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Unlocking new sources of adult plant resistance to wheat leaf rust

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Abstract

Leaf rust (LR), caused by *Puccinia triticina* Eriks., is among the most important fungal diseases of wheat (*Triticum aestivum* L.) crops globally. LR is prevalent in all wheat cultivating regions of Australia, inflicting both yield and grain quality losses. The most sustainable method for controlling rust diseases is to incorporate genetic resistance, particularly adult plant resistance (APR), in cultivars grown by farmers. APR is considered more durable than seedling resistance because resistance is often underpinned by multiple 'minor effect' genes providing partial resistance thereby reducing selection pressure on the pathogen. Seedling resistance is often conferred by a single major gene that is pathogen race specific. Yet, despite the significance of APRs only a limited number of APR genes are currently deployed for wheat cultivation. Therefore, additional genes are required to prolong resistance as use of multiple genes in different combinations slows down the pathogen to gain virulence. Hence, the overall aim of the study was to identify new sources of APR from historical wheat germplasm.

A diversity panel comprising of 295 bread wheat accessions was assembled, originally sourced from the N. I. Vavilov Institute of Plant Genetic Resources (VIR), a seed bank in St Petersburg, Russia. The panel comprised landraces, cultivars, and breeding lines, collected from 28 countries around the world over a period spanning from 1920 to 1990. The diversity panel was genotyped using the Diversity Arrays Technology genotyping-by-sequencing platform (DArT-seq) and the first genomic characterisation of wheat accessions from VIR was performed. This revealed a huge array of new alleles which were either fixed or absent in a sample of modern cultivars and breeding lines from Australia and the International Maize and Wheat Improvement Center (CIMMYT) in Mexico.

To enable evaluation of LR resistance all-year-round, a rapid phenotyping protocol that integrates assessment at both seedling and adult plant growth stages under controlled conditions was designed and validated. The method enables up to seven consecutive disease assays per year, compared to just one assessment in the field. The integrated method is more efficient requiring less time, space, and labour than traditional approaches where seedling and adult plants are assessed in separate assays.

To accelerate the discovery of new sources of APR, a novel approach was adopted, which involved screening the diverse wheat accessions using: 1) DNA markers linked to

known APR genes, 2) the rapid phenotyping method and 3) field evaluation using multiple *P. triticina* pathotypes. Based on DNA marker screening, 83 lines were deemed to carry known APR genes (*Lr34*, *Lr46*, and *Lr67*) thus were eliminated. Rapid phenotypic screening identified 50 lines carrying APR and field-testing of the subset using pathotypes with additional virulence for race-specific APR genes (*Lr13* and *Lr37*), identified 13 lines that consistently displayed high levels resistance across years and pathotypes. These lines provide useful sources for future research.

Next, genome-wide association studies (GWAS) were performed for the diversity panel using 10,748 polymorphic DArT-seq markers. The diversity panel was evaluated at both seedling and adult plant growth stages using three *P. triticina* pathotypes prevalent in Australia. GWAS was applied to 11 phenotypic data sets which identified a total of 52 significant marker-trait associations representing 31 quantitative trait loci (QTL). Among them, 29 QTL were associated with APR. Of the 31 QTL, 13 were considered potentially new loci, whereas four co-located with previously catalogued LR resistance genes (*Lr*) and fourteen aligned to regions reported in other GWAS and genomic prediction studies. Notably, highly resistant accessions carried a large number of alleles for resistance, thus highlighting the potential of allele stacking or pyramiding to strengthen resistance levels against *P. triticina*.

Major outcomes include the establishment and genetic characterisation of a Vavilov wheat diversity panel, development of a rapid phenotyping method, development of a new screening approach to mine seed bank accessions for disease resistance, and identification of new genomic regions underpinning LR resistance. This study provides open access seed and genetic resources, along with the insight and tools to exploit them in research, pre-breeding and breeding programs. This will help pathologists, geneticists and plant breeders to assemble improved wheat cultivars with long lasting resistance to LR.

Declaration by author

This thesis *is composed of my original work,* and *contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Riaz A, Periyannan S, Aitken E, Hickey L (2016) A rapid phenotyping method for adult plant resistance to leaf rust in wheat. Plant Methods 12(1):1-10

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Keywords

Wheat, leaf rust, germplasm, landraces, genetic resistance, adult plant resistance, phenotyping, genome-wide association studies, quantitative trait loci, gene pyramiding.

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List of Abbreviations used in the thesis

AFLP ..... Amplified fragment length polymorphism
AGC ..... Accelerated growth conditions
APR ..... Adult plant resistance
COI ..... Coefficient of infection
DH ..... Double haploid
DArT ..... Diversity Arrays Technology
DArT-seq GBS..... Diversity Arrays Technology genotyping-by-sequencing
FIGS .....Focused identification of germplasm strategy
GBS .....Genotyping-by-sequencing
GS ..... Genomic selection
GP ..... Genomic prediction
GWAS ..... Genome-wide association studies
HR ..... Hypersensitive response
IT ..... Infection type
LD ..... Linkage disequilibrium
LR ..... Leaf rust
MAF ..... Minor allele frequency
MAGIC ..... Multi-parent advanced generation inter-cross
MAS ..... Marker-assisted selection
NAM ..... Nested-association mapping
RAPD ..... Randomly amplified polymorphic DNA
RFLP ..... Restriction fragment length polymorphism
RIL .....Recombinant inbred line
SNP ..... Single nucleotide polymorphism
SSR ..... Simple sequence repeat
QTL .....Quantitative trait loci

Chapter 1 - General introduction

1.1 Introduction

Bread wheat (*Triticum aestivum* L. em. Thell.) is the third most produced food crop after maize and rice (Ray et al. 2013). Wheat provides ≥20% of the dietary calories (i.e. starch and proteins) to the human diet (Hawkesford et al. 2013). Given that wheat is the main food source, and the global population is set to increase from 7.3 billion to 9.7 billion by the year 2050 (UN 2015), wheat production has to be doubled to meet the projected demand. To complicate this grand challenge, wheat production is under constant threat of climate change (Asseng et al. 2015) and the emergence of highly virulent pests and pathogens (Chakraborty and Newton 2011).

In Australia, wheat is one of the major winter crops mostly grown in the states of Western Australia, Victoria, New South Wales, and Queensland. Australia is the ninth leading wheat producer and the fourth largest wheat exporter in the world (FAO 2015). The average wheat yield in Australia is 1.9 tonnes per hectare, which is low compared to other regions of the world (ABARES 2015). This is primarily due to low rainfall (varies from 250 to 650 mm), low soil fertility and biotic stresses such as diseases (i.e. stem rust, leaf rust, stripe rust, yellow spot, and crown rot) and nematodes (Murray and Brennan 2009).

Wheat leaf rust (LR) caused by the biotrophic fungal pathogen *Puccinia triticina* Eriks., is one of the most common and geographically widespread diseases of wheat in Australia and worldwide. LR causes 10–70% reduction in yield and also affects the grain quality (Huerta-Espino et al. 2011; Chaves et al. 2013; Niks et al. 2015). Each year in Australia, LR causes severe economic losses of AUD 12 million per annum, but has the potential to cause greater losses up to AUD 197 million per annum to the Australian cereal industry (Murray and Brennan 2009; Huerta-Espino et al. 2011). In Australia, the pathogen, *P. triticina* is prevalent in all wheat growing regions, and the populations of such are reported to be evolving rapidly to form new pathotypes (Park 1996, 2016).

Deployment of genetically resistant wheat cultivars is the most effective and environment-friendly strategy to control LR. To date, 77 genes for resistance to LR (i.e. *Lr*) have been catalogued including seedling resistance and adult plant resistance (APR) genes

(McIntosh et al. 2017). Typically, seedling resistance is detected at the seedling stage and remains effective through the lifecycle of the plant. Seedling resistance is often controlled by a single major gene effective against selective races of the pathogen (i.e. race-specific), and this places strong selection pressure on the pathogen population to overcome resistance through a selection of favourable genetic variants. In contrast, a more durable form of resistance is provided by APR, which is effective at post seedling growth stages, and is either controlled by multiple genes each with minor effect or single gene with a major effect (Lagudah 2011; McCallum et al. 2012; Burdon et al. 2014). APR genes typically confer partial resistance against all races of the pathogen (i.e. race-nonspecific). For instance, an APR gene *Lr34* provided effective resistance against LR for almost 100 years following deployment from a range of different breeding programs (Ellis et al. 2014). Few LR APR genes (i.e. *Lr34*, *Lr46*, and *Lr67*) have been identified to provide race non-specific APR to multiple pathogens. For instance, APR gene *Lr34* provides partial resistance for LR, stripe rust, stem rust, powdery mildew, spot blotch and resistance is associated with the morphological phenotype of leaf tip necrosis (i.e. *Lr34/Yr18/Sr57/Pm38/Sb1/Ltn1*) (Lagudah et al. 2011; Ellis et al. 2014). In the past, APRs were less preferred in wheat breeding programs as their selection requires multiple rounds of evaluation in the field and are difficult to detect as well. With the recent shift in the breeding approach for developing rust resistant cultivars, wheat breeders currently strive to deploy APR genes singly or in combinations with seedling resistance genes. Therefore, additional sources of rust resistances are needed to develop high-yielding resistant cultivars to cope with the rapidly evolving *P. triticina*.

Landraces preserved in seed banks around the world harbour new genetic diversity which can be introduced into modern wheat germplasm (Longin and Reif 2014). Many of these accessions have already been adapted to particular target environments carrying new genetic diversity for various biotic and abiotic stresses. In fact, *Lr* genes *Lr52* and *Lr67* were identified from landraces (Hiebert et al. 2010; Bansal et al. 2013). Nonetheless there are hundreds of thousands of viable wheat accessions preserved in seed banks, such as the Svalbard Global Seed Vault, Norway, collections based at CIMMYT, Mexico, the United States Department of Agriculture, USA, and John Innes Centre, UK. Although this represents a vast array of genetic diversity, identifying accessions carrying new sources of rust resistance is still challenging, such as screening for disease resistance.

Traditionally, phenotyping APR is performed at the adult plant stage in the field which is sometime challenging due to variable weather patterns (i.e. temperature, humidity), and

are limited to one assessment per year (Ortiz et al. 2007; Hickey et al. 2012). This slows research efforts to identify new APR genes and breeding of genetically resistant cultivars. Alternatively, various studies have reported the use of controlled environmental conditions (CEC) for phenotyping APR to foliar pathogens such as phenotyping APR to stripe rust, stem rust, and yellow spot (Hickey et al. 2012; Prins et al. 2016; Sørensen et al. 2016; Dinglasan et al. 2016). A key advantage is the controlled environmental factors, such as temperature and light, which minimises the response variation and also helps to accelerate the growth of wheat plants. Further, CEC allow disease screening all-year-round, thus making it time and resource efficient.

Next, the identification of the genomic regions controlling various traits in the wheat genome has always been a challenge due to the large genome size and presence of repetitive sequences. The development of low-cost high-throughput marker platforms such as genotyping-by-sequencing (GBS) or 90K single nucleotide polymorphism (SNP), offers a cost-effective tool for determining genetic diversity, especially in natural populations (Miller et al. 2007; Elshire et al. 2011). Similarly, Diversity Arrays Technology (DArT) offers a low-cost high-throughput and restriction enzyme-based complexity reduction system performed on a microarray platform. DArT detects DNA polymorphism in the form of a single base change (SNP) and insertions and deletions (InDels). Recent advancements replaced the microarray platform to the next generation sequencing platform, known as DArT-seq GBS platform (DArT-seq) (Kilian et al. 2012), offering very high marker densities. The adoption of whole genome high-density molecular markers has propelled the understanding of the genetic architecture of complex traits and also enabled the exploitation of germplasm collections for modern wheat improvement (Voss-Fels et al. 2015).

High-throughput marker systems have enabled genomic characterisation of hexaploid wheat cultivars from many parts of the world and facilitated genome-wide association studies (GWAS) (Cavanagh et al. 2013), which is a powerful approach to dissect the genetic architecture of complex traits in diverse collections or natural populations (Zhu et al. 2008; Hall et al. 2010). GWAS detects genomic regions in linkage disequilibrium (LD) affecting the trait of interest. Due to the greater number of historical chromosomal recombinations accumulated over a large number of generations in natural populations, GWAS can map genomic regions controlling the trait at a much higher resolution (Yu and Buckler 2006; Semagn et al. 2010). Thus, GWAS offers the unique opportunity of linking diversity analysis, identification of marker-trait associations and molecular breeding.

Improving wheat yield through sustainable means is a multifaceted challenge and therefore, less likely to be achieved by the use of a single technology or approach. An integrated approach is required which involves developing new breeding strategies, use of advanced tools (i.e. molecular and phenotypic) and utilising diverse germplasm resources. This would aid in the development of better wheat cultivars with not only increased yields and enhance nutritional quality but also reduces vulnerability to new and re-emerging pests and pathogens. Thus, the overall aim of this research was to discover new sources of APR to wheat leaf rust and to deliver robust tools to enable their deployment in wheat pre-breeding and breeding programs. For this purpose, a diversity panel of 295 bread wheat accessions sourced from N. I. Vavilov Institute of Plant Genetic Resources (VIR), St Petersburg, Russia was analysed in this thesis.

1.2 Hypothesis and aims

1.2.1 Overall thesis hypothesis

Seed bank accessions can be effectively mined to identify new sources of APR to LR in wheat.

1.2.2 Thesis aims

- Establish and genetically characterise a diverse bread wheat panel based on accessions sourced from the VIR.
- Develop a protocol that permits rapid phenotyping for adult plant resistance to LR in wheat grown under accelerated growth conditions.
- Identify new sources of APR to LR in wheat by applying a new screening approach involving effective elimination of known APR genes using linked DNA markers and rapid phenotyping.
- Identify quantitative trait loci underpinning LR resistance in the Vavilov wheat diversity panel via GWAS.

1.3 Significance of research

P. triticina is prevalent in all wheat growing regions of Australia and causes yield losses and poor grain quality. Historically, a LR epidemic was observed in Western Australia in 1999, where LR and stem rust caused a monetary loss of AUD 50 million (Hills et al. 1999). Under favourable environmental conditions, *P. triticina* has the potential to cause national losses up to AUD 197 million to the Australian cereal industry (Murray and Brennan 2009; Huerta-Espino et al. 2011). Most of the instances where *P. triticina* entered Australia as an exotic incursion, it has evolved further through single step mutation, thus giving rise to new pathotypes at an average of 10–15 pathotypes per year (Park 1996, 2016). Deployment of resistant cultivars is the most effective method to control rust diseases. Australia annually saves AUD 85 million and AUD 12 million through the deployment of genetically resistant cultivars and reduction in the cost of fungicide sprays, respectively (Ellis et al. 2014). Previously, breeding for genetic resistance to rust diseases was primarily focused on seedling resistance which was often overcome by the pathogen either through rapid evolution here in Australia or introduction of exotic compatible races. For instance, in Australia, a recent exotic introduction of *P. triticina* pathotype 104–1,3,4,6,7,8,10,12+*Lr37* carried virulence for five *Lr* genes (*Lr12*, *Lr13*, *Lr20*, *Lr27+31*, and *Lr37*) which were widely deployed in Australian cultivars (Cuddy et al. 2016; Park 2016).

Therefore, the exploitation of diverse wheat accessions preserved in seed banks such as the VIR, is considered a promising approach to identify new and durable resistance factors that can be utilised for the improvement of modern high-yielding cultivars.

1.4 Methodology

To identify new sources of APR, a wheat diversity panel comprising 295 accessions sourced from the VIR, was established. The purified accessions were genotyped using the DArT-seq platform. The diversity panel was compared with a selection of breeding material from Australia and CIMMYT to determine the population structure and benchmark the genetic diversity. A new method was developed that permits rapid phenotyping for APR to LR in wheat by exploiting ‘accelerated growth conditions’ (AGC) or ‘speed breeding’ to expedite plant development and involves two sequential inoculations to detect APR. Later, the wheat diversity panel was screened using PCR markers for known APR genes (*Lr34*, *Lr46*, and

Lr67) and the rapid phenotyping method, followed by field screening using multiple *P. triticina* pathotypes. This new screening strategy enabled the rapid identification of new sources of APR to LR. Furthermore, GWAS of LR resistance in the wheat diversity panel was conducted at seedling and adult plant stage, using a total of 11 phenotypic data sets against three most prevalent *P. triticina* pathotypes in Australia. The identified QTL were aligned with previously catalogued *Lr* genes and QTL regions reported in eight recent GWAS studies and two genomic prediction studies using high-throughput marker platforms. Haplotype analyses for a seedling and APR QTL revealed high linkage disequilibrium and fixation of alleles in the diversity panel. Finally, the potential of allele stacking, to strengthen resistance against *P. triticina*, was also demonstrated. Overall, different strategies were explored throughout the thesis aimed to rapidly identify diverse new sources of APR to LR that can be introgressed into elite genetic backgrounds for breeding future wheat cultivars.

1.5 Thesis outline

This thesis consists of seven chapters. Chapter 2 introduces background information relevant to the targeted research and highlights gaps that need to be fulfilled. The thesis includes four research chapters (i.e. Chapter 3–6), which have been published as original research articles in international refereed journals (i.e. Genetic Resources and Crop Evolution, Plant Methods, Plant Disease, and Theoretical and Applied Genetics). The four research chapters correspond to the four aims of the thesis, as detailed above. In Chapter 7, the main findings of the thesis are summarised and discussed, along with future directions and implications for pre-breeding and breeding programs.

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Chapter 2 - Review of literature

2.1 Wheat

Bread wheat (*Triticum aestivum L. em. Thell.*) is the third most important food crop after maize and rice (Hawkesford et al. 2013). Wheat provides ≥20% of dietary calories (i.e. starch and proteins) in the human diet. Also, wheat provides vitamins (notably B vitamins), dietary fibre and phytochemicals which are beneficial for human health (Shewry and Hey 2015). Wheat is cultivated in an area of 220 million hectares in diverse eco-geographical regions around the world, where cultivation extends from 67°N (North) in Scandinavia and Russia to 45°S (South) in Argentina (Gustafson et al. 2009). The annual production of wheat was 713 million metric tonnes in 2013, with an average wheat yield of 3 tonnes per hectare (Ray et al. 2013; Shiferaw et al. 2013; FAO 2015). The major wheat producing countries are China, India, United States of America, Russia, France, Canada, Germany, Pakistan, Australia, and Ukraine (FAO 2015). In Australia, wheat is the major cereal crop and places Australia as the ninth leading world producer and the fourth biggest exporter (Shiferaw et al. 2013).

In the Neothilic period, human societies transitioned from hunting-gathering to growing crops. In the beginning, only three cereals were domesticated namely einkorn (*T. monococcum* L.), emmer (*T. turgidum* ssp. *dicoccoides* (körn.) Thell), and barley (*Hordeum vulgare* L.) (Harlan and Zohary 1966). Wheat belongs to the *Triticeae* tribe in the *Poaceae* family and was most likely domesticated within the fertile crescent (i.e. west of Diyarbakir in southeastern Turkey) around 10,000 years ago (Heun et al. 1997; Ozkan et al. 2005; Luo et al. 2007). During evolution, the wheat genome evolved from multiple hybridisation events between three diploid progenitor genomes which have resulted in different ploidy levels, i.e. diploid (2n), tetraploid (4n) and hexaploid (6n). The first hybridisation event probably occurred between *T. urartu* (A^uA^u) and *Aegilops speltoides* (B^sB^s) and gave rise to wild emmer (*T. turgidum* ssp. *dicoccoides*) (genome AABB) (2n=4x=28) (Figure 2.1). Over the years, wild emmer was cultivated and domesticated to produce cultivated emmer (*T. turgidum* ssp. *dicoccum*) (genome AABB) (2n=4x=28) (Figure 2.1). The second hybridisation event occurred between cultivated emmer (*T. turgidum* ssp. *dicoccum*) (genome AABB) and the wild diploid *Ae. tauschii* (2n=2x=14, genome DD). After the second hybridisation, a chromosome doubling event occurred and resulted in the development of

present-day common hexaploid bread wheat (*T. aestivum*)(genome AABBDD) (Figure 2.1; Shewry 2009).

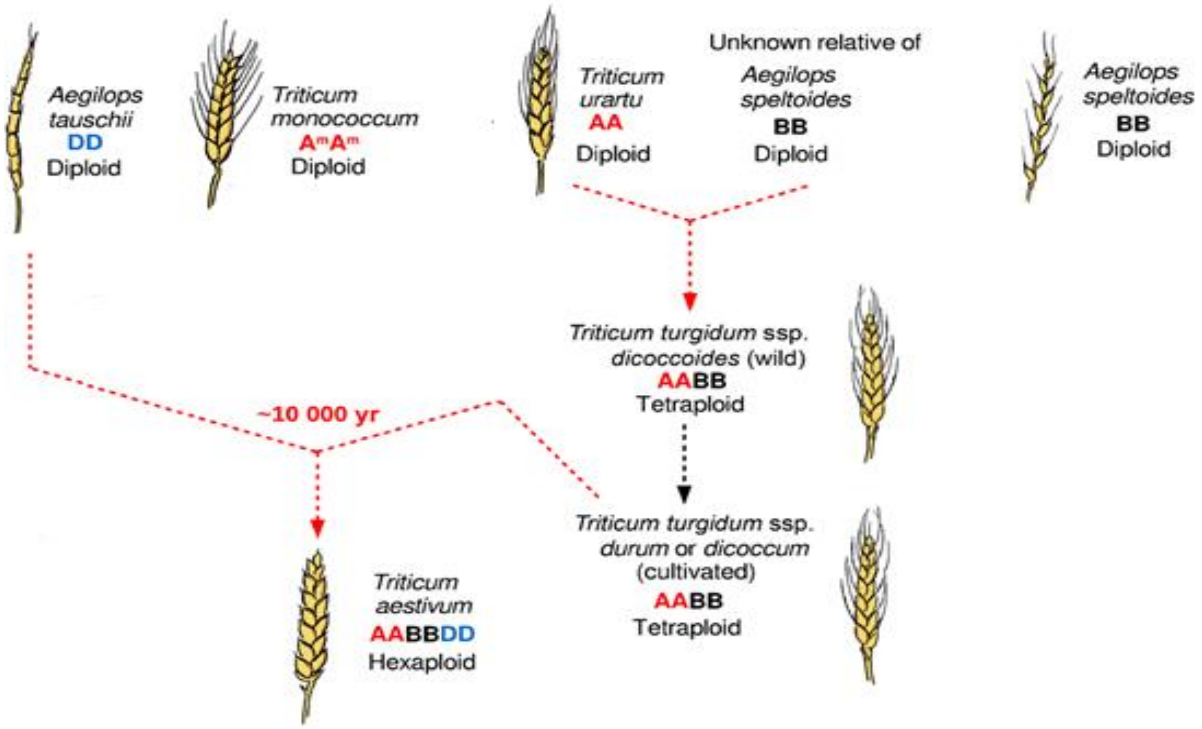

Figure 2.1 Domestication of wheat from wild relatives (from Krattinger and Keller 2016)

During domestication, the hexaploid, commonly known as bread wheat, was selected by humans for its superior traits such as non-brittle rachis, naked grains, large seed size, reduced number of tillers, increased straw strength, and reduced seed dormancy (Jantasuriyarat et al. 2004; Simons et al. 2006; Nalam et al. 2006; Dubcovsky and Dvorak 2007). Today, hexaploid wheat constitutes 95% of total wheat cultivated worldwide, while 5% is tetraploid durum wheat grown mostly in the Mediterranean region (Shewry 2009).

In the beginning of the 19th century, wheat breeding programs were initiated and largely focused on crossing and selection to improve yield, end-use quality, and resistance/tolerance to biotic and abiotic stresses. The conventional breeding strategy has successfully led to the development of modern cultivars with better yield, adaptation to different environments, disease resistance and nutritional quality. However, at present due to a rapid increase in human population, changes in consumption patterns and socioeconomic circumstances, especially in Africa and Asia, have increased global wheat

demand (Mondal et al. 2016). It is predicted that the world population will reach 9.7 billion by 2050 (UN 2015) and the current trends in wheat production are not on track to meet the future demand. If continued at the current rate of improvement of yield, a major shortage of wheat is expected. Furthermore, the stability of wheat production is vulnerable to climate change (i.e. low rainfall, and heat) and rapidly evolving pests and pathogens (Asseng et al. 2015).

2.2 Wheat rusts

In wheat, rusts are considered to be among the most damaging fungal diseases throughout history incurring regular losses to crop yield (Chester 1946; Kolmer et al. 2009). Three major rust diseases affect wheat; leaf rust, stripe rust and stem rust caused by *Puccinia triticina* Eriks., *P. striiformis* Westend. f.sp. *tritici* Eriks., and *P. graminis* Pers. f. sp. *tritici* Eriks. & E. Henn., respectively (Figure 2.2). Wheat rusts are highly specialised biotrophic fungal pathogens. However, they differ in disease symptomology, morphology and preference of environmental conditions favourable for host infection and re-infection.

Figure 2.2 Symptoms of (A) leaf rust, (B) stripe rust and (C) stem rust of wheat collected in the disease screening nurseries at the Redlands Research Facility and Gatton Research Facility, Queensland, Australia.

For instance, all three rusts require freely available moisture on the plant surface to help spore germination and infection; however, leaf and stem rust require higher temperatures for successful infection compared to stripe rust. The frequency of rust epidemics and damage caused to wheat vary in each country (Saari and Prescott 1985). The rust epidemics affect not only crop yield, but also the quality of grains, resulting in economic losses to the destruction of the whole wheat crop (Samborski 1985).

2.3 Wheat leaf rust

Leaf rust (LR) of wheat, caused by *P. triticina* Eriks., is regarded as the most common and geographically widespread disease of wheat (Webster 1980; Samborski 1985). *P. triticina* is a highly specific obligate parasite predominately infecting leaves of the wheat plant and is characterised by small (1.5 mm in diameter), round shape, orange to brown pustules called uredinia containing masses of urediniospores, which appear after rupturing the leaf epidermis (Bolton et al. 2008; Chaves et al. 2008). Under severe epidemic conditions, pustules may also appear on leaf blades, leaf sheaths, awns, glumes, peduncles and internodes (Knott 1989). In a compatible *P. triticina* interaction, large uredinia are produced while in an incompatible interaction no uredinia to small to medium size uredinia along with chlorotic and necrotic halos are typically observed (Bolton et al. 2008; Chaves et al. 2008).

2.3.1 Yield losses

P. triticina attacks the leaves of wheat and thereby directly affects the photosynthetic process which results in premature defoliation leading to a reduction in plants nutrient sink (Knott 1989). If defoliation occurs at the jointing stage, it can cause 95% of yield losses while defoliation at dough stage can result in 10% yield losses (Kolmer et al. 2009). Thus, plants get deprived of available nutrients leading to a reduction in grain number per spike, grain weight, floret setting, grain shrivelling, and grain quality by plummeting protein levels. However, losses caused by LR varies widely depending on the crop growth stage, environmental conditions, and degree of plant defence (Everts et al. 2001; Singh et al. 2002). LR infections are usually less damaging than those from stripe rust and stem rust, but it causes greater annual losses due to its more frequent and widespread occurrence nearly every year worldwide (Singh et al. 2002).

In Australia, LR is present in all wheat growing regions and causes severe economic losses to the Australian cereal industry. Estimated crop losses of up to 30% were reported in wheat cultivars susceptible to LR (Rees and Platz 1975) and up to 55% in cultivars susceptible to both stem rust and LR (Keed and White 1971). LR epidemics have been reported in Western Australia from 1990 to 2000 while incidence on plants was reported in South Australia and Victoria (Huerta-Espino et al. 2011). In 1992, a widespread LR epidemic in Western Australia caused yield losses of up to 37% in susceptible cultivars and an average loss of 15% across fields (McIntosh et al. 1995). Murray and Brennan (2009) estimated the financial losses due to LR in Australia were in the order of AUD 12 million per annum; however, has the potential to cause national losses up to AUD 197 million.

2.3.2 Taxonomy

P. triticina belongs to genus *Puccinia*, family *Pucciniaceae*, order *Uredinales,* class *Pucciniomycetes,* phylum *Basidiomycota* of the kingdom fungi (Bolton et al. 2008; Helfer 2014). Early reports of LR describe the causal agent as *Uredo rubigo-vera* (De Candolle 1815), *P. rubigo-vera* (Winter 1884) and latterly *P. recondita* f. sp. *tritici* (Wilson and Henderson 1966). However, Anikster et al. (1997) went on to distinguish the LR causal agent from *P. recondita* and consequently called it *P. triticina* Eriks. (Bolton et al. 2008). Furthermore, the *P. triticina* pathogen has two genetically incompatible groups (I and II) differing by infection and alternate hosts at the telial stage (Kolmer et al. 2009).

2.3.3 Host range

The primary hosts of *P. triticina* include bread wheat, durum wheat (*T. turgidum* L. var. durum), cultivated emmer wheat (*T. dicocc*um), wild emmer wheat (*T. dicoccoides*), *Ae. speltoides*, goatgrass (*Ae. cylindrical*), and triticale (*X Triticosecale*) (Roelfs 1992; Bolton et al. 2008). *P. triticina* requires an alternate host to complete the sexual stages of its life cycle, which helps in the evolution of new races or pathotypes through genetic recombination (Jackson and Mains 1921). The most common alternate host of *P. triticina* is *Thalictrum speciosissimum L.*, while *Isopyrum fumaroides* has been reported as an alternate host of LR pathogen only in Siberia, Russia (Chester 1946). However, the role of alternate host is not considered significant to the disease epidemics in the case of *P. triticina* (Zhao et al. 2016). The LR mostly infects and spreads through asexual spores (i.e. urediniospores) and

survives on the volunteer wheat plants to carry over the inoculum in the next season such as in Australia (McAlpine 1906; Waterhouse 1952).

2.3.4 Epidemiology

LR on wheat is characterised by the brownish uredinia, each produces 3,000 urediniospores per day (Roelfs et al. 1992). The urediniospores can travel long distances with the wind and also adhere to human clothing resulting in the introduction of a rust pathotype in a disease free area (Kolmer 2005). Once urediniospores land on the susceptible host, the spore germinates in the presence of free moisture and favourable temperature conditions. The urediniospores require an optimum temperature of 20°C and dew period (3 h or less) to initiate infection. Infection by *P. triticina* stops at a temperature lower than 2°C and higher than 32°C (Stubbs et al. 1986; Roelfs 1992). Following infection, after 3–4 days, colour variation (i.e. faint flecks) is visible. After 8–10 days urediniospores are visible while after 12–14 days maximum sporulation is observed under favourable temperatures (i.e. 15 to 25°C) (Stubbs et al. 1986; Kolmer et al. 2009). Fluctuations in temperature and moisture beyond optimum conditions affect the time of sporulation (Chaves et al. 2008). Urediniospores can survive without a host plant for several weeks and can endure freezing temperatures (Singh et al. 2002). When the host plant is about to senesce, teliospores are produced which can help the pathogen to overwinter (Roelfs 1992).

2.3.5 Life cycle

P. triticina is a macrocyclic fungus exhibiting five different spore stages including both asexual and sexual stages, of two un-related host species (i.e. wheat and *Thalictrum* spp.; Figure 2.3; Zhao et al. 2016). The five stages of the *P. triticina* life cycle are based on the spore type produced, including; spermatia (pycniospores), aeciospores, urediniospores, teliospores, and basidiospores (Webster 1980). The fungus overwinters as teliospores on wheat stubble, and when conditions become favourable in spring, it germinates. At first, teliospores are dikaryotic, but later undergo karyogamy followed by meiosis resulting in the production of basidiospores, which cause infection on *Thalictrum* spp. (Roelfs and Bushnell 1985; Anikster et al. 1997). When basidiospores come in contact with the alternate host (i.e. *Thalictrum* spp.), they germinate and penetrate directly giving rise to pycnium, which produce receptive hyphae and pycniospores (Singh et al. 2002). The sexual stage of *P.*

triticina involves pycniospores, where male '+' and female '-' hyphae raise to the top of pycnia and undergo genetic recombination, as a result of the sexual interaction (Figure 2.3). This gives rise to aeciospores, which cause infection on wheat under favourable conditions (Roelfs 1992). The aeciospore forms a germ tube, a primary structure that reaches stoma of the leaf and produces appressorium followed by a penetration peg leading to the development of a haustorium. The haustorium grows against the mesophyll cell and directly penetrates through the host cell wall in a compatible interaction between the host and the pathogen. In a resistant reaction, where the pathogen is incompatible on that host, the haustoria may die at this point or may develop only very gradually. In a susceptible plant, the initial infection resulting from an aeciospore gives rise to urediniospores in the host tissues, which can be followed by multiple asexual infection cycles and cause repetitive infection on wheat plants (Kolmer 2013). When conditions become unfavourable for the pathogen, it undergoes an overwintering stage and teliospores are once more formed.

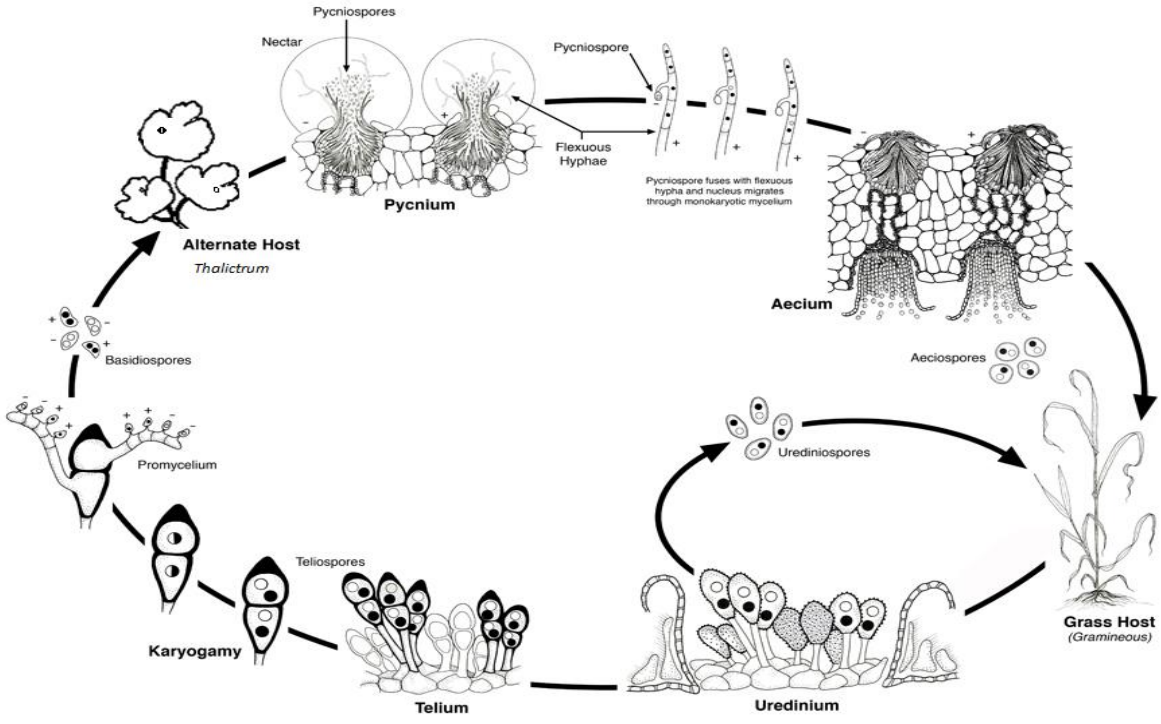

Figure 2.3 Life and disease cycle of *P. triticina* on wheat and its alternate host *Thalictrum* spp. exhibiting the sexual and asexual stages (from Roelfs 1992; Bolton et al. 2008).

2.3.6 Disease control

To control rust diseases, it is important to understand the epidemiology of the pathogen, particularly before initiating any control strategy. There are four main approaches implemented to control LR, either singly or in combinations. Firstly, the exclusion of the *P. triticina* from wheat growing regions by adopting strict quarantine practices such as preventing the entry of *P. triticina* spores through contaminated clothing or plant material (Roelf et al. 1992). Secondly, the removal of the alternate host helps to reduce sexual recombination and avoid the 'green bridge' (i.e. off-season wheat cultivation or self-sown volunteer wheat plants) thus lessen the opportunity for carryover inoculum across seasons (Zadoks and Bouwman 1985). However, the role of the alternate host species (i.e. *Thalictrum* spp., and *Isopyrum* spp.) is considered less significant in the evolution of new *P. triticina* pathotypes (Roelf et al. 1992; Zhao et al. 2016). Thirdly, manipulation of agronomic practices, such as early sowing and cultivation of early maturing cultivars can help in disease escape and lower inoculum pressure during earlier growth stages (Zadoks and Bouwman 1985). Fourthly, the use of fungicides is effective under high disease pressure, however, often multiple applications are required that increase production costs – leading to loss of efficacy (Uauy et al. 2005; Jørgensen et al. 2014). Also, fungicide applications are often hindered by management limitations such as torrential rains within the season which may prohibit the timely access of tractors and spray equipment to the field resulting in the non-effective timing of fungicide applications. By far, the deployment of cultivars carrying genetic resistance is the most efficient, durable and cost-effective approach to reduce the losses caused by *P. triticina* (Naz et al. 2008).

2.3.7 Genetic resistance

In the early 1900s, for the first time, Rowland Harry Biffen in Cambridge, UK, described the genetic basis of resistance to wheat rust (Biffen 1905). Since then, the discovery of genetic variation for rust resistance has been an ongoing component of wheat breeding programs and attracted significant monetary investment in the cereal industry. To discover genetic variation, plants are typically challenged with the rust pathogen at both seedling and adult growth stages, and the disease response is scored using the 0–4 scale (Stakman and Levine 1922). Once resistant lines are identified, they can be used as parents in the next breeding cycle. Often multiple rounds of selection are required to improve different agronomic, biotic and abiotic traits before an improved cultivar is released to farmers. To

date, 77 genes conferring LR resistance (*Lr*) (i.e. *Lr1–Lr77*) have been identified in wheat (McIntosh et al. 2017). A small number of these genes were found either duplicated or redundant, thus were removed from the 'Catalogue of gene symbols for wheat'. The redundant genes include: *Lr4* to *Lr8*, *Lr39* (=*Lr21*), *Lr41* (=*Lr39*), and *Lr43* (McIntosh et al. 2013). As the case for many cereal pathosystems, broadly there are two main types of genetic resistance to LR: seedling resistance and adult plant resistance (APR).

2.3.8 Genetic resistance: seedling resistance

Most of the catalogued resistance genes are 'seedling resistance' or 'R' genes, which are typically detected at the seedling growth stage and remain effective at all plant growth stages, thus sometimes referred to as 'all-stage resistance'. Seedling resistance is often controlled by a single major gene with large effect and confers a hypersensitive response (localised cell death at the infection site), such as *Lr28* (Figure 2.4) (McIntosh et al. 1995, 2017). However, in some cases, seedling genes may not provide complete resistance (Mondal et al. 2016). R genes are effective against selective races of the pathogen hence, referred as 'race-specific resistances' (Park et al. 2014). The R genes are principally governed by the gene-for-gene interaction, where each R gene confers resistance in the host to a pathogen race carrying the corresponding avirulence (*Avr*) gene (Flor 1971; Figure 2.4).

To date, three R genes conferring resistance to LR, namely *Lr1*, *Lr10*, and *Lr21* have been cloned (Feuillet et al. 2003; Huang et al. 2003; Cloutier et al. 2007; Loutre et al. 2009). These genes belong to the coiled-coil, nucleotide binding site and leucine-rich repeat domain (CC-NB-LRR) structure (Cloutier et al. 2007; Ellis et al. 2014). The cloning of these genes has provided perfect markers for breeders for selection of lines carrying the resistance via marker-assisted selection (MAS) in breeding programs. The benefits of using R genes from a plant breeder's perspective is that they can be easily phenotyped at seedling stage (i.e. two leaf stage) and perfect markers facilitate MAS. This allows elimination of plants lacking the phenotype of a gene at an early stage in the breeding cycle. One of the consequences of adopting R genes that follow gene-for-gene interaction is a strong selection pressure on the pathogen which drives an increase in the selection of virulent mutants that are sometimes already present at low frequency in the pathogen population (Burdon et al. 2014; Niks et al. 2015). Therefore, *P. triticina* as a population may evolve and have a selective advantage for genotypes with virulence to a specific R gene and

consequently render that resistance ineffective (McCallum et al. 2011). Typically a cultivar carrying a R gene operates in a “boom and bust cycle” (Singh et al. 2014). The cycle starts with a release of the resistant cultivar and due to its disease resistance is favoured by farmers and consequently cultivated across large acreages. In response, the selection pressure on the pathogen population results in favour of those genotypes that have gained virulence for the R gene and as the disease frequency increases farmers are forced to abandon the cultivar. To avoid this cycle and enhance the durability of resistance, pyramiding or stacking of R genes is a viable strategy where the pathogen has to undergo multiple mutations to gain virulence for multiple genes simultaneously.

Figure 2.4 A comparison of the seedling resistance in wheat leaves showing responses to leaf rust in susceptible (Thatcher-*Lr28*) and resistant (Thatcher+*Lr28*) (left to right) lines (from Singh et al. 2017).

2.3.9 Genetic resistance: adult plant resistance

Adult plant resistance (APR), is shown to be effective at the post-seedling stage. Among the 77 *Lr* genes catalogued, only 14 confer resistance specifically at the adult plant stage. These include *Lr12*, *Lr13*, *Lr22* (alleles a, and b), *Lr34*, *Lr35*, *Lr37*, *Lr46*, *Lr48*, *Lr49*, *Lr67*, *Lr68*, *Lr75*, and *Lr77* (McIntosh et al. 2017). Typically APR is controlled by either multiple genes each with minor effects or a single gene with large effect; such APR can confer partial resistance or “slow rusting” resistance (Parlevliet and Vanommeren 1985). The components of slow rusting resistance include a long latent period, low infection frequency, a low

sporulation rate, and small pustule size (Caldwell 1968). Some APR genes provide partial resistance that is effective against all races of a given pathogen species (i.e. race-nonspecific), for example, *Lr34*. However, there are exceptions, where some APR genes provide race-specific resistance (i.e. *Lr13*) or confer a hypersensitive response (i.e. *Lr48*) (Bansal et al. 2008; McCallum et al. 2012).

Only a few APR genes confer partial resistance against multiple pathogens, and these include *Lr34*, *Lr46*, and *Lr67*. These genes provide variable levels of resistance to LR, stripe rust, stem rust, powdery mildew disease of wheat and the morphological marker leaf tip necrosis (i.e. *Lr34/Yr18/Pm38/Sr57/Ltn1*, *Lr46/Yr29/Pm39/Sr57/Ltn2*, and *Lr67/Yr46/Pm46/Sr55/Ltn3*) (Lagudah et al. 2011; Figure 2.5).

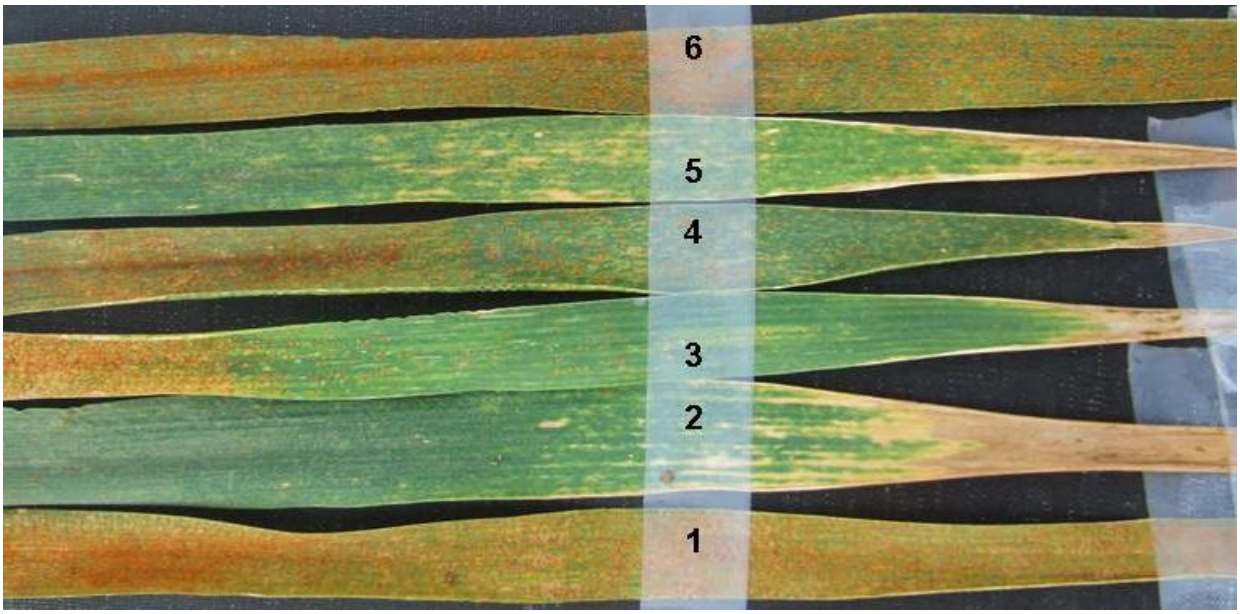

Figure 2.5 Resistance to LR expressed by wheat lines carrying different APR genes compared to the susceptible lines. The lines are Avocet-*YrA* (1), *Lr34* (2), *Lr46* (3), *Lr68* (Arula1) (4), Avocet-*YrA*/RL6077 (*Lr67*) (5) and Apav (6) in Mexico (from Herrera-Foessel et al. 2012)

The APR gene *Lr34* provides partial resistance to multiple pathogens at the flag leaf stage in the field (Figure 2.5; Krattinger et al. 2009). In case of LR, *Lr34* causes a reduction in density of urediniospores from the base to the tip of the leaf and also displays leaf tip necrosis – a senescence-like phenotype (Schnurbusch et al. 2004). The expression of *Lr34* is higher at cool temperatures compared with warm or high temperatures (Singh 1993;

Kolmer et al. 2008; Krattinger et al. 2009; Lagudah 2011). *Lr46* conditions a resistance response weaker than *Lr34* but with a small effect causing fewer and smaller uredinia, prolonged latency period in infected plants carrying *Lr46* and with variable chlorosis and necrosis around uredinia in adult plants (Figure 2.5) (Singh et al. 1998). Cool temperatures are considered favourable for the optimal gene expression and time of disease assessment is critical to detect the slow rusting nature of *Lr46*. The APR gene *Lr67* has a similar phenotypic response as that of *Lr34*, but resistance levels are often weaker (Figure 2.5) (Hiebert et al. 2010; Herrera-Foessel et al. 2011). The APR genes, if deployed singly may not confer adequate levels of resistance under high inoculum pressure or at high temperatures, however, if deployed in combination (e.g. 4–5 genes) they can confer resistance levels resembling near-immunity (Singh et al. 2011).

2.3.10 Cloning of APR genes and underlying mechanisms

Modern wheat breeding programs have realised the effectiveness of APR, and an increasing number of breeders are working towards their deployment in future wheat cultivars, in particular, the deployment of different gene combinations. However, gene pyramiding through conventional methods is often challenging and time-consuming, as it involves repeated phenotyping against different rust pathotypes across different environments and performing field-based selections. On the other hand, MAS provides the opportunity for fast identification and selection of the best gene combinations using linked molecular markers, thus helping the breeding programs for a quick release of rust-resistant cultivars. To date, three *Lr* APR genes have been cloned, namely *Lr22a* (Thind et al. 2017), *Lr34* (Krattinger et al. 2009), and *Lr67* (Moore et al. 2015). These genes differ in their resistance phenotype, effective growth stage and underlying mechanisms governing resistance, detailed below.

2.3.10.1 Lr22a

The APR gene *Lr22a* was originally detected in wild relative *Ae. tauschii* in 1960 (Dyck and Kerber 1970) and was later transferred into bread wheat and mapped to the short arm of chromosome 2D (Hiebert et al. 2007). The *Lr22a* gene confers partial resistance to a wide range of *P. triticina* pathotypes at post-seedling stages (i.e. three-leaf stage or ≥25 days after germination). Recently, *Lr22a* was cloned using cultivar-specific long-range chromosome assembly encoding an intracellular immune receptor homologous to the

Arabidopsis thaliana RPM1 protein. The predicted gene sequence consists of 2,739 base pairs (bp) comprising of a single exon and translates into a protein of 912 amino acids with a N-terminal coiled-coil domain, a central nucleotide-binding (NB-ARC) domain, and a C-terminal leucine-rich repeat domain (Thind et al. 2017). So far, the *Lr22a* gene has been used in wheat breeding programs in Canada in cultivars such as “AC Minto” which only occupied a small area from 1998 to 2006 (McCallum and Seto-Goh 2005).

2.3.10.2 Lr34

Lr34 was first described in the cultivar ‘Frontana’ in 1966 (Dyck 1966) and has been mapped to the short arm of chromosome 7D (Schnurbusch et al. 2004). *Lr34* confers resistance at the flag leaf stage in the field. The *Lr34* gene was cloned using map-based cloning approach, where the gene consists of 11,805 (bp) and 24 exons. The gene is predicted to contain 1,401 amino acid proteins that encode a putative ATP - binding cassette (ABC) transporter (Krattinger et al. 2009). The protein has a basic structure of two cytosolic nucleotide-binding domains (AAA) and two hydrophobic transmembrane domains. The *Lr34* has two alleles, namely *Lr34res* and *Lr34sus*, where the *Lr34res* allele resulted from two gain-of-function mutations in the ABC transporter. *Lr34res* allele is present in cultivated bread wheat and does not occur in the wild progenitors of wheat. Recently, the *Lr34res* allele was transferred into durum wheat conferring resistance to LR at the seedling stage (Rinaldo et al. 2017). Similarly, the *Lr34res* allele has been transferred into several crop species, namely, barley, rice, and sorghum, providing resistance against various important pathogens (i.e. barley leaf rust, barley powdery mildew, rice blast, sorghum anthracnose, and sorghum rust) (Risk et al. 2013; Krattinger et al. 2016; Schnippenkoetter et al. 2017).

2.3.10.3 Lr67

Lr67 was originally detected in a Pakistani bread wheat accession “PI250413”, which was transferred into a Thatcher accession “RL6077” (Dyck and Samborski 1979). The gene was mapped to the long arm of chromosome 4D (Hiebert et al. 2010; Herrera-Foessel et al. 2011). *Lr67* gene was cloned using comparative genomics, mutagenesis and transformation approach. *Lr67* has two alleles, *LR67res* (resistant) and *LR67sus* (susceptible). The *LR67res* encodes a predicted hexose transporter comprising of 514 amino acids, 12 predicted transmembrane helices and is most similar to the STP13 family of

H+/monosaccharide symporters, which facilitate the transport of hexoses across the plasma membrane. The two amino acids conserved in orthologous hexose transporters differ between the *LR67res* and *LR67sus* alleles. The resistant allele occurs in tall wheat varieties widely cultivated before the 'Green Revolution' but absent in the germplasm utilised at CIMMYT (Moore et al. 2015).

2.4 The Puccinia triticina population in Australia

The co-evolution of pathogen and host has occurred since the domestication of modern crop varieties (Liu et al. 2014). To this date, *P. triticina* continue to evolve into new pathogen races or pathotypes by undergoing single step mutations corresponding to resistance genes deployed in cultivars (Burdon et al. 2014). The variation in virulence of the pathogen population is the outcome of sexual recombination, migration of pathogens, accumulated mutations, genetic drift, gene flow and selection pressure (Anderson et al. 2010). In Australia, yearly surveys are conducted by the Australian Cereal Rust Control Program (ACRCP) led by the University of Sydney and have monitored pathogenic variability since the 1920s (Park et al. 2014). These surveys identify the variation in pathogen populations by inoculating a set of differential host genotypes (i.e. lines carrying known genes for rust resistance) at the seedling stage in glasshouses (Park 2016). In Australia, two sets of differentials are used to differentiate pathotypes (Table 2.1). The first is an international set comprising of four wheat genotypes, used to assign a standard race designation (e.g. 10, 76, 104), by following rules established by Stakman et al. (1962). The second set, known as the Australian supplemental differential set contains 13 wheat genotypes. Virulence on a given number is indicated by the inclusion of the number in pathotype formula while parenthesis shows partial virulence, i.e. pathotype (*pt*) 104–2,3,6,(7) (Table 2.1). Recent advances in molecular markers has enabled the characterisation of the rust populations determining their origin (Kolmer et al. 1995; Park et al. 1995; Kolmer 2001; Szabo and Kolmer 2007). However, the virulence surveys are considered more powerful in the identification of individual pathotypes (Park 2016). These surveys have provided a strong indication that periodic exotic incursions followed by selection pressure and somatic hybridisation determine the structure of Australian pathogen population (Park et al. 1995; Park et al. 1999).

Table 2.1 The Australian set of differential wheat genotypes used for identification of *P. triticina* pathotypes (Park 2016).

Differential set	Line	Key resistance gene (s)
International set	Tarsa	*Lr1*
	Webster	*Lr2a*
	Mediterranean	*Lr2a, Lr3a*
	Democrat	*Lr3a*
Australian supplementary differentials	1. Thew	*Lr20*
	2. Gaza	*Lr23*
	3. Spica	*Lr14a*
	4. K1483	*Lr15*
	5. Klein Titan	*Lr16*
	6. Gatcher	*Lr27+Lr31*
	7. Songlen	*Lr17a*
	8. CS 2A/2M	*Lr28*
	9. Mildress	*Lr26*
	10. Egret	*Lr13*
	11. Exchange	*Lr16*
	12. Harrier	*Lr17b*
	13. Agent	*Lr24*
Additional differential genotypes	Sunlin	*Lr37*
	Sun6B[a]	*Lr1, Lr3a, Lr27+Lr31*
	Naparoo[a]	*Lr13, Lr24*
	Agatha[a]	*Lr19*
	Norka[a]	*Lr1, Lr20*
	Mentana[a]	*Lr3bg*
	Morocco[a]	*Lr73*
	Thatcher +*Lr2c*[a]	*Lr2c*
	Thatcher +*Lr30*[a]	*Lr30*

[a] Not used in designating pathotypes.

In Australia, the *P. triticina* population is very diverse and rapidly evolving with 30 pathotypes belonging to six clonal lineages detected to date. Studies have reported that in Australia on average 10–15 *P. triticina* pathotypes are detected each year (Park et al. 1995). Most of the time *P. triticina* pathotypes have entered Australia as airborne spores followed by single step acquisition of virulence resulting in new and more virulent pathotypes (Huerta-Espino et al. 2011). The frequent pathogenic variations and the introduction of exotic pathotypes have rendered many R gene-carrying cultivars susceptible to LR. For instance, *P. triticina* pathotype *pt* 104–1,2,3,(6),(7),11,13 carries virulence for *Lr24* which had been deployed for 18 years in important Australian wheat cultivars; whereas in the USA virulence to *Lr24* was detected only a few years following deployment in a cultivar (Long et al. 1985; Park et al. 2002). Similarly, in India, virulence to *Lr26* was detected after six years. On the other hand, APR genes provide more durable resistance. However, the breakdown of resistance (i.e. race-specific APR) has also been reported (Ellis et al. 2014). In 2014, an exotic *P. triticina* pathotype (*pt* 104–1,3,4,6,7,8,9,10,12+*Lr37*) in Australia rendered many cultivars susceptible which possessed the race-specific APR genes *Lr13* and *Lr37*, which until that point were considered to have a high level of resistance against LR (Park et al. 2014).

The *P. triticina* pathotype *pt* 104–2,3,(6),(7),11 was first detected in Australia in 1984 and was considered an exotic incursion because it differed greatly to other local pathotypes. Following this, the pathotype appears to have undergone several single-step mutations resulting in virulence to *Lr20* (Park et al. 1995). This resultant pathotype was then designated *pt* 104–1,2,3,(6),(7),11) and was initially detected in New South Wales (Park et al. 1995). Additional variants were then frequently detected and isolated in all regions of Australia from 1989 to 2008. One of the important variants was *pt* 104–1,2,3,(6),(7),9,11, which acquired virulence for *Lr26* (discovered in New South Wales 1997), but later spread to all eastern states. In 2000, the *pt* 104–1,2,3,(6),(7),11,13 was detected; this specific pathotype had acquired virulence to *Lr24* in South Australia rendering that gene ineffective (Park et al. 2002). Later, *pt* 104–1,2,3,(6),(7),11,13 was detected in all eastern states as well as Western Australia, as at that time cultivars relying on *Lr24* were cultivated across 25% of the total wheat growing area in Australia (Park et al. 2002). Currently, *pt* 104–1,2,3,(6),(7),11,13 continues to be prevalent in all wheat growing regions of Australia. The *P. triticina* pathotype *pt* 104–1,2,3,(6),(7),11+*Lr37* (also known as “VPM”) was detected in 2002 in Western Australia (Park 2008); as the designated name implies, it has virulence for *Lr37*. Additionally, virulence to *Lr28* and *Lr39* has been detected in some Australian *P. triticina* pathotypes,

although this is currently of minimal concern as neither of these resistance genes are deployed in commercial cultivars.

The pathotypes that are now posing serious threats to wheat production are *pt* 76–1,3,5,7,9,10,12+*Lr37* (first detected in 2013) and *pt* 104–1,3,5,7,9,10,12+*Lr37* (incursion detected in 2014). The latter pathotype appears to have undergone a step mutation resulting in the new pathotype *pt* 104–1,3,4,6,7,8,10,12+*Lr37* which possesses virulence for the host R genes: *Lr12*, *Lr13*, *Lr20*, *Lr27+31*, and *Lr37* all of which have been widely used in Australian cultivars (Cuddy et al. 2016; Park 2016). As well as displaying virulence to the five *Lr* genes mentioned above *pt* 104–1,3,4,6,7,8,10,12+*Lr37* is also virulent on the for the host complimentary R genes: *Lr15*, *Lr27+31*, and *Lr28.* This poses a serious threat given the gain of virulence against so many of the available resistance genes. As a consequence, the LR ratings for several Australian cultivars changed from resistant to susceptible, for instance, *pt* 104–1,3,4,6,7,8,9,10,12+*Lr37* resulted in virulence for *Lr15* and *Lr28* in cultivar "SQP Revenue".

The frequent variations in the pathogen population imply that many of the resistances deployed in cultivars are underpinned by genes that provide a single genetic barrier, which have lost their effectiveness. This presents a continuous task for pathologists and breeders to identify and deploy new resistance genes, or allelic variations of known resistance genes, for utilisation in wheat breeding programs.

2.5 Importance and revival of genetic diversity in wheat breeding programs

Wheat was domesticated about 10,000 years ago and has a complex genome compared to other domesticated cereal crops, such as barley and emmer wheat. During the early domestication process, natural biodiversity was subject to an extreme bottleneck, and in this process, many potentially valuable alleles were lost (Feuillet and Eversole 2007). This has restricted modern breeding and selection to a relatively small genetic pool. As a consequence, this has resulted in low genetic diversity for resistance factors deployed in cultivars, thus making them vulnerable to evolving pathogens. Also, if the deployment of resistance genes is focused on a small number of R or APR genes, this risks the longevity of these genes. Further, if genetic diversity is not incorporated into modern germplasm, the potential of continued genetic gain for all traits is likely limited (Feuillet et al. 2008). The

introduction of new alleles or genes will enable protection against new pathogens or their evolved pathotypes (Mondal et al. 2016). For wheat improvement striving to introduce genetic diversity, breeders and researchers are seeking to utilise primary gene pools, as these carry homologous chromosomes and can readily be recombined within hexaploid wheat (Friebe et al. 1996; Wulff and Moscou 2014). The primary gene pool includes species from genera *Triticum* and *Aegilops*, which represent the progenitors of hexaploid wheat carrying the A, B, and D genomes, i.e. *T. urartu* (genome AA), *T. turgidum* (genome AABB), and *Ae. tauschii* (genome DD), and landraces. Additionally, synthetic hexaploids of wheat have been developed to incorporate useful genes from *T. turgidum* and *Ae. tauschii* into modern bread wheat. Despite this, genetic diversity present in the seed banks remains largely unexplored (Lopes et al. 2015).

2.5.1 Wild progenitor and non-progenitor species

Resistance to *P. triticina* can be achieved by identifying useful sources from wild relatives of wheat and subsequently transferring them into modern wheat cultivars through interspecific hybridisation (Wulff and Moscou 2014). To date, more than half of the available *Lr* genes have been identified from wild relatives (McIntosh et al. 2017). These include *Lr9* (*Ae. umbellulata*); *Lr19*, *Lr24*, and *Lr29* (*Thinopyrum ponticum*); *Lr37* (*Ae. ventricosa*); *Lr38* (*Th. intermedium*); *Lr28*, *Lr35*, *Lr36*, *Lr51,* and *Lr66* (*Ae. speltoides*); *Lr21*, *Lr22a*, *Lr32*, and *Lr39* (*Ae. tauschii*); *Lr57* (*Ae. geniculata*); *Lr58* (*Ae. triuncialis*); *Lr59* (*Ae. peregrina*); *Lr62* (*Ae. neglecta*); *Lr63* (*T. monococcum*), and *Lr53*, *Lr64* (*T. dicoccoides*); *Lr14a* and *Lr61* (*T. turgidum*) (McIntosh et al. 2013; Bansal et al. 2017). Although many genes were identified from wild relatives, a very few of these genes have been deployed in modern cultivars as wild progenitor crosses often result in problems associated with different ploidy levels, sexual incompatibility, hybrid inviability and sterility. Moreover, if the cross is successful the gene of interest often brings negative traits affecting yield and quality via linkage drag. This has limited the adoption of wild relatives in wheat breeding programs (Brown and Marshall 1986; Zamir 2001; Salamini et al. 2002; Dempewolf et al. 2017).

2.5.2 Synthetic hexaploid wheat

Synthetic hexaploid wheat (SHW) enables the transfer of genes from wheat progenitors into modern hexaploid wheat. SHW is generated by crossing durum wheat (*T. turgidum*; AABB)

with *Ae. tauschii* (DD), the latter being one of the ancestors of wheat (Mujeeb-Kazi et al. 1996). Synthetic wheat can be backcrossed to modern wheat lines potentially resulting in adapted varieties incorporating new genes for wheat development (Trethowan and Mujeeb-Kazi 2008). These lines have served to provide many important traits to cultivated wheat (Mujeeb-Kazi et al. 1996) such as resistance to stripe rust (Ma et al. 1995), LR (Assefa and Fehrmann 2000), and stem rust (Marais et al. 1994). While this approach can deliver new sources of resistance to rust pathogens, the SHW lines are problematic in that they have poor agronomic value; they are often tall and susceptible to lodging, difficult to thresh, have low and variable yields as well as poor grain quality (Trethowan and Mujeeb-Kazi 2008).

2.5.3 Wheat landraces

According to Casañas et al. (2017), landraces can be defined as:

"*Landraces consist of cultivated varieties that have evolved and may continue evolving, using conventional or modern breeding techniques, in traditional or new agricultural environments within a defined eco-geographical area and under the influence of the local human culture".*

Landraces represent an excellent source of untapped genetic diversity. These have been grown in farmer's fields for thousands of years and gone through natural and the farmer's induced selection. The natural selection is based on diverse environmental conditions while farmer selection was likely applied for various purposes including bread making, feed for cattle and straw strength for building roof-tops. Different studies have reported that landraces have contributed various agronomically important alleles in wheat, for instance, among the semi-dwarfing genes, *Rht8c* was sourced from landraces and was used in the 'Green Revolution' to develop varieties that can grow in dry regions. Similarly, the photoperiod insensitivity gene *Ppd*_D1 responsible for inducing flowering was derived from a Japanese landrace '*Aka Komugi*' (Worland et al. 1998; Ellis et al. 2007). Several resistance genes including resistance to rust diseases were also identified in wheat landraces (Cavanagh et al. 2013; Lopes et al. 2015; Vikram et al. 2016; Rinaldo et al. 2017). For instance, *Lr* genes *Lr52* and *Lr67* (Hiebert et al. 2010; Bansal et al. 2013), and stripe rust resistance gene *Yr47* (Bansal et al. 2011) were first identified in wheat landraces. Along with landraces, historic cultivars and old breeding lines are also considered a useful

resource of allelic diversity that was left behind many years ago (Lopes et al. 2015). Fortunately, many of these germplasm resources are well preserved in seed banks around the world.

2.5.4 *Ex-situ* seed banks

Global *ex-situ* seed banks (or gene banks) conserve plant material such as seed, away from their original environment. Conservation aims to preserve diversity at the genetic level and with the crop species, originating from diverse eco-geographical areas. According to the Food and Agriculture Organisation, there are 1,750 gene banks worldwide maintaining 7.4 million samples of seeds or plant tissues from thousands of crop species (including duplicate accessions) (FAO 2010). The seed banks contain valuable germplasm including wild crop relatives, landraces, old cultivars, and breeding lines (Bhullar et al. 2009). Some of the important wheat seed banks include the Svalbard Global Seed Vault, Norway; the National Center for Genetic Resources, USA; Seeds of Discovery, CIMMYT, Mexico; IPK Gatersleben, Germany; N. I. Vavilov Institute of Plant Genetic Resources, Russia and Australian Winter Cereals Collection, Australia.

2.5.4.1 The Watkins collection

A. E. Watkins, an English botanist, acquired 7,400 landraces, including both hexaploid (bread) wheat and tetraploid (durum) wheat, from local markets in 32 countries (mainly Europe and Asia) during the 1920s to 1930s (Miller et al. 2001; Wingen et al. 2014). In the aftermath of World War II, the collection was lost as only 826 bread wheat accessions are available as originating from the Watkins collection at present. To understand the genetic diversity, lines in the Watkins collection were genotyped with high-density 35K 'Wheat Breeders' Array comprising of 35,143 SNP markers (Allen et al. 2017). The results revealed a snapshot of huge amount of new allelic diversity which is absent in modern wheat breeding programs (Winfield et al. 2017). Studies have examined accessions belonging to the Watkins collection for LR, stripe rust, and eyespot resistance (Hiebert et al. 2010; Burt et al. 2014). These studies led to the discovery of *Lr52* (Hiebert et al. 2005) and *Yr47* (Bansal et al. 2011). Daetwyler et al. (2014) confirmed the presence of many known rust resistance genes such as *Lr34/Yr18/Sr57*, in the Watkins wheat landrace collection, and identified previously uncharacterized genome regions.

2.5.4.2 N. I. Vavilov Institute of Plant Genetic Resources, St Petersburg, Russia

The modern era of seed collection was revolutionised by the Russian botanist and geneticist Nikolai Ivanovich Vavilov (1887–1943). N. I. Vavilov was known for his theory of the 'centres of origin of cultivated plants', according to which the domestication of plants did not happen at random, but in regions where the domestication of the plants started. These regions are called the 'centres of origin' and are also regarded as the epicentre of diversity for that crop species. Vavilov and his colleagues, mainly between the 1920s and 1940s, made several expeditions to different regions of the world to collect various crop species including wheat (Fu and Somers 2009; Mitrofanova 2012). Vavilov focused on systematic representation of the variation within crop species at the population level and geographical area. The collected samples were preserved in the seed bank, now known as the N. I. Vavilov Institute of Plant Genetic Resources (VIR) in St Petersburg, Russia. Scientists at the Vavilov Institute are continuing his legacy and increasing their germplasm sets with samples around the world. Currently, VIR has approximately 38,430 wheat samples maintained in living conditions. The VIR collection is highly diverse regarding species of the genus *Triticum* , including some 27 species. About 76% of the total samples (29,209 samples) belong on *T. aestivum,* and 16.1% (6,199 samples) are *T. durum*. The collection also includes 3,022 wild wheat samples including primitive wheat representing 7.9% of the total collection. This collection has extensive intraspecific genetic diversity regarding various traits and information about the exclusivity of the sites and period of the collection (Mitrofanova 2012). It has been suggested that the use of the VIR wheat collection will reveal a promising basis for the genetic improvement of resistance to various biotic stresses (Mitrofanova 2012; Sadovaya et al. 2015). Despite this, the genetic diversity preserved in VIR is still largely an untapped resource harbouring many potentially new sources of resistance to LR as well as other important traits.

2.6 Breeding for resistant cultivars

2.6.1 Conventional breeding

To discover genetic variation for LR resistance, individual plants are challenged with the rust pathogen as seedlings in the glasshouse or adults in the field. In particular, assessment of resistance at the adult stage is conducted across environments and against multiple

pathotypes to identify durable forms of resistance. The identified resistance source is also crossed with the farmer preferred cultivar to improve rust resistance. Often multiple rounds of selection and crossing are required to improve different agronomic, biotic and abiotic traits before a cultivar is released. Traditional wheat breeding programs rely heavily on the selection of best plants possessing the most desirable traits (e.g. high yield or disease resistance) for making crosses. Although this has contributed to significant increases in yield, the development of wheat cultivars with effective and durable rust resistance remains the biggest challenge to wheat breeders and may take several years to resolve. Phenotyping in the field is considered an inexpensive method to understand interactions between the desired trait, plant growth stage and environment (Velu and Singh 2013). However, the problems associated with field-based phenotyping include weather dependency, low epidemic development, the introduction of unwanted diseases or pathotypes, time inefficiency, and labour intensiveness (Hickey et al. 2012). Further, field phenotyping was traditionally only performed once a year in the wheat growing season. When employing such an approach, it can take up to 10–15 seasons or years to develop an improved cultivar (Hickey et al. 2017). Despite this, many national breeding programs still adopt this approach around the world. Rapidly evolving rust pathogens can acquire virulence for deployed resistance genes in cultivars within a short timeframe, thus emphasising the need to speed up breeding cycles and be ahead of the pathogen.

2.6.2 Shuttle breeding

In 1968, Nobel Peace Prize Laureate, Dr Norman E. Borlaug, initiated shuttle breeding at CIMMYT, Mexico. The technique involved growing wheat populations at two different locations differing in geographical locations and environments in Mexico; Ciudad Obregon (Sonora) 27.5°N and Toluca (State of Mexico) 19°N (Borlaug 1968). Ciudad Obregon is dry, but an irrigated site situated 40 meters above sea level (masl) in the Yaqui Valley of north-west Mexico; and Toluca is a cool, humid highland at 2,640 masl near Mexico City. The screening of segregating breeding material against LR and stem rust is carried out in Obregon while stripe rust, *Septoria tritici* and *Fusarium* head blight screening in Toluca. Initially, the shuttle breeding approach was employed to speed up the breeding program, as they could grow two consecutive generations per year; from May to October at Toluca, and from November to April in Ciudad Obregon (Velu and Singh 2013). Selection and transfer of plant material between the two sites reduced the time required in variety development from 10–12 years to 5–6 years. The shuttle breeding approach also enabled selection for

wide adaptation and photoperiod insensitivity and durable disease resistance. This stabilised yield gains in the breeding program (Ortiz et al. 2007). The strategy also contributed to screening and evaluation of available germplasm, finding new genes for rust resistance and their frequent deployment in elite cultivars, and anticipation of variation in rust races.

2.6.3 Doubled haploid technology

Usually, cultivar development requires 10–15 years of conventional breeding (Hickey et al. 2017). From a breeder's perspective, the time reduction in homozygous line development and subsequent cultivar release is critical. This led to the development of doubled haploid (DH) technology where it reduces the time as much as four seasons, thus increasing the efficacy of the breeding program. Doubled haploids are genetically pure inbred plants in which F_1s are subjected to either anther culture, microscopic culture or wheat × maize cross method of haploid induction to rapidly achieve homozygosity (2n) in a single year (Collard et al. 2005). DH technology has been a revelation for plant breeding and been successfully implemented for decades in different crop species, including barley, rapeseed (*Brassica napus* L.), maize (*Zea mays* L.), and bread wheat (Li et al. 2013). In wheat, DH technology served as an alternative approach to shuttle breeding practised at CIMMYT, thus reducing the time frame required for the development of the improved cultivars. This improved cultivar can further be used as parents for future crosses to develop elite breeding lines in the breeding program. The DH method also allows the fixing of alleles or genes when crossed with wild relatives (Mujeeb-Kazi 2003).

Despite the benefits associated with rapid variety development, there are bottlenecks related to DH technology. The colchicine treatment required for chromosome doubling takes place in the F_1 which results in a single meiotic event with subsequently low recombination frequency being obtained (Humphreys and Knox 2015). Further, DH technology is relatively expensive, costing US$ 30 per line. A very small amount of seed is generated for each DH line, which means seed increase must be performed prior to field phenotyping. Although, the DH approach reduces the time required to generate fixed lines, in crops such as wheat, production of DH lines still requires around two years after production of the F_1 cross, and only saves one year compared to using off-season nurseries for generation advance (Li et al. 2013). The success rate of DH technology also varies between crosses and is entirely dependent upon genetic background and germplasm. DH technology requires expertise in

other technologies such as embryo rescue skills. Additionally, interspecific crosses and tissue culture tools including anther culture and generation of DH can result in albinism (i.e. loss of chlorophyll pigment and incomplete differentiation of chloroplast membranes) (Kumari et al. 2009).

2.6.4 Speed breeding

With the progression of molecular breeding techniques, predictive statistics, and breeding methodologies such as genomic selection (GS) the rate of wheat improvement has accelerated. However, a major limitation is the generation time of plants which have not been reduced significantly even after such technological advancements. On the other hand, studies have reported that the extended exposure to light can greatly reduce generation time for a broad range of plants (Sysoeva et al. 2010; Hickey et al. 2012). Such conditions can be optimised under controlled environmental conditions (CEC) to facilitate rapid generation advance (RGA) and rapidly develop recombinant inbred lines (RIL) via single seed descent (SSD), in less time and with viable seeds.

Such a CEC facility known as the 'Speed Breeding' system or accelerated growth conditions (AGC), was designed at the University of Queensland (UQ), St Lucia, Queensland, Australia, where crop plants are raised under controlled conditions using extended photoperiod and controlled temperature (Rowell et al. 1999; Velez-Ramirez et al. 2011). Speed breeding was first trialled at UQ in 2005 and 2006 to select wheat lines for resistance to tan spot *(Pyrenophora tritici-repentis*) (I. DeLacy, personal communication). Speed breeding promotes flowering and helps to attain up to six generations of spring wheat and barley plants per year; where seed to seed takes only nine weeks (Watson et al. 2017). Following crossing, this enables the rapid production and evaluation of fixed lines, thus has the potential to accelerate development of new cultivars (Hickey et al. 2017).

To date, speed breeding has been successfully applied to many crop species including spring bread wheat, barley, canola, pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.) and peanut (*Arachis hypogaea* L.) (O'Connor et al. 2013; Watson et al. 2017). Thus, speed breeding provides a useful tool for accelerated crop improvement. Despite this, extended photoperiods may cause injury in some plant species, such as tomato (*Solanum lycopersicum* L.) (Velez-Ramirez et al. 2011). However, over the past ten years, a number

of studies have reported techniques for assessing APR to pathogens in cereals grown under CEC (Hickey et al. 2012; Prins et al. 2016; Sørensen et al. 2016; Dinglasan et al. 2016; Hickey et al. 2017). For instance, evaluation of APR to wheat stripe rust in fixed lines and segregating populations was conducted using constant light and temperature, enabling phenotyping to be completed within just five weeks (Hickey et al. 2012).

To further broaden the application of speed breeding or AGC, different biotic (rusts, yellow spot and crown rot) and abiotic traits (root angle, root number, and seed dormancy) in wheat and barley have been studied, proving its effectiveness (Hickey et al. 2009, 2012; Ziems et al. 2014; Richard et al. 2015; Dinglasan et al. 2016). Compared to the conventional breeding pipeline, this nearly halves the length of breeding cycles and has potential to double the rate of genetic gain. Recently, speed breeding was used to introgress multiple disease resistance (i.e. barley leaf rust, net and spot forms of net blotch and spot blotch) through backcrossing into the barley cultivar ‘Scarlett’ (preferred for malting and brewing in Argentina), within two years (Hickey et al. 2017). The utility of such a breeding system would be further improved if coupled with high-throughput marker platforms.

2.7 High-throughput molecular marker platforms

Although traditional phenotypic characterisation of resistance genes is an effective strategy, at times it can be compromised by the presence of more than one gene, environmental variation, plant growth stage and genetic background. The use of polymerase chain reaction (PCR) based molecular markers provide aid in the detection, selection and introgression of useful alleles controlling desirable traits into modern wheat cultivars (Dreisigacker et al. 2016). Thus, reduction in the time and resources involved in the phenotypic screening of the genotypes in a breeding program is obtained. Numerous molecular marker technologies have been developed over the last few decades, including; restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR). The development of highly polymorphic SSR markers was considered a breakthrough which helped to develop high-density genetic maps for bread wheat (Somers et al. 2004). Despite higher numbers, the use of SSR markers in plant breeding was mostly limited to gene mapping and tagging. All of the above-mentioned genotyping methods are found to be labour intensive, of high cost per data point, restricted to low coverage, and constrained by their dependence on gel electrophoresis, resulting in low throughput (Rasheed et al. 2016). This makes the above

list of markers unsuitable for the latest genomic studies such as genome-wide association studies, which require higher marker density.

To overcome these limitations, the introduction of next-generation sequencing has allowed the development of high-throughput and low-cost array-based marker systems such as the single-nucleotide polymorphism (SNP) and diversity arrays technology (DArT) platforms, which are useful for plant breeding programs (Stodart et al. 2007). The SNP represents a single nucleotide base change at one locus. SNPs are the most abundant type of sequence variation in plant genomes and their application has been widely adopted by crop breeding programs (Mammadov et al. 2012). The use of these markers in a breeding program reduces the cost of genotyping (Gupta et al. 2010). Furthermore, the use of SNP markers provides numerous advantages regarding locus specificity, co-dominance, high throughput, and comparatively low genotyping errors (Rafalski 2002). In wheat, a bead-chip assay carrying SNPs has been developed, such as the 9K (Cavanagh et al. 2013) and 90K (Wang et al. 2014) by Illumina Infinium BeadChip. DArT is based on genome complexity reduction system using restriction enzymes followed by hybridisation to microarrays. This technology offers a rapid and DNA sequence-independent shortcut to genome scans, perfectly suitable for large genomes such as wheat (Akbari et al. 2006). A single DArT assay simultaneously types hundreds to thousands of SNPs and insertion/deletion polymorphisms spread across the genome.

Advancement in genotyping technologies has resulted in the development of genotyping-by-sequencing (GBS) marker platforms. GBS allows the use of SNP discovery and genotyping at the same time which is particularly useful in exploring the genetic diversity in crop germplasm (Elshire et al. 2011; Poland and Rife 2012). For instance, more than 40,000 wheat germplasm accessions were genotyped using GBS as part of CIMMYT Seeds of Discovery (SeeD) initiative (http://seedsofdiscovery.org/). These technologies have tremendously reduced the cost and increased the efficiency of identifying marker-trait associations in current years. Recently, DArT use GBS platform referred to as DArT-seq, which returns approximately 140,000 polymorphic markers (with the known sequence) across the bread wheat genome (Andrzej Kilian personal communication, Diversity Arrays Technology, Canberra, Australia). DArT-seq uses restriction enzyme-based complexity reduction system thus separating low-copy sequences from the repetitive fraction of the wheat genome. These low-copy sequences provide useful information in marker discovery. DArT-seq conducts genome profiling which requires high marker densities throughout the

genome and is scored either as presence or absence (i.e. 1 and 0) (Sansaloni et al. 2011). Moreover, DArT-seq markers can be easily converted to kompetitive allele-specific polymerase chain reaction (KASP) markers, which are increasingly getting popular among breeders to target key gene through MAS (Qureshi et al. 2017). Therefore, DArT-seq is particularly useful in carrying out linkage mapping, genome-wide association studies, genetic diversity studies, MAS and genomic selection in wheat and other crop species.

2.8 Strategies for discovery of genomic regions controlling LR resistance

The discovery of genomic regions conferring LR resistance is dependent on efficient phenotypic characterisation and then localisation of the gene in the wheat genome. Wheat has a large genome (~17 Gb) as compared to other cereal crops such as barley, maize, rice, and sorghum, which often leads to poor marker coverage. Likewise, linkage disequilibrium (LD) also varies across the genome in a given population (Breseghello and Sorrells 2006; Chao et al. 2007). With the advent of low-cost next-generation sequencing technologies, a whole genome scan is possible with higher marker coverage and marker density. Two approaches have been widely adopted to find the association between molecular markers and the desired trait. First is linkage, which is conducted using a bi-parental mapping population and second is association mapping or genome-wide association studies (Yu et al. 2006).

2.8.1 Linkage or QTL mapping

Linkage mapping is a conventional method to identify underlying genetic variations that co-segregate with the trait of interest using a bi-parental mapping population such as F_2, backcross, or RIL population (Koornneef et al. 2004; Zhu et al. 2008). However, RIL populations have an advantage over segregating F_2 populations because they can be subject to replicated testing of each genotype across environments and pathotypes, rather than the F_2 generation which relies on a single assay. The F_1 seed is subject to several generations of SSD to create inbred lines. A fast way to obtain fixed homozygous lines is either by subjecting F_1 seeds to DH technology or accelerating the SSD process via speed breeding (Hickey et al. 2017). Near-isogenic lines or introgression lines are typically developed to identify QTL from wild relatives. Although QTL mapping is successful in some cases, it is fundamentally limited to the comparative low allelic diversity of the two crossing

parents and low recombination events which impair the mapping resolution (Zhu et al. 2008). To date, hundreds of QTL have been detected using these mapping approaches.

2.8.1.1 Multi-parent mapping populations

Recent advances in mapping approaches have resulted in the development of multi-parent populations such as nested association mapping (NAM) and multi-parent advanced generation integrated crosses (MAGIC). In NAM, crosses are made between founder parents (donors) with an adapted parent that serves as a reference variety, usually a preferred cultivar. Small subpopulations of RILs are developed, from each donor-reference combination. In MAGIC populations, diverse founder lines are selected and inter-crossed until all founders have an equal probability of contributing to the genetic makeup of a line (Rakshit et al. 2012). This is followed by multiple generations of selfing to create RILs. These populations are excellent resource for understanding the genetic architecture of complex traits due to enhanced recombination, segregation of multiple alleles and high mapping resolution. For instance, 59 QTL were identified associated with resistance at adult plant stage against stem rust in a wheat NAM population comprising of 852 RILs (Bajgain et al. 2016). Similarly, a wheat MAGIC population comprising of 1579 RILs have been used for identification of QTL for hectolitre weight and plant height in wheat (Huang et al. 2012).

Despite successful application in various plant species, there are limitations associated with these mapping populations.Likewise, in MAGIC extensive segregation for agronomic traits (i.e. maturity and plant height) occurs which ultimately can affect complex traits such as yield or drought tolerance. The development and phenotyping of the large experimental populations is time-consuming, laborious and expensive. Although genetic diversity is higher than bi-parental linkage mapping population, it is still constrained by the lines selected as parents.

2.8.2 Genome-wide association studies of breeding and natural populations

Association mapping or genome-wide association studies (GWAS) is an alternative approach to overcoming limitations associated with bi-parental linkage mapping. With the advent of next-generation sequencing technologies, whole genome marker scans can provide very high marker density (Yu et al. 2006; Maccaferri et al. 2010) and can be used to

identify marker-trait associations referred as genome-wide association studies. GWAS can be applied to breeding populations or natural populations such as germplasm collections, and hence saves time, money and labour required to develop specially designed bi-parental mapping populations. In plants, GWAS was first performed in maize (Thornsberry et al. 2001), but is now widely adopted and applied to many different crop species (Huang et al. 2010; Morris et al. 2013; Ziems et al. 2014; Macaferri et al. 2015; Qian et al. 2016).

GWAS identifies marker-trait associations based on LD which utilises historical recombination events accumulated over several generations at the population level (Yu and Buckler 2006). LD is the non-random association of alleles between two genetic loci naturally occurring nearby. LD determined by estimating the deviation from the observed haplotype frequency from its corresponding allele frequencies expected under equilibrium. This estimation is known as the coefficient of correlation, represented by 'D'. The value of D can be positive or negative depending on the allele frequency of two loci. As allele frequencies cannot be negative, to overcome this problem, standardisation of LD (D′) is carried out by calculating a relative measure of the disequilibrium (D) compared to D maximum (Dmax) using formula D′=D/Dmax. This standardisation makes the value of D′ ranging between 0 and 1. However, correlation coefficient (r^2) is commonly used as a measure of LD (Devlin and Risch 1995; Pritchard and Przeworski 2001). LD varies with crop species depending on the mating system, historical recombination events, mutation, migration (admixture), genetic drift, selection and population structure (stratification) (Rafalski 2002; Myles et al. 2009). The mapping resolution of GWAS is highly dependent on the rate at which LD decays with the genetic distance (cM) or physical distance (kb). If LD decays rapidly in a natural population, higher marker density is required, which in turn will increase mapping resolution and vice versa.

In GWAS, the selection of germplasm is critical and often varies depending on the trait of interest or objective of the study. For instance, if the study aims to identify new loci for a given trait, then seed bank accessions are typically used, whereas if the objective is to find marker-trait associations that are relevant to breeding, then a panel of elite breeding lines is used (Breseghello and Sorrells 2006). In all natural populations, the distribution of genotypes is non-random which results in a population structure and leads the population to deviate from Hardy-Weinberg equation (i.e. p + q = 1). The population structure is due to the presence of admixtures, mating system, genetic drift, and natural or artificial selection during crop domestication and improvement (Hirschhorn and Daly 2005; Soto-Cerda and

Cloutier 2012). If not corrected, the population structure can cause spurious associations (Type I error) (Pritchard et al. 2000; Zhao et al. 2007; Myles et al. 2009). This can be corrected to a degree using statistical methods such as mixed linear model (MLM) (Q matrix) in natural populations where population structure is considered as a fixed effect (Price et al. 2006; Yu et al. 2006). Furthermore, use of a kinship matrix (K matrix) in MLM kinship to account for a proportion of phenotypic variation between pairs of individuals also reduces the false positives (Myles et al. 2009). Thus, a model which accounts for both population structure and kinship matrix (Q + K) gives more accurate results, in populations containing highly related individuals.

Spurious associations between marker and trait can also arise due to unbalanced allele frequencies. This is common in natural populations, and the statistical power to detect such rare alleles using the GWAS approach is poor (Brachi et al. 2011). Notably, these rare alleles are considered important in the natural variation detected in different species. Thus, plant breeders are often interested in rare alleles that frequently provide a yield or trait advantage. To precisely examine the phenotypic effects of such rare alleles, development of bi-parental mapping populations could serve as a better option where allele frequency is balanced in a structured scenario (Xu et al. 2017).

GWAS also has the potential to identify the underlying candidate genes for the trait of interest in a population (Hall et al. 2010). This approach has been found effective in the identification of candidate genes depicting a large phenotypic effect in wheat. For instance, candidate genes for pathogenicity and mycotoxin production for *Fusarium culmorum* were identified in wheat through a candidate gene approach (Castiblanco et al. 2017). The efficacy of such a study is dependent on the size of the population, the trait of interest, and number of markers available (Huang et al. 2010). However, to date, the wheat genome sequence is not available. A consensus map based on the genetic position of the molecular markers has been developed to locate different genes in the wheat genome. So, *in-silico* gene annotation is performed in other grass species such as *Brachypodium*, rice and barley, to identify candidate genes controlling the trait of interest and necessary conclusions are predicted in wheat. However, there is a need to isolate and characterise those target genes by developing mapping populations and subsequently clone them to decipher their functional role in wheat.

In wheat, GWAS has been successfully applied to identify QTL for resistance to various diseases such as rusts, bacterial leaf streak, *Fusarium* head blight and eyespot (Gurung et al. 2014; Yu et al. 2014; Arruda et al. 2016; Zanke et al. 2017). To date, eight GWAS studies have been performed to identify new genomic regions underpinning LR resistance in wheat using high-throughput marker platforms (Kertho et al. 2015; Jordan et al. 2015; Gao et al. 2016; Li et al. 2016; Auon et al. 2016; Pasam et al. 2017; Turner et al. 2017; Kankwatsa et al. 2017). Of these, most GWAS studies were focused on seedling resistance, while only a few studies were conducted on adult plant LR resistance. Notably, no studies have reported GWAS using the diverse wheat accessions from the seed bank in St Petersburg (i.e. VIR).

2.9 Conclusion

Diseases such as LR pose a continuous threat to global wheat production. As discussed, the rapid evolution of the pathogen can easily overtake the breeding progress and resistance genes deployed in cultivars. To reduce crop losses, plant breeders require a constant supply of new and durable sources of genetic resistance, such as APR. This will also help broaden the genetic basis of modern wheat breeding germplasm that has been constrained due to years of selection and inbreeding. Therefore, improving, and stabilising wheat yield is a complex challenge and is not likely to be answered by a single technology or approach. A multifaceted approach is required to integrate the latest breeding technologies to rapidly identify new resistance factors lying dormant in seed bank collections. Technology such as speed breeding can help to grow plants fast and achieve multiple generations in one year. If combined with trait phenotyping (i.e. LR resistance), such an approach could rapidly generate valuable information for diverse wheat accessions. Likewise, application of the latest genotyping platforms, such as DArT-seq, could lead to the rapid detection of novel genomic regions underpinning LR resistance. Thus, integrating such technologies will likely accelerate the discovery and deployment of these newly identified sources of resistance in wheat breeding programs. Such tools and resources will empower plant breeders to stay one step ahead of the rapidly evolving *P. triticina*.

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Chapter 3 - Into the vault of the Vavilov wheats: old diversity for new alleles

3.1 Abstract

Intensive selection in wheat (*Triticum aestivum* L.) breeding programs over the past 100 years has led to a genetic bottleneck in modern bread wheat. New allelic variation is needed to break the yield plateau, particularly in the face of climate change and rapidly evolving pests and pathogens. Landraces preserved in seed banks likely harbour valuable sources of untapped genetic diversity because they were cultivated for thousands of years under diverse eco-geographical conditions prior to modern breeding. We performed the first genetic characterization of bread wheat accessions sourced from the N. I. Vavilov Institute of Plant Genetic Resources (VIR) in St Petersburg, Russia. A panel comprising 295 accessions, including landraces, breeding lines and cultivars was subjected to single seed descent (SSD) and genotyped using the genotyping-by-sequencing Diversity Arrays Technology platform (DArT-seq); returning a total of 34,311 polymorphic markers (14,228 mapped and 20,083 unmapped). Cluster analysis identified two distinct groups; one comprising mostly breeding lines and cultivars, and the other comprising landraces. Diversity was benchmarked in comparison to a set of standards, which revealed a high degree of genetic similarity among breeding material from Australia and the International Maize and Wheat Improvement Center (CIMMYT). Further, 11,025 markers (1,888 mapped and 9,137 unmapped) were polymorphic in the diversity panel only, thus representing allelic diversity potentially not present in Australian or CIMMYT germplasm. Open-access to DArT-seq markers and seed for SSD lines will empower researchers, pre-breeders and breeders to rediscover genetic diversity in the VIR collection and accelerate utilisation of new alleles to improve wheat.

3.2 Introduction

Bread wheat is a staple food crop that was domesticated about 10,000 years ago in the Fertile Crescent of Western Asia (Shewry 2009; Ray et al. 2013). Wheat has a complex hexaploid genome (2n=6x=42) contributed by three different progenitors. The first hybridisation occurred between *T. urartu* Thum. (genome AA) and *Aegilops speltoides* Tausch (genome BB) and resulted in tetraploid Emmer (*T. turgidum* L.) (genome AABB). The second hybridisation event occurred between Emmer and *Ae. tauschii* Coss. (genome

DD) resulting in hexaploid bread wheat (genome AABBDD) (Salse et al. 2008). During wheat domestication, a limited number of hybridisation events occurred between progenitor species, subsequently leading to a relatively narrow genetic base in hexaploid wheat compared to its wild relatives (Brenchley et al. 2012). Moreover, trait-specific selection in wheat breeding programs has further reduced levels of genetic diversity (Doebley et al. 2006). Consequently, the rate of genetic gain for yield and some desirable traits within the modern wheat germplasm pool is approaching a plateau (Grassini et al. 2013).

Landraces have arisen through a combination of natural and artificial selection performed by farmers in a environment, thus are highly adapted to local conditions (Reif et al. 2005). However, landraces were developed under a lower selection pressure in comparison to modern cultivars, therefore collectively display a broader genetic base (Cavanagh et al. 2013). Immense genetic diversity for landrace collections has been reported for many crops, including; wheat, maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.*)*, barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.) and oats (*Avena sativa* L.) (Jilal et al. 2008; Huang et al. 2010; Cavanagh et al. 2013; Ignjatović-Micić et al. 2013; Pineda-Hidalgo et al. 2013; Mace et al. 2013; Montilla-Bascón et al. 2013; Pasam et al. 2014). Recent studies have examined landrace collections and identified new alleles for tolerance to abiotic and biotic stresses (McIntosh et al. 1998; Bansal et al. 2013; Jaradat 2013; Sthapit et al. 2014; Lopes et al. 2015; Maccaferri et al. 2015), which could be used by wheat breeding programs to improve yield stability.

Historical germplasm, such as landraces or old cultivars, represent a potentially valuable source of genetic variation (Motley 2006; Jones et al. 2008; Bhullar et al. 2009; Cavanagh et al. 2013). However, such material is rarely used in breeding programs, which typically target elite × elite crosses to improve the likelihood of developing higher yielding cultivars (Baenziger and DePauw 2009). Fortunately, a proportion of historical wheat germplasm has been maintained by gene banks. Approximately 850,000 viable wheat samples are stored in 229 independent collections worldwide (Mitrofanova 2012). Recent studies exploring the genetic diversity for landraces collected in the 1930s by renowned botanist A. E. Watkins have identified new sources of disease resistance, for instance, leaf rust resistance genes *Lr52* (Bansal et al. 2011) and *Lr67* (Hiebert et al. 2010) and stripe rust resistance gene *Yr47* (Bansal et al. 2011). The prominent Russian botanist and geneticist N. I. Vavilov, best known for his theory relating to ‘the centres of origin of cultivated plants’ (Vavilov, 1926), devoted his life to the improvement of cereal crops. During the early 19th

Century, Vavilov and his colleagues travelled around the world collecting seeds, including many wheat landraces, which were subsequently stored in a seed bank in Leningrad, now known as the N. I. Vavilov Institute of Plant Genetic Resources (VIR) in St Petersburg, Russia. Vavilov's collections represent a 'snap shot' of landraces cultivated around the world prior to modern breeding. Currently, the VIR seed bank consists of germplasm derived from almost 100 countries throughout Europe, Asia, Africa, America and Australia, where about 19% of the accessions are from various parts of Russia (Mitrofanova 2012).

The development of low-cost high-throughput DNA marker systems, such as genotyping-by-sequencing (GBS) (Elshire et al. 2011; Poland and Rife 2012) or the 90K single nucleotide polymorphisms (SNP) platform (Wang et al. 2014), offers a cost-effective way to explore the genetic diversity contained in landrace collections. Previous studies have examined VIR accessions for agronomic and disease traits, such as plant height, resistance to leaf rust, dark brown leaf spot-blotch and *Septoria* glume blotch (Tyryshkin and Tyryshkina 2003; Mitrofonova 2012); however, genetic analysis has been mostly limited to DNA markers specific for known genes. A whole-genome approach, such as GBS or SNP markers, would enable genome-wide association studies (GWAS) to identify marker-trait associations and discover new alleles for desirable traits (Lopes et al. 2015; Sukumaran et al. 2015). This way, useful genetic variation can be efficiently introgressed into modern wheat germplasm using marker-assisted breeding strategies.

In this study, we assembled 295 bread wheat accessions originally sourced from VIR. We genetically characterise this diversity panel using the Diversity Arrays Technology GBS platform (i.e. DArT-seq) and benchmark levels of genetic diversity using a set of standards comprising modern cultivars and elite breeding lines from Australia and CIMMYT. We anticipate that open-access to this global diversity panel, including DArT-seq marker profiles and seed for single seed descent (SSD) lines, will enable GWAS aiming to identify new alleles for important target traits and accelerate the use of genetically diverse material from VIR in modern wheat breeding programs.

3.3 Material and methods

3.3.1 Plant materials

Two hundred and ninety-five bread wheat accessions originally sourced from VIR were assembled to form a globally diverse panel. The 295 accessions were imported to Australia by the Australian Grains Genebank in Horsham, Victoria, Australia (Supplementary Table 1). The panel comprised 136 landraces, 32 cultivars, 10 breeding lines and 118 accessions with unknown classification regarding their cultivation status. The accessions were collected from different geographical regions of the world between 1922 and 1990. This panel also contains 56 accessions originally collected by A. E. Watkins, which were donated and registered at VIR in 1934 and 1936. Of the 295 accessions, 206 have known origin information, originating from 28 countries, spanning 5 different continents of the world, including; North America (n=4), South America (n=2), Africa (n=6), Europe (n=69), and Asia (n=125) (Figure 3.1). Although the exact geographical origin of the remaining 89 accessions was unknown - they were collected at the time of the former Union of Soviet Socialist Republics (USSR).

A set of standards comprising 20 cultivars and elite breeding lines from CIMMYT and Australian wheat breeding programs was assembled (Table 3.1), which was used to benchmark the genetic diversity in the panel of accessions from VIR.

3.3.2 Line purification

A single plant for each of the 295 VIR accessions and 20 standards was grown for line purification in a temperature controlled growth facility at The University of Queensland, St Lucia, Queensland, Australia. Seed bank collections (e.g. landraces) often contain mixtures of different genotypes (Newton et al. 2010). Thus a single random plant is not likely representative of the diversity contained within each accession. Although, this strategy aimed to maximise the number of accessions sampled from, rather than sampling the diversity within accessions. A generation of SSD was used to develop genetically stable lines for subsequent genotypic and phenotypic analyses. Seeds were imbibed in trays filled with potting media comprising composted pine bark fines (0–5 mm) (70%) and coco peat (30%) with a pH ranging 5.5–6.5 and placed at 4^{o}C for eight weeks to satisfy vernalization

requirements. Plants were transplanted into 140 mm (1.4L) ANOVAPot® (Anovapot Pty Ltd, Australia, www.anovapot.com) pots and grown under constant (24h) light and temperature (22^{o}C) to accelerate plant development (Hickey et al. 2009, 2012). During the growth cycle, notes were recorded for each accession, including leaf hairiness, the presence of awns, and seed shape following harvest. The progeny from each single plant was bulked and formed the pure seed source for all future experiments.

3.3.3 Field evaluation

The pure seed for each accession in the diversity panel, along with the set of standards, were sown in a field nursery located at The University of Queensland Research Station, Gatton, Queensland, Australia. Un-replicated hill plots were sown where each plot contained six seeds. At 113 days after sowing (DAS), the accessions were evaluated for growth habit, and plant height was recorded for genotypes exhibiting spring growth habit.

3.3.4 Genotyping

Young leaf tissue was sampled from the single plant selections, and genomic DNA was extracted using the CTAB (hexadecyl trimethyl ammonium bromide) method following the protocol recommended by DArT (https://www.diversityarrays.com/files/DArT_DNA_isolation.pdf). A total of 315 SSD lines (i.e. 295 VIR accessions and 20 standards) were genotyped using the DArT-seq wheat *PstI* microarray platform developed by Diversity Arrays Technology Pty Ltd, Canberra, Australia, as described by Li et al. (2015).

3.3.5 Analysis of genetic diversity and population structure

Clustering of individuals was performed using the partitioning around medoids (PAM) algorithm, the most common implementation of the k-medoids algorithm (Reynolds et al. 1992). The PAM algorithm attempts to partition a population into k clusters based on the levels of dissimilarity between individuals. The optimal number of clusters is determined using a graphical display called a silhouette plot. The algorithm then finds a representative individual (called a medoid) for each of the k clusters such that the average dissimilarity of that medoid to all other members of its cluster is minimised.

Clustering was initially performed for the 295 accessions forming the diversity panel using 34,311 dominant markers (DArT-seq). The 'Jaccards distance' (Jaccard 1908) between all 295 individuals was then calculated using R stats package (Team 2014). Using the resulting 295 × 295 dissimilarity matrix, the optimal number of clusters (i.e. k=2) was estimated using the fpc package (Hennig 2014). This estimate was then verified by running the PAM algorithm for a range of cluster sizes (k=1….5) and visually assessing the silhouette plots for each value of k. The cluster package was used to run the PAM algorithm and to generate the final biplots. Monomorphic markers were excluded from the analysis although there were no restrictions placed on rare alleles (i.e. alleles occurring at low frequency) as they were considered important in determining genetic diversity. The procedure was then repeated with the standards included in the population to investigate the diversity within the context of elite breeding lines and cultivars from Australia and CIMMYT. Accessions were classified according to their cultivation status (i.e. cultivar, breeding line, landraces, and unknown) and geographic origin (i.e. continent) to explore trends in genetic diversity based on the clustering analysis.

The 20 standards were also used to benchmark genetic diversity by identifying 'new' markers that were only polymorphic in the diversity panel (i.e. monomorphic in the standards). Markers were positioned based on the wheat DArT-seq consensus map and displayed on chromosomes using Mapchart software Version 2.2 (Voorrips 2002). To visualise the distribution of new markers at the genome level, markers that were polymorphic only in the diversity panel were coloured red and markers that were polymorphic in the standards were coloured black.

3.4 Results

3.4.1 Phenotypic diversity

During line purification in the glasshouse, the diversity panel was evaluated for morphological characters. Of the 286 accessions evaluated, 12.5% displayed the leaf hairiness trait. The majority of accessions (98.3%) displayed oblong seed shape, whereas only 1.7% displayed ovate (round) seed shape. The diversity panel was also evaluated for the presence of awns, where 25.2% were awnless, 4.9% were apically awnleted, 2.4% were awnleted, and 65.08% were awned. A sample of phenotypic variation in awn morphology

was displayed in Figure 3.2. Based on the classification defined by Dorofeev et al. (1979), the original accessions from which the pure lines were sampled, represented 5 species and 30 botanical varieties (Supplementary Table 1). It should be noted that some SSD lines developed in this study did not match the botanical variety assigned for the original accession. For example, original VIR accessions AUS38778 and AUS39503 both contain a mixture of var. *graecum* (Koern.) Mansf. and var. *pseudomeridionale* (Flaksb.) Mansf.; however according to our morphology results, the derived SSD lines (i.e. WLA-017 and WLA-039, respectively) belong to var. *graecum*.

In the field, the majority of accessions in the diversity panel displayed a spring growth habit (i.e. 80.1%), while 17.2% displayed a significantly delayed time to anthesis, indicating a winter growth habit. A small number of accessions (3.7%) failed to germinate, thus were not included in the phenotypic analysis. A total of 237 spring type accessions were observed, which included 61 accessions with an unknown origin (not presented in Figure 3.3). The remaining 176 spring type accessions originated from 27 countries, mostly from Russia (n=40), India (n=35) and Pakistan (n=32). Among the 51 winter type accessions, 25 were of unknown origin, while 26 originated from nine countries, with the largest samples from Russia (n=11), Ukraine (n=4) and Armenia (n=3) (Figure 3.3).

Plant height was measured 113 DAS, at which time most of the spring type accessions displayed growth stages ranging GS65 to GS71 (i.e. mid flowering to grain filling). In contrast, winter type accessions were depicting delayed growth, ranging GS21-GS29 (i.e. early to late tillering). Considering variation in maturity, the plant height data for winter type accessions was excluded from the analysis of the population distribution. The average height for the 237 spring type accessions was 103.8 cm, ranging 55–165 cm. The Australian and CIMMYT standards displayed an average height of 81.8 cm and 89.4 cm, respectively (Figure 3.4).

3.4.2 Genetic diversity

Genotyping of the diversity panel and standards using the DArT-seq GBS platform returned a total of 56,306 DArT-seq, of which 34,311 were polymorphic. Of the polymorphic markers, 14,228 were positioned on the current DArT-seq consensus map, while 20,083 were unmapped and their chromosomal position was unknown. Among the 14,228 mapped

polymorphic markers, 1,888 were found polymorphic only in the diversity panel, thus were considered “new” in comparison to the Australian and CIMMYT genotypes (Figure 3.5). Among the unmapped polymorphic markers, 9,137 were new to the diversity panel. The DArT-seq provided good coverage across the centromeric and pericentromeric regions of the seven homologous groups of chromosomes. A large portion of the new polymorphic markers were mapped to the A and B genomes (32% and 43%, respectively) compared to the D genome (25%). Also, marker density was higher for the A and B genome chromosomes (2.11 and 3.14 markers per cM, respectively) compared to the D genome chromosomes (1.7 markers per cM). The highest densities of new polymorphic markers were observed on chromosomes 2A, 2B, 3B, 6B and 7B and with considerably lower densities on chromosomes 1A, 1D, 4D, and 5D (Figure 3.5). Further, analysis of the DArT SNP data revealed low levels of heterozygosity in SSD lines forming the diversity panel, ranging from 0.7–1.8% per chromosome (Figure 3.6).

3.4.3 Population structure

The silhouette method revealed the optimum number of clusters (k=2) for the diversity panel. The PAM cluster analysis for two groups resulted in 171 accessions in cluster 1 and 124 accessions in cluster 2 (Figure 3.7). The 42 reported cultivars and breeding lines within the diversity panel were split across the two clusters, with 34 accessions (81%) in cluster 1. The 136 reported landraces were also divided across the two groups, with 90 accessions (66% of landraces) appearing in cluster 2. The population structure of the diversity panel was re-evaluated by adding standards to the PAM cluster analysis (Figure 3.8a and b). All of the Australian and CIMMYT standards were genetically similar and were positioned very close to one another and were all grouped within cluster 1 (Figure 3.8a and b). Cluster 2 mostly comprised landraces (Figure 3.8a).

3.4.4 Genetic diversity corresponding to geographic origin

Most of the accessions from Europe, all South American accessions and those of unknown origin, were grouped in cluster 1, along with the standards (Figure 3.9). Most of the accessions from Asia and all accessions from Africa were also grouped in cluster 1 (Figure 3.9). The North American accessions did not show a clear pattern and were equally distributed across both clusters (Figure 3.9). The genetically diverse landraces in cluster 2

were largely from Asia, mainly sourced from India and Pakistan. Accessions with unknown origin were found genetically similar to accessions from Europe, most of which were sourced from Russia.

3.5 Discussion

Through this study, we have gained an insight of the genetic diversity preserved in the wheat collection at VIR in St Petersburg, Russia. A high degree of new alleles were observed in comparison to a set of standards from Australia and CIMMYT. This diverse collection includes accessions from 28 countries, collected over a period spanning 70 years, presenting a potentially valuable open-access genetic resource for enriching diversity in modern breeding programs. We anticipate this will accelerate the discovery of new alleles for tolerance to abiotic and biotic stresses - needed to improve wheat productivity with the onset of climate change and anticipated new pests and diseases.

3.5.1 Diversity in the panel

The diversity panel was genotyped with 56,306 DArT-seq, of which 14,228 polymorphic markers had a chromosomal position, based on the current DArT-seq consensus map. Of these, 1,888 were deemed new to the diversity panel as they were monomorphic in the standards from Australia and CIMMYT. These new markers were distributed across all 21 chromosomes, but in particular clusters of new markers were located on chromosomes 2A, 2B, 3B, 6B, and 7B. It is important to note that a large number of unmapped polymorphic markers (i.e. 9,137) were also new in the diversity panel. While cluster analysis used 34,311 markers (mapped and unmapped), the chromosomal location of new markers could only be investigated using the subset of mapped markers. However, a genetic map is not required to identify marker-trait associations in wheat (Arief et al. 2014). Thus the entire set of polymorphic markers can be used in future GWAS studies.

Higher marker densities were observed in the A and B genome chromosomes (2.11 and 3.14 markers per cM, respectively) compared to the D genome chromosomes, which may be due to lower rates of recombination (Akbari et al. 2006; Allen et al. 2011; Cavanagh et al. 2013; Nielsen et al. 2014). Wang et al. (2014) used the 90K SNP chip to genotype 726 wheat accessions including landraces and found a similar trend, where only 15% of the

reported markers were in the D genome. Voss-Fels et al. (2015) also found large non-polymorphic chromosomal sections in the D genome, especially on 4D and 7D (>30 cM). The typically low genetic variation in the D genome of modern wheat means that breeding efforts essentially act to manipulate diversity largely in the A and B genomes (White et al. 2008; Jia et al. 2013; Henry and Nevo 2014; Voss-Fels et al. 2015). Accessions from this diversity panel could be used to increase genetic diversity, particularly for the D genome in modern germplasm.

In the future, we anticipate the development of an improved DArT-seq consensus map and positioning of unmapped polymorphic markers in this study. This could improve marker density for the D genome chromosomes, in particular, chromosomes 1D, 4D and 5D. Nevertheless, the mapped marker coverage using the current wheat consensus map is adequate for effective GWAS aiming to explore this genetic resource for target traits (Voss-Fels et al. 2015). The large number of new markers highlights the high degree of diversity and historical recombination among accessions. This coupled with the use of high-density DArT-seq will enable precise positioning of QTL in future GWAS studies.

It was clear that landrace accessions were genetically more diverse than breeding lines and cultivars, which tend to group in the cluster analyses. The group of most distinct landraces were those from India and Pakistan, which clustered in the upper section of cluster 2 (Figure 3.7 and 3.9). Landraces from India and Pakistan thus represent a great source of genetic variation for wheat improvement. Although there was no clear trend in the clustering of accessions based on growth habit (i.e. spring and winter types) according to cultivation status, although most winter type accessions originated from Russia, Ukraine and Armenia. These countries experience extremely low temperatures during winter and also relatively cool temperatures during the wheat growing season (Schierhorn et al. 2014). The study by Cavanagh et al. (2013) also found a lack of differentiation between spring and winter wheat using whole-genome profiles. This suggests that spring and winter wheat were selected side-by-side in farmers' fields and breeding programs. Flowering time in wheat is a complex trait, and many different genetic factors can lead to early flowering. Thus such differences between spring and winter genotypes may not be differentiated using a whole-genome marker scan.

3.5.2 Australian and CIMMYT breeding material have a narrow genetic base

Wheat breeding efforts for more than 100 years in Australia have increased farm yield from 0.5 t/ha to approximately 2 t/ha (Fischer 2009; Fischer et al. 2014). While a large improvement in yield was achieved via the transition to semi-dwarf varieties during the Green Revolution, the rate of gain for farm yield has slowed to just 1% per year (Fischer 2009; Fischer et al. 2014). However, this estimate includes both genetic gains resulting from breeding and improved management practices. It seems wheat yield around the world is beginning to plateau (Ray et al. 2013). While breeding strategies must improve to meet future demands, the intensive selection performed in modern breeding programs has resulted in bottlenecks in terms of genetic diversity (Cavanagh et al. 2013), which may restrict future genetic gains.

Since the early 1970s, CIMMYT material has been extensively used in wheat breeding programs in Australia. As a result, the majority of Australian cultivars are either direct CIMMYT lines or contain CIMMYT lines in their parentage (Brennan and Quade 2006). Of course, the set of 20 standards evaluated in this study does not capture all diversity in modern breeding programs around the world; nevertheless, it provides useful insight to gauge the diversity particularly within the context of wheat pre-breeding and breeding efforts in Australia. Widespread utilisation of CIMMYT material globally has led to significant yield gains, but simultaneously resulted in narrowing the genetic base of elite breeding material (Cavanagh et al. 2013). This can be problematic in the event of new pests or pathogens. A recent example is an emergence of a highly virulent stem rust pathotype Ug99 (Race TTKSK), first detected in Uganda in 1998, which rendered 90% of wheat cultivars susceptible worldwide (Singh et al. 2011).

The high degree of allelic variation in landraces can be used to broaden the genetic base of modern wheat germplasm and improve desirable traits (Smale et al. 2002; Rief et al. 2005; Lopes et al. 2015). Landraces have contributed many agronomically important traits in modern cultivars, such as the semi-dwarfing gene *Rht8c* and photoperiod insensitivity gene *Ppd_D1* (formerly known as *Ppd1*) from the Japanese landrace "*Aka Kamougi*" (Worland et al. 1998; Ellis et al. 2007). Similarly, disease resistance genes have been identified, such as leaf rust resistance gene *Lr67* from Pakistani landrace "PI250413" (Dyck and Sambroski 1979; Hiebert et al. 2010; Herrera-Foessel et al. 2011). While this study has genetically characterised 295 diverse wheat accessions from VIR, more

accessions could be genotyped and utilised for breeding, as a total of 29,209 bread wheat accessions are currently preserved at VIR (Mitrofanova 2012).

3.5.3 Exploiting the genetic resource

The diversity panel is currently being evaluated for root architecture traits (seminal root angle and number) and resistance to key foliar diseases, including; leaf rust, stripe rust, stem rust and yellow spot. The next step is to perform GWAS to identify new alleles for these traits. This information could then be used to profile the environments that contributed new alleles. This, in turn, would enable the identification of similar environments from which germplasm could be sampled to mine additional diversity from seed banks using the Focused Identification of Germplasm Strategy (FIGS) approach (Mackay 1995; Mackay and Street 2004; Bhullar et al. 2009).

This diversity panel is an open-access resource and available to researchers, pre-breeders and wheat breeders. A small quantity of pure seed can be requested from the Australian Grains Genebank in Horsham, Victoria, Australia (contact: sally.norton@ecodev.vic.gov.au) and will be provided under a Standard Material Transfer Agreement (SMTA). The DArT-seq marker data is available upon request from the corresponding author.

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3.7 Figures

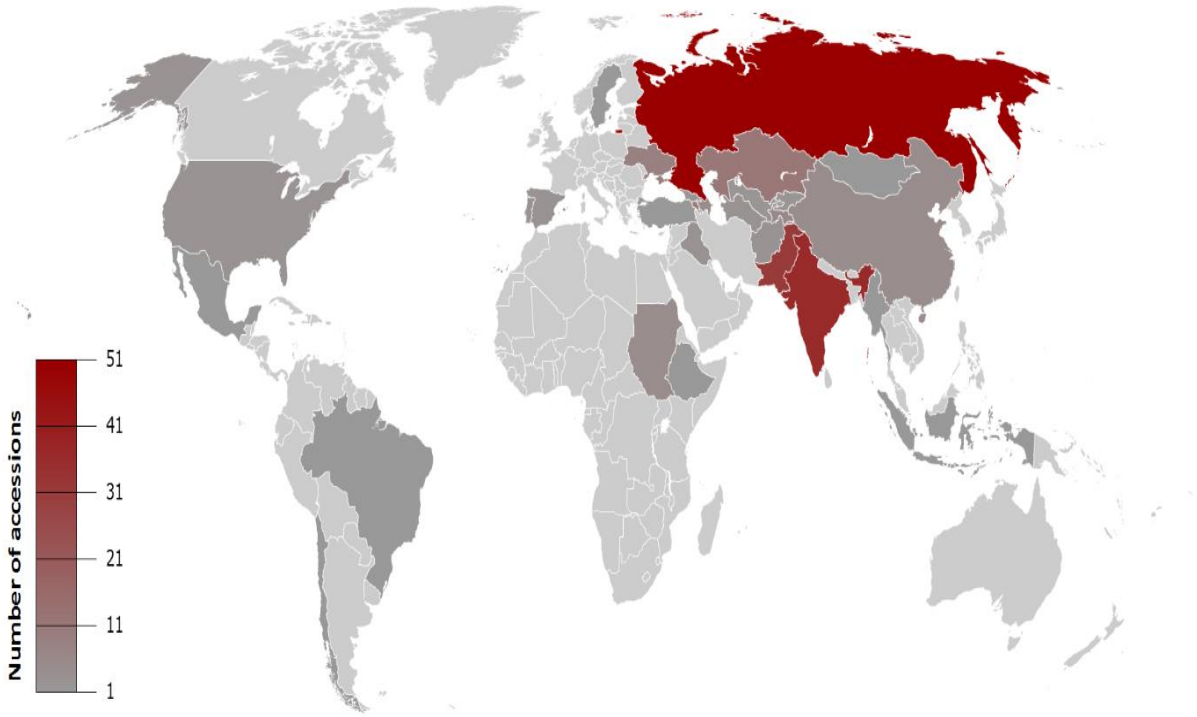
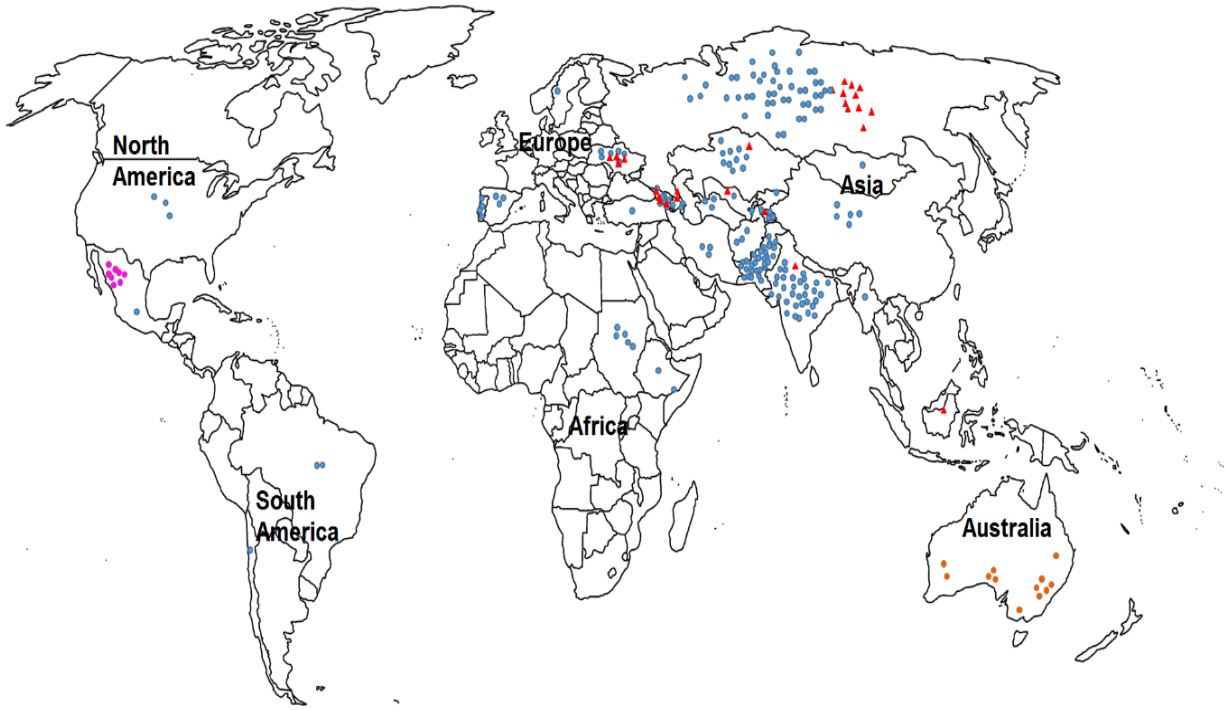


Figure 3.1 The geographical distribution of accessions with known origin in the diversity panel (206 out of 295).

Figure 3.2 A sample of phenotypic variation for awns in the diversity panel, where 1*) T. aestivum* var. *aureum* (Link) Mansf.; 2) *T. aestivum* var. *pseudomeridionale* (Flaksb.) Mansf.; 3) T. aestivum var. *ferrugineum* (Alef.) Mansf.; 4) *T. aestivum* var. *heraticum* (Vav. & Kob.) Mansf.; 5) *T. spelta* L.; 6) *T. erythrospermum (*Koern.) Mansf.; 7) *T. sphaerococcum* Perc.



1

2 **Figure 3.3** The geographical distribution of diversity panel accessions with the known origin
3 (i.e. 202 out of 295) displaying spring (blue) and winter (red) growth habits, along with
4 standards from Australia (brown) and the International Maize and Wheat Improvement
5 Center (pink).

6
7

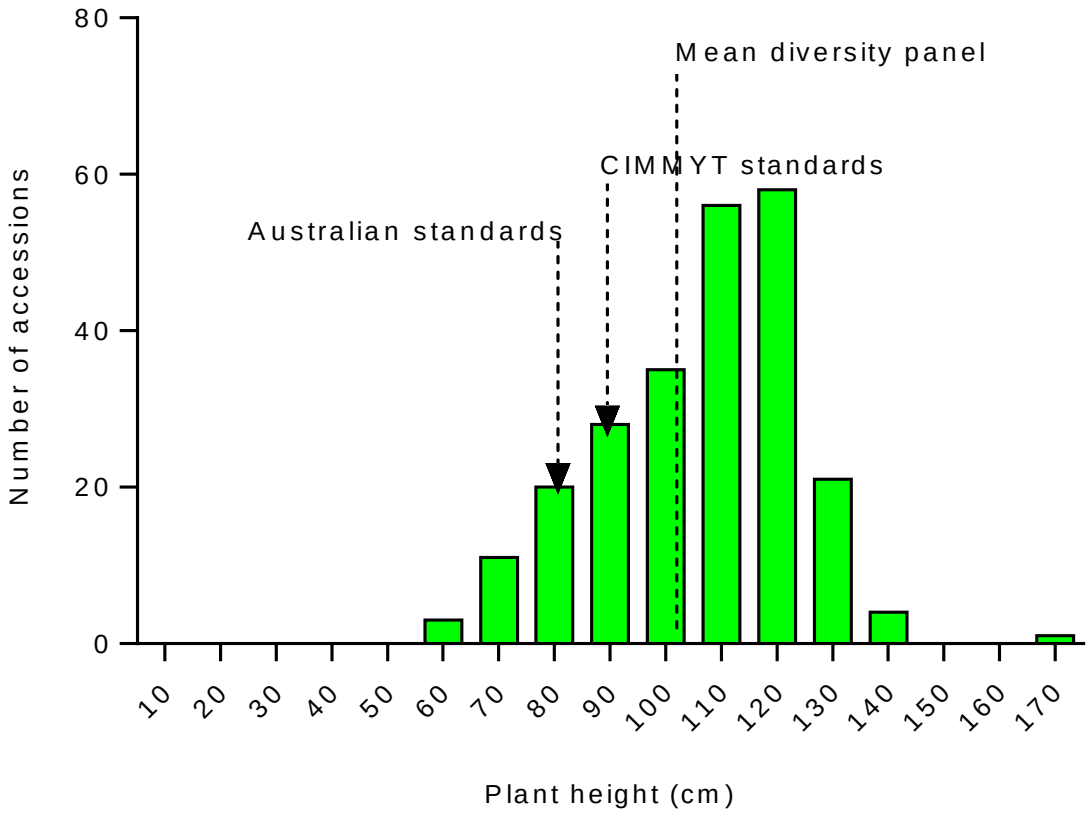


Figure 3.4 Distribution of plant height for accessions in the diversity panel. The population mean indicated by the dotted line (103.84 cm). The mean plant height for standards from Australia and the International Maize and Wheat Improvement Center (CIMMYT) is displayed by arrows (i.e. 81.81 cm and 89.38 cm, respectively).

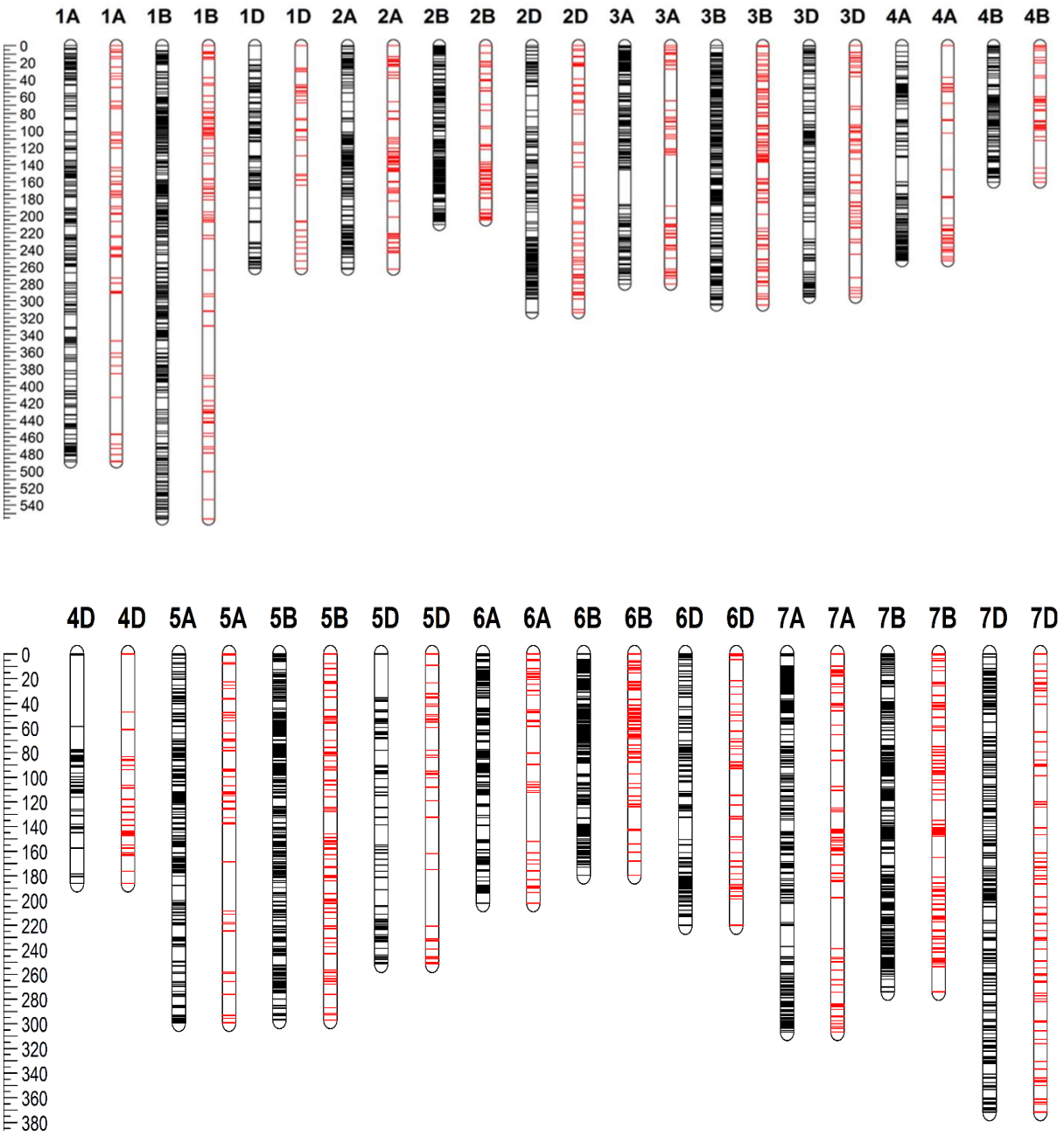


Figure 3.5 Distribution of polymorphic markers based on the wheat DArT-seq consensus map (version 4.0 provided by Dr. Andrezj Kilian, Diversity Array Techonology, Ltd, Canberra, Australia). Black bands on chromosomes indicate markers that were polymorphic in both the diversity panel and set of standards, while red bands indicate new markers which are monomorphic in the set of standards and polymorphic in the diversity panel. Length of the chromosome is presented in centimorgans (cM).

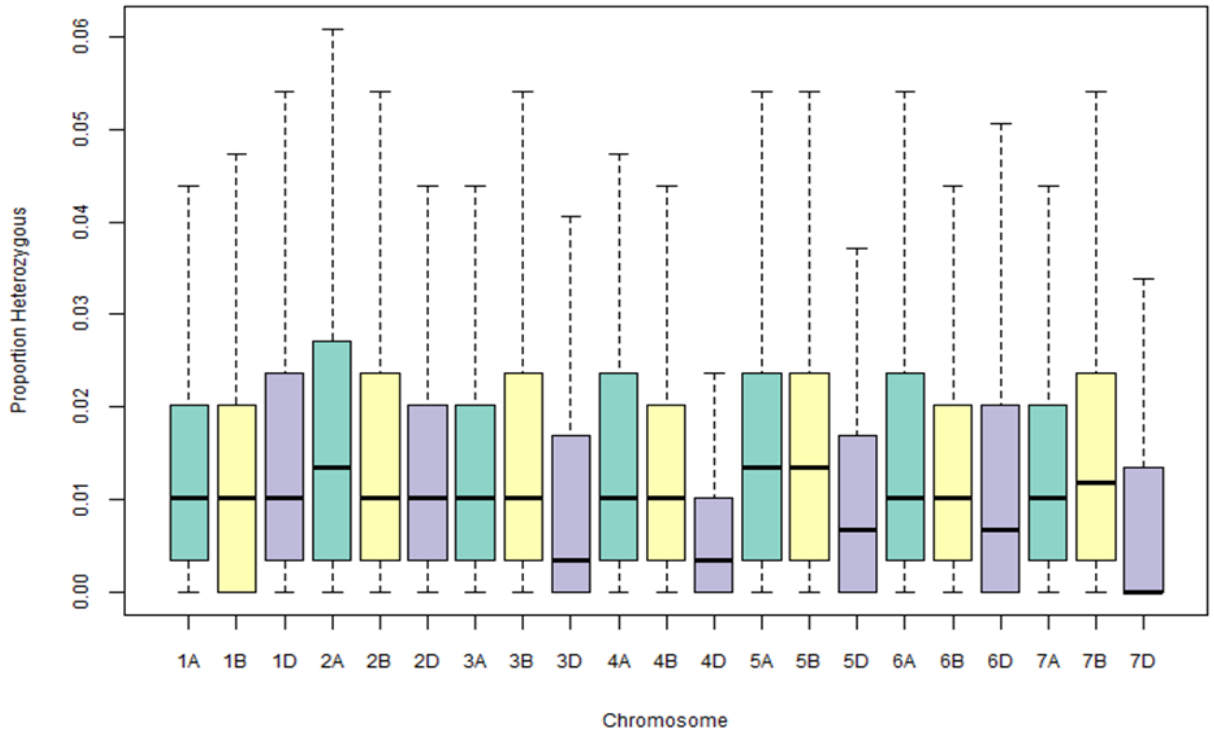


Figure 3.6 Box plot displaying the proportion of heterozygous SNP markers per chromosome in the diversity panel.

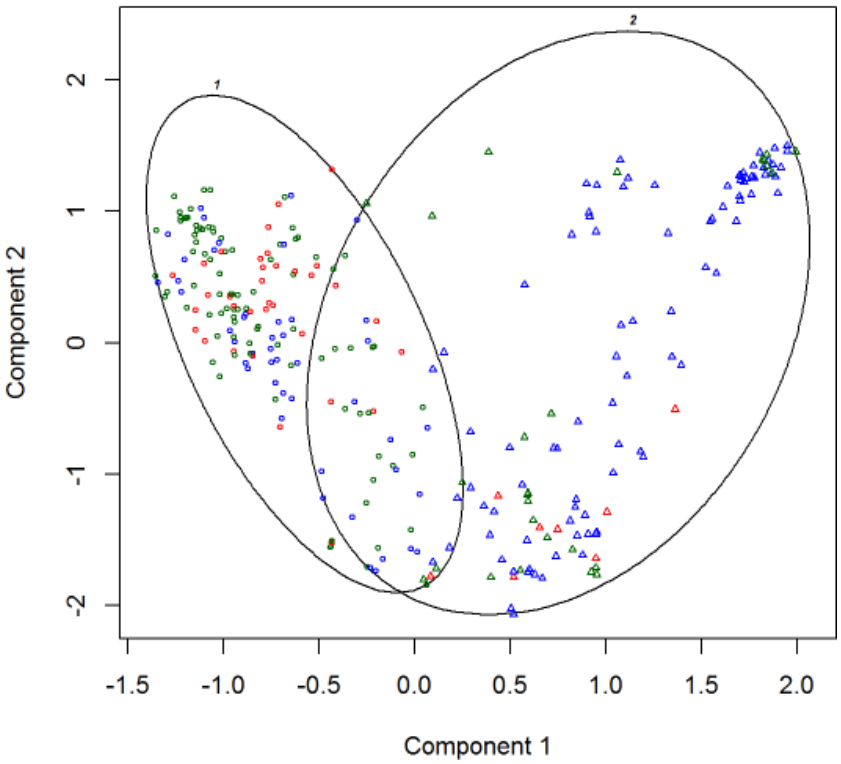
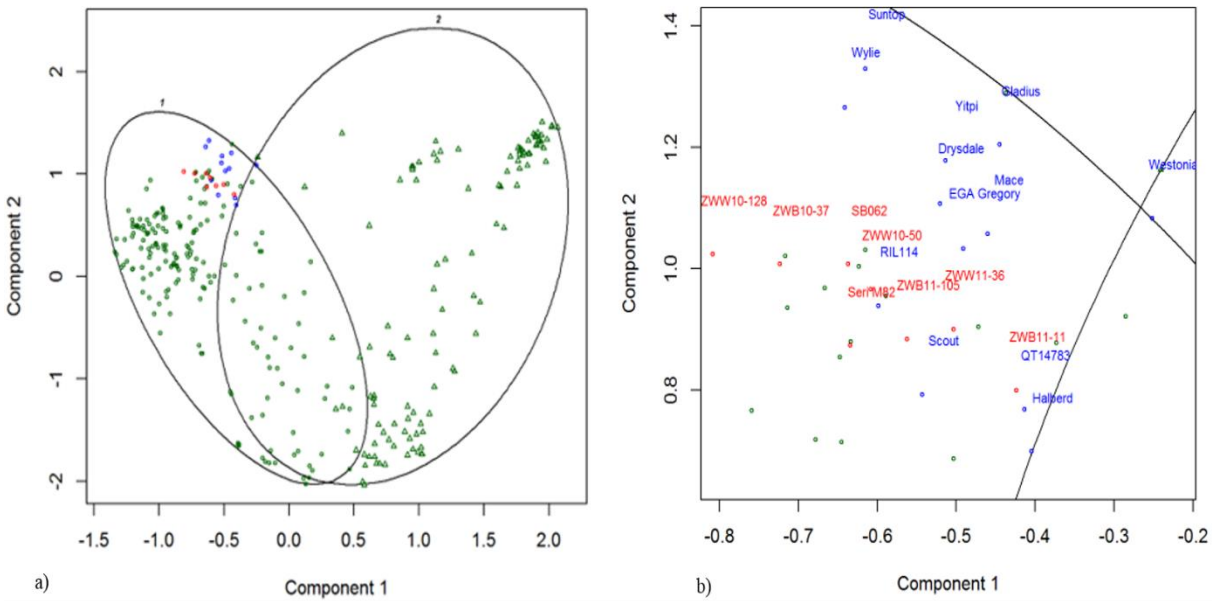


Figure 3.7 Biplot displaying results from cluster analysis of the 295 accessions in the diversity panel using the partitioning around medoids (PAM) algorithm. Members of cluster 1 denoted by circles and members of cluster 2 denoted by triangles. Colour coding of accessions is based on the following classifications: cultivars or breeding lines (red), landraces (blue) and 'unknown' (green).



1

2 **Figure 3.8 a)** Biplot displaying results from cluster analysis of the 295 accessions in the
3 diversity panel, plus the 20 standards from Australia and the International Maize and Wheat
4 Improvement Center (CIMMYT), using the partitioning around medoids (PAM) algorithm.
5 Members of cluster 1 denoted by circles and members of cluster 2 denoted by triangles.
6 Colour coding of accessions is based on the following classifications: diversity panel
7 accessions (green), Australian standards (blue) and CIMMYT standards (red). **b)** An
8 enlarged view of the 20 standards.

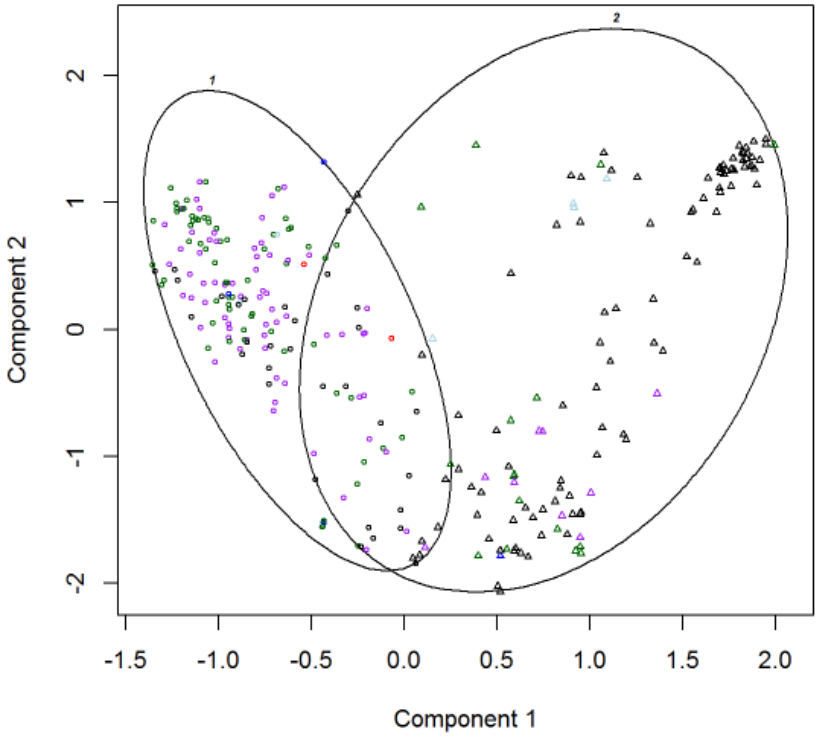


Figure 3.9 Biplot displaying results from cluster analysis of the 295 accessions in the diversity panel using the partitioning around medoids (PAM) algorithm. Members of cluster 1 denoted by circles and members of cluster 2 denoted by triangles. Accessions were colour coded according to geographic origin: Asia (black), Europe (purple), Africa (light blue), North America (dark blue), South America (red) and Unclassified (dark green).

3.8 Tables

Table 3.1 Pedigree information for the 20 standards from Australia and the International Maize and Wheat Improvement Center (CIMMYT).

Genotype	Status	Pedigree
Australia		
Drysdale	Cultivar	HARTOG*3/QUARRION
EGA Gregory	Cultivar	PELSART/2*BATAVIA
EGA Wylie	Cultivar	QT2327/COOK//QT2804
Gladius	Cultivar	RAC-875/KRICHAUFF//EXCALIBUR/KUKRI/3/RAC-875/KRICHAUFF/4/RAC-875//EXCALIBUR/KUKRI
Halberd	Cultivar	SCIMITAR/KENYA-C-6042//BOBIN/3/INSIGNIA-49
Mace	Cultivar	WYALKATCHEM/STYLET//WYALKATCHEM
QT14783	Breeding line	KENNEDY*2/QT8766
RIL114	Breeding line	UQ01484/RSY10//H45
Scout	Cultivar	SUNSTATE/QH71-6//YITPI
Suntop	Cultivar	SUNCO/2*PASTOR//SUN436E
Westonia	Cultivar	SPICA/TIMGALEN//TOSCA/3/CRANBROOK/BOBWHITE*2/JACUP
Yipti	Cultivar	C-8-MMC-8-HMM/FRAME
CIMMYT		
Seri M82	Breeding line	KAVKAZ/(SIB)BUHO//KALYANSONA/BLUEBIRD
SB062	Breeding line	SERI M82/BABAX

ZWB10–37	Breeding line	TACUPETO F2001/BRAMBLING//KIRITATI
ZWB11–11	Breeding line	ATTILA*2/PBW65*2/5/KAUZ//ALTAR 84/AOS/3/MILAN/KAUZ/4/HUITES
ZWB11–105	Breeding line	PFAU/SERI.1B//AMAD/3/WAXWING/4/BABAX/LR42//BABAX*2/3/KURUKU
ZWW10–50	Breeding line	ONIX/4/MILAN/KAUZ//PRINIA/3/BAV92
ZWW10–128	Breeding line	ESDA/KKTS
ZWW11–36	Breeding line	EGABONNIEROCK/4/MILAN/KAUZ//PRINIA/3/BAV92

Chapter 4 - A rapid phenotyping method for adult plant resistance to leaf rust in wheat

4.1 Abstract

The most sustainable method for controlling rust diseases is the deployment of cultivars incorporating adult plant resistance (APR). However, phenotyping breeding populations or germplasm collections for resistance in the field is dependent on weather conditions and limited to once a year. In this study, we explored the ability to phenotype APR to LR under accelerated growth conditions (AGC; i.e. constant light and controlled temperature) using a method that integrates assessment at both seedling and adult growth stages. A panel of 21 spring wheat genotypes, including disease standards carrying known APR genes (i.e. *Lr34* and *Lr46*), were characterised under AGC and in the field. Disease response displayed by adult wheat plants grown under AGC (i.e. flag-2 leaf) was highly correlated with field-based measures ($r = 0.83$, $P < 0.000$). The integrated method is more efficient - requiring less time, space, and labour compared to traditional approaches that perform seedling and adult plant assays separately. Further, this method enables up to seven consecutive adult plant LR assays compared to one in the field. The integrated seedling and adult plant phenotyping method reported in this study provides an excellent tool for identifying APR to LR. Assessing plants at early growth stages can enable selection for desirable gene combinations and crossing of the selected plants in the same plant generation. The method has the potential to be scaled-up for screening large numbers of fixed lines and segregating populations. This strategy would reduce the time required for moving APR genes into adapted germplasm or combining traits in top crosses in breeding programs. This method could accelerate selection for resistance factors effective across diverse climates by conducting successive cycles of screening performed at different temperature regimes.

4.2 Introduction

Wheat (*Triticum aestivum* L.) provides more than 20% of the calorific intake for almost two-thirds of the human population (Hawkesford et al. 2013). With an expected global population of 9–10 billion by the year 2050, world food security is paramount. *Puccinia triticina* Eriks., which causes leaf rust (LR), is regarded one of the most geographically widespread diseases of wheat and can incur yield losses ranging 10–70% (Samborski 1985). It results

in a reduction of kernels per head, lower kernel weight, degradation in grain quality and increased costs associated with chemical control (Everts et al. 2001; Bolton et al. 2008). In Australia, wheat diseases, including rusts, cause an estimated average annual loss of almost AUD 913 million to the wheat industry (Murray and Brennan 2009). Among the various control methods, the most profitable and sustainable disease minimization strategy is the deployment of genetically resistant cultivars (Pink 2002).

To date, research around the world has resulted in the designation of 77 genes for resistance to LR (i.e. *Lr*), which have been characterised and mapped to chromosomal locations (McIntosh et al. 2017). Genetic resistance is broadly classed into two forms: seedling and adult-plant resistance (APR). Seedling resistance, or 'all stage resistance' (R), is typically expressed at all growth stages, conferred by a single 'major effect' gene often associated with a hypersensitive response and is often race specific. On the other hand, APR is typically best expressed in adult plants and often polygenic in nature, controlled by multiple 'minor effect' genes that may influence factors such as pustule size, infection frequency, and latent period, thus commonly referred to as 'slow rusting' genes (Qi et al. 1998; Ellis et al. 2014). While APR is often non-race specific, there are exceptions where some genes provide race-specific resistance (e.g. *Lr13* and *Lr37*) (McIntosh et al. 2013; Ellis et al. 2014) and confer a hypersensitive response (e.g. *Lr48* and *Lr49*) (Bansal et al. 2008). Notably, some APR genes have been deployed for almost 100 years, such as *Sr2* and *Lr34*, which continue to provide resistance to stem rust (SR) and LR, respectively. Three well-characterized APR genes are now available to wheat breeders that appear to convey race-nonspecific resistance to LR (i.e. *Lr34*, *Lr46*, and *Lr67*), for which useful DNA markers are also available (Lagudah et al. 2006). However, additional sources of resistance are needed for stacking or pyramiding in new cultivars, which will serve to protect these highly valuable genes against the rapidly evolving nature of *P. triticina*.

APR to LR is typically identified by phenotyping wheat plants at the seedling stage in the glasshouse, then subsequently evaluating adult plants in the field (Ellis et al. 2014). However, the accuracy of phenotyping in the field can be compromised by environmental factors that influence the expression of APR, such as weather patterns, inoculum pressure, sequential infection, differences in plant maturity and the presence of other diseases (Hickey et al. 2012). Further, expression of LR resistance in wheat is sensitive to temperature (Kaul and Shaner 1989), resulting in variability across environments or years of testing (Risk et al. 2012). Some studies have successfully evaluated APR to foliar pathogens in cereals grown

under glasshouse or controlled environmental conditions (CEC) (Hickey et al. 2012; Singh et al. 2013). A key advantage is that environmental factors, such as temperature and light, can be controlled. Artificial lighting can also be used to impose an extended photoperiod or constant light to accelerate the growth of wheat plants. A plant management system providing accelerated growth conditions (AGC) could be used to speed up disease screening and plant selection.

In this study, we investigated the ability to rapidly phenotype APR to LR in wheat grown under AGC (i.e. constant light and controlled temperature). Using a panel of 21 spring wheat genotypes we compared LR response displayed by adult plants grown under AGC to levels displayed by adult plants grown in the field. We discuss opportunities to exploit this rapid phenotyping method to accelerate research and wheat breeding efforts to develop rust resistant wheat cultivars.

4.3 Material and methods

4.3.1 Plant materials

A panel comprising 21 spring wheat genotypes (Table 4.1) was used to generate a protocol for phenotyping resistance to LR in wheat grown under AGC. The panel comprised a selection of standards, cultivars and breeding lines from Australia, the International Center for Agriculture Research in the Dry Areas (ICARDA), and the International Maize and Wheat Improvement Center (CIMMYT).

4.3.2 Rust screening: seedling stage

The panel was evaluated for resistance to LR at the seedling stage in a glasshouse at The University of Queensland, St Lucia, Queensland, Australia. Seeds were imbibed for 24 h at room temperature and were placed in a refrigerator (4˚C) for 48 h to encourage synchronous germination across genotypes. Germinated seeds were transplanted into 140 mm ANOVApot® (Anovapot Pty Ltd, Australia, www.anovapot.com) pots filled with a potting media consisting of composted pine bark fines (0–5 mm) (70%) and coco peat (30%) with a pH ranging 5.5–6.5. Slow release Osmocote® fertilizer (NPK 13.4 : 1.5 : 4.9, Scotts Australia Pty Ltd, NSW, Australia) was applied at a rate of 2 g per pot. Each pot contained four

different positions (i.e. positions 1–4 clockwise from the pot tag), where each position contained four germinated seeds of the same genotype clumped together. Each genotype was replicated three times in a completely randomized design. Plants were grown at a temperature regime of 22/17°C (day/night) and a natural 12 h diurnal photoperiod. After 10 days, (i.e. two-leaf stage) plants were inoculated with *P. triticina* pathotype *pt* 104–1,2,3,(6),(7),11,13. This pathotype evolved from pathotype *pt* 104–1,2,3,(6),(7),11 via a single step mutation on wheat carrying the resistance gene *Lr24* and was first reported in Australia in 2000 (Park et al. 2002). It currently occurs in wheat production regions throughout the east coast of Australia. The rust isolate used in this study was developed using a single spore culture technique and spores increased using susceptible wheat cultivar Morocco. The inoculum was prepared by suspending urediniospores in light mineral oil (Isopar 6) at a rate of 0.005g per ml. Inoculum at the concentration of 6×10^5 spores/ml was applied to the leaves of wheat plants using an air brush (IWATA power jet lite). Plants were then lightly misted with deionized water and placed in a dew chamber maintained at 100% humidity using an ultrasonic fogger. After 18 h of incubation, plants were removed from the dew chamber and returned to the glasshouse for subsequent disease development. Twelve days post-inoculation seedlings were assessed for infection type (IT) using the 0–4 Stakman scale (Stakman et al. 1962). Genotypes that displayed an IT of <3 were considered resistant.

4.3.3 Rust screening: adult plant stage

In total, three adult plant experiments were conducted using the panel. Two phenotyping experiments, namely, “adult plant integrated” and “adult plant independent” were conducted under AGC, while phenotyping in the field was conducted in a disease screening nursery.

4.3.4 Adult plant experiment 1: integrated method under AGC

Following assessment of disease response at the seedling stage (as describe above), the plants were transferred to a fully-enclosed temperature controlled growth facility (dimensions 5m × 6m). The growth facility is fitted with 20 low-pressure sodium vapor lamps (400 watt each) generating 400–550 µmol $M^{-2}S^{-1}$ photosynthetically active radiation (PAR) at pot height and 900 µmol $M^{-2}S^{-1}$ at adult plant height (i.e. about 45 cm above pot level). AGC was achieved by adopting constant (i.e. 24 h) light (Hickey et al. 2009) and a 12 h cycling temperature regime of 22/17°C. Pots were positioned on a bench according to a completely

randomized design in a stainless steel tray (240 × 90 × 10 cm). Plants were grown for 2 weeks under AGC, and then re-inoculated with a suspension of *P. triticina* urediniospores (*pt* 104–1,2,3,(6),(7),11,13), as described above. Prior to inoculation, the developmental growth stage (GS) was recorded for each plant using the Zadoks decimal code scoring system (Zadoks et al. 1974). Twelve days post-inoculation IT was recorded for different leaves (i.e. flag, flag-1, and flag-2) on the primary/main tiller of each plant using the 0–4 Stakman scale (Stakman et al. 1962). Genotypes displaying an IT of <3 were considered resistant.

4.3.5 Adult plant experiment 2: independent method under AGC

As a control, a new batch of plants were sown for the panel and grown from day one under AGC. Environmental conditions and experimental design was consistent with adult plant experiment 1 (above). Three weeks after sowing, the majority of genotypes achieved the adult plant stage and were inoculated with *pt* 104–1,2,3,(6),(7),11,13, as outlined above. Prior to inoculation, the GS for all plants was recorded using the Zadoks scale. Twelve days later, plants were assessed for IT using the Stakman scale.

4.3.6 Adult plant experiment 3: in the field

The panel of wheat genotypes was evaluated for response to LR in the field at Redlands Research Facility, Queensland, Australia, from July to October 2014. Six seeds of each genotype was sown as un-replicated hill plots. The susceptible genotype Morocco was used as a disease spreader in the field nursery, where two rows of Morocco were sown between each bay compromising two rows of hill plots. LR epidemics were initiated by transplanting Morocco seedlings infected with *pt* 104–1,2,3,(6),(7),11,13 (as outlined above) into the field among the spreader rows about 5 weeks after sowing. The LR epidemic was promoted with sprinkler irrigation applied in the late evenings when temperatures were favorable for infection and high humidity and low winds at night were expected. Once the epidemic had sufficiently developed on LR standards to allow a clear differentiation between susceptible and resistant genotypes, disease response was assessed on a whole plot basis using the modified Cobb scale (Peterson et al. 1948). Multiple disease assessments were conducted from late tillering/stem elongation to early grain filling (i.e. 70, 77, 86, and 96 days after sowing; DAS). Host response and disease severity data were used to calculate the

coefficient of infection (CI), as per Loegring (1959). Genotypes that displayed a LR response from resistant (R) to moderately resistant-moderately susceptible (MRMS) were considered resistant.

4.3.7 Statistical analysis

For experiments performed under controlled conditions, LR response was evaluated using the 0–4 Stakman scale, which encompasses both numbers (e.g. 0, 1…4) and symbols (e.g. ;, +). This data was converted to the 0–9 scale, where 0 = immune and 9 = very susceptible, using a conversion table (Ziems et al. 2014). The IT were converted as follows: 0;, ;n, ;, 1−, 1, 1+, 2−, 2, 2+, 2++, 3−, 3, 3+, 3++ and 4 were coded as 0, 0.5, 1, 2.5, 3, 3.5, 4, 5, 6, 6.5, 7, 8, 8.5, and 9, respectively. For heterogeneous ITs, each score was converted individually to the 0–9 scale and the average calculated. The converted datasets were then used for further statistical analysis.

Data analysis was performed using GenStat 17.1 2000–2015 VSN International Ltd. Analysis of variance (ANOVA) was performed by fitting a linear model using the converted data was used for experiments including; seedling, adult plant integrated and adult plant independent. Mean disease response and standard error means (SEM) for each genotype were calculated for comparison of disease reactions.

Correlation analyses were performed to investigate the correlation between phenotypes observed for the different experiments and to determine which leaf (i.e. flag, flag-1, and flag-2) under AGC provided the best estimate for LR response in the field for each disease assessment (i.e. 70, 77, 86, and 96 DAS). For the field dataset, CI values obtained from the un-replicated hill plots were used for correlation analyses. The CI values were divided by 10 to convert to the 0–9 scale. The converted scores were used in the comparison of mean LR response and principal component analysis (PCA). To investigate trends in disease response displayed by genotypes across multiple experiments, a PCA was performed and results visualized in the form of a biplot using GenStat (17.1 2000–2015; VSN International Ltd.) (GenStat.co.uk). This was performed using the following phenotype datasets: 1) seedling, 2) adult plant integrated, 3) adult plant independent, and 4) adult plant in the field (i.e. fourth assessment at 96 DAS). The disease response for flag-2 was used for both adult plant experiments conducted under AGC. Heritability (H^2) for the adult plant

integrated and adult plant independent experiment was also conducted using flag-2 disease response. Variance component was measured via residual maximum likelihood (REML) algorithm and the best linear unbiased predictions (BLUPs) were formed for the random genotype effects. Data were analysed with ASReml-R (Butler et al. 2009).

4.4 Results

4.4.1 Rust screening: seedling stage

Of the 21 spring wheat genotypes in the panel, 8 displayed susceptibility, while 13 displayed resistance to *P. triticina* pathotype 104–1,2,3,(6),(7),11,13 at the seedling growth stage (Figure 4.1). Thatcher, Avocet, Avocet+*Lr34*, Avocet+*Lr46*, Dharwar dry, Drysdale, Lang, and Janz displayed susceptibility with characteristic symptoms of large uredia without chlorosis (i.e. mean disease responses ranging 7–9; Figure 4.1). The susceptible standard, Thatcher, lacks effective LR resistance genes and displayed a mean disease response of 9.0. Notably, Avocet carries a race specific APR gene *Lr13* (Singh and Park 2008) and displayed seedling susceptibility (9.0; Figure 4.1). The Indian cultivar Dharwar dry, previously uncharacterized for LR resistance genes, also displayed susceptibility (8.0). Drysdale carries *Lr1* (Table 4.1), which is ineffective against the pathotype used in this study (Wellings et al. 2012) and displayed a susceptible response (8.0; Figure 4.1). Janz and Lang displayed susceptibility at the seedling stage (i.e. 8.0; Figure 4.1); both genotypes carry *Lr24* and *Lr34* (Table 4.1). The seedling gene *Lr24* is ineffective against *pt* 104-1,2,3,(6),(7),11,13 (Park et al. 2002), whereas *Lr34* is an APR gene and best expressed at adult plant growth stages (Lagudah et al. 2006). Based on the Stakman scale, the IT of seedling susceptible genotypes range from 3–4 (Supplementary Table 2).

EGA Gregory carries *Lr1*, *Lr3a*, *Lr13*, *Lr23*, and *Lr34* (Table 4.1) and displayed a moderately resistant (MR) response (2.3; Figure 4.1). The seedling resistance displayed by EGA Gregory was likely due to *Lr13*, as both *Lr1, Lr3a*, and *Lr23* are ineffective against the pathotype. The MR response displayed by Mace (1.5; Figure 4.1) was also likely due to *Lr13* and *Lr37* (Table 4.1)*. Lr13* and *Lr37* are APR genes and are effective against the pathotype used in this study (Table 4.1). Previous studies have reported early expression of *Lr13* at the seedling stage (Pretorius et al. 1984). Scout carries *Lr1* and *Lr37* (Table 4.1), where *Lr1* is ineffective against this pathotype, while *Lr37* is effective. Scout displayed a MR response

(1.5) at the seedling stage, which could be due to an uncharacterized seedling resistance or early expression of *Lr37* at the seedling stage (Figure 4.1) (Kloppers and Pretorius 1994). EGA Wyile carries *Lr17a* and *Lr34* (Table 4.1) and displayed a MR response (2.2; Figure 1), as the pathotype used in this study is avirulent on *Lr17a*. SeriM82 and Zebu carry *Lr26* and both displayed a highly resistant response (1.5 and 0.0, respectively; Table 4.1 and Figure 4.1). The previously, uncharacterized ICARDA line (FAC10-16-1) displayed a MR response (2.1; Figure 4.1). Other genotypes previously uncharacterised for LR resistance genes, including RIL114, Suntop, SB062, ZWB10-37, and ZWW10-128 depicted high levels of resistance with mean disease response ranging 0-1.5 (Figure 4.1). Based on the Stakman scale, the IT of the seedling resistant genotypes ranged from 0; to 12+ (Supplementary Table 2).

4.4.2 Rust screening: adult stage under AGC

In both adult plant experiments performed under AGC (i.e. integrated and independent), 20 of the 21 genotypes in the panel displayed varying levels of resistance (Figure 4.1). In both experiments, Thatcher displayed a very susceptible (VS; 9.0) response with urediniospores freely sporulating on leaves (Figure 4.1). Avocet displayed a resistant-moderately resistant (RMR) response with a mean disease response ranging 3–4 (Figure 4.1). As mentioned earlier, Avocet carries race specific APR gene *Lr13*, which is effective against the pathotype used in this study. In the Avocet background, resistance to LR was slightly enhanced with the addition of *Lr34* and *Lr46* (i.e. *Avocet+Lr34* and *Avocet+Lr46*), which are considered multi-resistance APR genes (Figure 4.1). Avocet+*Lr34* displayed a RMR response with mean disease response ranging 2.8–3.0 and Avocet+*Lr46* displayed a MR response, ranging 4.4–5.3 in the adult plant independent and integrated experiments, respectively. On the Stakman scale, the IT displayed by Avocet+*Lr34* and Avocet+*Lr46* ranged ;n12- (independent) to 12- (integrated), where pustules were smaller in comparison to Avocet and some necrosis in case of *Lr34* (Supplementary Table 2). The Indian cultivar Dharwar dry displayed a resistant response in both AGC experiments (Figure 4.1). Dharwar dry has not been previously characterized for rust resistance genes, thus the underlying genes are unknown. Drysdale carries *Lr1* along with race specific APR *Lr13* and displayed resistance (Table 4.1 and Figure 4.1). Both Janz and Lang carry *Lr24* and *Lr34* in combination (Table 4.1) however *Lr24* was not effective against the pathotype used in this study. These genotypes displayed a MRMS response, likely due to expression of APR gene *Lr34* (Figure 4.1). The mean disease response for Janz and Lang was 3.3 and 5.5 in adult plant integrated

experiment, respectively, and displayed similar responses in the adult plant independent experiment (i.e. 5.3 and 5.2, respectively; Figure 4.1). EGA Gregory (1.7) and Mace (1.5) displayed a resistant response in both AGC experiments (Figure 4.1). EGA Gregory carries *Lr1*, *Lr3a*, *Lr13*, *Lr23,* and *Lr34* and Mace carries *Lr1*, *Lr23*, and *Lr37* (Table 4.1). The *P. triticina pt* 104-1,2,3,(6),(7),11,13 is virulent on both *Lr1, Lr3a*, and *Lr23*, but avirulent on APR genes *Lr13*, *Lr34*, and *Lr37*. Thus, resistance displayed at adult growth stages by EGA Gregory and Mace is likely a combination of these genes. Scout displayed resistance (1.5) (Figure 4.1), most likely attributable to *Lr37* (Table 4.1). EGA Wylie displayed a highly resistant (HR) response in the integrated (1.8) and independent (0.5) AGC experiments (Figure 4.1). This was most likely a result of the combined effect of seedling gene *Lr17a* and APR gene *Lr34* (Table 4.1). SeriM82 depicted a HR response in AGC experiments (Figure 4.1), most likely due to the presence of seedling gene *Lr26* (Table 4.1). Genotypes previously uncharacterised for LR resistance genes (including SB062, RIL114, Suntop, Zebu, ZWW10-50, ZWW10-37, ZWW10-128 and FAC10-16-1) displayed high levels of resistance in AGC experiments (Figure 4.1), indicating effective resistance to the pathotype used in this study. Overall, comparison of datasets from the integrated and independent experiments performed under AGC revealed only minor differences in infection and response types displayed by the panel of genotypes. Genotypes either displayed the same response or it varied within only one response type across both experiments. For instance, Drysdale displayed a RMR response in the independent experiment, but displayed R response in the integrated experiment (Figure 4.1). The GS of plants evaluated under AGC ranged between GS25–45 and GS23–43 (i.e. tillering to booting stage) for the integrated and independent experiments, respectively (Table 4.2).

4.4.3 Rust screening: in the field

All genotypes in the panel displayed varying levels of resistance to LR, with the exception of Thatcher, which consistently displayed a susceptible response (60 S). Avocet displayed a MRR response for the first three disease assessments; however on the fourth assessment, Avocet displayed a 50 MRMS response (Supplementary Table 2). In the Avocet background, the APR gene *Lr34* (i.e. Avocet+*Lr34*) displayed a 20 MRR response, while Avocet+*Lr46* displayed a MRMS response (40 MRMS). Dharwar dry displayed a MRMS response (30 MRMS), likely due to the presence of uncharacterised APR gene(s) (Supplementary Table 2). Drysdale displayed a MRR response in the field, likely due to race specific APR *Lr13* (50 MRR). Janz carries *Lr24* and *Lr34* in combination and displayed the MRMS response (30

MRMS). As the pathotype used in this study is virulent on *Lr24*, the resistance displayed by Janz is likely due to *Lr34*. CIMMYT lines (ZWW10-128 and SB062) both displayed a MRR response in the first three disease assessments, however, on the fourth assessment, each was considered MRMS (30 MRMS). ICARDA breeding line FAC10-16-1 was considered RMR (30 RMR) in the field. Other genotypes, such as EGA Gregory, EGA Wyile, Mace, Scout, RIL114, Suntop, Zebu, ZWW10-50, and ZWW10-37, displayed high levels of resistance (i.e. MRR) in the field with mean disease response ranging 30–40 MRR. Lang failed to germinate in the field. The detailed host response and disease severity data is provided in Supplementary Table 2.

4.4.4 Adult plant assessment under AGC is predictive of field response

Based on correlation analyses, the LR response for different leaves showed very good correspondence across the two adult plant AGC experiments: r = 0.85, (P < 0.000) (flag), 0.88 (P < 0.000) (flag-1), and 0.97 (P < 0.000) (flag-2). Despite all leaves showing good correspondence, the flag-2 leaf was considered to provide the most consistent LR response across AGC experiments. Correlation analysis was also performed using data from the adult plant integrated AGC experiment and the field. The highest correlation was found for the response displayed by the flag-2 leaf versus the fourth (final) disease assessment in the field (r=0.83, P < 0.000; Table 4.3). Correlations (r) for the other leaves (flag and flag-1) corresponding with the four disease assessments ranged between 0.45–0.46 and 0.71–0.73, respectively (Table 4.3).

Results from PCA displayed in the biplot (Figure 4.2) revealed a high correlation between both adult plant experiments conducted under AGC, where the adult plant integrated experiment appeared to be slightly more correlated to the field disease response. The field response was moderately correlated with the adult plant independent experiments performed under AGC (Figure 4.2). Notably, only a weak correlation was observed between field and seedling response (Figure 4.2). High heritabilities were observed for the adult plant integrated experiment (H^2=0.88) while for the adult plant independent experiment (H^2=0.90).

4.5 Discussion

This study presents a new method that permits rapid phenotyping for APR to LR in wheat by exploiting AGC to speed up plant development and involves two sequential inoculations to detect APR. Characterisation of a panel of 21 wheat genotypes revealed that the LR response displayed under AGC was indicative of levels expressed by adult plants grown in the field. Phenotyping for APR to LR can be completed within just seven weeks and performed all-year-round, thus provides a useful tool to accelerate breeding and research aiming to develop rust resistant cultivars.

4.5.1 Detection of APR to LR under AGC

Of the 21 spring wheat genotypes evaluated, 7 were determined to carry APR to LR, including; Avocet, Avocet+*Lr34*, Avocet+*Lr46*, Janz, Lang, Drysdale, and Dharwar dry. These genotypes were considered susceptible in the seedling experiment but displayed resistance in adult plant experiments. Genotypes known to carry APR genes, in particular, *Lr13*, *Lr34*, and *Lr46*, consistently displayed resistance at the adult plant stage under AGC - similar to levels displayed in the field. For instance, both Janz and Lang carry seedling gene *Lr24* and APR gene *Lr34* in combination; however *Lr24* is not effective against the pathotype used in this study. Therefore, these genotypes displayed a susceptible response in the seedling experiment, but a MRMS response under AGC at the adult plant stage, likely due to expression of *Lr34*. In some genotypes, the expression of *Lr34* was likely masked by the presence of effective seedling resistance genes, such as *Lr13* in EGA Gregory and *Lr17a* in EGA Wylie. Another good example of APR expression under AGC was observed for Avocet and the Avocet near-isogenic lines for *Lr34* (i.e. Avocet+*Lr34*) and *Lr46* (i.e. Avocet+*Lr46*). Notably, Avocet carries race specific APR gene *Lr13*, which is effective against the pathotype used in this study. The RMR response displayed by Avocet indicated that *Lr13* was successfully detected in the adult plant AGC experiments. In the Avocet background (*Lr13*), the addition of *Lr34* and *Lr46* enhanced the levels of resistance displayed in the adult plant experiments. This indicates the additive effect of APR genes can be detected under AGC. However, to detect the effectivity of the APR against different races the developed method can also be applied by conducting multiple screens using different pathotypes.

4.5.2 Disease response under AGC is related to field-based measures

The GS of plants evaluated under AGC ranged between tillering to booting stage at the time of inoculation with *P. triticina* and plants displayed adult plant phenotypes. This aligns well with previous studies on wheat that report the early expression of APR to stripe rust at mid-tillering growth stages in the field (Park and Rees 1987) and at the stem elongation stage in plants grown under controlled environment (Hickey et al. 2012). Correlation analyses for the panel revealed that the flag-2 leaf expressed levels of APR most similar to those observed in the field. The upper-most infected leaf (i.e. flag leaf) displayed increased susceptibility to the pathogen in comparison to lower leaves. Thus, it appears APR is best expressed in 'older' leaves (that are more aged) compared to 'younger' leaves.

In the field, the inoculum pressure fluctuates due to infection cycles of rust urediniospores and weather conditions. One of the advantages of phenotyping under AGC is the application of inoculum can be controlled. It might be expected that the inoculum concentration applied under AGC using a single inoculation would correlate better with disease assessment performed early in the season (i.e. low disease pressure) as opposed to late in the season (i.e. high disease pressure). However, our results under AGC correlated well with measurements early in the season (i.e. 70 DAS) and late in the season (i.e. 96 DAS). It is feasible that phenotyping based on IT on a single leaf using a single controlled inoculation is indicative of factors important for reducing overall disease severity in the field under polycyclic conditions; such as pustule size and infection frequency.

4.5.3 Importance of temperature and light to detect APR under AGC

AGC involves constant light and temperature regimes during the early plant growth phase to achieve adult plant stage rapidly. However, to assist a successful infection, diurnal light and temperature regime was implemented post-inoculation until disease assessment. Post-inoculation conditions are important for a successful host-pathogen interaction and become more important when plants are raised and inoculated in an artificial environment, such as the AGC adopted in this study. As discussed above, plant growth stage, along with temperature and light (i.e. quantity and quality) are considered key factors determining disease development (Hickey et al. 2012).

All known *Lr* genes are sensitive to fluctuating post-inoculation temperatures, for instance, expression of *Lr13* at the adult growth stage (Kaul and Shaner 1989). In the present study, plants were grown under a 12 h cycling temperature regime of 22/17°C. This temperature enabled rapid plant growth, and importantly, provided healthy plants prior to inoculation. Notably, this falls within the optimal temperature range for LR development (i.e. 10–25°C) (Dyck and Johnson 1983). Under AGC, a warmer growing temperature (e.g. >24°C) can compromise plant health, which is critical if plants are to be subjected to disease assays. The increase or decrease in temperature can also influence latent period (Eversmeyer et al. 1980; Kaul and Shaner 1989). The fluctuations in the latent period are critical in wheat rust infections, and AGC could serve as a tool to study the latent period under different temperature regimes.

Light is another key component of the rapid phenotyping method, where it not only affects plant photosynthetic activity but also plays a role in disease development. Under AGC, wheat plants were grown under constant (24 h) light to quickly obtain adult plants. The importance of light influencing disease development both pre- and post-inoculation has been previously reported for both LR and stripe rust in wheat (de Vallavieille-Pope et al. 2002). We employed a diurnal (12 h) photoperiod post-inoculation until disease assessment. High-quality light is important for disease development, particularly for good sporulation (Roelfs et al. 1992). In addition, the diurnal light appears to be important, as constant (24 h) light can impede pathogen development, thus reducing the ability to differentiate between resistant and susceptible genotypes (unpublished data).

4.6 Conclusion

Breeding for rust resistance requires a continuous effort to stay ahead of the rapidly evolving pathogen. This requires robust phenotypic screening and ongoing deployment of new resistance genes. The method reported in this study provides a great tool for detecting APR to LR at levels similar to those observed in the field. It can be scaled-up for screening large numbers of fixed lines and segregating populations, similar to that reported for stripe rust in wheat (Hickey et al. 2012). Using this technique, it is possible to conduct up to seven consecutive screens annually, compared to just one in the field. It is possible to phenotype APR prior to anthesis under AGC, as genotypes inoculated at or beyond GS30 display resistance representative of adult plants. Assessing plants at early growth stages can enable selection of desirable gene combinations for APR and crossing of the selected plants in the

same plant generation. This strategy would reduce the time required for moving APR genes into adapted germplasm (from donor sources) or combining traits in top crosses in breeding programs.

4.7 References

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4.8 Figures

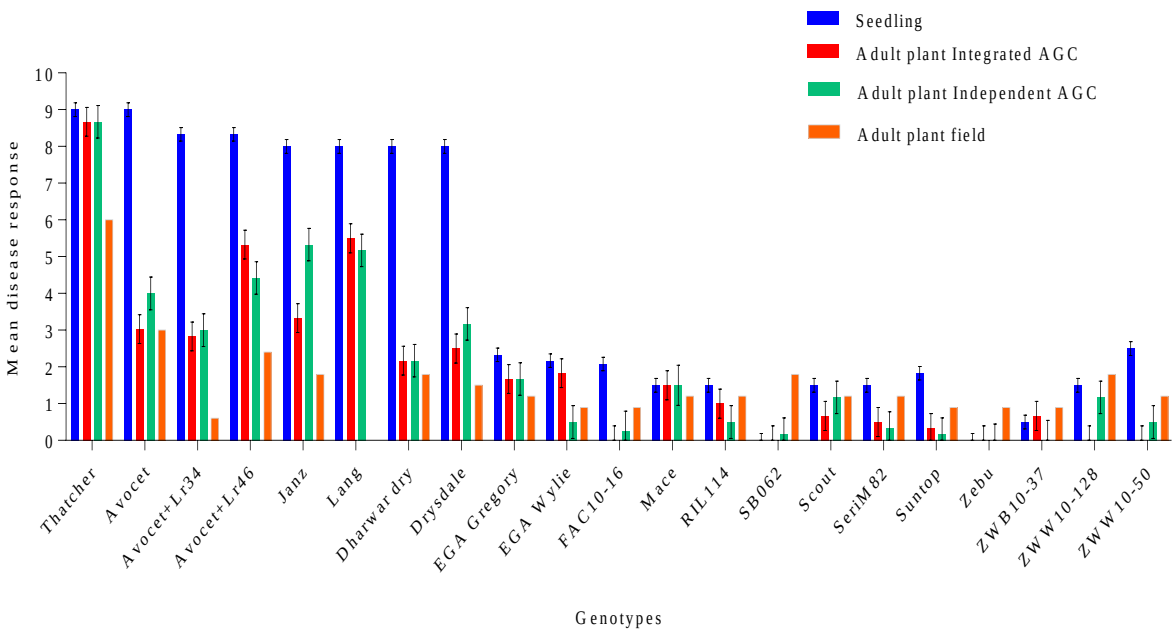
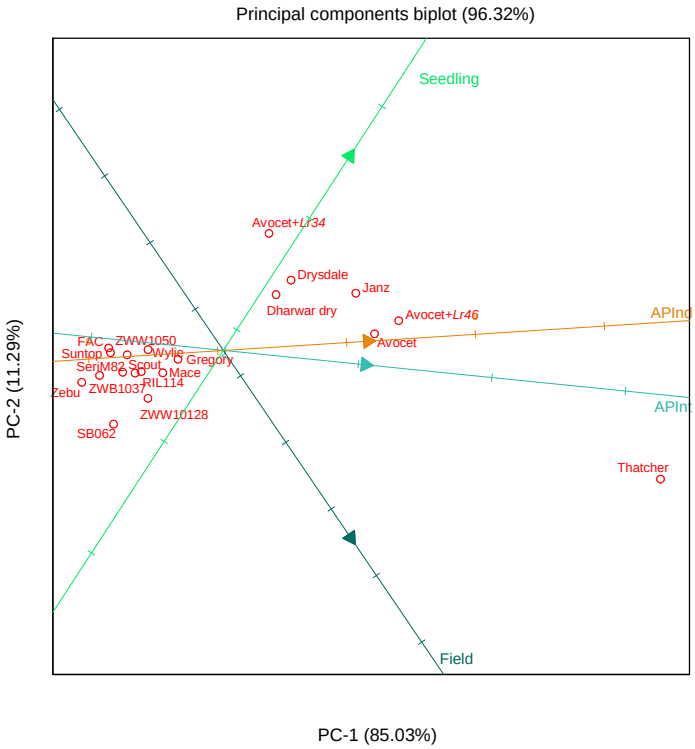


Figure 4.1 Mean leaf rust response and standard error means for the panel of 21 spring wheat genotypes evaluated in the following experiments: seedling (standard glasshouse), adult plant integrated and adult plant independent under accelerated growth conditions (AGC), and in the field. The disease response for the seedling and adult plant AGC experiments was collected using the 0–4 scale and converted to the 0–9 scale (displayed). Whereas, the disease response in the field was collected using the modified Cobb scale, which was used to calculate the coefficient of infection, and was converted to the 0–9 scale (displayed).



1

2 **Figure 4.2** Biplot displaying results from principal component analysis using leaf rust
3 response obtained in the following experiments: seedling (standard glasshouse), adult plant
4 integrated (APInt) under accelerated growth conditions (flag-2 leaf), adult plant independent
5 (APInd) under accelerated growth conditions (flag-2 leaf) and in the field (96 days after
6 sowing). The displayed principal components (i.e. PC1 and PC2) account for 96.32% of the
7 variation.

4.9 Tables

Table 4.1 Name, pedigree, breeding program and leaf rust resistance genes present in 21 spring wheat genotypes.

Genotypes	Pedigree	Type	Resistance genes		Breeding program	Source[1]
			Seedling	APR		
Thatcher	MARQUIS/IUMILLO DURUM//MARQUIS/KANRED	Cultivar	-[2]	-	North America	Hayes et al. (1936)
Avocet	THATCHER- *AGROPYRON ELONGATUM* TRANSLOCATION/3* PINNACLE//WW15/3/EGRET	Cultivar	-	*Lr13*	Australia	Fitzsimmons et al. (1983)
Avocet+*Lr34*	Avocet NIL[3] *LR34*	NIL	-	*Lr34*	-	Lillemo et al. (2007)
Avocet+*Lr46*	Avocet NIL *LR46*	NIL	-	*Lr46*	-	Lillemo et al. (2007)
Dharwar Dry	DWR39/C306//HD2189	Cultivar	-	-	India	-
Drysdale	HARTOG*3/QUARRION	Cultivar	*Lr1*	*Lr13*	Australia	Wellings et al. (2012)
Janz	3AG3/4*CONDOR//COOK	Cultivar	*Lr24*	*Lr34*	Australia	Wellings et al. (2012)
Lang	QT3765/SUNCO	Cultivar	*Lr24*	*Lr34*	Australia	Wellings et al. (2012)
EGA Gregory	PELSART/2*BATAVIA	Cultivar	*Lr1, Lr3a, Lr23*	*Lr13, Lr34*	Australia	Wellings et al. (2012)
EGA Wylie	QT2327/COOK//QT2804	Cultivar	*Lr17a*	*Lr34*	Australia	Wellings et al. (2012)
FAC10-16-1	10CB-F/W234	Breeding line	-	-	ICARDA	-

Mace	WYALKATCHEM/STYLET//WYALKATCHEM	Cultivar	*Lr23*	*Lr13, Lr37*	Australia	Wellings et al. (2012)
RIL114	UQ01484/RSY10//H45	Breeding line	-	-	Australia	-
SB062	SERI M82/BABAX	Breeding line	-	-	Australia	-
Scout	SUNSTATE/QH71-6//YITPI	Cultivar	*Lr1*	*Lr37*	Australia	Wellings et al. (2012)
Suntop	SUNCO/2*PASTOR//SUN436E	Cultivar	-	-	Australia	-
SeriM82	KAVKAZ/(SIB)BUHO//KALYANSONA/BLUEBIRD	Breeding line	*Lr23, Lr26*	-	CIMMYT	-
Zebu	-	Cultivar	*Lr26*	-	CIMMYT	Wellings et al. (2012)
ZWB10-37	TACUPETOF2001/BRAMBLING//KIRITATI	Breeding line	-	-	CIMMYT	-
ZWW10-128	ESDA/KKTS	Breeding line	-	-	CIMMYT	-
ZWW10-50	ONIX/4/MILAN/KAUZ//PRINIA/3/BAV92	Breeding line	-	-	CIMMYT	-

[1] Study reporting the status of leaf rust resistance genes.

[2] A dash (-) indicates data is unavailable or unknown.

[3] Near isogenic lines

Table 4.2 Zadoks growth stages for 21 spring wheat genotypes at inoculation under accelerated growth conditions.

Genotypes	Growth stage at inoculation	
	Adult plant integrated	Adult plant independent
Thatcher	31	37
Avocet	33	43
Avocet+*Lr34*	34	41
Avocet+*Lr46*	39	41
Dharwar dry	37	31
Drysdale	37	25
Janz	32	31
Lang	31	31
EGA Gregory	30	25
EGA Wylie	32	25
FAC10-16-1	33	25
Mace	30	25
RIL114	45	41
SB062	32	26
Scout	37	25
SeriM82	33	37
Suntop	39	37
Zebu	28	26
ZWB10-37	30	31
ZWW10-50	37	26
ZWW10-128	37	26

Table 4.3 Results from correlation analysis (r values) for 21 spring wheat genotypes evaluated for leaf rust response in the adult plant integrated experiment versus the field. Correlation analysis was performed for the disease response displayed by each leaf under accelerated growth conditions (i.e. Flag, Flag-1, and Flag-2) in comparison to the field response observed for each of the four assessment dates (i.e. 70, 77, 86 and 96 days after sowing, DAS).

Leaf number	Number of observations (n)	Days after sowing (DAS)			
		70	77	86	96
Flag	15	0.46*	0.05	0.31	0.45·
Flag-1	19	0.73***	0.52**	0.63**	0.71***
Flag-2	19	0.80***	0.60**	0.75***	0.83***

Level of significance (*P-value*): 0.000 '***', 0.001 '**', 0.01 '*', 0.05 '·', 0.1 ' '.

Chapter 5 - Mining Vavilov's treasure chest of wheat diversity for adult plant resistance to *Puccinia triticina*

5.1 Abstract

In the search for new sources of adult plant resistance (APR) to leaf rust (LR) caused by *Puccinia triticina*, here we explored a diversity panel sourced from the N. I. Vavilov Institute of Plant Genetic Resources (VIR). Based on DNA marker screening, 83 of the 300 lines were deemed to carry known APR genes, namely *Lr34*, *Lr46*, and *Lr67*. Interestingly, lines carrying *Lr67* were mostly landraces from India and Pakistan, reconfirming the likely origin of the gene. Rapid phenotypic screening using a method that integrates assessment at both seedling and adult growth stages under accelerated growth conditions (i.e. constant light and controlled temperature) identified 50 lines carrying APR. Levels of APR corresponded well with phenotypes obtained in a field nursery inoculated using the same pathotype (r=0.54, $P < 0.000$). The second year of field testing using a mixture of pathotypes with additional virulence for race-specific APR genes (*Lr13* and *Lr37*), identified a subset of 13 lines that consistently displayed high levels of APR across years and pathotypes. These lines provide useful sources of resistance for future research. A strategy combining rapid generation advance coupled with phenotyping under controlled conditions could accelerate introgression of these potentially new alleles into adapted genetic backgrounds.

5.2 Introduction

Bread wheat (*Triticum aestivum* L.) is considered the third most important food crop after maize and rice, providing a major source of carbohydrates and protein in the human diet (Ray et al. 2013). Along with the necessity to increase global wheat production to meet the needs of 9.7 billion people by 2050 (UN 2015), productivity is threatened by climate change (Asseng et al. 2015) and rapidly evolving diseases, such as rusts caused by fungi from the genus *Puccinia* (Chaves et al. 2013). Among the rust diseases, leaf rust (LR) caused by *Puccinia triticina* Eriks., is an ongoing threat; yield losses due to its incidence have been reported in almost all wheat growing regions, such as the USA, South America, Russia, Australia, China, India, South Africa, Mexico, Pakistan, Bangladesh, and Nepal. In these production environments, the annual yield loss due to LR ranges from 10 to 70%, which

varies greatly depending on the differences in crop growth stage, environmental conditions and the degree of plant defence (Huerta-Espino et al. 2011; Niks et al. 2015).

Cultural measures such as removal of the “green bridge” (i.e. off-season wheat cultivation), cultivation of early-maturing varieties, and use of fungicides can minimise losses due to LR; however, they have limitations (Jørgensen et al. 2014). The most effective method is the deployment of genetic resistance which, if managed correctly, can be sustainable.

Adult plant resistance (APR) is best expressed at the adult plant stage and is often underpinned by multiple genes, each quantitatively contributing minor effect to the plant defence level (Lagudah 2011; Ellis et al. 2014; Niks et al. 2015). APR is underpinned by genes that influence factors, such as latent period, pustule size and infection frequency in order to provide a “slow rusting” or partial resistance phenotype (Caldwell 1968; McIntosh et al. 1995; Spielmeyer et al. 2013; Ellis et al. 2014; Niks et al. 2015). Therefore, APR is considered more durable than all-stage resistance or seedling resistance, which is typically governed by a major gene providing a hypersensitive response (HR). Although APR is often non-race specific, there are exceptions, where some genes provide race-specific resistance (such as *Lr13*) (Ellis et al. 2014) and confer an HR (such as *Lr48*) (Bansal et al. 2008). When APR genes are combined, they often act additively, and high levels of resistance (or near-immunity) can be achieved (Singh et al. 2014). The cloning of *Lr34* (Krattinger et al. 2009) and *Lr67* (Moore et al. 2015) has provided perfect markers for marker-assisted selection (MAS) and facilitates deployment of these genes in cultivars. However, if these genes are deployed alone in cultivars, it could make them more vulnerable to pathogen evolution. This highlights the importance of searching for new or additional sources of resistance for creating gene stacks or pyramids, which if deployed in this form, will prolong the life of these valuable genes.

There are approximately 850,000 viable wheat accessions stored in seed banks worldwide (Mitrofanova 2012). While this represents a huge array of genetic diversity, identifying accessions carrying new sources of rust resistance is challenging. Traditionally, APR is determined by phenotyping seedlings in the glasshouse and adult plants in the field. However, the success of field-based phenotyping is dependent on weather conditions favorable for epidemic development (Hickey et al. 2012) and restricted to local pathotypes. Moreover, screening in the field is often limited to just once a year in the wheat growing

season. Using this approach to evaluate large numbers of seed bank accessions is a slow process. A new method reported in Chapter 4 permits rapid phenotyping for APR to LR in wheat grown under a controlled environment. The method exploits constant light and controlled temperature to rapidly obtain adult plants. The technique involves two sequential inoculations: one at the seedling stage and a second at the adult stage, to phenotype APR within 7 weeks and can be performed all year round.

Here, we search for new sources of APR to LR by mining a diverse panel of 300 wheat accessions sourced from the N. I. Vavilov Institute of Plant Genetic Resources (VIR) in St Petersburg, Russia. The institute was originally formed in 1901 and was later named after the great Russian botanist and Geneticist N. I. Vavilov, best known for his theory relating to ‘‘the centers of origin of cultivated plants’’. Vavilov and his colleagues led various expeditions to different parts of the world and collected a huge diversity of wheat. Currently, the VIR wheat collection contains 38,430 samples, of which 29,209 are bread wheat (i.e. *T. aestivum* L.), 6,199 are durum wheat (i.e. *T. durum* Desf.), and 3,022 are wild wheat (Mitrofanova 2012). In the present study, we apply DNA markers to screen for known APR genes (i.e. *Lr34*, *Lr46*, and *Lr67*) and perform rapid phenotyping under controlled conditions. We investigate the distribution of known APR genes in the diversity panel and provide insight on their likely origin. Based on initial screening, we evaluate a promising subset in the field over two years and identify valuable genetic materials for future research aiming to characterise new APR genes, which are required to diversify resistance factors in breeding programs.

5.3 Materials and methods

5.3.1 Plant materials

This study examined a diverse panel of wheat accessions comprising 300 single-seed descent (SSD) lines (295 hexaploid and 5 tetraploids) sourced originally from VIR, St. Petersburg, Russia. The 295 hexaploid accessions were previously characterized for genetic diversity and population structure using the genotyping-by-sequencing Diversity Arrays Technology platform (DArT-seq) (Chapter 3). For line purification, a single plant for each of the 300 VIR accessions was grown in the glasshouse and subjected to a generation of SSD, to develop genetically stable lines for subsequent genotypic and phenotypic analyses. The derived SSD lines were assigned new Australian Grain Genebank (AGG)

accession numbers (Supplementary Table 3). The panel includes landraces (n=136), cultivars (n=36), breeding lines (n=10) and lines with unknown cultivation status (n=118). The pure seed for SSD lines was used in all experiments conducted in this study. A set of disease standards were also included: Thatcher, Avocet, and near-isogenic lines (NILs) in the Avocet background (i.e. Avocet+*Lr34* and Avocet+*Lr46*).

5.3.2 Pathogen materials

Two *P. triticina* pathotypes were used in this study: *pt* 104–1,2,3,(6),(7),11,13 and *pt* 76–1,3,5,7,9,10,12,13+*Lr37* (Table 5.1). These pathotypes are prevalent in eastern and western wheat-growing regions of Australia (Park et al. 2002; Park and Wellings 2011; Park and Bariana 2013; Park et al. 2015). The rust cultures used in this study were maintained through single spore culture technique using the susceptible cultivar 'Morocco' wheat.

5.3.3 Polymerase chain reaction marker screening for known APR genes

Three hundred SSD lines in the diversity panel were screened with polymerase chain reaction (PCR) - based markers for previously reported LR APR genes, namely *Lr34*, *Lr46*, and *Lr67*. Genomic DNA for each accession was extracted using the method reported by Diversity Arrays Technology Pty. Ltd. (https://www.diversityarrays.com/files/DArT_DNA_isolation.pdf). The screening of *Lr34* was performed using the gene-specific cleaved amplified polymorphic sequence (CAPS) marker *cssfr5* (forward primer *Lr34SPF* and reverse primer *L34DINT13R2*), as described by Lagudah et al. (2009). The *cssfr5* marker enables accurate identification of the gene in diverse wheat germplasm (Lagudah et al. 2009). For detection of *Lr46*, the CAPS marker named *csLV46* was used (unpublished data). For detection of *Lr67*, a gene-specific single nucleotide polymorphic (SNP) marker (i.e. SNP1-TM4) was used (Moore et al. 2015).

5.3.4 Rapid phenotyping for seedling and APR

The integrated seedling and adult plant method developed by was employed to phenotype the 300 SSD lines at The University of Queensland, St Lucia, Queensland, Australia. To encourage synchronous germination for all genotypes, seeds were imbibed with water for 24 h at room temperature and were placed in a refrigerator (4°C) for 48 h. Germinated seeds

were transplanted into 140-mm ANOVApot® (Anovapot Pty Ltd, Australia, www.anovapot.com) pots filled with a potting medium consisting of 70% composted pine bark fines (0 to 5 mm) and 30% coco peat with a pH ranging of 5.5 to 6.5. Slow release Osmocote fertiliser was applied at a rate of 2 g/pot. Three seeds of each line were clumped together at one position, where each pot had four positions. Plants were grown under regular glasshouse conditions at temperatures of 22°C (day) and 17°C (night) and a diurnal photoperiod (12 h). After 10 days, seedlings were inoculated with pathotype *pt* 104–1,2,3,(6),(7),11,13, where urediniospores were suspended in light mineral oil (Isopar 6) at a concentration of 6×10^5 spores/ml (0.005 g/ml) and applied using an airbrush (IWATA power jet lite). At 12 days post-inoculation seedlings were assessed for infection type (IT) using the 0–4 Stakman scale (Stakman et al. 1962). Lines that displayed an IT of <3 were considered resistant (R).

Following the seedling assessment, plants were grown under accelerated growth conditions (AGC), which was achieved by adopting constant (24 h) light and a 12 h cycling temperature regime of 22 and 17°C (Chapter 4). The plants were grown for 2 weeks under AGC and were re-inoculated with a suspension of *P. triticina* urediniospores (i.e. *pt* 104–1,2,3,(6),(7),11,13), as described above. Inoculation of plants after this period of growth provided phenotypes similar to adult plants in the field (Chapter 4). At 12 days post-inoculation the LR response was recorded for the flag-2 leaf using 0–4 Stakman scale, where IT <3 was considered R.

5.3.5 Field evaluation

SSD lines displaying APR under AGC that lacked known APR genes (based on marker screening) were evaluated for resistance in the field over two consecutive years (from July to October 2014 and 2015) at Redlands Research Facility, Queensland, Australia. The LR-susceptible (S) genotype Morocco was used as a disease spreader, where two rows of Morocco were sown between each bay comprising two rows of hill plots. Lines were sown as non-replicated hill plots. A set of disease standards, including; Thatcher, Avocet, Avocet+*Lr34*, and Avocet+*Lr46*, were replicated throughout the nursery to monitor the LR epidemic progression. The LR epidemic was initiated by transplanting rust-infected Morocco seedlings into the field among the spreader rows about 5 weeks after sowing. Favorable conditions for the disease were maintained by applying sprinkler irrigation in the late evenings. Plants were assessed when the rust epidemic had sufficiently developed on

disease standards to allow a clear differentiation between susceptible and resistant genotypes.

In 2014, the LR nursery was inoculated with *P. triticina* pathotype *pt* 104–1,2,3,(6),(7),11,13 - the same pathotype used for screening under controlled conditions. The disease response was assessed on a whole plot basis using the modified Cobb scale (Peterson et al. 1948). Multiple disease assessments were conducted from late tillering to early grain filling (i.e. 70, 77, 86, and 96 days after sowing - DAS). Host response and disease severity data were used to calculate the coefficient of infection (CI), as per Loegering (1959). Lines that displayed a LR response between R to moderately resistant-moderately susceptible (MRMS) were considered resistant.

In 2015, the LR field nursery was inoculated with a mixture of two pathotypes: *pt* 104–1,2,3,(6),(7),11,13 and *pt* 76–1,3,5,7,9,10,12,13+*Lr37*. Notably, in comparison with the 2014 screening, this provided additional virulence for race-specific APR genes *Lr13* and *Lr37*. Plants were assessed using the 1–9 scale reported by Bariana et al. (2007). Two disease assessments (i.e. 78, 85, and 101 DAS) were conducted from late tillering to early grain filling. Lines that displayed a LR response ≤5 (i.e. MRMS) were considered resistant.

5.3.6 Statistical analysis

In the integrated seedling and adult plant experiment under AGC, disease response was evaluated using the 0-to-4 Stakman scale, which contains both numbers (e.g., 0, 1…4) and symbols (e.g., ;, +, and others). The symbols represent variations in the LR response, which were indicated by the use of “–” (i.e. lower than average for the class) and “+” (i.e. higher than average for the class), as well as “c” and “n” to indicate more than usual degrees of chlorosis and necrosis, respectively. The data were converted to a 0–9 scale, where 0 = immune and 9 = very susceptible. The converted ITs were as follows: 0;, ;n, ;, 1−, 1, 1+, 2−, 2, 2+, 2++, 3−, 3, 3+, 3++, and 4, were coded as 0, 0.5, 1, 2.5, 3, 3.5, 4, 5, 5.5, 6, 6.5, 7, 8, 8.5, and 9, respectively. For heterogeneous ITs, where no direct conversion value was available, each score was converted individually to the 0–9 scale and the average calculated. The converted datasets were used to generate frequency distributions and illustrate resistance levels for lines carrying known APR genes.

In order to compare disease response of the lines potentially carrying new sources of APR across the four experiments (i.e. seedling, AGC, and two years of field assessment) principal component analysis (PCA) was performed and results visualized in the form of a biplot using GenStat (17.1 2000–2015; VSN International Ltd.) (GenStat.co.uk). Correlation between disease response under AGC and field 2014 was also performed.

5.4 Results

5.4.1 Geographical distribution of known APR genes

A total of 83 lines in the diversity panel were deemed to carry known genes for APR (Supplementary Table 3). The APR genes *Lr34*, *Lr46*, and *Lr67* were present in 9, 13, and 48 lines with known origin information, respectively (Figure 5.1a, b, and c). Some lines with unknown origin also carried the APR genes: 3 carried *Lr34*, 12 carried *Lr46*, and 3 carried *Lr67* (not presented in Figure 5.1). Two lines carried *Lr34* and *Lr46* in combination, while three lines carried *Lr46* and *Lr67* in combination. The mean disease response for lines carrying known APR genes (*Lr34*, *Lr46*, and *Lr67*) evaluated under AGC was 4.2, 5.9, and 6.1 on the 0–9 scale, respectively (Figure 5.2). Notably, none of the lines carried all three APR genes. *Lr34* was detected in lines from Russia (n=3), unknown origin (n=3), China (n=2), Ukraine (n=2), Kazakhstan (n=1) and Sweden (n=1) (Figure 5.1a). *Lr46* was present in lines from Russia (n=4), Sudan (n=4), Kazakhstan (n=2), Ukraine (n=2), Armenia (n=1) and unknown origin (n=12) (Figure 5.1b). In case of *Lr67*, the allele for resistance was predominantly observed in lines from India (n=22) and Pakistan (n=18), and to a lesser extent it was present in lines from Iraq (n=3), Sudan (n=2), Myanmar (n=1), Russia (n=1), Mexico (n=1) and with unknown origin (n=3) (Figure 5.1c).

Of the 136 SSD lines classed as landraces, 53 carried known APR genes; *Lr34* (n=4), *Lr46* (n=7), and *Lr67* (n=42). Only one breeding line carried a known APR gene (i.e. *Lr46*). However, a number of lines classed as cultivars carried *Lr34* (n=4), *Lr46* (n=7) and *Lr67* (n=1). None of the five durum lines carried *Lr34*, *Lr46*, or *Lr67*.

5.4.2 Rapid phenotyping: seedling stage

Of the 300 lines, 73 displayed variable levels of resistance (<7) and 220 displayed susceptibility (≥7) against pathotype 104–1,2,3,(6),(7),11,13. Seven lines failed to germinate. Among the S lines, 13 were scored 7, 77 scored 8, 55 scored 8.5, and 75 scored 9 (based on 0–9 scale). The majority of the lines deemed seedling susceptible were of unknown origin (n=75), followed by lines originating from India (n=31), Pakistan (n=24), and Russia (n=29). The majority of lines that displayed seedling resistance were from Russia (n=21), Ukraine (n=6), and Pakistan (n=6). Nineteen lines of unknown origin also displayed resistance. Notably, two lines displayed a HR (i.e. IT 0;) and these originated from Pakistan and Tajikistan. The frequency distribution for seedling response was skewed towards susceptibility on the 0–9 scale (Figure 5.3a). The disease standards such as Thatcher, Avocet, Avocet+*Lr34*, and Avocet+*Lr46* were found to be seedling susceptible, with an IT ranging from 7 to 9 on the 0–9 scale.

5.4.3 Rapid phenotyping: adult stage

In the integrated adult plant experiment under AGC, the 300 lines displayed a complete range of LR response types; ranging from R to S, when inoculated with pathotype 104–1,2,3,(6),(7),11,13 (Figure 5.3b). As described above, seven lines failed to germinate. Therefore under AGC, 139 lines displayed resistance, while 153 depicted susceptibility. One line was evaluated at the seedling stage, but not at the adult stage. The majority of R lines were of unknown origin (n=43), followed by Russia (n=33), India (n=16), and Pakistan (n=12) (Figure 5.3b). Likewise, the majority of S lines were of unknown origin (n=51), followed by India (n=18), and Pakistan (n=18). By seedling susceptibility and resistance displayed at the adult growth stage, 86 lines were deemed to carry APR, while 54 lines displayed ASR, defined by resistance displayed at both seedling and adult growth stages. The frequency distribution of adult plant response to LR was more evenly distributed in comparison with the seedling response (Figure 5.3b).

Thatcher displayed a very susceptible response (VS; IT 9), with urediniospores freely sporulating on leaves. Avocet displayed a resistant-moderately resistant (RMR) response, with IT 4, because Avocet carries race-specific APR gene *Lr13*, which is effective against

the pathotype used for screening under AGC. Avocet+*Lr34* displayed an RMR response (i.e. IT 4), while Avocet+*Lr46* demonstrated a moderately resistant (MR) response (i.e. IT 6).

5.4.4 Identification of new sources of APR

A total of 86 lines were deemed to carry APR based on the integrated seedling and adult plant phenotyping performed under AGC. Of these, 36 lines carried known APR genes based on results from marker screening. Therefore, the screening process identified 50 wheat lines carrying potentially new sources of APR to LR.

5.4.5 Field evaluation

In 2014, the 50 lines carrying potentially new APR were evaluated in the field using the same pathotype (i.e. *pt* 104–1,2,3,(6),(7),11,13) used for initial screening of lines at seedling and adult stage under AGC. Of the 50 lines, 29 were considered R while 21 were considered S in the field; that is, 2 were moderately susceptible (MS; 6 on 0–9 scale), 3 were moderately susceptible to susceptible (MSS; 7 on 0–9 scale) and 16 were S (8 on 0–9 scale). Despite some variation in response, field phenotypes observed in 2014 corresponded well with those observed under AGC (r=0.54, $P < 0.000$).

In 2015, the set of 50 lines were again evaluated in the field, but using a mixture of two different pathotypes: *pt* 104–1,2,3,(6),(7),11,13 and *pt* 76–1,3,5,7,9,10,12,13+*Lr37*. In 2015, 13 lines were deemed resistant and 37 displayed varying levels of susceptibility. A total of 16 lines displayed resistance at the adult stage under AGC and in the field in 2014 but displayed susceptibility in 2015 (Figure 5.4). These lines likely carry race-specific APR *Lr13* and *Lr37*, because the pathotype mixture used in 2015 had additional virulence for *Lr13* and *Lr37*. Similarly, the disease standard Avocet, which carries *Lr13*, displayed APR in 2014; however, it displayed a VS response in 2015 (Figure 5.5). In contrast, Avocet+*Lr34* and Avocet+*Lr46* displayed stable resistance even against the *Lr13*-virulent pathotype and high inoculum pressure (Figure 5.5). Following the two years of field evaluation, 13 lines were deemed to carry stable APR and likely harbor new genes (Figure 5.5).

5.5 Discussion

In this study, we identified useful sources of APR to LR by effectively mining diverse wheat lines from the VIR. We anticipate this will accelerate the isolation of new genes, which are required to diversify resistance factors in the breeding material.

Of the three known APR genes screened using PCR markers, *Lr34* was the least common (i.e. only 12 lines). The gene was mostly observed in lines with unknown origin, followed by lines from China, Kazakhstan, Russia, Ukraine, and Sweden. A previous study by Dakouri et al. (2014) suggests that *Lr34* likely originated from Asia, specifically China and/or Japan. Although some *Lr34* - carrying lines in this study were from China, the presence of the gene in lines from other countries in Central Asia or Europe could have resulted from early movement of wheat germplasm around the world. One of the key cultivars that increased the spread and utilisation of *Lr34* was 'Frontana', which was used to first characterise the gene in 1966 (Dyck et al. 1966; Singh 1992).

Based on the *csLv46* marker, the resistance allele for *Lr46* was present in 25 of the 300 lines in the diversity panel. These lines were largely of unknown origin, followed by lines from Armenia, Kazakhstan, Russia, and Ukraine. It should be noted that *csLv46* sometimes provides false positives because it is not diagnostic; thus, it is difficult to infer the likely origin of *Lr46*. However, based on the *Lr46*-linked marker, the resistance allele was present in one landrace collected in the 1960s from Sudan and also Russian cultivars and breeding lines from 1952 onwards. Notably, *Lr46* was first characterized in the International Maize and Wheat Improvement Center (CIMMYT) cv. Pavon 76 (Singh et al. 1998).

Lr67 was present in 17% of the lines evaluated in this study (i.e. 51 lines). The majority of these lines were from India (n=22) and Pakistan (n=18), followed by Russia, Burma, and Iraq. Most of the lines carrying *Lr67* from India and Pakistan were landraces, suggesting that the gene originated from this region. Similar observations of the prevalence of *Lr67* in the Punjab were reported by Forrest et al. (2014) and Moore et al. (2015). The high frequency of *Lr67* in this diversity panel might be due to the higher proportion of lines collected from Asia (particularly India and Pakistan), likely a result of multiple expeditions conducted by N. I. Vavilov and A. E. Watkins from 1920 to 1930, followed by succeeding investigators to date. In this diversity panel, *Lr67* was also found in lines from Sudan and

Mexico, most likely a result of the early transfer of genetic material across continents. *Lr67* was originally detected and characterised in Pakistani landrace 'PI250413' (Dyck and Samborski 1979; Hiebert et al. 2010; Herrera-Foessel et al. 2012).

The integrated phenotyping performed under AGC identified wheat lines displaying both ASR (i.e. hyper-sensitive flecking and small uredia with necrosis) and varying levels of APR (i.e. restricted sporulation and chlorosis) to *P. triticina*. Following the elimination of lines that carried known APR genes, 50 lines were deemed to carry potentially new sources of APR. Of these, 37 displayed varying levels of susceptibility in at least one of the two field environments. Notably, 16 of the 37 lines appeared to carry race-specific APR *Lr13* and *Lr37* based on their increased susceptibility to the pathotype mix used in 2015 field screening. Other factors contributing to the variability in disease response across adult assays were likely differences in environmental conditions (e.g. temperature, light, and humidity) and growth stage at assessment, which are known to influence expression of APR (Kaul and Shaner 1989; Singh and Huerta-Espino 2003; Hiebert et al. 2010; Herrera-Foessel et al. 2012; Hickey et al. 2012). Cooler temperatures are known to enhance the effectiveness of *Lr34*, for instance, 13 to 18°C compared to 23°C (Singh and Huerta-Espino 2003). In this study, the rapid phenotyping assay was performed at a controlled temperatures of 17 and 22°C (night and day). Under these conditions, lines carrying *Lr34* displayed higher levels of resistance at the adult plant stage in comparison with lines carrying *Lr46* or *Lr67,* which is in agreement with previous field studies (Hiebert et al. 2010; Ellis et al. 2014). The assessment under AGC uses a single controlled inoculation, whereas assessment in the field was subject to variable weather conditions (e.g. fluctuating temperatures), plant growth stage, and polycyclic pathogen infection (Niks et al. 2015). Therefore, some resistance factors, such as a long latent period or small pustule size, may be phenotyped more precisely using a single controlled inoculation. APR genes often provide weak or low levels of resistance to LR, thus are often scored as MS or MSS in the field, such as APR gene *Lr67* (Hiebert et al. 2010). In this study, we applied strict criteria for resistance (i.e. ≤ MRMS). Thus, it is possible additional lines displaying low susceptibility scores in the field (i.e. MS or MSS) could carry weak APR. These factors might be useful under lower disease pressure or coupled with additional APR.

PCR marker screening and APR phenotypes observed under AGC initially identified 50 lines of interest. Of these, 13 lines consistently displayed moderate to high levels of resistance in the field using a mixture of pathotypes. Interestingly, these lines originated

from different geographical regions of the world, including; Russia (six), unknown origin (two), India (two), China (one), Chile (one), and Portugal (one). Therefore, they likely carry different sources of resistance. Furthermore, among the 13 lines, there was also diversity in terms of cultivation status; 5 cultivars, 5 landraces, 1 breeding line and 2 with unknown cultivation status. In addition to the APR genes screened in this study (i.e. *Lr13*, *Lr34*, *Lr37*, *Lr46,* and *Lr67*), there is a number of other race-specific APRs that have been catalogued, including *Lr12*, *Lr22* (alleles a and b), *Lr35*, *Lr48*, and *Lr49*, plus *Lr68* which is a race - nonspecific APR (McIntosh et al. 1995; Ellis et al. 2014; Li et al. 2014). Therefore, to determine whether the genes are new, fine mapping is required and, if positioned on the same chromosome as these previously catalogued genes, allele testing is needed.

This study highlights the value of historical germplasm to provide the much-needed genetic diversity to improve wheat productivity in the face of climate change and rapidly evolving pathogens. Although only a small selection of 300 wheat lines from VIR is screened, around the world there are hundreds of thousands of viable wheat accessions preserved in seed banks, such as the Svalbard Global Seed Vault and collections based at CIMMYT, the United States Department of Agriculture, and John Innes Centre.

The rapid phenotyping methodology performed under controlled conditions presents a number of advantages: (i) it only requires 7 weeks to complete, (ii) environmental factors are controlled, and (iii) it can be performed all year round. On the other hand, conventional field screening is time-consuming (i.e. 4 to 5 months), subject to weather conditions, and can only be performed once a year. Additional accessions in seed banks can be rapidly screened using this approach, which reduces the number for field-based evaluation of resistance. Further, it could be integrated with Focused Identification of Germplasm Strategy or other trait mining techniques (Mackay et al. 2016).

The sources of resistance identified in this study will be used to generate bi-parental populations for gene mapping and identification of linked DNA markers, which will assist gene pyramiding. Transfer of resistances could be accelerated by selecting for APR to LR in parallel with rapid generation advance under “speed breeding” or AGC (Mackay et al. 2016).

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5.7 Figures

Figure 5.1 Geographical distribution of **a)**, *Lr34*, **b)** *Lr46*, and **c)** *Lr67* in the diversity panel. Sizes of the circles are proportional to the number of lines carrying alleles for resistance. Lines with gene combinations were tallied individually for the respective gene total. Lines lacking origin information were not displayed.

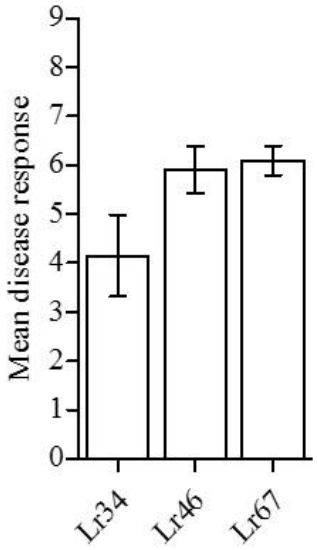


Figure 5.2 Mean leaf rust response observed under accelerated growth conditions (flag-2 leaf) for lines carrying known adult plant resistance genes: *Lr34* (12 lines), *Lr46* (25 lines), and *Lr67* (51 lines). Error bars display the standard error of the mean for lines carrying the respective gene.

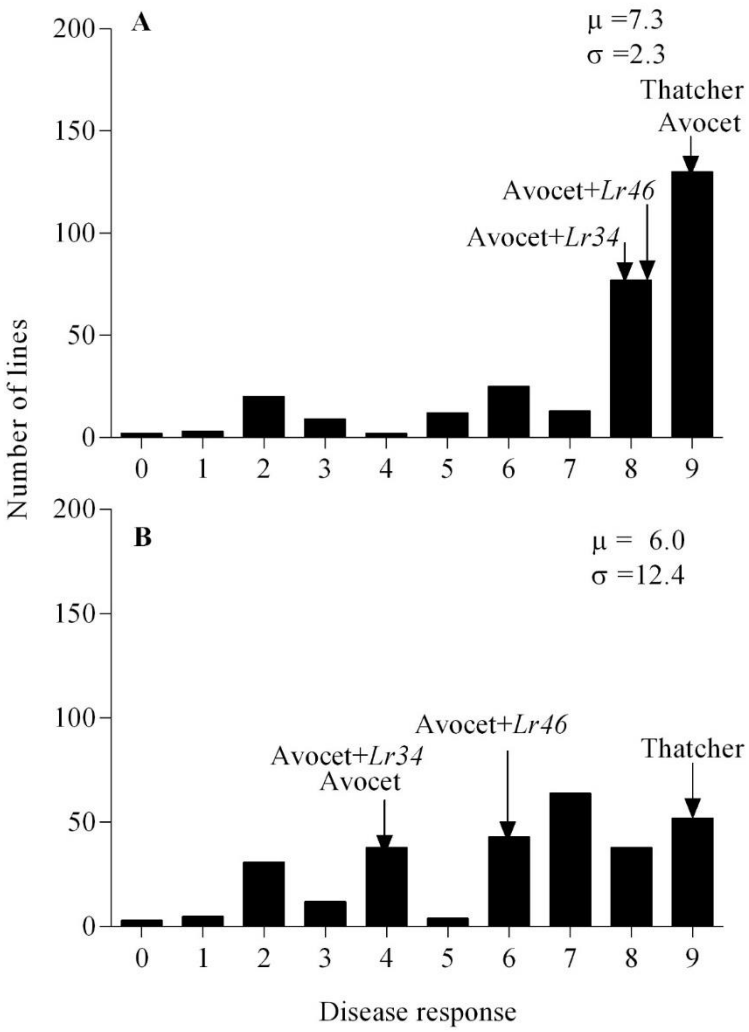
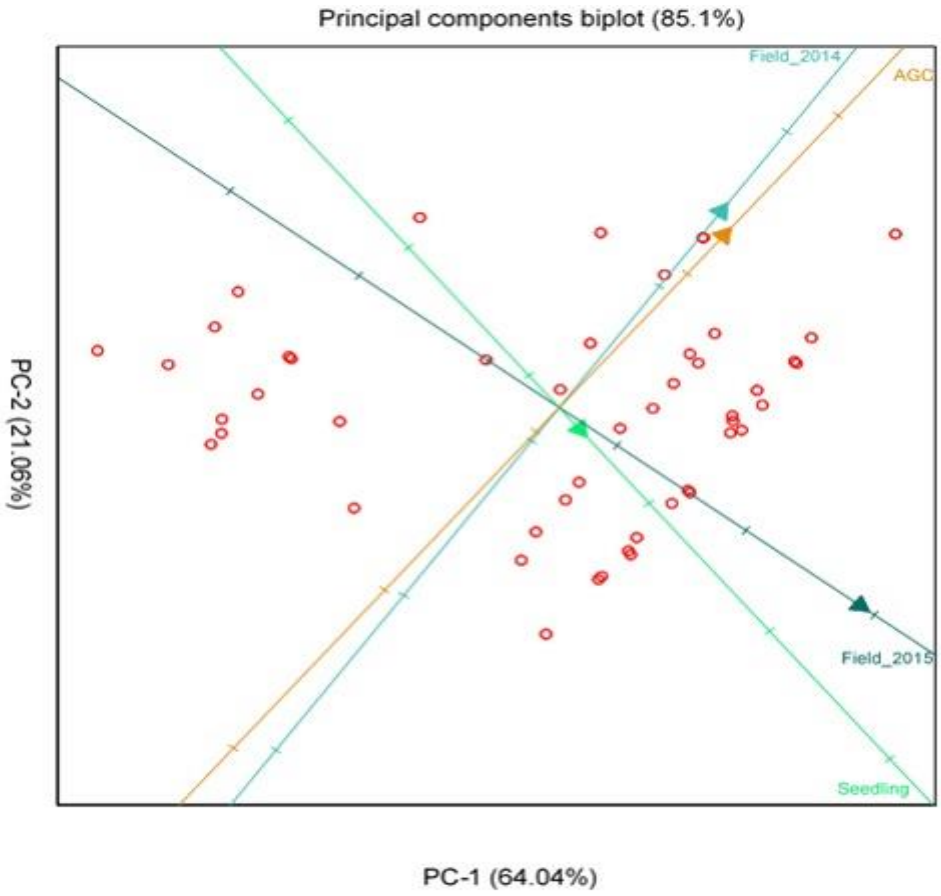


Figure 5.3 Frequency distribution of leaf rust response for 300 wheat lines evaluated at **a)**, seedling stage (standard glasshouse) and **b)**, adult stage under accelerated growth conditions (flag-2 leaf). The disease response for seedling and adult stage under accelerated growth conditions was collected using the 0–4 Stakman scale and converted to the 0–9 scale (displayed).



1

2 **Figure 5.4** Biplot displaying results from principal component (PC) analysis of leaf rust
3 response for the 50 lines identified following initial screening. Leaf rust response was
4 obtained in the following four experiments: (i) seedling, (ii) adult stage under accelerated
5 growth conditions, (iii) field in 2014, and (iv) field in 2015. Displayed PCs (i.e. PC1 and PC2)
6 account for 85.1% of the variation.

7

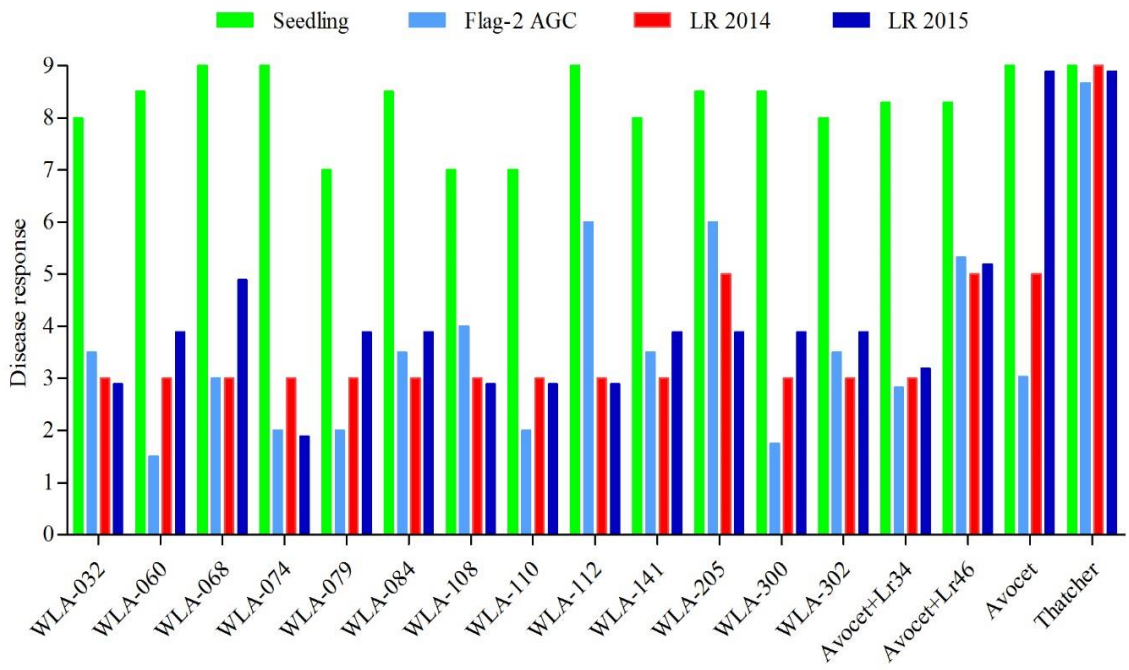


Figure 5.5 Comparison of leaf rust response for the 13 lines carrying new adult plant resistance, along with disease standards (Thatcher, Avocet, Avocet+*Lr34*, and Avocet+*Lr46*) evaluated at the seedling stage, adult stage (i.e. flag-2 leaf) under accelerated growth conditions (AGC), and in the field in 2014 and 2015.

5.8 Tables

Table 5.1 Virulence profile of *Puccinia triticina* pathotypes used in this study.

Pathotype	Virulent on genes[a]	Avirulent on genes
104–1,2,3,(6),(7),11,13[b]	*Lr1, Lr3a, Lr14a*, *Lr16*, *Lr17a**, *Lr20*, *Lr24, Lr27+31**	*Lr2a, Lr3ka, Lr13, Lr15, Lr17b, Lr23*, *Lr26*, *Lr28*, *Lr37*
76–1,3,5,7,9,10,12,13+*Lr37*[c]	*Lr3a, Lr3ka*, *Lr13*, *Lr14a*, *Lr17a*, *Lr17b*, *Lr20*, *Lr24*, *Lr26*, *Lr37*	*Lr1, Lr2a, Lr15*, *Lr16*, *Lr23*, *Lr27+31, Lr28*

[a] Asterisk indicates that the pathotype is partially virulent on the gene.

[b] Single pathotype used in rapid phenotyping seedling and APR under accelerated growth conditions and the 2014 field experiments.

[c] The additional pathotype used in the 2015 field experiment.

Chapter 6 - Genome-wide association study for leaf rust resistance in the Vavilov wheat diversity panel

6.1 Abstract

A diversity panel of 295 bread wheat accessions from the N. I. Vavilov Institute of Plant Genetic Resources (VIR) in St Petersburg, Russia was evaluated for leaf rust (LR) (*Puccinia triticina* Eriks.,) resistance and performed a genome-wide association studies (GWAS) using 10,748 polymorphic DArT-seq markers. The diversity panel was evaluated at seedling and adult plant growth stages using three *P. triticina* pathotypes prevalent in Australia. GWAS was applied to 11 phenotypic data sets which identified a total of 52 significant marker-trait associations representing 31 quantitative trait loci (QTL). Among them, 29 QTL were associated with adult plant resistance (APR). Of the 31 QTL, 13 were considered potentially new loci, whereas 4 co-located with previously catalogued *Lr* genes and 14 aligned to regions reported in other GWAS and genomic prediction studies. One seedling LR resistance QTL located on chromosome 3A showed pronounced levels of linkage disequilibrium among markers (r^2=0.7), suggested a high allelic fixation. Subsequent haplotype analysis for this region found 7 haplotype variants, of which 2 were strongly associated with LR resistance at seedling stage. Similarly, analysis of an APR QTL on chromosome 7B revealed 22 variants, of which 4 were associated with resistance at adult-plant stage. Furthermore, most of the tested lines in the diversity panel carried 10 or more combined resistance-associated marker alleles, highlighting the potential of allele stacking for long-lasting resistance.

6.2 Introduction

Wheat (*Triticum aestivum* L.) is a major source of calories and protein in the human diet (Shewry and Hey 2015). However, the current global production is insufficient to meet the demand of a rapidly growing world population (Grassini et al. 2013). At the same time, wheat yields are consistently threatened by increasing climatic variations (Asseng et al. 2015) and rapidly evolving pests and pathogens (Chaves et al. 2013). Leaf rust (LR) caused by *Puccinia triticina* Eriks., is one of the most common and geographically widespread wheat diseases worldwide. LR causes more annual yield losses globally compared to losses attributed to stem and stripe rust (Bolton et al. 2008; Huerta-Espino et al. 2011). Among

various disease management strategies, the cultivation of resistant wheat cultivars is the most effective and environment-friendly strategy (Kolmer et al. 2013).

Genetic resistance against LR is broadly categorised into seedling or all-stage resistance and adult plant resistance (APR). To date, 77 leaf rust resistance genes (*Lr*) have been successfully characterised of which the majority confer seedling resistance (McIntosh et al. 2017). Typically, seedling resistance is controlled by a single gene with the major effect that interacts with the pathogen in a 'gene-for-gene' relationship (Flor 1971). Usually, the seedling genes are pathogen race-specific and confer a hypersensitive response (HR) - a cell death phenomenon preventing the pathogen spread (Mondal et al. 2016). This exerts intense selective pressure on the pathogen population, thus quickly rendering the deployed resistance gene ineffective (Burdon et al. 2014; Li et al. 2014; Niks et al. 2015). In contrast, APR is usually effective at the post-seedling growth stages, is either controlled by multiple genes each with minor effect or single genes with major effect. Some APR genes provide partial resistance that is effective against all races of a given pathogen species (i.e. race-nonspecific) (Lagudah 2011; McCallum et al. 2012; Burdon et al. 2014). Mostly APR genes interact additively and enhance resistance to a level of immunity (Singh et al. 2014). Some APR genes confer pleiotropic resistance against multiple diseases. For instance, the APR genes *Lr34*, *Lr46*, and *Lr67* provide partial resistance to LR, stripe rust, stem rust, and powdery mildew disease of wheat (Lagudah 2011; Risk et al. 2012; Ellis et al. 2014).

To date, six *Lr* genes (including seedling and APR) have been cloned; *Lr1* (Cloutier et al. 2007), *Lr10* (Feuillet et al. 2003), *Lr21* (Huang et al. 2003), *Lr22a* (Thind et al. 2017), *Lr34* (Krattinger et al. 2009), and *Lr67* (Moore et al. 2015). This has enabled the development of gene-specific molecular markers for rapid gene identification via marker-assisted selection (MAS). Markers further assist in pyramiding of 4-5 APR or seedling resistance genes or in combinations to generate durable rust resistant wheat cultivars (Ellis et al. 2014; Singh et al. 2014). To maintain and/or broaden the genetic diversity of durable rust resistance, the identification of new genetic sources of resistance is required. One approach for the genetic enrichment of elite breeding pools is to exploit landraces by introducing genetic diversity from germplasm collections (Lopes et al. 2015; Sehgal et al. 2015; Kumar et al. 2016). More than 850,000 wheat accessions are stored in gene banks, representing a rich genetic resource to reinstate the variation of genetic bottlenecks (e.g. from domestication or selective breeding). Many of these accessions are already adapted to very specific target environments, possessing exclusive advantageous characteristics,

such as resistances towards specific biotic and abiotic stresses (Mitrofanova 2012; Huang and Han 2014; Lopes et al. 2015), including resistance to rust diseases (Cavanagh et al. 2013; Lopes et al. 2015; Rinaldo et al. 2016; Vikram et al. 2016).

For instance, the Lr genes *Lr52* and *Lr67* (Hiebert et al. 2010; Bansal et al. 2013), and the stripe rust gene *Yr47* (Bansal et al. 2011) were identified in wheat landraces from the Watkins collection. Another historical yet relatively unexploited wheat landrace collection is the “N. I. Vavilov Institute of Plant Genetic Resources” (VIR) in St Petersburg, Russia, collected by the Russian botanist and geneticist N. I. Vavilov and his colleagues in the early 1900s. Different studies have reported a large variety of new alleles in the VIR wheat collection, revealing the promising basis for the genetic improvement of resistances to various biotic and abiotic stresses (Mitrofanova 2012; Sadovaya et al. 2015). However, determining the genomic regions underpinning these resistances is challenging.

Traditionally, quantitative trait loci (QTL) mapping is used to identify underlying genetic variations that co-segregate with a trait of interest using a bi-parental mapping population (Koornneef et al. 2004; Zhu et al. 2008). Although QTL mapping has been proven successful in some cases, it is fundamentally limited to the comparative low allelic diversity of the two crossing parents and low recombination events which impair the mapping resolution (Zhu et al. 2008). Alternatively, genome-wide association studies (GWAS) represent a powerful tool to dissect the genetic architecture of complex traits in natural populations, such as germplasm collections (Zhu et al. 2008; Hall et al. 2010), by detecting genomic regions that are in linkage disequilibrium (LD) with genes affecting the trait of interest. Due to the greater number of historical chromosomal recombinations accumulated over a large number of generations in natural populations GWAS can map QTL at a much higher resolution (Yu and Buckler 2006; Semagn et al. 2010).

Here, we present a large-scale association study for seedling and APR to LR under controlled and field conditions in a highly diverse panel of 295 bread wheat lines from the VIR. Using high-density Diversity Arrays Technology (DArT-seq) markers and multi-year phenotypic data sets we were able to map previously undescribed QTL for resistance against three major *P. triticina* pathotypes that are prevalent in Australia. We anticipate that this study provides breeders with a rich basis for the improvement of durable LR resistances in future wheat cultivars. Ongoing work based on these findings will help to functionally validate the significance of candidate genes in the identified new genomic regions.

6.3 Materials and methods

6.3.1 Plant materials and genotyping

A diversity panel of 295 homozygous single seed descent (SSD) bread wheat lines from VIR, representing species-wide genetic diversity (Chapter 3) was selected for the assessment of LR response. DNA of each wheat line was extracted following the recommended Diversity Arrays Technology (DArT) protocol (www.diversityarray.com) and the whole panel was genotyped with the DArT genotyping-by-sequencing (GBS) platform using the DArT-seq wheat *PstI* complexity reduction method, as described by Li et al. (2015), which returned a total of 56,306 raw DArT-seq markers. The DArT-seq markers are presence-absence dominant markers extracted *in-silico* from sequences obtained from genomic representations. The raw marker data was filtered to retain only markers with ≤10% missing values, a minor allele frequency ≤3% and lines with ≤20% missing values, resulting in a selection of 10,748 high-quality, polymorphic DArT-seq markers for the subsequent genetic analyses. All used DArT-seq markers were ordered according to their genetic positions in a high-resolution DArT-seq consensus map (version 4.0), provided by Dr Andrzej Kilian (Diversity Arrays Technology Pty Ltd, Canberra, Australia).

As described in Chapter 5, the diversity panel was also screened for the polymerase chain reaction (PCR)-based markers *cssfr5* (Lagudah et al. 2009), *csLV46* (Lagudah, unpublished data) and SNP1-TM4 (Moore et al. 2015) which facilitated identification of the known LR APR genes *Lr34*, *Lr46*, and *Lr67*, respectively.

6.3.2 Evaluation of leaf rust resistance

For the resistance screening we used the three *P. triticina* pathotypes (*pt*), namely *pt* 104–1,2,3,(6),(7),11,13, *pt* 76–1,3,5,7,9,10,12,13+*Lr37* and *pt* 104–1,3,4,6,7,8,10,12+*Lr37* (Table 6.1), which are prevalent in the eastern and western wheat growing regions of Australia (Park 2016). A summary of the experiments performed in this study at the seedling and adult plant stage for scoring LR response across years and pathotypes is presented in Table 6.2.

6.3.3 Integrated seedling and adult plant phenotyping

The 295 SSD lines in the diversity panel were evaluated using the integrated seedling and adult plant phenotyping method under controlled conditions, as described in Chapter 5. For seedling infection, the *P. triticina* pathotype *pt* 104–1,2,3,(6),(7),11,13 was used (Table 6.2). Briefly, the diversity panel was sown in a standard glasshouse with diurnal temperatures (i.e. 22/17°C day/night) and 12 h photoperiod. Twelve days post-inoculation, seedlings were scored using the 0–4 Stakman scale (Stakman et al. 1962). Afterwards, plants were transferred to a temperature-controlled growth facility where the plants were subjected to “speed breeding” or “accelerated growth conditions” (AGC) by adopting a 12 h cycling temperature (22/17°C) and 24 h photoperiod, which helps the plants to attain the adult plant stage rapidly (Chapter 5; Watson and Ghosh et al. 2017). After two weeks, plants were re-inoculated using the same *P. triticina* pathotype *pt* 104–1,2,3,(6),(7),11,13 (Table 6.2). Twelve days post-inoculation LR response was recorded for the flag-2 leaf using the 0–4 Stakman scale (Stakman et al. 1962). This provides representative measures of the adult plant response to LR, which are associated to field-based measures (Chapter 4). The LR response in this experiment was converted from the 0–4 to 0–9 scale to standardise data sets across all experiments (Ziems et al. 2014) and for subsequent GWAS analyses. Lines that depicted a LR response <7 on the 0–9 scale were considered resistant.

6.3.4 Field trials

The SSD lines in the diversity panel were subjected to LR screening in the field over a three-year period (2014, 2015, and 2016) at the Redlands Research Facility (27°31'40.8"S 153°15'05.7"E), Queensland, Australia, as detailed in Chapter 4. Six seeds of each SSD lines were sown as un-replicated hill plots, whereas four standards with known disease responses (i.e. Thatcher, Avocet, Avocet+*Lr34*, and Avocet+*Lr46*) were replicated five times throughout the test material to detect spatial variation in the nursery. About five weeks after sowing, the LR epidemic was initiated by transplanting rust-infected wheat (Morocco) seedlings into the field among the spreader rows. When the disease was sufficiently established on susceptible standards (i.e. Thatcher was scored 20 moderately susceptible to susceptible (MSS) in the field in 2014, 9 in the field in 2015, and 8 in the field in 2016), all SSD lines were assessed for LR response.

In 2014, the diversity panel was assessed for disease response in the LR nursery inoculated with *P. triticina* pathotype *pt* 104–1,2,3,(6),(7),11,13 (Table 6.2). The disease response for each line was assessed on a whole plot basis using the modified Cobb scale (Peterson et al. 1948). The disease severity data and IT were used to calculate the coefficient of infection (CI), as reported by Loegering (1959). Disease scoring was conducted at 70, 77, 86, and 96 days after sowing (DAS). Therefore, these multiple phenotypic data sets represent different time-points during the epidemic development in the nursery. The CI values of each disease score was then divided by 10 to convert to 0–9 scale. The converted scores were used to visualize the density distribution of LR response across phenotypic data sets.

In 2015, the LR nursery was inoculated with a mixture of two *P. triticina* pathotypes, namely *pt* 104–1,2,3,(6),(7),11,13 and *pt* 76–1,3,5,7,9,10,12,13+*Lr37* (Table 6.2). Plants were assessed on a whole plot basis for disease response three times during the season (i.e. 78, 85, and 101 DAS) using the 1–9 scale where 1 = very resistant and 9 = very susceptible, as reported by Bariana et al. (2007). In 2016, the LR nursery was inoculated with a mixture of three *P. triticina* pathotypes, namely *pt* 104–1,2,3,(6),(7),11,13, *pt* 76–1,3,5,7,9,10,12,13+*Lr37* and *pt* 104–1,3,4,6,7,8,10,12+*Lr37* (Table 6.2). Therefore, the 2016 nursery comprised the most virulent composition of *P. triticina* pathotypes compared to nurseries conducted in 2014 and 2015. Plants were evaluated for disease response twice (i.e. 71 and 84 DAS) using the 1–9 scale, as detailed above.

In all the field trials, a threshold for ‘resistance’ to LR was determined as any line depicting a disease response ≤5 based on the 1–9 scale, where resistance was deemed as “moderately resistant to moderately susceptible” (MRMS) or better. Each disease reading within a field environment was regarded as a unique phenotypic dataset and subsequently used for GWAS. The field phenotypic data sets were referred as Field_2014_1, Field_2014_2, Field_2014_3, Field_2014_4, Field_2015_1, Field_2015_2, Field_2015_3, Field_2016_1, and Field_2016_2.

6.3.5 Population structure, genetic diversity, and linkage disequilibrium

The population structure and genetic diversity for the diversity panel were previously described in Chapter 3. Briefly, population structure was estimated using the partitioning

around medoids clustering algorithm and 'Jaccard distance' in R (Team 2014). The optimal number of clusters (i.e. k=2) was determined using the 'fpc' package (Hennig 2014). Pairwise LD between markers was measured as r^2 (Hao et al. 2007). LD decay, the relationship between LD and genetic map distance between marker pairs in cM, was estimated as a locally estimated scatterplot smoothing (LOESS) curve and the LD cut–off threshold was set at r^2=0.1. The LD decay for the A, B, and D genomes was estimated for the whole population and the previously described clusters.

6.3.6 Genome-wide association analysis, allele stacking, and haplotyping

Genome-wide marker-trait associations were calculated for data from a total of 11 phenotypic data sets (seedling, AGC and the field trials), using the R package GenABEL (Aulchenko et al. 2007). The applied mixed linear model was adjusted for population stratification by including identity-by-state estimates (kinship-matrix) for genotype pairs and a principal component adjustment that uses the first four principal components as covariates. The significance cut-off value was arbitrarily set at −log10(P) = 3.5. Overlapping significant markers for different environments that were located at the same chromosomal position within a 5 cM window were considered the same QTL. Based on the predicted direction of the allele effect on the resistance score (e.g. negative effect ≙ resistance allele) (Table 6.3) we assigned resistance alleles for each significant marker.

To investigate an effect of accumulated alleles for LR resistance at the independent loci on the disease score we assigned the lines to groups, based on the absolute number of resistance-associated alleles possessed and compared their relative disease indices that were calculated as

$$\mathrm{LRi} = \sum_{k}^{n} \frac{individual\ Dis.\ score[k]}{mean\ Dis.\ score\ [k]}$$

where the disease index LRi is the accumulated relative value of a line's disease score in experiment *k* in relation to the population mean in this experiment *k* over all *n* field experiments. Lines with high indices (above 0) are relatively more susceptible to LR infection than lines with indices below 0.

Two QTL were selected for haplotype analysis and subsequent network analysis: 1) a seedling QTL on chromosome 3A (*qNV.Lr-3A.3*) because it was deemed a new QTL with large effect, and 2) a QTL conferring APR (*qNV.Lr-7B.2*) on the long arm of chromosome 7B which was detected across many phenotypic data sets and reported by numerous previous studies. Haplotypes for seedling resistance and APR were constructed on the basis of LD around the respective identified QTL on chromosomes 3A (*qNV.Lr-3A.3*) and 7B (*qNV.Lr-7B.2*). All surrounding markers with pairwise r^2-values >0.8 were included in the haplotype analysis, resulting in 7 and 22 haplotype variants, respectively. Haplotype networks, showing TCS genealogies between haplotype variants (Clement et al. 2000), were calculated using PopART (http://popart.otago.ac.nz.) (Leigh and Bryant 2015). The network nodes were coloured according to the average disease rating in the respective haplotype groups. A Tuckey's test was performed to test for significant phenotypic differences between the haplotype groups. The origin information for lines within each haplo-group was used to visualise the geographic distribution of these haplotypes in the diversity panel.

6.3.7 Alignment of QTL identified in this study with previously reported *Lr* genes and QTL

For comparison, QTL identified in this study and already catalogued *Lr* genes (McIntosh et al. 2017) were projected onto the common integrated map developed by Maccaferri et al. (2015) using MapChart software version 2.3 (Voorrips 2002). A QTL was considered potentially new if the genetic distance was ≥5 cM of the reported *Lr* gene or QTL. Eight recent GWAS studies (Kertho et al. 2015; Jordan et al. 2015; Gao et al. 2016; Li et al. 2016; Auon et al. 2016; Pasam et al. 2017; Turner et al. 2017; Kankwatsa et al. 2017) and two genomic prediction studies (Daetwyler et al. 2014; Juliana et al. 2017) using high-throughput marker platforms were only considered for QTL comparison.

6.3.8 *In-silico* annotation of significant markers

The genomic regions identified in this study were subjected to homology search for syntenic regions in Brachypodium distachyon and rice (*Oryza sativa* L.) genome. The marker sequences were annotated against the protein sequences to determine putative molecular functions, which could lead to the possible identification of candidates for disease resistance

across species. The homology search was performed using EnsemblPlants; http://plants.ensembl.org/index.html (Kersey et al. 2016).

6.4 Results

6.4.1 Disease response

Of the 288 lines in the diversity panel tested for LR response, 76.4% lines were seedling susceptible, and 23.6% lines were resistant (Figure 6.1a and b). At the adult stage under AGC, 46.5% lines were resistant, and 53.1% showed a susceptible response (Figure 6.1a; Supplementary material 4). In the field trials conducted in 2014, 63%, 75.35%, and 63.0% of the tested 284 lines were resistant at the first three disease assessments (70, 77, and 86 DAS). At the fourth disease assessment (96 DAS) when the lines were already at flag leaf stage, and the inoculum pressure in the nursery was highest, 71.1% lines displayed susceptibility, while only 28.8% lines displayed resistance (Figure 6.1a). In 2015, 29.8% of the evaluated 288 lines showed resistance and 70.1% demonstrated susceptibility at the first disease assessment (78 DAS), while only 9% of the lines showed a resistant disease response at the third reading (101 DAS) (Figure 6.1a). In 2016, of the 261 tested lines, 56.7% and 27.6% were resistant for the disease assessments performed at 71 and 85 DAS, respectively. The full description of disease responses observed for all lines in the diversity panel are provided in Supplementary Table 4.

6.4.2 Marker properties, population structure, and linkage disequilibrium

After filtering, a total of 10,748 polymorphic mapped markers along with three PCR-based markers for known APR genes (*Lr34*, *Lr46*, and *Lr67*) were used for LD analysis and GWAS. Lower marker density and marker coverage was evident for the D genome compared to A and B genomes. Analysis of population structure in the diversity panel was previously described In Chapter 3, where distinct clustering was observed on the basis of cultivation status and geographical origin. The diversity panel was divided into two clusters (k=2), containing 171 and 124 lines, respectively. Analysis of LD decay revealed strong differences between the three subgenomes. Overall, LD between marker pairs decayed quickly in the A and B genomes, especially in the latter, where the r^2 LOESS-curve never exceeded the threshold line. In contrast, LD in the D genome was very pronounced, and LOESS curves

did not drop below the threshold line until 19 cM for cluster 1 and 21 cM for cluster 2 (Figure 6.2).

6.4.3 Marker-trait associations

A total of 52 significant markers ($p < 0.001$) were associated with LR resistance (Table 6.3). Six markers were detected at the seedling stage and 46 markers at the adult stage (Table 6.3). Most of the significant markers (n=32) were detected in 2015 field environments. Manhattan plots depicting association between significant markers and LR response in different environments were displayed in Supplementary material 3. By considering chromosome position and LD between adjacent markers, a total of 31 QTL regions were assigned. These QTL were located on chromosomes 1A, 1B, 2A, 2B, 3A, 3B, 4A, 5A, 5B, 6A, 6B, 7A, 7B, and 7D (Table 6.3). Of the 31 QTL, 29 were associated with resistance at the adult stage and one QTL each was found to be associated with seedling (i.e. detected only at the seedling stage) and all-stage resistance (i.e. detected both at the seedling and adult stage) (Table 6.3). The QTL *qNV.Lr-2B.3* (all-stage resistance) and *qNV.Lr-7B.2* (adult-plant stage) were detected in many of the environments. The gene-specific marker *cssfr5* for known APR gene *Lr34* on chromosome 7D (Lagudah et al. 2009) was the only loci among the three PCR markers used that could be detected in GWAS with –log10 (p-value) between 3.9–6.19 for different field trials, designated QTL *qNV.Lr-7D* (Table 6.3).

Out of the 31 QTL in total, 13 were identified as being new LR resistance loci (Table 6.3). Among the other 18 QTL, 4 were co-located with the catalogued *Lr* genes, namely *Lr3* on chromosome 6B, *Lr64* on 6A, *Lr14* (a and b alleles), *Lr68*, *LrBi16* and *LrFun* on 7B, and *Lr34* on 7D (McIntosh et al. 2017; Table 6.3 and Figure 6.4). The remaining 14 QTL identified in our study were in alignment with the candidate regions reported in other GWAS studies (Table 6.3, Figure 6.4). An *in-silico* annotation of the identified significant markers showed that most sequences were uncharacterised regarding their molecular function (Table 6.3). However, 12 markers corresponded to the putative proteins carrying domains involved in disease resistance mechanism, such as leucine rich repeat (LRR), NB-ARC, P-loop_NTPase, Zinc finger, CCHC-type, RNA-dependent DNA polymerase, Protein kinase-like domain, Cyclin-like F-box, Galectin, carbohydrate recognition domain, Glycosyl transferase family 29, Glycosyl transferase family 31, Ran GTPase, Small GTP-binding protein, ABC transporter and Domain of unknown function-DUF1618 (Table 6.3).

6.4.4 Haplotype analysis and allele stacking

A new QTL (*qNV.Lr-3A.3*) on the long arm of chromosome 3A (116.7–117.0 cM) represented by two highly significant markers for seedling LR resistance (–log10(p-value) =6.26/4.1) which were in high LD (r^2=0.7), was selected for subsequent haplotype analysis (Table 6.3, Figure 6.5a). This large effect QTL was considered a new genomic region confering seedling resistance because it did not align with any previously reported *Lr* genes or QTL (Fig. 6.5b). Screening of allelic variation in our diversity panel resulted in seven different haplotype variants (*qNV.Lr-3A.3* - hap1-hap7), where hap1 was the most frequent variant in our diversity panel (frequency=92.5%) (Figure 6.5b). Hap2 was present in 4.7% of the lines while all other variants only occurred in 1% of the lines each. Inter-group comparisons of the disease responses for the first three haplotype groups, showed that hap1 was associated with a significantly higher susceptibility to LR (8 on a 0–9 scale) than hap2 and hap3, where the median disease response ranged between 3.6 and 5.5, respectively (Figure 6.5c). The lines carrying hap1 are geographically widespread and originate from 28 countries, including Russia (n=48), India (n=37), and Pakistan (n=30). The lines carrying hap2 were from Armenia (n=3), Azerbaijan (n=3), Russia (n=2), Pakistan (n=1), Ethiopia (n=1), and 5 were of unknown origin while hap3 was from Ukraine (n=1) and 2 were of unknown origin (Figure 6.5d). Interestingly, of the 14 lines carrying the resistant haplotype (hap2), only one line was deemed to also carry the known APR genes *Lr34* and *Lr46* (Table 6.4).

We also constructed a haplotype on the basis of the identified APR QTL *qNV.Lr-7B.2* on the long arm of chromosome 7B (126.0–130.6 cM) represented by 11 highly significant markers associated with LR resistance at the adult stage. Interestingly, several previously reported *Lr* genes and QTL have been reported in the region, including *Lr14* (a and b alleles) (Dyck and Sambroski 1970; Terracciano et al. 2013), *Lr68* (Herrera-Foessel et al. 2012), *LrBi16* (Zhang et al. 2011), and *LrFun* (Xing et al. 2014) (Figure 6.6a). Around the identified QTL, the five DArT-seq markers (i.e. 1207290, 1117456, 1214960, 1134022, and 2304335) in very high LD (r^2>0.75) were used for the haplotype analysis (Table 6.3; Figure 6.6a). In total, 22 haplotype variants were identified in our panel, of which hap1 and hap2 were the most frequent (78.3% and 7.8%, respectively). To construct the TCS haplotype network, only the variants which occurred at least twice in the panel (i.e. hap1-hap9) were used (Figure 6.6b). Tuckey's test and a comparison of median values for seven haplotypes showed that genotypes in hap1 were significantly susceptible to LR (8 on a 1–9 scale) in all

screenings of 2015 (Figure 6.6c). Four haplotypes (hap2-hap5) displayed less susceptibility across three phenotypic data sets in 2015, where the median value of each haplotype across phenotypic data sets was variable i.e. hap2 (5 to 8 on a 1–9 scale), hap3 (4 to7 on a 1–9 scale), hap4 (3 to 8 on a 1–9 scale) and hap5 (4 to 5.5 on a 1–9 scale) (Figure 6.6c). The lines carrying hap2 originated from Russia (n=4), India (n=2), Armenia (n=1), and 16 were of unknown origin. The lines carrying hap3 were from Russia (n=2), unknown of origin (n=2) and one each from Iraq, Spain, and India. The hap4 originated from China (n=2), and one each from Russia, India, and Ukraine. The hap5 was present in line from Pakistan (n=1) (Figure 6.6d).

To test the effect of an accumulation of alleles for LR resistance at the independent loci we assigned the lines from the diversity panel to groups, based on the absolute number of resistance-associated alleles possessed. This resulted in 13 different groups, ranging from two lines that carried ≤5 resistance-associated alleles, up to three lines that carried 29 or more (Figure 6.7). A comparison of their indices which represent the average LR response of a line in relation to the overall population evaluated in field trials from 2014–2016 revealed a very clear linear trend. While lines that combined relatively few of the identified resistance-associated alleles showed a comparatively strong negative mean response to LR, resistance was continuously increasing with the number of resistance-associated alleles. In total, 51 lines were detected that carry 19 or more resistance-associated alleles and show index levels largely below zero (Figure 6.7).

6.5 Discussion

6.5.1 New sources of LR resistance

Deployment of resistant cultivars is the most economical and effective method to control rust diseases in the field (Ellis et al. 2014). However, deployed resistance genes can easily be overcome due to the rapid evolution of the pathogen and limited genetic diversity for resistance factors in modern wheat germplasm. Crop domestication and later selective breeding in modern breeding programs have led to a dramatic loss of genetic diversity in many important crop species, such as rice (*Oryza sativa* var. japonica) (Huang et al. 2012), maize (*Zea mays* subsp. mays L.) (Wright et al. 2005) and hexaploid bread wheat (Reif et al. 2005). This imposes the constant need for new effective genetic resistance sources in

modern wheat breeding. The exploitation of genetic resources from the primary gene pool of wheat is considered as a promising approach to identify new and durable resistance factors that can be utilised for the improvement of modern high-yielding varieties (Mujeeb-Kazi et al. 2014). The primary gene pool includes wild and early domesticated relatives of wheat, landraces, old cultivars and breeding lines. The use of landraces compared to wild relatives is advantageous as they carry homologous chromosomes that can easily recombine with hexaploid wheat (Wulff and Moscou 2014). As advances in genotyping technologies provide high-throughput genome information at an unprecedented resolution and low costs, vast germplasm collections stored in many gene banks worldwide represent a rich and now accessible genetic treasure chest for useful diversity in modern wheat improvement (Voss-Fels et al. 2016). We have identified potential new genomic regions that are highly associated with LR resistance at seedling and adult stage in the Vavilov wheat diversity panel. Analysis of LD for the three genomes revealed that LD decayed rapidly in the A and B genomes in both population clusters, reflecting the high level of allelic diversity in the diversity panel. In our study, the highest LD was estimated for the D genome, which was also reported in numerous previous studies (Nielsen et al. 2014; Wang et al. 2014; Zegeye et al. 2014; Voss-Fels et al. 2015). Across experiments, the lines identified as resistant include landraces, cultivars and breeding lines originating from different countries of the world (i.e. Russia, Kazakhstan, India, Pakistan, Ukraine, and China), thus providing diverse sources of LR resistance to achieve durable disease resistance in various eco-geographic contexts (Gurung et al. 2014; Maccaferri et al. 2015). These resources harbor promising new resistances against rapidly evolving pathogens. For instance, in Australia, a recent exotic introduction of *P. triticina* pathotype *pt* 104–1,3,4,6,7,8,10,12+*Lr37* carried virulence on five *Lr* genes (*Lr12*, *Lr13*, *Lr20*, *Lr27+31*, and *Lr37*) which were widely deployed in cultivars (Cuddy et al. 2016; Park 2016). Thus, the identification of resistant lines in this study provided not only new LR resistance sources, but likely different alleles for already known genes, which can help to enhance genetic diversity in modern wheat breeding programs.

6.5.2 Alignment of putative QTL to previously reported *Lr* genes and QTL

A large number of QTL were detected in our study (n=31). Almost half (n=13) of the identified QTL were considered new, while the remainder (n=18) aligned with previously reported QTL and/or catalogued *Lr* genes. Interestingly, a locus corresponding to the seedling resistance gene *Lr3* on chromosome 6B was detected in our study, despite the use of *P. triticina*

pathotypes that were virulent for *Lr3*. This, suggests the probable presence of alternate alleles of the already 'extinct' resistance loci or a tightly linked gene with distinct resistance functions. The lack of availability of tightly linked or gene-specific markers for the known *Lr* genes hinders the ability to precisely position these genes on the respective chromosome. Further, allelism testing can also be performed to determine the association between the detected loci and previously reported genes and/or the QTL. A large number of QTL (n=30) were identified in GWAS using more than one adult phenotype data set. Of these, six QTL were detected across different adult phenotypic data sets. This might be due to the fact the genomic regions underpinning APR often interact with the plant growth stage, inoculum pressure and the temperature conditions, thus affecting the resistance phenotype. An interesting region identified across both seedling, AGC and field data sets was QTL *qNV.Lr-2B.3* on chromosome 2B, which contained seven associated markers. Within this genomic region, we identified several candidate genes (i.e. NB-ARC, P-loop_NTPase, Zinc finger, CCHC-type, and RNA-dependent DNA polymerase) that are known to encode proteins involved in pathogen recognition and subsequent activation of innate immune responses that lead to programmed cell death. It is well known that R genes tend to occur in clusters in plant genomes and give rise to many haplotypes via recombination (Friedman and Baker 2007; van Ooijen et al. 2008). Such 'hotspots' for resistance QTL could involve various combinations of classical R genes and other race-nonspecific genes (Burdon et al. 2014). A good example is the QTL region *qNV.Lr-7B.2* which contains seedling resistance gene *Lr14b* (Dyck and Sambroski 1970) and APR gene *Lr68* (Herrera-Foessel et al. 2012). It should be noted that QTL detected at the adult plant stage could also harbour genes regulating physiological characteristics, rather than classical R genes. For instance, in sorghum (*Sorghum bicolor* (L.) Moench), several QTL for resistance to rust (*Puccinia purpurea*) were found to co-locate with QTL for maturity and plant height (Wang et al. 2014). These genetic factors could offer durable resistance to rust diseases.

We compared the genomic location of QTL associated LR resistance reported in this study to those already reported in the recent GWAS studies and with catalogued *Lr* genes.

Chromosome 1A

The QTL *qNV.Lr-1A* (146.3 cM) located on chromosome 1A was detected using two *P. triticina* pathotypes in the field in 2015 (Field_2015_1) and did not align with any previously reported QTL or *Lr* genes, thus was considered new.

Chromosome 1B

The QTL *qNV.Lr-1B.1* (51.3 cM) was detected in the field in 2015 (Field_2015_2) and did not align with any previously reported QTL or *Lr* genes, thus was considered new. Another QTL *qNV.Lr-1B.2* on chromosome 1BL (269.3 cM) was detected in the field in 2016 (Field_2016_2). The chromosome arm 1BL carries *Lr* gene *Lr51*, which is a seedling resistance gene located within a 15–30 cM translocation from *T. speltoides* (Dovrak 1977; Helguera et al. 2005). As the gene is introgressed from a wild relative, it is unlikely to be the gene of interest in this hexaploid wheat diversity panel. The region (*qNV.Lr-1B.2*) also aligns with the locus *IWA6512* for resistance to THBL pathotype of *P. triticina* prevalent in North Dakota, USA (Kertho et al. 2015).

Chromosome 2A

The QTL *qNV.Lr-2A.1* (67.8 cM) was detected in the field in 2015 (Field_2015_1) and co-located with the locus *IWA3235*, which is associated with seedling resistance to *P. triticina* pathotype KFBJ prevalent in the USA (Turner et al. 2017). The short arm of chromosome 2A (2AS) harbours a previously known race-specific APR gene, *Lr37* (commonly known as *VPM1*). *Lr37* is located on a 25–38 cM translocation from *T. ventricosum* (2NS) (Bariana and McIntosh 1993). However, in the current study, we used *P. triticina* pathotypes virulent for *Lr37*. Thus, the APR mapped in this region is likely conferred by a different locus or allele. Two QTL *qNV.Lr-2A.2* (115.8 cM) and *qNV.Lr-2A.3* (123.7 cM) were detected in the field in 2014 (Field_2014_4) and in 2015 (Field_2015_3), respectively, and did not align with any previously reported QTL or *Lr* genes, thus were considered new.

Chromosome 2B

Two QTL *qNV.Lr-2B.1* (76.6 cM) and *qNV.Lr-2B.2* (86.2 cM) located on chromosome 2B were associated with resistance in the field in 2016 (Field_2016_1 and Field_2016_2, respectively). The QTL *qNV.Lr-2B.1* co-located with two loci *IWA5128* and *IWA207*, which are *a*ssociated with resistance at the seedling and adult stage in the USA, respectively (Turner et al. 2017). The QTL *qNV.Lr-2B.1* also co-located with loci *QLr.stars-2BL1* (*IWA1488*), which is associated with resistance at the seedling stage in the USA (Li et al. 2016). The QTL *qNV.Lr-2B.2* did not align with any previously reported QTL or *Lr* gene, thus

was considered new. A QTL *qNV.Lr-2B.*3 (107.0 cM) on chromosome 2B was associated with all stage resistance detected in seedling, accelerated growth conditions (AGC) and in the field (Field_2015_3). The QTL was in proximity to locus *IWA1668*, associated with resistance at the seedling stage in the USA (Turner et al. 2017). The long arm of chromosome 2B also carries two known *Lr* genes (i.e. *Lr35* and *Lr50*). *Lr35/Sr39* was introgressed from *Ae. speltoides* Tausch (chromosome 2S) to the short arm of chromosome 2B of bread wheat (Kerber and Dyck 1990; Friebe et al. 1996). *Lr50* is *a* seedling resistance gene introgressed from a wild relative of wheat *T. timopheevii* ssp. *armeniacum* (Brown-Guedira et al. 2003). However, the length and position of the alien segment is unclear, and we are unable to position the gene on our common integrated map.

Chromosome 3A

The QTL *qNV.Lr-3A.1* (47.7 cM) located on chromosome 3A was associated with resistance in the field in 2015 (Field_2015_3). The QTL co-located with three loci namely, *IWA3546*, *IWA8374*, and *IWA4851*, which are associated with resistance at the seedling stage against three *P. triticina* pathotypes in the USA (Kertho et al. 2015; Turner et al. 2017). Two QTL *qNV.Lr-3A.2* (109.5 cM) and *qNV.Lr-3A.3* (116.7–117.0 cM) located on chromosome 3A were associated with resistance in the field in 2014 (Field_2014_1) and at the seedling stage, respectively. Both of these regions did not align with any previously reported QTL or *Lr* genes. The chromosome 3A also carries two known *Lr* genes (i.e. *Lr63* and *Lr66*) (McIntosh et al. 2013). The *Lr* gene *Lr63* was introgressed from *T. monococcum* L. while *Lr66* was transferred from *Ae. speltoides* Tausch (Kolmer et al. 2010; Marais et al. 2010). As the gene is introgressed from a wild relative, it is unlikely to be the gene of interest in the current study.

Chromosome 3B

Four QTL were detected on chromosome 3B in our study. The QTL *qNV.Lr-3B.1* (5.9 cM) was detected in the field in 2014 (Field_2014_1) and did not align with any previously reported QTL or *Lr* genes, thus was considered new. The *qNV.Lr-3B.2* (49.2-53.6 cM) was detected at the adult stage in the field in 2014 (Field_2014_2) and in 2016 (Field_2016_1), The *qNV.Lr-3B.2* was co-located with locus *IWA6244*, which is associated with seedling resistance to *P. triticina* pathotype MCDL prevalent in the USA (Kertho et al. 2015). This

depicts that the region is associated with resistance at all stages of plant growth. The *qNV.Lr-3B.3* (60.4 cM) was associated with resistance in AGC. The QTL *qNV.Lr-3B.2* and *qNV.Lr-3B.3* were located in proximity to two loci *IWA6633* and *IWA2494,* which are associated with resistance at the seedling and adult stage in the USA (Turner et al. 2017). Both the regions were also co-located with locus wsnp_Ex_c6223_10857649, which is associated with adult stage resistance to *P. triticina* pathotypes prevalent in New South Wales, Australia (Daetwyler et al. 2014). The QTL *qNV.Lr-3B.4* (94.1 cM) was associated with resistance in the field in 2015 (Field_2015_3) and was co-located with locus *IWA8053*, which is associated with resistance to *P. triticina* pathotypes prevalent in the Australia (Kankwatsa et al. 2017).

Chromosome 4A

One QTL *qNV.Lr-4A* (133.9–135.1 cM) were detected in the field in 2014 (Field_2014_4). The QTL *qNV.Lr-4A.1* co-located with locus *IWB3569*, which is associated with resistance at the seedling and adult stage to *P. triticina* pathotype BBBQD present in the USA (Gao et al. 2016). The QTL is also co-located with *IWB4030,* which is associated with seedling resistance to LR pathotype MCDL in the USA (Kertho et al. 2015). Thus, the *qNV.Lr-4A* genomic region might harbour multiple loci and/or alleles conferring resistance at seedling and adult growth stages.

Chromosome 5A

A QTL *qNV.Lr-5A* (112.0 cM) was detected in AGC and co-located with locus *IWA7014*, which is associated with resistance to *P. triticina* pathotype TDBG in the USA (Kertho et al. 2015).

Chromosome 5B

Three QTL namely *qNV.Lr-5B.1*, *qNV.Lr-5B.2,* and *qNV.Lr-5B.3* were detected on chromosome 5B. The QTL *qNV.Lr-5B.1* (8.5 cM) was detected in AGC and co-located with locus *IWA6567*, which is associated with resistance at the seedling stage in the USA (Turner et al. 2017). Two QTL *qNV.Lr-5B.2* (37.9 cM) and *qNV.Lr-5B.3* (136.4 cM) were detected in

the field in 2015 (Field_2015_1), however, did not align with any previously reported QTL or *Lr* genes, thus were considered new.

Chromosome 6A

The QTL *qNV.Lr-6A.1* (9.4 cM) was detected in the field in 2014 (Field_2014_3 and Field_2014_4). Another QTL *qNV.Lr-6A.2* (27.5–28.0 cM) was detected in the field in 2015 (Field_2015_3). The QTL *qNV.Lr-6A.1* and *qNV.Lr-6A.2* were positioned in proximity with locus *IWB40242,* which is associated with resistance at the adult stage in the USA (Gao et al. 2016). The QTL *qNV.Lr-6A.3* (83.1 cM) was detected in the field in 2016 (Field_2016_2) and was mapped near to seedling gene *Lr64*. Notably, *Lr64* was transferred from *T. dicoccoides* to the 6A chromosome of bread wheat (Kolmer 2008; McIntosh et al. 2013). The region also co-located with locus *IWA260*, which is associated with resistance at the adult stage in the USA (Turner et al. 2017).

Chromosome 6B

Two QTL *qNV.Lr-6B.1* and *qNV.Lr-6B.2* on chromosome 6B were detected in AGC and in the field in 2014 (Field_2014_2), respectively. The *qNV.Lr-6B.1* (18.9 cM) did not align with any previously reported QTL or *Lr* genes, thus was considered new. The QTL *qNV.Lr-6B.*2 (79.8 cM) co-located with known *Lr* genes (i.e. *Lr3* and *Lr9*) (McIntosh et al. 2013). *Lr3* is a seedling resistance gene on the long arm of chromosome 6B of bread wheat, and three alleles are known, including *Lr3a*, *Lr3bg*, and *Lr3ka* (Haggag and Dyck 1973; McIntosh et al. 1995). *Lr9* is a seedling resistance gene positioned on a translocation from the wild relative of wheat *Ae. Umbellulata* (Sears 1956, 1961), however, it is unlikely to be present in this diversity panel. The region also co-located with two loci *IWB3292* and *IWB6474*, which are associated with resistance to *P. triticina* pathotypes BBBDB and BBNQD in the USA (Gao et al. 2016).

Chromosome 7A

Two QTL *qNV.Lr-7A.1* and *qNV.Lr-7A.2* were detected on chromosome 7A. The QTL *qNV.Lr-7A.1* (21.0 cM) was detected in the field in 2016 (Field_2016_2) and did not align with any previously reported QTL or *Lr* genes, thus was considered new. The QTL *qNV.Lr-*

7A.2 (40.3 cM) was detected in AGC. The loci was co-located with *Lr47,* which is a seedling gene introgressed from *T. speltoides* (7S) to the short arm of chromosome 7A of bread wheat (Dubcovsky et al. 1998). So the gene is unlikely to be the gene of interest in the diversity panel. The region also co-located with QTL *QLr.stars-7AS1* (*IWA3760*), which is associated with seedling resistance to *P. triticina* pathotype Pt52-2 in Oklahoma, USA (Li et al. 2016).

Chromosome 7B

Two QTL were detected on chromosome 7B in our study. The QTL *qNV.Lr-7B.1* (67.3 cM) was detected in AGC and did not align with any of the previously reported QTL or *Lr* genes, thus was considered new. The QTL *qNV.Lr-7B.2* (126.0-130.6 cM) was detected in the field in 2015 across multiple disease readings (Field_2015_1, Field_2015_2, and Field_2015_3). The QTL co-located with an important region known to harbour four *Lr* genes, including *Lr14*, *Lr68*, *LrBi16*, and *LrFun* (McIntosh et al. 2013). *Lr14* is a seedling resistance locus introgressed from *T. turgidum* and has two alleles, namely *Lr14a* and *Lr14b* (Dyck and Sambroski 1968; Herrera-Foessel et al. 2008). However, in the current study, we used pathotypes virulent for *Lr14a*. Thus, the APR mapped in this region is likely conferred by *Lr14b,* which is also closely linked to *Lr68*. *Lr68* is an APR gene on the long arm of chromosome 7B in bread wheat (Herrera-Foessel et al. 2012). On the other hand, *LrBi16* and *LrFun* are seedling resistance genes isolated from cultivars Bimai16 and Fundulea 900, respectively. The two genes *LrBi16* and *LrFun*, share the same chromosomal position. However, cultivar Bimai16 (*LrBi16*) was susceptible to *P. triticina* pathotype PHTT in China, whereas *LrFun* was resistant (Xing et al. 2014; Zhang et al. 2015). The QTL also co-located with two loci *IWA5000* and *IWA4803,* which are associated with resistance to *P. triticina* pathotypes prevalent in the USA (TDBG and BBBBD), respectively (Kertho et al. 2015; Turner et al. 2017).

Chromosome 7D

The QTL *qNV.Lr-7D* on chromosome 7D (56.6 cM) was associated with *cssfr5* - the gene-specific marker for catalogued APR gene *Lr34* (Lagudah et al. 2009), which is associated with resistance at the adult stage in the field (Krattinger et al. 2009; McIntosh et al. 2013).

The QTL was detected in the field in 2015 (Field_2015_1, Field_2015_2, and Field_2015_3) and in 2016 (Field_2016_2).

6.5.3 Haplotype analysis

In GWAS, single marker scans are performed to understand the underlying genetic architecture of disease resistance in natural populations. In addition, a more powerful approach is to perform a haplotype analysis based on closely linked markers which are more likely to be inherited together as a block (Hayes et al. 2007). Haplotype analyses which typically depict marker-trait associations at a higher resolution due to an increased information content compared to bi-allelic molecular markers, like SNPs or DArT markers have also been successfully applied in identifying genomic regions involved in effective *Fusarium* head blight resistance on wheat chromosome 3BS (Hao et al. 2012). In the present study, we performed haplotype analyses for two QTL, the seedling QTL *qNV.Lr-3A.3* and the APR QTL *qNV.Lr-7B.2*. Therefore, we followed previous studies and jointly defined markers in strong LD with the two identified QTL as a haplotype block (Hao et al. 2012; Diaz et al. 2011). Analysis of LD around the seedling QTL (*qNV.Lr-3A.3*) displayed a high level of LD between two associated markers, suggesting a high level of allelic fixation. One of the lines carrying the resistant haplotype (hap2) of QTL *qNV.Lr-3A.3* was also found positive to carry known APR genes *Lr34* and *Lr46*, thus providing a combination of R and APR genes/QTL. Such gene/QTL combinations are promising to achieve longer lasting resistance in elite cultivars. Similarly, out those markers located in the APR QTL *qNV.Lr-7B.2* on chromosome 7B, five markers located in a block with very high LD were considered a haplotype block. The results revealed a broad allelic variation for this chromosomal fragment and showed that four haplotype groups (hap2-hap5) were associated with a reduction in susceptibility across three phenotypic data sets in 2015. This might be explained by the fact that this chromosomal region is known to carry *Lr* genes such as *Lr14* (*a* and *b* alleles), *Lr68*, *LrBi16i,* and *LrFun* (McIntosh et al. 2017).

6.5.4 Pyramiding of resistance-associated alleles for durable rust resistance

It has been well described that durable rust resistance in wheat can be achieved by pyramiding multiple QTL (Ellis et al. 2014; Mundt 2014). In our study, a comparatively high number of loci with variable LR resistance in the field was detected with a high fraction of

lines carried more than 20 resistance-associated alleles. The detection of a large number of favourable alleles is in alignment with previously reported studies (Kollers et al. 2014; Naruoka et al. 2015; Muleta et al. 2017). The landraces are the traditional varieties which were selected by the farmers in the field preferably for agronomic traits but at the same time they also indirectly selected for disease resistance (Zeven 2002). Since the rust pathogen has coevolved with landraces for thousands of years in the same environment, therefore, diverse resistant alleles and their combinations exist in the host population keeping epidemic development in check (Thrall and Burdon 2000; Ordonez and Kolmer 2007). Since the landraces in the seed bank were removed from their environmental context, it is hypothesised that they might hold new allelic variations against the modern *P. triticina* pathotypes. Detection of a large number of resistance alleles showed that this resistance-associated allele has accumulated in landraces over time and occured in variable frequencies (i.e. high, low, and rare) in the population. In particular, rare alleles are known to provide resistance to diseases and environmental stresses (Vikram et al. 2016). Therefore, the introgression of landraces may greatly increase the genetic diversity and frequency of rare alleles into modern wheat breeding programs. In our study, we were able to show that there is a close relationship between the level of LR resistance and number of resistance alleles from independent loci, highlighting the high potential of allele stacking for rust improvement in future cultivars. Combining R and APR alleles is most effective and promising to provide sustainable resistance levels and also reducing the fitness cost associated with APR (Nelson 1978; Ellis et al. 2014; Consortium 2016). For example, durable resistance using combinations of resistances (seedling and APR) against stripe rust was achieved in Western Europe, while combining multiple minor genes provided durable resistance to stem rust and powdery mildew at The International Maize and Wheat Improvement Center (CIMMYT), Mexico (Singh et al. 2011; Basnet et al. 2014; Brown 2015; Ellis et al. 2014; Singh et al. 2014).

The detection of a large number of favourable alleles is promising. However, simultaneous consideration of all alleles in a breeding program is often challenging. To overcome this challenge a small subset of alleles can be targeted by designing specific crosses in a breeding program thus, supporting recombination of favourable alleles at many loci. Furthermore, implementing trait introgression via MAS allows selection for various traits in early generations and can easily eliminate undesirable allele combinations. Recent advancements in genomic approaches such as marker assisted backcrossing, whole-genome scans, genomic prediction and genomic selection enable the rapid combination of

multiple alleles in a single variety (Liu et al. 2014). Together with the latest advancements in plant phenotyping approaches and rapid generation advance systems, such as “speed breeding” (Watson et al. 2017), the breeding of rust resistant wheat cultivars can be accelerated and simplified (Hickey et al. 2012). In these phenotyping approaches, the individuals can be screened and selected by “phenotyping-on-the-go” during line development. The identified resistance can be rapidly introgressed using the rapid generation advance system in breeding programs. This approach could help fast-track the introgression of new LR resistance from the Vavilov wheat diversity panel into elite genetic backgrounds for future cultivars. This strategy is not limited to LR, as phenotyping methods adapted to speed breeding have been designed for other important traits in wheat, including stripe rust (Hickey et al. 2012), yellow spot (Dinglasan et al. 2016), seed dormancy (Hickey et al. 2009), and root architecture (Richard et al. 2015).

6.6 References

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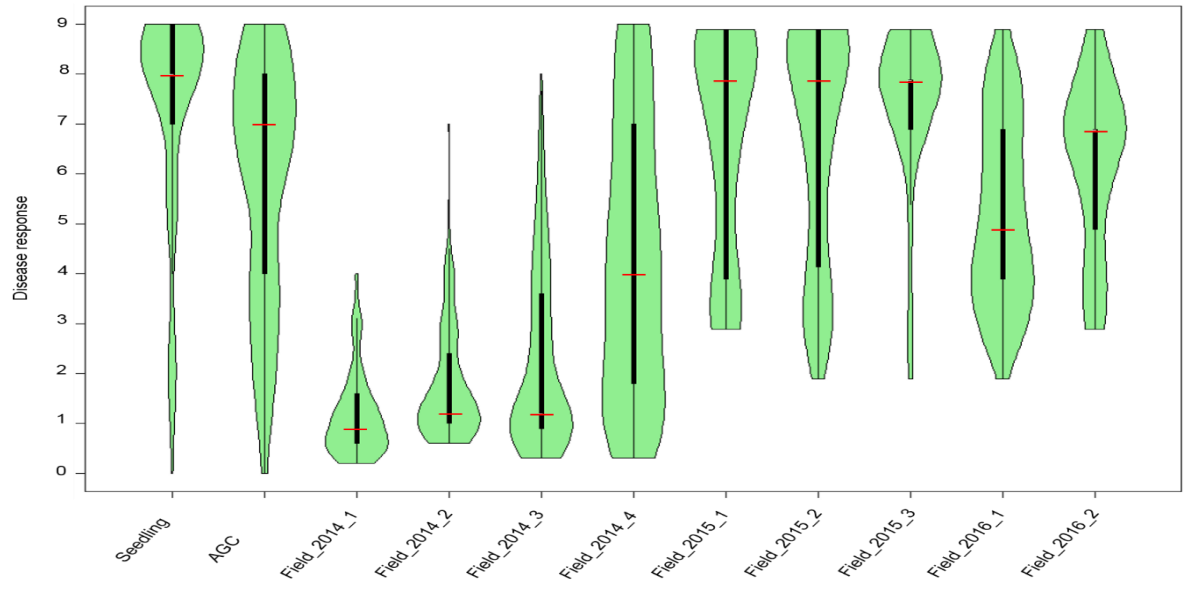
6.7 Figures



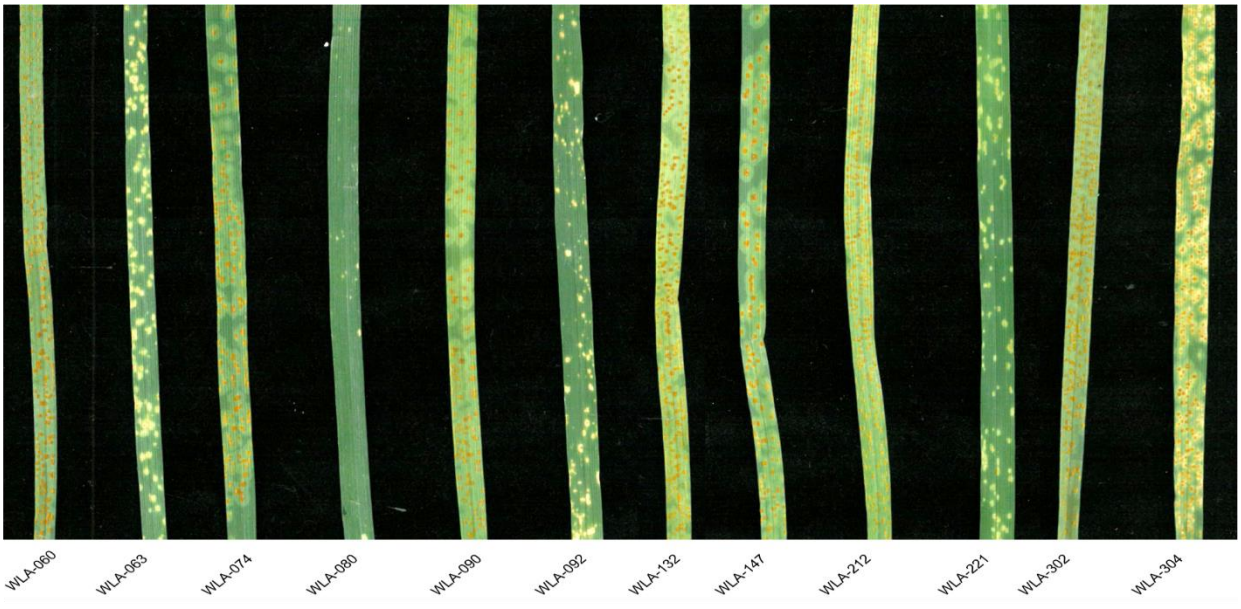


Figure 6.1 a) Violin plots illustrating the density distribution of leaf rust response for lines in the diversity panel based on 11 phenotypic datasets. The disease data for environments AGC and field (2014, 2015 and 2016) were converted on to the 0–9 scale (9 = very susceptible) to allow comparison across all datasets. The red line displays the median, the top and bottom of the thick vertical bars represent first and third quartiles, respectively, and the green fill shows disease density estimates (n=248). **b)** A sample of the seedling leaf rust responses observed for the diversity panel.

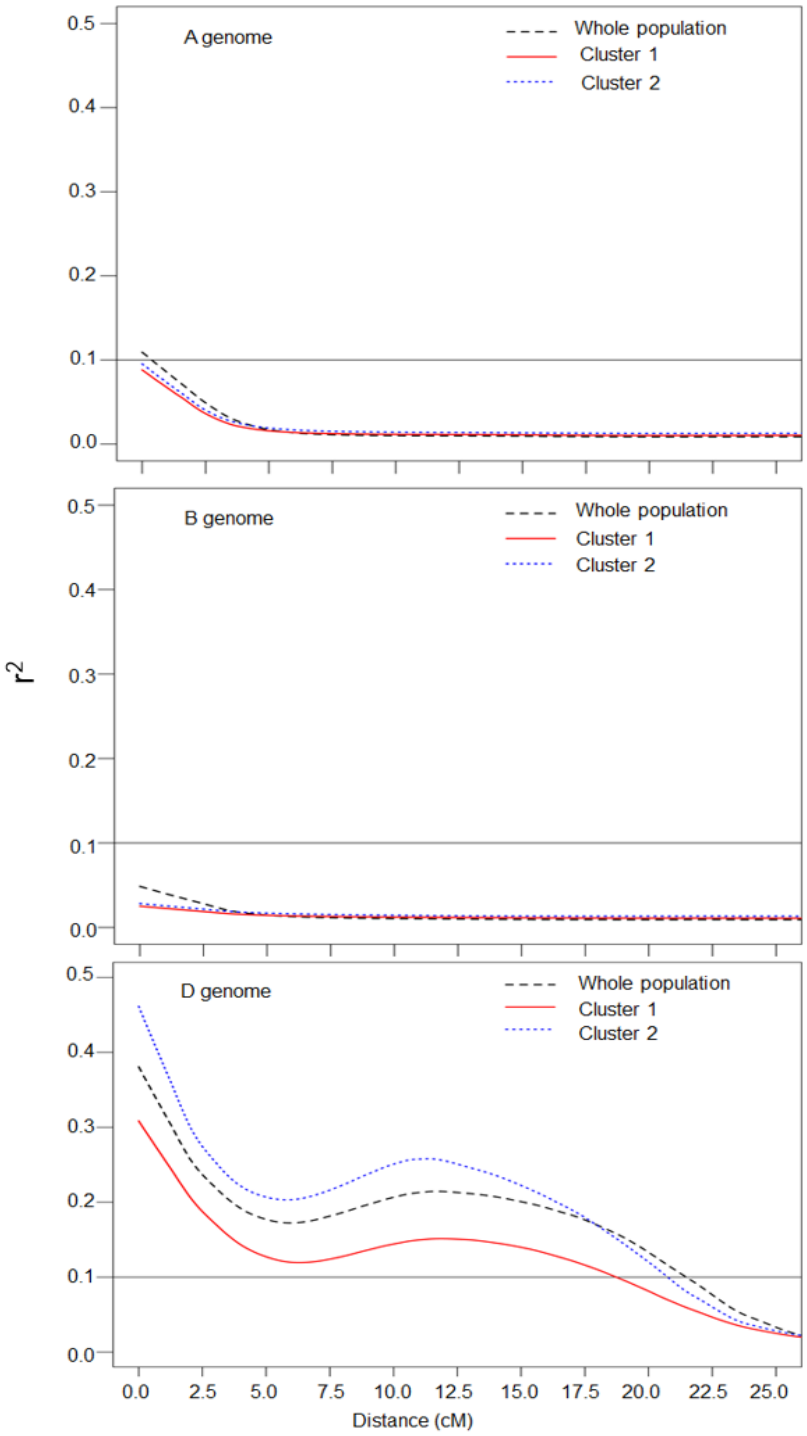


Figure 6.2 Linkage disequilibrium (LD) decay as a function of genetic distance (cM) in A, B and D genomes for the diversity panel. LD was estimated for the whole population (black dotted line), and cluster 1 (red line) and cluster 2 (blue dotted line) as defined in Chapter 3. The LD decay was the point where the locally estimated scatterplot smoothing (LOESS) curves intersect the LD, whereas the threshold for LD decay was at $r^2 = 0.1$ (black line).

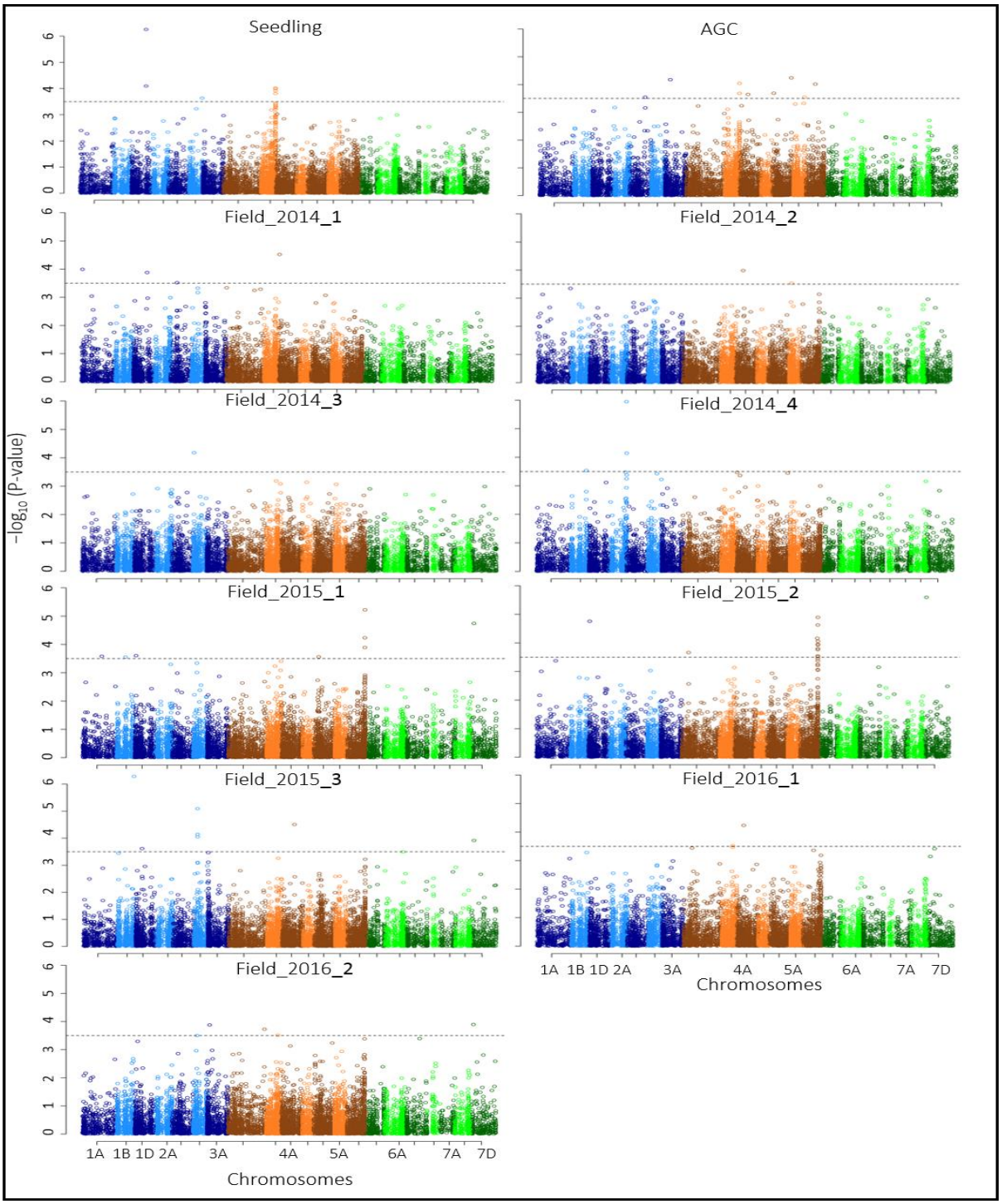
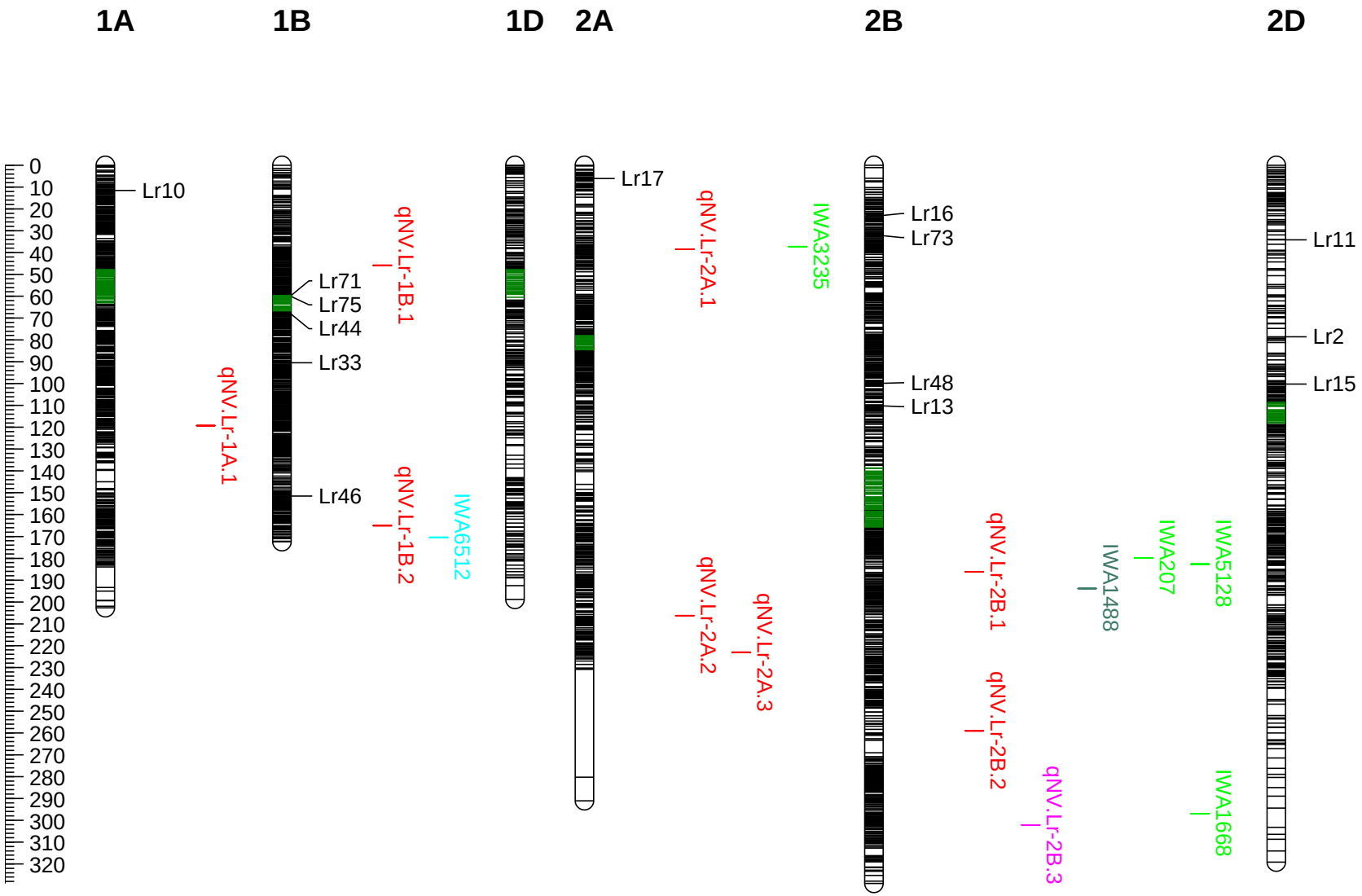


Figure 6.3 Manhattan plots are displaying results from genome-wide association studies for leaf rust resistance in the Vavilov wheat collection. Associations are displayed for the 11 phenotypic data sets used in this study. The dotted line represents the threshold for significant marker-trait associations (–log10 P-value >3.5).


1A
1B
1D
2A
2B
2D
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290
300
310
320
Lr10
qNV.Lr-1A.1
Lr71
Lr75
Lr44
Lr33
Lr46
qNV.Lr-1B.1
qNV.Lr-1B.2
IWA6512
Lr17
qNV.Lr-2A.1
qNV.Lr-2A.2
qNV.Lr-2A.3
IWA3235
Lr16
Lr73
Lr48
Lr13
qNV.Lr-2B.1
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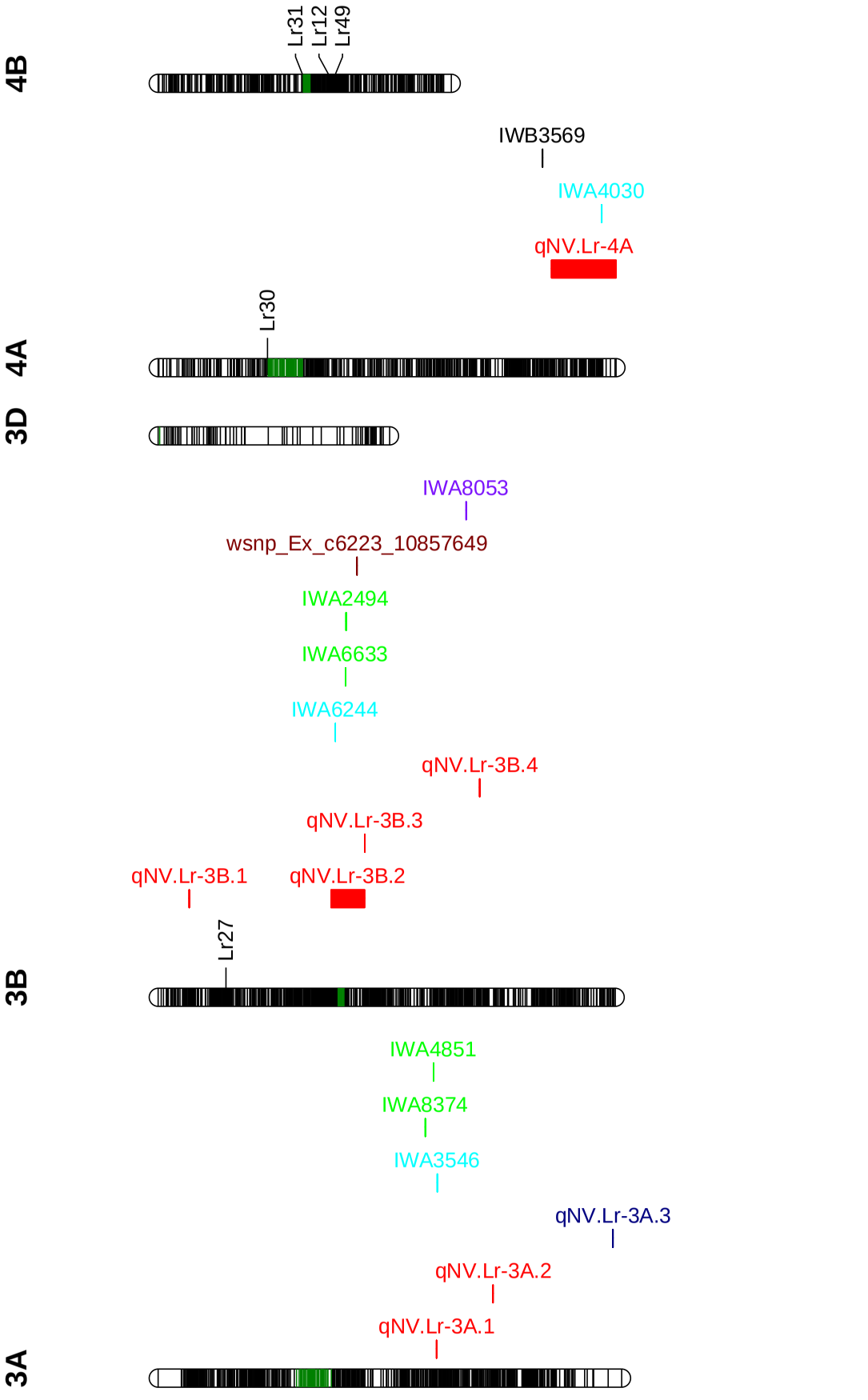
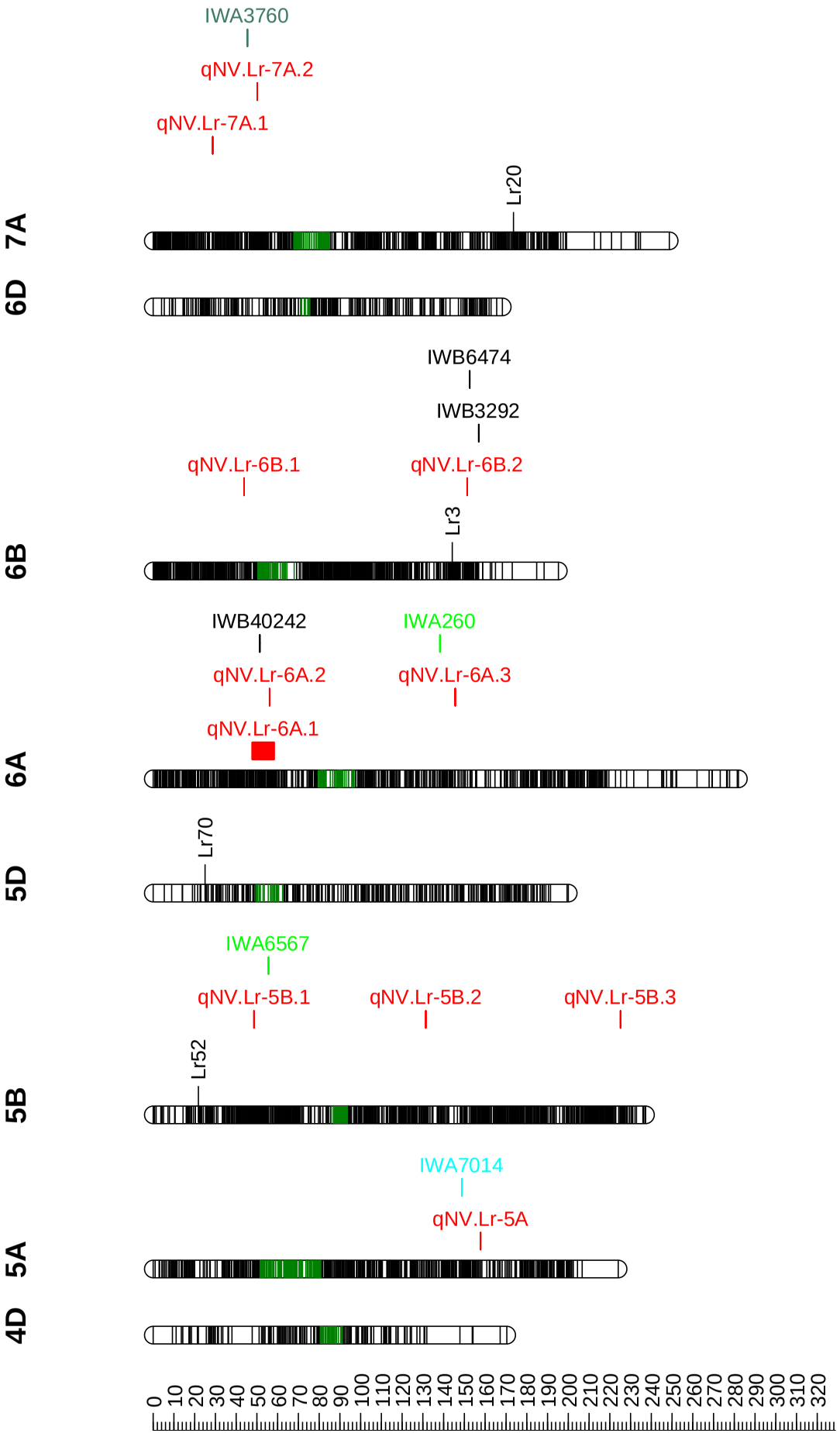
IWA5128
IWA1668
Lr11
Lr2
Lr15


4B
Lr31
Lr12
Lr49
IWB3569
IWA4030
qNV.Lr-4A
4A
Lr30
3D
IWA8053
wsnp_Ex_c6223_10857649
IWA2494
IWA6633
IWA6244
qNV.Lr-3B.4
qNV.Lr-3B.3
qNV.Lr-3B.1
qNV.Lr-3B.2
3B
Lr27
IWA4851
IWA8374
IWA3546
qNV.Lr-3A.3
qNV.Lr-3A.2
qNV.Lr-3A.1
3A
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290
300
310
320


IWA3760
qNV.Lr-7A.2
qNV.Lr-7A.1
Lr20
7A
6D
IWB6474
IWB3292
qNV.Lr-6B.1
qNV.Lr-6B.2
Lr3
6B
IWB40242
IWA260
qNV.Lr-6A.2
qNV.Lr-6A.3
qNV.Lr-6A.1
6A
Lr70
5D
IWA6567
qNV.Lr-5B.1
qNV.Lr-5B.2
qNV.Lr-5B.3
Lr52
5B
IWA7014
qNV.Lr-5A
5A
4D
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320

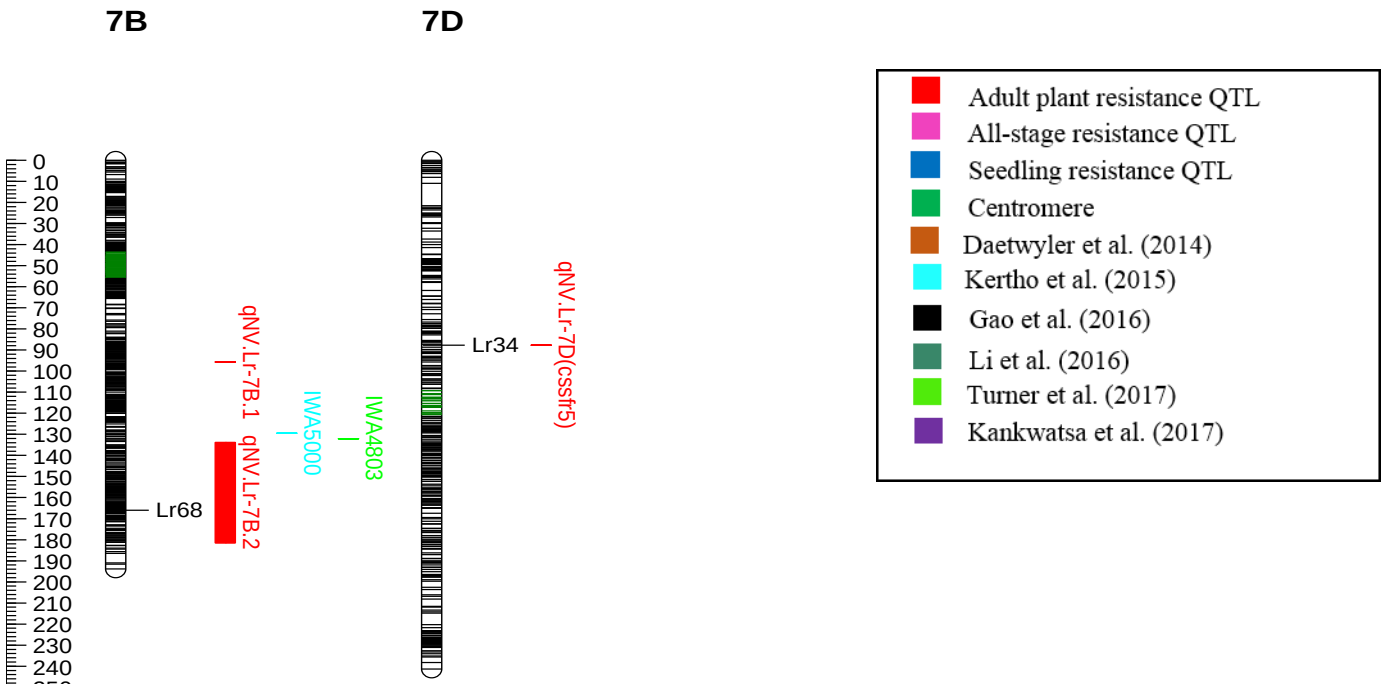


Figure 6.4 Genomic regions associated with leaf rust resistance in the wheat diversity panel projected on the common integrated map developed by Macafferri et al. (2015). The quantitative trait loci (QTL) from the present study are depicted in legend. To determine the co-location of potentially new loci, previously known QTL and catalogued *Lr* genes were projected on the integrated map using markers in common between the DArT-seq consensus map (version 4.0 provided by Dr. Andrezj Kilian) and the common integrated map developed by Macafferri et al. (2015), using the 'bridge marker' technique outlined by Mace et al. (2009). All catalogued *Lr* genes from wild relatives of wheat were not projected on the map due to unknown length of alien segments (i.e. *Lr9*, *Lr14a*, *Lr19*, *Lr21*, *Lr22a*, *Lr23*, *Lr24*, *Lr25*, *Lr26*, *Lr28*, *Lr29*, *Lr32*, *Lr35*, *Lr36*, *Lr37*, *Lr38*, *Lr39*, *Lr40*, *Lr41*, *Lr42*, *Lr45*, *Lr47*, *Lr50*, *Lr51*, *Lr54*, *Lr55*, *Lr56*, *Lr57*, *Lr58*, *Lr59*, *Lr61*, *Lr62*, *Lr63*, *Lr64*, *Lr65*, *Lr66*, *Lr72*, and *Lr76*).

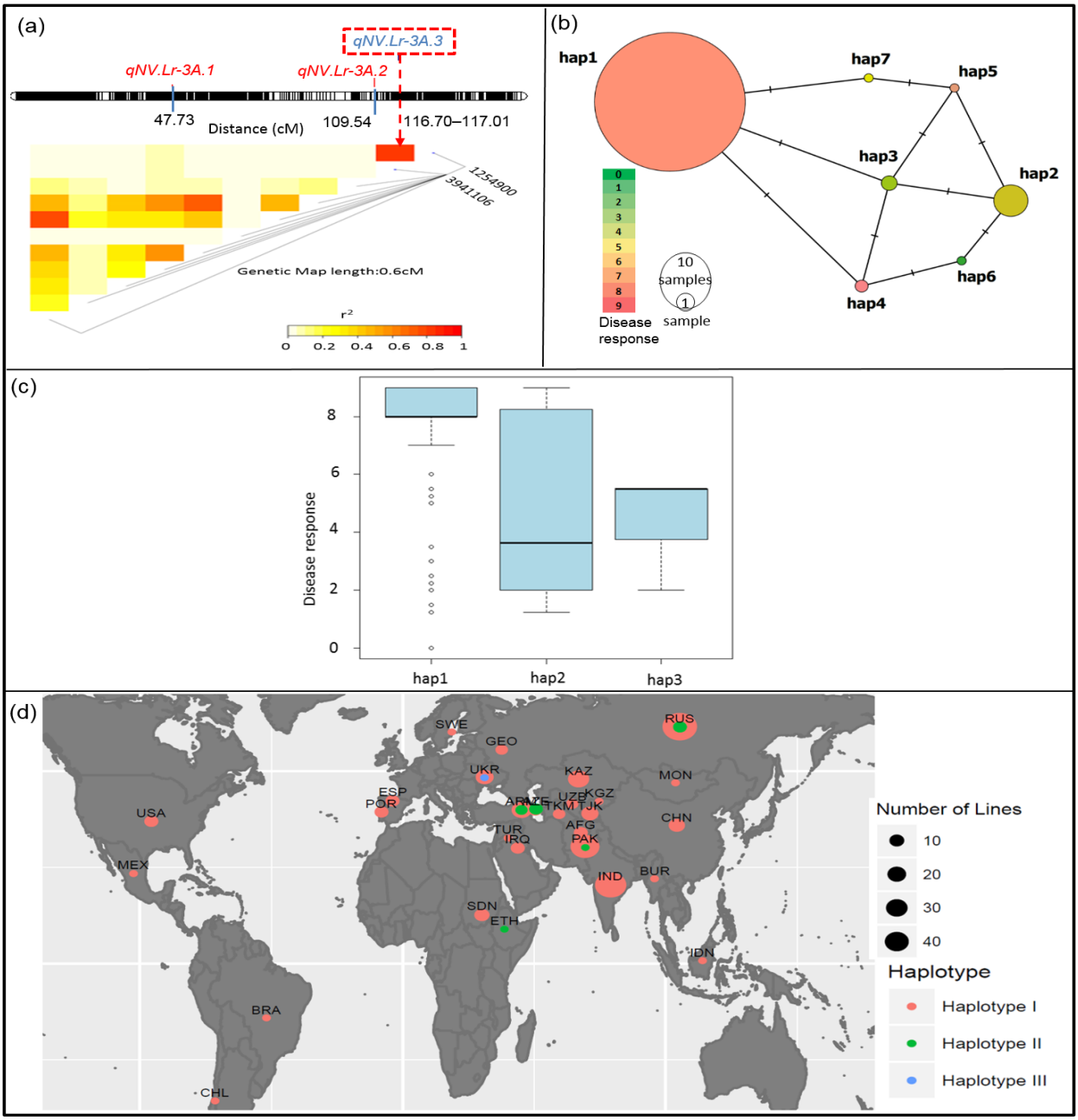


Figure 6.5 Haplotype analysis of QTL *qNV.Lr-3A.3* on chromosome 3A associated with resistance to leaf rust at the seedling stage. **a)** Chromosomal position of QTL *qNV.Lr-3A.3* (116.7–117.0 cM based on the DArT-seq consensus map version 4.0 provided by Dr. Andrezj Kilian) and linkage disequilibrium for associated markers. **b)** Haplotype network displaying 7 haplotype variants, where the size of the node is proportional to the number of lines carrying that haplotype variant while color indicates the mean disease response for those lines (0–9 scale, where 9 = very susceptible). **c)** Boxplots displaying the disease response for the lines carrying the three most common haplotypes. **d)** The geographic distribution of the three most common haplotypes in the diversity panel.

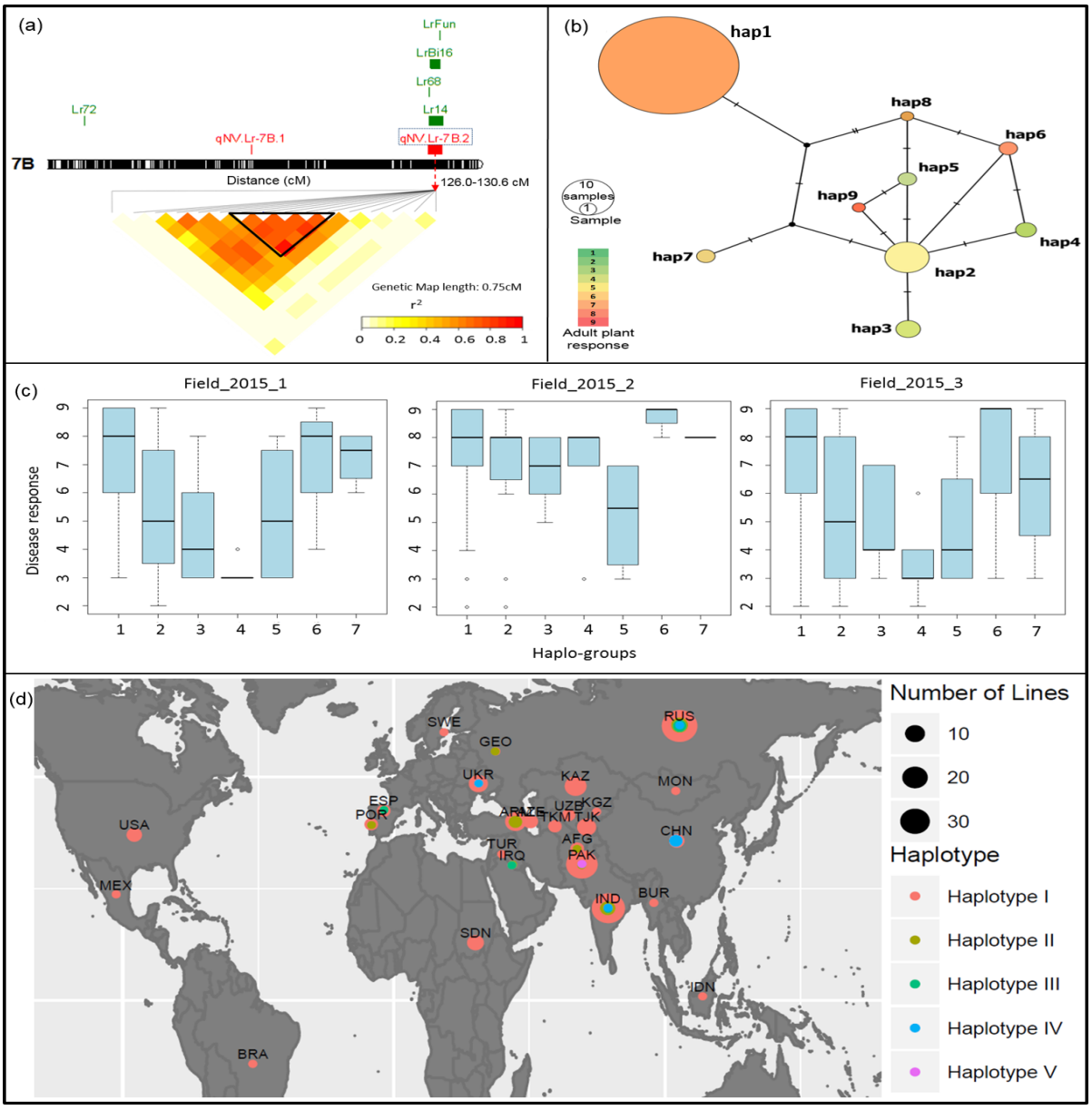
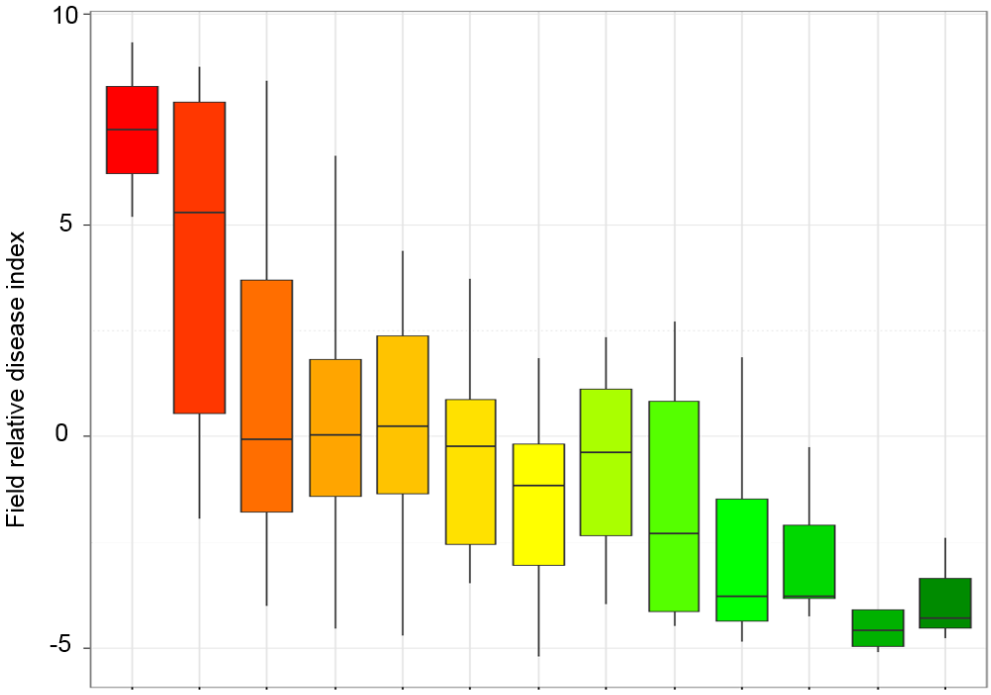


Figure 6.6 Haplotype analysis of QTL *qNV.Lr-7B.2* on chromosome 7B associated with resistance to leaf rust at the adult plant stage. a) Chromosomal position of the QTL *qNV.Lr-7B.2* (128.6–130.6 cM based on the DArT-seq consensus map version 4.0 provided by Dr. Andrezj Kilian) and comparison with catalogued *Lr* genes. The linkage disequilibrium block highlighted for the five associated markers. **b)** Haplotype network displaying the 9 most common haplotype variants, where the size of the node is proportional to the number of lines carrying that haplotype variant while colors indicate mean disease response for those lines (1–9 scale, where 9 = very susceptible). **c)** Boxplots displaying the disease response by lines carrying seven most common haplotypes in three phenotypic datasets in 2015. **d)** The geographic distribution of the five most common haplotypes in the diversity panel.



Number of resistance alleles	≤5	≤7	≤9	≤11	≤13	≤15	≤17	≤19	≤21	≤23	≤25	≤27	29/31
Frequency (n)	2	15	45	86	52	25	19	9	12	14	5	8	3

Figure 6.7 The effect of resistance-associated favourable alleles at quantitative trait loci for resistance to leaf rust response in the diversity panel. The field relative disease index is calculated using phenotypic data sets from field environments only. The frequency of lines carrying favourable alleles is also presented.

6.8 Tables

Table 6.1 Virulence and avirulence profile of leaf rust pathotypes used in this study.

Leaf rust pathotype*	Virulent on genes	Avirulent on genes
104–1,2,3,(6),(7),11,13	*Lr1, Lr3a, Lr14a*, *Lr16*, *Lr17a***, *Lr20*, *Lr24, Lr27+31***	*Lr2a, Lr3ka, Lr13, Lr15, Lr17b, Lr23*, *Lr26*, *Lr28*, *Lr37*
76–1,3,5,7,9,10,12,13+*Lr37*	*Lr3a, Lr3ka*, *Lr13*, *Lr14a*, *Lr16*, *Lr17a*, *Lr17b*, *Lr20*, *Lr24*, *Lr26*, *Lr27+31, Lr37*	*Lr1, Lr2a, Lr15*, *Lr23*, *Lr28*
104–1,3,4,6,7,8,10,12+*Lr37*	*Lr1, Lr3a, Lr12, Lr13, Lr14a, Lr15, Lr17a*, *Lr17b*, *Lr20*, *Lr27+31, Lr28, Lr37*	*Lr2a, Lr3ka, Lr16, Lr23, Lr24, Lr26*

* The virulence/avirulence status of the leaf rust pathotype was reported by Park (2016)

** Pathotype is partially virulent on the gene

Table 6.2 Summary of experiments performed in this study at the seedling and adult stage for scoring leaf rust response across years and pathotypes used.

Growth stage	Environment	Year tested	Phenotypic data sets	Number of lines assessed (n)	Leaf rust pathotypes
Seedling	Glasshouse	2014	Seedling	288	104–1,2,3,(6),(7),11,13
Adult	AGC*	2014	AGC	288	104–1,2,3,(6),(7),11,13
	Field	2014**	Field_2014_1 Field_2014_2 Field_2014_3 Field_2014_4	284	104–1,2,3,(6),(7),11,13
		2015**	Field_2015_1 Field_2015_2 Field_2015_3	288	104–1,2,3,(6),(7),11,13; 76–1,3,5,7,9,10,12,13+ *Lr37*
		2016**	Field_2016_1 Field_2016_2	261	104–1,2,3,(6),(7),11,13; 76–1,3,5,7,9,10,12,13+ *Lr37*; 104–1,3,4,6,7,8,10,12+ *Lr37*

* Accelerated growth conditions

** Multiple phenotypic datasets were recorded in each of the field environment

Table 6.3 Summary of the leaf rust resistance QTL identified at both the seedling and adult plant stage in the diversity panel.

QTL name	Marker	Chr.	Position (cM)[a]	Phenotypic datasets[b]	–log$_{10}$ (p-value)	Allele for res[c]	Effect on trait	Resistance growth stage	Co-located *Lr* gene	Gene annotation
qNV.Lr-1A	1133392	1A	146.3	Field_2015_1	3.58	1	-0.73	Adult	[g]	-
qNV.Lr-1B.1	1219818	1B	51.3	Field_2015_2	3.66	0	-0.89	Adult	[g]	-
qNV.Lr-1B.2	2276699	1B	269.3	Field_2016_2	3.73	1	-0.61	Adult	[f]	-
qNV.Lr-2A.1	1164339	2A	67.8	Field_2015_1	3.56	1	-0.63	Adult	[f]	-
qNV.Lr-2A.2	1242099	2A	115.8	Field_2014_4	3.54	1	-7.97	Adult	[g]	-
qNV.Lr-2A.3	1687763	2A	123.6	Field_2015_3	6.28	1	-1.22	Adult	[g]	-
qNV.Lr-2B.1	1232931	2B	76.6	Field_2016_1	3.52	1	0.59	Adult	[f]	P-loop_NTPase
qNV.Lr-2B.2	1140050	2B	86.2	Field_2016_2	3.51	1	0.47	Adult	[g]	-
qNV.Lr-2B.3	1092839	2B	107.0	Seedling	4.02	1	-0.78	All stage	[f]	-
	1126885			Seedling	4.00	1	-0.76			NB-ARC , P-loop_NTPase
	2290750			Seedling	3.92	1	-0.80			Zinc finger, CCHC-type
	1109454			Seedling	3.81	1	-0.75			-
	1686496			AGC	4.05	1	-0.61			RNA-dependent DNA polymerase
	1095829			AGC	3.68	1	-1.10			-
	1112316			Field_2015_3	4.16	1	-0.81			-
qNV.Lr-3A.1	1126760	3A	47.7	Field_2015_3	3.62	1	-0.52	Adult	[f]	-
qNV.Lr-3A.2	1116501	3A	109.5	Field_2014_1	3.88	1	3.23	Adult	[g]	-

qNV.Lr-3A.3	1254900	3A	116.7	Seedling	4.10	1	-1.23	Seedling	g	-
	3941106		117.0	Seedling	6.26	1	-1.36			-
qNV.Lr-3B.1	1203924	3B	5.9	Field_2014_1	4.52	1	2.31	Adult	g	Domain of unknown function DUF1618
qNV.Lr-3B.2	1101980	3B	49.2	Field_2016_1	4.23	1	0.56	Adult	f	-
	1107825		53.6	Field_2014_2	3.98	1	-3.31			-
qNV.Lr-3B.3	1696461	3B	60.4	AGC	3.65	0	-1.07	Adult	f	LRR, Protein kinase-like domain
qNV.Lr-3B.4	1127641	3B	94.1	Field_2015_3	4.51	1	-0.92	Adult	f	-
qNV.Lr-4A	1266809	4A	133.9	Field_2014_4	5.95	1	11.04	Adult	f	-
	1122735		135.1	Field_2014_4	4.15	1	9.40			-
qNV.Lr-5A	1118643	5A	112.9	AGC	3.55	1	-0.93	Adult	f	-
qNV.Lr-5B.1	1139539	5B	8.5	AGC	3.69	1	-1.16	Adult	f	-
qNV.Lr-5B.2	1085450	5B	37.9	Field_2015_1	3.56	1	-0.53	Adult	g	-
qNV.Lr-5B.3	1106570	5B	136.4	AGC	4.24	1	0.64	Adult	g	Glycosyl transferase, family 31, Galectin, carbohydrate recognition domain
qNV.Lr-6A.1	1138518	6A	9.4	Field_2014_4	4.02	1	0.33	Adult	f	Cyclin-like F-box
				Field_2014_3	4.18	1	5.06			
qNV.Lr-6A.2	1056198	6A	27.5	Field_2015_3	4.05	0	-0.55	Adult	f	-
	1242792		27.8	Field_2015_3	4.15	1	-0.96			-
	1308654		28.0	Field_2015_3	5.09	1	-1.02			Glycosyl transferase family 29

qNV.Lr-6A.3	3950708	6A	83.0	Field_2016_2	3.50	1	0.41	Adult	*Lr64*	-
qNV.Lr-6B.1	1228013	6B	18.9	Field_2014_2	3.54	1	2.59	Adult	[g]	-
qNV.Lr-6B.2	2341955	6B	79.7	AGC	3.55	1	-1.61	Adult	*Lr3*	LRR, NB-ARC, P-loop_NTPase, Zinc finger
qNV.Lr-7A.1	3021391	7A	21.0	Field_2016_2	3.88	0	-0.43	Adult	[g]	P-loop_NTPase, Ran GTPase, Small GTP-binding protein
qNV.Lr-7A.2	3384641	7A	40.3	AGC	4.18	0	0.64	Adult	[f]	-
qNV.Lr-7B.1	1119801	7B	67.3	AGC	4.02	1	-0.69	Adult	[g]	-
qNV.Lr-7B.2	1140798	7B	126.0	Field_2015_2	4.17	1	-0.88	Adult	*Lr14b,*	-
	2303264		128.6	Field_2015_2	3.77	1	-0.82		*Lr68,*	-
	1073236			Field_2015_2	3.55	1	-0.82		*LrBi16,*	-
	1207290		129.9	Field_2015_2	3.80	1	-0.83		*LrFun*	-
	1048655		130.6	Field_2015_1	4.24	1	-0.85			-
	1200909			Field_2015_1	3.90	1	-0.87			-
				Field_2015_2	3.53	1	-0.81			
	1079125			Field_2015_2	4.63	1	-0.93			-
	1117456			Field_2015_3	4.91	1	-0.91			-
				Field_2015_2	4.90	1	-0.93			
	1214960			Field_2015_1	5.23	1	-0.96			-
				Field_2015_2	3.93	1	-0.83			
	1134022			Field_2015_2	4.07	1	-0.89			-
	2304335			Field_2015_2	3.95	1	-0.83			-

qNV.Lr-7D	*cssfr5*[d]	7D	56.6	Field_2015_3	6.19	1	-1.70	Adult	*Lr34*	ABC transporter
				Field_2015_1	4.74	1	-1.53			
				Field_2015_2	5.60	1	-1.64			
				Field_2015_3	3.92	1	-0.96			
				Field_2016_2	3.90	1	-1.03			

[a] The markers were positioned on the latest DArT-seq consensus map (version 4.0) provided by Dr. Andrzej Kilian (Diversity Arrays Technology, Pty, Ltd).

[b] LR pathotype used in seedling, AGC and in the field in 2014 *pt* 1041,2,3,(6),(7),11,13; 2015 - *pt* 104–1,2,3,(6),(7),11,13 and *pt* 76–1,3,5,7,9,10,12,13+*Lr37*; 2016 – 104–1,2,3,(6),(7),11,13; 76–1,3,5,7,9,10,12,13+*Lr37* and 104–1,3,4,6,7,8,10,12+ *Lr37*

[c] Allele for resistance, where 1 = present, 0 = absent

[d] *cssfr5* was projected using closest DArT marker *wPt-7171*

[e] Alignment with catalogued *Lr* genes

[f] Alignment with previously reported QTL from eight GWAS and two genomic prediction studies using high-throughput marker platforms

[g] QTL new to this study.

Table 6.4 Leaf rust phenotypic response and APR gene information of lines in the diversity panel carrying resistance haplotype (hap2) of QTL *qNV.Lr-3A.3* on chromosome 3A.

UQ SSD line number	Cultivation status	Marker associated with *qNV.Lr-3A.3*			Seedling	*Lr* APR
		1254900	3941106	Sequence		
WLA-007	-	1	1	11	NA	-
WLA-011	-	1	1	11	8.5	-
WLA-043	Landrace	1	1	11	6	-
WLA-117	-	1	1	11	8	-
WLA-125	-	1	1	11	5	-
WLA-165	-	1	1	11	2.25	-
WLA-176	-	1	1	11	1.5	-
WLA-231	Landrace	1	1	11	8.5	-
WLA-250	Breeding Line	1	1	11	2	-
WLA-253	-	1	1	11	2	*Lr46/Lr34*
WLA-275	-	1	1	11	NA	-
WLA-303	Breeding Line	1	1	11	2	-
WLA-309	Landrace	1	1	11	9	-
WLA-315	Landrace	1	1	11	1.25	-

Chapter 7 - General Discussion

7.1 Context

Global food production has shown a significant increase since the 'Green Revolution', which led to the massive adoption of short, early maturing and high yielding rice and wheat cultivars. This process saved billions of lives from starvation and helped to maintain food security especially in developing countries such as India, Pakistan and Bangladesh. To further enhance and sustain wheat yields across diverse environments this breeding strategy became the biggest focus of wheat breeding programs around the world. This has led to an intensive selection for fewer genetic loci and ultimately has reduced genetic diversity in modern wheat germplasm, which may limit future increases in wheat yields (Fu et al. 2015). Continued increases in wheat yields are essential to feed a rapidly growing human population. It is anticipated the human population will exceed 9 billion by the year 2050 (UN 2015). To feed such a large population would be the greatest challenge ever faced by our global food production systems. Moreover, climate change is posing a constant threat to wheat yield, in particular, due to increases in global temperature and more frequent drought episodes, not to mention the possible emergence of new pests and pathogens (Chakraborty and Newton 2011; Chaves et al. 2013; Asseng et al. 2015).

Wheat breeding programs are largely focused on improving wheat yields by enhancing their adaptability to heat and drought stresses. While gains need to be made for yield and abiotic tolerance, yield potential and stability can only be realised if cultivars are deployed with effective genetic resistance to diseases. Also, breeding progress for genetic resistance is compromised if durable forms of resistance are not adopted, because the pathogens can easily undergo genetic variation and overcome deployed race-specific resistances, sometimes even before they are deployed in a cultivar. A good example is the wheat cultivars, Mitch and Dart, in Australia which were released after the new virulent *P. triticina* pathotypes 76–1,3,5,7,9,10,12+*Lr37* and 104–1,3,4,6,7,8,10,12+*Lr37* had rendered them susceptible to LR before even deployed in the farmer's field (NVT 2015; Cuddy et al. 2016; Park 2016). Thus, the identification of resistant lines in this study provides not only new LR resistance sources but also different alleles for already known adult plant resistance (APR) genes, which can help to boost diversity for resistance factors in modern wheat germplasm.

7.2 Overview of research achievements

The present study identified new sources of durable forms of resistance to LR from a bread wheat diversity panel from N. I. Vavilov Institute of Plant Genetic Resources (VIR), in St Petersburg, Russia. Moreover, new insights and tools are also provided to exploit new sources of LR resistance in breeding programs. Chapter 3 reported the first genetic characterisation of wheat accessions from the VIR. The genotyping and partitioning around medoids (PAM) cluster analysis revealed a huge array of new alleles that are unique to the Vavilov diversity panel, which are either fixed or absent in a subset of modern breeding materials from Australia and the International Maize and Wheat Improvement Center (CIMMYT). The analysis also revealed that accessions classified as landraces in the diversity panel were more diverse than accessions classified as cultivars or breeding lines. Notably, the most diverse were landraces collected from India and Pakistan. Overall, the diversity panel harbours an enormous amount of genetic diversity, which is anticipated to provide new allelic variations for different traits such as disease resistance and drought tolerance. The seed source and marker information for this diversity panel is now provided as open-access to the scientific community.

Chapter 4 reported a new method to rapidly phenotype APR to leaf rust (LR) under 'speed breeding' or accelerated growth conditions (AGC). The method integrates LR response assessment on the same genotypes first at the seedling and then at the adult plant stage. The method can be completed in just seven weeks and enables up to seven consecutive assays in a year compared to just one or two in the field. Along with phenotypic selection, marker-assisted selection (MAS) can also be performed and desired crosses can be made all in the same plant generation. Developing populations in the speed breeding system reduces the time required to develop recombinant inbred line (RIL) QTL mapping populations and subsequent introgression into an adapted genetic background for use in a breeding program. Such methodologies would also be helpful in phenotyping large germplasm collections and could be done all year round. However, in the context of a breeding program, such activities must be integrated with field testing to select for other agronomic traits along with the disease resistance.

Chapter 5 reported a new approach to rapidly identify new sources of APR to LR from the Vavilov wheat collection. This involved: 1) screening the diversity panel using polymerase chain reaction (PCR) markers, 2) rapid phenotyping under controlled conditions

and 3) field evaluation of a subset of lines using multiple pathotypes in multiple environments. Through this screening approach, thirteen new sources of APR to LR were identified by effectively mining diverse wheat lines from the VIR. These resources include landraces, cultivars, and breeding lines, which originated from different geographical regions of the world, representing diverse sources of LR resistance. However, allelism testing is needed to confirm whether the LR resistance carried by each accession is unique and the underlying genetics should be examined in bi-parental mapping populations to precisely map the gene(s) in the wheat genome. The approach employed here could be further applied to screen large numbers of genebank accessions in search for additional sources of LR resistance. Further, this approach could be scaled-up if combined with the Focused Identification of Germplasm Strategy (FIGS; Bhullar et al. 2009) or genomic prediction (GP; Juliana et al. 2017). This would accelerate the isolation of new genes from germplasm collections; a requirement to diversify resistance factors in modern breeding materials.

Chapter 6 reported the first GWAS study using diverse wheat accessions from VIR and focused on identification of the genomic regions underpinning LR resistance. The GWAS revealed 13 potentially new genomic regions conferring LR resistance in the Vavilov wheat diversity panel, where most of them were associated with LR resistance at the adult plant stage. Four of the identified QTL were co-located with the catalogued LR genes while 14 were aligned with previously reported QTL in recent GWAS or GP studies. Six loci were associated with LR resistance at the adult stage and were detected using multiple adult plant phenotypic data sets. Haplotype analysis of two important QTL on chromosomes 3A (*qNV.Lr-3A.3*) and chromosome 7B (*qNV.Lr-7B.2*) revealed strongly fixated allelic variants that are highly associated with seedling and APR, implying strong prior selection during LR stress adaptation. Haplotype analysis and allele stacking revealed a clear linear trend in resistance with an increasing number of resistance alleles from independent loci, where most of the lines in the diversity panel carried 20 or more combined resistance-associated marker alleles, highlighting the enormous potential of allele stacking to provide long-lasting resistance to LR disease of wheat.

7.3 Identification of genetic diversity is key to breeding for genetic resistance

Breeding for genetic resistance to rust diseases often faces many challenges. Firstly, a genetic bottleneck was imposed on modern wheat during early crop domestication where limited hybridisation events left behind a vast genetic diversity present in the wild progenitors

of wheat. The genetic diversity was further narrowed-down due to intensive selective breeding in modern breeding programs. Consequently, the modern wheat breeding germplasm has limited genetic diversity for rust resistance factors (Wulff and Moscou 2014). Secondly, in the past, breeding for genetic resistance was mostly focused on often short-lived race-specific resistance. Thirdly, the development of a genetically resistant cultivar is a slow process; it can take 10–15 years (Hickey et al. 2017). Moreover, the *P. triticina* population is constantly evolving and can readily gain virulence for the already deployed resistance genes (Chaves et al. 2013; Park 2016). The breakdown of genetic resistance in a widely adopted wheat cultivar can result in huge yield and subsequently economic losses, which can affect the livelihood of rural communities. This necessitates the ongoing discovery and introgression of new effective genetic resistance sources into modern wheat cultivars.

When it comes to breeding for genetic resistance to rust diseases in wheat, Pretorius et al. (2017) stated two important objectives, which must be considered in a breeding program. The first objective is pre-breeding or germplasm development, which involves the identification and characterisation of genetic resources conferring resistance against the pathogen. The second objective is the introgression of the identified genetic resistances into the adapted cultivars to provide effective and durable resistance in the farmer's field. Both objectives require different breeding strategies to implement and thus result in different outcomes but ultimately contribute to the development of resilient wheat cultivars.

Chapter 3, revealed the immense genetic diversity preserved in the wheat accessions from VIR. This was highlighted by the vast number of alleles either absent or fixed in the modern wheat germplasm from Australia and CIMMYT. The genomic analysis also demonstrated that many Australian and CIMMYT elite wheat lines share common genetics, which aligns with observations from Brennan and Quade (2006), who claimed that most Australian wheat cultivars have direct or indirect parentage from the CIMMYT germplasm. This overutilization of CIMMYT germplasm in Australia has narrowed the genetic base of resistant factors deployed and makes them vulnerable to new pathotypes and perhaps even new pathogens. To overcome this limitation, fortunately, hundreds and thousands of wheat accessions have been preserved in different seed banks all over the world representing huge diversity regarding origin, history, and cultivation status of accessions. With the advancement in cost-effective genotyping platforms, these germplasm collections can be genotyped to provide rich and accessible genetic diversity for modern wheat improvement (Voss-Fels et al. 2015). Until now, the majority of *Lr* genes were identified from wild relatives

of wheat, but very few of them have been used in breeding programs due to problems such as deleterious traits through linkage drag (Wulff and Moscou 2014). However, with the latest rapid gene cloning approaches, such as mutational resistance gene enrichment sequencing (MutRenSeq), the gene of interest can be rapidly cloned to enable more targeted breeding and much-reduced linkage drag (Steuernagel et al. 2016).

Alternatively, the use of germplasm accessions such as landraces, historic cultivars, and old breeding lines, often provide advantages over the use of wild relatives' due to the same ploidy level and ease in recombination with hexaploid wheat (Wulff and Moscou 2014). In particular, landraces have been found to be genetically diverse and widely adapted to various eco-geographic conditions. Such genetic resources carry either new genes or new allelic variations of already known genes thus increasing the genetic basis of not only the resistance factors but also of other agronomic traits. In the past, landraces had provided various important genes for several agronomic and disease traits. For instance, the APR gene *Lr67* was identified from a landrace accession, which still provides durable resistance against multiple pathogens and is widely used for durable genetic resistance (Moore et al. 2015). Likewise, landraces also contribute agronomically important alleles, for instance, the semi-dwarfing gene *Rht8c* which was identified from a Japanese landrace and played a significant role in the development of short stature and early maturing wheat varieties during the 'Green Revolution' in the 1960s (Worland et al. 1998; Ellis et al. 2007). The advancement in genotyping technologies such as DArT-seq has provided cost-effective whole genome marker scans with extremely high marker coverage and density. With regards to diversity, the A and B genomes were observed to have much greater diversity than the D genome which is in alignment to previous studies (Akhunov et al. 2009; Nielsen et al. 2014) where low marker coverage on the D genome has always been a problem in performing genetic studies. This low diversity in the D genome may be due to the short domestication history of the D genome, since it was the last hybridisation event which added the D genome to the cultivated bread wheat, and this has consequently presented a genetic bottleneck in the polyploidization of wheat. Moreover, the D genome has low effective recombination sites due to the prevention of homoeologous chromosomes pairing (Akhunov et al. 2010; Peng et al. 2011). However, to increase marker coverage on the D genome, specific crosses can be carried out with *Aegilops tauschii* (DD), D genome progenitor of wheat, to increase the recombination sites (Mujeeb-Kazi et al. 1996). In the past, useful stem rust resistance genes such as *Sr33* and *Sr45* have been isolated from *Ae. tauschii* (Periyannan et al. 2013; Steuernagel et al. 2016) and are now widely adopted in the modern wheat cultivars. Apart

from VIR, there are also hundreds and thousands of wheat accessions lying dormant in several seed banks around the world, and when genetically characterised, they would be an invaluable resource for new alleles in modern wheat. It is anticipated the new alleles identified in this study will be an excellent resource for broadening the genetic basis of modern wheat germplasm.

Although the germplasm collections carry huge genetic diversity, often working with them is problematic. Some of the problems associated with germplasm collections include duplicity of accessions, variety admixtures, lack of passport information about the accessions, missing genotype and phenotype information, poor yield traits (i.e. unfit for breeding context), and often target genes are accompanied by deleterious traits or linkage drag (Kilian and Garner 2012). Moreover, screening a large number of accessions for a trait of interest is not feasible in the context of a breeding program. Therefore, development of a core collection is a viable approach for the efficient exploration and utilisation of new allelic variations in the genetic resources (Brown 1986). The core collection comprises of a set of accessions with a maximum genetic diversity of a crop species and its wild relatives with minimum accession repetitiveness and redundancy. For instance, in the USDA-ARS National Small Grains Collection, a bread wheat core collection was curated comprising of 4,007 accessions which is 10% of the 42,138 accessions available in that collection based on the origin information (Bonman et al. 2015). The core subsets can also be identified using FIGS which utilises both the trait and environmental data. FIGS has been successfully applied to discover *Pm3*; a new allele associated with resistance to powdery mildew in wheat (Bhullar et al. 2009). Furthermore, the latest robust, high throughput and cost-effective genotyping platforms have facilitated the genotyping of germplasm collections. Consequently, the genomic approaches such as genomic selection (GS) or GP can facilitate the selection of superior accessions for the core collections (Juliana et al. 2017). For instance, GS was successfully applied for the selection of rust resistance (particularly APR) in diverse wheat landraces (Daetwyler et al. 2014) and in advanced wheat lines at CIMMYT (Juliana et al. 2017).

The development of a rapid phenotyping method in Chapter 4 provides a new opportunity to expedite the exploration of germplasm collections. For instance, the seedling and adult plant phenotypic response for LR can be obtained in just seven weeks, which can also go in tandem with an appropriate crossing and population development strategy. Furthermore, the new screening approach adopted in Chapter 5 efficiently identified a

subset of accessions for further field validation using multiple pathotypes and environments. This approach can be applied directly to smaller germplasm collections where screening of accessions is routinely carried out under field conditions. The approach can also be applied in developing core collections by performing FIGS, and GS, where the collection of rust response is often time-consuming.

7.4 Identification of genomic regions underpinning leaf rust resistance

With the advances in genotyping and analytical software to handle large datasets, GWAS has become a valuable tool for the dissection of the genetic architecture of important traits in crop species. Applied to diversity panels, as done in this thesis, GWAS exploits historical recombination to determine the allelic variation at much higher resolution compared to bi-parental linkage mapping approaches. GWAS depends on linkage disequilibrium (LD) between two non-randomly associated alleles. Therefore, estimation of LD is a good indicator to determine the accuracy of the association analysis based on a number of markers required to find a true association. In Chapter 6, LD was estimated in the diversity panel, where LD decayed rapidly in the A and B genomes, as both genomes have high rates of recombination, and as expected much higher marker coverage and marker density was observed. On the other hand, high LD was detected on the D genome due to low recombination sites which is in alignment with previous wheat genomic studies (Nielsen et al. 2014). The extent of LD decay in a population can differ with crop species and marker system used. Likewise, LD in the breeding populations represents the exclusive breeding history and selection pressures targeted to various genomic regions during cultivar development and crop improvement (Crossa et al. 2007). In this study, while there were two distinct subpopulations in the diversity panel representing mostly landraces, and cultivars and breeding lines (i.e. cluster 1 and cluster 2, respectively) no difference in LD was observed between the two groups.

Often performing GWAS on germplasm collections is challenging due to large population structures resulting from high genetic relatedness among the individuals according to origin and cultivation status. Consequently, this can lead to the detection of spurious associations or false positives (Type I error) (Yu and Buckler 2006; Zhu et al. 2008). To reduce the number of false positives, linear mixed models are used, where population structure (Q matrix) and genetic relatedness (Kinship matrix) are considered as a fixed effect and random effect, respectively (Yu and Buckler 2006). Thus, an ideal GWAS analysis

requires a large population size, high marker density and a mixed linear model to identify true associations with the trait of interest such as LR resistance (Bulli et al. 2016). Although such GWAS offer high mapping resolution, they have low power to detect rare alleles (Brachi et al. 2011). Advanced QTL mapping strategies that exploit multi-parent populations, such as nested-association mapping (NAM), can improve the power to detect rare alleles because allele frequency is balanced within each donor-reference subpopulation (Xu et al. 2017), however, the number of diverse donors that can be used as parents is limited based on resources available for population development and costs for genotyping. The LR resistant accessions identified in this study could be used to generate a NAM population, where the large number of resistance alleles would segregate, and they could be closely examined within the context of an adapted genetic background.

In Chapter 6 of this study, a large number of QTL were detected (n=31) associated with LR resistance at seedling and adult plant stage. Of these, 13 QTL were deemed newly identified genomic regions associated with LR resistance. Detection of a significant number of resistance alleles is common with diversity panels. However, simultaneously considering a large number of QTL in a breeding program is challenging. To overcome this limitation, and prioritise the QTL, the identified QTL can be subjected to a broader range of LR pathotypes and can be tested in multiple environments to determine which QTL confer stable resistance. This objective could be achieved by sending accessions to various disease screening nurseries established by CIMMYT or other research organizations around the world. Alternatively, the speed breeding facility provides the opportunity to test various pathotypes in a controlled environment; even the pathotypes which are not prevalent in the field. Moreover, with the latest advances in molecular genetics including MAS, marker-assisted backcrossing, and GS can effectively support prioritising and combining multiple alleles in a single variety (Liu et al. 2014). The QTL identified in this study were also aligned with previously reported QTL and catalogued *Lr* genes. Allelism tests are required to confirm whether the co-located QTL detected here correspond to the known *Lr* genes and QTL. This confirmation would be of great importance to convert these DArT-seq markers into breeder friendly kompetitive Allele-Specific PCR (KASP) markers for implementing MAS in wheat breeding programs.

Across experiments, the lines identified as resistant include landraces, cultivars and breeding lines originating from different countries of the world. Therefore, they likely represent diverse sources of LR resistance and could be exploited to achieve durable

disease resistance in various eco-geographic contexts (Gurung et al. 2014; Maccaferri et al. 2015). This correlation of resistance and geographical origin is valuable information for future exploration of accessions from these regions. The molecular analysis of the identified resistant lines holds great potential for the characterisation and isolation of new LR resistance genes. Once characterised, such genes can be cloned using a conventional map-based cloning approach, or using modern approaches such as MutRenSeq (Steuernagel et al. 2016) and targeted chromosome-based cloning via long-range assembly (Thind et al. 2017). This would allow the development of gene-specific markers for R or APR genes which can be used for gene pyramiding through MAS in breeding programs.

7.5 Pyramiding resistance genes/QTL for durable resistance

In the past, breeding for genetic resistance was mostly focused on R genes rather than APR genes. This is because plants carrying R genes are easy to identify and plant breeders find it hard to resist a clean, green plant. However, resistance is short-lived as the pathogen can rapidly gain virulence for R genes, particularly when they are deployed alone. Research groups around the world are striving to combine 4–5 R genes in the form of a gene cassette through genetic engineering which can be transferred to high yielding cultivars for incorporating durable rust resistance. However, no success has yet been reported. In contrast, current breeding programs are much more focused in the deployment of APR genes. A single APR gene may not confer adequate resistance levels under high inoculum pressure or at high temperatures. The APR genes can work in an additive manner, and if deployed together (e.g. a combination of four to five genes) they can enhance the resistance to the level of immunity (Singh et al. 2011). Thus, pyramiding or stacking multiple alleles is a more useful approach for to achieving high levels of resistance and will extend the life of the resistant cultivar.

In Chapter 6 of this thesis, it was revealed that a large portion of the lines in the diversity panel carried a high number of resistance-associated alleles. For instance, 42 lines carried more than 20 resistance-associated alleles. Landraces are the traditional varieties that have been grown in different geographical regions for thousands of years and were selected by farmers primarily for yield, but also inadvertently for disease resistance (Zeven 2002). As the majority of the lines in the diversity panel were landraces, these resistance-associated alleles likely accumulated over time since wheat and the rust pathogen would have co-evolved in the same place and time. It is very likely that the landraces harbour

exclusive combinations of resistance alleles to limit the disease. Secondly, the landraces examined in this study were sourced from a seed bank, thus have been removed from their environmental context for almost a hundred years and so likely harbour new alleles to which the modern rust pathogen populations are not exposed.

Traditionally, gene or QTL pyramiding is performed by crossing a single recipient parent to few donor parents carrying the target loci, followed by population development and phenotypic selection. This strategy has proved successful for providing durable resistance. For instance, the combination of four to five APR genes for stem rust and powdery mildew in wheat cultivars in CIMMYT has resulted in a higher level of disease resistance (Singh et al. 2011; Singh et al. 2014). Although the pyramids prolong the life of deployed resistant cultivars, these pyramids are not necessarily everlasting. Instances have been reported where gene pyramids were effectively providing resistance to disease for a few years, but later succumbed to the pathogen. This might be due to various reasons, which include: 1) widespread exposure of the pyramided genes to the pathogen population; 2) pre-existence of virulence for the pyramided genes in the pathogen population; 3) genetic variation in the pathogen population due to sexual recombination; and 4) lack of genetic diversity in combined resistance genes (Burdon et al. 2014; Mundt 2014; Brown 2015). For instance, wheat cultivars with resistance gene combinations were providing effective resistance against stem rust until the 1990s. In 1998, wheat stem rust race TTKSK (synonym Ug99) was detected for the first time, and to date, 13 variants of the Ug99 have been detected (http://www.rusttracker.org). This stem rust race has rendered 90% of the wheat grown in the world susceptible to stem rust (Singh et al. 2011; Singh et al. 2014). This scenario often leads to some frequently asked questions. How many genes are enough to achieve durable resistance? How rapidly will the genetic erosion take place? These questions are yet to be answered.

More recently, pyramiding of both R and APR genes has been proposed as a viable strategy to attain durable resistance and has been successfully practised in different environmental contexts. For instance, in Western Europe, durable resistance to stripe rust in wheat was achieved for more than 15 years by combining R and APR genes/QTL (Basnet et al. 2014; Brown 2015). This strategy has some positive outcomes to offer. Firstly, it provides multiple barriers against the rust pathogen that are effective at all stages of plant growth. Usually, resistance conferred by these genes increases to a level of immunity, but in case the pathogen gains virulence for the race-specific R gene, there is still an APR gene

intact that offers partial resistance. Secondly, it reduces the evolution of new pathotypes. Thirdly, it reduces the inoculum load in the field during the cropping season, thus reducing the number of asexual cycles. Finally, it minimises the yield penalty associated with the deployment of APR genes, which usually serve as a trade-off between resistance and the yield. The plant remains healthy throughout the life cycle and improves the wheat yield.

7.6 Future application of speed breeding - a perspective

The process of developing wheat cultivars with improved genetic resistance to rust diseases takes many years before they reach the farmer's field. In a typical breeding program, elite breeding lines are used as donor parents due to better adapted genetic backgrounds. In contrast, use of historical germplasm requires more time as they are first introgressed in to adapted genetic backgrounds (referred as pre-breeding) and are later used as donor parents in the breeding program. In a pre-breeding program, after selection of the parents with the desired resistance response, they would be subjected to crossing to produce subsequent populations. In the case of seedling resistance genes, segregating F2 populations are developed, where seedlings are screened for LR response and genotyped with molecular markers to map and clone the gene using map-based cloning or MutRenSeq approach. However, in the case of APR, RIL development is preferred for gene mapping. After crossing, the development of RILs requires four to six generations of single seed descent (SSD) to achieve a desirable level of homozygosity for the evaluation of the rust response (Chahal and Gosal 2002). RIL development is mainly carried out in the field, and if available, off-season nurseries and glasshouses are also used to accelerate the process. Once the appropriate level of homozygosity is achieved (i.e. F4 or F6), the RILs are tested at multiple locations and years in the field nurseries, where individuals are screened and selected not only for rust resistance but also for other agronomic traits. Thus, field-based generation advancement and phenotyping slow down the process of developing rust-resistant cultivars. However, if SSD is applied in under speed breeding conditions, it can effectively accelerate the development of RILs in a shorter timeframe of 1.5 years compared to 3–4 years in the field.

Speed breeding or AGC enables a shorter plant generations for spring wheat. This technique uses extended photoperiods and controlled temperatures that help the plants to grow fast and rapidly attain the adult plant stage. The plant management system enables up to six generations of wheat in a year (Watson et al. 2017). Speed breeding facilities have

been used for wheat pre-breeding and breeding at The University of Queensland for the past ten years, and have recently been established at the John Innes Centre, and the system is currently being trialled at CIMMYT in Mexico. Although, the extended photoperiod is successful in accelerating plant development in many species there are still some plant species such as eggplant (*Solanum melongena*) and tomato (*S. lycopersicum*), in which extended photoperiods disturb the circadian clock and cause injury (Velez-Ramirez et al. 2011). Consequently, the development of appropriate protocols for other plant species could broaden the application of speed breeding to improve the productivity of other important crops. The speed breeding system provides a key advantage, in that environmental factors are controlled, such as temperature and light, and these conditions can be tuned according to the objective of the study. The utility of such a plant management system increases many folds if combined with screening and selection of traits.

In Chapter 4 of this thesis, a rapid phenotyping method for LR resistance adapted to the speed breeding system was reported. It integrates assessment of LR response at the seedling and adult plant stage and can be completed in just seven weeks. Initially, the constant light was applied to help the wheat plants attain the adult plant stage quickly, but post-inoculation plants are exposed to diurnal light and controlled temperature regimes to favour disease development. Numerous studies report that post-inoculation conditions are critical for pathogen infection and development, particularly for expression of APR (Hickey et al. 2012; Dinglasan et al. 2016). The high-quality diurnal light pattern is essential for disease development and sporulation, while constant (24 h) light can impede pathogen development (Roelfs et al. 1992; de Vallavieille-Pope et al. 2002; Hickey et al. 2012). This protocol overcomes limitations associated with field-based assessment techniques, such as variation in phenotypes due to unfavourable weather conditions, spatial variation in the field, multiple infection cycles (i.e. usually four to five asexual cycles of LR), and unwanted pathotypes or other pathogens. Moreover, these controlled growth facilities allow screening of genetic material using multiple pathotypes, and exotic pathotypes in secure facilities. Phenotyping under AGC can be carried out throughout the year, thus providing an advantage over traditional field-based screening that can be conducted only once a year (Hickey et al. 2012; Ellis et al. 2014). Moreover, controlled facilities enable the study of the host-pathogen interaction, the effect of temperature on gene expression and understanding the mechanism of resistance genes.

In recent years, the utility of speed breeding or AGC has improved greatly. Particularly in wheat, numerous phenotyping methods have been developed for biotic traits (i.e. rust and yellow spot resistance) (Hickey et al. 2012; Dinglasan et al. 2016) and abiotic traits (i.e. root angle and grain dormancy) (Hickey et al. 2009; Richard et al. 2015). The application of such a plant management system can be further scaled up by developing integrated breeding approaches where individuals are selected simultaneously for multiple traits such as screening for triple rust resistance in wheat (Hickey and Dieters 2013). A trait introgression strategy was implemented in the speed breeding system to successfully transfer grain dormancy and triple rust resistance into two Australian spring wheat cultivars within a two-and-a-half year period (Hickey and Dieters 2013). Similarly, a modified backcrossing strategy was implemented for spring barley cultivar 'Scarlett' (preferred for malting and brewing in Argentina), to introgress multiple disease resistance (i.e. leaf rust, net and spot forms of net blotch, and spot blotch) within a two-year period (Hickey et al. 2017). A similar strategy could be adapted to fast-track the introgression of new LR resistance sources from the Vavilov diversity panel into elite genetic backgrounds for future wheat cultivars.

While speed breeding or AGC has several advantages, there are a number of factors that limit widespread adoption of this technology. Firstly, the development of a growth facility with all the services (i.e. reliable electricity supply and air-conditioning), along with the labour, could be expensive depending upon the objective and scale of the breeding program. However, expenses could be reduced by replacing sodium vapour lamps with modern LED lighting, and by transforming standard glasshouse facilities to effective speed breeding systems (Watson et al. 2017). These modifications will reduce set up and operating costs associated with speed breeding. Furthermore, the cost of phenotyping under speed breeding can be reduced by screening multiple traits in one plant generation, thus reducing the overall cost per data point. Speed breeding is less labour intensive, however, can become labour intensive at times, such as times of sowing and harvesting each generation. One strategy is to use speed breeding facilities in the off-season when field conditions are not favourable, which would further reduce the operating cost of the facility throughout the year. Moreover, complex traits such as yield and quality cannot be selected for in the speed breeding system. Therefore, yield and quality traits still need to be evaluated in the field. However, speed breeding and rapid phenotyping methodologies offer opportunities to develop and select inbred lines enriched with desirable traits such as disease resistance. This way, a 'better' set of fixed lines can be evaluated in the field. If applied to segregating

populations, resistant plants can be identified and crossed at each plant generation. Phenotypic screening of the individual plants can be performed at F_2 or can be subjected to SSD to reach F_4 or F_6, while screening can be performed in parallel. For instance, the large number of F_2 seeds can be raised at higher densities using a cell-based system which allows the growth of up to 900 plants/m^2 (Watson et al. 2017).

Speed breeding along with the rapid phenotyping methods developed as part of this study can also accelerate the development and subsequent screening of mutant populations. To date, the development and screening of mutant populations are the biggest limitations to fully harness the benefits of modern gene cloning techniques such as MutRenSeq and targeted chromosome-based cloning via long-range assembly. Speed breeding and phenotypic screening can also be integrated with GWAS to rapidly identify genomic regions and develop KASP markers to be used in MAS. When integrated with high throughput marker platforms, speed breeding could allow the selection of disease traits using the GS approach.

7.7 Conclusion and future directions

This study reported the first genomic characterisation of wheat accessions from the VIR gene bank. The results demonstrated that a huge array of allelic diversity was present in this panel which could potentially hold new alleles for various biotic and abiotic traits. The new sources of APR and the genomic regions identified in this study should be tested against multiple *P. triticina* pathotypes and multiple pathogens through international disease screening nurseries established by CIMMYT and various collaborators around the world. This will help to prioritise new regions associated with multiple disease resistance for gene cloning using map-based cloning approaches, MutRenSeq or targeted chromosome-based cloning via long-range assembly. The development of gene specific markers will allow the implementation of MAS to accelerate the selection of these genes in breeding programs. Once identified, these genes can be rapidly introgressed into modern wheat cultivars using speed breeding to hasten the process. The LR resistant accessions can also be used as donor parents for disease resistance in family-based populations such as nested association mapping (NAM) populations. This would allow the simultaneous discovery and utilisation of genes in an adapted genetic background. Apart from disease resistance, the Vavilov wheat diversity panel could also be tested for other valuable traits, such as drought or heat tolerance to develop climate resilient varieties. Further, VIR is just one seed bank – there

are numerous of seed banks around the world which would be expected to contain an abundance of new allelic variations that are relatively unexplored. Without a doubt, more information and improved access to genetic resources will improve utilisation by the wheat pre-breeding and breeding community. The pure seed source and marker information for this diversity panel is open-access for the scientific community. We hope this serves as an invitation to breeders and researchers around the world to harness new sources of disease resistance in this diversity panel, and fast-track introgression into modern wheat. The discovery of new resistance alleles is important to maintain and increase the productivity of wheat crops globally.

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Appendices

Supplementary Table 1

See link: https://link.springer.com/article/10.1007/s10722-016-0380-5#SupplementaryMaterial

Supplementary Table 2

See link: https://plantmethods.biomedcentral.com/articles/10.1186/s13007-016-0117-7

Supplementary Table 3

See link: http://apsjournals.apsnet.org/doi/suppl/10.1094/PDIS-05-16-0614-RE/suppl_file/PDIS-05-16-0614-RE.st1.pdf

Supplementary Table 4 Phenotypic response the wheat diversity panel comprising 295 single seed descent (SSD) lines originally sourced from the N. I. Vavilov Institute of Plant Genetic Resources (VIR) in St Petersburg, Russia. Details for the SSD lines include; the University of Queensland (UQ) SSD line number, and cultivation status. The leaf rust response for each SSD line obtained in each experiment is presented. A dash (–) indicates data is unavailable or unknown.

UQ SSD line number	Cultivation status	Seedling	AGC	Field_2014_1			Field_2014_2			Field_2014_3			Field_2014_4			F_15_1	F_15_2	F_15_3	F_16_1	F_16_2
		0-9 scale	0-9 scale	SEV	IT	CI	SEV	IT	CI	SEV	IT	CI	SEV	IT	CI					
WLA-001	Landrace	8.5	6	20	MRMS	12	30	MSS	27	40	S	40	50	MS	40	9	8	7	4	4
WLA-002	Landrace	-	-	15	MSMR	9	20	MRMS	12	20	MRR	6	30	MS	24	5	9	7	4	7
WLA-003	-	8	7	20	MRMS	12	30	MR	12	30	MRR	9	100	S	100	7	8	9	7	9
WLA-004	-	9	8	15	MRR	5	20	MR	8	20	MRR	6	20	MRR	6	4	5	2	4	4
WLA-005	-	8	3.25	20	S	20	40	S	40	50	S	50	30	S	30	9	9	9	4	7
WLA-006	-	8	8	20	S	20	40	S	40	50	S	50	30	S	30	9	9	9	4	8
WLA-007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	8	6	-	-
WLA-008	-	5.5	4.25	20	RMR	6	25	MRR	7.5	30	MRMS	18	20	MRR	6	3	3	4	5	5
WLA-009	-	8	4	20	S	20	30	S	30	50	S	50	60	S	60	9	9	9	5	7
WLA-010	-	8.5	5.5	30	S	30	40	S	40	50	S	50	90	S	90	9	9	8	8	8
WLA-011	-	8.5	9	20	MSS	18	40	MSS	36	30	MRR	9	50	S	50	9	9	8	6	6
WLA-012	-	9	4	20	MRMS	12	25	MR	10	40	MRMS	24	60	S	60	8	9	7	7	7
WLA-013	-	8	6	20	MRMS	12	25	MR	10	40	MRMS	24	40	MSS	36	7	8	8	6	7
WLA-014	-	8	9	20	RMR	6	25	MRR	7.5	30	MRR	9	40	MSS	36	4	6	8	5	6
WLA-015	-	2.5	6	20	MRMS	12	20	MR	8	30	MRR	9	20	MRR	6	4	4	2	4	3
WLA-016	-	5.5	9	10	MRMS	6	20	MRMS	12	30	MRR	9	40	MS	32	8	8	7	6	6
WLA-017	Landrace	8.5	8	20	MSS	18	20	MSS	18	30	S	30	40	S	40	9	9	8	-	-
WLA-018	Landrace	8	8.5	40	S	40	30	S	30	50	S	50	90	S	90	9	9	9	7	7
WLA-019	Landrace	5.5	9	30	MSS	27	70	S	70	50	S	50	100	S	100	9	9	9	7	9

WLA-020	Landrace	9	6	20	MSS	18	40	S	40	40	S	40	70	S	70	9	9	9	6	9
WLA-021	Landrace	9	7	15	MS	12	30	MR	12	30	MRMS	18	50	S	50	4	8	9	5	6
WLA-022	Landrace	9	8	15	MS	12	30	MRMS	18	30	MRR	9	60	S	60	7	7	9	4	6
WLA-023	Landrace	8	8	20	MSMR	12	30	MRMS	18	40	MRMS	24	40	MSS	36	9	8	7	7	7
WLA-024	Landrace	8	7	20	MRMS	12	20	MR	8	20	MRR	6	40	MSS	36	7	8	8	4	4
WLA-025	Landrace	9	7.25	20	RMR	6	20	MR	8	20	MRR	6	20	MS	16	4	3	2	4	4
WLA-026	Landrace	9	7	20	RMR	6	20	MR	8	20	MRR	6	20	MS	16	4	3	2	4	5
WLA-027	Landrace	9	6	20	MSMR	12	20	MRMS	12	30	MRR	9	70	S	70	8	9	9	7	5
WLA-028	Landrace	9	7.25	20	MS	16	20	MRMS	12	40	MSS	36	50	MSS	45	7	8	7	5	3
WLA-029	Landrace	8	8	20	MSMR	12	30	MR	12	30	MRR	9	40	MSS	36	4	6	8	5	5
WLA-030	Landrace	8.5	8	20	MSMR	12	30	MRMS	18	40	S	40	80	S	80	9	8	8	7	7
WLA-031	Landrace	8	6	20	MRMS	12	30	MR	12	40	MRMS	24	30	MRMS	18	7	7	8	5	5
WLA-032	Landrace	8	3.5	10	MR	4	30	MR	12	30	MRR	9	30	MS	24	4	3	7	5	5
WLA-033	Landrace	6	2	15	MRR	5	30	MR	12	30	MRR	9	40	MRMS	24	8	9	8	3	3
WLA-034	Landrace	8	8.5	10	MR	4	30	MR	12	30	MRR	9	30	MRMS	18	6	7	8	4	7
WLA-035	Landrace	8.5	5.5	10	RMR	3	25	MR	10	30	MRR	9	40	MR	16	4	3	8	4	5
WLA-036	Landrace	8.5	8	30	MS	24	50	S	50	60	S	60	90	S	90	9	9	9	8	8
WLA-037	Landrace	8.5	9	20	MRMS	12	30	MRMS	18	40	S	40	90	S	90	9	8	7	6	7
WLA-038	Landrace	8.5	4.25	20	MR	8	30	MR	12	30	MRR	9	30	MRR	9	3	5	7	4	4
WLA-039	Landrace	9	4.25	10	MR	4	20	MR	8	30	MRR	9	40	MS	32	9	9	7	6	7
WLA-040	Landrace	9	9	25	MSMR	15	30	MRMS	18	50	S	50	80	S	80	9	9	7	8	8
WLA-041	Landrace	8	6.5	15	MRMS	9	30	MRMS	18	30	MRR	9	50	S	50	9	9	8	5	7
WLA-042	Landrace	8.5	8	15	MRMS	9	30	MR	12	30	MRR	9	50	S	50	3	4	7	4	7
WLA-043	Landrace	6	3	10	MR	4	20	MRR	6	30	RMR	9	30	MS	24	3	3	4	2	3
WLA-044	Landrace	7	8	40	S	40	50	S	50	40	S	40	100	S	100	9	9	8	8	8
WLA-045	Landrace	8.5	9	30	MRMS	18	30	MRMS	18	30	MRR	9	80	S	80	7	9	9	5	7
WLA-046	Landrace	9	9	30	S	30	40	S	40	40	S	40	80	S	80	9	9	8	8	8
WLA-047	Landrace	8	7	20	MS	16	30	S	30	40	S	40	80	S	80	9	9	8	6	7

WLA-048	Landrace	9	8	30	S	30	40	S	40	50	S	50	90	S	90	9	9	9	7	7
WLA-049	Landrace	6	3.5	20	MS	16	30	MRMS	18	30	MRR	9	50	S	50	7	9	8	6	6
WLA-050	Landrace	6	8	15	MSMR	9	30	MR	12	30	MRMS	18	50	S	50	8	2	8	4	7
WLA-051	Landrace	8.5	9	20	MSMR	12	30	MRMS	18	40	MRMS	24	30	MS	24	9	8	8	4	7
WLA-052	Landrace	8.5	8	10	MSMR	6	30	MR	12	30	MRR	9	40	S	40	7	4	7	4	6
WLA-053	Landrace	8	8	30	MS	24	30	MSS	27	40	MRMS	24	20	S	20	9	9	8	8	8
WLA-054	Landrace	8	6	20	MSMR	12	25	MR	10	30	MRR	9	40	S	40	6	8	7	4	6
WLA-055	Landrace	5.5	9	20	MS	16	30	S	30	50	S	50	100	S	100	9	7	8	8	7
WLA-056	Landrace	7	9	20	MR	8	30	MR	12	30	MRR	9	30	S	30	3	9	7	5	4
WLA-057	Landrace	9	7	10	MSMR	6	30	MRMS	18	40	MRR	12	50	S	50	8	6	8	7	8
WLA-058	Landrace	9	9	20	MS	16	30	MSS	27	50	S	50	70	S	70	9	9	8	8	7
WLA-059	Landrace	9	7	15	MSMR	9	30	MR	12	30	MRR	9	30	MSS	27	3	7	7	5	4
WLA-060	Landrace	8.5	1.5	10	MR	4	25	MRR	7.5	30	MRR	9	20	MR	8	3	4	5	4	4
WLA-061	Landrace	9	8	15	MS	12	40	S	40	60	S	60	90	S	90	9	9	8	7	8
WLA-062	Landrace	9	3.5	10	MRMS	6	30	MRMS	18	40	MSMR	24	70	S	70	7	9	8	4	4
WLA-063	Landrace	0	0	15	MR	6	30	MR	12	30	MRR	9	20	MRR	6	7	8	7	4	3
WLA-064	Landrace	9	6	10	MSMR	6	30	MR	12	50	S	50	40	MRMS	24	5	7	7	3	4
WLA-065	Landrace	9	7	30	S	30	40	S	40	50	S	50	30	S	30	9	9	8	8	3
WLA-066	Landrace	8	9	10	MS	8	30	S	30	60	S	60	70	S	70	9	9	9	4	3
WLA-067	-	8.5	6	20	S	20	40	S	40	30	MRR	9	80	S	80	9	9	8	3	3
WLA-068	Landrace	9	3	20	MRMS	12	30	MRMS	18	40	MRR	12	40	MSS	36	5	5	7	3	3
WLA-069	Landrace	5	4.25	20	MR	8	25	MR	10	30	MRR	9	30	MS	24	7	5	8	3	3
WLA-070	Landrace	8	8.5	10	S	10	20	MRMS	12	30	MRR	9	30	MS	24	4	7	8	3	4
WLA-071	Landrace	8.5	7	5	MRMS	3	40	MRR	12	30	MRR	9	50	MSS	45	4	7	8	3	4
WLA-072	Landrace	8.5	7	10	MS	8	30	MRMS	18	25	MRR	8	30	MS	24	9	9	7	3	3
WLA-073	Landrace	5.5	4.25	5	MRMS	3	30	MRMS	18	30	MRMS	18	50	MSS	45	3	3	9	3	4
WLA-074	Landrace	9	2	10	RMR	3	30	MR	12	25	MRR	8	10	MR	4	3	2	3	4	4
WLA-075	Landrace	9	6	15	MS	12	30	MRMS	18	30	MS	24	40	MSS	36	9	9	8	-	-

WLA-076	Landrace	9	7	10	MS	8	30	MRMS	18	40	S	40	80	S	80	8	8	8	7	7
WLA-077	Landrace	8.5	8	20	MS	16	30	MRMS	18	40	S	40	40	MS	32	9	8	7	5	7
WLA-078	Cultivar	8	3.5	5	MR	2	30	MRMS	18	30	MRR	9	20	MRR	6	9	9	9	4	6
WLA-079	Landrace	7	2	5	MR	2	30	MR	12	30	MRR	9	20	MRR	6	3	4	7	3	3
WLA-080	Landrace	0	0.5	10	MR	4	20	MR	8	30	MRR	9	20	MRR	6	8	8	8	2	3
WLA-081	Landrace	1.5	0.5	15	MRMS	9	30	MR	12	30	MRR	9	20	MRR	6	6	5	9	3	4
WLA-082	Cultivar	9	3	10	MR	4	30	MR	12	30	MRR	9	30	S	30	6	8	8	8	7
WLA-083	Cultivar	9	2	20	MRMS	12	40	MRMS	24	60	S	60	50	MRMS	30	4	6	8	3	3
WLA-084	Cultivar	8.5	3.5	10	MRMS	6	30	MRMS	18	40	MRR	12	40	S	40	3	4	8	3	6
WLA-085	Landrace	8	9	20	MS	16	40	MSS	36	50	S	50	70	S	70	8	8	9	7	8
WLA-086	Cultivar	8	8	10	MR	4	30	MR	12	30	MRMS	18	20	MR	8	3	4	6	8	7
WLA-087	Breeding Line	5.5	3	10	MRMS	6	30	MRMS	18	40	MRR	12	30	MR	12	4	3	8	6	8
WLA-088	Landrace	9	6	20	MRMS	12	40	MRMS	24	50	S	50	60	S	60	8	7	9	8	8
WLA-089	Landrace	9	8.5	40	S	40	40	S	40	70	S	70	100	S	100	8	9	9	8	9
WLA-090	Landrace	9	8.5	20	MSS	18	40	S	40	40	S	40	90	S	90	9	9	9	8	8
WLA-091	Cultivar	8.5	9	40	S	40	40	S	40	80	S	80	100	S	100	9	9	9	8	9
WLA-092	Cultivar	1.5	0	15	RMR	5	30	MR	12	30	MRR	9	30	MR	12	3	3	3	4	4
WLA-093	Cultivar	2	0.5	15	RMR	5	30	MR	12	40	MRR	12	30	MR	12	3	3	5	3	4
WLA-094	Cultivar	8.5	9	30	MS	24	30	MRMS	18	40	SMS	36	90	S	90	9	9	9	9	8
WLA-095	Cultivar	5.5	2	20	RMR	6	25	MRMS	15	30	MRR	9	30	MS	24	7	7	7	-	-
WLA-096	Landrace	3.5	2	15	MR	6	30	MRR	9	40	MRR	12	30	MS	24	3	4	2	5	4
WLA-103	Cultivar	5	NA	20	RMR	6	30	MR	12	40	MRR	12	40	MR	16	3	7	8	3	6
WLA-104	Cultivar	8.5	7	30	MR	12	20	MR	8	30	MRR	9	40	MSS	36	3	4	8	8	7
WLA-105	Cultivar	2	NA	20	MRMS	12	20	MRMS	12	30	MRR	9	70	S	70	9	8	9	7	7
WLA-106	Cultivar	2	2	30	MR	12	20	MRMS	12	30	MRR	9	40	MSS	36	9	8	9	7	6
WLA-107	Cultivar	7	4.25	20	MRMS	12	30	MRMS	18	30	SMS	27	40	MS	32	9	8	6	4	5
WLA-108	Cultivar	7	4	20	MRMS	12	30	MR	12	40	MRR	12	30	MSMR	18	3	3	8	4	7
WLA-109	Cultivar	5	2	15	RMR	5	30	MR	12	30	MRR	9	40	MR	16	3	4	7	5	5

WLA-110	Cultivar	7	2	20	MR	8	25	MRR	7.5	40	MRR	12	40	MRMS	24	3	3	8	6	6
WLA-111	Cultivar	7	7	15	MR	6	20	MR	8	40	MRR	12	30	MR	12	3	3	6	3	4
WLA-112	Cultivar	9	6	10	MR	4	20	MR	8	40	MRR	12	30	MR	12	3	3	8	3	3
WLA-113	Landrace	8	7	20	S	20	30	S	30	30	S	30	70	S	70	9	9	7	6	7
WLA-114	-	9	8	30	S	30	30	MSS	27	40	MSS	36	80	S	80	9	9	9	8	8
WLA-115	-	8	7	10	MR	4	20	MR	8	40	MRMS	24	40	MS	32	3	3	7	3	3
WLA-116	-	8.5	7	20	MS	16	30	MSS	27	30	S	30	70	S	70	4	6	7	-	-
WLA-117	-	8	7	30	S	30	30	MSS	27	40	S	40	70	S	70	4	6	7	8	7
WLA-118	Landrace	8	6	20	MS	16	30	MSS	27	40	S	40	40	S	40	7	6	7	7	7
WLA-121	-	6	7	15	MR	6	20	MRMS	12	30	MRR	9	40	MSS	36	9	8	7	-	-
WLA-122	-	8	2	20	MRMS	12	40	MRMS	24	30	MRR	9	70	S	70	9	9	9	7	8
WLA-123	-	8	7	15	MSMR	9	20	MRMS	12	30	MRMS	18	70	S	70	3	3	7	6	6
WLA-124	-	8	9	15	MRMS	9	30	MRMS	18	30	MRR	9	60	S	60	9	9	9	8	7
WLA-125	-	5	5	-	-	-	-	-	-	-	-	-	-	-	-	9	9	8	8	7
WLA-126	-	9	8.5	15	MRMS	9	15	MRMS	9	30	MRR	9	50	S	50	8	8	7	7	7
WLA-128	Landrace	8	4	10	MS	8	25	MRMS	15	30	SMS	27	40	S	40	5	6	6	-	-
WLA-129	-	8.5	8	10	S	10	20	MRMS	12	30	MRR	9	50	MS	40	9	9	8	6	7
WLA-130	Landrace	8	2	-	-	-	-	-	-	-	-	-	-	-	-	3	6	7	4	6
WLA-131	Landrace	9	2	15	MRMS	9	40	MRMS	24	30	MRR	9	50	MSS	45	8	6	7	5	3
WLA-132	Landrace	8.5	1.75	15	MR	6	30	MR	12	40	MRR	12	30	MRR	9	3	3	3	4	3
WLA-133	-	8	3.5	15	MR	6	30	MR	12	40	MRR	12	40	MRMS	24	5	3	7	4	3
WLA-134	-	9	7	20	MS	16	30	S	30	50	S	50	80	S	80	8	9	9	6	7
WLA-135	Cultivar	5	2	10	MSMR	6	30	MSS	27	40	MRMS	24	50	MS	40	8	7	8	6	8
WLA-136	-	8.5	4.67	20	MS	16	25	MRMS	15	40	MRR	12	50	S	50	8	7	8	5	7
WLA-137	-	9	9	30	S	30	40	S	40	70	S	70	90	S	90	8	9	9	7	9
WLA-138	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
WLA-139	-	8	2	20	MSMR	12	30	MRR	9	30	S	30	80	S	80	4	3	8	4	7
WLA-140	-	9	8	30	S	30	40	S	40	60	S	60	80	S	80	9	9	9	8	9

WLA-141	-	8	3.5	10	MR	4	25	MR	10	40	MRR	12	20	MRR	6	4	4	6	4	4
WLA-142	Cultivar	8	4.25	30	S	30	25	MSS	23	40	S	40	70	S	70	9	9	9	7	8
WLA-143	Cultivar	8	8	30	S	30	40	MRMS	24	60	S	60	70	S	70	8	9	9	5	8
WLA-144	-	8.5	8	20	MRMS	12	20	MRR	6	10	RMR	3	50	S	50	6	5	8	6	8
WLA-145	Landrace	8	7	20	MSMR	12	20	MRR	6	30	MRR	9	40	S	40	6	6	7	6	7
WLA-146	Landrace	3	4.25	15	MRR	5	30	MR	12	40	MRR	12	30	MR	12	3	3	3	3	5
WLA-147	Landrace	8.5	1.75	20	MRR	6	30	MR	12	40	MRR	12	40	MR	16	3	3	3	3	4
WLA-148	Landrace	8.5	2	20	MS	16	30	MR	12	40	MRMS	24	60	S	60	7	2	3	4	7
WLA-149	Landrace	8.5	3.25	20	MSMR	12	25	MR	10	30	MRR	9	40	S	40	6	8	8	4	6
WLA-150	Landrace	8	7	20	S	20	25	MR	10	30	MRR	9	60	S	60	6	6	7	5	6
WLA-151	Landrace	8	8.5	20	MRMS	12	30	MR	12	30	MRR	9	60	S	60	7	9	9	4	7
WLA-152	Landrace	8	4	20	MSMR	12	20	MRR	6	30	MR	12	50	S	50	8	8	9	3	8
WLA-153	Landrace	8.5	9	10	MR	4	30	MR	12	40	MRR	12	40	S	40	6	8	8	3	7
WLA-154	Landrace	7	9	20	MSMR	12	30	MR	12	50	S	50	40	S	40	6	4	8	4	8
WLA-155	Landrace	9	8.5	20	MRMS	12	30	MRMS	18	40	MRMS	24	50	S	50	4	7	8	3	7
WLA-156	Landrace	9	7	20	MSMR	12	30	MSS	27	70	S	70	70	S	70	7	4	7	6	8
WLA-157	Landrace	9	7	20	MS	16	40	MRMS	24	60	S	60	70	S	70	8	5	8	7	9
WLA-158	Landrace	8	3.5	20	MRMS	12	30	MR	12	30	MRMS	18	50	S	50	6	3	6	5	7
WLA-159	Landrace	1.5	0.5	20	MSMR	12	30	MRMS	18	30	MR	12	70	S	70	7	5	8	3	7
WLA-160	Landrace	9	7	10	MSS	9	30	MSS	27	40	S	40	90	S	90	8	9	9	5	8
WLA-161	-	8	8.5	20	S	20	20	MSS	18	30	S	30	30	MSS	27	9	7	8	-	-
WLA-163	-	8	8	20	S	20	30	S	30	40	S	40	60	S	60	9	7	8	8	7
WLA-164	-	9	9	20	S	20	30	S	30	40	S	40	60	S	60	-	-	-	-	-
WLA-165	-	2.25	8.5	20	MRMS	12	30	MR	12	40	S	40	50	S	50	6	7	7	6	7
WLA-166	-	9	7	20	S	20	30	S	30	40	S	40	60	S	60	9	9	8	-	-
WLA-168	-	2.25	1.75	15	MR	6	25	MRR	7.5	30	S	30	10	MRR	3	7	8	8	6	7
WLA-169	Landrace	9	9	15	S	15	30	S	30	40	S	40	50	S	50	9	9	9	-	-
WLA-170	-	5.25	3	10	MR	4	30	MR	12	30	MRR	9	30	MRR	9	9	9	8	7	7

WLA-171	Landrace	8.5	7	20	S	20	40	S	40	60	S	60	70	S	70	9	9	9	7	8
WLA-172	Landrace	8	7	15	S	15	30	S	30	40	S	40	50	S	50	8	7	7	-	-
WLA-173	-	8.5	6	10	S	10	30	S	30	40	S	40	50	S	50	9	9	9	-	-
WLA-174	-	6	6	10	MS	8	30	MR	12	40	S	40	50	S	50	9	9	8	-	-
WLA-176	-	1.5	4	10	MR	4	30	MRR	9	30	MR	12	10	MRR	3	8	7	7	7	7
WLA-177	-	6	9	20	S	20	30	MRMS	18	30	MS	24	50	S	50	-	-	-	-	-
WLA-178	-	8	9	15	S	15	30	S	30	30	S	30	50	S	50	9	9	8	-	-
WLA-179	-	NA	NA	10	S	10	20	MRMS	12	30	MRMS	18	30	S	30	-	-	-	-	-
WLA-180	-	8.5	8.5	10	MS	8	30	S	30	40	S	40	70	S	70	9	9	8	7	8
WLA-181	-	8	7	10	MS	8	30	MRMS	18	30	MSS	27	60	S	60	9	8	8	-	-
WLA-182	-	8	6	10	MS	8	20	MRMS	12	40	S	40	60	S	60	7	7	7	-	-
WLA-183	-	8	6	10	MSS	9	30	MRMS	18	40	SMS	36	50	S	50	7	8	7	7	7
WLA-184	-	7	4	10	MS	8	30	MRMS	18	40	S	40	50	S	50	9	7	8	5	7
WLA-185	-	8.5	8	10	MSS	9	25	MRMS	15	40	MRMS	24	50	S	50	9	7	8	-	-
WLA-186	-	8	7	10	MRMS	6	20	MRR	6	40	MRMS	24	50	S	50	8	8	8	4	7
WLA-187	-	7	6	10	MS	8	30	MRMS	18	30	S	30	50	S	50	8	8	7	5	7
WLA-189	-	7	8	10	MSMR	6	20	MRMS	12	30	S	30	50	S	50	-	-	-	-	-
WLA-190	-	9	6	10	MS	8	25	MRMS	15	30	S	30	50	S	50	8	8	8	7	7
WLA-191	-	8	5.75	10	MSMR	6	25	MRMS	15	40	MRMS	24	50	S	50	9	9	8	7	8
WLA-192	-	8.5	8	10	MRMS	6	20	MR	8	40	MRMS	24	50	S	50	8	8	7	-	-
WLA-193	-	8.5	8	10	MS	8	30	MRMS	18	30	MR	12	90	S	90	6	4	8	5	4
WLA-194	-	8	7	15	MR	6	20	MR	8	30	MRR	9	90	S	90	9	9	9	5	6
WLA-195	-	8	7	20	MS	16	20	MRR	6	40	MRR	12	50	S	50	8	9	8	5	7
WLA-196	-	9	9	20	MRMS	12	30	MRMS	18	40	MSS	36	60	S	60	9	9	9	-	-
WLA-197	-	9	8.5	10	MSMR	6	25	MRMS	15	40	MRR	12	80	S	80	4	3	8	4	3
WLA-198	-	8	7	20	MR	8	25	MRMS	15	40	MRR	12	40	S	40	6	6	8	6	6
WLA-199	-	5.5	7	20	MR	8	25	MRMS	15	30	MRR	9	30	S	30	7	7	8	5	6
WLA-200	-	9	6	10	MRMS	6	30	MR	12	40	MRMS	24	30	MR	12	8	7	9	5	6

WLA-201	-	9	7	20	MSMR	12	25	MRMS	15	30	MRMS	18	20	MR	8	5	3	8	3	4
WLA-202	-	8	7	10	RMR	3	20	MR	8	30	MRR	9	30	MR	12	3	3	4	3	6
WLA-203	-	8.5	7	20	MS	16	25	MRMS	15	40	SMS	36	30	MSMR	18	7	7	9	8	7
WLA-204	-	9	4	20	MRMS	12	20	MRMS	12	30	MRR	9	30	MSMR	18	3	6	8	6	7
WLA-205	-	8.5	6	20	MR	8	20	MRMS	12	40	MRMS	24	30	MR	12	3	4	5	5	7
WLA-206	-	2.5	3	15	MRR	5	20	MR	8	30	MRR	9	30	MR	12	3	3	6	4	5
WLA-207	-	8.5	6	10	MRMS	6	20	MRMS	12	40	MRR	12	70	S	70	8	3	8	5	8
WLA-208	-	9	4.25	10	MR	4	20	MRR	6	20	MRR	6	30	MRR	9	7	7	7	4	6
WLA-209	Landrace	8.5	7	10	MR	4	20	MR	8	20	MRR	6	30	MRMS	18	8	9	8	5	8
WLA-210	Landrace	9	8	40	MS	32	30	S	30	50	S	50	90	S	90	9	9	9	8	8
WLA-211	Landrace	9	7	10	MR	4	30	MR	12	30	MRR	9	30	MRR	9	4	3	8	3	4
WLA-212	Landrace	9	6	10	RMR	3	25	MR	10	20	MRR	6	30	MRMS	18	3	3	3	3	5
WLA-213	Landrace	8	3.25	10	MR	4	20	MR	8	25	MRR	8	30	MR	12	4	3	5	4	6
WLA-214	Landrace	9	3	10	MRMS	6	20	MRMS	12	30	MRMS	18	50	S	50	8	9	7	6	6
WLA-215	Landrace	9	3.5	10	MR	4	20	MR	8	30	MRR	9	40	MSMR	24	7	8	8	5	6
WLA-216	Landrace	8	6	10	MR	4	20	MR	8	25	MRR	8	30	MR	12	7	7	8	4	6
WLA-217	Landrace	9	2	10	MSMR	6	25	MRR	7.5	40	MRMS	24	50	MSMR	30	8	9	8	6	5
WLA-218	Landrace	8	7.25	10	MR	4	20	MR	8	40	MRR	12	30	MRR	9	4	3	8	4	5
WLA-219	Cultivar	5	5.75	10	MSMR	6	15	MR	6	30	S	30	90	S	90	8	7	8	6	6
WLA-221	Cultivar	5.5	8	10	MR	4	20	MRR	6	20	MRR	6	10	MRR	3	5	5	2	3	3
WLA-222	Cultivar	8	2	10	MR	4	20	MR	8	30	MRR	9	20	MRMS	12	7	6	8	7	7
WLA-223	Breeding Line	2.5	6	10	MR	4	15	MR	6	20	MRR	6	10	MR	4	7	6	8	8	7
WLA-224	Breeding Line	5.5	7	40	MSS	36	25	MRMS	15	40	S	40	70	S	70	8	8	9	8	8
WLA-225	Landrace	8.5	8	30	MSS	27	30	S	30	30	S	30	40	S	40	9	9	8	8	7
WLA-226	-	8	9	20	MSS	18	30	S	30	30	S	30	40	S	40	9	8	8	8	7
WLA-227	Breeding Line	5.5	7	20	S	20	30	S	30	30	S	30	40	S	40	9	8	8	8	7

WLA-228	Landrace	8	7	20	MS	16	30	S	30	40	S	40	70	S	70	9	9	9	8	8
WLA-229	Breeding Line	2.25	7	20	MR	8	20	MR	8	20	MR	8	10	MRR	3	2	2	8	-	-
WLA-230	Landrace	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
WLA-231	Landrace	8.5	8	20	MSMR	12	30	MSS	27	30	SMS	27	30	S	30	8	8	7	7	7
WLA-232	Landrace	8	8	10	MRMS	6	20	MRMS	12	30	MRR	9	40	S	40	8	8	7	5	7
WLA-233	Landrace	8.5	9	30	S	30	40	S	40	60	S	60	90	S	90	9	9	9	9	9
WLA-234	Landrace	8	3.25	20	MRMS	12	20	MRMS	12	30	MS	24	20	MRR	6	4	8	8	3	4
WLA-235	-	2	3.5	10	MRR	3	20	MR	8	10	MR	4	20	MRR	6	4	3	8	4	6
WLA-236	Landrace	8	9	-	-	-	-	-	-	-	-	-	-	-	-	7	9	8	7	7
WLA-237	-	8	4	20	S	20	30	S	30	30	S	30	90	S	90	8	7	8	8	7
WLA-238	Landrace	5.25	8	10	MRMS	6	25	MR	10	30	MRR	9	30	S	30	8	7	8	7	7
WLA-239	Landrace	8.5	8	10	MSMR	6	20	MRMS	12	20	MRR	6	30	S	30	8	8	8	-	-
WLA-241	-	2	8.5	20	S	20	30	S	30	40	S	40	50	S	50	8	7	8	8	7
WLA-242	Landrace	5	1.75	10	MR	4	20	MRR	6	20	MRR	6	30	MSMR	18	7	8	8	4	6
WLA-243	Landrace	9	7.25	10	MSS	9	30	MR	12	40	S	40	40	S	40	6	7	8	8	7
WLA-246	Landrace	8.5	7	10	MSMR	6	30	S	30	20	MRR	6	60	S	60	8	9	8	4	4
WLA-247	Landrace	2	7.25	-	-	-	-	-	-	-	-	-	-	-	-	7	9	8	8	7
WLA-248	-	5	9	10	MSMR	6	25	MRMS	15	30	MRMS	18	70	S	70	8	9	8	7	8
WLA-249	Landrace	9	7	10	MSMR	6	30	S	30	10	S	10	10	S	10	9	9	8	6	7
WLA-250	Breeding Line	2	2	15	MR	6	20	MR	8	30	S	30	30	S	30	7	7	7	4	7
WLA-251	-	2.5	0.5	10	MR	4	20	MR	8	30	MRR	9	30	MR	12	3	3	4	3	4
WLA-252	-	8	8	30	S	30	40	S	40	70	S	70	100	S	100	9	9	9	9	9
WLA-253	-	2	6	10	MR	4	25	MR	10	30	MRR	9	10	MRR	3	6	5	8	3	3
WLA-254	-	8.5	6	20	S	20	30	MRMS	18	30	MRMS	18	50	S	50	6	7	8	3	4
WLA-255	-	8	6	30	S	30	40	S	40	70	S	70	90	S	90	9	9	9	9	9
WLA-256	-	8	3.5	10	MR	4	20	MR	8	20	MRR	6	30	MSMR	18	5	6	8	4	7
WLA-257	-	9	7	10	MR	4	25	MRMS	15	20	MRR	6	70	S	70	8	7	8	6	8

WLA-258	-	5	9	20	MRMS	12	20	MR	8	30	MRMS	18	90	S	90	8	8	9	3	7
WLA-259	-	7	7.25	30	S	30	30	S	30	40	S	40	80	S	80	8	8	9	6	8
WLA-260	-	5.5	3.5	20	MRMS	12	20	MRMS	12	30	MSS	27	60	S	60	8	8	8	8	8
WLA-261	-	NA	NA	10	MRMS	6	20	MR	8	20	MR	8	60	S	60	4	3	8	3	3
WLA-262	-	9	7.25	20	MS	16	20	MRMS	12	30	MRR	9	50	S	50	4	3	7	2	5
WLA-263	-	9	7	20	MRMS	12	40	MRMS	24	40	MRR	12	70	S	70	8	9	9	4	8
WLA-264	-	9	9	30	S	30	40	S	40	70	S	70	100	S	100	-	-	-	-	-
WLA-265	-	8.5	7	30	S	30	40	S	40	70	S	70	100	S	100	9	9	9	9	9
WLA-266	-	8	7	10	MR	4	20	MR	8	20	MRR	6	10	MR	4	5	5	7	3	6
WLA-268	-	8	8.5	20	MR	8	20	MR	8	30	MRR	9	40	MSS	36	7	8	8	6	6
WLA-269	-	8	9	20	MSMR	12	30	MRMS	18	40	S	40	70	S	70	9	9	9	8	8
WLA-270	-	9	9	10	MS	8	20	MRMS	12	30	S	30	80	S	80	8	9	9	9	9
WLA-271	-	5.5	6	20	MRMS	12	20	MR	8	30	MRR	9	60	S	60	8	8	8	6	7
WLA-273	-	8	8.5	20	MSMR	12	20	MR	8	20	MRR	6	30	MSS	27	9	8	9	5	7
WLA-274	Landrace	8	8	20	MS	16	20	MRMS	12	30	S	30	40	S	40	9	9	8	5	6
WLA-275	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	4	8	-	-
WLA-276	Cultivar	2.5	3.625	10	MRR	3	20	MR	8	30	MRR	9	20	MR	8	9	9	9	-	-
WLA-277	Cultivar	9	9	20	S	20	30	S	30	40	S	40	70	S	70	9	9	9	9	9
WLA-278	-	7	8	20	MSMR	12	30	MRMS	18	40	S	40	60	S	60	9	9	8	-	-
WLA-279	Landrace	9	7	10	MS	8	30	S	30	40	S	40	50	S	50	9	9	9	8	8
WLA-280	Cultivar	1.5	0	-	-	-	-	-	-	-	-	-	-	-	-	5	3	4	3	4
WLA-281	-	2.25	8	-	-	-	-	-	-	-	-	-	-	-	-	5	3	7	3	4
WLA-283	-	5.5	5	20	MR	8	30	MRMS	18	30	MRR	9	20	MRR	6	5	5	4	-	-
WLA-285	-	9	9	30	S	30	30	S	30	30	S	30	70	S	70	9	9	9	9	9
WLA-286	-	8	6	10	MR	4	15	MR	6	10	MRR	3	15	MR	6	6	5	8	3	7
WLA-287	-	9	6	10	MR	4	15	MR	6	10	MRR	3	10	MR	4	5	5	7	3	7
WLA-288	-	9	3.5	10	MRMS	6	15	MR	6	15	MRR	5	20	MR	8	5	4	5	4	7
WLA-289	-	8.5	6	10	RMR	3	15	MR	6	15	MRR	5	25	MR	10	5	4	8	3	8

WLA-290	-	8.5	6	10	MR	4	20	MR	8	20	MRR	6	30	MR	12	5	4	8	3	7
WLA-291	-	8.5	7	20	MRMS	12	20	MRMS	12	20	MRR	6	60	S	60	9	9	9	4	7
WLA-292	-	5	7	20	MSMR	12	30	MRMS	18	20	MRR	6	60	S	60	9	9	9	5	8
WLA-293	-	9	6	20	MS	16	30	MRMS	18	30	S	30	30	S	30	9	9	7	5	6
WLA-294	Cultivar	8.5	4	20	MRMS	12	30	MR	12	40	MRR	12	60	MSS	54	9	9	8	3	6
WLA-295	-	8	6	10	MS	8	30	MRMS	18	30	MRMS	18	30	S	30	9	8	8	-	-
WLA-296	Landrace	5.5	5.25	20	MSMR	12	20	MR	8	30	MRR	9	20	MRR	6	9	8	9	8	7
WLA-297	-	1.5	1.5	20	MSMR	12	40	MRMS	24	40	MRR	12	50	MRMS	30	9	9	9	3	6
WLA-298	Landrace	2.5	1.75	10	MSMR	6	20	MR	8	30	MRR	9	50	S	50	9	9	9	6	8
WLA-299	Cultivar	9	1.75	10	MRMS	6	20	MR	8	30	MRMS	18	50	S	50	8	8	9	5	6
WLA-300	Cultivar	8.5	1.75	10	MR	4	25	MR	10	30	MRR	9	30	MR	12	4	4	6	4	8
WLA-301	Breeding Line	2.5	4.25	20	MR	8	20	MR	8	25	MRR	8	30	MR	12	4	4	8	3	6
WLA-302	Breeding Line	8	3.5	10	MR	4	15	MR	6	10	MRR	3	50	MR	20	4	4	7	3	6
WLA-303	Breeding Line	2	3	10	RMR	3	20	MR	8	30	MRR	9	30	MR	12	3	3	7	4	6
WLA-304	Breeding Line	1.25	2	20	MR	8	30	MR	12	40	MRR	12	30	MR	12	3	3	5	4	5
WLA-305	Landrace	8.5	7	10	MR	4	30	MR	12	40	MRR	12	30	MR	12	3	3	3	3	3
WLA-306	Landrace	9	8.5	10	MR	4	30	MR	12	40	MRR	12	30	MR	12	3	3	6	4	3
WLA-307	Landrace	9	7	10	MSMR	6	30	MR	12	40	MRMS	24	50	MSS	45	8	8	7	6	8
WLA-308	Landrace	9	7	20	MS	16	25	MRMS	15	40	MSMR	24	50	S	50	8	8	8	4	7
WLA-309	Landrace	9	9	-	-	-	-	-	-	-	-	-	-	-	-	9	9	8	7	7
WLA-310	Landrace	8.5	7	10	MSS	9	30	MSS	27	30	MRR	9	40	S	40	8	9	9	5	8
WLA-311	Landrace	8	6	10	MRMS	6	30	MR	12	30	MRR	9	40	MRMS	24	3	3	8	4	4
WLA-312	Landrace	5.5	4.25	20	MSS	18	30	MSS	27	40	S	40	40	S	40	8	8	9	-	-
WLA-313	Landrace	9	5.5	10	MS	8	20	MRMS	12	30	MRR	9	20	MRMS	12	8	7	7	4	7
WLA-314	Landrace	5.5	4.25	10	MS	8	30	MSS	27	50	S	50	90	S	90	9	9	9	7	9
WLA-315	Landrace	1.25	6	5	MRMS	3	30	MRMS	18	30	MRR	9	30	MRMS	18	7	5	7	4	7

WLA-318	Landrace	8	4.25	10	MS	8	30	MRMS	18	30	MR	12	30	MRMS	18	9	9	8	7	7