# Characterising and mapping a potential new source of wheat stem rust resistance

by

**Kholosa Maqolo**

Dissertation submitted in fulfilment of the requirements in respect of the Master's degree in Plant Breeding in the Department of Plant Sciences in the Faculty of Natural and Agricultural Sciences at the University of the Free State, Bloemfontein, South Africa

**September 2020**

**Supervisor:**

Prof Liezel Herselman (PhD)

**Co-supervisors:**

Dr Willem Boshoff (PhD)

Dr Ansori Maré (PhD)

# Declaration

I, Kholosa Maqolo, declare that the dissertation that I herewith submit for the Master's 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.

_____ _____

Kholosa Maqolo Date

# Dedication

This dissertation is dedicated in memory of my late grandmother,

**Lumka Majiya**

# Acknowledgements

I would like to extend my sincerest gratitude to the following people and organisations who played a part in the completion of this dissertation:

- My parents for all their love, wisdom, and support throughout this journey
- Prof Liezel Herselman for granting me this opportunity, her guidance and support
- Dr Willem Boshoff for his support and guidance
- Dr Ansori Maré for her patience, words of encouragement and support
- Dr Chrisna Steyn for assisting me with seed germination and fertilising
- Dr Steven Xu for the STARP marker analysis performed in the United States Department of Agriculture laboratory
- Mrs. Sadie Geldenhuys and the entire Plant Breeding staff for their support and motivational words
- Plant Breeding students for the support, love and well-needed laughs
- Grain SA for financial support
- The University of the Free State for financial support

And most of all, the Lord Almighty, for granting me strength and resilience throughout this journey

# Table of contents

# List of tables

# List of figures

# List of abbreviations

| | |
|---|---|
| α | Alpha |
| AFLP | Amplified fragment length polymorphism |
| AMAS | Asymmetrically modified allele-specific |
| APR | Adult plant resistance |
| APS | Ammonium persulfate |
| ASR | All stage resistance |
| Avr | Avirulence |
| β | Beta |
| bp | Base pairs |
| BC | Before Christ |
| $BC_1$ | Backcross one generation |
| BSA | Bulk segregant analysis |
| CAPS | Cleaved amplified polymorphic sequence |
| CIMMYT | International Center for Wheat and Maize Improvement |
| cM | CentiMorgan(s) |
| CTAB | Hexadecetyltrimethylammonium bromide |
| DAFF | Department of Agriculture, Forestry and Fisheries |
| DArT | Diversity array technology |
| dCAPS | Derived cleaved amplified polymorphic sequence |
| DMI | 14α-demethylation inhibitor |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| EDTA | Ethylene-diaminetetraacetate |
| EMS | Ethyl methanosulfonate |
| EcoTILLING | Ecotype targeting induced local lesions in genomes |
| eSNP | Electronic SNP |
| EST | Expressed sequence tag |
| $F_1$ | First filial generation |
| $F_2$ | Second filial generation |
| $F_3$ | Third filial generation |
| f. sp. | Forma specialis |

| | |
|---|---|
| *g* | Gravitational force |
| gDNA | Genomic DNA |
| GS | Genomic selection |
| GWAS | Genome-wide association study |
| ISRTN07 | 2nd International Stem Rust Trap Nursery |
| IT | Infection type |
| K | Potassium |
| KASP | Kompetitive allele specific PCR |
| Kbp | Kilobase pair |
| L. | Linnaeus |
| LOD | Logarithm of odds |
| *Lr* | Leaf rust resistance gene |
| LRGS | Low-resolution genome scan |
| MAS | Marker-assisted selection |
| $MgCl_2$ | Magnesium chloride |
| MR | Moderately resistant |
| MRR | Moderately resistant and resistant |
| MS | Moderately susceptible |
| N | Nitrogen |
| NaCl | Sodium chloride |
| NGS | Next-generation sequencing |
| $(NH_4)_2SO_4$ | Ammonium sulfate |
| NIL | Near-isogenic line |
| P | Phosphorus |
| P1 | Donor line |
| P2 | Recipient line |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| PEA | Priming element-adjustable |
| *Pgt* | *Puccinia graminis* f. sp. *tritici* |
| pH | Power of hydrogen |
| PVE | Phenotypic variation explained |
| QoI | Quinone outside inhibitor |
| QTL | Quantitative trait loci |

| | |
|---|---|
| ® | Registered |
| R | Resistant |
| RAPD | Random amplified polymorphic DNA |
| RFLP | Restriction fragment length polymorphism |
| RGA | Resistance gene analog |
| RNA | Ribonucleic acid |
| S | Susceptible |
| SA | South Africa |
| SAGL | South African Grain Laboratory |
| SCAR | Sequence characterised amplified region |
| SDHI | Succinate dehydrogenase inhibitor |
| SNP | Single nucleotide polymorphism |
| spp. | species pluralis |
| *Sr* | Stem rust resistance gene |
| SSR | Simple sequence repeat |
| STS | Sequence tagged site |
| STARP | Semi-thermal asymmetric reverse polymerase chain reaction |
| $T_{a/e}$ | Annealing/extension temperature |
| $T_m$ | Annealing temperature |
| *Taq* | *Thermus aquaticus* |
| TBE | Tris-HCl/Boric acid/EDTA |
| TE | Tris-HCl/EDTA |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| TILLING | Targeting induced local lesions in genomes |
| Tris-HCl | Tris(hydroxymethyl) aminomethane hydrochloride |
| UFS | University of the Free State |
| UK | United Kingdom |
| USA | United States of America |
| USDA-ARS | United States Department of Agriculture-Agricultural Research Service |
| *Yr* | Stripe rust resistance gene |

# List of SI units

| | |
|---|---|
| % | Percentage |
| ℃ | Degrees Celsius |
| λ | Lambda |
| cm | Centimetre(s) |
| g | Gram(s) |
| h | Hour(s) |
| ha | Hectare(s) |
| kPa | Kilopascal |
| l | Litre(s) |
| m | Metre(s) |
| M | Molar(s) |
| mg | Milligram(s) |
| min | Minute(s) |
| ml | Millilitre(s) |
| mm | Millimetre(s) |
| mM | Millimolar(s) |
| ng | Nanogram(s) |
| nM | Nanomolar(s) |
| r/s | Revolutions per second |
| s | Second(s) |
| µE/m²/s | Microeinsteins per second per square meter |
| µl | Microlitre(s) |
| µM | Micromolar(s) |
| U/µl | Unit per microlitre |
| V | Volt(s) |
| v/v | Volume per volume |
| w/v | Weight per volume |

# Abstract

Stem or black rust caused by *Puccinia graminis* f. sp. Pers. *tritici* Eriks. & E. Henn (*Pgt*) is an important disease of wheat (*Triticum aestivum* L.) as outbreaks of the disease contribute to increased input costs through the application of fungicides and often result in lower yield and quality of the crop. Upon appearance of *Pgt* race Ug99 in Uganda, known for its virulence against the once widely deployed stem rust resistance (*Sr*) gene *Sr31*, there has been an increased urgency among wheat scientists to identify and deploy new sources of resistance. The aim of this study was to characterise and map a potential new source of stem rust resistance in two durum derived wheat lines M3283_4 and M3192_7. Parental lines, M3283_4 and M3192_7 (resistant) and Line 37-07 (susceptible) were screened with molecular markers for the presence of 13 designated *Sr*-genes (*Sr2*, *Sr13*, *Sr22*, *Sr25*, *Sr26*, *Sr31*, *Sr32*, *Sr36*, *Sr39*, *Sr42*, *Sr55*, *Sr57* and *Sr58*). Of these only *Sr13*, located on chromosome 6A, was detected in the two resistant parental lines.

Crosses were made between Line 37-07 and the resistant lines (M3283_4 and M3192_7) to produce $F_2$ mapping populations. To determine how rust resistance segregated in these populations, phenotypic evaluation was performed by inoculating the seedlings with urediniospores of *Pgt* race PTKST, the most virulent member of the Ug99 race group in South Africa. The phenotypic evaluation of the $F_2$ populations and $F_3$ families produced Mendelian segregation ratios evident of a single dominant *Sr*-gene. Consequently, an allelism test was performed by making a top cross between the two resistant parents. Phenotyping of the derived $F_2$'s indicated that either the same *Sr*-gene or a closely linked gene is present in the two resistant parents.

Genotyping was performed to detect polymorphic loci between the three parental lines. In total, 170 simple sequence repeat (SSR) markers were screened, of which 86 and 85 were polymorphic between M3283_4 and M3192_7, respectively. Bulk segregant analysis (BSA) was used to screen the polymorphic markers on the Line37-07/M3283_4 and Line37-07/M3192_7 $F_2$ bulks, individuals within the bulks and the entire $F_2$ populations to identify markers closely linked to stem rust resistance. Two markers, Sr13 and gwm427 (both located on chromosome 6A) were consistently found closely linked to the gene(s) conferring resistance. A partial linkage map was drawn depicting the position of the resistance gene(s) relative to the closely linked markers. Marker Sr13 explained 62% and

22% of the phenotypic variation for *Pgt* race PTKST in populations Line37-07/M3283_4 and Line37-07/M3192_7, respectively. Furthermore, marker gwm427 explained 76% and 39% of the phenotypic variation for *Pgt* race PTKST, in populations Line37-07/M3283_4 and Line37-07/M3192_7, respectively.

The effectiveness of the two markers was successfully validated using $F_3$ families. Semi-thermal asymmetric reverse polymerase chain reaction (STARP) marker analysis was also performed to detect which haplotype of *Sr13* was present. The *Sr13a* haplotype was detected in both resistant parents. The combined results from phenotyping and genotyping suggest that *Sr13* is present in both M3283_4 and M3192_7 as it is known to confer resistance to *Pgt* race PTKST. Furthermore, results from the SSR markers used to detect the *Sr13* gene was supportive and the diagnostic STARP marker Rwgsnp37 confirmed the presence of *Sr13*. Future work should include fine mapping through screening molecular markers known to map in the distal region of chromosome 6A. The resistance gene could be combined with other known effective *Sr*-genes in an effort to prolong resistance.

**Keywords:** Bulk segregant analysis, *Puccinia graminis* f. sp. *tritici*, Resistance breeding, Semi-thermal asymmetric reverse PCR, Simple sequence repeats, *Sr13*, *Triticum aestivum*

# Chapter 1

# General introduction

Wheat is an important grain crop used as a staple food worldwide. Wheat is important economically as it is a great source of nutrients for humans and is used as feed for livestock. It is also used in the textile industry to create baskets as well as in the bioethanol production industry (Mohanty and Swain 2019). There has been an unstable fluctuation in the production of wheat in South Africa (SA), therefore there is a need to import from other countries. The decrease in wheat yield is caused by multiple factors such as rainfall, climate change, pests and diseases (Department of Agriculture, Forestry and Fisheries (DAFF) 2016).

Wheat rusts are one of the major diseases causing a decrease in wheat yield. There are three types of rust: stem rust, leaf rust and stripe rust. The most devastating is stem rust, also known as black rust, caused by the fungal pathogen *Pgt*. The first epidemic of stem rust to occur in SA was in 1726 in the Western Cape Province (Pretorius et al. 2007). Stem rust race Ug99 was first detected in Uganda in 1998 and described in 1999 (Pretorius et al. 2000). The last epidemic outbreak recorded in SA was in 1985. Currently, five of the 13 known Ug99 race group members have been found in SA (Terefe et al. 2019).

The spread of this disease can be controlled by fungicide applications and through resistance breeding (Wanyera et al. 2009; Bender et al. 2016; Langridge 2017). The most effective control method is resistance breeding as it is environmentally and economically friendly, whereas fungicide applications add to input costs and considered harmful to the environment (Terefe et al. 2010). Resistance breeding usually involves using molecular markers to identify the gene conferring resistance against the disease through an approach named marker-assisted selection (MAS). The molecular markers often used are microsatellites, also known as SSRs and single nucleotide polymorphisms (SNPs). These markers indicate the location of the gene in a segregating population, using various strategies, including BSA (Michelmore et al. 1991; Gupta and Varshney 2000; Jehan and Lakhanpaul 2006).

To date, more than 70 *Sr*-genes have been identified in wheat and its wild relatives (Saini et al. 2018). The gene *Sr31* was the main source of stem rust resistance used to control stem

rust across the world (Zurn et al. 2018). The emergence of *Pgt* race Ug99 (also known as TTKSK) resulted in virulence to *Sr31* and *Sr38* and as more races developed, more *Sr*-genes became susceptible to the disease (Xu et al. 2017; Lewis et al. 2018; Bhavani et al. 2019). Over time, the pathogen overcomes *Sr*-genes through mutations and introductions from other areas through wind dispersion (Bhavani et al. 2019; Visser et al. 2019). This creates a need for breeders and plant pathologists to deploy new sources of stem rust resistance (Visser et al. 2011). Combining *Sr*-genes is considered an effective method for obtaining prolonged resistance to the disease (Singh et al. 2002; Hussain et al. 2016).

The aim of this study was to characterise and map a potential new source of stem rust resistance in the spring wheat lines M3283_4 and M3192_7. The wheat lines were provided by Dr Julian Thomas (Morden Research and Agri-Food Canada) and were derived from a cross that was made between common wheat and a stem rust resistant AABB tetraploid (durum) line. The objectives of the study were as follows:

(i) Screen for the presence of known *Sr*-genes in wheat lines M3283_4 and M3192_7 using SSR markers;

(ii) Use of a low-resolution genome scan (LRGS) marker set to identify polymorphisms between M3283_4 and M3192_7 (resistant) and Line 37-07 (susceptible);

(iii) Phenotypic evaluation of $F_2$ populations of Line37-07/M3283_4 and Line37-07/M3192_7;

(iv) Evaluation of $F_2$ populations using polymorphic SSR markers to map the *Sr*-gene(s) to specific wheat chromosome(s);

(v) Phenotypic evaluation of $F_3$ families to validate segregation ratios and polymorphic markers.

# References

Bender, C. M., Prins, R., and Pretorius, Z. A. (2016). Development of a greenhouse screening method for adult plant response in wheat to stem rust. *Plant Disease,* 100: 1627-1633.

Bhavani, S., Hodson, D. P., Huerta-Espino, J., Randhawa, M. S., and Singh, R. P. (2019). Progress in breeding for resistance to Ug99 and other races of the stem rust fungus in CIMMYT wheat germplasm. *Frontiers of Agricultural Sciences and Engineering*, 6: 210-224.

Department of Agriculture, Forestry and Fisheries. (DAFF) (2016). *Production Guideline for Wheat.* Pretoria: Department of Agriculture, Forestry and Fisheries.

Gupta, P. K., and Varshney, R. K. (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica,* 113: 163-185.

Hussain, M., Khan, M. A., Hussain, M., Javed, N., and Khaliq, I. (2016). Phenotypic characterization of wheat selected lines for resistance against leaf, stripe and stem rust through phenotypic markers application. *The Journal of Animal and Plant Sciences,* 26: 185-193.

Jehan, T., and Lakhanpaul, S. (2006). Single nucleotide polymorphism (SNP) - Methods and applications in plant genetics: A review. *Indian Journal of Biotechnology*, 5: 435-459.

Langridge, P. (2017). *Achieving Sustainable Cultivation of Wheat: Wheat Diseases, Pests and Diseases.* Volume 1. Burleigh Dodds Science Publishing, Cambridge, pp 1-629.

Lewis, C. M., Persoons, A., Bebber, D. P., Kigathi, R. N., Maintz, J., Findlay, K., Bueno-Sancho, V., Corredor-Moreno, P., Harington, S. A., Kangara, N., Berlin, A., Garcia, R., German, S. E., Hanzalova, A., Hodson, D. P., Hovmøller, M. S., Huerta-Espino, J., Imtiaz, I., Mirza, J. I., Justesen, A. F., Niks, R. E., Omrani, A., Patpour, M., Pretorius, Z. A., Roohparvar, R., Sela, H., Singh, R. P., Seteffenson, B., Visser, B., Fenwick, P. M., Thomas, J., Wulff, B. B. H., and Saunders, D. G. (2018). Potential for re-emergence of wheat stem rust in the United Kingdom. *Communications Biology*, 1: 1-9.

Michelmore, R. W., Paran, I., and Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences of the United States of America,* 88: 9828-9832.

Mohanty, S. K., and Swain, M. R. (2019). Bioethanol production from corn and wheat: Food, fuel, and future. In: Ray, R. C., Ramachandran, C. (eds) *Bioethanol Production from Food Crops*, Volume 1. Elsevier, London, pp 45-59.

Pretorius, Z. A., Singh, R. P., Wagoire, W. W., and Payne, T. S. (2000). Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis* f. sp. *tritici* in Uganda. *Plant Disease,* 84: 203.

Pretorius, Z. A., Pakendorf, K. W., Marais, G. F., Prins, R., and Komen, J. S. (2007). Challenges for sustainable cereal rust control in South Africa. *Australian Journal of Agricultural Research,* 58: 593-601.

Saini, J., Faris, J. D., Zhang, Q., Rouse, M. N., Jin, Y., Long, Y., Klindworth, D. L., Elias, E. M., McClean, P. E., Edwards, M. C., and Xu, S. S. (2018). Identification, mapping, and marker development of stem rust resistance genes in durum wheat 'Lesbock'. *Molecular Breeding,* 38: 77.

Singh, R. P., Huerta-Espino, J., and Roelfs, A. P. (2002). The wheat rusts. In: Curtis, B. C, Rajaram, S., Gómez Macpherson, H. (eds) *Bread Wheat: Improvement and Production*. Plant Production and Protection Series no. 30. FAO, Rome, pp 317-330.

Terefe, T., Pretorius, Z. A., Paul, I., Mebalo, J., Meyer, L., and Naicker, K. (2010). Occurrence and pathogenicity of *Puccinia graminis* f. sp. *tritici* on wheat in South Africa during 2007 and 2008. *South African Journal of Plant and Soil,* 127: 63-167.

Terefe, T., Pretorius, Z. A., Visser, B., and Boshoff, W. H. (2019). First report of *Puccinia graminis* f. sp. *tritici* race PTKSK, a variant of wheat stem rust race Ug99 in South Africa. *Plant Disease,* 103: 1-3.

Visser, B., Herselman, L., Park, R. F., Karaoglu, H., Bender, C. M., and Pretorius, Z. A. (2011). Characterization of two new *Puccinia graminis* f. sp. *tritici* races within the Ug99 lineage in South Africa. *Euphytica,* 179: 119-127.

Visser, B., Meyer, M., Park, R. F., Gilligan, C. A., Burgin, L. E., Hort, M. C., Hodson, D. P., and Pretorius, Z. A. (2019). Microsatellite analysis and urediniospore dispersal simulations support the movement of *Puccinia graminis* f. sp. *tritici* from Southern Africa to Australia. *Phytopathology,* 109: 133-144.

Wanyera, R., Macharia, J. K., Kilonzo, S. M., and Kamundia, J. W. (2009). Foliar fungicides to control wheat stem rust, race TTKS (Ug99), in Kenya. *Plant Disease,* 93: 929-932.

Xu, X. F., Li, D. D., Liu, Y., Gao, Y., Wang, Z. Y., Ma, Y. C., Yang, S., Cao, Y. Y., Xuan, Y. H., and Li, T. Y. (2017). Evaluation and identification of stem rust resistance genes *Sr2*, *Sr24*, *Sr25*, *Sr26*, *Sr31* and *Sr38* in wheat lines from Gansu Province in China. *Peer Journal*, 5: e4146.

Zurn, J. D., Rouse, M. M., Chao, S., Aoun, M., Macharia, G., Hiebert, C. W., Pretorius, Z. A., Bonman, J. M., and Acevedo, M. (2018). Dissection of the multigenic wheat stem rust resistance present in the Montenegrin spring wheat accession PI 362698. *BMC Genomics*, 19: 1-11.

# Chapter 2

# Literature review

## 2.1 Introduction

Bread wheat is one of the most important cereal crops used as a staple food globally (Shewry 2009). Wheat is a nutrient source of proteins and starch. Damaged raw starch of good quality wheat and wheat with low economic value are used for bioethanol production (Suresh et al. 1999; Sayaslan et al. 2008; Mohanty and Swain 2019). Wheat is also used in the spirits industry to produce alcoholic beverages (Nhemachena and Kirsten 2017).

Wheat is produced in all nine provinces of SA, with the highest producing provinces being the Western Cape, Northern Cape and Free State (South African Grain Laboratory (SAGL) 2018). Over the past years an unstable fluctuation in wheat production was observed in these provinces with an overall decrease in wheat production (Figure 2.1; SAGL 2018). Globally wheat production has increased since the 1960s because of the Green Revolution and helped to minimise hunger and malnutrition (Rehman et al. 2013). As mentioned before, wheat yield losses are due to multiple factors (Barnard and Smith 2009; Barnard and Smith 2012; Sosibo et al. 2017).

There are several wheat diseases affecting yield, but one of the most devastating and destructive wheat diseases is stem rust (also known as black rust) caused by *Pgt* (Tsilo et al. 2009; Terefe et al. 2010; Li et al. 2016; Xu et al. 2017; Xu et al. 2018). Fungicides are used to control the disease in the field, however, the most effective method to control stem rust infections is resistance breeding (Pathan and Park 2007; Hussain et al. 2016). Before 1991 the resistance gene *Sr31* was the main resistance gene used against stem rust across the world but the emergence of Ug99 was problematic (Zurn et al. 2018). The new race Ug99 (TTKSK) discovered in Uganda in 1998, became virulent to *Sr31* and as more races have developed, more *Sr*-genes were overcome by *Pgt* (Xu et al. 2017; Lewis et al. 2018).

Marker-assisted selection uses molecular markers, e.g. SSRs and SNPs, to identify the desired gene. This approach is used in resistance breeding programmes to improve wheat resistance against stem rust. Durable resistance can be achieved when two or more resistance genes are combined into one wheat cultivar (Liu et al. 2010; Hussain et al. 2016).

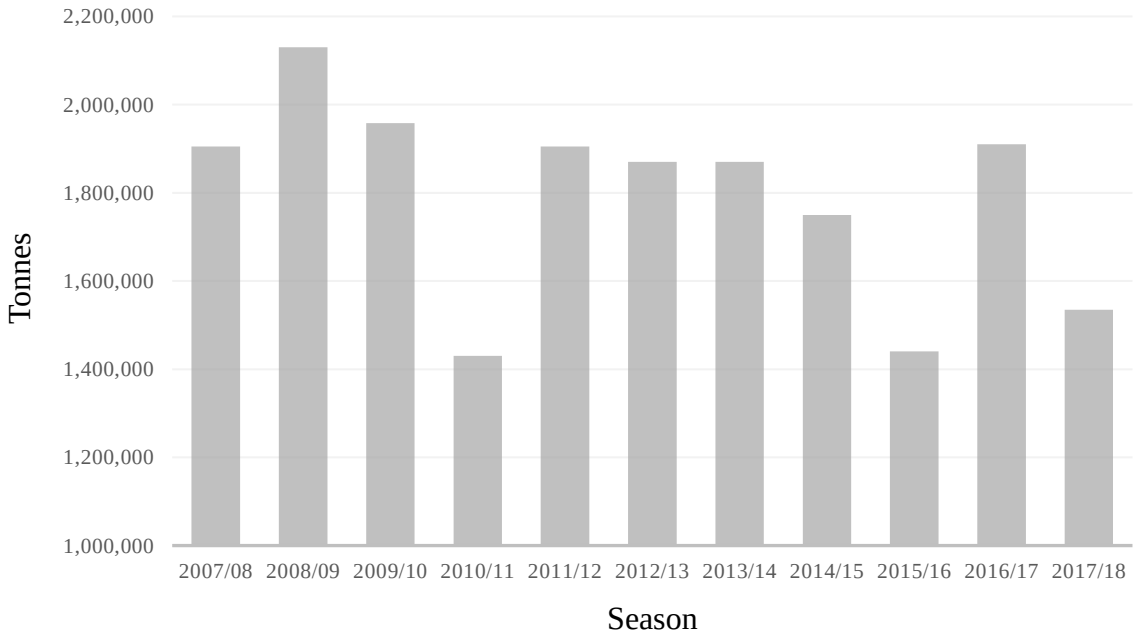


**Figure 2.1 Wheat production in South Africa from 2007/2008 to 2017/2018 seasons** (SAGL 2018).

## 2.2 Wheat

Wheat belongs to the genus *Triticum* that forms part of the grass family Poaceae. Wheat originated in the Near East, a region now known as Syria, Turkey, Afghanistan, Iraq and Iran (also known as the Fertile Crescent; Shewry 2009). Following this, wheat was cultivated across the world including countries such as India, China, United States of America (USA) and the African continent. Wheat was introduced to SA upon arrival of Jan van Riebeeck in the Cape in 1652 (Nhemachena and Kirsten 2017).

### 2.2.1 Wheat production in South Africa

Wheat is the fourth most important produced field crop in SA and ranks second as the most important produced cereal crop, following maize (*Zea mays* L.). Wheat production occurs during different planting seasons in SA. The wheat planting season in the winter rainfall regions occurs between mid-April and mid-June and in the summer rainfall regions between mid-May to July and is harvested from November until January. Mainly bread wheat is produced in SA and durum wheat (*T. turgidum* L. ssp. *durum*) is produced in small quantities in certain areas (Nhemachena and Kirsten 2017).

In 2006, wheat was mainly produced in the Western Cape (dryland production), Northern Cape (irrigation) and Free State (irrigation and dryland) provinces. These three provinces

were the largest producers that accounted for about 84% of SA's wheat production. Reports by DAFF in 2016 stated that wheat was planted on an area of about 508 365 ha of which 323 000 ha (64%) was in the Western Cape and 110 000 ha (22%) in the Free State provinces (DAFF 2017). Both provinces showed an increase in wheat yield over years. In the 2017/2018 season wheat production was at 1.54 million tonnes that was lower than production in the 2016/2017 season (1.83 million tonnes).

The crop was planted on a total area of 491 600 ha and the average yield was 3.12 t/ha in the 2017/2018 season (SAGL 2018). Due to the fluctuation in wheat production in SA over the years, the country has imported wheat from other countries. South Africa is a net importer of wheat from Germany, Lithuania, and the Russian Federation (DAFF 2018). In the 2017/2018 season, SA imported 1.68 million tonnes for local consumption (SAGL 2018).

For wheat to grow well and produce high yields, the environment must be favourable. Environmental factors influencing wheat production are soil type, temperature and precipitation. Wheat grows well in a variety of soils but for optimum growth, loamy to sandy loam soils are preferred (Sosibo et al. 2017). Wheat is sensitive to acidic soils, especially during plant developmental stages, which contains a high aluminium ($Al^{3+}$) content. The required pH of soil is 6.0 to 7.5 (Monsanto 2015).

Winter wheat needs to be exposed to low temperatures before flowering can take place. Spring wheat does not require vernalisation and can be grown in areas with warmer temperatures when compared to winter wheat. An ideal environment for growing wheat is cool temperatures and moist soil during planting and grain development stages, and warm and dry climate conditions at harvesting (Nhemachena and Kirsten 2017). Another environmental factor influencing the production of wheat in dry land areas is lack of precipitation during the growing season and at critical growth stages (Barnard and Smith 2009).

The water requirement for growing wheat optimally is 600 mm per year in SA (DAFF 2016). In dry areas, practices like no-tillage are used to conserve soil moisture. Frost during flowering in early spring can reduce yield. Environmental factors are unpredictable and differ between seasons, which add to production risk (DAFF 2018). However, yield can be increased by optimising environmental factors.

### 2.2.2 Economic importance of wheat

Wheat production is important to communities as it serves as a staple food to many people across the world. Wheat is used to produce flour that is used in the baking industry (DAFF 2016). Wheat is also used as livestock feed for poultry and pigs, as straw in the artistry industry to make hats and rugs, pastes and sizing textiles, in the paper manufacturing industry and in alcoholic beverage production (Goldblatt 2011; Nhemachena and Kirsten 2017). Wheat production plays an important role in the economy of SA, however the area planted with wheat has shown a downward trend since the late nineties although wheat consumption increased. South Africa's wheat consumption is the highest in sub-Saharan Africa and is expected to increase annually with the increasing population and urbanisation (DAFF 2018). The decreased production forces SA to import wheat produced in other countries.

Importing wheat causes a financial burden on the country in addition to the strain caused by the high production costs used to maintain the wheat grown in the country. For example, in 2013 alone, African countries spent more than 13 billion USA dollars on imported wheat. The narrow profit margins forces local wheat farmers to switch from wheat production to crops like sunflower, maize, soybean, canola or livestock production (DAFF 2018). The trend of decreased wheat production has been sporadic over the past 20 years due to unpredictable climate changes (AgriOrbit 2018).

### 2.2.3 Genetic structure of wheat

Wild diploid wheat, *T. urartu* Thumanian (2n = 2x = 14) is the A-genome donor and *T. searsii* Feldman and Kislev the B-genome donor of wild tetraploid wheat, *T. turgidum* L. that was cultivated to produce emmer wheat (2n = 4x = 28; AABB genome). Hexaploid wheat (bread wheat; 2n = 6x = 42; AABBDD genome) originated from two different species, tetraploid emmer wheat (AABB) and diploid *Aegilops tauschii* Cosson (also known as *T. tauschii* L.) as the donor of the D-genome (Raman et al. 2010). The combination of these two species produced hexaploid wheat with superior characteristics such as having strong glumes that enclose the grains.

According to Shewry (2009), since the occurrence of hexaploid wheat, little divergence occurred between the D-genome in hexaploid and diploid wheat species. Furthermore, tetraploid and hexaploid wheat contain B-genomes that were derived from the S-genome that is present in the Sitopsis section of *Aegilops* (Shewry 2009). Common bread wheat has

a complex and large genome containing six copies of each chromosome (Zimin et al. 2017). This complexity has caused a challenge in genomic research of wheat. Agronomic traits like yield, quality and disease resistance are controlled by multiple genes (Zimin et al. 2017). Furthermore, these traits are influenced by environmental conditions and challenge the process of identifying genes linked to these specific traits.

## 2.3 Stem rust

Stem rust is a disease affecting small grain crops and occurs worldwide wherever wheat is grown. Crops that are also affected by stem rust include barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.) and rye (*Secale cereale* L.). The fungal pathogen *Pgt*, causing stem rust on wheat, durum wheat, triticale (*xTriticosecale* Wittmack) and barley, is an obligate biotroph, meaning it extracts nutrients only from living plant tissues and cannot exist apart from their hosts (Leonard and Szabo 2005) and is heteroecious implying that it requires two hosts for a complete life cycle. Stem rust thrives in warm (25-30°C) to mild (15-20°C) temperatures, with adequate moisture to produce night dew (Singh et al. 2002).

### 2.3.1 Stem rust in South Africa

Stem rust occurs worldwide in countries where susceptible hosts are grown and has been traced back to the time of Aristotle (384-322 BC; Schumann and Leonard 2000). This disease has been a problem to wheat in ancient Rome and Greece and has spread across the world, including SA, where it causes major yield losses of wheat (Schumann and Leonard 2000; Pretorius et al. 2007).

The first stem rust epidemic in SA was documented in 1726, occurring in the Western Cape region (Pretorius et al. 2007). As wheat production expanded, epidemics became a recurrent phenomenon and caused stem rust to become a devastating wheat disease in the winter-rainfall production regions of the Eastern- and Western Cape. Stem rust epidemics also became devastating in the summer-rainfall regions of the Free State (Figlan et al. 2014). The last widespread stem rust outbreaks were recorded in 1985 on *Sr24*-derived cultivars in the Western Cape province. There have been new races of *Pgt* resulting in the sporadic occurrence of the disease in wheat fields (Boshoff et al. 2000; Figlan et al. 2014).

Since the 1980s, more than 30 *Pgt* pathotypes have been characterised on wheat and triticale in SA (Figlan et al. 2014; Terefe et al. 2016; Terefe et al. 2019). In 2011 it was reported that SA had four of the eight Ug99 pathotypes; TTKSF, TTKSP, PTKST and TTKSF+ (Figlan et al. 2014). TTKSF was the first pathotype of the Ug99 lineage that was

detected in SA (Boshoff et al. 2002). TTKSF, locally known as 2SA88, was found to be genetically different from other local rust isolates and more closely related to the Ug99 lineage (Pretorius et al. 2010; Terefe et al. 2010).

The second detected Ug99 pathotype was TTKSP (Western Cape in 2007) that is known as virulent against *Sr24*. The third SA Ug99 pathotype, PTKST, was detected in 2009 in KwaZulu-Natal province (Pretorius et al. 2010; Figlan et al. 2014). Race PTKST is virulent against the resistance genes *Sr24* and *Sr31*. The fourth recorded Ug99 pathotype detected in SA was TTKSF+ in 2010 in the eastern Free State (Pretorius et al. 2012; Figlan et al. 2014). More recently, a fifth Ug99 pathotype PTKSK, avirulent to *Sr24* and virulent to *Sr31*, was discovered by Terefe et al. (2019) in the Free State province.

### 2.3.2 Economic importance of stem rust

Wheat production is often affected by stem rust epidemics late in the growing season (Leonard and Szabo 2005; Figlan et al. 2014). An infected wheat crop is characterised by the presence of urediniospores at the initial stages of the infection and black teliospores in the pustules towards the end of the season (Roelfs et al. 1992; Schumann and Leonard 2000).

Extreme stem rust infections disrupt the transportation of nutrients to developing wheat heads (Leonard and Szabo 2005). This causes shrivelled grains and loss of grain due to stem lodging. In addition to stem lodging, grain yield losses are also caused by the decreased photosynthetic area available and loss of water and nutrients from lacerated lesions (Pradhan et al. 2016). The extent in which wheat can be affected by stem rust depends on whether the environment is suitable for infection to take place, level of available inoculum and the presence of susceptible hosts (McIntosh 2009).

Stem rust infections influence yield potential and the quality of infected crops (Figueroa et al. 2018; Soko et al. 2018). For example, a new *Pgt* race, virulent to the wheat cultivar Digalu caused almost 100% yield loss in southern Ethiopia during 2013-2014 (Olivera et al. 2015; Xu et al. 2018). In Sicily, there was a large stem rust outbreak caused by a new and unusually devastating race in 2016 (Bhattacharya 2017; Xu et al. 2018), causing yield losses of durum and bread wheat. Grain yield losses due to major stem rust epidemics have a detrimental effect on a country's economy as wheat needs to be imported due to a higher demand than supply and could lead to food price increases (Macauley 2015). Control strategies have been put in place to prevent yield losses and secure wheat quality.

### 2.3.3 Control strategies

Strategies to control *Pgt* disease outbreaks were developed to limit disease infection and prevent yield losses and poor grain quality. Over the years, different methods have been used to control disease infections, however, these methods are not effective when used individually. These control methods include cultural practices, chemical control and genetic control (Roelfs and Bushnell 1985; Pradhan et al. 2016).

Cultural practice is a method that can be used to control stem rust epidemics (Pradhan et al. 2016). It is known that moisture on leaves and excess foliar nitrogen are favourable conditions for rust infections (Schumann and Leonard 2000). Applying different cultural practices can prevent the development of favourable conditions for rust pathogens (Roelfs and Bushnell 1985). These cultural practices include change in planting dates, eradication of the alternate host, planting different cultivars with different characteristics linked to maturing dates as well as using varietal mixtures that help in reducing levels of the inoculum and decreasing disease pressure (Pradhan et al. 2016).

Stem rust infections can be prevented by avoiding excess nitrogen application (Roelfs and Bushnell 1985). Roelfs and Bushnell (1985) also stated that in areas where the disease over summers, susceptible grasses and volunteer wheats were eradicated weeks before planting to reduce inoculum levels, leading to a delay in initial infection. In areas where both winter and spring wheat are grown, rust infections can be controlled by rotational planting with non-host crops and avoiding overlapping or adjoining fields (Roelfs and Bushnell 1985; Pradhan et al. 2016).

Previous studies state that the focus should be on green bridges because they transport the disease from one crop to another (Pradhan et al. 2016). A green bridge refers to weeds and/or other crop volunteers that promote growth and development of diseases in crop fields (Leonard and Szabo 2005). The effect of green bridges increases when crops are planted late in the season and when minimum to no-tillage occurs. The green bridge effect can be reduced using tillage and herbicides that are known as effective methods to control disease infection and spread. Although these cultural practices seem easy to perform, they require extensive knowledge and if the inoculum levels are already too high, the method will not be helpful (Roelfs et al. 1992).

The use of chemicals to control stem rust infections was initiated by 1968 (Roelfs and Bushnell 1985). Chemical control is applied by spraying fungicides on crops. Fungicides

are an effective method of controlling rust diseases (leaf, stem and stripe rust) on wheat (Wanyera et al. 2009; Tadesse et al. 2010; Soko et al. 2018). Fungicides are most effective when applied timely and contain active ingredients such as triazoles and strobilurins or both (Pradhan et al. 2016).

The inhibition of sterol synthesis was used as a control strategy however, this routine was not ideal, as it was time consuming and costly (Schumann and Leonard 2000). Bayleton (triadimefon) and Indar (fenbuconazole) were effective fungicides in 1985, however, Indar was most effective against leaf rust only, whereas Bayleton controlled the other rust diseases (Pradhan et al. 2016). The use of these chemicals had a minor impact on stem rust infection and their use are expensive and considered ecologically unsafe (Chen 2005; Wanyera et al. 2009). This was due to three reasons; (i) the effectiveness of host resistance, (ii) the high rate of disease increment for stem rust of wheat under ideal conditions and (iii) the relatively low economic return per ha in contrast to the cost of the application of fungicides (Roelfs and Bushnell 1985).

Stem rust outbreaks in Kenya have led to an increase in fungicide usage until new resistant cultivars could be developed (Wanyera et al. 2009). Another consideration is that over time pathogens may become resistant to fungicides (Tadesse et al. 2010; Pradhan et al. 2016). Thus, new chemical formulations were developed to combat disease spread and control infection rates of crops. Chemical formulations are classified based on their activity to pathogens and three classes exist for rust fungi namely, quinone outside inhibitors (QoIs), 14α-demethylation inhibitors (DMIs) and recently succinate dehydrogenase inhibitors (SDHIs). These classes were approved to control rust based on their effectiveness (Oliver 2014; Figueroa et al. 2018).

Large scale farmers apply fungicides preventatively and often repetitively to ensure investment returns, whereas in low yielding situations the use of these chemicals becomes harder to justify (McIntosh et al. 1995). The most effective way to apply fungicides is during early heading, as application after flowering is not considered economically feasible (Pradhan et al. 2016). The application of fungicides against stem rust is a preventative method more than curative.

Although fungicides are biologically effective, they are not economically sustainable to prevent wheat rust infections (Pradhan et al. 2016). Therefore, fungicides are used as an emergency procedure when favourable environmental conditions for disease development

occur to prevent rust outbreaks. Fungicides should be carefully used because of the negative impact they can have on the environment, high development cost, and with consideration to the likelihood that they may become ineffective through pathogen resistance (Langridge 2017). Based on these considerations, in the longer run genetic control through the development of resistant cultivars, is the preferred method to prevent disease infection of wheat (Pathan and Park 2007; Singh et al. 2015; Bender et al. 2016; Langridge 2017).

## 2.4 Stem rust resistance breeding

Stem rust resistance is another method used to control the spread of infections caused by *Pgt* in wheat (Ellis et al. 2014). Resistance breeding effectively controls stem rust infection when a single *Sr*-gene or a combination of genes are present in commercial cultivars (Roelfs and Bushnell 1985). Rust resistance genes are divided into two classes based on their phenotypic effects. These two classes are race specific or commonly known as all stage resistance (ASR) and race specific/non-specific resistance known as adult plant resistance (APR). According to Ellis et al. (2014) ASR genes are expressed and effective from seedling to adult plant stage while APR genes are only expressed and functional during the adult plant stage.

### 2.4.1 Phenotypic disease evaluation

Conventional disease resistance breeding involves field-based phenotyping (Riaz and Hickey 2017). A plant breeder uses phenotyping to characterise the plant's performance (Velu and Singh 2013). It is a traditional method used for selection in breeding programmes, accelerating genetic gains and monitoring a plant's health status (Chawade et al. 2019). According to Velu and Singh (2013) phenotyping of wheat lines is an important strategy for developing widely adaptable, stable and disease or pest resistant germplasm. However, there are limitations that compromise the success of field phenotyping by slowing down breeding and research programme progress. These limitations are environmental factors, plant developmental stage, progression rate of an epidemic and unexpected non-target disease outbreaks (Riaz and Hickey 2017). When breeding for disease resistance, relying on only phenotypic screening methods can result in a lack of information on the underlying genetics, especially when more than one trait is present and effective against the disease (Tsilo et al. 2009).

In the study of rust genetics and resistance breeding, it is important to employ efficient and reproducible phenotypic methods to produce infection under greenhouse and field

conditions. During rust resistance breeding, all plants involved in the test are infected and the test must accurately determine infection types (Velu and Singh 2013). Different inoculation techniques including injection, dusting, transplanting and spraying were developed for infecting seedlings with urediniospores. Commonly applied is spraying seedlings with spores mixed with Soltrol 170, which is a light mineral oil that is a known successful carrier of spores and has low phototoxicity (Velu and Singh 2013).

Inoculation is initiated 6 to 8 days post planting and 12 to 14 days post inoculation infection responses are measured by scoring the size of the rust pustules and associated chlorosis or necrosis. Appropriate scoring of disease severity and host response provides useful information such as epidemic status and field level expression of host resistance (Ali and Hodson 2017). Seedling infection types (ITs) are used to differentiate between different diseases and races of a pathogen (e.g. stem rust races). Infection types are characters that represent phenotypic expression when wheat varieties are infected with different rust races under controlled environmental conditions (Stakman et al. 1962). Variation in ITs against different rust races may result from single or multiple resistance genes present in a cultivar (McIntosh 2009).

Stakman et al. (1962) proposed a nomenclature system to differentiate between the ITs visible on stem rust hosts (Xu et al. 2018). The scale (Table 2.1) proposed is a 0 to 4 scale (Stakman et al. 1962). Low ITs of 0, 0;, 1 and 2 or a combination of these are considered resistant whereas high ITs of 3 and 4 are considered susceptible. However, in some studies, this scale was modified to a 0 to 9 qualitative scale (McIntosh 2009). Roelfs et al. (1992) categorised the APR responses into four groups, resistant (R), moderately resistant (MR), moderately susceptible (MS) and susceptible (S). Infection response ranging between two categories is denoted by a hyphen (e.g. R-MR; MS-S).

The severity of the rust disease is measured using the Cobb scale with ratings from 0 to 100% (Peterson et al. 1948). Understanding partial resistance with its molecular mechanisms is through a definitive disease phenotype (Lowe et al. 2011). Plant phenotyping is still considered as a bottleneck as the development for precise and accurate recording of important agronomical traits and crop monitoring are lagging (Chawade et al. 2019).

**Table 2.1 Major infection type classes for stem rust** (McIntosh et al. 1995)

| Infection type | Disease | Host response | Symptoms |
|---|---|---|---|
| 0 | Stem rust | Immune | No visible uredia |
| 0; | Stem rust | Highly resistant | Hypersensitive flecks |
| 1 | Stem rust | Resistant | Small uredia with necrosis |
| 2 | Stem rust | Resistant/moderately resistant | Small to medium-sized uredia with green islands and surrounded by necrosis or chlorosis |
| 3 | Stem rust | Moderately resistant/ moderately susceptible | Medium-sized uredia with or without chlorosis |
| 4 | Stem rust | Susceptible | Large uredia without chlorosis |

## 2.4.2 Sources of resistance

### 2.4.2.1 All stage resistance

Seedling resistance or ASR, also known as gene-for-gene resistance, major gene resistance or race-specific resistance, is one of the two classes of genetic resistance used to control *Pgt* infections on wheat. All stage resistance genes protect wheat during all plant stages, from seedling to adult plant stage (Spanic et al. 2015). According to Ellis et al. (2014), ASR genes conform mostly to the gene-for-gene hypothesis described by Flor (1971) that states that the avirulent (Avr) effector gene (in the pathogen) has an equivalent resistance (ASR) gene in the host (Li et al. 2016). This means that the resistance gene confers resistance against the pathogen containing the equivalent Avr effector gene. Some ASR genes are classified as broad spectrum because they confer resistance to all the evaluated races of the pathogen. However, this statement is questionable as it implies that these genes differ from the narrow spectrum genes that confer resistance to only some isolates of the pathogen (Ellis et al. 2014). The statement also suggests that ASR genes are more durable than APR genes and that is false although they are suitable for disease control. It is stated that ASR genes were the first class of resistance genes to be genotypically identified (Ellis et al. 2014).

All stage resistance genes were discovered to express clear phenotypic effects with high resistance levels that made selection simple and economical, which led to their interest by wheat breeders (Ellis et al. 2014). After their introduction in breeding programmes in the middle of the 20$^{th}$ century, it was evident that pathogens overcome resistance controlled by single genes. The virulent races or strains were either present at low frequencies in the population of the pathogens or were detected later by sexual re-assortment of genetic variations or mutations (Ellis et al. 2014). However, researchers found a way of using ASR genes effectively to control stem rust in other parts of the world e.g. Australia and North America (Ellis et al. 2014; Chen et al. 2018).

The long-term success of resistance breeding is achieved by using varieties that carry a combination of effective genes against local races also known as gene pyramiding (Singh et al. 2002). Cultivars carrying single resistance genes should not dominate the planted area as single genes are not durable in agriculture. Stem rust infections can be controlled by applying good agronomic practices to extend durability (Ellis et al. 2014). A good agronomic approach is planting resistant cultivars and preventing the inter-seasonal survival of rust races as this decreases the pathogen population. In addition to good agronomic practices, global and national rust control programmes have surveillance programmes to monitor the frequencies of *Pgt* races that are virulent to specific ASR genes and combinations of ASR genes (Singh et al. 2011). Surveillance information is useful to breeders to anticipate and respond to new virulent rust races identified across the world.

The response to the anticipated increase in the occurrence of new and more virulent *Pgt* races should be timely and vigorous. Waldron and Clark (1936) stated that it is difficult to persuade farmers to stop planting high yielding cultivars and to plant new stem rust resistant cultivars based on the potential of future epidemics unless the cultivar has the same or higher yielding potential. The challenge with resistance breeding is to ensure that the best durable combination of resistance genes are deployed effectively across epidemiological regions and to keep in mind that new pathogen races regularly spread across countries (Ellis et al. 2014).

#### 2.4.2.2 Adult plant resistance

Previously it was stated that rust resistance involves the gene-for-gene hypothesis between the host and the pathogen (Flor 1942). Resistance genes are involved in the recognition of the pathogen initiating a hypersensitive response that programme cell death to prevent the

spread of infection (Rutkoski et al. 2011). A single change (mutation) in the pathogen can render a specific resistance gene ineffective. In a genotype containing several resistance genes, breakdown would require simultaneous mutations in the corresponding Avr genes in rust fungi that is more unlikely (Rutkoski et al. 2011).

When resistance sources used in a gene pyramid do occur singly in other cultivars, the pathogen can become virulent to each cultivar in a stepwise manner, thus rendering the resistance of the cultivar carrying the gene pyramid ineffective. For this reason, it was proposed that resistance sources other than those with a hypersensitive response should be used to prevent stem rust resistance breakdown and the use of race non-specific APR genes was considered a promising mechanism. Adult plant resistance is evaluated in the field instead of the greenhouse because the genes are detected at post-seedling growth stages. However, some APR genes can be induced to express resistance in seedlings by modifying the growth temperatures as well as the light conditions (Lagudah 2011).

Race non-specific APR genes are frequently used in wheat for protection against stem rust (Mago et al. 2014). These genes provide partial resistance and are associated with a slow rusting effect without necrosis and therefore, associated with a non-hypersensitive response (Lagudah 2011; Rutkoski et al. 2011). The combination of three or four APR genes increases the level of resistance and durability (Njau et al. 2013). Rutkoski et al. (2011) suggested that improved APR levels will enhance long term resistance to *Pgt*. However, wheat breeding using APR genes is more complex than using ASR genes. Firstly, the contribution of APR genes is masked in the presence of ASR genes. Secondly, selecting for APR with stronger resistance require access to field sites either with regular natural infections or where epidemics can be induced to facilitate effective selection for resistance (Ellis et al. 2014). Individual APR genes exhibit different levels of partial resistance but near immunity can be achieved when several APR genes are combined (Singh et al. 2011).

Although combined APR genes lead to high levels of disease resistance, transferring these genes to adapted wheat germplasm is difficult during backcrossing because some effective genes may be lost during the process even though molecular markers are used. The challenges with regards to APR evaluation force breeders to develop new breeding strategies to evaluate APR effectively (Rutkoski et al. 2011). A proposed method by Rutkoski et al. (2011) is genomic selection (GS) that aims to improve quantitative traits by

using genome-wide marker coverage to predict breeding values of selected candidates and to improve traits such as APR that are based on multiple genes.

Marker-assisted selection and GS are tools used to combine several APR genes from different resistance sources. The advantage of GS for stem rust APR is that the genes do not need to be mapped and the quantitative trait loci (QTL) that do not exceed significant thresholds can be selected (Rutkoski et al. 2011). However, there are complications with using genome-wide association study (GWAS) and these include; (i) the appearance of false positives as a result of population structure, (ii) it requires a large population size, (iii) it requires high marker density and, (iv) very rare alleles are not detected in genome scans although the trait is tested and has a large effect (Rutkoski et al. 2011; Cockram and Mackay 2018).

#### 2.4.2.3 Frequently used stem rust resistance genes/quantitative trait loci

More than 70 stem rust resistance genes and many QTL have been identified in wheat and its wild relatives against stem rust. About half of the known *Sr*-genes are ineffective against *Pgt* races in the Ug99 linage including the widely used genes *Sr24, Sr31, Sr36*, and *Sr38* (Pretorius et al. 2000; Jin et al. 2008; Singh et al. 2011; Pretorius et al. 2012; Saini et al. 2018). There are at least 33 *Sr*-genes that are effective against one or more races in the Ug99 lineage: *Sr2, Sr9h, Sr13, Sr21, Sr22, Sr24, Sr25, Sr26, Sr27, Sr28, Sr32, Sr33, Sr35, Sr36, Sr37, Sr39, Sr40, Sr42, Sr43, Sr44, Sr45, Sr46, Sr47, Sr50, Sr51, Sr52, Sr53, Sr57, SrTA10171, SrTA10187, SrTA1662, SrTmp* and *Sr1RS$^{Amigo}$* (Kielsmeier-Cook et al. 2015). Some of these genes are contributed by tetraploid wheats (Saccomanno et al. 2018). Studies have been performed previously to identify resistance genes and QTL in common bread wheat and tetraploid wheats.

Among the 33 *Sr*-genes, eight genes (*Sr2, Sr13, Sr22, Sr25, Sr26, Sr35, Sr39* and *Sr40*) have been reported to be the most effective (Aktar-Uz-Zaman et al. 2017; Umar et al. 2019). One of the most effective genes, *Sr2* is categorised as an APR gene and is located on the short arm of chromosome 3B. This gene confers moderate resistance against all stem rust races, however, strong resistance is obtained when combined with other effective *Sr*-genes. Haile et al. (2012) identified a QTL region (*QSr.ipk*-3B) on chromosome 3BS and suggested that the QTL could be *Sr2* because the gene was translocated from tetraploid to hexaploid wheat.

*Sr13* is a gene conferring moderate resistance to *Pgt* race in durum wheat cultivars. *Sr13* is characterised as an ASR gene located on chromosome 6A (Zhang et al. 2017). Another gene located on this chromosome is *Sr26* that was introgressed into wheat from *Thinopyrum ponticum* (Podp.) Barkworth and Dewey (Knott 1961; Liu et al. 2010). In a study by Saini et al. (2018), two QTL associated with resistance were identified in North Dakota durum wheat. However, only QTL *QSr.rwg-6A.2* was located in a region known to be associated with *Sr13*, thus concluding that in 'Lebsock' the QTL is underlied by *Sr13*.

*Sr22* provides ASR resistance to race Ug99 and is located on chromosome 7A. *Sr22* was introgressed into wheat from *T. monococcum* L. *QSr.umn-7A* was the only QTL located on chromosome 7A in a study by Bajgain et al. (2016). In a study by Zurn et al. (2018), two QTL (*QSr.ace-7A.2* and *QSr.abr-7AL*) were reported on this chromosome. The two QTL mapped to the same location and *QSr.abr-7AL* was detected by Pujol et al. (2016) who suggested that the QTL could be an allele of *Sr15*.

Two QTL (*QSr.umn-7D.1* and *QSr.umn-7D.2*) detected on chromosome 7D mapped close to one another (Bajgain et al. 2016). Stem rust resistance genes *Sr25*, *Sr43* and *Sr44* are located on the long arm of chromosome 7D. These genes provide resistance to different variants of Ug99, however, *Sr43* and *Sr44* are not used in wheat breeding. *Sr57/Lr34/Yr18* is an APR gene that confers partial resistance to races that belong to the Ug99 lineage and is used to enhance the resistance of other genes. This gene is also located on the distal end of chromosome 7D. Another APR gene, *Sr55/Lr67/Yr46*, confers partial resistance to race TTKSK and is located on the long arm of chromosome 4D.

Several QTL that confer resistance to race Ug99 have been detected in spring wheat and durum wheat on chromosomes 2A, 2B and 2D. *Sr32* that has been introgressed into chromosomes 2A, 2B and 2D and genes that are located on chromosome 2B (*Sr9h*, *Sr28*, *Sr39*, *Sr40* and *Sr47*), are effective against Ug99 and its derivatives. However, the resistance of gene *Sr9h* was overcome by race TTKSF+ (Rouse et al. 2014). *QSr.umn-2A.5* is a QTL located 600 kilobase pairs (Kbp) from the distal end of chromosome 2AL, however no genes or QTL were previously reported in that position (Bajgain et al. 2016).

*Sr40* was transferred from *T. timopheevii* spp. *araraticum* Jakubz. and is located on the short arm of chromosome 2B (Aktar-Uz-Zaman et al. 2017). Quantitative trait locus *QSr.rwg-2B* was associated with minor effect resistance to *Pgt* race TRTTF in the same region associated with *Sr9h* and *Sr28*. These two genes confer resistance to race TTKSK

however, the newly detected QTL was not associated with resistance to race TTKSK (Saini et al. 2018). *Sr35* is an effective gene to race Ug99 categorised as an ASR gene. This gene was transferred from *T. monococcum* and is mapped on the long arm of chromosom 3A. Another gene that is used in *Pgt* resistance is *Sr42* that is located on the short arm of chromosome 6D. This gene is located in the same region as *SrCad* (Ghazvini et al. 2012).

The most effective way to decrease yield losses due to the spread of new races of stem rust is to deploy cultivars containing durable resistance sources and identifying QTL through wheat breeding. Quantitative trait loci analysis identifies chromosome regions associated with a desired phenotypic trait (Yu et al. 2014). Most traits are controlled by multiple genes and indepth research of the loci associated with the trait is necessary to completely understand the QTL's effect on a population (Njau et al. 2013). An accurate mapping of the QTL and genes across different environments and genetic backgrounds is required to use in MAS studies as closely linked markers should be used to determine the status of traits (Yu et al. 2014).

### 2.4.3 Genotyping methods

Genotyping is a process used to detect differences in the genetic make-up of individuals by analysing the deoxyribonucleic acid (DNA) sequence using biological assays. Genotyping using molecular markers dates to the early 1990s. The first DNA-based molecular marker that was developed was restriction fragment length polymorphisms (RFLPs) followed by random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs) and SSRs. Newer technologies such as SNPs and diversity array technologies (DArTs) were also developed. These methods are applied in various science disciplines such as fingerprinting and MAS.

#### 2.4.3.1 Overview of older molecular marker techniques

The application of molecular markers dates to the early 1990s when RFLP markers were used in wheat for gene mapping, identifying variation, identifying homoeologous chromosome arms and characterising wheat-rye recombinants. Restriction fragment length polymorphism is a non-polymerase chain reaction (PCR)-based marker detected by hybridisation-based techniques where a DNA fragment is labelled and used as a probe and hybridisation is detected using a Southern blot (William 1989). The different samples are digested using proteins called restriction enzymes to obtain sequence differences at cleavage sites of the enzymes. According to Garrido-Cardenas et al. (2018) RFLP markers

detect both alleles in a heterozygous sample and these alleles can be identified from point mutations such as single nucleotide bp changes.

Although the technique was successful in developing linkage groups in wheat, there were limitations. Limitations include that applying the technique was time consuming, had a high cost and low frequency of markers in bread wheat (Williams et al. 1990; Rasheed and Xia 2019). Other limitations include the requirement of large amounts and high quality DNA and labelling of the probes using radioisotope $^{32}P$ (Garrido-Cardenas et al. 2018). Due to these limitations, RFLPs did not become the marker of choice and this eventually led to the development of PCR-based techniques (e.g. RAPDs and SSRs) to be the focus of researchers.

Reseachers were interested in the RAPD technique due to its simplicity and applicability. Random amplified polymorphic DNA is a PCR-based technique described as an assay based on the amplification of random DNA segments using short synthetic 10 bp nucleotides (Williams et al. 1990; Badarcki 2001). The technique was used to obtain fragments of different lengths after carrying out a PCR reaction on the genomic DNA. Random primers are designed to attach at different regions of the DNA so that a profile is obtained for each pair of primers (Garrido-Cardenas et al. 2018).

The main advantage of the RAPD technique is that it is easy to perform as it does not need prior knowledge of the genome and this received attention from population geneticists who used the technique to detect genetic variation at DNA level (Williams et al. 1990; Badarcki 2001). Due to the analysis being fast and efficient, scientists were encouraged to produce high-density maps (Vierling and Nguyen 1992; Badarcki 2001). In cases where RAPDs were used to map QTL, the marker was converted into sequence tagged site (STS) and sequence characterised amplified region (SCAR) markers (Rasheed and Xia 2019). In addition, the technique was mostly used to identify markers linked to desired traits without mapping the whole genome and to identify strains and varieties using genomic fingerprinting. However, RAPDs were not used extensively in wheat due to lack of reproducibility and the absence of information on their locations in the genome (Devos and Gale 1992). Other disadvantages of using RAPD include the requirement of good quality DNA template, the reaction conditions must be more established and most of these markers are dominant (Badarcki 2001; Garrido-Cardenas et al. 2018).

The AFLP technique was initially described as a method for DNA fingerprinting (Vos et al. 1995). The technique is based on PCR amplification of genomic DNA digested with two restriction enzymes and can be used for DNA of any origin or complexity. An AFLP marker can be considered a mixture of both RFLPs and RAPDs. However, in contrast to RAPDs, the amplification is selective and not random (Garrido-Cardenas et al. 2018). The technique consists of three basic steps: (i) the genomic DNA is digested using two restriction enzymes, a rare cutter and frequent cutter, and the halfsite-adaptors are ligated to all restriction fragments, (ii) selective amplification of the fragments with two primers that have complementary adaptor and restriction-site sequences as their target site and (iii) gel analysis of the amplified fragments (Vos et al. 1995; Vuylsteke et al. 2007).

The AFLP technique has proven to be useful in the assessment of genetic differences among individuals and populations (Mueller and Wolfenbarger 1999). The technique was not only applicable in DNA fingerprinting, it was also used in systematics, population and conservation genetics, and QTL mapping. Amplified fragment length polymorphism requires no prior information of the sequence, thus has a low start-up cost (Janssen et al. 1996; Vuylsteke et al. 2007). The technique is also automable and can be multiplexed and this allows increased performance of markers (Vuylsteke et al. 2007; Garrido-Cardenas et al. 2018). These markers are also reliable and robust because stringent PCR conditions are used. However, the AFLP technique has a number of limitations such as, in a case of low sequence homology between samples, AFLP fingerprints shared will be low and the technique will no longer be useful. AFLP markers are less likely to out-compete co-dominant markers due to their dominant nature.

#### 2.4.3.2 Newer molecular marker techniques

Simple sequence repeats, also known as microsatellites, are short tandem repeats of 1 to 8 nucleotide sequences. This technique is the most used PCR-based marker in comparison to the older techniques that were used previously in wheat. Microsatellites occur randomly and frequently in eukaryotes, as well as prokaryotes (Pestsova et al. 2000). These markers are present in both the coding and non-coding regions of a genome (Garrido-Cardenas et al. 2018). Microsatellite markers detect higher levels of polymorphisms than other markers and can be assayed easily using PCR (Röder et al. 1995; Jaccoud et al. 2001; Wenzl et al. 2004).

According to Garrido-Cardenas et al. (2018), primers used in the PCR reaction are usually labelled with a fluorophore, a radioactive element or they are not labelled and these options have different detection methods (Garrido-Cardenas et al. 2018). Microsatellites are utilised in MAS and gene tagging. The availability of closely linked markers to the desired trait facilitates breeding by saving time and expense, thus making SSRs the marker of choice (Gupta and Varshney 2000). These markers are also used for DNA fingerprinting and anchoring chromosome loci in population studies due to their heritability. They are used in genetic diversity as they are suitable for variety identification because they are able to detect large numbers of discrete alleles repetitively, accurately and efficiently (Gadaleta et al. 2009).

In addition, SSRs are also used to research if germplasm accessions maintain their genetic fidelity and in cytogenetics research. Another advantage of SSRs is that they have a co-dominant nature and are highly reproducible (Röder et al. 1998; Li et al. 2017). Although SSRs are multi-allelic and can be used in gene mapping there is a limited potential for use in breeding (Ganal and Röder 2007). This is because, (i) it is a challenge to gather information in terms of multi-alleles, (ii) it is difficult to integrate or compare SSR data retrieved from different populations, (iii) the number of markers are finite in a genome and are not evenly distributed, and (iv) gel-based SSR analysis is cost ineffective as genotyping is laborious and time-consuming (Xu and Crouch 2008; Rasheed and Xia 2019).

The most common form of DNA polymorphisms are SNPs. Single nucleotide polymorphisms are defined as single nucleotide bp changes between different DNA sequences of different individuals within a species. These markers are found within coding and non-coding regions of genes or in intergenic regions between genes at different frequencies in different chromosomes (Xu 2010). Single nucleotide polymorphism markers are classified into three categories based on the type of mutations: (i) transversions with changes in nucleotides of C/G, A/T, C/A or G/T, (ii) transitions, appearing as C/T or G/A changes, and (iii) indels, produced by insertions or deletions of single nucleotides (Garrido-Cardenas et al. 2018). Multiple methods have been devised to detect and genotype SNPs. One of the requirements is to identify and detect the presence of SNPs in DNA samples (Jehan and Lakhanpaul 2006).

The discovery methods used include re-sequencing of PCR amplicons with or without pre-screening, electronic SNP (eSNP) discovery in genomic libraries and eSNP discovery in

expressed sequence tag (EST) libraries (Rafalski 2002). According to Mammadov et al. (2012), the development of next-generation sequencing (NGS) technologies such as Roche 454, HiSeq and Ion Torrent have limited the problems that were associated with low throughput and high cost of SNP discovery approaches and these methods have been successful in wheat.

The most used high throughput assays and genotyping platforms that are used for validation are Illumina's Bead Array technology-based Golden Gate and Infinium assays, Life Technologies Taqman assay coupled with the Open Array platform and KBiosciences Kompetitive Allele Specific PCR (KASP) combined with the SNP-line platform (now known as KASP by LGC bioresearch technologies) (Mammadov et al. 2012). These modern genotyping assays and platforms differ from each other in their chemistry, cost, equipment required, difficulty of assay development, potential of multiplexing and throughput (Rafalski 2002). The biallelic and stable nature of SNPs makes them amenable to automated high throughput genotyping and is a useful tool in QTL mapping and MAS in plant breeding (Langridge and Chalmers 2005). The SNP genotyping assays are also applied in pharmacogenomics, they have potential for DNA fingerprinting and are used in selecting desirable traits in plant breeding programmes (Jehan and Lakhanpaul 2006).

Genetic marker analysis using diversity arrays offers a low cost, high throughput, robust system with minimal DNA sample. Diversity array technology is a method used to discover and score genetic polymorphic markers that detects single bp changes within restriction sites or one of the selective bases of the PCR primer. The technique is based on microarray hybridisations that detect the presence or absence of individual fragments in genomes. According to Huttner et al. (2005), DArTs were successfully developed in wheat.

Wenzl et al. (2004) stated that DArTs enable whole-genome profiling of crops without the need of sequence information. These markers are also used to create medium-density genetic maps for plants with complex genomes. Being high throughput enables DArTs to be applied in plant breeding programmes such as, fingerprinting, QTL identification, tracking methylation changes and marker-assisted breeding (Jaccoud et al. 2001; Wenzl et al. 2004). Although the marker is biallelic and behaves in a dominant and co-dominant way, its weakness includes marker dominance and being technically demanding (Xu 2010).

### 2.4.4 Application of molecular marker technologies in resistance breeding

The development of molecular markers gave rise to MAS. Marker-assisted selection is a selection method that selects a trait of interest based on the molecular marker linked to the trait and enhances plant breeding. Molecular markers are effective because they are linked to genes of interest in a genome. These markers are used to increase the efficiency of selection in marker-assisted backcrossing. Molecular markers also allow scientists to construct genetic maps indicating the distances between markers and the gene, and chromosome location and identity.

#### 2.4.4.1 Marker-assisted selection

Marker-assisted selection is an indirect selection process using molecular markers linked to the trait of interest rather than directly selecting the trait. A critical component in plant breeding is to select plants in segregating populations that contain a desired combination of traits (Collard et al. 2005). Identification and development of different molecular marker types over the past years improved molecular genetics and enhances plant breeding programmes across the world e.g. by mapping and tagging of important agronomical genes (Mohan et al. 1997; Xu and Crouch 2008; Cobb et al. 2019). In plant breeding, MAS increases the efficiency and effectiveness of selection compared to conventional breeding.

Marker-assisted selection can be used to identify recessive traits that can be difficult to evaluate phenotypically (Gupta et al. 2010). Molecular markers must meet some requirements to be successfully used in breeding programmes. The markers must be closely linked to the trait of interest, effective means must be available to screen large populations and markers must be reproducible (Mohan et al. 1997). Marker-assisted selection has advantages over phenotypic selection as it is simple and saves time including effort and resources. In addition, selection of genotypes can be carried out at seedling stage and single plants can be selected (Mohan et al. 1997; Collard and Mackill 2008). These advantages can be used to accelerate the breeding process.

Molecular markers are used as chromosome landmarks for selecting segments carrying the gene of interest and assist breeders to combine several genes linked to the same or different characteristic that can mask the presence of one another (Dubcovsky 2004). An example is the combination of multiple disease resistance genes/QTL into a single genotype to increase durability, also known as gene pyramiding (Collard and Mackill 2008). Marker-assisted

selection can also be used to eliminate the transfer of deleterious genes and helps to select for phenotypes that are unreliably expressed in field trials, due to environmental effects.

Phenotypic evaluation of crops infected simultaneously with different pathotypes and biotypes is difficult. Markers closely linked to traits of interest assist scientists to identify the status of the gene in the host plant without exposing the host plant to the pathogen. Therefore, MAS has made it possible for breeders to evaluate the status of multiple genes in the absence of pathogens or pests (Mohan et al. 1997). Marker-assisted selection is also applied in marker-assisted backcrossing. This approach is used to transfer the desired traits into various lines (Collard and Mackill 2008; Gupta et al. 2010). The use of molecular markers in backcrossing increases the efficiency of selection.

These advantages allow plant breeders to exploit the genome to speed up breeding. However, the lack of reliable markers linked to abiotic stress tolerance as well as the need for polymorphic or diagnostic markers, such as SSRs that can be expensive to develop, limit the use of MAS (Gazal et al. 2016; Chukwu et al. 2019). Other limitations of MAS directly linked to wheat are caused by the complexity of the wheat genome that requires approaches that allow management of the large linkage blocks and dealing with many loci that can be an issue (Gupta et al. 2010). In addition, cost, equipment and consumables are factors limiting the use of MAS (Chukwu et al. 2019).

Although there are limitations to MAS, it has accelerated the breeding process compared to conventional techniques that were used before MAS was introduced two decades ago. According to Collard et al. (2005), once disease resistance markers have been developed for MAS, the approach becomes cheaper than the conventional methods. However, when conventional breeding is combined with MAS, breeders can select defined sets of genes in segregating progenies effectively and accurately (William et al. 2007). The exploitation of the advantages of MAS can improve crops and newer marker technologies will lower the cost of MAS for example SNPs and NGS.

#### 2.4.4.2 Gene targeting strategies

Gene targeting is defined as the process of targeting a specific gene region to find a gene of interest without having to scan the entire genome. Plant breeding makes use of gene targeting strategies such as aneuploids, chromosome specific libraries, near isogenic lines (NILs), BSA, induced mutations, targeting induced local lesions in genomes (TILLING), transposon tagging and resistance gene analogs (RGAs) can be used to target the location

of the trait or gene of interest. Aneuploids are described as cells that have an abnormal number of chromosomes, either more or less than what is expected. They are used to determine the chromosomal location of a gene and transfer a specific chromosome or chromosome regions from one cultivar to the next (Shimelis and Spies 2011). A complete set of hexaploid wheat aneuploid cultivars (cytogenetic stocks) were produced by Sears (1954).

Aneuploids of bread wheat are divided into monosomics (2x-1), nullisomics (2x-2), trisomics (2x+1) and tetrasomics (2x+2). On the other hand, aneuploids of durum wheat are categorised into monosomics (4x-1=27), D-genome substitution monosomics (4x-1+1=28), ditelomonotelosomics (2x=27+2t+t), double ditelosomics (2x=27+2t+2t) and D-genome disomic substitutions (4x-2+2=28; Shimelis and Spies 2011). Monosomic lines are used to localise genes in both hexaploid and tetraploid wheats.

The most common type of experimental population used for analysis of quantitative traits is NILs (Keurentjes et al. 2007). Near isogenic lines can be constructed through a variety of methods. Introgression lines carry a single target locus from a donor variety in a recurrent background (Kooke et al. 2012). According to Tanksley et al. (1995) NILs are created when breeders cross a donor line (P1) with a recipient line (P2). The resulting $F_1$ is backcrossed to the recipient line to produce the first backcross generation ($BC_1$). A single individual in the $BC_1$ population that contains the dominant allele of the target gene from P1 is selected. The selection is made based on the phenotype followed by the $BC_1$ individual being crossed back to P2 and the cycle is repeated for several generations (Kooke et al. 2012). In the $7^{th}$ generation, most of the genome will be derived from P2, except for a small chromosomal segment containing the selected dominant allele that is derived from P1. In plant breeding, the recipient parent is usually an enduring variety or inbred line that is durable in the field. The NIL technique is used to confirm previously identified genetic loci, for fine mapping and for targeting genes for cloning (Keurentjes et al. 2007). However, it is time-consuming to develop a NIL population. To overcome this problem, the BSA technique was developed.

Bulk segregant analysis is a technique used for targeting genes or QTL of interest by identifying a genomic region that contains genetic loci that affect the trait of interest (Michelmore et al. 1991; Becker et al. 2011; Magwene et al. 2011). The technique involves screening for differences between two pools of DNA samples of individuals from a segregating population that originated from a single cross. In each bulk the individuals

carry the same trait of interest but are random for all other traits, for example, two DNA bulks with one consisting of 10 susceptible and the other 10 resistant individuals (Quarrie et al. 1999; Uma et al. 2016). Markers that are polymorphic between the pools are linked to the loci determining the trait used to construct the pools (Michelmore et al. 1991). The two bulks are screened in the same way as NILs with several probes or markers, however, BSA overcomes the limitations that occur with using NILs to identify markers linked to the gene of interest (Michelmore et al. 1991; Tanksley et al. 1995).

To increase the efficiency of targeting strategies, plant breeders combine several techniques. Another gene targeting strategy is induced mutations that have been used to improve major crops that are seed propagated e.g. rice, wheat, barley and cotton, etc. Induced mutations are used in basic research where mutants are used to analyse the function of genes. In plant breeding, nuclear techniques have been used to induce mutations. This strategy has been used to improve well-adapted varieties by altering one or two major traits (Ahloowalia and Maluszynski 2001). A method that allows direct identification of mutations in specific genes was developed in the 2000s, namely TILLING.

The TILLING technique is a reverse genetic method used in the rapid detection of small mutations and natural polymorphisms. This method identifies single bp changes of a target gene and has been demonstrated in the model plant, *Arabidopsis thaliana* L. Heynhold and extended to improve crop plants (Slade and Knauf 2005). According to Simsek and Kacar (2010) TILLING is suitable for most plants. The technique consists of several major steps. Firstly, single bp mutations are induced in genomes using chemical mutagens such as ethyl methanosulfonate (EMS). Secondly, DNA samples are prepared and extracted for mutational screening. Thirdly, the samples are pooled and arrayed into a microtiter plate and lastly, mutations are discovered using PCR-based markers such as SNPs (Till et al. 2003; Simsek and Kacar 2010). TILLING is described as a high throughput, sensitive, cost-effective and a rapid means to find genetic variation (Slade and Knauf 2005). A closely related technique to TILLING is ecotype TILLING (ECOTILLING). ECOTILLING is described as an approach that refers to a high throughput screening technique for the discovery of polymorphisms in natural populations. This technique also serves as a cheaper alternative to full DNA sequencing. In addition, ECOTILLING is used for mapping, association analysis, mutational profiling and biodiversity studies (Kaur et al. 2008).

Resistance gene analogs are disease resistance genes identified based on their structure. This is an effective tool for identifying and isolating resistance genes and is efficient in building durable resistance (Khan 2009). Well known RGAs include nucleotide binding sites, leucine-rich repeats, receptor-like kinases and receptor-like proteins (Sekhwal et al. 2015). Several RGAs have been identified from sequenced plant genomes using bioinformatics tools. According to Sekhwal et al. (2015), high density genome wide RGA genetic maps are useful in the identification of QTL or markers that are linked to disease resistance. After identification and mapping, RGAs can be cloned using transposon tagging and map-based cloning.

Transposon tagging is a cloning strategy that relies on transposons to provide DNA tags with a known sequence. Transposons are defined as mobile genetic elements that amplify in a genome (Settles 2009). Transposable elements were initially discovered in maize and later found in all organisms (Zale and Steber 2002). The transposon's sequence is used to identify DNA sequences adjacent to the transposable elements that are useful only when the sequence of the transposon is known (Settles 2009). Mutants are characterised based on their phenotypes and tagged alleles are identified to explain the molecular cause of phenotypes. Transposons are used in reverse genetics where mutations that affect the sequence are identified through the use of PCR. In wheat, a transposon tagging system is used because of its large genome (Zale and Steber 2002).

## 2.5 Summary and motivation for this study

Although SA is not self-sufficient in wheat production, it is the biggest wheat producer in sub-Saharan Africa and the fourth biggest wheat producer on the African continent (Figlan et al. 2014). The decline in wheat production area planted since the late nineties is mostly the result of an increase in production costs and abiotic constraints such as rising winter temperatures in combination with changing rain patterns in the Western Cape and Free State provinces. Wheat production under irrigation in all provinces has remained stable during this time. Biotic factors such as rust diseases contribute to the risk of wheat production and planting susceptible cultivars increases input costs due to the application of fungicides. Although no recent stem rust epidemic has been reported in SA, the continuous detection of new stem rust races is a concern.

In SA, stem rust has evolved, and five races within the Ug99 linage have been reported that are virulent towards *Sr8b*, *Sr24*, *Sr31*, *Sr38* and *Sr9h*. Although *Sr24* virulence has been reported previously in SA, the combination of *Sr24* and *Sr31* virulence by race PTKST is a concern. To mitigate the risk of *Pgt*, it is important that wheat breeders continuously identify and incorporate new sources of stem rust resistance. Mapping novel sources of rust resistance and the development of closely linked reliable molecular markers would allow the rapid deployment of such sources in more complex gene stacks that may contribute towards durability of resistance.

## 2.6 References


AgriOrbit (2018). South African wheat production under the spotlight. AgriOrbit. Available at: https://www.agriorbit.com/south-african-wheat-production-under-the-spotlight/ (Accessed: 8 April 2019).

Ahloowalia, B. S., and Malusyznski, M. (2001). Induced mutation - A new paradigm in plant breeding. *Euphytica,* 118: 167-173.

Aktar-Uz-Zaman, M., Tuhina-Khatun, M., Hanafi, M. M., and Sahebi, M. (2017). Genetic analysis of rust resistance genes in global wheat cultivars: an overview. *Biotechnology and Biotechnological Equipment,* 31: 431-445.

Ali, S., and Hodson, D. (2017). Wheat rust surveillance: Field disease scoring and sample collection for phenotyping and molecular genoyping. In: Periyannan, S. (ed) *Wheat Rust Diseases: Methods and Protocols*, *Methods in Molecular Biology*.Volume 1659. Springer, New York, pp 3-11.

Badarcki, F. (2001). Random amplified polymorphic DNA (RAPD) markers. *Turkish Journal of Biology,* 25: 185-196.

Bajgain, P., Rouse, M. N., Tsilo, T. J., Macharia, G. K., Bhavani, S., Jin, Y., and Anderson, J. A. (2016). Nested association mapping of stem rust resistance in wheat using genotyping by sequencing. *PLoS ONE,* 11: e0155760.

Barnard, A., and Smith, M. F. (2009). The effect of rainfall and temperature on the preharvest sprouting tolerance of winter wheat in the dryland production areas of the Free State Province. *Field Crops Research,* 112: 158-164.

Barnard, A., and Smith, M. F. (2012). Determination of the influence of climate on falling number of winter wheat in the dryland production areas of the Free State Province of South Africa. *Euphytica,* 188: 15-24.

Becker, A., Chao, D.-Y., Zhang, X., Salt, D. E., and Baxter, I. (2011). Bulk segregant analysis using single nucleotide polymorphism microarrays. *PLoS ONE,* 6: e15993.

Bender, C. M., Prins, R., and Pretorius, Z. A. (2016). Development of a greenhouse screening method for adult plant response in wheat to stem rust. *Plant Disease,* 100: 1627-1633.

Bhattacharya, S. (2017). Deadly new wheat disease threatens Europe's crops. *Nature,* 542: 145-146.

Boshoff, W. H., Pretorius, Z. A., van Niekerk, B. D., and Komen, J. S. (2002). First report of virulence in *Puccinia graminis* f. sp. *tritici* to wheat stem rust resistance genes *Sr8b* and *Sr38* in South Africa. *Plant Disease,* 86: 922.

Boshoff, W. H., van Niekerk, B. D., and Pretorius, Z. A. (2000). Pathotypes of *Puccinia graminis* f. sp. *tritici* detected in South Africa during 1991-1997. *South African Journal of Plant and Soil,* 17: 60-62.

Chawade, A., van Ham, J., Blomquist, H., Bagge, O., Alexandersson, E., and Ortiz, R. (2019). High-throughput field-phenotyping tools for plant breeding and precision agriculture. *Agronomy,* 9: 1-18.

Chen, S., Zhang, W., Bolus, S., Rouse, M. N., and Dubcovsky, J. (2018). Identification and characterization of wheat stem rust resistance gene *Sr21* effective against the Ug99 race group at high temperature. *PLoS Genetics,* 14:e1007287.

Chen, X. M. (2005). Epidemiology and control of stripe rust [*Puccinia striiformis* f. sp. *tritici*] on wheat. *Canadian Journal of Plant Pathology,* 27: 314-337.

Chukwu, S. C., Rafii, M. Y., Ramlee, S. I., Ismail, S. I., Oladosu, Y., Okporie, E., Onyishi, G., Utobo, E., Ekwu, L., Swaray, S., and Jalloh, M. (2019). Marker-assisted selection and gene pyramiding for resistance to bacterial leaf blight disease of rice (*Oryza sativa* L.). *Biotechnology and Biotechnological Equipment,* 33: 440-455.

Cobb, J. N., Biswas, P. S., and Platten, J. D. (2019). Back to the future: revisiting MAS as a tool for modern plant breeding. *Theoretical and Applied Genetics, 132,* 647-667.

Cockram, J., and Mackay, I. (2018). Genetic mapping populations for conducting high-resolution trait mapping in plants. *Advances in Biochemical Engineering/Biotechnology*, 164: 109-138.

Collard, B. C., and Mackill, D. J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society*, 363: 557-572.

Collard, B. C., Jahufer, M. Z., Brouwer, J. B., and Pang, E. C. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica*, 142: 169-196.

Department of Agriculture, Forestry and Fisheries. (DAFF) (2016). *Production Guideline for Wheat.* Pretoria: Department of Agriculture, Forestry and Fisheries.

Department of Agriculture, Forestry and Fisheries. (DAFF) (2017). *Trends in the Agricultural Sector*. Pretoria: Department of Agriculture, Forestry and Fisheries.

Department of Agriculture, Forestry and Fisheries. (DAFF) (2018). *Trends in the Agricultural Sector*. Pretoria: Department of Agriculture, Forestry and Fisheries.

Devos, K. M., and Gale, M. D. (1992). The use of random amplified polymorphic DNA markers in wheat. *Theoretical and Applied Genetics,* 84: 567-572.

Dubcovsky, J. (2004). Marker-assisted selection in public breeding programs. *Crop Science, 44*, 1895.

Ellis, J. G., Lagudah, E. S., Spielmeyer, W., and Dodds, P. N. (2014). The past, present and future of breeding rust resistant wheat. *Frontiers in Plant Science*, 5: 1-13.

Figlan, S., Le Roux, C., Terefe, T., Botes, W., Visser, B., Shimelis, H., and Tsilo, T. J. (2014). Wheat stem rust in South Africa: Current status and future research directions. *African Journal of Biotechnology*, 13: 4188-4199.

Figueroa, M., Hammond-Kosack, K. E., and Solomon, P. S. (2018). A review of wheat diseases - A field perspective. *Molecular Plant Pathology*, 19: 1523-1536.

Flor, H. H. (1942). Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology,* 32: 653-669.

Flor, H. H. (1971). Current status of the gene-for-gene concept. *Annual Review of Phytopathology,* 9: 275-296.

Gadaleta, A., Giancaspro, A., Giove, S. L., Zacheo, S. A., Mangini, G., Simeone, R., Signorile, A., and Blanco, A. (2009). Genetic and physical mapping of new EST-derived SSRs on the A and B genome chromosomes of wheat. *Theoretical and Applied Genetics,* 118: 1015-1025.

Ganal, M. W., and Röder, M. S. (2007). Microsatellite and SNP markers in wheat breeding. In: Varshney, R. K., Tuberosa, R. (eds) *Genomics-Assisted Crop Improvement, Genomics Applications in Crops*. Volume 2. Springer, Dordrecht, pp 1-24.

Garrido-Cardenas, J. A., Mesa-Valle, C., and Manzano-Agugliaro, F. (2018). Trends in plant research using molecular markers. *Planta,* 247: 543-557.

Gazal, A., Dar, Z. A., Wani, S. H., Lone, A. A., Shikari, A. B., Ali, G., and Abidi, I. (2016). Molecular breeding for enhancing resilience against biotic and abiotic stress in major cereals. *SABRAO Journal,* 48: 1-32.

Ghazvini, H., Hiebert, C. W., Zegeye, T., Liu, S., Dilawari, M., Tsilo, T., Anderson, J. A., Rouse, M. N., Jin, Y., and Fetch, T. (2012). Inheritance of resistance to Ug99 stem rust in wheat cultivar Norin 40 and genetic mapping of *Sr42*. *Theoretical and Applied Genetics,* 125: 817-824.

Goldblatt, A. (2011). *Agriculture: Facts and Trends.* Cape Town: World Wide Fund. pp 1-32.

Gupta, P. K., and Varshney, R. K. (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica,* 113: 163-185.

Gupta, P. K., Langridge, P., and Mir, R. R. (2010). Marker-assisted wheat breeding: present status and future possibilities. *Molecular Breeding,* 26: 145-161.

Haile, J. K., Nachit, M. M., Hammer, K., Badebo, A., and Röder, M. S. (2012). QTL mapping of resistance to race Ug99 of *Puccinia graminis* f. sp. *tritici* in durum wheat (*Triticum durum* Desf.). *Molecular Breeding,* 30: 1479-1493.

Hussain, M., Khan, M. A., Hussain, M., Javed, N., and Khaliq, I. (2016). Phenotypic characterization of wheat selected lines for resistance against leaf, stripe and stem

rust through phenotypic markers application. *The Journal of Animal and Plant Sciences*, 26: 185-193.

Huttner, E., Wenzl, P., Akbari, M., Caig, V., Carling, J., Cayla, C., Evers, M., Jaccous, D., Peng, K., Patarapuwadol, S., Uszynski, G., Xia, L., Yang, S., and Kilian, A. (2005). Diversity arrays technology: A novel tool for harnessing the genetic potential of orphan crops. In: Serageldin, I., Persley, G. J. (eds) *Discovery to Delivery: BioVision Alexandria.* CABI Publishing, Alexandria, pp 145-155.

Jaccoud , D., Peng, K., Feinstein, D., and Kilian, A. (2001). Diversity arrays: a solid state technology for sequence information independent genotyping. *Nucleic Acids Research,* 29: 1-7.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M., and Kersters, K. (1996). Evaluation of the DNA figerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology,* 142: 1881-1893.

Jehan, T., and Lakhanpaul, S. (2006). Single nucleotide polymorphism (SNP) - Methods and applications in plant genetics: A review. *Indian Journal of Biotechnology,* 5: 435-459.

Jin, Y., Szabo, L. J., Pretorius, Z. A., Singh, R. P., Ward, R., and Fetch, T. J. (2008). Detection of virulence to resistance gene *Sr24* within race TTKS of *Puccinia graminis* f. sp. *tritici*. *Plant Disease,* 92: 923-926.

Kaur, N., Street, K., Mackay, M., Yahiaoui, N., and Keller, B. (2008). Molecular approaches for characterization and use of natural disease resistance in wheat. *European Journal of Plant Pathology,* 121: 389-397.

Keurentjes, J. J., Bentsink, L., Alonso-Blanco, C., Hanhart, C. J., Blankestijn-De Vries, H., Effgen, S., Vreugdenhil, D., and Koornneef, M. (2007). Development of a near-isogenic line population of *Arabidopsis thaliana* and comparison of mapping power with a recombinant inbred line population. *Genetics,* 175: 891-905.

Khan, M. A. (2009). Importance and use of resistance gene analogs. *The Open Plant Science Journal,* 3: 54-60.

Kielsmeier-Cook, J., Danilova, T. V., Friede, B., and Rouse, M. N. (2015). Resistance to the Ug99 race group of *Puccinia graminis* f. sp. *tritici* in wheat-intra/intergeneric hybrid derivatives. *Plant Disease,* 99: 1317-1325.

Knott, D. R. (1961) The inheritance of rust resistance VI. The transfer of stem rust resistance from *Agropyron elongatum* to common wheat. *Canadian Journal of Plant Science,* 41:109-123 .

Kooke, R., Wijnker, E., and Keurentjes, J. J. (2012). Backcross populations and near isogenic lines. In: Rifkin, S. C. (ed) *Quantitative Trait Loci (QTL): Methods and Protocols,* Methods in Molecular Biology. Volume 871. Springer Science and Business Media, New York, pp 3-16.

Lagudah, E. S. (2011). Molecular genetics of race non-specific rust resistance in wheat. *Euphytica,* 179: 81-91.

Langridge, P. (2017). *Achieving Sustainable Cultivation of Wheat; Volume 1: Wheat Diseases, Pests and Diseases.*Volume 1. Burleigh Dodds Science Publishing, Cambridge, pp 1-629.

Langridge, P., and Chalmers, K. J. (2005). The principle: Identification and application of molecular markers. In: Lörz, H., and Wenzel, G. (eds) *Molecular Marker Systems in Plant Breeding and Crop Improvement, Biotechnology in Agriculture and Forestry*, Volume 55. Elsevier, Berlin, pp 3-22.

Leonard, K. J., and Szabo, L. J. (2005). Stem rust of small grains and grasses caused by *Puccinia graminis*. *Molecular Plant Pathology,* 6: 99-111.

Lewis, C. M., Persoons, A., Bebber, D. P., Kigathi, R. N., Maintz, J., Findlay, K., Bueno-Sancho, V., Corredor-Moreno, P., Harington, S. A., Kangara, N., Berlin, A., Garcia, R., German, S. E., Hanzalova, A., Hodson, D. P., Hovmøller, M. S., Huerta-Espino, J., Imtiaz, I., Mirza, J. I., Justesen, A. F., Niks, R. E., Omrani, A., Patpour, M., Pretorius, Z. A., Roohparvar, R., Sela, H., Singh, R. P., Seteffenson, B., Visser, B., Fenwick, P. M., Thomas, J., Wulff, B. B. H., and Saunders, D. G. (2018). Potential for re-emergence of wheat stem rust in the United Kingdom. *Communications Biology,* 1: 1-9.

Li, L., Fang, Z., Zhou, J., Chen, H., Hu, Z., Gao, L., Chen, L., Ren, S., Ma, H., Lu, L., Zhang, W., and Peng, H. (2017). An accurate and efficient method for large-scale SSR genotyping and applicatons. *Nucleic Acids Research*, 45: 1-12.

Li, T. Y., Cao, Y. Y., Wu, X. X., Xu, X. F., and Wang, W. L. (2016). Seedling resistance to stem rust and molecular marker analysis of resistance genes in wheat cultivars of Yunnan, China. *PLoS ONE*, 11: e0165640.

Liu, S., Yu, L.-X., Singh, R. P., Jin, Y., Sorrells, M. E., and Anderson, J. A. (2010). Diagnostic and co-dominant PCR markers for wheat stem rust resistance genes *Sr25* and *Sr26*. *Theoretical and Applied Genetics*, 120: 691-697.

Lowe, I., Cantu, D., and Dubcovsky, J. (2011). Durable resistance to the wheat rusts: Integrating systems biology and traditional phenotype-based research methods to guide the deployment of resistance genes. *Euphytica*, 179: 69-79.

Macauley, H. (2015). 'Cereal Crops: Rice, Maize, Millet, Sorghum, and Wheat'. *Abdou Diouf International Conference Center*. Dakar, Senegal, 21 - 23 October 2015.

Mago, R., Tabe, L., Vautrin, S., Simkova, H., Kubalakova, M., Upadhyaya, N., Berges, H., Kong, X., Breen, J., Dolezel, J., Appels, R., Ellis, J., and. Spielmeyer, W. (2014). Major haplotype divergence including multiple germin-like protein genes, at the wheat *Sr2* adult plant resistance locus. *BMC Plant Biology*, 14: 379.

Magwene, P. M., Willis, J. H., and Kelly, J. K. (2011). The statistics of bulk segregant analysis using next generation sequencing. *PLoS ONE*, 7: e1002255.

Mammadov, J., Aggarwal, R., Buyyarapu, R., and Kumpatla, S. (2012). SNP markers and their impact on plant breeding. *International Journal of Plant Genomics*, 2012: 1-11.

McIntosh, R. A. (2009). History and status of the wheat rusts. *Proceedings of the 2009 Technical Workshop of the Borlaug Global Rust Initiative*. Oral Papers, Sonora, pp 11-23.

McIntosh, R. A., Wellings, C. R., and Park, R. F. (1995). *Wheat rusts: An atlas of resistance gene*. Springer, Dordrecht, pp 1-200.

Michelmore, R. W., Paran, I., and Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect

markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences of the United States of America,* 88: 9828-9832.

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

Mohanty, S. K., and Swain, M. R. (2019). Bioethanol production from corn and wheat: Food, fuel, and future. In: Ray, R. C., Ramachandran, C. (eds) *Bioethanol Production from Food Crops*, Volume 1. Elsevier, London, pp 45-59.

Monsanto. (2015). *Soil and Nutrient Management for Wheat.* Monsanto, Illinois. pp 1-2.

Mueller, U. G., and Wolfenbarger, L. L. (1999). AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution,* 14: 389-394.

Nhemachena, C. R., and Kirsten, J. (2017). A historical assessment of sources and uses of wheat varietal innovations in South Africa. *South African Journal of Science,* 113: 1-8.

Njau, P. N., Bhavani, S., Huerta-Espino, J., Keller, B., and Singh, R. P. (2013). Identification of QTL associated with durable adult plant resistance to stem rust race Ug99 in wheat cultivar 'Pavon 76'. *Euphytica*, 190: 33-44.

Oliver, R. P. (2014). A reassessment of the risk of rust fungi developing resistance to fugicides. *Pest Management Science,* 70: 1641-1645.

Olivera, P., Newcomb, M., Szabo, L. J., Rouse, M., Johnson, J., Gale, S., Luster, D. G., Hodson, D., Cox, J. A., Burgin, L., Hort, M., Gilligan, C. A., Patpour, M., Justesen, A. F., Hovmøller, M. S., Woldeab, G., Hailu, E., Hundie, B., Tadesse, K., Pumphrey, M., Singh, R. P., and Jin, Y. (2015). Phenotypic and genotypic characterization of race TKTTF of *Puccinia graminis* f. sp. *tritici* that caused a wheat stem rust epidemic in Southern Ethiopia in 2013-14. *Phytopathology,* 105: 917-928.

Pathan, A. K., and Park, R. F. (2007). Evaluation of seedling and adult plant resistance to stem rust in European wheat cultivars. *Euphytica*, 155: 87-105.

Pestsova, E., Ganal, M. W., and Röder, M. S. (2000). Isolation and mapping of microsatellite markers specific for the D genome of bread wheat. *Genome*, 43: 689-697.

Peterson, R. F., Campbell, A. B., and Hannah, A. E. (1948). A diagrammatical scale for estimating rust intensity on leaves and stems of cereals. *Canadian Journal of Research,* 26: 496-500.

Pradhan, S., Sapkota, M., and Thapa, D. B. (2016). Wheat rust: The devastation and attempts to control over the years. *International Journal of Experimental Research and Review,* 8: 9-22.

Pretorius, Z. A., Bender, C. M., Visser, B., and Terefe, T. (2010). First report of a *Puccinia graminis* f. sp. *tritici* race virulent to the *Sr24* and *Sr31* wheat stem rust resistance genes in South Africa. *Plant Disease*, 94: 784.

Pretorius, Z. A., Pakendorf, K. W., Marais, G. F., Prins, R., and Komen, J. S. (2007). Challenges for sustainable cereal rust control in South Africa. *Australian Journal of Agricultural Research,* 58: 593-601.

Pretorius, Z. A., Singh, R. P., Wagoire, W. W., and Payne, T. S. (2000). Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis* f. sp. *tritici* in Uganda. *Plant Disease,* 84: 203.

Pretorius, Z. A., Szabo, L. J., Boshoff, W. H., Herselman, L., and Visser, B. (2012). First report of a new TTKSF race of wheat stem rust (*Puccinia graminis* f. sp. *tritici*) in South Africa and Zimbabwe. *Plant Disease*, 96: 590.

Pujol, V., Robeles, P., Wang, J., Taylor, P., Tabe, L. Z., and Lagudah, E. (2016). Cellular and molecular characterization of a stem rust resistance locus on wheat chromosome 7AL. *BMC Research Notes,* 9: 502.

Quarrie, S. A., Lazic-Jancic, V., Kovacevic, D., Steed, A., and Pekic, S. (1999). Bulk segregant analysis with molecular markers and its use for improving drought resistance in maize. *Journal of Experimental Biology,* 50: 1299-1306.

Rafalski, A. (2002). Applications of single nucleotide polymorphisms in crop genetics. *Current Opinion in Plant Biology,* 5: 94-100.

Raman, H., Stodart, B. J., Cavanagh, C., Mackay, M., Morell, M., Milgate, A., and Martin, P. (2010). Molecular diversity and genetic structure of modern and traditional landrace cultivars of wheat (*Triticum aestivum* L.). *Crop and Pasture Science,* 61: 222-229.

Rasheed, A., and Xia, X. (2019). From markers to genome-based breeding in wheat. *Theoretical and Applied Genetics,* 132: 767-784.

Rehman, A. U., Sajjad, M., Khan, S. H., and Ahmad, N. (2013). Prospects of wheat breeding for durable resistance against brown, yellow and black rust fungi. *International Journal of Agriculture and Biology*, 15: 1209-1220.

Riaz, A., and Hickey, L. T. (2017). Rapid phenotyping adult plant resistance to stem rust in wheat grown under controlled conditions. In: Periyannan, S. (ed) *Wheat Rust Diseases: Methods and Protocols,* Methods in Molecular Biology. Volume 1659. Springer, New York, pp 183-196.

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

Röder, M. S., Plaschke, J., Konig, S. U., Borner, A., Sorrells, M. E., Tanksley, S. D., and Ganal, M. W. (1995). Abundance, variability and chromosomal location of microsatellites in wheat. *Molecular Genetics,* 246: 327-333.

Roelfs, A. P., and Bushnell, W. R. (1985). *The Cereal Rusts: Diseases, Distribution, Epidemiology, and Control.* Volume 2. Academic Press, Orlando, pp 1-630.

Roelfs, A. P., Singh, R. P., and Saari, E. E. (1992). *Rust Diseases of Wheat: Concepts and Methods of Disease Management.* CIMMYT, Mexico, pp 1-81.

Rouse, M. N., Nirmala, J., Jin, Y., Chao, S., Fetch Jr, T. G., Pretorius, Z. A., and Hiebert, C. W. (2014). Characterization of *Sr9h*, a wheat stem rust resistance allele effective to Ug99. *Theoretical and Applied Genetics*, 127: 1681-1688.

Rutkoski, J. E., Heffner, E. L., and Sorrells, M. E. (2011). Genomic selection for durable stem rust resistance in wheat. *Euphytica,* 179: 161-173.

Saccomanno, A., Matny, O., Marone, D., Laido, G., Petruzzino, G., Mazzucotelli, E., Desiderio, F., Blanco, A., Gadaleta, A., Pecchioni, N., de Vita, P., Steffenson, B., and Mastrangelo, A. M. (2018). Genetic mapping of loci for resistance to stem rust

in a tetraploid wheat collection. *International Journal of Molecular Sciences,* 19: 1-15.

Saini, J., Faris, J. D., Zhang, Q., Rouse, M. N., Jin, Y., Long, Y., Klindworth, D. L., Elias, E. M., McClean, P. E., Edwards, M. C., and Xu, S. S. (2018). Identification, mapping, and marker development of stem rust resistance genes in durum wheat 'Lesbock'. *Molecular Breeding,* 38: 77.

Sayaslan, A., Koyuncu, M., Turker, S., Irkh, Y., Serin, A., and Orhan, F. G. (2008). Use of durum wheat clear flour in vital gluten and bioethanol production. *Journal of Cereal Science,* 80: 50-56.

Schumann, G. L., and Leonard, K. J. (2000). Stem rust of wheat. *The Plant Health Instructor,* doi:10.1094/PHI-I-2000-0721-01.

Sears, E. R. (1954). The aneuploids of common wheat. *Missouri Agricultural Experiment Station Research Bulletin, 572,* 59.

Sekhwal, M. K., Li, P., Lam, I., Wang, X., Cloutier, S., and You, F. M. (2015). Disease resistance gene analogs (RGAs) in plants. *International Journal of Molecular Science,* 16: 19248-19290.

Settles, A. M. (2009). Transposon tagging and reverse genetics. In: Kriz, A. L., Larkins, B. A. (eds) *Molecular Genetic Approaches to Maize Improvement Biotechnology in Agriculture and Forestry*. Volume 63. Springer-Verlag, Berlin, pp 143-159.

Shewry, P. R. (2009). Wheat. *Journal of Experimental Botany,* 60: 1537-1553.

Shimelis, H., and Spies, J. J. (2011). Aneuploids of wheat and chromosomal localization of genes. *African Journal of Technology,* 10, 5545-5551.

Simsek, O., and Kacar, Y. A. (2010). Discovery of mutations with TILLING and ECOTILLING in plant genomes. *Scientific Research and Essays,* 24: 3799-3802.

Singh, R. P., Hodson, D. P., Huerta-Espino, J., Jin, Y., Bhavani, S., Njau, P., Herrera-Foessel, S., Singh, P. K., Singh, S., and Govindan, V. (2011). The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. *Annual Review of Phytopathology,* 49: 465-481.

Singh, R. P., Hodson, D. P., Jin, Y., Lagudah, E. S., Ayliffe, M. A., Bhavani, S., Rouse, M. N., Pretorius, Z. A., Szabo, L. J., Huerta-Espino, J., Basnet, B. R., Lan, C., and Hovmøller, M. S. (2015). Emergence and spread of new races of wheat stem rust fungus: continued threat to food security and prospects of genetic control. *Phytopathology,* 105: 872-884.

Singh, R. P., Huerta-Espino, J., and Roelfs, A. P. (2002). The wheat rusts. In: Curtis, B. C, Rajaram, S., Gómez Macpherson, H (eds) *Bread Wheat: Improvement and Production*. Plant Production and Protection Series no. 30. FAO, Rome, pp 317-330.

Slade, A. J., and Knauf, V. C. (2005). TILLING moves beyond functional genomics into crop improvement. *Transgenic Research,* 14: 109-115.

Soko, T., Bender, C. M., Prins, R., and Pretorius, Z. A. (2018). Yield loss associated with different levels of stem rust resistance in bread wheat. *Plant Disease,* 102: 2531-2538.

Sosibo, N. Z., Muchaonyerwa, P., Visser, L., Barnard, A., Dube, E., and Tsilo, T. J. (2017). Soil fertility constraints and yield gaps of irrigation wheat in South Africa. *South African Journal of Science,* 113: 1-9.

South African Grain Laboratory. (SAGL) (2018). *South African Wheat Crop Quality Report 2017/2018.* South African Grain Laboratory, Pretoria, pp 1-147.

Spanic, V., Rouse, M. N., Kolmer, J. A., and Anderson, J. A. (2015). Leaf and stem seedling rust resistance in wheat cultivars grown in Croatia. *Euphytica,* 203: 437-448.

Stakman, E. C., Stewart, D. M., and Loegering, W. Q. (1962). *Identification of Physiologic Races of Puccinia graminis var tritici.* U.S.D.A. Beureau of Entomology, Minnesota, pp E1-E617.

Suresh, K., Kiransree, N., and Venkateswar Rao, L. (1999). Production of ethanol by raw starch hydrolysis and fermentation of damaged grains of wheat and sorghum. *Bioprocess Engineering,* 21: 165-168.

Tadesse, K., Ayalew, A., and Badebo, A. (2010). Effect of fungicide on the development of wheat stem rust and yield of wheat varieties in highlands of Ethiopia. *African Crop Science Journal,* 18: 23-33.

Tanksley, S. D., Ganal, M. W., and Martin, G. B. (1995). Chromosome landing: A paradigm for map-based gene cloning in plants with large genomes. *Trends in Genetics,* 11: 63-68.

Terefe, T., Pretorius, Z. A., Boshoff, W. H., and Visser, B. (2019). First report of *Puccinia graminis* f. sp. *tritici* race PTKSK, a variant of wheat stem rust race Ug99 in South Africa. *Plant Disease,* 103: 1-3.

Terefe, T., Pretorius, Z. A., Paul, I., Mebalo, J., Meyer, L., and Naicker, K. (2010). Occurrence and pathogenicity of *Puccinia graminis* f. sp. *tritici* on wheat in South Africa during 2007 and 2008. *South African Journal of Plant and Soil,* 27: 163-167.

Terefe, T. G., Visser, B., and Pretorius, Z. A. (2016). Variation in *Puccinia graminis* f. sp. *tritici* detected on wheat and triticale in South Africa from 2009 to 2013. *Crop Protection,* 86: 9-16.

Till, B. J., Colbert, T., Tompa, R., Enns, L. C., Codomo, C. A., Johnson, J. E., Reynolds, S. H., Henikoff, J. G., Greene, E. A., Steine, M. N., Comai, L., and Henikoff, S. (2003). High-throughput TILLING for functional genomics. In: Grotewold, E. (ed) *Methods in Molecular Biology*. Volume 236. Humana Press, New Jersey, pp 205-220.

Tsilo, T. J., Chao, S., Jin, Y., and Anderson, J. A. (2009). Identification and validation of SSR markers linked to the stem rust resistance gene *Sr6* on the short arm of chromosome 2D in wheat. *Theoretical and Applied Genetics,* 118: 515-524.

Uma, M. S., Hedge, N., and Hittalmani, S. (2016). Identification of SSR markers associated with rust resistance in cowpea (*Vigna unguiculata* L.) using bulk segregant analysis. *Legume Research,* 39: 39-42.

Umar, M., Nawaz, R., Hussain, R., Khalid, M. W., and Siddique, M. S. (2019). Molecular basis and genetics of stem rust resistance in wheat. *Asian Journal of Research in Crop Science,* 4: 1-9.

Velu, G., and Singh, R. P. (2013). Phenotyping in wheat breeding. In: Panguluri, S. K., Kumar, A. A. (eds) *Phenotyping for Plant Breeding: Applications of Phenotyping Methods for Crop Improvement*. Springer, New York, pp 41-71.

Vierling, R. A., and Nguyen, H. T. (1992). Use of RAPD markers to determine the genetic diversity of diploid wheat genotypes. *Theoretical and Applied Genetics,* 84: 835-838.

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

Vuylsteke, M., Peleman, J. D., and van Eijk, M. J. (2007). AFLP technology for DNA fingerprinting. *Nature Protocols,* 2: 1387-1398.

Waldron, L. R., and Clark, J. A. (1936). Breeding rust-resistant spring wheats. *Science,* 83: 106-108.

Wanyera, R., Macharia, J. K., Kilonzo, S. M., and Kamundia, J. W. (2009). Foliar fungicides to control wheat stem rust, race TTKS (Ug99), in Kenya. *Plant Disease,* 93: 929-932.

Wenzl, P., Carling, J., Kudrna, D., Jaccoud, D., Huttner, E., Kleinhofs, A., and Kilian, A. (2004). Diversity Arrays Technology (DArT) for whole-genome profiling of barley. *Proceedings of the National Academy of Sciences of the United States of America,* 101: 9915-9920.

William, R. C. (1989). Restriction fragment length polymorphism (RFLP). *Yearbook of Physical Anthropology,* 32: 159-184.

William, H. M., Trethowan, R., and Crosby-Galvan, E. M. (2007). Wheat breeding assisted by markers: CIMMYT's experience. *Euphytica*, 157: 307-319.

Williams, J. G., Kubelik, A., Livak, K. J., Rafalski, J. A., and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research,* 18: 6531-6535.

Xu, X. F., Li, D. D., Liu, Y., Gao, Y., Wang, Z. Y., Ma, Y. C., Yang, S., Cao, Y. Y., Xuan, Y. H., and Li, T. Y. (2017). Evaluation and identification of stem rust resistance genes *Sr2, Sr24, Sr25, Sr26, Sr31* and *Sr38* in wheat lines from Gansu Province in China. *Peer Journal,* 5: e4146.

Xu, X., Yuan, D., Li, D., Gao, Y., Wang, Z., Liu, Y.,Wang, S., Xuan, Y., Zhao, H., Li, T., and Wu, Y. (2018). Identification of stem rust resistance genes in wheat cultivars in China using molecular markers. *Peer Journal,* 6: e4882.

Xu, Y. (2010). *Molecular Plant Breeding.* CABI, Beijing, pp 1-734.

Xu, Y., and Crouch, J. H. (2008). Marker-assisted selection in plant breeding: From publications to practice. *Crop Science,* 48: 391-407.

Yu, L., Barbier, H., Rouse, M. N., Singh, S., Singh, R. P., Bhavani, S., Huerta-Espino, J., and Sorrells, M. E. (2014). A consensus map for Ug99 stem rust resistance loci in wheat. *Euphytica,* 127: 1561-1581.

Zale, J. M., and Steber, C. M. (2002). Transposon-related sequences in the Triticeae. *Cereal Research Communications,* 30: 237-244.

Zhang, W., Chen, S., Abate, Z., Nirmala, J., Rouse, M. N., and Dubcovsky, J. (2017). Identification and characterization of *Sr13*, a tetraploid wheat gene that confers resistance to the Ug99 stem rust race group. *Proceedings of the National Academy of Sciences of the United States of America,* E9483-E9492.

Zimin, A., Puiu, D., Hall, R., Kingan, S., Clavijo, B. J., and Salzberg, S. L. (2017). The first-near complete assembly of the hexaploid bread wheat genome, *Triticum aestivum*. *GigaScience,* 6: 1-7.

Zurn, J. D., Rouse, M. M., Chao, S., Aoun, M., Macharia, G., Hiebert, C. W., Pretorius, Z. A., Bonman, J. M., and Acevedo, M. (2018). Dissection of the multigenic wheat stem rust resistance present in the Montenegrin spring wheat accession PI 362698. *BMC Genomics,* 19: 1-11.

# Chapter 3

# Molecular marker evaluation of wheat lines M3283_4 and M3192_7 for selected *Sr*-genes

## 3.1 Introduction

Stem rust also known as black rust, caused by the fungal pathogen *Pgt*, is an important disease of wheat worldwide. Stem rust is the most feared disease based on historic outbreaks and reported yield losses. In SA the presence of stem rust affects the commercial production of wheat by decreasing yield (Pretorius et al. 2000; Pretorius et al. 2012). Race Ug99 (TTKSK) is the first race of *Pgt* known to be virulent to the widely deployed resistance gene *Sr31* and was first detected in 1998 in Uganda (Pretorius et al. 2000).

To date, five out of 13 Ug99 races reported worldwide, have been detected in SA and these are TTKSF, TTKSP, PTKSK, PTKST and TTKSF+ (Terefe et al. 2019). These *Pgt* races vary in virulence to resistance genes *Sr9h*, *Sr21*, *Sr24* and *Sr31*. Of these races, PTKST is known as the most virulent and predominant in SA (Pretorius et al. 2010; Boshoff et al. 2019). The most efficient way to control the disease is through the deployment of resistant cultivars.

To date, more than 70 *Sr*-genes have been identified from wheat and its wild relatives (Saini et al. 2018). About 27 of these genes are still effective against stem rust races in the Ug99 lineage with *Sr13*, *Sr22*, *Sr25*, *Sr35*, *Sr39*, *Sr40* (all ASR) and *Sr2* (APR) being the most effective genes (Aktar-Uz-Zaman et al. 2017). The resistant wheat lines used in this study were derived from a cross between tetraploid (durum) and common bread wheat. According to Simons et al. (2011), several durum cultivars carry *Sr13*, which is the only characterised gene reported to confer resistance to stem rust in USA durum cultivars. *Sr13* also confers resistance to virulent stem rust races that occur in African countries (Zhang et al. 2017).

Marker-assisted selection is an effective approach of resistance breeding as it utilises molecular markers that are closely linked to the trait of interest and can be used to combine multiple disease resistance genes/QTL into a single genotype to increase durability (Collard and Mackill 2008; Pandey et al. 2019). This approach is advantageous in screening for known *Sr*-genes because it can be easily applied at the seedling stage and is inexpensive (Miedaner and Korzun 2012). The benefits and limitations of MAS are further discussed

by Mohan et al. (1997). One of the most effective marker types for MAS in wheat is SSRs. Simple sequence repeats are advantageous because they are highly informative and can be diagnostic for the targeted trait. Using closely linked markers to screen for the desired gene is inexpensive and saves time (Powell et al. 1996; Gupta and Varshney 2000). In this chapter, the stem rust resistant wheat lines M3283_4 and M3192_7, derived from a cross between tetraploid and common bread wheat, were analysed for certain known *Sr*-genes using molecular markers. The aim was to identify which, if any, of the known *Sr*-genes are present in the resistant wheat lines.

## 3.2 Materials and methods

### 3.2.1 Plant material

Seeds from the stem rust susceptible wheat variety Line 37-07 (Kasyob/Genaro-81//Cham4) and the two Canadian resistant wheat lines M3283_4 and M3192_7 (pedigrees: unavailable) were planted in 2 l pots filled with red garden top soil in the greenhouse at the University of Free State (UFS). Seeds were grown under greenhouse conditions with temperatures ranging between 18-22°C. Line 37-07 was selected from entry 37 of the 2$^{nd}$ International Stem Rust Trap Nursery (ISRTN07) based on its susceptibility to stem rust in field trials in SA. Seeds of the durum wheat cultivar Kronos were germinated in a petri dish as a positive control for *Sr13* stem rust resistance. The positive controls for the rest of the selected *Sr*-genes were supplied as DNA samples by the UFS.

Pots were watered daily and two weeks after emergence, seedlings were fertilised once weekly using 100 ml of micro-elements [Hyperfeed®, NPK Analysis 3:1:5 (9), 2.5 g/l water]. Four weeks after planting, seedlings were fertilised with macro elements [10 ml/l Effekto® Wonder Slow Release 3:2:1 (28)]. Four weeks after planting, ten leaf samples per wheat line were collected in 2 ml tubes. Scissors and tweezers used to collect leaf material were sterilised using 70% (v/v) ethanol between samples to avoid contamination. Tubes were placed on ice during sampling, then leaf samples were freeze-dried for three days using the Alpha 1-2 LD plus (Martin Christ, Osterode am Hartz, Germany) freeze-drier and stored at -20°C.

### 3.2.2 Genomic DNA isolation for genotyping

Genomic DNA (gDNA) was extracted from freeze-dried leaves using a modified hexadecyltrimethylammonium bromide (CTAB) method described by Saghai-Maroof et al. (1984). Two 3 cm pieces of the dried leaves (±250 mg) were homogenised to a fine powder

by adding two 5 mm stainless steel balls to each sample followed by homogenising using Qiagen's TissueLyser (Haan, Germany) for 1 min at 30 r/s. A total of 750 µl 2x 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 each sample and incubated for 1 h at 65°C in a water bath (Memmert, Buechenbach, Germany). A volume of 500 µl chloroform:isoamylalcohol [24:1 (v/v)] was added and centrifuged at 12 000 *g* for 5 min at 4°C using a Sigma 2-16K centrifuge [Sigma, Shropshire, United Kingdom (UK)]. The supernatant from each sample was transferred to a new 1.5 ml tube and DNA precipitated using 500 µl (0.66 volumes) isopropanol. The solution was mixed and left to incubate at room temperature (22°C) for 20 min for precipitation. The solution was centrifuged at 12 000 *g* for 5 min at 4°C and the supernatant discarded. A total of 500 µl of ice cold 70% (v/v) ethanol was added to wash the pellet. The solution was incubated for 20 min at room temperature (22°C) and centrifuged at 12 000 *g* for 5 min and the supernatant discarded. The pellet was air-dried for 1 h at room temperature (22°C). The air-dried pellet was resuspended in 200 µl of 1x TE (Tris-HCl/EDTA) buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) and left overnight at 4°C. The following day 100 mg/ml DNase-free RNase was added after which the sample was incubated at 37°C for 1 h to digest ribonucleic acid (RNA).

### 3.2.3 Determination of genomic DNA quality and quantity

Following extraction, 5 µl gDNA was diluted with 495 µl double distilled water for quantification of each DNA sample. The quantity of gDNA was measured using a Jenway 7315 spectrophotometer (Staffordshire, UK). The absorbance was measured at $A_{260}$ and $A_{280}$ and the $A_{260}$:$A_{280}$ ratio was used to determine the quality of the extracted DNA. The quality of the gDNA was visualised on a 0.8% (w/v) agarose gel stained with ethidium bromide. The agarose gel was loaded with a mixture containing 3 µl of 10x Ficoll loading dye [15% (w/v) Ficoll; 0.24% (w/v) bromophenol blue] and 3 µl of gDNA, and ran for 50 min at 80 V using 1x UNTAN buffer (40 mM Tris-HCl, pH 8.0; 2 mM EDTA, pH 8.0; pH was adjusted to 7.4 using acetic acid). Lambda (λ) DNA digested with *Eco*RI and *Hind*III was used as a size marker. After the run, the DNA was visualised using the Gel Doc™ EZ Imager (BioRad, CA, USA). The gDNA was diluted to a total volume of 200 µl using 1x TE buffer, pH 8.0 to a working concentration of 20 ng/µl. After dilution to 20 ng/ul, 10

DNA samples per genotype were bulked in 1.5 ml Eppendorf tubes to obtain an accurate genomic representation of each genotype.

### 3.2.4 Screening of molecular markers

#### 3.2.4.1 Polymerase chain reactions

All PCR reactions were performed using a T100$^{Tm}$ Thermal Cycler (BioRad, CA, USA), using molecular markers linked to 13 known *Sr*-genes of which all except *Sr31* are effective against *Pgt* in SA. The 13 known *Sr*-genes that were evaluated are listed in Table 3.1. The PCR kit used was KAPA*Taq* ReadyMix (KAPABiosystems, Wilmington, MA, USA) that contained all the components needed for PCR [0.5 U per 25 µl KAPA *Taq* DNA polymerase; KAPA *Taq* Buffer, 0.2 mM deoxynucleotide triphosphates (dNTPs); 1.5 mM magnesium chloride ($MgCl_2$); and stabilisers] except for the template and primers. However, TEMPase Hot Start 2x Master Mix (Ampliqon; Denmark) [Tris-HCl pH 8.5; ammonium sulfate ($(NH_4)_2SO_4$); 3.0 mM $MgCl_2$; 0.2% Tween® 20; 0.4 mM of each dNTP; 0.2 U/µl TEMPase DNA polymerase; and stabilisers] was used for the PCR reaction of cssfr5. All PCR primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA). The PCR cycling conditions of all the molecular markers are presented in Table 3.2. A multiplex PCR reaction was performed for *Sr39* and *Sr55*.

#### 3.2.4.2 Polyacrylamide gel electrophoresis analysis

All PCR products for the markers used, except for products from the multiplexed markers Sr39F2/R3 and BE500705, were screened using the Gel-Scan 3000 Real Time DNA Fragment Analysis system with software version 8.00.01 (Corbett Research, Sydney, Australia). The 5% (w/v) non-denaturing polyacrylamide gel was prepared to a total volume of 25 ml consisting of 1x TBE (Tris-HCl/Boric acid/EDTA) buffer (89 mM Tris base; 89 mM boric acid; 2 mM EDTA, pH 8.0) and 5% (w/v) acrylamide: bis-acrylamide (19:1). After filtration, 0.08% (v/v) APS (ammonium persulfate) and 0.12% (v/v) TEMED (N,N,N',N'-tetramethylethylenediamine) were added. The gel was left to polymerise overnight at room temperature (22°C).

The polyacrylamide gel electrophoresis (PAGE) apparatus consisted of a lower and upper chamber. The lower and upper chamber buffer contained 0.5x TBE buffer prepared using deionised water with the lower chamber buffer containing an additional 1% (v/v) ethidium bromide. For the pre-run, 1 µl of deionised formamide loading dye [98% (v/v) formamide; 10 mM EDTA, pH 8.0; 0.05% (w/v) bromophenol blue)] was loaded every fourth well and run at 800 V for 45 min at 37°C.

**Table 3.1 Markers used to screen for the presence of known stem rust resistance genes in two wheat lines M3283_4 and M3192_7**

| Targeted gene | Marker name | Forward primer (5'-3') | Reverse primer (5'-3') | Reference |
|---|---|---|---|---|
| *Sr2* | csSr2 | CAAGGGTTGCTAGGATTGGAAAAC | AGATAACTCTTATGATCTTACATTTTTCTG | Mago et al. 2011 |
| *Sr13* | Sr13 | TTCTTGGCTCAGAAGACACATG | AAGTCATCATCATCATTCCCGC | Zhang et al. 2017 |
| *Sr22* | wmc633 | ACACCAGCGGGGATATTTGTTAC | GTGCACAAGACATGAGGTGGATT | Olson et al. 2010 |
| | cfa2123 | CGGTCTTTGTTTGCTCTAAACC | ACCGGCCATCTATGATGAAG | |
| *Sr25* | PSY-D1 | TTGCAGTGCAATGGTTTTCCA | GACTCCTTTGACGATGTCTTC | Zhang and Dubcovsky 2008 |
| | PSY-E1 | CTACGTTGCGGGCACCGTT | AGAGAAAACCATTGCATCTGTA | |
| *Sr26* | Sr26#43 | AATCGTCCACATTGGCTTCT | CGCAACAAAATCATGCACTA | Mago et al. 2005 |
| | BE518379 | AGCCGCGAAATCTACTTTGA | TTAAACGGACAGAGCACACG | Liu et al. 2010 |
| *Sr31* | Iag95 | CTCTGTGGATAGTTACTTGATCGA | CCTAGAACATGCATGGCTGTTACA | Mago et al. 2002 |
| *Sr32* | csSr32#1 | GGTTTGGTGGCAACTCAGGT | CATAAGCCAAAGAGGCACCA | Mago et al. 2013 |
| | csSr32#2 | CAAATGAATAGAAAAACCCGTGCT | CACACACTGTTTTCCGTTGC | |
| *Sr36* | wmc477 | CGTCGAAAACCGTACACTCTCC | GCGAAACAGAATAGCCCTGATG | Tsilo et al. 2008 |
| | stm773-2 | ATGGTTTGTTGTGTTGTGTGTAGG | AAACGCCCCAACCACCTCTCTC | |
| *Sr39* | Sr39F2/R3 | AGAGAGAGTAGAAGAGCTGC | AGAGAGAGAGCATCCACC | Gold et al. 1999 |
| | BE500705 | ATCTGTGGCAGTGTGCTCCT | TCCTGCAAATGCTTGTCGTT | Mago et al. 2009 |
| *Sr42* | FSD_RSA | GTTTTATCTTTTTATTTC | CTCCTCCCCCCA | Ghazvini et al. 2012 |
| *Sr55/Lr67/Yr46* | cfd71 | CAATAAGTAGGCCGGGACAA | TGTGCCAGTTGAGTTTGCTC | Hiebert et al. 2011 |
| | cfd23 | TAGCAGTAGCAGCAGCAGGA | GCAAGGAAGAGTGTTCAGCC | |
| *Sr57/Lr34/Yr18* | cssfr5 | GGGAGCATTATTTTTTTCCATCATG | ACTTTCCTGAAAATAATACAAGCA | Lagudah et al. 2009 |
| | | TTGATGAAACCAGTTTTTTTTCTS | TATGCCATTTAACATAATCATGAA | |
| *Sr58/Lr46/Yr29* | wmc44 | GGTCTTCTGGGCTTTGATCCTG | GTTGCTAGGGACCCGTAGTGG | Singh et al. 1998 |
| | gwm259 | AGGGAAAAGACATCTTTTTTTTC | CGACCGACTTCGGGTTC | |
| | barc80 | GCGAATTAGCATCTGCATCTGTTTGAG | CGGTCAACCAACTACTGCACAAC | |

**Table 3.2 Polymerase chain reaction conditions for the molecular markers used in the study**

| Marker name and targeted gene | Reaction conditions and total PCR volume | PCR cycling conditions | Marker type, controls and fragment sizes |
|---|---|---|---|
| csSr2<br>*Sr2* | 100 ng DNA<br>1x KAPA*Taq* ReadyMix DNA polymerase<br>75 ng csSr2F primer<br>75 ng csSr2R primer<br>Total reaction volume: 20 µl | 1 cycle: 3 min, 93°C;<br>40 cycles: 45 s, 95°C; 45 s, 57.6°C; 45 s, 72°C;<br>1 cycle: 10 min, 72°C | Dominant marker<br>Positives and false positives (337 bp) |
| CAPS marker<br>*Sr2* | 1x Cutsmart® buffer<br>10 U *Bsp*HI | Incubate for 60 min, 37°C | Co-dominant marker<br>Resistant control: Kingbird (172 bp, 112 bp, 53 bp)<br>Susceptible control: 2S#/163 (225 bp and 112 bp) |
| Sr13<br>*Sr13* | 80 ng DNA<br>1x KAPA*Taq* ReadyMix DNA polymerase<br>25 ng Sr13F primer<br>25 ng Sr13R primer<br>Total reaction volume: 10 µl | 1 cycle: 2 min, 95°C;<br>35 cycles: 45 s, 94°C; 45 s, 58°C; 60 s, 72°C;<br>1 cycle: 4 min, 72°C | Dominant marker<br>Single allele amplifies in all genotypes (265 bp) |
| CAPS marker<br>*Sr13* | 1x Cutsmart® buffer<br>10 U *Hha*I | Incubate for 60 min, 37°C | Co-dominant marker<br>Resistant control: Kronos (244 bp)<br>Susceptible control: Line 37-07 (265 bp) |

**Table 3.2 Polymerase chain reaction conditions for the molecular markers used in the study (continued)**

| Marker name and targeted gene | Reaction conditions and total PCR volume | PCR cycling conditions | Marker type, controls and fragment sizes |
|---|---|---|---|
| wmc633<br>*Sr22* | 80 ng DNA<br>1x KAPA*Taq* ReadyMix DNA polymerase<br>25 ng WMC633F primer<br>25 ng WMC633R primer<br>Total reaction volume: 10 µl | 1 cycle: 2 min, 95°C;<br>35 cycles: 30 s, 94°C; 30 s, 56°C; 45 s, 72°C;<br>1 cycle: 5 min, 72°C | Co-dominant marker<br>Resistant control: Sr22Tb (117 bp)<br>Susceptible allele size range: 170 - 260 bp |
| cfa2123<br>*Sr22* | 80 ng DNA<br>1x KAPA*Taq* ReadyMix DNA polymerase<br>25 ng WMC633F primer<br>25 ng WMC633R primer<br>Total reaction volume: 10 µl | 1 cycle: 3 min, 95°C;<br>decrease 1°C/cycle for 15 cycles: 45 s, 95°C; 45 s, 65-51°C; 60 s, 72°C;<br>25 cycles: 45 s, 95°C; 45 s, 50°C; 60 s, 72°C;<br>1 cycle: 4 min, 72°C | Co-dominant marker<br>Resistant control: SwSr22Tb (234 bp)<br>Susceptible allele size range: 245 - 260 bp |
| PSY-D1<br>*Sr25* susceptible allele | 60 ng DNA<br>1x KAPA*Taq* ReadyMix DNA polymerase<br>25 ng PSY1_DF2 primer<br>25 ng PSY1_R3 primer<br>Total reaction volume: 10 µl | 1 cycle: 4 min, 94°C;<br>decrease 0.5°C/cycle for 10 cycles: 20 s, 94°C; 20 s, 63°C; 1 min 20 s, 72°C;<br>35 cycles: 20 s, 94°C; 20 s, 58°C; 1 min 20 s, 72°C;<br>1 cycle: 7 min, 72°C;<br>standby, 15°C | Dominant marker<br>Susceptible control: Kariega and CS-*Lr19*-149-299 (175 bp) |

**Table 3.2 Polymerase chain reaction conditions for the molecular markers used in the study (continued)**

| Marker name and targeted gene | Reaction conditions and total PCR volume | PCR cycling conditions | Marker type, controls and fragment sizes |
|---|---|---|---|
| PSY-E1<br>*Sr25* resistant allele | 60 ng DNA<br>1x KAPA*Taq* ReadyMix DNA polymerase<br>25 ng PSY1_DF2 primer<br>25 ng PSY1_R3 primer<br>Total reaction volume: 10 µl | 1 cycle: 4 min, 94°C;<br>decrease 0.5°C/cycle for 10 cycles: 20 s, 94°C; 20 s, 63°C; 1 min 20 s, 72°C;<br>35 cycles: 20 s, 94°C; 20 s, 58°C; 1 min 20 s, 72°C;<br>1 cycle: 7 min, 72°C;<br>standby, 15°C | Dominant marker<br>Resistant control: LcSr25Ars (191 bp) |
| Sr26#43<br>*Sr26* resistant allele | 80 ng DNA<br>1x KAPA*Taq* ReadyMix DNA polymerase<br>50 ng Sr26#43F primer<br>50 ng Sr26#43R primer<br>Total reaction volume: 10 µl | 1 cycle: 3 min, 94;<br>45 cycles: 45 s, 94°C; 45 s, 60°C; 75 s, 72°C;<br>1 cycle: 10 min, 72°C | Dominant marker<br>Resistant control: Avocet*YrSp* and Blade (207 bp) |
| BE518379<br>*Sr26* susceptible allele | 80 ng DNA<br>1x KAPA*Taq* ReadyMix DNA polymerase<br>50 ng BE518379F primer<br>50 ng BE518379R primer<br>Total reaction volume: 10 µl | 1 cycle: 3 min, 94°C;<br>35 cycles: 1 min, 94°C; 1 min, 58°C; 2 min, 72°C;<br>1 cycle: 10 min, 72°C | Dominant marker<br>Susceptible control: Kariega and CS-*Lr19*-149-299 (303 bp) |

**Table 3.2 Polymerase chain reaction conditions for the molecular markers used in the study (continued)**

| Marker name and targeted gene | Reaction conditions and total PCR volume | PCR cycling conditions | Marker type, controls and fragment sizes |
|---|---|---|---|
| Iag95 | 80 ng DNA | 1 cycle: 2 min, 95°C; | Dominant marker |
| *Sr31* | 1x KAPA*Taq* ReadyMix DNA polymerase | 35 cycles: 45 s, 94°C; 45 s, 50°C; 60 s, 72°C; | Resistant control: WRT- 2 (1 100 bp) |
| | 25 ng Iag95F primer | 1 cycle: 4 min, 72°C | |
| | 25 ng Iag95R primer | | |
| | Total reaction volume: 10 µl | | |
| csSr32#1 | 80 ng DNA | 1 cycle: 2 min, 95°C; | Dominant marker |
| *Sr32* | 1x KAPA*Taq* ReadyMix DNA polymerase | 30 cycles: 30 s, 95°C; 40 s, 58°C; 50 s, 72°C; | Resistant control: Angas, Aroona and |
| | 25 ng csSr32#1F primer | 1 cycle: 5 min, 72°C | Westonia (184 bp) |
| | 25 ng csSr32#1R primer | | |
| | Total reaction volume: 10 µl | | |
| csSr32#2 | 80 ng DNA | 1 cycle: 2 min, 95°C; | Dominant marker |
| *Sr32* | 1x KAPA*Taq* ReadyMix DNA polymerase | 30 cycles: 30 s, 95°C; 40 s, 60°C; 50 s, 72°C; | Resistant control: Angas, Aroona and |
| | 25 ng csSr32#2F primer | 1 cycle: 5 min, 72°C | Westonia (152 bp) |
| | 25 ng csSr32#2R primer | | |
| | Total reaction volume: 10 µl | | |
| wmc477 | 80 ng DNA | 1 cycle: 10 min, 94°C; | Co-dominant marker |
| *Sr36* | 1x KAPA*Taq* ReadyMix DNA polymerase | 35 cycles: 31 min, 94°C; 1 min, 61°C; 2 min, 72°C; | Resistant control: W2691-StTt (190 bp) |
| | 25 ng WMC477F primer | 1 cycle: 10 min, 72°C | Susceptible control: Kariega and CS- |
| | 25 ng WMC477R primer | | *Lr19*-149-299 (160 bp) |
| | Total reaction volume: 10 µl | | |

**Table 3.2 Polymerase chain reaction conditions for the molecular markers used in the study (continued)**

| Marker name and targeted gene | Reaction conditions and total PCR volume | PCR cycling conditions | Marker type, controls and fragment sizes |
|---|---|---|---|
| stm773-2 | 80 ng DNA | 1 cycle: 10 min, 94℃; | Co-dominant marker |
| *Sr36* | 1x KAPA*Taq* ReadyMix DNA polymerase | 7 cycles: 1 min, 92℃; 1 min, 64℃; 1 min, 72℃; | Resistant control: W2691-SrTt1 (155 bp) |
| | 25 ng STM773-2F primer | 5 cycles: 1 min, 92℃; 1 min, 57℃; 1 min, 72℃; | Susceptible control: Kariega and CS-*Lr19*-149-299 (190 bp) |
| | 25 ng STM773-2R primer | 10-25 cycles: 30 s, 92℃; 1 min, 55℃; 2 min, 72℃; | |
| | Total reaction volume: 10 µl | 1 cycle: 10 min, 72℃ | |
| Sr39F2/R3 and | 60 ng DNA | 1 cycle: 3 min, 94℃; | Co-dominant marker |
| BE500705 | 1x KAPA*Taq* ReadyMix DNA polymerase | 30 cycles: 30 s, 92℃; 30 s, 56℃; 40 s, 72℃; | Resistant controls: Line 2S#/163 (900 bp) |
| *Sr39* | 50 ng Sr39F2 primer | 1 cycle: 10 min, 72℃ | Susceptible controls: Kariega, Avocet*YrSp*, Blade and CS-*Lr19*-149-299 (166 bp) |
| | 50 ng Sr39R3 primer | | |
| | 25 ng BE500705F primer | | |
| | 25 ng BE500705R primer | | |
| | Total reaction volume: 11 µl | | |
| FSD_RSA | 50 ng DNA | 1 cycle: 3 min, 94℃; | Co-dominant marker |
| *Sr42* | 1x KAPA*Taq* ReadyMix DNA polymerase | 35 cycles: 30 s, 94℃; 1 min 45 s, 44℃; 2 min, 72℃; | Resistant control: Norin40 (275 bp) |
| | 75 ng FSD primer | | Susceptible allele size range: 300 - 310 bp |
| | 75 ng RSA primer | 1 cycle: 10 min, 72℃ | |
| | Total reaction volume: 10 µl | | |

**Table 3.2 Polymerase chain reaction conditions for the molecular markers used in the study (continued)**

| Marker name and targeted gene | Reaction conditions and total PCR volume | PCR cycling conditions | Marker type, controls and fragment sizes |
|---|---|---|---|
| cfd71 and cfd23 | 80 ng DNA | 1 cycle: 2 min, 94°C; | Dominant marker |
| *Sr55/Lr67/Yr46* | 1x KAPA*Taq* ReadyMix DNA polymerase | 30 cycles: 1 min, 95°C; 60 s, 60°C; 1 min, 72°C; | Resistant control: RL6077 (214 and 211 bp) |
| | 25 ng CFD71F primer | 1 cycle: 5 min, 73°C | |
| | 25 ng CFD71R primer | | |
| | 25 ng CFD23F primer | | |
| | 25 ng CFD23R primer | | |
| | Total reaction volume: 11 µl | | |
| Cssfr5 | 60 ng DNA | 1 cycle: 5 min, 94°C; | Co-dominant marker |
| *Sr57/Lr34/Yr18* | 1x KAPA2G Fast ReadyMix PCR Kit | 5 cycles: 1 min, 94°C; 1 min, 58°C; 2 min, 72°C; | Resistant control: Kariega (751 bp) |
| | 25 ng L34SPF primer | 35 cycles: 30 s, 94°C; 30 s, 58°C; 50 s, 72°C; | Susceptible control: Blade (523 bp) |
| | 25 ng L34DINT9F primer | 1 cycle: 30 s, 94°C; 30 s, 58°C; 5 min, 72°C | |
| | 25 ng L34DINT13R2 primer | | |
| | 25 ng L34MINUSE primer | | |
| | Total reaction volume: 10 µl | | |
| wmc44 | 80 ng DNA | 1 cycle: 3 min, 94°C; | Co-dominant marker |
| *Sr58/Lr46/Yr29* | 1x KAPA*Taq* ReadyMix DNA polymerase | 45 cycles: 30 s, 94°C; 30 s, 61°C; 90 s, 72°C; | Resistant control: Pavon 76 (260 bp) |
| | 25 ng WMC44F primer | 1 cycle: 10 min, 72°C | Susceptible allele size range: 250 - 300 bp |
| | 25 ng WMC44R primer | | |
| | Total reaction volume: 10 µl | | |

**Table 3.2 Polymerase chain reaction conditions for the molecular markers used in the study (continued)**

| Marker name and targeted gene | Reaction conditions and total PCR volume | PCR cycling conditions | Marker type, controls and fragment sizes |
|---|---|---|---|
| gwm259 | 80 ng DNA | 1 cycle: 3 min, 94°C; | Co-dominant marker |
| *Sr58/Lr46/Yr29* | 1x KAPA*Taq* ReadyMix DNA polymerase | 45 cycles: 30 s, 94°C; 30 s, 56°C; 90 s, 72°C; | Resistant control: Pavon 76 (120 bp) |
| | 25 ng WMS259F primer | 1 cycle: 10 min, 72°C | Susceptible allele size range: 75 - 125 bp |
| | 25 ng WMS259R primer | | |
| | Total reaction volume: 10 µl | | |
| barc80 | 80 ng DNA | 1 cycle: 3 min, 94°C; | Co-dominant marker |
| *Sr58/Lr46/Yr29* | 1x KAPA*Taq* ReadyMix DNA polymerase | 45 cycles: 30 s, 94°C; 30 s, 50°C; 60 s, 72°C; | Resistant control: Pavon 76 (115 bp) |
| | 25 ng BARC80F primer | 1 cycle: 10 min, 72°C | Susceptible allele size range: 100 - 125 bp |
| | 25 ng BARC80R primer | | |
| | Total reaction volume: 10 µl | | |

CAPS = cleaved amplified polymorphic sequence

PCR products were mixed with approximately 2 µl deionised formamide loading dye depending on the intensity of PCR samples and 1 µl of the mixture loaded into the wells. The size marker, a 25 bp HyperLadder V of Bioline (Taunton, MA, USA), was loaded on both ends of the gel. A pulse run was performed for 16 sec and excess product was rinsed off. The gel was run at 1 200 V for 45 min at 37°C.

#### 3.2.4.3 Agarose gel electrophoresis

Polymerase chain reaction products for *Sr39* were separated on a 2% (w/v) agarose gel. Products were mixed with 10x Ficoll loading dye before loading on the gel. The fragment sizes were compared against a 100 bp DNA ladder (Promega, Madison, WI, USA). The gel was stained using ethidium bromide and run at 100 V for 45 min in 1x UNTAN buffer. Fragments were visualised using the Gel Doc™ EZ Imager.

### 3.2.5 Data analysis

The data was captured on Microsoft Excel version 2007 for each of the molecular markers that were screened. The main aim of MAS was to determine if the known *Sr*-genes were present or absent, thus only the fragment linked to the resistance allele was scored. Thus, both dominant and co-dominant markers were scored based on the presence (1) and absence (0) of the amplified resistance allele. For co-dominant markers the absence of the resistance allele was confirmed by the presence of the susceptible allele (data not shown).

## 3.3 Results

The presence or absence of the selected 13 known *Sr*-genes was successfully evaluated using molecular markers (Table 3.1). These markers were used to determine whether any of the three parental lines namely Line 37-07, M3283_4 and M3192_7 possess any of the known *Sr*-genes. Most of the genes were screened using SSR markers while cleaved amplified polymorphic sequence (CAPS) markers were used for *Sr2* and *Sr13*. The molecular markers used are closely linked to the screened *Sr*-genes, which are all known to be effective against *Pgt* race PTKST except for *Sr31*. The four APR genes, *Sr2, Sr55*, *Sr57* and *Sr58*, conferring stem rust resistance, were also evaluated, because if present, they may result in an enhanced resistant phenotype when the target gene(s) in the two M-lines are evaluated under field conditions. Table 3.3 represents the data generated from molecular marker analysis.

**Table 3.3 Data generated from screening markers linked to 13 known stem rust resistance genes in control and parental lines**

| Gene | Marker name | Positive control | Line 37-07 | M3283_4 | M3192_7 |
|---|---|---|---|---|---|
| *Sr2* | csSr2 | 1 | 0 | 0 | 0 |
| *Sr13* | Sr13 | 1 | 0 | 1 | 1 |
| *Sr22* | wmc633 | 1 | 0 | 0 | 0 |
| *Sr22* | cfa2123 | 1 | 0 | 0 | 0 |
| *Sr25* | PSY-E1 and | 1 | 0 | 0 | 0 |
| | PSY-D1 | 0 | 1 | 1 | 1 |
| *Sr26* | Sr26#43 and E518379 | 1 | 0 | 0 | 0 |
| *Sr31* | Iag95 | 1 | 0 | 0 | 0 |
| *Sr32* | csSr32#1 | 1 | 0 | 0 | 0 |
| *Sr32* | csSr32#2 | 1 | 0 | 0 | 0 |
| *Sr36* | wmc477 | 1 | 0 | 0 | 0 |
| *Sr36* | stm773-2 | 1 | 0 | 0 | 0 |
| *Sr39* | Sr39F2/R3 and BE500705 | 1 | 0 | 0 | 0 |
| *Sr42* | FSD-RSA | 1 | 0 | 0 | 0 |
| *Sr55/Lr67/Yr46* | cfd71 and cfd23 | 1 | 0 | 0 | 0 |
| *Sr57/Lr34/Yr18* | cssfr5 | 1 | 0 | 0 | 0 |
| *Sr58/Lr46/Yr29* | wmc44 | 1 | 0 | 0 | 0 |
| *Sr58/Lr46/Yr29* | gwm259 | 1 | 0 | 0 | 0 |
| *Sr58/Lr46/Yr29* | barc80 | 1 | 0 | 0 | 0 |

Diagnostic marker csSr2 amplified a fragment of approximately 337 bp in the *Sr2* positive control before enzyme digestion. No fragment amplified in the three parental lines and it was thus not necessary to do the enzyme digestion step for this marker. The Sr13 marker is a perfect CAPS marker that yields a 244 bp fragment in resistant haplotypes and a 265 bp fragment in susceptible haplotypes after digestion with the *Hha*I restriction enzyme. The 244 bp fragment linked to resistance was observed in Kronos and the M-lines while the 265 bp fragment linked to susceptibility was present in Line 37-07. Co-dominant markers, wmc633 and cfa2123, linked to *Sr22*, amplified fragment sizes of 117 bp and 234 bp for the positive controls, respectively. Although a fragment amplified in all three parental lines for both markers, the fragment size did not correlate with the fragment size linked to

resistance in the positive controls thus indicating the absence of the *Sr22* gene in the parental lines.

Dominant markers, PSY-E1 and PSY-D1 produce fragment of sizes 191 bp and 175 bp, respectively. Marker PSY-E1 is linked to the resistance allele while PSY-D1 is linked to the susceptible allele. Marker PSY-E1 amplified the resistant allele in the control and no amplification was observed for the parental lines. On the other hand, PSY-D1 amplified the susceptible allele in all the parental lines but no amplification was observed for the *Sr25* positive control. Results therefore suggest that *Sr25* was not present in the parental lines. The presence or absence of *Sr26* was determined using a combination of two markers, where Sr26#43 amplifies the resistance allele and BE518379 the susceptible allele. The combined results of these two markers indicated the absence of *Sr26* in all three parental lines.

Iag95, a dominant marker linked to *Sr31,* showed amplification in the positive control WRT-2 but no amplification was observed for the parental lines. CsSr32#1 and csSr32#2 are perfect dominant markers amplifying fragment sizes of 184 bp and 152 bp respectively in lines containing *Sr32*. CsSr32#1 amplified the expected 184 bp fragment in the positive control however the parental lines showed no amplification. CsSr32#2 displayed amplification only in the positive control and no amplification in the parental lines. Results from both markers indicated the absence of *Sr32* in the parental lines. Co-dominant markers wmc477 and stm773-2 linked to *Sr36,* amplified fragment sizes of 190 bp and 155 bp in the positive control W2691-SrTt1, respectively. The markers amplified the susceptible allele with fragment sizes of 160 bp and 190 bp, respectively for the three parental lines. Results from both primer sets indicated the absence of *Sr36* in the parental lines.

The two markers linked to the *Sr39* gene, Sr39F2/R3 and BE500705, were multiplexed to produce a co-dominant marker. Sr39F2/R3 is a dominant marker linked to the presence of the resistance allele and amplifies a 900 bp fragment. BE500705 is a dominant marker linked to the susceptible allele and amplifies a 166 bp fragment. The resistance allele was amplified in the positive control while the susceptible allele was present in the parental lines, indicating the absence of *Sr39* in the parental lines.

FSD_RSA is a co-dominant marker that is tightly linked and co-segregates with *Sr42*. *Sr42* is located in the same chromosomal region as *SrCad* and the marker is used to identify both genes. In lines carrying *Sr42,* FSD-RSA amplifies a 275 bp fragment. The same fragment

size was not amplified in any of the parental lines. Cfd71 and cfd23, linked to the resistance allele of *Sr55/Lr67/Yr46*, amplify fragment sizes of 214 bp and 211 bp, respectively. The cfd23 fragment size was amplified in the resistant control. However, in the parental lines, the 214 bp fragment size was observed, thus the allele present in these lines was that of cfd71. Cssfr5 is a co-dominant and perfect marker for *Sr57/Lr34/Yr18* that amplifies the resistance allele at 751 bp and the susceptible allele at 523 bp. The resistance allele was present in the positive control and the 523 bp fragment was amplified in all the parental lines including the susceptible control line, thus indicating the presence of the susceptible allele and thus the absence of the *Sr57/Lr34/Yr18* gene.

Three co-dominant markers were used to identify the *Sr58/Lr46/Yr29* resistance gene/gene complex. Wmc44 amplified the expected fragment size of 260 bp in the positive control. However, a smaller fragment size was amplified in the parental lines. Gwm259 amplified the expected fragment size of 120 bp and barc80 the expected 115 bp in the positive control. However, a different fragment size was amplified by both markers in all the parental lines. Markers wmc44 and gwm259 work together as flanking markers and barc80 can be used as alternative to gwm259. Based on these results, *Sr58/Lr46/Yr29* was not present in any of the parental lines.

## 3.4 Discussion

Several *Sr*-genes have been identified previously in different wheat cultivars. The genes screened for in this study were selected based on their effectiveness against *Pgt* race PTKST. Race PTKST is the most virulent race in SA and the resistant M-lines used in this study showed effective resistance against this race. Molecular markers closely linked to these genes have been developed to simplify MAS. Molecular markers linked to the 13 selected *Sr*-genes were successfully screened in the three wheat parental lines. Most of these genes are ASR genes, however *Sr2* is an APR gene known to confer resistance against all *Pgt* races (Mago et al. 2011). The other APR genes, *Sr55/Lr67/Yr46, Sr57/Lr34/Yr18* and *Sr58/Lr46/Yr29, also* confer resistance against some Ug99 variants and contribute to durable stem rust resistance.

Of the 13 known *Sr*-genes, only gene *Sr13* was detected in the two M-lines. *Sr13* was initially detected in tetraploid wheat and confers moderate resistance against *Pgt* race TTKSK in durum wheat (Simons et al. 2011). This gene maps to the long arm of chromosome 6A. The resistant lines used in this study were derived from a cross between

durum and common bread wheat, and because of this, the presence of *Sr13* was suspected. The gene was furthermore detected using a perfect marker developed based on the gene sequence, thus producing accurate results with no possibility of false positives and negatives. Other perfect markers used in this study include csSr2 and cssfr5 linked to *Sr2* and *Sr57/Lr34/Yr18*, respectively. In a previous study by Mago et al. (2011), csSr2 accurately predicted the presence of *Sr2* in 95% of the lines that were identified as resistant. Cssfr5 was developed by Lagudah et al. (2009) to identify the presence and absence of the *Sr57/Lr34/Yr18* gene. However, both APR genes were absent in the M-lines.

The use of SSR markers in this study was effective as these markers are highly informative, reproducible and cost ineffective (Powell et al. 1996). Simple sequence repeats markers wmc44 and gwm259, flanking the chromosome region of *Sr58*/*Lr46*/*Yr29*, and barc80, an alternative distal marker for this gene/gene complex, were all absent in the M-lines indicating the absence of this gene/gene complex. In a study by Sivasamy et al. (2014), wmc44 was successfully used to link APR gene (*Lr46*) to leaf tip necrosis in Indian wheat. In addition, gwm259 was used to locate *Lr46* in a study by Mateos-Hernandez et al. (2006). Closely linked or flanking markers were used to screen for the presence of the genes *Sr22*, *Sr25*, *Sr26*, *Sr31*, *Sr32*, *Sr36*, *Sr39*, and *Sr42*. These markers produced accurate results in the positive and negative control lines and were thus successful in indicating the absence of these genes in the parental lines.

Overall, the markers used were effective in detecting the presence and absence of the *Sr*-genes as they amplified correctly in the positive and negative controls. The absence of the slow-rusting APR genes means these lines do not possess genes that delay the spread of infection in the variety. As mentioned before, *Sr13* provides moderate resistance and the absence of the other APR and/or ASR genes means the resistance will not be durable. Therefore, should a new *Pgt* race occur that is virulent to *Sr13*, these M-lines could be infected.

The possibility exists that other *Sr*-genes not included in this study, effective against race TTKSK and its variants, especially race PTKST, might be present in these lines. *Sr31* was absent in the M-lines. However, a variety known to carry *Sr31* was susceptible to race PTKST (Chapter 4), as a result, the use of this gene in SA is not encouraged. Multiple genes (*Sr8*, *Sr14* and *Sr17*) have been introgressed into wheat from durum wheat. However, these genes are ineffective against race TTKSK and its variants (Laido et al. 2015; Saini et al.

2018) and could not be responsible for the observed resistance in the M-lines. Therefore, none of the durum wheat genes effective against race PTKST were overlooked in this study.

## 3.5 Conclusions

In this study, molecular markers were successfully applied and used to evaluate the status of known *Sr*-genes in the resistant parental lines, M3283_4 and M3192_7. These lines were derived from a cross made between durum wheat and hexaploid wheat. *Sr13* has been reported as the only known effective *Sr*-gene in durum wheat cultivars in the USA to race TTKSK (Simons et al. 2011). Of the 13 evaluated *Sr*-genes, marker Sr13 showed amplification in both the resistant parental lines. Therefore, *Sr13* might be the stem rust resistance gene present in these Canadian derived germplasm sources that provides effective resistance against *Pgt* race PTKST. However, other unknown or novel *Pgt* resistance genes might also be present.

## 3.6 References


Aktar-Uz-Zaman, M., Tuhina-Khatun, M., Hanafi, M. M., and Sahebi, M. (2017). Genetic analysis of rust resistance genes in global wheat cultivars: an overview. *Biotechnology and Biotechnological Equipment*, 31: 431-445.

Boshoff, W. H., Bender, C. M., and Pretorius, Z. A. (2019). Reaction of South African rye, triticale and barley forage cultivars to stem and leaf rust. *South African Journal of Plant and Soil*, 36: 77-82.

Collard, B. C., and Mackill, D. J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society*, 363: 557-572.

Gold, J., Harder, D., Townley-Smith, F., Aung, T., and Procunier, J. (1999). Development of a molecular marker for rust resistance genes *Sr39* and *Lr35* in wheat breeding lines. *Electronic Journal of Biotechnology*, 2: 1.

Ghazvini, H., Hiebert, C. W., Zegeye, T., Liu, S., Dilawari, M., Tsilo, T., Anderson, J. A., Rouse, M. N., Jin, Y., and Fetch, T. (2012). Inheritance of resistance to Ug99 stem rust in wheat cultivar Norin 40 and genetic mapping of *Sr42*. *Theoretical and Applied Genetics*, 125: 817-824.

Gupta, P. K., and Varshney, R. K. (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica,* 113: 163-185.

Hiebert, C. W., Fetch, T. G., Zegeye, T., Thomas, J. B., Somers, D. J., Humphreys, D. G., McCallum, B. D., Cloutier, S., Singh, D., and Knott, D. R. (2011). Genetics and mapping of seedling resistance to Ug99 stem rust in Canadian wheat cultivars 'Peace' and 'AC Cadillac'. *Theoretical and Applied Genetics,* 122: 143-149.

Lagudah, E. S., Krattinger, S. G., Herrera-Foessel, S., Singh, R. P., Huerta-Espino, J., Spielmeyer, W., Brown-Guedira, G., Selter, L. L., and Keller, B. (2009). Gene-specific markers for the wheat gene *Lr34/Yr18/Pm38* which confers resistance to multiple fungal pathogens. *Theoretical and Applied Genetics,* 119: 889-898.

Laido, G., Panio, G., Marone, D., Russo, M. A., Ficco, D. B., Giovanniello, V., Cattivelli, L., Steffenson, B., de Vita, P., and Mastrangelo, A. M. (2015). Identification of new resistance loci to African stem rust race TTKSK in tetraploid wheats based on linkage and genome-wide association mapping. *Frontiers in Plant Science,* 6: 1033.

Liu, S., Yu, L., Singh, R. P., Jin, Y., Sorrells, M. E., and Anderson, J. A. (2010). Diagnostic and co-dominant PCR markers for wheat stem rust resistance genes *Sr25* and *Sr26*. *Theoretical and Applied Genetics,* 120: 691-697.

Mago, R., Bariana, H. S., Dundas, I. S., Spielmeyer, W., Lawrence, G. J., Pryor, A. J., and Ellis, J. G. (2005). Development of PCR markers for the selection of wheat stem rust resistance genes *Sr24* and *Sr26* in diverse wheat germplasm. *Theoretical and Applied Genetics,* 111: 496-504.

Mago, R., Brown-Guedira, G., Dreisigacker, S., Breen, J., Jin, Y., Singh, R., Appels, R., Lagudah, E. S., Ellis, J., and Spielmeyer, W. (2011). An accurate DNA marker assay for stem rust resistance gene *Sr2* in wheat. *Theoretical and Applied Genetics,* 122: 735-744.

Mago, R., Spielmeyer, W., Lawrence, J., Lagudah, E. S., Ellis , G., and Pryor, A. (2002). Identification and mapping of molecular markers linked to rust resistance genes located on chromosome 1RS of rye using wheat-rye translocation lines. *Theoretical and Applied Genetics,* 104: 1317-1324.

Mago, R., Verlin, D., Zhang, P., Bansal, U., Bariana, H., Jin, Y., Ellis, J., Hoxha, S., and Dundas, I. (2013). Development of wheat *Aegilops speltoides* recombinants and simple PCR-based markers for *Sr32* and a new stem rust resistance gene on the 2S#1 chromosome. *Theoretical and Applied Genetics*, 126: 2943-2955.

Mago, R., Zhang, P., Bariana, H. S., Verlin, D. C., Bansal, U. K., Ellis, J. G., and Dundas, I. S. (2009). Development of wheat lines carrying stem rust resistance gene *Sr39* with reduced *Aegilops speltoides* chromatin and simple PCR markers for marker-assisted selection. *Theoretical and Applied Genetics*, 124: 65-70.

Mateos-Hernandez, M., Singh, R. P., Hulbert, S. H., Bowden, R. L., Huerta-Espino, J., Gill, B. S., and Brown-Guedira, G. (2006). Targeted mapping of ESTs linked to the adult plant resistance gene *Lr46* in wheat using synteny with rice. *Functional and Integrative Genomics*, 6: 122-131.

Miedaner, T., and Korzun, V. (2012). Marker-assisted selection for disease resistance in wheat and barley breeding. *Phytopathology*, 102: 560-566.

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

Olson, E. L., Brown-Guedira, G., Marshall, D., Stack, E., Bowden, R. L., Jin, Y., Rouse, M., and Pumphrey, M. O. (2010). Development of wheat lines having a small introgressed segment carrying stem rust resistance gene *Sr22*. *Crop Science,* 50: 1823-1830.

Pandey, K., Dangi, R., Prajapati, U., Kumar, S., Maurya, N. K., Singh, A. V., Singh, J., and Rajan, R. (2019). Advance breeding and biotechnological approaches for crop improvement: A review. *International Journal of Chemical Studies,* 7: 837-841.

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

Pretorius, Z. A., Bender, C. M., Visser, B., and Terefe, T. (2010). First report of a *Puccinia graminis* f. sp. *tritici* race virulent to the *Sr24* and *Sr31* wheat stem rust resistance genes in South Africa. *Plant Disease*, 94: 784.

Pretorius, Z. A., Singh, R. P., Wagoire, W. W., and Payne, T. S. (2000). Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis* f. sp. *tritici* in Uganda. *Plant Disease,* 84: 203.

Pretorius, Z. A., Szabo, L. J., Boshoff, W. H., Herselman, L., and Visser, B. (2012). First report of a new TTKSF race of wheat stem rust (*Puccinia graminis* f. sp. *tritici*) in South Africa and Zimbabwe. *Plant Disease,* 96: 590.

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences of the United States of America,* 81: 8014-8018.

Saini, J., Faris, J. D., Zhang, Q., Rouse, M. N., Jin, Y., Long, Y., Klindworth, D. L., Elias, E. M., McClean, P. E., Edwards, M. C., and Xu, S. S. (2018). Identification, mapping, and marker development of stem rust resistance genes in durum wheat 'Lesbock'. *Molecular Breeding,* 38: 77.

Simons, K., Abate, Z., Chao, S., Zhang, W., Rouse, M., Jin, Y., Elias, E., and Dubcovsky, J. (2011). Genetic mapping of stem rust resistance gene *Sr13* in tetraploid wheat (*Triticum turgidum* spp. *durum* L.). *Theoretical and Applied Genetics,* 122: 649-658.

Singh, R. P., Mujeeb-Kazi, A., and Huerta-Espino, J. (1998). *Lr46*: A gene conferring slow-rusting resistance to leaf rust in wheat. *Phytopathology,* 88: 890-894.

Sivasamy, M., Aparna, M., Kumar, J., Jayaprakash, P., Vikas, V. K., John, P., Satyaprakash, R. N., Sivan, K., and Punniakotti, E. (2014). Phenotypic and molecular confirmation of durable adult plant leaf rust resistance (APR) genes *Lr34+*, *Lr46+* and *Lr67+* linked to leaf tip necrosis (LTN) in select registered Indian wheat (*T. aestivum*) genetic stocks. *Cereal Research Communications,* 42: 262-273.

Terefe, T., Pretorius, Z. A., Visser, B., and Boshoff, W. H. (2019). First report of *Puccinia graminis* f. sp. *tritici* race PTKSK, a variant of wheat stem rust race Ug99 in South Africa. *Plant Disease,* 103: 1-3.

Tsilo, T. J., Jin, Y., and Anderson, J. A. (2008). Diagnostic microsatellite markers for the detection of stem rust resistance gene *Sr36* in diverse genetic backgrounds of wheat. *Crop Science*, 48: 253-261.

Zhang, W., and Dubcovsky, J. (2008). Association between allelic variation at the Phytoene synthase 1 gene and yellow pigment content in wheat grain. *Theoretical and Applied Genetics*, 116: 635-645.

Zhang, W., Chen, S., Abate, Z., Nirmala, J., Rouse, M. N., and Dubcovsky, J. (2017). Identification and characterization of *Sr13*, a tetraploid wheat gene that confers resistance to the Ug99 stem rust race group. *Proceedings of the National Academy of Sciences of the United States of America*, 114: E9483-E9492.

# Chapter 4

# Phenotypic evaluation of $F_2$ populations and $F_3$ families of Line37-07/M3283_4 and Line37-07/M3192_7 and selected *Sr*-control varieties

## 4.1 Introduction

Stem rust, also known as black rust, caused by the fungal pathogen *Pgt* is considered an important disease of wheat in SA. The disease is stated to be devastating based on the ability of *Pgt* to cause significant losses in wheat yield when epidemic outbreaks occur (Bender et al. 2016). The stem rust pathogen originated in the Fertile Crescent and spread to wheat producing regions with warm and moist environmental conditions that favour disease outbreaks (Singh et al. 2015; Bhavani et al. 2019).

The appearance of *Pgt* race TTKSK (North American race nomenclature) in Uganda during the 1998 wheat growing season posed a threat to the wheat crop at that time because of increased virulence to numerous cultivars carrying the widely deployed *Sr31* resistance gene (*Sr*-gene) (Lopez-Vera et al. 2013). Ug99 stem rust races are distributed over 11 countries on the African continent namely Uganda, Kenya, Ethiopia, SA, Tanzania, Eritrea, Zimbabwe, Rwanda, Egypt, Mozambique and Sudan (Singh et al. 2008; Visser et al. 2011; Pretorius et al. 2012; Bhavani et al. 2019). Ug99 is the first known stem rust race group to be virulent to the *Sr*-genes *Sr31* and *Sr38*, as well as many other *Sr*-genes commonly used in breeding programmes (Pretorius et al. 2000; Singh et al. 2011). This race group has evolved rapidly, through mutations, genetic recombination, and new introductions from other countries by wind dispersion (Singh et al. 2011; Figlan et al. 2014).

Five variants of the known Ug99 races were detected in Kenya (Bhavani et al. 2019). Four of the 13 Ug99 race group members (TTKSF, TTKSP, PTKST and TTKSF+) occur in SA (Boshoff et al. 2000; Pretorius et al. 2010; Terefe et al. 2010; Mukoyi et al. 2011; Pretorius et al. 2012). However, a recent paper by Terefe et al. (2019) reported a fifth Ug99 race, PTKSK, which was previously detected in Yemen, Kenya, and Ethiopia (Figlan et al. 2014; Bhavani et al. 2019). The newly discovered race PTKSK is virulent to *Sr31* and avirulent to *Sr24*. Race PTKST, detected in SA in 2009, is potentially the most destructive of these races based on its combined virulence to the *Sr*-genes *Sr24* and *Sr31* (Pretorius et al. 2010).

Stem rust is controlled by traditional, chemical, and genetic methods. In some countries, the traditional method for controlling the spread of the disease was by eradicating the alternate host, common barberry (Simons et al. 2011). However, because there has been no recorded occurrence of *Pgt* on plants of the alternate host in SA, the widely used method of control in SA and other African countries like Kenya is through fungicide applications (Wanyera et al. 2009; Figlan et al. 2014). Fungicides are used to prevent disease outbreaks on susceptible cultivars. In addition to being a preventative method rather than a curative, this method adds to input cost and may negatively impact the environment (Simons et al. 2011). The deployment of resistance sources through cultivar development is therefore considered a more environmentally friendly and economical sustainable method to control the disease (Terefe et al. 2010).

According to Saini et al. (2018) more than 70 *Sr*-genes have been characterised. In bread wheat, the *Sr*-genes *Sr36*, *Sr9h* and *SrTmp* confer resistance to stem rust race PTKST (Visser et al. 2011; Bhavani et al. 2019). *Sr13* provides effective resistance against race TTKSK in durum wheat (Simons et al. 2011; Olivera et al. 2012; Zhang et al. 2017). Phenotyping is a traditional method used for selection in breeding programmes, accelerating genetic gains, and monitoring a plants health status (Chawade et al. 2019). Phenotyping platforms used for screening of wheat to virulent races of stem rust in African countries have shown an increase in resistance in the International Center for Wheat and Maize Improvement (CIMMYT) wheat germplasm (Bhavani et al. 2019). The aims of this study were to (i) determine the seedling ITs, segregation ratios and test the fit to genetic models for $F_2$ populations and $F_3$ families originating from the crosses Line37-07/M3283_4 and Line37-07/M3192_7 to Ug99 race PTKST and (ii) evaluate stem rust resistant control lines to determine their seedling ITs to stem rust race PTKST to phenotypically compare with the IT responses of the two M-lines.

## 4.2 Phenotypic evaluation of $F_2$ populations of Line37-07/M3283_4 and Line37-07/M3192_7

### 4.2.1 Materials and methods

#### 4.2.1.1 Plant material and stem rust field responses

Crosses were made between a stem rust susceptible wheat parent (Line 37-07) and two resistant (M3283_4 and M3192_7) parents. Line 37-07 (pedigree and details given in Chapter 3) was selected based on its susceptibility to stem rust race PTKST in SA field

trials. Wheat lines M3283_4 and M3192_7 originated from crosses between stem rust resistant tetraploid wheat (AABB) and common wheat (AABBDD) by Dr J Thomas, a since retired wheat researcher previously based at the Morden Research and Development Centre - Agriculture and Agri-Food Canada. The exact details of the tetraploid and wheat parents used by Dr Thomas in his crosses are unfortunately unknown. However, seed from the crosses were screened by Prof ZA Pretorius (UFS) for stem rust seedling resistance using race PTKST and selections among inbred lines were made based on low seedling ITs and agrotype including normal wheat head appearance of plants.

The stem rust field response of the parental lines was determined in a wheat stem rust nursery planted near Greytown, KwaZulu-Natal in SA during the 2018 season. The trial was established by planting the wheat parents in 1 m observation rows with regular irrigation and sufficient fertiliser to support optimum plant development. Adjacent stem rust spreader rows planted with either the susceptible wheat line McNair or Line 37-07 were inoculated six weeks after planting, using an ultralow-volume sprayer (ULVA; Micron Group, Bromyard, England), with a suspension of urediniospores of stem rust race PTKST in Soltrol® 130 isoparaffinic oil (± 3 mg/ml) (Chevron Phillips Chemical Co., Borger, TX, USA) to achieve early infection and consequent epidemic development. The modified Cobb scale (Peterson et al. 1948) combined with a host reaction type (Roelfs et al. 1992) was used to determine the field response of the parents after heading.

#### 4.2.1.2 Population development of $F_2$

Twelve $F_1$ seeds obtained from each of the Line37-07/M3283_4 and Line37-07/M3192_7 crosses were planted in 2 l pots containing steam-sterilised soil to generate two $F_2$ populations. Heads on individual plants were covered with glassine crossing bags to prevent out-crossing during flowering. Following harvesting, 500 $F_2$ seeds from each $F_2$ population were sown in 10 cm diameter pots, 25 seeds per pot, containing Mikskaar Professional Potting Soil. Planted seeds were incubated in a growth chamber set at 25°C for 4 days to allow even germination before being moved to a greenhouse cubicle set at a mean night/day temperature regime of 18-23°C under natural light. Emerging seedlings were fertilised with 50 ml of 0.2% (w/v) Multifeed-Classic water-soluble fertiliser per tray of five pots [Effekto® NPK 19:8:6 (43)] before inoculation followed by a second application after inoculation.

#### 4.2.1.3 Seedling inoculation of $F_2$ populations

Seven days post planting, $F_2$ seedlings were inoculated with freshly collected urediniospores of stem rust race PTKST, increased on the wheat variety Federation*4/Kavkaz that is resistant to most of the other SA stem rust races. The urediniospores were suspended in Soltrol® 130 isoparaffinic oil (± 1 mg/ml). Seedlings were spray inoculated onto their primary leaves using a pressure pump at 25 kPa pressure setting (Vacuubrand® pump - model MZ2) connected to an inoculation device. The inoculated seedlings were then dried for 1 h in a growth chamber set at 25°C (200 µE/m²/s light), followed by an 18 h overnight incubation in a dark dew chamber set at ±18°C. Seedlings were removed and dried for 3 h under light in a growth chamber set at 25°C (200 µE/m²/s light). Finally, the seedlings were moved to the greenhouse set at a mean night/day temperature regime of 18-23°C under natural light.

#### 4.2.1.4 Determination of seedling infection types

The wheat seedlings were evaluated and grouped as either resistant or susceptible 10 to 12 days after inoculation for each of the $F_2$ populations. The seedling ITs were assessed using a 0 to 4 scale as described by Stakman et al. (1962). Infection types in the scale of 0 to 2 were considered as low or resistant to stem rust race PTKST and ITs of 3 to 4 as high or susceptible, suggesting that the corresponding *Sr*-gene is not present.

#### 4.2.1.5 Data analysis

The data collected from the phenotypic evaluation was used to determine the segregation ratios of the respective $F_2$ populations and the goodness of fit to genetic models were tested using a chi-square test calculated using Excel version 2007. A Mendelian segregation ratio of 3:1 was expected for a single dominant resistance gene.

### 4.2.2 Results

The two resistant parents M3283_4 and M3192_7 expressed similar severity and infection responses in the field (20MRR) compared to the susceptible parent Line 37-07 (60S) that was clearly high in response to stem rust race PTKST (Figure 4.1). Considering their seedling response, the stem rust resistant parents, M3283_4 and M3192_7, expressed seedling ITs ranging between a 1+ to 2- with the presence of some chlorosis against stem rust race PTKST. Line 37-07 consistently expressed a high seedling IT score of 3+, indicating susceptibility to stem rust race PTKST (Figures 4.2 and 4.3).

From the 500 $F_2$ seeds that were planted per cross combination, a total of 483 and 453 seedlings belonging to the populations Line37-07/M3283_4 and Line37-07/M3192_7, germinated respectively. For the Line37-07/M3283_4 population, of the 483 $F_2$ seedlings, 357 were found resistant (ITs 1+ to 2-) and 126 susceptible (ITs 3 to 3+) to race PTKST (Figure 4.2). The observed segregation ratio for this population of 2.8:1 was not significantly different from the expected Mendelian ratio of 3:1 (Table 4.1) for a single dominant gene. The chi-square value for the fit of this $F_2$ population to a 3:1 ratio was 0.304 (*P* value = 0.581).

For the Line37-07/M3192_7 population, 347 of the 453 $F_2$ seedlings were resistant (ITs 1+ to 2-) and 106 were susceptible (ITs 3 to 3+) to stem rust race PTKST (Figure 4.3). The observed segregation ratio for this population was 3.3:1 thus consistent with the expected Mendelian ratio of 3:1 for a single dominant gene (Table 4.1). The chi-square value for the fit of this $F_2$ population to a 3:1 ratio was 0.619 (*P* value = 0.431).


**Figure 4.1 Adult plant field response of the parental lines M3283_4, M3192_7 and Line 37-07 used to study the inheritance of resistance to stem rust race PTKST**

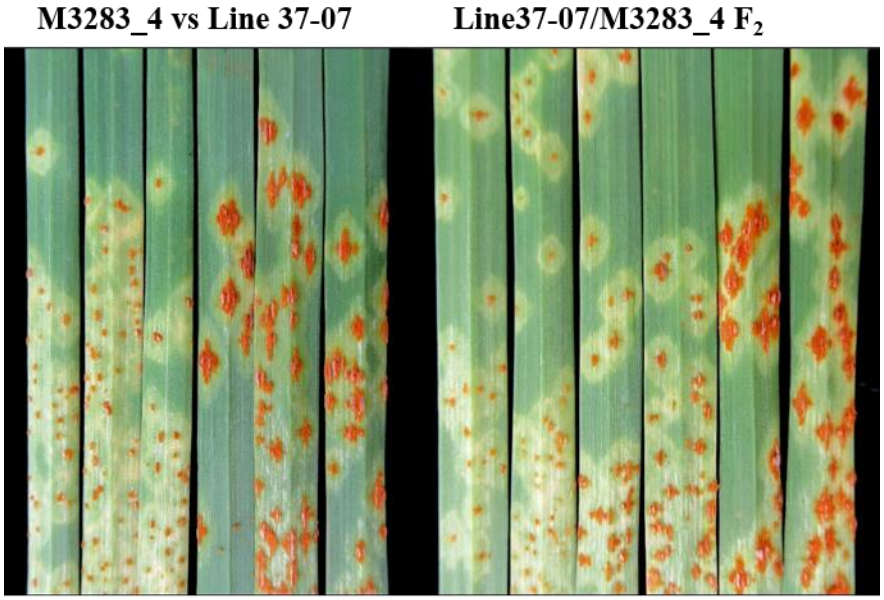


**Figure 4.2 Seedling infection types of the parental lines M3283_4 and Line 37-07 (three leaves each on the 1st plate) and segregation observed in the $F_2$ population indicating resistant (four leaves left on the 2nd plate) and susceptible (two leaves right on the 2nd plate) to stem rust race PTKST**

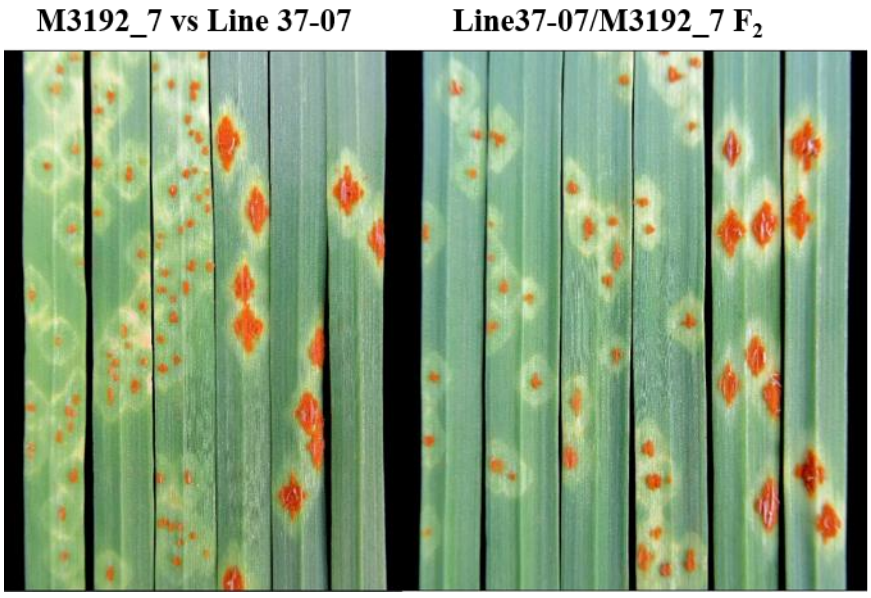


**Figure 4.3 Seedling infection types of the parental lines M3192_7 and Line 37-07 (three leaves each on the 1st plate) and segregation observed in the $F_2$ population indicating resistant (four leaves left on the 2nd plate) and susceptible (two leaves right on the 2nd plate) to stem rust race PTKST**

**Table 4.1 Greenhouse phenotypic evaluation of $F_2$ mapping populations based on seedling infection types to stem rust race PTKST**

| $F_2$ populations | Number of seedlings | Observed frequency | Expected ratio | Chi-square value | *P* value |
|---|---|---|---|---|---|
| Line37-07/M3283_4 | 483 | 2.8:1 (357:126) | 3:1 | 0.304 | 0.581 |
| Line37-07/M3192_7 | 453 | 3.3:1 (347:106) | 3:1 | 0.619 | 0.431 |

### 4.2.3 Discussion

Stem rust resistance of the two donor parents used in this study was confirmed under field conditions. The field- and seedling data confirmed that both parents contain an effective source of resistance to stem rust race PTKST and that Line 37-07 is a suitable susceptible parent to use for mapping of the *Sr*-gene(s). The chi- square values for both Line37-07/M3283_4 and Line37-07/M3192_7 $F_2$ populations conformed to the expected Mendelian segregation ratio for a single dominant gene conferring resistance to stem rust race PTKST. A similar study was previously done by Tsilo et al. (2009), where the $F_2$ population derived from a cross between Chinese Spring and ISr6-Ra segregated in the ratio 3:1, which is expected for a single dominant gene which conferred resistance against stem rust race QFCS.

## 4.3 Phenotypic evaluation of the M3283_4/M3192_7 intercross $F_2$ population and selected stem rust control varieties

### 4.3.1 Materials and methods

#### 4.3.1.1 Plant material

The stem rust resistant parents M3283_4 and M3192_7 were crossed with each other to generate $F_1$ seed. Following harvesting of the $F_1$ seed, kernels were germinated and vernalised through incubation for three weeks in a cold room set at ±5°C in petri dishes containing two sheets of Munktell filter paper and 5 ml of distilled water. Twenty $F_1$ seedlings originating from a single cross were transplanted into 2 l pots filled with steam sterilised soil in the greenhouse at 18-22°C and fertilised weekly with 50 ml of a 0.2% (w/v) Multifeed-Classic water-soluble fertiliser [Effekto®, NPK Analysis 19:8:16 (43)]. Spikes were covered using glassine crossing bags before flowering to prevent outcrossing between

plants. Following ripening, the $F_2$ seeds from each $F_1$ plant were harvested individually and stored in the germplasm bank at the UFS.

Wheat seed stocks with selected designated *Sr*-genes were obtained from the wheat germplasm bank maintained at the Plant Pathology division of the UFS to determine comparative seedling ITs to stem rust race PTKST. These included Combination VII, Leeds, Kronos, SWSr22, LcSr25, Kite, Federation*4/Kavkaz, CnsSr32, W2691SrTt-1 and RWG-1 (Table 4.2).

#### 4.3.1.2 Seedling inoculation and determination of seedling infection types

The same planting, inoculation and incubation procedures were followed as described in sections 4.2.1.2 and 4.2.1.3 to determine the seedling ITs of the M3283_4/M3192_7 $F_2$ population as described in section 4.2.1.4. The determination of seedling ITs for selected stem rust resistant varieties were carried out by planting five entries, six seeds per entry, in clumps in a 10 cm diameter pot filled with Mikskaar Professional Potting Soil. Inoculation and incubation procedures followed were as described above. The experiment was replicated in an independent trial to confirm the seedling ITs.

**Table 4.2 Seedling infection types (ITs) recorded for the two resistant parents M3283_4 and M3192_7 and selected wheat varieties with known *Sr*-genes to stem rust race PTKST**

| Wheat variety | *Sr*-gene(s) | ITs[a] |
|---|---|---|
| M3283_4 | Unknown | 2- |
| M3192_7 | Unknown | 2- |
| Combination VII | *Sr17*, *Sr13a* | 2 |
| Leeds | *Sr8b*, *Sr9e*, *Sr13b* | 0; |
| Kronos | *Sr13* | 1 |
| SWSr22 | *Sr22* | 1+ |
| LcSr25 | *Sr25* | 1 |
| Kite | *Sr26* | ;1 |
| Federation*4/Kavkaz | *Sr31* | 4 |
| CnsSr32 | *Sr32* | 1+ |
| W2691SrTt-1 | *Sr36* | 0; |
| RWG-1 | *Sr39* | 1- |

[a]Seedling infection types scored according to Stakman et al. (1962)

#### 4.3.1.3 Data analysis

The segregation ratio was determined for the $F_2$ population of the M3283_4/M9192_7 intercross. If a segregation ratio of 15:1 is observed in the $F_2$ population it would imply that the two resistant parents carry two independent *Sr*-genes to stem rust race PTKST. However, if no segregation is observed in the $F_2$ population it would show that the two parents carry the same *Sr*-gene or very closely linked *Sr*-genes. Data was analysed as described in section 4.2.1.5.

### 4.3.2 Results

A total of 482 $F_2$ seedlings from the M3283_4/M3192_7 intercross were inoculated with stem rust race PTKST. All seedlings showed a resistant reaction (2- to 2) to stem rust (Figure 4.4). The seedling IT data for the stem rust resistant parent lines as well as for 10 additional control varieties carrying known *Sr*-genes to stem rust race PTKST is summarised in Table 4.2. As observed before, the two wheat lines M3283_4 and M3192_7 were found resistant with dominantly 2- seedling ITs as presented in Table 4.2. Federation*4/Kavkaz showed a seedling IT of 4. The remaining varieties were found resistant with seedling ITs ranging between 0; to 2 (Figure 4.5).

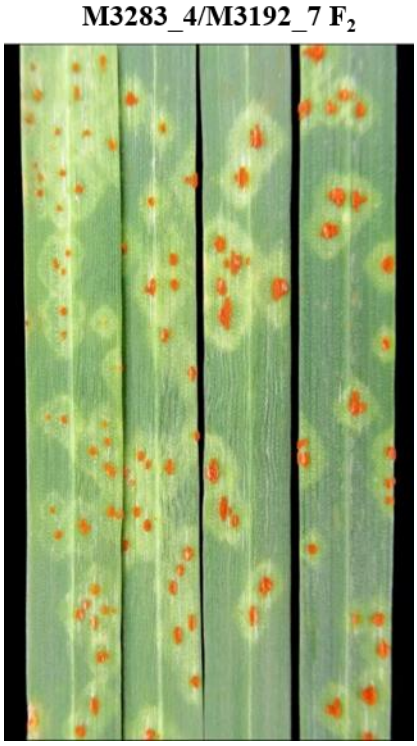


**Figure 4.4 Seedling infection types range (2- on the left to 2 on the right) to stem rust race PTKST expressed by $F_2$ seedlings from an intercross between the two resistant parents M3283_4 and M3192_7**

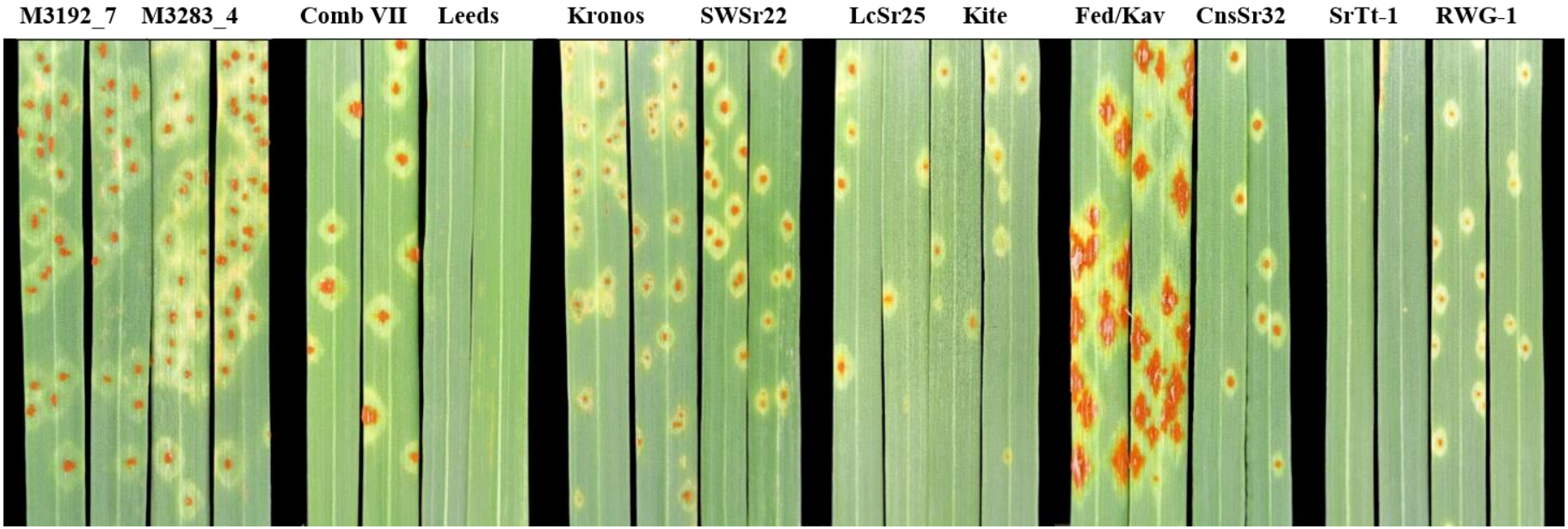


**Figure 4.5 Greenhouse determined seedling infection types for the resistant parents M3192_7 and M3283_4 and 10 selected wheat varieties carrying catalogued stem rust resistance genes to race PTKST**

Combination VII (*Sr17*, *Sr13a*), Leeds (*Sr8b*, *Sr9e*, *Sr13b*), Kronos (*Sr13*), SWSr22 (*Sr22*), LcSr25 (*Sr25*), Kite (*Sr26*), Federation*4/Kavkaz (*Sr31*), CnsSr32 (*Sr32*), W2691SrTt-1 (*Sr36*) and RWG-1 (*Sr39*)

### 4.3.3 Discussion

A total of 482 $F_2$ seedlings from the intercross M3283_4/M3192_7 were successfully inoculated with urediniospores of stem rust race PTKST. All $F_2$ seedlings were resistant to stem rust race PTKST with no susceptible seedlings observed. The presence of only resistant seedlings in the $F_2$ population suggested that the two resistant lines M3283_4 and M3192_7 carry the same or a closely linked *Sr*-gene(s) to stem rust race PTKST. Federation*4/Kavkaz carrying *Sr31* was susceptible to stem rust race PTKST. This outcome was expected as the race is known to be virulent against *Sr31* and avirulent for genes *Sr22, Sr26, Sr33, Sr35, Sr36* and *Sr39* (Pretorius et al. 2010; Prins et al. 2016).

Allelism tests have been performed in previous studies. For example, in a study by Olivera et al. (2013) the $F_2$ population of a cross made between two resistant accessions (PI 271074 and PI 410803) carrying uncharacterised genes produced only resistant progenies to race TTKSK. This suggested that race TTKSK resistance in the accessions was allelic. In a similar study, an allelism test was performed to validate that the resistance genes carried by Webster and Gabo 56 (known to map at the region of *SrWeb*), are most likely the same gene (Rouse et al. 2014). It was confirmed that *SrWeb* is an allele of *Sr9h*.

Allelism tests have also been performed for resistance to leaf and stripe rust. In a recent study by Kthiri et al. (2018), progenies derived from a cross involving Amria and Bylos suggested that the genotypes carry allelic or closely linked genes as no susceptible $F_2$ plants were observed. However, in other crosses involving Amria, susceptible plants were observed, thus making the gene carried by Amria and Bylos different from the gene carried by Geromtel_3 and Tunsyr_2. It was reported that Geromtel_3 and Tunsyr_2 carry the same, allelic, or tightly linked genes to *Lr61*.

## 4.4 Phenotypic evaluation of $F_3$ families of Line37-07/M3283_4 and Line37-07/M3192_7

### 4.4.1 Materials and methods

#### 4.4.1.1 Plant material

One hundred $F_2$ seeds were randomly taken from each of the Line37-07/M3283_4 and Line37-07/M3192_7 crosses and planted in 2 l pots containing steam-sterilised soil to generate $F_3$ families. From the hundred $F_2$ seeds planted per cross, 97 and 83 seedlings emerged for Line37-07/M3283_4 and Line37-07/M3192_7, respectively. Heads on

individual plants were covered with glassine crossing bags to prevent out-crossing during flowering. Following harvesting of individual plants, $F_3$ seeds were sown in 10 cm diameter pots, 20 seeds per pot originating from an individual plant, containing Mikskaar Professional Potting Soil. Pots were incubated in a growth chamber set at 25°C for 4 days to allow even germination before being moved to a greenhouse cubicle set at a mean night/day temperature regime of 18-23°C under natural light. Seedlings were fertilised with 50 ml of 0.2% (w/v) Multifeed-Classic water-soluble fertiliser per tray of five pots before inoculation [Effekto® NPK 19:8:6 (43)] followed by a second application after inoculation.

#### 4.4.1.2 Seedling inoculation and determination of seedling infection types

The inoculation procedure was followed as described in sections 4.2.1.3 and 4.2.1.4 to determine the seedling ITs of the Line37-07/M3283_4 and Line37-07/M3192_7 $F_3$ families.

#### 4.4.1.3 Data analysis

The data collected from the phenotypic evaluation was used to determine the segregation ratios of the respective $F_3$ families. Based on the seedling ITs for seed from individual $F_3$ plants in each family, plants were rated as either homozygous resistant, homozygous susceptible or heterozygous in their stem rust response. The total number of stem rust resistant and susceptible seedlings, over plants, per $F_3$ family was also determined. The data was used to test the goodness of fit to genetic models using the chi-square test for each $F_3$ family using Excel version 2007.

### 4.4.2 Results

The stem rust resistant parents, M3283_4 and M3192_7 expressed seedling ITs ranging between 1+ to 2- with the presence of some chlorosis against stem rust race PTKST. Consistent with previous results, Line 37-07 expressed a susceptible seedling IT score of 3+ (Figures 4.6 and 4.7). From the total number of $F_3$ seeds that were planted per cross combination, a total of 1 731 and 1 436 seedlings belonging to the Line37-07/M3283_4 and Line37-07/M3192_7 families, germinated respectively.

For Line37-07/M3283_4, there was an $F_3$ population distribution of 1 107 resistant and 624 susceptible seedlings. The 97 $F_3$ families segregated in a 26 homozygous resistant, 48 heterozygous and 23 homozygous susceptible ratios (Table 4.3). The observed segregation ratio for this family was 1.1:2.1:1 and when tested for the goodness of fit to a single dominant gene ratio of 1:2:1, with a chi-square value of 0.196 (*P* value = 0.907).

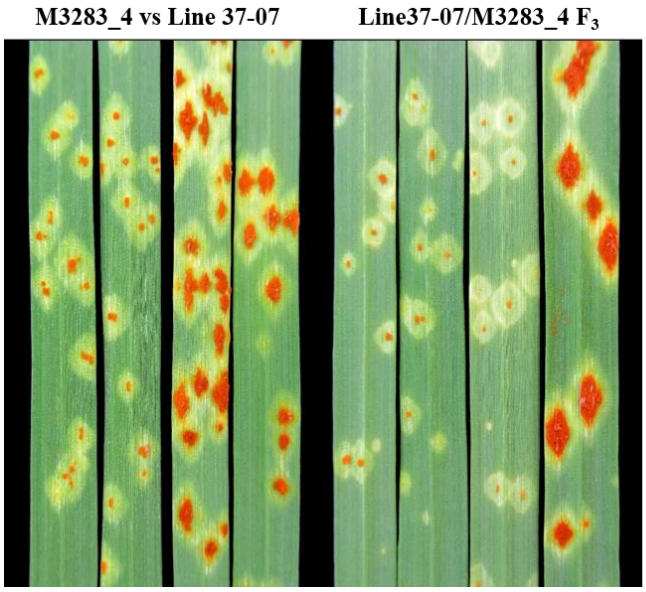


**Figure 4.6 Seedling infection types of the parental lines M3283_4 and Line 37-07 (two leaves each on the 1st plate) and segregation observed in the $F_3$ population indicating resistant (three leaves left on the 2nd plate) and susceptible (one leaf right on the 2nd plate) to stem rust race PTKST**

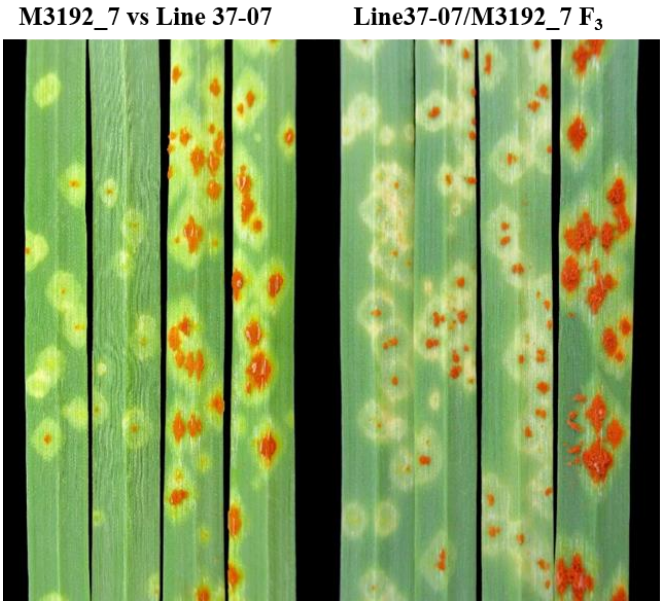


**Figure 4.7 Seedling infection types of the parental lines M3192_7 and Line 37-07 (two leaves each on the 1st plate) and segregation observed in the $F_3$ population indicating resistant (three leaves left on the 2nd plate) and susceptible (one leaf right on the 2nd plate) to stem rust race PTKST**

**Table 4.3 Greenhouse phenotypic evaluation of $F_3$ segregating families based on their seedling infection type response to stem rust race PTKST**

| $F_3$ family | Number of families | Observed frequency (R:H:S) | Expected ratio (R:H:S) | Chi-square value | *P* value |
|---|---|---|---|---|---|
| Line37-07/M3283_4 | 97 | 1.1:2.1:1 (26:48:23) | 1:2:1 | 0.196 | 0.907 |
| Line37-07/M3192_7 | 83 | 1:2.2:1.2 (19:41:23) | 1:2 1 | 0.398 | 0.820 |

R = homozygous resistant; H = heterozygous; S = homozygous susceptible

For Line37-07/M3192_7, there was an $F_3$ population distribution, of 848 resistant and 588 susceptible seedlings. The 83 $F_3$ families segregated in a 19 homozygous resistant, 41 heterozygous and 23 homozygous susceptible ratios (Table 4.3). The observed segregation ratio for this family was 1:2.2:1.2 and when tested for the goodness of fit to the single dominant gene ratio of 1:2:1, the chi-square value was 0.398 (*P* value = 0.820).

### 4.4.3 Discussion

The $F_3$ families were successfully inoculated with stem rust race PTKST. The observed ratio for Line37-07/M3283_4 was consistent with the expected ratio of 1:2:1 (homozygous resistant : heterozygous : homozygous susceptible) for a single dominant resistance gene. The chi-square value of the population indicated that the population conformed to the expected Mendelian segregation ratio for a single dominant gene conferring resistance to stem rust race PTKST. The observed segregation ratio for Line37-07/M3192_7 was consistent with the expected Mendelian ratio of 1:2:1. Based on the chi-square value, this population also conformed to the expected Mendelian segregation ratio for a single dominant gene conferring resistance to stem rust race PTKST.

## 4.5 Conclusions

In summary, the evaluation of the $F_2$ populations from the crosses Line37-07/M3283_4 and Line37-07/M3192_7 fitted the Mendelian ratio of 3:1 for the presence of a single dominant gene. The evaluation of the two $F_3$ families, randomly derived from the two $F_2$ populations, conformed to the segregation ratio of 1:2:1. This confirmed the presence of a single dominant gene present in both populations that confers resistance to stem rust race PTKST in the seedling stage in both M3283_4 and M3192_7. Due to the two resistant parents being developed from the offspring of tetraploid crosses with common wheat it was important to

test whether they carry the same *Sr*-gene or not. Therefore, an intercross evaluation had to be performed for validation. The intercross results confirmed that the same gene or a closely linked gene is present in the two resistant wheat lines. Phenotypic comparisons of the seedling ITs for M3283_4 and M3192_7 did not reveal identical phenotypes to each other, although the dominant phenotype recorded for both was a 2-, or when individually compared with the phenotypes recorded for the 10 resistant wheat varieties carrying catalogued resistance genes. The genetic background in which a *Sr*-gene is deployed might however result in small deviations in the observed phenotype, especially when no backcrossing to a universal susceptible recurrent parent is involved. Unfortunately, no pedigree information is available for the M-lines thereby not allowing any postulation based on genetic relatedness. Considering the results presented in Chapter 3, the markers linked to *Sr13* produced the same fragment sizes in the two M-lines and the *Sr13* positive control Kronos. The combined results from these two chapters therefore do not exclude *Sr13* as the candidate gene. The gene, or closely linked genes, that is present in lines M3283_4 and M3192_7 can be confirmed using genetic mapping.

## 4.6 References


Bender, C. M., Prins, R., and Pretorius, Z. A. (2016). Development of a greenhouse screening method for adult plant response in wheat to stem rust. *Plant Disease,* 100: 1627-1633.

Bhavani, S., Hodson, D. P., Huerta-Espino, J., Randhawa, M. S., and Singh, R. P. (2019). Progress in breeding for resistance to Ug99 and other races of the stem rust fungus in CIMMYT wheat germplasm. *Frontiers of Agricultural Sciences and Engineering,* 6: 210-224.

Boshoff, W. H., Pretorius, Z. A., van Niekerk, B. D., and Komen, J. S. (2000). First report of virulence in *Puccinia graminis* f. sp. *tritici* to wheat stem rust resistance genes *Sr8b* and *Sr38* in South Africa. *Plant Disease,* 86: 922.

Chawade, A., van Ham, J., Blomquist, H., Bagge, O., Alexandersson, E., and Ortiz, R. (2019). High-throughput field-phenotyping tools for plant breeding and precision agriculture. *Agronomy,* 9: 1-18.

Figlan, S., Le Roux, C., Terefe, T., Botes, W., Visser, B., Shimelis, H., and Tsilo, T. J. (2014). WSheat stem rust in South Africa: Current status and future research directions. *African Journal of Biotechnology*, 13: 4188-4199.

Kthiri, D., Loladze, A., MacLachlan, P. R., N'Diaye, A., Walkowiak, S., Nilsen, K., Dreisigacker, S., Ammar, K., and Pozniak, C. J. (2018). Characterization and mapping of leaf rust resistance in four durum wheat cultivars. *PLoS ONE,* 13: e0197317.

Lopez-Vera, E. E., Nelson, S., Singh, R. P., Basnet, B. R., Haley, S. D., Bhavani, S., Huerta-Espino, J., Xoconostle-Cazares, B. G., Ruiz-Medrano, R., Rouse, M. N., and Singh, S. (2013). Resistance to stem rust Ug99 in six bread wheat cultivar maps to chromosome 6DS. *Theoretical and Applied Genetics,* 127: 231-239.

Mukoyi, F., Soko, T., Mulima, E., Hodson, D., Herselman, L., Visser, B., and Pretorius, Z. A. (2011). Detection of variants of wheat stem rust race Ug99 (*Puccinia graminis* f. sp. *tritici*) in Zimbabwe and Mozambique. *Plant Disease,* 95: 1188.

Olivera, P. D., Jin, Y., Rouse, M. N., Badebo, A., Fetch Jr, T., Singh, R. P., and Yahyaoui, A. (2012). Races of *Puccinia graminis* f. sp. *tritici* with ombined virulence to *Sr13* and *Sr9e* in a field stem rust screening nursery in Ethiopia. *Plant Disease,* 96: 623-628.

Olivera, P. D., Pretorius, Z. A., Badebo, A., and Jin, Y. (2013). Identification of resistance to races of *Puccinia graminis* f. sp. *tritici* with broad virulence in triticale (×*Triticosecale*). *Plant Disease,* 97: 479-484.

Peterson, R. F., Campbell, A. B., and Hannah, A. E. (1948). A diagrammatical scale for estimating rust intensity on leaves and stems of cereals. *Canadian Journal of Research,* 26: 496-500.

Pretorius, Z. A., Bender, C. M., Visser, B., and Terefe, T. (2010). First report of a *Puccinia graminis* f. sp. *tritici* race virulent to the *Sr24* and *Sr31* wheat stem rust resistance genes in South Africa. *Plant Disease,* 94: 784.

Pretorius, Z. A., Singh, R. P., Wagoire, W. W., and Payne, T. S. (2000). Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis* f. sp. *tritici* in Uganda. *Plant Disease,* 84,: 203.

Pretorius, Z. A., Szabo, L. J., Boshoff, W. H., Herselman, L., and Visser, B. (2012). First report of a new TTKSF race of wheat stem rust (*Puccinia graminis* f. sp. *tritici*) in South Africa and Zimbabwe. *Plant Disease*, 96: 590.

Prins, R., Dreisigacker, S., Pretorius, Z., van Schalkwyk, H., Wessels, E., Smit, C., Bender, C., Singh, D., and Boyd, L. A. (2016). Stem rust resistance in a geographically diverse collection of spring wheat lines collected from across Africa. *Frontiers in Plant Science,* 7: 973.

Roelfs, A. P., Singh, R. P., and Saari, E. E. (1992). *Rust Diseases of Wheat: Concepts and Methods of Disease Management.* CIMMYT, Mexico, pp 1-81.

Rouse, M. N., Nirmala, J., Jin, Y., Chao, S., Fetch Jr, T. G., Pretorius, Z. A., and Hiebert, C. W. (2014). Characterization of *Sr9h*, a wheat stem rust resistance allele effective to Ug99. *Theoretical and Applied Genetics*, 127: 1681-1688.

Saini, J., Faris, J. D., Zhang, Q., Rouse, M. N., Jin, Y., Long, Y., Klindworth, D. L., Elias, E. M., McClean, P. E., Edwards, M. C., and Xu, S. S. (2018). Identification, mapping, and marker development of stem rust resistance genes in durum wheat 'Lesbock'. *Molecular Breeding*, 38: 77.

Simons, K., Abate, Z., Chao, S., Zhang, W., Rouse, M., Jin, Y., Elias, E., and Dubcovsky, J. (2011). Genetic mapping of stem rust resistance gene *Sr13* in tetraploid wheat (*Triticum turgidum* spp. *durum* L.). *Theoretical and Applied Genetics*, 122: 649-658.

Singh, R. P., Hodson, D. P., Huerta-Espino, J., Jin, Y., Bhavani, S., Njau, P., Herrera-Foessel, S., Singh, P. K., Singh, S., and Govindan, V. (2011). The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. *Annual Review of Phytopathology*, 49: 465-481.

Singh, R. P., Hodson, D. P., Huerta-Espino, J., Jin, Y., Njau, P., Wanyera, R., Herrera-Foessel, S. A., and Ward, R. W. (2008). Will stem rust destroy the world's wheat crop? *Advances in Agronomy*, 98: 271-309.

Singh, R. P., Hodson, D. P., Jin, Y., Lagudah, E. S., Ayliffe, M. A., Bhavani, S., Rouse, M. N., Pretorius, Z. A., Szabo, L. J., Huerta-Espino, J., Basnet, B. R., Lan, C., and Hovmøller, M. S. (2015). Emergence and spread of new races of wheat stem rust

fungus: continued threat to food security and prospects of genetic control. *Phytopathology,* 105: 872-884.

Stakman, E. C., Stewart, D. M., and Loegering, W. Q. (1962). *Identification of Physiologic Races of Puccinia graminis var tritici.* U.S.D.A. Beureau of Entomology, Minnesota, pp E1-E617.

Terefe, T., Pretorius, Z. A., Paul, I., Mebalo, J., Meyer, L., and Naicker, K. (2010). Occurrence and pathogenicity of *Puccinia graminis* f. sp. *tritici* on wheat in South Africa during 2007 and 2008. *South African Journal of Plant and Soil,* 27: 163-167.

Terefe, T., Pretorius, Z. A., Visser, B., and Boshoff, W. H. (2019). First report of *Puccinia graminis* f. sp. *tritici* race PTKSK, a variant of wheat stem rust race Ug99 in South Africa. *Plant Disease,* 103: 1-3.

Tsilo, T. J., Chao, S., Jin, Y., and Anderson, J. A. (2009). Identification and validation of SSR markers linked to the stem rust resistance gene *Sr6* on the short arm of chromosome 2D in wheat. *Theoretical and Applied Genetics,* 118: 515-524.

Visser, B., Herselman, L., Park, R. F., Karaoglu, H., Bender, C. M., and Pretorius, Z. A. (2011). Characterization of two new *Puccinia graminis* f. sp. *tritici* races within the Ug99 lineage in South Africa. *Euphytica,* 179: 119-127.

Wanyera, R., Macharia, J. K., Kilonzo, S. M., and Kamundia, J. W. (2009). Foliar fungicides to control wheat stem rusts race TTKS (Ug99), in Kenya. *Plant Disease,* 93: 929-932.

Zhang, W., Chen, S., Abate, Z., Nirmala, J., Rouse, M. N., and Dubcovsky, J. (2017). Identification and characterization of *Sr13*, a tetraploid wheat gene that confers resistance to the Ug99 stem rust race group. *Proceedings of the National Academy of Sciences of the United States of America,* 114: E9483-E9492.

# Chapter 5

# Identification and mapping of markers linked to stem rust resistance in the wheat lines M3283_4 and M3192_7

## 5.1 Introduction

Various methods have been developed to select genotypes carrying a specific gene. Bulk segregant analysis is a method used to locate the target gene using molecular markers and segregating populations (Xu and Crouch 2008; Zou et al. 2016). The analysis can be performed using various populations with phenotypic differences including populations derived from biparental crosses. In a study that focuses on qualitative traits (e.g. disease resistance), samples are bulked into two pools that contain different phenotypes (Michelmore et al. 1991). This method is advantageous as it is less tedious to perform than for example NILs and reduces genotyping costs (Michelmore et al. 1991; Vikram et al. 2012).

Multiple DNA marker technologies have been used in MAS and genetic mapping. Simple sequence repeats have been used widely as they provide an effective marker system for molecular mapping. However, other effective marker systems have also been developed, such as SNP genotyping technologies. The SNP technologies are categorised into micro-array based and PCR-based. The SNP genotyping technologies that were initially developed had limitations such as inaccuracy, high cost and low throughput. However, a recently developed SNP genotyping method, semi-thermal asymmetric reverse PCR (STARP), provides high accuracy and throughput and is inexpensive (Long et al. 2017). The STARP marker system has been used to detect resistance genes and to facilitate gene pyramiding. For example, this marker system has been used in the detection of *Sr13* alleles (Saini et al. 2018).

In this chapter, SSR analysis combined with BSA of a biparental $F_2$ mapping population was used to identify stem rust resistance gene(s) providing resistance in the durum derived wheat lines M3283_4 and M3192_7. Simple sequence repeat markers were used to identify polymorphisms in the parental wheat lines; Line 37-07 (susceptible parent), M3283_4 and M3192_7 (resistant parents). Secondly, markers polymorphic between the parental lines were screened on the bulks of two $F_2$ populations, namely Line37-07/M3283_4 and Line37-07/M3192_7. Markers that showed possible linkage to stem rust resistance loci were

screened on the individuals of the bulks. The informative markers were then confirmed in the entire $F_2$ populations and homozygous resistant and susceptible $F_3$ families. Thirdly, using the $F_2$ population data, a partial linkage map was constructed. Lastly, STARP markers were used to identify the *Sr13* allele present in the resistant lines.

## 5.2 Materials and methods

### 5.2.1 Plant material collection and DNA isolation

For the identification of polymorphic loci between parental lines [Line 37-07 (susceptible) and M3283_4 and M3192_7 (resistant lines)], leaf samples collected as described in section 3.2.1, were used. Leaf material of the two $F_2$ populations (Line37-07/M3283_4 and Line37-07/M3192_7) was collected 14 days post inoculation as described in section 4.2.1.3 and used for the evaluation of polymorphic markers. Between 198 and 200 (approximately 50 susceptible and 150 resistant) plants were randomly sampled per $F_2$ population for molecular marker analysis. For BSA, 10 resistant and 10 susceptible $F_2$ plants were randomly selected per cross combination for DNA extraction to form the resistant and susceptible bulks, respectively.

The informative markers identified on the $F_2$ population were validated in the $F_3$ families. The plant material was developed and inoculated as described in sections 4.4.1.2 and 4.4.1.3. Five plants were randomly sampled from each homozygous susceptible and homozygous resistant $F_3$ family. In total, Line37-07/M3283_4 had 97 $F_3$ families (26 homozygous resistant, 48 heterozygous and 23 homozygous susceptible) and Line37-07/M3192_7 had 83 $F_3$ families (19 homozygous resistant, 41 heterozygous and 23 homozygous susceptible).

Genomic DNA was isolated using the modified CTAB method of Saghai-Maroof et al. (1984), as described in section 3.2.2. The quality and quantity of the isolated gDNA was evaluated as described in section 3.2.3. All gDNA samples were diluted to final working concentrations of 20 ng/µl. To obtain a representative sample of each parental line, equal amounts of diluted DNA of 10 samples per parental line were mixed and used as parental samples for BSA. Equal amounts of diluted DNA of 10 resistant and 10 susceptible samples were mixed to develop the resistant and susceptible bulks for the $F_2$ populations.

### 5.2.2 Evaluation of SSR markers

A LRGS marker set consisting of 104 SSR markers was initially used to identify polymorphic loci between the susceptible and resistant parents (Appendix 1). Each of the 21 wheat chromosomes were scanned with five SSR markers, except for chromosome 4A where only four markers were screened. The LRGS marker set was constructed by Wessels and Prins (2017) based on results obtained for SA wheat cultivars. To obtain at least four polymorphic markers per chromosome, 54 additional SSR markers, covering chromosome areas that lacked polymorphic markers, were selected from literature (Somers et al. 2004; GrainGenes) and screened (Appendix 2). Markers polymorphic between the parental lines were selected and screened on the resistant and susceptible $F_2$ bulk samples to identify informative markers. These informative markers were used to evaluate the individuals used to constitute the bulk samples for consistent linkage. Two markers (Sr13 and gwm427), which showed strong linkage in the individuals of the bulk samples, were used to genotype the entire Line37-07/M3283_4 and Line37-07/M3192_7 $F_2$ populations.

To validate the predictive ability of the two identified closely linked markers (Sr13 and gwm427) for the *Sr*-gene, the markers were only tested on plants selected based on phenotypic data, as homozygous resistant and homozygous susceptible. The informative markers were evaluated on 245 and 210 $F_3$ plants obtained from the Line37-07/M3283_4 and Line37-07/M3192_7 mapping populations, respectively. PCR reactions and PAGE of amplified fragments were done as described in sections 3.2.4.1 and 3.2.4.2. The PCR cycling conditions for all markers used were obtained from literature (Somers et al. 2004; Wessels and Prins 2017; GrainGenes) including the expected PCR fragment sizes and annealing temperatures. The PCR conditions of the informative markers are given in Table 5.1.

### 5.2.3 Data analysis

The data obtained after PAGE of both the $F_2$ populations and $F_3$ families was captured on Microsoft Excel version 2007. The resistance allele was scored as A, susceptible allele as B and heterozygotes as H. A chi-square test was performed on the informative markers to test for marker segregation distortion. The co-dominant markers were expected to segregate in a 1:2:1 ratio in the $F_2$ populations.

**Table 5.1 Polymerase chain reaction conditions for the informative markers used to evaluate the $F_2$ and $F_3$ individuals**

| Marker name | Chromosome number | Reaction conditions and total reaction volume | PCR cycle conditions | Marker type, controls and fragment sizes |
|---|---|---|---|---|
| Sr13 | 6A | 80 ng DNA<br>1x KAPATaq ReadyMix DNA polymerase<br>25 ng Sr13F primer<br>25 ng Sr13R primer<br>Total reaction volume: 10 µl | 1 cycle: 2 min, 95°C;<br>35 cycles: 45 s, 94°C; 45 s, 58°C; 60 sec, 72°C;<br>1 cycle: 4 min, 72°C | Dominant marker<br>One allele in both resistant and susceptible plants (265 bp) |
| Sr13<br>CAPS marker | | 1x Cutsmart® buffer<br>10 U *Hha* I | Incubate for 60 min, 37°C | Co- dominant marker<br>Resistant control: Kronos (244 bp)<br>Susceptible control: Line 37-07 (265 bp) |
| gwm427 | 6A | 1x KAPATaq ReadyMix DNA polymerase<br>25 ng GWM427F primer<br>25 ng GWM427R primer<br>Total reaction volume: 10 µl | 1 cycle: 3 min, 94°C;<br>35 cycles: 1 min, 94°C; 1 min, 50°C; 2 min, 72°C;<br>1 cycle: 10 min, 72°C | Co- dominant marker<br>Resistant control: Kalka, Tamaroi and Gundaroi (200 bp)<br>Susceptible control: Bansi (226 bp) |

CAPS = cleaved amplified polymorphic sequence

### 5.2.4 Genetic linkage mapping

For Line37-07/M3283_4 and Line37-07/M3192_7 $F_2$ populations, mapping was done using Map Manager QTXb20 (Manly et al. 2001) with minimum logarithm of odds (LOD) of 3.0. The genetic distance was calculated using the Kosambi mapping function (Kosambi 1944). The data used was scored as A for the resistance allele, B for susceptible allele and H for heterozygotes. A partial linkage map of chromosome 6A was drawn using Map Chart version 2.30 software (Voorrips 2002) and to determine the position of the resistance gene on the chromosome. To better visualise the map positions of the two markers and the resistance genes on chromosome 6A, the map positions of the two distal markers on wheat chromosome 6A (gwm459 and wmc59) as well as the map position of gwm427, as obtained from Somers et al. (2004), were included in the Map Chart data.

### 5.2.5 *Sr13* allele test

Three allele specific STARP markers namely, Rwgsnp37, Rwgsnp38 and Rwgsnp39 were used to detect and discriminate between the three *Sr13* alleles in durum and tetraploid related wheat germplasm (Table 5.2). These markers were developed based on the functional SNPs of the cloned *Sr13* haplotypes R1 (*Sr13a*), R2 (*Sr13b*) and R3 (*Sr13c*). Rwgsnp37 detects the presence of *Sr13*. Rwgsnp38 discriminates between R1/R3 and R2, and Rwgsnp39 differentiates between R1 and R3. These markers were developed by Saini et al. (2018) and Zhang et al. (2020) and were evaluated on DNA samples from the varieties Combination VII (*Sr13a*), Leeds (*Sr13b*), Kronos, Line 37-07, M3283_4 and M3192_7.

The *Sr13a* control belongs to the R1 haplotype and the *Sr13b* control to the R2 haplotype. In addition, CAPS marker Sr13 and SSR marker gwm427 were previously screened on the parental lines (Chapter 3). The PCR reactions for the STARP markers were performed in the laboratory of Dr Steven Xu, United States Department of Agriculture - Agricultural Research Service (USDA-ARS, Edward T. Schafer Agricultural Research Center, Fargo, ND, USA). Based on previous studies, genotyping was performed using two-universal priming element-adjustable primers (PEA-primers), two asymmetrically modified allele-specific primers (AMAS-primers) and one common reverse primer (Long et al. 2017; Zhang et al. 2020). The sequences of the STARP primers and the melting temperatures are summarised in Table 5.2.

**Table 5.2 Semi-thermal asymmetric PCR markers used to identify the *Sr13* allele present in M3283_4 and M3192_7**

| Marker | Primer | Primer sequence (5'-3') | $T_m$ (°C) | Reference |
|---|---|---|---|---|
| Rwgsnp37 | Sr13-F1 | [Tail 2] AAACCTTTGTTCTCTAACTCTGC | 56.99 | Saini et al. 2018 |
| | Sr13-F2 | [Tail 1] AAACCTTTGTTCTCTAACTACGT | 55.83 | |
| | Sr13-R1.1 | GCGTCAGCAAGAAGTCATCATCA | 61.47 | |
| Rwgsnp38 | Sr13ab-F1 | [Tail 1] GAATGTATATGTCATGTCCAACG | 55.57 | Zhang et al. 2020 |
| | Sr13ab-F2 | [Tail 2] GAATGTATATGTCATGTCCGCCT | 58.43 | |
| | Sr13ab-R | GCGACTGTAATCTTCAGTTATCCTC | 59.31 | |
| Rwgsnp39 | Sr13ac-F1 | [Tail 1] GCCTGAGGAAGTTTAAATATTGG | 57.18 | Zhang et al. 2020 |
| | Sr13ac-F2 | [Tail 2] GCCTGAGGAAGTTTAAATACTAT | 56.52 | |
| | Sr13ac-R | CGTACGCAGAGGATAACTGAAGA | 59.94 | |

Tail 1 and Tail 2 universal sequences are 5'- GCAACAGGAACCAGCTATGAC-3′ and 5'- GACGCAAGTGAGCAG TATGAC-3′, respectively (Long et al. 2017); $T_m$ = annealing temperature

According to Long et al. (2017), STARP behaves as a 2-plex PCR where allele 1 is specifically amplified using PEA-primer 1 coupling with AMAS-primer 1 in combination with the reverse primer and allele 2 is specifically amplified using PEA-primer 2 coupling with AMAS-primer 2 in combination with the common reverse primer. The STARP reactions were carried out in a total volume of 10 µl containing 1x $NH_4^+$ buffer [16 mM Ammonium sulphate $(NH_4)_2SO_4$ and 67 mM Tris-HCl, pH 8.3 at 25°C], 0.8 M betaine, 0.04% (w/v) bovine serum albumin, 1.5 mM $MgCl_2$, 50 µM of each dNTP, 200 nM common reverse primer, 200 nM of each PEA-primer (PEA-primer 1 and PEA-primer 2), and 40 nM of each AMAS-primer (AMAS-primer 1 and AMAS-primer 2), 1 U of *Taq* DNA polymerase (without 3′ → 5′ exonuclease activity), and gDNA in the range from 10 to 100 ng.

Touchdown PCR was performed with a denaturation step at 94°C for 3 min, followed by six cycles of a two-step touchdown PCR programme starting at 94°C for 20 s and then 56°C for 2 min, with the annealing/extension temperature ($T_{a/e}$) being decreased by 1°C per cycle. This touchdown PCR programme was immediately followed by another two-step PCR programme of 94°C for 20 s and then at 62°C for 2 min with 32-36 cycles for gel-based size separation. The extension step was carried out for 2 min at 62°C. The STARP markers were evaluated on 6.5% denaturing polyacrylamide gels in an IR2 4300/4200 DNA Analyzer (LI-COR, Lincoln, NE, USA).

## 5.3 Results

### 5.3.1 Molecular marker analysis

The chromosomal location of the resistance gene was successfully determined using molecular markers combined with BSA. One hundred and seventy SSR markers (104 from the LRGS set, 54 additional SSR markers and 12 markers linked to known *Sr*-genes screened in Chapter 3) were screened on the parental lines for the identification of polymorphic loci. As presented in Table 5.3 and Appendices 1 and 2, 89 of all the SSR markers screened were polymorphic between the resistant and susceptible parent lines. Although the aim was to obtain at least four polymorphic markers per chromosome, some chromosomes only had three polymorphic markers covering the entire chromosome. The information obtained in Chapter 3 regarding *Sr13* was also incorporated in this chapter.

**Table 5.3 Simple sequence repeat markers screened and polymorphic in the $F_2$ populations**

| Population | Markers screened | No. of polymorphic markers | Percentage (%) |
|---|---|---|---|
| Line37-07/M3283_4 | 170 | 86 | 50.6 |
| Line37-07/M3192_7 | 170 | 85 | 50.0 |

Eighty-six and 85 polymorphic markers were used to evaluate the resistant and susceptible bulks of the two $F_2$ populations (Line37-07/M3283_4 and Line37-07/M3192_7), respectively. Three markers that showed possible linkage to the resistance gene(s) were screened on the individuals of the bulks for validation. Based on the evaluation of these three markers, markers Sr13 and gwm427, located on chromosome 6A, showed close linkage to the gene in both $F_2$ populations. In addition, marker wmc617, located on chromosome 4B, showed linkage in the individuals of the bulk samples of the $F_2$ population of Line37-07/M3283_4. However, when marker wmc617 was evaluated on individuals of the entire $F_2$ population, linkage could not be confirmed. In contrast, the evaluation of markers Sr13 and gwm427 in individual plants from both $F_2$ populations showed close linkage to the gene conferring resistance in the two M-lines. Genotyping data for Sr13 did not fit to the expected segregation ratio of 1:2:1 in both $F_2$ populations. On the other hand, gwm427 only produced a good fit to the expected segregation ratio of 1:2:1 in the Line37-07/M3192_7 mapping population (Table 5.4).

Markers Sr13 and gwm427 were validated on all individuals selected from the $F_3$ families (Table 5.5). Marker Sr13 had a 99% and 82% prediction accuracy for the resistant and susceptible plants, respectively, in Line37-07/M3283_4. On the other hand, for the Line37-07/M3192_7 population, the marker had a 72% and 100% prediction accuracy for the resistant and susceptible plants, respectively. Marker gwm427 predicted the resistant and susceptible plants with 87% and 96% accuracy, respectively in Line37-07/M3283_4. On the other hand, for the Line37-07/M3192_7 population, the marker predicted the resistant and susceptible plants with 97% and 98% accuracy, respectively.

**Table 5.4 Evaluation of the informative markers Sr13 and gwm427 on the entire $F_2$ population**

| Population name | Marker | No. of plants screened | A | H | B | Chi-square (1:2:1) |
|---|---|---|---|---|---|---|
| Line37-07/M3283_4 | Sr13 | 200 | 83 | 67 | 50 | 32.7 |
| Line37-07/M3283_4 | gwm427 | 199 | 86 | 69 | 44 | 36.4 |
| Line37-07/M3192_7 | Sr13 | 196 | 59 | 50 | 87 | 55.0 |
| Line37-07/M3192_7 | gwm427 | 198 | 56 | 96 | 46 | 1.2 |

A = Homozygous resistant; H = Heterozygous; B = Homozygous susceptible

**Table 5.5 Validation of the informative markers Sr13 and gwm427 on the $F_3$ individuals**

| $F_3$ population | Markers | HR families | | Accuracy (%) | HS families | | Accuracy (%) |
|---|---|---|---|---|---|---|---|
| | | Resistant genotypes | Resistant phenotypes | | Susceptible genotypes | Susceptible phenotypes | |
| Line37-07/M3283_4 | Sr13 | 91 | 92 | 99 | 84 | 102 | 82 |
| | gwm427 | 113 | 129 | 87 | 100 | 104 | 96 |
| Line37-07/M3192_7 | Sr13 | 68 | 94 | 72 | 113 | 113 | 100 |
| | gwm427 | 85 | 88 | 97 | 110 | 112 | 98 |

HR = Homozygous resistant; HS = Homozygous susceptible

The STARP markers were successfully evaluated in the six control varieties. Rwgsnp37 showed that Line 37-07 was negative for *Sr13*, whereas the rest of the varieties tested positive for *Sr13* including Kronos, M3283_4 and M3192_7. The additional markers, Rwgsnp38 and Rwgsnp39 were further used to confirm which of the *Sr13* alleles were present. Rwgsnp38 analysis showed that Leeds (*Sr13b* control) grouped alone, however the other varieties amplified the same fragment as for the *Sr13a/c* genotype. The Rwgsnp39 analysis showed that Kronos, M3283_4 and M3192_7 amplified the same fragment as the *Sr13a* haplotype.

### 5.3.2 Mapping

A partial linkage map for chromosome 6A was drawn for both $F_2$ populations: Line37-07/M3283_4 and Line37-07/M3192_7 using Map Manager QTXb20. The traits were labelled as *SrM3283* and *SrM3192*, respectively. The two traits associated with resistance to *Pgt* race PTKST were identified towards the distal end on the long arm of chromosome 6A. These traits were confirmed to be closely linked to the informative markers, Sr13 and gwm427.

The traits, *SrM3283* and *SrM3192* mapped 17.4 cM and 62.0 cM away from Sr13 (Table 5.6) with LOD values of 41.9 and 10.6, respectively. Marker gwm427 mapped 24.2 cM and 28.5 cM away from *SrM3283* and *SrM3192* with LOD values of 61.1 and 21.1, respectively. The coefficient of variation was calculated, and it was deduced from the results that marker Sr13 explained 61.9% of the phenotypic variation for race PTKST in the Line37-07/M3283_4 population and 22% of the phenotypic variation in the Line37-07/M3192_7 population (Table 5.6). Furthermore, marker gwm427 analysis showed that it explained 75.7% of phenotypic variation in the Line37-07/M3283_4 population and 38.8% of the phenotypic variation in the Line37-07/M3192_7 population (Table 5.7).

**Table 5.6 Mapping position, logarithm of odds (LOD) and coefficient of variation for the stem rust resistance traits in the two $F_2$ populations relative to marker Sr13**

| Trait | Chromosome arm | Position from marker Sr13 (cM) | LOD | PVE (%) |
|---|---|---|---|---|
| *SrM3283* | 6AL | 17.4 | 41.9 | 61.9 |
| *SrM3192* | 6AL | 62.0 | 10.6 | 22 |

cM = centiMorgan; PVE = phenotypic variation explained by marker Sr13

**Table 5.7 Mapping position, logarithm of odds (LOD) and coefficient of variation for the stem rust resistance traits in the two $F_2$ populations relative to marker gwm427**

| Trait | Chromosome arm | Position from marker gwm427 (cM) | LOD | PVE(%) |
|---|---|---|---|---|
| *SrM3283* | 6AL | 24.2 | 61.1 | 75.7 |
| *SrM3192* | 6AL | 28.5 | 21.1 | 38.8 |

cM = centiMorgan; PVE = phenotypic variation explained by marker gwm427

To visualise the mapping results and because data was only available for two markers plus the trait, maps were drawn using marker information from Somers et al. (2004). The map positions of the two markers at the distal ends of chromosome 6A, gwm459 and wmc59, as well as the map position of gwm427 from the map of Somers et al. (2004) was used and marker Sr13 and the two traits, *SrM3283* and *SrM3192* was drawn relative to these three markers (Figure 5.1).

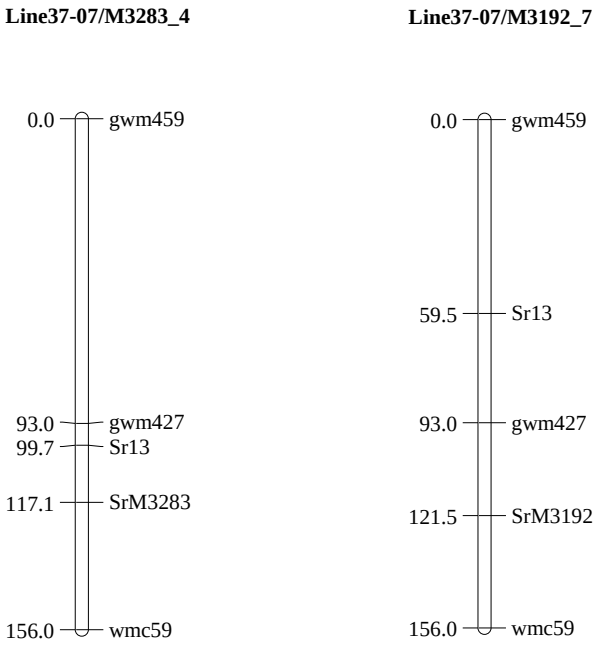


**Figure 5.1 Partial linkage map of chromosome 6A depicting distances between the traits and informative markers for Line37-07/M3192_7 and Line37-07/M3283_4**. Marker names are indicated on the right and marker positions are shown on the left in centiMorgans (cM)

## 5.4 Discussion

The aim of the study was to identify and map markers linked to stem rust resistance gene(s) providing resistance in the durum derived wheat lines M3283_4 and M3192_7. Based on the results obtained, the resistance gene was mapped successfully using polymorphic markers. Two markers, Sr13 and gwm427 were confirmed to be informative as results showed linkage in both $F_2$ populations. Linkage was not observed for the third marker wmc617 during the evaluation of the individuals of the $F_2$ population, thus the marker was discarded as false linkage was suspected in the bulks. Markers Sr13 and gwm427 are located on the long arm of chromosome 6A (Simons et al. 2011). According to literature, both markers are closely linked to resistance gene *Sr13* (Qamar et al. 2009; Simons et al. 2011). *Sr13* mapped within 1.2-2.8 cM of the flanking markers and recombination frequencies of 8.1 ± 3.0%, 10.7 ± 3.4% and 11.3 ± 3.1 % were observed between gwm427 and the three desired genes.

In a study by Saini et al. (2018), it was confirmed that the derived CAPS (dCAPS) marker Sr13 (Zhang et al. 2017) is diagnostic, thus the marker was expected to predict with 100% accuracy resistant and susceptible lines. However, this was not the case in the susceptible lines of the Line37-07/M3283_4 and the resistant lines of Line37-07/M3192_7 populations. The genotyping data of the Line37-07/M3192_7 population was difficult to score (due to incomplete enzyme digestion), and this was suspected to be the cause of the observed inaccuracy. Furthermore, marker gwm427 predicted the expected results in both populations with a high accuracy and the less than 100% is expected because gwm427 is not a perfect marker and recombination between the gene and the marker can be expected.

The stem rust resistance gene *Sr13* was initially identified in tetraploid wheat (e.g. emmer and durum wheat) and has since been used to develop stem rust resistant common wheat varieties (Simons et al. 2011; Sharma et al. 2019). Due to *Sr13* having different alleles, STARP marker Rwgsnp37 was developed based on SNPs between the susceptible and resistant haplotypes of this gene. Results from the marker analysis in this study confirmed that Line 37-07 did not carry *Sr13*, however, Kronos, Combination VII (*Sr13a*), Leeds (*Sr13b*), M3283_4 and M3192_7 tested positive for *Sr13*. The haplotypes detected by Rwgsnp37 matched the haplotypes of the dCAPS marker Sr13 indicating the presence of the *Sr13* gene, thus this marker is diagnostic as well. Rwgsnp38 differentiates between R1/R3 and R2 and Rwgsnp39 differentiates between R1 and R3 (Zhang et al. 2020). The observed result from the Rwgsnp38 analysis showed that the varieties grouped with *Sr13a/c*

and consequently with the *Sr13a* genotype for marker Rwgsnp39. These results thus eliminated the presence of the other alleles thus confirming that the allele present in Kronos, M3283_4 and M3192_7 is *Sr13a*.

Based on the results obtained from Map Manager, *SrM3283* was positioned 17.4 cM and *SrM3192* 62.0 cM from marker Sr13. The genetic distance between marker Sr13 and *SrM3283* suggest that the resistant trait mapped in line M3283_4 could possibly be *Sr13* or closely linked to the *Sr13* gene while the genetic distance between marker Sr13 and *SrM3192* suggests that the marker is not linked (>50 cM) to the gene in line M3192_7. However, based on the STARP results, as well as the marker data of Chapter 3 and phenotypic data of Chapter 4, the *Sr13a* allele is the likely source of *Pgt* race PTKST stem rust resistance present in durum derived lines, M3283_4 and M3192_7 as well as in the *Sr13* control durum variety Kronos. According to Somers et al. (2004), molecular breeding is most effective when the map is densely populated with markers. The presence of only two informative markers suggested that the map results are not 100% accurate. The map distance between the trait and marker Sr13 could thus decrease when additional markers are mapped to this chromosome region. Due to incomplete digestion of the dCAPS marker, especially in the Line37-07/M3192_7 population, lines were probably scored as heterozygotes instead of homozygotes, causing marker Sr13 to map further from the trait than expected. This could also have caused the change in marker order between the two populations. Furthermore, there was segregation distortion in the markers. According to Coulton et al. (2020), this can make genetic mapping difficult as it skews the data away from the desired genotype.

## 5.5 Conclusions

The stem rust resistance source in the wheat lines M3283_4 and M3192_7 was partially mapped to the long arm of chromosome 6A. The dCAPS marker Sr13 and the STARP markers indicated the presence of *Sr13* as the stem rust resistance source to *Pgt* race PTKST in the wheat lines M3283_4 and M3192. Linkage mapping of markers Sr13 and gwm427 and the trait confirmed linkage to the gene on the same chromosome. Due to the limited number of polymorphic markers detected and mapped to the long arm of chromosome 6A, it was not possible to 100% confirm that traits, *SrM3283* and *SrM3192* are indeed *Sr13*. It can be concluded that the results provided enough information to give insight for future possibilities for research.

Although the LRGS marker set provided a good basis to scan the entire genome and did detect the *Sr13* gene region as the source of resistance and no other chromosome regions, for future studies, the entire genome should be screened with more markers to ensure that a minor gene or QTL was not missed. It might also be worthwhile to screen more markers on chromosome 4B to elucidate the results obtained with marker wmc617 in the bulk samples. To generate a finer map, more markers such as additional SSRs, SNPs and DArTs, known to map to chromosome 6A, could be screened to get closer to the gene and confirm if it is indeed *Sr13*. To increase the accuracy of the map, the population size could be increased. The identified markers should also be screened in other genetic backgrounds by creating mapping populations using different susceptible parent lines. Whether the *Sr*-gene mapped in this study is *Sr13a*, another allele of *Sr13* or a novel source of stem rust resistance, it is unlikely to provide durable resistance to *Pgt*. The resistance gene should therefore be stacked with other effective stem rust resistance genes to improve the likelihood of durable stem rust resistance (Singh et al. 2015).

## 5.6 References


Coulton, A., Przewieslik-Allen, A., Burridge, A. J., Shaw, D. S., Edwards, K. J., and Barker, G. L. A. (2020). Segregation distortion: Utilizing simulated genotyping data to evaluate statistical methods. *PLoS ONE*, 15: e0228951.

GrainGenes, A database for Triticaea and Avena, Available at https://wheat.pw.usda.gov/, (Accessed 20 July 2020).

Kosambi, D. D. (1944). The estimation of map distances from recombination values. *Annals of Eugenics, 12*, 172-175.

Long, Y. M., Chao, W. S., Ma, G. J., Xu, S. S., and Qi, L. L. (2017). An innovative SNP genotyping method adapting to multiple platforms and throughputs. *Theoretical and Applied Genetics*, 130: 597-607.

Manly, K. F., Cudmore Jr, R. H., and Meer, J. M. (2001). Map Manager QTX, cross-platform software for genetic mapping. *Mammalian Genome*, 12: 930-932.

Michelmore, R. W., Paran, I., and Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings*

*of the National Academy of Sciences of the United States of America,* 88: 9828-9832.

Qamar, Z., Bansal, U. K., and Bariana, H. S. (2009). Genetics of stem rust resistance in three durum wheat cultivars. *International Journal of Plant Breeding,* 3: 99-102.

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences of the United States of America,* 81: 8014-8018.

Saini, J., Faris, J. D., Zhang, Q., Rouse, M. N., Jin, Y., Long, Y., Klindworth, D. L., Elias, E. M., McClean, P. E., Edwards, M. C., and Xu, S. S. (2018). Identification, mapping, and marker development of stem rust resistance genes in durum wheat 'Lesbock'. *Molecular Breeding,* 38: 77.

Sharma, J. S., Zhang, Q., Rouse, M. N., Klindworth, D. L., Friesen, T. L., Long, Y., Olivera, P. D., Jin, Y., McClean, P. E., Xu, S. S., and Faris, J. D. (2019). Mapping and characterization of two stem rust resistance genes derived from cultivated emmer wheat accession PI 193883. *Theoretical and Applied Genetics,* 132: 3177-3189.

Simons, K., Abate, Z., Chao, S., Zhang, W., Rouse, M., Jin, Y., Elias, E., and Dubcovsky, J. (2011). Genetic mapping of stem rust resistance gene *Sr13* in tetraploid wheat (*Triticum turgidum* spp. *durum* L.). *Theoretical and Applied Genetics,* 122: 649-658.

Singh, R. P., Hodson, D. P., Jin, Y., Lagudah, E. S., Ayliffe, M. A., Bhavani, S., Rouse, M. N., Pretorius, Z. A., Szabo, L. J., Huerta-Espino, J., Basnet, B. R., Lan, C., and Hovmøller, M. S. (2015). Emergence and spread of new races of wheat stem rust fungus: continued threat to food security and prospects of genetic control. *Phytopathology,* 105: 872-884.

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

Vikram, P., Mallikarjuna Swamy, B. P., Dixit, S., Ahmed, H., Sta Cruz, M. T., Singh, A. K., Ye, G., and Kumar, A. (2012). Bulk segregant analysis: "An effective approach

for mapping consistent-effect drought grain yield QTLs in rice". *Field Crops Research,* 134: 185-192.

Voorrips, R. E. (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. *Journal of Heredity,* 93: 77-78.

Wessels, E., and Prins, R. (2017). Development of a low- resolution genome scan marker set for the acceleration of major gene or QTL mapping in wheat. *South African Journal of Plant and Soil,* 34: 155-158.

Xu, Y., and Crouch, J. H. (2008). Marker-assisted selection in plant breeding: From publications to practice. *Crop Science,* 48: 391-407.

Zhang, J., Long, Y., Gill, B. K., Sharma, J. S., Zhang, Q., Klindworth, D. L., Cai, X., Friesen, T. L., Faris, J. D., and Xu, S. S. (2020) Development and Validation of Semi-Thermal Asymmetric Reverse PCR Markers for Ug99-effective Stem Rust Resistance Genes in Wheat. Poster presented at: Plant and Animal Genome XXVIII Conference, 11-15 January 2020, San Diego, California.

Zhang, W., Chen, S., Abate, Z., Nirmala, J., Rouse, M. N., and Dubcovsky, J. (2017). Identification and characterization of *Sr13*, a tetraploid wheat gene that confers resistance to the Ug99 stem rust race group. *Proceedings of the National Academy of Sciences of the United States of America,* 114: E9483-E9492.

Zou, C., Wang, P., and Xu, Y. (2016). Bulked sample analysis in genetics, genomics and crop improvement. *Plant Biotechnology Journal,* 14,:1941-1955.

# Chapter 6

# General conclusions and recommendations

Wheat is one of the important staple foods in SA. However, there is a fluctuating production of this crop, leading SA to import wheat from other countries such as Germany. The production of wheat is affected by multiple factors including the occurrence of fungal diseases such as stem rust. Devastating outbreaks of the disease, often resulting from the appearance and subsequent spread of more virulent *Pgt* races, have encouraged breeders to deploy new sources of resistance. These sources are characterised through the development of closely linked molecular markers and then manipulated in breeding programmes to achieve gene stacks through marker-assisted breeding.

The phenotypic evaluation performed in this study indicated that a single dominant gene is present in the M-lines. Furthermore, the allelism test performed between the M-lines indicated that the two resistant lines carry the same, allelic or closely linked genes. From past experiences we know that the presence of a single rust resistance gene is unlikely to produce durable resistance. Therefore, when new resistance genes are characterised they should preferably be stacked with existing genes known to be effective against virulent races of *Pgt*, in an effort to prolong resistance. The presence/absence of known *Sr*-genes was successfully determined using closely linked molecular markers. A total of 13 genes were screened for in the M-lines and *Sr13* was the only gene detected. To confirm the absence of any other minor genes/QTL known to confer effective resistance against *Pgt* race Ug99 and its variants, such as PTKST, markers closely linked to these genes/QTL should in future studies also be screened.

Bulk segregant analysis with the use of SSR markers and a segregating $F_2$ population successfully identified the region carrying the resistance gene. Two SSR markers (Sr13 and gwm427), both located on the long arm of chromosome 6A, showed linkage to the resistance gene(s). The location of the resistance gene was further confirmed by mapping the closely linked SSR markers on chromosome 6A. The STARP marker analysis successfully identified the presence of haplotype *Sr13a* in the M-lines. Taking into consideration both phenotyping and genotyping data, the gene present in lines M3283_4 and M3192_7 is most likely *Sr13*. Although at least four polymorphic markers per

chromosome were screened on each $F_2$ mapping population, it might not have been effective in detecting minor genes/QTL due to the large and complex nature of the wheat genome. To confirm that *Sr13* is the only effective *Sr*-gene present in these M-lines, more polymorphic markers (SSRs, SNPs and DArTs) covering the entire genome should be screened using the BSA approach.

Although mapping of the markers was successful, the mapping results were not considered 100% accurate as only two informative markers were found for chromosome 6A. Furthermore, Sr13 is a diagnostic marker and a 100% accuracy rate was expected in the $F_2$ populations and $F_3$ families. However, the prediction accuracy for both resistant and susceptible lines were not 100%. This inaccuracy was caused by incomplete enzyme digestion which led to difficulties in scoring of homozygous resistant and heterozygous plants as well as in some susceptible plants. In future studies, this could be avoided by increasing the enzyme concentration or the incubation time.

To create more accurate mapping results, the population sizes could be increased in future studies and more markers known to map in or near the identified region of chromosome 6A could be evaluated to get closer to the gene(s). To create a denser map, genotyping could be performed using DArT and SNP technologies, such as the 90K SNP chip array. Furthermore, markers Sr13 and gwm427 should be screened in other genetic backgrounds to assess the gene(s) prevalence and to validate the accuracy of these markers. The identified gene could also be combined with other effective stem rust resistance genes/QTL (including APR genes) to prolong resistance in case of an outbreak that is virulent against the gene. The information obtained should be shared with other researchers for use in their breeding programmes.

**Appendix 1: The set of 104 low-resolution genome scan markers used to evaluate polymorphisms in the parental lines**

| Chromosome | Marker name | $T_m$ (°C) | Size range (bp) |
|---|---|---|---|
| 1A | barc148 | 60 | 190-210 |
| | barc119*** | 60 | 200-235 |
| | psp3027*** | 62 | 155-165 |
| | wmc59*** | 64 | 175-205 |
| | cfa2219 | 63 | 195-250 |
| 2A | wmc407** | 57 | 130-140 |
| | wmc177 | 55 | 170-195 |
| | wmc474*** | 62 | 130-140 |
| | gwm312*** | 64 | 190-235 |
| | wmc181*** | 65 | 250-265 |
| 3A | wmc11*** | 67 | 160-195 |
| | barc356 | 48 | 140-165 |
| | gwm5* | 65 | 170-180 |
| | gwm218*** | 65 | 135-155 |
| | gwm155 | 63 | 120-150 |
| 4A | barc106*** | 62 | 125-140 |
| | wmc48*** | 64 | 240-260 |
| | barc343*** | 63 | 140-155 |
| | wmc313 | 64 | 185-210 |
| 5A | wmc524 | 67 | 160-210 |
| | cfd39 | 59 | 165-190 |
| | gwm156 | 55 | 280-320 |
| | barc10 | 57 | 260-295 |
| | wmc410 | 58 | 120-200 |
| 6A | psp3152*** | 62 | 220-250 |
| | barc113 | 56 | 115-135 |
| | wmc243** | 66 | 155-175 |
| | wmc179* | 63 | 195-240 |
| | gwm427*** | 50 | 195-215 |

**Appendix 1: The set of 104 low-resolution genome scan markers used to evaluate polymorphisms in the parental lines (continued)**

| Chromosome | Marker name | $T_m$ (°C) | Size range (bp) |
|---|---|---|---|
| 7A | gwm526 | 56 | 140-165 |
| | gwm276 | 58 | 80-90 |
| | gwm635 | 60 | 95-110 |
| | wmc422*** | 64 | 305-315 |
| | gwm282*** | 50 | 190-230 |
| 1B | gdm126 | 62 | 190-200 |
| | barc61 | 62 | 115-160 |
| | barc188 | 53 | 270-290 |
| | wmc44*** | 64 | 205-270 |
| | gwm140*** | 60 | 205-250 |
| 2B | wmc35 | 61 | 265-275 |
| | barc160*** | 58 | 100-115 |
| | gwm148 | 61 | 135-170 |
| | wmc154*** | 61 | 120-165 |
| | wmc445*** | 65 | 240-270 |
| 3B | gwm389 | 57 | 110-210 |
| | gwm493*** | 61 | 140-205 |
| | barc164*** | 62 | 175-210 |
| | barc344*** | 55 | 265-285 |
| | gwm181*** | 58 | 120-160 |
| 4B | wmc491*** | 63 | 200-220 |
| | gwm192*** | 53 | 125-145 |
| | gwm495*** | 63 | 150-180 |
| | wmc617*** | 63 | 210-290 |
| | wmc68 | 61 | 205-220 |
| 5B | barc32*** | 60 | 175-190 |
| | wmc235*** | 67 | 215-235 |
| | gwm335*** | 51 | 200-220 |
| | wmc27 | 55 | 385-400 |
| | barc340 | 65 | 200-225 |

**Appendix 1: The set of 104 low-resolution genome scan markers used to evaluate polymorphisms in the parental lines (continued)**

| Chromosome | Marker name | $T_m$ (°C) | Size range (bp) |
|---|---|---|---|
| 6B | wmc486 | 63 | 200-205 |
| | wmc494*** | 55 | 210-230 |
| | barc79*** | 54 | 135-170 |
| | barc354*** | 60 | 235-265 |
| | gwm219*** | 65 | 175-190 |
| 7B | wmc323*** | 66 | 160-185 |
| | wmc76*** | 61 | 250-270 |
| | gwm333*** | 61 | 145-155 |
| | wmc517*** | 67 | 185-205 |
| | gwm577*** | 52 | 150-170 |
| 1D | wmc147*** | 65 | 145-155 |
| | psp3000*** | 67 | 210-270 |
| | cfd48 | 65 | 250-265 |
| | wmc216*** | 54 | 85-135 |
| | gdm111 | 65 | 195-210 |
| 2D | cfd43 | 45 | 165-200 |
| | wmc25 | 47 | 125-225 |
| | cfd233 | 65 | 265-290 |
| | gwm382 | 59 | 85-130 |
| | gwm311*** | 65 | 115-160 |
| 3D | cfd4*** | 65 | 255-265 |
| | gwm52*** | 62 | 140-155 |
| | barc6*** | 55 | 455-475 |
| | gwm645*** | 62 | 145-170 |
| | cfd34 | 62 | 195-205 |
| 4D | wmc285 | 62 | 270-300 |
| | psp3103 | 67 | 170-185 |
| | wmc419*** | 67 | 135-145 |
| | wmc825*** | 45 | 145-190 |
| | gwm251 | 65 | 100-110 |

**Appendix 1: The set of 104 low-resolution genome scan markers used to evaluate polymorphisms in the parental lines (continued)**

| Chromosome | Marker name | $T_m$ (°C) | Size range (bp) |
|---|---|---|---|
| 5D | gwm190*** | 65 | 200-220 |
| | barc286*** | 62 | 250-260 |
| | barc110*** | 64 | 175-205 |
| | cfd26*** | 64 | 230-270 |
| | barc144 | 65 | 220-245 |
| 6D | cfd49*** | 64 | 155-220 |
| | gwm469 | 67 | 170-195 |
| | barc173*** | 55 | 225-240 |
| | barc21*** | 52 | 205-215 |
| | cfd47*** | 47 | 185-195 |
| 7D | cfd31*** | 65 | 210-235 |
| | barc352*** | 67 | 240-255 |
| | gwm437*** | 61 | 100-130 |
| | barc235*** | 66 | 300-315 |
| | barc126 | 54 | 115-130 |

$T_m$ = annealing temperature; bp = base pairs; *Polymorphic in Line37-07/M3283_4; **Polymorphic in Line37-07/M3192_7; ***Polymorphic in both populations, No * = monomorphic in both lines

**Appendix 2: Additional simple sequence repeat markers used to evaluate polymorphisms in the parental lines**

| Chromosome | Marker name | $T_m$ (°C) | Size (bp) |
|---|---|---|---|
| 2A | gwm512 | 60 | 185 |
| | gwm614 | 60 | 126 |
| | gwm497*** | 55 | 137 |
| 3A | wmc674 | 61 | 228 |
| | cfa2193 | 60 | 195 |
| | wmc153*** | 61 | 177 |
| | barc69 | 55 | - |
| | cfa2134*** | 60 | 210 |
| 4A | wmc219*** | 61 | 204 |
| 5A | gwm291 | 60 | 158-160 |
| | gwm129*** | 50 | 217-220 |
| | gwm186 | 60 | 106-132 |
| | wmc489* | 51 | 232 |
| | gwm126*** | 60 | 196 |
| | wmc445*** | 55 | 205 |
| | gwm293*** | | |
| 6A | barc3 | 52 | - |
| | gwm334*** | 50 | 110-114 |
| | barc206 | 52 | - |
| | wmc254 | 51 | 193 |
| | wmc59*** | 64 | 175-205 |
| 7A | wmc388*** | 61 | - |
| | wmc17 | 51 | 181 |
| | barc154 | 50 | - |
| | cfa2049** | 60 | 164 |
| | cfa2028 | 60 | 261 |
| | wmc179* | 63 | 195-240 |

**Appendix 2: Additional simple sequence repeat markers used to evaluate polymorphisms in the parental lines (continued)**

| Chromosome | Marker name | $T_m$ (°C) | Size (bp) |
|---|---|---|---|
| 1B | gwm264*** | 60 | 157-165 |
| | gwm18 | 50 | 182-188 |
| | cfa2129*** | 60 | 159 |
| | wmc818*** | 61 | 107 |
| 4B | wmc125 | 61 | 251 |
| | gwm538*** | 60 | 149-168 |
| 2D | gwm261*** | 55 | 164-194 |
| | wmc18*** | 61 | 230 |
| 4D | barc98*** | 55 | 150 |
| | cfd39*** | 51 | 163 |
| | wmc48*** | 51 | 179 |
| | wmc617*** | 60 | 187 |
| | wmc222 | 60 | 200 |
| | wmc89 | 51 | 172 |
| | cfd106 | 61 | 232 |
| | cfd160 | | |
| | wmc399 | | |
| | wmc206 | | |

$T_m$ = annealing temperature; bp = base pairs; *Polymorphic in Line37-07/M3283_4; **Polymorphic in Line37-07/M3192_7; ***Polymorphic in both populations, No * = monomorphic in both lines