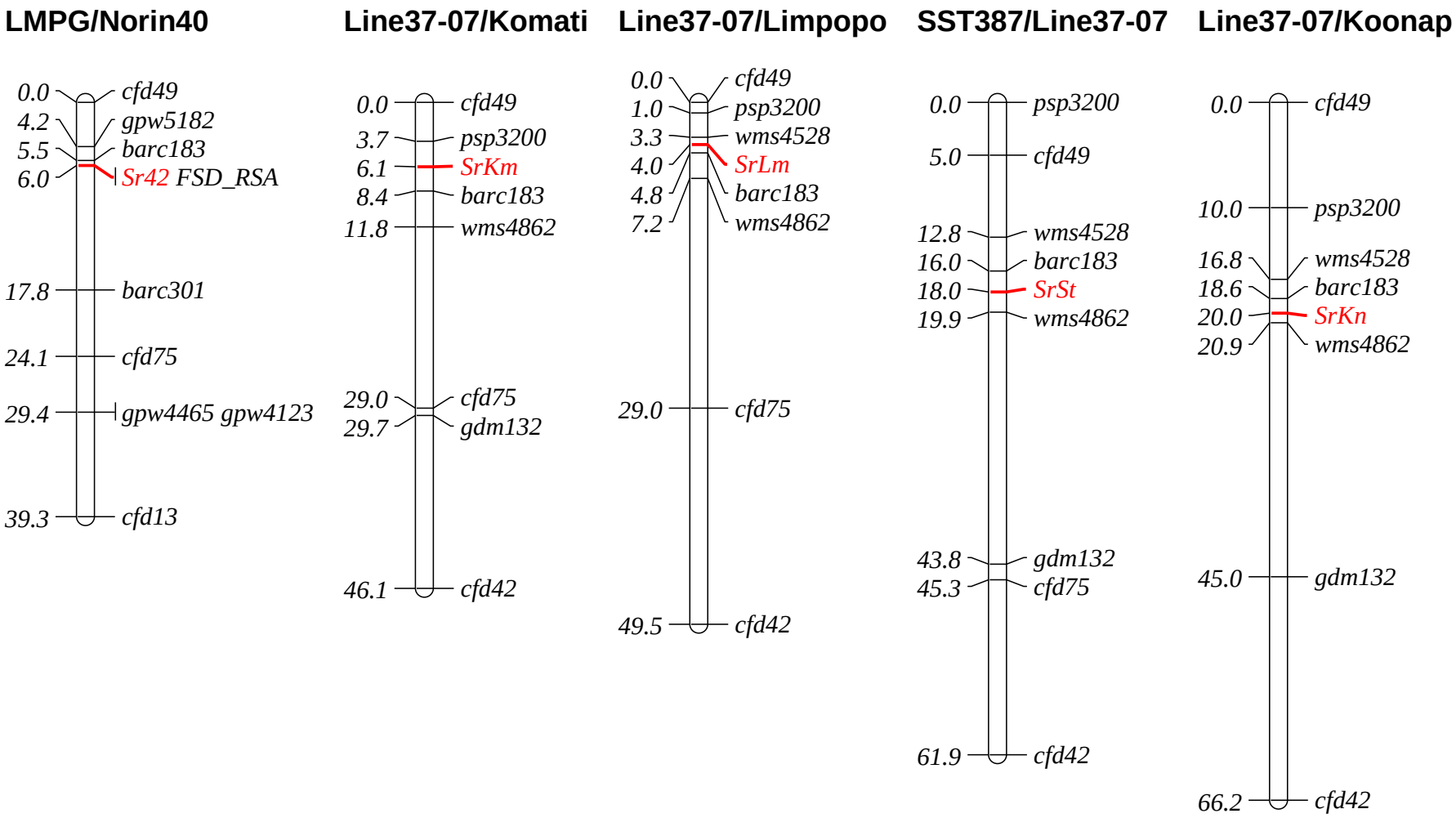


Figure 4.1 Partial linkage maps of chromosome 6DS for four South African winter wheat populations and control population LMPG/Norin40 (Ghazvini et al., 2012) based on microsatellite markers. Marker names are indicated on the right and marker positions are shown on the left in centiMorgans (cM), SSR markers are indicated in black and resistance gene(s) as red

Table 4.11 Segregation ratios for single nucleotide polymorphism markers in Line37-07/Koonap, Line37-07/Komati, Line37-07/Limpopo and SST387/Line37-07 mapping populations

Population	Marker name	No. of plants	No. allele calls	% Missing data	AA	BB	AB	χ2
Line37-07/Koonap	*BS00085937*	200	190	5.0	44	56	90	2.04
	BS00076638	200	195	2.5	45	60	90	3.46
	BS00075409	200	190	5.0	42	60	88	4.44
	BS00022481	200	192	4.0	42	57	93	2.53
	BS00085929	200	197	1.5	44	60	93	3.21
Line37-07/Komati	*BS00085937*	200	193	3.5	46	59	88	3.90
	BS00076638	200	193	3.5	46	60	87	3.82
	BS00075409	200	189	5.5	45	58	86	3.32
	BS00022481	200	197	1.5	47	62	88	4.52
	BS00085929	200	193	3.5	44	60	89	3.82
Line37-07/Limpopo	*BS00085937*	200	197	1.5	47	56	94	1.23
	BS00076638	200	194	3.0	46	55	93	1.16
	BS00075409	200	196	2.0	47	55	93	1.01
	BS00022481	200	194	3.0	47	54	93	0.84
	BS00085929	200	196	2.0	47	56	93	1.34
SST387/Line37-07	*BS00085937*	200	199	0.5	54	47	98	0.54
	BS00076638	200	199	0.5	54	48	97	0.49
	BS00075409	200	197	1.5	54	46	97	0.61
	BS00022481	200	197	1.5	54	48	95	0.70
	BS00085929	200	197	1.5	54	47	96	0.62

Co-dominant marker scored as AA, AB and BB. χ2 = Chi-square value.

Table 4.12 Sequences of chromosome 6DS single nucleotide polymorphism markers mapped in the South African winter wheat varieties

SNP name	Forward primer	Forward primer	Reverse primer
BS00085937	ATCGAGATGGCTATGCGACC**G**	ATCGAGATGGCTATGCGACC**C**	ATATCGGCCATGCTGCCTTTGGAA
BS00022481	TCAAGGCCCTTATCTACGAGTTT**A**	CAAGGCCCTTATCTACGAGTTT**C**	GTGCAGCCATTTGTCCAAGTTTCCAT
BS00075409	GCCTTAATAGGCCTTCATCTAGAT**C**	GCCTTAATAGGCCTTCATCTAGAT**T**	GAGGAAAAGGGCCTTCCAGTTCATT
BS00076638	GCCTTAATAGGCCTTCATCTAGAT**C**	GCCTTAATAGGCCTTCATCTAGAT**T**	GAGGAAAAGGGCCTTCCAGTTCATT
BS00085929	ATATGCTTAATTATATGTATAATAACGCGGT**T**	ATGCTTAATTATATGTATAATAACGCGGT**C**	CTACATACATAATTGAGAAGATTGCTCGTT

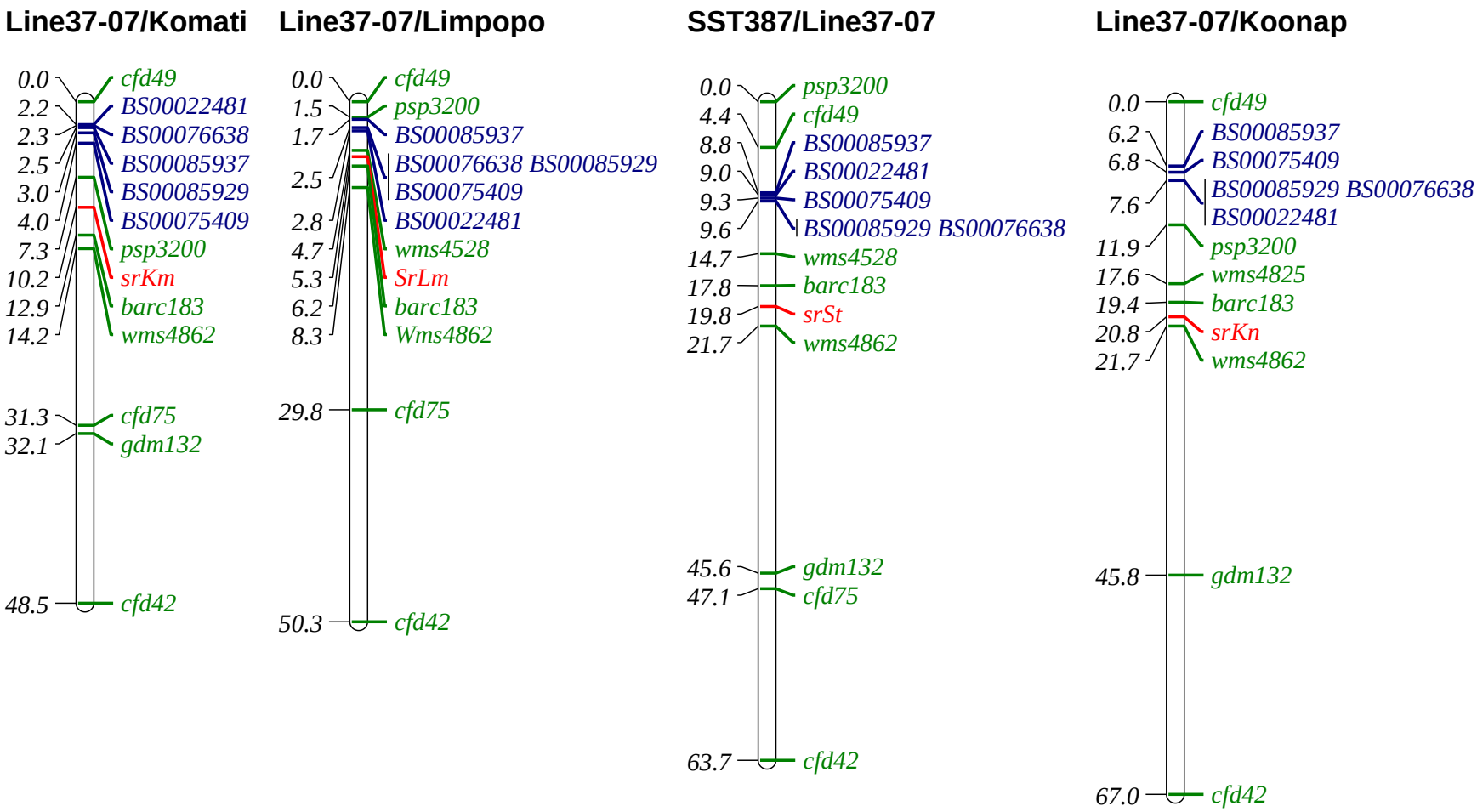


Figure 4.2 Mapping of stem rust resistance gene(s) on chromosome 6DS in four populations using microsatellite and single nucleotide polymorphism markers. Marker names are indicated on the right and marker positions are shown on the left in centiMorgans (cM), SNP markers are indicated in blue, resistance gene(s) as red and SSR markers in green

All five SNP markers mapped distally to the stem rust resistance gene(s) in the four mapping populations (Figure 4.2). This study also reports two SNP markers *BS00085929* and *BS00085937* that mapped distally to stem rust resistance gene(s) at an average of 8.4 and 9.2 cM, respectively (Figure 4.2). All five SNP markers mapped distal to SSR marker *barc183* (Figure 4.2) and thus did not map closer to the stem rust resistance gene(s) than the SSR markers. For instance, the closest SNP markers to stem rust resistance genes *SrKm, SrLm, SrSt* and *SrKn* mapped at 6.2, 2.5, 10.2 and 13.2 cM, respectively (Figure 4.2). Slight differences in marker orders were detected between the four mapping populations. All five SNP markers mapped within a narrow region of 1.8, 1.1, 0.8, and 1.4 cM in the Line37-07/Komati, Line37-07/Limpopo, SST387/Line37-07 and Line37-07/Koonap mapping populations (Figure 4.2). Total mapped distances varied across different mapping populations with Line37-07/Komati, Line37-07/Limpopo, SST387/Line37-07 and Line37-07/Koonap and showing 48.5, 50.3, 63.7, 67.0 cM, respectively (Figure 4.2).

SSR markers *psp3200* and *barc183, wms4528* and *bar183,* and *wms4862* and *barc183* were closely linked and flanked the resistance gene(s) *SrKm, SrLm,* and *SrSt* and *SrKn* in the Line37-07/Komati, Line37-07/Limpopo, and SST387/Line37-07 and Line37-07/Koonap mapping populations (Figure 4.2). *Psp3200* mapped distally to stem rust resistance gene(s) *SrKm, SrLm, SrSt and SrKn* at 2.9, 3.8, 19.8 and 8.9 cM, respectively. *Barc183* mapped proximally to resistance gene(s) *SrKm and SrLm* at 2.7 and 0.9 cM, respectively and distally to resistance gene(s) *SrSt and SrKn* at 2.0 and 1.4 cM, respectively. SSR marker *wms4528* mapped distally to resistance gene(s) *SrLm, SrSt* and *SrKn* at 0.6, 5.1 and 3.2 cM, respectively, while *wms4862* mapped proximally to *SrKm, SrLm, SrSt* and *SrKn* at 4.0, 3.0, 1.9 and 0.9 cM, respectively. SSR marker *cfd49* mapped distally to stem rust resistance gene(s) *SrKm, SrLm, SrSt and SrKn at* 10.2, 5.3, 15.4 and 20.8 cM, respectively (Figure 4.2).

The resistant parents, susceptible parent and stem rust resistant control lines carried different SNP haplotypes (Table 4.13). The stem rust resistant control line Norin 40 and the susceptible parent Line 37-07 possessed the same haplotype G-G-T-T-C for these five SNPs (Table 4.13). The resistant parents carried a C-A-C-C-T haplotype whereas, Triumph 64 and AC Cadillac shared the same haplotype of G-G-C-C-T (Table 4.13).

Table 4.13 Parental and control varieties and their respective single nucleotide polymorphism (SNP) haplotypes for five single nucleotide polymorphism markers closely linked to the chromosome 6DS stem rust resistance locus

		SNP markers								
Genotype	**Gene**	***BS00085937***	***BS00085929***	***BS00076638***	***BS00075409***	***BS00022481***	***BS00021867***	***BS000151031***	***BS00181153***	***BS00111704***
Koonap	*SrKn*	C	A	C	C	T	A	C	T	A
Komati	*SrKm*	C	A	C	C	T	A	C	T	A
Limpopo	*SrLm*	C	A	C	C	T	A	C	T	A
SST 387	*SrSt*	C	A	C	C	T	A	C	T	A
Line 37-07	-	G	G	T	T	C	G	T	C	C
Norin 40	*Sr42*	G	G	T	T	C	G	T	C	C
Triumph 64	*SrTmp*	G	G	C	C	T	G	T	C	C
AC Cadillac	*SrCad*	G	G	C	C	T	G	T	C	A/C

Six SNP markers *BS00085937*, *BS00085929*, *BS00021867*, *BS000151031*, *BS00181153* and *BS00111704* carried alleles in the resistant parents that were completely different from those in the susceptible parent Line 37-07 and the stem rust resistant control lines Norin 40, Triumph 64 and AC Cadillac (Table 4.13).

4.5.8 Quantitative trait loci analysis

The resistance derived from Komati, SST 387, Koonap and Limpopo was each associated with a single resistance gene/QTL against *Pgt* race PTKST located on the short arm of chromosome 6D (Table 4.14). This QTL was flanked by either *barc183*/*wms4862, psp3200/barc183* or *wms4528* and *barc183*. The chromosome 6DS QTL from Koonap, Komati, Limpopo and SST 387 explained 71.4, 73.0, 85.2 and 96.2%, respectively, of the phenotypic variation for stem rust resistance to race PTKST. Komati and Limpopo's QTL were the most distal and peaked at 9.0 and 11.0 cM, respectively. SST 387's QTL peaked at position 19.0 cM while Koonap's QTL was more proximal and peaked at 27.0 cM. The LOD scores associated with the QTL were high and in the range of 56.0-143.8. All four QTL had negative additive effects of between 0.66 and 0.89 (Table 4.14) indicating that resistance alleles were contributed by the resistant parents.

4.4.9 Validation of putative markers linked to stem rust resistance in F_3 populations for marker-assisted selection

To assess the usefulness of the two markers linked to stem rust resistance for MAS, 102 F_3 lines derived from the four mapping populations Line37-07/Komati, Line37-07/Koonap, Line37-07/Limpopo and SST387/Line37-07 were genotyped using the two closely linked markers, *barc183* and *wms4862* (Table 4.15). These closely linked markers were perfect (100%) in predicting resistant phenotypes in all four populations. *Barc183* and *wms4862* had an accuracy of 71-100% in predicting susceptible phenotypes.

4.4.10 Genotyping of parental and control lines with single nucleotide polymorphism markers linked to *Sr42, SrTmp* and *SrCad* on chromosome 6DS

To confirm whether or not the identified gene(s)/QTL on chromosome 6DS were either *Sr42*, *SrTmp* or *SrCad*, parents of the four mapping populations and control lines Norin 40, Triumph 64 and AC Cadillac were genotyped using nine SNP markers reported to be linked to *Sr42*,

Table 4.14 Additive quantitative trait loci for seedling resistance to stem rust caused by *Pgt* race PTKST detected by inclusive composite mapping

Resistant parent	QTL	Chr	Position(cM)	Marker interval	QTL range	LOD	PVE (%)	Add
Komati	*Qsr.ufs-6DS.1*	6D	9.0	*Psp3200-barc183*	8.0-10.5	58.48	73.04	-0.73
SST 387	*Qsr.ufs-6DS.4*	6D	19.0	*barc183-wms4862*	17.5-19.5	143.79	96.24	-0.89
Koonap	*Qsr.ufs-6DS.3*	6D	27.0	*barc183-wms4862*	25.5-27.5	56.01	71.36	-0.66
Limpopo	*Qsr.ufs-6DS.2*	6D	11.0	*Wms4528-barc183*	10.5-12.5	81.20	85.17	-0.67

QTL= Quantitative trait loci named according to McIntosh et al. (2003), Chr = Chromosome, LOD = Logarithm of odds for peak of the given QTL, PVE (%) = Phenotypic variance explained by each QTL, Add = Additive effects of QTL, the negative value means that the favourable allele was contributed by the resistant parent.

Table 4.15 Validation of microsatellite markers linked to stem rust resistance identified in four F_2 populations in F_3 populations

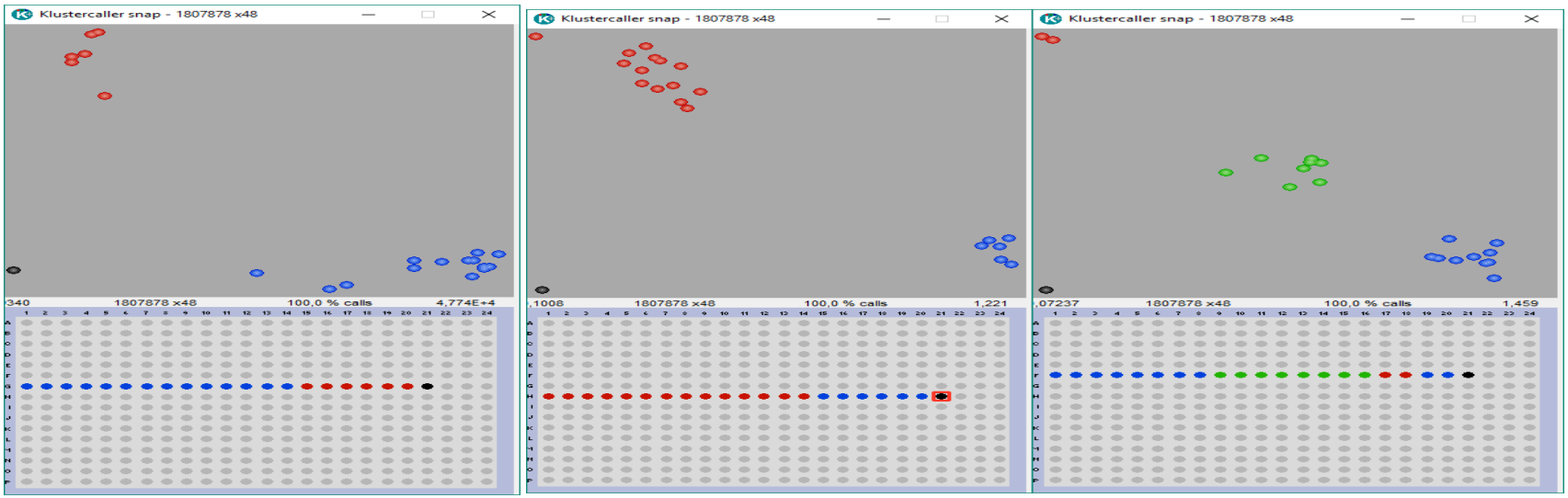
F_3 population	Markers	HR families		HS families			Accuracy (%)
		Resistant genotypes	Resistant phenotypes	Resistant genotypes	Susceptible genotypes	Susceptible phenotypes	
Line37-07/Koonap	*barc183*	13	13	4	12	16	75
	wms4862	13	13	4	12	16	75
Line37-07/Komati	*barc183*	14	14	0	8	8	100
	wms4862	14	14	0	8	8	100
Line37-07/Limpopo	*barc183*	11	11	2	12	14	86
	wms4862	11	11	2	12	14	86
SST387/Line37-07	*barc183*	15	15	4	10	14	71
	wms4862	15	15	4	10	14	71

HR =Homozygous resistant, HS= Homozygous susceptible.

SrTmp and *SrCad* (Gao et al., 2015; Kassa et al., 2016). Seven of the nine SNP markers were monomorphic among the four resistant parents and susceptible parent Line 37-07.

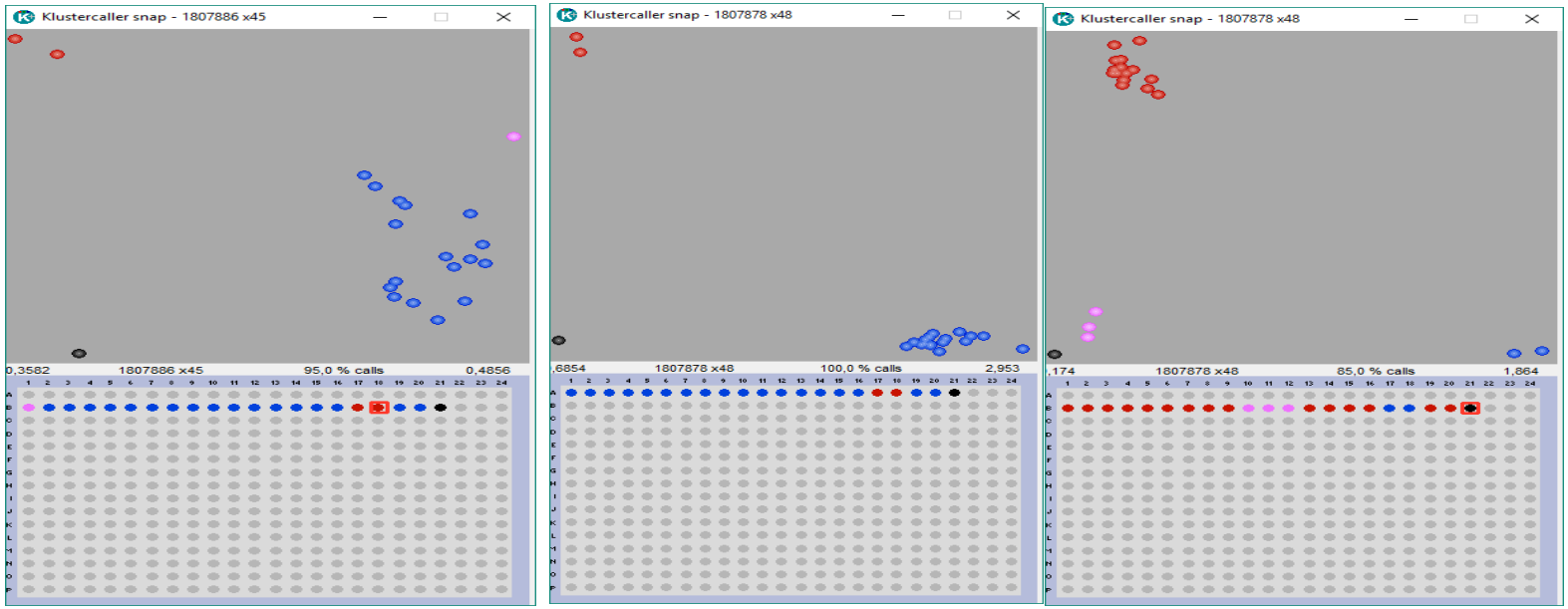
However, these markers amplified a different allele in the resistant control lines compared to the parental lines (see two distinctive clusters in Figures 4.3-4.5). The other two markers, *kwm994* and *IWB31561* produced ambiguous plots detecting heterozygotes among the tested parental and control homozygous lines (Figures 4.3c and 4.5a). Five of the nine tested SNP markers *IWB15852*, *IWB31561*, *IWB36391*, *TP43472* and *TP93838*, selected because of their reported close linkage to the *Sr42* gene, produced different haplotypes in the resistant parents compared to the resistant controls (Table 4.16). Markers *IWB36391* and *TP43472* differentiated between the parents (resistant and susceptible) of the four mapping populations with a T-C haplotype and the resistant control lines Norin 40, Triumph 64 and AC Cadillac with a C-T haplotype (Table 4.16). *IWB36391* and *TP43472* were the only selected SNP markers informative for *Sr42*. The resistant control line Norin 40 and the susceptible parent Line 37-07 were heterozygous for SNP marker *IWB31561* (Table 4.16 and Figure 4.3c). SNP markers for Norin 40 and Triumph 64 co-segregated, while AC Cadillac was different from Norin 40 and Triumph 64 for three SNP markers (*IWB15852*, *IWB31561* and *TP93838*). *Sr42*, *SrTmp* and *SrCad* co-segregated for markers *IWB36391* and *TP43472* (Table 4.16). The haplotype for *SrCad* for the five SNP markers *IWB15852*, *IWB31561*, *IWB36391*, *TP43472* and *TP93838* was different from the resistant and susceptible parents (Table 4.16).

Komati, Koonap, Limpopo, SST387 and Line 37-07 had different haplotypes for *SrCad* SNP markers using all four *SrCad* diagnostic markers; *kwm907*, *kwm987*, *kwm994* and *kwm997* (Table 4.17). Susceptible parent Line 37-07 was heterozygous for *kwm994* (Table 4.17 and Figure 4.5a). The resistant parents, susceptible parent, Norin 40 and Triumph 64 shared the same haplotype (A-T-T-G), while AC Cadillac had a different haplotype (T-C-C-T) for all four *SrCad* diagnostic SNP markers (Table 4.17; Figures 4.4 and 4.5 a).



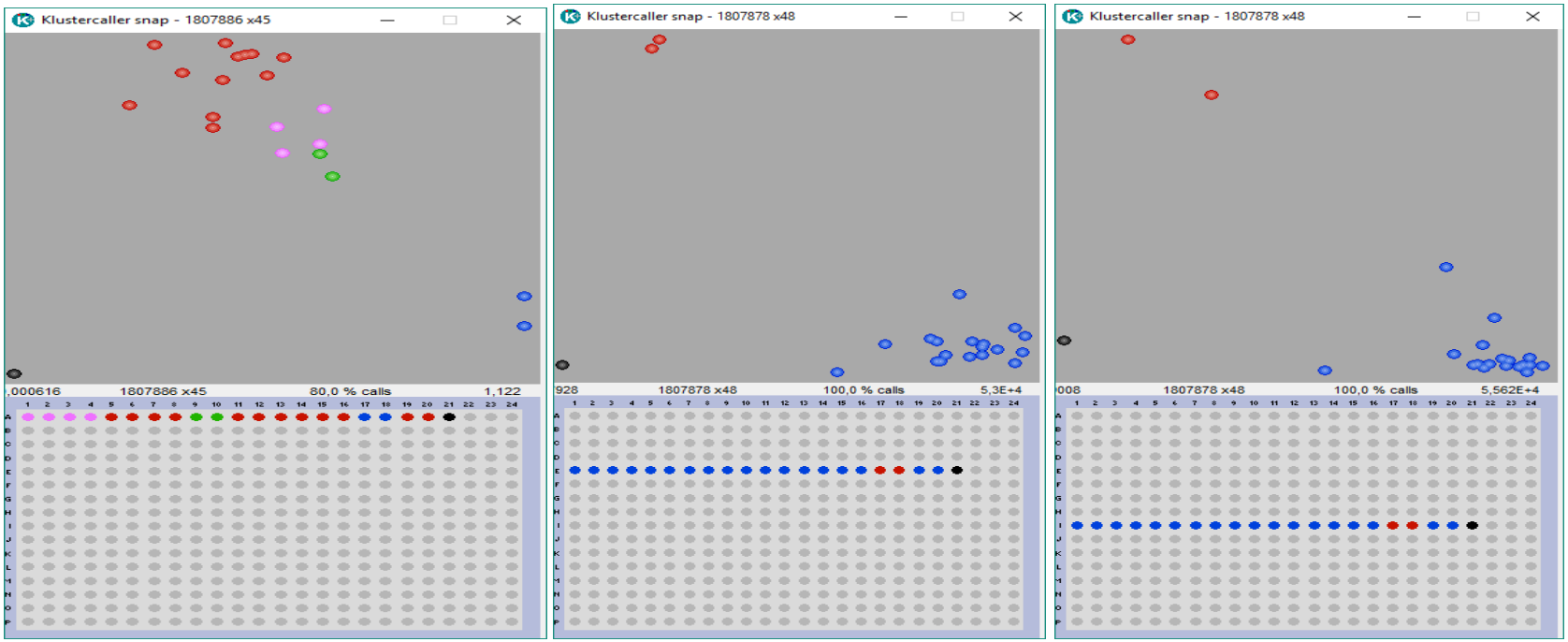
(a) *IWB36391* (b) *TP43472* (c) IWB31561

Figure 4.3 Single nucleotide polymorphism sequence based, co-dominant assays for *Sr42*. The names next to a, b and c represent SNP marker names. (a) Red (HEX-labelled) dots represent homozygous resistant control lines Norin 40, AC Cadillac and Triumph 64, while blue (FAM-labelled) dots represent resistant and susceptible parents. (b) Red (HEX-labelled) dots represent resistant and susceptible parents, while the blue (FAM-labelled) dots represent the resistant control lines. (c) Red (HEX-labelled) dots represent homozygous resistant control line AC Cadillac, the blue (FAM-labelled) dots represent homozygous resistant parent Koonap, Komati, Limpopo, SST 387 and resistant control line Triumph 64, while the green dots represent heterozygous resistant parents SrCad_1 and SrCad_2, susceptible parent Line 37-07 and resistant control line AC Cadillac. The black dot represents the no template control (NTC). Rows 1-21 represent samples loaded as: 1, 2 = Koonap; 2, 3 = Komati; 4, 5 = Limpopo; 7, 8 = SST 387; 9, 10 = Line 37-07; 11, 12 = SrCad_1; 13, 14 = SrCad_2; 15, 16 = Norin 40; 17, 18 = AC Cadillac; 19, 20 = Triumph 64 and 21 = NTC



(a) *kwm987* (b) *kwm907* (c) *kwm997*

Figure 4.4 Single nucleotide polymorphism sequence based, co-dominant assays for *Sr42*. The names next to a, b and c represent SNP marker names. (a) and (b) Red (HEX-labelled) dots represent homozygous resistant control AC Cadillac, while the blue (FAM-labelled) dots represent resistant, susceptible parents and resistant control lines Norin 40 and Triumph 64. (c) Blue (FAM-labelled) dots represent homozygous resistant control parent AC Cadillac, while the red (HEX-labelled) dots represent homozygous resistant, susceptible parents and resistant control lines Norin 40 and Triumph 64. The black dot represents the no template control (NTC). Rows 1-21 represent samples loaded as: 1, 2 = Koonap; 2, 3 = Komati; 4, 5 = Limpopo; 7, 8 = SST 387; 9, 10 = Line 37-07; 11, 12 = SrCad_1; 13, 14 = SrCad_2; 15, 16 = Norin 40; 17, 18 = AC Cadillac; 19, 20 = Triumph 64 and 21 = NTC



(a) *kwm994* (b) *IWB15852* (c) *TP93838*

Figure 4.5 Single nucleotide polymorphism sequence based, codominant assays for *SrCad*. The names next to a, b and c represent SNP marker names. (a) Blue (FAM-labelled) dots represent homozygous resistant control line AC Cadillac, the green dots represent heterozygous resistant parent SrCad_1 while the red (HEX-labelled) dots represent homozygous resistant, susceptible parents and resistant control lines Norin 40 and Triumph 64. (b) and (c) Red (HEX-labelled) dots represent homozygous resistant control line AC Cadillac while blue (FAM-labelled) dots represent homozygous resistant, susceptible parents and resistant control lines Norin 40 and Triumph 64. The black dot represents the no template control (NTC). Rows 1-21 represent samples loaded as: 1, 2 = Koonap; 2, 3 = Komati; 4, 5 = Limpopo; 7, 8 = SST 387; 9, 10 = Line 37-07; 11, 12 = SrCad_1; 13, 14 = SrCad_2; 15, 16 = Norin 40; 17, 18 = AC Cadillac; 19, 20 = Triumph 64 and 21 = NTC

Table 4.15 Parental and control lines and their haplotypes for five single nucleotide polymorphism markers near the chromosome 6DS-PTKST locus reported to be linked to *Sr42*

		SNP markers				
Genotype	**Gene**	***IWB15852***	***IWB31561***	***IWB36391***	***TP43472***	***TP93838***
Koonap	*SrKn*	T	T	T	C	T
Komati	*SrKm*	T	T	T	C	T
Limpopo	*SrLm*	T	T	T	C	T
SST 387	*SrSt*	T	T	T	C	T
Line 37-07	-	T	T/C	T	C	T
Norin 40	*Sr42*	T	T/C	C	T	T
Triumph 64	*SrTmp*	T	T	C	T	T
AC Cadillac	*SrCad*	C	C	C	T	C

Table 4.16 Parental and control lines and their haplotypes for four *SrCad* diagnostic single nucleotide polymorphism markers near the chromosome 6DS-PTKST locus.

		SNP markers			
Genotype	**Gene**	***Kwm907***	***Kwm987***	***Kwm994***	***Kwm997***
Koonap	*SrKn*	A	T	T	G
Komati	*SrKm*	A	T	T	G
Limpopo	*SrLm*	A	T	T	G
SST 387	*SrSt*	A	T	T	G
Line 37-07	-	A	T	T/C	G
Norin 40	*Sr42*	A	T	T	G
Triumph 64	*SrTmp*	A	T	T	G
AC Cadillac	*SrCad*	T	C	C	T

4.5 Discussion

4.5.1 Development of mapping populations

All four populations were considered for mapping as the resistant parents expressed similar low but not identical resistant phenotypes. It was therefore necessary to determine if it was the same gene(s) involved in their resistant responses. F_2 populations are a result of a single meiosis event and were considered as it takes a shorter time and are less costly to develop (Schneider, 2005; Semagn et al., 2006). It is also possible to measure dominant or additive gene effects in F_2 populations (Meng et al., 2015). Four background SSR markers were successful in identifying all 47 lines as true F_1 hybrids. These markers were selected as they displayed large allelic differences between the resistant and susceptible parents. This process ensured that the resulting F_2 mapping populations were derived from true F_1 hybrids. This is important so that the F_2 populations should differ in the traits under study (Schneider, 2005), hence accurate identification of polymorphic markers.

4.5.2 Identification of polymorphic microsatellite markers using genome-wide screening

In this study the whole wheat genome was screened to identify polymorphic markers across all the 21 chromosomes. The 104 LRGS marker set was selected for efficient and effective identification of polymorphic markers as this marker set was developed for South African wheat cultivars. Wessel and Prins (2016) reported that only this small set of markers is effective in identifying major gene(s)/QTL within a very short time and at a reduced cost. Wessel and Prins (2016) indicated that a minimum of four SSR markers spread across each of 21 wheat chromosomes were identified. As these SSR markers covered the entire length of wheat chromosomes, it means there is a high possibility of locating the uncharacterised genes.

Michelmore et al. (1991) stated that BSA is capable of identifying linked markers within a 0-25 cM window to either side of locus of wheat chromosomes. In addition, Somers et al. (2004) indicated that the wheat consensus map contained between 59 to 184 markers for chromosome 4B and 5A, respectively. Indeed, considering an average of four markers per chromosome would partition chromosomes 4B and 5A into 14.75 cM and 46 cM regions, respectively (Somers et al., 2004). This represents effective map distances within which BSA can detect a gene of interest. From initial screening of parental lines with 104 LRGS SSR markers, the number of polymorphic markers was 57-70, representing a polymorphism rate of 54.8-67.3%. This identified level of polymorphism of LRGS is lower than the 82% reported by Wessels and Prins (2016), but higher than the polymorphism rate of 20-40% as reported by Somers et al.

(2004) to be present in many single-wheat mapping populations. Therefore, to increase the polymorphism rates, an additional 31 SSR markers were screened on the parents of the mapping populations.

In total, 135 polymorphic SSR markers were identified and selected for further analyses. This resulted to an improved level of polymorphism across the different populations with Komati, Koonap, Limpopo and SST 387 possessing 68, 71, 75 and 87 polymorphic markers, respectively. In addition, ultimately each chromosome had a minimum of four SSR markers spread along its length, hence increasing the possibility of finding the resistance gene using BSA. The cross between SST 387 and Line 37-07 had the highest polymorphism rate of 67.3% compared to 54.8% for Komati and Line 37-07 population. Wider crosses have higher polymorphism rate >60% while narrow crosses are usually <40% (Somers et al., 2004). The polymorphism rates obtained in the current study indicate that the parents of mapping populations are genetically from wider crosses. The highest number of polymorphic markers between the parents was detected for genome A (35.7%), followed by B (32.6%) and D (31.7%). These results are consistent with previous studies that have indicated the D genome has the lowest levels of polymorphisms (Chao et al., 2009; Allen et al., 2011).

4.5.3 Identification of chromosome location linked to stem rust resistance using bulked segregant analysis

Following the low-resolution genome scan, using 135 polymorphic SSR markers on the parents and bulk lines, BSA indicated that only markers known to map to the short arm of chromosome 6D showed linkage in parents and bulks, indicating possible association with stem rust resistance in all four tested mapping populations. Therefore, BSA successfully identified the short arm of chromosome 6D as potentially carrying stem rust resistance gene(s). Initially, BSA identified three SSR markers *cfd49*, *gwm649* and *barc183* located on chromosome 6DS as linked to resistance in all four mapping populations. These results suggest that the same gene confers resistance against *Pgt* race PTKST in all four South African winter wheat varieties Komati, SST 387, Koonap and Limpopo. BSA efficiently identifies markers linked to genes of interest such as diseases resistance, hence allowing their rapid placement on genetic maps (Michelmore et al., 1991). It is likely that BSA could have missed to detect minor QTL in the current study, because BSA is basically for detection of major genes. However, several studies have reported the successful application of combination of LRGS and BSA in identifying both minor and major QTL in wheat (Smit et al., 2016; Prins et al., 2016; Wessels et al., 2019). The

LRGS set of markers with a minimum of four markers per chromosome was therefore effective in detecting the major stem resistance rust resistance gene(s).

4.5.4 Construction of linkage maps for chromosome 6DS using both microsatellite and single nucleotide polymorphism markers

The winter wheat stem rust resistance gene was initially mapped in the four mapping populations using only eight SSR markers. Genotyping of the populations using both SSR and SNP markers increased the total number of mapped markers from 8 to 13, hence improving the accuracy of detection of the gene/QTL using MAS. The total genetic distances increased in all four mapping populations when linkage maps containing both SSR and SNP markers were used. Map distances increased by 2.4, 0.8, 1.8 and 0.8 cM for Line37-07/Komati, Line37-07/Limpopo, SST387/Line37-07 and Line37-07/Koonap, respectively. Mapping results indicated that marker order and positions were similar and consistent with previously published wheat maps (Stephenson et al., 1998; Paillard et al., 2003; Somers et al., 2004; Allen et al., 2013, 2017; Olson et al., 2013; Lopez-Vera et al., 2014). The large gaps between markers in the linkage maps were expected as the D chromosomes of wheat are known for limited recombination and thus creating difficulties during mapping with limited marker coverage compared to A and B genomes.

SSR markers *psp3200*, *wms4528*, *barc183* and *wms4862* were either closely linked or flanked resistance gene(s) in the mapping populations. It is important to identify flanking markers to gene(s), because recombination can happen between the marker and gene(s). So ideally if both flanking markers are present there is a good chance that the gene(s) is still present. *Barc183* mapped either distally or proximally in different populations perhaps due to different recombination events that took place in different lines or varying segregation distortion of all other mapped markers.

To aid comparison, the linkage maps of South African varieties were compared with the map created to map *Sr42* previously to chromosome 6DS in Norin 40 (Ghazvini et al., 2012). SSR marker *cfd49* has previously being mapped distally at 6.0 cM to *Sr42* (Ghazvini et al., 2012) and at 7.7 cM to *SrCad* (Hiebert et al., 2011). *Cfd49* mapped distally to the stem rust resistance gene(s) at 5.3-20.8 cM from the resistance gene(s).

Evaluation of marker profiles revealed that *cfd49* amplified a product in all four resistant parents and susceptible parent that was different from that amplified in Norin 40, the carrier of *Sr42* (Ghazvini et al., 2012). *Barc183* mapped distally at 0.5 cM from *Sr42* (Ghazvini et al., 2012) and amplified a fragment in the four resistant parents and susceptible parent that was different from that amplified in Norin 40. Two SSR markers *wms4862* and *wms4528* identified in the current study as closely linked to stem rust resistance gene(s) amplified fragments in the four resistant parents that were different from that in Norin 40.

Presently, five *Pgt* resistance loci have been identified and mapped to wheat chromosome 6DS, namely *Sr5*, *Sr42*, *SrCad*, *SrTmp* and *SrTA10187* (McIntosh et al., 1995; Hiebert et al., 2011; Ghazvini et al., 2012; Olson et al., 2013; Gao et al., 2015; Babiker et al., 2016, 2017; Kassa et al., 2016; Wiersma et al., 2016). Four of these genes *Sr42*, *SrCad*, *SrTmp* and *SrTA10187* are effective against stem rust race TTKSK (Jin et al., 2007). However, *Pgt* races PTKTK, TTKTK and TTKTT virulent to *SrTmp* have been detected (Fetch et al., 2016; Newcomb et al., 2016; Patpour et al., 2016b). Hiebert et al. (2016) mapped SSR marker *gpw5182* distally at 0.8 cM from *SrTmp*. *Gpw5182* amplified the same fragment in all four resistant parents as well as the susceptible parent indicating that the segregation at the stem rust locus was possibly not associated with *SrTmp* because the fragment amplified in the parental lines was different in size from that in the positive resistant control line Triumph 64.

Olson et al. (2013) mapped *psp3200* at 7.3 cM from *SrTA10187* to the distal end of chromosome 6DS as a co-dominant marker. However, in the present study this marker was inherited dominantly and was linked to the resistance gene(s) in coupling phase in the four populations. *Psp3200* mapped at relatively different positions (2.9-19.8 cM) from the resistance gene(s). Given the similarity in map positions between *psp3200*, *SrTA10187* and the newly mapped stem rust resistance gene(s), results suggest that the mapped stem rust resistance(s) could be similar to *SrTA10187*. However, *SrTA10187* is less likely the same gene as that mapped in the four South African winter wheat varieties because the donor parent for *SrTA10187*, TA10187 is a wild wheat relative (*Ae. tauschii*) that is unlikely present in the pedigrees of South African winter wheat varieties.

Pedigree data indicate that both Komati and Limpopo share the winter wheat variety Betta as a parent, directly in Limpopo and indirectly through the use Molopo in Komati. Unfortunately, pedigree data for Koonap and SST 387 could not be retrieved as this is proprietary information.

Multi-race phenotyping ruled out the involvement of *Sr42* and *SrCad*, but suggested the likely presence of *SrTmp* among the four varieties. All four resistant parents expressed low infection types against races TTKSF, TTKSF+Sr9h, TTKSP, PTKSK, PTKST, QCCJB, QTHJC, TTKSK and TTKTT. However, the resistant parents performed differently, expressing either resistant or susceptible phenotypes against race TRTTF. Given the similar and narrow genetic region and markers that flanked the mapped resistance gene(s) coupled with the above stem rust phenotypes, it is probable that the same gene or alleles of the same gene confers resistance in all four varieties.

Of these five mapped SNP markers, only *BS00085937* has previously been associated with chromosome 6DS stem rust resistance gene(s). Wiersma et al. (2016) mapped SNP marker *BS00085937* at 3.4 cM distally to *SrTA10187*. This marker *BS00085937* mapped distally at 3.6-10.4 cM from the mapped stem rust resistance gene(s) in the current study. Similar to observations in the current study, the resistant parent TA10187 and susceptible parent KS05HW14 possessed the C and G alleles, respectively for *BS00085937*. Wiersma et al. (2016) fine mapped *SrTA10187* to a 1.1 cM region and reported that SNP marker *6DS0039*, was located 0.2 cM proximal to *SrTA10187* and STS marker *6DS0050*, mapped 1 cM distal to *SrTA10187,* as suitable markers for MAS.

A critical analysis of the haplotypes of the five mapped SNP markers revealed that Norin 40 (containing *Sr42)* and the susceptible parent Line 37-07 possessed the same haplotype G-G-T-T-C for these five SNPs, confirming SSR results that indicated that the mapped resistance gene(s) are different from *Sr42*. Furthermore, the four resistant parents carried a C-A-C-C-T haplotype whereas, Triumph 64 (*SrTmp)* and AC Cadillac (*SrCad*) shared the same haplotype of G-G-C-C-T. These SNP haplotype analyses results suggest that the mapped resistance gene(s) are different from *Sr42*, *SrTmp* and *SrCad*. SNP markers *BS00085937* and *BS00085929* detected a different allele than the parental lines, but were present in all resistant parents and should be validated for MAS of this resistance gene(s) compared to the other three SNP markers that amplified the sample allele in the four resistant parents as well as Triumph 64 and AC Cadillac.

4.5.5 Genotyping of parental and control lines with microsatellite and single nucleotide polymorphism markers linked to *Sr42, SrTmp* and *SrCad* on chromosome 6DS

The parents of the four mapping populations were tested for the possible presence of stem rust resistance genes commonly deployed in winter wheat breeding programmes in South Africa using published closely linked molecular markers. All parents tested negative for SSR markers linked to stem rust resistance genes *Sr2, Sr15, Sr24, Sr25, Sr31, Sr42/SrTmp/SrCad* and *Sr57*. However, all control lines tested positive for the respective stem rust resistance genes screened indicating that the correct amplification procedures were followed. These results indicated that the stem rust resistant parents Komati, SST 387, Koonap and Limpopo and the susceptible parent Line 37-07 lacked *Sr2, Sr15, Sr24, Sr25, Sr31, Sr42/SrTmp/SrCad* and *Sr57*. These molecular data combined with stem rust phenotying data of Pretorius et al. (2012) indicated that resistance observed in the parents could possibly be a result of uncharacterised resistance gene(s).

Because SSR and SNP mapping data could not with 100% accuracy indicate whether the resistance gene(s) on chromosome 6DS in the four evaluated mapping populations is either *Sr42, SrTmp* and/or *SrCad* or not, SNP markers linked to these genes were evaluated. Nine SNP markers closely linked to *Sr42, SrTmp* and *SrCad* were tested on the parents of the four mapping populations as well as Norin 40 (*Sr42*), Triumph 64 (*SrTmp*) and AC Cadillac (*SrCad*). Unfortunately, seven of these markers were monomorphic and two produced ambiguous results. None of these SNP markers could thus be mapped in the current study. However, five of these SNP markers could distinguish between the parental and control lines. *IWB36391* and *TP43472* clearly differentiated between the parents of the four mapping populations and the resistant control lines Norin 40, Triumph 64 and AC Cadillac. Gao et al. (2015) validated SNP markers *IWB36391* and *TP43472* as co-segregating with *Sr42* in the PI 410954 DH mapping population. Gao et al. (2015) reported that although SNP marker *IWB31561* co-segregated with *Sr42*, it could not clearly separate alleles and this was attributed to the presence of an additional sequence variation at the target locus or possibly due to interference from a homeologous locus. Similarly, in this study SNP marker *IWB31561* amplified two alleles in the susceptible parent Line 37-07 as well as Norin 40. Markers *IWB15852* and *TP93838* were reported to be informative in the PI 59566 population but monomorphic in the PI 410954 DH population (Gao et al., 2015) and were similarly, monomorphic in the current study and this underscores their utility.

In an attempt to make comparisons between linkage maps developed by Gao et al. (2015) and Kassa et al. (2016), Wiersma et al. (2016) tested SNP markers *IWB31561* and *Kwm112* on their mapping populations containing the *SrTA10187* stem rust resistance gene. Both markers were monomorphic between the resistant (TA10187) and susceptible (KS05HW14) parents with both carrying the C allele. This is contrary to the T allele carried by Koonap, Komati, Limpopo and SST 387 for the same SNP marker *IWB3561*. Gao et al. (2015) reported that three SNP markers (*IWB31561*, *IWB36391* and *TP43472*) co-segregated with the *Sr42* gene and proposed their use to form haplotypes that aid selection of *Sr42* (PI 59566 and Norin 40), *SrTmp* (Triumph 64) and *SrCad* (AC Cadillac). Their findings showed that: (1) PI 59566 shared the same haplotype of C-C-T with AC Cadillac (*SrCad*) and Peace (*SrCad*) for the three KASP assays, (2) Triumph 64 (*SrTmp*) and CnSSrTmp (*SrTmp*) had the T-C-T haplotype that was also present in PI 410954, Robin, Digalu, Blouk and Pfunye and (3) Norin 40 (*Sr42*) possessed a different haplotype T*-C-T that was also present in genotypes Guard, Shield, Eagle, Ripper and Ember. T* represented the *IWB31561* allele intermediate between T and C but much closer to T. The present analysis of the four resistant parents Koonap, Komati, Limpopo and SST 387 with these three SNP markers (*IWM31561*, *IWB36391* and *TP43472*) showed that these genotypes carried a different haplotype (T-T-C).

Kassa et al. (2016) identified four SNP markers as being completely predictive of *SrCad* in 50 different wheat varieties and breeding lines. Of these, *Kwm987* co-segregated with *SrCad* in the LMPG/ACCadillac mapping population. Both *Kwm994* and *Kwm997* were mapped proximally at 0.37 cM, while *Kwm907* mapped at 1.52 cM proximally to *SrCad* in LMPG/ACCadillac. Parents of the mapping populations were also genotyped with *SrCad* specific markers *Kwm907*, *Kwm987*, *Kwm994* and *Kwm997* (Kassa et al., 2016) and results clearly indicated that Komati, Koonap, Limpopo and SST 387 carried a A-T-T-G haplotype different from the T-C-C-T haplotype in AC Cadillac (*SrCad*) for these four diagnostic markers.

Rafalski (2002) stated haplotype analysis is more useful than individual SNP analysis and possesses greater power in analysing phenotype associations. Gao et al. (2015) recommended that breeders should consider the possibility of analysing and combining many SNP assays to form longer haplotypes so as to increase accuracy in selections. Similarly, Yu et al. (2017) have also stated that they increased the prediction accuracy of *Sr25* in 277 wheat genotypes by forming haplotypes using four SNP markers. Hence, by forming haplotypes with all nine tested

SNP markers closely linked to Ug99-6DS resistance, the resistant parents carried the A-T-T-G-T-T-C-T-T haplotype compared to Norin 40 (A-T-T-G-T/C-C-T-T-T), Triumph 64 (A-T-T-G-T-C-T-T-T) and AC Cadillac (T-C-C-T-C-C-T-C-C) for *Kwm907, Kwm987, Kwm994, Kwm997*, *IWB31561*, *IWBI36391*, *TP43472*, *IWB15852* and *TP93838*, respectively.

SNP markers (*IWB31561*, *IWB36391* and *TP43472*) co-segregated with the *Sr42* gene while among the *SrCad* linked markers, *Kmw987* co-segregated with *SrCad*. Therefore, these co-segregating SNP markers would not result in false positives or negatives, indicating that the lack of association with stem rust resistance gene(s) mapped in current study. The other SNP markers *Kwm907, Kwm994* and *Kwm997* are closely linked to *SrCad* and there exists a possibility of recombination happening between these markers and *SrCad* resulting in false positives and negatives. With the above evidence taken together, it is clear that the gene(s) mapped here are different and possibly novel or alleles of *Sr42*, *SrTmp* and/or *SrCad.*

4.5.6 Quantitative trait loci analysis

QTL analysis on all four mapping populations indicated that all four resistant parents possibly possess the same single dominant resistance gene(s) to stem rust race PTKST. The peak QTL location was at 9-27 cM on chromosome 6DS, LOD scores of 56-143 and PVE of 71-96% were reported. Komati's, Limpopo's, SST 387's and Koonap's QTL peaked at different locations of 9, 11, 19 and 27 cM, respectively. On average, QTL in all mapping populations spanned a distance of 2.0-2.5 cM. The small differences in QTL peak positions and distance spanned by the QTL could be a result of different recombination events (unequal crossovers) or alleles of same gene in the four mapping populations. For QTL mapping of seedling stem rust resistance gene(s), usually single greenhouse phenotypic data is sufficient to obtain an accurate QTL (Saini et al., 2018; Wessels et al., 2019). Field phenotyping data across different locations in replicated trials could not be recorded since F_2 mapping populations were used. Therefore, RIL populations can in future be created from the available F_2 populations to generate accurate field phenotypic data for QTL mapping.

4.5.7 Validation of putative markers linked to stem rust resistance in F_3 populations

Linkage mapping identified markers *barc183* and *wms4862* as closely linked to the stem rust resistance gene(s) conferring resistance to stem rust race PTKST. The effectiveness of these markers linked to the QTL region was validated by predicting the phenotypes of F_3 lines. Both these markers predicted the resistant phenotypes with 100% accuracy but were only 71-100%

accurate in predicting susceptible phenotypes. This was expected because these two markers were ≤4.0 cM from the gene thus allowing recombination to occur leading to false positives and/or negatives. These markers are thus not 100% diagnostic for the stem rust resistance against *Pgt* race PTKST. The marker prediction accuracy also depends on the quality of stem rust phenotypes being evaluated. For each F_3 family, 20-30 individuals were phenotyped but leaf-material was sampled from only 10 individuals to create F_3 bulks for DNA analysis. F_3 individuals are still heterogenous compared to homozygous cultivars. Therefore, the bulking of only a sub-set of all phenotyped individuals could have also affected the marker prediction accuracy in some populations. These flanking markers could therefore result in higher prediction accuracies in homozygous genotypes like cultivars. With a 100% prediction accuracy obtained in the F_3 Line37-07/Komati population lines, it is probable that these flanking markers could be useful in MAS. Because marker validation was only done using F_3 lines, these markers will in future have to be tested on a diverse set of cultivars and breeding lines to assess their effectiveness in MAS.

4.6 Conclusions and recommendations

BSA with SSR markers identified chromosome 6DS as the region carrying the PTKST stem rust resistance gene and this information was also confirmed by the successful mapping of SNP markers on chromosome 6DS. SSR markers were more closely linked to stem rust resistance gene(s) than the SNP markers hence were more effective in identifying the mapped stem rust resistance gene(s). SSR and SNP markers were sparsely spaced in the four linkage maps. This was expected given the reported low levels of polymorphism recorded for the D-genome compared to both the A- and B-genomes (Chao et al., 2009; Allen et al., 2011).

Therefore, to further saturate the chromosome 6D linkage maps developed in the current study, these mapping populations should be genotyped using high-marker density SNP platforms such as 90K and/or DArTs to identify closely linked or co-segregating markers. As the molecular markers identified in the current study resulted from linkage and QTL mapping using F_2 populations, these populations should be advanced to develop RILs to facilitate the evaluation of effectiveness of resistance gene(s) across various locations. South African winter wheat varieties and breeding lines should be assessed with molecular markers *psp3200*, *wms4528*, *barc183* and *wms4862* identified in the current study as closely linked to stem rust resistance gene(s) to postulate its local prevalence. Wheat germplasm from other regions of the world can also be tested for prevalence of these stem rust resistance gene(s). This is vital to generate a

critical source of information that will ultimately facilitate sharing of these resistant South African winter wheat varieties with other researchers and breeding institutions. South African winter wheat varieties tested negative with diagnostic SNP markers for known chromosome 6DS stem rust resistance genes *Sr42*, *SrTmp* and *SrCad* implying the mapped stem rust resistance gene(s) is novel or an allele of *Sr42*, *SrTmp* and/or *SrCad*. It is also critical to conduct further research to determine if the South African winter wheat varieties carry similar gene(s) or not. This can be achieved by performing extensive allelism tests and phenotyping with multiple pathogen races with different virulence spectrums.

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CHAPTER 5

IDENTIFICATION AND MOLECULAR MAPPING OF AN ADULT PLANT RESISTANCE GENE/QTL IN THE SOUTH AFRICAN WINTER WHEAT VARIETY PAN 3161

5.1 Abstract

Stem rust poses a threat to wheat production in SA and other countries. This study was conducted to identify and map an APR gene in the South African winter wheat variety PAN 3161. An F_2 population and F_3 families were developed from a cross between the resistant parent PAN 3161 and the susceptible parent Line 37-07 and tested against *Pgt* race PTKST in the field during the 2017 and 2018 seasons. SSR marker genotyping combined with BSA on 128 F_2 plants identified markers on the short arm of chromosome 4D as linked to a stem rust resistance gene. SSR marker *wmc720* flanked the APR gene *SrPan3161* distally at 1.8 cM. Another set of four co-segregating SSR markers *gpw7414*, *gpw8038*, *wmc52* and *cfd23* flanked the APR gene proximally at 1.8 cM. Although the pleiotropic APR locus *Lr34/Yr18/Sr57/Pm38/Ltn1* was detected in PAN 3161 using a gene-specific marker, no linkage to this adult plant stem rust resistance locus was detected during BSA. QTL analysis was performed to confirm results of linkage mapping and the identified single major QTL explained 71.5% of the phenotypic variation for resistance to *Pgt* race PTKST. The flanking SSR markers *wmc52*, *cfd23* and *wmc720* were predictive of *SrPan3161* in F_3 families thus validating the chromosome location and possible effectiveness in MAS in this population.

5.2 Introduction

Stem rust, caused by *Pgt*, is an economically important disease of wheat globally (Ghazvini et al., 2012). Yield losses up to 100% were reported during sporadic epidemic outbreaks (Park, 2007). Yield losses caused by *Pgt* during the mid of the 20th century reached 20-30% in eastern and Central Europe and many other countries including Australia, China and India (Leonard and Szabo, 2005). In SA, Soko et al. (2018) have reported that stem rust caused yield losses of 6.4% to 47.9% in resistant and susceptible varieties, respectively. The Ug99 group of races is virulent on many race-specific genes such as *Sr31*, *Sr24*, *Sr36* and *Sr38* that are prevalent in common wheat worldwide (Pretorius et al., 2000; Jin et al., 2007, 2009; Singh et al., 2008b, 2011a). Basnet et al. (2015) stated that one of the viable approaches to achieve durable stem

rust resistance is by combining multiple non-race specific genes that are mainly expressed in adult plants. Currently, at least five APR genes have been characterised and deployed against *Pgt* namely *Sr2* (McFadden, 1930), *Sr55* (Herrera-Foessel et al., 2014), *Sr56* (Bansal et al., 2014), *Sr57* (Lagudah et al., 2006, 2009; Krattinger et al., 2009) and *Sr58* (Singh et al., 2013; McIntosh et al., 2014). Of these, *Sr2* is the oldest and most widely characterised and utilised APR gene (Sunderwirth and Roelfs, 1980; Singh et al., 2008b). Though the use of APR genes singly may have small effects against rust diseases (Singh et al., 2008b), combinations of three to five APR genes/QTL are considered more effective and durable (Singh et al., 2000, 2011b). Enhanced stem rust resistance can also be attained by combining race-specific genes with APR genes (Singh et al., 2008a, 2011b). Most APR genes are non-race specific in nature, although examples of race-specific APR genes have been identified such as *Yr48* and *Yr49* (Bansal et al., 2008). Due to the limited number of previously characterised APR genes and given their significance in managing virulent stem rust races, it is therefore necessary to search for new APR genes (Bajgain et al., 2015) especially among cultivated wheat varieties. The wheat variety PAN 3161 has been observed to express APR to stem rust race PTKST. However, the genetic base of this resistance is unknown. Therefore, this study was conducted to analyse inheritance, map and develop closely linked markers for APR in the South African winter wheat variety PAN 3161.

5.3 Materials and methods

5.3.1 Plant material

Population development, field stem rust phenotyping and stem rust data collection were described in Chapter 3 (sections 3.3.1 and 3.3.4.3). Following stem rust phenotyping, rust-free leaf material was sampled from parents and 128 F_2 individuals and 21 F_3 families of the Line37-07/PAN3161 mapping population.

5.3.2 DNA extraction

Genomic DNA extractions were performed as described in Chapter 4 (section 4.3.5) using freeze-dried leaf material sampled from 128 F_2 individuals and 21 F_3 families. DNA was also isolated from the stem rust resistant control lines Kingbird (*Sr2*) (Mago et al., 2011), RL6077 (*Sr55*) (Herrera-Foessel et al., 2014) and Kariega (*Sr57*) (Lagudah et al., 2009). DNA concentrations and purity were determined using a spectrophotometer as described in Chapter 4 (section 4.3.5). Each DNA sample was diluted to a working concentration of 20 ng/µl. DNA quality was validated on a 0.8% (w/v) standard agarose gel run at 100 V for 15 min.

5.3.3 Polymerase chain reactions

All PCRs were performed as described in Chapter 4 (section 4.3.6). A complete list of background markers developed and published by different scientific groups such as *gdm*, *wmc*, *cfd*, *cfa*, *gdm*, *psp* and *wms* (https://.wheat.pw.usda.gov/GG3/) were tested on the parents of the mapping population Line37-07/PAN3161.

5.3.4 Polymerase chain reaction product visualisation

PCR products were visualised and analysed using the Gel Scan 3000 Real-Time DNA Fragment Analysis System (Corbett Research, Sydney, Australia) as described in Chapter 4 (section 4.3.6).

5.3.5 Screening of parental lines for presence of known adult plant resistance genes

PAN 3161 and Line 37-07 were screened using markers *gwm165*, *gwm192*, *cfd23* and *cfd71* closely linked to *Lr67/Yr46/Sr55* (Herrera-Foessel et al., 2011) and gene-specific markers *csSr2* linked to *Sr2* (Mago et al., 2011) and *Cssfr5* linked to *Lr34/Yr18/Sr57/Pm38/Ltn1* (Lagudah et al., 2009) for the possible presence of these genes in the parents.

5.3.6 Screening of parental lines to identify polymorphic markers and bulked segregant analysis

The parents were screened using the 104 LRGS marker set (Wessels and Prins, 2016) to identify polymorphic markers. To conduct BSA, two bulks of 10 resistant and 10 susceptible individuals each were formed, respectively. Including the resistant and the susceptible parental DNA bulks, a total of six DNA bulks were genotyped.

5.3.7 Construction of partial linkage map

The F_2 population (128 individuals) was genotyped using SSR markers identified in BSA to be possibly linked to the stem rust resistance gene/QTL. Data was scored and analysed as described in Chapter 4 (section 4.3.11). The linkage map of Line37-07/PAN3161 combined with stem rust phenotyping data were used to locate chromosome regions associated with race PTKST resistance.

5.3.8 Quantitative trait loci analysis and validation of identified linked stem rust resistance markers for marker-assisted selection

QTL analysis was done as described in Chapter 4 (section 4.3.12). SSR markers that were identified to be closely linked to stem rust resistance gene(s)/QTL in the current study were

validated by testing their robustness in predicting the phenotypes of F_3 plants as described in Chapter 4 (section 4.3.13). SSR markers that were identified to be closely linked to stem rust resistance gene(s)/QTL in the current study were validated by testing their robustness in predicting the phenotypes of F_3 plants as described in Chapter 4 (section 4.3.13).

5.4 Results

5.4.1 Genotyping of parental lines with *Sr2, Sr55*and *Sr57* markers

To postulate the presence of *Sr*2, the stem rust resistant parent PAN 3161, susceptible parent Line 37-07 and *Sr2* positive control (Kingbird) were tested with a diagnostic CAPS (cleaved amplified polymorphic sequence) marker csSr2. With initial amplification, PAN 3161 and Line 37-07 showed the null allele, while Kingbird showed the 337 bp allele linked to *Sr*2. The 337 bp allele is also carried by some genotypes that are false postives; hence to confirm the *Sr*2 status in Kingbird, PCR products were digested with the enzyme *Bsp*HI. Results indicated that Kingbird carries the resistant alleles of 172, 112 and 53 bp. PAN 3161 and Line 37-07 possessed the null allele from the initial amplification that is linked to susceptibility.

The two parents of the mapping population as well as the control line RL6077 were genotyped using molecular markers *cfd23*, *cfd71, gwm165* and *gwm192* reported to be linked to *Sr55* (Table 5.1). *Cfd23* amplified a different fragment in PAN 3161 compared to that of RL6077 and Line 37-07. SSR marker *cfd71* amplified a fragment in RL6077 similar to that of resistant parent PAN 3161 but different from that in susceptible parent Line 37-07. Similarly, *gwm165* and *gwm192* also detected different amplicons between the parents and the *Sr55* control line RL6077.

To assess the possible presence of the *Lr34/Yr18/Sr57/Pm38/Ltn1* locus, the parents and postive control (Kariega) were genotyped with the co-dominant diagnostic marker Cssfr5. The 751 bp fragment linked to the APR locus *Lr34/Yr18/Sr57/Pm38/Ltn1* was detected in PAN 3161 and Kariega. A 552 bp fragment indicating the absence of *Lr34/Yr18/Sr57/Pm38/Ltn1* was detected in the susceptible parent Line 37-07.

5.4.2 Identification of polymorphic markers

A total of 135 SSR markers were screened on both the resistant and the susceptible parent to identify polymorphic markers. Sixty-five SSR markers (Appendix D) showed polymorphisms between PAN 3161 and Line 37-07.

Table 5.1 Genotyping of parental lines with *Sr55* linked microsatellite markers

	Response of *Lr67/Yr46/Sr55* linked markers			
Entry	***cfd23***	***cfd71***	***gwm165***	***gwm192***
PAN 3161	-	+	-	-
Line 37-07	+	-	-	-
RL6077 (Control)	+	+	+	+

+ = Indicates the presence of the marker allele, - = Indicates the absence of the marker allele.

5.4.3 Identification of the adult plant stem rust resistance gene(s) chromosome location(s)

A total of 170 F_2 plants were phenotyped for stem rust responses of which leaf material of 112 resistant and 16 susceptible F_2 plants were sampled for genotyping. However, for BSA, two resistant and two susceptible DNA bulks were formed yielding four F_2 DNA bulks. Each F_2 DNA bulk consisted of between 8-10 individuals. In addition, a single DNA bulk (consisting of 10 individual plants) were also formed for each of the resistant and susceptible parents. The 65 SSR markers identified to be polymorphic between the parents of the Line37-07/PAN3161 mapping population were tested on resistant and susceptible bulks as well as the resistant and susceptible parents to detect linkage to resistance.

SSR markers known to map to the short arm of chromosome 4D showed linkage between parents and bulks, indicating possible association with stem rust resistance. From the initial 65 SSR markers identified as polymorphic among the parents, only SSR marker *psp3103* showed putative linkage to the stem rust resistance gene/QTL. To confirm this possible linkage, this chromosome region was screened with 13 additional SSR markers and a single PCR marker (*Rht-D1*). Fourteen SSR markers *psp3103*, *cfd23*, *cfd71*, *wmc52*, *wmc720*, *barc105*, *wms3000*, *wms4464*, *wms4726*, *wms4760*, *wms4866*, *gpw4315*, *gpw7414* and *gpw8038* showed consistent linkage with the APR locus (Table 5.2).

As expected for a segregating F_2 mapping population with a dominant resistant phenotype, the resistant and susceptible bulks contained both homozygous and heterozygous alleles. The possible resistance linkage on chromosome 4DS was confirmed by screening all individuals constituting the bulks with the 15 markers that showed possible linkage. All 15 markers showed consistent linkage to resistance when screened on all individuals constituting the bulks. Ultimately, these 15 markers were genotyped on all 128 individuals of the Line37-07/PAN3161 mapping population. Table 5.3 shows the sequences of some PCR and SSR markers identified

as linked to the stem rust resistance gene. The sequences of five SSR markers (*wms3000*, *wms4464*, *wms4726*, *wms4760* and *wms4866*) are proprietary and therefore not indicated in Table 5.3.

Table 5.2 Microsatellite markers linked to the adult plant stem rust resistance gene located on the short arm of chromosome 4D

Marker	Expected allele sizes (bp)	Locus	Ta	Inheritance
Rht-D1	254	4D	60	Dominant
psp3103	162	4D	64	Co-dominant
cfd23	187	4D	60	Co-dominant
cfd71	216	4A, 4D	60	Co-dominant
wmc52	192	1B, 4D	61	Co-dominant
wmc720	117	4D	60	Co-dominant
barc105	139	3A, 4D, 7A, 7D	50	Co-dominant
wms3000	188	4D	60	Co-dominant
wms4464	227	4D	55	Co-dominant
wms4726	131	4D	55	Co-dominant
wms4670	144	4D	60	Co-dominant
wms4866	176	4D	60	Co-dominant
gpw4315	150	4A, 4B, 4D	60	Co-dominant
gpw7414	222	4D	60	Co-dominant
gpw8038	150	3A, 4D	60	Co-dominant

bp = Base pairs, Ta = Annealing temperature.

5.4.4 Construction of partial linkage map for chromosome 4D

SSR markers that showed consistent linkage to resistance were genotyped on 128 F_2 individuals of the mapping population. Most of the SSR markers segregated according to a Mendelian ratio of 1:2:1 for co-dominant inheritance, except *cfd71*, *wms3000*, *wms4464* and *wms4726*. *Rht-D1b* segregated according to a 1:1 ratio for dominant gene inheritance (Table 5.4).

Table 5.3 Primer sequences of some microsatellite markers linked to an adult plant stem rust resistance gene on chromosome 4D

Marker	Forward sequence: 5'-3'	Reverse sequence: 5' - 3'
Rht-D1	CGCGCAATTATTGGCCAGAGATAG	CCCCATGGCCATCTCGAGCTGCTA
psp3103	CGGCTGGCATATTGGAATGTGC	GCTTCCGTGCTGTGGTTGGTC
cfd71	CAATAAGTAGGCCGGGACAA	TGTGCCAGTTGAGTTTGCTC
cfd23	TAGCAGTAGCAGCAGCAGGA	GCAAGGAAGAGTGTTCAGCC
wmc52	TCCAATCAATCAGGGAGGAGTA	GAACGCATCAAGGCATGAAGTA
wmc720	CACCATGGTTGGCAAGAGA	CTGGTGATACTGCCGTGACA
barc105	CAGGAAGAAAAGGAAAGCATGCGACAA	GCGGTGTGGCAATAATTACTTTTT
gpw4315	TCGGGGACAGAGGGTTCT	ACGTGCCAGGGAGGTATCTT
gpw7414	ACTGAACCAAGCAGCAGGAT	CTTCTCTCCTCACGTCCCTG
gpw8038	GTCAAGCCAAGCAATCTTTAGC	TCATACAAATTCAGCAAGGGC

A partial linkage map of chromosome 4D was generated covering 20.0 cM (Figure 5.1), where the *SrPan3161* gene was flanked distally and proximally by *wmc720* (1.8 cM) and co-segregating markers *wmc52, cfd23*, *psp3103*, *gpw8038* and *gpw7414* (1.8 cM), respectively. These flanking SSR markers were easy to score, for instance Wmc720 amplified a 100 and 150 bp fragment in the resistant parent PAN 3161 and the susceptible parent Line 37-07, respectively. SSR markers *psp3103, wmc52, wmc720, cfd23* and *cfd71* showed consistent linkage to resistance and have previously been mapped on the short arm of chromosome 4D hence confirming that *SrPan3161* is located on the short arm of chromosome 4D.

5.4.5 Quantitative trait locus analysis

The PAN 3161 QTL designated as *Qsr-ufs-4DS* had a LOD score of 34.91 and was flanked by SSR markers *wmc720* and *wmc52*. This QTL explained 71.54% of the phenotypic variation for resistance to race PTKST (Table 5.5). QTL analysis confirmed the position of the gene/QTL detected using linkage analysis.

Table 5.4 Segregation of microsatellite markers located on chromosome 4DS in the Line37-07/PAN3161 mapping population

Marker	Number of plants	AA	BB	AB	AB/AA	$\chi 2$
Rht-D1b	128	0	33	0	95	0.04
wmc720	128	40	28	60	0	2.75
barc105	128	36	32	60	0	0.75
gpw4315	128	39	31	58	0	2.13
gpw8038	128	43	28	57	0	5.05
gpw7414	128	43	28	57	0	5.05
psp3103	128	43	28	57	0	5.05
cfd23	128	43	28	55	0	5.05
wmc52	128	43	28	57	0	4.57
cfd71	128	44	29	55	0	6.05*
wms3000	128	44	30	54	0	6.19*
wms4464	128	44	28	56	0	6.00*
wms4670	128	43	27	60	0	5.13
wms4726	128	44	28	56	0	6.00*
wms4866	128	43	27	58	0	5.13

Co-dominant markers were scored as AA, AB and BB, Dominant marker were scored as BB, AB/BB, $\chi 2$ = Chi-square value and *$p \leq 0.05$.

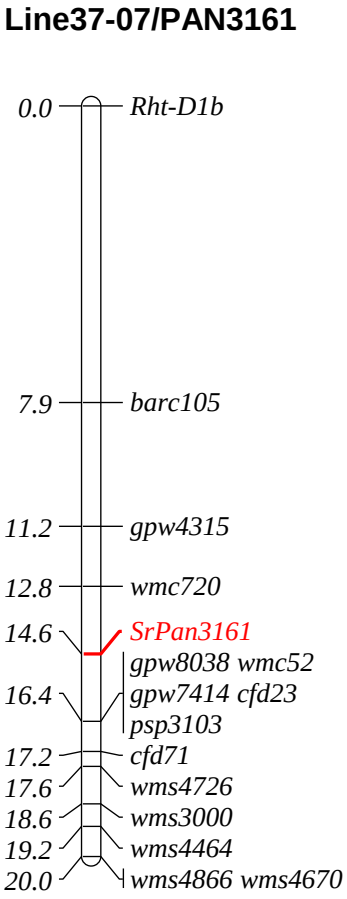

Figure 5.1 Partial genetic linkage map showing the position of the adult plant resistance gene *SrPan3161* on chromosome 4DS constructed using the Line37-07/PAN3161 F_2 mapping population. Marker names are indicated on the right and marker positions are shown on the left in centiMorgans (cM), SSR markers are indicated in black and the resistance gene in red

Table 5.5 Additive quantitative trait loci (QTL) for adult plant resistance to *Puccinia graminis* f. sp. *tritici* race PTKST detected by inclusive composite mapping

Resistant parent	QTL	Chr	Marker-interval	QTL range (cM)	LOD	PVE (%)	Add	Position
PAN 3161	*Qsr-ufs-4DS*	4D	*wmc720-wmc52*	2	34.91	71.54	-0.27	6.0

QTL = Named according to McIntosh et al. (2003), Chr = Chromosome, LOD = Logarithm of odds for peak of the given QTL, PVE (%) = Phenotypic variance explained by QTL, Add = Additive effects of QTL, the negative value means that the favourable allele was contributed by the resistant parent.

5.4.6 Validation of flanking markers for marker-assisted selection in the Line37-07/PAN3161 population

SSR markers that were closely linked to the stem rust resistance gene identified in the current study were validated for MAS by testing their robustness in predicting the phenotypes of F_2 plants using F_3 homozygous resistant and homozygous susceptible plants. The SSR markers flanking the *SrPan3161* gene/QTL were genotyped on 21 DNA bulks containing 10 homozygous F_3 adult plants each (Table 5.6). *Wmc720* had an accuracy of 87.5% in predicting resistant phenotypes. The other flanking markers *cfd23*, *wmc52* and *psp3103* had an accuracy of 81.3% in predicting resistant phenotypes. *Wmc720*, *cfd23*, *wmc52* and *psp3103* had an accuracy of between 20-60% in predicting susceptible phenotypes. Gene-specific marker *Cssfr5* was genotyped on all F_3 bulks to postulate the presence/absence of the *Lr34/Yr18/Sr57/Pm38/Ltn1* locus. The resistant and susceptible phenotypes tested 60% and 75% positive for presence of *Lr34/Yr18/Sr57/Pm38/Ltn1*, respectively.

5.5 Discussion

5.5.1 Genotyping parental lines for presence of known adult plant resistance genes

Only one (*cfd71*) of the four SSR markers reported to be linked to the *Lr67/Yr46/Sr55* locus amplified the same fragment in the resistant parent PAN 3161 and the positive control line RL6077. Analysis for the presence of *Sr2* also indicated the absence of this gene. Based on these results, it is likely that PAN 3161 does not carry either the *Lr67/Yr46/Sr55* or *Sr2* loci.

Table 5.6 Validation of microsatellite markers linked to the adult plant resistance gene identified initially in the Line37-07/PAN3161 F_2 population in F_3 families

		Marker data				
Phenotype	**Field score**	***Cssfr5***	***wmc720***	***cfd23***	***wmc52***	***psp3103***
Resistant	0R	+	+	+	+	+
Resistant	0-5MR	+	+	+	+	+
Resistant	10MS	+	-	-	-	-
Resistant	20MS	+	+	+	+	+
Resistant	5-10MR	+	+	+	+	+
Resistant	5MR	-	-	-	-	-
Resistant	20-30MS	+	+	+	+	+
Resistant	0-20MS	-	+	+	+	+
Resistant	0-20MS	+	+	+	+	+
Resistant	0-20MS	+	+	+	+	+
Resistant	20MS	+	+	+	+	+
Resistant	0-20MS	+	+	+	+	+
Resistant	0R	-	+	+	+	+
Resistant	0R	-	+	+	+	+
Resistant	0R	+	+	+	+	+
Resistant	Trace-20MS	+	+	-	-	-
Susceptible	60S	+	-	+	+	+
Susceptible	50S	-	+	+	+	+
Susceptible	40S	+	+	+	+	+
Susceptible	60S	-	-	+	+	+
Susceptible	40MSS	-	-	-	-	-

+ indicates the presence of marker alleles, - indicates the absence of marker allele, R = Resistant, MR = Moderately resistant, S = Susceptible, MS = Moderately susceptible, MSS = Moderately susceptible and susceptible.

Although PAN 3161 tested positive for the APR locus *Lr34/Yr18/Sr57/Pm38/Ltn1* using the gene-specific marker *Cssfr5*, the phenotypic response of PAN 3161 to race PTKST indicated the expression of a hypersensitive response observed in genotypes carrying major genes compared to the slow rusting response associated with APR genes like *Lr34/Yr18/Sr57/Pm38/Ltn1*. Results thus indicated that PAN 3161 should carry an additional stem rust resistance gene/QTL besides *Lr34/Yr18/Sr57/Pm38/Ltn1*.

Although gene-derived markers have increased the selection accuracy for certain traits, like durable stripe, leaf and stem rust resistance gene *Lr34/Yr18/Sr57/Pm38/Ltn1* (Lagudah et al., 2009), these markers also detect the *Lr34/Yr18/Sr57/Pm38/Ltn1* locus in some suscetible wheat lines (Lagudah et al., 2009). Although these are not perfect markers

as they are believed to be, they are much more accurate than previously used molecular markers. Hiebert et al. (2011) indicated that genotypes with a combination of *SrCad* and *Lr34* showed the lowest stem rust severities compared to those containing only one of these genes. They revealed that *Lr34*, although important, at best can provide basal resistance to novel stem rust races in some wheat varieties. Similarly, Vanegas et al. (2008) evaluated different Thatcher genotypes containing *Lr34* and reported that the presence of this locus enables expression of additional APR genes in segregating populations. However, wheat lines Terenzio, Chinese Spring and Sumai 3, all possessing *Lr34*, still showed susceptibility to stem rust (Vanegas et al., 2008; Hiebert et al., 2011).

5.5.2 Identification of polymorphic markers

Although 95 SSR markers spread across the entire chromosome 4D were tested for polymorphisms in the Line37/PAN3161 mapping population, low levels of polymorphism resulted in only 15 markers being mapped. These results are consistent with findings from other studies showing that the D-genome has the lowest level of polymorphism among all three wheat genomes (Chao et al., 2009; Allen et al., 2011). Similarly, Somers et al. (2004) stated that individual wheat mapping populations greatly vary in levels of polymorphism (20-40%), hence limiting the number of markers that can be added to a specific genetic map. Some of the tested SSR markers were polymorphic among the parents but due to their multi-locus nature were not linked to stem rust resistance. These markers could in the current study not be mapped to chromosome 4D. This could indicate that the identified polymorphic allele was actually mapping to a different locus other than chromosome 4D.

5.5.3 Bulked segregant analysis and molecular mapping of adult plant resistance gene(s)

BSA identified a resistance locus on the short arm of chromosome 4D in the wheat variety PAN 3161. However, BSA did not detect any linkage for stem rust resistance on chromosome 7DS, the map location for *Lr34/Yr18/Sr57/Pm38/Ltn1*, even when the gene-specific marker *Cssfr5* had detected its presence in PAN 3161. Similary, Zurn et al. (2018) also could not detect a significant QTL on chromosome 7DS in spring wheat land race PI 362698 that tested positive for *Lr34/Yr18/Sr57/Pm38/Ltn1* using marker *csLV34*. This scenerio possibly also happened in the current study because phenotyping did not target the *Lr34* phenotype per se. BSA mainly targets major genes and QTL and may have failed to detect slow-rusting QTL such as *Lr34/Yr18/Sr57/Pm38/Ltn1*. *Lr34* has been reported

to be effective against the original race Ug99 (Rouse et al., 2014). Stem rust field data captured over years against *Pgt* race PTKST in the Greytown rust nursery has shown that wheat varieties that carry *Lr34* generally show slower stem rust development combined with moderately susceptible to susceptible host reaction types.

5.5.4 Construction of a partial linkage map for chromosome 4DS

The adult plant stem rust resistance gene was mapped on the short arm of chromosome 4D using 14 co-dominant SSR and a single dominant SNP marker. The total mapped distance was 20.0 cM. Mapping results indicated that marker order and positions were similar and consistent with previously published wheat maps (Stephenson et al., 1998; Guyomarc'h et al., 2002; Somers et al., 2004; Sourdille et al., 2004; Ganal and Röder, 2007; Allen et al., 2011; Alaux et al., 2018). The two flanking markers *wmc720* and *wmc52* spanned a region of 3.6 cM. It is important to identify flanking markers for a QTL region or when a tightly linked marker or perfect marker is not available because recombination can happen between the marker and the gene(s). So ideally if both flanking markers are present there is a good chance that the gene(s)/QTL is still present.

So far, two stem rust resistance genes, *Sr41* and *Lr67/Yr46/Sr55*, have been identified on chromosome 4D. *Sr41* was detected using monosomic and cytological techniques in the spring wheat cultivar Waldron (Line WDR-B1) (Riede et al., 1995) while *Lr67/Yr46/Sr55* had been mapped using molecular markers (Herrera-Foessel et al., 2014). However, *Sr41* is not effective against the Ug99-lineage stem rust races such as PTKST (Pretorius et al., 2010; Prins et al., 2016). Furthermore, Waldron, containing *Sr41*, possesses ASR and APR to stem rust (Riede et al., 1995), unlike PAN 3161 that only expresses adult plant stem rust resistance. Hiebert et al. (2010) obtained a strong association of SSR markers *cfd23* and c*fd71* with the *Lr67* gene and suggested that these markers can be used for selection of the *Lr67/Yr46* locus. This locus, *Lr67*/*Yr46*, was later reported to carry the adult plant stem rust resistance gene, *Sr55* (Herrera-Foessel et al., 2014). The work of Hiebert et al. (2010) did not map the *Lr67/Yr46* locus but reported that SSR marker *cfd71* was closely associated and suggested that it was approximately 1.5 cM from the *Lr67/Yr46* locus. Other studies attempted to map *Lr67/Yr46* for instance, in the evaluation of Thatcher x RL6077 where SSR marker *cfd71* mapped 6.0 cM distally to the *Lr67/Yr46* locus (Forrest et al., 2014). Similarly, Herrera-Foessel et al. (2011) found that SSR marker *cfd71* mapped

5.0 cM distally from the *Lr*67/*Yr*46 locus using an Avocet x RL6077 mapping population. Liu et al. (2014) mapped SSR marker *cfd71* 5.5 cM distally from *Lr67/Yr46*.

In the present study, *cfd71* mapped 2.6 cM proximally to *SrPan3161*, suggesting that *SrPan3161* is different from the *Lr67/Yr46/Sr55* locus. Herrera-Foessel et al. (2011) reported two closely linked co-segregating markers *gwm165* and *gwm192* that mapped 0.4 cM distally from *Lr67/Yr46*. Similarly, Liu et al. (2014) mapped *gwm165* 0.5 cM distally to the *Lr67/Yr46* locus. A comparison between the present map and the map developed by Herrera-Foessel et al. (2011) suggests that *gwm165* and *gwm192* would map proximally to the identified APR. Given the map location of *cfd71* and mapped APR region, it is apparent that PAN 3161 does not posseses the *Lr67/Yr46/Sr55* locus but carries a novel gene. This confirms results obtained from screening PAN 3161 using four SSR markers reported to be closely linked to *Lr67/Yr46/Sr55* that indicated the absence of *Lr67/Yr46/Sr55* in PAN 3161.

Recently Moore et al. (2015) cloned the *Lr67* gene and showed that it encodes a predicted hexose transporter protein variant that confers resistance through inhibiting hexose up-take in host cells. Moore et al. (2015) developed and validated gene-specific SNP markers *SNP1-TM4* and *SNP2-TM10* for MAS of *Lr67*. These markers should be tested in future to cofirm the presence/absence of *Sr55* in the current mapping population.

Screening of a bread wheat nursery containing 340 entries of the International Center for Maize and Wheat Improvement (CIMMYT) with gene-specific SNP markers showed that all screened CIMMYT wheat lines did not carry the *Lr67* resistance allele. Interestingly, the *Lr67* resistance allele was found in older and tall wheat varieties such as Yaqui 53, Chapingo 48 and Chapingo 53 cultivated before the development of semi-dwarf wheat lines (Moore et al., 2015). As no yield drag has been reported in wheat varieties carrying the *Lr67* resistance allele (Hiebert et al., 2010), Moore et al. (2015) suggested that the rigorous selection for the semi-dwarf gene *Rht-D1b* has simultaneously led to the fixation of the *Lr67* susceptible alleles in modern CIMMYT wheat lines. Because *Lr67* has been cloned, Moore et al. (2015) recommended the use of *Lr67* diagnostic markers to develop modern wheat lines containing both *Lr67* and *Rht-D1b* genes.

Marker *Rht-D1b* mapped distally at 14.6 cM from the APR gene identified in the present study. It is possible that selection for the *Rht-D1b* gene in PAN 3161 could also have led to the fixation of *Lr67* susceptible alleles. *Rht-D1b* is a perfect dominant SNP marker for the plant height dwarfing gene (*Rht2*) that accounts for 44% of plant height reduction (Ellis et al., 2002). *Rht2* was initially mapped on chromosome 4D, 28.0 cM distally from SSR marker *gwm165* (Börner et al., 1997). A comparison between the linkage map of Börner et al. (1997) and the present map suggests that the APR gene mapped distally at 13.4 cM from the *Lr67/Yr46/Sr55* locus, further evidence that the mapped APR gene is different from *Lr67/Yr46/Sr55*. Semi-dwarfing genes have been used worldwide in many wheat breeding programmes since the onset of the Green Revolution (Liu et al., 2014). Apparently, over 90% of the worlds' wheat varieties possess these semi-dwarfing genes *Rht-B1b* (*Rht1*) and *Rht-D1b* (*Rht2*) (Worland et al., 1998). The presence of *Rht2* in PAN 3161 makes it a valuable genetic resource that can be used for crosses with plants lacking *Rht2* and that its selection can easily be tracked using the perfect marker *Rht-D1b*.

5.5.5 Quantitative trait locus analysis and validation of identified closely linked markers for marker-assisted selection

QTL analysis of the Line37-07/PAN3161 population identified a single major effect QTL designated as *Qsr-ufs-4DS* with a LOD score of 34.9. This QTL confers a high level of resistance as it explained 71.5% of the phenotypic variation for stem rust resistance to race PTKST. QTL mapping indicated that the APR QTL *Qsr-ufs-4Ds* is flanked by SSR markers *wmc720* and *wmc52*, 2 cM apart hence could be useful in MAS. This QTL spanned a distance of 2 cM quite similar to 3.6 cM around *SrPan3161* as identified through linkage mapping. These markers flanking the QTL were tested for their effectiveness in predicting the phenotypes of F_3 families derived from the Line37-07/PAN3161 mapping population. *Wmc720, wmc52* and *cfd23* had prediction accuracies of between 81.3% and 87.5% for the phenotypes of F_3 families. A number of false positives and negatives were detected among the tested F_3 families. This was expected given that these markers are closely linked to resistance gene/QTL and are therefore not diagnostic for the phenotypes. Besides, phenotying of the F_3 families was quite difficult due to variation in growth stages between and within families, and the presence of *Lr34/Yr18/Sr57/Pm38/Ltn1* known for partial expression. Relatively closely linked molecular markers such as *wmc720, wmc52* and *cfd23* could be effective for selection and deployment of the *SrPan316* gene. It is important to note that SSR marker *wmc52* co-segregated with *cfd23, psp3103, gpw8038*

and *gpw7414*. Therefore, this set of co-segregating SSR markers can be useful in MAS of *SrPan3161*.

5.6 Conclusions and recommendations

BSA successfully identified the short arm of chromosome 4D as linked to resistance against the Ug99-lineage *Pgt* race, PTKST. Both linkage and QTL mapping were effective in the identification of SSR markers *wmc720* and *wmc52* as flanking the resistance gene designated as *SrPan3161* in the South African winter wheat variety PAN 3161. *Wmc720* and *wmc52* were predictive of resistance in various homozygous Line37-07/PAN3161 F_3 plants, thus validating the chromosome location of the resistance gene and their effectiveness for MAS. In addition, this study indicated that PAN 3161 carries the plant height dwarfing gene (*Rht2*) and APR gene *Lr34/Yr18/Sr57/Pm38/Ltn1*. PAN 3161 should thus be a useful genetic stock that can be exploited in further crosses to combine these three genes and other useful traits in elite breeding materials. The long arm of chromosome 4D of wheat has been reported to carry the non-hypersensitive *Lr67/Yr46/Sr55* locus that was absent in PAN 3161. Because no other stem rust resistance gene has been mapped to the short arm of chromosome 4D, it is likely that the *Sr* gene mapped here is a novel adult plant stem rust resistance gene. Future work should include studies on the expression of this gene at different growth stages. The seedling IT of 3 for PAN 3161 did not allow for mapping in the seedling stage. Although the resistance gene in PAN 3161 has been found effective in the field to other South African stem rust races including TTKSF, TTKSF+ and TTKSP (data not presented), further testing against a wider range of stem rust races is required to determine whether it confers race-specific resistance.

Mapping in the current study was done using an F_2 population that was only phenotyped in one season and location. This population should be advanced to a RIL population to facilitate the evaluation of the robustness of this APR gene, *SrPan361,* across different locations and seasons. Mapping in a RIL will enable the addition of molecular markers to the current map creating a high-density map and hence further aid the identification of closely linked or co-segregating markers. The availability of a RIL population will also lead to improved phenotyping data (over seasons and locations) as several plants of each RIL will be tested that will improve the QTL mapping step. To achieve this, the RIL mapping population of Line37-07/PAN3161 should be genotyped using high-density genotyping platforms such as DArTs or 90K SNP chip.

South African wheat varieties and breeding lines should be assessed using molecular markers *wmc720*, *wmc52*, *cfd23*, *psp3103*, *gpw7414* and *gpw8038* identified in the current study as closely linked to stem rust resistance, to postulate the local prevalence of *SrPan3161*. Wheat germplasm from other regions of the world can also be tested for the prevalence of this resistance gene. This is vital to generate a critical source of information that will ultimately facilitate the sharing of resistant South African winter wheat varieties with other researchers and breeding institutions. The stem rust resistant parent PAN 3161 possesses APR to the most virulent Ug99-lineage stem rust race in SA, PTKST. This highlights the importance of the mapped resistance gene in protecting wheat against virulent stem rust races. PAN 3161 can also be tested with virulent stem rust races from other regions of the world to assess the broad effectiveness of this resistance gene or to possibly identify other new gene(s).

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CHAPTER 6

GENERAL CONCLUSIONS AND RECOMMENDATIONS

SA is one of the major producers of wheat in sub-Saharan Africa. However, the country remains a net importer of wheat. The productivity of wheat in SA is constrained by both biotic and abiotic factors. Among the biotic factors, stem rust is of great significance adding to production risk and input cost when chemical control is required on susceptible varieties. The risk of stem rust outbreaks is further compounded by the emergence of new, more virulent stem rust races such as Ug99 and its lineages. Therefore, it is vital to characterise existing and new sources of stem rust resistance so as to develop molecular markers that can effectively and efficiently be used in MAS to manage stem rust genetically.

Stem rust inheritance in this study indicated that a single dominant gene confers ASR to *Pgt* race PTKST in each of the four winter wheat varieties Komati, Koonap, Limpopo and SST 387. Allelism tests among these resistant parents indicated that it is the same or closely linked genes or alleles of the same gene in all four varieties. The test varieties are only protected by a single gene/QTL and thus at risk of pathogen adaptation and resistance breakdown. Therefore, to broaden the resistance base in South African winter wheat varieties, new sources of resistance should be characterised and combined with identified resistance gene(s). Allelism tests between the four resistant varieties Komati, Koonap, Limpopo and SST 387 and two controls Norin 40 (*Sr42*) and CnsSrTmp (*SrTmp*) indicated that the test varieties possess either novel gene(s) or very closely linked alleles to *Sr42* and *SrTmp*. However, the allelism and multi-race phenotyping results rule out the involvement of either *Sr42* or *SrCad* and seem to suggest the likely presence of *SrTmp* in South African winter wheat varieties.

To map the resistance gene identified during stem rust inheritance work, BSA combined with SSR markers identified chromosome 6DS as the region carrying the ASR stem rust resistance gene. This information was confirmed by the successful mapping of SNP markers on chromosome 6DS. SSR markers were more closely linked to the stem rust resistance gene than SNP markers hence in this study SSR markers were more effective in identifying the mapped stem rust resistance gene(s) than SNP markers. SSR and SNP

markers were sparsely spaced in the four linkage maps. This was expected given the reported low levels of polymorphism recorded for the D-genome compared to both the A- and B-genomes. Therefore, to further saturate the chromosome 6DS linkage maps developed in the current study, these mapping populations should be genotyped using high-marker density SNP platforms such as 90K chip and/or DArTs to identify closely linked or co-segregating markers. As the molecular markers identified in the current study resulted from linkage and QTL mapping using F_2 populations, these populations should be advanced to develop recombinant inbred line (RILs) to facilitate the evaluation of effectiveness of resistance gene(s) across various locations.

South African wheat winter varieties and breeding lines should be assessed with molecular markers *psp3200*, *wms4528*, *barc183* and *wms4862* identified in the current study as closely linked to stem rust resistance gene(s) to postulate its local prevalence. Wheat germplasm from other regions of the world can also be tested for prevalence of these stem rust resistance gene(s). This is vital to generate a critical source of information that will ultimately facilitate sharing of these resistant South African winter wheat varieties with other researchers and breeding institutions. South African winter wheat varieties tested negative to diagnostic SNP markers linked to known chromosome 6DS stem rust resistance genes *Sr42, SrTmp* and *SrCad* implying the mapped stem rust resistance gene(s) is novel or a closely linked allele of *Sr42, SrTmp* and/or *SrCad*. Evidence from allelism tests, multi-race analysis and marker analysis data suggests the absence of *Sr42* and *SrCad* in Komati, Koonap, Limpopo and SST 387. The most likely source of stem rust resistance in these varieties is therefore *SrTmp*. The single resistance gene theory is further supported by the narrow genetic pool from which South African winter wheats have been developed. Future work to resolve the exact identity of the resistance gene involved may include more extensive allelism tests for instance with larger F_2 populations and phenotyping with multiple pathogen races with different virulence profiles or ultimately cloning of the gene. This is necessary to plan and deploy different gene combinations aimed at protecting the South African wheat varieties from resistance breakdown resulting from deployment of a single or a few major stem rust resistance gene(s).

Results from screening the Line37-07/PAN3161 F_2 population indicated the presence of a single APR gene that was contradicted by results obtained for the 56 F_3 families that indicated the presence of two APR genes. In future studies the population size for the field

stem rust resistance work should be increased to include a minimum of 100 F_3 families to improve the validity of inheritance work. To further aid the characterisation of the ASR and APR sources identified in this study, the F_2 populations should be advanced to develop RIL populations that will result in effective and efficient inheritance studies across different seasons and locations.

The South African winter wheat variety PAN 3161 expressed near-immune stem rust response during field trials. Therefore, to characterise this APR gene, BSA in combination with SSR markers were utilised and this process successfully identified the short arm of chromosome 4D as linked to resistance against the Ug99-lineage *Pgt* race, PTKST. Both linkage and QTL mapping were effective in the identification of SSR markers *wmc720* and *wmc52* as flanking the resistance gene designated as *SrPan3161* in the South African winter wheat variety PAN3161. *Wmc720* and *wmc52* were predictive of resistance in various homozygous Line37-07/PAN3161 F_3 families, thus validating the chromosome location of the resistance gene and their effectiveness for MAS. In addition, this study indicated that PAN 3161 carries the plant height dwarfing gene (*Rht2*) and APR gene *Lr34/Yr18/Sr57/Pm38/Ltn1*, indicating that PAN 3161 should be a useful genetic stock that can be exploited in further crosses to combine these three genes and other useful traits in elite breeding materials.

The long arm of chromosome 4D of wheat has been reported to carry the non-hypersensitive *Lr67/Yr46/Sr55* locus. This locus was absent in PAN 3161 because reported closely linked markers amplified different fragment sizes in PAN 3161 and the positive control line RL6077. Because no other stem rust resistance gene has been mapped to the short arm of chromosome 4D, it is likely that the *Sr* gene mapped here is a novel adult plant stem rust resistance gene. Mapping in the current study was done using an F_2 population. This population should be advanced to a RIL population to facilitate the evaluation of the robustness of this APR gene, *SrPan361,* across different locations and seasons. Mapping in a RIL will facilitate the addition of molecular markers to the current map creating a high-density map and hence further aid the identification of closely linked or co-segregating markers. The availability of a RIL population will also lead to improved phenotyping data (over seasons and locations) that will improve the QTL mapping step. To achieve this, the RIL mapping population of Line37-07/PAN3161 should be genotyped using high-density genotyping platforms such as DArTs or 90K SNP chip.

South African wheat varieties and breeding lines should be assessed using molecular markers *wmc720*, *wmc52*, *cfd23*, *psp3103*, *gpw7414* and *gpw8038* identified in the current study as closely linked to stem rust resistance, to postulate the local prevalence of *SrPan3161*. Wheat germplasm from other regions of the world can also be tested for the prevalence of this resistance gene. This is vital to generate a critical source of information that will ultimately facilitate the sharing of resistant South African winter wheat varieties with other researchers and breeding institutions. The stem rust resistant parent PAN 3161 possesses APR to theUg99-lineage stem rust race, PTKST in SA. This highlights the importance of the mapped resistance gene in protecting wheat against virulent stem rust races. PAN 3161 can also be tested with virulent stem rust races from other regions of the world to assess the broad effectiveness of this resistance gene or to possibly identify other new gene(s).

Appendix A *Puccinia graminis* f. sp. *tritici* infection types for all 200 F_2 seedlings inoculated with stem rust race PTKST

Entry	Koonap	Komati	Limpopo	SST 387
1	3++	3++	3c	3+
2	3++	3++	3c	3+
3	3++	3+	3+c	3+
4	3++	3++	3+	3+
5	3++	3+	3+c	3+
6	3++	3+	3+c	3+
7	3++	3+	3+	3
8	3++	3+	3+c	3
9	3++	3++	3	3
10	3++	3+	3c	3
11	3++	3+	3	3
12	3++	3+	3+c	3
13	3++	3+	3c	3
14	3++	3+	3c	3
15	3++	3+	3c	3+
16	3++	3+	3+	3+
17	3++	3+	3+	3+
18	3++	3+	3+c	3
19	3++	3+	3c	3+
20	3++	3+	3c	3
21	3++	3+	3c	3
22	3++	3+	3+c	3+
23	3+	3++	3	3+
24	3++	3++	3	3+
25	3++	3+	3c	3+
26	3++	3+	3c	3+
27	3++	3+	3+	3+
28	3++	3+	3+c	3+
29	3++	3+	3c	3
30	3++	3++	3	3
31	3++	3++	3c	3
32	3++	3+	3c	3
33	3+	3+	3	3
34	3++	3+	3	3
35	3++	3+	3+	3
36	3++	3+	3+c	3
37	3++	3++	3c	3+
38	3++	3++	3	3
39	3++	3+	3+	3
40	3++	3+	3	3
41	3++	3+	3+c	3
42	3++	3++	3c	3+

Entry	Koonap	Komati	Limpopo	SST 387
43	3++	3+	3+c	3+
44	3++	3+	3	3+
45	3+	3++	3	3+
46	3++	3+	3	3+
47	3+	3+	3c	3+
48	3++	3+	3+	3+
49	3++	3++	3c	3
50	3++	3++	3c	3+
51	;12	;1	;;1	;1
52	;12	12	;1-	;1
53	12	12	;1+	;1
54	12	;1	;1	;1+
55	12	12	;;1	;1-
56	;12	;1	;1	;1-
57	12	12	;1-	;1-
58	12	;12	;1	;1-
59	12	12	;1+	;1
60	12-	;1+	;1	;1
61	;1	12	;1	;1+
62	;1+	;1	;1	;1-
63	;1+	;1+	;1	;1+
64	;1	;1	;1	;1
65	;12-	12	;1	;1
66	12	;1	;1+	;1=
67	12	12	;1	;1
68	;1+	;1+	;1	;1
69	12	;1	;1	;1+
70	;12	;1	;1+	;1
71	;1+	;1	;1	;1+
72	;1+	;1+	;1+	;1=
73	;12	;1	;1	;1
74	;12	12	;1	;1=
75	;1+	;1	;1-	;1=
76	;1	;1+	;1	;1-
77	;12	12	;1	;1=
78	;12	12	;1+	;1-
79	;12	12	;;1	;1+
80	;12	12	;1	;1
81	;1+	;1	;1	;1
82	;1+	;1	;1	;1+
83	;1+	;1	;;1	;1
84	;1	;1	;;1	;1
85	;1	;12	;1+	;1
86	;1+	;12	;1+	;1-
87	;12	;1	;12	;1

Entry	Koonap	Komati	Limpopo	SST 387
88	12	;12	;12	;1-
89	;1+	;1	;1+	;1+
90	12+	;1	;1	;1+
91	12	12	;1	;1
92	12	;1	;;1	;1
93	12	;1+	;1	;1-
94	;1+	;1+	;1	;1-
95	12	;1	;1	;1-
96	12	;1	;1+	;1
97	12	;1	;1-	;1
98	1	;1	;1	;1=
99	12-	;1	;1+	;1+
100	12	;1	;12	;1
101	;1	;1	;1-	;1
102	1+	;1	;1	;1
103	12	;1	;1	;1
104	12	;12	;;1	;1=
105	12	;1+	;;1	;1-
106	12	;1	;1	;1
107	;12	;1	;1-	;;1
108	12	12	;1-	;1
109	12	;1	;1+	;1
110	12	;12	;1	;1
111	12	;1	;1+	;1
112	;12	;1	;1	;1
113	;12	1+	;1	;;1
114	;1	;1	;1+	;1
115	;12	;12	;1-	;1
116	12	;1	;1-	;1
117	12	;1+	;1-	;;1
118	;1	;1	;1	;1
119	12	;12	;1+	;1
120	12	;1	;1+	;1
121	;1+	;1+	;1	;1+
122	;12	;1+	;1	;1
123	;1	;1	;1	;1
124	;1	;12	;;1	;1-
125	12	;1	;;1	;1-
126	12	;1	;12	;1-
127	;12	;12	;1	;1=
128	;1	;1	;1	;1=
129	;1+	;1	;12	;1-
130	;12	;1-	;1	;1
131	;1+	;1	;1+	;1-
132	;1+	;1	;1-	;1+

Entry	Koonap	Komati	Limpopo	SST 387
133	;12	12	;;1	;1=
134	;1	1	;1+	;1
135	;1+	;12	;1+	;1
136	;1+	;1	;1	;1
137	;12	;1	;1+	;;1
138	;1	12	;1+	;1
139	;1	;12	;1	;1+
140	12	;1	;;1	;1
141	1+	;1	;12	;1
142	;1	;1	;1+	;1
143	;1	;1	;1	;1
144	;1+	12	;1	;1
145	;12	12	;12	;1
146	;12	12	;1	;1
147	12	;1-	;1	;1
148	12	12	;1	;1
149	;12	1+	;1+	;1+
150	;12	;12	;1+	;1
151	12	12	;1	;1
152	;1	2	;1	;1
153	12	;1	;;1	;;1
154	;1	;1	;1+	;1+
155	;1+	;1	;1	;1
156	;1+	;1	;;1	;;1
157	;1+	12	;1	;1
158	1+	;12	1	;1+
159	;1	12	;1	;1+
160	;1+	;1	;1	;1
161	;1	1	;;1	;1
162	12	12	;1	;1+
163	;1	;1	;1	;1
164	1	;1	1	;1
165	;1	12	1+	;1-
166	1	;1	;1	;1
167	1+	;1	;12	;1
168	1	12	;12	;1
169	;1	;1+	;12	;1
170	12	;1	;1+	;1
171	;1	;1	;1+	;1
172	1	1	;1+	;1
173	;1	1++	;12	;1+
174	;1	;1	;1-	;;1
175	;12	;1	;12	;;1
176	12	1	;1+	;1
177	;1	;12	1+	;;1

Entry	Koonap	Komati	Limpopo	SST 387
178	;1	;1	;12	;;1
179	12	;1	;12	1
180	;12	12	12	;1+
181	;12	;1	;1	;1+
182	12	12	;1	;1
183	12	;1	;1	;1
184	12	12	;1	;1
185	;12	12	;1	;1
186	12	12	12	;1
187	12	12	;1	;1
188	;12	;1+	12	;1+
189	;12	12	;1+	;1
190	;1	12	1+	;;1
191	;1	;12	;1+	;;1
192	;1	;1+	;1	;1
193	;1	;1	;12	;;1
194	;1	12	1+	;1
195	;1	;1	;1	;1
196	;12	12	;1+	;1
197	;12	12	;1+	;1
198	;1	;12	1+	;;1
199	12	12	;1	;1
200	;1	;1	;1	;;1

Stem rust seedling infection types are according to Stakman et al. (1962).

Appendix B ***Puccinia graminis*** **f. sp.** ***tritici*** **seedling infection types (ITs) of individual F_2 plants used to constitute bulks for Koonap, Komati, Limpopo and SST 387**

Entry	Koonap		Komati		Limpopo		SST 387	
	Bulk 1 RR	**IT**	**Bulk 1 RR**	**IT**	**Bulk 1 RR**	**IT**	**Bulk 1 RR**	**IT**
1	61	;1	51	;1	51	;;1	66	;1=
2	76	;1	69	;1	57	;1-	77	;1=
3	84	;1	82	;1	83	;;1	98	;1=
4	85	;1	87	;1	84	;;1	104	;1=
5	118	;1	92	;1	92	;;1	107	;;1
6	124	;1	100	;1	101	;1-	113	;;1
7	128	;1	103	;1	104	;;1	117	;;1
8	134	;1	109	;1	105	;;1	127	;1=
9	142	;1	120	;1	108	;1-	137	;;1
10	152	;1	125	;1	115	;1-	153	;;1
Entry	**Bulk 2 RR**	**IT**	**Bulk 2 RR**	**IT**	**Bulk 2 RR**	**IT**	**Bulk 2 RR**	**IT**
1	154	;1	130	;1-	116	;1-	156	;;1
2	159	;1	131	;1	124	;;1	174	;;1
3	165	;1	141	;1	125	;;1	175	;;1
4	171	;1	147	;1-	132	;1-	177	;;1
5	173	;1	155	;1	133	;;1	178	;;1
6	174	;1	163	;1	140	;;1	190	;;1
7	190	;1	174	;1	153	;;1	191	;;1
8	192	;1	179	;1	156	;;1	193	;;1
9	194	;1	193	;1	161	;;1	198	;;1
10	200	;1	200	;1	174	;1-	200	;;1
Entry	**Bulk 1 SS**	**IT**	**Bulk 1 SS**	**IT**	**Bulk 1 SS**	**IT**	**Bulk 1 SS**	**IT**
1	1	3++	1	3++	3	3+c	2	3+
2	4	3++	2	3++	4	3+	3	3+
3	7	3++	4	3++	5	3+c	5	3+
4	10	3++	6	3+	6	3+c	6	3+
5	14	3++	9	3++	7	3+	15	3+
6	16	3++	11	3+	8	3+c	16	3+
7	18	3++	16	3+	12	3+c	17	3+
8	19	3++	20	3+	16	3+	22	3+
9	22	3++	23	3++	17	3+	23	3+
10	25	3++	24	3++	18	3+c	25	3+
Entry	**Bulk 2 SS**	**IT**	**Bulk 2 SS**	**IT**	**Bulk 2 SS**	**IT**	**Bulk 2 SS**	**IT**
1	28	3++	25	3+	22	3+c	26	3+
2	31	3++	30	3++	27	3+	27	3+
3	32	3++	31	3++	28	3+c	28	3+
4	35	3++	33	3+	31	3c	37	3+
5	38	3++	37	3++	35	3+	42	3+
6	41	3++	38	3++	36	3+c	43	3+
7	44	3++	42	3++	39	3+	46	3+
8	46	3++	45	3++	41	3+c	47	3+
9	49	3++	49	3++	43	3+c	48	3+
10	50	3++	50	3++	48	3+	50	3+

Stem rust seedling infection types are according to Stakman et al. (1962). Bulk 1 = First bulk population, Bulk 2 = Second bulk population, RR = resistant seedlings and S = susceptible seedlings.

Appendix C Microsatellite markers used for chromosome 6D bulked segregant analysis in Line37-07/Koonap, Line37-07/Komati, Line37-07/Limpopo and SST387/Line37-07 F_2 populations

Entry	6D markers	Locus	Ta
1	*cfd49*	6D	60
2	*barc173*	3B, 6D	50
3	*cfd47*	5A, 6B, 6D	60
4	*gwm469*	1B, 5D, 6D	64
5	*cfd13*	6B, 6D	60
6	*barc196*	6D	50
7	*cfd37*	5D, 6D	60
8	*gdm98*	6D	60
9	*cfd38*	6D	60
10	*gdm141*	6D	60
11	*barc202*	6D	52
12	*cfd5*	5B, 6D	60
13	*cfd45*	6D	60
14	*barc123*	6D, 7B	52
15	*cfd60*	5B, 6D	60
16	*cfd15*	1A, 1D	60
17	*barc21*	5B, 6D	52
18	*cfd219*	3B, 5B, 6D	60
19	*cfd19*	ID, 5D, 6D	60
20	*gwm133*	IB, 3A, 4D, 5B, 6B, 6D, 7D	60
21	*cfd287*	6D	60
22	*barc183*	2B, 6D	58
23	*cfd33*	6D	60
24	*gwm132*	6D	51
25	*gdm132*	6D	60
26	*gpw1034*	6D	60
27	*cfd1*	6A, 6B, 6D	60
28	*wmc749*	6D	60
29	*cfd135*	6D	50
30	*psp3200*	6D	58
31	*cfd75*	6D	60
32	*wms1662*	6D	55
33	*wms4528*	6B, 6D	60
34	*wms4862*	6D	60
35	*wms4451*	6D	60
36	*wms1391*	6D	60
37	*wms904*	6D	60

Ta = Annealing temperature.

Appendix D Microsatellite markers used for chromosome 4D bulked segregant analysis in the Line37-07/Pan3161 F_2 population

Entry	Marker	Locus	Ta
1	*wmc52*	IB, 4D	61
2	*psp3103*	4D	64
3	*cd23*	4D	60
4	*cfd71*	4A, 4D	60
5	*wmc285*	4D	58
6	*wmc48*	4A, 4B, 4D	61
7	*wmc617*	4A, 4B, 4D	61
8	*wmc419*	1B, 4B, 4D, 6B	64
9	*cfd39*	4B, 4D, 5A	60
10	*gwm133*	1B, 3A, 4D, 5B, 6B, 6D, 7B	60
11	*gwm165*	4A, 4B, 4D	60
12	*gwm251*	4B, 4D	60
13	*barc225*	4D	51
14	*barc98*	2B, 4D	51
15	*Rht-D1b*	4D	60
16	*Rht-D1a*	4D	60
17	*gpw2220*	4D	60
18	*gpw2046*	IA, 2A, 4D	60
19	*gpw2066*	1A, 4D	60
20	*gpw2180*	1A, 4D	60
21	*wmc89*	4A, 4B, 4D	61
22	*cfd106*	4D	60
23	*gpw1209*	4D, 7B	60
24	*gpw2271*	4B, 4D	60
25	*gpw336*	4D	60
26	*psp007*	4D	50
27	*psp3112*	3B, 4D, 6B	61
28	*cfd81*	4D, 5D, 7B	60
29	*wmc720*	4D	60
30	*gdm40*	4D	60
31	*barc334*	4D	57
32	*wmc331*	4D	61
33	*gwm608*	2D, 4D	60
34	*wmc818*	1A, 1B, 4D	61
35	*gdm129*	4D	60
36	*barc217*	4D	52
37	*barc1118*	4D	52
38	*cfd160*	2D, 4D	60
39	*gdm61*	4D	60
40	*wmc489*	4D	51
41	*cfd193*	2D, 3A, 3D, 4D, 7A, 7D	60
42	*gwm264b*	1B, 3B, 4D	60
43	*gwm293*	4D, 5A, 5B, 7B	55

Entry	Marker	Locus	Ta
44	*wmc182*	3B, 4D, 6B, 6A, 7A, 7D	51
45	*wmc206*	1B, 3B, 4B, 4D, 5D, 6A	61
46	*wmc399*	4D, 7B	51
47	*cfd84*	4D	60
48	*gwm194*	4D	50
49	*gwm624*	4D	50
50	*cfd39b*	4B, 4D, 5A	60
51	*gwm6*	4B, 4D, 5A	55
52	*gwm149*	4B, 4D	55
53	*gwm538*	4B, 4D	55
54	*gwm192*	4B, 4D	60
55	*gdm120*	3B, 4D	63
56	*wmc497a*	4A, 4D, 7A	61
57	*wmc473a*	4D, 6B, 7D	61
58	*gwm213*	4D, 5B	60
59	*wmc457*	4A, 4D	61
60	*gwm193*	4D, 6B, 7D	60
61	*barcM118*	4D, 6A	52
62	*barc105*	3A, 4D, 7A, 7D	50
63	*barc93*	4D, 5D	60
64	*wsr11*	4D	57
65	*wsr17*	4D	57
66	*wsr65*	4D	57
67	*cl 167*	4D	63
68	*stsj-15*	4D	47
69	*bg263898*	4D	53
70	*gdm40*	4D	60
71	*wms3000*	4D	60
72	*wms4726*	4D	55
73	*wms4346*	4D	60
74	*wms4693*	4D	60
75	*wms4901*	2D, 4B, 4D	60
76	*wms4464*	4D	55
77	*wms4670*	4D	60
78	*wms4555*	4D	60
79	*wms4866*	4D	60
80	*wms4190*	4D	60
81	*wms819*	4D	50
82	*wms888*	4B, 4D	60
83	*gpw4248*	4D	60
84	*wmc574*	2D, 4D, 5D	61
85	*gpw4189*	4D	60
86	*gpw4315*	4A, 4B, 4D	60
87	*gpw7196*	4D	60
88	*gpw8038*	3A, 4D	60

Entry	Marker	Locus	Ta
89	*barc91*	2A, 2B, 4D	50
90	*gpw5072*	4D	60
91	*gpw5062*	2B, 4D	60
92	*gpw7666*	4D	60
93	*gpw5185*	4D	60
94	*gpw4040*	4A, 4D	60
95	*gpw7414*	4D	60

Ta = Annealing temperature.