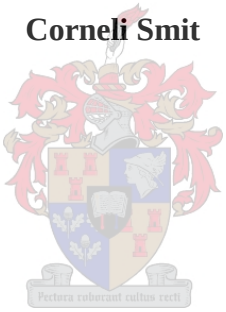
# Pyramiding of novel rust resistance genes in wheat, utilizing marker assisted selection and doubled haploid technology

**By:**

**Corneli Smit**



Thesis presented in fulfilment of the requirements for the degree of Master of Science in the faculty of Agricultural Sciences at Stellenbosch University

Study leader: Willem C. Botes

December 2013

**DECLARATION**

By submitting this thesis electronically, I declare that the entirety of the work contained herein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2013

## ABSTRACT

Wheat rust, caused by the *Puccinia* spp., is a global biotic cause of wheat yield losses. This disease can effectively be combatted by implementing rust resistant wheat cultivars. The release of new resistant wheat cultivars is however prolonged due to the time needed to fix resistance genes in a good quality background and develop pure breeding wheat lines. The aim of this study was the pyramiding of novel species derived leaf and stripe rust resistance genes in bread wheat lines through the utilization of high throughput marker assisted selection and microspore derived doubled haploid technology.

Wheat lines containing unknown numbers of novel leaf and stripe rust resistance genes formed the F1 population in this study. This material was screened for the presence of species derived resistance genes (*Lr53*/*Yr35*, *Lr54*/*Yr37*, *Lr56*/*Yr38*, *Lr59* and *Lr62/Yr42*). It was also screened with a molecular marker set routinely utilized by the SU-PBL for the screening of nurseries for stem, leaf and stripe rust resistance genes. The genes were detected with two multiplex PCR reactions, enabling high throughput marker assisted selection. The molecular analysis showed the initial material of this study to contain a number of species derived rust resistance genes in various frequencies and combinations together with the APR genes, *Sr2* and *Lr34*.

Wheat ears from the F1 generation were harvested to be used for microspore tissue culturing. The rest of the material was left for single seed descent. Seed obtained in this manner formed the subsequent F2 and F3 generations. All the plants in the F2 and F3 generations were also screened for the presence of species derived rust resistance genes and ears were continually harvested from these plants to be used for microspore tissue culturing.

The microspore tissue culture technique was employed to produce haploid wheat plants. Chromosome doubling was induced by treating the plants with colchicine to create pure breeding homozygous hexaploid wheat plants. The green plant regeneration frequency obtained in this study was between one and two percent. Induction media supplements such as cefotaxime and anti-oxidants did not improve the green plant regeneration rate.

A doubled haploid plantlet obtained in this study contained *Lr53/Yr35*, *Lr54*/*Yr37*, *Lr56*/*Yr38* and *Lr62*/*Yr42* together with *Lr34* and *Lr37*. A phylogenetic analysis indicated it to be

genetically most similar to the *Lr62/Yr42* donor parent. This plant was the end product of the successful application of marker assisted selection for rust resistance genes and the utilization of the microspore tissue culture technique. This doubled haploid plant can potentially be utilized in the PBL MS-MARS pre-breeding program as a recurrent parent.

## OPSOMMING

Koringroes het wêreldwyd verliese in koringopbrengste tot gevolg. Dit word veroorsaak deur die *Puccinia* fungi. Hierdie siekte kan effektief beveg word deur die verbouing van roesbestande kultivars. Die vrystel van nuwe weerstandbiedende kultivars is egter ‘n langdurige proses weens die tyd verbonde daaraan om weerstandsgene te fikseer in ‘n genetiese agtergrond met ‘n goeie kwaliteit en om dan suiwertelende lyne te ontwikkel. Die doelwit van hierdie studie was om nuwe spesie-verhaalde blaar- en streeproes weestandsgene in koringlyne te stapel met behulp van merker bemiddelde seleksie en mikrospoor geassosieerde verdubbelde haploïede tegnologie.

Koringlyne met ‘n onbekende aantal nuwe blaar- en streeproes weerstandsgene het die F1 populasie in hierdie studie gevorm. Dié materiaal is getoets vir die teenwoordigheid van spesie-verhaalde weerstandsgene (*Lr53*/*Yr35*, *Lr54*/*Yr37*, *Lr56*/*Yr38*, *Lr59* en *Lr62/Yr42*). Dit is ook geanaliseer met ‘n stel molekulêre merkers wat in die roetine analises van die US Planteteelt Laboratorium se kwekerye gebruik word om te toets vir die teenwoordigheid van stam-, blaar-, en streeproes weerstandsgene. Die teenwoordigheid van al hierdie gene is met behulp van twee multipleks PKR reaksies bepaal om sodoende hoë deursettingstempo merker bemiddelde seleksie teweeg te bring. Die molekulêre analise het verskillende weerstandsgeen-kombinasies en hoeveelhede in die begin materiaal van die studie getoon. Hierdie geenkombinasies het onder andere die APR gene *Sr2* en *Lr34* ingesluit.

Koringare van die F1 generasie is geoes om in die *in vitro* mikrospoor kultuur gebruik te word. Die res van die materiaal is vir enkelpit-nageslag gelos. Saad wat op hierdie manier verkry is, het die daaropvolgende F2 en F3 generasies gevorm. Al die plante in die F2 en F3 generasies is ook getoets vir die teenwoordigheid van spesie-verhaalde roesweerstandsgene en are is ook geoes om in die mikrospoor kultuur te gebruik.

Die mikrospoor weefselkultuur tegniek is toegepas om haploïede plante te produseer. Die plante is met kolgisien behandel om chromosoom verdubbeling te induseer en sodoende suiwertelende homosigotiese heksaploïede koringplante te vorm. Die groen plant regenerasie frekwensie in hierdie studie was tussen een en twee persent. Toevoegings tot die induksie media, soos kefotaksiem en anti-oksidante, het nie die regenerasie frekwensie verbeter nie.

‘n Verdubbelde haploïede plant wat in hierdie studie verkry is, het *Lr53/Yr35*, *Lr54*/*Yr37*, *Lr56*/*Yr38* en *Lr62*/*Yr42* sowel as *Lr34* en *Lr37*, bevat. ‘n Filogenetiese analise het getoon dat hierdie plant geneties die meeste ooreenstem met die *Lr62/Yr42* kruisingsouer. Die plant was die eindproduk van die suksesvolle toepassing van merker bemiddelde seleksie vir roes-weerstandsgene en die gebruik van die *in vitro* mikrospoor kultuur tegniek. Die verdubbelde haploïede plant kan potensieel in die Planteteelt Laboratorium se MS-MARS voortelings-program gebruik word as ‘n terugkruisingsouer.

## ACKNOWLEDGEMENTS

I would like to thank the following people and institutions for their assistance in completing this study:

- My study leader, Willem Botes, for his guidance, patience and willingness to share his knowledge and insight;
- Aletta Ellis, for her valuable technical assistance, motivation and friendship;
- The staff and students of the SU-PBL;
- My parents, for their constant support, love and motivation;
- The Winter Cereal Trust, for financial support;
- He who daily provides us with the bread of life.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percent |
| 2,4D | 2,4-Dichlorophenoxyacetic acid |
| ABC | ATP binding cassette |
| AFLP | Amplified Fragment Length Polymorphism |
| Amp | ampicillin |
| APR | Adult Plant Resistance |
| ARC | Agricultural Research Council |
| Avr | Avirulence |
| BGRI | Borlaug Global Rust Initiative |
| bp | Base pairs |
| °C | Degrees Celsius |
| CGIAR | Consultative Group on International Agricultural Research |
| CIMMYT | International Maize and Wheat Improvement Centre |
| cm | Centimetre |
| CS | Chinese Spring |
| CTAB | N-Cetyl-N, N, N-trimethyl Ammonium Bromide |
| DH | Doubled Haploid |
| $dH_2O$ | Distilled water |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxyribonucleotidetriphosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| EtBr | Ethidium Bromide |
| F | Forward primer |
| $F_n$ | Filial generation |
| f. sp. | *forma specialis* |

| | |
|---|---|
| g | Gram |
| GA | Gibberellic Acid |
| gDNA | Genomic Deoxyribonucleic Acid |
| g/l | Grams per litre |
| HCl | Hydrochloric acid |
| HMW-GS | High Molecular Weight Glutenin Subunit |
| HR | Hypersensitive response |
| ICARDA | International Center for Agricultural Research in the Dry Areas |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| L | Litre |
| LB | Luria Bertani |
| LMW-GS | Low Molecular Weight Glutenin Subunit |
| LRR | Leucine-rich repeat |
| M | Molar |
| MAS | Marker assisted selection |
| mb | Mega bases |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| min | Minutes |
| ml | Millilitre |
| mm | Millimeter |
| mM | Millimolar |
| MS-MARS | Male Sterility Mediated Marker Assisted Recurrent Selection |
| MS-medium | Murashige & Skoog medium |
| n | Haploid |
| 2n | Diploid |
| NaCl | Sodium chloride |

| | |
|---|---|
| $Na_2EDTA$ | Disodium Ethylenediaminetetraacetic acid |
| NaOAc | Sodium Acetate |
| NBS | Nucleotide Binding Site |
| ng | Nanogram |
| ng/µl | Nanogram per microlitre |
| $NH_4No_3$ | Ammonium Nitrate |
| NJ | Neighbour Joining |
| NtBHA | N-t-butyl hydroxylamine |
| PBL | Plant Breeding Laboratory |
| PCR | Polymerase chain reaction |
| pH | Percentage hydrogen |
| PIC | Polymorphic Information Content |
| PRR | Pattern recognition receptors |
| Pty Ltd | Propriety Limited |
| QTL | Quantitative Trait Loci |
| R | Reverse primer |
| RAPD | Random Amplified Polymorphic DNA |
| Rcf | Relative centrifugal force |
| ROA | Reactive oxygen species |
| Rpm | Revolutions per minute |
| RSA | Republic of South Africa |
| SCAR | Sequence Characterized Amplified Region |
| SGI | Small Grain Institute |
| spp. | Species |
| SSR | Simple Sequence Repeat |
| SU | Stellenbosch University |
| SU-PBL | Stellenbosch University Plant Breeding Laboratory |

| | |
|---|---|
| $T_a$ | Annealing temperature |
| $T_m$ | Melting temperature |
| TE | Tris-EDTA |
| UPGMA | Unweighted Pair Group Method with Arithmetic Mean |
| U.S.A. | United States of America |
| UV | Ultra Violet |
| UVPgt | Universiteit Vrystaat *Puccinia graminis* f. sp. *tritici* |
| UVPrt | Universiteit Vrystaat *Puccinia recondita* f. sp. *tritici* |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |
| V | Volt |
| v/v | Volume per volume |
| w/v | Weight per volume |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

## LIST OF SPECIES NAMES AND ABBREVIATIONS

| | |
|---|---|
| *Ae. kotschyi* | *Aegilops kotschyi* |
| *Ae. neglecta* | *Aegilops neglecta* |
| *Ae. peregrine* | *Aegilops peregrina* |
| *Ae. sharonensis* | *Aegilops sharonensis* |
| *Ae. speltoides* | *Aegilops speltoides* |
| *Ae. tauschii* | *Aegilops tauschii* |
| *P. graminis* | *Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn. |
| *P. triticina* | *Puccinia triticina* Eriks. |
| *P. striiformis* | *Puccinia striiformis* West. f. sp. *tritici* Eriks. & E. Henn. |
| T. *aestivum* L. | *Triticum aestivum* |
| *T. dicoccoides* | *Triticum dicoccoides* |
| *T. dicoccus* | *Triticum dicoccus* |
| *T. timopheevii* | *Triticum timopheevii* |
| *T. urartu* | *Triticum urartu* |
| *Z. mays* | *Zea mays* |

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

# CHAPTER 1: INTRODUCTION

Wheat is an essential food crop of great economic importance. It provides about a fifth of the calories consumed by man and acts as a staple food in many developing as well as developed countries. The world population is expected to reach 9 billion in 2050. With this growing world population, the world wheat demand is estimated to increase by 60% in 2050. It is thus evident that wheat production rapidly needs to be increased (FAOSTAT Trade: Crops and livestock products, 2013; How to feed the world in 2050, 2009).

There are two potential ways to increase global wheat production. This could either be through agricultural extensification or intensification. Agricultural extensification is not a viable option since there is only a limited amount of arable farming land available for crop production. The agricultural sector has to compete for this land with biofuel industries and governmental housing strategies, among others. Additional land clearing may also lead to significant ecological impacts and biodiversity concerns. The best way to supply the ever growing wheat demand is through agricultural intensification. This involves the attainment of higher crop yields through the utilization of improved cultivars, accompanied by the implementation of better agronomic practices and lower production costs (Tilman *et al*., 2011). The Consultative Group on International Agricultural Research (CGIAR), including the International Maize and Wheat Improvement Centre (CIMMYT) and International Center for Agricultural Research in the Dry Areas (ICARDA), are globally assisting both wheat breeders and producers to achieve this goal (Joshi *et al*., 2010; CGIAR Research Centres, [S.a.]).

Even the yields achieved by improved wheat cultivars are however impaired by several abiotic as well as biotic stress factors. Abiotic stressors may include conditions such as drought, extreme temperatures and soil salinity (Tester & Bacic, 2005). The biotic stresses exerted on wheat crops include several pests in the form of insects, fungi, bacterial pathogens and plant viruses (Duveiller *et al*., 2012). One of the biggest biotic stresses causing global wheat yield losses, is the *Puccinia* fungi, responsible for stem, leaf and yellow rust symptoms.

Genetic resistance is an effective way of combatting the rust diseases. Breeding programs have successfully implemented molecular markers to assist in the development of cultivars with stem, leaf and stripe rust resistance genes. Rust pathotypes however overcome resistance genes through mutation and genetic recombination (Dadkhodaie *et al*., 2011). It is thus necessary to constantly improve the genetic resistance of commercially grown wheat cultivars and to explore novel sources of genetic resistance.

When new rust resistance genes are to be deployed in wheat breeding programs, it unfortunately takes several years before the new sources of resistance will become available in commercial wheat cultivars. This is due to the long process involved in the establishment of pure breeding wheat lines. Biotechnology based techniques are available to accelerate the breeding process via doubled haploid production. By utilizing these techniques, an acceptable level of homozygosity for lines in a breeding program can be achieved in one plant cycle, opposed to the four to six plant generations needed when using the conventional breeding practices currently employed. One of the most promising techniques for the production of doubled haploid plants is the isolated microspore tissue culturing technique (Ferrie & Caswell, 2011).

The aim of this study was the pyramiding of novel species derived leaf and stripe rust resistance genes in bread wheat lines through the utilization of high throughput marker assisted selection and microspore derived doubled haploid technology.

In order to achieve the aim, the following objectives have been identified:

(a) The molecular characterization of the initial material that formed the F1 base population in this study. This involved screening the wheat material with a standardised panel of rust resistance gene markers utilized in the routine screening of nurseries at the SU-PBL, as well as screening the material for the presence of novel species derived leaf and stripe rust resistance genes, namely: *Lr53/Yr35, Lr54/Yr37, Lr56/Yr38, Lr59* and *Lr62/Yr42*.

(b) The Screening of all wheat material in the subsequent generations for the presence of novel species derived leaf and stripe rust resistance genes utilizing marker assisted selection.

(c) The identification, adaptation and implementation of the microspore tissue culture technique to develop haploid plants from wheat lines identified through marker assisted selection (MAS) to contain a number of novel species derived leaf and stripe rust resistance genes. The haploid plants were then to be treated with colchicine to induce chromosome doubling and essentially yield complete homozygotic hexaploid wheat lines.

(d) The molecular characterization of doubled haploid plants. This involved screening the plants with the marker set routinely used by the lab for detection of rust resistance genes, screening the plants for the presence of species derived resistance genes and evaluating the genetic diversity of the doubled haploid plants with a minimum subset of microsatellite markers. These doubled haploids could potentially be utilized in the SU-PBL wheat pre-breeding program.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 WHEAT

### 2.1.1 Global wheat production

Bread wheat (*Triticum aestivum* L.) is a significant agricultural product. It is firstly an essential world food crop. Secondly, it is an important economic commodity for wheat producing countries (FAOSTAT Trade: Crops and livestock products, 2013).

Globally, wheat is the most widely consumed cereal after rice and provides about 20% of the calories consumed by man. It is a staple in many developed, but especially developing countries like China and India. The global wheat consumption for 2013/2014 is estimated at 691 million tons (FAO Cereal Supply and Demand Brief, 2013). Despite the fact that wheat and other cereal crops contribute to almost 50% of the energy in the average African diet, almost 250 million people are annually undernourished in sub-Saharan African countries alone (Dimensions of need – An atlas of food and agriculture, 1995; Africa Hunger and Poverty Facts, 2013). There is thus a continued demand for wheat as a primary food crop.

Wheat is cultivated in a number of countries across the globe under dryland conditions as well as on irrigated land. Total world wheat production for 2013/2014 is estimated at about 709 million tons. The world's largest wheat producer is the European Union (EU-27), contributing about 20% of the world's wheat stock. Other major wheat producing countries in descending order include China (17%), India (13%), the USA (8.2%) and the Russian federation (7.7%), with South Africa (0.24%) ranked around number 30 on the global wheat production list. The largest net exporter of wheat is America, exporting about 50% of its home-grown wheat. The world wheat export commodity trade in 2011 was valued at around 46.8 billion US dollars, which highlights the economic importance of this valuable food crop (FAOSTAT Trade: Crops and livestock products, 2013; Index mundi: Wheat production by country in 1000 MT, 2013).

In South Africa, wheat is the largest cultivated winter cereal crop. Total production was estimated at 1.87 million tons for 2012/2013. About 80% of this was produced under dryland conditions, mainly in the Western Cape and Free State areas, and about 20% came from irrigation areas, largely in the Northern Cape (South African Wheat Crop Quality Report

2012/2013 Season, 2013). Wheat contributes about 2.5% to the gross value of agricultural production. This is about the same contribution as the viticulture industry (2.6%) but significantly less than poultry and poultry products (22.6%) which is regarded as the largest monetary contributor to agricultural GDP in South Africa (Agriculture production, 2012).

Despite these wheat production figures, South Africa remains a net importer of wheat and an additional 1.59 million tons had to be imported during the 2012/2013 season, in combination with reserve stocks from the previous season, to meet the local demand. This brought South Africa to a self-sufficiency index of about 50% (Protein Research Foundation: Oil seed & Grain supply in the RSA, 2013). South Africa's population currently stands at around 50 million, necessitating imports of agricultural commodities such as wheat to feed all the people. For the period of 1960-1999, the world population doubled in size from 3 million to 6 million people. The global population recently reached 7 billion and although it is not expected to ever double again, it is set to reach more than 9 billion by 2050. Together with the ever increasing population comes the inevitable growing demand for food and the global wheat demand is estimated to increase by 60% by 2050. It is thus evident that wheat production needs to be dramatically increased (How to feed the world in 2050, 2009).

Wheat production can be increased in two ways to supply the ever growing demand – either through agricultural extensification or intensification. Extensification involves the clearing of additional arable land that can be utilized for crop production. This method however leads to a number of environmental concerns. Land clearing induces habitat fragmentation which threatens biodiversity. It is also responsible for almost one third of global greenhouse gas emissions and the extensive use of fertilizer on increased areas of land can be detrimental to several types of marine and terrestrial ecosystems. Furthermore, there is only a limited amount of arable land available. The agricultural sector has to compete for this land with other sectors like the biofuel industry (Tilman *et al*., 2011), as well as governmental housing strategies in rural areas and increased urban developments (Davis, 2013).

Agricultural intensification involves the attainment of higher yields through the utilization of better agronomic practices, superior crop cultivars together with lower input costs. This possibility offers a solution with much less environmental concerns. A large part of the global area currently utilized for crop production delivers yields far under its potential. There is thus extensive opportunity for intensification. If the existing crop area in under-yielding wheat countries can be exploited at its maximum potential through intensification, most of the

global wheat demand can be met by 2050. Unfortunately, in developing countries, extensification is still the predominant choice to obtain increased agricultural production. A concerted effort towards greater technology transfer and agricultural awareness is thus essential (Tilman *et al*., 2011). Global institutions like CIMMYT in Mexico and ICARDA have launched initiatives to develop and distribute seed with improved disease resistance and improved adaptability to specific agricultural conditions to farmers in developing countries (Joshi *et al*., 2010). These institutions are part of the fifteen research centres associated with CGIAR (CGIAR Research Centres, [S.a.]). This is a fine example of global collaboration to improve agricultural progress worldwide.

### 2.1.2 The local wheat crop

The Western Cape is one of the three major wheat producing areas in South Africa, contributing about 38% of the country's annual wheat crop (South African Grain Laboratory Wheat Report 2011/2012, 2012). The predominant wheat producing areas in this region include the Swartland, Namaqualand and the Rûens.

Wheat is traditionally categorized according to growth habit into spring, facultative (intermediate) or winter types. In the Western Cape, spring wheat is sown in autumn. This is common practice in countries that experience milder winters associated with a Mediterranean climate and lacking the period of cold temperatures (0-5ºC) required for the heading of winter wheat. The Western Cape is situated in a winter rainfall region, providing enough soil moisture for the germination of spring wheat. By planting spring wheat in autumn, wheat seedlings that are sensitive to soil temperature are also protected from the high temperatures often experienced during the Western Cape summer (Curtis, 2002).

### 2.1.3 Wheat on the molecular level

*Triticum aestivum* has a hexaploid genome, consisting of three subgenomes: A, B and D. Each subgenome consists of 14 chromosomes in the diploid state, making the total chromosome number of hexaploid bread wheat 42.

Historically wheat dates back thousands of years. Wild diploid wheat (*Triticum urartu*) hybridized with diploid goat grass, *Aegilops speltoides*, which is the closest grass relative to wheat. They respectively contributed the A and B genomes to wheat and this natural hybridization event led to the formation of tetraploid wild emmer wheat, *Triticum*

*dicoccoides*, with an AABB genome (figure 2.1). During years of wild emmer cultivation, several populations of domesticated emmer wheat, *Triticum dicoccus*, emerged. Another hybridization event of domesticated emmer wheat with another goat grass, *Aegilops tauschii*, which contributed the D genome, led to the formation of the hexaploid bread wheat (Peng *et al*., 2011).

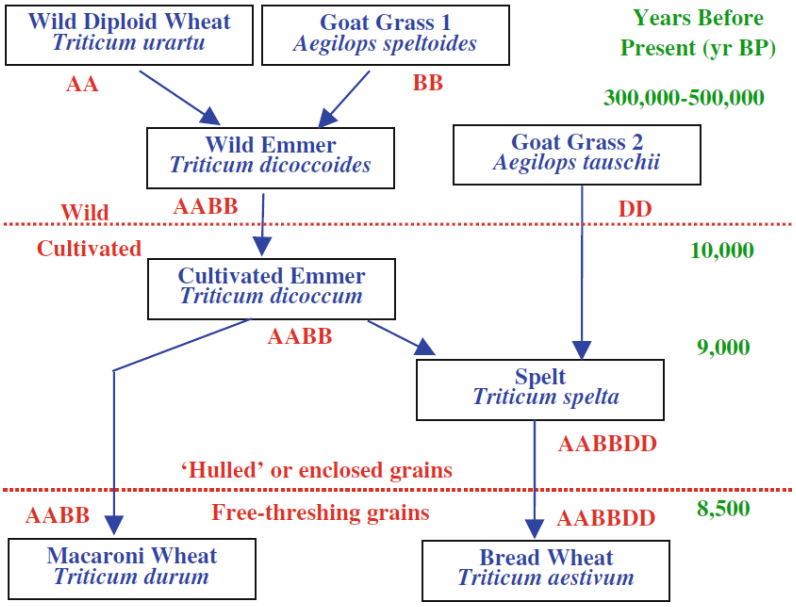



**Figure 2.1: Domestication of bread wheat (Figure reproduced from http://www.newhallmill.org.uk/wht-evol.htm)**

The domestication of wheat brought about several morphological changes to the wheat plant that facilitates modern crop production. For example, emmer wheat contains a brittle rachis gene (*Br*), located on the short arms of chromosomes 3A and 3B. This gene causes a brittle morphology of the spikes which leads to the scattering of grain when the wind blows and impairs effective harvesting (figure 2.2 A). During domestication, this trait was eliminated. Another important wheat domestication event was the loss of tough glumes, transforming hulled wheat into free-threshing wheat. The primary genetic determinants of the free-threshing habit are recessive mutations at the *Tg* (tenacious glume) loci. *Tg1* is situated on chromosome 2DS of hexaploid wheat. It has however been found that several QTL on chromosomes 2A, 2B, 2D, 5A, 6A, 6D and 7B also affect the free-threshing quality of cultivated wheat. Furthermore, the *Q* locus on chromosome 5AL also has a modifying effect on the threshability, glume texture and rachis composition. The dominant allele (*Q*) promotes

threshability, softer glumes and toughness of the rachis and is therefore favoured to the recessive allele (*q*). The *Tg* gene however supresses the expression of *Q*, so the ideal free-threshing hexaploid wheat would have a *tgtg*, *QQ* genotype, phenotypically resembling figure 2.2 C and D (Peng *et al*., 2011).

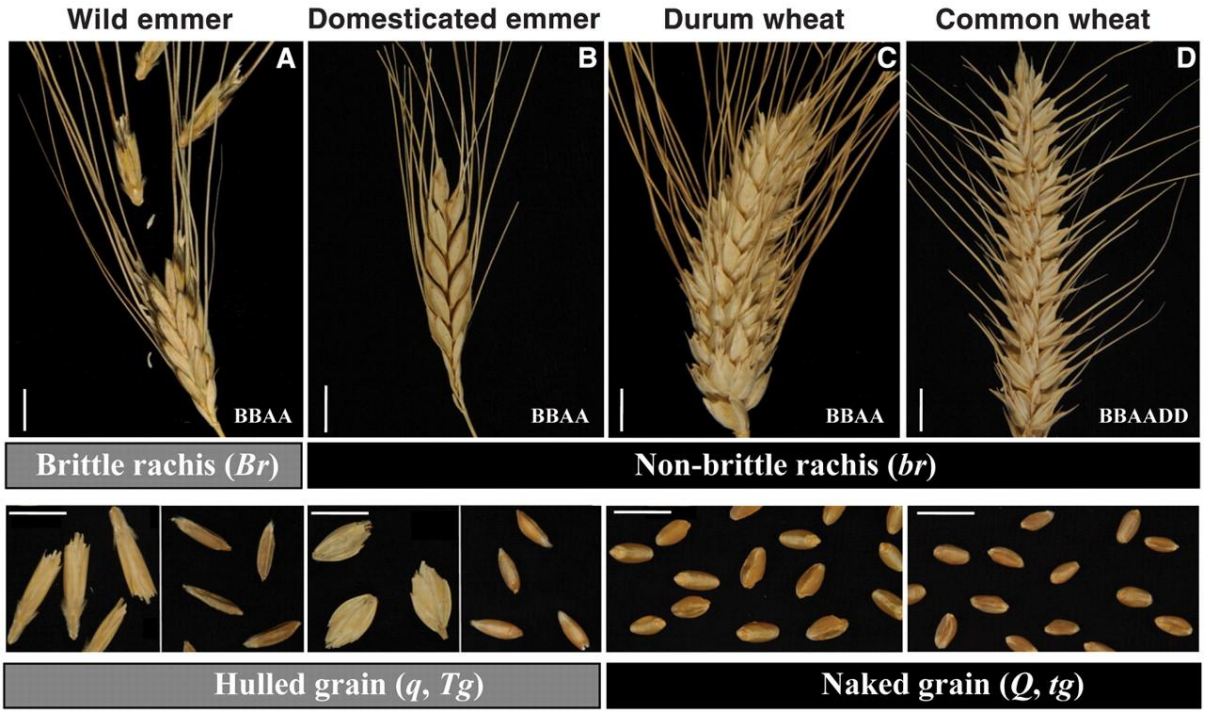



**Figure 2.2: Wheat spikes showing (A) brittle rachis, (B to D) nonbrittle rachis, (A and B) hulled grain, and (C and D) naked grain. (A) Wild emmer wheat, (B) domesticated emmer, (C) durum wheat, and (D) common wheat. (Figure reproduced from Dubcovsky & Dvorak, 2007).**

The entire wheat genome consists of more than 16 000mb. It is about 5 times larger than the human genome and consists of large repetitive sequences. The genome was recently sequenced by an international collaboration of researchers using a whole genome shotgun approach with next generation 454 pyro-sequencing. This yielded a five-fold coverage of the reference Chinese Spring wheat genome. Detailed annotation of the genome still needs to be done, but between 94000 and 96000 genes have already been identified and more than 60% of these were assigned to the three subgenomes, A, B and D (Brenchley *et al*., 2012). This information is publicly available to plant breeders and molecular scientist across the world and may be utilized to improve selective breeding to maximise commercial wheat yields.

### 2.1.4 Biotic and abiotic stresses

Biotic and abiotic stresses are among the number of factors that influence agricultural yields. These factors can either cause a direct yield reduction or it can damage the quality of the produce that would essentially also affect the yield and financial turnover.

Abiotic stressors include extreme temperatures, water stress, the sub-optimal concentration of inorganic solutes in the soil and abnormal photon irradiance. Other factors that are also becoming significantly bigger concerns in the cultivation of cereals and forage grasses are ground salinity and acidity. The majority of cereals are moderately sensitive to a wide variety of abiotic stresses. Several abiotic stresses may simultaneously be exerted under certain circumstances and this may hamper the crop's ability to effectively handle the stress conditions. High temperatures may for example often be associated with high light intensity, coupled with water shortages and soil salinity. Unfortunately, man contributes greatly to increased soil salinization since the beginning of crop cultivation. When irrigation water contains a large number of solutes and insufficient drainage of these accumulated soil salts occur, intolerable salinity levels are quickly reached. Low temperatures and excess water may also lead to impaired plant growth. Grasses and cereal crops may suffer low temperature damage during anthesis, compromising fertility. Poor soil drainage may in turn lead to waterlogging causing poor soil aeration and root suffocation (Tester & Bacic, 2005; Taiz & Zeiger, 2006).

Biotic stresses that influence wheat crops may include weeds, insect damages or pathogenic infections by fungi, bacteria or plant viruses. An example of a weed problem commonly experienced in the Western Cape area is rye grass. Among the most common insects that infest wheat plants are the Russian wheat aphid, stalk borers and mites. One of the biggest biotic stresses affecting wheat yields in South Africa, and globally, are the wheat rust diseases caused by the *Puccinia* fungi. Other fungal diseases observed in wheat crops include karnal bunt, powdery mildew, septoria diseases, fusarium leaf blotch and head blight as well as take-all (Duveiller *et al*., 2012).

In order to maximise yields in accordance with the agricultural intensification approach, yield losses caused by disease should be minimized as much as possible. In the Western Cape, where favourable conditions for the development of fungal diseases often prevail, producers largely utilize chemical control to keep these pests at bay (Smit *et al*., 2008).

## 2.2 THE WHEAT RUSTS

Wheat rust diseases are some of the most destructive abiotic stresses that affect wheat crops across the globe, resulting in major yield losses. The *Puccinia* spp. are obligate parasites of wheat that cause a variety of wheat rust symptoms (Singh, 2002; Dadkhodaie *et al*., 2011). All three of the rust pathogens, respectively causing stem, leaf and yellow rust, occur in South Africa.

### 2.2.1 The *Puccinia*

The genus *Puccinia* belongs to the *Basidiomycota* phylum of the fungi kingdom. This genus consists of approximately 4000 species with three of them being of particular importance in this study: *Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn., producing stem rust symptoms, *Puccinia triticina* Eriks., causing leaf rust symptoms and *Puccinia striiformis* West. f. sp. *tritici* Eriks. & E. Henn. responsible for yellow rust.

Obtaining sufficient nutrients like carbohydrates and amino acids from their hosts is crucial for the survival of these fungal parasites. The rust pathogens have developed specialized structures, called haustoria, which are utilized to absorb nutrients from the plant host and presumably also play a role in the inhibition of host defence responses (Du Plessis *et al*., 2011; Voegele & Mendgen, 2011). Draft sequences of the *P. graminis*, *P. triticina* and *P. striiformis* genomes were released in 2007, 2009 and 2011 (*Puccinia* Group Database, 2010; Cantu *et al*., 2011). In 2011 the 88.6 Mb genome of *P. graminis f. sp tritici* was re-sequenced by Du Plessis *et al*. (2011) and among the 17 773 predicted proteins of this genome, a large number of effector-like small secreted proteins was identified. Other genomic features showed impaired sulphur and nitrogen assimilation pathways in the *Puccinia* species. These findings were in accordance with a typical obligatory biotrophic parasitic lifestyle. The genomes of *P. triticina* and *P. striiformis* that are not yet as fully annotated as the *P. graminis* genome are expected to portray a similar array of expressed proteins and biochemical pathways.

The *Puccinia* species have a complex lifecycle consisting of two distinct phases taking place on two phylogenetically unrelated hosts (Du Plessis *et al*., 2011). The basic asexual lifecycle of the rust pathogen starts with the primary inoculum, either urediniospores or aeciospores, making contact with the wheat host (figure 2.3). When continually exposed to moisture for longer than six hours, the spores germinate and infect the host plant by forming fungal

mycelia. At a period of between 10-20 days after infection, new dikaryotic urediniospores are produced in uredinial structures sprouting from the mycelia. These spores may be distributed to infect neighbouring plants or wheat fields (Markell, 2000). Rust spores can be distributed by the wind over very long distances. Even some intercontinental transfer of rust pathotypes has been noted, some of which was presumably caused by urediniospores stuck to the clothing of international travellers (Wellings, 2011). This is the asexual phase of the *Puccinia* life cycle.

When the wheat host approaches the end of its growing season, the *Puccinia* fungi need a strategy to survive until the next season. *Puccinia* can over winter on a green bridge, which primarily consists of volunteer wheat plants. Wheat cultivated in Lesotho throughout the year may also act as a green bridge for rust in South Africa (Pretorius *et al*., 2007). On a green bridge, the *Puccinia* will continue its asexual lifecycle until the new crop season arrives and inoculum will once again spread to cultivated wheat. The rust fungi can however also survive on alternative hosts. In order to achieve this, the fungi go into a sexual phase and reproduce on the alternative hosts until the next cropping season.

Black, thick-walled dikaryotic teliospores (figure 2.3) are produced in special structures, known as the telia. These spores undergo karyogamy where the two nuclei fuse. This process is then followed by meiosis through which four haploid basidiospores are produced. It is also during this stage that sexual recombination in the *Puccinia* genome takes place. Basidiospores are thin-walled and colourless and they infect an alternative grass host. When the basidiospores germinate, they form a network of haploid mycelia that infiltrate the host leaf. The mycelia then produce pycnia structures that may protrude through the upper leaf surface. Pycnia produce pycniospores in a sticky honeydew as well as receptive hyphae and pycniospores can be spread via insects that are attracted by the honey-like dew or by splattering raindrops. When a pycniospore cross fertilizes a receptive hyphae, the mycelium becomes a dikaryotic dividing structure which grows toward the abaxial side of the leaf where it forms an aecium at the bottom of the leaf surface. At this point dikaryotic aeciospores are released. Aeciospores can then again germinate on the wheat host in a similar way to the urediniospores. This completes the sexual phase of the *Puccinia* life cycle (Schumann & Leonard, 2000; Jin *et al*., 2010). There are however currently no known alternative hosts of the *Puccinia* fungi present in South Africa and therefore the sexual phase of the *Puccinia* lifecycle is thought not to be taking place in this country (Scott, 1990).

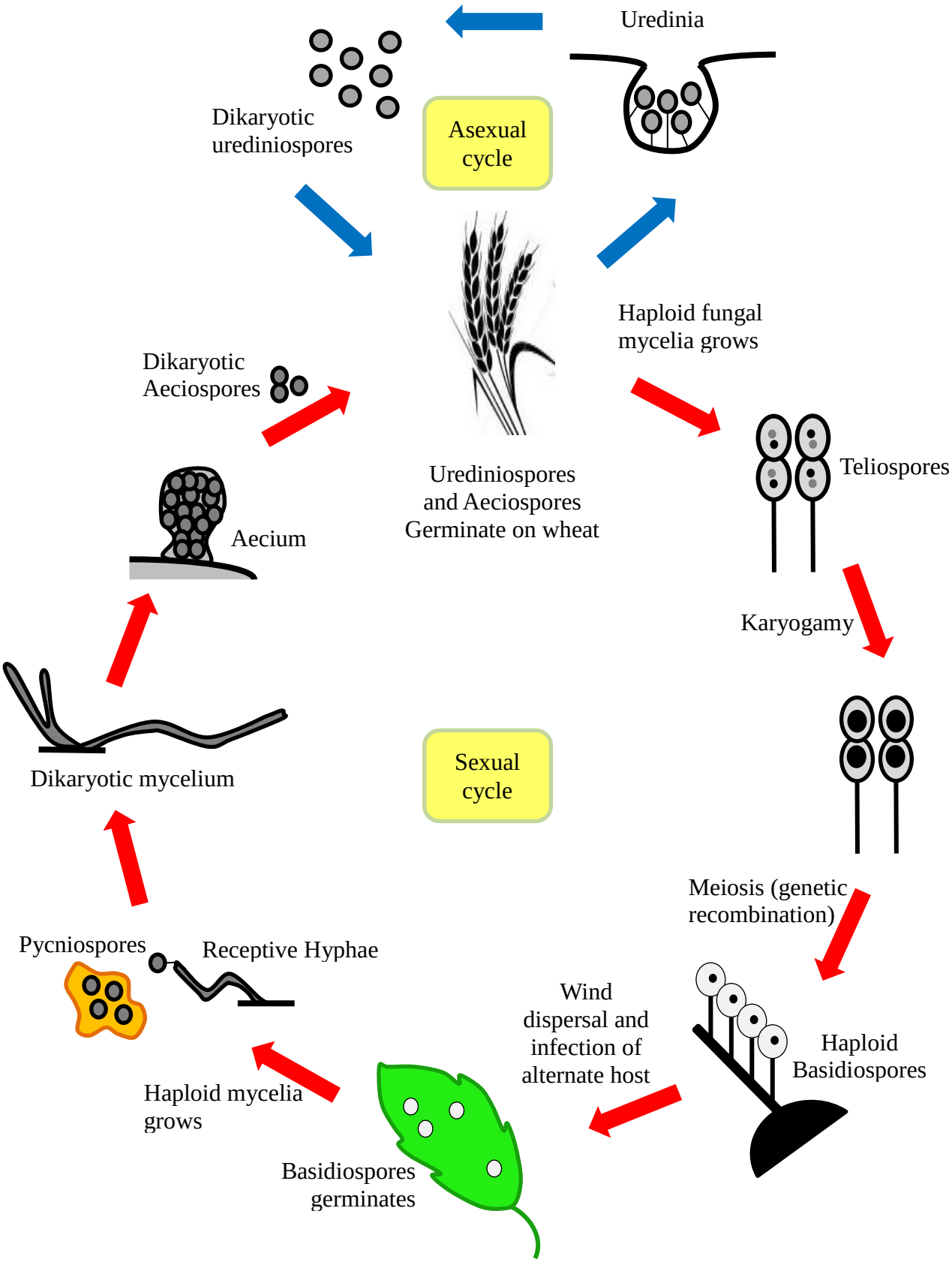



**Figure 2.3: The sexual and asexual phase of the *Puccinia* life cycle**

### 2.2.2 Leaf rust

The most common of the wheat rust pathogens, *Puccinia triticina* Eriks., is responsible for the development of leaf rust symptoms. This pathogen can tolerate a wide range of temperatures, varying from 2ºC during germination to 35ºC during sporulation, with the optimal temperature for development ranging between 15 and 25ºC. This unique quality facilitates the ability of leaf rust to emerge at almost any time during the entire crop season. Internationally leaf rust causes the most significant yield losses, not because of the extensive damage per crop plant, but because it is the most widespread occurring of the three rust pathotypes. Losses in grain yield are primarily caused by grain shrivelling and decreased floret set (Huerta-Espino *et al*., 2011).

*P. triticina* produces orange-brown lesions on the upper side of leaves and on the leaf sheaths of wheat crops. Many individual pustules containing urediniospores can be formed on one particular leaf. These pustules are up to 1.5mm in size, thus relatively small when compared to stem rust. Nearing the end of the crop season, black teliospores will spring from telia on the lower leaf surfaces (Singh, 2002). Teliospores are unable to infect the wheat host, but may be carried by the wind to infect an alternative host and continue to the sexual part of the *P. triticina* lifecycle. The primary alternative host to *P. triticina* is considered to be the *Thalictrum* species, also known as meadow-rue (Huerta-Espino *et al*., 2011).

### 2.2.3 Stem rust

*Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn., the causal agent of stem rust, prefers more humid conditions and slightly warmer temperatures. Hot days with temperatures between 25 and 30°C and mild nights ranging between 15 and 20°C, are considered optimal conditions for the development of stem rust. It thus favours temperatures on average 5.5ºC higher than the leaf rust pathogen (Singh, 2002). Stem rust affects both the stems and leaves of crops. Red-brown urediniospores form on stems and on both sides of the leaves. Pustules on the leaves are oval shaped, measuring up to 9.5mm in length. These pustules are much larger than those formed by leaf rust and it is visible on both sides of the leaves, contrary to leaf rust pustules predominantly found on the adaxial side of the leaves. On the stems, pustules are elongated, and at close observation one may see pieces of shredded epidermis alongside the pustules where it burst through the upper cell layers of the stem (Markell, 2000). Stem rust is sometimes also referred to as black rust due to the large numbers of shiny black teliospores

that are produced near the end of the harvest season. Teliospores infect the alternate host, which in the case of *P. graminis*, is barberry. Worldwide campaigns have been launched towards the eradication of barberry in an attempt to control the spreading of stem rust.

The fungus causes yield losses in a number of ways. Firstly it absorbs essential nutrients from the plant tissue that would normally be channelled towards grain development. It also becomes difficult for the wheat plant to regulate transpiration since the stem rust pustules break through the epidermal tissue. The plants' metabolism thus becomes less efficient with greater water loss. When the vascular system of a wheat plant is damaged, it may also lead to shrivelled grains. In cases of severe stem rust infection, wheat plants may also be prone to lodging due to weakened stems. Heavy wind and rain may contribute to this phenomenon and in some cases of severe unanticipated lodging, the mechanical harvesting of wheat crops may be impeded (Schumann & Leonard, 2000).

### 2.2.4 Stripe rust

Stripe rust, also commonly referred to as yellow rust, symptoms are caused by *Puccinia striiformis* West. f. sp. *tritici* Eriks. & E. Henn. Symptoms of this disease involve yellow coloured urediniospores in a characteristic stripe pattern on the leaves of the wheat plant. The onset of these symptoms is typically associated with cooler temperatures. *P. striiformis* can endure temperatures between 0 and 23ºC, with optimal temperature for development ranging from 9-15ºC. This is on average about 10ºC cooler than that of *P. triticina* and *P. graminis*. *P striiformis*' early attack in the season leads to the occurrence of underdeveloped wheat plants and grain losses are attributed to damaged tillers and shrivelled grain (Singh, 2002). Infection from this pathogen also damages the crops' transpiration and stunts the growth of the plants (Bux *et al*., 2012). This fungus is particularly dangerous, since it is the only rust pathogen that spreads through plant tissue beyond the point of infection. After infection the fungus grows inside the leaf, absorbing nutrients from the plant, typically along the venation pattern of the leaf. This gives rise to the yellow stripes, instead of pustules, typically associated with stripe rust. A small number of spores may thus cause large crop damages with only a few infection points.

For many years it was believed that the *P. striiformis* lifecycle did not involve a sexual phase since no alternative host could be identified. In 2010, Jin *et al*. proved that *Berberis* species

were able to host the aecial form of this fungus. This discovery proved that *P. striiformis* does indeed follow a heteroecious lifecycle.

### 2.2.5 Disease control

A number of strategies varying in effectivity exist to control the spread of the *Puccinia* pathogens and limit yield losses due to disease symptoms. Chemical control via the use of fungicides has been successfully utilized to eradicate rust epidemics. This method is however expensive and not an economically viable option if the producer has to spray two or three times per season. Chemical control also has a large environmental impact (Roelfs *et al*., 1992; Bux *et al*., 2012).

The implementation of better farming practices may also contribute to the reduction of rust disease occurrence. This may for example involve the cultivation of early maturing cultivars to avoid the onset of stem rust later in the season or planting early maturing cultivars downwind from late-maturing cultivars. Some more preventative measures involve the eradication of all alternative hosts and volunteer wheat and also timing the amount and application of irrigation and fertilization. Changing cultural practices has a reduced environmental impact and may add to the effectiveness of resistance genes and fungicides when it is used. It may also delay the onset of disease and thus limit its severity. However, even when implementing all of these measures, the absence of disease is not guaranteed. A large inoculum that reaches a wheat field can cause significant damage, especially when no other preventative measures are in place (Roelfs *et al*., 1992).

The most effective way to control yield losses caused by stem, leaf and yellow rust, is by implementing genetic resistance in wheat cultivars. This reduces the need for fungicide usage and thus offers an environmentally friendly alternative. By planting cultivars containing rust resistance genes, the input cost of the producer is somewhat reduced since the need for chemical control is decreased. The proper combination of resistance genes should also offer protection during the whole planting season, minimizing the risk of infection. This method however involves prior knowledge of the *Puccinia* pathotypes present in a specific area, which highlights the importance of pathogen monitoring (Pretorius *et al*., 2007). New molecular tools, for example SSR typing, enable pathologists to accurately determine the origin and spread of novel pathotypes. This method was used in the identification of stem rust pathotypes related to Ug99 in South Africa (Visser *et al*., 2011). Le Maitre (2010) also used

several molecular markers to aid in a population study of *Puccinia* species affecting wheat production in the Western Cape. Rust resistance genes may, however, only be of value for a limited duration of time since the wheat rusts mutate, and overcome resistance genes used in commercial wheat cultivars (Dadkhodaie *et al*., 2011). This reiterates the need to exploit new genetic sources of rust resistance that can be implemented in wheat breeding programs.

### 2.2.6 Why is rust such a devastating disease?

New pathotypes, overcoming the resistance genes utilized in commercial cultivars are constantly emerging. During the sexual phase of the *Puccinia* lifecycle, when the fungus resides on an alternative host, genetic recombination during meiosis can take place to produce new *Puccinia* pathotypes. The known alternative hosts for the various leaf and stem rust pathogens have largely been eradicated in wheat growing areas around the world as an attempt to prevent this phenomenon. New pathotypes however still arise from mutations in the *Puccinia* genome, presenting a constant source of new virulence. Although the rate at which new virulent pathotypes emerge due to mutations are much slower than the rate that could be achieved via genetic recombination, the practice of monoculture, the indiscriminate use of fungicides and constant selection pressure from resistant host crops, induce the mutation rate of the *Puccinia* species (Goyeau *et al*., 2007; Hovmøller & Justesen, 2007). In South Africa there are no known alternative hosts for the rust fungi. Mutations in the *Puccinia* genomes are thus considered as the primary causal agent for the development of novel pathotypes in South Africa.

Hovmøller and Justesen (2007) found an average mutation rate in *P. striiformis* for acquiring of virulence in the absence of host selection to be $4.7x10^{-4}$ per AFLP locus per generation. This mutation rate is approximately 100 times more than the average mutation rate of other AFLP loci not related to any resistance genes, suggesting that mutations at avirulence loci are more frequent than other random mutations in the genome. Wheat rust is therefore an extremely dangerous disease since the responsible pathogens can mutate at a rapid pace and evolve to overcome resistance. It takes as little as one mutation event to achieve virulence for a specific rust resistance gene. The average resistance gene employed in a commercial wheat cultivar is only expected to remain effective for about 3-5 years before a new pathotype mutates to overcome the resistance.

In 1999 a discovery was made that threatened to destroy the wheat crops of Eastern Africa. A previously unknown variant of a new virulent stem rust pathotype was detected in Uganda by Pretorius *et al*. (2000). This pathotype was designated Ug99, referring to the locality at which it was first detected. It is part of the TTKSK race, according to the North American nomenclature. Ug99 was reported to show virulence for a number of stem rust resistance genes, including *Sr31* and *Sr24*, which at that point were commonly utilized resistance genes in commercially grown wheat cultivars. It was estimated that Ug99 had the ability to destroy up to 70% of a wheat crop within a month's time. This historic example is a clear illustration of how dangerous the *Puccinia* species can be when overcoming trusted sources of resistance. Since its discovery in Uganda in 1999, Ug99 has spread northward through many African countries such as Kenya, Ethiopia, Sudan and out of East Africa up to Yemen and Iran (Joshi *et al*., 2010). It also spread southward, and in 2007 the first South African pathotype related to Ug99 was detected in the Western Cape. It was designated UVPgt59, which is equivalent to 2SA106 and TTKSP. In 2009 another pathotype related to Ug99, namely UVPgt60 (also PTKST), was detected in KwaZulu-Natal (Visser *et al*., 2010). These stem rust pathotype relations were determined by utilizing SSR markers.

### 2.2.7 Pathotype monitoring and nomenclature

The wheat rust situation is internationally monitored by a number of collaborations between plant breeders, pathologists and wheat growers across the globe. Monitoring of rust pathotypes are primarily done through the utilization of trap nurseries or by pathotype surveys. Platforms have been established where wheat rust pathotype information can interactively be obtained or shared on a real time basis. Some of these systems include the Borlaug Global Rust Initiative (BGRI), the Rust SPORE web portal established by the FAO and software like RustMapper, developed by CIMMYT (Park *et al*., 2011). From a plant breeder's perspective, some of the most valuable data to obtain would be information on which resistance genes remain effective against novel rust pathotypes in specific agricultural areas.

The Global Cereal Rust Monitoring System was founded in order to establish standardized protocols to be used in rust surveys and to serve as a platform to share information on virulence testing, rust management and data collection. The ideal would be for all the different parties involved with wheat rust to actively participate and contribute to this system's operation (Park *et al*., 2011). Although these systems provide valuable data for

wheat breeders and pathologists about the distribution of the latest rust outbreaks, new observed pathotypes and rust resistance genes, there are still some limitations regarding an internationally recognized pathotype monitoring system. Different methods are used across the globe to detect rust pathotypes and different pathotype nomenclature systems are used by different institutions. Novel pathotypes are classified according to a set of differential virulence/avirulence patterns expressed by each isolate. Every nomenclature system does not necessarily have an exact equivalent in another type of system as the differentials utilized may differ. This complicates the application of internationally shared pathogen information (Park *et al*., 2011).

In South Africa there are two main nomenclature systems used for the classification of wheat rust pathogens: the Agricultural Research Council (ARC) system and the University of the Free State (UV) nomenclature. In the ARC system, the number "2" denotes a stem rust pathogen and the number "3" a leaf rust pathogen. 2SA106 would for example indicate stem rust pathogen number 106 with a virulence/avirulence pattern as shown in table 2.1. The University of the Free State's system uses "Pgt" and "Prt" to indicate stem rust and leaf rust pathogens, followed by a number to identify the pathotype. UVPgt60 would thus be *P. graminis* pathotype number 60 with the virulence/avirulence pattern also indicated in table 2.1. Internationally, *Puccinia striiformis* has the most standardized nomenclature system of the three wheat rust causing fungi. This system is based on a differential of wheat cultivars that show different resistance or susceptibility patterns *to P. striiformis* pathotypes across the globe. The first number in the system indicates in which of the possible seven world differential sets the pathogen is classified. This number is followed by a letter indicating the regional classification of the pathotype as well as another number indicating the regional differential set used. Pathotype 6E22A would thus fall into world differential set 6, but more precisely into differential set 22 of the European (E) classification (Johnson *et al*., 1972).

## 2.3 GENETIC RESISTANCE

### 2.3.1 How does genetic resistance work?

When a plant is attacked by a pathogen it can react in one of two ways: a disease susceptible plant will start to show stress symptoms whereas a resistant plant will sense the presence of the pathogen and elicit an appropriate defence response.

Pathogen and host interactions, or more specifically the wheat and *Puccinia* interaction, can be described as either a direct or an indirect mode of action. The direct mode of action involves the release of certain effectors from the pathogen upon infection, which bind with resistance receptors in the host cells and these receptors activate a signalling pathway for the defence response in the plant to be expressed. This type of receptor-ligand model was first described in 1971 by Flor who postulated the gene-for-gene concept in the interaction between flax and the rust pathogens. Today, this concept is also known as effector-triggered immunity (ETI). It is a typical avirulence reaction where the avirulence proteins, encoded by genes from the pathogen infecting the host, are recognized by specific receptors, encoded by the plant (Catanzariti *et al*., 2010). The mechanism of resistance genes in plant material can thus not be considered separately from the elicitors present in the pathogens that evoke the resistance response in a plant under attack.

Another commonly recognized theory on the mechanism of a host resistance reaction is the guard hypothesis which exemplifies the indirect mode of reaction. Resistance proteins encoded by the host plant associate with another plant protein that is targeted by pathogen effectors. They effectively "guard" this protein, known as the "guardee". When the guardee protein is ultimately modified by the effector proteins secreted by the pathogen during a state of infection, the associated guard proteins will sense this change and be triggered to signal the resistance response. The avirulence factors thus indirectly stimulate the resistance response. Many different types of elicitors will most likely lead to the same kind of resistance strategy being expressed (Catanzariti *et al*., 2010).

In some instances however, the pathogen effectors are not recognized by any of the plant's sensing mechanisms. The resistance response will thus not be activated in a direct or indirect manner and the pathogen will proceed to infect the host and produce disease symptoms. These unrecognisable effectors are known as virulence factors, enabling the pathogen to cause disease by avoiding activation of the plant defence system (Soosaar *et al*., 2005).

The resistance reaction in plants is commonly associated with a hypersensitive response (HR). This includes a form of programmed cell death around the infection site which phenotypically results in tiny necrotic flecks surrounded by healthy, uninfected plant tissue. It prevents the proliferation of the pathogen, localizes it to the dead plant cells and restricts spreading beyond the point of infection through the rest of the plant. Pathogens rarely survive a strong resistance response such as the HR (Soosaar *et al*., 2005; Catanzariti *et al*., 2010).

Resistance genes encode proteins that can be divided into different classes according to their structure. The main groups include cell surface pattern recognition receptors (PRR) and cytoplasmic nucleotide binding leucine-rich repeat (LRR) proteins that have a LRR domain at the C-terminal of the protein. The nucleotide binding site (NBS)-LRR proteins are localized within the plant cells, contrary to the cell surface PRR receptors, and can be subdivided into two further groups based on protein morphology. These proteins either contain a coiled coil (CC) domain or a *Toll*/*Interleukin-1* receptor (TIR) domain, similar to the TIR found in mammals, located at the N-terminal of the protein (Catanzariti *et al*., 2010). It is thus evident that resistance proteins share structural similarities. Avirulence proteins on the other hand demonstrate sequence, and thus structural, diversity.

By studying the flax-rust interaction, it was determined that the specificity of resistance genes is conferred specifically by the LRR domains of the encoded proteins which mean that this domain consequently regulates the protein-protein interactions (Chisholm *et al*., 2006). The LRR domain consists of 24-29 amino acids and alterations within this sequence are responsible for the most variation present in resistance receptor genes (Catanzariti *et al*., 2010).

Pathogen recognition clearly plays an essential role in disease resistance. Unfortunately only one mutation in the pathogen genome that changes the avirulence protein's tertiary structure will render it unrecognisable to the LRR domain of the resistance receptor and prevent the activation of the resistance response. On a molecular level, resistance can thus easily be overcome and virulence can be conferred to a pathogen. This intricate balance of receptor-ligand recognition causes selective pressure on the pathogen that drives the diversification, or even deletion, of avirulence genes to become virulence genes. In fact, the co-evolution of plants to develop more diverse pathogen receptors and pathogens to develop more divers and unrecognisable effectors, are a constant competition (Catanzariti *et al*., 2010).

### 2.3.2 Rust resistance genes in wheat

By 2012, more than 180 resistance genes for stem (57), leaf (71) and yellow (54) rust as well as a number of QTL's have been identified in wheat (McIntosh *et al*., 2012). Rust resistance genes include two types: pathotype specific resistance genes and pathotype non-specific resistance genes. Pathotype non-specific genes only confer partial resistance to the *Puccinia* fungi, but are effective against a broad range of pathotypes (Lagudah, 2011).

Furthermore, the rust resistance genes can be categorised as either seedling resistance genes or adult plant resistance (APR) genes. The former is expressed during the seedling stage of the wheat plant's lifecycle and may in many instances be expressed during the mature phase of the plant as well. It is more commonly associated with pathotype specific resistance genes. The latter is of course expressed after the seedling stage and is also known as field resistance. It is more commonly associated with pathotype non-specific resistance genes. Although these associations are considered a rule of thumb, gene expression can be altered under artificial conditions by manipulating the growth temperature and light exposure of the wheat plants (Lagudah, 2011).

The *Sr2* gene for resistance to stem rust and the *Lr34* gene that confers resistance to leaf rust, are two examples of valuable sources of pathotype non-specific APR. *Sr2* has been widely utilized in breeding programs since it was first transferred from wild emmer wheat to bread wheat in the 1920's. This source of stem rust resistance is situated on chromosome 3B of the wheat genome (Mago *et al*., 2011a). *Lr34* is situated on wheat chromosome 7D. Apart from providing valuable resistance to leaf rust, this gene is also associated with resistance to stripe rust (*Yr18*) and powdery mildew (*Pm38*). The *Lr34* gene has been sequenced and found to encode an ATP-binding cassette (ABC) transporter. This type of molecule is associated with the active transmembrane transport of a variety of biochemical compounds. In *Arabidopsis*, homologs of the *Lr34* ABC transporter are postulated to actively remove toxins from the plant cell. When considering the gene's function, it may explain how a single genetic factor can provide durable resistance against a number of pathogens (Krattinger *et al*., 2009). Neither of these genes, *Sr2* or *Lr34*, has been overcome by *Puccinia* pathotypes yet and is widely utilized in wheat breeding programs as the basis for building rust resistance in new cultivars. The pathotype non-specific resistance genes confer a so called slow rusting phenotype to wheat. This phenotypic response is associated with smaller as well as less uredinia and longer latent periods of rust activity post inoculation compared to susceptible plants (Lagudah, 2011).

The duration and efficiency of the rust resistance genes utilized within a wheat cultivar can be improved by pyramiding multiple resistance genes in the same cultivar (Simons *et al*., 2011). This strategy, used by plant breeders, aims to largely prevent pathogens from overcoming resistance by increasing the genetic barrier the fungi need to overcome. The pyramiding strategy also prolongs the shelf life of the individual resistance genes utilized in

the gene combinations. In some cases cultivars are marketed in a region specific manner, according to the resistance gene combinations they carry that will be effective in that area. This is done regardless of the slight yield deficit that may be associated with the improved resistance in cultivars since it contributes to the effective management of resistance resources.

It is important to utilize the correct combination of resistance genes in a wheat cultivar to achieve the maximum effect. Not all gene combinations will yield a much greater additive effect when compared to the individual effects, but genes utilizing different resistance mechanisms or genes working at different optimal temperatures may ensure resistance among a broader range of conditions and thus achieve a greater additive effect (Lagudah, 2011). Ideally one would also combine pathotype non-specific resistance genes conveying APR with several pathotype specific genes. Such combinations have in many instances ensured complete resistance to wheat rust.

### 2.3.3 Utilization of rust resistance genes in breeding programs through marker assisted selection

Of the more than 180 rust resistance genes identified in wheat, only a small amount remain effective against the rust fungi and have not yet been overcome by new virulent pathotypes. It is of vital importance to know which of these genes can still be utilized effectively in wheat cultivars and plant breeders strive to incorporate these genes in their breeding programs.

Molecular markers, closely linked to the various resistance genes, are used to screen potential novel wheat cultivars for the presence of resistance genes. There is also a perfect marker available for screening of the *Lr34* leaf rust resistance gene since the gene has been fully sequenced as previously mentioned (Krattinger *et al.*, 2009; Lagudah *et al.*, 2009). It is necessary to validate the markers utilized in a breeding program from time to time by performing rust inoculation tests on the plants putatively carrying the resistance genes. This ensures that the linked markers still segregate with the resistance genes and that genetic recombination did not separate the marker from the gene. In this way the resistance genes are maintained in the germplasm.

In a breeding program, an ideal wheat line will contain a maximum number of resistance genes, combined with other favourable agronomic traits from a good genetic background.

These traits include good baking quality and a semi-dwarf plant height. The SU-PBL currently utilizes a standardized panel of molecular markers (table 3.1) in the routine molecular screening of wheat nurseries. This panel includes markers for rust resistance genes, glutenin genes, which influence the baking quality of the wheat, and reduced height genes, which determine plant height. The PBL also employs a subset of six microsatellite markers that have been identified as a minimum marker set to be used in the genetic diversity assessment of wheat lines and backcrossing material in the nurseries (Honing, 2007).

The reduced height genes *Rht-B1b* and *Rht-D1b* that are included in this panel of markers, confer a semi-dwarfing phenotype to the wheat lines. This involves wheat lines with shorter, stronger stems which in turn permits high-yielding cultivars to better resist lodging prompted by heavier grain. *Rht-B1b* and *Rht-D1b,* previously known as *Rht1* and *Rht2*, were originally introduced into American wheat germplasm from a Japanese cultivar, Norin10, as part of a wheat improvement strategy in the 1960s. In 2002, these genes were sequenced and perfect markers for the different alleles of the reduced height genes were developed, hence *Rht-B1b* and *Rht-D1b*.Today *Rht-B1b* and *Rht-D1b* are present in approximately 70% of the world's commercially grown wheat cultivars (Ellis *et al*., 2002; Rebetzke *et al*., 2011)

In South Africa bread is a primary staple for a large part of the population and thus the major form in which wheat is consumed. The baking quality of wheat flour is an important factor to consider in the cultivation of commercial wheat cultivars. This is primarily determined by the gluten proteins found in the wheat endosperm that affect the visco-elasticity and the water retention capabilities of the dough. The baking industry requires wheat flour to meet a certain standard of protein quality in order for the industry to produce a high quality standardized product with the minimum input cost. Currently, the Sensako cultivar SST027 is the South African baking quality standard for wheat and the baking quality of new cultivars is measured relative to this standardized quality. Gluten consists of two components, namely glutenin and gliandin. Glutenin in its turn consists of high molecular weight subunits (HMW-GS) and low molecular weight subunits (LMW-GS). The SU-PBL's routine marker set contains markers for both the HMW-GS and the LMW-GS as it is the combinations of these sub-units present in the wheat endosperm that determine the protein quality of the wheat flour and essentially influence the baking quality (Xu *et al*., 2006; Yan *et al*., 2008).

MAS holds several advantages over traditional phenotypic selection in a plant breeding program. Firstly, MAS saves time in a breeding program. DNA can be extracted and screened

as soon as viable seedlings are developed, whereas phenotypic selection requires an entire growth cycle for sufficient phenotypic responses to be observed. Selection can also be completed before anthesis and informed crossings can thus be made in a breeding program. Furthermore, MAS can analyse traits that cannot be observed on a phenotypic level (Ben-Ari & Lavi, 2012). Screening of breeding material with molecular markers is a highly repeatable process and the results are independent of individuals' experience which may be a factor during the phenotypical scoring of plant material.

Lastly the SU-PBL also utilizes a minimum set of six microsatellite markers to assess the genetic diversity of the material within the wheat pre-breeding program. Microsatellites, also known as simple sequence repeats (SSR), are short repeating sequencing consisting of 2-6 nucleotides, which occur throughout the genomes of prokaryotes and eukaryotes. These molecular markers have a wide allelic diversity and are co-dominant as well as selectively neutral. Microsatellites offer a high level of polymorphism and are reproducible under different laboratory conditions, rendering it a marker of choice to be utilized in genetic diversity assessments (Li *et al*., 2001). Information on the genetic diversity of a crop is useful in the determination of novel genetic resources and germplasm conservation. It is also important in breeding programs to provide a basis for the identification of crossing parents and the prediction of progeny performance (Haile *et al*., 2013).

### 2.3.4 Pathotype virulence/avirulence patterns of SA wheat rust

A number of stem, leaf and stripe rust pathotypes have been identified in South Africa. These pathotypes' virulence/avirulence patterns are indicated in table 2.1. The introduction of pathotypes related to Ug99 in South Africa emphasised the need for plant breeders to employ effective resistance genes in wheat cultivars.

**Table 2.1: Leaf, stem and stripe rust pathotypes present in South Africa (adapted from Le Maitre, 2010)**

| | Pathotype | Avirulence genes | Virulence genes |
|---|---|---|---|
| **Leaf Rust** | UVPrt2 | *Lr1, Lr2a, Lr2b, Lr3ka, Lr11, Lr15,Lr17, Lr20, Lr24, Lr26, Lr30* | *Lr2c, Lr3a, Lr3bg, Lr10, Lr14a, Lr16* |
| | UVPrt3 (3SA123) | *Lr3a, Lr3bg, Lr3ka, Lr10, Lr11, Lr14a, Lr16, Lr17, Lr20, Lr26, Lr30* | *Lr1, Lr2a, Lr2b, Lr2c, Lr15, Lr24* |
| | UVPrt4 | *Lr1, Lr2a, Lr2b, Lr3bg, Lr11, Lr15, Lr16, Lr17, Lr24, Lr26* | *Lr2c, Lr3a, Lr3ka, Lr10, Lr14a, Lr20, Lr30* |
| | UVPrt5 | *Lr1, Lr2a, Lr3bg, Lr10, Lr11, Lr14a, Lr15, Lr17, Lr24, Lr26* | *Lr2b, Lr2c, Lr3a, Lr3ka, Lr16, Lr20, Lr30* |
| | UVPrt8 (3SA132) | *Lr3a, Lr3bg, Lr3ka, Lr11, Lr16, Lr20, Lr26, Lr30* | *Lr1, Lr2a, Lr2b, Lr2c, Lr10, Lr14a, Lr15, Lr17, Lr24* |
| | UVPrt9 (3SA133) | *Lr2a, Lr2b, Lr3bg, Lr15, Lr16, Lr17, Lr26* | *Lr1, Lr2c, Lr3a, Lr3ka, Lr10, Lr11, Lr14a, Lr20, Lr30* |
| | UVPrt10 (3SA126) | *Lr3a, Lr3bg, Lr3ka, Lr10, Lr11, Lr16, Lr20, Lr24, Lr26, Lr30* | *Lr1, Lr2a, Lr2b, Lr2c, Lr10, Lr14a, Lr15, Lr17* |
| | UVPrt13 (3SA140) | *Lr3a, Lr3bg, Lr3ka, Lr11, Lr16, Lr20, Lr30* | *Lr1, Lr2a, Lr2b, Lr2c, Lr10, Lr14a, Lr15, Lr17, Lr24, Lr26* |
| | *UVPrt20 (3SA145) | *Lr1, Lr2a, Lr2b, Lr2c, Lr9, Lr11, Lr16, Lr18, Lr19, Lr21, Lr23, Lr24, Lr25, Lr28, Lr29, Lr32, Lr36, Lr38, Lr45, Lr47, Lr50, Lr51, Lr52* | *Lr3,3bg,3ka,10,14a,15,17,20 26,27+31,30,33,B* |
| **Stem Rust** | UVPgt50 (2SA4) | *Sr8b, Sr9g, Sr13, Sr15, Sr21, Sr22, Sr24, Sr25, Sr26, Sr27, Sr29, Sr31, Sr32, Sr33, Sr35, Sr36, Sr38, Sr39, Sr43, SrEm, SrKiewiet, SrSatu* | *Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr9a, Sr9b, Sr9d, Sr9e, Sr9f, Sr10, Sr11, Sr12, Sr14, Sr16, Sr17, Sr18, Sr19, Sr20, Sr23, Sr28, Sr30, Sr34, Sr37, SrGt, SrLc* |
| | UVPgt51 (2SA36) | *Sr8b, Sr9e, Sr9g, Sr13, Sr15, Sr21, Sr22, Sr23, Sr24, Sr25, Sr26, Sr27, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr37, Sr38, Sr39, Sr43, SrAgi, Srdp2, SrEm, SrGt* | *Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr9a, Sr9b, Sr9d, Sr9f, Sr10, Sr11, Sr12, Sr14, Sr16, Sr17, Sr19, Sr20, Sr28, Sr34, Sr36, SrLc* |
| | UVPgt52 (2SA100) | *Sr8b, Sr9e, Sr9g, Sr13, Sr15, Sr21, Sr22, Sr25, Sr26, Sr27, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr36, Sr37, Sr38, Sr39, Sr43, SrAgi, Srdp2, SrEm, SrGt, SrKiewiet, SrSatu* | *Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr9a, Sr9b, Sr9d, Sr9f, Sr10, Sr11, Sr12, Sr14, Sr16, Sr17, Sr18, Sr19, Sr20, Sr23, Sr24, Sr28, Sr34, SrLc* |
| | UVPgt53 (2SA102) | *Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr11, Sr13, Sr15, Sr17, Sr21, Sr22, Sr23, Sr24, Sr25, Sr26, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr36, Sr37, Sr38, Sr39, Sr43, SrEm, SrGt, SrKiewiet, SrSatu* | *Sr7a, Sr7b, Sr8a, Sr9a, Sr9d, Sr9f, Sr9g, Sr10, Sr12, Sr14, Sr16, Sr19, Sr20, Sr27, Sr30, Sr34, SrLc, SrTobie* |
| | UVPgt54 (2SA55) | *Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr9g, Sr13, Sr15, Sr21, Sr22, Sr23, Sr24, Sr25, Sr26, Sr27, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr38, Sr39, Sr43, SrEm, SrGt* | *Sr7a, Sr8a, Sr9a, Sr9d, Sr9f, Sr10, Sr11, Sr12, Sr14, Sr16, Sr19, Sr20, Sr34, SrLc* |
| | UVPgt55 (2SA88) | *Sr13, Sr14, Sr21, Sr22, Sr24, Sr25, Sr26, Sr27, Sr29, Sr31, Sr32, Sr33, Sr35, Sr36, Sr37, Sr39, Sr42, Sr43, Sr44, SrEm, SrTmp, SrSatu* | *Sr5, Sr6, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr16, Sr17, Sr30, Sr34, Sr38, Sr41, SrMcN* |

**Table 2.1 continued from previous page.**

| | Pathotype | Avirulence genes | Virulence genes |
|---|---|---|---|
| **Stem rust** | UVPgt56 (2SA104) | *Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr11, Sr17, Sr21, Sr24, Sr30, Sr31, Sr36, Sr38, SrEm, SrSatu, SrTobie* | *Sr8a, Sr9g, Sr27, SrKiewiet* |
| | **UVPgt58 (2SA103) | *Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr9g, Sr11, Sr13, Sr15, Sr17, Sr21, Sr22, Sr23, Sr24, Sr25, Sr26, Sr29, Sr30, Sr31, Sr32, Sr33, Sr35, Sr36, Sr37, Sr38, Sr39, Sr43, SrEm, SrGt, SrKiewiet, SrSatu* | *Sr7a, Sr7b, Sr8a, Sr9a, Sr9d, Sr9f, Sr10, Sr12, Sr14, Sr16, Sr19, Sr20, Sr27, Sr30, Sr34, SrLc, SrTobie* |
| | **UVPgt59 (2SA106) | *Sr13, Sr14, Sr21, Sr22, Sr25, Sr26, Sr27, Sr29, Sr31, Sr32, Sr33, Sr35, Sr36, Sr37, Sr39, Sr42, Sr43, Sr44, SrEm, SrTmp, SrSatu* | *Sr5, Sr6, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr16, Sr17, Sr24, Sr30, Sr34, Sr38, Sr41, SrMcN* |
| | ▪UVPgt60 (2SA107) | *Sr13, Sr14, Sr21, Sr22, Sr25, Sr26, Sr27, Sr29, Sr32, Sr33, Sr35, Sr36, Sr37, Sr39, Sr42, Sr43, Sr44, SrEm, SrTmp, SrSatu* | *Sr5, Sr6, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr16, Sr17, Sr24, Sr30, Sr31, Sr34, Sr38, Sr41, SrMcN* |
| | [a]Ug99 (TTKS) | *Sr21, Sr22, Sr25, Sr26, Sr27, Sr29, Sr32, Sr33, Sr35, Sr39, Sr40, Sr42, Sr43, SrAgi, SrEm* | *Sr5, Sr6, Sr7b, Sr8a, Sr8b, Sr9b, Sr9e, Sr9g, Sr11, Sr15, Sr17, Sr24, Sr30, Sr31, Sr36, Sr38* |
| **Yellow Rust** | 6E16A- | *Yr1, Yr3a, Yr4a, Yr4b, Yr5, Yr9, Yr10, Yr15, Yr25, Yr27, YrA, YrCle, YrCv, YrHVII, YrMor, YrSd, YrSp, YrSu* | *Yr2, Yr6, Yr7, Yr8, Yr17* |
| | 6E22A- | *Yr1, Yr3a, Yr4a, Yr4b, Yr5, Yr9, Yr10, Yr15, Yr27, YrA, YrCle, YrCv, YrHVII, YrMor, YrSd, YrSp, YrSu* | *Yr2, Yr6, Yr7, Yr8, Yr17, Yr25* |
| | ▪▪6E22A+ | *Yr1, Yr3a, Yr4a, Yr4b, Yr5, Yr9, Yr10, Yr15, Yr27, YrSp,* | *Yr2, Yr6, Yr7, Yr8, Yr17, Yr25, YrA* |
| | 7E22A- | *Yr3a, Yr4a, Yr4b, Yr5, Yr9, Yr10, Yr15, Yr27, YrA, YrSp* | *Yr2, Yr6, Yr7, Yr8, Yr17, Yr25* |

[a]Ug99 does not occur in South Africa (as of November 2009) and is only included to illustrate the relatedness of other pathotypes

* (Terefe *et al*., 2011) ** (Visser *et al*., 2011)

▪ (Pretorius *et al*., 2010) ▪▪ (Marais *et al*., 2009)

### 2.3.5 New sources of genetic resistance

Commercial bread wheat is an inbred crop. In terms of genetic resistance to wheat rust, the *T. aestivum* gene pool is fairly depleted. Wild relatives of wheat serve as an abundant genetic resource for improving the genetic variation in cultivated wheat (Dadkhodaie *et al*., 2011). Plant breeders have access to the primary (*Triticum* spp.*)*, secondary (*Aegilops* spp.*)* and tertiary (*Tritiaceae* spp.) gene pools of wheat, primarily constituted of wild wheat species and related grasses. These gene pools may provide sources of insect resistance, heat and cold tolerance, drought resistance as well as saline tolerance (Marais *et al*., 2006).

The polyploid nature of the wheat genome facilitates the survival of genetically unbalanced genomic material within the nucleus. This permits the introgression of foreign DNA into the wheat genome since even the addition of whole chromosome arms from a different genome may be tolerated (Marais *et al.*, 2006; Endo, 2007).

As early as 1989 it was shown that plants have morphologically and functionally similar resistance genes for related pathogens (Kobayashi *et al*., 1989; Cook, 1998). An experiment was performed where an avirulence gene from a tomato pathogen, *P. syringae* pv. *tomato*, was transformed to a soybean pathogen, *P. syringae* pv. *glycinea*. This pathogen elicited a resistance response in a soybean cultivar even though it did not carry the corresponding resistance gene from tomato. If plants as different as tomato and soybean can have almost identical responses to pathogen elicitors, it provides ever the more reason that closely related genotypes to wheat may contribute greatly to the search of new sources of rust resistance in wheat breeding.

## 2.4 RESISTANCE GENES TRANSFERRED TO WHEAT

Over a period of several years, Marais *et al.* (2003; 2005a; 2005b; 2008; 2010a) transferred a number of novel leaf rust and stripe rust resistance genes from related species into the hexaploid wheat genome. These translocations included the following genes: *Lr53/Yr35*, *Lr54/Yr37*, *Lr56/Yr38*, *Lr59* and *Lr62/Yr42* (table 2.2).

**Table 2.2: Origin of the species derived leaf and stripe rust genes utilized in this study**

| Original Introgession (i)/ Translocation (t) | Leaf and stripe rust resistance genes | SU-PBL entry | Pedigrees |
|---|---|---|---|
| S8 i | *Lr53/Yr35* | 05M64 | CS*4/*T. dicoccoides*//3*CS-S/3/CS/4/2*Thatcher |
| S12 i | *Lr56/Yr38* | 05M65 | CS*9/*Ae. sharonensis*//3*W84-17/3/CS/4/W84-17/5/2*Thatcher |
| S14 t | *Lr54/Yr37* | 03M130 | CSDM2D//CS*4/*Ae. kotshyi*/3/2*Thatcher |
| S15 t | *Lr59* | 05M16 | CS*2/*Ae. peregrina*//10*W84-17 |
| S20 t | *Lr62/Yr42* | 03M119-71 | CSDM3B-5B//CS*5/*Ae. neglecta*/3/W84-17 |

(CS = Chinese Spring)

### 2.4.1 *Lr53/Yr35*

In 2003, Marais *et al*. identified two genes that conferred resistance to leaf and stripe rust, derived from *Triticum dicoccoides*, present in wheat lines that originally came from Israel. These genes were designated as *Lr53* and *Yr35*. *T. dicoccoides*, or wild emmer wheat, has a tetraploid AABB genome and is considered as part of the primary gene pool for hexaploid bread wheat (Dadkhodaie *et al*., 2011; Peng *et al*., 2011). Several other rust resistance genes have been introduced into wheat from other *Triticum* species such as *Lr18* from *T. timopheevii* situated on wheat chromosome 5BL ; *Yr15* from *T. dicoccoides* situated on wheat chromosome 1BS and *Lr36* together with *Yr36* also from *T. dicoccoides* located on chromosome 6BS.

*Lr53* and *Yr35* were introgressed into a Chinese Spring background. Chromosome analysis showed *Lr53/Yr35* to be located on the short arm of wheat chromosome 6B. This may have resulted due to the homology of the B-genomes present in both *T. dicoccoides* and *T.*

*aestivum*. Although *Lr53* and *Yr35* were at first reported to co-segregate and therefore be tightly linked (Marais *et al*., 2003; Marais *et al*., 2005b; Dadkhodaie *et al*., 2011), some genetic recombination between the genes were later identified. Dadkhodaie *et al*. (2011) observed a recombination frequency of 3% between the two genes while Marais (2005b) found an 11% recombination in a backcross population. This information implies that the recombination rate between *Lr53* and *Yr35* may vary between different crosses and that the genetic background of the relevant wheat lines may influence the recombination ability.

Resistant plants, homozygous for *Lr53* and *Yr35*, proved to be agronomically sustainable. They were fertile, presented a normal phenotype and they produced plump seeds. Studies performing reciprocal crossings indicated that *Lr53/Yr35* had a strong preferential pollen transmission of between 96-98%. The female transmission varied according to the genetic background of the ovary donor, but ranged between 41-66% (Marais *et al*., 2005b).


**Figure 2.4: (a) *T. dicoccoides* ears on the left compared to (b) a wheat ear on the right. (Danin, 2008; 2011)**

### 2.4.2 *Lr54/Yr37*

The linked genes *Lr54* and the *Yr37* originate from *Aegilops kotschyi*. *Ae. kotschyi* is a wild tetraploid grass with a UUSS genome. It's naturally native to Northern Africa, the Mid-East and Western Asia. The spikelets of this wild grass usually contain two fertile florets with rough outer glumes (Clayton, 2006).

Breeders rely on complex molecular mechanisms, like cross species hybridization and homologous chromosome recombination when trying to transfer resistance genes from wild species to domesticated crops like wheat. Unpaired meiotic chromosomes are predisposed to undergo centric misdivision during cell division. These chromosomes are inclined to break at the centromeres to form telocentric chromosomes and then arbitrarily fuse to another chromosome, establishing a whole arm translocation. This is also the hypothesis put forward by Marais *et al*. (2005a) of how the chromosome fragment containing *Lr54/Yr37* was transferred to the wheat genome from a wheat line with disomic addition chromosomes from *Ae. kotschyi*, when crossed with monosomic Chinese Spring wheat. Marais *et al*. (2005a) found that the entire chromosome arm containing *Lr54/Yr37* replaced wheat chromosome 2DL.

Such a large amount of foreign chromatin in a wheat line is undesirable in a breeding program since it is associated with linkage drag and could present unwanted agronomic effects. This particular translocation seemingly included an uncharacterized gene for reduced plant height which, in combination with the reduced height gene *Rht-B1b* present in the Chinese Spring genetic background, produced a double dwarf phenotype that impeded the use of lines containing *Lr53/Yr37* in breeding programs (Marais *et al*., 2005a). Plants containing *Lr53/Yr37* also showed an earlier flowering time that had presumably been caused by a photoperiod insensitivity gene transferred with the resistance genes. The shortening of the translocation containing *Lr53/Yr37* was thus of paramount importance.

The structural discrepancies between chromosome 2DL of wheat and the chromosome arm inherited from *Ae. kotschyi* however prevented homologous recombination during meiosis and caused the entire chromosome arm to be inherited as a single large linkage block. Fortunately, enough homoeology existed between the translocated chromosome arm and wheat chromosome 2DL so that homoeologous recombination could be induced in *Ph1* mutants. The *Pairing homoeologous 1* (*Ph1*) gene is situated on wheat chromosome 5B and induces homoeologous chromosome pairing in wide crosses (Al-Kaff *et al*., 2008). In 2011, Heyns *et al*. reported on a developed wheat line with a shortened *Ae. kotschyi* translocation on chromosome 2DL. This line contained both *Lr54* and *Yr37*, but lacked the uncharacterized reduced height gene, which enabled the use of this translocation in wheat breeding programs. These genes showed preferred pollen transmission of about 96%, but a relatively normal expected female transmission (Marais *et al*., 2005a).


**Figure 2.5: (a) *Ae. kotschyi* ear compared to (b) wheat ear. (c) *Ae. kotschyi* on the left progressively looking more like wheat as continual backcrossing took place and the *Ae. kotschyi* derived chromatin became less. (d) Wheat leaves showing severe leaf rust symptoms on the left, compared to a wheat plant containing *Lr53/Yr37* on the right, showing signs of the hyper sensitive resistance response. (Danin, 2007)**

### 2.4.3 *Lr56/Yr38*

The *Lr56/Yr38* gene combination was transferred by Marais *et al*. (2006) from *Aegilops sharonensis* into a wheat line. *Ae. sharonensis* is a goat grass with a limited natural habitat in the coastal plain of Israel and a few spots in the south of Lebanon. This diploid goat grass (SS) is a valuable source of genetic resistance that can be employed in wheat, but due to the rapid urbanization and expansion of agriculture into its natural habitat, the genetic diversity of this wild species is actually under threat itself (Olivera *et al*., 2007).

*Ae. sharonensis* forms part of the secondary gene pool of wheat and its genome is therefore not homologous to that of wheat, which complicated the transfer of genes across genomes. Before the transfer of *Lr56/Yr38*, no other resistance genes have been sourced from the *Ae. sharonensis* genome although the closely related *Ae. speltoides* contributed greatly to leaf rust resistance genes utilized in wheat breeding. Some of these include *Lr28*, *Lr36*, *Lr47* and *Lr51*.

The original accession containing *Lr56/Yr38* that was obtained from Israel was backcrossed several times into a Chinese Spring genome. Genetic mapping showed *Lr56/Yr38* to be located on wheat chromosome 6AL (Marais *et al*., 2010a). Under artificial conditions, plants that were homozygous for these rust resistance gene displayed an acceptable phenotype and

high levels of fertility. These genes were however associated with alien DNA that translocated from the *Ae. sharonensis* genome and were undesirable for breeding purposes. Marais *et al.* (2010a) successfully managed to remove large parts of foreign DNA from the wheat genome in order to prevent deleterious agronomic effects in potential wheat cultivars. This was also achieved through the induction of homoeologous recombination in crossings with *Ph1* mutants. The agronomic quality of lines containing the *Lr56/Yr38* genes however still needs to be verified in elite germplasm.

As the case was with the previously mentioned resistance genes utilized in this study, *Lr56/Yr38* also did not follow a pattern of Mendelian inheritance. It showed a decreased female transmission of 35% and an exceptional preferential male transmission of 100% (Marais *et al*., 2006).

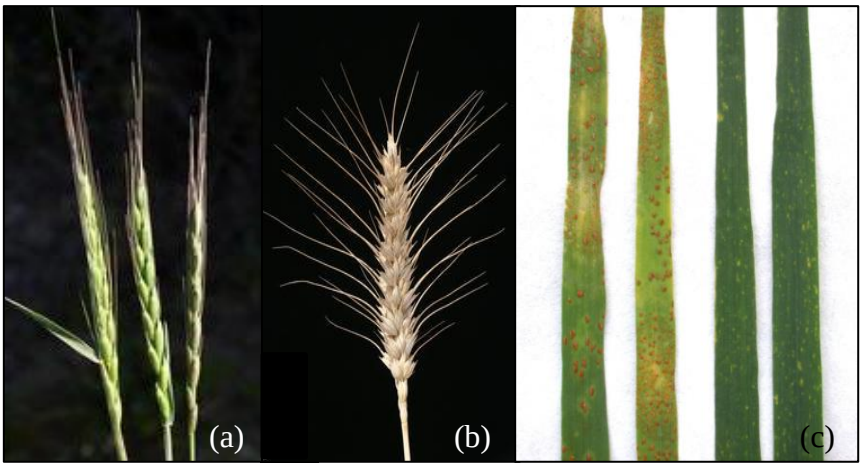



**Figure 2.6: (a) *Ae. Sharonensis* ears compared to (b) wheat ear. (c) Wheat leaves showing leaf rust symptoms on the left, compared to a wheat plant containing *Lr56/Yr38* on the right showing small necrotic flecks as part of the resistance response (Danin, 2007; 2011)**

### 2.4.4 *Lr59*

The leaf rust resistance gene *Lr59* was transferred to wheat from *Aegilops peregrina.* The translocation of this leaf rust resistance gene is said to have occurred spontaneously when Marais *et al*. (2008) backcrossed a leaf rust resistant line from Israel with hexaploid bread wheat.

*Aegilops peregrina* is also more commonly known as one of the goat grass species. It has a tetraploid genome consisting of U and S subgenomes. Its natural geographic distribution and phenotype is similar to that of *Ae. kotschyi* which belongs to the same grass family.

The transfer of *Lr59* most likely resulted from centromeric breaks and fusion when *Ae. peregrina* was backcrossed to hexaploid wheat. Microsatellite and monosomic analysis indicated that this gene is present on wheat chromosome 1AL (Marais *et al*., 2008). Female transmission of this gene seemed to be normal although the male transmission appeared to be higher than the expected Mendelian pattern of inheritance.

In 2009 Kotze attempted to identify wheat lines with the shortest possible amount of *Ae. peregrina* chromatin still containing the *Lr59* resistance gene. In that study, shorter fragments of foreign DNA containing *Lr59* were successfully identified with the utilization of microsatellite and SCAR markers. The shorter fragments were obtained via homoeologous recombination between the wheat chromosome 1AL and the donor *Ae. peregrina* chromosome. It is speculated that the S-genome of *Ae. peregrina* is the donor genome for *Lr59* rather than the U genome, since the S-genome is more similar to wheat and would therefore allow greater homoeologous pairing and effectively more recombination to shorten the *Lr59* fragment. It was however also postulated that some of the recombination events arose from abnormal meiotic pairing events and may have led to genomic instability. When the *Lr59* genes are introduced into a breeding program, special care should be taken with regard to the agronomic quality of the resulting progeny to ensure that unfavourable traits from possible genetically unstable donor lines aren’t inherited with the leaf rust resistance gene (Kotze, 2009).

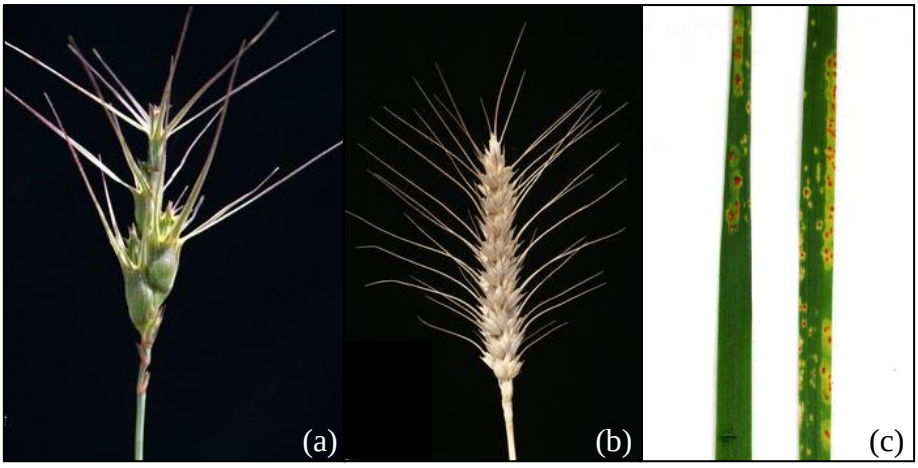



**Figure 2.7: (a) *Ae. Peregrina* ear compared to (b) a wheat ear. (c) Wheat leaves showing leaf rust symptoms on the right, compared to a wheat plant containing *Lr59* on the left showing less pustules and small necrotic flecks as part of the resistance response. (Danin, 2006; 2007).**

### 2.4.5 *Lr62/Yr42*

The last combination of rust resistance gene utilized in this study is the *Lr62* and *Yr42* genes. These genes were translocated from *Aegilops neglecta* to hexaploid wheat (Marais *et al*., 2009). *Ae. neglecta* is another member of the goat grass family.

The rust resistance genes *Lr62* and *Yr42* were obtained from a Californian wheat line that showed leaf and stripe rust resistance. The original translocated region containing these genes also contained a large amount of foreign DNA from *Ae. neglecta*. It was suspected to have replaced the entire wheat chromosome 6AS as well as the proximal part of 6AL. The *Lr62* and *Yr42* resistance genes were later found to be located on the distal end of wheat chromosome 6AS. Marais *et al*. (2010b) managed to reduce the amount of *Ae. neglecta* chromatin in the translocation and replaced it with wheat chromatin.

The original *Ae. neglecta* translocation showed strong preferential female transmission, contrary to the other resistance genes utilized in this study that mainly showed preferred pollen transmission. The *Lr62/Yr42* genes however showed different patterns of preferred transmission that varied according to the genetic background of the heterozygote in question.

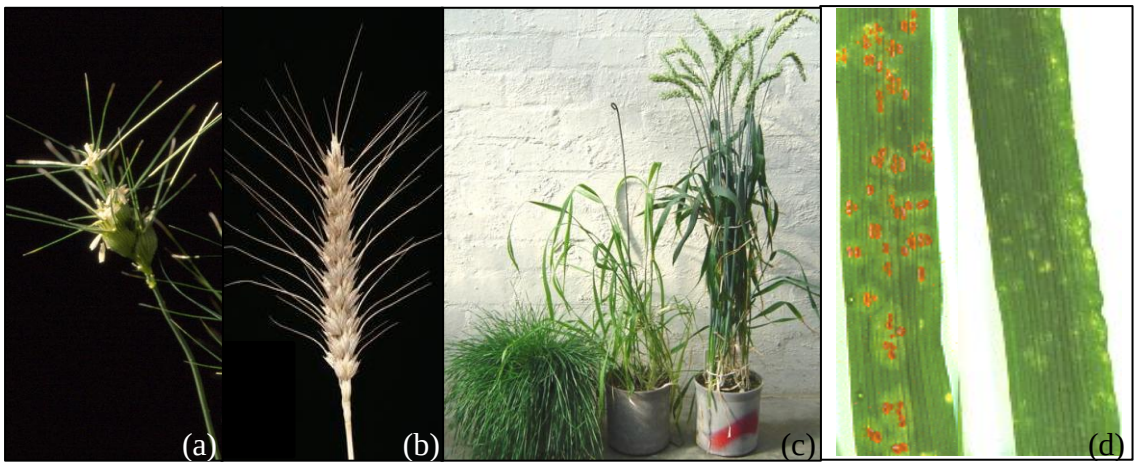



**Figure 2.8: (a) *Ae. neglecta* ear compared to a (b) wheat ear. (c) *Ae. neglecta* on the left progressively looking more like wheat as continual backcrossing took place and the *Ae. neglecta* derived chromatin became less. (d) Wheat leaves showing leaf rust symptoms on the left, compared to a wheat plant containing *Lr62/Yr42* on the right, showing signs of the hyper sensitive resistance response. (Wheat Genetic and Genomic Resources Centre, 2012; Danin, 2007)**

### 2.4.6 Segregation distortion

All of the resistance genes utilized in this study show some form of preferential transmission, mostly through male pollen cells. The resulting genotypes of progeny that carry the resistance genes thus deviate from the expected Mendelian ratio whenever the male crossing parents are carriers of the resistance genes. This deviation from the anticipated ratio is known as segregation distortion. It is a common occurrence in the plant kingdom and was first described in 1926 by Mangelsdorf and Jones who observed it in maize. Segregation distortion can be caused by a number of physiological or genetic factors (Hao *et al*., 2013; Tang *et al*., 2013).

During meiosis, certain alleles may be overexpressed in the gametes. This meiotic drive thus ensures that a larger number of gametes containing the gene of interest will potentially fertilize the ovary compared to gametes without it. The progeny will also have a greater chance of containing the gene of interest and will thus exhibit segregation distortion. Zygotic selection may also contribute to segregation distortion. In this case the normal expected genotypic ratio of zygotes is formed, but differential mortality in the embryo causes a distorted genotypic ration in the mature progeny (Tang *et al*., 2013).

Another, and perhaps the most probable cause of segregation distortion, is viability selection in gametes (Tang *et al*., 2013). This may be caused by gametocidal chromosomes or segregation distortion loci, both considered as "selfish" genetic elements. Genes that are beneficial to organisms are often maintained in the genome because it confers survival fitness under natural selection. Some genes however are maintained in the genome via other "selfish" mechanisms.

A certain group of chromosomes in wheat are known as gametocidal chromosomes (*Gc*). These chromosomes were introduced into the wheat genome during crosses made with the wild *Aegilops* species. *Gc* genes causes sterility in gametes that lack the presence of this gene. It causes chromosome breakage in those gametes ensuring "selfish" survival in the progeny. Different *Gc* genes show varying degrees of intensity and are also influenced by the genetic background of the relevant wheat line (Endo, 2007).

There are also segregation distortion (*Sd*) genes that alter the Mendelian pattern of inheritance. A well-known example of these *Sd* genes are those that were associated with the leaf rust resistance gene, *Lr19*. *Lr19* was originally transferred to wheat from *Thinopyrum ponticum* in 1966 (Sharma & Knott, 1966). The original translocation showed preferential pollen transmission, caused by the *Sd1* gene located proximally to *Lr19* on the translocated segment (Zhang & Dvořák, 1990). Gametes without the *Sd1* gene aborted, thus effectively ensuring preferential transmission of the *Lr19* gene (Marais *et al*., 2001). The translocation containing *Lr19* was shortened and the *Sd1* gene was eliminated. The leaf rust gene however still showed non-Mendelian inheritance which was found to be the cause of the *Sd2* gene, also associated with *Lr19*. *Sd2* initiated self-elimination which distorted the inheritance of *Lr19* in the opposite direction of *Sd1* (Prins *et al*., 1997). In a different genetic background, *Sd2* however also showed preferential transmission (Prins & Marais, 1998; Prins & Marais, 1999). Segregation distortion in wheat lines specifically containing the *Lr19* gene was also observed more recently by Wessels (2010) in a study on the creation of a wheat nursery with pyramided rust resistance genes. This emphasises the effect of the wheat genome in the expression of certain segregation distortion factors and the potential difficulty of introducing species derived genes into the wheat genome.

Lastly, maternal cytoplasmic elements may also play a role in segregation distortion. In plants the cell cytoplasm contains a number of organelles. Amongst these are mitochondria and chloroplasts which both have their own DNA. The expression of these genes in the cell

may have an epistatic effect on the nuclear expressed genes and influence gamete viability. The cytoplasm is furthermore the milieu for a number of biochemical metabolic reactions which may differentially affect gene expression in gametes (Tang *et al*., 2013).

## 2.5 MICROSPORE TISSUE CULTURE AND DOUBLED HAPLOID PRODUCTION

### 2.5.1 The application of DH technology

The ever increasing food demand requires producers to deliver more agricultural commodities to sustain the globe. Due to the current financial climate and limited natural resources, producers want to maximise their yields and profit margin with the minimum capital and resource input. Plant breeders are therefore required to constantly improve crop yields as well as resistance to disease and abiotic stresses in new wheat cultivars. Almost half of the crop yield increases in the last fifty years were obtained through genetic improvement of cultivars with the remainder attributed to agro-chemistry, irrigation and mechanization (Germanà, 2011). Both traditional and biotechnological techniques are employed to create wheat cultivars with novel gene combinations, and thus improved traits, to fulfil the requirements of producers.

A wheat breeding program requires homozygous pure breeding plants to ensure fixation of individual genes in subsequent generations before selection of a potential new cultivar can take place. Through conventional pedigree, single seed descent and backcrossing it takes between four and six generations to obtain what can be considered as homozygous individuals. Doubled haploid (DH) technology has however enabled breeders to accelerate this rate. A hundred percent homozygosity can be achieved in one generation when using DH technology compared to the several generations required when employing conventional breeding protocols (Germanà, 2011). Throughout the last decades the frequency of haploid production has increased and a variety of tissue culture protocols for a number of plant species and agricultural crops have been established. Doubled haploid technology is thus a valuable biotechnological technique that can be utilized to accelerate the breeding cycle and ultimately, the release of new cultivars (Germanà, 2011).

Haploid plants, and essentially doubled haploids, can be produced via the wide crossing method or from either male or female gametes through androgenesis or gynogenesis (figure 2.9).

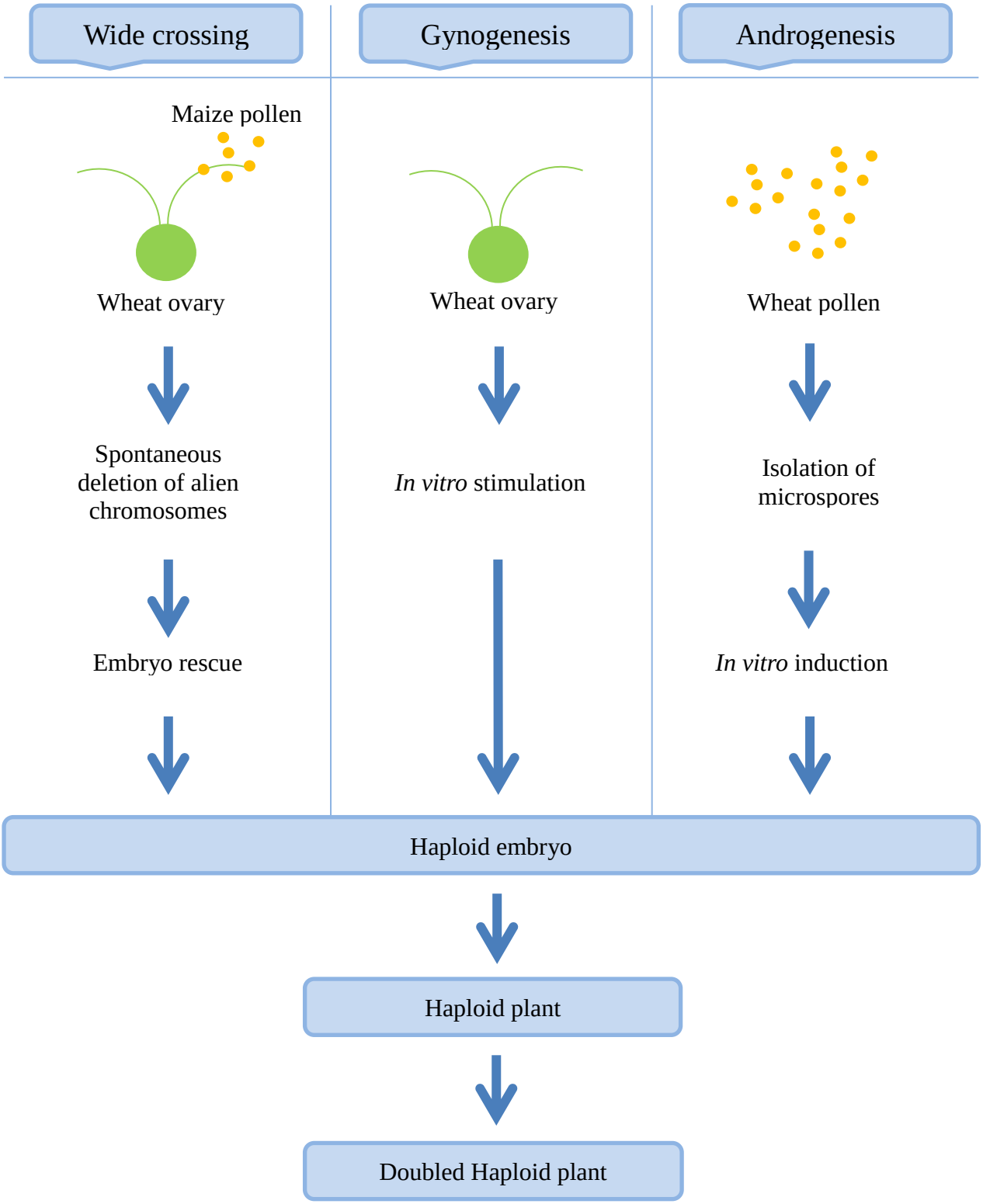



**Figure 2.9: Doubled haploid techniques involving wide crosses, gynogenesis or androgenesis**

### 2.5.2 Wide crossing

Success has been achieved at the SU-PBL, as well as other institutions, with the use of the wide crossing technique where wheat ovaries are pollinated with *Zea Mays* (Eudes & Chugh, 2009; Wessels, 2010). After pollination, the paternal chromosomes are spontaneously eliminated from the hybrid embryo. Haploid embryos are then rescued because of poor endosperm development preventing the embryos from maturing in the caryopsis. The embryos are cultivated *in vitro*, followed by colchicine treatment to induce chromosome doubling. This technique is less genotype dependant than the anther culture techniques (Eudes & Chugh, 2009), however, with wide crossing, the production of haploids is limited to only one plant per pollinated floret. In rare cases, some of the paternal chromosomes may also survive in the embryo, leading to the presence of foreign DNA in the DH plants.

### 2.5.3 Gynogenesis

Gynogenesis, where plant flowers are stimulated *in vitro* to undergo embryogenesis without pollination, is not a widely utilized technique for creating haploid plants in wheat or other cereals. It is primarily used for species that are unresponsive to androgenesis. With this technique, the production of haploid plants is also limited to the number of ovaries utilized, usually far less than the number of anthers used in androgenesis (Wędzony *et al*., 2009).

### 2.5.4 Androgenesis

Androgenesis has become a standard research tool in breeding of plant species such as wheat, barley and rapeseed (Germanà, 2011). It involves the diversion of microspores under optimal culture conditions to undergo a sporophytic pathway instead of following the normal gametophytic lifecycle. The haploid microspores are manipulated to undergo embryogenesis and ultimately develop into a haploid plant. Chromosome doubling can then be induced in the haploid plant cells by treating plants with colchicine. This will effectively produce a complete homozygous diploid, or then hexaploid in the case of wheat (Ferrie & Caswell, 2011).

Androgenesis in wheat can be achieved via two techniques: anther culture and the isolated microspore tissue culture method. Under perfect conditions, both of these techniques are theoretically able to produce vast numbers of haploid plants from a small number of anthers due to the thousands of microspores contained in them. In this study the microspore tissue

culturing method was utilized as it holds several advantages over anther culture. There is no risk of somatic cells from the anther wall developing into callus or ultimately embryos. The microspore tissue culture system permits better nutrient uptake for the dividing microspores since they are in direct contact with the media. Anther culture also requires micro surgical skills and may be very time consuming. The microspore tissue culturing method provides a faster alternative (Ferrie & Caswell, 2011).

The application of doubled haploid technology utilizing the isolated microspore technique however also has its complications. Not all species respond equally well to microspore *in vitro* propagation. Studies have found wheat to respond particularly poorly in terms of embryo formation and the large percentage of albino embryos generated. Within the wheat species, differential responses to microspore tissue culturing have also been observed between different genotypes (Cistue & Echa, 2009; Mago *et al*., 2011b). It thus seems necessary to optimize the isolated microspore tissue culturing protocol for each donor species and perhaps even further for each particular breeding program to accommodate the particular genotypes utilized in it. Difficulty has previously been encountered with genotype interactions when employing the wide crossing technique on wheat material containing species derived chromatin at the SU-PBL (Wessels, 2010). It was nevertheless decided to use the microspore tissue culturing technique in this study. The theoretical advantages of this technique outweighed any of the alternatives.

### 2.5.5 Factors to consider when applying microspore tissue culture in wheat.

The basic microspore propagation technique consists of three phases. Firstly, the pre-treatment phase which includes the cultivation and preparation of the donor plants, then the induction phase where the microspores are isolated and put into tissue culture induction media and lastly, the regeneration phase where the formed embryos develop into haploid plants and are hardened off. These haploid plants may then proceed to the chromosome doubling colchicine treatment. In rare cases spontaneous chromosome doubling may occur. A negative correlation has however been found between embryogenesis rate and spontaneous chromosome doubling (Ferrie & Caswell, 2011).

The regeneration frequency, also referred to as the embryo recovery rate indicates the percentage of embryos that developed into green plants and is determined by a number of factors influencing the different phases of the microspore process. During the pre-treatment

phase, a number of factors play a role in the eventual wheat embryo recovery rate. Whether donor plants are grown in greenhouses or growth chambers has been found to play a particularly large role in the micro-propagation of especially cereal crops, where albinism is a big concern. In a growth chamber the temperature can be controlled which affects the development of the donor ears.

Field grown donor material may also be more prone to contamination during the culturing stage opposed to greenhouse grown plants (Cistue & Echa, 2009; Ferrie & Caswell, 2011; Santra *et al*., 2012). *In vitro* contamination of tissue culture has been known to obstruct the study of microspore derived haploid production. The media utilized in these studies are rich in sugars, amino acids and other macro and micro nutritional elements. It is thus a good source of nutrients for microorganisms. Microbial contamination could include bacteria, yeast and fungi. A potential source of microbial contamination is infected donor plants, which would be more probable when utilizing field grown material compared to material from a greenhouse. These plants could contain microorganism that are resistant to the surface sterilization of the material prior to the initiation of the microspore isolation process or it could retain endophytic bacteria that are nearly impossible to remove via surface sterilization. Other sources of contamination could involve inadequate sterile conditions or contamination deriving from the operator. Bacteria and yeast are prone to rapid colony growth during the first day or two in enriched media. Growth can be observed as white, yellow or pink coloration of the media. Some bacteria like *Pseudomonads* are undetectable with the naked eye, but may also cause microspore growth impairment. Bacteria and yeast are particularly adaptable to the culturing conditions required for microspore growth. This rapid growth of microbes may result in plant cell death and entire microspore cultures may be lost. Several antibiotics have been evaluated in triticale and wheat microspore tissue culturing studies. Cefotaxime and Vancomycin were found to be the most successful in limiting microbial growth without compromising microspore development. Cefotaxime has in fact been found to increase ELS production and reduce the occurrence of albinism. Not all microbial growth can however be controlled by utilizing these antibiotics. Neither Cefotaxime nor Vancomycin was effective in the control of yeast and certain *Pseudomonas* species isolates. Furthermore, significant genotype x antibiotics interaction was noted in wheat microspore cultures (Asif *et al*., 2013a)

The collection period of the donor spikes is also significant since the photoperiod and light intensity the plants are exposed to may influence the microspore division. The physiology of the donor spikes is important as it may have a big influence on the initial microspore concentration during the induction phase. Treatments like fertilizer may indirectly play a role in the embryo recovery rate since it directly influences the quality of the donor spikes used. Once the donor spikes are harvested, optimally when the microspores are at the mid to late uni-nucleate or early bi-nucleate state, the spikes are subject to a cold incubation treatment prior to microspore isolation. This cold treatment may last between 21 and 28 days with the length of the incubation period affecting the growth stage of the microspores during isolation, and essentially also embryo formation (Cistue & Echa, 2009; Ferrie & Caswell, 2011; Santra *et al*., 2012).

During the microspore induction phase, the primary influence on the embryo recovery rate is most likely the composition of the induction media (Santra *et al*., 2012). A fine balance of macro- and micro-elements is necessary to sustain the development of the fragile spores. Cell death of microspores starts as early as the induction phase and typically continues throughout the process. It is mainly due to oxidative damage to the cell organelles, caused by reactive oxygen species (ROS) generated during the stress treatments applied to the microspores. This may include pre-treatment stressors as well as the physical stress exerted on the cells by the microspore isolation procedure. ROS mainly target cell plastids, which in the case of wheat is the mitochondria and chlorophyll. These organelles have a direct influence on the cell's viability and green plant regeneration, effectively controlling the embryo recovery rate (Asif *et al*., 2013b).

As previously mentioned, albinism is a big concern during monocot androgenesis, particularly in the production of wheat haploids. The production of albino embryos is a heritable trait, controlled by the nuclear genome. Several quantitative trait loci (QTL) for albinism have been identified in the wheat genome, respectively on chromosomes 1BL, 2AL, 2BL and 5BL. Culturing conditions however also have an effect on albinism in tissue culture embryos (Wędzony *et al*., 2009).

By adding anti-oxidants to the tissue culture media, the oxidative stress damages can be minimized and essentially the embryo recovery rate can be improved. Various anti-oxidative substances have been evaluated in induction media that either act as mitochondrial anti-oxidants, improving the viability of embryos, or as plastid anti-oxidants, preserving

chlorophyll molecules and minimizing the albino frequency. Among these substances were ascorbic acid, salicylic acid, glutathione, proline, methylene blue and N-t-butyl hydroxylamine (NtBHA). Glutathione was found to be the most successful supplement to the induction media due to its dual effect on the mitochondria and the chlorophyll. It keeps the endogenous glutathione redox potential of the cells in the oxidative state which increases the number of embryos produced. It also induces constant cell division which enhances embryo development and it reduces oxidation in the chlorophyll that leads to more green plants being formed (Asif *et al*., 2013b).

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 INTRODUCTION

Seed containing species derived leaf and yellow rust resistance genes were obtained from a previous project at the SU-PBL. It was used as the F1 base population in this study. The starting material thus already contained a number of species derived rust resistance genes in unknown combinations.

Genomic DNA was extracted from the plants and the material from the F1 base population was screened for the presence of rust resistance genes with the SU-PBL routine marker set utilized in the molecular characterization of nurseries. The F1 generation was also screened for the presence of novel species derived leaf and yellow rust resistance genes. Ears from the F1 generation were harvested to be used for microspore tissue culturing. Embryo rescue was also performed on some of the material in order to accelerate the generation time so that more ears could be harvested for the isolated microspore tissue culture. The rest of the material was left for single seed decent.

The material gained from the embryo rescue formed part of the F2 generation. Seed obtained via the single seed descent were planted to form the rest of the F2 generation. Similarly, seed obtained via single seed descent form the F2 generation was planted to form the subsequent F3 population.

All wheat lines in the subsequent F2 and F3 generations were also screened for the presence of novel species derived leaf and stripe rust resistance genes utilizing marker assisted selection. Material from the F2 and F3 generations were continually harvested for microspore tissue culturing.

The doubled haploid plant obtained was also molecularly characterized with the marker set routinely used by the lab for detection of rust resistance genes. It was also screened for the presence of species derived resistance genes and the genetic diversity of this plant was evaluated with a minimum subset of microsatellite markers. The workflow for this study is indicated in figure 3.1.

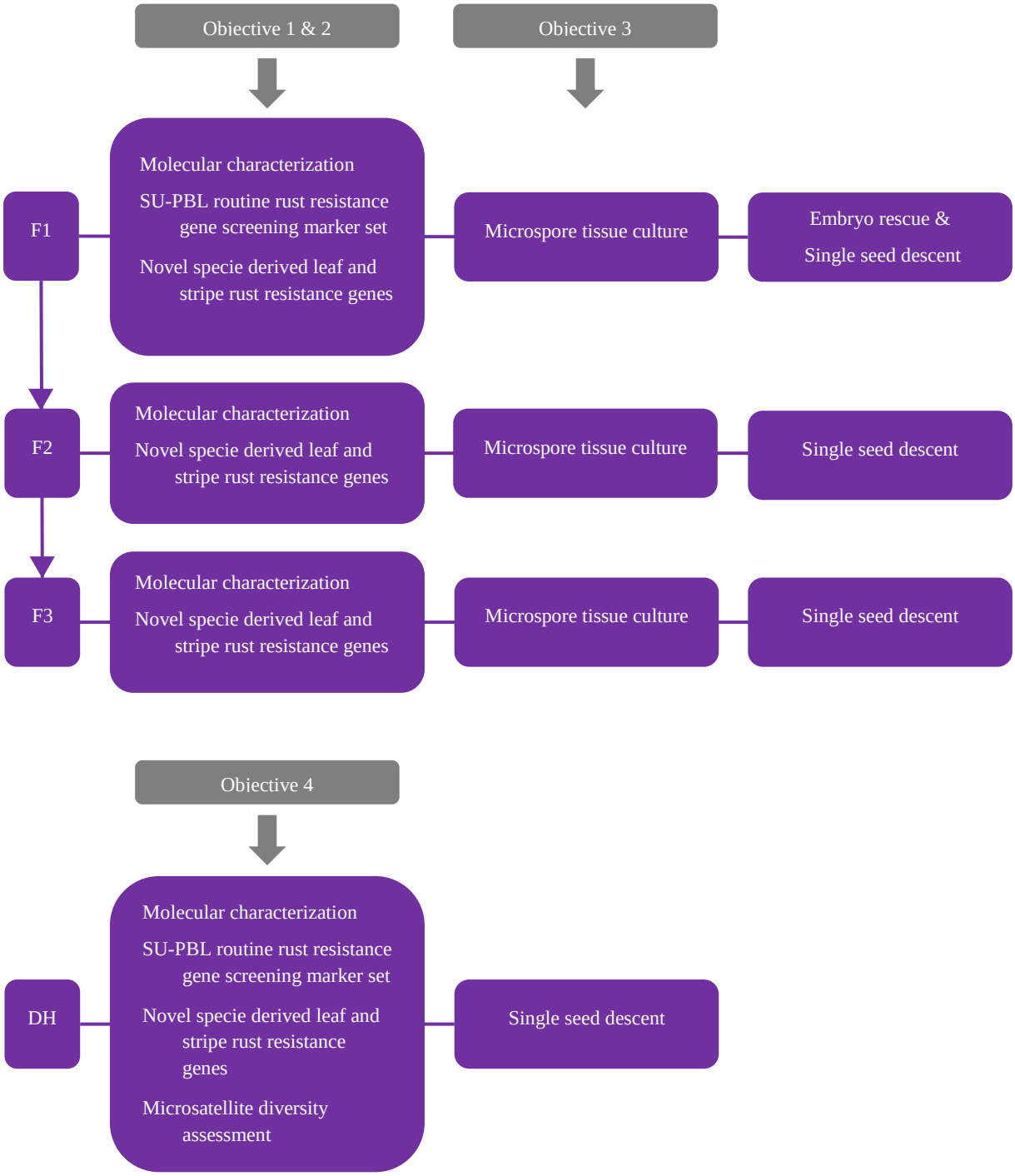



**Figure 3.1: An illustration of the workflow during this study**

## 3.2 PLANT MATERIAL

### 3.2.1 Donor material

Plant material was grown in a glass house. Four seeds were planted per 3L pot filled with a coarse sand mixture. The plants were irrigated with a feeding mixture of 0.164% (w/v) Sol-u-fert (Kynoch Fertilizers (Pty) Ltd, Milnerton, South Africa), 0.002% (w/v) Microplex (Ocean Agriculture (Pty) Ltd, Muldersdrift, South Africa) and 0.077% (v/v) calcium nitrate. A natural light-dark photoperiod was followed and temperatures ranged from 10 to 25ºC.

### 3.2.2 Embryo rescue

Embryo rescue was performed on self-fertilized wheat ears from the F1 generation at the soft-dough stage. This protocol was adapted from Pienaar *et al*. (1997). Seeds were removed from the ears and the outer glumes were taken off. Seeds were surface sterilized in 10% bleach (3.5% m/v sodium hypochlorite) for 3 min, rinsed twice with $dH_2O$, soaked in 70% ethanol for 1 min and finally rinsed with $dH_2O$ again. Under sterile conditions, a small incision was made through the pericarp of the seeds with a spear point and the seed was gently squeezed with sterilized forceps to reveal the embryo. The embryos were placed on Murashigue and Skoog (1962) media (MS), with a ten times $NH_4NO_3$ concentration, in magenta vessels with about 10 embryos per bottle.

When the embryos developed into plantlets with sufficient roots and leaves, they were transferred to 3L pots in the glasshouse containing a course sand mixture.

## 3.3 MOLECULAR CHARACTERIZATION OF WHEAT LINES

### 3.3.1 Plant genomic DNA extraction

An adjusted Doyle and Doyle (1990) protocol was used to extract genomic DNA (gDNA) from the seedlings at the two to three leaf stage.

Approximately 100mg of plant tissue was cut up and placed into a micro centrifuge tube. Five hundred microlitres of 2% (m/v) CTAB extraction buffer [1.4 M NaCl, 20 mM $Na_2EDTA$ (pH 8), 100 mM Tris-HCl (pH 8)] and three sterilized steel bearings were added to each sample. A Qiagen®TissueLyser (Qiagen (Pty) Ltd; local distributer: Southern Cross Biotechnology, Claremont, RSA) was used to grind the samples for 2 minutes (min) at 30Hz. This mixture was then incubated in a water bath for 20 min at 60°C. Two hundred and fifty microlitres of chloroform:isoamyl-alcohol (C:I::24:1) was added and the solution was centrifuged for 5 min at 12000 rcf. The supernatant was transferred to a clean centrifuge tube and another 250µl of chloroform:isoamyl-alcohol was added, followed by centrifugation for 5 min at 12000 rcf. The supernatant was once again transferred to a clean centrifuge tube.

Fifty microlitres of 3M Sodium acetate (pH 5.0) was added, followed by 500µl of ice cold 100% ethanol. The tubes were carefully inverted and the gDNA was precipitated. The tubes were centrifuged for 5 min at 12000 rcf. The supernatant was discarded and the pellet was left to air dry. The dry pellet was re-suspended in 50µl of TE buffer (pH 8.0) containing 40µg/ml RNase A and incubated for 30 min at 37ºC. After incubation the pellet underwent two wash steps with 70% ethanol. The supernatant was discarded and the pellet was left to air dry. The pellet was finally re-suspended in 30µl of DNase/RNase-free water and incubated at 60 ºC for 2 min.

The extracted gDNA was quantified using a Nanodrop® ND-1000 spectrophotometer. The DNA was diluted with DNase/RNase-free water to a concentration of 100ng/µl and stored at -20ºC.

### 3.3.2 SU-PBL routine marker screening for stem, leaf and yellow rust, reduced height genes and baking quality genes

PCR reagents were supplied by KapaBiosystems (distributed by Lasec SA (Pty) Ltd, Cape Town, RSA). All primers were manufactured by Integrated DNA Technologies (distributed by Whitehead Scientific, (Pty) Ltd, Stikland, RSA). PCR reactions were performed in a GeneAmp® PCR System 2720 Thermal cycler (supplied by Applied Biosystems® Life Technologies, Johannesburg, RSA) or a TECHNE TC-5000 (distributed by Lasec, Cape Town, RSA).

The primer sets indicated in table 3.1 are used in the routine screening of material in the wheat nurseries at the SU-PBL. It contains genes for stem, leaf and stripe rust resistance as well as reduced height genes and glutenin genes for determining the baking quality of wheat. For the purpose of this study, only the rust resistance makers indicated with an asterix (*), as well as the *Sr2* (CAPS) marker were utilized. The F1 base population and the doubled haploid plant was characterized with this marker set.

The multiplex reaction contained the following final concentrations in a 25µl reaction: one times KAPA2G$^{TM}$ Fast Multiplex PCR Mix, 0.5µM of Iag95 forward and reverse primer, 0.25µM of every other forward primer, 0.25µM of every other reverse primer, 120ng gDNA and dH2O to bring the volume to 25µl. The PCR cycling conditions were set at 94ºC for 3 min for denaturation, followed by 30 cycles of 30 seconds at 94ºC, 30 seconds at an annealing temperature of 57ºC, 1 min at 72 ºC and then 72ºC for 10 min. The PCR products were stored at 4ºC. All PCR products were electrophoresed on 1.8% agarose gels and visualized by staining it with 0.05g/L ethidium bromide.

The *Sr2* reaction performed with the most recent CAPS marker contained the following final concentrations in a 25µl reaction: one times KAPA2G$^{TM}$ Fast Multiplex PCR Mix, 0.2µM of each forward primer, 0.2µM of each reverse primer, 120ng gDNA and dH2O to bring the volume to 25µl. The PCR cycling conditions were set at 95ºC for 3 min for denaturation, followed by 40 cycles of 15 seconds at 95ºC, 15 seconds at an annealing temperature of 60ºC, 15 seconds at 72ºC and then 72ºC for 7 min. The PCR products were stored at 4 ºC. Five microlitres of PCR products were electrophoresed on a 1% agarose gel and visualized by staining it with 0.05g/L ethidium bromide to determine if the PCR amplification was successful before proceeding to the enzyme digestion step. For this step 2µl dH2O, 2.5µl of

Buffer O and 0.5µl PagI enzyme (Thermo Scientific) was added to the original DNA samples and incubated at 37 ºC for one hour. Three microlitres of loading dye was added to the sample and it was electrophoresed on 2.5% agarose gels. Samples showing a band pattern of 53bp, 112bp and 172bp were considered to be positive for *Sr2*.

**Table 3.1: SU-PBL standardised molecular marker panel utilized in the routine screening of wheat nurseries**

| Screen-ing traits | Genes | Primers | Primer sequences | Ta (°C) | Fagment size (bp) | References |
|---|---|---|---|---|---|---|
| Disease resistance genes | *Sr2* (positive) | Xgmw533-F<br>Xgmw533-R | 5'-AAGGCGAATCAACAGGAATA-3'<br>5'-GTTGCTTTAGGGGAAAAGCC-3' | 62 | 120 | Spielmeyer *et al.* (2003) |
| | *Sr2*(negative) | X3B028-F<br>X3B028-R | 5'-ACGAACAAGGGGAAGACG-3'<br>5'-TTTCGGTAGTTGGGGATGC-3' | 62 | 243 | McNeil *et al.* (2008) |
| | *Sr2 (CAPS)* | csSr2 F<br>csSr2 R | 5'-CAAGGGTTGCTAGGATTGGAAAAC-3'<br>5'-AGATAACTCTTATGATCTTACATTTTTCTG-3' | 60 | 53, 112, 172 | Mago *et al.*, 2011a |
| | *Sr24/Lr24** | SCS719-F<br>SCS719-R | 5'-TCGTCCAGATCAGAATGTG-3'<br>5'-CTCGTCGATTAGCAGTGAG-3' | 55 | 719 | Cherukuri *et al.* (2003) |
| | *Sr26** | Sr26#43F<br>Sr26#43R | 5'-AATCGTCCACATTGGCTTCT-3'<br>5'-CGCAACAAAATCATGCACTA-3' | 60 | 207 | Mago *et al.* (2005) |
| | *Sr31** | lag 95-F<br>lag 95-R | 5'-CTCTGTGGATAGTTACTTGATCGA-3'<br>5'-CCTAGAACATGCATGGCTGTTACA-3' | 55 | 1030 | Mago *et al.* (2005) |
| | *Lr19** | 12C-F<br>12C-R | 5'-CATCCTTGGGGACCTC-3'<br>5'-CCAGCTCGCATACATCCA-3' | 60 | 119 | Prins *et al.* (2001) |
| | *Lr37/Sr38/Yr17** | VENTRUIP<br>LN2 | 5'-AGGGGCTACTGACCAAGGCT-3'<br>5'-TGCAGCTACAGCAGTATGTACACAAAA-3' | 65 | 259 | Helguera *et al.* (2003) |
| | *Lr34/Yr18/Pm38** | L34DINT9-F<br>L34PLUS-R | 5'-TTGATGAAACCAGTTTTTTTTCTA-3'<br>5'-GCCATTTAACATAATCATGATGGA-3' | 58 | 517 | Lagudah *et al.* (2009) |
| Dwarfing genes | *Rht-B1* | BF<br>MR1 | 5′-GGTAGGGAGGCGAGAGGCGAG-3′<br>5′-CATCCCCATGGCCATCTCGAGCTA-3′ | 58 | 237 | Ellis *et al.* (2002) |
| | *Rht-D1* | DF<br>MR2 | 5′-CGCGCAATTATTGGCCAGAGATAG-3′<br>5′-CCCCATGGCCATCTCGAGCTGCTA-3′ | 58 | 254 | |
| Glutenin Gene | *Glu-Dx5* | P1<br>P2 | 5'-GCCTAGCAACCTTCACAATC-3'<br>5'-GAAACCTGCTGCGGACAAG-3' | 63 | 450 | Ahmad (2000) |
| | *Glu-Dy10/ Glu-Dy12* | P3<br>P4 | 5'-GTTGGCCGGTCGGCTGCCATG-3'<br>5'-TGGAGAAGTTGGATAGTACC-3' | 63 | 576/ 612 | Ahmad (2000) |
| | *Glu-A3* | Xpsp2999 F<br>Xpsp2999 R | 5'-TCCCGCCATGAGTCAATC-3'<br>5'-TTGGGAGACACATTGGCC-3' | 55 | 133-157 | Manifesto *et al.* (2001) |

*Genes analysed in one multiplex reaction

### 3.3.3 Molecular screening for species derived leaf and stripe rust resistance genes

The primer sets indicated in table 3.2 were used in a multiplex PCR analysis to screen for the species derived leaf and yellow rust resistance genes in the wheat germplasm. This analyses was performed on the F1, F2 and F3 wheat populations in this study as well as on the doubled haploid plant obtained.

The multiplex reaction contained the following final concentrations in a 20µl reaction: one times KAPA2G$^{TM}$ Fast Multiplex PCR Mix, 0.25µM of each forward primer, 0.25µM of each reverse primer, 100ng gDNA and 5µl of dH2O to bring the volume to 20µl.

The PCR cycling conditions were set at 95ºC for 3 min for denaturation, followed by 30 cycles of 15 seconds at 95 ºC, 30 seconds at an annealing temperature of 60 ºC, 30 seconds at 72 ºC and then 72 ºC for 10 min. The PCR products were stored at 4 ºC.

All PCR products were electrophoresed on 1.5% agarose gels and visualized by staining it with 0.05g/L ethidium bromide.

**Table 3.2: Primers utilized in molecular characterization of wheat lines**

| Resistance translocation | Primer | Primer sequence | Ta (ºC) | Amplified fragment size (bp) | Reference |
|---|---|---|---|---|---|
| *Lr53/Yr35* | S8N1-OF<br>S8N1-OR | 5'-CACGTTGGTAACTGAACATT-3'<br>5'-CTCACGTTGGACTTAAA-3' | 48 | 500 | Eksteen (2009) |
| *Lr54/Yr37* | S14 275F<br>S14 252R | 5'-CATGCAGAAAACGACACACC-3'<br>5'-GGTAAGTGGTCAGGCGTTGT-3' | 60 | 297 | Section 3.3.4 |
| *Lr56/Yr38* | S8N1-OF<br>S8N1-OR | 5'-CACGTTGGTAACTGAACATT-3'<br>5'-CTCACGTTGGACTTAAA-3' | 48 | 500 | Eksteen (2009) |
| *Lr59* | S15 T3F<br>S15 T3R | 5'-GTCACTTGCTTGAATTTAATG-3'<br>5'-TCCATAGCTGGTAGCTAGATG-3' | 52 | 622 | Eksteen (2009) |
| *Lr62/Yr42* | Opw 7.2F<br>Opw 7.2R | 5'-CAGGAGCATAGTCATACTTGGG-3'<br>5'-CTGGACGTCAACAATGGC-3' | 60 | 700 | Eksteen (2009) |

### 3.3.4 Alternative *Lr54/Yr37* marker design

The original *Lr54/Yr37* marker developed by Heyns (2010) could not be optimized in the multiplex PCR reaction because of the fragment size of the 410bp amplified product that overlapped with an irremovable background band of the *Lr53/Yr35* marker. A new set of primers were developed to accommodate screening for *Lr54/Yr37* in the multiplex reaction.

The *Lr54/Yr37* gene was amplified in a single PCR reaction with the original S14-410 marker (forward primer: 5'-ACCAATTCAACTTGCCAAGAG-3'; reverse primer: 5'-GAGTAACATGCAGAAAACGACA-3') (Heyns, 2010). The PCR contained the following final concentrations in a 25µl reaction: one times KAPA2G™ Fast Multiplex PCR Mix, 0.4µM of each forward primer, 0.4µM of each reverse primer, 100ng gDNA and 9.5µl of dH2O to bring the volume to 25µl. The PCR cycling conditions were set at 94ºC for 5 min for denaturation, followed by 35 cycles of 30 seconds at 94 ºC, 30 seconds at an annealing temperature of 61 ºC, 40 seconds at 72 ºC and then 72 ºC for 10 min.

The PCR product was electrophoresed on a 1.8% agarose gel for 150 min at 100V. The 410bp fragment was excised under ultra violet light and the DNA was extracted from the agarose gel using the Sigma Aldrich GenElute™ DNA extraction kit (NA1111) as per manufactures' instructions.

The DNA fragment was ligated into a pGEM®-T plasmid using the pGEM®-T and pGEM®-T Easy Vector System I by Promega (A1360) according to the accompanying manufacturer's manual. Three microlitres of the DNA insert was used in the ligation reaction. A control reaction was also set up with 2µl of the control insert. Both of these ligation reactions were incubated overnight at 4°C to ensure the maximum number of transformants.

Plasmids were transformed into competent DH5α *E. coli* cells. The ligation reaction was added to 100µl of competent cells in a 1.5ml microcentrifuge tube and mixed very carefully. The microcentrifuge tube was incubated on ice for 20 min, followed by 1 min incubation at 42°C in a water bath to heat shock the cells in order to facilitate the uptake of the plasmid. The heat shock was immediately followed by 2 min incubation on ice. Nine hundred microlitres of Luria Bertani (LB) media (Bertani, 1951) was added to the competent cell mixture and it was incubated at 37°C for 90 min while being shaken at 115rpm. A 100µl of the transformation culture was plated in duplicate on LB media containing 100mg/ml

ampicillin as antibiotic selector as well as 100µl of 0.1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 20µl of 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal) per plate. This was also done for the control insert reaction. LB plates were incubated overnight at 37ºC. Other control reactions included a normal LB agar plate inoculated with untransformed competent DH5α *E. coli* cells. Colony formation after the incubation period proved the cells to be competent. An LB/Amp control plate was also inoculated with untransformed competent DH5α *E. coli* cells. The absence of colony formation after the incubation period indicated that the ampicillin was working and that the competent cells did not contain any plasmids conferring ampicillin resistance prior to transformation.

After the incubation period, the plates were wrapped with parafilm. Transformed colonies were identified through blue-white selection. Five white colonies together with one blue colony were marked on each plate. The plates were stored at 4°C. Five of the marked white colonies from each transformation that remained white after 24 hours and one blue colony were utilized in a colony PCR reaction to confirm transformation with the plasmid containing the ligated *Lr54/Yr37* fragment.

The colony PCR contained the following final concentrations in a 10µl reaction: one times KAPA ReadyMix & Dye, 0.25µM of T7 forward primer (5'-TAATACGACTCACTATAGGG-3'), 0.25µM of Sp6 reverse primer (5'-ATTTAGGTGACACTATAG-3'), 1.5mM $MgCl_2$, 5µl of dH2O to bring the volume to 10µl and one half of a colony. The PCR cycling conditions were set at 94ºC for 5 min for denaturation, followed by 30 cycles of 30 seconds at 94 ºC, 30 seconds at an annealing temperature of 55 ºC, 30 seconds at 72 ºC and then 72 ºC for 5 min. PCR products were electrophoresed on a 1.5% agarose gel. The amplified fragment of interest was identified as the band having a 180 base pair larger size (590bp) than the original insertion (410bp) due to the primer borders in the plasmid.

Verified transformed colonies were picked and incubated overnight at 37°C in a glass tube containing 5ml of LB broth with 100mg/µl of ampicillin. The tubes were shaken lightly at 225rpm during incubation. Plasmids were extracted from 2ml of these transformed cultured colonies using the Wizard ®Plus SV Miniprep DNA Purification System from Promega (A1330). A cleared bacterial lysate solution was produced according to the accompanying protocol and the plasmid DNA was purified using microcentrifugation.

Purified DNA was sent to the Central Analytical Facility (CAF) for sequencing on an ABI 3730xl Genetic Analyser. The sequencing data received from CAF was viewed in Chromas 1.45 (McCarthy, 1998) and the fragments were analysed using BioEdit version 7.0.4.1 (Hall, 1999). Primer 3 (v. 0.4.0) (Rozen & Skaletsky, 2000) was used to design new primers for the isolated fragment with an annealing temperature of 60ºC and a GC content of 40-60% to function optimally in the multiplex PCR screening reaction.

The primers were validated by comparing scoring results with results of the original *Lr54/Yr37* marker.

### 3.3.5 Molecular diversity assessment

The genetic diversity of the doubled haploid plant was analysed using the minimum set of six microsatellite markers routinely employed by the lab (Honing, 2007). The original backcrossing parents from the 2009 SU-PBL wheat rust resistance nursery was also included in the analysis, as well as the crossing parents that served as the original donors of the species derived leaf and stripe rust resistance genes. Three other control cultivars, namely SST027, SST047 and Kariega were also included in the genetic diversity assessment.

Each of the primer sets indicated in table 3.3 were utilized in a single PCR reaction to screen for the different microsatellite markers. The PCR contained the following final concentrations in a 25µl reaction: one times KAPA2G$^{TM}$ Fast Multiplex PCR Mix, 0.2µM of each forward primer, 0.2µM of each reverse primer, 180ng gDNA and dH2O to bring the volume to 25µl.

The PCR cycling conditions were set at 94ºC for 1 min for denaturation, followed by 45 cycles of 1 min at 94 ºC, 1 min at the annealing temperature ($T_a$), 2 min at 72 ºC and then 72ºC for 10 min. The PCR products were stored at 4 ºC. All PCR products were electrophoresed on 6% polyacrylamide gels as described by Le Maitre (2010).

The microsatellite data analysis was performed using PowerMarker v3.25 (Liu & Muse, 2005). Data obtained was analysed as haplotype data using all the default settings in the program. The sub-dataset was used to compute the summary of statistics. This indicated the allele frequencies as well as the polymorphic information content (PIC) values. The C.S. Chord distance matrix (Cavalli-Sforza & Edwards, 1967) was used to calculate the genetic distance between the wheat lines. Both Neighbour Joining (NJ) and Unweighted Pair Group

Method with Arithmetic Mean (UPGMA) phylogenetic trees were constructed from the data. These trees were visualized in *MEGA* version 5 (Tamura *et al*., 2011).

**Table 3.3: Microsatellite markers utilized in genetic diversity assessment of wheat lines**

| Primer name | Primer Sequence | Ta (ºC) | Repeat sequence |
|---|---|---|---|
| Xgwm190-5D F<br>Xgwm190-5D R | 5'-GTG CTT GCT GAG CTA TGA GTC-3'<br>5'-GTG CCA CGT GGT ACC TTT G-3' | 55 | CT |
| Xgwm437-7D F<br>Xgwm437-7D R | 5'-GAT CAA GAC TTT TGT ATC TCT C-3'<br>5'-GAT GTC CAA CAG TTA GCT TA-3' | 47 | CT |
| Xgwm539-2D F<br>Xgwm539-2D R | 5'-CTG CTC TAA GAT TCA TGC AAC C-3'<br>5'-GAG GCT TGT GCC CTC TGT AG-3' | 60 | GA |
| Xwmc11-1A, 3A F<br>Xwmc11-1A, 3A R | 5'-CAC CCA GCC GTT ATA TAT GTT GA-3'<br>5'-GTT GTG ATC CTG GTT GTG TTG TGA-3' | 56 | CT |
| Xwmc59-1A, 6A F<br>Xwmc59-1A, 6A R | 5'-TCA TTC GTT GCA GAT ACA CCA C-3<br>5'-TCA ATG CCC TTG TTT CTG ACC T-3' | 58 | CA |
| Xwmc177-2A F<br>Xwmc177-2A F | 5'-AGG GCT CTC TTT AAT TCT TGC T-3'<br>5'-GGT CTA TCG TAA TCC ACC TGT A-3' | 52 | CA |

Table references: Xgwm (Röder *et al*., 1998) and Xwmc (GrainGenes: A Database for Triticeae and Avena – Genetic Markers, [S.a.])

## 3.4 MICROSPORE TISSUE CULTURE

### 3.4.1 Triticale microspore tissue culture protocol

The microspore tissue culturing technique utilized in this study was originally developed by Eudes *et al*. (2005) to be utilized in the doubled haploid production of triticale. Given the novelty of the technique at the SU-PBL, it was first validated on triticale before testing it on any wheat genotypes. A number of triticale ears (cultivar US2007) was grown in the glass house under the exact same conditions as the wheat lines and were harvested to undergo the microspore tissue culturing procedure. The triticale ears were thus utilized as a control to validate the proficiency of the technique and the competency of the operator.

Triticale ears were harvested when the microspores were at the late uni-nucleate or early bi-nucleate stages, approximately when the spike was about half way emerged from the boot. The microspore stage was determined under a light microscope (Zeiss Axioscope) after staining the pollen with 0.5% acetocarmine. The ears were wrapped in foil with about 5cm of stem still emerging at the bottom, and incubated at 4ºC in a glass beaker with distilled water. After about 28 days of cold-incubation, the microspore isolation protocol was performed.

Awns of the triticale ears were removed with scissors. These ears were surface sterilized with a treatment of 10% bleach (3.5% m/v sodium hypochlorite) for 3 min, two 1 min washes with autoclaved distilled water, another 1 min wash with 50% ethanol and a final wash step with autoclaved distilled water for 1 min. The ears were constantly shaken during the sterilization process. In a laminar flow hood the ears were aseptically cut into 1cm pieces and placed in a sterile blender cup with 50ml sterilized extraction solution (NPB99 media supplemented with 72.9g.$L^{-1}$ D-mannitol, and 250mg.$L^{-1}$ MES hydrate). This plant material was blended twice for 7 seconds on the low setting of a Warring commercial blender (distributed by Healthcare Technologies, Table View, Cape Town, RSA) to release the microspores contained in the anthers. This solution was poured through a sterile 0.7mm sieve to remove large pieces of plant debris. Fifty millilitres of extraction solution was used to rinse the blender cup and also poured through the sieve. This solution was divided into two 50ml conical tubes by pouring it through a 100µm nylon mesh sterile cell strainer. The tubes were centrifuged at 850 rcf for 5 min (Hettich Universal 21, distributed by Labotec, Pinelands, Cape Town, RSA) in order to obtain microspore pellets. The supernatant was discarded and the pellet washed by adding 30ml of extraction solution and centrifuging it under the same conditions. The supernatant was subsequently discarded, and the pellets from the two conical tubes were pooled into one 10ml centrifuge tube. The tube was filled with NPB99-media (Konzak 1999, without nicotinic acid, pyridoxine-HCl and thiamine) and centrifuged once more under the same conditions. The supernatant was discarded and the cells were resuspended in 2ml of 3M D-mannitol. This cell suspension was transferred to a new 10ml centrifuge tube containing 21% maltose monohydrate so that the cell suspension formed a layer on top of the maltose. This tube was centrifuged at 850 rcf for 5 min. The maltose formed a gradient that separated the isolated microspores into a pellet at the bottom of the tube, containing dead cells, and a white layer between the maltose and the mannitol, containing cells at the optimal stage for induction. The white layer of cells were pipetted out of the solution and placed into a clean 10ml centrifuge tube. The tube was filled with NPB99 and the cells were pelleted by

centrifugation for 5 min at 850 rcf. The supernatant was discarded and the cells were resuspended in 1ml NPB99. The cell concentration was determined using a haemocytometer. Cells were adjusted to a concentration of approximately $10^5$ cells per ml. Two hundred microlitre of this cell suspension was added to 35mm petri dishes containing 3.3ml of NPB99 and 10% ficoll PM400 (Sigma, F4375).

Ovaries were removed from either wheat or triticale (depending on availability) ears and surface sterilized by rinsing it in 50% ethanol for 3 min. It was then washed twice with autoclaved distilled water for 1 minute. Only light shaking was applied when sterilizing the ovaries.

Three to five sterilized ovaries were placed aseptically into each 35mm petri dish. The petri dished were wrapped with parafilm and placed in a large 14cm petri dish. One such large petri dish contained 7 small petri dishes with microspores and one small open petri dish with distilled water. The large petri dishes were also wrapped in parafilm and incubated in the dark at 28°C.

After about 40 days any developed embryo-like structures (ELS) were aseptically removed from the induction media and placed onto GEM media (Eudes *et al*., 2005) in 10cm petri dishes with no more than 25 ELS per plate. The plates were incubated at 16°C with a 16h light – 8h dark cycle. ELS that developed into embryos with small shoots were transferred aseptically to magenta bottles containing 30ml of rooting media (Eudes *et al*., 2005). When the green plants developed adequate roots and shoots, they were transferred to small pots with a soil composition of 3:1::sand:potting soil, and placed in the glass house. Each pot was covered with a plastic bag and over a period of about 8 days the bag was gradually opened and removed to harden off the plant.

The triticale microspores were only cultivated up to the point of haploid green plants. The chromosomes in the root tips were counted in order to determine the ploidy level of the tissue culture propagated plants.

### 3.4.2 Counting of root tip chromosomes

About 4 days prior to the cutting of root tips, the foliage of seedlings were trimmed to promote new growth. Four days later the roots were rinsed to remove the soil and the root tips were cut and incubated on ice for 29 hours in small 18mm x 50 mm glass vials containing 4-

5ml $dH_2O$. The root tips were fixated in 3:1::methanol:propionic acid solution. Approximately 4ml were used per vial. These roots were kept at 4ºC for two to three weeks.

The fixation solution was replaced with $dH_2O$ for 30 minutes. Root tips were transferred to 1N HCl at 60ºC for 7.5 minutes. Root tips were transferred back to $dH_2O$ and rinsed continually for 1-2 minutes. The last $dH_2O$ was replaced with Feulgen and the root tipes were stored overnight at 4ºC.

The Feulgen was discarded and the root tips were rinsed with $dH_2O$ for 5 minutes. The $dH_2O$ was replaced with sodium acetate (NaOAc) buffer, pH 4.5, for 5 minutes. After the removal of the NaOAc buffer, fresh 2.5% (w/v) pecticlear solution was added to the root tips followed by incubation at 37ºC for 30 minutes. The pecticlear solution was replaced with $dH_2O$ and roots were stored at 4ºC until analysis.

The chromosomes were analysed by cutting of the root tip and placing it on a glass slide with a drop of 1% (w/v) acetocarmine solution. A cover slip was placed on top and firm pressure was exerted on the cover slip to create an even layer of cells underneath, but not as to shatter the cover slip. Excess acetocarmine was removed with filter paper and the fixated chromosomes were analysed under a 100x magnification using a Zeiss Axioscope

### 3.4.3 Wheat microspore tissue culture protocol

Given the success of the triticale haploid production utilizing the microspore tissue culturing technique, the same protocol as previously described was followed during the wheat microspore isolation. The wheat lines were grown under the exact same conditions as the triticale plants. Wheat ears were harvested when the microspores were at the late uni-nucleate or early bi-nucleate stages, approximately just when the spike was about to emerge from the boot.

The wheat microspores were subject to three different induction media compositions as indicated in table 3.4. Treatment A was the same induction media utilized in the triticale experiments. Treatment B and C contained additional supplement in an attempt to improve the ELS.

**Table 3.4: Three types of induction media utilized during wheat microspore isolation protocol**

| Induction media | Composition |
|---|---|
| A | NPB-99 with 10% Ficoll |
| B | NPB-99 with 10% Ficoll + 1mg/l glutathione |
| C | NPB-99 with 10% Ficoll + 1mg/l glutathione + 25mg/l ascorbic acid + 100mg/l cefotaxime + 0.085mg/l PSK-alpha peptide |

The ploidy levels of the putative haploids were determined by checking for seed set. Haploid plants were treated with colchicine, as described by Pienaar *et al*. (1997), to induce chromosome doubling. Seed was harvested from the DH.

# CHAPTER 4: RESULTS AND DISCUSSION

## 4.1 MOLECULAR CHARACTERIZATION OF WHEAT LINES

### 4.1.1 SU-PBL standardised rust resistance screening panel

As stated in objective one, The F1 base population was characterized with the SU-PBL's routine panel of rust resistance gene markers. The APR, pathotype non-specific, stem and leaf rust resistance genes, *Sr2* and *Lr34*, were present in the material with frequencies of 25% and 65%. This is favourable if these wheat lines are potentially to be included in a breeding program. The linked genes *Sr24* and *Lr24* had a gene frequency of 88%. *Lr19* and *Sr26* were not present in the material. *Sr31* only occurred in 5% of the plants. The low frequency of, *Sr31* and *Lr19* is not a concern since both of these resistance genes have been overcome by virulent rust pathotypes and are therefore not desirable in new wheat cultivars. *Sr26* have shown a negative correlation with yield and therefore material containing this gene has been disregarded in the breeding program, leading to its absence from the F1 base population.

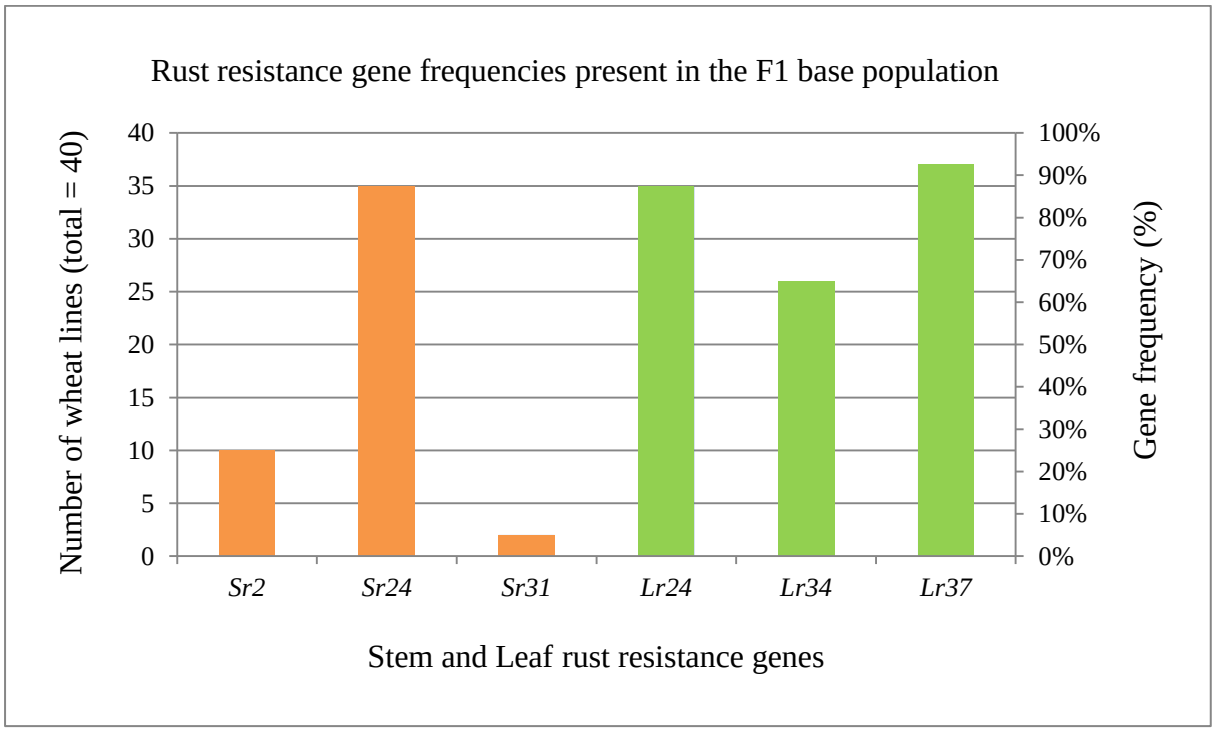



**Figure 4.1: Rust resistance gene frequencies in F1 generation**

### 4.1.2 Alternative *Lr54/Yr37* marker design

The 410bp *Lr54/Yr37* gene amplicons obtained from the PCR reaction with the original S14-410 marker, was excised from the agarose gel and the DNA was purified. The isolated DNA fragment was ligated into a pGEM®-T plasmid and the plasmids were transformed into competent DH5α *E. coli* cells. Each LB Agar plate with ampicillin that was plated with transformed DH5α-cells contained blue and white colonies. The blue colonies represented cells without the *Lr54/Yr37* genes inserted since the *lacZ* gene from the pGEM®-T plasmid was still intact and metabolised the X-gal to the blue substance. The cells were however successfully transformed since they survived on the ampicillin media and must therefore have contained the pGEM®-T plasmid with an ampicillin resistance gene. The white colonies represented potentially successfully transformed cells with successful ligation of the *Lr54/Yr37* fragment into the plasmid since the *lacZ* was interrupted due to the gene insert and couldn't metabolise the X-gal to produce the blue substance.

The control LB agar plate without ampicillin was plated with untransformed competent DH5α-cells. Colonies were observed which proved the cell viability. A control LB agar plate with ampicillin was also plated with untransformed competent cells. No colony growth had been observed, thus proving that the competent cells that were utilized had no inherent resistance to ampicillin.

A colony PCR was performed on putatively transformed white colonies as well as some blue colonies. The inserted fragment had to amplify to a size of about 180bp larger than the original fragment due to the primer borders from the ligation reaction that had to be taken into consideration. Colonies W17 and W18 (figure 4.2) had the amplified product of about 600bp since the original inserted fragment was 410bp long. The plasmids extracted from colonies W17 and W18 were sent to the Central Analytical Facility (at Stellenbosch University) and a sequence of 393bp indicated in figure 4.3, was obtained.

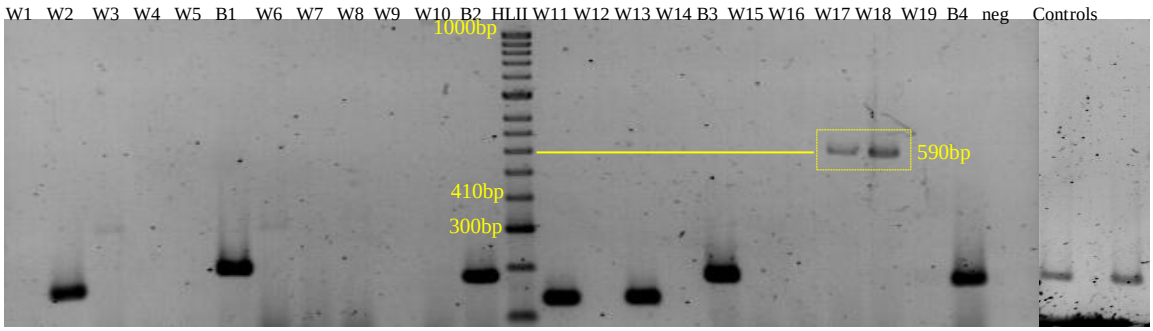



**Figure 4.2: Colony PCR of white (W) and blue (B) putative transformed colonies.**

According to a BLAST analysis and a Clustal W alignment performed in BioEdit (Hall, 1999), the sequence indicated in figure 4.3 showed a 95% similarity to the sequence of *Lr54/Yr37* previously obtained by Heyns (2010).

GAGTAACATGCAGAAAACGACACACCTTGTGTGCCCGCTCCACGAAGGTGTCGT
TACCTTCATCGTCAGCAACCATGGTGACTTCCGTATGTGCCTCGCACCGATTTGCT
AGATCGTGTCTGTGGACTCACCAACCCTCGCGACATGTTCGCGGTCGAGCTTCGG
CCCATGACGGTAATTTGGGCTCCAAAAATCCGGAGACACCTACTAGCAGACCAT
GGATGAGCATCTGCAAAATGTGAATGGGCACAAAACTAGCGCATGGTCAGATTT
GTCTGGCCAGACAACGCCTGACCACTTACCTCCTACTCAGAGGACGGATATATTG
CAGCAATAGACGTGGTTGGCATGGGAATATGGTGAAACCACCTCCTCTTGGCAA
GTTGAATTGGT

**Figure 4.3: Sequenced fragment obtained from CAF. Highlighted sections indicate the new primers designed to amplify a 297bp fragment.**

Five potential primers were designed to amplify the isolated gene sequence and they were evaluated in a PCR reaction. Each primer combination was tested on an *Lr54/Yr37* negative (-) and positive (+) wheat line (Addendum B). The new primer combination was chosen on the grounds that it amplified a distinct band in the positive control that was not amplified in the negative control. It also did not amplify a lot of background bands in the positive control. This new primer set with forward primer 5'-CATGCAGAAAACGACACACC-3' and reverse primer 5'-GGTAAGTGGTCAGGCGTTGT-3' amplified a band with a size of 297bp and was optimized to work in a multiplex reaction with the other species derived resistance gene markers. This multiplex reaction is indicated in figure 4.4.

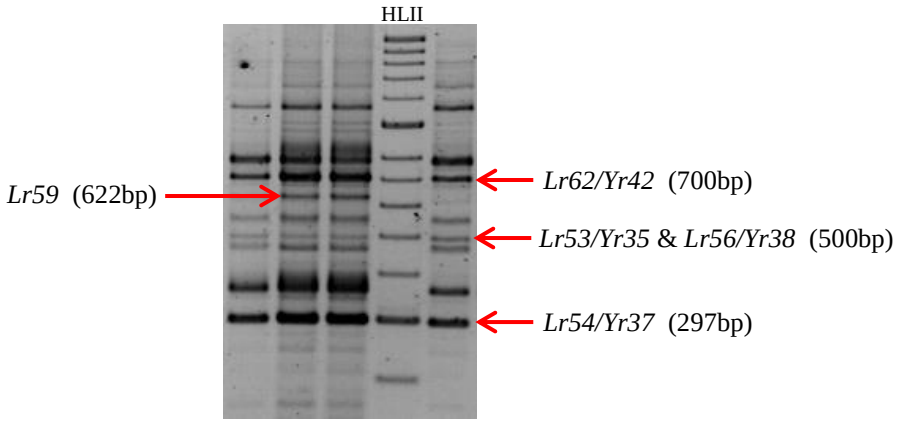



**Figure 4.4: Multiplex reaction for the screening of species derived leaf and stripe rust resistance genes.**

### 4.1.3 Species derived leaf and stripe rust resistance genes

In the F1 base population there was a number of wheat lines that contained more than one of the species derived rust resistance genes. Fourteen lines carried all four of the species derived leaf and stripe rust resistance genes and 15 lines carried a combination of three of the genes. There were two lines that didn't contain any of the species derived resistance genes. Embryos were rescued from wheat ears containing combinations of three of four resistance genes. The rest of the seed was left for single seed decent. Only seed obtained from plants shown to contain three or more resistance genes were planted to form the next generation. The F2 generation was thus selected to only come from parental lines with three or more resistance genes present. The same concept was applied when the F3 generation was selected.

As shown in figure 4.5, the frequency of wheat lines shifted towards lines containing three species derived resistance genes in the subsequent generations. This was the expected result since selection towards lines with a higher resistance gene frequency took place.

Even though selection for wheat lines carrying more pyramided resistance genes was implemented, the frequencies of wheat lines testing positive for the individual the species derived resistance genes didn't vary significantly from generation to generation (figure 4.6). This assumption was validated by a test of Least Significant Difference (LSD) calculated at 0.05%.

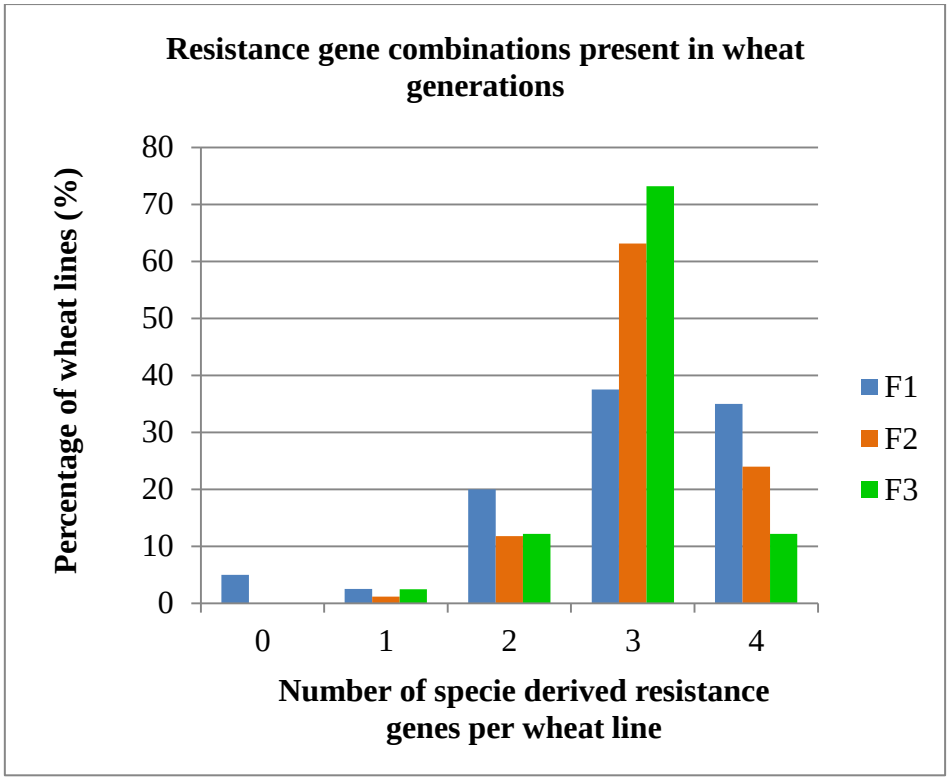



**Figure 4.5: Number of species derived resistance genes present per wheat line in the different generations: F1,F2 and F3. (Addendum A, C and D)**

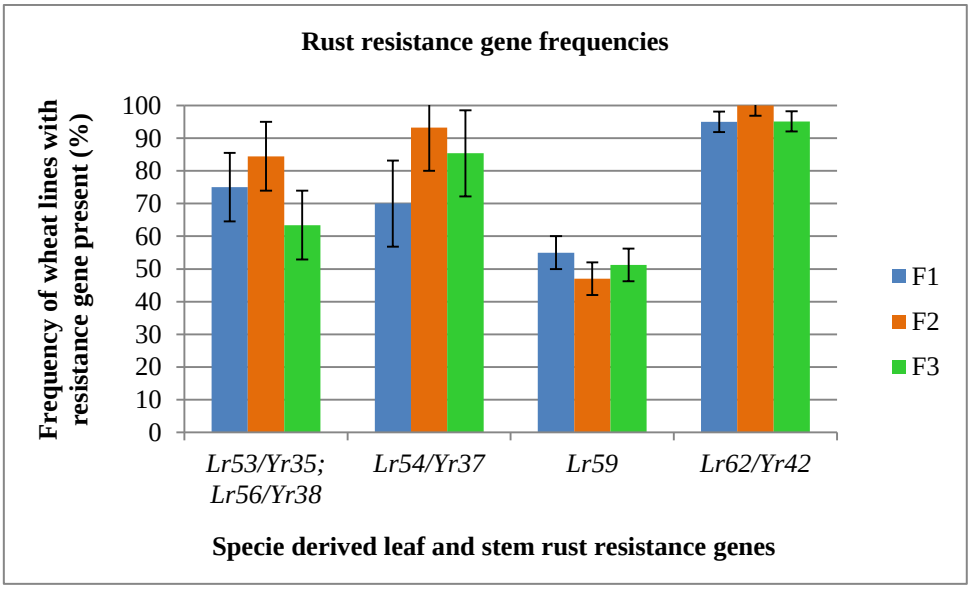



**Figure 4.6: Frequencies of wheat lines containing the individual species derived leaf and stripe rust resistance genes in the three different generations. Error bars indicate the LSD values at 0.05 (Addendum A, C and D).**

The inheritance of all the individual resistance genes did not always follow a Mendelian pattern of inheritance. Chi-square tests were conducted for each of the different resistance genes in each of the wheat generations. This was done assuming the individual resistance genes were present in a heterozygous state. The significant p-values (<0.005) in table four are indicated in red. In these instances the resistance genes did not follow a traditional pattern of Mendelian inheritance. This was especially true for the F2 population. In most cases the observed ratios deviated from the expected because the resistance genes were more prevalent in the wheat lines. The only exception to this was in the F2 population where the wheat lines containing *Lr59* was less than the expected amount.

**Table 4.1: P-values (significant <0.05) for F2 and F3 generations, derived from Chi-squared values testing Mendelian pattern of inheritance.**

| | ***Lr53/Yr35; Lr56/Yr38*** | ***Lr54/Yr37*** | ***Lr59*** | ***Lr62/Yr42*** |
|---|---|---|---|---|
| F2 population | $1.85 \times 10^{-4}$ | $3.01 \times 10^{-11}$ | $2.89 \times 10^{-7}$ | $9.89 \times 10^{-15}$ |
| F3 population | 0.7 | 0.0804 | 0.224 | $1.6 \times 10^{-3}$ |

The deviation of the expected Mendelian ratio in subsequent generations could be due to a number of reasons associated with segregation distortion. The original transfer experiments of the leaf and stripe rust resistance genes to wheat, conducted by Marais *et al*. (2003, 2005a, 2006, 2008, 2009), showed all of the genes to be associated with preferential pollen transmission. All of the progeny in this study was obtained through self-fertilization and there are still some amounts of alien DNA associated with the resistance genes present in the wheat material. One can thus assume that the preferential pollen transmission originally observed in the species derived resistance genes may still occur.

Segregation distortion as was observed in this study is a common phenomenon in plants (Hao *et al*., 2013). Some of the genetic elements identified to influence this include gametocidal genes (*Gc*) and segregation distortion genes, such as *Sd1* and *Sd2*.

Gametocidal genes were introduced into the wheat genome via the *Aegilops* species (Endo, 2007). Four of the five resistance genes utilized in this study, namely *Lr54*/*Yr37*, *Lr56*/*Yr38*, *Lr59*, *Lr62*/*Yr42* were derived from *Aegilops* species (Marais *et al*., 2005a, 2006, 2008,

2009). *Gc* genes cause gametes that lack these genes to be eliminated. Gametes containing *Gc* genes thus have a favoured chance of survival and a heightened likelihood of being the pollen donor in the fertilization of wheat embryos. It is thus possible that there may be *Gc* genes present in the wheat material associated with the remaining fragments of alien DNA linked to the individual resistance genes.

The segregation distortion gene *Sd1* that was originally associated with *Lr19* derived from a species of tall wheat grass, *Thinopyrum ponticum* (Sharma & Knott, 1966; Marais *et al*., 2001). It has a similar effect to the gametocidal genes as it promotes self-survival and gametes without *Sd1* abort. Other wild grass species or related wild wheat species are thus likely carriers of similar segregation distortion genes. The novel species derived leaf and stripe rust resistance genes in this study may also be linked with unknown segregation distortion genes favouring the transmission of the resistance genes to subsequent generations.

Not all segregation distortion genes however act in a selfish manner to promote personal proliferation. *Sd2*, also associated with *Lr19* and derived from *T. ponticum*, has been shown to cause self-elimination in gametes containing this gene (Prins *et al*., 1997). This will then distort the associated genes' frequencies in the subsequent generation to be less than the expected Mendelian ratios. In the case of *Lr59* in the F2 generation the observed lines containing this gene was significantly less than expected. *Lr59* was also derived from the wild *Aegilops* grass species. It can thus be speculated that *Lr59* is linked with a different type of segregation distortion gene that does not favour its transmission to subsequent generations.

It is interesting to note that wheat lines containing species derived leaf and stripe rust resistance genes in the F3 generation followed the expected frequencies except where *Lr62/Yr42* was concerned.

The normal inheritance patterns may be attributed to the fact that in an additional generation the specific DNA fragments containing the rust resistance genes may have been shortened through genetic recombination to exclude possible genetic segregation distortion factors.

Lines containing *Lr62/Yr42* were more prevalent than expected in both the F2 and F3 generations. This could be due to the presence of genetic distortion factors as discussed. It could also be because the *Lr62/Yr42* gene could already be fixated in a homozygotic state in

these wheat lines. This would lead to these resistance genes being present in all subsequent self-fertilized progeny.

### 4.1.4 Molecular profile of doubled haploid wheat

The single doubled haploid wheat plant that was obtained via the microspore tissue culturing method contained four of the five novel species derived leaf and stripe rust resistance genes: *Lr53/Yr35*, *Lr54/Yr37*, *Lr56/Yr38* and *Lr62/Yr42*. It also contained the additional leaf rust resistance genes *Lr34* and *Lr37* when screened with the SU-PBL standardized rust panel. This doubled haploid plant is thus a valuable source of leaf and stripe rust resistance.

The genetic diversity assessment of the doubled haploid plant yielded 35 different alleles over the six utilized markers. The polymorphic information content of the markers ranged between the values of 0.4275 and 0.8532 (table 4.2).

**Table 4.2: Microsatellite genetic diversity assessment results**

| Marker | Nr of alleles | Allele range (bp) | PIC |
|---|---|---|---|
| Xgwm190-5D | 3 | 207-233 | 0.4275 |
| Xgwm437-7D | 6 | 106-151 | 0.7186 |
| Xgwm539-2D | 6 | 138-164 | 0.7786 |
| Xwmc11-1A, 3A | 6 | 172-208 | 0.7406 |
| Xwmc59-1A, 6A | 9 | 176-228 | 0.8532 |
| Xwmc177-2A | 5 | 194-215 | 0.7161 |

The UPGMA phylogenetic dendogram (figure 4.7) showed the backcrossing parents from the SU-PBL wheat rust resistance nursery, 09US001, 09US002, 09US003, 09US004, and 09US005 to cluster together. The Kariega control also fell within this group. The two Sensako cultivars, SST027 and SST047, clustered together. Given the similar genetic background of these commercial cultivars from the same breeding program, this clustering was expected. It also served as an internal control reaction in the marker analysis. The donor parents for the species derived resistance genes, namely W14-24 (*Lr53/Yr35*), W14-25 (*Lr56/Yr38*), W14-26 (*Lr54/Yr37*), W14-27 (*Lr59*) and W14-29 (*Lr62/Yr42*), also clustered together. These parental lines all share Chinese Spring DNA in their pedigrees which explains the clustering.

The doubled haploid plant was shown to be genetically the most similar to W14-29, the donor parent for *Lr62/Yr42*. From a breeding perspective, it would have been more favourable for the doubled haploid plant to cluster closer to the backcrossing parents from the SU-PBL nursery. The doubled haploid plant possibly contains large parts of Chinese Spring DNA. Alternatively it could contain larger than anticipated parts of species derived chromatin. This will hamper the usage of this valuable new source of leaf and stripe rust resistance genes in the development of new rust resistant cultivars.

The low bootstrap values obtained in this analysis indicated that there were not distinct genetic differences between the wheat lines that were analysed in this study. The purpose of the microsatellites were however rather to show similarity between the wheat lines and not divergence. The values were therefore considered to be acceptable. To further validate the statistical soundness of the dendogram, another phylogenetic tree was computed using the Neighbour Joining algorithm. Although the branch topology differed, essentially the same clustering was observed with the alternative method (Addendum E, Table E.1).

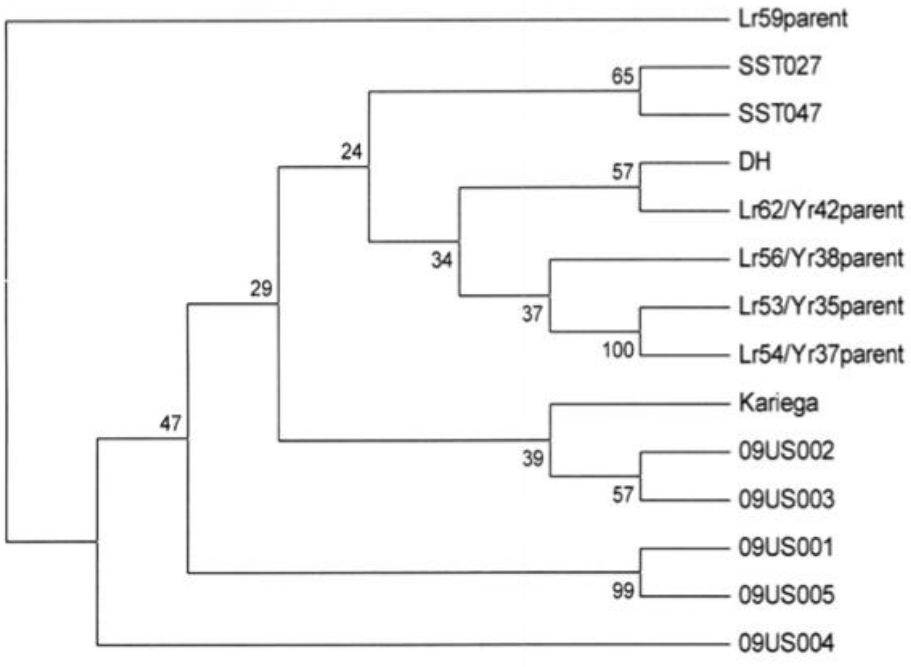



**Figure 4.7: UPGMA phylogenetic tree for doubled haploid plant**

## 4.2 EMBRYO RESCUE

The embryo rescue was very successful. The survival rate was high in most instances with survival rates of up to 100% observed. Seed from parent line 5.2 and 7.3 were not at the optimal stage for embryo rescue. The seed was still a long way from the soft dough stage. The embryos were extremely small and that is presumably why embryos from parent line 5.2 did not survive and embryos from parent line 7.3 only had a 24% survival rate. The embryos from parent line 3.4 also did not respond well to the embryo rescue. A possible reason could be that the parent line has a genotype that is recalcitrant toward the MS media and *in vitro* conditions.

**Table 4.3: Embryo rescue survival rate**

| Parent line | 1.5 | 2.3 | 2.4 | 3.4 | 3.5 | 4.7 | 4.8 | 5.2 | 5.3 | 5.4 | 5.5 | 5.6 | 5.8 | 6.2 | 7.3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Embryos rescued | 18 | 28 | 6 | 10 | 13 | 15 | 31 | 5 | 14 | 11 | 19 | 14 | 2 | 17 | 17 |
| Mature Plants obtained | 17 | 19 | 5 | 1 | 13 | 15 | 26 | 0 | 14 | 11 | 11 | 13 | 1 | 12 | 2 |
| Survival (%) | 94 | 68 | 83 | 10 | 100 | 100 | 84 | 0 | 100 | 100 | 58 | 93 | 50 | 71 | 24 |

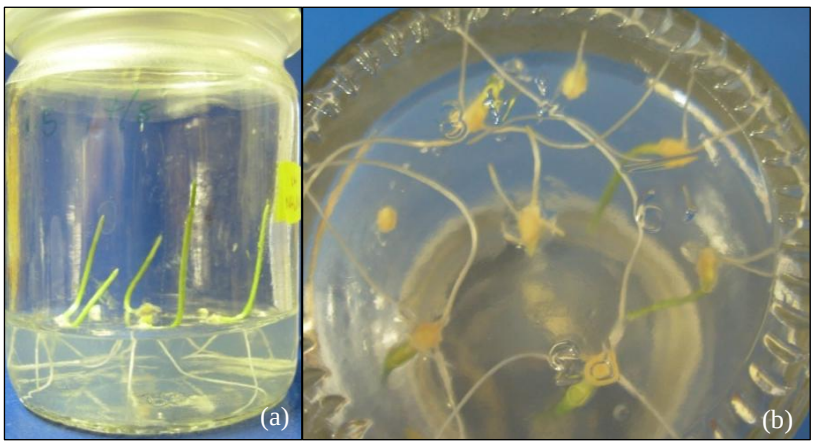



**Figure 4.8: (a) Rescued embryos growing *in vitro* on MS media; (b) rescued embryos as seen from underneath the media bottle.**

## 4.3 MICROSPORE TISSUE CULTURE

### 4.3.1 Triticale control experiments

The triticale control experiments comprised 15 batches of ears over 13 experimental runs. All of the triticale experiments were performed using induction medium A, containing NPB-99 with 10% ficoll. Five of these batches showed uncontaminated growth of callus and ELS. The percentage of haploid green plants obtained was 79.35% (table 4.4). The triticale plants served as a control for the microspore tissue culture experimental design and weren't maintained up to the stage of seed setting. These plants were confirmed to be haploid by counting the root tip chromosomes. Chromosome counts of 21 proved the plants obtained via tissue culture to be haploid. The plants thus originated form the isolated microspores and not from some debris plant material.

**Table 4.4: Microspore tissue culture for triticale from successful batches, subject to treatment A with no additional supplements.**

| Total batches | Induction dishes | Total ELS | Green haploid plants | % Haploids |
|---|---|---|---|---|
| 5 | 18 | 247 | 196 | 79.35 |

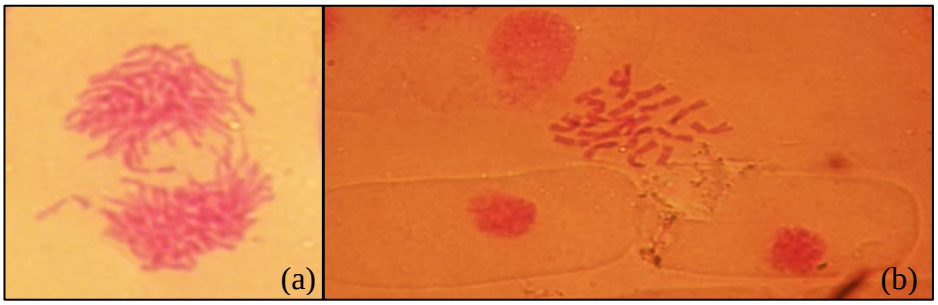



**Figure 4.9: Triticale root tip chromosomes, (a) dividing cell in metaphase, (b) ruptured haploid cell with 21 chromosomes.**

With a triticale haploid percentage of almost 80%, it was assumed that the experimental design was effective and that the operator was proficient in performing the technique.

### 4.3.2 Wheat tissue culture

Over the course of this study, three different induction media compositions were utilized. Induction media A consisted of the basic NPB-99 with 10% ficoll, induction media B consisted of the basic media with 1mg/l glutathione added, and media C had 1mg/l glutathione, 25mg/l ascorbic acid, 100mg/l cefotaxime and 0.085mg/l PSK-alpha peptide added to the basic media. A total of 36 experimental runs were performed on wheat ears consisting of 57 individual batches of ears. These batches mostly contained wheat ears deriving from the same parental lines, thus having similar genotypes, but mixtures were also used depending on the availability of the ears in the cold incubation.

Of the 57 batches of wheat microspores that were placed in the induction media, only 7 batches showed growth of ELS (table 4.5). About half of the total batches had some form of contamination that stunted microspore growth. This included pink yeast, yellow bacterial and fungal growth. The contamination could be attributed to some endogenous contamination in the plant material as well as some contamination that might have occurred during the handling of the material outside of the laminar flow cabinet during the microspore isolation process. About one third of the batches were uncontaminated after six weeks of incubation, but had no indication of any ELS growth.

In the wheat microspore tissue culture, only induction media A yielded haploid green plants (table 4.6). The haploid percentage of 2.91%, is an indication of the number of green haploids obtained as a percentage of the total number of ELS removed from the induction media, cultivated on the regeneration media and placed in bottles with rooting media. The number of doubled haploids (DH) obtained were the number of green plants that survived the colchicine treatment and produced seed.

**Table 4.5: Microspore tissue culture for wheat**

| | Total | Clean tissue culture with ELS growth | Clean tissue culture with no ELS growth | Contaminated tissue culture |
|---|---|---|---|---|
| Number of runs | 36 | 7 | 11 | 18 |
| Number of batches | 57 | 7 | 20 | 30 |
| Induction media A | 27 | 4 | 6 | 17 |
| Induction media B | 5 | 1 | 0 | 4 |
| Induction media C | 25 | 2 | 14 | 9 |

**Table 4.6: Wheat microspore growth from successful batches**

| Induction media | Total batches | Induction dishes | Total ELS | | | Green haploids | % Haploids | DH obtained |
|---|---|---|---|---|---|---|---|---|
| | | | Green | Albino | Callus | | | |
| A | 4 | 28 | 3 | 11 | 89 | 3 | 2.91 | 1 |
| B | 1 | 8 | 0 | 1 | 6 | 0 | 0 | 0 |
| C | 2 | 15 | 0 | 1 | 9 | 0 | 0 | 0 |
| Total | 7 | 51 | 3 | 13 | 104 | 3 | 2.5 | 1 |

The number of ELS obtained per induction dish for the wheat, over all induction media types, and the triticale were compared. A two-tailed t-test assuming equal variances was performed on the data and a p-value of less than 0.05 was considered as significant. A p-value of 0.025 was obtained which indicated the amount of ELS obtained per induction dish for the wheat microspore tissue culture was significantly less than the number obtained in the triticale tissue culture. This was however not a comparative study between wheat and triticale and the triticale just served as a control reaction for the experimental design. With a triticale haploid percentage of almost 80%, it was assumed that the experimental design was effective.

When comparing the different induction media types for the wheat microspore tissue culture, media A was the most effective. Neither induction media B with added glutathione (1mg/l), nor induction media C, with added glutathione (1mg/l), ascorbic acid (25mg/l), cefotaxime (100mg/l) and PSK-alpha peptide (0.085mg/l) yielded any green haploid embryos.

Figure 4.10 shows the physical differences between the successful and unsuccessful microspore tissue culture experimental runs. The initial cell morphology of the successful and unsuccessful batches was phenotypically similar. Both consisted of healthy dividing cells prior to placing the cells in the induction media. The concentration of the microspores was adapted to approximately $10^5$ cells per millilitre in every batch and treatment, so the amount of cells per induction dish were more or less the same, regardless of the amount of cells obtained from the specific microspore isolation run. At about four weeks of incubation in the induction media, clear differences were visible between the successful and unsuccessful batches (figure 4.10b). A large amount of ELS growth was visible in the successful batches. The unsuccessful wheat batch however contained only the ovaries used as co-culture and a very small amount of tiny calli. When these dishes were examined under the microscope, the induction dishes of the unsuccessful batches did indeed contain isolated microspores, but no growth was observed. This indicated that the isolation protocol was effective, but the induction of cell division and growth were not.

The successful microspore batches consisted of ELS that developed into green embryos (figure 4.10d) and albino embryos (figure 4.10e). The unsuccessful batches that had some ELS growth, only contained calli and no embryos. Of the three haploid green plants obtained via the microspore technique, only one survived the chromosome doubling colchicine treatment. Seed set confirmed the plant to be doubled haploid

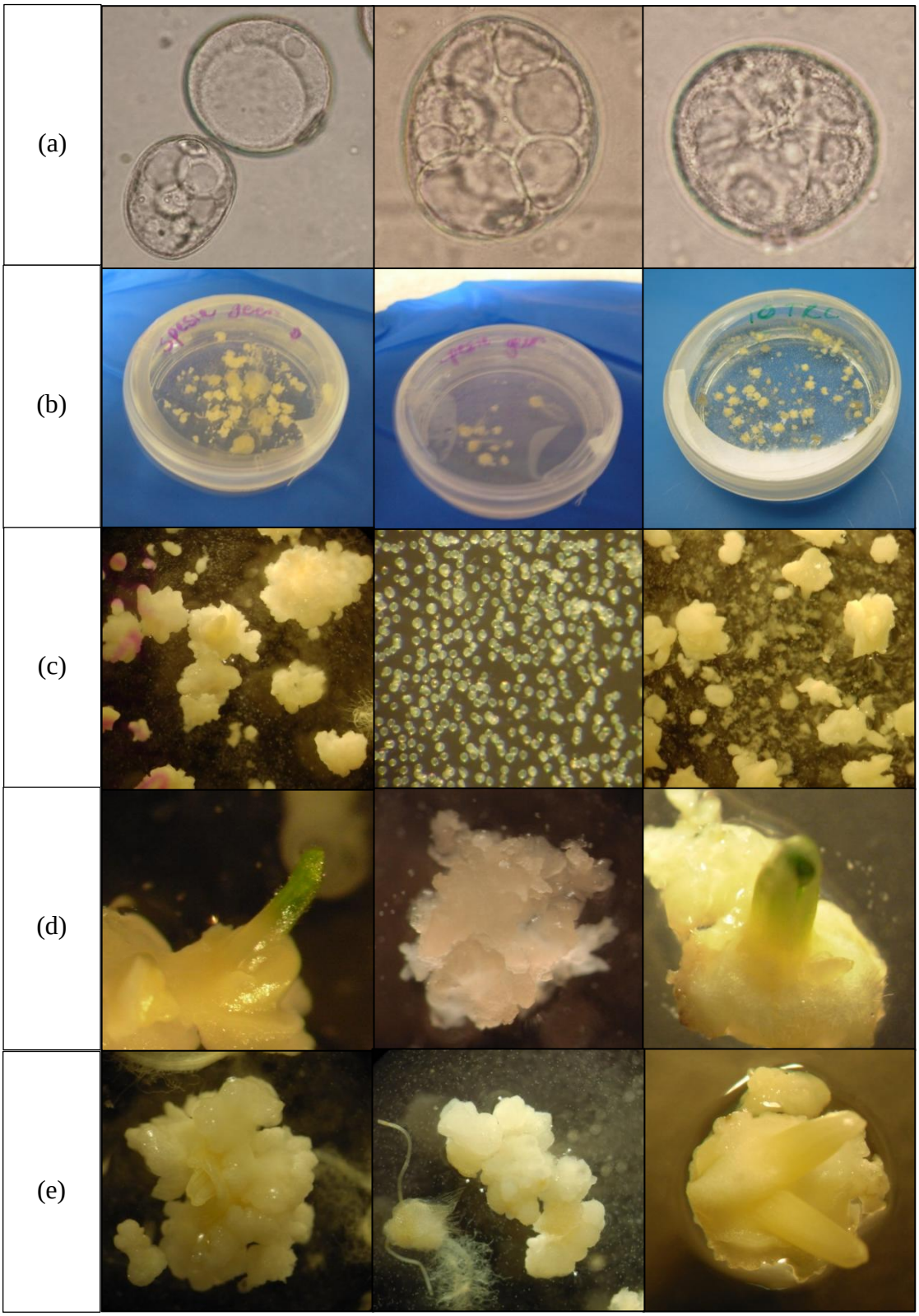



**Figure 4.10: Comparison between successful wheat microspore cycle (left), unsuccessful wheat microspore cycle (middle) and successful triticale microspore cycle (right). Different stages of isolated microspore protocol: (a) dividing isolated microspores prior to induction, (b) uncontaminated induction dishes, (c) microscopic view of microspore growth in induction media, (d) green ELS in successful batches and callus in the unsuccessful batch, (e) albino ELS in successful batches and callus in the unsuccessful batch.**

A common indicator utilized in *in vitro* haploid production and doubled haploid studies, whether it be anther culture or isolated microspore tissue culture, is the green plant regeneration rate or the embryo recovery rate. This gives an indication of the number of doubled haploid green plants obtained expressed as a percentage of the number of ELS formed. In this study for wheat microspores utilizing induction media A, a green plant regeneration rate of 1% was achieved.

The first factor leading to such a small green plant regeneration rate, was the relatively small number of ELS formation. The second problem was that a large percentage of the total ELS, about 80%, remained callus and never developed into embryos. Another problem was that 81% of the developed embryos were albino plants that could not survive *in vivo*. Lastly, the colchicine treatment entailed a harsh chemical shock to the fragile haploid plants and only the strongest plants survived. Only one third of the haploid plants in this study survived this treatment.

According to Asif *et al*. (2013b), cell death of the isolated microspores already starts at the early phase of induction and continues throughout the tissue culture protocol. This is possibly due to excessive oxidative damages suffered by the cells during the stresses exuded by the pre-treatment and isolation phases of the process. This type of cell death is a likely contributor to the small number of ELS formation observed in the wheat microspore tissue culture. It is also possible that the isolated microspores in the unsuccessful batches (figure 10c middle) simply have suffered too much oxidative stress which halted any further *in vitro* cell growth.

One of the largest limiting factors in the production of wheat haploids is considered to be the large occurrence of albinism. A large number of albino haploids were observed in this study, as well as several other studies conducted on cereal microspore tissue culture (Ferrie & Caswell, 2011; Asif *et al*., 2013b). It has generally been found that anti-oxidant supplements like glutathione and ascorbic acid reduce the number of albino embryos obtained (Cistue & Echa, 2009; Santra *et al*., 2012; Asif *et al*., 2013b). Different concentrations of glutathione between 0.6mg/l (Asif *et al*., 2013b) and 500mg/l (Cistue & Echa, 2009) has been sighted in literature, but no quantitative studies have been done to determine the optimal dosage for maximum effect on the microspores in the induction media. The additional supplements added to induction media B and C in this study, namely glutathione and a combination of glutathione, ascorbic acid, cefotaxime and PSK-alpha peptide, did not improve the green

embryo formation compared to the original induction media. This can possibly be because the optimal working concentration for these substances is higher than that which had been utilized in this study. It has also been found that different genotypes respond differently to anti-oxidant treatment (Asif *et al*., 2013b). The addition of extra anti-oxidants may thus not be effective for inducing green embryo formation in all wheat cultivars or lines, especially in wheat containing alien chromatin.

The wheat material containing species derived resistance genes utilized in this study were found to be extremely recalcitrant towards microspore tissue culturing. It was also previously reported that cereals crops, particularly wheat, responded poorly to microspore propagation (Ferrie & Caswell, 2011). A similar green plant regeneration rate of 0.1-1% was obtained in a previous study by Ascough *et al*. (2006) who analysed the androgenic competence of South African wheat germplasm, utilizing four PANNAR (Pty) Ltd cultivars. When analysing microspore tissue culture techniques in spring wheat cultivars, Santra *et al*. (2012) found a green plant regeneration frequency of between 4 and 55%. Among wheat cultivars that respond very well to microspore tissue culturing are the spring wheat cultivars Pavon 76 and Chris (Ascough *et al*., 2006; Ferrie & Caswell, 2011). Green plant regeneration rates as high as 76% for a Spring wheat cultivar called Macon, has been reported in literature (Ferrie & Caswell, 2011). It is thus evident that a significant interaction exists between the wheat genotype and the *in vitro* induction parameters utilized. All wheat genotypes over the world are variable in terms of the response to microspore cultivation. Extreme variation of between 0-100% has also been observed in the success rate of chromosome doubling with colchicine treatment (Mago *et al*., 2011). The physical health of the plant plays a role in this statistic, but different genotypes may possibly also respond differently to the colchicine effect as it does with growth stimulants and anti-oxidants.

Doubled haploid production is commonly practised in plant breeding laboratories around the world. The microspore tissue culturing method for wheat however does not have a high enough success rate to be economically viable in a commercial wheat breeding program (Ascough *et al*., 2006; Ferrie & Caswell, 2011; Santra *et al*., 2012; Asif *et al*., 2013). The interaction between wheat genotypes and induction conditions is considered as the greatest limiting factor in the establishment of an effective microspore protocol for wheat germplasm around the world (Ascough *et al*., 2006; Cistue & Echa, 2009; Santra *et al*., 2012; Asif *et al*., 2013). There are no universal protocols available that can ensure an effective green plant

regeneration rate in wheat cultivars. In this study, the wheat lines containing small parts of alien DNA alongside species derived leaf and stripe rust resistance genes exhibited a particularly large genotype x induction conditions interaction.

# CHAPTER 5: CONCLUSION

The F1 base population was molecularly characterized with the SU-PBL's routine marker set to test for the presence of stem, leaf and stripe rust resistance genes. This population was also screened for the presence of novel species derived leaf and stripe rust resistance genes. A number of rust resistance genes were present in the F1 base population, including the APR genes for stem and leaf rust, namely *Sr2* and *Lr34*. A number of species derived leaf and stripe rust resistance genes were also present in various quantities and combinations. The lines varied from containing zero to four of the markers for the screened species derived genes.

The subsequent F2 and F3 generations also contained the species derived resistance genes. A shift towards lines testing positive for three of the four species derived rust resistance markers were observed. These genes did not follow a Mendelian pattern of inheritance in the F2 generation, possibly due to the presence of segregation distortion factors in the alien chromatin associated with the species derived genes. The observed gene frequencies returned to the expected values in the F3 population, except for *Lr62/Yr42*. This was likely due to the elimination of possible segregation factors via shortening of the alien chromatin associated with the resistance genes.

Both of the marker sets used for the molecular characterization of the wheat lines in terms of rust resistance genes, consisted of efficient multiplex reactions, ensuring a high throughput screening technique in the laboratory.

The doubled haploid wheat plant was also molecularly characterized with the previously mentioned marker sets. It contained species derived resistance genes *Lr53/Yr35*, *Lr54*/*Yr37*, *Lr56*/*Yr38* and *Lr62*/*Yr42*. It also contained the additional leaf rust resistance genes *Lr34* and *Lr37*. Furthermore, the genetic diversity of the doubled haploid line was assessed by means of a minimum microsatellite marker set routinely utilized by the lab. The doubled haploid plant was found to be genetically the most similar to the donor parent of the *Lr62/Yr42* leaf and stripe rust resistance genes.

The adapted microspore tissue culturing technique for the creation of haploid plants was validated in triticale. A haploid green plant regeneration rate of almost 80% proved the

proficiency of the technique and the operator. The wheat microspore tissue culture, utilizing the same induction media as the triticale control, yielded a haploid green plant percentage of 2.91%. After the colchicine treatment to induce chromosome doubling, the green plant regeneration rate was 1%. None of experiments with additional supplements in the induction media, including anti-oxidants and antibiotics, yielded any green haploid plants. Similar results were obtained by other studies utilizing South African wheat germplasm (Ascough *et al*., 2006), but a lot of variation in terms of the green plant regeneration rates were found all over the globe, with the highest being 76% (Ferrie & Caswell, 2011). It has been shown that the wheat genotype interaction with *in vitro* microspore culturing conditions is the largest constraint of haploid production worldwide. The wheat material with the species derived DNA in this study proved to be especially recalcitrant towards the tissue culturing conditions. The microspore isolation protocol thus has to be customized according to wheat genotype. Other factors that influenced the green plant regeneration rate in this study were the large incidence of albinism, commonly associated with the *in vitro* cultivation of crops, and contamination.

Future improvements to the microspore isolation protocol may include the optimization of the supplement concentrations utilized in the induction media. This may involve using higher concentration of cefotaxime, glutathione and ascorbic acid.

The objectives of this study were achieved. The high throughput screening of wheat material for stem, leaf and stripe rust resistance was successfully implemented. The microspore tissue culturing technique was utilized in the production of wheat haploids, although the success rate was too low for the technique to be economically viable in a breeding program. A 100% homozygous wheat plant containing several species derived leaf and stripe rust resistance genes was produced and can potentially be implemented in the SU-PBL wheat pre-breeding program.

# REFERENCES

*Africa Hunger and Poverty Facts. 2013*. [Online] Available: http://www.worldhunger.org/articles/Learn/africa_hunger_facts.htm Accessed: 11 May 2013.

*Agriculture Production. 2012*. [Online] Available: http://www.info.gov.za/aboutsa/agriculture.htm Accessed: 11 May 2013.

Ahmad, M. 2000. Molecular marker-assisted selection of HMW glutenin alleles related to wheat bread quality by PCR-generated DNA markers. *Theoretical and Applied Genetics*, 101:892-896.

Al-Kaff, N., Knight, E., Bertin, I., Foote, T., Hart, N., Griffiths, S. & Moore, G. 2008. Detailed dissection of the chromosomal region containing the *Ph1* locus in wheat *Triticum aestivum*: With deletion mutants and expression profiling. *Annals of Botany*, 101:863-872.

Ascough, G.D., Bakos, F., Balázs, E., Barnabás, B. & Van Staden, J. 2006. Screening South African wheat germplasm for androgenic competence. *South African Journal of Bottany*, 72:40-45.

Asif, M., Eudes, F., Goyal, A., Amundsen, E., Randhawa, H. & Spaner, D. 2013b. Organelle antioxidants improve microspore embryogenesis in wheat and triticale. *In Vitro Cellular & Developmental Biology*. [Electronic Journal] Available: DOI 10.1007/s11627-013-9514-z Accessed: 4 July 2013.

Asif, M., Eudes, F., Randhawa, H., Amundsen, E., Yanke, J. & Spaner, D. 2013a. Cefotaxime prevents microbial contamination and improves microspore embryogenesis in wheat and triticale. *Plant Cell Reports*, 32:1627-1646.

Ben-Ari, G. & Lavi, U. 2012. Marker-assisted selection in plant breeding. In: Smith, A.A.R.H. & Moser, P.M.H.B.C. (eds.), *Plant Biotechnology and Agriculture Prospects for the 21st Century*. [Electroninc book] Available: http://www.sciencedirect.com/science/article/pii/B9780123814661000110 Accessed: 28 February 2012.

Bertani, G. 1951. Studies on lysogenesis - I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology*, 62:293-300.

Brenchley, R., Spannagl, M., Pfeifer, M., Barker, G.L.A., D'Amore, R., Allen, A.M., McKenzie, N., Kramer, M., Kerhornou, A., Bolser, D., Kay, S., Waite, D., Trick, M., Bancroft, I., Gu, Y., Huo, N., Luo, M., Sehgal, S., Gill, B., Kianian, S., Anderson, O., Kersey, P., Dvorak, J., McCombie, W.R., Hall, A., Mayer, K.F.X., Edwards, K.J., Bevan, M.W. & Hall, N. 2012. Analysis of the bread wheat genome using whole-genome shotgun sequencing. *Nature*, 491(7426):705-710.

Bux, H., Ashraf, M., Hussain, F., Rattu, A.U.R. & Fayyaz, M. 2012. Characterization of wheat germplasm for stripe rust (*Puccinia striiformis* f. sp. *tritici*) resistance. *Australian Journal of Crop Science*, 6(1):116-120.

Cantu, D., Govindarajulu, M., Kozik, A., Wang, M., Chen, X., Kojima, K.K., Jurka, J., Michelmore, R.W. & Dubcovsky, J. 2011. Next Generation Sequencing Provides Rapid Access to the Genome of *Puccinia striiformis* f. sp. *tritici*, the Causal Agent of Wheat Stripe Rust. *PloS one*, 6(8):1-8.

Catanzariti, A.M., Dodds, P.N., Ellis, J.G. & Staskawicz, B.J. 2010. The interaction of avirulence and resistance gene products in flax rust disease – providing advances in rust research. *Canadian Journal of Plant Pathology*, 32(1):11-19.

Cavalli-*S*forza, L.L. & Edwards, A.W.F. 1967. Phylogenetic analysis: models and estimation procedures. *American Journal of Human Genetics*, 19:233–*257*.

*CGIAR Research Centres*. [S.a.]. [Online] Available: http://www.cgiar.org/cgiar-consortium/research-centers/ Accessed: 22 September 2013.

Cherukuri, D.P., Gupta, S.K., Charpe, A., Koul, S., Prabhu, K.V., Singh, R.B., Haq, Q.M.R. & Chauhan, S.V.V. 2003. Identification of a molecular marker linked to an *Agropyron elongatum*-derived gene *Lr19* for leaf rust resistance in wheat. *Plant Breeding*, 122:204-208.

Chisholm, S.T., Coaker, G., Day, B. & Staskawicz, B.J. 2006. Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell*, 124:803-814.

Cistue, L. & Echa, B. 2009. Improvements in the production of doubled haploids in durum wheat (*Triticum turgidum L.*) through isolated microspore culture. *Plant cell Reproduction,* 28(5):727-735.

Clayton, W.D., Vorontsova, M.S., Harman, K.T. & Williamson, H. 2006. *GrassBase - The Online World Grass Flora*. [Online] Available: http://www.kew.org/data/grasses-db/www/imp00042.htm Accessed: 4 June 2013.

Cook, R.J. 1998. The molecular mechanisms responsible for resistance in plant-pathogen interaction of the gene-for-gene type function more broadly than previously imagined. *Proceedings of the National Academy of Sciences*, 95:9711-9712.

Curtis, B.C. 2002. Wheat in the world. In: Curtis, B.C., Rajaram, S. & Gómez Macpherson, H. (eds.), *FAO Plant Production and Protection Series: Bread wheat improvement and production*. Rome: Food and Agriculture Organization of the United Nations.

Dadkhodaie, A., Karaoglou, H., Wellings, C.R. & Park, R.F. 2011. Mapping genes *Lr53* and *Yr35* on the short arm of chromosome 6B of common wheat with microsatellite markers and studies of their association with *Lr36*. *Theoretical and Applied Genetics*, 122:479-487.

Danin, A. 2006. *Flora of Israel:* Aegilops peregrina *(Hack.) Maire & Weiller*. [Online] Available: http://flora.huji.ac.il/browse.asp?lang=en&action=specie&specie=AEGPER&fileid=13105 Accessed: 23 September 2013.

Danin, A. 2007. *Flora of Israel:* Triticum aestivum L. [Online] Available: http://flora.huji.ac.il/browse.asp?lang=en&action=specie&specie=TRIAES&fileid=17388 Accessed: 23 September 2013.

Danin, A. 2008. What shall we eat today. In: *Plant Stories*. [Online] Available: http://flora.huji.ac.il/static/9/93/0024939.002.jpg Accessed: 23 September 2013.

Danin, A. 2011. Sands and their destabilization. In: *Vegetation of Israel and neighbouring countries*. [Online] Available: http://flora.huji.ac.il/browse.asp?action=content&keyword=local_veg_d5 Accessed: 23 September 2013.

Davis, R. 2013. *The battle for Cape Town's farmland.* [Online] Available: http://www.dailymaverick.co.za/article/2013-08-01-the-battle-for-cape-towns-farmland/#.Uj2vnX-ynHc Accessed: 22 September 2013.

*Dimensions of need – An atlas of food and agriculture*. 1995. [Online] Available: http://www.fao.org/docrep/u8480e/u8480e07.htm Accessed: 11 May 2013.

Doyle, J.J. & Doyle, J.L. 1990. A rapid total DNA preparation procedure for fresh plant tissue. *Focus*, 12:13-15.

Du Plessis, S., Cuomo, C.A., Lin, Y.C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., Joly, D.L., Hacquard, S., Amselem, J., Cantarel, B.L., Chiu, R., Coutinho, P.M., Feau, N., Field, M., Frey, P., Gelhaye, E., Goldberg, J., Grabherr, M.G., Kodira, C.D., Kohler, A., Kües, U., Lindquist, E.A., Lucas, S.M., Mago, R., Mauceli, E., Morin, E., Murat, C., Pangilinan, J.L., Park, R., Pearson, M., Quesneville, H., Rouhier, N., Sakthikumar, S., Salamov, A.A., Schmutz, J., Selles, B., Shapiro, H., Tanguay, P., Tuskan, G.A., Henrissat, B., Van de Peer, Y., Rouzé, P., Ellis, J.G., Dodds, P.N., Schein, J.E., Zhong, S., Hamelin, R.C., Grigoriev, I.V., Szabo, L.J. & Martin, F. 2011. Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proceedings of the National Academy of Sciences of the United States of America*, 108(22):9166-9171.

Dubcovsky, J. & Dvorak, J. 2007. Genome plasticity a key factor in the success of polyploid wheat under domestication. *Science*, 316:1862-1866.

Duveiller, E., Singh, P.K., Mezzalama, M., Singh, R.P. & Dababat, A. 2012. *Wheat diseases and pests: A guide for field identification, Second Edition*. Mexico: CIMMYT.

Eksteen, A. 2009. *Die ontwikkeling van molekulêre merkers vir wilde-spesie-verhaalde weerstandsgeenkomplekse van gewone koring*. MSc Thesis, Stellenbosch University.

Ellis, M.H., Spielmeyer, W., Gale, K.R., Rebetzke, G.J. & Richards, R.A. 2002. "Perfect" markers for the *Rht-B1b* and *Rht-D1b* dwarfing genes in wheat. *Theoretical and Applied Genetics*, 105:1038-1042.

Endo, T.R. 2007. The gametocidal chromosome as a tool for chromosome manipulation in wheat. *Chromosome research*, 15:67-75.

Eudes, F. & Amundsen, E. 2005. Isolated microspore culture of Canadian 6x triticale cultivars. *Plant Cell, Tissue and Organ Culture*, 82:233-241.

Eudes, F. & Chugh, A. 2009. An Overview of Triticale Doubled Haploids. In: Touraev, A., Forster, B.P. & Jain, S.M. (eds), *Advances in Haploid Production in Higher Plants*. Netherlands: Springer. pp 87-96.

*FAO Cereal Supply and Demand Brief*. 2013. [Online] Available: http://www.fao.org/worldfoodsituation/wfs-home/csdb/en/ Accessed: 11 May 2013.

*FAOSTAT Trade: Crops and livestock products*. 2013. [Online] Available: http://faostat3.fao.org/home/index.html#DOWNLOAD Accessed: 22 May 2013.

Ferrie, A.M.R. & Caswell, K.L. 2011. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. *Plant Cell, Tissue Organ Culture*, 104:301-309.

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

Germanà, M.A. 2011. Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Reproduction*, 30:839-857.

Goyeau, H., Halkett, F., Zapater, M., Carlier, J. & Lannou, C. 2007. Clonality and host selection in the wheat pathogenic fungus *Puccinia triticina*. *Fungal genetics and biology*, 44(6):474-483.

*GrainGenes: A Database for Triticeae and Avena – Genetic Markers*. [S.a.]. [Online] Available: http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi?class=marker;begin=1;query=x* Accessed: 27 September 2013.

Haile, J.K., Hammer, K., Badebo, A., Nachit, M.M. & Röder, M.S. 2013. Genetic diversity assessment of Ethiopian tetraploid wheat landraces and improved durum wheat varieties using microsatellites and markers linked with stem rust resistance. *Genetic Resources and Crop Evolution*, 60:513-527.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41:95-98.

Hao, M., Chen, J., Zhang, L., Luo, J., Yuan, Z., Yan, Z., Zhang, B., Chen, W., Wei, Y., Zhang, H., Zheng, Y. & Liu, D. 2013. The genetic study utility of a hexaploid wheat DH population with non-recombinant A- and B-genomes. *SpringerPlus*, 2:131.

Helguera, M., Khan, I.A., Kolmer, J., Lijavetzky, D., Zhong-qi, L. & Dubcovsky, J. 2003. PCR assays for the *Lr37-Yr17-Sr38* cluster of rust resistance genes and their use to develop isogenic hard red spring wheat lines. *Crop Science*, 43:1839-1847.

Heyns, I.C. 2010. *Mapping and restructuring of an* Ae. Kotschyi *derived translocation segment in common wheat*. PhD Thesis, Stellenbosch University.

Heyns, I., Pretorius, Z. & Marais, F. 2011. Derivation and characterization of recombinants of the *Lr54/Yr37* translocation in common wheat. *The Open Plant Science Journal*, 5:1-8.

Honing, J. 2007. *Evaluation and implementation of DNA-based diagnostic methodology to distinguish wheat genotypes*. MSc Thesis, Stellenbosch University.

Hovmøller, M.S. & Justesen, A.F. 2007. Rates of evolution of avirulence phenotypes and DNA markers in a northwest European population of *Puccinia striiformis* f. sp. *tritici*. *Molecular ecology*, 16:4637-4647.

*How to feed the world in 2050*. 2009. [Online] Available: http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf Accessed: 3 March 2013.

Huerta-Espino, J., Singh, R.P., Germán, S., McCallum, B.D., Park, R.F., Chen, W.Q., Bhardwaj, S.C. & Goyeau, H. 2011. Global status of wheat leaf rust caused by *Puccinia triticina*. *Euphytica*, 179:143-160.

*Index mundi: Wheat production by country in 1000 MT*. 2013. [Online] Available: http://www.indexmundi.com/agriculture/?commodity=wheat&graph=production Accessed: 22 September 2013.

Jin, Y., Szabo, J. & Carson, M. 2010. Century-old mystery of *Puccinia striiformis* life history solved with the identification of *Berberis* as an alternative host. *Phytopathology*, 100:432-435.

Johnson, R., Stubbs, R., Fuchs, E. & Chamberlin, N. 1972. Nomenclature for physiologic races of *Puccinia striiformis* infecting wheat. *Transactions of the British Mycological Society*, 58:475-480.

Joshi, A.K., Azab, M., Mosaad, M., Moselhy, M., Osmanzai, M., Gelalcha, S., Bedada, G., Bhatta, M.R., Hakim, A., Malaker, P.K., Haque, M.E., Tiwari, T.P., Majid, A., Jalal Kamali, M.R., Bishaw, Z., Singh, R.P., Payne, T. & Braun, H.J. 2010. Delivering rust resistant wheat to farmers: a step towards increased food security. *Euphytica*, 179(1):187-196.

Kobayashi, D.Y., Tamaki, S.J. & Keen, N.T. 1989. Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean. *Proceedings of the National Academy of Sciences*, 86:157-161.

Konzak, C.F., Polle, E.A., Liu, W. & Zheng, Y. 1999. Methods for generating doubled-haploid plants. *International Patent Application* PCT/US99/19498.

Kotze, L. 2009. *Verkorting van die* Ae. peregrina-*verhaalde* Lr59-*translokasie van koring*. MSc Thesis, Stellenbosch University.

Krattinger, S.G., Lagudah, E.S., Spielmeyer, W., Singh, R.P., Huerta-Espino, J., McFadden, H., Bossolini, E., Selter, L.L. & Keller, B. 2009. A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science*, 323:1360-1363.

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

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

Le Maitre, N.C. 2010. *Molecular genetic study of wheat rusts affecting cereal production in the Western Cape*. MSc Thesis, Stellenbosch University.

Li, C., Fatokun, C.A., Ubi, B., Singh, B.B. & Scoles, G.J. 2001. Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. *Crop Science*, 41(1):189-197.

Liu, K. & Muse, S.V. 2005. PowerMarker: Integrated analysis environment for genetic marker data. *Bioinformatics*, 21(9):2128-2129.

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

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

Mago, R., Lawrence, G. & Ellis, J. 2011b. The application of DNA marker and doubled-haploid technology for stacking multiple stem rust resistance genes in wheat. *Molecular Breeding*, 27(3):329-335.

Mangelsdorf, P.C. & Jones, D.F. 1926. The expression of Mendelian factors in the gametophyte of maize. *Genetics*, 11(5):423-455.

Manifesto, M.M., Schlatter, A.R., Hopp, H.E., Suarez, E.Y. & Dubcovsky, J. 2001. Quantitative evaluation of genetic diversity in wheat germplasm using molecular markers. *Crop Science*, 41(3):682-690.

Marais, G.F., Badenhorst, P.E., Eksteen, A. & Pretorius, Z.A. 2010a. Reduction of *Aegilops sharonensis* chromatin associated with resistance genes *Lr56* and *Yr38* in wheat. *Euphytica*, 171:15-22.

Marais, G.F., Marais, A.S. & Groenewald, J.Z. 2001. Evaluation and reduction of *Lr19-149*, a recombined form of the *Lr19* translocation of wheat. *Euphytica*, 121:289-295.

Marais, G.F., Marais, A.S., Eksteen, A., Pretorius, Z.A. 2010b. Modification of the *Aegilops neglecta*-common wheat *Lr62*/*Yr42* translocation through allosyndetic pairing induction. *South African Journal of Plant and Soil*, 27(2):142-151.

Marais, G.F., Marais, A.S., McCallum, B. & Pretorius, Z. 2009. Transfer of leaf rust and stripe rust resistance genes *Lr62* and *Yr42* from *Aegilops neglecta* Req. ex Bertol. to common wheat. *Crop Science*, 49:871-879.

Marais, G.F., McCallum, B. & Marais, A.S. 2006. Leaf rust and stripe rust resistance genes derived from *Aegilops sharonensis*. *Euphytica*, 149:373-380.

Marais, G.F., McCallum, B. & Marais, A.S. 2008. Wheat leaf rust resistance gene *Lr59* derived from *Aegilops peregrina*. *Plant Breeding*, 127:340-345.

Marais, G.F., McCallum, B., Snyman, J.E., Pretorius, Z.A. & Marais, A.S. 2005a. Leaf rust and stripe rust resistance genes *Lr54* and *Yr37* transferred to wheat from *Aegilops kotschyi*. *Plant Breeding*, 124:538-543.

Marais, G.F., Pretorius, Z.A., Marais, A.S. & Wellings, C.R. 2003. Transfer of rust resistance genes from *Triticum* species to common wheat. *South African Journal of Plant and Soil*, 20(4):193-198.

Marais, G.F., Pretorius, Z.A., Wellings, C.R., McCallum, B. & Marais, A.S. 2005b. Leaf rust and stripe rust resistance genes transferred to common wheat from *Triticum dicoccoides*. *Euphytica*, 143:115-123.

Markell, S., Milus, G., Cartwright, R. & Hedge, J. 2000. *Rust diseases of wheat. University of Arkansas. CES FSA7547*. [Online] Available: http://plantpathology.uark.edu/FSA-7547_rusts.pdf Accessed: 18 May 2013.

McCarthy, C. 1998. Chromas Version 1.45. [Online] Available: http://www.technelysium.com.au/chromas/html Accessed: 15 June 2012.

McIntosh, R.A., Dubcovsky, J., Rogers, W.J., Morris, C., Appels, R. & Xia, X.C. 2012. *Catalogue of gene symbols for wheat: 2010 supplement*. [Online] Available: www.shigen.nig.ac.jp/wheat/komugi/genes/macgene/supplement2012.pdf Accessed: 27 September 2013.

McNeil, M.D., Kota, R., Paux, E., Dunn, D., McLean, R., Feuillet, C., Li, D., Kong, X., Lagudah, E., Zhang, J.C., Jia, J.Z., Spielmeyer, W., Bellgard, M. & Appels, R. 2008. BAC-derived markers for assaying the stem rust resistance gene, *Sr2*, in wheat breeding programs. *Molecular Breeding*, 22:15–24.

Murashigue, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15:473-497.

Olivera, P.D., Kolmer, J.A., Anikster, Y. & Steffenson, B.J. 2007. Resistance of Sharon Goatgrass (*Aegilops sharonensis*) to fungal disease of wheat. *Plant Disease*, 91(8):942-950.

Park, R., Fetch, T., Hodson, D., Jin, Y., Nazari, K., Prashar, M. & Pretorius, Z. 2011. International surveillance of wheat rust pathogens: progress and challenges. *Euphytica*, 170(1):109-117.

Peng, J.H., Sun, D. & Nevo, E. 2011. Domestication evolution, genetics and genomics in wheat. *Molecular Breeding*, 28:281-301.

Pienaar, R. de V., Horn, M. & Lesch, A.J.G. 1997. A reliable protocol for doubled haploid accelerated wheat breeding. *Wheat Information Service*, 85:49-51.

Pretorius, Z.A., Bender, C.M., Visser, B. & Terefe, T. 2010. First report of a *Puccinia graminis* f. sp. *tritici* race virulent to the *Sr24* and *Sr31* wheat stem rust resistance genes in South Africa. *Plant Disease*, 94(6):784.

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

Pretorius, Z.A., Singh, R.P., Wagoire, W.W. & Payne, T.S. 2000. Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis*. f. sp. *tritici* in Uganda. *Plant disease*, 84(2):203.

Prins, R. & Marais, G.F. 1998. An extended deletion map of the *Lr19* translocation and modified forms. *Euphytica*, 103:95-102.

Prins, R. & Marais, G.F. 1999. A genetic study of the gametocidal effect of the *Lr19* translocation of common wheat. *South African Journal of Plant and Soil*, 16:10-14.

Prins, R., Groenewald, J.Z., Marais, G.F., Snape, J.W. & Koebner, R.M.D. 2001. AFLP and STS tagging of *Lr19*, a gene conferring resistance to leaf rust in wheat. *Theoretical and Applied Genetics*, 103:618-624.

Prins, R., Marais, G.F., Pretorius, Z.A., Janse, B.J.H. & Marais, A.S. 1997. A study of modified forms of the *Lr19* translocation of common wheat. *Theoretical and Applied Genetics*, 95:424-430.

*Protein Research Foundation: Oil seed & Grain supply in the RSA*. 2013. [Online] Available: http://www.proteinresearch.net/index.php?dirname=html_docs_030statistics_and_estimates/05000protein_statistics/050002013/00600march2013_D20130406.php Accessed: 11 May 2013.

*Puccinia Group Database*. 2010. [Online] Available: http://www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html Accessed: 27 May 2013.

Rebetzke, G.J., Ellis, M.H., Bonnet, D.G., Condon, A.G., Falk, D. & Richard, R.A. 2011. The *Rht13* dwarfing gene reduces peduncle length and plant height to increase grain number and yield of wheat. *Field Crop Research*, 124:323-331.

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

Roelfs, A.P., Singh, R.P. & Saari, E.E. 1992. *Rust Diseases of Wheat: Concepts and methods of disease management*. Mexico: Cimmyt.

Rozen, S. & Skaletsky, H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawets, S. & Misener, S. (eds), *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. New Jersey: Humana Press. pp 365-386.

Santra, M., Ankrah, N., Santra, D.K. & Kidwell, K.K. 2012. An improved wheat microspore culture technique for the production of doubled haploid plants. *Crop Science*, 52:2314-2320.

Schumann, G.L. and Leonard, K.J. 2000. *Stem rust of wheat (black rust)*. [Online] Available: http://www.apsnet.org/edcenter/intropp/lessons/fungi/Basidiomycetes/Pages/StemRust.aspx Accessed: 27 May 2013.

Scott, D.B. 1990. *Wheat diseases in South Africa*. Pretoria: CTP Book Printers.

Sharma, D. & Knott, D.R. 1966. The transfer of leaf rust resistance from *Agropyron* to *Triticum* by irradiation. *Canandian Journal of Genetics and Cytology*, 8:137-143.

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

Singh, R. 2002. *Bread wheat: Improvement and production*. Rome: Food and Agricultural Organization of the United Nations.

Smit, H.A., Tolmay, V.L., Barnard, A., Jordaan, J.P., Koekemoer, F.P., Otto, W.M., Pretorius, Z.A., Purchase, J.L. & Tolmay, J.P.C. 2008. An overview of the context and scope of wheat (*Triticum aestivum*) research in South Africa from 1983 to 2008. *South African Journal of Plant and Soil*, 27(1):81-96.

Soosaar, J.L.M., Burch-Smith, T.M. & Dinesh-Kumar, S.P. 2005. Mechanisms of plant resistance to viruses. *Nature*, 3(10):789-798.

*South African Grain Laboratory Wheat report 2011/2012*. 2012. [Online] Available: http://www.sagl.co.za/Portals/0/Wheat%20crop%202011%202012/Average%20yield%20per%20province.pdf Accessed: 28 February 2012.

*South African Wheat Crop Quality Report 2012/2013 Season*. 2013. [Online] Available: http://www.sagl.co.za/Portals/0/Wheat%20Report%202012%202013%2009%2009%202013%20web.pdf Accessed: 21 September 2013.

Spielmeyer, W., Sharp, P.J. & Lagudah, E.S. 2003. Identification and validation of markers linked to broad-spectrum stem rust resistance gene *Sr2* in wheat (*Triticum aestivum* L.). *Crop Science*, 43:333-336.

Taiz, L. & Zeiger, E. 2006. *Plant Physiology, Fourth Edition*. Sunderland: Sinauer Associates Incorporated.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. *2011*. *MEGA5*: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28:2731-2739.

Tang, Z.X., Wang, X.F., Zhang, M.Z., Zhang, Y.H., Deng, D.X. & Xu, C.W. 2013. The maternal cytoplasmic environment may be involved in the viability selection of gametes and zygotes. *Heredity*, 110:331-337.

Terefe, T., Pretorius, Z.A., Bender, C.M., Visser, B., Herselman, L. & Negussie, T.G. 2011. First report of a new whet leaf rust (*Puccinia triticina*) race with virulence for *Lr12*, *13*, and *37* in South Africa. *Plant Disease*, 95(5):611.

Tester, M. & Bacic, A. 2005. Abiotic stress tolerance in grasses. From model plants to crop plants. *Plant Physiology*, 137:791-793.

*The evolution of wheat – Introduction*. [S.a.]. [Online] Available: http://www.newhallmill.org.uk/wht-evol.htm Accessed: 22 September 2013.

Tilman, D., Balzer, C., Hill, J. & Befort, B.L. 2011. Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, 208(50):20260-20264.

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

Voegele, R.T. & Mendgen, K.W. 2011. Nutrient uptake in rust fungi: how sweet is parasitic life? *Euphytica*, 179:41-55.

Wędzony, M., Forster, B.P., Żur, I., Golemiec, E., Szechyńska-Hebda, M., Dubas, E. & Gotębiowska, G. 2009. Progress in doubled haploid technology in higher plants. In: Touraev, A., Forster, B.P. & Jain, S.M. (eds), *Advances in Haploid Production in Higher Plants*. Netherlands: Springer. pp 1-33.

Wellings, C.R. 2011. Global status of stripe rust: a review of historical and current threats. *Euphytica*, 179(1): 129-141.

Wessels, E. 2010. *Ontwikkeling van 'n koringkwekery met gestapelde, spesie-verhaalde roesweerstand*. MSc Thesis, Stellenbosch University.

*Wheat Genetic and Genomic Resources Center:* Aegilops neglecta *Req. Ex Bertol*. 2012. [Online] Available: http://www.k-state.edu/wgrc/Germplasm/Aegilops/neglecta.html Accessed: 23 September 2013.

Xu, H., Wang, R.J., Shen, X., Zhao, Y.L., Sun, G.L., Zhao, H.X. & Guo, A.G. 2006. Functional properties of a new low-molecular-weight glutenin subunit gene from a bread wheat cultivar. *Theoretical and Applied Genetics*, 113:1295-1202.

Yan, Z.H., Dai, S.F., Liu, D.C., Wei, Y.M. & Zheng, Y.L. 2008. Isolation and Characterization of a Novel HMW-GS *Glu-Dx* allele from Tibet bread wheat landrace. *Cereal Research Communications*, 36(4):523-531.

Zhang, H.B. & Dvořák, J. 1990. Characterization and distribution of an interspersed repeated nucleotide sequence from *Lophopyrum elongatum* and mapping of a segregation distortion factor with it. *Genome*, 33:927-936.

## ADDENDUM A.

**Table A.1**: **Molecular characterization of the F1 base population with the standardized SU-PBL marker set for rust resistance and the novel species derived leaf and stripe rust resistance genes**

| F1 lines | Rust resistance genes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Stem rust | | | Leaf rust | | | Species derived leaf and stripe rust | | | | |
| | *Sr2* | *Sr24* | *Sr31* | *Lr24* | *Lr34* | *Lr37* | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
| 1.1 | 0 | 1 | 0 | 1 | 1 | 1 | | | 0 | | 3 |
| 1.2 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | | 1 |
| 1.3 | 0 | 1 | 0 | 1 | 1 | 1 | | | 0 | | 3 |
| 1.4 | 1 | 1 | 0 | 1 | 1 | 1 | | 0 | 0 | | 2 |
| 1.5 | 1 | 1 | 0 | 1 | 0 | 1 | | | 1 | | 4 |
| 1.6 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | | 2 |
| 1.7 | 1 | 1 | 0 | 1 | 1 | 1 | | 0 | 0 | | 2 |
| 1.8 | 0 | 1 | 0 | 1 | 0 | 1 | | 0 | 0 | | 2 |
| 2.1 | 0 | 1 | 0 | 1 | 1 | 1 | | | 1 | | 4 |
| 2.2 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | | 1 | | 3 |
| 2.3 | 1 | 1 | 0 | 1 | 1 | 1 | | | 1 | | 4 |
| 2.4 | 0 | 1 | 0 | 1 | 0 | 1 | | | 1 | | 4 |
| 3.1 | 0 | 1 | 0 | 1 | 1 | 1 | | | 1 | | 4 |
| 3.3 | 0 | 1 | 0 | 1 | 0 | 1 | | | 0 | | 3 |
| 3.4 | 0 | 1 | 0 | 1 | 1 | 1 | | | 1 | | 4 |
| 3.5 | 0 | 1 | 0 | 1 | 1 | 1 | | | 0 | | 3 |
| 3.6 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | | 3 |
| 3.7 | 0 | 1 | 0 | 1 | 0 | 1 | | | 0 | | 3 |
| 3.8 | 0 | 1 | 0 | 1 | 1 | 1 | | | 0 | | 3 |
| 4.1 | 1 | 1 | 0 | 1 | 1 | 1 | | | 1 | | 4 |
| 4.2 | 0 | 1 | 0 | 1 | 1 | 1 | | | 0 | | 3 |
| 4.4 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | | 2 |
| 4.5 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | | 2 |
| 4.6 | 1 | 1 | 0 | 1 | 1 | 1 | | 0 | 0 | | 2 |
| 4.7 | 0 | 1 | 0 | 1 | 1 | 1 | | | 1 | | 4 |
| 4.8 | 0 | 0 | 0 | 0 | 0 | 0 | | | 0 | | 3 |
| 5.1 | 0 | 1 | 0 | 1 | 1 | 1 | | | 0 | | 3 |
| 5.1 | 0 | 1 | 0 | 1 | 0 | 1 | | | 1 | | 4 |
| 5.3 | 0 | 1 | 0 | 1 | 1 | 1 | | | 1 | | 4 |
| 5.4 | 0 | 1 | 0 | 1 | 1 | 1 | | | 1 | | 4 |
| 5.5 | 0 | 1 | 0 | 1 | 1 | 1 | | 0 | 1 | | 3 |
| 5.6 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | | 1 | | 3 |
| 5.7 | 0 | 1 | 0 | 1 | 1 | 1 | | | 0 | | 3 |
| 5.8 | 0 | 1 | 1 | 1 | 1 | 1 | | | 1 | | 4 |
| 6.1 | 0 | 0 | 0 | 0 | 0 | 1 | | | 1 | | 4 |
| 6.2 | 0 | 1 | 1 | 1 | 1 | 1 | | | 1 | | 4 |
| 6.3 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | | 1 | | 3 |
| 6.4 | 0 | 0 | 0 | 0 | 1 | 1 | | | 0 | | 3 |
| 7.2 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | | 2 |
| 7.3 | 0 | 1 | 0 | 1 | 0 | 1 | | | 0 | | 3 |
| Total | 10 | 35 | 2 | 35 | 26 | 37 | 32 | 30 | 22 | 40 | |

**ADDENDUM B.**

**Table B.1: Potential new designed primers for *Lr54/Yr37***

| | | Primer name | Primer sequence | Reference |
|---|---|---|---|---|
| Original Lr54/Yr37 primers | 1. | S14 (410 F) | 5'- ACC AAT TCA ACT TGC CAA GAG -3' | (Heyns, 2010) |
| | 2. | S14 (410 R) | 5'- GAG TAA CAT GCA GAA AAC GAC A -3' | (Heyns, 2010) |
| New designed Lr54/Yr37 primers | 3. | S14-300F | 5'- CGT TAC CTT CAT CGT CAG CA -3' | |
| | 4. | S14-300R | 5'- CAT GCC AAC CAC GTC TAT TG -3' | |
| | 5. | S14-275F | 5'- CAT GCA GAA AAC GAC ACA CC -3' | |
| | 6. | S14-275R | 5'- GGC CAG ACA AAT CTG ACC AT -3' | |
| | 7. | S15-252R | 5'- GGT AAG TGG TCA GGC GTT GT -3' | |

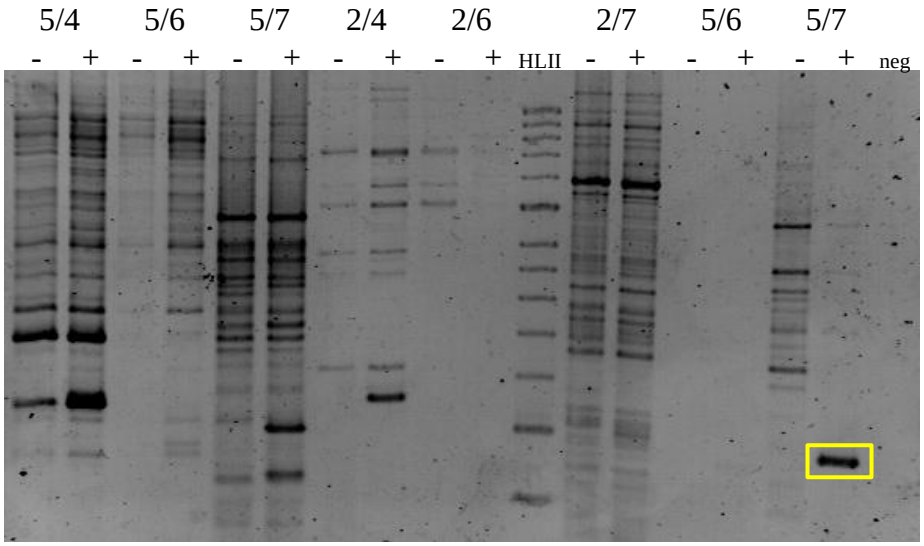



**Figure B.1: Primer combinations evaluated and visualized on a 1.5% agarose gel. Primer combination 5/7 on the right of the gel was identified as the new primer set as it amplified a distinct band in the positive reaction that was not present in the negative control reaction.**

**ADDENDUM C.**

**Table C.1: Molecular characterization of the F2 generation**

| F2 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| 1.5.1 | 0 | 1 | 1 | 1 | 3 |
| 1.5.2 | 1 | 1 | 0 | 1 | 3 |
| 1.5.3 | 0 | 1 | 1 | 1 | 3 |
| 1.5.4 | 0 | 1 | 1 | 1 | 3 |
| 1.5.5 | 1 | 1 | 1 | 1 | 4 |
| 1.5.6 | 1 | 1 | 1 | 1 | 4 |
| 1.5.7 | 1 | 1 | 0 | 1 | 3 |
| 1.5.8 | 0 | 1 | 1 | 1 | 3 |
| 1.5.9 | 1 | 1 | 1 | 1 | 4 |
| 1.5.10 | 1 | 1 | 0 | 1 | 3 |
| 1.5.11 | 0 | 1 | 1 | 1 | 3 |
| 1.5.12 | 1 | 1 | 1 | 1 | 4 |
| 1.5.13 | 1 | 1 | 0 | 1 | 3 |
| 1.5.14 | 1 | 1 | 0 | 1 | 3 |
| 1.5.15 | 1 | 1 | 1 | 1 | 4 |
| 1.5.16 | 1 | 1 | 0 | 1 | 3 |
| 1.5.17 | 1 | 1 | 0 | 1 | 3 |
| 2.3.1 | 1 | 1 | 1 | 1 | 4 |
| 2.3.2 | 0 | 1 | 1 | 1 | 3 |
| 2.3.3 | 1 | 1 | 0 | 1 | 3 |
| 2.3.4 | 0 | 1 | 1 | 1 | 3 |
| 2.3.5 | 1 | 1 | 1 | 1 | 4 |
| 2.3.6 | 0 | 1 | 1 | 1 | 3 |
| 2.3.7 | 1 | 1 | 0 | 0 | 2 |
| 2.3.8 | 1 | 1 | 0 | 0 | 2 |
| 2.3.9 | 1 |  | 0 | 0 | 1 |
| 2.3.10 | 1 | 1 | 0 | 0 | 2 |
| 2.3.11 | 0 | 1 | 1 | 1 | 3 |
| 2.3.12 | 1 | 1 | 1 | 1 | 4 |
| 2.3.13 | 1 | 1 | 1 | 1 | 4 |
| 2.3.14 | 1 | 1 | 0 | 1 | 3 |
| 2.3.15 | 1 | 1 | 0 | 0 | 2 |
| 2.3.16 | 0 | 1 | 1 | 1 | 3 |
| 2.3.17 | 1 | 1 | 1 | 1 | 4 |
| 2.3.18 | 1 | 1 | 0 | 1 | 3 |

| F2 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| 2.3.19 | 1 | 1 | 0 | 1 | 3 |
| 2.4.1 | 1 | 1 | 1 | 1 | 4 |
| 2.4.2 | 1 | 1 | 0 | 0 | 2 |
| 2.4.3 | 1 | 1 | 1 | 1 | 4 |
| 2.4.4 | 0 | 1 | 0 | 0 | 1 |
| 2.4.5 | 0 | 1 | 1 | 1 | 3 |
| 3.4.1 | 1 | 1 | 1 | 1 | 4 |
| 3.5.1 | 1 | 1 | 0 | 1 | 3 |
| 3.5.2 | 1 | 1 | 0 | 1 | 3 |
| 3.5.3 | 1 | 1 | 0 | 1 | 3 |
| 3.5.4 | 1 | 1 | 0 | 1 | 3 |
| 3.5.5 | 1 | 1 | 0 | 1 | 3 |
| 3.5.6 | 1 | 1 | 0 | 1 | 3 |
| 3.5.7 | 1 | 1 | 0 | 1 | 3 |
| 3.5.8 | 1 | 1 | 0 | 1 | 3 |
| 3.5.9 | 1 | 1 | 0 | 1 | 3 |
| 3.5.10 | 1 | 1 | 0 | 1 | 3 |
| 3.5.11 | 1 | 1 | 0 | 1 | 3 |
| 3.5.12 | 1 | 1 | 0 | 1 | 3 |
| 3.5.13 | 1 | 1 | 0 | 1 | 3 |
| 4.7.1 | 1 | 1 | 0 | 1 | 3 |
| 4.7.2 | 1 | 1 | 1 | 1 | 4 |
| 4.7.3 | 1 | 1 | 1 | 1 | 4 |
| 4.7.4 | 1 | 1 | 1 | 1 | 4 |
| 4.7.5 | 0 | 1 | 1 | 1 | 3 |
| 4.7.6 | 0 | 1 | 1 | 1 | 3 |
| 4.7.7 | 1 | 1 | 0 | 1 | 3 |
| 4.7.8 | 1 | 1 | 1 | 1 | 4 |
| 4.7.9 | 1 | 1 | 0 | 1 | 3 |
| 4.7.10 | 1 | 1 | 0 | 1 | 3 |
| 4.7.11 | 1 | 1 | 0 | 1 | 3 |
| 4.7.12 | 1 | 1 | 0 | 1 | 3 |
| 4.7.13 | 1 | 1 | 0 | 1 | 3 |
| 4.7.14 | 1 | 1 | 1 | 1 | 4 |
| 4.7.15 | 1 | 1 | 0 | 1 | 3 |

**Table C.1 continued from previous page**

| F2 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| 4.8.1 | 1 | 1 | 0 | 1 | 3 |
| 4.8.2 | 1 | 1 | 0 | 1 | 3 |
| 4.8.3 | 1 | 1 | 0 | 1 | 3 |
| 4.8.4 | 1 | 1 | 0 | 1 | 3 |
| 4.8.5 | 1 | 1 | 0 | 1 | 3 |
| 4.8.6 | 1 | 1 | 0 | 1 | 3 |
| 4.8.7 | 1 | 1 | 0 | 1 | 3 |
| 4.8.8 | 1 | 1 | 0 | 1 | 3 |
| 4.8.9 | 1 | 1 | 0 | 1 | 3 |
| 4.8.10 | 1 | 1 | 0 | 1 | 3 |
| 4.8.11 | 1 | 1 | 0 | 1 | 3 |
| 4.8.12 | 1 | 1 | 0 | 1 | 3 |
| 4.8.13 | 1 | 1 | 0 | 1 | 3 |
| 4.8.14 | 1 | 1 | 0 | 1 | 3 |
| 4.8.15 | 1 | 1 | 0 | 1 | 3 |
| 4.8.16 | 1 | 1 | 0 | 1 | 3 |
| 4.8.17 | 1 | 1 | 0 | 1 | 3 |
| 4.8.18 | 1 | 1 | 0 | 1 | 3 |
| 4.8.19 | 1 | 1 | 0 | 1 | 3 |
| 4.8.20 | 1 | 1 | 0 | 1 | 3 |
| 4.8.21 | 1 | 1 | 0 | 1 | 3 |
| 4.8.22 | 1 | 1 | 0 | 1 | 3 |
| 4.8.23 | 1 | 1 | 0 | 1 | 3 |
| 4.8.24 | 1 | 1 | 0 | 1 | 3 |
| 4.8.25 | 1 | 1 | 0 | 1 | 3 |
| 4.8.26 | 1 | 1 | 0 | 1 | 3 |
| 5.3.1 | 0 | 1 | 1 | 1 | 3 |
| 5.3.1 | 1 | 1 | 1 | 1 | 4 |
| 5.3.3 | 1 | 1 | 0 | 1 | 3 |
| 5.3.4 | 1 | 1 | 0 | 1 | 3 |
| 5.3.5 | 1 | 1 | 1 | 1 | 4 |
| 5.3.6 | 1 | 1 | 0 | 1 | 3 |
| 5.3.7 | 1 | 1 | 0 | 1 | 3 |
| 5.3.8 | 1 | 1 | 1 | 1 | 4 |
| 5.3.9 | 1 | 1 | 1 | 1 | 4 |

| F2 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| 5.3.10 | 0 | 0 | 1 | 1 | 2 |
| 5.3.11 | 1 | 0 | 0 | 1 | 2 |
| 5.3.12 | 1 | 1 | 0 | 1 | 3 |
| 5.3.13 | 1 | 1 | 0 | 1 | 3 |
| 5.3.14 | 1 | 1 | 1 | 1 | 4 |
| 5.4.1 | 1 | 1 | 1 | 1 | 4 |
| 5.4.2 | 1 | 1 | 1 | 1 | 4 |
| 5.4.3 | 1 | 1 | 0 | 1 | 3 |
| 5.4.4 | 1 | 1 | 0 | 1 | 3 |
| 5.4.5 | 1 | 1 | 0 | 1 | 3 |
| 5.4.6 | 1 | 1 | 0 | 1 | 3 |
| 5.4.7 | 1 | 1 | 0 | 1 | 3 |
| 5.4.8 | 0 | 0 | 0 | 1 | 1 |
| 5.4.9 | 0 | 1 | 1 | 1 | 3 |
| 5.4.10 | 1 | 1 | ? | 1 | 3 |
| 5.4.11 | 1 | 0 | 0 | 1 | 2 |
| 5.5.1 | 1 | 0 | 0 | 1 | 2 |
| 5.5.2 | 1 | 0 | 0 | 1 | 2 |
| 5.5.3 | 1 | 0 | 1 | 1 | 3 |
| 5.5.4 | 1 | 0 | 0 | 1 | 2 |
| 5.5.5 | 1 | 0 | 1 | 1 | 3 |
| 5.5.6 | 0 | 0 | 1 | 1 | 2 |
| 5.5.7 | 1 | 0 | 0 | 1 | 2 |
| 5.5.8 | 1 | 0 | 0 | 1 | 2 |
| 5.5.9 | 1 | 1 | 1 | 1 | 4 |
| 5.5.10 | 1 | 0 | 1 | 1 | 3 |
| 5.5.11 | 1 | 0 | 0 | 1 | 2 |
| 5.6.1 | 0 | 1 | 1 | 1 | 3 |
| 5.6.2 | 0 | 0 | 1 | 1 | 2 |
| 5.6.3 | 0 | 1 | 1 | 1 | 3 |
| 5.6.4 | 0 | 1 | 1 | 1 | 3 |
| 5.6.5 | 0 | 0 | 1 | 1 | 2 |
| 5.6.6 | 0 | 1 | 1 | 1 | 3 |
| 5.6.7 | 0 | 1 | 1 | 1 | 3 |
| 5.6.8 | 0 | 1 | 1 | 1 | 3 |

**Table C.1 continued from previous page**

| F2 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| 5.6.9 | 0 | 1 | 1 | 1 | 3 |
| 5.6.10 | 0 | 0 | 1 | 1 | 2 |
| 5.6.11 | 0 | 0 | 1 | 1 | 2 |
| 5.6.12 | 0 | 1 | 1 | 1 | 3 |
| 5.6.13 | 0 | 1 | 1 | 1 | 3 |
| 6.2.1 | 1 | 1 | 1 | 1 | 4 |
| 6.2.2 | 1 | 1 | 1 | 1 | 4 |
| 6.2.3 | 1 | 1 | 0 | 1 | 3 |
| 6.2.4 | 1 | 1 | 1 | 1 | 4 |
| 6.2.5 | 1 | 1 | 1 | 1 | 4 |
| 6.2.6 | 1 | 1 | 1 | 1 | 4 |
| 6.2.7 | 1 | 1 | 1 | 1 | 4 |
| 6.2.8 | 1 | 1 | 0 | 1 | 3 |
| 6.2.9 | 0 | 1 | 1 | 1 | 3 |
| 6.2.10 | 1 | 0 | 1 | 1 | 3 |
| 6.2.11 | 1 | 0 | 0 | 1 | 2 |
| 6.2.12 | 1 | 1 | 1 | 1 | 4 |
| 7.3.1 | 1 | 1 | 0 | 1 | 3 |
| 7.3.3 | 1 | 1 | 0 | 1 | 3 |
| C1.1.1 | 1 | 1 | 0 | 1 | 3 |
| C1.1.2 | 1 | 1 | 0 | 1 | 3 |
| C.1.1.3 | 1 | 1 | 0 | 1 | 3 |
| C1.1.4 | 1 | 1 | 0 | 1 | 3 |
| C1.1.5 | 1 | 1 | 0 | 1 | 3 |
| C1.1.6 | 0 | 1 | 0 | 1 | 2 |
| C1.1.7 | 1 | 1 | 0 | 1 | 3 |
| C1.1.8 | 1 | 1 | 0 | 1 | 3 |
| C1.5.1 | 0 | 1 | 1 | 1 | 3 |
| C1.5.2 | 1 | 1 | 1 | 1 | 4 |
| C1.5.3 | 1 | 1 | 0 | 1 | 3 |
| C1.5.4 | 1 | 1 | 1 | 1 | 4 |
| C1.5.5 | 0 | 1 | 1 | 1 | 3 |
| C1.5.6 | 0 | 1 | 1 | 1 | 3 |
| C1.5.7 | 0 | 0 | 1 | 1 | 2 |
| C1.7.8 | 1 | 1 | 1 | 1 | 4 |

| F2 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| C2.1.1 | 1 | 1 | 0 | 1 | 3 |
| C2.1.2 | 1 | 1 | 1 | 1 | 4 |
| C2.1.3 | 1 | 1 | 1 | 1 | 4 |
| C2.1.4 | 1 | 1 | 1 | 1 | 4 |
| C2.1.5 | 0 | 1 | 1 | 1 | 3 |
| C2.1.6 | 1 | 1 | 0 | 1 | 3 |
| C2.1.7 | 1 | 1 | 0 | 1 | 3 |
| C2.1.8 | 0 | 1 | 1 | 1 | 3 |
| C2.3.1 | 1 | 1 | 0 | 0 | 2 |
| C2.3.2 | 1 | 1 | 0 | 0 | 2 |
| C.2.3.2 | 1 | 1 | 1 | 1 | 4 |
| C2.3.4 | 1 | 1 | 0 | 0 | 2 |
| C2.3.5 | 0 | 1 | 1 | 1 | 3 |
| C2.3.6 | 0 | 1 | 1 | 1 | 3 |
| C2.3.7 | 0 | 1 | 1 | 1 | 3 |
| C2.3.8 | 0 | 1 | 1 | 1 | 3 |
| C3.8.1 | 1 | 1 | 0 | 1 | 3 |
| C3.8.2 | 1 | 1 | 0 | 1 | 3 |
| C3.8.2 | 1 | 1 | 0 | 1 | 3 |
| C3.8.2 | 1 | 0 | 0 | 1 | 2 |
| C3.8.5 | 1 | 1 | 0 | 1 | 3 |
| C3.8.6 | 1 | 1 | 0 | 1 | 3 |
| C3.8.7 | 1 | 0 | 0 | 1 | 2 |
| C3.8.8 | 1 | 1 | 0 | 1 | 3 |
| C4.7.1 | 1 | 1 | 0 | 1 | 3 |
| C4.7.2 | 1 | 1 | 1 | 1 | 4 |
| C4.7.3 | 1 | 1 | 0 | 1 | 3 |
| C4.7.4 | 1 | 1 | 0 | 1 | 3 |
| C4.7.5 | 1 | 1 | 1 | 1 | 4 |
| C4.7.6 | 1 | 1 | 0 | 1 | 3 |
| C4.7.7 | 0 | 1 | 1 | 1 | 3 |
| C4.7.8 | 0 | 1 | 1 | 1 | 3 |
| C.5.3.1 | 0 | 1 | 1 | 1 | 3 |
| C5.3.2 | 1 | 1 | 1 | 1 | 4 |
| C5.3.3 | 1 | 1 | 0 | 1 | 3 |

**Table C.1 continued from previous page**

| F2 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| C5.3.4 | 1 | 1 | 1 | 1 | 4 |
| C5.3.5 | 0 | 1 | 1 | 1 | 3 |
| C5.3.6 | 1 | 1 | 0 | 1 | 3 |
| C5.3.7 | 1 | 1 | 1 | 1 | 4 |
| C5.4.1 | 1 | 1 | 0 | 1 | 3 |
| C5.4.2 | 1 | 1 | 1 | 1 | 4 |
| C5.4.3 | 1 | 1 | 1 | 1 | 4 |
| C5.4.4 | 1 | 1 | 0 | 1 | 3 |
| C5.4.5 | 1 | 1 | 1 | 1 | 4 |
| C5.4.6 | 0 | 0 | 1 | 1 | 2 |
| C5.4.7 | 1 | 1 | 0 | 1 | 3 |
| C5.4.8 | 0 | 0 | 1 | 1 | 2 |
| C6.2.1 | 1 | 1 | 0 | 1 | 3 |
| C6.2.2 | 1 | 1 | 1 | 1 | 4 |
| C6.2.3 | 1 | 1 | 0 | 1 | 3 |
| C6.2.4 | 1 | 0 | 1 | 1 | 3 |
| G1.1.1 | 1 | 1 | 0 | 1 | 3 |
| G1.1.2 | 1 | 1 | 0 | 1 | 3 |
| G1.1.3 | 1 | 1 | 0 | 1 | 3 |
| G1.1.4 | 1 | 1 | 0 | 1 | 3 |
| G1.5.1 | 1 | 1 | 1 | 1 | 4 |
| G1.5.2 | 1 | 1 | 1 | 1 | 4 |
| G1.5.3 | 0 | 1 | 1 | 1 | 3 |
| G1.5.4 | 1 | 1 | 1 | 1 | 4 |
| G2.1.1 | 1 | 1 | 1 | 1 | 4 |
| G2.1.2 | 1 | 1 | 0 | 1 | 3 |
| G2.1.3 | 1 | 1 | 1 | 1 | 4 |
| G2.1.4 | 1 | 1 | 1 | 1 | 4 |
| G2.3.1 | 1 | 1 | 0 | 0 | 2 |
| G2.3.2 | 1 | 1 | 0 | 0 | 2 |
| G2.3.3 | 1 | 1 | 1 | 1 | 4 |
| G2.3.4 | 1 | 1 | 1 | 1 | 4 |
| G3.8.1 | 1 | 1 | 0 | 1 | 3 |
| G3.8.2 | 1 | 1 | 0 | 1 | 3 |
| G3.8.3 | 1 | 1 | 0 | 1 | 3 |
| G3.8.4 | 1 | 1 | 0 | 1 | 3 |
| G4.7.1 | 1 | 1 | 1 | 1 | 4 |
| G4.7.2 | 1 | 1 | 1 | 1 | 4 |
| G4.7.3 | 1 | 1 | 1 | 1 | 4 |
| G47..4 | 1 | 1 | 1 | 1 | 4 |
| G5.3.1 | 1 | 1 | 0 | 1 | 3 |
| G5.3.2 | 1 | 1 | 0 | 1 | 3 |
| G5.3.3 | 1 | 0 | 1 | 1 | 3 |
| G5.3.4 | 1 | 1 | 0 | 1 | 3 |
| G5.4.1 | 1 | 1 | 0 | 1 | 3 |
| G5.4.2 | 1 | 1 | 0 | 1 | 3 |
| G5.4.3 | 1 | 1 | 1 | 1 | 4 |
| G5.4.4 | 1 | 1 | 1 | 1 | 4 |
| G6.2.1 | 1 | 1 | 0 | 1 | 3 |
| G6.2.2 | 1 | 1 | 0 | 1 | 3 |
| G6.2.3 | 1 | 1 | 0 | 1 | 3 |
| G6.2.4 | 1 | 1 | 1 | 1 | 4 |

**ADDENDUM D.**

**Table D.1: Molecular characterization of the F3 generation**

| F3 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| 15.5.1 | | 0 | | | 3 |
| 15.5.2 | 0 | | | | 3 |
| 15.5.3 | | | | | 4 |
| 15.5.4 | 0 | 0 | | | 2 |
| 23.1.1 | | | 0 | 0 | 2 |
| 23.1.2 | | | 0 | 0 | 2 |
| 23.1.3 | | | | | 4 |
| 23.1.4 | 0 | | | | 3 |
| 34.1.1 | 0 | | | | 3 |
| 34.1.2 | 0 | | | | 3 |
| 34.1.3 | 0 | | | | 3 |
| 34.1.4 | 0 | | | | 3 |
| 35.7.1 | | | 0 | | 3 |
| 35.7.3 | | | 0 | | 3 |
| 35.7.4 | | | 0 | | 3 |
| 47.2.1 | | | | | 4 |
| 47.2.2 | | | 0 | | 3 |
| 47.2.3 | | | | | 4 |
| 47.2.4 | | | | | 4 |
| 48.4.1 | | | 0 | | 3 |
| 48.4.2 | | | 0 | | 3 |
| 48.4.3 | | | 0 | | 3 |

| F3 lines | *Lr53/Yr35 & Lr56/Yr38* | *Lr54/Yr37* | *Lr59* | *Lr62/Yr42* | Total |
|---|---|---|---|---|---|
| 48.4.4 | | | 0 | | 3 |
| 53.12.1 | | | 0 | | 3 |
| 53.12.2 | | | 0 | | 3 |
| 53.12.3 | | | 0 | | 3 |
| 53.12.4 | | | 0 | | 3 |
| 54.3.1 | | | 0 | | 3 |
| 54.3.2 | | | 0 | | 3 |
| 54.3.3 | | | 0 | | 3 |
| 55.9.1 | | 0 | | | 3 |
| 56.4.1 | 0 | 0 | | | 2 |
| 56.4.2 | 0 | | | | 3 |
| 56.4.3 | 0 | 0 | | | 2 |
| 56.4.4 | 0 | | | | 3 |
| 58.2.1 | | 0 | 0 | | 2 |
| 58.2.3 | | 0 | 0 | | 2 |
| 58.2.4 | | 0 | 0 | | 2 |
| 62.9.1 | 0 | | | | 3 |
| 62.9.2 | 0 | | | | 3 |
| 62.9.3 | 0 | 0 | 0 | | 1 |
| 62.9.4 | 0 | | | | 3 |
| 73.1.1 | | | 0 | | 3 |
| 73.1.3 | | | 0 | | 3 |

**ADDENDUM E.**

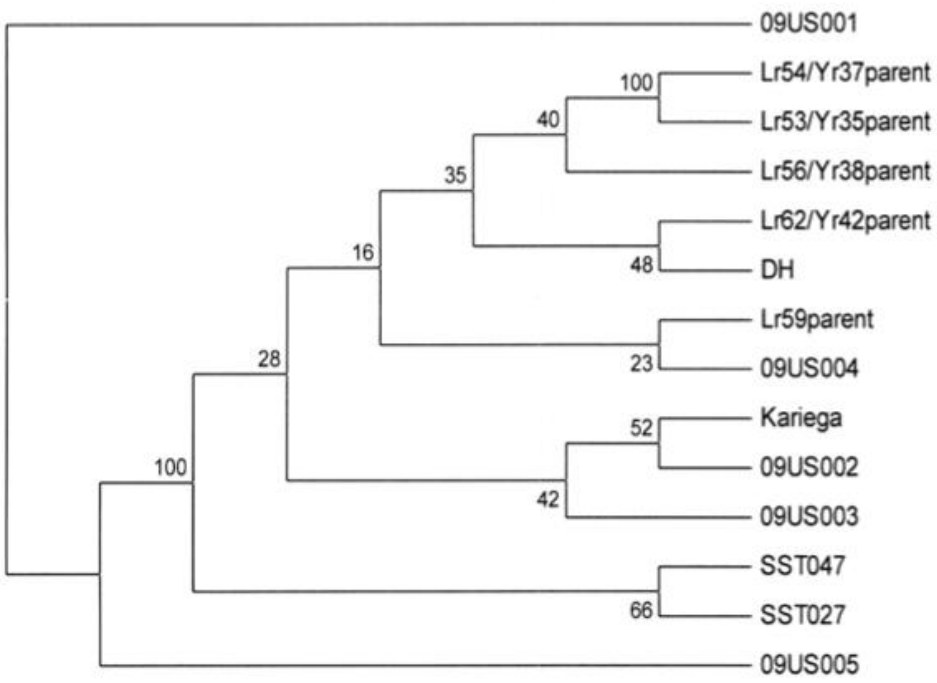



**Figure E.1: Neighbour Joining phylogenetic tree**

**ADDENDUM F.**

Article as submitted to the South African Journal of Plant and Soil on 16 August 2013:

**Optimized microspore tissue culture protocol for creating DH wheat**

Wheat doubled haploids are predominantly produced via the wide crossing method utilizing maize (*Zea mays* L.) pollen, but doubled haploids can also be produced via anther culturing or microspore tissue culturing. This study was performed to optimize, implement and evaluate a microspore tissue culture protocol that can be applied to wheat genotypes. Wheat ears with microspores at late uni-nucleate or early bi-nucleate stages were harvested and cold-incubated for 28 days after which the microspore isolation took place. After 40 days embryo-like structures (ELS) were removed from the induction media and placed onto GEM media. Sixty seven percent of embryos that developed were albinos and only 33% were green plantlets that could be considered as having viable roots and shoots. These plantlets were transplanted into a growth house and treated with colchicine after adequate development took place. Doubled haploid seed were subsequently harvested from fertile tillers. The green plant regeneration rate was about 2%. Although higher than previously reported South African attempts at employing microspore culturing, the success rate is still lower than the currently utilized wide crossing protocol. Further media optimization attempts are therefore on-going and include among others the use of a microbial peptide to lower the albino frequency.

**Keywords**: accelerated breeding-time, germplasm specific, microspore isolation, *Triticum aestivum*

Bread wheat (*Triticum aestivum* L.) is one of the most important globally consumed staples. Wheat's demand is estimated to increase with 60% to 880 million tons by 2050 (Singh *et al*. 2011). Plant breeding, together with improved crop husbandry, is the primary mechanism to improve yields to meet this predicted global demand. It typically takes up to six generations, following initial hybridization between parents, to obtain pure breeding lines. Employing doubled haploid (DH) technology has however enabled breeders to accelerate this rate and achieve complete homozygosity in a single generation, thus potentially improving the rate of year-on-year yield gain achieved in a breeding program (Germanà 2011).

Doubled haploids can be produced via the wide crossing method (Pienaar *et al.* 1997) where wheat ovaries are pollinated by maize followed by embryo rescue. Other methods include

producing DH from either male or female gametes through androgenesis or gynogenesis (Eudes and Chugh 2009). Gynogenesis is not a widely utilized technique for creating DH plants in cereals. Androgenesis involves the diversion of microspores under optimal culture conditions to undergo a sporophytic pathway, instead of following the normal gametophytic lifecycle. Androgenesis in wheat can be achieved via two techniques: anther culture and the isolated microspore tissue culture method (Ferrie and Caswell 2011).

The isolated microspore technique holds several advantages over anther culture: it minimizes the risk of somatic cells developing from the anther wall; it permits better nutrient uptake for the dividing microspores; it does not require micro surgical skills and is less time consuming than anther culture (Eudes and Chugh 2009). This technique however also has complications: not all genotypes respond equally well to microspore *in vitro* propagation which leads to poor embryo formation and a large percentage of albino embryos (Ascough *et al*. 2006, Cistue 2009, Mago 2011). It is thus necessary to optimize the protocol for each specific breeding program's germplasm in order to maintain an economically viable success rate.

This study was performed to optimize, implement and evaluate a microspore tissue culture protocol that can be applied to wheat genotypes containing (high frequencies) specie derived rust resistance genes. An adapted microspore isolation protocol from Eudes *et al*. (2005) was followed.

Plant material was grown in a mechanically cooled growth house with a natural light-dark photoperiod and temperatures that ranged from 10 to 25ºC. The microspores' developmental stage was determined under a light microscope (Zeiss Axioscope) after staining the pollen with 0.5% acetocarmine. Ears containing microspores at the late uni-nucleate or early bi-nucleate stages were subsequently harvested, wrapped in foil and incubated at 4ºC in a glass beaker with distilled water.

After about 28 days of cold-incubation, the microspore isolation protocol was performed under sterile conditions in a laminar flow hood. Awns were removed and ears were surface sterilized with a treatment of 10% bleach (3.5% m/v sodium hypochlorite stock) for 3 minutes, two 1 minute washes with autoclaved distilled water, another 1 minute wash with 50% ethanol and a final wash step with autoclaved distilled water for 1 minute. The ears were constantly shaken during the sterilization process. The ears were aseptically cut into 1cm pieces into a refrigerated blender cup containing 50ml sterilized extraction solution (NPB99

media supplemented with 72.9g.$L^{-1}$ D-mannitol, and 250mg.$L^{-1}$ MES hydrate) kept at 4°C. Plant material was subsequently blended twice for 7 seconds on the low setting of a Warring commercial blender (distributed by Healthcare Technologies, Table View, Cape Town, RSA) to release the microspores contained in the anthers. This solution was poured through a sterile 0.7mm sieve. Fifty millilitres of extraction solution was used to rinse the blender cup and also poured through the sieve. This solution was divided into two 50ml conical tubes by pouring it through a sterile 100µm nylon mesh cell strainer. The tubes were centrifuged in a Hettich Universal 21 at 850rcf for 5 minutes (distributed by Labotec, Pinelands, Cape Town, RSA) in order to obtain microspore pellets. The supernatant was discarded and the pellet washed by adding 30ml of extraction solution and centrifuging it under the same conditions. The supernatant was subsequently discarded and the pellets from the two conical tubes were pooled into one 10ml centrifuge tube. The tube was filled with NPB99-media (Konzak 1999, without nicotinic acid, pyridoxine-HCl and thiamine) and centrifuged once more under the same conditions. The supernatant was discarded and cells were resuspended in 2ml of 3M D-mannitol. This cell suspension was transferred to a new 10ml centrifuge tube containing 21% maltose monohydrate so that the cell suspension formed a layer on top of the maltose. This tube was centrifuged at 850rcf for 5 minutes. Viable microspores at the optimal stage for induction formed a white interphase between the maltose mannitol gradient. This layer of cells were collected and placed into a clean 10ml centrifuge tube. The tube was filled with NPB99 and the cells were pelleted by centrifugation for 5 minutes at 850rcf. The supernatant was discarded and the cells resuspended in 1ml NPB99. The cell concentration was determined using a haemocytometer. Cells were adjusted to a concentration of approximately $10^5$ cells per ml. Two hundred microlitre of this cell suspension was added to 35mm petri dishes with 3.3ml of NPB99 containing 10% ficoll PM400 (Sigma, F4375).

Ovaries were removed from either wheat or triticale (depending on availability) ears and surface sterilized by rinsing it in 50% ethanol for 3 minutes. It was then washed twice with autoclaved distilled water for 1 minute. Only light shaking was applied when sterilizing the ovaries.

Three to five sterilized ovaries were added to each petri dish containing induction media with isolated microspores. The petri dishes were wrapped with parafilm and placed into a large 15cm petri dish that was also wrapped in parafilm and subsequently incubated at 28°C in the dark. One such large petri dish contained 7 small petri dishes with microspores and one small

open petri dish with distilled water. The eight wheat ears that we utilized in this study yielded eight small petri dishes with induction media. Four petri dishes contained only induction media and four of the dishes were supplemented with 20µg/ml Vancomycin.

After 40 days only the petri dishes without the antibiotic showed developing embryo-like structures. The ELS were aseptically removed from the induction media and placed onto GEM media (Eudes *et al*. 2003) in 10cm petri dishes with no more than 25 ELS per plate. The plates were incubated at 16°C with a 16h light – 8h dark cycle. Ten days later the ELS that developed into embryos with small shoots were transferred to magenta bottles containing 30ml of rooting media (Eudes *et al*. 2003). Sixty seven percent of these embryos were albinos. The small green plants that developed adequate roots and shoots were transferred to small pots with a soil composition of 3:1::sand:potting soil and placed in a growth house. The pots were covered with plastic bags and over a period of about 8 days the bags were gradually opened and removed to harden off the plants. Plants were treated with colchicine, as described by Pienaar *et al*. (1997), to induce chromosome doubling. Seed was harvested from the DH plants.

The green plant regeneration rate after colchicine treatment was 2%. This corresponds to findings of Ascough *et al*. (2006) who evaluated the androgenic competence of South African germplasm. They found a green plant regeneration rate of 0.1-1% when utilizing the isolated microspore technique.

Current work includes further optimization of the induction media. Additional supplements like PSK-alpha peptide (www.polypeptide.com, SC1465), glutathione, larcoll and ascorbic acid are being evaluated as agents to reduce the albino frequency and callus oxidation. As an additional measure to prevent contamination, antibiotics like cefotaxime, which also stimulates callus formation, could also be added to the media.

References:

Ascough GD, Bakos F, Balázs E, Barnabás B, Van Staden J. 2006. Screening South African wheat germplasm for androgenic competence. South African Journal of Bottany 72: 40-45.

Cistue L, Echa B. 2009. Improvements in the production of doubled haploids in durum wheat (*Triticum turgidum L.*) through isolated microspore culture. *Plant cell Reproduction* 28(5): 727-735.

Eudes, F., Acharya, S., Laroche, A., Selinger, L.B. & Cheng, K.J. 2003. A novel method to induce direct somatic embryogenesis, secondary embryogenesis and regeneration of fertile green cereal plants. *Plant Cell, Tissue and Organ Culture*, 73: 147-157.

Eudes F, Amundsen E. 2005. Isolated microspore culture of Canadian 6x triticale cultivars. *Plant Cell, Tissue and Organ Culture* 82: 233-241.

Eudes F, Chugh. 2009. An Overview of Tritical Doubled Haploids. In: Touraev A, Forster BP, Jain SM (eds), *Advances in Haploid Production in Higher Plants*. Netherlands: Springer. pp 87-96.

Ferrie AMR, Caswell KL. 2011. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. *Plant Cell, Tissue Organ Culture* 104: 301-309.

Germanà MA. 2011. Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Reproduction* 30: 839-857.

Konzak CF, Polle EA, Liu W, Zheng Y. 1999. Methods for generating doubled-haploid plants. *International Patent Application* PCT/US99/19498.

Mago R, Lawrence G, Ellis J. 2010. The application of DNA marker and doubled-haploid technology for stacking multiple stem rust resistance genes in wheat. *Molecular Breeding* 27(3): 329-335.

Pienaar RV, Horn M, Lesch AJG. 1997. A reliable protocol for doubled haploid accelerated wheat breeding. *Wheat Information Series* 85: 49-51.

Singh RP, Hodsons DP, Huerta-Espino J, Jin Y, Bhavani S, Njau P, Herrera-Foessel S, Singh PK, Singh S, Govindan V. 2011. The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. *Annual Review of Phytopathology* 49(13): 1-17.