Pyramiding of rust resistance genes in wheat utilizing male sterility mediated marker-assisted recurrent selection

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Declaration

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Abstract

Wheat production is globally affected by several different wheat rust diseases. The rust diseases can effectively be controlled by the deployment of multiple resistance genes that confer durable resistance. One of the most effective strategies to incorporate resistance genes is by the implementation of recurrent mass selection as it maximizes opportunities for gene pyramiding. The implementation of a recurrent mass selection program in wheat can effectively be enhanced with the use of genetic male sterility and the incorporation of maker assisted-selection (MAS).

The aim of the study was to pyramid wheat rust resistance genes in wheat lines by utilizing a male sterile mediated marker-assisted recurrent selection breeding (MS-MARS) scheme. An existing segregating MS-MARS base population and resistance donor lines carrying genes of interest (*Sr26*, *Sr35* and *Sr45*) were used as female and male crossing parents. Potential markers for the genes of interest were first identified and validated on the male population. PCR based markers tested for *Sr26* and *Sr45* easily distinguished between resistant and non-resistant plants in the study, while markers tested for the detection of *Sr35* and *Sr45* in most instances failed to do so.

The identified *Sr26* marker (Sr26#43) was successfully added to the SU-PBL's standardized marker set in a multiplex reaction. The standardized marker set and the co-dominant PCR marker for *Sr45* were used to screen male and female populations before and after cross-pollination. Several wheat rust resistance genes were present in various frequencies in both male and female populations prior to the first crossing cycle, except *Sr26* and *Sr45*. Increases in gene frequencies and combinations were obtained after the first crossing cycle, highlighting the effectiveness of the MS-MARS breeding strategy to improve gene frequencies of desirable genes.

Two MS-MARS crossing cycles were successfully completed and large numbers of hybrid seeds were produced in a short period of time by selecting male sterile plants based on distinct characteristics induced by the dominant male sterility gene.

Future studies will include the wide deployment of *Sr26* and *Sr45* in the MS-MARS breeding program as markers are now available and can be included in the SU-PBL's standardized marker set for the effective detection of these genes, the development of gene-

specific markers for *Sr35* to ascertain the presence of the gene in the MS-MARS population and the specific selection of male sterile plants with wide open glumes to maximize outcrossing rates.

Uittreksel

Koring produksie word wêreldwyd aansienlik deur koringroes siektes geaffekteer. Die siektes kan doeltreffend beheer word deur die ontplooing van veelvuldige weerstandsgene, wat langdurige weerstand tot gevolg het. Een van die mees doeltreffendste strategieë om weerstandsgene in n koring plant te inkorporeer is deur die implementering van herhalende massa seleksie (HMS), siende dat dit geleenthede vir geen stapeling maksimaliseer. Die implementering van 'n HMS program in koring kan effektief aangewend word met behulp van genetiese manlike steriliteit en merker bemiddelde seleksie (MBS).

Die doelwit van hierdie studie was om veelvuldige koringroes weerstandsgene in koring lyne te stapel met behulp van die manlik steriliteits merker bemiddelde herhalende seleksie (MS-MBHS)-telingsskema. ‘n Gevestigde segregerende MS-MARS basis populasie en donor lyne, wat die gene (*Sr26*, *Sr35* en *Sr45*) van belang dra, was onderskeidelik as vroulike en manlike kruisingsouers gebruik. Potensiële molekulêre merkers vir die gene van belang was eers geidentifiseer in literatuur en op die donor lyne getoets, voordat dit vir die opsporing van die gene in die nageslag gebruik was. Polimerase ketting reaksie (PKR)-gebaseerde merkers wat getoets was vir *Sr26* en *Sr45*, kon maklik tussen weerstand en nie-weerstandbiedende plante in die studie onderskei, terwyl ander merkers vir die opsporing van *Sr35* en *Sr45* nie so doeltreffend was nie.

Die geidentifiseerde *Sr26* merker was suksesvol bygevoeg tot die SU-PBL se gestandardiseerde merkerpaneel, in ‘n multipleks reaksie. Die gestandardiseerde merkerpaneel en die ko-dominante PKR merker vir *Sr45* was gebruik om die manlike en vroulike populasie te analiseer vir die teenwoordigheid van verskeie weerstandsgene voor en na kruisbestuiwing. Merker analise het die teenwoordigheid van verskeie koringroes weerstandsgene in verskillende frekwensies in beide die manlike en vroulike populasie voor die eerste kruising siklus aangedui. *Sr26* en *Sr45* was egter afwesig in beide populasies. ‘n Toename in geen frekwensies en kombinasies was waargeneem na die eerste kruising siklus. Dit het gevolglik die doeltreffendheid van die MS-MARS teling strategie beklemtoon.

Twee herhalende kruising siklusse was suksesvol voltooi en groot hoeveelhede bastersaad was verkry vanaf steriele plante wat geselekteer was op grond van unieke eienskappe wat hulle vertoon as gevolg van die manlike steriliteits geen.

Toekomstige studies sluit in, die groot skaalse gebruik van *Sr26* en *Sr45* in die MS-MARS teelprogram aangesien merkers nou beskikbaar is en gebruik kan word in die MS-MARS teelprogram vir die doeltreffende opsporing van hierdie gene, die ontwikkeling van ‘n geen-spesifieke merker vir *Sr35* om die teenwoordigheid van die geen in die MS-MARS populasie vas te stel, en die selektering van manlike steriele plante met wyd oop kaffies om kruisbestuiwing te verhoog.

List of abbreviations

%	Percent
ABC	ATP binding cassette
AFLP	Amplified Fragment Length Polymorphism
AgNO3	Silver Nitrate
Amp	Ampicillin
APR	Adult Plant Resistance
ARC	Agricultural Research Council
BC	Backcrossing
bp	Base pairs
˚C	Degrees Celsius
cM	CentiMorgan
CO2	Carbon dioxide
CMS	Cytoplasmic male sterility
CS	Chinese Spring
CTAB	N-Cetyl-N, N, N-trimethyl Ammonium Bromide
DH	Doubled Haploid
dH2O	Distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotidetriphosphate

EDTA	Ethylenediaminetetraacetic acid
EMS	Ethylmethane sulfonate
EtBr	Ethidium Bromide
F	Forward primer
f. sp.	Forma specialis
g	Gram
gDNA	Genomic Deoxyribonucleic Acid
ha	Hectare
HCl	Hydrochloric acid
HR	Hypersensitive response
HZ	Hertz
LB	Luria Bertani
Lr	Leaf rust resisance gene
LTN	Leaf tip necrosis
M	Molar
MAS	Marker assisted selection
Mb	Mega bases
MgCl2	Magnesium chloride
min	Minutes
ml	Millilitre

mm	Millimeter
Mm	Millimolar
MS-MARS	Male Sterility Mediated Marker Assisted Recurrent Selection
n	Haploid
2n	Diploid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Ng	Nanogram
ng/µl	Nanogram per microlitre
OECD	Organisation for Economic Co-operation and Development
PAGE	Polyacrylamide gel electrophoresis
Pa	Pavon
PBC	Pseudo black chaff
PBL	Plant Breeding Laboratory
PCR	Polymerase chain reaction
pH	Percentage hydrogen
Pty Ltd	Propriety Limited
QTL	Quantitative Trait Loci
R	Reverse primer
RAPD	Random Amplified Polymorphic DNA

RFLPs	Restriction fragment length polymorphisms
R-gene	Resistance gene
RMS	Recurrent mass selection
Rpm	Revolutions per minute
RSA	Republic of South Africa
SAGL	South African Grain Laboratory
SCAR	Sequence Characterized Amplified Region
sec	Seconds
SGI	Small Grain Institute
SNP's	Single nucleotide polymorphisms (SNP)
spp.	Species pluralis
Sr	Stem rust resistance gene
SSD	Single seed descent
SSR	Simple Sequence Repeat
STSs	Sequence tagged sites
SU	Stellenbosch University
SU-PBL	Stellenbosch University Plant Breeding Laboratory
t/ ha	Tons per hectare
TKM	Thousand kernels mass
Tris-Cl	Tris-chloride

U	Unit
UK	United Kingdom
U.S.A.	United States of America
USDA	Agricultural Research Service of the US department of Africulture
UV	Ultra Violet
UVPgt	Universiteit Vrystaat *Puccinia graminis* f. sp. *tritici*
UVPrt	Universiteit Vrystaat *Puccinia recondita* f. sp. *tritici*
Mg	Microgram
µl	Microlitre
µM	Micromolar
V	Volt
v/v	Volume per volume
WES	Welgevallen Experimental Station
WRRN	Wheat rust resistance nursery
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Yr	Stripe rust resistance gene

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Chapter 1: Introduction

Wheat is a staple food crop in many countries, and contains micro and macro nutrients for a healthy diet and feeds about 35% of the world population (Curtis, 2002). During 2012 global wheat production dropped significantly with 5.5% from the 699.4 million ton to 661.2 million tons (Food and Agriculture Organization (FAO), 2012). Moreover, there is an ever-increasing demand for wheat because of the annual growth in the human population with increasing nutrional standards and stagnating productivity. The Organisation for Economic Co-operation and Development-FAO (2013) predicted that the world population will increase with between 30 and 50% by 2022, and in order to meet the growing demand of the world population, wheat production must also increase at a similar rate in the coming decades. Therefore, wheat production can be considered a major concern to the global food security and needs to be rapidly and continuously improved.

Nevertheless, various abiotic and biotic stressors are affecting the normal functioning and development of wheat. Among the biotic stresses, fungal diseases of wheat and the rusts have been of the most important biotic constraints in most of the wheat growing areas worldwide and continue to pose a major threat to sustainable wheat production (Singh *et al*., 2002). The rust diseases are devastating and capable of destroying wheat crops in weeks, resulting in significant yield losses (Leonard & Szabo, 2005).

Breeding for disease resistance is a key factor in the battle to maintain adequate food supplies for the increasing world population (Stuthman *et al*., 2007). It is an important strategy for the protection of crops from damages due to biotic stresses (Singh *et al*., 2004) and reduces the need for other methods of control that are environment unfriendly and costly i.e. spraying fungicides (Roelfs *et al*., 1992). This breeding strategy has been followed for several decades and significant progress has already been made (Todorovska *et al*., 2009).

However, significant variation for virulence to specific resistance genes in the pathogen, combined with the rapid development of new wheat rust pathotypes through genetic changes such as mutation, migration and recombination has made it more difficult for breeders to develop cultivars with durable resistance. The fact that rusts pathogens can easily change and adapt to resistance genes of commercially produced cultivars results in the resistance genes being ineffective and susceptible. Consequently, the numbers of available resistance genes

that are effective are greatly reduced with an increase in new virulence combinations (Singh *et al*., 2002).

Because of this, the need for cultivars with durable resistance is ever-increasing. The use of multiple resistance genes has been put forward as one of the best strategies for the genetic control of wheat rusts (Kolmer, 2005; Singh *et al*., 2002). This strategy can be achieved by combining (pyramiding) several effective resistance genes into one cultivar to improve durability of the plant's resistance to wheat rust attacks. The pyramiding approach makes it difficult for the pathogen to overcome multiple resistance genes (Todorovska *et al*., 2009).

Pyramiding strategies are mostly based on backcrosses or convergent crosses that enforce yield ceilings and make it difficult to improve on existing gene pyramids (Marais & Botes, 2003). Recurrent selection instead, allows the continuous addition of more genes onto existing pyramids without the loss of selection progress for other genetic qualities (Pretorius *et al*., 2007). Furthermore, it is the most suitable breeding technique for the improvement of rust resistance, yield and quality, as it allows breeders to increase the frequencies of desirable alleles in the breeding population so that opportunities to extract superior genotypes are maximised (Marais & Botes, 2009).

Recurrent selection schemes for population improvement are naturally suited to cross-pollinating crops, but can also be facilitated in self-pollinating crops like wheat, by using genetic male sterility (Stuthman *et al*., 2007). Marais *et al.* (2000) have implemented a recurrent wheat mass selection (RMS) program at the Stellenbosch University's Plant breeding laboratory (SU-PBL), based on the dominant male sterility gene *Ms3*. A hydroponic system was developed in which male sterile tillers were cut at the flowering stage and pollinated with male fertile tillers in order to produce large hybrid populations (Marais *et al*., 2000).

A RMS program can effectively be enhanced with the incorporation of marker-assisted selection (MAS). MAS enable us to inspect wheat lines and the genes each carries, before it gets incorporated into the mass crossings. By only selecting lines with desired genes to cross with each other in successive cycles, allele enrichment and breeding for multi-gene resistance in wheat can be facilitated (Marais & Botes, 2009). In view of the fact that the process of breeding is very time consuming, the application of MAS in a breeding program can effectively help to reach a breeding objective in a shorter period of time (Ye & Smith, 2008).

The aim of this study was to pyramid several wheat rust resistance genes in wheat lines utilizing a male sterility mediated marker-assisted recurrent selection (MS-MARS) scheme. In order to achieve the stated aim of the study, the following objectives were identified:

a) Identification, validation and optimization of molecular markers for *Sr26, Sr35* and *Sr45*. This entailed the molecular screening of the male population containing the particular genes of interest as well as ensuring that the markers were validated and their use in MAS evaluated.
b) Implementation of molecular markers (together with an existing panel of markers) in a male sterile mediated marker-assisted selection scheme (MS-MARS). This entailed the screening of male and female populations for the presence of desirable wheat rust resistance genes, prior to recurrent selection cycles, with new and current markers used by the SU-PBL.
c) Validation of MS-MARS breeding scheme. This entailed the evaluation of an established recurrent mass selection strategy, based on the hydroponic system to pollinate and maintain male sterile spikes and the development of a molecular marker linked to the dominant male sterility gene *Ms3*.

Chapter 2: Literature review

2.1. Wheat

2.1.1. World wheat production

Bread wheat (*Triticum aestivum* L.) is an essential food crop to humans and offers more nourishment than any other food source. It provides the primary source of carbohydrates and contains minerals, vitamins and protein for a healthy diet (Curtis, 2002). As the main ingredient of bread and noodles it provides 20% of the calories consumed as food (Brencley *et al*., 2012).

World wheat consumption was estimated at 693.8 million tons in the 2013/2014 marketing year, with a 1.1% increase from the previous year (2012/2013) (FAO: Food Outlook, 2013). It is predicted that total wheat consumption will resume growing after a decline in 2012/2013. This increase places a large burden on the global food system as it is predicted that 742 million people needing food will inhabit the planet by 2022 (The OECD-FAO Agricultural Outlook, 2013).

Wheat is being produced on almost 217 million hectares (ha) in a number of countries worldwide (Pena, 2002). World wheat production was estimated at 702 million tons in 2013/2014 with a 6.5% increase from 2012/2013's reduced harvests. The top five countries responsible for driving the world's wheat production to a record 702 million tons include the European Union with a 20.4% contribution, China (17.2%), India (13.2%), United States of America (8.2%) and the Russian Federation (7.3%). South Africa is in the 28th position, contributing 0.24% towards global production (Index mundi: Wheat production by country in 1000 MT, 2013).

Recent estimates by the FAO indicate that 36 countries around the world require external assistance for food, mainly due to crop failures and food insecurities. Twenty-eight of these countries form part of Africa, whose population is still growing at over 2.3% annually (FAO: Crop Prospects and Food Situation, 2013). The ever-increasing growth in population thus leads to an increase in food demand. With the global wheat demand estimated to increase by 60% by 2050, wheat production therefore needs to be rapidly and continuously improved (How to feed the world in 2050, 2009).

2.1.2. Wheat production in South Africa

Wheat was planted for the first time in South Africa on January 13th 1653, after the arrival of Jan van Riebeeck in the Cape during the year 1652. Initially, wheat farming was not very successful as farmers attempted to grow cultivars that were adapted to Northern European conditions at the Cape, with its Mediterranean climate. It was only later discovered that the crop progressed better in some parts where the winter seasons were not too cold and rainy. Wheat farming did however only become successful during the 1960's (de Villiers, 2012).

In South Africa wheat is the second most important cereal after maize. Most of the wheat produced is bread wheat with small quantities of durum wheat. It is mainly produced for human consumption with the remaining being used for seed and animal feed.

Wheat is produced in the summer and winter rainfall regions. It is planted between middle of April and middle of June in the winter rainfall region and between middle May and the end of July in the summer rainfall region. Harvesting usually takes place between the month of November and December. Wheat is planted in 32 of the 36 crop producing areas, in which South Africa (consisting of nine provinces) are divided (The Southern African Grain Laboratory, 2012). The leading wheat-producing provinces in South Africa are the Western Cape (winter rainfall), Free State (summer rainfall) and the Northern Cape (irrigation). These three provinces account for more than three quarters of wheat produced in South Africa (Wheat focus, 2014).

In 2013, wheat was cultivated on an estimated area of 505 500 ha, the lowest cultivated area over the past 40 years (figure 2.1). Almost three quarters (367 350 ha) of the estimated total area cultivated to wheat was planted under dryland conditions with 138 150 ha under irrigation. Wheat production was estimated at 1,751 million tons in 2013, which is 119 150 tons less than the 1,870 million tons produced in the previous season. Approximately 48% of the total wheat produced was grown under irrigation with an average wheat yield of 6.13 tons/ha, while 52% was produced under dryland conditions with an average yield of 2.46 tons/ha.

The Western-Cape is currently the main wheat producing province, producing 49% (852 500 tons) of total wheat production, while the Northern-Cape and Free State respectively produce 16% (285 600 tons) and 15% (265 500 tons) of wheat in South Africa.

It is also predicted that the declining trend in ha planted with wheat will continue in the 2013/2014 marketing year as wheat will be planted on only 480, 000 ha. This reduction has led to an estimated wheat production of 1.6 million tons for the 2013/2014 year and subsequently created a major gap between production and demand for wheat in South Africa (Esterhuizen, 2013).

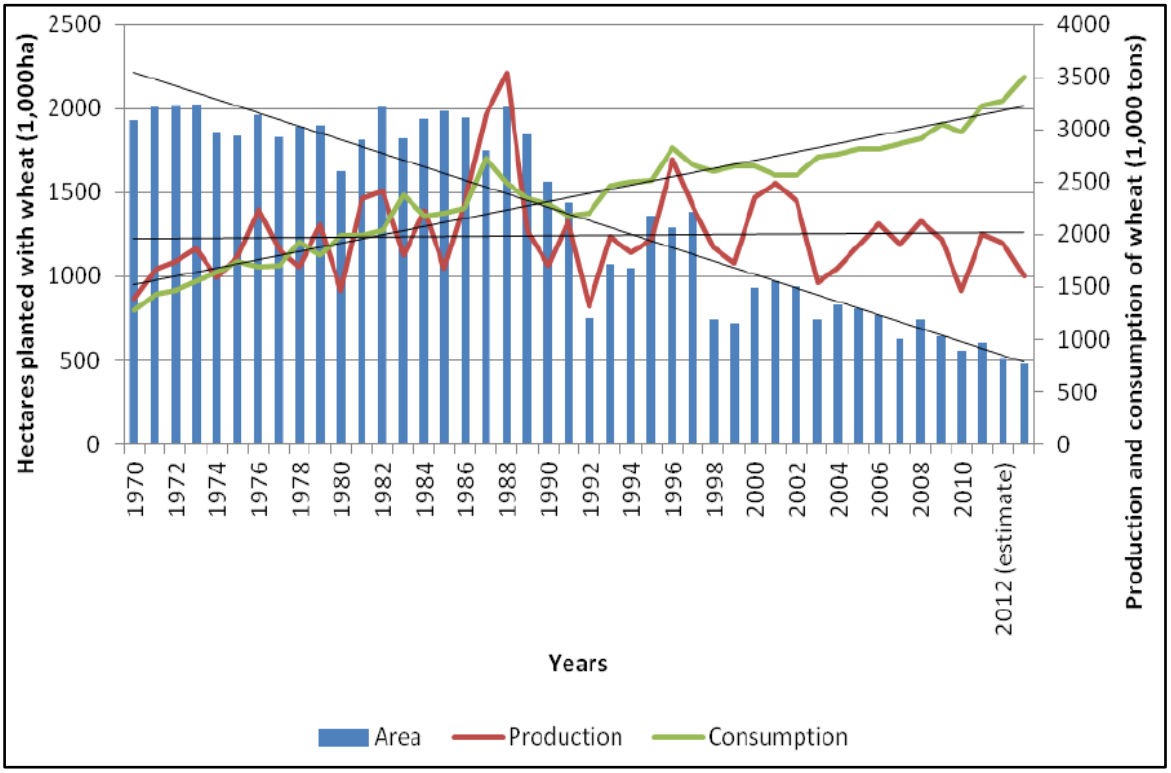

Figure 2.1.Wheat production, consumption and area planted over the past 40 years (Esterhuizen, 2013).

Wheat production locally is not enough for domestic requirements and South Africa has to import approximately one million tons of wheat each year to meet its domestic consumption. As wheat consumption is expected to increase by 7% to 3.5 million tons in the 2013/2014 marketing year, wheat imports are therefore expected to increase by almost 25% to 2 million tons. The major wheat suppliers for South Africa include Ukraine, Brazil, Argentina and Australia. Apart from the fact that South Africa is a net importer of wheat, it also exports wheat to the Southern African region and function as a network for imported grain (Esterhuizen, 2013).

2.1.3. The need to increase production

The challenge to achieve the projected increase in food production can be met either by increasing the area of cultivated land to generate products (extensification) or by increasing the input of resources, especially labour and technology, to produce more on the same area of land (intensification). The technique of extensification is associated with major environmental consequences like the production of greenhouse gasses especially increased carbon dioxide (CO_2) outputs, the adverse modification of the physical properties of soil, and the fragmentation of habitats, which lead to reduced biodiversity. Moreover, the available arable land is limited and estimates showed that utilization of it will only contribute 51 million ha to cereal production on a global basis by 2020 (Gregory & Ingram, 2003).

Although intensification also results in environmental consequences, just to a lesser extent, it is considered the most powerful technique for increasing production in the future. As significant increases in cereal yields have already been observed, a further increase estimated at 1.4% per year is predicted (OECD-FAO, 2013). Success thus far achieved through intensification has been through changes in crop management and genotype. It is believed that efficient and accurate breeding and selection systems will play an important role in improving global food security and helping to meet the ever-increasing food demand (Whitford *et al*., 2013).

2.1.4. Domestication and genetics of wheat

The genus *Triticum* is made up of six species: *Triticum monococcum* L and *Triticum urartu* forming the diploid AA genome species; *Triticum turgidum* L and *Triticum timopheevii* forming the tetraploid AABB and AAGG genome species); and *Triticum aestivum* L. and *Triticum zhukovskyi* forming the hexaploid AABBDD and AAAAGG genome species (Matsuoka, 2011). These *Triticum* species are further divided in sections Monococcon (diploid species, 2n=2x=14), Dicoccoidea (tetraploid species, 2n=4x=28) and Triticum (hexaploid species, 2n =6x=42) (table 2.1). Species *T. aestivum* and *T. zhukovskyi* exist only as cultivated forms, while *T. urartu* exists only in its wild form. The other three species (*T. monococcum, T. turgidum* and *T. timopheevii)* exist in wild and cultivated forms (Matsuoka, 2011).

The evolution of *Triticum* species can be dated millions of years back to the prehistoric Stone Age grasses. It is believed that wild diploid wheat *T. urartu* hybridized with the B-genome progenitor *Aegilops speltoides* to produced two tetraploid species, *T. turgidum* subsp. *dicoccoides* and *T. timopheevii* subsp. *armeniacum*. After several years of domestification, cultivated forms of these tetraploid species emerged namely *T. turgidum* subsp. *dicoccon* and *T. timopheevii* subsp. *timopheevii*. Thereafter hexaploid species, *T. aestivum*, emerged through hybridization between a tetraploid wheat species (*T. turgidum*) and a diploid wheat species, *Aegilops tauschii* (*Ae. tauschii*) with the DD-genome under natural conditions. *T. zhukovsky*i emerged through a hybridization event between *T. timopheevii* and cultivated diploid wheat *T.* monococcum. The T. *timopheevii* lineage that consists of *T. timopheevii* and *T. zhukovskyi* has limited distribution and are only found in the Transcauscasus. Also, relatively few studies have been done around their evolution and a lot is still unknown about domestication and diversification (Matsuoka, 2011).

During the domestication process of wheat, several morphological modifications in wheat traits occurred that effectively distinguished domesticated species from their wild progenitors. An essential morphological modification includes the non-brittle rachis trait (figure 2.2). Domesticated emmer wheat contains a non-brittle rachis that is controlled by recessive alleles at loci *Br*-A1 and *Br*-B1 on chromosomes 3AS and 3BS respectively. The non-brittle rachis feature enables the rachis to not (naturally) break at maturity level and is regarded as an important factor in preventing yield losses (Matsuoka, 2011). Another important domestication event was the change in glume tenacity. Wild wheat florets contain tough glumes and hulled seeds that make harvesting difficult, while florets of cultivated wheat's have soft glumes and are free-threshing. Several quantitative trait loci (QTL) located on chromosomes 2A, 2B, 2D, 5A, 6A, 6D and 7B have been found to affect the free-threshing characteristic. However, the free-threshing characteristic is predominantly controlled by a partially recessive allele at loci *Tg* (tenacious glume) and a partially dominant allele at loci *Q* on chromosomes 2DS and 5AL respectively (Peng *et al*., 2011). The morphology of a wheat spike is greatly affected by the interaction of the *Tg* and *Q* loci. The *Tg* gene has an epistatic effect on locus *Q* and is therefore in control of glume toughness, while the *Q* gene influences multiple traits like glume toughness, glume shape, spike length, plant height and the time of spike development. An excellent example of the epistatic effect of *Tg* is illustrated in figure 2.2b where domesticated emmer wheat, recently found to carry the dominant Q allele instead of the q allele with genotype *QQTgTg*, has no effect on

threshability (Matsuoka, 2011). Studies indicated that the modification of genotype *qqTgTg* to genotype *QQtgtg* was important to the emergence of the free-threshing characteristic in tetraploid and hexaploid wheat (figures 2.2c-d).

The genome size of bread wheat is relatively large, 17 000 MB, and consists of repetitive DNA sequences (mostly retrotransposons) of up to 80%. More than 94 000 genes were identified and assigned to the three genomes A, B and D, of which each is almost twice the size of the human genome (Brenchley *et al*., 2012).

Table 2.1. Classification of Triticum (Matsuoka, 2011).

Section	Species and subspecies	Genome constitution	Common names
Monococcon	*Triticum monococcum* L.	AA	
	subsp. *Aegilopoides*		
	subsp. *Monococcum*		Wild eirnkorn
Dicoccoidea	*Triticum turgidum* L.	AABB	
	subsp. *Dicoccoides*		Wild emmer
	subsp. *Dicoccon*		Cultivated emmer
	subsp. *Durum*		Durum or macaroni wheat
	subsp. *Polonicum*		Polish wheat
	subsp. *Turanicum*		Khorassan wheat
	subsp. *Turgidum*		Rivet wheat
	subsp. *Carthlicum*		Persian wheat
	subsp. *Paleocolchicum*		Georgian wheat
	Triticum timopheevii	AAGG	
	subsp. *Armeniacum*		Wild timopheevii
	subsp. *Timopheevii*		Cultivated timopheevii
Triticum	*Triticum aestivum* L.	AABBDD	Common wheat
	subsp. *Aestivum*		Bread wheat
	subsp. *Compactum*		Club wheat
	subsp. *Sphaerococcum*		Indian dwarf wheat
	subsp. *Macha*		
	subsp. *spelta* (L.)		Spelt
	Triticum zhukovskyi	AAAAGG	

Figure 2.2. Wheat spikes showing (A) brittle rachis, (B to D) nonbrittle rachis, (A and B) hulled grain, and (C and D) naked grain. (A) Wild emmer wheat, (B) domesticated emmer, (C) durum wheat, and (D) common wheat. (Figure reproduced from Dubcovsky & Dvorak, 2007).

The development of a wheat plant involves four main growth stages: tillering, stem elongation, heading, and ripening. The duration of each growth stage depends on several factors, including temperature, genotype, day-length, and sowing date (Acevedo & Silva, 2002).

Several different methods exist for the identification of the different wheat growth stages, such as the Feekes scale and Zadoks scale. The Feekes scale which designates the growth stages on a scale of one (seedling growth) to eleven (ripening) (figure 2.3), is the most common and widely used method. The Zadoks scale is a detailed system of identification of various wheat growth stages and use a two digit system to identify the different growth stages (Simmons *et al*., 1995).

Wheat seeds usually start germinating between temperatures of 4°C and 37°C. Germination starts when the seeds absorb adequate water from the soil, following the appearance of the seedling and primary roots along with the coleoptile that subsequently protect the roots. As soon as the coleoptile appears above the soil, it stops growing and the

first true leaves appear followed by seedling growth. Germination can occur within seven days, if growth conditions are favourable (Simmons *et al*., 1995).

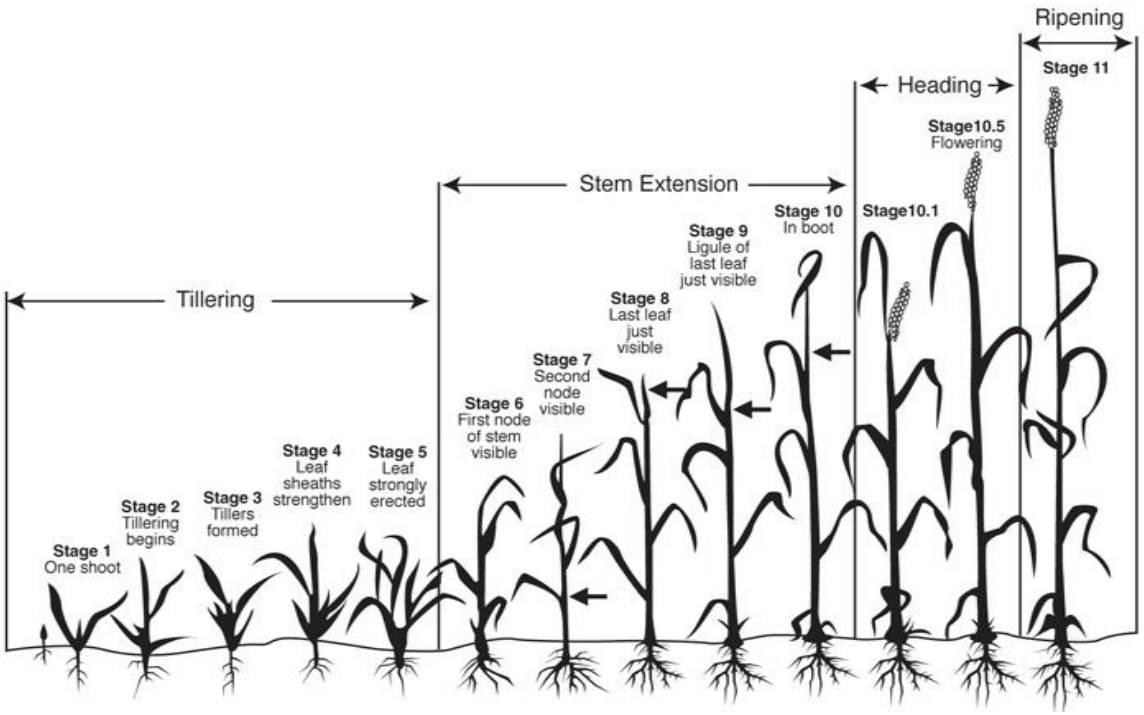

Figure 2.3. The Feekes scale of wheat development (Marsalis and Goldberg, 2006).

Tillering is considered an important stage during wheat development as the tillers give rise to grain-bearing heads that are important for wheat yield. The tillering stage starts after the development of the third leaf in the seedling stage. Tillers develop from the axils of the main shoot leaves and are usually dependent on the main stem for nourishment during development. The tillers are only independent after three or more leaves develop. The potential number of tillers formed varies with genotype (especially among flowering types) and environment. A high number of tillers usually develop in winter wheat (Acevedo *et al*., 2002). However, not all of the tillers will produce grain and contribute to yield. Tillers that contain more than three leaves and initiate their own root system mostly survive. On the other hand, tillers formed at a later stage during development usually tend to abort with no production of grain (Simmons *et al*., 1995).

The wheat plant continues to grow as temperatures rapidly increase in the spring. The leaf sheaths are growing and forms a pseudostem (not a real stem). At this stage the actual stem has not elongated and is still below ground level. As the plant continues to grow, stem elongation or jointing occurs as a result of internode elongation. The stem nodes and

internodes appear above the soil surface and leaves develop and appear as the stem elongates. Stem elongation is usually complete when the last leaf, called the flag leaf appears from the whorl (Simmons *et al*, 1995).

After the appearance of the flag leaf, the boot stage takes place and indicates the appearance of the head. The boot stage ends when the awns are first visible at the flag leaf collar and the leaf sheath open by the head. The heading stage is characterized by the appearance of the complete head which consists of two spikelets positioned on opposite sides of a central rachis. Each spikelet contains a floret made up of underlying structures (ovary, stigma, stamen and anthers) that is important for reproduction (Australian Government Department of Health and Ageing, Office of the Gene Technology Regulator, 2008).

Within a few days after heading, florets on the tillers of the main stem open first and flowering progresses both up and down the spike from the middle. The time and duration of flowering is dependent on the environment and temperatures of 11-13˚C are usually required (Simmons *et al*., 1995; Australian Government Department of Health and Ageing Office of the Gene Technology Regulator, 2008).

As wheat is primarily a self-pollinating crop, pollen is shed before the flowers opens thereby reducing the rates of outcrossing. After pollen is released it attaches to the stigmatic branches. Absorption of water by the pollen grain then enables the pollen tube to grow, which in turn enables fertilisation. The stigma is receptive for 6-13 days, while the viability of pollen may be less than 30 minutes in field conditions.

After fertilisation, swelling of the tissues surrounding the fertilised embryo takes place and the seeds start to grow very rapidly. Products of photosynthesis as well as proteins and starches previously produced and stored in the leaves are transferred to the grain. Approximately 21 days after fertilisation the grain reaches their maximum fresh weight.

2.2. Wheat rusts

Wheat rust diseases have historically been one of the major biotic stresses limiting production wherever wheat is cultivated. Three distinct diseases namely stem rust, leaf rust and stripe/ yellow rust caused by wheat rust pathogens *Puccinia graminis* f. sp. *tritici* Eriks.

& E. Henn., *Puccinia triticina* Eriks and *Puccinia striiformis* West. f. sp. *tritici* Eriks. & E. Henn. respectively, occur in South Africa. The wheat rust pathogens belong to phylum Basidiomycetes, order Uridenales and family Puccinacea. The family contains approximately 6000 species of which the majority, including the three of particular interest in this study, belongs to the genus *Puccinia* (Leonard & Szabo, 2005; Du Plessis *et al*., 2011). The rust pathogens are obligate parasites and require intimate interaction with their hosts in order to survive. Rust pathogens develop specialized infection structures within the lumen of the host cell of which the haustorium that is directly linked to the haustorial mother cell are crucial for enhancing availability of nutrients that get extracted from the host plant. It also secretes an array of effector proteins that inhibit the defences of the host.

The genome sizes of wheat rust fungi are relatively large compared to other fungi in the class Basidiomycetes. The genomes of *P. graminis* and *P. triticina* are 88.64 Mb and 135.34 Mb respectively (Cuomo *et al.*, 2013). Approximately 1100 genes encoding effector-like small secreted proteins were identified in the genome sequence of *P. graminis*, of which 84% are unique to the rust fungus. This together with the finding of deficiencies in the nitrate and sulphate assimilation pathways of rust fungi showed consistency with their obligate biotrophic lifestyle (Du Plessis *et al*., 2011). Similar findings were recently made when the complete genome of 117.3 Mb of *P. striiformis* was sequenced using data obtained from next generation sequencing platforms (Illumina and 454) which gave a 81 x coverage of the genome. Five secreted effector proteins were found highly expressed in the haustorium and polymorphic between two isolates (PST-87/7 and PST-08/21) from the United Kingdom (UK). Isolates only differed in virulence to two differential wheat cultivars known as Robigus (*YrRob*) and Solstice (*YrSol*) (Cantu *et al*., 2013). Researchers believe that the validation of polymorphisms, between the two isolates as virulence/ avirulence effectors, in the corresponding wheat cultivars might lead to better understanding of the pathogenicity of *P. striiformis* that will aid in developing more effective breeding strategies.

2.2.1. Life cycle of rust pathogens and disease development

The life cycle of wheat rust pathogens is very complex and consists of five different spore stages and two taxonomically unrelated hosts. It consists of both asexual production, which occur primarily on wheat and sexual production which occur on a different host (figure 2.4). For leaf rust the alternate host is meadow rue (*Thalictrum speciosissium*) and for stem rust the alternate host is barberry (*Berberis vulgaris*) (Leonard & Szabo, 2005; Bolton *et al*.,

2008). Recently, barberry was also reported to serve as alternate host for stripe rust (Jin *et al*., 2008). These alternative hosts are however not prevalent in South Africa and no sexual reproduction has been reported (Visser *et al*., 2009).

The development of wheat rust diseases starts with the introduction of either aeciospores or urediniospores as primary inoculum to a wheat plant, depending on the region, in which the wheat is grown. During the growing season urediniospores are produced on the wheat host. As the urediniospores develop, they will burst the host epidermis, producing rusty-coloured urediospores on the plant. This is the most damaging stage, as urediospores are wind dispersed and can re-infect the host. As the host matures, the uredium gradually converts into the telium and begins to produce two celled teliospores, which are black. The teliospore stage is usually the over wintering stage of the fungus and will remain dormant for the winter. Each cell is capable of germination to produce a single haploid basidiospore (Singh *et al*., 2002).

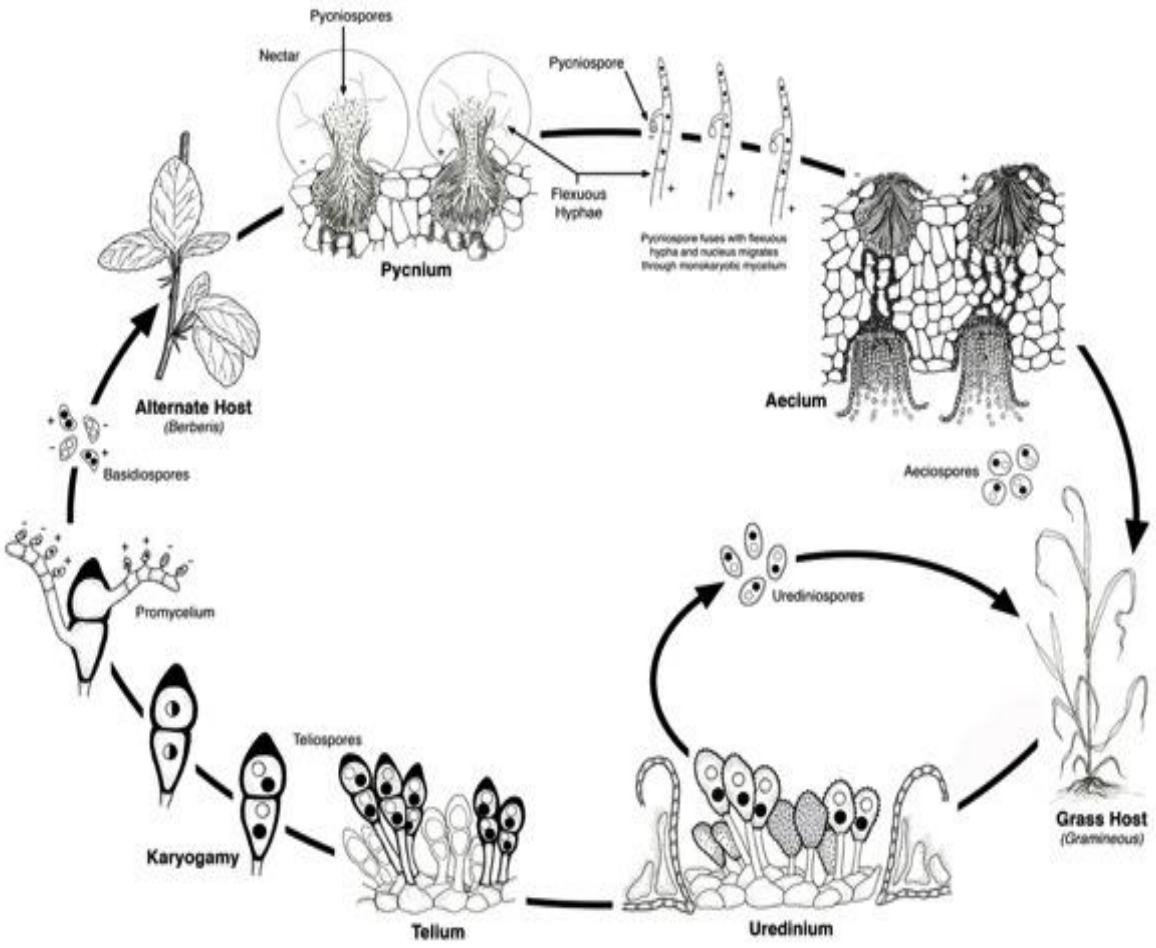

Figure 2.4. The lifecycle of wheat rust pathogens (Leonard & Szabo, 2005).

The basidiospores directly infect the alternate host and produce flask-shaped pycnia. Each pycnium consist of female (receptive hyphae) and male (pycniospores) gametes. The pycnium produces sweet nectar that attracts insects. As an insect move from pycnium to pycnium, sucking the nectar, pycniospores become attached to the insect and are transferred to the receptive hyphae to initiate aeciospore development. Single celled dikaryotic aeciospores are produced in chains and are wind dispersed to wheat. When conditions are favourable, aeciospores successfully infects the wheat plant. Germination of the spores takes place and enters the plant through the stomata, resulting in the production of single celled dikaryotic urediospores (Singh *et al*., 2002; Leonard & Szabo, 2005). To date no known alternative hosts of the wheat rust fungi have been reported in South Africa and sexual reproduction is believed not to occur (Visser *et al*., 2009).

2.2.2. Leaf rust

Of the three rust diseases, Leaf rust caused by *Puccinia triticina* Eriks, is the most common and widely distributed disease of wheat. It is an important disease of wheat in South Africa in most areas, but distribution and severity are influenced by climatic conditions during the growing period (Terefe *et al*., 2009). *P. triticina* grows rapidly at temperatures between 10°C and 30°C, with dew periods of 6h or more (Singh *et al*., 2002). The severity of leaf rust is generally high on autumn sown spring wheat in the winter rainfall areas of the Western Cape, but moist conditions and high temperatures during spring also contributes towards severe leaf rust on winter wheat in the Free State (Pretorius *et al*., 2007).

Losses due to leaf rust are usually less than 10%, but can be more than 30% in severe cases. Yield losses in grain are generally a result of reduced floret set and grain shrivelling, and it can reach 40% in susceptible cultivars (Todorovska *et al*., 2009). Leaf rust of wheat is a major problem particularly in countries like North and South America, as over 70 pathotypes of *P. triticina* are reported every year in North America. It mainly occurs on the leaf blades, but may also affect leaf sheaths under favourable conditions. It causes small orange pustules on the leaf surface (figure 2.5a). The leaves normally stay green, but as it ages, pustules begin to produce dark black spores, that can be seen on the lower leaf surface and leaf-sheaths (Kolmer, 2005; Bolton *et al*., 2008). *P. triticina* is primarily a pathogen of wheat (*T. aestivium*), but is also found on other grasses such as triticale (X *Triticosecale* Wittmack).

2.2.3. Stem rust

Stem rust also known as black rust, due to the abundant production of teliospores is caused by *Puccinia graminis.* f. sp. *tritici* (Todorovska *et al*., 2009). It has been a serious disease of wheat in most wheat growing areas, and was particularly feared for its ability to turn a healthy crop into black broken stems before harvest (Kolmer, 2005). Wheat stem rust infections are mainly found on stems and leaf sheaths, but occasionally on leaf blades and glumes as well (USDA, 2010). A small chlorotic fleck usually appears as the first symptom of the disease, but after a few days of infection brick red linear or oval shape pustules occur on the stems and leaves (figure 2.5b)

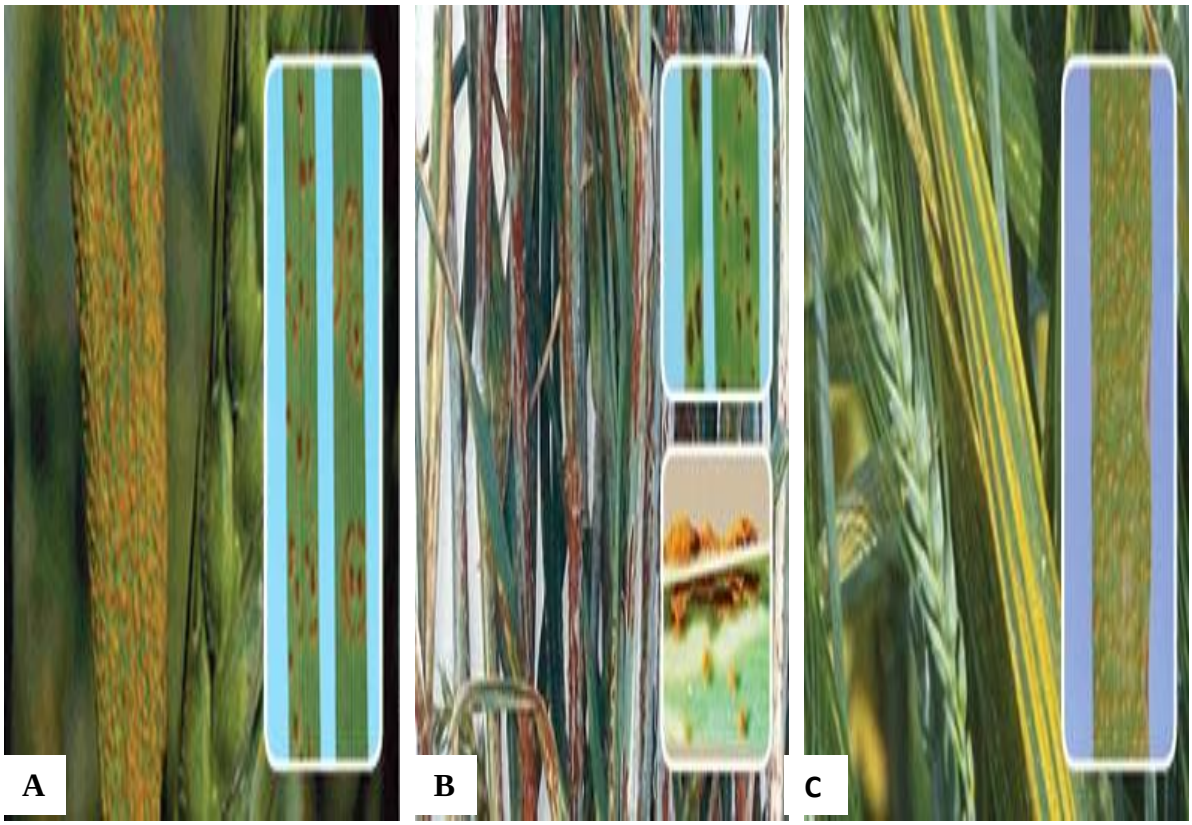

Figure 2.5. Distinct features of the three wheat rust diseases. a) Leaf rust. b) Stem rust. c) Stripe rust/ Yellow rust (USDA, 2010).

Black teliospores are produced as the leaves age, which cause the stems to take on a black colour. Stem rust development in wheat is favoured by warm temperatures of 18°C-30°C with relative high humid conditions. It requires a dew period of about 6-8 hours, compared to the shorter dew period of leaf rust (Leonard & Szabo, 2005; Singh *et al*., 2002).

Stem rust is very damaging and can cause losses of 50% in one month when conditions for its growth and development are favourable (Singh *et al*., 2002). The first documented rust epidemic in South Africa was in the year 1926. During that period more than 16 different pathotypes were identified (Pretorius *et al*., 2007). Heavy stem rust outbreaks were also documented in North America and Australia during 1900 and 1960. Thereafter stem rust has successfully been controlled and prevented by the use of resistant wheat cultivars, and the eradication of the alternate host (Leonard & Szabo, 2005).

However, during 1999 the disease was brought to the forefront by the appearance of a new stem rust pathotype, also known as Ug99 that was first detected in Uganda. Later the pathotype also spread to other regions of Africa and the Middle East (Fesher *et al*., 2010; Admassu *et al*., 2011; Visser *et al*., 2009).

Ug99, also known as TTKS (according to the North American nomenclature system) is virulent for a number of resistance genes used in breeding programmes in wheat producing countries. Amongst other, Ug99 shows virulence against *Sr31* and *Sr38* for which virulence had never been previously reported (Admassu *et al*., 2011). More recently, the potential threat of Ug99 to wheat production worldwide has been intensified by the movement of Ug99 to Yemen in 2007 and Iran in 2008. More seriously is that Ug99 has gradually developed further by accumulating additional virulence's and making it more forceful and dangerous (Fesher *et al*., 2010).

Seven variants (TTKSK , TTKSF, TTKST, TTTSK, TTKSP, PTKSK and PTKST) belonging to the Ug99 lineage are now known with almost identical DNA profiles, but slightly different avirulence/ virulence profiles. However, these pathotypes are very different from other known pathotypes found in the United States and South Africa (Singh *et al*., 2011; Visser *et al*., 2009). It is believed that TTKSF (also UVPgt55 and 2SA88), the first Ug99 variant detected in South Africa in 2000, was an exotic introduction into South Africa. Using SSR and AFLP analyses, it was shown that TTKSF has an identical virulence profile to Ug99, with the exception of avirulence towards *Sr31* (Visser *et al*., 2009). A few years after the detection of TTKSF, two new Ug99 related pathotypes, TTKSP (also 2SA106 and UVPgt59) and PTKST (also UVPgt60) were detected in South Africa in 2007 and 2009 respectively. TTKSP was detected in the Western Cape and is virulent to *Sr24* while PTKST, with virulence towards both *Sr24* and *Sr31*, was detected in KwaZula-Natal (Visser *et al*., 2010).

2.2.4. Stripe rust

Stripe rust (yellow rust) is caused by *Puccinia Striiformis* West. f. sp. *tritici* Eriks, and can be as damaging as stem rust. In comparison to the other wheat rust disease, stripe rust is a low-temperature disease that is usually found at higher altitudes and in cooler environments. The optimum temperature for the development of stripe rust disease is between 10 and 15˚C. Infections occur normally on the leaf blades and heads when diseases are very severe. It is characterized by long stripes of small yellowish orange pustules on the leaves (figure 2.5c) (USDA, 2010).

P. striiformis is more different than the other two rusts as it develops systemically. The pathogen spreads beyond the infection site within the plant leaf tissue, extracting nutrients as it grows inside after infection and ultimately giving rise to parallel yellow stripes. Thus, the pathogen is very dangerous as epidemics can result from only a small number of uredineospores. Stripe rust can cause significant reductions in yield losses and grain quality. The seeds produced usually have low vigour and as a result develop poorly after germination. Early infections could result in yield losses of up to 100% in susceptible cultivars (Afzal *et al*., 2007).

Stripe rust was first reported on spring wheat in the Western Cape, South Africa in 1996, with the introduction of pathotype 6E16A. After the initial detection in South Africa, the disease has spread rapidly to wheat producing regions and within two wheat seasons the stripe rust pathogen was widely distributed in major wheat producing areas of South Africa (Boshoff *et al*., 2002). Losses caused by stripe rust during 1996 amounts to $22.5 million (Pretorius, 2004).

2.2.5. Managing rust diseases

Several strategies to limit damages caused by wheat rusts exist. However, it is important to fully understand the epidemiology of the disease in order to apply any of the existing control strategies (Singh *et al*., 2002).

In South Africa and various other countries the chemical control strategy through the use of fungicides has been successfully used to manage rust epidemics in areas where disease pressure was high. Fungicides have been found effective at specific growth stages and have

resulted in high wheat yield and improved quality. An advantage of this control method is that the chemicals can be applied when needed. However, most available fungicides provide inadequate control on susceptible cultivars when environmental conditions for disease development are favourable (Wanyera *et al.*, 2010; Roelfs *et al.*, 1992). Moreover, researchers are concerned that the long term use of fungicides can lead to the development of resistant-fungal populations of wheat rust pathogens, thereby declaring fungicides ineffective (Milus *et al.*, 2009). Also, the timing of fungicide application is very critical in managing wheat rust. Crops that are poorly sprayed suffer as much damage as untreated crops. The activity of the rusts, especially stem rust, needs to be closely monitored both in individual fields and across areas. Chemical control is also very expensive and R28 million was spent on fungicides to control stripe rust epidemic in 1996 in South Africa (Boshoff *et al*., 2002).

Another method for at least partial control is cultural practices. This involves the use of early maturing cultivars to limit the length of time for stem rust epidemics to develop. Some other preventative methods involve the removal of volunteer wheat by tillage and/or herbicides. This method greatly helps to prevent disease carryover from one growing period to the next. It also enhances the effectiveness of chemicals when used, but requires the cooperation of farmers in a region (Roelfs *et al*., 1992).

The elimination of the alternate host was a very successful method in countries like Europe and the North Central States of the USA. The method is effective for delaying disease onset and initial disease severities. It removes a significant early source of inoculum and reduces genetic variation in the fungal population by eliminating the sexual cycle of the fungal pathogen. However, this method is not often economically feasible and only reduces loss to some degree (Schumann & Leonard, 2000; Roelfs *et al.*, 1992).

The simplest and most cost effective method for wheat rust control is through the use of resistant cultivars. It is seen by breeders as the main mechanism to minimize the impact of rust diseases (Singh *et al*., 2002). Many cultivars such as ‘Thatcher’, ‘Hope’, ‘Americano’, ‘Surpreza’, ‘Frontana’, ‘Wilhelmina’, ‘Cappelle-Desprez’, ‘Manella’, ‘Juliana’, and ‘Carstens’ have remained resistant for several years and great success in breeding programs for wheat rust resistance have been achieved, especially against stem rust (Roelfs *et al.*, 1992).

2.3. Genetic plant resistance

The genetic inheritance of pathogenicity and host response was first illustrated by Flor (1942) while working with flax rust, *Melampsora lini,* and its host *Linum usitatissimum*. Flor's study dealt with a gene in the pathogen matching the gene in the host. Based on his results he developed a gene-for-gene theory that is mainly found with pathogens such as wheat rusts that are highly specialized biotrophic parasites. These parasites lives in close proximity with their hosts and relies on the ability to transfer effectors (proteins known to enhance disease development in the host but promoting growth processes in the pathogen) into host cells (Leonard & Szabo, 2005). Flor (1956) created his ideal model of the gene-for-gene theory on the generalization that each gene operates independently of the other corresponding gene and that resistance in the host and avirulence in the pathogen is controlled by dominant genes, while susceptibility in the host and virulence in the pathogen is controlled by recessive genes.

In a typical gene-for-gene interaction between wheat and rust, the characteristic feature of wheat can be describe as a reaction which may be resistant or susceptible whereas the pathogen has pathogenicity that may be seen as virulent or avirulent. Resistance genes in the wheat plant provide resistance against pathotypes of the pathogen that have an avirulence gene that matches the host resistance gene, but not against pathogen races lacking the matching avirulence gene. Therefore, for each resistance gene in the plant there is a corresponding avirulence gene in the avirulent pathotype of the pathogen or a corresponding virulence gene in the virulent pathotype of the pathogen. In actual fact, every gene for resistance present in the plant can be overcome by a virulence gene that is present in some of the pathotypes of the pathogen population (Bhardwaj, 2013). The gene-for-gene interaction is well documented in wheat rust pathogens and the theory has been used to postulate genotypes for resistance in wheat cultivars (Loegering & Powers, 1962).

2.3.1. Types of resistance systems

2.3.1.1. Pathotype-specific resistance

Specific resistance occurs when a single pathogen isolate interacts with a single host genotype to produce a different disease response than another isolate with the same host in the same environment. In other words a genotype may be highly resistant to one isolate and susceptible to another. Specific resistance or vertical resistance as described by Van der Plank (1963) provides the basis for the gene-for-gene-theory and is inherited through oligogenes.

These genes are known as major genes. Specific resistance type can also be associated with a hypersensitive response (HR), a programmed cell death response that avoids distribution of the pathogen (Singh *et al*., 1992).

2.3.1.2. Non-pathotype specific resistance

Non-pathotype specific type of resistance, also known as horizontal resistance, functions against all pathotypes of a pathogen, and is characterized by a series of intermediate infection responses. These responses can be attributed to the polygenic nature of the resistance mechanism, where several genes with minor to intermediate effects quantitatively contribute towards resistance (Singh *et al*., 1992). Minor resistance genes do not generally provide high levels of resistance, but their effects are of sustainable and durable nature. Non-pathotype specific resistance is also associated with partial and slow rusting resistance genes that prevent the development of epidemics by reducing infection rate and spore production in the plant.

2.3.1.3. Adult plant resistance

Adult plant resistance (APR) or post seedling resistance are not expressed during the seedling stage, but rather at a later stage of plant development. It often provides a partial resistance response and is associated with non-specific resistance (Rutkoski *et al*., 2011). APR-genes have also been referred to as “slow rusting”, which is a type of resistance where disease progresses at a slow rate and results in low infection levels against all pathotypes of a pathogen (Duveiler *et al.*, 2007).

APR is also in most cases a complex trait conditioned by many genes of which most are minor genes, acting as (QTL) mostly. Pyramiding of APR-genes has the effect of producing high levels of resistance in plants (Singh *et al*., 2011).

2.3.1.4. Durable resistance

Durable resistance remains effective in a genotype for a long period of time (beyond five years), despite exposure to the pathogen. It is associated with non-specific resistance, governed by minor genes. In some cases a single resistance gene are required to effectuate resistance, while more than one are require in other cases. Plant breeders usually combine minor genes with major genes to achieve durable resistance (Prabhu, 2008).

2.3.2. Sources of resistance and genetic diversity

The most important resource in any breeding program is the availability of suitable germplasm. The main sources of resistance for wheat breeding are *T. aestivum*, wild relatives of *T. aestivum*, and the grass species of the family Triticeae Dum (Moustafa, 2011). These species sources are grouped in primary (1°), secondary (2°) and tertiary (3°) gene pools, from which genetic transfers can be accomplished for wheat improvement.

The gene pools are structured upon the genomic constitution of the species and the different levels indicate the accessibility of the species to be utilized in wheat breeding programs. The primary gene pool is the most accessible, with the tertiary gene pool being less accessible. Hexaploid land pathotypes and wild tetraploids of the turgidum group and diploid ancestors of the A and D genomes all represent the primary gene pool. The secondary and tertiary gene pool includes species of the genus *Aegilops sitopsis* and species (diploid and polyploid) that share non-homologous genomes with wheat respectively (Lagudah, 2008).

The transfer of resistance from non-related material is technically complex, due to the fact that it could result in the transfer of undesirable genes or gene complexes. When genetic sources other than *T. aestivum* are used the following requirements should first be met in order to successfully incorporate resistance. Techniques for selection should be available to search for the genes, should be able to make a cross between wheat and the donor species, the targeted genes should be fixated in a stable genetic background, and the genes of interest should be phenotypically expressed in the genetic background (Moustafa, 2011; Merker, 1992).

Several resistance genes have been transferred from related species, including the *Sr2* gene from *Triticum turgidum*, *Sr24* from *Thinopyrum elongatum*, *Sr22* from *Triticum beoticum* and *Triticum monococcum* and *Sr31* from *Secale cereale*. The leaf rust resistance gene *Lr19* was the first gene introduced from a *Thinopyrum* sp. (Moustafa, 2011).

2.4. Breeding for wheat rust resistance

Wheat breeding in South Africa started during the early years of the 20^{th} century when major efforts were made to incorporate the stem rust resistance of cultivar ‘Rieti’ in locally adopted

land pathotypes. These efforts were followed by attempts to use rust resistance obtained from sources like *Agropyron elongatum* and *T. timopheevi*, but in many instances the vulnerability of the vertical resistance used was highlighted in typical boom-and-bust cycles (Pretorius *et al*., 2007).

Genetic resistance appears to fail mostly due the fact that rust fungi can easily overcome resistance genes of commercially cultivated cultivars. These changes occur when naturally occurring genetic changes such as mutation, migration, natural selection and recombination allow pathotypes to overcome the genetic resistance of the plant (USDA, 2011).

The degree to which mutation contributes to the diversity of pathogen populations mainly depends on the inherent rate of mutation, the ploidy level, the size of the pathogen population, and the selective advantage conferred by the mutant phenotype (Burdon & Silk, 1997). Mutation is considered a powerful source of new variation in plant pathogenic fungi and is postulated to be the primary origin of new pathotypes, especially in South Africa, where no alterative host for wheat rust fungi exist. Many pathotypes belonging to the Ug99 lineage were found to have arisen from the same ancestor through single-step mutation as they only differed in one or two virulence attributes and are otherwise genetically similar (Pretorius *et al*., 2010). The virulent race Ug99, originally detected in Uganda in 1999, has the ability to destroy up to 80% of a wheat crop, and was consequently recognized as a major threat to wheat production.

New rust populations have also been reported through the process of migration. These pathogens are spread by dikaryotic uredineospores that are dispersed over wide areas from initial infection sites (Kolmer, 2005). Stem rust spores are distributed by wind over 800 km annually across the North American Great Plains and 2000 km from Australia to New Zealand (Singh *et al*., 2002).

Recombination (both sexual and asexual) has significant importance in genotypic diversity in a rust pathogen population. In regions where alternative hosts of wheat rust pathogens exist, sexual recombination can take place leading to the production of an enormous number of incipient clonal lineages. More than 17 pathotypes were detected annually in the Great Plains during the early 1920's, before the removal of the alternative host barberry. Since then only six to seven pathotypes are now detected on a regular basis (Burdon & Silk, 1997).

Asexual recombination has been proposed to involve the exchange of nuclei between fungal hyphae. As rust fungi are a dikaryotic basidiomycete, two genetically distinct nuclei usually exist together in a stable state. This process of exchange of nuclei can create a maximum of two new combinations of characters (Burdon & Silk, 1997). More variation may be created when nuclear exchange is followed by re-association of intact nuclei, nuclear fusion and segregation (parasexuality and somatic meiosis). Nuclear exchange and parasexuality has been reported in nine *Puccinia* spp., including *P. triticina* (Wang & McCallum, 2009).

2.4.1. Resistance breeding strategies

Breeding for resistance is a major objective for plant breeders and several requirements must be met to ensure the successful incorporation of resistance. Firstly, a resistance source to an important disease must be found; the resistance must be introduced into an agronomically desirable genotype; the resistant line must be widely deployed in the state; lastly the resistance should be as durable as possible.

Various breeding methods such as bulk selection, backcrossing, pedigree selection and recurrent selection exist for the successful incorporation and improvement of resistance gene (Aquaah, 2007). These methods, especially the pedigree, backcross, and recurrent selection method has been widely used in resistance breeding, and the majority of disease resistant genotypes have been developed by them (Pretorius *et al.*, 2007).

2.4.1.1. Bulk population breeding

Bulk population breeding is a strategy of crop improvement that relies on natural selection to shift gene frequencies. Superior lines are often produced, because many characteristics that contribute to the survival of the wild species are associated with the productivity of the cultivated species (Campbell, 1999).

The procedure of the bulk method entails that seeds from all F_1 plants are grown in bulk to produce enough seeds for the F_2 generation, which are then planted in a relative large plot to accommodate all the plants. Seeds are harvested from the plants and samples are planted in bulk again until the F_4 is reached or when a desired percentage of homozygosity has been accomplished in the population. By the sixth generation, homozygosity (order of 96%) would occur for all traits and individual selections of potential phenotypes, which have desirable

recombination of traits from their parents, can be made. Selection after F_6 generations strongly relies on progeny performances (Campbell, 1999).

This method is very simple and convenient to conduct. It also allows large amounts of segregating material to be handled. Frequencies of desirable genotypes are increased by the end of the bulking period, through natural selection, which make the method of bulk population very useful in breeding programs. A major disadvantage of this method is that desirable genotypes may be lost to natural selection, while undesirable genotypes are advanced during the early generations. It is also difficult to determine the genetic characteristics of the populations from generation to generation (Acquaah, 2007).

2.4.1.2. Pedigree selection

The pedigree method is based on the combination of desirable genes that are found in two or more different genotypes. It is especially useful if the two parents display characteristics such as high yield, adaptability and resistance to a disease, with the knowledge of the genetic control of the disease. The breeder usually keeps record of the ancestry of a cultivar (Moustafa, 2011).

Selection starts in the second generation, when the progeny are highly heterozygous. The level of homozygosity starts to increase by the third and fourth generation, and family characteristics become visible. Only the best plants in the best families are selected, as heterozygosity between the families are still high. Homozygosity becomes wide spread between families by the fifth generation and selection within most families gets ineffective (Acquaah, 2007).

The pedigree method has been showed to be more efficient than the bulk selection method, due to the high chance of obtaining a genotype of great quality. Another advantage of this method is that selection is based on both phenotype and genotype, making it an effective method for the selection of lines with suitable quality from among segregating plants. The pedigree method is however not best suited for quantitative disease breeding. It is ineffective to accumulate the number of minor genes needed to provide horizontal resistance. This method is also not suitable for species in which individual plants are difficult to isolate and characterize (Acquaah, 2007).

2.4.1.3. Single seed decent

The single seed descent (SSD) method is a modification of the pedigree method, and was originally developed to speed up breeding programs. The process of the SSD method starts with the selection of parents, which are crossed, to create the base population. After crossing, single seeds from each plant are randomly selected and sampled in subsequent generations. Segregating generations can then be grown in the glasshouse allowing two to three generations per year (Campbell, 1999).

This method concentrates mainly on the early stages of the procedure to rapidly accomplish homozygosity, without selection. Other advantages of the SSD method is that it reduced the duration of breeding programs by several years through the use of single seed descent and it increase the genetic diversity in each generation, since every plant originates from a different F_2 plant. The weakness of this method is however that selection is not based on progeny performance, but rather on individual phenotypes (Acquaah, 2007).

2.4.1.4. Backcrossing method

The underlying principle of backcrossing is to replace a specific undesirable gene with a desirable alternative, while preserving all other qualities of an adapted cultivar or breeding line (Acquaah, 2007). It is a method of transferring disease resistance into a commercial susceptible variety with good agronomical background such as yield and adaptation (but lacks disease resistance).

In a breeding program the susceptible cultivar, which is mostly dominant, is crossed with a germplasm accession which is the donor of resistance, to produce the F_1. The progeny of the F_1 would be heterozygous and resistant to the disease. F_1 seeds are grown and backcrossed to the recurrent parent, to obtain the first backcross (BC_1). The progeny of BC_1 will segregate: 50% (Rr) resistant plants and 50% susceptible plants (rr). The resistant plants are then selected and backcrossed to the recurrent parent to produce BC_2, BC_3 and BC_4 until resistance is confirmed (Moustafa, 2011).

The backcross method is an important method to overcome rapid development of new pathotypes of diseases. It is extremely useful for the integration of specific genes from wide-crosses and it provides new cultivars, which are phenotypically similar to the desirable

parent. Backcrossing has been successfully employed in many wheat breeding programs. It has shown to reduce the population sizes and increase gene frequencies in the populations (Bonnet *et al*., 2005).

2.4.1.5. Recurrent mass selection and male sterility

Recurrent mass selection is a well-established breeding technique whereby cross-pollinating as well as self-pollinating species can be genetically improved. It was developed mainly for the improvement of quantitatively inherited traits, and the overall goal is to increase the frequency of genes of interest in a breeding population so that the chances of selecting superior genotypes are increased (Marais & Botes, 2009). The high level of heterozygosity in the breeding population together with the large amount of crossings that can be made, allow for a more complete investigation of polygenic recombination potential.

Due to the ability of self-pollinating crops to rapidly fix genes, the chance of genetic recombination is dramatically reduced. In dealing with polygenic traits, it is therefore absolutely unlikely to expect that the polygenic recombination potential of a cross can be adequately investigated in a single cycle of crossing and selection. Employing recurrent mass selection for breeding in regards to polygenic traits in cross-pollinating crops has shown to be successful, and breeders and researchers have considered ways to incorporate RMS to wheat and other self-pollinating crops. The inability of self-pollinating crops to pollinate on a large scale limits the successful use of RMS in self-pollinating crops (Marais *et al.*, 2000). This limitation was overcome by the introduction of male sterility as a tool for the production of hybrid wheat.

2.4.1.5.1. Male sterility systems

Male sterility is described as a biological characteristic commonly found in nature. It is the inability of plants to produce functional anthers, pollen or male gametes. It is caused by several factors such as mutations, diseases, or unfavourable environmental and growth conditions. The identification and creation of male sterile lines that is genetically unable to produce functional pollen is an easy way to create a female line for the production of hybrid seed. Moreover, male sterile plants offer breeders an option to produce and develop seeds of hybrid cultivars with much greater agronomic value than its parents (European Seed Association, 2013). Thus, male sterility is of significant importance and benefit for plant

breeders and has been identified in more than 150 plant species. It can be found in crops like maize, oilseed rape, sunflower, rye, rice, barley, pepper and wheat. Different types of sterility systems have been explored in wheat including cytoplasmic male sterility (CMS), chemical induced male sterility and genic male sterility.

2.4.1.5.1.1. Cytoplasmic male sterility

Cytoplasmic type of male sterility (CMS) is a maternally inherited characteristic, controlled by genes of the cytoplasm. It is caused by changes of the mitochondrial genome that creates an open reading frame. Sequence analysis of the CMS derived mitochondrial genome from wheat showed that the open reading frame is made up of a chimeric sequence produced by gene fusion between a native gene and an unknown sequence. CMS can occur both naturally and following mutagenesis or it can occur due to inter-and intraspecific crosses as well as intergeneric crosses. In many cases of CMS, fertility in the male sterile line can be bringing in effect again by the introduction of nuclear encoded fertility-restorer (*Rf*) genes. Two to three major loci in wheat are usually necessary for complete fertility restoration.

The cytoplasm of *T. timopheevii* and its derivatives or related species such as Aegilops are normally used for creating male sterility in wheat. However, only male sterile cytoplasms derived from *T. timopheevii* Zhuk have been used thus far for commercial production of hybrid wheat as it contains several important factors/ genes of fertility restoration for CMS as compared to other wild relatives and species (Mahajan & Nagarajan, 1998).

In order to maintain male sterility, a male sterile line is required to cross with a maintainer line containing an exact nuclear genotype but a fertile cytoplasm derived form an elite adapted line. Therefore sterile progeny is produced when the maintainer line, which carries a recessive restorer allele (*rf*), is crossed to a sterile CMS plant (Whitford *et al*, 2013).

Despite the fact that CMS technology have been successfully used for producing F1 hybrids for enhancing crop productivity in numerous cereal crops, CMS relies on the availability of mutants and effective fertility restorer genes in a given crop to produce hybrid seed. Furthermore, the mutations should not cause yield problems or unwanted phenotypic effects. CMS systems are also sensitive to the environment (Whitford *et al*, 2013).

2.4.1.5.1.2. Chemical induced male sterility

Chemical induced male sterility is caused by chemicals known as gametocides. It is a rapid non-heritable method that can be induced simply by spraying. The male gametocides selectively sterilize the male part without distressing the rest of the plant and can therefore be used to produce female parents for hybrid wheat seed production (Whitford *et al*, 2013).

However, gametocides must meet certain prerequisites in order to be useful for commercial hybrid seed production: Firstly, it must selectively induces male sterility and not female sterility; it must be genotype independent; have complete activity and continuity to allow different stages of development among the treated plants; non-phytotoxic; non mutagenic; environmentally safe; practically useful; cost-effective to produce and lastly it must have no effect on the quality of F1 seeds and seedling vigour. These strict prerequisites have led to a small number of gametocides being taken up by commercial seed companies (Whitford *et al*, 2013).

Male gametocides used in wheat include maleic hydrazide, ethephon and glibberens (Mahajan and Nagarajan, 1998). These chemicals were however phytotoxic and unable to show adequate male sterility. Thus new effective chemicals such as fenridazon-potassium (RH-0007, HYBREX®), the sogital compound SC2053 (Orsan) , azetidine-3- carboxylic acid (WL 84811) from Shell, clofencet (Genesis®) from Monsanto, and sintofen (Croisor®100) from Saaten Union Recherche were developed. However commercial deployment of these new chemicals is still hindered by several challenges such as environmental conditions under which complete sterility is expressed. RH-007 was only used for a certain period of time because it only worked in certain genotypes and in a narrow application window. Azetidine-3- carboxylic acid (WL 84811) was used effectively in Europe, USA, South Africa, China, Australia and New Zealand until it was considered risky and immediately stopped because of toxic residues detected in F1 seeds produced on treated plants. Genesis and Croisor®100 were the most useful gametocides in Europe and USA. In 2007, Genesis was discontinued and Croisor®100 is the only gametocide still in use today for commercial production of hybrid wheat (Whitford *et al*., 2013; Mahajan & Nagarajan, 1998). Genetic male sterility also known as nuclear male sterility is caused by mutations in nuclear-encoded genes. GMS can arise spontaneously or may be induced through exposure to physical or chemical mutagens like ethylmethane sulfonate (EMS). EMS-induced male sterile wheat's include FS2, FS20 and KS87UP9 controlled by genes *ms1d*, *ms5* and *ms3* respectively (Fossati & Ingold, 1970;

Driscoll & Barlow, 1976; Sasakuma *et al*., 1978; Maan *et al*., 1987). Spontaneous mutants observed by wheat breeders over the years include Pugsley's, Langzhou, BNY-S, and Taigu, which affect *Ms1*, *Wtms1*, and *Ms2* fertility loci (Pugsley & Oram, 1959; Deng & Huang, 1993; Xing *et al*., 2003; Zhou *et al*., 2008).

The genes causing male sterility are either recessive or dominant and only three have been located to specific chromosomes. Cornerstone, x- ray induced male sterile wheat is controlled by a recessive gene *ms1*, located on the short arm of chromosome 4A. A disadvantage of the *ms1* gene is that the amount of sterile plants is not always sufficient for recombination in breeding techniques like wheat recurrent selection (Whitford *et al*, 2013).

In 1972, a wheat mutant Taigu was found in China. This mutant was controlled by *Ms2*, a dominant male sterile gene located on chromosome 4DS at a distance of 31.1cM from the centromere. *Ms2* exists in wheat plants always in a heterozygous state of *Ms2ms2*, and receives pollen from normal wheat cultivars with a homozygous genotype *ms2ms2* (Zhang *et al*., 2014). The progeny of sterile wheat plants always segregates 1:1 for male sterility/ male fertility plants. However, during its application in wheat breeding, no difference in plant height between sterile and fertile plants could be found. This shortcoming was soon overcome by the development of dwarf male sterile wheat (DMSW) and later a molecular marker (*MS2-WMC617*) to easy identify between sterile plants at an early stage during development (Liu & Yang., 1991; Cao *et al*., 2009). Since then *Ms2* has been widely used by wheat breeders to develop several wheat cultivars with improved adaptation and quality by means of backcrossing and recurrent selection techniques (Liu *et al*., 2002; Zhai & Liu, 2009).

Another important dominant male sterility gene, *Ms3*, was found after EMS treatment of the seeds of alloplasmic common wheat "Chris" with *Aegilops squarrosa* cytoplasm. The gene was later transferred to euplasmic common wheat and is equally effective in producing dominantly inherited male sterility in both the euplasmic and alloplasmic common wheat cultivars (Maan & Williams, 1984). *Ms3* is closely located to the centromere on chromosome 5AS. A molecular marker, WG341, closely associated with this dominant gene was identified at a genetic distance of 0.8 cM (Qi & Gill, 2001). This marker could effectively be used to identify the dominant male sterility gene at an early stage in wheat breeding.

Male sterility with *Ms3* is however only stable under normal greenhouse temperatures of 16ºC-21ºC as high temperatures of 21ºC-35ºC results in the incomplete expression of the gene leading to the production of functional pollen (Maan & Williams, 1984). *Ms3* cannot be used satisfactorily to effect cross-pollination among plants grown in the field. Seed set on male sterile ears is normally low, selection needs to be done prior to flowering when foliar diseases are not yet fully developed and the extent of cross hybridization of spatially scattered selected plants cannot be predicted. Therefore, a hydroponic system was developed to facilitate the pollination of large numbers of male-sterile (female) spikes with selected fertile (male) spikes. Marais *et al*. 2000 introduced the dominant *Ms3* from a winter wheat accession KS87UP9 into the spring wheat 'Inia 66'. The F_1 female progeny obtained from the cross segregated for the dominant *Ms3* gene. Male sterile lines were selected out of the segregating progeny and used in a recurrent selection crossing cycle with male fertile wheat lines in order to facilitate large amount of different crossing combinations. With the application of MAS, male and female lines can be screen for desirable genes before it is incorporated into the mass crossings. By selecting only wheat lines with desirable genes to cross with each other in successive cycles, allele enrichment and breeding for multi-gene resistance in wheat can be facilitated. This technique came to be known as male sterility mediated marker-assisted recurrent selection (MS-MARS) (figure 2.6).

In the MS-MARS scheme, male fertile plants are allowed to self and the F_2 are grown in a single seed descent manner. After four to six generations wheat lines are field tested for agrotype, disease resistance, yield and quality. Lines with the best performance can then be selected and used as male parents in the MS- MARS scheme. F_1 seeds can also be planted in the field and double haploids (DH) can be produced (Marais *et al*., 2000)

2.4.2. Molecular markers in breeding

2.4.2.1. Marker assisted selection

Over the last ten years, plant breeding programs have embraced modern biotechnology tools that make the process more efficient and speedy. Marker assisted selection (MAS) is the use of known molecular markers to track the location of desirables genes in a breeding crop, such as resistance to a disease. The markers are either directly or closely linked to the target gene or regions on the chromosome (QTL).

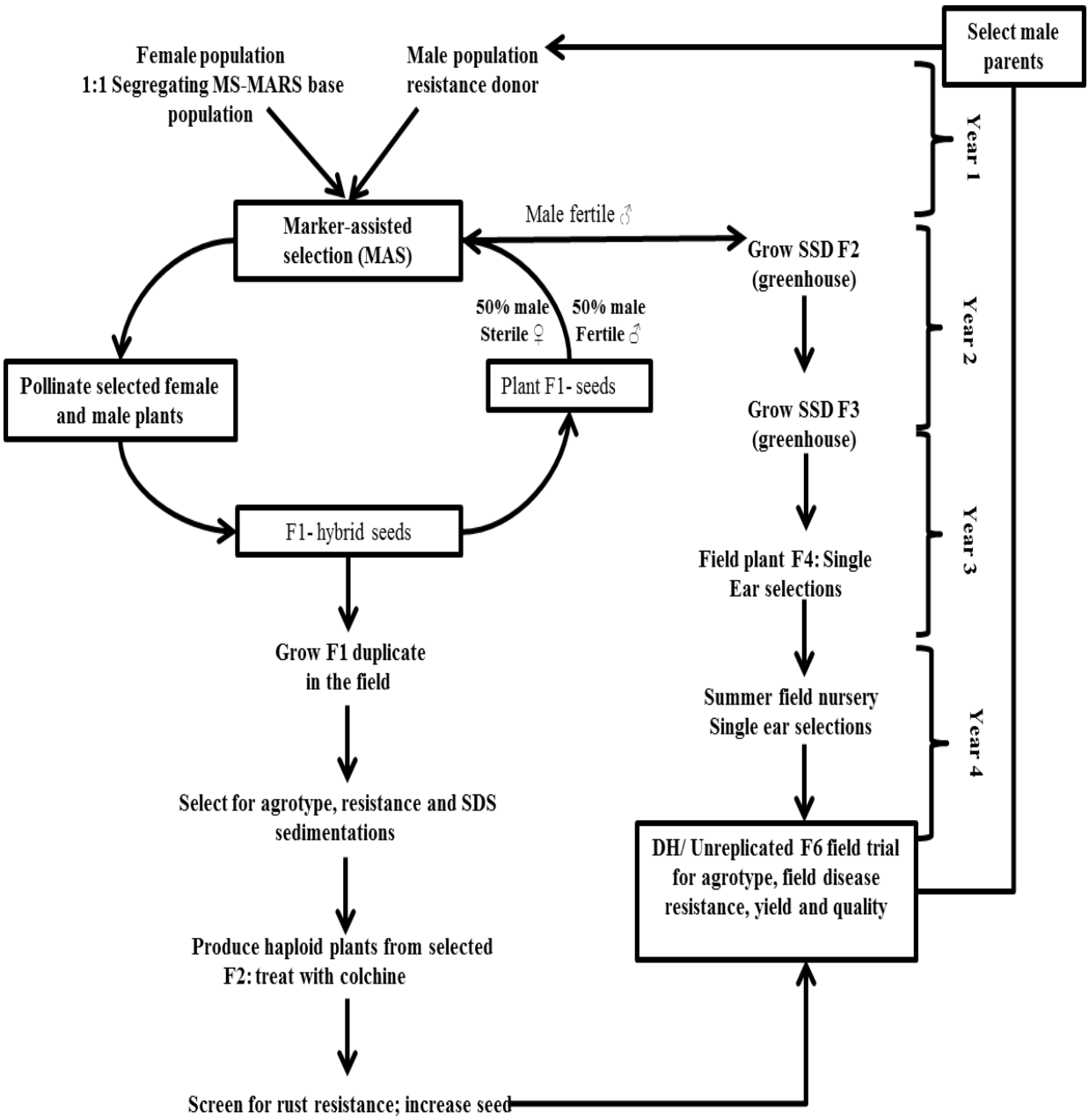

Figure 2.6. MS-MARS breeding scheme (Marais & Botes, 2009).

Once the traits are mapped, a closely linked marker can be used to screen large numbers of sample to rapidly identify progeny that contain desirable characteristics. MAS offer an opportunity to select desirable genes based on genotype rather than phenotype (Rana *et al*., 2011). It can effectively be used to speed up development of wheat cultivars. It can be used to differentiate between various genes for the same characteristic in gene pyramiding of different resistance and developing multi-line cultivars targeting for durable resistance to the disease (Rana *et al*., 2011). Advantages from MAS increase with decreasing heritability of the trait. Traits that show low heritability will reach a threshold when QTL detection gets difficult, or when interaction between a QTL and the environment influenced the reliability of the markers (William *et al*., 2007).

MAS can be an attractive alternative in cases where large numbers of seed are required for analysis that is not usually available in a breeding program. MAS based on protein profiles or DNA based markers can be applied to make early selections (William *et al*., 2007). MAS are now routinely used in breeding strategies in commercial breeding programs to increase gain from selection per unit (Rutkoski, 2011). Breeding strategies using molecular markers include: predicting an individual's breeding value for selection and backcrossing favourable alleles into elite germplasm where one gene of a donor species are transfer to the genetic background of the recipient. In this case, markers are used to monitor and speed up the presence of the target gene, through the selection of lines with the smallest amount of donor chromatin, which can be done with the use of linkage maps. MAS can be seen as an example of the successful integration of conventional breeding techniques, with modern molecular resources (Hospital, 2009).

2.4.2.2. Factors considered for selecting markers

The successful application of molecular markers to aid in breeding procedures relies on the following factors:

- An accurate genetic map with molecular markers linked to the major genes or QTLs of interest.
- A tight association between the markers and the major genes or the QTLs. Markers located directly is most favorable and commonly requires the availability of the target gene

cloned. Markers linked to the gene should preferably have a genetic distance of 1 cM or less to decrease linkage drag (undesirable alleles of other genes linked to the target gene) and recombination, and polymorphic markers should preferably flanked the desirable QTL, and or be within the QTL region (Menzo, 2010).

- The level of polymorphisms (effective discrimination between different genotypes);
- The possibility of screening large populations in a time and cost effective manner. High-throughput simple and quick methods are desirable.

2.4.2.3. Types of molecular markers

Molecular markers can be broadly classified in the following groups:

- Hybridization based DNA markers-Restriction fragment length polymorphisms (RFLP)
- Polymerase Chain Reaction (PCR) based markers- Amplified fragment length polymorphism (AFLP), Random amplified polymorphic DNA (RAPD), Sequenced-characterized amplified regions (SCARS), Microsatellites (SSR's)
- DNA chip and sequencing based DNA markers- Single nucleotide polymorphisms (SNP) and Sequence-tagged sites (STS).

All of these marker techniques are being used extensively to identify specific traits and genes in many important crops (Gupta *et al*., 1999). RFLP markers were the first to be developed and were initially used to map human genomes. It was also later adapted for plant genome mapping. The RFLP technique is however time consuming and labour intensive.

RAPD was a very simple and inexpensive method for diversity studies in most crops, but is now less frequently used due to its weaknesses such as poly AFLP's and SSR's are very effective in detecting polymorphisms and are the most popular markers in cereals. Microsatellites are more promising markers for the identification and differentiation of genotypes, within a species and are extremely ideal for different applications in wheat breeding. SSR's and SNP's are highlighted for the rapid development of new markers (Rana *et al*., 2011).

The high frequency, wide distribution in genomes, and adaptation to highly multiplexed detection systems has made SNP's excellent markers for most organisms. However, the availability of SNP markers for wheat is limited (Yu *et al*., 2009).

Microsatellite markers, also known as simple sequence repeats (SSR's) are one of the most popular markers in cereals. It is ideal for detecting differences between and within species of genes of all eukaryotes. It consist of simple sequence repeats of only one of a few (maximum of six) base pairs, that are arranged in repeats of mono-, di-, tri- ,tetra- , and penta-nucleotides. The tandem repeats are less that 100bp in length with a minimal length of 12 bp (Paux *et al*., 2012). These repetitive sequences are widely distributed throughout the genomes of plants and animals that display a high level of genetic variation based on differences in the number of tandem repeating units of a locus (Jonah *et al*., 2011). The variation in the number of repeats results in highly polymorphic banding patterns. These patterns can be detected by PCR, through the use of locus specific flanking region primers where they are known.The development of microsatellite primers is complex and requires considerable technical ability, like cloning and sequencing. It is also time consuming, labour intensive and costly (Hayden *et al*., 2004; Korzun, 2002).

2.5. Breeding for durable resistance

Alternative strategies for the deployment of resistance genes have been encouraged by several authors, in order to achieve durable resistance. These strategies include, the piling of resistance genes by "stacking" or pyramiding, and the deployment of several resistance genes in a heterogeneous crop as multi-lines or mixtures of cultivars (Pink, 2002)

2.5.1. Multi-lines and mixtures

Multi-lines are cultivars that consist of a mixture of genotypes that are agronomically similar, but differ in resistance to different pathotypes of the same pathogen (Pink, 2002). These genotypes are called near isogenic lines (NIL's) and are developed through a series of backcrosses where the F_1 generation is repeatedly backcrossed to the recurrent parent. The recurrent parent is mostly a dominant cultivar and do not contain the targeted gene (Acquaah, 2007). Cultivar mixtures on the contrary are the concurrent use of different cultivars that differ from each other phenotypically, as well as on the basis of resistance they confer. Multi-lines and mixtures appear to manage disease epidemics through the combination of several effects (Garreth & Mundt, 1999). Firstly it reduces the density of susceptible plants & only a fraction of a sowed land will be susceptible to a pathotype of a disease. Secondly, resistant

plants poses a barrier effect against infection, as resistant plants interrupt spores of incompatible pathotypes. Lastly, infections by spores of incompatible pathotypes on resistant plants, results in the activation of induced resistance mechanism which provide cross protection against later infection by compatible pathotypes (Pink, 2002).

Significant evidence for disease reductions, caused by mixtures have been reported (Smithson & Lenne, 1996). The disease reduction of wheat was up to 60% and from 29 cases reported of mixture in wheat and barley, disease reductions of more than 30% was observed. According to Pink (2002), the main reason for the restricted use of mixtures is crop heterogeneity. Practical problems of mixtures include the determination of harvest maturity and quality, legal constraints and seed production and distribution.

The durability of resistance provided by multiline cultivars depends on the rate at which complex pathogen pathotypes evolve. Factors that influence the rate at which pathogens evolve include firstly the selection of stabilization, to prevent the development of complex pathotypes in a multiline. Secondly, a significant number of cultivars or lines should be incorporated in a mixture, to increase its buffering effects against corrosion of resistance genes. The limited number of components used in multiline cultivars with low degree of stabilizing selection is insufficient to keep the pathogen population composed of simple pathotypes. Epidemiological situations are a third factor that may affect the durability of a multiline cultivar. If the same genes in the mixture are carried by pure line cultivars, the situation could then result in the rapid evolution of complex pathotypes (Moustafa, 2011).

2.5.2. Pyramiding of genes

Gene pyramiding in plant breeding is described as the simultaneous deployment of multiple resistance genes in the same cultivar. The incorporation of new resistance genes into a cultivar potentially provide more durable resistance since mutational events at several avirulence loci would be required to produce new virulent pathotypes. However the incorporation of more than one effective resistance gene at a time has not been practical using traditional breeding methods as epistatic effects made it difficult to distinguish between resistant plants carrying one or more effective resistance gene. Therefore, breeders have initially leaned towards the pyramiding of resistance genes that were already overcome by the pathogen, since the matching virulence/avirulence profile of the genes enabled breeders to distinguish between the genes. With the development of MAS, breeders can now stack

resistance genes that have not been overcome by the pathogen to prolong the time of effectiveness of the gene pyramid. However, gene pyramids are vulnerable and the use of genes in the pyramid as single genes in other cultivars will result in the occurrence of virulence to individual resistance genes. The number of resistance genes stacked in a cultivar is not directly proportional to the durability of its resistance. Thus, the durability of resistance cannot be predicted based on the number of resistance gene possessed by a cultivar (Pink, 2002).

It is emphasized that the combination of several minor genes, responsible for non-specific resistance, will lead to cultivars with durable resistance. Breeders and scientist are now focussing on the deployment of several minor genes with additive effects to combat rust diseases (Prins *et al*., 2011). It is reported that recurrent selection strategies (MS-MARS) are effective to accumulate several minor genes in a single genetic background. Wheat lines containing resistance genes can be selected continuously, thereby increasing the level of durable resistance (Marais & Botes, 2003).

Pyramiding of different genes and traits, through the use of MAS has been reported in several studies. Three leaf rust resistance genes *Lr13*, *Lr34* and *Lr37* were successfully transferred by Kloppers and Pretorius in 1997. Another successful pyramiding of three powdery mildew resistance genes was published by Liu *et al*. (2000). These reports pove that gene pyramiding with MAS is possible and can be successfully deployed to stack several resistance genes to provide durable resistance.

When resistance genes are successfully incorporated and detected by use of MAS in wheat lines, the fixing of the genes in a suitable genetic background and the subsequent development of inbred lines can however take up to several years before a new cultivar is ready for field trials (Wessels & Botes, 2014). The doubled haploid (DH) method can shorten the inbreeding process to just one generation, without compromising the integrity and agronomical quality of the lines. Several DHs production methods are available in wheat such as the androgenesis method, which uses anther/microspore culture, the gynogenesis method that uses ovary/ovule culture, chromosome elimination following wide hybridization, haploid inducer genes and chemicals (Niroula & Bimb 2009). The wide hybridization method is the most employed DH method in wheat breeding programs. The development of DH through this method was shown to be particularly practical through the *bulsom* method in barley and the wheat X maize method. The latter method is currently the most extensively used method

for the production of haploids in wheat all over the world, since it is very simple and does not have the high genotype dependent interaction shown by other techniques. The combined use of DH and MAS has already been explored internationally and locally, and several lines with very different genetic profiles have been produced (Yonezawa & Ishii, 2005; Gupta *et al*., 2010; Wessels & Botes, 2014).

2.5.3. Wheat rust resistance genes used in this study

To date, 71 designated leaf rust resistance genes, 58 stem rust resistance genes and 53 yellow rust resistance genes have been identified and named in wheat (Liu *et al*., 2014). Of the 58 stem rust resistance genes, at least 27 are effective or partially effective to the Ug99 group of pathotypes. These genes include *Sr2*, *Sr13*, *Sr14*, *Sr22*, *Sr25*, *Sr26*, *Sr32*, *Sr33*, *Sr35*, *Sr36*, *Sr37*, *Sr39*, *Sr42*, *Sr43*, *Sr45*, *Sr46*, *Sr47*, *Sr51*, *Sr52*, *Sr53*, *Sr55* (/*Lr67*/*Yr46*/*Pm46*), *Sr57* (/*Lr34*/*Yr18*/*Pm38*) and *Sr58* (/*Lr46*/*Yr29*/*Pm39*). A lot of these genes have been effectively used to incorporate genetic resistance to stem rust, leaf rust and stripe rust over the years (Xi-Yu *et al*., 2014).

2.5.3.1. *Sr2*

Sr2 is a critical gene for stem rust resistance in many breeding programs the world-wide, and has been effectively employed in wheat from the 1940s against stem rust. It has offered durable, broad-spectrum resistance for more than 80 years in the wheat breeding industry against all known stem rust pathotypes, including the virulent pathotype Ug99 and its variants (Mago *et al*., 2011). *Sr2* is a recessive, non-race specific resistance gene, and confers a slow rusting response when deployed on its own (Singh *et al*., 2002). Therefore, deployment of *Sr2* with other rust resistance minor genes, known as the *Sr2*-complex, can provide resistance against most of the stem rust pathotypes (Singh *et al*., 2006).

The *Sr2* gene was originally transferred from ‘Yaroslav’ emmer wheat (*Triticum. dicoccum* Schronk) into the cultivar ‘Hope’ (hexaploid wheat), and it is located on the short arm of chromosome 3B. *Sr2* is also linked to leaf rust resistance gene *Lr27* and pseudo-black chaff (PBC) which involves melanin pigmentation of the glumes and stem of the wheat plant, particularly below the uppermost node. PBC has been used as morphological marker for screening *Sr2* in rust breeding programs, but use of this marker for the detection of *Sr2* is

limited due to its partial dominance and variable level of expression in different genetic and environmental backgrounds (Sharp *et al*., 2001).

Various gene specific and closely linked markers for the detection of *Sr2* have been reported and utilized in stem rust breeding programs, including the SSR gwm533 and CAPS based csSr2 marker (Spielmyer *et al*., 2003; Mago *et al*., 2011). Marker Xgwm533 is associated with a 120 bp product for the presence of *Sr2*. However, a lot of difficulty with this SSR marker was experienced as several lines predicted not to carry the gene also amplified a 120 bp product (Hayden *et al*., 2004). The CAPS marker, highly accurate in the detection of *Sr2* is associated with fragments of 53 bp, 112 bp and 172 bp for the presence of *Sr2*, while products of 112 bp and 225 bp are associated with the absence of *Sr2*. The CAPS marker showed to be much closer to the *Sr2* gene than the Xgwm533 marker (Mago *et al*., 2011). However, it is concluded that the combined use of these two markers provides accurate information at a molecular level and may also enhance accuracy of *Sr2* gene pyramiding during a breeding program (Malik *et al*., 2013).

2.5.3.2. *Sr24*

The stem rust resistance gene *Sr24* was found to reside on the long arm of chromosome 3D, and is postulated to be a natural translocation from the 3Ag chromosome of *Thinopyrum ponticum* (syn. *Agropyron elongatum*) when the hexaploid wheat 'Agent' was examined by Smith *et al.* (1968). The 3Ag segment in Agent, also containing leaf rust resistance gene *Lr24*, was found closely associated with the red grain colour gene. By using induced homologous recombination, Sears *et al.* (1973) introduced a reduced *Th. ponticum* translocation segment carrying *Sr24* into the 3DL chromosome in wheat. This reduced segment has led to the breakage of the linkage between *Sr2*4 and red grain colour (which is not preferred by consumers) in 'Agent', allowing the subsequent introduction of *Sr24* into white grained wheat.

Sr24 is a valuable source of resistance worldwide to most stem rust pathotypes, including Ug99. However, stem rust pathotypes, including variants of Ug99, with virulence to this effective resistance gene has been reported in countries like South-Africa (not pre-dominant in all production areas) and India (Jin *et al*., 2008; Visser *et al*., 2009).

A number of molecular markers are available for the detection of *Sr24*, including a SSR marker (BARC71) and two AFLP markers (Sr24#12 and Sr24#50). AFLP marker Sr24#12 is completely associated with the *Sr24* gene and amplified a fragment of 500 bp, while the other AFLP marker failed to predict the presence of *Sr24* in some germplasm. A SCAR marker, $SCS73_{719,}$ originally developed from a RAPD marker ($S73_{728}$) for *Lr19* can also be used for the detection of the *Lr24*/*Sr24* translocation. The SCAR marker was found to be specific to *Lr24* and closely associated with *Sr24* (Cherukuri *et al*., 2003; Prabhu *et al*., 2004).

2.5.3.3. *Sr26*

The *Sr26* resistance gene was originally transferred from *Th. ponticum* into the hexaploid wheat chromosome 6AL (Dundas *et al*., 2007). The *Th. ponticum* segment containing *Sr26* has been used for many years only as a source of resistance in Australia where the cultivar, 'Eagle', was released (Martin, 1971). However, the use of *Sr26* has been limited to Australia due to a reported 9% yield reduction associated with the original 6AS.6AL-6Ae#1L segment. Despite the yield reduction, several cultivars carrying *Sr26* in addition to 'Eagle' were developed and released (McIntosh *et al.*, 1995). Evidence of the limited deployment of *Sr26* was reflected in a study by Liu *et al.* (2010) where none of the 170 diverse wheat lines around the world, tested for the presence/absence of *Sr26*, contained the gene. New wheat lines with reduced alien segments have been developed in recent years by programs inside and outside of Australia that do not display the reported yield reduction of the original *Sr26* containing lines (Dundas *et al.*, 2007).

The effectiveness of *Sr26* against the Ug99 pathotype group, its low frequency among modern cultivars and the availability of donor lines with reduced alien segments makes it ideal for deployment in breeding programs following a strategy of marker-assisted gene pyramiding in response to the presence of Ug99 and its variants (Liu *et al.*, 2010).

A dominant STS marker (Sr26#43), associated with a 207 bp product for the presence of *Sr26*, was developed by Mago *et al.* (2005). This marker has been used successfully for MAS in breeding programs (Bariana *et al*., 2007). Liu *et al.* (2010) developed a co-dominant marker by combining the dominant STS marker with a chromosome 6A-specific marker (BE518379) to distinguish between homozygotes and heterozygotes. Marker BE518379 amplifies a 303bp product in lines without *Sr26*. The co-dominant marker Sr26#43/

BE518379 showed to be very diagnostic for *Sr26* and tend to be useful in the detection of heterozygotes during early segregating generations.

2.5.3.4. *Sr35*

Sr35 is potentially one of the most important and effective genes against the virulent pathotype Ug99 and its variants. It was originally transferred from *T. monococcum* to hexaploid wheat and is located on the long arm of chromosome 3A in wheat. Remapping of *Sr35* by Zhang *et al.* (2010) in two *T. monococcum* and two *T. aestivum* populations showed that it is located between markers XBF483299 and XCJ656351 in a region of 2.2 to 3.1cM in the diploid mapping population. The location of *Sr35* was further validated in two backcross derived hexaploid populations, segregating for *Sr35,* and the length of the *T. monococcum* fragment transferred into hexaploid wheat were reduced ten-fold.

Sr35 was cloned by Saintenac *et al.* (2013) due to additional effectiveness against the TRTTF group of pathotypes from Africa, Yemen, and Pakistan. The TRTTF group has a broad virulence profile, but it differs from the virulence profile of the Ug99 pathotype group. Also, *Sr35* is effective against the same virulent pathotypes (Ug99, Ug99 variants and TRTTF) when transferred to hexaploid wheat by crossing and recombination. Map based cloning of *Sr35* showed that it codes for a coiled coil-nucleotide binding-leucine rich repeat product (CNL). CNL proteins are involved in the detection of pathogen attacks of different types. The annotated 307519 bp sequence, obtained from three overlapping bacterial artificial chromosomes (BAC's) spanning the *Sr35* region, also includes five intact genes (*CNL1*, *CNL2*, *CNL4*, *CNL6*, and *CNL9*), two pseudogenes (*pCNL3* and *pCNL10*), three small gene fragments (*pCNL5*, *pCNL7*, and *pCNL8*) and two unrelated genes (*SFGH* and *APGG1*). Phylogenetic analyses of the complete CNL gene showed that CNL4 and *CNL9* are the closest members of the CNL cluster group. However, after refining the *Sr35* region, and re-sequencing of the four candidate genes (*CNL4*, *CNL6*, *CNL9* and *APGG1)* in a *T. monococcum* collection including 24 Ug99 resistant accessions (carrying *Sr35*) and 25 susceptible accessions (without *Sr35*), it was found that all susceptible accessions contained mutations in the *CNL9* gene with five accessions having mutations in only the *CNL9* gene. Saintenac *et al.* (2013) then analysed mutants induced by ethyl-methanesulfonate and found two mutant families segregating for susceptibility. Sequencing results of the four candidate genes in the two mutant families confirmed the presence of the mutations only in the *CNL9* gene. This gene was then validated as the *Sr35* gene as transgenic hexaploid wheat plants

carrying the *CNL9* gene were observed to be resistant to Ug99 while plants without the gene were susceptible (figure 2.7a). Also, similar race specificity between the transgenic and normal *Sr35* were observed when transgenic plants inoculated with OTHJC (virulent for *Sr35*) all showed susceptibility, regarding the presence or absence of the *CNL9* transgene (figure 2.7b).

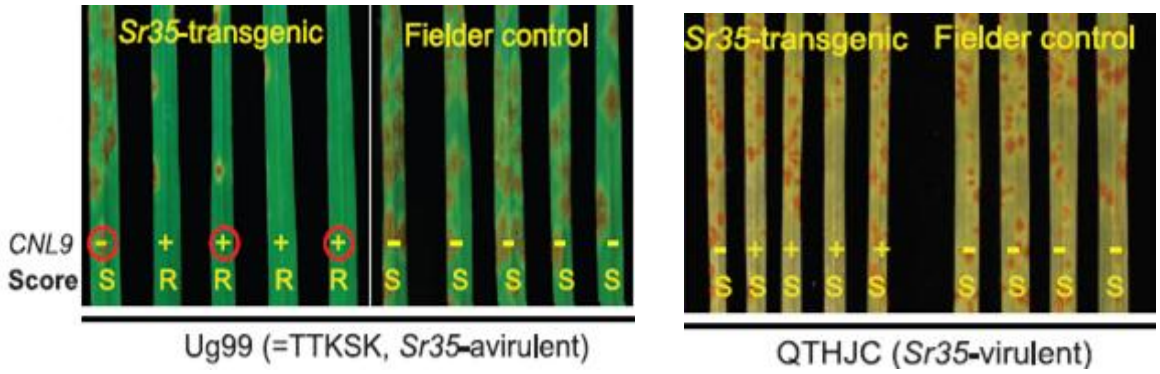

Figure 2.7. Infection types produced on transgenic seedlings inoculated with pathotypes Ug99 and QTHJC. A) Plants carrying the CNL9 transgene (+) were resistant to Ug99 (R) and plants without the transgene (-) were susceptible. B) All *Sr35* plants were susceptible to QTHJC (Saintenac *et al*., 2013).

A number of markers closely linked to *Sr35* were identified in wheat. The markers include four indel types and six microsatellites. SSR markers xcfa2170, xcfa2076 and xcfa2193 were reported to be useful for MAS as the *T. monococcum* fragment has reduced recombination in hexaploid wheat. However, it is advised to use a marker that is closely linked to *Sr35* to decrease the risk of irregular recombination (Babiker *et al*., 2009; Zhang *et al*., 2010).

2.5.3.5. *Sr45*

The stem rust resistance gene *Sr45* was originally described in *Aegilops tauschii*, the D-genome donor of *T. aestivum*. The close relationship between *Ae. tauschii* and *T. aestivum* has facilitated the rapid transfer of several important traits, including resistance to wheat stem rust from *Ae. tauschii* to *T. aestivum*. This can be achieved either through synthetic wheat's produced from a cross between *T. turgidum* with the AABB-genome and *Ae. tauschii* or direct crossing between *T. aestivum* and *Ae. tauschii* followed by backcrossing.

Sr45 is located on chromosome 1DS at a locus proximal to *Sr33* (Marais *et al*., 1998). It was found in *Ae. tauschii* accession RL5289, which also carries leaf rust resistance gene *Lr21*. RL5406, a synthetic hexaploid wheat produced from a crossed between RL5289 and an

experimental line 'TetraCanthatch' was used as a source in the transfer of *Sr45* and *Lr21* to other hexaploid cultivars. The use of RL5406 for stem rust resistance studies was limited due to previous reports of susceptibility to Canadian stem rust pathotypes, until *Sr45* was found effective against South African stem rust pathotypes (Marais *et al*., 1994). *Sr45* also provides effective resistance against Australian and Indian stem rust pathotypes as well as the Ug99 pathotype group (Periyannan *et al*., 2014).

Sr45 has not been extensively used in breeding programs, but deployment is possible due to the uses of *Ae. tauschii* derived germplasm in many programs. Successful pyramiding of *Sr45* with other effective stem rust resistance genes would accomplish durable rust resistance (Rouse *et al*., 2011).

Several molecular markers closely linked to *Sr45* were identified by Sambasivum *et al.* (2008). The marker cluster wmc222/gwm106/stm694tgag/cfa2158/cfd21 was identified at a locus 3.3 cM distal from *Sr45* and marker xbarc229 was 3.7 cM proximal from *Sr45*. A co-dominant PCR marker, cssu45 were developed by Periyannan *et al.* (2014) after fine mapping of *Sr45* in a high resolution mapping population between Chinese Spring and CD1D5406 (Chinese Spring with a disomic substitution line carrying *Sr45*). Chromosome 1D-specific microsatellites, expressed sequence tags and AFLP markers were useful in the identification of this marker that is linked with *Sr45* at a distance of 0.39 cM. This novel marker distinguished the presence of *Sr45* in different genetic backgrounds and can be combined with a *Lr21* specific marker to select for both stem and leaf rust resistance in breeding populations (Periyannan *et al*., 2014).

2.5.3.6. *Lr34*

Leaf rust resistance gene *Lr34* has been one of the few durable rust resistance genes genetically identified in wheat (Risk *et al*., 2012). It owes its longevity to the fact that it confers partial resistance to all pathotypes of *P. triticina* and *P. striiformis*. It allows rust pathogens to develop slowly during the stages of infection by reducing the rate of haustorium formation and intercellular hyphal growth (Krattinger *et al*., 2009).

Lr34 is also associated with other characteristics and genes like resistance to powdery mildew (*Pm38*), leaf tip necrosis (*Ltn1*) on the flag leaf, improved tolerance to barley yellow dwarf virus (*Bdv1*), and enhanced resistance to stem rust in some genetic backgrounds (Keller

et al., 2013). LTN can also be used as phenotypic marker for *Lr34*, but can be problematic due to the fact that it is environment dependent, and can differ significantly in different environmental conditions and genetic backgrounds (Keller *et al*., 2013; Spielmeyer *et al*., 2005).

Lr34 was first describe in the cultivar 'Frontana' in 1966, but has been incorporated into more than 50% of wheat cultivars worldwide including 'Jupateco 73', 'Forno', 'Sunvale' and 'Chinese Spring'. Wheat cultivars carrying *Lr34* is planted on more than 26 million ha in several developing countries alone (Krattinger *et al.*, 2009).

The *Lr34* gene has remained effective for more than 100 years, and no increased virulence has been observed (Keller *et al*., 2013). However, *Lr34* alone cannot provide adequate protection (resistance), and needs to be used in combination with other resistance genes to achieve high levels of resistance (Krattinger *et al*., 2009).

In 2009, Krattinger *et al.* successfully cloned the *Lr34* gene using bi-parental mapping and positional cloning techniques. It was found that the nucleotide sequence of *Lr34* is 11805 bp in length and consists of 24 exons. The predicted protein consists of 1401 amino acids and forms part of the ABCG (pleiotropic drug resistance) subfamily of ABC transporters. An interesting finding was made that cultivars with or without *Lr34*-based resistance both carry a functional *Lr34* gene on the short arm of chromosome 7D. Only three polymorphisms separate the alleles of the resistant and susceptible cultivars: An A/ T single nucleotide polymorphism in intron 4, a 3 bp (TTC) deletion in exon 11 and a C/T single nucleotide polymorphism in exon 12.

The successful cloning of *Lr34* has led to the development of gene specific molecular markers based on the sequence changes in exons 11 and 12. Initially, Krattinger *et al.* (2009) had some limitations while developing gene specific markers. The limitation was however soon overcome when they combined the tightly linked co-dominant sequence tagged site marker, csLV34 (Lagudah *et al*., 2006) in a multiplex reaction with one of the gene-specific markers cssfr1 to detect *Lr34*. The cssfr1 marker ensures the effective detection of *Lr34* (Krattinger *et al*., 2009).

Chapter 3: Materials and Methods

3.1. Introduction

The male-sterile (female) population were sourced from the SU-PBL's MS-MARS pre-breeding program's F_1 segregating population, and the male population was sourced from the SU-PBL's 8th (2013) Wheat Rust Resistance Nursery (8thWRRN) as well as donor lines containing desirable rust resistance genes (*Sr26, Sr35* and *Sr45*). The donor lines which were previously backcrossed into a desirable background, 07M38, were obtained from the SU-PBL germplasm bank. *Sr26* was backcrossed from cultivar 'Kite', *Sr35* donor lines were backcrossed over four generations from the resistance source W269, and donor lines carrying *Sr45*, derived from *Triticum tauschi*, was backcrossed over four generations from 87M66.

DNA extractions were performed on the male and female populations. Thereafter, both populations were screened, prior to cross-pollination, with the SU-PBL's standardized set that have been developed for the molecular characterization of its wheat rust nurseries (Wessels & Botes, 2014). The male population (donor lines) was additionally screened with markers for stem rust resistance genes *Sr26, Sr35* and *Sr45*. At the same time the markers were validated and their use in MAS were evaluated with the aim of adding them to the standardized set of routinely used markers.

Female (male sterile) plants were carefully selected from the segregating population, cut open, and cross pollinated with male plants forming the male population, to produce F_1 seeds. Male fertile plants were allowed to self. According to the protocol developed by Marais & Botes (2003) and shown in figure 2.6. F_1 seeds obtained from the female plants were planted to form a new female population (segregating population). The F_1 female population was screened for gene combinations utilizing MAS. A new male population was planted and used to facilitate a second round of mass crossings with the new F_1 female population.

An attempt was also made to develop a molecular marker from a RFLP marker (WG341) linked to the dominant male sterility gene *Ms3*. Plasmid extraction and colony PCR was performed to obtain the sequence needed for marker development. In addition, SSR analyses were performed with markers, located on the same chromosome as the male sterility gene, to detect polymorphisms among sterile and fertile plants in order to identify a possible novel SSR marker for the *Ms3* gene.

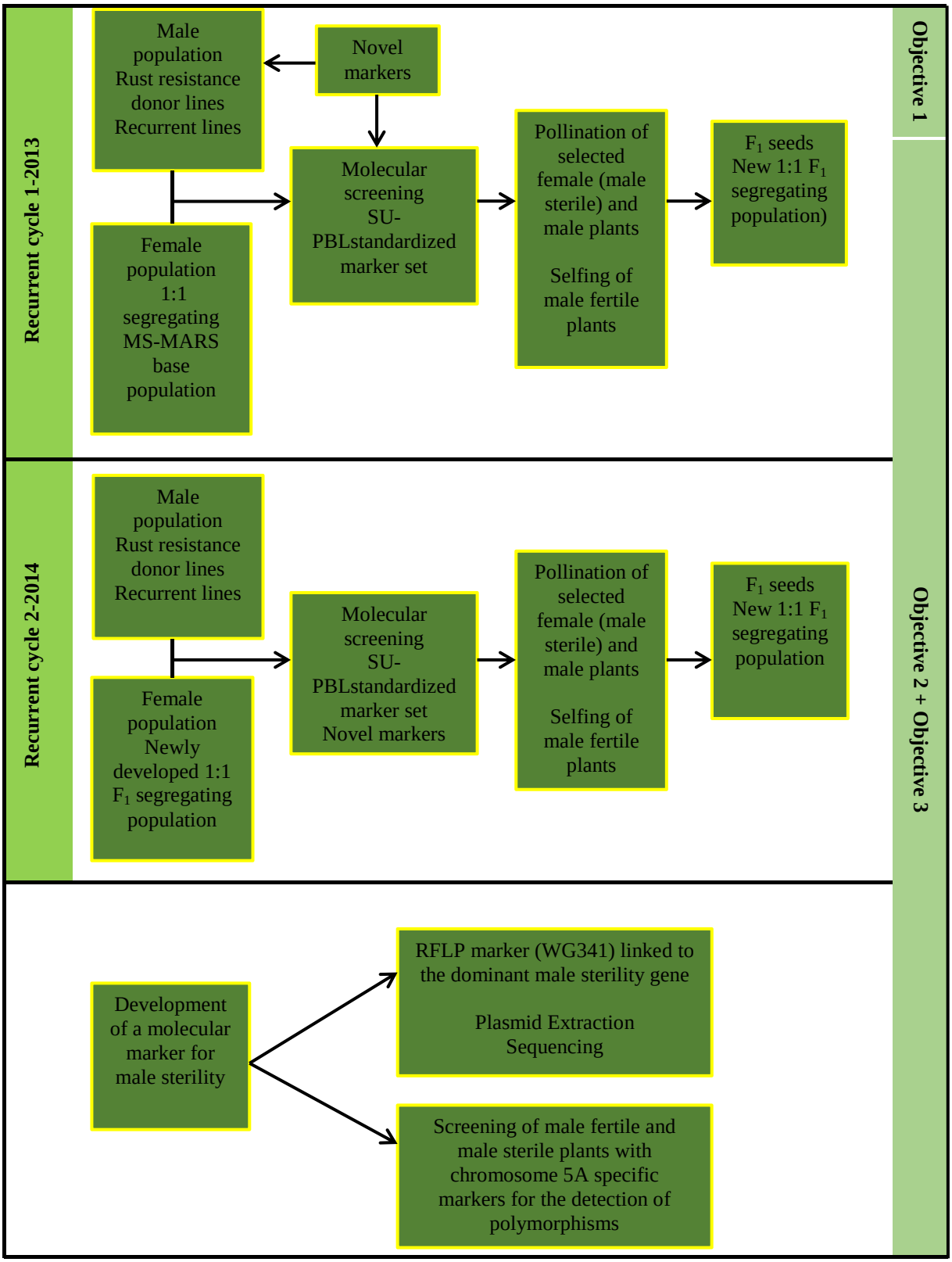

Figure 3.1. Schematic overview of the study.

3.2. Plant material

Plant material used in this study for the female parent group came from a highly diverse MS-MARS pre-breeding base population. This population was established in 1999 (and segregates for an array of different rust resistance genes/complexes including *Lr19, Lr21, Sr2, Sr31/Lr26/Yr9, Lr24/Sr24, Lr37/Sr38/Yr17* and *Lr34/Yr18* (Botes, 2001). Seeds sourced from the MS-MARS base population and which segregates 1:1 for male sterility/ fertility were planted in a greenhouse at Welgevallen Experimental Station (WES), Stellenbosch.

Plant material used in this study for the male parent group consisted of donor lines carrying desired stem rust resistance genes (*Sr26, Sr35* and *Sr45*) and recurrent lines obtained from the SU-PBL's 2013 8WRRN (figure 3.1). Resistance gene *Sr26* were backcrossed from cultivar 'Kite', *Sr35* donor lines were backcrossed over four generations from the resistance source W269, and donor lines carrying *Sr45,* derived from *Triticum tauschi,* was backcrossed over four generations from 87M66 using 07M38 as recurrent parent.

Male and female populations were planted in the same greenhouse with temperatures ranging from 10-25ºC. Plants were irrigated daily with a nutrient solution of 164g Sol-u-fert T3T (Kynoch Fertilizers Pty Ltd, Milnerton, South Africa), 2g Microplex (Ocean Agriculture Pty Ltd, Muldersdrift, South Africa) and 77 ml potassium nitrate in 100 l H_2O), 0.05% Jik (household detergent containing 3.5% sodium hypochlorite, Reckitt and Colman South Africa Pty Ltd., Elandsfontein, South Africa) and tap water.

3.2.1. Plant genomic DNA extractions

An adapted shortened DNA extraction protocol based on Doyle & Doyle (1990) was followed for the extraction of genomic DNA (gDNA). Leaf material was sampled and cut into smaller pieces into a 2 ml micro centrifuge tube, containing three stainless steel ball bearings. Five hundred microlitres of 2% cetyltrimethylammoniumbromide (CTAB) buffer [100mM Tris-Cl (pH 8.0) 1.4 M NaCl, 20mM EDTA (pH 8.0)], were added to the leaf tissue. The tubes containing the leaf tissue and CTAB mixture were put in a Qiagen® Tissue Lyser (Qiagen, Southern Cross Biotech, Claremont, RSA), where the leaf material was ground three times for 60 seconds (sec) at 30 HZ. After grinding, the mixture was incubated in a water bath for 15 minutes at 60°C. A volume of 500 µl chloroform: isoamylalcohol (1:1) was added to the mixture and centrifuged for five minutes at 14 000 rpm. Approximately 400µl

supernatant was transferred to a new clean 1.5 ml micro centrifuge tube and the same volume chloroform: isoamyl alcohol (1:1) was added. The tubes were then centrifuged for five minutes at 14 000 rpm and the supernatant were transferred to a new clean 1.5 ml micro centrifuge tube. Fifty microliters of 3M Ammonium acetate and 500 µl ice cold 100% ethanol were added to the tubes. The tubes were then inverted several times to precipitate the DNA. The tubes were centrifuge for ten minutes (4ºC, 12000 rpm) and the supernatant was discarded. The pellet was then washed twice with 70% ethanol and centrifuged for ten minutes (4ºC, 12000 rpm). Afterwards the pellet was allowed to air dry, and re-suspended in 30 µl distilled water (dH_2O) The DNA was then stored at -20°C. The concentration of the extracted DNA was determined using a Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Kempton Park, RSA). All the DNA monsters were diluted, using distilled water (dH_2O), to a final concentration of 100 ng/µl.

3.3. Molecular screening of wheat lines

3.3.1. Marker identification and validation

The primer sequences and molecular markers used in this study for the genes of interest are listed in Table 3.3. All primer pairs were obtained from Integrated DNA Technologies (Whitehead Scientific Inc, Stikland, RSA).

Microsatellite markers for *Sr35* and *Sr45* were tested on the male population, consisting of wheat lines postulated to carry *Sr35* and *Sr45*, according to protocols obtained from published articles (Babiker *et al*., 2009; Zang *et al*., 2010; Sambasivam *et al*., 2008;). All the SSR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, Fairlands, RSA). The reaction volumes and conditions for both *Sr35* and *Sr45* are listed in Table 3.1 and Table 3.2 respectively. A PCR marker, cssu45, was also tested and used as final validation for the detection of *Sr45*. The PCR reaction contained 5.25 µ1 dH_2O, 6.25 µl 2X KAPA 2G Fast Multiplex mix, 0.25µl of each of cssu45-F primer and cssu45-R primer and 0.5 µl DNA (100 ng/ µl). The reaction conditions were as follow: 3 minutes at 95ºC, 40 cycles of 15 s at 95ºC, 15 s at 60ºC and 15 s at 72ºC, and a final extension step of 7 minutes at 72ºC.

Table 3.1. PCR reaction volumes for *Sr35* and *Sr45* markers.

Reagents	***Sr35***	***Sr45***
dH_2O	5.0	5
2X 2GFast Multiplex Mix	6.25	6.25
(10 µM) Forward primer (Table	0.25	0.25
(10 µM) Reverse primer (Table	0.25	0.25
DNA (100ng/ µL)	1.0	0.75
Total	**12.75**	**12.5**

Table 3.2. Optimal PCR reaction conditions for *Sr35* and *Sr45* markers.

Sr35		***Sr45***	
Temperature (°C)	**Duration**	**Temperature**	**Duration**
94	5	95	3
94	0.5	95	0.5
60	0.5	60	0.5
72	0.5	72	0.5
72	10	72	7
30 Cycles		35 Cycles	

A multiplex reaction was performed for *Sr26* with markers Sr26#43 and BE518379. The markers were tested on both the male and female populations. The reaction volumes and conditions for the multiplex reaction are shown in Table 3.3 and Table 3.4 respectively

3.3.2. Screening of crossing parents for wheat rust resistance genes in the MS-MARS population

Molecular markers for *Sr2*, *Sr24*, *Sr26* and *Lr34* were used to screen the male (recurrent wheat lines and donor lines carrying desired rust resistance genes) and female (1:1 segregating MS-MARS base population and the F_1 progeny) populations prior to cross-pollination. The markers form part of a standard panel of markers (Wessels & Botes, 2014) routinely used by the SU-PBL for the detection of respective genes and/or gene translocations in the MS-MARS population. The *Sr45* marker was used to screen the male population additionally as well as the F_1 female progeny.

PCR reactions were performed in multiplex, except for the CAPS (*csSr2*) and *Sr45* (cssu45) marker. All the PCR reactions were performed using a 2720 Thermal Cycler

(Applied Biosystems, Fairlands, RSA). The magnesium chloride was supplied by Bioline (distributed by Celtic Molecular Diagnostics (Edms) Bpk, Mowbray, RSA) and the Green Readymix by KapaBiosystems (distributed byLasec SA (Pty) Ltd, Cape Town, RSA).

The multiplex reaction contained 3.3 µl dH_2O, 12.5 µl 2X KAPA 2G Fast Multiplex mix, 0.5 µl of each of dint9-F primer, L34plus-R primer, SCS719-F primer, SCS719-R primer, Sr26#43-F primer, Sr26#43- R primer and 1.2 µl DNA (100ng/ µl).

Table 3.3. Molecular markers and primer sequences for the wheat rust resistance genes used in this study.

Rust resistance gene	Marker name	Primer sequences	Annealing temperatures	Expected fragment size in bp	References
Lr34	L34DINT9 L34PLUS	F: 5'- TTGATGAAACCAGTTTTTTTTCTA- 3' R: 5'- GCCATTTAACATAATCATGATGGA- 3'	57°C	517	Krattinger *et al.*, 2009
Sr2	Xgwm533	F: 5'- AAGGCGAATCAAACGGAATA- 3' R: 5'- GTTGCTTTAGGGGAAAAGCC- 3'	61°C	120	Spielmeyer *et al.*, 2003
	X3B028	F: 5'- ACGAACAAGGGGAAGACG- 3' R: 5'- TTTCGGTAGTTGGGGATGC-3'	61°C	243	McNeil *et al.*, 2008
	csSr2	F: 5'- CAAGGGTTGCTAGGATTGGAAAAC- 3' R: 5'- AGATAACTCTTATGATCTTACATTTTTCTG- 3'	60°C	53, 112, 172	Mago *et al.*, 2011
Sr24	SCS719	F: 5'- TCGTCCAGATCAGAATGTG- 3' R: 5'- CTCGTCGATTAGCAGTGAG- 3'	57°C	719	Cherukuri *et al*, 2003
Sr26	*Sr26*#43	F: 5'- AATCGTCCACATTGGCTTCT- 3' R: 5'- CGCAACAAAATCATGCACTA-3'	60°C	207	Mago *et al.*, 2005
	BE518379	F: 5'- AGCCGCGAAATCTACTTTGA- 3' R: 5'- TTAAACGGACAGAGCACACG- 3'	60°C	303	Liu *et al.*, 2010
Sr35	Xcfa2076	F: 5' - CGAAAAACCATGATCGACAG- 3' R: 5' - ACCTGTCCAGCTAGCCTCCA- 3'	60°C	Pos- 210 ; Neg- 200	Babiker *et al.*, 2009
	Xcfa2193	F: 5' - ACATGTGATGTGCGGTCATT- 3' R: 5' - TCCTCAGAACCCCATTCTTG- 3'	60°C	Pos- 200 , 210 ; Neg- 200	
	Xcfa2170	F: 5'-TGGCAAGTAACATGAACGGA- 3' R: 5'-ATGTCATTCATGTTGCCCT- 3'	60°C	Pos- 180 ;199 Neg- 190	Zhang *et al.*, 2010
Sr45	Xwmc222	F: 5'- AAAGGTGCGTTCATAGAAAATTAGA- 3' R: 5' - AGAGGTGTTTGAGACTAATTTGGTA- 3'	60°C		Sambasivam *et al.*, 2008
	Xcfa2158	F: 5'- TTTCGTCTTCAAAATGCACTG- 3' R: 5'- TGGTAGCTTACAAAGGTGCG- 3'	60°C	229	
	Xbarc229	F: 5'- GGCCGCTGGGGATTGCTATGAT- 3' R: 5'- TCGGGATAAGGCAGACCACAT- 3'	60°C		
	Cssu45	F: 5′- CGAGTTTCAATACTTCGCCC- 3′ R: 5′- GATTACTATGCAATAGGGCCC- 3′	60°C	Pos: 220	Periyannan *et al.*, 2014

The reaction conditions were as follow: 3 minutes at 94ºC, 30 cycles of 30 s at 94ºC, 30 s at 57ºC and 1 minute at 72ºC, and a final extension step of 10 minutes at 72ºC

Table 3.4. Multiplex PCR reaction volumes for markers *Lr34*, *Sr2* and *Sr26.*

Sr2		***Sr26***	
Reagents	**Volume**	**Reagents**	**Volume**
dH_2O	4.7 µl	dH_2O	1.7 µl
2X Green	10.0 µl	2X Green	8.5 µl
50 mM $MgCl_2$	0.5 µl	10 µM Sr26 #43-	0.6 µl
10 µM	0.75 µl	10 µM Sr26 #43-	0.6 µl
10 µM Xgwm533	0.75 µl	10 µM	1 µl
10 µM X3B028-	1.0 µl	10µM	1 µl
10 µM X3B028-	1.0 µl	50 mM $MgCl_2$	0.1 µl
DNA (100ng/ µl)	1.3 µl	DNA (100ng/µl)	1.5 µl
	20 µl		**15** µl

Table 3.5. Optimal PCR reaction conditions for markers *Lr34*, *Sr2* and *Sr26*.

Sr2		***Sr26***	
Temperature (°C)	**Duration (minutes)**	**Temperature (°C)**	**Duration (minutes)**
94	5	94	5
94	0.5	94	1
61	0.5	60	1
72	0.5	72	1
72	5	72	5
35 Cycles		30 Cycles	

The CAPS marker was used as final validation for the detection of stem rust resistance gene *Sr2*, after a first round of testing with markers *Xgwm533* and *X3B028* in a multiplex reaction (table 3.4 and 3.5). The PCR reaction was performed in reaction volume of 12.5µl containing 5.25 µ1 dH_2O, 6.25 µl 2X KAPA 2G Fast Multiplex mix, 0.25µl of each of cSSr2-F and cSSr2-R primer and 0.5 µl DNA (100 ng/ µl). The reaction conditions were as follow: 3 minutes at 95ºC, 40 cycles of 15 s at 95ºC, 15 s at 60ºC and 15 s at 72ºC, and a final extension step of 7 minutes at 72ºC. After amplification, 5 µl of each product was mixed with 3 µl Cressol loading dye and run on a 1.5% (w/v) agarose gel. The remaining 7.5 µl PCR product of samples showing a 337-bp band was digested with 10U *PagI* (*Bsp*HI) (Fermentas, Life Sciences) by adding 1 µl nuclease- free water, 1.25 µl 10X Buffer O and 0.25 µl restriction enzyme (*PagI*) to each PCR reaction tube. The PCR reaction tubes were then incubated at 37ºC for one hour and the CAPS products were electrophoresed on a 2% (w/ v)

agarose gel following visualization under UV- light by using the Uvitec gel imaging system (distributed by Whitehead Scientific Inc, Stikland, RSA).

3.3.3. Polyacrylamide gel- electrophoresis (PAGE)

Polyacrylamide gel-electrophoresis was performed in four steps: plate preparation, gel preparation, loading of samples and silver staining.

3.3.3.1. Plate preparation

A 125 µl of plate glue was diluted in 25 ml 100% ethanol. Following it 500 µl of the diluted plate glue was further diluted in 1500 µl 100% ethanol. A volume of 1740 µl of diluted plate glue was added to 140 µl 10% acetic acid in a 2 ml micro centrifuge tube. The long and short glass plates were first thoroughly cleaned with 100% ethanol after that the long glass plate was wiped with Wynn's C- thru and the short glass plate with plate glue. The long glass plate was allowed to dry for three minutes and the short plate for 30 sec. The plates were then assembled by placing 1 mm spacers between them. The bottom of both plates were levelled with one another and clamped to hold them in place.

3.3.3.2. Gel preparation

An acrylamide stock solution of 40% was made, consisting of 5.3 M acrylamide, 0.129 M bis-acrylamide and sufficient distilled water to a final volume of 200 ml. The stock solution was placed in a foil-covered flask. From the 40% acrylamide stock solution, a volume of 37.5 ml was used to prepared a 6% sequencing gel mix, containing 6 M urea and 50 ml 5X TBE. A 10% solution of ammonium persulfate (APS) was prepared by dissolving 0.1g of APS in 1 ml of distilled water. The gel solution was prepared by adding 800 µl APS solution and 160 µl N, N, N‟, N‟-Tetramethylethylenediamine (TEMED) to 160 ml leading to a 6% gel mix. The solution was mixed thoroughly and casted immediately thereafter.

3.3.3.3. Loading of samples

The PCR samples (microsatellite products) were mixed with 10 µl loading dye (98% formamide, 10 mM EDTA, pH 8.0, 0.05% (w/v) bromo phenol blue, and 0.05% (w/v) xylene cyanol FF). The samples were then denatured by incubating for five minutes at 95°C and immediately placed on ice prior to loading.

3.3.3.4. Silver staining

The fixing solution was prepared by adding 210 ml 100% ethanol to 1879.50 ml distilled water; 10.5 ml acetic acid was added to the solution just before use. The staining solution was prepared by dissolving 2.1g of silver nitrate ($AgNO_3$) in 2100ml distilled water. The developing solution was prepared by dissolving 31.5 g of sodium hydroxide (NaOH) in 2100 ml distilled water; 8.505 ml of formaldehyde was added to the developing solution just before use.

The spacers and the short plate were carefully removed, and the gel was placed in the fixing solution for 20 minutes on a shaker. The gel was then rinsed twice, for 5 minutes, in distilled water on a shaker. It was then placed in the staining solution for 20 minutes on the shaker. The gel was then rinsed with distilled water for 10 sec, and after that it was placed in the developing solution on the shaker until bands became observable. The gel was then rinsed in distilled water a final time.

3.3.4. Agarose gels

For the visualization of PCR products, three microliters of Cresolsulfonephthalein (Cresol red) were added to each PCR tube and loaded on agarose gels. Agarose gel electrophoresis was performed on 2%, 3% and 1.8% (w/ v) agarose gels in 1X TBE buffer [5X TBE stock solution: 0.5 M Tris (hydroxymetyl) Aminomethane, 0.5 m Boric acid, 0.5 M Ethylenediamine tetra acetic acid disodium salt dehydrate (EDTA)] at 120V for 45 minutes for the products of *Sr2* , *Sr45* and *Sr26*, *Sr24* and *Lr34* respectively. The PCR products were stained with Ethidium bromide for 10-15 minutes and visualised under UV- light, using the Uvitec gel imaging system (distributed by Whitehead Scientific Inc, Stikland, RSA).

3.4. Validation of MS-MARS scheme

3.4.1. Phenotypic validation of MS-MARS breeding scheme

In order to pyramid several wheat rust resistance genes, a breeding strategy based on male sterile mediated marker-assisted recurrent selection (MS-MARS) was followed and the degree of cross-pollination was evaluated.

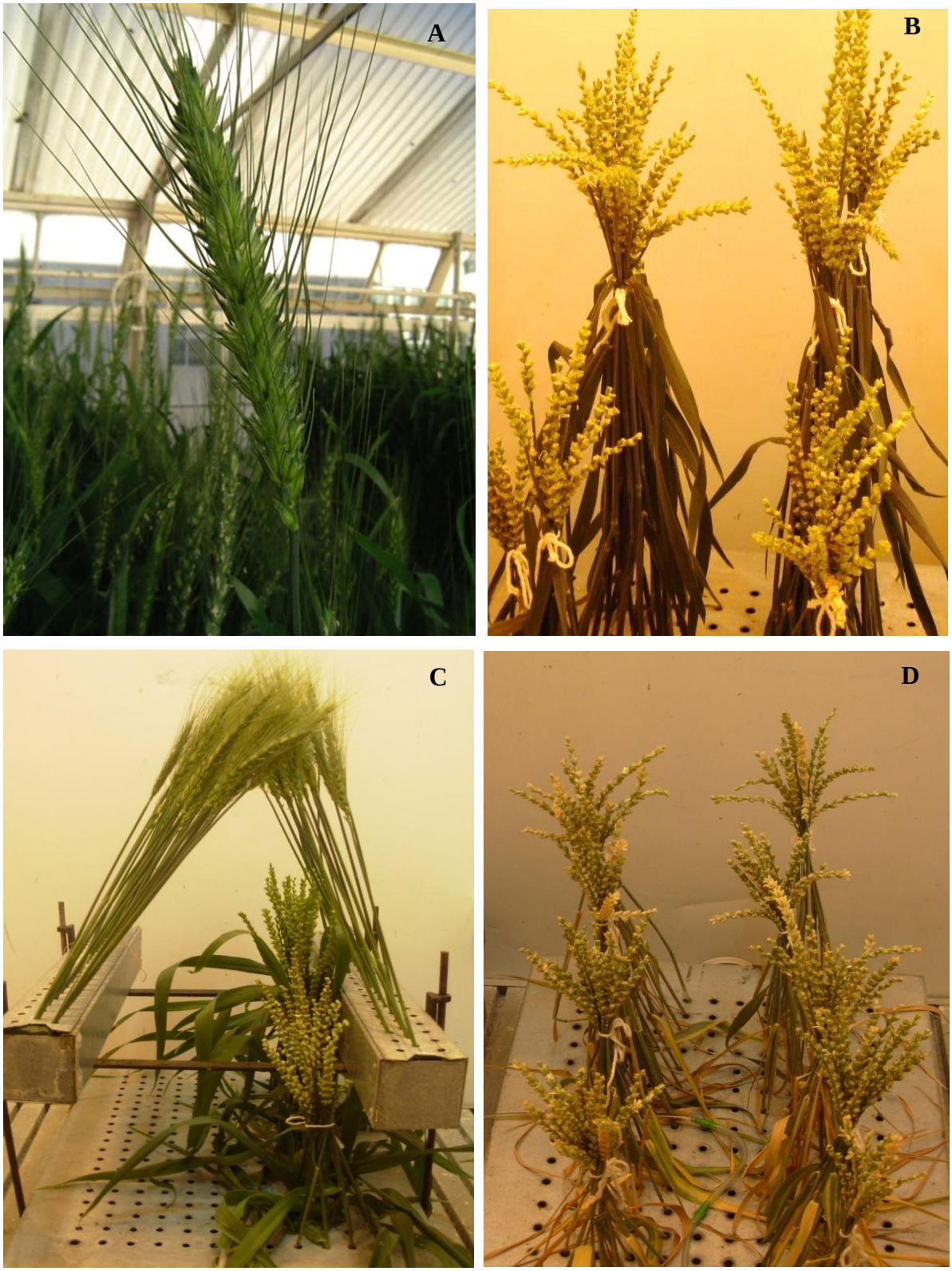

Figure 3.2. Cross-pollination in wheat utilizing male sterility. A) Male sterile ear with wide open glumes to enable cross- pollination. B) Male sterile wheat tillers cut open. C) Male fertile tillers arranged above the sterile tillers. D) Seed development on sterile tillers after cross- pollination.

Male sterile (female) plants (figure 3.1a) were carefully selected from the 1:1 male sterile/male fertile segregating population and one wheat tiller from each plant were cut at the flowering stage. Female tillers were then immediately placed in water, before being transferred to galvanized steel trays that were 600 mm x 450 mm x 160 mm in dimension and painted on the inside with black antifungal paint.

The flag leaf of each selected tiller was carefully kept intact, while the other leaves were removed. Florets on female tillers were cut open to maximise cross pollination (figure 3.1b). The tillers were then cut shorter in equal lengths and transferred to galvanized trays that can each contain 230 female spikes. The galvanized steel trays were then filled with a standard nutrient solution.

Male tillers were cut just before shedding pollen. The tillers were collected in buckets, filled with water and all the leaves were removed. Tillers were then arranged into two narrow galvanized steel trays. The trays were positioned approximately 600 mm above the female tillers on either side of the bottom tray and filled with nutrient solution (figure 3.1c). Male and female tillers were then allowed to pollinate for one week, after which the male tillers were discarded (figure 3.1d). Female tillers were trimmed and transferred to fresh nutrient solution every two weeks until ripening of seeds. After five to six weeks, when seeds were fully ripened, the female tillers were taken out of the trays and placed into brown paper bags. The bags were incubated at 21° C for one week allowing seeds to dry before threshing by hand occurred.

3.4.2. Development of a molecular marker for male sterility

3.4.2.1. Sequencing of probe WG341

A PCR based marker was designed from a RFLP probe, WG341, closely associated with the dominant male sterility gene *Ms3*. On two occasions the plasmid DNA probe was kindly provided by the Wheat Genetics Resource Center, Department of Plant Pathology, Plant Science Centre, Kansas State University, USA. One millilitre of the probe was inoculated in erlenmeyer flasks containing 150 ml Luria broth with 100mg/µl ampicillin. The flasks were incubated at 37°C overnight and lightly shaken at 180 rpm on a shaker. Plasmid extraction was carried out using the GeneJET ™ plasmid miniprep kit (Fermentas, Life Science).

Once the plasmid had been extracted and purified according to the manufactures protocol, a colony PCR was performed to confirm the plasmid containing the insert. A colony PCR was performed in a 20 µl reaction containing the following reagents with final concentrations: 2X Kapa Green mix, 0.25µM T7 primer, 0.25 µM Sp6 primer, 2.5mM $MgCl_2$ 3µl dH_2O and 1µl plasmid DNA. The cycling conditions were as follows: one cycle of 5 min at 94°C, 25 cycles of 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C followed by one cycle at 72 for 7 min. The PCR products were electrophoresed on a 1.5% agarose gel and visualised under ultra-violet light.

A restriction digestion reaction was also performed. The reaction contained 5µl plasmid DNA, 9µl dH_2O, 10X buffer (Bioline Once the insert was confirmed it was submitted for automated DNA sequencing at the Central Analytical Facility (CAF), University of Stellenbosch.

DNA sequences were analysed using Chromas (version 1.45) and BioEdit Sequence Alignment Editor (version 7.0.4 Copyright 1997-2005). Known sequences of sequencing primers T7 and Sp6 flanking the insert of interest, which is inserted in a pGEM4 vector, was used as reference for detecting the insert in the obtained sequences. Obtained sequences were aligned in BioEdit and nucleotide blast analyses were performed to identify regions of similarity between the obtained sequences and sequences in the NCBI sequence database.

In addition, molecular markers located on the same chromosome (5A) as the male sterility gene were selected and tested on the 1:1 MS-MARS segregating base population for the detection of unique polymorphisms between male sterile and male fertile lines. Marker analyses were performed with markers Xcfe34, Xcfe37 and Xcfe229 on 58 male sterile and 58 male fertile lines, randomly selected from the MS-MARS segregating population.

PCR reactions were performed in a 12.75µl reaction containing 5µl dH2O, 6.25µl 2X KAPA 2G Fast Multiplex mix, 0.25µl each of the forward and reverse primers (table 3.6) and 1µl DNA (100ng/µl). The reaction conditions were as follow: 3 minutes at 94ºC, 30 cycles of 0.5 minutes at 94ºC, 0.5 minutes at 57ºC and 1 minute at 72ºC, and a final extension step of 10 minutes at 72ºC.

DNA sequences were analysed using Chromas (version 1.45) and BioEdit Sequence Alignment Editor (version 7.0.4 Copyright 1997- 2005).

3.4.2.2. Chromosome marker analyses

Molecular markers located on the same chromosome (5A) as the male sterility gene were selected and tested on the MS-MARS segregating base population. Marker analyses were performed with markers Xcfe34, Xcfe37 and Xcfe229 on male sterile and male fertile plants to test for unique fragments.

PCR reactions were performed in a 12.75µl reaction containing 5µl dH2O, 6.25µl 2X KAPA 2G Fast Multiplex mix, 0.25µl each of the forward and reverse primers (table 3.6) and 1µl DNA (100ng/µl). The reaction conditions were as follow: 3 minutes at 94ºC, 30 cycles of 0.5 minutes at 94ºC, 0.5 minutes at 57ºC and 1 minute at 72ºC, and a final extension step of 10 minutes at 72ºC.

Table 3.6. Chromosome 5A-specific molecular markers.

Marker name	Primer sequences	Annealing temperatures
Xcfe34	F: TCCTCGTTCAACTACTGCGG	60˚C
	R: GGATGGATTGTAGATCATGCG	
Xcfe37	F:ACAGCAGGCATCCACTATACG	60˚C
	R: GCCTAGTTCGATGACAAGCA	
Xcfe229	F:TCACAGGGATGATGACGACGAT	60˚C
	R:GAGCGACGAGGAGCTGAG	

Chapter 4: Results and Discussion

4.1. Molecular screening of wheat lines

4.1.1. Marker identification and validation

Potentially useful markers closely associated with stem rust resistance genes *Sr26*, *Sr35* and *Sr45* were identified from literature. PCR tests were optimized and validated. Two markers were used for *Sr26*, three for *Sr35* and four for *Sr45*.

4.1.1.1. Markers for *Sr26*

A co-dominant marker was used for *Sr26* and PCR tests were performed on both male and female plant material. Marker Sr26#43, dominant for the presence of *Sr26* amplifies a 207 bp product, while marker *BE518379*, dominant for the absence of *Sr26* amplifies a 303 bp product. 'Eagle' was used as positive control.

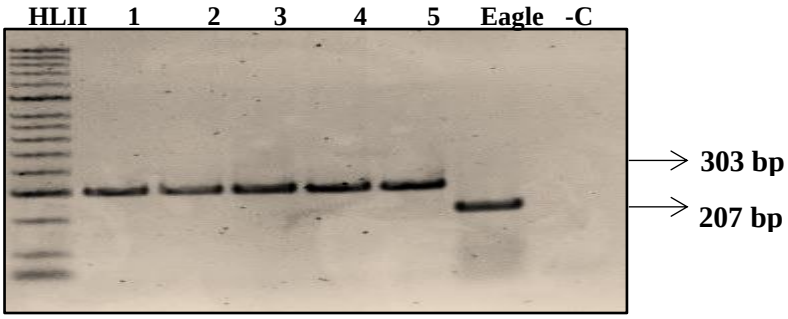

Figure 4.1. Optimization and validation of a co-dominant marker for *Sr26*. Lane 1: HyperladderII, Lanes 2-6: Plants tested for the presence of *Sr26*. Lane 7: 'Eagle' (positive control), Lane 8: Negative (dH_2O) control.

A 207 bp fragment was observed only in the positive control ('Eagle'), while fragments of 303 bp in size were observed in lanes 2-6, indicating the absence of *Sr26* in the tested plants (figure 4.1). No contamination was observed as no bands were visible in the negative (dH_2O) control. The marker successfully distinguished between resistant and non-resistant plants and can be used as a diagnostic co-dominant marker for the detection of *Sr26* as well as MAS.

4.1.1.2. Markers for *Sr35*

SSR markers Xcfa2076, Xcfa170 and Xcfa2193 were tested on male plants obtained from two donor lines (*Sr35*-A and *Sr35*-B) believed to carry *Sr35*. A *Sr35* carrying line (C35) was

included as a positive control (reference line for tested plants carrying *Sr35*) as well as non-*Sr35* control lines ('Chinese Spring' (CS), and two *Sr45* carrying lines (C3 and C6)).

4.1.1.2.1. Xcfa2076

PCR reactions performed with Xcfa2076 primer pair produced five fragments of 230 bp, 220 bp, 175 bp, 150 bp and 143 bp in size, in the positive control line (C35) (figure 4.2). The same banding patterns were observed in some of the plants tested for *Sr35*. However, in a similar study by Bernardo *et al.* (2013) two of the five fragments (150 bp and 143 bp) were observed in plants without *Sr35*. Also, no amplification was observed in some of their non-*Sr35* lines.

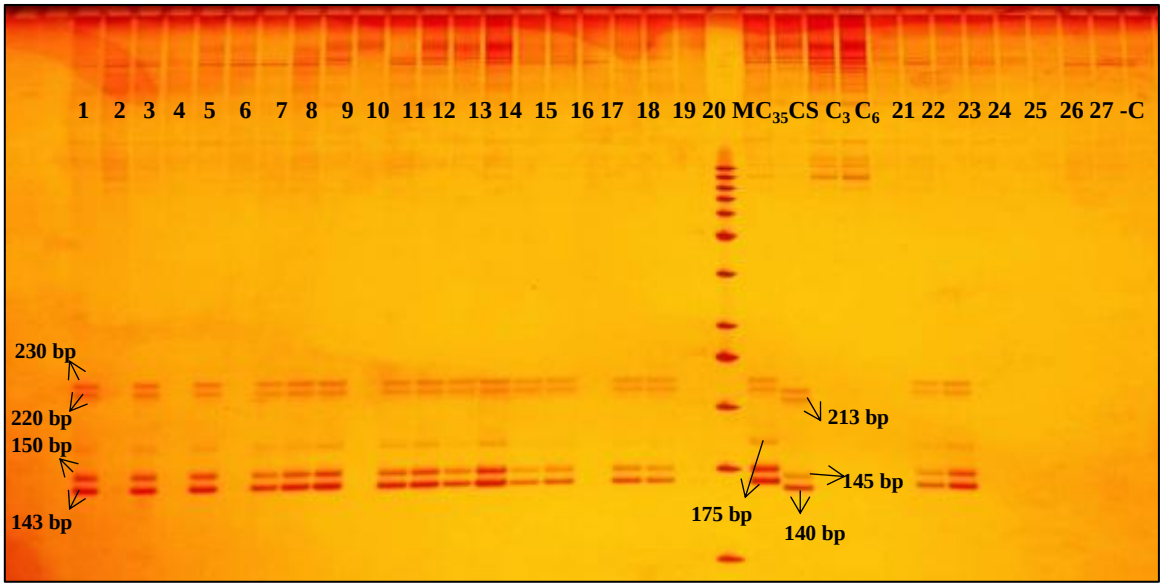

Figure 4.2. Marker analysis of *Xcfa2076*. Lanes 1-20: plants tested for the presence of *Sr35*. Lane 21: 50 bp marker. Lane 22: C35-Positive control (*Sr35*-carrying line). Lane 23-25: non-*Sr35* control lines. Lane 33: Negative (dH_2O) control.

This result was also observed in the non-*Sr35* control lines (lane 24-25) as well as some of the plants (lanes 2, 4, 6, 10, 17, 20, 25, 26 and 27) tested for *Sr35* in this study. Fragments of 220 bp, 213 bp, 145 bp and 140 bp in size were observed in another non-*Sr35* control line (CS). The 213 bp product corresponds to the non-*Sr35* associated fragment also observed by Bernardo *et al.* (2013). The 220 bp product was also observed in the *Sr35* positive control line (figure 4.2). However, none of the fragments produced by this marker corresponded to reported fragments associated with the presence of *Sr35*. Different fragment sizes have been observed for this marker in different genetic backgrounds (Bernardo *et al*., 2013; Babiker *et*

al., 2009; MASWHEAT). It has been reported that molecular markers identified in a particular mapping population may not always be effective in other genetic and breeding populations, as the genetic variation detected in a particular mapping population may not be shared by other breeding populations due to allelic diversity. Thus, it is difficult to conclude whether the plants tested in this study were carrying *Sr35*.

4.1.1.2.2. Xcfa2170

Despite several optimizing attempts, multiple poor amplicons were produced by marker Xcfa2170 (fig 4.3 b). Still, a clear fragment of 276 bp was observed in the *Sr35* positive control line (C35). This fragment was also observed with two very close fragments of 267 bp and 268 bp in the non-*Sr35* control line (CS) (fig 4.3a). Some of the tested plants showed both the 276 bp and 268 bp fragments, while other plants only showed the 276 bp fragment or the 267 and 268 bp fragments (Addendum A). However, these fragments were not of the expected sizes associated with the presence/ absence of *Sr35* as indicated by Haile *et al.* (2013) and Liu *et al.* (2014). Moreover, poor amplification was observed, especially in the 150– 200 bp region reported for the expected fragments (fig 4.3b). Although clear differences were observed between the positive and non-*Sr35* controls, more closely linked markers together with rust screening tests are required to accurately identify this gene amongst the plants as the marker produces different fragment sizes in different backgrounds.

4.1.1.2.3. Xcfa2193

Clear fragment sizes of 206 bp, 220 bp, 267 bp and 452 bp were observed in the positive control line (C35) (figure 4.3c). The same banding pattern was observed in the non-*Sr35* control line (CS) as well as in most of the plants tested for the presence of *Sr35*. Additional fragments observed in some of the tested lines was 210 bp, 230 bp, 369 bp and 385 bp in size. The 230 bp product associated with the absence of *Sr35*, as indicated by Zhang *et al.* (2010), was observed together with the 210 bp product associated with the presence of *Sr35* (Babiker *et al*., 2009) in some of the tested plants. However, the 210 bp and 230 bp products were absent in both the positive control (C35) and non-*Sr35* control line (CS). None of the other fragments produced by this marker corresponded to expected fragments associated with the presence/ absence of *Sr35*. Marker Xcfa2193 could not distinguished between *Sr35* carrying lines and non-*Sr35* control lines and are therefore not diagnostic for the detection of *Sr35* and MAS.

4.1.1.3. Markers for *Sr45*

Potential SSR markers Xwmc222, Xbarc229 and Xcfa2158 were tested on male plants obtained from two donor lines (*Sr45*a and *Sr45*b) believed to carry *Sr45*. A *Sr45* carrying line (C45) was included as a positive control (reference line for tested plants carrying *Sr45*) as well as non-*Sr45* carrying lines (Chinese Spring (CS), *Sr35* carrying line (C35) and Pavon (Pav)).

4.1.1.3.1. Xcfa2158

Multiple fragments were produced by marker xcfa2158. However, an expected fragment of 229 bp in size reported on the Graingenes website (http://wheat.pw.usda.gov/cgi-bin/graingenes/report.cgi?class=marker&name-Xcfa2158-1D), was observed in the non-*Sr45* carrying lines (CS and C35) and some of the plants tested for the presence of *Sr45*, but not in the positive control. A unique fragment of 206 bp (figure 4.4a) was continuously observed in the *Sr45* carrying line, but none of the plants tested showed the unique fragment. Fragments observed in the tested plants mostly corresponded to observed fragments in the non-*Sr45* control lines. Based on these results alone, it is difficult to predict the presence of the gene among the tested plants. More closely linked markers are required as marker xcfa2158 is still distant (3.3 cM) (Sambasivam *et al*., 2008)) from *Sr45*. Thus, the marker has reduced selection efficiency and may possibly indicate an association with the allele at the resistance gene, while in actual fact there is no association (producing false positives).

4.1.1.3.2. Xwmc222

Multiple fragments were observed in both the positive and non-*Sr45* control lines (figure 4.4b). However, a clear and distinct fragment of 156 bp in size was continuously observed in the positive control, but in none of the control lines or tested plants. The banding patterns observed in the tested plants mostly corresponded to the banding patterns observed in the non-*Sr45* carrying lines. The xwmc222 marker together with marker xcfa2158 forms part of a cluster of markers reported to possibly indicate low resolution; due to the small mapping population (Sambasivam *et al*., 2008). Therefore, more closely linked markers along with rust screening tests are required to ascertain the presence/ absence of *Sr45* amongst the tested plants.

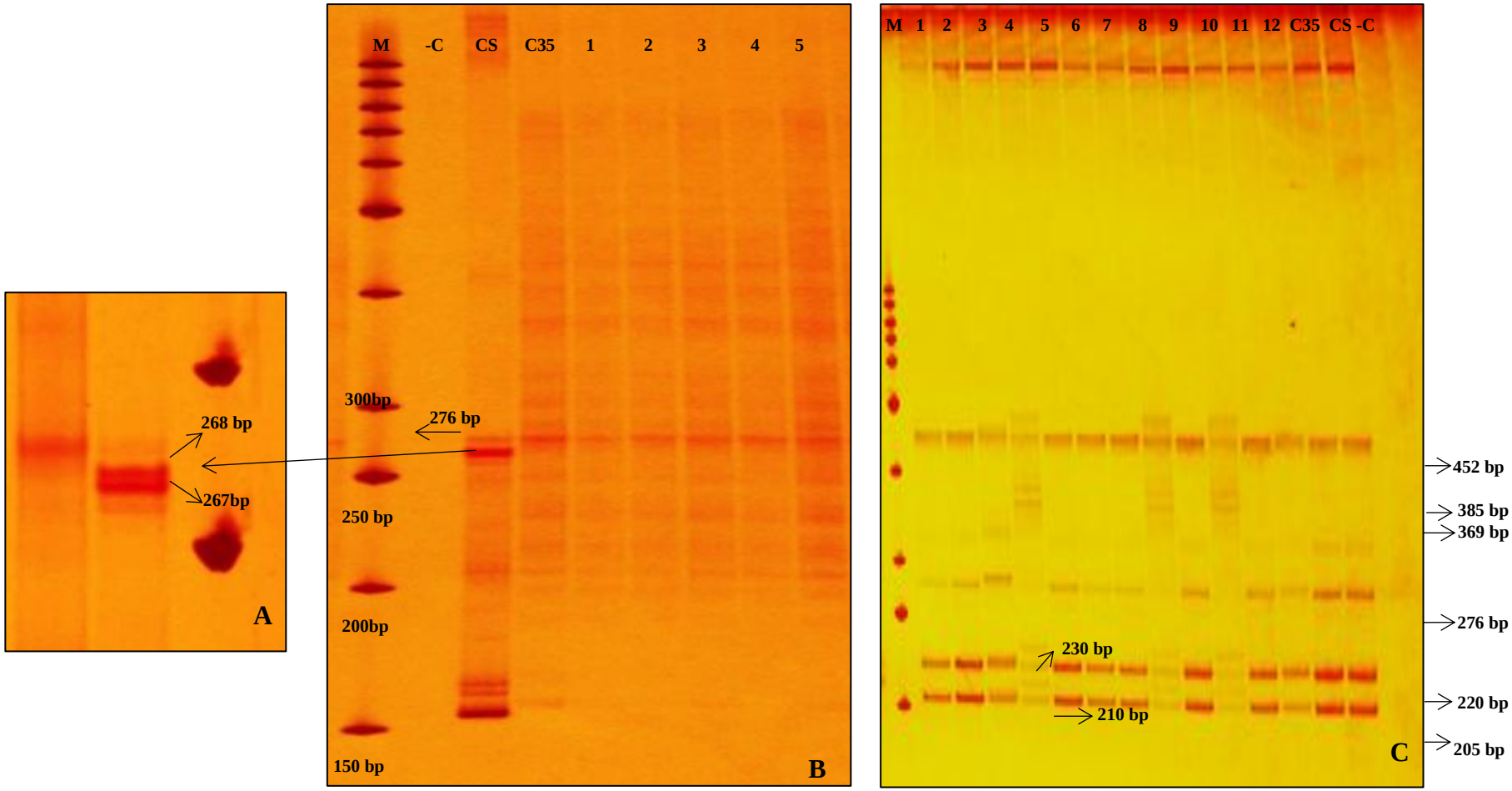

Figure 4.3. Marker analyses of Xcfa2170 and Xcfa2193 for the detection of *Sr35*. A)Enlargement of the two fragments produced by marker Xcfa2170 in CS. B)Fragments produced by marker Xcfa2170. Lane 1: 50 bp marker, lane 2: negative (dH_2O), lane 3: Chinese Spring (negative control), lane 4: *Sr35*-carrying line (positive control), lanes 5-9: Plants tested for the presence of *Sr35*.C)Fragments produced by marker Xcfa2193. Lane 1:50 bp marker, lanes 2-13: plants tested for the presence of *Sr35*,lane 14: *Sr35* carrying line (positive control), lane 15: Chinese Spring (negtive control line), lane 16: negative (dH_2O) control.

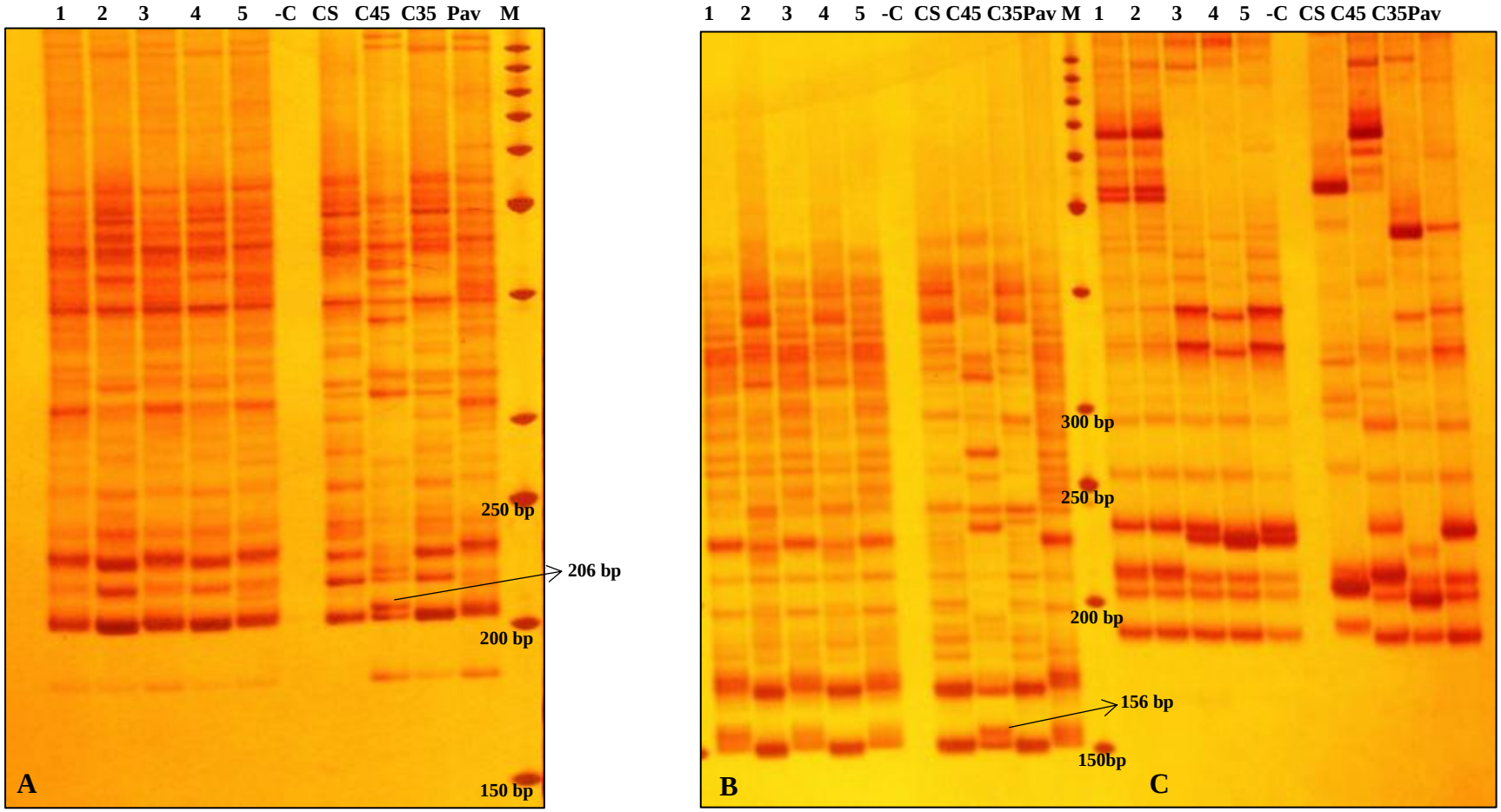

Figure 4.4. Marker analyses of A) Xcfa2158, B) Xwmc222 and C) Xbarc229 for the detection of *Sr45*.A)Lanes 1-5: Plants tested for the presence of *Sr45*, Lane 6: negative (dH_2O) control, lane 7: Chinese Spring; lane 8; *Sr45* carrying line (postive control), lane 9: *Sr35* carrying line (negative contol), lane 10: Pavon and lane 11: 50 bp marker. B)Lanes 1-5: Plants tested for the presence of *Sr45*, lane 6: negative (dH_2O) control,lane 7: Chinese Spring, lane 8: Pavon. C) Lanes 1-5: Plants tested for the presence of *Sr45*, lane 6: negative (dH_2O) control,lane 7: Chinese Spring, lane 8: Pavon. C) Lanes 1-5: Plants tested for the presence of *Sr45*, lane 6: negative (dH_2O) control,lane 7: Chinese Spring, lane 8: Pavon.

4.1.1.3.3. Xbarc229

Multiple fragments were observed in the positive and non-*Sr45* control lines for this marker (figure 4.4c). Fragments produced in the positive control line were also observed in the non-*Sr45* control lines. The marker could not clearly distinguish between the positive and negative plants and is therefore not diagnostic for the detection of *Sr45*.

4.1.1.3.4. Cssu45

A newly developed co-dominant PCR marker was tested as final validation for the detection of *Sr45* amongst the male plants. Amplification of *cssu45* produces a 220 bp fragment in backgrounds with *Sr45*, and a 238 bp fragment in backgrounds without *Sr45* (Periyannan *et al*., 2014).

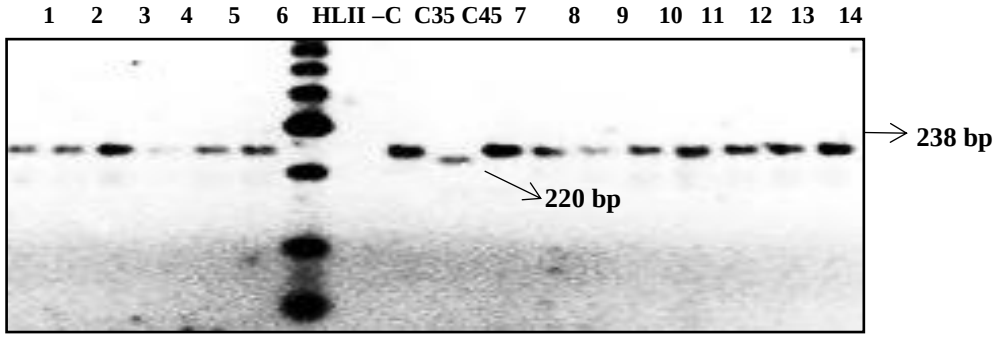

Figure 4.5.Marker cssu45 . Lanes 1- 6 and 11-18: Male plants tested for the presence of *Sr45*. Lane 8: Negative (dH_2O) control. Lane 9: Non-*Sr45* line. Lane 10: *Sr45*-carrying line.

A clear 220 bp product was observed only in the positive control (C45), while a 238 bp product was observed in the negative control (CS). The 238 bp product was also observed in plants tested for *Sr45*, indicating the absence of the gene amongst the plants. No contamination occurred as no fragments were visible in the negative (dH_2O) control. The cssu45 marker easily distinguished between positive and negative plants and is therefore diagnostic for the detection of *Sr45* as well as MAS.

4.1.2. Screening of crossing parents for wheat rust resistance genes in the MS-MARS population

Male and female populations were screened with a panel of molecular markers routinely used by the SU-PBL, together with closely linked markers for *Sr26*, *Sr35* and *Sr45*.

The male population was screened for *Lr34 Sr2*, *Sr24*, *Sr26*, *Sr35* and *Sr45* prior to cross-pollination. Gene frequencies of 23%, 87% and 66% were observed for *Sr2*, *Sr24* and *Lr34* prior to the first crossing cycle (figure 4.6). *Sr26* and *Sr45* were absent in the initial plant material. The presence of *Sr35* is unknown in the population due to the failure of linked markers to accurately identify the gene. Male plants used in the second crossing cycle showed gene frequencies of 33%, 87% and 56% for *Sr2*, *Sr24* and *Lr34* respectively. *Sr26* and *Sr45* were again not present in the population.

The female populations were screened for *Sr2*, *Sr24*, *Sr26* and *Lr34* prior to the first and second crossing cycles. The newly produced F_1 population was additionally screened for *Sr35* and *Sr45*. Gene frequencies observed in the base population for *Sr2*, *Sr24* and *Lr34* were 38%, 87% and 62% (figure 4.7). *Sr26* was not present in the female material screened before or after cross-pollination. After the first crossing cycle, *Sr45* was not present in the F_1 population, as expected, due to its absence in the male population. The absence of stem rust resistance genes *Sr26* and *Sr45*, after the first crossing cycle, can be explained by the fact that they are newly introduced genes in the MS-MARS breeding program and have not yet been established. A few recurrent selection cycles are thus needed to increase the frequencies of the genes in the breeding program. Marais & Botes (2009) reported that a minimum of four recurrent selection cycles are needed to relatively increase low gene frequencies (5%) to a high level of 70%. Thus, after four generations, the genes may be observed more frequently as the population will be improved and sustainable.

At the same time, it is important to take into account that the absence of genes in the population, shown by molecular marker analyses after the first crossing cycle, does not necessarily mean that the genes are totally absent in the population. Given that only 192 plants obtained from a total of 11 428 seeds produced after the first crossing cycle (table 4.1) were randomly selected for the detection of multiple genes, one has to bear in mind that the absence of the genes in the screened population is only true for less than 2% of the produced MS-MARS population. Thus, the desirable genes might be present in the untested 98% of the MS-MARS population.

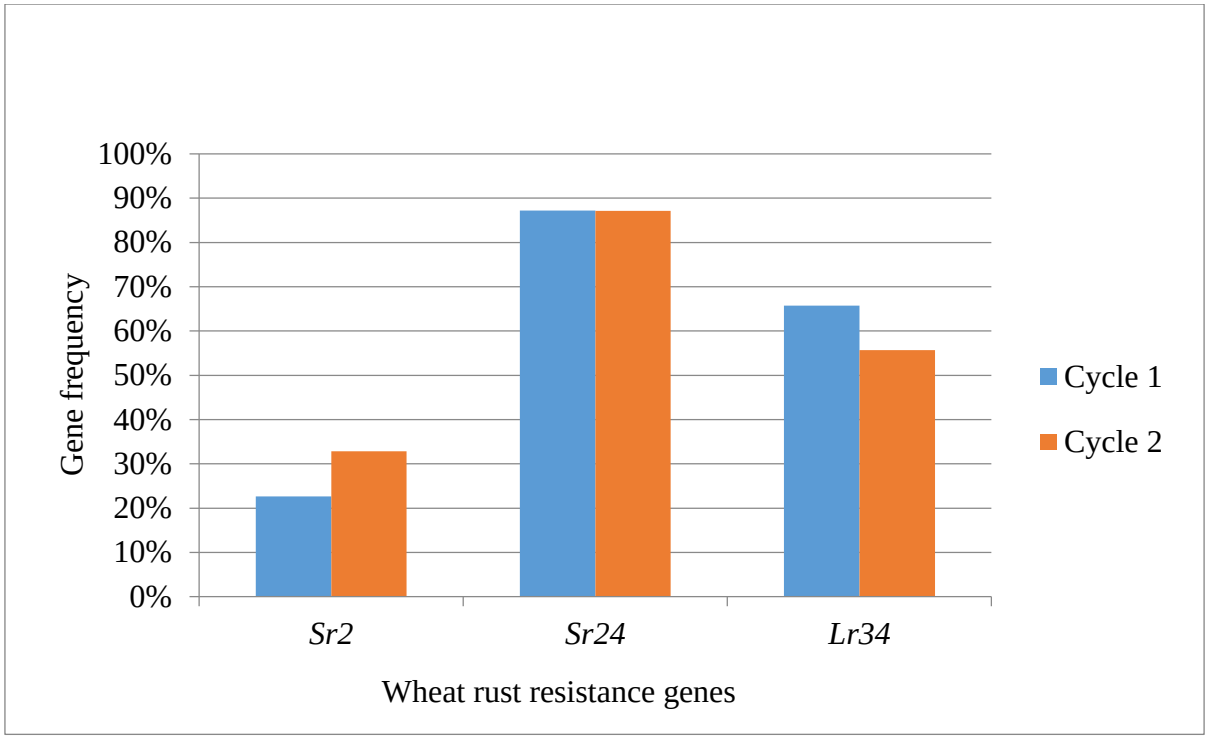

Figure 4.6.Wheat rust resistance gene frequencies in the male populations used in crossing cycles one and two.

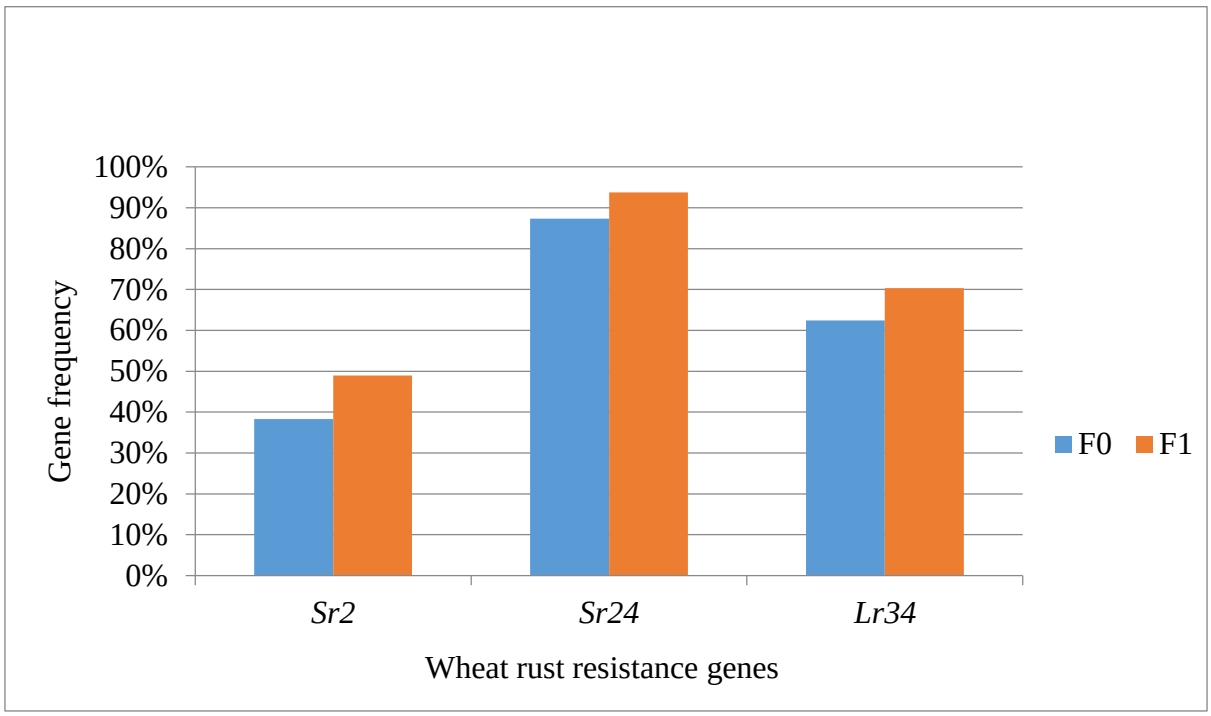

Figure 4.7. Wheat rust resistance gene frequencies present in the female populations before and after cross-pollination.

Increases of 11%, 7% and 8% in gene frequencies were observed for *Sr2*, *Sr24* and *Lr34* over one cycle respectively. The observed increases consequently showed the effectiveness of recurrent mass selection after just one crossing cycle. Marais & Botes (2009) illustrated gradual changes in the frequencies of desirable alleles selected in F_1 after several crossing cycles assuming dominance. Thus, further increases in gene frequencies can be expected in the new F_1 MS-MARS population, after the second crossing cycle. The gene frequency for *Sr2* especially may increase significantly, considering the increased frequency of *Sr2* in the male population used in the second crossing cycle (figure 4.6).

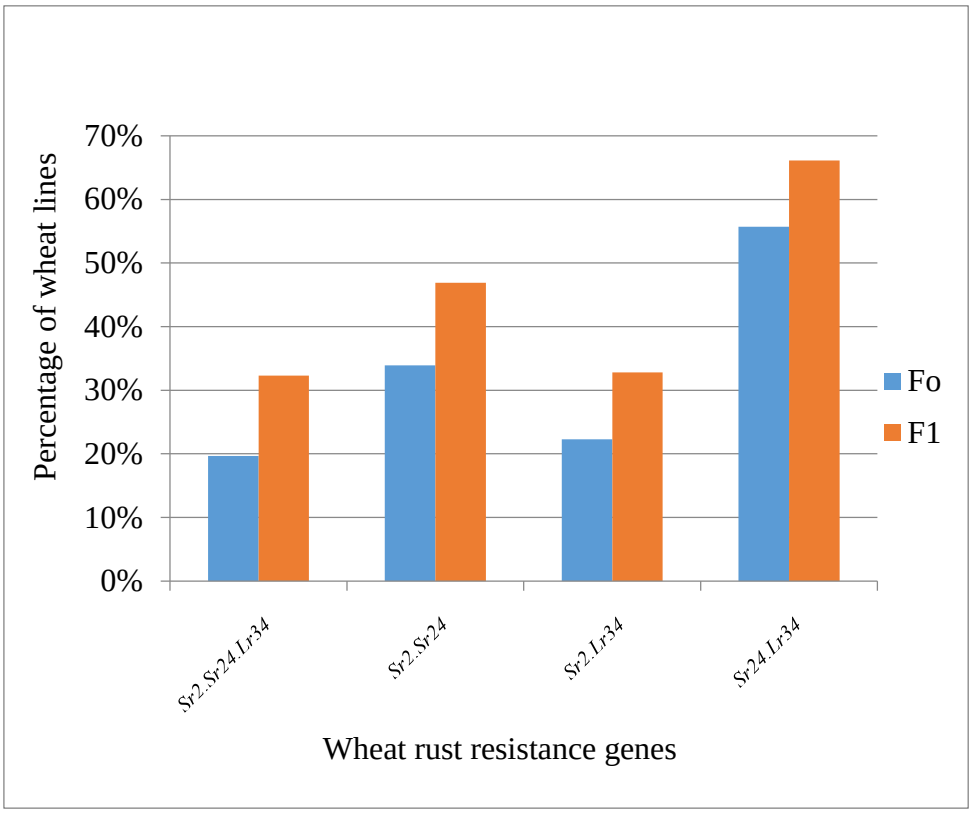

Figure 4.8.Wheat rust resistance gene combinations observed before and after pollination.

Significant increases in the percentage of wheat lines carrying more than one resistance gene were observed in the F_1 population due to high gene frequencies observed after the first crossing cycle. Thus pyramided genotypes in the base population (F_0) improved significantly after the first crossing cycle (figure 4.8). Although *Sr24* was reported ineffective in South Africa, it is reported to still provide adequate protection when used in combination with other stem rust resistance genes, since some of the stem rust pathotypes is still avirulent for *Sr24* (Pretorius *et al*., 2007). Therefore, lines containing high frequencies of *Sr24* in combination with other stem rust resistance genes, such as *Sr2* in this study can greatly contribute towards

resistance in the MS-MARS breeding program. Also lines containing *Sr2* and *Lr34* in combination with each other are favorable as it will increase broad spectrum resistance in the MS-MARS breeding program.

4.2. Validation of MS-MARS breeding scheme

4.2.1. Phenotypic validation

Careful selections based on distinct characteristics display by male sterile wheat plants were made prior to cross-pollination.

4.2.1.1. Recurrent cycle 1-2013

The female (male sterile) population consisted of seeds obtained from a segregating base population established in a MS-MARS breeding program in 2000 at the SU-PBL. Seeds were planted on five tables in a greenhouse. Four of the tables consisted of three rows each with eighteen pots per row and one table consisted of two rows with eighteen pots per row. Four to six seeds were planted per pot.

The male (fertile) population consisted of 100 recurrent lines obtained from the SU-PBL's 2013 wheat rust resistance nursery and six donor lines carrying desired resistance genes (*Sr2*/*24*, *Sr26*, *Sr35* and *Sr45*). A total of 216 and 36 pots were planted with recurrent and donor lines respectively.

After molecular screening of male and female plants for desired resistance genes, tillers were cut from both populations twice a week for seven consecutive weeks. Fourteen cutting sessions were successfully completed and the minimum-maximum amount of female and male tillers, selected during the seven weeks for mass crossings, varied from 80-193 and 93-177 respectively. A total of 868 male plants were used to facilitate cross-pollination on a total of 780 female tillers. After four harvesting periods, 11428 seeds were obtained from 86 % of the female plants selected for cross-pollination. An overall average of 58 % successful cross-pollination was obtained. The seeds were shriveled and small with an average of 13.6g. However, the germinating quality of the seeds was quite high when planted in the green-house.

Table 4.1. Recurrent selection cycle 1-2013

Week	Male fertile	Male sterile	Possible combinations	Harvest	Number of sterile plants sourced from	Total seeds/ harvest	Average % cross pollination	T.k.m (g)
1	114	93	10602					
2	121	146	17666	1	232	3927	55	18
3	142	169	23998					
4	177	112	19824	2	214	4052	61	14.4
5	99	93	9306	3	93	1448	60	13.2
6	122	87	10614					
7	93	80	7440	4	133	2001	54	8.8
	868	780	99450		672	11428	58	13.6

*T.k.m= Total kernel mass

Table 4.2. Recurrent selection cycle 2-2014

Week	Male fertile	Male sterile	Possible combinations	Harvest	Number of sterile plants sourced from	Total seeds/ harvest	Average % cross pollination	T.k.m (g)
1	140	135	18900	1	134	885	41	18.8
2	184	248	45632	2	211	1275	23	16.8
3	208	251	52208	3	243	2307	40	18
4	196	279	54684	4	271	2820	50	13.2
5	234	230	53820	5	204	1657	45	14.8
	962	1143	225244		1063	8944	40	16.3

4.2.1.2. Recurrent cycle 2- 2014

The female population consisted of F_1 seeds sourced from the female plants in the first MS-MARS cycle. Seeds were planted on four tables consisting of four rows each with 18 pots per row. Five seeds were planted per pot.

The male population consisted of 100 recurrent lines obtained from the SU-PBL's 2013 nursery and three donor lines containing resistance genes *Sr26, Sr35* and *Sr45*. Two separate tables consisting of 144 pots were planted with recurrent lines and donor lines each. Five seeds were planted per pot, but only 41 donor plants were obtained due to low germinating quality of the seeds.

Ten cutting sessions were successfully completed and the minimum-maximum amount of female and male tillers, selected during the five weeks for mass crossings, varied from 135-279 and 140-234 respectively. A total of 962 male plants were used to facilitate cross-pollination on a total of 1143 female tillers. After five harvesting periods, a total of 8944 seeds were obtained from 93 % of the female plants selected for cross-pollination. An overall average of 40 % cross-pollination was obtained. An average t.k.m of 16.3g was obtained.

Despite the increase in the amount of male sterile and fertile plants selected for cross-pollination in crossing cycle two, the average cross-pollination obtained in the first crossing cycle was almost 20 % higher than that in crossing cycle two. This can be explained by the ratio of male fertile to male sterile plants that were cut per week. A very low average percentage cross-pollination were obtained during week two in cycle two when fertile plants were pollinated on almost twice as much sterile plants. Thus, it is evident that the average percentage cross-pollination increase when more fertile plants are selected to facilitate cross-pollination on sterile plants (table 4.1 and table 4.2). At the same time, it is important to arrange the spikes of male plants not too high above the open cut sterile plants, to ensure that each sterile plant gets the chance to be pollinated.

During harvesting periods, it was observed that seed set on sterile plants with wide opened glumes were always much higher than on sterile plants with narrow glumes (figure 4.9). This might therefore be another reason for the low average percentage cross-pollination obtained in cycle two, as the structure of a sterile wheat plant are essential to maximize pollen reception (Whitford *et al*., 2013).

Figure 4.9. Wheat ears of male sterile plants. A) Florets with narrow glumes. B) Florets with wide open glumes.

Chi-square analyses were conducted to determine the inheritance of the male sterility gene in each of the two segregating populations (female populations) used in the two MS-MARS crossing cycles. Chi-square tests were conducted for each of the tables on which segregating populations were planted. All of the tables, consisting of segregating populations in both crossing cycles, showed good fit to ratio of 1 male fertile:1 male sterile (table 4.2 and 4.3) These results confirmed that male sterility is controlled by a single dominant gene in the heterozygous state in both populations used for cross-pollination.

Table 4.3. Inheritance of male sterility in the segregating base population.

Table number	Number of plants (Cycle 1)		X^2	Probability of fit to a 1:1 ratio
	Male sterile	Male fertile		
1	97	103	0.18	0.67
2	78	86	0.39	0.53
3	77	79	0.03	0.87
4	65	89	3.74	0.05
5	48	40	0.73	0.39
Overall	365	397	1.34	0.25

Table 4.4. Inheritance of male sterility in the F_1-segregating population.

Table	Number of plants		X^2	Probability of fit to a 1:1
	Male	Male		
1	109	121	0.80	0.370
2	108	118	0.44	0.506
3	110	126	0.72	0.396
4	121	139	1.18	0.278
Overall	448	504	3.29	0.070

4.3.2 Developent of a molecular marker for male sterility

The availability of a molecular marker to easily distinguish between male sterile and male fertile plants would result in more efficient recurrent selection cycles, within the MS-MARS breeding program. Therefore, a PCR based marker was developed from an RFLP probe marker, WG341, closely associated with the male sterility gene.

The DNA sequence of the RFLP probe was however not available in literature and plasmid extracion was performed in order to obtain the sequence. A colony PCR test performed with plasmid DNA showed a 600 bp poduct. This result was very promising as the expected insert size was 500 bp. Restriction digestion of the DNA (figure 4.10) then confirmed the correct insert size reported on the Graingenes database (http://wheat.pw.usda.gov/cgi-bin/graingenes/report.cgi?class=probe;name=WG341). However, sequence analyses of the submitted clones showed near complete homology with the pGEM4-vector and the insert was not found in the sequence. This indicates that the plasmids obtained from Kansas State University were empty and contained no insert of interest.

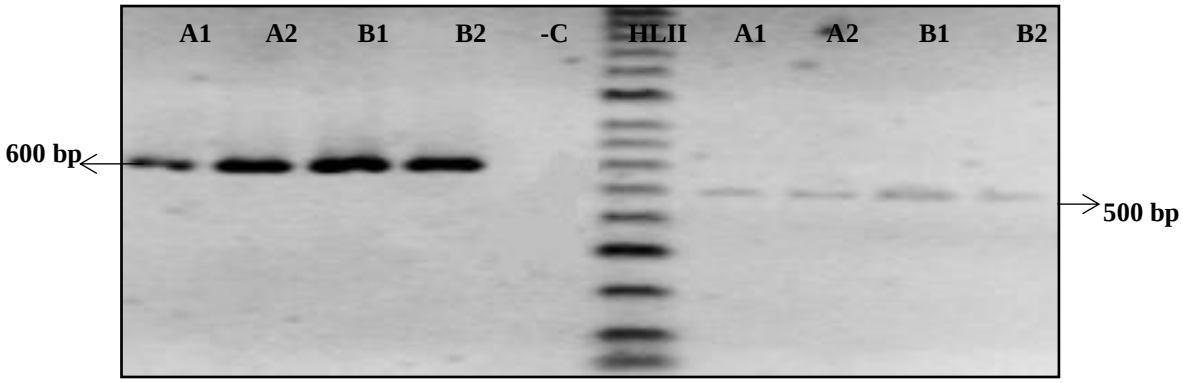

Figure 4.10. Colony PCR and restriction digest tests showing fragments of 600 bp and 500 bp in two clones A and B performed in duplicate (A1/A2 and B1/B2).

Additional tests performed with chromosome 5A-specific markers showed no unique polymorphisms between the sterile and fertile plants. Marker cfe34 was the only marker found to be polymorphic, as the other two markers (cfe229 and cfe37) produced monomorphic bands. However, the polymorphic fragments produced by marker cfe34 were not unique to the sterile or the fertile plants (Addendum E). As these markers were randomly selected with no association between the markers and the *Ms3* gene it is highly possible that no unique bands were detected.

Chapter 5: Conclusions

Molecular markers closely associated with stem rust resistance genes *Sr26*, *Sr35* and *Sr45* were identified in literature and tested on rust resistance donor lines, which formed part of the male population. Two co-dominant markers, one for *Sr26* (Sr26#43/BE518379) and one for *Sr45* (cssu45) was validated on the *Sr26* and *Sr45* resistance donor lines. The markers were able to successfully discriminate between homozygotes and heterozygotes. In contrast, SSR markers associated with *Sr35* and *Sr45* could not be validated on the *Sr35* and *Sr45* resistance donor lines as the markers produced fragments that differed from the reported fragment sizes, associated with the presence/absence of the respective genes, in several published articles. In most cases the SSR markers were unable to discriminate between positive and negative plants and consequently failed to accurately detect the respective stem rust resistance genes amongst the tested plants. These results can be explained by the proximity of the markers to the genes as well as the specific populations used to map the markers.

Marker Sr26#4, dominant for the presence of *Sr26*, was successfully used in a multiplex reaction with markers routinely used by the SU-PBL for the detection of stem, leaf and stripe rust resistance genes. The multiplex reaction was used to screen the MS-MARS segregating populations (female F_0 and F_1 population) as well as the male populations for the presence of *Sr2*, *Sr26*, *Lr24*/*Sr24* and *Lr34*/*Yr18* prior to the first and second crossing cycle. The male population was additionally screened with the validated co-dominant marker, cssu45, for the detection of *Sr45*. Stem rust resistance genes *Sr26* and *Sr45* were absent in both the male and female populations used in the first and second crossing cycle. The *Sr24* and *Lr34* genes were present in high frequencies in both the male and female populations prior to the first crossing cycle. Significant increases in gene frequencies were observed after the first crossing cycle and prior to the second crossing cycle in the female population. Also increases of more than 10% were observed in the percentage of wheat lines containing combinations of resistance genes, indicating the effectiveness of the recurrent selection breeding strategy to increase the frequencies of desirable genes in a pre-breeding population.

Male sterile (female) plants were effectively selected from the 1:1 MS-MARS segregating populations prior to the first and second crossing cycle. An overall average of almost 60% success of crosses was made in the first crossing cycle and a large number of hybrid seeds were produced. Although the average t.k.m was relatively low, the germinating quality of the seeds was very high and therefore supports the finding made by Botes (2001) in a similar

study. The overall average percentage cross- pollination obtained in the second crossing cycle was much lower than in the first crossing cycle. This is most probably due to the number of male fertile plants used in relation to the number of male sterile plants selected to facilitate cross-pollination as increases in the average percentage cross-pollination were observed when large numbers of male fertile plants in relation to male sterile plants were cut to facilitate cross-pollination during several cutting sessions. Other factors such as the height at which male fertile plants are arranged above the male sterile plants and the floral structure of sterile plants might also contribute to the low average percentage cross-pollination observed in the second crossing cycle.

An attempt to develop a molecular marker, based on sequence information of a RFLP probe closely associated with the dominant male sterility gene, *Ms3*, was made in order to accurately detect sterile plants from fertile plants in the MS-MARS breeding population and to validate phenotypic observations. The sequence of the RFLP probe was however not found in literature and wg341 plasmid DNA probes were obtained from Kansas State University. Plasmid extraction was performed followed by colony PCR and restriction digestion. A 500 bp product of the expected insert size was observed. However, the sequence of interest was not obtained and the development of a PCR based male sterility marker failed even after obtaining a second copy from Kansas State University.

Despite that, the study proved that cross-pollination between male sterile and male fertile plants can still effectively be achieved based on the distinct floral structure portray by the male sterile plants due to the dominant male sterility gene (*Ms3*). It will however require highly skilled labor in order to avoid incorrect selections of male sterile plants prior to pollination, as it might result in self-pollination. Molecular markers were also validated for stem rust resistance genes *Sr26* and *Sr45*. Although *Sr26* and *Sr45* were absent in the female population tested for the presence of the respective genes after the first crossing cycle, it is highly probable that the genes may be present in the larger MS-MARS population, as the absence of the genes was only detected in less than 2% sample size of the MS-MARS seeds produced after the first crossing cycle (table 4.1). Therefore, increase in the sample size of MS-MARS population is recommended for future studies of *Sr26* and *Sr45*. Increases in the percentage of existing gene pyramided lines were obtained. Increases observed in the frequency of APR genes such as *Sr2* and *Lr34* present in combination with other genes in the

MS-MARS population might add considerably to the durability of resistance in the pre-breeding population.

Stem rust resistance genes *Sr26* and *Sr45* are among a limited number of genes still effective against the Ug99 group of pathotypes therefore attempts to deploy them in the MS-MARS pre-breeding program should be intensified in the future, and the validated markers can then be used effectively to detect the respective genes in the SU-PBL's MS-MARS wheat pre-breeding program. Given that the *Sr35* resistance gene has been recently cloned, a sequence specific marker can be developed to accurately ascertain whether the particular gene was successfully introduced in the population. The marker will improve the selection efficiency of the gene in the SU-PBL's wheat pre-breeding program. Cross-pollination between male and female plants can be improved by specifically selecting male sterile plants with wide open glumes so that pollen reception by the stigmatic hair is maximized when the florets are cut open.

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Addendum A

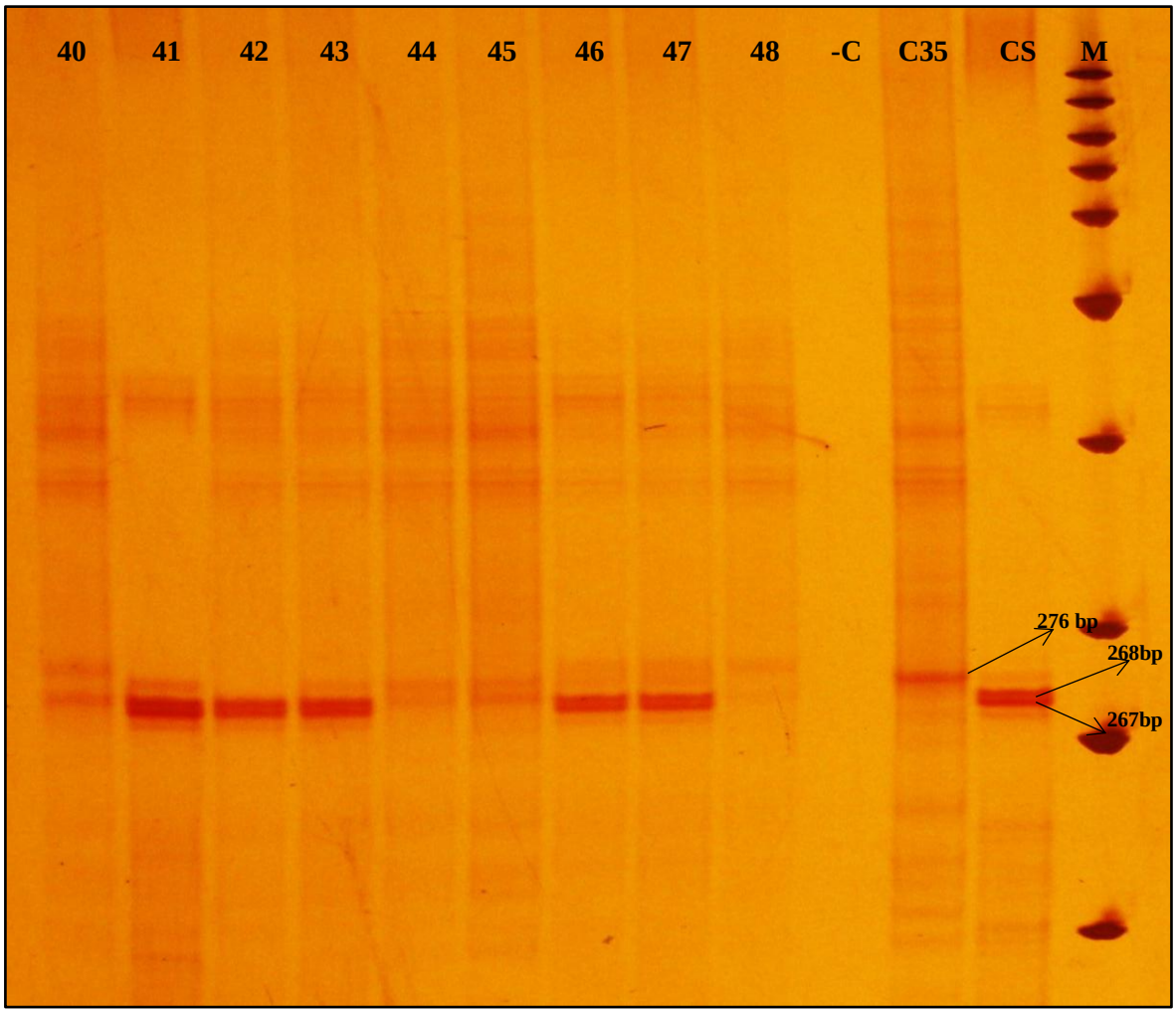

Figure A.1**:** Marker xcfa2170 discriminating between the plants. Lane 1-9: Plants tested with marker xcfa2170, Lane 10: Negative (dH_2O) control (-C), Lane 11: *Sr35*-carrying line (positive control), Lane 12: Non-*Sr35* line-'Chinese Spring', Lane 13: 50 bp marker.

Addendum B

Table B.1: Genes in genotypes of the male population prior to the first crossing cycle.

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
Sr2/24-1	0			0	0	2
Sr2/24-2	0			0	0	2
Sr2/24-3	0			0	0	2
Sr2/24-4	0			0	0	2
Sr2/24-5	0			0	0	2
Sr2/24-6	0			0	0	2
Sr2/24-7	0			0	0	2
Sr2/24-8	0			0	0	2
Sr2/24-9	0			0	0	2
Sr2/24-10	0			0	0	2
Sr26-1	0			0	0	2
Sr26-2	0		0	0	0	1
Sr26-3	0		0	0	0	1
Sr26-4	0		0	0	0	1
Sr26-5	0		0	0	0	1
Sr26-6	0			0	0	2
Sr26-7	0			0	0	2
Sr26-8	0			0	0	2
Sr26-9	0			0	0	2
Sr26-10	0			0	0	2
Sr26-11	0			0	0	2
Sr26-12	0			0	0	2
Sr35A-1	0			0	0	2
Sr35A-2	0			0	0	2
Sr35A-3	0			0	0	2
Sr35A-4	0			0	0	2
Sr35A-5	0			0	0	2
Sr35A-6	0			0	0	2
Sr35A-7	0			0	0	2
Sr35A-8	0			0	0	2
Sr35A-9	0			0	0	2
Sr35A-10	0			0	0	2
Sr35A-11	0			0	0	2
Sr35A-12	0			0	0	2
Sr35A-13	0			0	0	2

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
Sr35A-14	0			0	0	2
Sr35A-15	0			0	0	2
Sr35A-16	0			0	0	2
Sr35A-17	0			0	0	2
Sr35A-18	0			0	0	2
Sr35A-19	0			0	0	2
Sr35A-20	0	0	0	0	0	0
Sr35A-21	0	0	0	0	0	0
Sr35A-22	0			0	0	2
Sr45A-1	0		0	0	0	1
Sr45A-2	0		0	0	0	1
Sr45A-3	0	0	0	0	0	0
Sr45A-4	0	0	0	0	0	0
Sr45A-5	0		0	0	0	1
Sr45A-6	0		0	0	0	1
Sr45A-7	0		0	0	0	1
Sr45A-8	0			0	0	2
Sr45A-9	0		0	0	0	1
Sr45A-10	0			0	0	2
Sr45A-11	0			0	0	2
Sr45A-12	0			0	0	2
Sr45A-13	0			0	0	2
Sr45A-14	0			0	0	2
Sr45A-15	0			0	0	2
Sr45A-16	0			0	0	2
Sr45A-17	0			0	0	2
Sr45A-18	0			0	0	2
Sr45A-19	0			0	0	2
Sr45A-20	0			0	0	2
Sr45A-21	0			0	0	2
Sr2/24-11	0			0	0	2
Sr2/24-12	0			0	0	2
Sr26-13	0	0		0	0	1
Sr26-14	0			0	0	2
Sr26-15	0	0		0	0	1

Table B.1 Continued

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
Sr26-16	0	0		0	0	1
Sr26-17	0	0	0	0	0	0
Sr26-18	0	0		0	0	1
Sr26-19	0	0		0	0	1
Sr35A-23	0			0	0	2
Sr35A-24	0			0	0	2
Sr35A-25	0			0	0	2
Sr35A-26	0			0	0	2
Sr35A-27	0			0	0	2
Sr35A-28	0			0	0	2
Sr35A-29	0			0	0	2
Sr35A-30	0			0	0	2
Sr35A-31	0			0	0	2
Sr35A-32	0			0	0	2
Sr35A-33	0			0	0	2
Sr35A-34	0			0	0	2
Sr35A-35	0			0	0	2
Sr35A-36	0		0	0	0	1
Sr35A-37	0			0	0	2
Sr45A-22	0	0		0	0	1
Sr45A-23	0	0		0	0	1
Sr45A-24	0	0		0	0	1
Sr45A-25	0	0		0	0	1
Sr45A-26	0	0	0	0	0	0
Sr45A-27	0	0		0	0	1
Sr45A-28	0	0		0	0	1
Sr45A-29	0	0	0	0	0	0
Sr45A-30	0			0	0	2
Sr45A-31	0			0	0	2
Sr45A-32	0			0	0	2
Sr45A-33	0			0	0	2
Sr45A-34	0			0	0	2
Sr45A-35	0			0	0	2
Sr45A-36	0			0	0	2
Sr45A-37	0			0	0	2
Sr45A-38	0		0	0	0	1
Sr45A-39	0			0	0	2
Sr45A-40	0			0	0	2

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
Sr45A-41	0		0	0	0	1
Sr45A-42	0			0	0	2
Sr45A-43	0			0	0	2
Sr45A-44	0			0	0	2
Sr45A-45	0		0	0	0	1
Sr45A-46	0		0	0	0	1
Sr45A-47	0		0	0	0	1
Sr45A-48	0		0	0	0	1
Sr45A-49	0		0	0	0	1
Sr45A-50	0			0	0	2
Sr45A-51	0		0	0	0	1
Sr45A-52	0			0	0	2
Sr45A-53	0			0	0	2
Sr45A-54	0			0	0	2
Sr45A-55	0			0	0	2
Sr45A-56	0			0	0	2
Sr45A-57	0			0	0	2
Sr45A-58	0		0	0	0	1
Sr45A-59	0			0	0	2
Sr45A-60	0			0	0	2
Sr45A-61	0			0	0	2
Sr45A-62	0	0	0	0	0	0
Sr45A-63	0			0	0	2
Sr45A-64	0			0	0	2
Sr45A-65	0			0	0	2
Sr45A-66	0		0	0	0	1
Sr45A-67	0			0	0	2
Sr45A-68	0			0	0	2
Sr45A-69	0			0	0	2
Sr45A-70	0		0	0	0	1
Sr45A-71	0			0	0	2
Sr45A-72	0		0	0	0	1
Sr45A-73	0			0	0	2
Sr45A-74	0	0		0	0	1
Sr45A-75	0			0	0	2
Sr45A-76	0		0	0	0	1
Sr35-37	0			0	0	2
Sr35-38	0			0	0	2

Table B.1 Continued

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
Sr45A-59	0			0	0	2
Sr45A-60	0			0	0	2
Sr45A-61	0			0	0	2
Sr45A-62	0	0	0	0	0	0
Sr45A-63	0			0	0	2
Sr45A-64	0			0	0	2
Sr45A-65	0			0	0	2
Sr45A-66	0		0	0	0	1
Sr45A-67	0			0	0	2
Sr45A-68	0			0	0	2
Sr45A-69	0			0	0	2
Sr45A-70	0		0	0	0	1
Sr45A-71	0			0	0	2
Sr45A-72	0		0	0	0	1
Sr45A-73	0			0	0	2
Sr45A-74	0	0		0	0	1
Sr45A-75	0			0	0	2
Sr45A-76	0		0	0	0	1
Sr35-37	0			0	0	2
Sr35-38	0			0	0	2
Sr35-39	0			0	0	2
Sr35-40	0			0	0	2
Sr35-41	0			0	0	2
Sr35-42	0			0	0	2
Sr35-43	0			0	0	2
Sr35-44	0			0	0	2
Sr35-45	0			0	0	2
Sr35-46	0			0	0	2
Sr35-47	0			0	0	2
Sr35-48	0			0	0	2
Sr35-49	0			0	0	2
Sr35-50	0			0	0	2
Sr35-51	0			0	0	2
Sr35-52	0			0	0	2
Sr35-53	0			0	0	2
Sr35-54	0			0	0	2
Sr35-55	0			0	0	2
Sr35-56	0			0	0	2

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
Sr35-57	0			0	0	2
Sr35-58	0			0	0	2
Sr35-59	0			0	0	2
Sr35-60	0			0	0	2
US13001				0	0	3
US13002			0	0	0	2
US13003	0	0		0	0	1
US13004				0	0	3
US13005	0	0		0	0	1
US13006			0	0	0	2
US13007			0	0	0	2
US13008	0			0	0	2
US13009	0	0	0	0	0	0
US13010			0	0	0	2
US13011			0	0	0	2
US13012				0	0	3
US13013			0	0	0	2
US13014	0			0	0	2
US13015	0			0	0	2
US13016	0			0	0	2
US13017	0		0	0	0	1
US13018	0			0	0	2
US13019	0			0	0	2
US13020	0		0	0	0	1
US13021	0			0	0	2
US13022	0			0	0	2
US13023	0			0	0	2
US13024				0	0	3
US13025				0	0	3
US13026	0		0	0	0	1
US13027	0			0	0	2
US13028	0			0	0	2
US13029	0			0	0	2
US13030	0			0	0	2
US13031				0	0	3
US13032	0			0	0	2
US13033				0	0	3
US13034	0			0	0	2

Table B.1 Continued

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
US13035	0		0	0	0	1
US13036			0	0	0	2
US13037			0	0	0	1
US13038			0	0	0	2
US13039			0	0	0	1
US13040			0	0	0	2
US13041			0	0	0	2
US13042			0	0	0	1
US13043			0	0	0	1
US13044			0	0	0	1
US13045			0	0	0	2
US13046			0	0	0	1
US13047			0	0	0	1
US13048				0	0	3
US13049				0	0	2
US13050			0	0	0	1
US13051		0	0	0	0	1
US13052	0		0	0	0	1
US13053	0	0		0	0	1
US13054				0	0	3
US13055			0	0	0	2
US13056			0	0	0	2
US13057			0	0	0	2
US13058	0			0	0	2
US13059	0			0	0	2
US13060	0	0		0	0	1
US13061	0		0	0	0	1
US13062	0			0	0	2
US13063	0			0	0	2
US13064	0	0	0	0	0	0
US13065	0			0	0	2
US13066	0			0	0	2
US13067	0			0	0	2

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
US13068	0		0	0	0	0
US13069	0		0	0	0	1
US13070	0	0		0	0	1
US13071				0	0	3
US13072	0	0		0	0	1
US13073			0	0	0	2
US13074			0	0	0	2
US13075	0		0	0	0	1
US13076	0	0	0	0	0	0
US13077			0	0	0	2
US13078			0	0	0	2
US13079				0	0	3
US13080			0	0	0	2
US13081	0	0		0	0	1
US13082	0			0	0	2
US13083	0			0	0	2
US13084	0		0	0	0	1
US13085	0			0	0	2
US13086	0			0	0	2
US13087	0			0	0	2
US13088	0			0	0	2
US13089	0		0	0	0	1
US13090	0			0	0	2
US13091	0			0	0	2
US13092	0			0	0	2
US13093	0			0	0	2
US13094	0		0	0	0	1
US13095	0			0	0	2
US13096	0			0	0	2
US13097	0		0	0	0	1
US13098	0			0	0	2
US13099	0		0	0	0	1
US13100	0			0	0	2

Table B.2: Genes in genotypes of the female population prior to the first crossing cycle.

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-1	1	0		0	0	2
F_0-2	0			0	0	2
F_0-3			0	0	0	2
F_0-4	0		0	0	0	1
F_0-5		0		0	0	2
F_0-6		0	0	0	0	1
F_0-7	0			0	0	2
F_0-8	0			0	0	2
F_0-9				0	0	3
F_0-10				0	0	3
F_0-11	0			0	0	2
F_0-12				0	0	3
F_0-13	0	0		0	0	1
F_0-14	0			0	0	2
F_0-15	0			0	0	2
F_0-16	0			0	0	2
F_0-17	0	0		0	0	1
F_0-18				0	0	3
F_0-19	0			0	0	2
F_0-20				0	0	3
F_0-21	0	0		0	0	1
F_0-22	0	0		0	0	1
F_0-23		0		0	0	2
F_0-24	0	0		0	0	1
F_0-25	0	0		0	0	1
F_0-26	0	0		0	0	1
F_0-27	0			0	0	2
F_0-28				0	0	3
F_0-29	0			0	0	2
F_0-30				0	0	3
F_0-31				0	0	3
F_0-32	0			0	0	2
F_0-33		0		0	0	2
F_0-34	0			0	0	2
F_0-35		0		0	0	2
F_0-36		0		0	0	2
F_0-37	0	0		0	0	1
F_0-38	0			0	0	2

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-39	0			0	0	2
F_0-40	0			0	0	2
F_0-41		0		0	0	2
F_0-42	0			0	0	2
F_0-43	0	0	0	0	0	0
F_0-44				0	0	3
F_0-45	0			0	0	2
F_0-46				0	0	3
F_0-47	0		0	0	0	1
F_0-48	0	0		0	0	1
F_0-49	0			0	0	2
F_0-50			0	0	0	2
F_0-51	0	0		0	0	1
F_0-52	0	0		0	0	1
F_0-53		0		0	0	2
F_0-54	0	0	0	0	0	0
F_0-55	0	0	0	0	0	0
F_0-56	0		0	0	0	1
F_0-57	0	0		0	0	1
F_0-58				0	0	3
F_0-59			0	0	0	2
F_0-60	0	0		0	0	1
F_0-61	0	0	0	0	0	0
F_0-62		0		0	0	2
F_0-63		0		0	0	2
F_0-64	0	0		0	0	1
F_0-65	0			0	0	2
F_0-66				0	0	3
F_0-67	0	0		0	0	1
F_0-68	0	0		0	0	1
F_0-69	0	0		0	0	1
F_0-70		0		0	0	2
F_0-71	0			0	0	2
F_0-72	0			0	0	2
F_0-73	0			0	0	2
F_0-74				0	0	3
F_0-75		0		0	0	2
F_0-76		0		0	0	2

Table B.2 Continued

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-77	0	0		0	0	1
F_0-78		0		0	0	2
F_0-79				0	0	3
F_0-80	0			0	0	2
F_0-81				0	0	3
F_0-82				0	0	3
F_0-83		0		0	0	2
F_0-84				0	0	3
F_0-85	0			0	0	2
F_0-86		0		0	0	2
F_0-87	0	0		0	0	1
F_0-88		0		0	0	2
F_0-89			0	0	0	2
F_0-90	0			0	0	2
F_0-91	0		0	0	0	1
F_0-92	0		0	0	0	1
F_0-93	0			0	0	2
F_0-94		0		0	0	2
F_0-95	0	0		0	0	1
F_0-96	0	0		0	0	1
F_0-97	0			0	0	2
F_0-98	0			0	0	2
F_0-99	0		0	0	0	1
F_0-100				0	0	3
F_0-101	0			0	0	2
F_0-102				0	0	3
F_0-103				0	0	3
F_0-104	0	0		0	0	1
F_0-105	0		0	0	0	1
F_0-106				0	0	3
F_0-107	0			0	0	2
F_0-108	0			0	0	2
F_0-109	0			0	0	2
F_0-110		0		0	0	2
F_0-111	0			0	0	2
F_0-112				0	0	3
F_0-113	0	0		0	0	1

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-114				0	0	3
F_0-115	0	0		0	0	1
F_0-116	0			0	0	2
F_0-117	0			0	0	2
F_0-118	0	0		0	0	1
F_0-119	0	0		0	0	1
F_0-120		0		0	0	2
F_0-121		0		0	0	2
F_0-122		0		0	0	2
F_0-123		0		0	0	2
F_0-124		0		0	0	2
F_0-125	0			0	0	2
F_0-126	0			0	0	2
F_0-127		0		0	0	2
F_0-128		0		0	0	2
F_0-129	0	0		0	0	1
F_0-130	0	0		0	0	1
F_0-131				0	0	3
F_0-132				0	0	3
F_0-133				0	0	3
F_0-134	0			0	0	2
F_0-135	0			0	0	2
F_0-136				0	0	3
F_0-137	0			0	0	2
F_0-138	0	0		0	0	1
F_0-139				0	0	3
F_0-140			0	0	0	2
F_0-141				0	0	3
F_0-142	0			0	0	2
F_0-143	0			0	0	2
F_0-144	0			0	0	2
F_0-145	0	0		0	0	1
F_0-146	0	0	0	0	0	0
F_0-147		0	0	0	0	1
F_0-148			0	0	0	2
F_0-149	0	0	0	0	0	0
F_0-150		0		0	0	2

Table B.2 Continued

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-151				0	0	3
F_0-152		0		0	0	2
F_0-153	0			0	0	2
F_0-154				0	0	3
F_0-155	0			0	0	2
F_0-156	0	0		0	0	1
F_0-157	0	0		0	0	1
F_0-158	0			0	0	2
F_0-159		0		0	0	2
F_0-160	0	0	0	0	0	0
F_0-161		0		0	0	2
F_0-162	0			0	0	2
F_0-163			0	0	0	2
F_0-164	0			0	0	2
F_0-165				0	0	3
F_0-166	0			0	0	2
F_0-167	0			0	0	2
F_0-168	0			0	0	2
F_0-169	0			0	0	2
F_0-170	0		0	0	0	1
F_0-171	0	0		0	0	1
F_0-172		0		0	0	2
F_0-173				0	0	3
F_0-174				0	0	3
F_0-175	0	0		0	0	1
F_0-176				0	0	3
F_0-177				0	0	3
F_0-178	0			0	0	2
F_0-179	0			0	0	2
F_0-180	0	0		0	0	1
F_0-181		0		0	0	2
F_0-182	0			0	0	2
F_0-183	0		0	0	0	1
F_0-184			0	0	0	2
F_0-185	0			0	0	2
F_0-186	0			0	0	2
F_0-187	0			0	0	2

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-188	0			0	0	2
F_0-189	0			0	0	2
F_0-190	0			0	0	2
F_0-191		0		0	0	2
F_0-192	0	0		0	0	1
F_0-193	0			0	0	2
F_0-194		0		0	0	2
F_0-195		0	0	0	0	1
F_0-196				0	0	3
F_0-197				0	0	3
F_0-198	0	0		0	0	1
F_0-199	0		0	0	0	1
F_0-200	0			0	0	2
F_0-201				0	0	3
F_0-202	0			0	0	2
F_0-203	0			0	0	2
F_0-204	0	0	0	0	0	0
F_0-205	0			0	0	2
F_0-206		0	0	0	0	1
F_0-207	0			0	0	2
F_0-208	0			0	0	2
F_0-209		0		0	0	2
F_0-210	0			0	0	2
F_0-211	0			0	0	2
F_0-212	0			0	0	2
F_0-213	0	0		0	0	1
F_0-214		0		0	0	2
F_0-215	0	0		0	0	1
F_0-216	0			0	0	2
F_0-217	0	0		0	0	1
F_0-218				0	0	3
F_0-219		0		0	0	2
F_0-220				0	0	3
F_0-221	0			0	0	2
F_0-222				0	0	3
F_0-223	0			0	0	2
F_0-224				0	0	3

Table B.2 Continued

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-225	0			0	0	2
F_0-226				0	0	3
F_0-227	0			0	0	2
F_0-228	0		0	0	0	1
F_0-229	0			0	0	2
F_0-230	0	0		0	0	1
F_0-231		0		0	0	2
F_0-232		0		0	0	2
F_0-233	0	0		0	0	1
F_0-234	0			0	0	2
F_0-235				0	0	3
F_0-236	0	0		0	0	1
F_0-237	0			0	0	2
F_0-238	0	0		0	0	1
F_0-239	0			0	0	2
F_0-240	0			0	0	2
F_0-241	0	0		0	0	1
F_0-242	0			0	0	2
F_0-243				0	0	3
F_0-244	0	0		0	0	1
F_0-245		0		0	0	2
F_0-246	0			0	0	2
F_0-247	0	0	0	0	0	0
F_0-248	0	0		0	0	1
F_0-249				0	0	3
F_0-250		0		0	0	2
F_0-251	0			0	0	2
F_0-252	0			0	0	2
F_0-253	0			0	0	2
F_0-254		0		0	0	2
F_0-255				0	0	3
F_0-256				0	0	3
F_0-257				0	0	3
F_0-258	0	0		0	0	1
F_0-259	0			0	0	2
F_0-260	0	0		0	0	1
F_0-261	0			0	0	2
F_0-262			0	0	0	2
F_0-263		0		0	0	2

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-264				0	0	3
F_0-265				0	0	3
F_0-266		0	0	0	0	1
F_0-267		0	0	0	0	1
F_0-268	0	0		0	0	1
F_0-269	0			0	0	2
F_0-270				0	0	3
F_0-271	0	0		0	0	1
F_0-272	0			0	0	2
F_0-273	0	0		0	0	1
F_0-274				0	0	3
F_0-275		0		0	0	2
F_0-276				0	0	3
F_0-277	0			0	0	2
F_0-278	0			0	0	2
F_0-279				0	0	3
F_0-280	0			0	0	2
F_0-281		0		0	0	2
F_0-282	0			0	0	2
F_0-283	0	0		0	0	1
F_0-284	0			0	0	2
F_0-285	0			0	0	2
F_0-286	0			0	0	2
F_0-287				0	0	3
F_0-288				0	0	3
F_0-289				0	0	3
F_0-290		0		0	0	2
F_0-291	0	0		0	0	1
F_0-292	0			0	0	2
F_0-293	0			0	0	2
F_0-294				0	0	3
F_0-295	0	0		0	0	1
F_0-296	0	0		0	0	1
F_0-297	0			0	0	2
F_0-298	0	0		0	0	1
F_0-299	0	0	0	0	0	0
F_0-300	0	0		0	0	1
F_0-301	0			0	0	2
F_0-302	0			0	0	2

Table B.2 Continued

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-303	1	0		0	0	2
F_0-304	0	0		0	0	1
F_0-305				0	0	3
F_0-306	0			0	0	2
F_0-307	0			0	0	2
F_0-308		0		0	0	2
F_0-309	0		0	0	0	1
F_0-310	0			0	0	2
F_0-311	0			0	0	2
F_0-312	0	0		0	0	1
F_0-313				0	0	3
F_0-314	0			0	0	2
F_0-315	0	0		0	0	1
F_0-316	0			0	0	2
F_0-317		0		0	0	2
F_0-318				0	0	3
F_0-319	0			0	0	2
F_0-320		0		0	0	2
F_0-321	0	0	0	0	0	0
F_0-322				0	0	3
F_0-323	0	0		0	0	1
F_0-324				0	0	3
F_0-325	0	0	0	0	0	0
F_0-326			0	0	0	2
F_0-327	0			0	0	2
F_0-328	0			0	0	2
F_0-329	0		0	0	0	1
F_0-330		0		0	0	2
F_0-331				0	0	3
F_0-332	0			0	0	2
F_0-333	0			0	0	2
F_0-334	0			0	0	2
F_0-335				0	0	3
F_0-336	0			0	0	2
F_0-337	0			0	0	2
F_0-338	0	0		0	0	1
F_0-339		0		0	0	2
F_0-340		0		0	0	2
F_0-341		0		0	0	2

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-342				0	0	3
F_0-343	0	0		0	0	1
F_0-344	0			0	0	2
F_0-345	0			0	0	2
F_0-346				0	0	3
F_0-347	0	0	0	0	0	0
F_0-348	0	0	0	0	0	0
F_0-349		0		0	0	2
F_0-350	0	0	0	0	0	0
F_0-351				0	0	3
F_0-352	0		0	0	0	1
F_0-353	0			0	0	2
F_0-354				0	0	3
F_0-355	0	0		0	0	1
F_0-356	0			0	0	2
F_0-357	0	0		0	0	1
F_0-358	0			0	0	2
F_0-359	0	0		0	0	1
F_0-360		0	0	0	0	1
F_0-361	0			0	0	2
F_0-362				0	0	3
F_0-363	0			0	0	2
F_0-364	0			0	0	2
F_0-365	0	0	0	0	0	0
F_0-366	0			0	0	2
F_0-367	0			0	0	2
F_0-368	0			0	0	2
F_0-369	0		0	0	0	1
F_0-370	0			0	0	2
F_0-371	0			0	0	2
F_0-372	0			0	0	2
F_0-373	0			0	0	2
F_0-374	0			0	0	2
F_0-375				0	0	3
F_0-376	0		0	0	0	1
F_0-377	0			0	0	2
F_0-378	0			0	0	2
F_0-304	0			0	0	1
F_0-305	1			0	0	3

Table B.2 Continued

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-306	0			0	0	2
F_0-307	0			0	0	2
F_0-308		0		0	0	2
F_0-309	0		0	0	0	1
F_0-310	0			0	0	2
F_0-311	0			0	0	2
F_0-312	0	0		0	0	1
F_0-313				0	0	3
F_0-314	0			0	0	2
F_0-315	0	0		0	0	1
F_0-316	0			0	0	2
F_0-317		0		0	0	2
F_0-318				0	0	3
F_0-319	0			0	0	2
F_0-320		0		0	0	2
F_0-321	0	0	0	0	0	0
F_0-322				0	0	3
F_0-323	0	0		0	0	1
F_0-324				0	0	3
F_0-325	0	0	0	0	0	0
F_0-326			0	0	0	2
F_0-327	0			0	0	2
F_0-328	0			0	0	2
F_0-329	0		0	0	0	1
F_0-330		0		0	0	2
F_0-331				0	0	3
F_0-332	0			0	0	2
F_0-333	0			0	0	2
F_0-334	0			0	0	2
F_0-335				0	0	3
F_0-336	0			0	0	2
F_0-337	0			0	0	2
F_0-338	0	0		0	0	1
F_0-339		0		0	0	2
F_0-340		0		0	0	2
F_0-341		0		0	0	2
F_0-342		1		0	0	3

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-343	0	0		0	0	1
F_0-344	0			0	0	2
F_0-345	0			0	0	2
F_0-346				0	0	3
F_0-347	0	0	0	0	0	0
F_0-348	0	0	0	0	0	0
F_0-349		0		0	0	2
F_0-350	0	0	0	0	0	0
F_0-351				0	0	3
F_0-352	0		0	0	0	1
F_0-353	0			0	0	2
F_0-354				0	0	3
F_0-355	0	0		0	0	1
F_0-356	0			0	0	2
F_0-357	0	0		0	0	1
F_0-358	0			0	0	2
F_0-359	0	0		0	0	1
F_0-360		0	0	0	0	1
F_0-361	0			0	0	2
F_0-362				0	0	3
F_0-363	0			0	0	2
F_0-364	0			0	0	2
F_0-365	0	0	0	0	0	0
F_0-366	0			0	0	2
F_0-367	0			0	0	2
F_0-368	0			0	0	2
F_0-369	0		0	0	0	1
F_0-370	0			0	0	2
F_0-371	0			0	0	2
F_0-372	0			0	0	2
F_0-373	0			0	0	2
F_0-374	0			0	0	2
F_0-375				0	0	3
F_0-376	0		0	0	0	1
F_0-377	0			0	0	2
F_0-378	0			0	0	2
F_0-379	1			0	0	3

Table B.2 Continued

Female lines	*Sr2*	*Lr34*	*Sr24*	*Sr26*	*Sr45*	Total resistance genes/ line
F_0-380	0			0	0	2
F_0-381				0	0	3
F_0-382	0			0	0	2
F_0-383	0			0	0	2
F_0-384				0	0	3
F_0-385	0			0	0	2
F_0-386	0			0	0	2

Addendum C

Table C.1: Genes in genotypes of the male population prior to the second crossing cycle.

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
Sr26-1	0		0	0	0	1
Sr26-2	0		0	0	0	1
Sr26-3	0		0	0	0	1
Sr26-4	0		0	0	0	1
US13001				0	0	3
US13002			0	0	0	2
US13003	0	0		0	0	1
US13004				0	0	3
US13005	0	0		0	0	1
US13006			0	0	0	2
US13007			0	0	0	2
US13008	0			0	0	2
US13009	0	0	0	0	0	0
US13010			0	0	0	2
US13011			0	0	0	2
US13012				0	0	3
US13013			0	0	0	2
US13014	0			0	0	2
US13015	0			0	0	2
US13016	0			0	0	2
US13017	0		0	0	0	1
US13018	0			0	0	2
US13019	0			0	0	2
US13020	0		0	0	0	1
US13021	0			0	0	2
US13022	0			0	0	2
US13023	0			0	0	2
US13024				0	0	3
US13025				0	0	3
US13026	0		0	0	0	1
US13027	0			0	0	2
US13028	0			0	0	2
US13029	0			0	0	2
US13030	0			0	0	2
US13031				0	0	3
US13032	0			0	0	2
US13033	1			0	0	3

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
US13034	0			0	0	2
US13035	0		0	0	0	1
US13036			0	0	0	2
US13037			0	0	0	1
US13038			0	0	0	2
US13039			0	0	0	1
US13040			0	0	0	2
US13041			0	0	0	2
US13042			0	0	0	1
US13043			0	0	0	1
US13044			0	0	0	1
US13045			0	0	0	2
US13046			0	0	0	1
US13047			0	0	0	1
US13048				0	0	3
US13049				0	0	2
US13050			0	0	0	1
US13051		0	0	0	0	1
US13052	0		0	0	0	1
US13053	0	0		0	0	1
US13054				0	0	3
US13055			0	0	0	2
US13056			0	0	0	2
US13057			0	0	0	2
US13058	0			0	0	2
US13059	0			0	0	2
US13060	0	0		0	0	1
US13061	0		0	0	0	1
US13062	0			0	0	2
US13063	0			0	0	2
US13064	0	0	0	0	0	0
US13065	0			0	0	2
US13066	0			0	0	2
US13067	0			0	0	2
US13068	0	0	0	0	0	0
US13069	0		0	0	0	1
US13070	0	0		0	0	1

Table C.1 Continued

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
US13071				0	0	3
US13072	0	0		0	0	1
US13073			0	0	0	2
US13074			0	0	0	2
US13075	0		0	0	0	1
US13076	0	0	0	0	0	0
US13077			0	0	0	2
US13078			0	0	0	2
US13079				0	0	3
US13080			0	0	0	2
US13081	0	0		0	0	1
US13082	0			0	0	2
US13083	0			0	0	2
US13084	0		0	0	0	1
US13085	0			0	0	2
US13086	0			0	0	2
US13087	0			0	0	2
US13088	0			0	0	2
US13089	0		0	0	0	1
US13090	0			0	0	2
US13091	0			0	0	2
US13092	0			0	0	2
US13093	0			0	0	2
US13094	0		0	0	0	1
US13095	0			0	0	2
US13096	0			0	0	2
US13097	0		0	0	0	1
US13098	0			0	0	2
US13099	0		0	0	0	1
US13100	0			0	0	2
Sr26-7	0	0	0	0	0	0
Sr35-1	0	0	0	0	0	0
Sr35-2	0	0	0	0	0	0
Sr35-3	0			0	0	2

Male lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
Sr35-4	0		0	0	0	1
Sr35-5	0	0	0	0	0	0
Sr45-1	0			0	0	2
Sr45-2	0			0	0	2
Sr45-3	0			0	0	2
Sr45-4	0			0	0	2
Sr45-5	0			0	0	2
Sr45-6	0			0	0	2
Sr45-7	0			0	0	2
Sr45-8	0			0	0	2
Sr45-9	0			0	0	2
Sr45-10	0			0	0	2
Sr45-11	0			0	0	2
Sr45-12	0			0	0	2
Sr45-13	0		0	0	0	1
Sr45-14	0		0	0	0	1
Sr45-15	0			0	0	2
Sr45-16	0		0	0	0	1
Sr45-17	0			0	0	2
Sr45-18	0		0	0	0	1
Sr45-19	0		0	0	0	1
Sr45-20	0			0	0	2
Sr45-21	0			0	0	2
Sr45-22	0			0	0	2
Sr45-23	0			0	0	2
Sr45-24	0			0	0	2
Sr45-25	0		0	0	0	1
Sr45-26	0		0	0	0	1
Sr45-27	0			0	0	2
Sr45-28	0		0	0	0	1
Sr45-29	0			0	0	2
Sr45-30	0			0	0	2
Sr45-31	0			0	0	2
Sr45-32	0			0	0	2

Table C.2: Genes in genotypes of the female population prior to the second crossing cycle.

Female lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
F_1-1	0			0	0	2
F_1-2	0		0	0	0	1
F_1-3	0		0	0	0	1
F_1-4			0	0	0	2
F_1-5	0			0	0	2
F_1-6		0	0	0	0	1
F_1-7			0	0	0	2
F_1-8	0			0	0	2
F_1-9	0		0	0	0	1
F_1-10	0		0	0	0	1
F_1-11				0	0	3
F_1-12				0	0	3
F_1-13	0			0	0	2
F_1-14				0	0	3
F_1-15				0	0	3
F_1-16				0	0	3
F_1-17				0	0	3
F_1-18	0			0	0	2
F_1-19	0			0	0	2
F_1-20			0	0	0	2
F_1-21	0			0	0	2
F_1-22	0			0	0	2
F_1-23				0	0	3
F_1-24			0	0	0	2
F_1-25	0		0	0	0	1
F_1-26	0			0	0	2
F_1-27	0		0	0	0	1
F_1-28	0		0	0	0	1
F_1-29	0			0	0	2
F_1-30				0	0	3
F_1-31				0	0	3
F_1-32				0	0	3
F_1-33				0	0	3
F_1-34			0	0	0	2
F_1-35				0	0	3
F_1-36		0	0	0	0	1

Female lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
F_1-37	0		0	0	0	1
F_1-38	0	0		0	0	1
F_1-39				0	0	3
F_1-40	0	0	0	0	0	0
F_1-41		0		0	0	2
F_1-42				0	0	3
F_1-43				0	0	3
F_1-44	0			0	0	2
F_1-45	0			0	0	2
F_1-46	0			0	0	2
F_1-47	0			0	0	2
F_1-48	0		0	0	0	1
F_1-49	0	0		0	0	1
F_1-50	0	0		0	0	1
F_1-51				0	0	3
F_1-52	0		0	0	0	1
F_1-53	0	0		0	0	1
F_1-54	0			0	0	2
F_1-55	0			0	0	2
F_1-56	0			0	0	2
F_1-57	0			0	0	2
F_1-58	0			0	0	2
F_1-59				0	0	3
F_1-60			0	0	0	2
F_1-61			0	0	0	2
F_1-62				0	0	3
F_1-63				0	0	3
F_1-64	0			0	0	2
F_1-65				0	0	3
F_1-66			0	0	0	2
F_1-67	0			0	0	2
F_1-68	0		0	0	0	1
F_1-69	0	0		0	0	1
F_1-70				0	0	3
F_1-71	0			0	0	2
F_1-72				0	0	3

Table C.2 Continued

Female lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
F_1-73				0	0	3
F_1-74	0			0	0	2
F_1-75	0			0	0	2
F_1-76			0	0	0	2
F_1-77				0	0	3
F_1-78				0	0	3
F_1-79			0	0	0	2
F_1-80	0	0		0	0	1
F_1-81				0	0	3
F_1-82			0	0	0	2
F_1-83				0	0	3
F_1-84			0	0	0	2
F_1-85	0		0	0	0	1
F_1-86	0			0	0	2
F_1-87	0			0	0	2
F_1-88				0	0	3
F_1-89				0	0	3
F_1-90				0	0	3
F_1-91				0	0	3
F_1-92	0			0	0	2
F_1-93	0			0	0	2
F_1-94	0			0	0	2
F_1-95	0			0	0	2
F_1-96	0			0	0	2
F_1-97				0	0	3
F_1-98				0	0	3
F_1-99				0	0	3
F_1-100				0	0	3
F_1-101				0	0	3
F_1-102	0		0	0	0	1
F_1-103				0	0	3
F_1-104	0			0	0	2
F_1-105	0			0	0	2
F_1-106			0	0	0	2
F_1-107	0			0	0	2
F_1-108	0			0	0	2
F_1-109	0			0	0	2
F_1-110	0			0	0	2
F_1-111				0	0	3

Female lines	*Sr2*	*Sr24*	*Lr34*	*Sr26*	*Sr45*	Total resistance genes/ line
F_1-112				0	0	3
F_1-113				0	0	3
F_1-114	0		0	0	0	1
F_1-115				0	0	3
F_1-116	0			0	0	2
F_1-117				0	0	3
F_1-118	0			0	0	2
F_1-119				0	0	3
F_1-120			0	0	0	2
F_1-121	0			0	0	2
F_1-122	0			0	0	2
F_1-123	0			0	0	2
F_1-124				0	0	3
F_1-125			0	0	0	2
F_1-126			0	0	0	2
F_1-127				0	0	3
F_1-128				0	0	3
F_1-129			0	0	0	2
F_1-130				0	0	3
F_1-131			0	0	0	2
F_1-132	0			0	0	2
F_1-133	0			0	0	2
F_1-134				0	0	3
F_1-135	0			0	0	2
F_1-136	0			0	0	2
F_1-137			0	0	0	2
F_1-138			0	0	0	2
F_1-139	0			0	0	2
F_1-140	0		0	0	0	1
F_1-141	0			0	0	2
F_1-142				0	0	3
F_1-143				0	0	3
F_1-144	0			0	0	2
F_1-145	0			0	0	2
F_1-146	0		0	0	0	1
F_1-147	0			0	0	2
F_1-148	0		0	0	0	1
F_1-149	0			0	0	2
F_1-150	0			0	0	2

Table C.2 Continued

F_1-151			0	0	0	2
F_1-152			0	0	0	2
F_1-153				0	0	3
F_1-154	0			0	0	2
F_1-155	0		0	0	0	1
F_1-156				0	0	3
F_1-157	0			0	0	2
F_1-158				0	0	3
F_1-159	0			0	0	2
F_1-160	0			0	0	2
F_1-161	0			0	0	2
F_1-162	0		0	0	0	1
F_1-163	0			0	0	2
F_1-164	0		0	0	0	1
F_1-165				0	0	3
F_1-166	0			0	0	2
F_1-167	0			0	0	2
F_1-168	0	0		0	0	1
F_1-169				0	0	3
F_1-170	0			0	0	2
F_1-171	0			0	0	2
F_1-172			0	0	0	2
F_1-173			0	0	0	2
F_1-174			0	0	0	2
F_1-175				0	0	3
F_1-176				0	0	3
F_1-177	0		0	0	0	1
F_1-178	0		0	0	0	1
F_1-179	0		0	0	0	1
F_1-180				0	0	3
F_1-181	0		0	0	0	1
F_1-182			0	0	0	2
F_1-183				0	0	3
F_1-184				0	0	3
F_1-185	0		0	0	0	1
F_1-186				0	0	3
F_1-187	0			0	0	2
F_1-188		0	0	0	0	1
F_1-189			0	0	0	2
F_1-190			0	0	0	2
F_1-191				0	0	3
F_1-192	0			0	0	2

Addendum D

Figure D.1: Sequences of the submitted plasmid DNA obtained from both the T7 and Sp6 primer sites.

Clone A-Sp6

AAAAWMARRCYGGGTWCCGGGGATCCTCTAGAGTCGACCTGCAGAGTAATAGTTGTAGTACAGAAAAAGTGCCCATGAT
ACCGTTGGATGCAGCCTGTGAGCTGTGCTCTGCCCCAATGCTTAGGGTAAATCTATCATGTTTTTCATGTTCTGTTTGGATG
TTCTTTGCAAATTAAGATGATTTCTTCCTGTTATGTGTCGTACCATTTTCTCAATGTGGTTTTAACAATCGTTGGGTTTCTAA
CTGAGACAAAAGTTCAGGTGAGAACTTTAGAATAATCCCAAGTTCAGGTAAGCACTCCGGGAGCTCGCCCACATCCAAAT
GGTATGTGCAAAACCTCTTATTCGCACTGCTCCTGGAGTTATAGATAGCATACACAACATGAGATTTTGTAACAACGTTCTA
AAATTTGCAGTGAATGACACAGGAGATTAAGATATGGCATGCAGTTGAGATTATAAGAGTTTGTCACACTAAACAAATTTA
CTAATGCTTAGTGAATTTCCCATGTATTGATTTTTTGTGTGACCTGCAGGCATGCAAGCTTGTCTCCCTATAGTGAGTCGTAT
TA

Clone A-T7

ARRMMWGAMGCCTGCAGTCACACAAAAATCAATACATGGGAAATTCACTAAGCATTAGTAAATTTGTTTAGTGTGACAA
ACTCTTATAATCTCAACTGCATGCCATATCTTAATCTCCTGTGTCATTCACTGCAAATTTTAGAACGTTGTTACAAAATCTCA
TGTTGTGTATGCTATCTATAACTCCAGGAGCAGTGCGAATAAGAGGTTTTGCACATACCATTTGGATGTGGGCGAGCTCCC
GGAGTGCTTACCTGAACTTGGGATTATTCTAAAGTTCTCACCTGAACTTTTGTCTCAGTTAGAAACCCAACGATTGTTAAAA
CCACATTGAGAAAATGGTACGACACATAACAGGAAGAAATCATCTTAATTTGCAAAGAACATCCAAACAGAACATGAAAA
ACATGATAGATTTACCCTAAGCATTGGGGCAGAGCACAGCTCACAGGCTGCATCCAACGGTATCATGGGCACTTTTTCTGT
ACTACAACTATTACTCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTATTCTATAGTGTCACCTAA
AT

Clone A-Sp6

CAAWYRARMYSKGGWCCCGGGGGGATCCTCTAGAGTCGACCTGCAGAGTAATAGTTGTAGTACAGAAAAAGTGCCCATG
ATMCCGTTGGATGCAGCCTGTGAGCTGTGCTCTGCCCCAATGCTTAGGGTAAATCTATCATGTTTTTCATGTTCTGTTTGGA
TGTTCTTTGCAAATTAAGATGATTTCTTCCTGTTATGTGTCGTACCATTTTCTCAATGTGGTTTTAACAATCGTTGGGTTTCT
AACTGAGACAAAAGTTCAGGTGAGAACTTTAGAATAATCCCAAGTTCAGGTAAGCACTCCGGGAGCTCGCCCACATCCAA
ATGGTATGTGCAAAACCTCTTATTCGCACTGCTCCTGGAGTTATAGATAGCATACACAACATGAGATTTTGTAACAACGTTC
TAAAATTTGCAGTGAATGACACAGGAGATTAAGATATGGCATGCAGTTGAGATTATAAGAGTTTGTCACACTAAACAAATT
TACTAATGCTTAGTGAATTTCCCATGTATTGATTTTTTGTGTGACCTGCAGGCATGCAAGCTTGTCTCCCTATAGTGAGTCGT
ATTA

Clone A-T7

GRRMYKSYMTGYTKCTGCAGGTCMACAAAAATCAATACATGGGAAATTCACTAAGCATTAGTAAATTTGTTTAGTGTGAC
MAACTCTTATAATCTCAACTGCATGCCATATCTTAATCTCCTGTGTCATTCACTGCAAATTTTAGAACGTTGTTACAAAATC
TCATGTTGTGTATGCTATCTATAACTCCAGGAGCAGTGCGAATAAGAGGTTTTGCACATACCATTTGGATGTGGGCGAGCT
CCCGGAGTGCTTACCTGAACTTGGGATTATTCTAAAGTTCTCACCTGAACTTTTGTCTCAGTTAGAAACCCAACGATTGTTA
AAACCACATTGAGAAAATGGTACGACACATAACAGGAAGAAATCATCTTAATTTGCAAAGAACATCCAAACAGAACATGA
AAAACATGATAGATTTACCCTAAGCATTGGGGCAGAGCACAGCTCACAGGCTGCATCCAACGGTATCATGGGCACTTTTTC
TGTACTACAACTATTACTCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTATTCTATAGTGTCACCT
AAAT

Clone B-Sp6

ACAWMSRRRCYYGGTWCGGGGATCTCTAGAGTCGACCTGCAGAGTAATAGTTGTAGTACAGAAAAAGTGCCCATGATAC
CGTTGGATGCAGCCTGTGAGCTGTGCTCTGCCCCAATGCTTASGGTAAATCTATCATGTTTTTCATGTTCTGTTTGGATGTTC
TTTGCAAATTAAGATGATTTCTTCCTGTTATGTGTCGTACCATTTTCTCAATGTGGTTTTAACAATCGTTGGGTTTCTAACTG
AGACAAAAGTTCAGGTGAGAACTTTAGAATAATCCCAAGTTCAGGTAAGCACTCCGGGAGCTCGCCCACATCCAAATGGT
ATGTGCAAAACCTCTTATTCGCACTGCTCCTGGAGTTATAGATAGCATACACAACATGAGATTTTGTAACAACGTTCTAAA
ATTTGCAGTGAATGACACAGGAGATTAAGATATGGCATGCAGTTGAGATTATAAGAGTTTGTCACACTAAACAAATTTACT
AATGCTTAGTGAATTTCCCATGTATTGATTTTTTGTGTGACCTGCAGGCATGCAAGCTTGTCTCCCTATAGTGAGTCGTATT
A

Clone B-T7

GGAAGCSATGCCTGCTGMAGTMCACAAAAATCAATACATGGAAATTCACTAAGCATTAGTAAATTTGTTTAGTGTGACAAAY
TYTTATAATCTCAACTGCATGCCATATCTTAATCTCCTGTGTCATTCACTGCAAATTTTAGAACGTTGTTACAAAATCTCATGT
TGTGTATGCTATCTATAACTCCAGGAGCAGTGCGAATAAGAGGTTTTGCACATACCATTTGGATGTGGGCGAGCTCCCGGAG
TGCTTACCTGAACTTGGGATTATTCTAAAGTTCTCACCTGAACTTTTGTCTCAGTTAGAAACCCAACGATTGTTAAAACCACA
TTGAGAAAATGGTACGACACATAACAGGAAGAAATCATCTTAATTTGCAAAGAACATCCAAACAGAACATGAAAAACATGA
TAGATTTACCCTAAGCATTGGGGCAGAGCACAGCTCACAGGCTGCATCCAACGGTATCATGGGCACTTTTTCTGTACTACAAC
TATTACTCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTATTCTATAGTGTCACCTAAAT

Addendum E

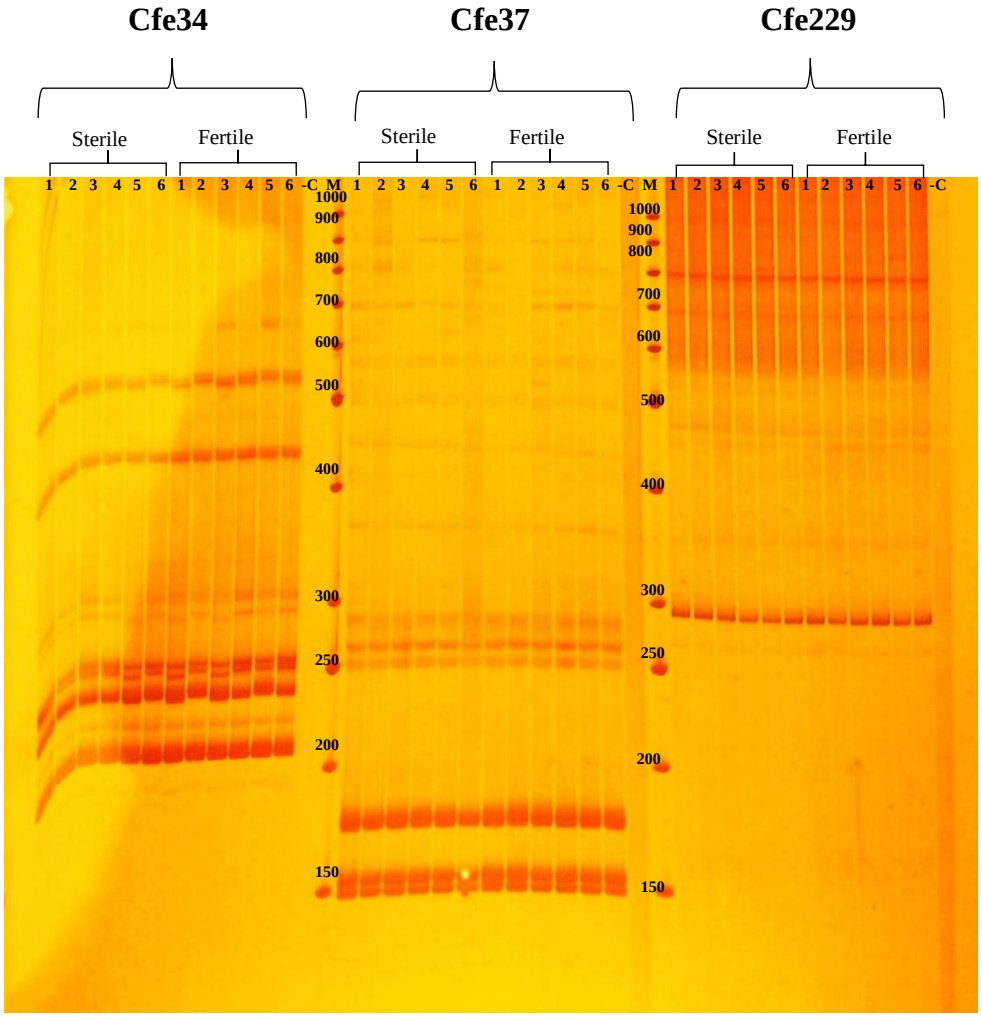

Figure E.1: SSR analysis with chromosome 5A-specific markers. Lane 1-6: Sterile plants tested with marker cfe34, Lane 7-12: Fertile plants tested with marker cfe34, Lane 13: Negative (dH_2O) control (-C), Lane 14: 50 bp marker (M), Lane 15-20: Sterile plants tested with marker cfe37, Lane 21-26: Sterile plants tested with marker cfe37, Lane 27: Negative (dH_2O) control (-C), Lane 28: 50 bp marker (M), Lane 29-34: Sterile plants tested with marker cfe229, Lane 35-40: Fertile plants tested with marker cfe229, Lane 41: Negative (dH_2O)control (-C).