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Genetic analysis and development of molecular markers linked with rust resistance in wheat

Vallence Nsabiyera

A thesis submitted in fulfilment of the requirements for the degree of
DOCTOR OF PHILOSOPHY



THE UNIVERSITY OF
SYDNEY

Plant Breeding Institute, Cobbitty
Faculty of Science
The University of Sydney
October 2017

Statement of originality

I declare that the work presented in this thesis is original to me. All contributions including molecular and cytogenetic procedures and analyses, technical and supervisory advice have clearly been acknowledged. None of this thesis material either in part or in whole except as cited in the text has ever been submitted to this or any other academic institution for the award of a degree or diploma.

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Vallence Nsabiya
October 2017

Author Attribution Statement

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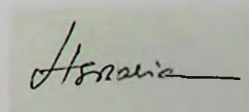
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Quote

“If you can't fly, run. If you can't run, walk. If you can't walk, crawl, but by all means, keep moving”

Martin Luther King Jr.

Dedication

Dedicated with love to my dear daughter Theopheria M. Uwera and all those who have encouraged and supported me to complete this project

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Abstract

The main aim of this study was to develop closely linked markers for rust resistance genes *Lr48*, *Lr49* and *Sr48*. In addition, mapping of adult plant stripe rust resistance in a landrace Aus27284 was also performed.

Close SNP-*Lr48* associations were identified using the iSelect 90K Infinium array. Five SNP markers co-segregated with *Lr48* and *IWB70147* mapped 0.3 cM proximal to this gene. Kompetitive allele-specific PCR (KASP) assays were developed for linked SNPs. In contrast, the KASP markers developed from the iSelect 90K Infinium SNP array for *Lr49* did not result in close marker-trait association. Sequence comparison of flow sorted chromosome 4B from parents VL404 (*Lr49*) and WL711 (*lr49*) resulted in close association of *sunKASP_21* (0.4 cM) with *Lr49*.

The Arina/Cezanne RIL population was used to develop markers linked with *Sr48* using the DArTseq platform and the iSelect 90K Infinium SNP array. DArTseq based linkage map located *Sr48* on the short arm of chromosome 2D. Marker *sun590* derived from a DArTseq marker mapped 0.4 cM distal to *Sr48*. *Sr48* was earlier mapped in the long arm of chromosome 2A in the Arina/Forno RIL population based on repulsion linkage with *Yr1*. The detection of 2AL-2DS translocation in Forno through genomic in-situ hybridisation (GISH) appears to have resulted in pseudo-linkage to locate *Sr48* in chromosome 2AL.

Aus27284 was susceptible to stripe rust at the seedling stage and exhibited resistance in field experiments. Genetic analysis showed monogenic segregation and the resistance gene was temporarily designated *YrAW11*. *YrAW11* was located on

chromosome 3BL near the centromere and *KASP_65624/KASP_58449* and *KASP_53113* flanked this locus.

The closely linked markers identified in this study were tested on a set of Australian and/or Nordic wheat genotypes to validate their suitability for marker-assisted selection (MAS). The results indicated that *IWB70147*, *sunKASP_21*, *sun590* and *KASP_53113* can be used for MAS of *Lr48*, *Lr49*, *Sr48* and *YrAW11*, respectively.

Chapter 1

Introduction

The human population is projected to reach 9.6 billion by 2050 (UN-DESA 2013) and 70% increase in food production by 2050 is required (World Bank 2008; Godfray et al. 2010; Ray et al. 2013). Increased food production is expected from new technologies related to increased yield potential and resistance to biotic and abiotic stresses, agronomic practices and mechanisation (Sharma 2012).

Common wheat is among the most important global food crops providing proteins, carbohydrates, and minerals (IWGSC 2014). Several biotic and abiotic stresses often hinder wheat productivity (Edae et al. 2014). The adverse effects of climate change; such as emergence of new diseases, shift in the virulence of pathogens and variations in temperatures and humidity, affect wheat crop at different growth stages thereby limiting its productivity (Rosenzweig et al. 2001; Chakraborty and Newton 2011; Elad and Pertot 2014).

Three rust diseases namely; leaf rust caused by *Puccinia triticina* Erikss. (Pt), stem rust caused by *P. graminis* f. sp. *tritici* (Pgt) and stripe rust caused by *P. striiformis* f. sp. *tritici* (Pst) are the among the major biotic constraints to global wheat production. These diseases have resulted in severe yield losses globally varying from 5 to 70% (McIntosh et al. 1995; Chen 2005; Singh et al. 2012) depending on the susceptibility of cultivars grown. Stripe rust and stem rust have the potential to cause 100% yield loss if susceptible cultivars are grown under favourable conditions for disease development (Chen 2005; Leonard and Szabo 2005; Singh et al. 2012).

The cultivation of rust resistant cultivars (Biffen 1905; Knott 1989; McIntosh et al. 1995; McIntosh and Brown 1997) along with other control and/management practices; such as chemical (Knott 1989; Wanyera et al. 2006; Hodson 2011) and cultural (Knott 1989; Roelfs et al. 1992; Kolmer 2005) measures have been used to protect wheat crop against rust diseases. However, the deployment of resistance genes has been the most preferred means of control over other measures (Bariana 2003; Chen 2005; Singh et al. 2006).

Many rust resistance genes have been described and formally named and majority of these condition all stage resistance (ASR) (McIntosh et al. 1995; 2013; 2015), which is prone to be defeated by evolution of virulence among pathogen populations. In contrast, only a few adult plant resistance (APR) genes have been formally named; *Lr34/Sr57/Yr18* (Dyck 1977, 1987; Singh et al. 2000), *Lr46/Sr58/Yr29* (Singh et al. 1998; William et al. 2003), *Lr67/Yr46* (Hiebert et al. 2010; Herrera-Foessel et al. 2011), *Lr68* (Herrera-Foessel et al. 2012) and *Sr2/Lr27/Yr30* (Singh et al. 2000; Mago et al. 2011; Li et al. 2015) and these genes are considered to be durable. When present alone, APR genes do not provide adequate protection and four to five genes have to be pyramided to achieve near-immune levels of resistance (Singh et al. 2000). Combinations of ASR and APR genes can enhance cultivar durability against wheat rusts (Johnson 1984; Bariana and McIntosh 1995; Pedersen and Leath 1988; Mundt 2014; McCallum et al. 2016). Such pyramiding is expected to make deployed resistance genes to be more difficult for the pathogen to overcome as multiple mutations in pathogens will be required (Schafer and Roelfs 1985; Ayliffe et al. 2008). Therefore, a continued search for new sources of ASR and APR genes is necessary.

Combining ASR and APR in cultivars can be challenging as the presence of more than one ASR genes can be difficult to confirm without genetic analysis (Bariana 2003). Molecular markers closely linked with resistance genes facilitate accelerated selection of effective gene combinations in new high yielding rust resistant wheat cultivars in an anticipatory manner (Bariana et al. 2007a; Singh et al. 2008). The development of high throughput genotyping platforms (Akbari et al. 2016; Cavanagh et al. 2013; Semagn et al. 2014; Wang et al. 2014) and publicly available genomic resources (IWGSC 2014 and reference sequence of wheat <https://urgi.versailles.inra.fr/blast-iwgsc/blast.php>) have enabled the construction of highly saturated genetic linkage maps for accelerated gene discovery.

This investigation was planned to develop molecular markers linked with resistance to rust diseases for their marker-assisted pyramiding with other widely effective and marker-tagged loci in future wheat cultivars. The objectives of this investigation include:

- Development of molecular markers closely linked with adult plant leaf rust resistance genes *Lr48* and *Lr49*
- Identification of molecular markers closely linked with wheat stem rust resistance gene *Sr48*
- Characterization and chromosomal location of adult plant stripe rust resistance loci in Aus27284

Chapter 2

Review of literature

2.1 Rust diseases of wheat

Wheat production is constrained by various biotic factors which include diseases and insects. Fungal pathogens are the major causes of wheat diseases (Rajaram and Van Ginkel 1996; McIntosh 1998) and rust diseases namely; leaf or brown rust caused by *Puccinia triticina* (formerly known as *P. recondita* f. sp. *tritici*), stem or black rust caused by *P. graminis* f. sp. *tritici* and stripe or yellow rust caused by *P. striiformis* f. sp. *tritici* are estimated to cause A\$ 12 million, 8 m and 127 m in losses in Australia, respectively (Murray and Brennan 2009).

Leaf rust is the most widely distributed disease in all areas where wheat is grown (Chester 1946; Roelfs et al. 1992). It has the potential to cause yield losses more than 15% (Samborski 1985) and in the event of severe epidemics losses can reach 50% (Roelfs et al. 1992; McIntosh et al. 1995). Significant losses by wheat stem rust have been reported with the most severe epidemic that occurred in Australia in 1973 (Park 2007). Upto 50% losses due to stem rust are estimated to occur in the USA (Leonard and Szabo 2005). China loses about 1 million metric tons of wheat production from stripe rust infection annually (Chen 2005). Stripe rust also affects wheat production in Central Asia (Ziyaev et al. 2011) and South Asia (Singh et al. 2004) annually. Wellings (2007) estimated the annual expenditure of SAUD 40-90 million in chemical control of stripe rust in Australia.

Therefore, breeding for rust resistance is a major aim of wheat breeding programs worldwide and it relies on availability of genetically diverse and marker-tagged sources of resistance (Bariana et al. 2007a)

2.2 Control of wheat rust diseases

Several means of control can be used to mitigate rust diseases including the use of rust resistant cultivars (Biffen 1905; Knott 1989; McIntosh et al. 1995; McIntosh and Brown 1997), chemical (Knot 1989; Wanyera et al. 2006; Hodson 2011), cultural (Knott 1989; Roelfs et al. 1992; Kolmer 2005) and biological (Fleming 1980; Spencer and Atkey 1981; Roh et al. 2009; Henk et al. 2011) measures. The use of rust resistant cultivars is preferred over other measures because its efficient, economical and environmentally friendly nature (Bariana 2003; Chen 2005; Singh et al. 2006).

2.3 Resistance to rust diseases in wheat

2.3.1 Types of resistance

Resistance to rust diseases is broadly divided into two categories; simply inherited and complex resistance and these types are often referred to as all stage resistance (ASR) and adult plant resistance (APR), respectively (Bariana 2003; Fu and Chen 2005).

2.3.1.1 All stage resistance (ASR)

The ASR genes interact differentially with pathogen isolates to follow 'gene-for-gene' hypothesis (Flor 1946) and express hypersensitive responses (McIntosh et al. 1995, 1998). A single ASR gene can confer complete protection at all growth stages of plant and is prone to breakdown as a result of pathotypic evolution to acquire virulence (Singh et al. 2000). Several rust resistance genes that condition ASR have been

identified (76 for leaf rust, 75 for stripe rust and 60 for stem rust) and formally named

(McIntosh et al. 2013, 2015).

2.3.1.2 Adult plant resistance (APR)

This category of resistance can be either race specific or race non-specific. The race-specific APR genes are characterized by hypersensitive response similar to ASR genes. Leaf rust resistance genes *Lr12*, *Lr22a* and *Lr22b* represent this category (Kolmer 1996; Rubiales and Niks 1995). Like ASR, race specific APR can become ineffective in a short period of time due to the emergence of new virulent pathotypes.

The race non-specific APR is also known as polygenic, non-hypersensitive, horizontal, general, minor, slow rusting (Caldwell 1968; Johnson 1984), partial (Parlevliet 1985) and durable (Johnson 1984). The genes conditioning race non-specific APR do not provide adequate protection when used singly and hence use of combinations of such genes is essential (Bariana and McIntosh 1995; Singh et al. 2000). *Lr34/Sr57/Yr18* on chromosome 7D (Dyck 1977; Singh et al. 2000) represents an example of race non-specific pleiotropic APR locus that conditions APR to all three rust diseases. In contrast, *Yr71* on chromosome 3D (Bariana et al. 2016), *Lr75* on chromosome 1B (Singla et al. 2017) and *Sr56* (Bansal et al. 2014a) on chromosome 5B only reported to impart APR to stripe rust, leaf rust and stem rust, respectively. Future studies are needed to prove their pleiotropic nature.

2.4 Identification and characterization of new rust resistance genes

2.4.1 Screening of diverse germplasm and genetic analysis

The process of discovering new genetic variation involves screening of diverse germplasm against an array of isolates of the respective pathogen differing in virulence phenotypes at the seedling stage for ASR genes and at multilocations under field conditions for APR genes (Bariana 2003). The genotypes that showed resistance to all pathogen isolates and scored resistant at all field locations were then crossed with susceptible parent to develop segregating populations.

An ideal mapping population should be genetically diverse with sufficient phenotypic contrast for the trait of interest (Saxena et al. 2010). Bi-parental mapping populations are derived from hybridising two parents possessing contrasting alleles for the trait under study. These include F_2 backcrosses (BC), doubled haploid (DH) and recombinant inbred line (RIL) populations. The heterozygous nature of F_2 and backcross populations limits their use in experiments that require to be replicated at various sites; for example APR to rust diseases (Collard et al. 2005). In contrast, DH and RIL populations can be used in replicated trials to enhance the power of QTL detection (Varshney et al. 2009). A new generation of genetic-mapping populations has been generated from multi-parent cross designs (MpCD) to overcome many limitations of biparental QTL mapping and association mapping (Morrell et al. 2012; Dell'Acqua et al. 2015). These include collaborative cross, diversity outbred, heterogeneous stock, nested association mapping (NAM) and multi-parent advanced generation inter cross (MAGIC) or recombinant inbred advanced intercross lines (RIAIL) (Cavanagh et al

2008; Morrell et al. 2012; Dell'Acqua et al. 2015). The NAM and MAGIC populations have been used to map traits in several crops including wheat (Morrell et al. 2012; Bandillo et al. 2013).

Randhawa et al. (2016) screened a set of Nordic wheat cultivars against three rust diseases both under the greenhouse and field conditions to identify genotypes for detailed genetic analysis. There are many other studies published on identification of putatively new sources of resistance (Pathan and Park 2006; Pathan et al. 2008). Qureshi et al. (2017b) identified durum landraces carrying resistance against durum-specific pathotypes of the leaf rust pathogen and showed that geneotypes Aus26579 and Aus26582 carried one and two leaf rust resistance genes, respectively.

2.4.2 Identification of genomic location of new rust resistance loci

Genetic analysis is followed by identification of chromosomal location of resistance gene(s). The process is simple if the resistance is controlled by a single locus and in the case of involvement of more than one locus conditioning rust resistance, development of single gene mapping populations is required (Bariana and Bansal 2017). These researchers reviewed the use of different technologies used for genomic locations of disease resistance loci. Bi-parental linkage mapping is an approach that can successfully map loci (Mackay et al. 2009). Association mapping was recently introduced to overcome some limitations of bi-parental populations by taking advantage of linkage disequilibrium defined as the non-random association of alleles at two loci (Falconer and MacKay 1996) from many more generations of recombination into a

single analysis. This increases mapping resolution and also avoids the generations of time-consuming crosses used for linkage mapping (Myles et al. 2009). This approach is however limited by the high probability of false-positives (Type I error) and false negatives (type II error) resulting from confounding effects of the population structure and phenology (Brachi et al. 2011). It also excludes markers with low allele frequencies from the association analysis (Edae et al. 2014).

2.4.3 Mapping of ASR genes

2.4.3.1 Bulk Segregant Analysis (BSA)

Michelmoore et al. (1991) described the 'BSA' method. It was initially applied to resistance controlled by ASR genes (Michelmoore et al. 1991). It has been extended to quantitative trait loci (QTL) (Wang and Paterson 1994). It involves genotyping of pooled DNA of segregating progenies corresponding to the two phenotypic extremes (Michelmore et al. 1991) to identify markers linked with the gene of interest through marker-trait associations. The DNA pooling strategies to identify QTL in different mapping populations were discussed by Wang and Paterson (1994). The polymorphic markers between the two pools are used to genotype the whole mapping population to identify markers linked with the trait. Although this method is cheap and quick (Michelmoore et al. 1991), the use of extreme tails may fail to detect QTL with smaller effects and the interactions between different loci (Vikram et al. 2012). Various markers for rust resistance in wheat have been developed using BSA including, *Yr4* (Bansal et al. 2010), *Yr47* (Bansal et al. 2011), *Yr51* (Randhawa et al. 2014) and *Yr57* (Randhawa et al. 2015) and *Lr72* (Herrera-Foessel et al. 2014).

2.4.3.2 Selective genotyping

The term selective genotyping (SG) was coined by Lebowitz et al. (1987). In this method progenies are grouped into different classes based on the trait values and marker allele frequencies and are compared between the classes. Lander and Botstein (1989) used this approach for QTL mapping based on selected groups of progeny instead of the whole population. It can be unidirectional (one tail of distribution is used) or bidirectional (two tails of the distribution is used) (Navabi et al. 2009). Bidirectional SG approach is more effective and commonly used because it eliminates the effect of segregation distortion, but unidirectional SG is appropriate for traits that have been subjected to strong negative or lethal selection pressure in unfavorable environments (Sun et al. 2010). In bidirectional SG, individuals from two tails are genotyped and marker-trait association is inferred by analyzing the differences in allelic frequency between the two tails (Stuber et al. 1980, 1982). Several loci have been mapped using this approach including seed size in wheat (Ammiraju et al. 2001), α -amylase activity in rye grain (Masojć et al. 2011), stripe rust resistance in barley (Vales et al. 2005) and rust resistance in wheat (Bariana et al. 2010; Qureshi et al. 2017a).

2.4.4 Mapping of APR genes

Understanding the genetic control of complex traits such as APR to rust diseases requires genotyping and phenotyping of large mapping populations (Vikram et al. 2012). It is necessary to phenotype mapping populations over multiple environments to ensure identification of QTL with stable effects (Vikram et al. 2012). Marker trait associations in most QTL mapping experiments are determined by genotyping the whole population with markers evenly covering the whole genome (Soller et al.

1976; Huang et al. 2014; Edae et al. 2016). Although there is no substitute for whole population phenotyping, two approaches; bulk segregant analysis (BSA) and selective genotyping (SG) have been suggested to reduce on the extra cost of whole population genotyping.

In plants, QTL mapping was initiated more than 20 years ago (Bernardo 2008) and has been successfully used in the genetic analysis of several traits including abiotic and biotic stress tolerance (Crossa et al. 2014). QTL are detected based on marker-trait associations (Collard et al. 2005).

Various QTL mapping methodologies have been developed. These include: single marker analysis (SMA) (Sax 1923 ; Soller et al. 1976; Mott et al. 2000), simple interval mapping (SIM) (Lander and Botstein 1989), multiple trait/QTL mapping (MQM) (Jansen 1994); composite interval mapping (CIM) (Zeng 1994; Jansen 1993), multiple interval mapping (MIM) (Kao et al. 1999), whole genome average interval mapping (WGAIM) (Verbyla et al. 2007; Verbyla et al. 2012) and Bayesian model (Corander et al. 2003). SMA is the simplest method that compares mean trait values among genotypic classes at each marker locus (Edwards et al. 1992). The limitation of this approach includes difficulty to detect and accurately estimate the effects of QTL located far from the markers because of recombination between the markers and the QTL. It needs prior knowledge of marker positions in order to detect the QTL position relative to a marker locus (Collard et al. 2005). SIM involves testing marker-trait associations at positions within marker intervals along each chromosome (Lander and Botstein 1989). Other methods CIM, MQM and MIM extend SIM to include

additional markers outside a defined window as co-factors to the variation associated with other QTL present within the genome. WGAIM also extends from SIM and uses R package (R Development Core Team 2013) where all intervals of the linkage map are simultaneously considered for QTL analysis (Verbyla et al. 2007; Verbyla et al. 2012).

2.5 Genomic resources for marker development in wheat

Despite the large genome size of common wheat, ample progress has been made to develop saturated maps for all seven chromosome groups (Somers et al. 2004; Röder et al. 1998). The genome of wheat has been sequenced and assembled (IWGSC-RefSeq-v1) (IWGSC 2014). This resource has been successfully utilised to identify, fine map and develop markers closely linked with genes of interest (Hulbert et al. 2007; Houde and Diallo 2008; He et al. 2009; Cao et al. 2012; Gunnaiah et al. 2012; Bansal et al. 2014a, 2015; Qureshi et al. 2017a) and cloning (Fu et al. 2009; Periyannan et al. 2013; Saintenac et al. 2013).

Several bioinformatics tools have also been developed and applied to identify and predict gene functions such as sequence alignment: (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=MegaBlast&PROGRAM=blastn&BLAST-PROGRAMS=megaBlast&PAGE-TYPE=BlastSearch&BLAST-SPEC=blast2seq&DATABASE=n/a&QUERY=&SUBJECTS=>), BLAST search (<https://urgi.versailles.inra.fr/blast/?dbgroup=wheat-all&program=blastn>), phylogenetic analysis, and domain and motif analysis (Bailey et al. 2006) using programs such as PrimerHaploSNPer (Tang et al. 2008) and primer3 (Untergasser et al. 2012).

2.6 Isolating (cloning) wheat rust candidate genes and development of robust markers

Gene cloning allows the development of diagnostic molecular markers for rapid introgression of genes into new cultivars (Fu et al. 2009; Krattinger et al. 2009). Map-based cloning (MBC) or positional cloning is the method that has been used to isolate resistance genes of economic importance from different crop plants. The MBC strategy relies on the genetic to physical distance, the size of the population, accurate genetic map and accurate phenotyping to correctly position a gene of interest between close flanking markers (Peters et al. 2003). It has also benefitted from the availability of arrayed bacterial artificial chromosome (BAC) libraries of the wheat diploid ancestors in particular with respect to *Ae. tauschii*, the donor of the D genome (Luo et al. 2003) and the hexaploid wheat BAC libraries including high-density filters and PCR screenable BAC pools (Nilmagoda et al. 2003). The high-density maps have greatly improved to narrowdown the number of possible bacterial artificial clones (BACs) harbouring the gene.

In wheat, a majority of the so-far isolated and sequenced seedling resistance genes encode for CC-NBS-LRR group of proteins that are similar in a range of plants (Collins et al. 1998; Mago et al. 1999; Meyers et al. 2003). These include: *Lr1* (Cloutier et al. 2007), *Lr10* (Feuillet et al. 2003), *Lr21* (Huang et al. 2009), *Yr10* (Temel et al. 2008), *Sr33* (Periyannan et al. 2013) and *Sr35* (Saintenac et al. 2013). The cloned race non-specific resistance genes encode different proteins; *Lr34* is an ABC transporter (Krattinger et al. 2009), *Yr36* is Kinase-START (Fu et

al. 2009), *Lr67* is a predicted hexose transporter (LR67res) that differs from the susceptible form of the same protein (LR67sus) by two amino acids that are conserved in orthologous hexose transporters (Moore et al. 2015). Other method (MutRenSeq) that reduces the complexity of the wheat genome has been recently used to clone stem rust resistance genes *Sr22* and *Sr45* (Steuernagel et al. 2016). Markers for many rust resistance genes have been developed in the last decade and protocols are posted on MAS Wheat website (<http://maswheat.ucdavis.edu/protocols/>).

2.7 Marker validation

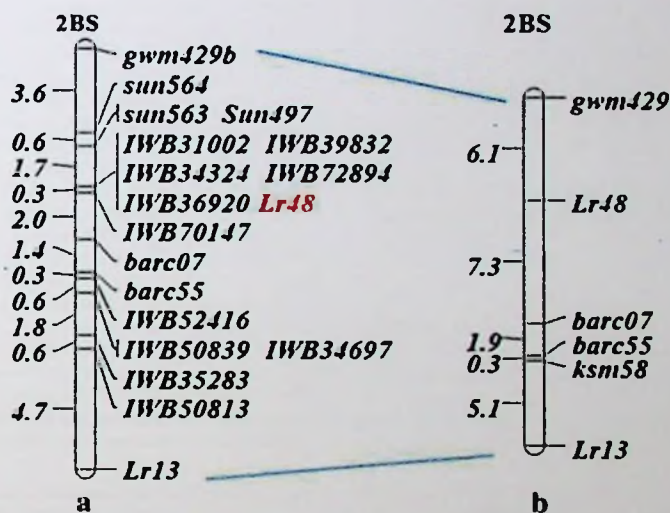
Marker validation studies are conducted to confirm allelic associations to ensure that markers are diagnostic and useful in different backgrounds (Cakir et al. 2003; Collins et al. 2003). A robust marker for marker-assisted selection (MAS) should not amplify gene-linked allele in genotypes lacking the target gene. Molecular markers can be background specific depending upon the extent of linkage. Loose linkage results in recombination between the marker and gene/QTL (Sharp et al. 2001; Thomas 2003). To ensure greater effectiveness in MAS, marker validation should be performed across diverse genetic backgrounds (Sharp et al. 2001; Bariana 2003; Spielmeier et al. 2003).

Chapter 3

Molecular markers for adult plant leaf rust resistance
gene *Lr48* in wheat

(This chapter is published in a peer-reviewed journal)

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Molecular markers for adult plant leaf rust resistance gene *Lr48* in wheat

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Abstract Leaf rust of wheat, caused by *Puccinia triticina*, is an important disease throughout the world. The adult plant leaf rust resistance gene *Lr48* reported in CSP44 was previously mapped in chromosome 2B, but the marker-gene association was weak. In this study, we confirmed the location of *Lr48* to be in the short arm of chromosome 2B and identified closely linked markers suitable for use in breeding. The CSP44 WL211 recombinant inbred line (RIL) population (90 lines) showed monogenic segregation for *Lr48*. Twelve resistant and 12 susceptible RILs were used for selective genotyping using an iSelect 90K Infinium SNP assay. Closely linked SNPs were converted into Kompetitive allele-specific primers (KASP) and tested on the parental lines. KASP markers giving clear clusters for alternate genotypes were assayed on the entire RIL population. SNP markers *WB31002*, *WB39832*, *WB34324*, *WB72894* and *WB36920* co-segregated with *Lr48* and the marker *WB70147* was mapped 0.3 cM proximal to

this gene. Closely linked KASP markers were tested on a set of Australian and Nordic wheat genotypes. The amplification of SNP alleles alternate to those linked with *Lr48* in the majority of the Australian and Nordic wheat genotypes demonstrated the usefulness of these markers for marker-assisted pyramiding of *Lr48* with other rust resistance genes.

Keywords KASP genotyping · Leaf rust resistance · Adult plant resistance · SNP markers · Marker-assisted selection · Wheat

Introduction

Common wheat (*Triticum aestivum* L.) is the second most important global cereal crop after rice (Ortiz et al. 2008; Khan and Iqbal 2011) and is affected by many biotic stresses that hinder its production (Meintosh 1998). Among the biotic stresses, fungal diseases including the three rust diseases: leaf rust, stripe rust and stem rust adversely affect the yield and quality of wheat grain (Gowda et al. 2014). Leaf rust caused by *Puccinia triticina* (Pt) is the most widely spread among the rust diseases and occurs in all wheat-growing areas of the world (Roelfs et al. 1992; Bolton et al. 2008). Because of its regular and worldwide occurrence, global losses from leaf rust damage surpass those from stem rust and stripe rust (Huerta-Espino et al. 2011). Losses due to leaf rust vary from

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region to region. For example, in the USA, leaf rust caused yield losses worth US\$350 million dollars between 2000 and 2004 (Huerta-Espino et al. 2011). In Australia, actual losses of AUS12 million and estimated potential national losses of AUS197 million were reported by Munay and Brennan (2009).

Leaf rust can be controlled by chemical application and/or through the deployment of genetic resistance in commercial cultivars. Use of resistant cultivars is preferred over chemical control due to its economic and eco-friendly nature (Bariana et al. 2007; Singh et al. 2013). Leaf rust resistance can be conditioned by either all stage resistance (ASR) or adult plant resistance (APR) genes. ASR is controlled by major genes which confer a high level of resistance (Kolmer 2005), but can be easily overcome by the evolution of new virulent pathotypes (Kolmer et al. 2013). Consequently, cultivars carrying ASR genes usually become susceptible within a few years of their release (Priyamvada et al. 2011). In contrast, APR is often controlled by non rice-specific genes that are effective at the post-seedling stages. APR genes are considered to be more durable. Many wheat breeding programs around the world have recently shifted from ASR to APR (Bansal et al. 2008), and deployment of combinations of more than two APR genes is necessary to achieve adequate levels of protection (Bariana and McIntosh 1995; Singh et al. 2000). APR genes that condition leaf rust resistance can be either hypersensitive (e.g., *Lr22h*) or non-hypersensitive in expression (e.g., *Lr34*) (McIntosh et al. 1995).

Currently, more than 70 leaf rust resistance genes have been identified and formally named (McIntosh et al. 1995, 2013, 2014). Most of these genes condition ASR, and only a few have remained effective over a long period (Singh and Dubin 1997; Lowe et al. 2011). To achieve durable leaf rust control, there is an ongoing need to identify and incorporate new sources of APR into modern cultivars along with other major and/or minor genes. Combining both types of resistance in cultivars can be challenging as the presence of more than one gene can be difficult to confirm without genetic analysis. Closely linked molecular markers are best suited to confirm gene combinations in breeding material and potential donor sources.

The advent of next-generation sequencing and high-throughput genotyping technologies has revolutionized wheat research (Morozova and Mana 2008; Trick et al. 2012). High-throughput

sequence-based markers such as Diversity Arrays Technology (www.diversityarrays.com) and single-nucleotide polymorphism (SNP) (Wang et al. 2014) have become marker system of choice due to their cost-effectiveness (Mammadov et al. 2012) and have contributed to a rapid increase in the identification of markers closely linked with disease resistance traits (Randhawa et al. 2014).

The adult plant leaf rust resistance gene *Lr48* was identified by Sami et al. (2002) in Cador single plant selection 44 (CSP44). It is reported to be effective against *P. tritici* pathotypes prevalent in India (Dhariwal et al. 2015) and Australia (Bansal and Bariana unpublished). Bansal et al. (2008) mapped *Lr48* on chromosome arm 2BS, flanked by markers *gwm429b* (6.1 cM) and *bare7* (7.3 cM). Samsampour et al. (2010) reported closely linked RAPD markers *S3450* and *S33699* in a *Tc* × *Lr251* Agra Local F_2 population. Singh et al. (2011) located *Lr48* in 2BL with *S336738* (10.3 cM) and *wmc332* (2.5 cM) as flanking markers. This study was undertaken to identify closely linked markers for use in breeding and to clarify disagreements among published reports for the position of *Lr48* in chromosome 2B.

Materials and methods

Host material

The mapping population used comprised 90 $F_{8,R}$ recombinant inbred lines (RILs) developed by crossing the resistant parent CSP44 and the susceptible parent WL711. In addition, susceptible parent WL711 carries a temperature-sensitive seedling resistance gene *Lr3* (Bansal et al. 2008; Pretorius et al. 1984). Parents and RILs were screened for leaf rust resistance in the greenhouse. To test the utility of the developed markers for marker-assisted selection (MAS), closely linked markers were tested on the Australian and Nordic wheat genotypes listed in Table 3.

Greenhouse leaf rust resistance screening

Two sets of RILs (4–5 seeds each) and parents were sown in 9-cm-diameter pots as four lines per pot. Twenty grams of Aquasol fertilizer dissolved in 10 L of tap water was applied to pots filled with potting mix.

before sowing. One-week-old seedlings were fertilized using urea at the same rate as Aquasol. One set was inoculated at the 2-leaf stage with Pt pathotype 104-1,(2),3,(6),(7),11,13 (culture no 547) to observe segregation for *Lr13*. The second set was grown to 4-leaf stage at 20 °C in a rust-free microclimate room prior to inoculation. Urea was applied to this set every week prior to inoculation with Lr3-virulent Pt pathotype 76-1,3,5,10,12 (culture no 539). The inoculation procedure described in Bansal et al. (2008) was followed. Rust response assessments were made 14 days post-inoculation using the scale as detailed in McIntosh et al. (1995).

Molecular mapping

DNA isolation

DNA was extracted from the entire RIL population using a CTAB method (Bansal et al. 2014a). The quality and quantity of DNA were assessed on 1 % agarose gel and using a Nanodrop spectrophotometer (Nanodrop Technologies).

SNP genotyping

Selective genotyping was carried out on 12 resistant and 12 susceptible RILs using an Illumina iSelect 90K Infinium SNP genotyping array (Wang et al. 2014). Closely linked SNPs were converted into Kompetitive allele-specific primers (KASP) and genotyped on the parents using KASP technology (LGC genomics, UK). Genotyping assays were carried out in a final volume of 8 µl consisting of 3 µl genomic DNA (30 ng/µl), 4 µl 2× KASP mix [a mixture of FAM and HEX Fluorescence Resonance Energy Transfer (FRET) cassettes, ROX™ passive dye, an optimized buffer containing MgCl₂, Taq polymerase and dNTPs], 0.11 µl primer mix containing two allele-specific forward primers each 12 µM and 30 µM of common primer and 0.89 of sterile water. PCR amplification was performed in a T100™ thermal cycler (BioRad) using the cycling conditions: 95 °C for 15 min for hot-start Taq DNA polymerase activation, followed by a touchdown profile of 9 cycles at 94 °C for 20 s and 61 °C for 1 min with a 0.6 °C reduction per cycle, followed by 38 cycles at 94 °C for 20 s and 55 °C for 1 min. The reactions were held at 12 °C for 3 min. End-point fluorescent images were

visualized using the CTX96 Touch™ real-time PCR detection system (BioRad, USA), and the data were analyzed using BioRad CTX Manager Software (BioRad, USA).

Genotyping of SSR and RAPD markers

The method described by Samsampour et al. (2010) was used to amplify the reported RAPD markers, while the SSR markers were amplified using the conditions described in Bansal et al. (2014b).

Data analyses and genetic mapping

Alleles for the SNP and SSR markers were scored as present (A and B for the resistant and susceptible allele, respectively) or '-' for missing values (failed to amplify). Chi-squared test was performed to test the goodness of fit of the observed segregation to the expected genetic ratio. Map Manager Version Q1Xb20 (Manly et al. 2001) was used to detect trait-marker associations. The Kosambi map function (Kosambi 1943) was used to convert recombination fractions to centimorgans. The linkage map diagram was constructed using MapChart software (Voorrips 2002). Markers with LOD scores of 3.0 and higher were considered to be linked.

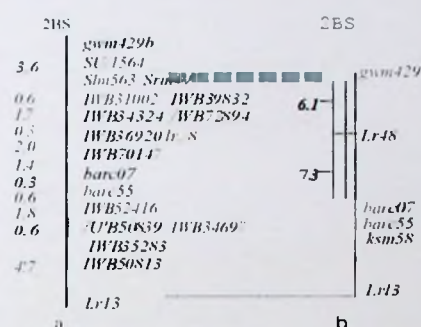
Results

Leaf rust tests on CSP44 W1711 RIL population in the greenhouse

The resistant parent CSP44 (*Lr48*) produced infection types (ITs) 7 to 2 at the 4-leaf stage against Pt pathotype 76-1,3,5,10,12, and W1711 was scored susceptible (IT3+). In contrast, W1711 exhibited ITs 23N to X against Pt pathotype 104-1,(2),3,(6),(7),11,13 at the 2-leaf stage and CSP44 was rated susceptible (IT3+). The RIL population segregated for *Lr48* (4-leaf stage) against pathotype 76-1,3,5,10,12 (36 resistant, 54 susceptible, $\chi^2 = 3.6$, non-significant at $P = 0.05$ and 1 df) and for *Lr13* (2-leaf stage) against pathotype 104-1,(2),3,(6),(7),11,13 (41 resistant, 47 susceptible, $\chi^2 = 0.41$, non-significant at $P = 0.05$ and 1 df).

Table 2 Primer sequences of SSR markers developed from chromosome survey sequence (CSS) contigs from new-sorted chromosomes of cultivar Chinese Spring

CSS contig no.	SSR	Repeat	Forward primer ^a	Reverse primer
5185093	<i>sun496</i>	(CTC) ₄	actcaccctcttcccttggt	gaggagcagctcccttgt
5209173	<i>sun497</i>	(AAT) ₅	tgcaaacagcagagaatcaaa	aacctctg tegtccacttc
5209173	<i>sun498</i>	(GTC) ₅	agcgggcccgaactaattt	cagagctgagggagggaggg
5195980	<i>sun563</i>	(GAG) ₉	aaactattccacacacacacg	agtgccattctttctcttc
5209756	<i>sun564</i>	(CTC) ₄	tctctgcaatctatgttgc	gtctgaaacagctcaggt

^a Forward primers were labeled with M13: caagacctgttaaaacagc**Fig. 1** Genetic linkage map of chromosome 2BS of CSP44 WL711 RIL population **a**, present study and **b** Bansal et al. (2008)

appears to be a better choice for marker-assisted selection of this gene.

Discussion

Identification of tightly linked markers

Breeding for rust resistance is a continuous process as rust pathogens evolve to produce pathotypes with new virulence phenotypes that overcome ASR genes. Deployment of diverse sources of resistance in commercial cultivars and breeding material is necessary to sustain/increase wheat production. The conventional method of deploying combinations of resistance genes is difficult, especially in the case of similar resistance expression and absence of epistatic

additive interactions (Randhawa et al. 2014). Closely linked molecular markers can facilitate the creation of gene combinations in breeding material and potential donor sources.

To develop diagnostic markers for *Lr48*, the previously published map (Bansal et al. 2008) was enriched with SNP markers. Five SNP markers *IWB1002*, *IWB39832*, *IWB34324*, *IWB36920* and *IWB72894* co-segregated with *Lr48*. The first four markers were previously assigned to Chinese Spring flow-sorted chromosome survey sequence (CSS) contig 2BS_5209173 (Wang et al. 2014) and the last marker corresponded to the contig 2BS_5185093. We also queried the TGACv1 shotgun assembly of Chinese Spring (<https://urgi.versailles.inra.fr/blast/>) and the synthetic W7984 whole genome sequence scaffolds (Chapman et al. 2015; <http://dx.doi.org/10.5447/1PK/2014114>), each of which contains scaffolds much longer than the CSS contigs. Markers *IWB39832*, *IWB1002*, *IWB36920*, *IWB34324* and *IWB72894* (which co-segregate with *Lr48*) were located on a single Chinese Spring TGACv1 scaffold (*Trinomea_acevium_CS42_TGACv1* scaffold 147085 2BS) and a single synthetic W7984 scaffold (Scaffold1352988), while the flanking markers were located on other scaffolds. Additionally, three markers that co-segregated with *Lr48* (*IWB39832*, *IWB1002* and *IWB72894*) were all found at a CRT500 distance of 1012.4 on a radiation hybrid map of 2BS, which was reported to reflect physical distance more accurately than genetic maps (Tiwari et al. 2016). Comparison of marker order against the NIAB 8-way MAGIC map (Cockram et al. 2015) also showed that these markers are located within a 4.5-cM interval. Taken together, all evidence suggests that these markers are closely linked to *Lr48* and are suitable for marker-assisted selection.

Table 3 Validation of closely linked SNP markers on Australian and Nordic wheats

Cultivar	Marker haplotype					
	14836920	14872894	14831002	14839832	14834324	117870347
CSP44	G	T	T	A	A	C
WL711	T	C	C	G	C	T
Australian wheats						
Atol, Katana, Ave Banks, Baster, Beaufort, Bolac, Calingiri, Catalina, Chara, Corack, Correll, Crusader Dart, Derrimut, Diamondbird, EGA Bonnie Rock, EGA Bounty, EGA Wedgetail, Elmore CL Plus, Emu Rock, Envoy, Espada, Estoc, Forest, Fortune, Gauntlet, Gazelle, GBA Sapphire, Giles, Gladus, Grenade CL Plus, Harper, Impala, Impose CL Plus, Janz, Justice CL Plus, King Rock, Kord CL Plus, Lancer, Lang, Lincoln, Livingston, Mare, Mackellar, Magenta, Mansfield, Merinda, Merlin, Orion, Preston, Scout, SF Adagio, SF Scenario, Spitting, SQP, Revenue, Sunzell, Ventura, Waapon, Wallup, Wedin, Wyalkatchem, Wylah, Yitpi, Young	T	C	C	G	C	T
Sunco	G/T	T	T/C	G	C	T
Sungai rd			T/C	A	A	T
Sunvale	G/T	T/C	T/C	A	A	T
Sunvex	T	T	C	A	A	T
Batavia, Camamah, Cobra, EGA Gregory, EGA Wylie, Kunjin, Phantom, Sentinel, Shield, Strzelecki, Suntop, Trojan, Westonia	G	T	T	A	A	C
EGA Burke, Napator, Yandanooka	G	C	T	A	A	T
Nordic wheats						
Körn	T	C	C	G	C	C
Krakka, Magnifik, Nana, Rudolf, Rubin, Runar, Steiner	G	C	T	G	C	T
Apu, Ator, Atorn, Avre, Bostrom, Botati, Botne, Bräcke, Blanka, Brysum, Boru, Brons, Canon, Dacke, Dala, Dalarna, Diamant II, Drabant, Dragon, ELS 6404-102-3, Ergo, Eroica, Extra, Kolben, Tagett, Fram I, Fram II, Fylgia I, Fylgia II, Huorapuu M10102, Apu, Holland, Hildun, Holger, Housmanaha M1201, Timontti, J-93, Juvenskylä M10302, Timantti JO 3524, JO 8028, Jekkylä M10505, Apu, Kaden, Kalle, Kanzler, Kinn II, Krimmo, Kuru, Laitala M10103, Landvaikette, Lantvete från Dalarna, Lantvete från Halland, Laven, Mami, Monda M1136, Målsåed, MS 274 - 150, Naxos, Nematos, Nera, Nourma, Pallas, Pansar II, Pansar III, Polka, Pompe, Pondus, Prime, Progress, Pudel, Rang, Reno, Renslät, Sammetvete, Rida, Ring, Rival, Rollo, Rubin, Rusu, Saffran, Safir, Sappo, Sibirian, Skirne, Snögg II, Snögg I, Soler, Sol II, Sol IV, Sopu, Sport, Stava, Stovik, Sunde, Svenno, Tasso, Thule II, Thule III, Timantti, Timantti Paavo, Tjalve, Touko, Troll, Trym, Ulla, Vakka, Viking, Vinjon, Vitus, Walter, William, WW 20200, Zelma, Diamant, Kesack, Sign II,	T	C	C	G	C	T

When relatively small-sized populations are used, like in the present study, marker–trait associations observed among mapping populations need to be validated on a diverse set of germplasm to assess their value in marker-assisted selection. These five SNP markers amplified alleles alternate to that linked with *Lr48* in 63 (76 %) Australian and 111 (94 %) Nordic cultivars. Although the marker *WB70147* exhibited recombination (0.3 cM) with *Lr48*, it was more informative than the above five markers when tested on the same set of genotypes (Table 3). This marker amplified the *Lr48*-linked allele 'C' in 13 Australian cultivars (Batavia, Camamah, Cobra, FGA Gregory, FGA Wylie, Kunjin, Phantom, Sentinel, Shield, Strzelecki, Suntop, Trojan and Westonia) and one Nordic wheat cultivar. All Australian cultivars that carried the *Lr48*-linked allele contain Condor in their pedigree and hence can be expected to carry *Lr48* originally identified in a Condor single plant selection 44 (CSP44). These results clearly showed the robustness of marker *WB70147* in marker-assisted selection of *Lr48*. It also demonstrated that the identification of trait–marker associations based on small populations followed by validation on a diverse set of germplasm could replace the use of large population for initial detection.

Genomic location of *Lr48*

Genetic independence of markers *5336^{rrs}*, *53₁₀* and *wmc332* shown to be linked with *Lr48* by Samsampour et al. (2010) and Singh et al. (2011) suggested that the target gene in mapping populations used by these workers was different. The tight linkage of chromosome 2BS derived SNPs with *Lr48* supported the results of Bansal et al. (2008).

Conclusions

The present study identified closely linked markers for adult leaf rust resistance gene *Lr48* and confirmed its location in chromosome 2BS. The haplotypes revealed by six KASP markers showed strong association with *Lr48* in a large set of Australian and Nordic wheat genotypes, demonstrating their utility in marker-assisted pyramiding of *Lr48* with other marker-tagged resistance genes. The KASP marker *WB70147* was observed to be the most robust of the six markers. Five

other KASP markers (*WB31002*, *WB39832*, *WB34324*, *WB72894* and *WB36920*) can also be used for marker-assisted selection of *Lr48* following assessment of parental polymorphisms.

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Author contributions YN drafted the manuscript. YN, HB and UB developed segregating population. YN, HB and UB did rust phenotyping. YN and NQ did marker work. UB, KI and MH designed primers. UB, HB and MH edited the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 4

Molecular mapping of adult plant leaf rust resistance gene *Lr49* using high throughput technologies and flow sorted chromosomes

4.1 Introduction

The ever-increasing global population, diminishing land resource and climate change threaten food and nutrition security. By 2050, the world population is projected to reach 9.6 billion and more than 70% increase in food will be needed (Bank 2008; World Godfray et al. 2010; UN-DESA 2013; Ray et al. 2013). Common wheat (*Triticum aestivum* L.) is one of the protein, carbohydrate and mineral rich staple food source for

30% of the global population (IWGSC 2014). The Green Revolution in the mid-1960's through the incorporation of reduced height genes together with improved production technologies led to significant increase in wheat production (Khush 2003; Pingali 2012). Wheat production is affected by many biotic and abiotic stresses. Wheat diseases alone are estimated to cause loss of 22 million tonnes in annual production (Kosina et al. 2007).

Leaf rust, caused by *Puccinia triticina* is among the important diseases of wheat that affect its productivity and quality throughout the world (Kolmer 2005, Singh et al. 2012) leading to losses varying from 5 to 70% (McIntosh et al. 1995). Wheat has inherent defense mechanisms to resist diseases; however, emphasis on selection for high yield and other desirable traits resulted in narrow genetic base for disease resistance (Davis et al. 1990; Khush 2001; Borlaug 2007). Consequently, farmers started using

synthetic chemicals to control diseases which resulted in environmental pollution and higher production costs (Goutam et al. 2015). The best strategy to control diseases is to deploy genetic resistance in new cultivars to reduce production costs and risk of environmental pollution due to fungicide usage (Singh et al. 2006).

Many leaf rust resistance genes have been identified and named in wheat (McIntosh et al. 1995; 2015). These genes represent two categories; all stage resistance (ASR) and adult plant resistance (APR). A majority of the formally named genes confer ASR (McIntosh et al. 1995; 2013). ASR genes exhibit hypersensitive reaction conditioning high levels of resistance against avirulent pathogen isolates and can be identified at the seedling stage under greenhouse conditions (McIntosh et al. 1995). ASR genes are prone to breakdown due to evolution in pathogen to acquire virulence. On the contrary, APR genes express at the post-seedling stages and confer low level of resistance singly. APR genes *Lr34/Sr57/Yr18* (Dyck 1977, 1979, 1987; Singh et al. 2000), *Lr46/Sr58/Yr29* (Singh et al. 1998; William et al. 2003), *Lr67/Yr46/Sr55* (Hiebert et al. 2010; Herrera- Foessel et al. 2011; Herrera-Foessel et al. 2014), *Lr68* (Herrera-Foessel et al. 2012) and *Lr75* (Singla et al. 2017) are race non-specific and are considered durable. *Lr12* (McIntosh et al. 1995) and *Lr22h* (Dyck 1979; Pretorius et al. 1989; Hiebert et al. 2007) belong to the race-specific hypersensitive APR gene category. Saini et al. (2002) identified adult plant resistance genes *Lr48* and *Lr49* and based on monocyclic flag leaf tests assigned them to the hypersensitive category. However, Bariana and Bansal (unpublished results) observed these genes to be slow rusting under polycyclic infection conditions in the field.

Traditionally, the development of new wheat cultivars has followed conventional phenotypic selection of desirable traits. Although this approach remains effective, it has significant number of challenges including a lengthy cycle to release a new cultivar (Lammerts et al. 2011; Shimelis and Laing 2012; Forster et al. 2015). Selection for disease resistance is further hampered by lack of appropriate pathogen isolates and the effect of environment on disease expression (Singh et al. 2012). The recent advances in wheat genomics have led to the development of more efficient and precise molecular tools to revolutionize marker-assisted selection of economic traits in wheat improvement programs (Varshney et al. 2006; Dubcovsky and Dvorak 2007; Fleury et al. 2010). The identification of DNA markers linked with rust resistance genes provided alternative selection technology to overcome the limitations of phenotypic selection and to pyramid two or more genes in breeding programs (Bariana et al. 2007a; Singh et al. 2012; Bariana et al. 2013, Bariana and Bansal 2017).

The availability of high-throughput DArTseq (Diversity Arrays Technology, Bruce, Australia; Cruz et al. 2013) and iSelect 90k Infinium Arrays (Wang et al. 2014) has expedited mapping of new loci (Bariana and Bansal 2017). Conversion of SNP to kompetitive allele specific PCR (KASP; LGC Genomics, UK) assays for routine use has provided gel-free alternative. DArTseq (Cruz et al. 2013; Bansal et al. 2014a; Randhawa et al. 2014; Li et al. 2015; Ren et al. 2015; Chhetri et al. 2016) and iSelect 90k Infinium platform followed by KASP assays (Cabral et al. 2014; Bariana et al. 2016; Nsabiya et al. 2016; Qureshi et al. 2017a; Tan et al. 2017) have been used recently for developing closely-linked markers for various disease resistance genes. The Centre of Plant Structural and Functional Genomics, the Institute of Experimental Botany,

Czech Republic has developed high-throughput chromosome isolation using flow cytometry (Vrána et al. 2000). Chromosome flow sorting enables sequencing of individual chromosomes. This approach reduces the complexity associated with the assembly of samples and allows analysis of next generation sequencing data (NGS) data of complex polyploid genomes at the subgenomic level (Doležel et al. 2007). This technology has added new dimensions to marker development and gene cloning in allopolyploids including wheat (Šimková et al. 2011; Doležel et al. 2012; IWGSC 2014; Sánchez-Martín et al. 2016).

An Indian cultivar VL404 was susceptible at the two-leaf stage but showed resistance at the adult stage against Indian leaf rust pathotypes and the underlying resistance locus was formally named *Lr49* (Saini et al. 2002). *Lr49* was mapped in the long arm of chromosome 4B (Bansal et al. 2008b) using VL404/WL711 RIL population. The flanking markers were not close enough for marker-assisted selection of *Lr49*. This study covers molecular mapping of the *Lr49*-carrying genomic region using the cutting-edge genomic resources to identify closely linked markers for marker-assisted selection of *Lr49* in breeding programs.

4.2 Materials and Methods

4.2.1 Plant and pathogen material

The F_{6:8} derived recombinant inbred line (RIL) population used in this study comprised of 181 lines developed from a cross between two Indian cultivars; the resistant parent VL404 (Pedigree; Kentana/Bage//Frontana/General Urquiza/3/ST464/PI74106) and the susceptible parent WL711 (Pedigree; S308/Chris//Kalyansona). A set of Australian (75)

and European (90) cultivars was used to test the usefulness of closely-linked markers in marker-assisted selection.

4.2.2 Green house tests

Eight to 10 seeds of each RIL and parents were sown in 9 cm-diameter pots as four lines per pot. The greenhouse screening protocol is described in Chapter 3.

4.2.3 Molecular mapping

The sequences of previously published SSR markers in linkage map (Bansal et al. 2008b) were obtained from the GrainGenes Database (<http://wheat.pw.usda.gov/>). These SSR markers were amplified following conditions given in Bansal et al. (2014a). Methods used for molecular mapping are detailed in Chapter 3.

4.2.4 Flow cytometric chromosome sorting and sequencing library preparation for chromosome 4B

Suspensions of intact mitotic chromosomes were prepared from synchronized root meristems of parental lines VL404 and WL711 according to Vrána et al. (2000, 2012) and GAA microsatellites on chromosomes were labelled in suspension by fluorescein isothiocyanate (FITC) using the protocol for Fluorescent In Situ Hybridization In Suspension (FISHIS) (Giorgi et al. 2013). Genomic DNA was stained by 4',6-diamidino-2-phenylindole (DAPI) and chromosomes were analysed on a FACSaria II SORP high speed flow sorter (BD Biosciences, San José, USA) as described in Kubaláková et al. (2002). One thousand chromosomes from each cluster were sorted onto a microscopic slide into a drop of 10 µl of PRINS buffer supplemented with 5% sucrose (Kubaláková et al. 1997). To assign chromosomes to an individual cluster on a dot plot, FISH with probes for GAA and *Afa* repeats was used to identify flow-sorted chromosomes and to

assess purity of sorted chromosome fraction. Chromosomal DNA of 4B was Multiple Displacement Amplified (MDA) using the Illustra GenomiPhi V2 DNA amplification kit (GE Healthcare, <http://www.gehealthcare.com>) as described in Šimková et al. (2008). Sequencing libraries were generated using Nextera DNA Sample preparation kit (Illumina Inc, San Diego, CA, USA) using 50ng of amplified DNA according to the manufacturer's instructions. Only difference was usage of 3µl of TDE1 for DNA fragmentation compared to the protocol given by manufacturer. Libraries with insert sizes 600-800 bp were selected for sequencing. The insert sizes were verified by Agilent DNA 1000 Kit (Agilent Technologies, Inc.) and concentrations were assessed by KAPA Library Quantification Kit (Kapa Biosystems, Woburn, USA). The libraries were sequenced as pair-end reads using HiSeq Rapid SBS Kit v2 (2x250 bp) (Illumina Inc, San Diego, CA, USA).

4.2.5 Detection of polymorphism across flow sorted chromosome 4B reads and development of KASP markers

GYDLE software (Gyde Inc. Bioinformatics Service, Quebec City, Canada; <http://www.gyde.com>) was used to quality filter the raw sequence reads (minimum phred score 20; minimum read length 50 bp) derived from flow sorted 4B chromosome sequences of VL404 and WL711, resistant and susceptible parent, respectively and to align the filtered reads to the IWGSC-RefSeq-v1 Chromosomes Chinese Spring contigs (<https://urgi.versailles.inra.fr/blast-iwgsc/blast.php>). SNP variant discovery was performed using the GYDLE findsnp function. Single-marker KASP assays were designed to interrogate nucleotide variation between the resistant and susceptible

parental samples. The KASP markers from this experiment were prefixed with 'sun' to differentiate them from iSelect 90K Infinium SNPs.

4.2.6 KASP assay

All KASP markers were tested on parental lines and population using a protocol described in Chapter 3 Materials and methods.

4.2.7 Data analyses

The RIL population was categorized as homozygous resistant or homozygous susceptible based on the phenotypic scores of respective parents. The marker alleles were scored as A (VL404 allele), B (WL711 allele) or missing [-] (failed to amplify). Chi-squared (χ^2) tests were used to determine the goodness of fit of observed segregation to the expected segregation ratios.

A genetic map was established by MapManager Version QTXb20 (Manly et al. 2001) at $P = 0.05$ linkage criterion. Recombination frequencies were converted to centiMorgans using the Kosambi map function (Kosambi 1943). The linkage map was drawn using MapChart software v2.2 (Voorrips 2002). A LOD score of 3.0 was used as the threshold for linkages among loci.

4.3 Results

4.3.1 Genetic analysis

VL404 (*Lr49*) exhibited infection type (IT) X and WL711 was scored IT 3+ when inoculated with Pt pathotype 76-1,3,5,10,12 at the 5th leaf stage under the greenhouse conditions. The RIL population segregated at a single leaf rust resistance locus (99 resistant: 82 susceptible; $\chi^2_{1,1} = 1.59$, non-significant at $P = 0.05$ and 1 d.f.).

4.3.2 Chromosome sorting and sequencing

Thirty five thousand copies each from VL404 and WL711 chromosome 4B were sorted with 97 and 98% purity, respectively. Chromosomal DNA was amplified by MDA according to Šimková et al. (2008). To minimize the risk of representation bias, the products from three independent amplification reactions were pooled. Amplification and pooling of 4B chromosomal DNA from VL404 and WL711 yielded 7.88 and 8.36 µg DNA and pair-end sequencing provided 70519221 and 70685036 reads representing 19.78 and 19.83 fold coverage, respectively.

4.3.3 Molecular mapping

Forty five SNPs from the iSelect 90K Infinium array showed linkage with *Lr49*. KASP primers (Table 4.1) were designed from the Infinium sequences flanking SNP. Twenty KASP markers produced clear discrimination between parents and were subsequently tested on the entire RIL population. Data generated from these markers defined the *Lr49* interval (Fig. 4.1a). The SSR markers (*barc199*, *gwm251*, *gwm495*, *gwm513* and *wmc34*) were also included in the map with the aim to refine map locations. Chromosome sorting and sequencing identified 40 SNPs between VL404 and WL711 within the *Lr49* interval. Twenty-eight *sunKASP* markers were developed (Table 4.2) and flanking sequences around SNP were not enough to design KASP for 12 SNPs. Seven out of 28 KASP markers produced clear clusters between parents and were unambiguously scored on the entire VL404/WL711 RIL population. In total, 32 markers including 5 SSRs (*barc199*, *gwm251*, *gwm495*, *gwm513* and *wmc349*) generated a partial linkage map of *Lr49* covering a distance of 30.5 cM (Fig. 4.1a). KASP marker

sunKASP_21 mapped 0.4 cM proximal and *sunKASP_24*, *KASP_20289*, *KASP_20288*, *KASP_35049*, *KASP_21440* and *KASP_39484* mapped 0.6 cM distal to *Lr49* (Fig. 4.1a).

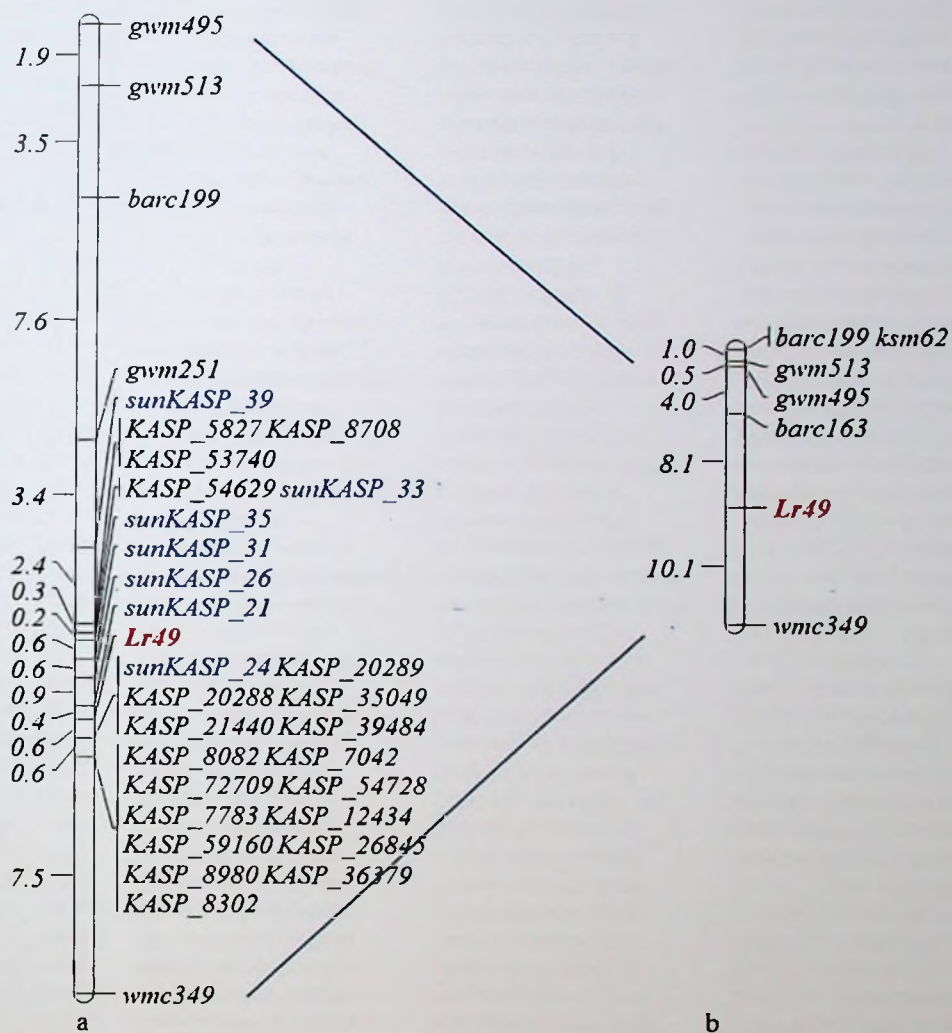


Figure 4.1 Genetic linkage map of chromosome 4BL of VI.404/WL711 RIL population (a) Present study; (b) Bansal et al. 2008b

Table 4.1 Primer sequences for KASP markers designed from SNP sequences linked with *Lr49* region on chromosome 4BL

KASP marker	Forward (Allele 1) primer ^a	Forward (Allele 2) primer ^b	Common reverse primer
KASP_1109	tattcatttacgattttaaatacttccaat	cattttacgattttaaatacttccaac	ccagggttggtgaccttcttatt
KASP_1606	atcagaaaagctccttcaaggggt	cagaaaagctccttcaaggggc	gtgtcacaaaagttctgagagtaacatt
KASP_1998	gagcttgatgacgaagaaggagat	agcttgatgacgaagaaggagac	tgacgtgtcgttgacgccgaa
KASP_5827*	gaagcagctggcagcactca	gaagcagctggcagcactcg	gtcagcctcaaggctgggtgtt
KASP_6418	actacatttaccagactttatatgactgc	gtactacatttaccagactttatatgactgt	tcacatgcagagacaccacttcat
KASP_6562	gatcagaactttgctgacgacg	ctgatcagaactttgctgacgaca	gctttgcttgattctgcgcacatt
KASP_7042*	tccaagttgactcaagagacgaga	ccaagttgactcaagagacgagg	ccctctacgaacaaccgacacaa
KASP_7077	gcagttccgtccaccacaca	cagttccgtccaccacag	cgaccttgaagtgacctcggtt
KASP_7783*	agtaagaggcactaccgttcagatt	aagaggcactaccgttcagatc	agagggcgctgtttccaaagtgaat
KASP_8082*	ttgttggtgtttagaataccctc	gctttggtgtgtttagaatacccta	cttgcactaacatcacacccccat
KASP_8302*	accctttacaacaacttcatctgc	ctaccctttacaacaacttcatctgt	gggaataatcagggttagacctcta
KASP_8517	gcggcggcggcggcga	gcggcggcggcggcg	gttgccttaccttagtagaggtgaa
KASP_8708*	ggctcgtggtgacgcacgcaa	gtcgtggtgacgcacgcag	ttcatatgacctatggcaggagat
KASP_8945	atgccaaactgacgaacaattctagttt	gccaaactgacgaacaattctagttc	gcgggatattggattaaagatgtaattagctt
KASP_8980*	agagcagaattgattgctgcaact	agagcagaattgattgctgcaacc	ctccacttctcactgtcactctgtt
KASP_10342	catagtgtcaaaattacaagcaatcgcat	agtgtcaaaattacaagcaatcgcac	ccatgtgtccaaatgaagattcagtat
KASP_12434*	cgcacgacgagtgctgccca	gcacgacgagtgctgccca	gtcaaggctgcaccaaccctgaa
KASP_20288	gtctcaatttggagatgcgctt	gtctcaatttggagatgcgctg	atcaagatgtaattctggattcagaagaa
KASP_20289*	tctttatactacacatttgaatcaggat	ctttatactacacatttgaatcaggac	ctccgagatgcaaaatacaagaagtgttt
KASP_21440	gtccccagctaatctgtggaa	ccccagctaatctgtggag	tagtttctgtagcttgggtgatacctt
KASP_21707	gtctcctctggagttgagtt	ctgctcctctggagttgagtc	tctgtaagacctgttaacatctgagaaa
KASP_26845*	cagtttaatatgcagcagcactta	ctcagtttaatatgcagcagcacttc	gcgcagatccatctcagaaggtaa
KASP_31674	cacatccaaagatttgattgacaaaaggga	catccaaagatttgattgacaaaaggg	ccaaacttaccacaatgaatgattaaaa
KASP_35049	aagcgaaagagaaactattttacagt	ctaagcgaaagagaaactattttacagc	tttagacacagcgatactgtgacatgtt
KASP_35851	ggctcggcgtaacgacagca	gtcggcgtaacgacagcg	ctccttatggatggtggcctggat
KASP_36054	gagatcccccaaggagacagaaggt	agatcccccaaggagacagaaggc	ccatgccggccgcctcccat
KASP_36379*	gtgaccttatgactggttagaag	cctgtgaccttatgactggttagaaa	aaatcgcaatttsaatgaattcagctt
KASP_38423	caaagtttcacgggttgggcataat	aaagtttcacgggttgggcataac	ttaagtgccatagagaacaaccagaagaa
KASP_39484*	agtcgaatgcaaggaggagaaa	ctagtcgaatgcaaggaggagaag	gccgctgttttaggcttctgctt
KASP_39515	gcattgtctacatcgcttgaccca	catgtctacatcgcttgacaccg	gcatatgccactacagaactgagaa
KASP_42264	aattctctgttgtaacgaacttgggt	cctgtggttaacgaacttggc	gaggggaaccaccgataaggat
KASP_51855	cgcattgaatttctgtaagggaagct	gcattgaatttctgtaagggaagcc	caagctctgcgcacactctaaaggat
KASP_51856	gcaagggtgtgcaaaaactggct	caagggtgtgcaaaaactggcc	aaatgagattgcaagggtgtgtgcaat
KASP_53740*	tgctcatttcatcttgcacatctgca	gtcatttcatcttgcacatctgcg	gatacagaggagaaagggttctgcta
KASP_54629*	gtgtctcaagtgacagttgaatgt	gtctcaagtgacagttgaatgc	atccagcttcttgcacagcgagaa
KASP_54642	atgtcgaccacagacaatacagatt	gtcgaccacagacaatacagatc	atcgctgacgtcattctgtgtgat
KASP_54728*	gatgacatcgacggcgaaactga	atgacatcgacggcgaaactgg	acacggctggtgatgccaccua
KASP_59160*	aagatttgccttgcacccgacttca	agatttgccttgcacccgacttccg	gtlaacaacgattcaaatgtggacacgaaa
KASP_72709*	cgatcctaataacacgacttattgt	gatcctaataacacgacttattgc	accgtagctgacttgggtgcagaa
KASP_74301	accagttaccttctgacttggaca	ccagttaccttctgacttggacg	gtgatgacctgatggcgccat
KASP_76214	gaacaagtgctgcttggaaatgatgt	aaacaagtgctgcttggaaatgatgc	gattgcaatgaagcaaggagtattgactt
KASP_76849	aaaacaagctgatgcttccgt	aaaacaagctgatgcttccgc	gaaaaaaaacygtgttctaatacagagat
KASP_77275	tagaaaaggcaaaagcatgaagcca	agaaaaggcaaaagcatgaagccg	gtgtttaccacagcatctactctgaa
KASP_77276	aacacccaagcctccacacgt	cacacccaagcctccacacgc	ctgtctcactgtccacggactt
KASP_77324	gacgacatctaccgcacgtgaca	acgacatctaccgcacgtgacg	cgaaggcgcaaggacatcgaggaa

^aA1 primer labelled with FAM: GAAGGTGACCAAGTTCATGCT; ^bA2

GAAGGTCCGAGTCAACGGATT

primer labelled with HEX:

*marker mapped in *Lr49* region

Table 4.2 Primer sequences for KASP markers designed from SNP discovered from the flow sorted chromosome 4B of parental genotypes

KASP marker	Allele 1 primer	Allele 2 primer	Common reverse primer
<i>sunKASP_19</i>	ttctataaaactcaaaaacaaggaaaaag	aactcaaaaacaaggaaaaagagaaa	tagagcccagtgccaatttc
<i>sunKASP_21</i>	gattegaatgtttttgtaggatttc	ttcagatctaaaatcacggcact	ctattaacgtagagcccagtg
<i>sunKASP_23</i>	gtgcttgggtgggtggga	ttcgattaccggggagc	atcaaacaaaccccatcagg
<i>sunKASP_25</i>	caaaaagaaaacattctgtagtaatctgta	agtaccaaatgcagcaaaaaa	cagtgatgggttgatgaga
<i>sunKASP_27</i>	cgcacaacaggtecatcattc	ttcttgacaccagcattgagag	aggcagttcctgaacaaaatg
<i>sunKASP_29</i>	cagaaattcctatattttactggaacg	aacacaagagagcatcatgaaatac	tgggttaagggaagaaca
<i>sunKASP_31</i>	tcaatcatttacttcatcgga	gaaatgtaattttttgtttgtgtgt	cacgtccaccaatttatgtca
<i>sunKASP_33</i>	catgtcaatagttatgcaatcaaat	aatctttttgtagccttcattc	ctttggcccaagttgtgtct
<i>sunKASP_35</i>	ccaaatcctaaaagccaagatgt	gagtagcggcggtaatagggtatt	gattgatgtccatgacctct
<i>sunKASP_37</i>	ccttttgaggeaaggagcaa	atagtttcatgatgtccctt	aaaaaggttcgggtgtgttg
<i>sunKASP_39</i>	caccatctectctcattatcg	catctctagaacaatgggtggt	aaaaattctgaacattacgaaaaca
<i>sunKASP_41</i>	gactcttctgggtccatctca	tcacaagttcagttgcagaaga	tccecaaaaattgtttctcaca
<i>sunKASP_43</i>	tttatatgcatgatttttatagcctca	actacatgcgcaaaaagcaat	caagcgcttttctttaacgtc
<i>sunKASP_45</i>	tggagaccgggtgtctct	ttatcaatgaccttttgaattggctg	ggtagctatgtgaggactctctt
<i>sunKASP_19</i>	ttctataaaactcaaaaacaaggaaaaac	aactcaaaaacaaggaaaaagagaaac	cactgaataatgcaacactagtagga
<i>sunKASP_21</i>	gattegaatgtttttgtaggatttt	cagatctaaaatcacggcacc	cgcattctggcttttaggat
<i>sunKASP_23</i>	tgtgtgtgggtgggtggg	ttcgattaccggggagt	aggatggcacagaaaatccg
<i>sunKASP_25</i>	caaaaagaaaacattctgtagtaatctgtt	cagtagcaaatgcagcaaaaaat	cttttcaattcgggacttgg
<i>sunKASP_27</i>	cgcacaacaggtecatcattt	ttcttgacaccagcattgagac	tccegtgtacctcaagaac
<i>sunKASP_29</i>	gcagaaattcctatattttactggaaca	aacacaagagagcatcatgaaatag	gttaggcgatgatgttcaatg
<i>sunKASP_31</i>	tcaatcatttacttcatcgag	aatgtaattttatgtttgtgttgc	cacggaccaccattgttcta
<i>sunKASP_33</i>	catgtcaatagttatgcaatcaattg	aatctttttgtagccttcattc	tggccaagtacagggtctacca
<i>sunKASP_35</i>	caaatcctaaaagccaagatgc	ttcatttgggacttggga	cggagctatttttgaccag
<i>sunKASP_37</i>	ccttttgaggeaaggagcag	atgtttcatgatgtccctc	ttgcacaggggtgagcactta
<i>sunKASP_39</i>	caccatctectctcattatca	catctctagaacaatgggtggtc	cttctccgttgcagaat
<i>sunKASP_41</i>	actcttctgggtccatctcgg	cacaagttcagttgcagaagc	tgtcgaataccaccttgcag
<i>sunKASP_43</i>	cttatatgcatgatttttatagcctcg	ctacatgcgcaaaaagcaac	cacgggcacgaacaaatag
<i>sunKASP_45</i>	tggagaccgggtgtctctc	ttatcaatgaccttttgaattgggtca	atccgaagtggcaatttgac

4.3.4 Validation of *Lr49* linked markers

Markers that showed close associations with *Lr49* were tested on a set of European and Australian cultivars and compared to the parental genotypes as checks. The marker allele variation among these cultivars and parental genotypes is presented in Table 4.3. Markers *sunKASP_21* amplified WL711 (T:T) haplotype in all Australian cultivars,

Table 4.3 Validation of closely_linked KASP markers on Australian and European wheat varieties/genotypes

Cultivars/lines	<i>sunKASP_21</i>
VL404 (<i>Lr49</i>)	C:C
WL711	T:T
AGT Katana, Axe, Baxter, Beaufort, Bolac, Calingiri, Camamah, Catalina, Chara, Cobra, Corack, Correll, Crusader, Dart, Derrimut, Diamondbird, EGA Bonnie Rock, EGA Bounty, EGA Burke, EGA Gregory, EGA Wedgetail, EGA Wylie, Elmore CL Plus, Emu Rock, Envoy, Espada, Estoc, Forrest, Fortune, Gauntlet, GBA Sapphire, Giles, Gladius, Grenade CL Plus, Impala, Impose CL Plus, Janz, Justica CL Plus, King Rock, Kord CL Plus, Kunjin, Lang, Lincoln, Livingston, Mace, Mackellar, Magenta, Merinda, Merlin, Naparoo, Orion, Phantom, Preston, Scout, Sentinel, Shield, Spitfire, SQP Revenue, Strzelecki, Sunco, Sunguard, Suntop, Sunvale, Sunvex, Sunzell, Ventura, Waagan, Wallup, Wedin, Westonia, Wyalkatchem, Wylah, Yandanooka, Yitpi, Young	T:T
Gazelle, JO 8023, Safir	C:C
Apu, Aros, Atson, Avle, Bastian, Bjarne, Blanka, Børsum, Boru, Canon, Dala, Dalarna, Diamant, Diamant II, Drabant, Dragon, ELS 6404 _ 102 _ 3, Ergo, Eroica, Extra Kolben, Fagott, Fram I, Fram II, Fylgia I, Fylgia II, Haarajärvi ME0102 Apu, Halland, Horsmanaho ME201 Timantti, J_03, Järvenkylä ME0302 Timantti, JO 3524, Jokikylä ME0505 Apu, Kadett, Kärn II, Kenya Farmer, Kimmo, Kiuru, Kota, Laitiala AP0103, Landvårkveite, Lantvete från Dalarna, Lantvete från Halland, Lavett, Manu, Monola ME1301, Møystad, MS 273 _ 150, Naxos, Nemaes, Nora, Norrøna, østby, Polkka, Pompe, Pondus, Prins, Progress, Rang, Reno, Ring, Rival, Rollo, Rubin, Runar, Ruso, Saffran, Sappo, Sibirian, Skirne, Snøgg II, Snøgg I, Sober, Söpu, Sport, Svenno, Timantti , Timantti Paavo, Tjalve, Touko, Troll, Trym, Ulla, Vinjett, Vitus, Walter, William, WW 20299, Zebra,	T:T

except Gazelle which carried the VL404 allele (C:C). Markers *KASP_20289*, *KASP_20288*, *KASP_35049*, *KASP_21440* and *KASP_39484* showed the presence of VL404 allele (A:A) in nineteen cultivars (data not given). Marker *sunKASP_21* amplified WL711 haplotype in 88 European cultivars and genotypes Safir and JO 8023 carried the VL404 haplotype (C:C) (Table 4.3). These results demonstrated the usefulness of *sunKASP_21* in marker-assisted selection of *Lr49* following assessment of parental polymorphism.

4.4 Discussion

Conventional phenotype based selection for disease resistance has been performed in various wheat breeding programs around the globe. While cultivars carrying durable resistance to diseases were released, the understanding of genetic basis of resistance was obscure. It is challenging to combine ASR and APR genes with confidence in a single genotype based on phenotypic assay due to high levels of resistance conditioned by effective ASR genes singly. The retrospective genetic analysis can however confirm the genetic basis of resistance. Bariana and McIntosh (1995) reported the involvement of four genes for resistance to stripe rust in cultivar Hybride-de-Bersee that showed durable resistance.

Rust response-linked molecular markers can facilitate pyramiding of two or more rust resistance genes in a single genotype. This investigation identified and validated close *Lr49-sunKASP_21* association. Previously, molecular marker development was based on very laborious low throughput marker systems. The identification of a high number of SSR markers assigned to all chromosomes in wheat

(Roder et al. 1998) made the process faster than that based on RFLP and RAPD technology. SSR markers linked with several economic traits in wheat were developed and protocols are available on the MASWheat website (<http://maswheat.ucdavis.edu/>). The technological developments including high throughput genotyping platforms and the next-generation sequencing (Zhang et al. 2011; Visendi et al. 2013) in the last decade have offered opportunities for developing ‘designer’ cultivars through marker-assisted selection (MAS) of economic traits. SNP markers linked rust resistance genes *Yr57* (Randhawa et al. 2016), *Lr48* (Nsabiya et al. 2016) and *Lr67* (Forrest et al. 2014) were identified.

Although 90K SNP markers mapped close to *Lr49* in this study, none of these markers produced in useful information when tested on a validation panel of 165 genotypes. The flow sorted chromosome technology has made identification of trait-marker associations and cloning of loci of economic interest faster in many crops including wheat (Vrána et al. 2000; Sanchez-Martin et al. 2016). This technology was also employed in this study and resulted in close association of marker *sunKASP_21* with *Lr49*. This marker amplified VL404 haplotype in three genotypes (Gazelle, JO 8023 and Safir), whereas the alternate haplotype was carried by 162 genotypes. These results confirmed the close association of *sunKASP_21* with *Lr49*.

To achieve durable control of leaf rust resistance in wheat, deployment of combinations of ASR and APR genes is essential. Marker linked with ASR genes *Lr1* (Cloutier et al. 2007), *Lr23* (Chhetri et al. 2017), *Lr24* (Mago et al. 2005; Bariana et al. 2016) and *Lr37* (Helguera et al. 2003; Blaszczyk 2004) are available and combinations

of these genes are effective against all Australian Pt pathotypes. Markers for APR genes *Lr34* (Lagudah et al. 2006), *Lr46* (E. Lagudah personal communication), *Lr67* (Forrest et al. 2014) and *Lr68* (Herrera-Foessel et al. 2012) are also available. All of these genes are associated with leaf tip necrosis (Singh 1992; Rosewarne et al. 2006; Herrera-Foessel et al. 2012; Spielmeyer et al. 2013). Leaf tip necrosis is associated with slight yield reduction in disease free environments (Singh and Huerta-Espino 1997). Therefore, it is not advisable to combine two-three slow rusting genes. Combinations of APR genes with effective ASR genes should be deployed in new cultivars. Stacking *Lr49* with another APR gene and at least two ASR genes can provide long lasting resistance that is hard to overcome by a single step mutation in the pathogen.

Overall, this investigation demonstrated the use of different genomic resources for identification of close trait-marker associations. The molecular map generated in this population will be useful for cloning of *Lr49*. Phenotypic assays were almost impossible to track *Lr49* among breeding populations segregating for ASR genes and the identification of *sunKASP_21* will be useful for pyramiding of this gene with other marker-tagged ASR and APR genes.

Chapter 5

Identification of molecular markers closely linked with wheat stem rust resistance gene *Sr48*

5.1 Introduction

Common wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD) is the most widely grown crop with an annual global acreage of 220 million ha (Curtis 2002; Shiferaw et al. 2013) and in the developing countries its acreage is ranked second. Its global trade volume exceeds all other major food crops combined (Atchison and Head 2010). Wheat provides about 20% of daily calories and its contribution to protein is more than maize (Shiferaw et al. 2013).

Stem rust also known as black rust, caused by *Puccinia graminis* f. sp. *tritici* (Pgt), has historically been a devastating disease of wheat in Africa, Asia, Australia, Europe, Middle East and New Zealand (Watson 1974, 1981; Luig 1977; Roelfs 1985; Saari and Prescott 1985; Leonard and Szabo 2005). Yield losses of 20-30% from stem rust were reported in the middle of the 20th century in many wheat growing regions in Asia, Australia, Europe and the United States (Zadoks 1963; Roelfs 1978; Leonard and Szabo 2005). Under favourable environment for disease development and cultivation of susceptible cultivars, stem rust has the potential to destroy the whole crop within three weeks of its onset (Leonard and Szabo 2005; Singh et al. 2012).

The detection of Pgt pathotype Ug99 (TTKSK) in Uganda in 1998 (Pretorius et al. 2000) rendered a high proportion (90%) of global wheat cultivars susceptible (Singh et al. 2006; Singh et al. 2011). From Uganda, TTKSK rapidly migrated to neighbouring

wheat growing countries including Tanzania, Kenya, Ethiopia, and Sudan and it has subsequently been detected in the Middle East and South Africa (Singh et al. 2006; Nazari et al. 2009; Pretorius et al. 2010; Visser et al. 2011). This pathotype acquired virulence for stem rust resistance genes *Sr24* and *Sr36* in Kenya in 2006 and 2007, respectively (Jin 2008, 2009). In 2013-2014, a Pgt pathotype TKTTF acquired virulence for *SrTnp* and caused an epidemic that destroyed 10,000 ha of the widely cultivated wheat variety Digalu in Ethiopia (Olivera et al. 2015).

Various stem rust control measures have been used and these included the removal of alternate host (*Berberis vulgaris* L.) of Pgt coupled with the use of host plant resistance (Kolmer et al. 1991; Singh et al. 2011) and application of fungicides (Wanyera et al. 2009; Macharia et al. 2013). To date, more than 60 stem rust resistance genes have been identified, located to specific chromosome positions and formally named in wheat (McIntosh et al. 2015). The deployment of rust resistance genes to control stem rust was estimated to save Australian wheat farmers AUD 12 million annually (Murray and Brennan 2009). The deployment of resistance genes singly resulted in boom and bust cycles through the emergence of new pathogen variants in Australia (Watson 1984). Watson and Singh (1952) emphasized the deployment of combinations of resistance genes following the demise of singly deployed genes *Sr6* (Eureka) and *Sr11* (Gabo) in Australia. Although it is possible for the pathogen to acquire virulence for more than one gene at once, the probability of such an event is very low.

A successful breeding for disease resistance program relies on the continuous supply of genetically diverse sources of resistance and development of selection technologies to deploy them in combinations (Bariana 2003; Bariana et al. 2007a; Bariana and Bansal 2017). Bansal et al. (2009) mapped stem rust resistance gene *Sr48* on the long arm of chromosome 2A based on its repulsion linkage (16.5 cM) with stripe rust resistance gene *Yr1* using the Arina/Forno recombinant inbred line (RIL) population. Attempts to identify markers closely-linked with *Sr48* using several genomic resources available in the distal region of chromosome 2AL failed to identify close *Sr48*-marker association (Bansal and Bariana unpublished). In this study, we used the Arina/Cezanne RIL population to identify markers closely-linked with *Sr48* for use in marker-assisted pyramiding of this gene with other marker-tagged resistance genes.

5.2 Materials and methods

5.2.1 Host material

The Swiss winter wheat cultivar Arina (Zenith/Moisson) was crossed with a stem rust susceptible winter wheat cultivar Cezanne (Thesee/87B29). F₂ seeds generated from a single F₁ plant were used to produce F_{6,7} recombinant inbred line (RIL) population. This population comprised of 172 RILs. A panel of 82 Australian and 90 Nordic wheat cultivars was used for validation of markers linked with *Sr48*.

5.2.2 Greenhouse screening

Five seedlings of cultivars Arina and Cezanne were raised in 9 cm diameter pots filled with potting mix consisting of pine bark and coarse sand (2:1). The stem rust susceptible cultivar Morocco was included as a control. The experimental material was

fertilized at sowing with Aquasol[®] (25g/10L of water) and six days after germination with Urea (25g/10L of water). Inoculations were performed according to Bariana and McIntosh (1993) using the Pgt pathotype 98-1,2,3,5,6,7 (PBI culture no. 580). Inoculated seedlings were incubated in the greenhouse at 18-20°C for 48 h on water-filled steel trays covered with hoods made from translucent polythene sheets to allow natural light and to provide 100% relative humidity. After incubation, infected seedlings were transferred to two microclimate growth rooms maintained at low (17±2°C) and high (27±2°C) temperatures to observe the effect of temperature on the expression of *Sr48*. Rust response assessments were performed 14-16 days after inoculation using the 0 to 4 infection type (IT) scale described by Stakman et al. (1962).

5.2.3 Mapping of *Sr48*

5.2.3.1 DNA extraction and genotyping

Genomic DNA was extracted from seedlings of each Arina/Cezanne RIL according to Bansal et al. (2014b). The quality and quantity of DNA were measured on 1% agarose gel and using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies), respectively. The DNA samples of 92 RILs and parents were sent to Diversity Arrays Technology Pty Ltd, Canberra, Australia for wheat DArTseq 1.0.

5.2.3.2 Development of PCR based SSR and sequence-tagged-site (STS) markers

Phenotypic data for segregation at *Sr48* were converted to genotypes (A = *Sr48* from Arina and B = *sr48* from Cezanne) and incorporated into the DArTseq map. The sequences (about 40 bp) of *Sr48*-linked DArTseq markers were used to identify Chinese Spring (CS) Chromosome Survey Sequence (CSS) contigs using the BlastN

(<https://urgi.versailles.inra.fr/blast/?dbgroup=wheat-all&program=blastn>) query. The CSS contigs were searched for repeats using the simple sequence repeat (SSR) identification tool (<http://archive.gramene.org/db/markers/ssrtool>). Primers were designed using the Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/primer3>). Three STS and 11 SSR markers were designed and named with the prefix 'sun' denoting Sydney University followed by consecutive number on the marker list (Table 5.1). The forward primers were labelled with M13 sequence: CACGACGTTGTAAAACGAC. These markers were tested for polymorphism on parental DNA. Polymorphic markers were tested on the entire RIL population according to the procedure described in Bansal et al. (2014a). In addition to 'sun' markers mentioned above, we also used 2DS specific markers; *barc124*, *barc95*, *gdm5*, *gwm296*, *gwm455*, *xib3*, *xib58*, *xib59*, *xib66*, *xib87*, *xib89*, *xib90*, *xib100* and *xib128* reported by Cao et al. (2012) and *gwm210*, *cf36* and *wmc111* reported by Yu et al. (2015).

5.2.3.3 Development of SNP markers

Genomic DNA (300ng/μl) from 14 resistant and 14 susceptible RILs and parents were sent to AgriBioscience, La Trobe University, Melbourne for selective genotyping using an Illumina iSelect 90K Infinium SNP array to identify SNPs linked with *Sr48*. KASP primers were designed using BatchPrimer3 v1.0 software (<http://probes.pw.usda.gov/batchprimer3/>). The detailed procedure of designing primers and running KASP assays is given in Chapter 3.

5.2.3.4 Genomic in situ hybridisation

Cultivars Arina, Forno and Cezanne were subjected to genomic in situ hybridisation (GISH) following the procedure of Zhang et al. (2001). Total genomic DNA from *T. urartu* (AA genome) and *Aegilops tauschii* (DD genome) were labelled with Fluorescein-12-dUTP and Rhodamine-5-dUTP (Roche Diagnostic Australia, Castle Hill, NSW, Australia), respectively using nick translation. The unlabelled total genomic DNA of *Ae. speltoides* (SS genome) was used as a blocker. The probe to blocker ratio was ~1:10. Chromosomes were counterstained with 4', 6-diamidino-2-phenylindole (Invitrogen Life Science, Carlsbad, CA) and fluoresced blue. Slides were analysed with a Zeiss Axio Imager epifluorescence microscope. Images were captured with a Retiga EXi CCD (charge-coupled device) camera (QImaging, Surrey, BC, Canada) operated with Image-Pro Plus version 7.0 software (Media Cybernetics Inc., Bethesda, MD) and processed with Photoshop version CS6 software (Adobe Systems, San Jose, CA).

5.2.4 Statistical analysis

Chi-squared test of goodness of fit was used to compare the observed stem rust response segregation against the expected genetic ratio for a single gene. Map Manager Version QTXb20 (Manly et al. 2001) was used to create linkage groups using a LOD score of 3 as threshold for significance. Genetic distances were determined using the Kosambi mapping function (Kosambi 1943) by transforming recombination fractions into centimorgans (cM). MapChart software v2.2 (Voorrips 2002) was used to generate linkage map.

5.3 Results

5.3.1 Rust response assessments

Cultivar Arina displayed IT 22⁻ at 17±2°C and IT 23C at 27±2°C while the susceptible parent Cezanne displayed IT 3⁺ at both temperature regimes (Fig. 5.1). The Arina/Cezanne RIL population was evaluated at 17 °C and showed monogenic segregation for seedling stem rust response against the Pgt pathotype 98-1,2,3,5,6,7 (84 RILs with ITs 22⁻ to 22⁺ and 88 RILs with ITs 33⁺ to 3⁻; $\chi^2_{1:1} = 0.29$, non-significant at $P = 0.05$ and 1 d.f.).

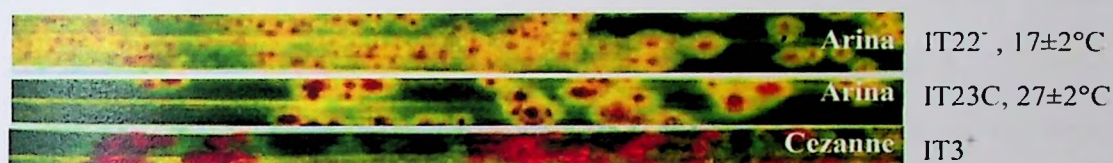


Figure 5.1 Infection types produced by Arina (*Sr48*) and Cezanne against pathotype Pgt 98-1,2,3,5,6,7

5.3.2 Construction of DArTseq linkage map

In total, 32,679 DArTseq markers were received and were subjected to χ^2 analysis to identify markers with significant deviations from the expected 1:1 ratio. Overall, 6576 markers displayed segregation distortion ($\chi^2 > 3.84$), 8606 markers had more than 10% missing data and 9940 markers did not differentiate between parents. These markers were excluded. Out of 7557 markers that passed the quality test for linkage map construction, 4498 markers were not assigned to any known chromosome and 3059 markers were distributed across 21 chromosomes. After hiding the redundant loci, 2202 markers were used for constructing the linkage map. A total of 1391 markers constituted 49 linkage groups, whereas 811 markers remained unlinked. Nineteen of the 21 chromosomes (except chromosomes 5D and 6B) had good marker coverage. The Arina/Cezanne linkage map covered a genetic distance of 3795.3 cM with marker

density of at least one marker per 2.7 cM. The number of markers mapped varied, with chromosome 4B having the smallest number of markers (9) while chromosome 1B had the largest number (458). The A, B and D genomes had 454, 722 and 215 mapped loci, respectively with the A genome exhibiting the most uniform coverage.

5.3.3 Marker discovery

Sr48 was incorporated in the DArTseq linkage map. *Sr48* showed co-segregation with 12 markers located in the chromosome arm 2DS and 2AL (Fig. 5.2b). A BlastN search of the DArTseq marker sequences linked with *Sr48* was performed. Markers *1211448*, *1094958*, *1148632*, *1000216*, *1064890*, *1093453*, *1104800* and *1246029* did not have good match with CSS contigs and the remaining four markers (*1109505*, *2258266*, *1141405* and *1375819*) identified four CSS contigs (Table 5.1). These contigs were used to develop 3 STS and 11 SSR markers. These markers were tested for polymorphism between the parents. Markers *sun590* and *sun592*, both from the CSS contig 2DS-5324961 were polymorphic. Marker *sun590* was co-dominant and amplified 161 bp and 157 bp fragments in Arina and Cezanne, respectively and *sun592* was dominant and amplified 159+153 bp amplicons in Arina and 153 bp amplicon in Cezanne.

Twenty seven KASP primers were designed from the *Sr48*-linked SNPs identified by selective genotyping (Table 5.2). Four markers, *KASP_56436* (2A), *KASP_6976* (2A), *KASP_42132* (2A) and *KASP_78815* (2D) differentiated parents clearly and were genotyped on the entire RIL population. In addition, chromosome 2DS specific markers *xib58*, *xib59* and *efd36* (Cao et al. 2012; Yu et al. 2015) were also tested on the RIL population. The final *Sr48* map (Fig. 5.2c) covered a 29.9 cM interval comprised of markers *sun590*, *sun592*, *KASP_56436*, *KASP_6976*, *KASP_42132*,

KASP_78815, *xib58*, *xib59* and *cfld36*. Markers *sun590* and *sun592* mapped distal and the rest of the markers mapped proximal to *Sr48*. Markers *sun590* and *xib59* flanked *Sr48* at 0.4 cM and 3.5 cM, respectively.

Table 5.1 Primer sequences of STS and SSR markers derived from the wheat chromosome survey sequences (CSS) that matched *Sr48*-linked DArTseq marker sequences

DArTseq marker	CSS contig	Repeat	Marker name	Forward ^a primer	Reverse primer
1109505	2AL-6421726	STS	<i>sun582</i>	agtacagggtgtggtgctt	atgtgccaaagttcgcatag
			<i>sun583</i>	ggcaaaatggcaggatattg	tccatccatgttctgatga
2258266	2DS-4785040	STS	<i>sun584</i>	aaggtgacatttgacctga	cgcacatcaccgggtacat
1141405	2DS-5324961	(GCG)3	<i>sun585</i>	gtgaatcgtacaagtggatg	actctgcacgtgaagtcacc
		(CCG)3	<i>sun586</i>	ttaaggctaaaacaatcagg	cgtacaaaaggcagatgg
		(AGA)3	<i>sun587</i>	aagtagcatttctggaacaca	aatacttgcacatctctttg
		(AGAGA)5	<i>sun588</i>	gcttgttccagttccag	ctcaagcaangeaaatagtcac
		(TCGCCG)6	<i>sun589</i>	ccgtcccaacagggtgtctc	tctgaacaaaacacatctac
		(TCCT)4	<i>sun590</i>	tactctagtgaaccaaccaaca	ggccattacatggtactagttt
		(AGCT)4	<i>sun591</i>	ttatccatttcaatctgagc	cctcactaataattgaaacacg
		(GTGTGC)6	<i>sun592</i>	tctggtgattttgttaggaa	aactctgtgcactgtagtttc
		(CTGGAA)6	<i>sun593</i>	tctctcactctctctctctc	tttatgggtacgtgctgtatt
1375819	2AL-6422050	(AG)6	<i>sun594</i>	ttagctctctaccatccagaa	aacatattgactgaaggacccaa
		(CGG)4	<i>sun595</i>	gtacagcgagacatgtgagac	gatgtggagaaaccaagtcac

5.3.4 Molecular cytogenetic analysis

The linkage of markers from chromosome 2D with *Sr48* was in disagreement with the original location of this gene in chromosome 2A (Bansal et al. 2009) based on its repulsion linkage with stripe rust resistance gene *Yr1*. These results suggested the involvement of a chromosomal rearrangement. Genomic in situ hybridisation (GISH) of cultivars Arina, Forno and Cezanne was carried out to determine if there were any

translocations between chromosomes 2A and 2D. GISH results indicated an interstitial translocation of A genome chromatin in the short arm of chromosome 2D in Forno (Fig. 5.3). In addition, the common 4AL-5AL-7BS cyclic translocation was observed in all three cultivars. These results indicated that translocation in Forno might have played a role in the location of *Sr48* on 2AL in Arina/Forno RIL population. The genetic association of SNPs located in chromosomes 2A and 2D also supported this observation.

Table 5.2 List of KASP markers developed from the *Sr48*-linked SNPs identified from selective genotyping of resistant and susceptible RILs using 90K SNP array

KASP marker	Chromosome	PopSeq position cM*	Scaffold**	Allele 1 primer ¹	Allele 2 primer ²	Common reverse primer
KASP_6982	-	-	-	tctacgatagcccaaccac	tctacgatagcccaaccac	cgaatgccagccaggttcta
KASP_8331	-	-	-	ctagagcttctctctccata	ctagagcttctctctccata	tcttcaggttggcaatctct
KASP_47559	2A	4.6	33664	atcggctggattattttgtt	atcggctggattattttgtt	taacttctgaaactacgggc
KASP_77910	2A	11.3	73235	tgtcaacaagataaaaggagg	tgtcaacaagataaaaggagg	aggagttagaataagccactaac
KASP_25308	2A	11.9	60956	gggcatttctcaatcaccacaa	gggcatttctcaatcaccacaa	ctcttctgtatctcttgggc
KASP_53165	2A	11.9	60956	ccggggagcagaattatggct	ccggggagcagaattatggct	catgttcttcagcagggtt
KASP_56436	2A	11.9	60956	aaaggatggaggatctgggt	aaaggatggaggatctgggc	aggaccgagacagcagaaac
KASP_65059	2A	11.9	60956	tgccecaagatacatggcga	tgccecaagatacatggcga	cccttgggttcttgggttgg
KASP_6976	2A	11.9	7356	acgtgtgtctgggtgttaa	acgtgtgtctgggtgttaa	atgtccactttgaagacaca
KASP_42132	2A	12.6	108478	ccagatgaagaaccaccagtata	ccagatgaagaaccaccagtata	ggacagtaagaataccacattcag
KASP_73038	2A	49.4	82164	gcagcttcattggatactctctta	gcagcttcattggatactctctt	aacaatgtcttcttcttctctt
KASP_10489	2A	52.2	46174	gaggtagtatgtcacaatgaacaa	gaggtagtatgtcacaatgaacag	tgcattgcaagcttaccgagt
KASP_71620	2A	59.2	58116	acttgaatcttattgttccacaata	acttgaatcttattgttccacaatg	agccagagggaggatgc
KASP_8846	2A	59.2	47476	ggcccccaacacacatt	ggcccccaacacacattg	ttctaccgggtgcatgagtt
KASP_65638	2A	59.2	22440	tcttatacttaggaacatggcttca	tcttatacttaggaacatggcttcg	ctgactgtctacatgtctattgg
KASP_23044	2A	59.2	117850	agccaccgagtcctgttagt	agccaccgagtcctgttagg	tgagcactactgtgtgttccc
KASP_41062	2A	74.4	78889	gattgaggaagggggaaagcgga	gattgaggaagggggaaagcggg	atcttcttctatccgtactctg
KASP_36583	2A	75.9	52744	tgtcagcatgtgtccctgt	tgtcagcatgtgtccctgc	cccccttagccaagaatgcac
KASP_36584	2A	75.9	52744	ggctgttcgaaagcttttggtta	ggctgttcgaaagcttttggtg	tcttggctaaagggggagaaa
KASP_12110	2D	7.5	32556	gagtcctctgttctgcca	gagtcctctgttctgccg	cataacaacaatctgtgcgac
KASP_12364	2D	7.5	32556	gtccgccgaagactgcaa	gtccgccgaagactgcag	ttatgaagcgtctctgtcc
KASP_32369	2D	7.5	32556	accggatgactttgacaataaaca	accggatgactttgacaataaacc	cccgggtctggagaaactcaa
KASP_7748	2D	9.7	87109	ggatcttccagttagagaaaggga	ggatcttccagttagagaaagggg	tctggccgttttcaatcg
KASP_8864	2D	9.7	87109	gagaatagcccaattggctga	gagaatagcccaattggctgg	tatgaggtgtccggggcag
KASP_10460	2D	9.7	87109	gagcgcggggagaggtat	gagcgcggggagaggtac	ttatgacctctgttaccggc
KASP_78506	2D	9.7	87109	gggtgatacttcccttgcgt	gggtgatacttcccttgcgc	cagtttctacttgaagcacaat
KASP_78815	2D	9.7	87109	gccttgcctgaaggagagaaa	gccttgcctgaaggagagaa	caggtcttcaggtatgcttag

*based on iselect 90K Infinium genotyping array

**based on IWGSC RefSeq_v1

¹A1 primer labelled with FAM: GAAGGTGACCAAGTTCATGCT, ²A2 primer labelled with HEX: GAAGGTCGGAGTCAACGGATT

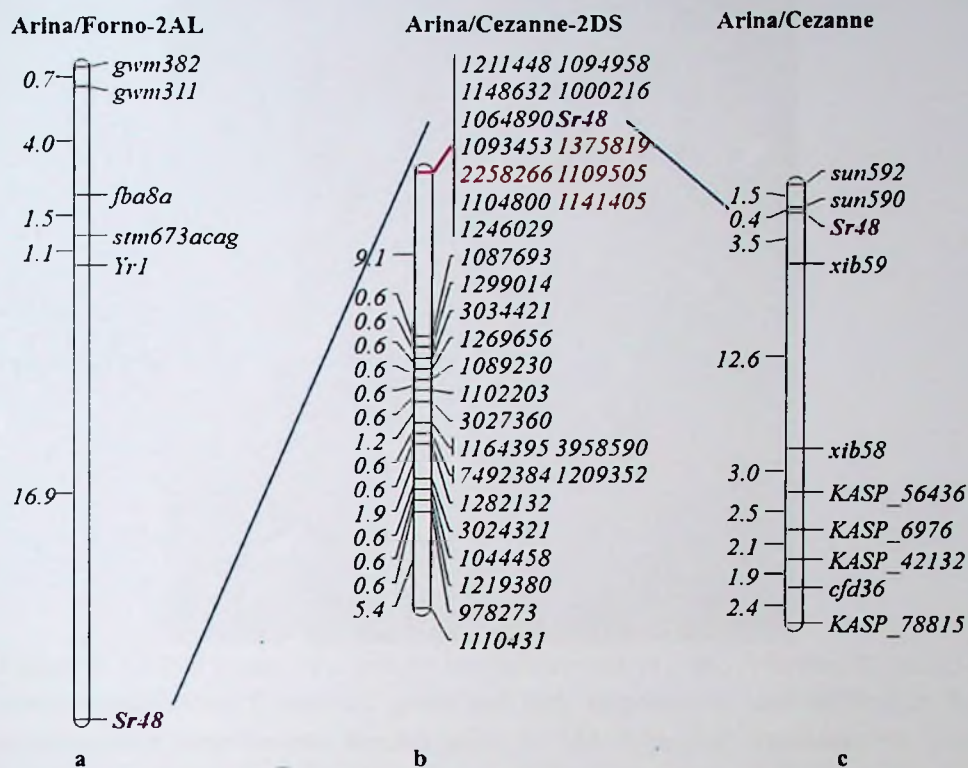


Figure 5.2 Genetic linkage maps showing location of *Sr48* a) Arina/Forno - Bansal et al. (2009), b) Arina/Cezanne - DArTseq of 92 RILs and c) Arina/Cezanne - PCR based marker map of 172 RILs

5.3.5 Comparison of *Sr48* with *Sr46*

The revised location of *Sr48* on the short arm of chromosome 2D suggested that it could either be *Sr46* or an allele at that locus. Genotypes carrying *Sr48* and *Sr46* were tested against Pgt pathotypes 98-1,2,3,5,6,7 and 34-1,2,7+*Sr38*. Pathotype 34-1,2,7+*Sr38* carries virulence for *Sr48*. Both genes exhibited resistance against pathotype 98-1,2,3,5,6,7. The ineffectiveness of *Sr48* and effectiveness of *Sr46* against Pgt pathotype 34-1,2,7+*Sr38* demonstrated the uniqueness of these loci (Table 5.3).

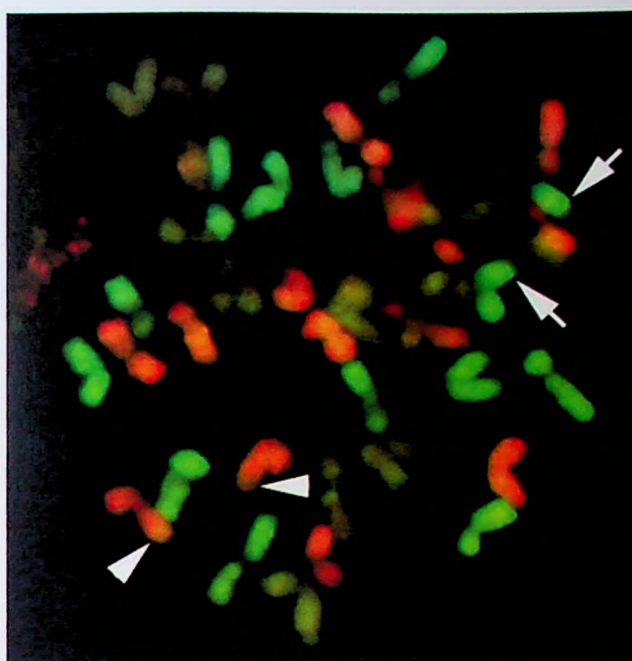


Figure 5.3 GISH image of a mitotic metaphase cell of cultivar Forno. A- and D-genome chromosomes were fluoresced green and red, respectively, and unlabelled B-genome chromosomes were brown. Arrows point to 4AL-5AL-7BS translocations found in all hexaploid wheats. Arrowheads point to a short segment of chromosome 2A (green colour) interstitially translocated to chromosome 2D (red colour)

Table 5.3 Comparison of resistance expression by stem rust resistance genes *Sr46* and *Sr48*

Temperature	27°C		17°C	
Pathotype	34-1,2,7 +Sr38	98-1,2,3,5,6	34-1,2,7 +Sr38	98-1,2,3,5,6
Genotype				
Aus18913 (<i>Sr46</i>)	2	2+	2=	2-
Arina (<i>Sr48</i>)	33+	23-	33+	22+
Morocco	3+	3+	3+	3+

5.3.6 Validation of *Sr48*-linked markers

Markers *sun590* and *sun592* mapped close to *Sr48*. These markers were tested on a set of Australian and Nordic cultivars (Table 5.4) to determine their diagnostic value in marker-assisted selection of *Sr48*. Marker *sun590* amplified the Cezanne allele (157 bp) in all Australian cultivars except a winter wheat cultivar Naparoo which carried the

Arina allele (161 bp). Thirty three Australian cultivars amplified Arina allele (159 bp) when tested with *sun592* indicating that this marker is not as close as observed in this study. Among the Nordic cultivars, both markers amplified the Arina alleles in nine genotypes (Bastian, Blanka, Dacke, Lavett, Nemares, Sport, Troll, Zebra and Pompe). Lantvete från Halland and Prins carried the Arina allele with *sun590* and the Cezanne allele with *sun592*. These results indicated that *sun590* is more robust in differentiating cultivars and can be used for marker-assisted selection following parental polymorphism checks.

Table 5.4 Validation of closely-linked SSR markers on Australian and Norwegian wheat cultivars

Cultivar	Amplicon size	
	<i>sun590</i>	<i>sun592</i>
Arina*	161	153+159
Cezanne**	157	153
Australian cultivars		
Baxter, Beaufort, Bolac, Calingiri, Carnamah, Chara, Derrimut, Diamondbird, EGA Bonnie Rock, EGA Bounty, EGA Gregory, EGA Wedgetail, EGA Wylie, Envoy, Espada, Estoc, Forrest, Fortune, Gazelle, Gladius, Grenade CL Plus, Harper, Impala, Justica CL Plus, King Rock, Kord CL Plus, Kunjin, Lancer, Lincoln, Livingston, Mackellar, Magenta, Mansfield, Merinda, Phantom, Preston, Scout, Sentine, SF Adagio, SF Scenario, Shield, SQP Revenue, Strzelecki, Suntop, Sunzell, Ventura, Wedin, Yandanooka, Yitpi, Young.	157	153
AGT Katana, Axe, Catalina, Cobra, Corack, Correll, Crusader, Dart, EGA Burke, Elmore CL Plus, Emu Rock, Gauntlet, GBA Sapphire, Giles, Impose CL Plus, Janz, Lang, Mace, Merlin, Orion, Spitfire, Sunco, Sunguard, Sunvale, Sunvex, Trojan, Waagan, Wallup, Westonia, Wyalkatchem, Wylah.	157	153+159
Naparoo	161	153+159
Norwegian cultivars		
Bastian, Blanka, Dacke, Lavett, Nemaes, Pompe, Sport, Troll, Zebra.	161	153+159
Apu, Atson, Avle, Batalj Bjarne, Borsum, Boru, Brons, Canon, Dala, Dalarna, Diamant, Diamant II, Drabant, Dragon, ELS 6404 - 102 - 3, Extra Kolben, Fagott, Fram I, Fram II, Fylgia I, Fylgia II, Haarajärvi ME0102 Apu, Halland, Horsmanaho ME201 Timantti, J-03, Järvenkylä ME0302 Timantti, JO 3524, JO 8023, Jokikylä ME0505 Apu, Kadett, Kärn, Kärn II, Kimmo, Kiuru, Kota, Laitiala AP0103, Landvårkveite, Lantvete från Dalarna, Manu, Monola ME1301, Moystad, MS 273 - 150, Naxos, Nora, Norrøna, Østby, Polkka, Pondus, Progress, Rang, Reno, Ring, Rival, Rollo, Rubin, Runar, Ruso, Saffran, Safir, Sappo, Sibirian, Skirne, Snøgg II, Snøgg I, Sober, Söpu, Svenno, Timantti, Timantti Paavo, Tjalve, Touko, Trym, Ulla, Vinjett, Vitus, Walter, William, WW 20299.	157	153
Prins, Lantvete från Halland.	161	153

*Resistant control and **Susceptible control

5.4 Discussion

This study demonstrated better expression of *Sr48* at a lower temperature ($17\pm 2^{\circ}\text{C}$). This resulted in robust phenotyping of Arina/Cezanne RIL population in contrast to Arina/Forno population where the presence of *Sr17* in Forno may have interfered with evaluation of that population at a low temperature regime. The temperature-sensitive expression of stem rust resistance genes *Sr6* (Green et al. 1960; Tsilo et al. 2009), *Sr12* (Singh and McIntosh 1986), *Sr15* (Watson and Luig 1966), *Sr17* (Roelfs and McVey 1979), *Sr21* (Chen et al. 2015), *Sr38* (Bariana and McIntosh 1993), *Sr43* (Niu et al. 2014), *Sr46* (Yu et al. 2015) and *Sr52* (Qi et al. 2011) has been reported.

Arina/Cezanne RIL population was produced to Mendelize the QTL *Q_{Sr.sun-5BL}* which was named *Sr56* (Bansal et al. 2014a). Surprisingly, *Sr48* was mapped to the short arm of chromosome 2D using the Arina/Cezanne DArTseq map. This finding was contrary to the previous location of *Sr48* on the long arm of 2A in Arina/Forno RIL population (Bansal et al. 2008, 2009). PCR based markers (*sun590* and *sun592*) developed from the CSS contig 2DS-5324961 that showed homology with DArTseq markers also confirmed the location of *Sr48* in 2DS in Arina/Cezanne RIL population. This observation prompted us to test Arina and Cezanne with markers previously reported to map on chromosome 2DS (Somers et al. 2004; Cao et al. 2012; Yu et al. 2015) and the marker *xib59* mapped 3.3 cM proximal to *Sr48*. Based on the microsatellites in the deletion bins on the Chinese Spring deletion map (Sourdille et al. 2004) and the microsatellite consensus map (Somers et al. 2004), the 2DS specific markers linked with *Sr48* are located in the 2DS5-0.47-1.00 deletion bin. These results supported the location of *Sr48* in the distal region of the short arm of chromosome 2D.

GISH results confirmed an interstitial translocation of chromosome 2A at the distal end of the short arm of chromosome 2D in cultivar Forno. According to Endo and Gill (1996), the 2A and 2D chromosomes of wheat are sub-metacentric and metacentric, respectively. Hence, the observed sub-metacentric nature of chromosome 2D in Forno could have resulted from the interstitial translocation from the long arm of chromosome 2A to chromosome 2DS. This translocation might be producing quadrivalents and resulting in pseudo linkage between *Yr1* and *Sr48* observed by Bansal et al. (2009).

By applying two different marker systems (SSRs and SNPs) using different next-generation sequencing and genotyping technologies (GBS, 90K Illumina iSelect SNP chip), this study has failed to ascertain the exact location of *Sr48* based on Arina/Cezanne RIL because of the locations of linked markers on chromosomes 2A and 2D. However, because *Sr48* was flanked by markers on chromosome 2DS, examining its uniqueness from other stem rust resistance loci reported in this region was necessary. Two other stem rust resistance genes; *Sr6* (Tsilo et al. 2009, 2010) and *Sr46* (Yu et al. 2015) have been mapped to chromosome 2DS (bin 2DS5-0.47-1.00). *Sr6* produces low seedling infection type (IT 0-;) (Tsilo et al. 2009, 2010) contrary to the intermediate IT displayed by *Sr48* (IT 22⁻ to 22⁺) at low temperatures. In addition, *Sr6* is linked with marker *wmc453* which was mapped at 69 cM position (Somers et al. 2004). In the present study, *cfd36* mapped 25.6 cM proximal to *Sr48*, whereas it was located at the 7 cM position by Somers et al. (2004). Marker *cfd36* mapped 62 cM distal to *wmc453*. Therefore, *Sr6* and *Sr48* represent different loci. *Sr46* was first identified from *Ae. tauschii* accession Aus18913 and was later observed in another *Ae. tauschii* accession Clae 25 which was originally derived from Aus18913 (Yu et al. 2015). The origin,

pathogenic specificity and comparative location of *Sr46* and *Sr48* made us believe that these genes are different.

SSR markers were breeders choice in the late 1990s and at the beginning of the 21st century (Mammadov et al. 2012) due to their abundance, co-dominance, high rate of polymorphism and genome specificity (Röder et al. 1998). Despite the low level of polymorphism and highly repetitive nature of the wheat genome, SSRs have been widely used for linkage analysis studies (Röder et al. 1998; Somers et al. 2004). However, their major drawback has been the high cost of their detection due to the lack of amenability for high-throughput systems (Cortés et al. 2011). The recent discovery of the next-generation sequencing technologies enables sequencing, discovery and genotyping of thousands of markers in hundreds of individuals at a much faster rate (Davey et al. 2011). SNP markers are replacing SSRs because of their amenability to high and ultra-high-throughput automation (Mammadov et al. 2012). The 90K SNP Illumina chip (Wang et al. 2014) and subsequent development of KASP (LGC Genomics) genotyping assays were used to identify breeder friendly SNP markers. However, none of the *Sr48*-linked KASP markers were close enough for use in marker-assisted selection. One would expect the *KASP-78815* marker developed from the 2D genome to map closer to *Sr48* than those developed from the 2A genome (*KASP-56436*, *KASP-6976* and *KASP-42132*) but it mapped more distant than the above 2A markers suggesting the involvement of chromosomal rearrangement.

Close trait-marker associations need validation across a diverse set of genotype to assess their usefulness in marker-assisted selection. The level of polymorphism of

such markers may vary among populations and/or cultivars. The co-dominant marker *sm590* demonstrated its usefulness in marker-assisted selection of *Sr48* in all Australian wheat backgrounds in the panel except Naparoo. Naparoo is a winter wheat and it is difficult to assess whether it carries *Sr48* or not due to the presence of *Sr24* which is effective against all Australian Pgt pathotypes. Pedigree of Nordic wheat cultivars was not informative enough to determine the presence of *Sr48* in a small set of genotypes that amplified the Arina allele. However, the presence of *Sr48* in these cultivars can be expected due to the European origin of Arina. Overall, a marker that can facilitate marker-assisted pyramiding of Ug99-effective stem rust resistance gene *Sr48* with other marker-tagged genes was developed and validated.

Chapter 6

Characterization and mapping of adult plant stripe rust resistance in Aus27284

6.1 Introduction

Stripe rust, caused by a biotrophic fungus *Puccinia striiformis* Westend. f. sp. *tritici* Erikss. (Pst) incurs severe damage to wheat production in many regions worldwide (Chen 2005; Milus et al. 2008). It was previously considered to be endemic in regions with cool and temperate climatic conditions and higher altitudes; such as Europe, Australia, Western USA, China and East African highlands (Zadoks 1963; Roelfs et al. 1992). It has recently spread to areas with warm temperatures (Milus et al. 2008; Hovmöller et al. 2010; Wellings 2011) making it a threat to all wheat growing regions. Losses from stripe rust vary from 10 to 70 % depending on prevailing climatic conditions, cultivars grown and disease pressure. However, 100% losses can occur on susceptible cultivars under favourable climatic conditions for disease development (Chen 2005; Bariana et al. 2016). The crop protection costs due to shift in Pst population in 2003 in Australia were estimated to be \$40-90 million annually (Wellings 2007). In another estimate, annual losses from stripe rust infection were reported to be \$127 million (Murray and Brennan 2009).

The use of fungicides and cultivation of resistant cultivars are the two most widely used measures to control stripe rust on large scale production systems. Although the timely application of fungicides can effectively control the disease (Chen 2005), it imposes additional cost and causes environmental pollution. The use of fungicides often fails to prevent yield losses in highly susceptible cultivars under high disease pressure

(Zegeye et al. 2014). Consequently, deployment of genetic resistance in cultivars is viewed to be the most attractive option to control stripe rust (Pu et al. 2010; Chen 2013) and it is dependent on the availability of genetically diverse sources of resistance.

Resistance to stripe rust can be classified into two types based on its expression at different growth stages; seedling/all stage resistance (ASR) and adult plant resistance (APR) (Bariana 2003; Chen 2005; Rosewarne et al. 2013). ASR is often controlled by genes that condition high level of resistance individually at all growth stages of the plant against an avirulent pathogen isolate and are prone to be defeated by evolution in pathogen population through acquisition of virulence (Bariana et al. 2007a). On the other hand, APR is controlled by genes that express at the post-seedling stages and often provide low level of resistance when present alone. More than 75 stripe rust resistance genes have been formally catalogued and most of these condition ASR (McIntosh et al. 1995; 2015). Rosewarne et al. (2013) summarized 140 quantitative trait loci (QTL) for stripe rust resistance identified in various studies.

Continuous evolution in Pst population gives rise to new pathotypes with different virulence and avirulence phenotypes and this evolution is reported to be caused by step-wise mutation in the initial exotic introductions in Australia (Wellings 2007; Park and Wellings 2012). Several stripe rust epidemics have occurred in various regions of the world including Ethiopia, USA, China and Australia causing loss of several million dollars (Wellings 2011) due to virulence on major genes including *YrA*, *Yr2*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr17* and *Yr27* genes (Wellings and McIntosh 1990; Chen 2007;

Singh et al. 2008). This constant evolution and subsequent breakdown of resistance especially of major genes necessitates continuous identification and deployment of diverse sources of resistance in new cultivars.

Cytogenetic techniques were used to map genes in the 20th century, but they were ineffective in determining location of APR genes (Chhetri et al. 2016). The recent discovery of high-throughput single nucleotide polymorphism (SNP) 90K array has revolutionized mapping of economic traits in wheat (Wang et al. 2014). The development of kompetitive allele specific PCR (KASP) genotyping system makes it even more robust due to its simplicity, cost-effectiveness and flexibility for in-house use (Semagn et al. 2014). Most of the genes in the 21st century have been characterized and mapped in classical bi-parental populations through bulk segregant analysis (BSA), selective genotyping or genotyping the whole population using high-throughput genotyping technologies.

Aus27284 was identified to be resistant under field conditions across three crop seasons by Bariana and Bansal (unpublished results). This investigation was planned to determine the inheritance and chromosomal location of stripe rust resistance in Aus27284.

6.2 Materials and methods

6.2.1 Plant material and mapping population development

The mapping population used in this study comprised 137 F_{5:6} RILs developed by crossing a susceptible genotype Avocet S (AvS) and the landrace Aus27284. Aus27284 was collected from Afghanistan and was received from the Australian

Winter Cereal Collection, Tamworth (now Australian Grains Genebank, Horsham). Three F₁ plants were grown in field to produce F₂ seed. F₂ seeds were sown and harvested as whole plant individually to generate F₃ population. The material was enhanced to F₅ generation through single head descent and single F₅ plants were harvested to generate F₆ generation. A set of 81 Australian wheat cultivars were used to test the effectiveness of closely-linked markers for marker-assisted selection.

6.2.2 Pathogen material and resistance phenotyping

The RIL population was phenotyped under the greenhouse and field conditions. A mixture of Pst pathotypes (134 E16A+Yr17+Yr27+, 134 E16A+YrJ+YrT+, 150 E16A+ and 110 E143A+) was used for field inoculations, while seedling evaluation was carried out using the predominant Pst pathotype 134 E16A+Yr17+Yr27+. Details of the virulence pattern of these pathotypes are provided in Table 6.1.

Table 6.1 *Puccinia striiformis* f. sp. *tritici* pathotypes used for artificial inoculation in the field and their virulence on the known genes

PBI culture no	Pathotype	Virulence for genes*
444	110 E143A+	Yr2, Yr3, Yr4, Yr6, Yr7, Yr25, YrA
615	134 E16A+YrJ+YrT+	Yr2, Yr6, Yr7, Yr8, Yr9, Yr25, YrJ, YrT, YrA
617	134 E16A+Yr17+Yr27+	Yr2, Yr6, Yr7, Yr8, Yr9, Yr17, Yr27, Yr25, YrA
598	150 E16A+	Yr2, Yr6, Yr7, Yr8, Yr9, Yr10, Yr24, Yr25, YrA

*based on reactions on Australian *P. striiformis* f. sp. *tritici* differential lines (McIntosh et al. 1995) having Yr1, Yr2, Yr3, Yr4, Yr5, Yr7, Yr8, Yr9, Yr10, Yr15, Yr17, Yr25, Yr27, Yr32, Yr33, YrA, YrSP resistance genes

6.2.3 Seedling stripe rust screening

The Aus37284/AvS RIL population, parents and the susceptible control (Morocco) were evaluated at the seedling stage in the greenhouse. The material was sown following the procedure given in Nsabiyera et al. (2016). At the two-leaf stage, seedlings were inoculated by atomising two mg of urediniospores of Pt 134 E16A+Yr17+Yr27+ suspended in 10 mL light mineral oil (IsoparL, Univar) using a hydrocarbon propellant atomizer. Inoculated seedlings were then transferred to enclosed hoods filled with warm water to create humid environment and incubated in dark conditions at 9-12°C for 24 h. Pots were then shifted to a temperature-controlled ($17\pm 2^\circ\text{C}$) microclimate growth room. Seedling rust response assessment was carried out 14-16 days after inoculation following a 0-4 scale outlined in McIntosh et al. (1995).

6.2.4 Adult plant stripe rust screening

The RIL population and parents were evaluated for adult plant stripe rust response at the Plant Breeding Institute, Cobbitty experimental sites; Karalee (KAR), Horse Research Unit (HRU) and Lansdowne (LDN) during the 2016 crop season. These locations are each separated by a radius of approximately 1KM and therefore, have similar climatic or environmental conditions; however, soil texture varies. Sowing was carried out in mid-May towards the end of autumn and beginning of winter. The susceptible cultivar, Nyabing 3 (Nyb-3) was planted after every 4 blocks of 35 experimental lines and on both sides throughout the field to facilitate uniform disease development and spread. The experiment was artificially inoculated three times (at 2 week intervals) using a mixture of pathotypes (Table 6.1) starting from the 3-leaf stage. About 30 g of urediniospores were mixed in 300 ml of light mineral oil (Isopar-L, Univar) and sprayed

on the experimental block using a Herbiflex4 hand-held sprayer (Micron Sprayers Ltd., Bromyard, Herefordshire, UK). The experimental field was irrigated twice a week with overhead sprinklers throughout the cropping season to promote disease development.

Stripe rust response variation was scored on a 1-9 scale (Bariana et al. 2007b) with 1 being very resistant and 9 very susceptible. Rust responses were noted at weekly intervals after the emergence of flag leaf till the susceptible check was scored 8 to 9. The final rating of each line was used for analysis.

6.2.5 Molecular mapping

6.2.5.1 DNA extraction and bulked segregant analysis (BSA)

Genomic DNA was extracted from young leaves collected from F₆ plants using the modified CTAB (cetyltrimethyl ammonium bromide) method (Bansal et al. 2014b). The DNA quality and quantity were assessed using NanoDrop ND-1000 (Thermo scientific, Wilmington, DE, USA) and diluted to a concentration of 300 ng/50 µl for BSA. Equal amount of DNA from 10 RILs each with scores 5 and 9 were bulked to make resistant and susceptible bulks, respectively. An artificial F₁ was generated by pooling equal amounts of DNA from 50 RILs. DNA from parents, artificial F₁ and the two bulks were subjected to genotyping using the iSelect 90K Infinium array (Wang et al. 2014) to identify SNPs linked with resistance in Aus27284. The linked SNPs were converted into KASP primers and were used to test the entire RIL population according to Nsabiyeira et al. (2016). The end-point fluorescent images were visualized using the CFX96 Touch™ real-time PCR detection system (Bio-Rad Laboratories Pty.

Ltd., USA) and allelic discrimination analysed using Bio-rad CFX Manager Software (Bio-Rad Laboratories Pty. Ltd., USA).

6.2.5.2 Genotyping with markers linked with other characterized stripe rust APR genes

The RIL population was screened with markers linked with previously characterized pleiotropic APR genes; *SNPLr46G22* for *Yr29/Lr46/Sr58/Pm39/Ltn2* (Evans Lagudah, unpublished), *csLv34* for *Yr18/Lr34/Sr57/Pm38/Ltn1* (Lagudah et al. 2006) and *csSNP-856* for *Yr46/Lr67/Sr55/Pm46/Ltn3* (Forrest et al. 2014).

6.2.6 Statistical analysis

The RIL population was scored susceptible at the seedling stage and therefore the phenotypic status of each RIL was deduced from the adult plant stripe rust response data. The final disease score for each RIL in the field over the three test locations were used for further analysis. The resistance was classified into two classes based on the rating of the parental types: homozygous resistant (RILs exhibiting score 5 to 7) and homozygous susceptible (8 to 9). Pearson's correlation coefficients (r) for the severity scores were calculated between the locations to evaluate the consistency in disease expression. The goodness of fit of the observed segregation with the expected genetic segregation ratios of adult plant stripe rust response variation and marker data at a single locus among RIL population was performed using the χ^2 test. Map Manager QTX version b20 (Manly et al. 2001) was used to develop linkage group using linkage criteria set at 0.1% probability level ($P = 0.001$). Kosambi (1943) mapping function was used to convert recombination fractions between markers into map distances in

centimorgans (cM). The final linkage map was constructed and drawn using MapChart (Voorrips 2002) software.

6.3 Results

6.3.1 Stripe rust response assessments

Aus27284 expressed seedling stripe rust infection type (IT) 33⁺ C, while AvS was produced IT 3+. The entire Aus27284/AvS RIL population exhibited ITs 33⁺ to 3+. These results indicated that stripe rust resistance in Aus27284 was not controlled by ASR genes.

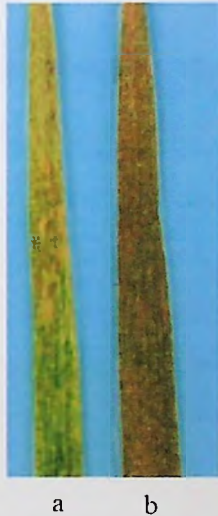


Figure 6.1 Disease severity of parental line a) Aus27284 and b) Avocet S

The resistant parent Aus27284 was consistently scored lower (5) than the susceptible parent AvS (8 to 9) at all locations (Fig 6.1). RILs were grouped into two categories; homozygous resistant and homozygous susceptible based on rust response variation at different locations and monogenic inheritance of adult plant stripe rust resistance was observed (Table 6.2). Sixteen RILs showing heterozygosity were excluded from the analysis. The resistance locus was temporarily named *YrAW11*.

6.3.2 Co-efficient of correlation

The Pearson correlation coefficients (r) for stripe rust responses across locations were highly significant ($p < 0.0001$) with r values ranging from 0.88 to 0.93 (Table 6.3).

Table 6.2 Distribution of Aus27284/AvS RILs when tested against stripe rust under field conditions

Location	Rust response of RILs		No of Missing RILs	$\chi^2_{1:1}$
	Homozygous resistant (4-6)	Homozygous susceptible (8-9)		
KAR	57	60	4	0.08
HRU	56	59	6	0.08
LDN	57	59	5	0.03

χ^2 table value at $P = 0.05$, 1 d.f = 3.84

6.3.3 Genotyping with gene specific/linked markers

Aus27284 and Avocet S amplified alleles alternate to those linked with the target genes when tested using markers linked with APR genes *Yr18* (*csLv34*), *Yr29* (*SNPLr46G22*) and *Yr46* (*csSNP-856*) indicating their absence in both parents.

Table 6.3 Pearson's correlation co-efficient across various stripe rust response data sets of Aus27284/AvS RIL population during the 2016 crop season

Location	KAR	HRU
LDN	0.88***	0.92***
KAR	-	0.93***

*** Highly significant ($P < 0.001$)

6.3.4 Molecular mapping of *YrAW11*

Linkages of 155 SNPs from chromosome 3B with *YrAW11* was observed in BSA using 90K SNP assay. Markers from the 60-75 cM region (Wang et al. 2014; total map length of chromosome 3B is 156 cM) in the 90K consensus map of chromosome 3B showed the strongest association with *YrAW11*. Many SNPs were clustered together at one location and a set of 28 KASP primers (Table 6.4) were designed by selecting a single SNP from each cluster. These KASP markers were tested for clustering on the resistant and susceptible bulks together with parents. Fourteen markers produced good clusters and were tested on the entire RIL population.

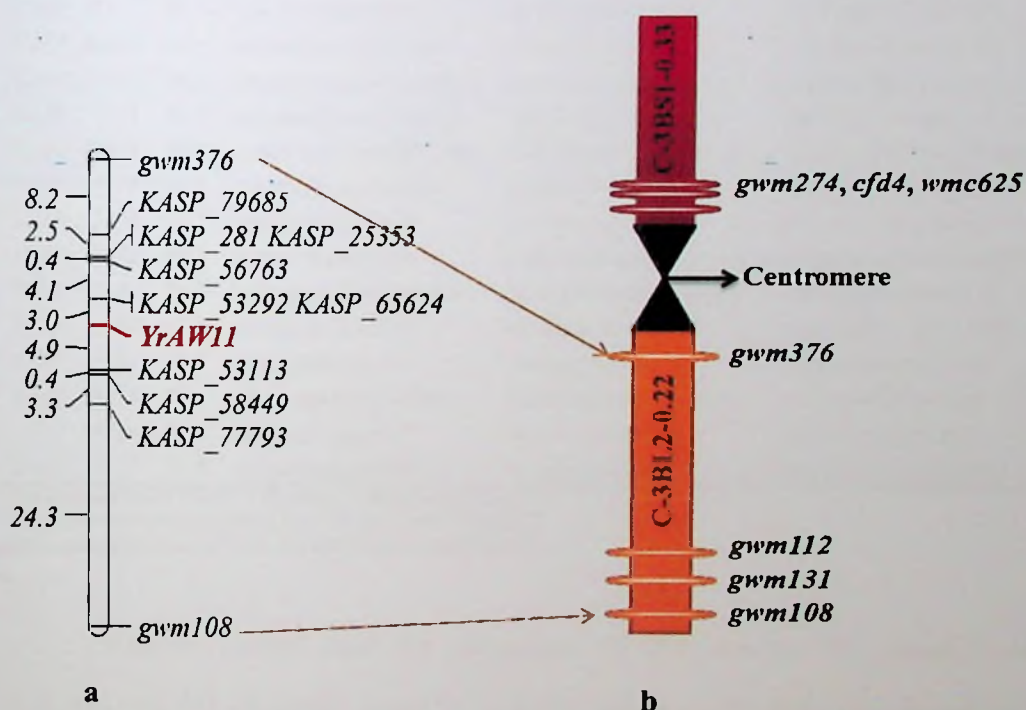


Figure 6.2 a) Partial genetic linkage map showing the location of resistance gene *YrAW11* on the long arm of chromosome 3B b) SSR marker location in chromosome 3B deletion bins (Sourdille et al. 2001).

Table 6.4 KASP primer sequences of *YrAW11*-linked SNPs and their map locations

KASP marker	cM*	Allele 1 primer ^y	Allele 2 primer ^z	Common reverse primer
<i>KASP_79920</i>	60.3	acaggattggcggtctttctat	acaggattggcggtctttctac	tgttgtgaaaaactttgagcagtag
<i>KASP_54174</i>	60.6	tagcatcgtgtgtgcccgat	tagcatcgtgtgtgcccgaac	gcattccttcggaaacagcc
<i>KASP_58591</i>	60.7	taatagtgtccctcgcctgt	taatagtgtccctcgcctgc	tttcatectcagtcgccaacg
<i>KASP_26756</i>	62.3	agacgttgttgcgcctgt	agacgttgttgcgcctgc	cctagcgccctcgtgattcag
<i>KASP_8418</i>	62.6	ttcctgcgatgggtgatgt	ttcctgcgatgggtgatgg	gtgggttgggtggtgtgt
<i>KASP_24225</i>	63.8	tgcagtgaggcaaatccct	tgcagtgaggcaaatcccc	tgataaaccactgcaccaca
<i>KASP_42364</i>	64.8	attggactggaaggcttgaca	attggactggaaggcttgacg	cccgtggcctagcaaatattt
<i>KASP_38240</i>	65.6	gttagtcggattctgttctca	gttagtcggattctgttctcg	tgggacaaattcagaccaaaact
<i>KASP_35317</i>	65.7	ctactgtcagccaccatcac	ctactgtcagccaccatcag	ggaacctacgccctgatttacta
<i>KASP_46345</i>	67.5	cctcctcctggatctcatca	cctcctcctggatctcatcg	gttgattgtctgctggcc
<i>KASP_7502</i>	67.7	ctggccaggatgcaccgt	ctggccaggatgcaccgg	aatggagctcctcgggtgt
<i>KASP_66016</i>	67.8	gtcagtaaccgcgatagattgt	gtcagtaaccgcgatagattgc	cagagatatatggcgttggaaat
<i>KASP_79685</i>	68.1	actggaaagtggagagctctt	actggaaagtggagagctctc	aagatccgcccttgtccttg
<i>KASP_25353</i>	68.4	ctccttcaagctcaagggttat	ctccttcaagctcaagggttag	gctggtcagagtttgggtgc
<i>KASP_281</i>	68.7	gcagagaagggttgcgtttct	gcagagaagggttgcgtttcc	ccttttatagagcttctgtgacct
<i>KASP_63444</i>	68.9	aggtagtttggagtgtgaggt	aggtagtttggagtgtgaggg	tcctaccaatctcagaagttccg
<i>KASP_56763</i>	88.0	gaggtaaaccgctatcatcataatt	gaggtaaaccgctatcatcataatc	gtgatalcaggagctgcaactg
<i>KASP_73763</i>	68.9	cgttggataggaaagctatgtgt	cgttggataggaaagctatgtgc	gtgagtataaccagtcgcaca
<i>KASP_77336</i>	69.4	acacctctaaataacattggaagga	acacctctaaataacattggaaggc	gccatttaactgcacagataaagac
<i>KASP_37750</i>	69.5	gatgttttgtctgggcaagttat	gatgttttgtctgggcaagttac	caagtaggtgtgacagggc
<i>KASP_53292</i>	69.7	gttgccgcgtgaaagagt	gttgccgcgtgaaagagc	gctggctatcggatcactacc
<i>KASP_48415</i>	70.1	accactgtggcatgagtc	accactgtggcatgagtcg	gcagatcaagatttcagagatcg
<i>KASP_65624</i>	71.3	ttgtaagactgttgagttaagga	ttgtaagactgttgagttaaggg	tcgaggctggctatttactg
<i>KASP_28376</i>	71.7	ctccagcaccggaacctt	ctccagcaccggaacctc	gtagtcaaggctcttatttccatg
<i>KASP_28184</i>	72.2	ttacaggggtatgtctctgt	ttacaggggtatgtctctgc	gtgtgccaaggatagggccc
<i>KASP_53113</i>	73.9	tgtacaatgactcctcgactaaca	tgtacaatgactcctcgactaacg	gccacgcaatcacaccatcg
<i>KASP_58449</i>	74.2	aacctcgcgaagctcat	aacctcgcgaagctcac	agtalccaatcacgacgacg
<i>KASP_77793</i>	75.0	acctgctgtgacaaatagtactt	acctgctgtgacaaatagtactc	ggatgagttaaaacgagacct

* Marker position in 3B 90K SNP consensus map (Wang et al. 2014)

^yA1 primer labelled with FAM: GAAGGTGACCAAGTTCATGCT

^zA2 primer labelled with HEX: GAAGGTCGGAGTCAACGGATT

Markers located near the centromere of chromosome 3B *gwm274*, *cfld4*, *bare73*, *wmc625*, *gwm108*, *gwm112*, *gwm131* and *gwm376* were also tested on the population. Nine KASP markers (*KASP_79685*, *KASP_25353*, *KASP_56763*, *KASP_281*, *KASP_65624*, *KASP_53292*, *KASP_53113*, *KASP_58449* and

KASP_77793) and two SSR markers *gwm376* and *gwm108* that showed linkage with *YrAW11* were used to construct the genetic linkage map. *KASP_65624*, *KASP_53292* mapped at 3 cM distal to *YrAW11* and *KASP_53113* mapped at 4.9 cM proximal to this resistance locus (Fig 6.2).

6.3.5 Polymorphism of *YrAW11*-linked markers

KASP markers *KASP_65624*, *KASP_53113* and *KASP_58449* were tested on a set of 81 Australian wheat cultivars (Table 6.5). *KASP_53113* produced *YrAW11*-linked 'A:A' haplotype in 5 cultivars Catalina, Mackellar, Naparoo, SF Scenario and Estoc, while *KASP_58449* produced *YrAW11*-linked 'G:G' haplotype in 14 cultivars and one cultivar was heterozygous. *KASP_65624* carried the *YrAW11*-linked (C:C) haplotype in more than 50% of the cultivars. These results indicated that the *KASP_53113* can be used for marker-assisted selection of *YrAW11* in 94% of these cultivars.

Table 6.5 Haplotype of Australian cultivars at KASP markers flanking *YrAW11*

Genotypes	<i>KASP_53113</i>	<i>KASP_58449</i>	<i>KASP_65624</i>
Aus26582 (<i>YrAW11</i>)	A:A	G:G	C:C
Avocet S	G:G	A:A	T:T
Catalina, Mackellar, Naparoo	A:A	G:G	C:C
SF Scenario	A:A	G:G	T:T
Estoc	A:A	A:A	T:T
AGT Katana, Axe, Beaufort, Fortune, Gauntlet, Merlin, SQP Revenue, Wedin, SF Adagio	G:G	G:G	C:C
Lincoln	G:G	G:G	C:T
Bolac, Calingiri, Correll, Diamondbird, Emu Rock, Envoy, Espada, Gazelle, Impose CL Plus, Justica CL Plus, King Rock, Magenta, Mansfield, Orion, Scout, Shield, Strzlecki, Sunzell, Ventura, Wallup, Wyalkatchem, Yandanooka, Yitpi	G:G	A:A	C:C
Spitfire	G:G	A:G	C:T
Carnamah, EGA Bonnie Rock, EGA Bounty, EGA Burke, EGA Wedgetail, GBA Sapphire, Gladius, Mace	G:G	A:A	C:T
Baxter, Chara, Cobra, Corack, Crusader, Dart, Derrimut, EGA Gregory, EGA Wylie, Elmore CL Plus, Forrest, Giles, Grenade CL Plus, Harper, Impala, Janz, Kord CL Plus, Kunjin, Lancer, Lang, Livingston, Merinda, Phantom, Preston, Sentinel, Sunco, Sunguard, Suntop, Sunvale, Sunvex, Trojan, Waagan, Wylah, Young	G:G	A:A	T:T

6.4 Discussion

Disease resistance is an integral component of grain yield in wheat and hence is a major objective in wheat improvement programs worldwide. This study identified a new source of APR to stripe rust on the long arm chromosome 3B of Aus27284 and it was temporarily named *YrAW11*. Aus27284 produced adult plant stripe rust response 5 at all experimental sites and the response scores among homozygous resistance RILs varied from 4 to 6. Although monogenic segregation for adult plant stripe rust resistance among Aus27284/AvS RILs was observed, the variation in expression of *YrAW11*

among RILs points to the fact that another very minor-effect gene may be present and interacts to lower its expression to 4. Since adult plant stripe rust response data across three sites in 2016 were consistent, we plan to conduct BSA using bulks comprising scores of 5 versus 9. The absence marker-trait association on any other chromosome in addition to chromosome 3B also points to the very minor contribution of this locus. Williams et al. (2006), Lillimo et al. (2008) and Rosewarne et al. (2012) reported presence of minor genes contributed by Avocet S in chromosomes 4B, 6A and 7A, respectively. The expression of adult plant response 4 produced by some RILs in Aus27284/AvS could be due to contribution of minor gene from AvS. Other stripe rust resistance genes with intermediate effects have been reported and include *Yr31* (Rosewarne et al. 2012) and *Yr60* (Herrera-Foessel et al. 2015). Some APR genes are classified as high temperature adult plant resistance (HTAP); for example *Yr36* (Uauy et al. 2005), *Yr39* (Lin and Chen 2007) and *Yr52* (Ren et al. 2012). Genetic stocks carrying these genes show susceptible responses at the seedling stage and show high levels of resistance at the adult plant stages under high temperature incubation regimes (Lin and Chen 2007). Stripe rust resistance genes *Yr58* (Chhetri et al. 2016), *Yr75* (McIntosh et al. 2015) and *YrSen* (Chemayek 2017) express at the 3rd to 4th leaf stage. *YrAW11* more appropriately fits into the APR category as it failed to express under different conditions in the greenhouse (data not presented).

YrAW11 was mapped using SNPs from the 90K map (Wang et al. 2014) and the comparison of marker positions in the consensus map indicated that *YrAW11* was located near the centromere. SSR markers *gwm376* and *gwm108* located in deletion bin

C-3BL2-0.22 (Sourdille et al. 2001) mapped proximal and distal to *YrAW11* confirming its location in the long arm. Recently 90K SNPs have been assigned to chromosome arms (M. Hayden Pers comm). Markers *KASP_65624* and *KASP_58449* belong to scaffolds, chr3B-super103 and chr3B-super108, respectively, in chromosome 3BL and *YrAW11* is present between these two scaffolds. No seedling or adult plant stripe rust resistance gene and/or QTL has been reported in chromosome 3BL (Rosewarne et al. 2013; McIntosh et al. 2016;), therefore *YrAW11* represents a new stripe rust resistance locus.

The usefulness of a molecular marker in MAS depends on its close linkage with the gene of interest and its ability to predict the gene in diverse genetic backgrounds. The flanking marker *KASP_53113* showed polymorphism in 94% of the Australian cultivars indicating that it can be used for MAS after testing the parental polymorphism. *YrAW11* provides moderate resistance at adult plant stage and can be utilised along with other marker-tagged ASR and APR genes; such as *Yr18/Lr34* (Lagudah et al. 2006), *Yr46/Lr67* (Moore et al. 2015), *Yr47/Lr52* (Qureshi et al. 2017a), *Yr70/Lr76* (Bansal et al. 2017), *Yr51* (Randhawa et al. 2014), *Yr57* (Randhawa et al. 2015), *Yr58* (Chhetri et al. 2016b) and *Yr71* (Bariana et al. 2016) to achieve durable stripe rust control.

Aus27284 exhibits resistance to leaf rust under Australian conditions and was scored 5 at Kenya against stem rust (Bariana and Bansal unpublished data). *YrAW11* is either pleiotropic or an additional locus for resistance to leaf rust and stem rust is present in Aus27284. Some APR loci described previously have been shown to be

pleiotropic (McIntosh et al. 2016). Screening of Aus27284/AvS RIL population at a stripe rust free location is needed to examine whether *YrAW11* is a pleiotropic locus or not. The stripe rust susceptible parent Avocet S carries *Sr26* and hence only 50% of the population will be useful for screening against stem rust. The stem rust response variation among 60 to 70 RILs that would lack *Sr26* will be useful for initial evaluation.

Chapter 7

General discussion

Wheat breeders have been selecting combinations of APR genes based on additive interaction and this type of selection relies on good epidemics in field nurseries (Bariana et al. 2003, 2007a). Similarly, selection for combinations of all stage stem rust resistance gene *Sr38* (IT X) with other *Sr* genes that express ITs varying from 1 to 2+ based on a lower IT ; have been made (Bariana and McIntosh 1993). This would however require greenhouse screening facilities and collection of appropriate pathogen isolates. The simultaneous selection of ASR and APR genes among segregating populations is not feasible in a single step using conventional technologies. Hence, development of markers linked with diverse sources of APR and ASR will be important to resolve this issue. This investigation focused on identification of markers linked with adult plant leaf rust resistance genes *Lr48*, *Lr49* and all stage stem rust resistance gene *Sr48* using diverse genomic resources. In addition, a new adult plant stripe rust resistance locus *YrAW11* was described and markers linked with this locus were identified.

Markers linked with pleiotropic APR genes *Lr34/Yr18/Sr57* (Lagudah et al. 2006), *Lr46/Yr29/Sr58* (Evans Lagudah Unpublished) and *Lr67/Yr46/Sr55* (Forrest et al. 2014) have previously been reported and are being used in marker-assisted selection (MAS) of these genes in breeding programs. The development of robust rust resistance linked markers in this study is a significant addition to breeder's toolbox. Pyramiding of at least two of these resistance genes with marker-tagged and currently effective linked

resistance genes *Lr52/Yr47* (Qureshi et al. 2017), *Lr57/Yr40* (Kuraparthi et al. 2009) and *Lr53/Yr35* (Dadkhodaie et al. 2011) can provide durable leaf rust and stripe rust control.

Markers closely linked with a Ug99-effective resistance gene *Sr48* have also been identified in this study. Various workers previously reported markers linked with stem rust resistances *Sr2* (Spielmeyer et al. 2003), *Sr13* (Peyiannan et al. 2014), *Sr26* (Mago et al. 2005), *Sr22* (Periyannan et al. 2011), *Sr36* (Bariana et al. 2007a), *Sr39* (Mago et al. 2009), *Sr45* (Periyannan et al. 2014), *Sr50* (Mago et al. 2015) and *Sr56* (Bansal et al. 2014). Deployment of combinations of more than one gene in new cultivars will provide sustained stem rust control. Transfer of stem rust resistance genes in genotypes carrying marker-tagged linked stripe rust and leaf rust resistance mentioned in the previous paragraph can assure triple rust resistance in future wheat cultivars. Markers for many other resistance genes listed on MAS Wheat website could be useful in diversifying the genetic base of rust resistance in wheat.

A continuous effort on detection and mapping of diverse sources of resistance is important to combat highly evolving rust pathogens. This study identified a putatively new APR locus, *YrAW11*, from a landrace Aus27284 to increase genetic diversity among a very narrow APR gene pool among common wheat cultivars. Marker *KASP_53113* showed genetic association with *YrAW11* and did not show the resistance-linked allele in 94% wheat cultivars in the validation experiment. This marker can be used for marker assisted selection following polymorphism test between the recurrent parent and donor source. Many other rust resistance genes identified from

pre-Green Revolution landraces include *Sr49*, *Yr47/Lr52*, *Yr51*, *Yr57*, *Yr58*, *Yr63* and *Yr72* (Bariana and Bansal 2017). Similarly, wild relatives of wheat have also contributed several rust resistance loci and these include *Yr37/Lr54* from *Aegilops kotschyii* (Marais et al. 2005), *Yr40/Lr57* from *Ae. geniculata* (Kuraparthi et al. 2009), *Yr42/Lr62* from *Ae. neglecta* (Marais et al. 2010) and *Yr70/Lr76* from *Ae. umbellulata* (Bansal et al. 2017).

In conclusion, the objectives set out for this study were successfully achieved. The genomic location of *Lr48* on 2BS was confirmed. Markers closely-linked with resistance genes *Lr48*, *Lr49* and *Sr48* were developed using different genomic resources. These markers can be used for marker-assisted pyramiding of respective genes with other marker-tagged genes for other economic traits. *Sr48* has been relocated from 2AL to 2DS; however, this observation needs to be confirmed. A putatively new resistance gene *YrAW11* conditioning moderate resistance in a landrace Aus27284 was characterized and mapped.

Recommendations

This study provided markers for pyramiding of ASR and APR genes. Some suggestions for future work are:

- Markers linked with *Lr48*, *Lr49* and *Sr48* should be made available to breeders for MAS
- Saturation of the *YrAW11* region should be carried out to identify markers more closely linked with this locus

- A permanent gene symbol should be requested for *YrAW11*
- The Aus27284/AvS RIL population should be screened against leaf rust and stem rust to assess if *YrAW11* is a pleiotropic locus
- High resolution families should be screened with markers developed in this study to conduct map based cloning of *Lr48*, *Lr49* and *Sr48*

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