THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Molecular genetic characterisation of triple rust resistance in *Aegilops tauschii*

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Master of Science (Biochemistry)

A thesis submitted for the degree of Doctor of Philosophy at
The University of Queensland in 2018

Centre for Crop Science
Queensland Alliance for Agriculture and Food Innovation (QAAFI)

Abstract

Bread wheat (*Triticum aestivum)* is one of the top three cultivated crops, and a major caloric source for humanity. Global wheat production is under threat due to the rapid evolution of highly virulent fungal pathogens such as *Puccinia* spp. that cause rust diseases. Losses due to rusts are routinely minimised through the deployment of host-mediated genetic resistance. However, the rust pathogens have the ability to evolve virulence and overcome the host resistance. Therefore, a continuous supply of new sources of resistance is essential for sustainable rust management. Wheat wild relatives are a valuable resource as they provide resistance against diverse rust forms.

In this study, CPI110672, an accession of the D-genome progenitor *Aegilops tauschii,* was chosen for in-depth analysis as it resists three wheat rust diseases, namely leaf, stem and stripe rust. To determine whether the triple rust resistance is pleiotropic or involves multiple genes conferring specific resistance, we conducted genetic analysis using a mapping population derived from a cross between CPI110672 and CPI110717 (susceptible) accessions. Through rust infection screening, we determined the triple rust resistance was conferred by multiple genes. Two independent genes *(Sr672a* and *Sr672b)* segregated for stem rust resistance, while we identified monogenic segregation for stripe *(Yr672)* and leaf rust *(Lr672)* resistance. Genotyping by 90K Infinium single nucleotide polymorphism (90K SNP) chip analysis confirmed independent segregation where each of the resistances were linked with different SNP markers. Based on closely-associated SNPs and their physical position, the leaf rust resistance gene *Lr672* and one of the stem rust resistance genes *(Sr672a)* were mapped to the short arm of chromosome 2D, whereas the stripe rust resistance gene mapped to chromosome arm 4DS. Converting the SNPs into Kompetitive Allele Specific (KASP) genotyping markers and mapping on the segregating population resulted in the identification of flanking markers for all three resistance genes.

Anchoring the *Sr672a* flanking markers on to the Chinese Spring (CS) IWGSC RefSeq v1.0 and *Ae. tauschii* AL8/78 v4.0 reference genome sequences identified one candidate gene belonging to *CC-NBS-LRR (CNL)*, the major class of resistance genes known in plants for rust resistance. The candidate gene was identified as a homologue of the recently cloned *Sr46* gene. Screening using a gene specific marker for *Sr46*

confirmed that *Sr672a* was an allele of *Sr46* with a single amino acid difference and hence designated as *Sr46b.* Validation of *Sr46b* by a transgenic complementation test confirmed that rust resistance is conferred by this allele, and indicated that the difference in one amino acid did not alter the rust resistance function. Further, we deployed *Sr46b* into a commercial cultivar through marker assisted selection. Also, we attempted to stack *Sr46b* with other stem rust resistance genes (*Sr33* and *Sr45)* isolated from *Ae. tauschii* using speed breeding technology. Through speed breeding and marker assisted selection, we were able to select recombinant lines with multiple resistance genes combinations within 180 days.

We used traditional map-based cloning in conjunction with a comparative genomics approach to fine mapping the leaf rust *(Lr672)* and stripe rust *(Yr672)* resistance genes. The whole genome sequence assemblies of parental accessions CPI110672, CPI110717 from the open wild wheat consortium (OWWC), John Innes Centre, UK and the recent version of reference sequences of CS-RefSeq v1.0 and AL8/78 v4.0 were used to fine map and identify candidate genes for *Lr672* and *Yr672.*

Based on previous studies, we identified the accession CPI110672 as synonymous with TA1675, the donor for the leaf rust resistance gene *Lr39*, thus confirming that *Lr672* is *Lr39*. Anchoring the closely linked flanking markers in the CS-RefSeq v1.0 and AL8/78 v4.0 reference sequences delimited a 1.2 Mb genomic region in both reference genomes. Additionally, we also used the CPI110672 genome sequences to predict *CNL* genes mapped within the 1.2 Mb physical region of *Lr39.* Markers specific to the candidate genes co-segregated with the leaf rust phenotype in the CPI110672xCPI110717 $F_{2:3}$ mapping population and hence was useful for marker assisted deployment of *Lr39* resistance.

Similarly, anchoring the *Yr672* flanking markers narrowed down an approximately 500 kb region in both reference sequences. Evaluation of the CPI110672 *CNL* genes mapped collinear to the 500 kb reference sequences identified one candidate gene. Markers specific to the candidate *CNL* gene co-segregated with the stripe rust resistance phenotype in the $F_{2:3}$ mapping population. Cloning and transgenic complementation tests confirmed the stripe rust resistance. Further, we deployed the *Yr672* in the commercial cultivar using marker assisted backcross strategy.

The major outcomes of this study include:

- Understanding the genetic architecture of triple rust resistance in accession CPI110672
- Cloning and validation of *Sr46b* and *Yr672* rust resistance candidate genes
- Fine mapping a leaf rust resistance *(Lr39)* gene
- Development of breeder-friendly molecular markers for *Sr46, Yr672* and *Lr39*
- Marker assisted introgression of *Sr46b* and *Yr672* into a commercial cultivar
- Marker assisted pyramiding of *Sr33/Sr45/Sr46b* resistance genes using speed breeding technology

Declaration by author

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

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications included in this thesis

No publications included

Conference presentations

Athiyannan N, McIntosh R, Zhang P, Hewitt T, Hoxha S, Forrest K, Kay P, Upadhyaya N, Hayden M, Hickey L, Lagudah E, Periyannan, S (2017) Dissecting the genetics of multiple rust disease resistance in a wild relative of bread wheat using comparative genomics. **Oral** presentation at a satellite meeting on Plant-Microbe Interactions at Queenstown Research Week held at Queenstown, New Zealand. September 3-4 2017.

Athiyannan N, McIntosh R, Zhang P, Hewitt T, Chandramohan S, Hoxha S, Forrest K, Kay P, Upadhyaya N, Steuernagel B, Wulff B, Richardson T, Louis S, Bhatt D, Ayliffe M, Hayden M, Hickey L, Long Y, Xu S, Lagudah E, Periyannan, S (2018) Molecular genetic characterisation of triple rust resistance in *Aegilops tauschii.* **Poster** presentation at the Plant and Animal Genome Conference XXVI held at San Diego, USA. January 13-17 2018

Submitted manuscripts included in this thesis

No manuscripts submitted for publication

Other publications during candidature

Peer-reviewed articles

Riaz A, **Athiyannan N,** Periyannan S, Mitrofanova O, Afanasenko O, Platz G, Aitken E, Snowdon R, Lagudah E, Hickey L, Voss-Fels K (2018) Unlocking novel alleles for resistance to leaf rust in the Vavilov wheat collection. Theoretical and Applied Genetics. 131:127-144

Riaz A, **Athiyannan N,** Periyannan S, Afanasenko O, Mitrofanova O, Aitken E, Lagudah E, Hickey L (2016) Mining Vavilov's treasure chest of wheat diversity for adult plant resistance to *Puccinia triticina*. Plant Disease 101:317-23

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Jacob J, Senthil K, **Naveenkumar A** (2016) Evaluation and Biochemical Characterization of Casuarina Clones Resistant to the Bark Feeder *Indarbela quadrinotata* Walker (Lepidoptera: Metarbelidae). In: Nicodemus A, Pinyopusarerk K, Zhong C, Franche C (eds) Fifth International Casuarina Workshop on Casuarina Improvement for Securing Rural Livelihoods. Institute of Forest Genetics and Tree Breeding, Coimbatore, India. pp. 191-197

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Jambuthenne D, Riaz A, Ng WL, Ziems Laura, **Athiyannan N**, Afanasenko O, Mitrofanova O, Periyannana S, Lagudah E, Aitken E, Godwin I, Hickey L (2017) A Genome Wide Association Study of Resistance to Stripe Rust in the Vavilov Wheat Collection. Science Protecting Plant Health 2017, 26-28 September 2017, Brisbane, Australia.

Riaz A, Voss-Fels K, **Athiyannan N,** Periyannan S, Mitrofanova O, Afanasenko O, Platz G, Aitken E, Snowdon R, Lagudah E, Hickey L (2017) Unlocking novel alleles for resistance to leaf rust in the Vavilov wheat collection. Science Protecting Plant Health 2017, 26-28 September 2017, Brisbane, Australia.

Riaz A, Voss-Fels K, **Athiyannan N,** Hathorn A, Mitrofanova O, Afanasenko O, Dinglasan E, Ziems L, Richard C, Singh D, Snowdon R, Periyannan S, Aitken E, Godwin I, Lagudah ES, Hickey L (2017) Mining Vavilov’s treasure chest of wheat diversity for resistance to leaf rust. Oral presentation in the “Genomics of Genebanks”

workshop, the International Plant and Animal Genome Conference XXV, January 14-18, 2017 San Diego, USA.

Riaz A, **Athiyannan N,** Mitrofanova O, Afanasenko O, Periyannan S, Aitken E, Lagudah ES, Hickey L (2017) Speeding up the discovery of novel sources of leaf rust resistance in wheat. Oral presentation in the “New approaches for developing disease resistance to cereals” workshop, the International Plant and Animal Genome Conference XXV, January 14-18, 2017 San Diego, USA.

Riaz A, **Athiyannan N,** Mitrofanova O, Afanasenko O, Periyannan S, Aitken E, Lagudah ES, Hickey L (2016) Speeding up the discovery of novel sources of leaf rust resistance in wheat. Oral presentation at the IV International Conference on Modern Problems of Plant Resistance to Disease and Pests, 11-13 October 2016, St. Petersburg, Russia.

Riaz A, **Athiyannan N,** Dieters MJ, Lagudah ES, Aitken EAB, Periyannan SK, Hickey LT (2015) Phenotyping adult plant resistance to leaf rust in wheat under accelerated growth conditions. Oral presentation at the Borlaug Global Rust Initiative workshop, 17-19 September 2015, Sydney, Australia.

Contributions by others to the thesis

- Prof Robert McIntosh, Dr Peng Zhang and Mr Sami Hoxha assisted in rust phenotyping at the University of Sydney Plant Breeding Institute, Cobbitty
- The 90K SNP genotyping for the Bulk Segregant Analysis (BSA) was carried out by Dr Kerrie Forrest and Dr Matthew Hayden at AgriBio Centre, Latrobe University, Victoria, Australia.
- Dr Burkhard Steuernagel, Dr Sanu Arora and Dr Brande Wulff from John Innes Centre, Norwich, UK provided the whole genome shotgun sequence assemblies of the *Aegilops tauschii* accessions CPI110672 and CPI110717.
- Dr Mehran Patpour and Prof Mogens Hovmoller for phenotyping Sr46b transgenic lines for stem rust resistance at Global Rust Reference Centre, Denmark
- Ms Dhara Bhatt, Ms Smitha Louis and Ms Terese Richardson generated transgenic wheat lines.
- Mr Timothy Hewitt and Dr Narayana Upadhyaya assisted with the bioinformatics analysis.
- Dr Kathy Dibley and Dr Ricky Milne critically reviewed the thesis.

Statement of parts of the thesis submitted to qualify for the award of another degree

No works submitted towards another degree have been included in this thesis.

Research Involving Human or Animal Subjects

No animal or human subjects were involved in this research.

Acknowledgements

My profound gratitude to the Almighty God for showering me with all the blessings. I express my sincere thanks to my teachers for enlightening my knowledge. I especially thank my supervisors Dr Sambasivam Periyannan, Dr Lee Hickey and Professor Evans Lagudah for giving me this opportunity, invaluable guidance, support and motivation during my PhD duration. I sincerely thank the Department of Education and Training, the Australian government and the University of Queensland for the Research Training Program and UQ-Centennial scholarships to undertake my PhD studies. I also thank the Queensland Alliance for Agriculture and Food Innovation (QAAFI) for a travel award to attend the Plant and Animal Genome Conference (PAG 2018), USA.

I am extremely grateful to Dr Narayana Upadhyaya, Mr Tim Hewitt for their support and patience helping in bioinformatics analysis. I greatly acknowledge the collaborators, Prof Bob McIntosh, Dr Peng Zhang, Mr Sami Hoxha (University of Sydney), Dr Matthew Hayden, Dr Kerrie Forrest (AgriBio, LaTrobe University), Dr Burkhard Steuernagel, Dr Sanu Arora, Dr Brande Wulff (John Innes Centre, UK) and Dr Mehran Patpour, Prof Mogens Hovmoller (Global Rust Reference Centre, Denmark) for their time and valuable contribution to my thesis. I am thankful to Ms Dhara Bhatt, Ms Smitha Louis and Ms Terese Richardson for their tireless help in generating transgenic plants. I would also like to thank Dr Ricky Milne for his critical review and valuable comments in my thesis. My special thanks to Dr Kathy Dibley for her lovely friendship, mentorship, selfless support and motivation.

I am also thankful to Dr Dharmendra Singh, Dr Adnan Riaz, Ms Dilani Jambuthenne and Mr Samir Alahamad for their memorable friendship, advice and hospitality during my stay in Brisbane. I extend my sincere thanks to all my colleagues Mr Raghvendra Sharma, Ms Jianping Zhang, Ms Hongyu Li, Mr Jian Chen, Dr Diana Ortiz, Dr Matthias Jost, Ms. Sutha Chandramohan, Ms Xiaodi Xia, Ms Kim Newell, Ms Soma Chakraborty, Mr Luch Hac, Mr Andy Moore, Ms Stephanie Gollasch, Ms Lisa Molvig, Dr Chunhong Chen, Dr Wendelin Schnippenkoetter, Dr Rohit Mago, Dr Mick Ayliffe, Dr Peter Dodds, Dr TJ Higgins, Dr Jeff Ellis, Dr Tony Ashton, Dr Linda Tabe and Dr Greg Lawrence for their support in day to day activities during my PhD.

My lovable thanks to Mr Fahim, Mr Jayaprakash and family, and all my close friends whose support and care are precious. Finally, I have no words to express my gratitude for the unconditional love, and support by family members Mr Kaliappa Gounder, Ms Athayammal, Mr Athiyannan, Ms Malleswari, Mr Sivakumar, Dr Sivakami, Ms Thenmozhi, Ms Ratchambika and Ms Sivambika. My study would not have been possible without their sacrifice, patience and understanding.

Financial support

This research was supported by an Australian Government Research Training Program Scholarship. My living allowances were supported by the University of Queensland Centennial Scholarship. A travel grant was awarded (QAAFI-HDR travel award) to present the work at Plant and Animal Genome Conference 2018 in San Diego, USA.

This research was partly funded by Durable Rust Resistance in Wheat (DRRW) project administered by Cornell University with funds from Bill and Melinda Gates Foundation, and Grain Research and Developmental Corporation (GRDC), Australia.

Keywords

Aegilops tauschii, wheat rust, all-stage resistance, map-based cloning, reference genome, NBS-LRR, *Lr39, Sr46, Yr672,* triple rust resistance

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060412, Quantitative Genetics (incl. Disease and Trait Mapping Genetics), 40%

ANZSRC code: 060702, Plant Cell and Molecular Biology, 40%

ANZSRC code: 070308, Crop and Pasture Protection (Pests, Diseases and Weeds), 20%

Fields of Research (FoR) Classification

FoR code: 0604, Genetics, 40%

FoR code: 0607, Plant Biology, 40%

FoR code: 0703, Crop and Pasture Production, 20%

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List of Abbreviations used in the thesis

°C	Degree Celsius
µl	Microliter
ACRCP	Australian Cereal Rust Control Program
AFLP	Amplified Fragment Length Polymorphism
AgRenSeq	Association genetics with Resistance gene enrichment Sequencing
APR	Adult Plant Resistance
ASR	All Stage Resistance
AU	Australian
Avr	Avirulence
BAC	Bacterial Artificial Chromosome
BC	Backcross
Blast	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bulk Segregant Analysis
BSMV	Barley Stripe Mosaic Virus
CAPS	Cleaved Amplified Polymorphic Sequence
CC	Coiled-Coil
Chr	Chromosome
CIMMYT	The International Maize and Wheat Improvement Centre
CNL	CC-NBS-LRR
CS	Chinese Spring
CSIRO	The Commonwealth Scientific and Industrial Research Organisation
Csq	CSIRO and QAAFI
DArTseq	Diversity Array Sequence Technology
DH	Double Haploid
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxynucleotide
dpi	Days Post Infection
EMS	Ethyl Methane Sulphonate
FSWC	Flow Sorted Wheat Chromosome
GBS	Genotyping by Sequencing
GRRC	Global Rust Reference Centre
GWAS	Genome Wide Association Studies
HR	Hypersensitive Response
InDel	Insertions and Deletions
IT	Infection Type
IWGSC	International Wheat Genome Sequencing Consortium
JIC	John Innes Centre, United Kingdom
KASP	Kompetitive Allele Specific PCR
Kb	Kilobase
L1	Lineage 1
L1E	Lineage 1 East
L1W	Lineage 1 West
L2	Lineage 2
L2E	Lineage 2 East
L2W	Lineage 2 West
LD	Linkage Disequilibrium
LED	Light Emitting Diode

LOD	Logarithm of Odds
Lr	Leaf Rust
LRR	Leucine Rich Repeat
MAS	Marker Assisted Selection
Mb	Megabase
$MgCl_2$	Magnesium Chloride
mM	Millimolar
MT	Million Tons
MutRenSeq	Mutagenesis, Resistance gene enrichment and Sequencing
MY	Million Years
NBS	Nucleotide Binding Site
ng	Nanogram
NHR	Non-Host Resistance
NIL	Near Isogenic Lines
nM	Nanomolar
OWWC	The Open Wild Wheat Consortium
PBI	Plant Breeding Institute, the University of Sydney
PCR	Polymerase Chain Reaction
Pgt	*Puccinia graminis* f. sp*. tritici*
Pst	*Puccinia striiformis* f. sp*. tritici*
Pst79	*P. striiformis* f. sp*. tritici* lineage identified in 1979
Pt	*Puccinia triticina*
QAAFI	Queensland Alliance for Agriculture and Food Innovation
QTL	Quantitative Trait Loci
R	All stage or seedling resistance genes
RAPD	Random Amplified Polymorphic DNA
RenSeq	Resistance gene enrichment Sequencing
RFLP	Restriction Fragment Length Polymorphism
RIL	Recombinant Inbred Lines
RNA	Ribonucleic Acid
SCAR	Sequence Characterised Amplified Region
SHW	Synthetic Hexaploid Wheat
SNP	Single Nucleotide Polymorphism
Sr	Stem rust resistance
SSD	Single Seed Descent
SSR	Simple Sequence Repeat
STS	Sequence-Tagged Site
Syn	Synonymous
TACCA	Targeted Chromosome-based Cloning via long-range Assembly
Taq	*Thermus aquaticus*
TILLING	Targeting Induced Local Lesions IN Genomes
TIR	Toll-Interleukin-1-Receptor
U	Unit
UK	United Kingdom
UQ	The University of Queensland
US or USA	United States of America
UTR	Untranslated Region
VIGS	Virus-Induced Gene Silencing
YAC	Yeast Artificial Chromosome
Yr	Yellow rust resistance

Chapter 1 - General introduction

Over 9000 years ago, spontaneous hybridisation between grasses in the Fertile Crescent led to the origin of bread or hexaploid wheat (*Triticum aestivum L.* 2n=6x=42, AABBDD), the staple food crop feeding the global human population. The hybridisation occurred between the cultivated tetraploid wheat (*T. turgidum* 2n=4x 28, AABB) and a wild goat grass *Aegilops tauschii* (2n=14, DD) (Feuillet et al. 2008; Rasheed et al. 2018). Addition of the D genome to the cultivated tetraploid resulted in hexaploid wheat with adaptability to diverse environments and better bread making qualities. Thus, it is the world's third most cultivated crop after maize and rice with an annual yield of 759 million tons (MT) in 2016/17 (Ray et al. 2013; FAOSTAT). Globally nearly 35% of the human population consume wheat products for protein, and wheat provides about 20% of the total calories (Chaves et al. 2013). Wheat production has increased on average by about 0.85% every year for the last 25 years, from about 545 MT in 1991 to 759 MT in 2017 (FAOSTAT - http://www.fao.org/faostat/en/#home). However, this is well below the required output to feed a rapidly growing population. Wheat production needs to increase at least 2.4% every year to feed 10 billion people by 2050 (Ray et al. 2013).

Increasing production by 2.4% annually will be challenging due to factors such as climate change, declining water resources and biotic pathogens. For instance, pest and disease alone accounted for about 20% of the average annual loss of wheat in Australia (Murray and Brennan 2009b). Fungal pathogens cause the majority of wheat diseases; these include seven necrotrophic and six biotrophic leaf diseases, eight root and crown diseases, and four inflorescence diseases (McIntosh 1998; Murray and Brennan 2009b). Among them, the rust diseases are considered some of the most damaging due to rapid evolution and emergence of new races (Singh et al. 2006). The three forms of wheat rust diseases are leaf, stem and stripe rust, caused by *Puccinia triticina*, *Puccinia graminis* f. sp. *tritici* and *Puccinia striiformis* f. sp. *tritici,* respectively*.* Rusts can cause yield losses from 10% to a total loss under highly disease-favorable conditions. Rust-causing fungi are biotrophic pathogens, highly diverse and new races emerge rapidly either by undergoing single step mutation, sexual recombination or somatic recombination (Park 2015). The ability to evolve new variants, their rapid spread across continents, and their ability to overcome host resistance of commercial

wheat varieties makes rusts as an ever-present threat to wheat production. Records describing the extent of rust damage to wheat crops in Australia date back to the very early days of agricultural practices by the first settlers. The first record of rust in Australia was:

"October 21, 1803 - A more beautiful appearance of a successful harvest never flattered the expectations of a farmer within three weeks of being ripe; ears were full, plump, and well coloured and in every respect gratifying to look at. In three days it was completely destroyed by rust. The produce of 266 acres was not worth 20 pounds – a loss of $4000 was made" - (Waterhouse 1929).

In the 18th century, after the initial crop introduction into Australia, continuous damage and yield loss caused by rust led delegates from different states to organise a series of conferences to initiate rust proof wheat varieties. During the 1890's, James William Farrer initiated wheat breeding for rust disease resistance and released the first rust resistant variety "Federation" in 1903 (Waterhouse 1929). Despite the success of wheat breeding programs for rust resistance in the early 20th century, rust damage still remains a threat to global wheat production. Wheat yield loss due to stem rust in Australia was 2 - 3 million Australian (AU) pounds in 1889, 0.4 million pounds in 1903, 2 million pounds in 1916, 7 million pounds in 1947, and the most devastating loss of about AU $200 - 300 million in 1973 (Park 2007). Similarly, leaf rust also causes significant yield losses in Australia. It is prevalent in all wheat growing regions of Australia and causes losses up to $12 million per year (Murray and Brennan 2009b). Since the first incursion in 1979, stripe rust became a major limiting factor for wheat production in Australia causing yield losses up to $127 million to Australian wheat industries between 1998 and 2008 (Murray and Brennan 2009b). Stripe rust also threatens global wheat production since about 88% of wheat varieties are deemed susceptible to the highly virulent stripe rust races detected recently in Europe, USA, South Asia and Africa (Schwessinger 2017). Therefore, it is essential to strengthen the disease management programs to minimize yield losses due to disease epidemics caused by the frequent evolution of virulent rust races.

Deployment of host-mediated genetic resistance in commercial cultivars is the most preferred, environmentally safe and economical strategy for rust disease control. Collectively about 204 genes have been catalogued for resistance to leaf, stem and

stripe rust diseases from wheat and its relatives (Park 2016c; McIntosh et al. 2017). Rust resistance is conferred by genes from two distinct groups - all stage resistance *(R)* genes and adult plant resistance *(APR)* genes. So far, the majority of the cloned *R* genes have been shown to encode proteins containing coiled coil, nucleotide binding site and leucine-rich repeat (*CC-NBS-LRR* or *CNL*) domains (Pretorius et al. 2017a; Keller et al. 2018). *R* genes confer resistance throughout plant development, right from the seedling stage, and are most commonly race specific, although some *R* genes may confer resistance to multiple races of a single pathogen species. Hence *R* genes may also be referred to as seedling, race-specific, major and gene-for-gene resistance. In contrast, *APR* genes function mainly at the adult plant stage and confer a partial resistance phenotype, hence also referred to as minor resistances (Ellis et al. 2014). Transporter genes and a kinase start domain gene are the commonly known genes that confer broad spectrum *APR* (reviewed by Pretorius et al. 2017).

Deployment of a limited number of resistance genes in commercial varieties poses a risk for outbreaks of rust epidemics. This was exemplified by the Ug99 stem rust race from Uganda in 1999 that overcame the long-term durable resistance gene *Sr31* (Pretorius et al. 2000), resulting in widespread losses. Over 80% of European wheat varieties tested recently were susceptible to another stem rust race Digalu, which emerged in Turkey and spread to Europe (Lewis et al. 2018). Aggressive races of stripe rust (such as Warrior) are also emerging with virulence to several useful genes (Hovmoller et al. 2016; Hovmoller et al. 2017). Intensive wheat breeding programs for other agronomic traits such as yield in the past several years have reduced genetic diversity for disease resistance, posing further threats of epidemics (Feuillet et al. 2008).

Wild relatives of wheat and landraces possess higher genetic diversity than cultivated varieties and are useful resources for improvement of various traits. *Aegilops tauschii,* the D genome progenitor of bread wheat, possesses great genetic diversity among wheat relatives and landraces (Borner et al. 2015) and can be introgressed into cultivated wheat through conventional breeding approaches. With respect to disease resistance, accessions of *Ae. tauschii* have been identified as an excellent source for resistance to all three rust diseases. At present, however, only a few resistance genes such as *Lr21, Lr22a, Lr32, Lr39, Lr42, Sr33, Sr45, Sr46,*

SrTA1662, SrTA10187, Yr28, and *YrAs2388* have been catalogued and introgressed into commercial cultivars. Transfer of traits from the D-genome diploid grass is achieved either by direct hybridisation of *Ae. tauschii* with the hexaploid wheat or by a two-step process through the production of synthetic hexaploid wheat (crossing *Ae. tauschii* with a tetraploid) and introgression into elite cultivars (Olson et al. 2013b). Developing an elite cultivar with improved rust resistance is often a slow process as the conventional approach can take about 10 to 12 years.

In recent years, with advancement in molecular breeding, coupled with speed breeding techniques, the time required to characterise and breed host-mediated resistance into elite cultivars may be shortened. Speed breeding shortens generation time via an extended photoperiod (22 hours) enabling up to six generations per year and can be effectively combined with phenotyping or marker assisted selection (MAS) (Watson et al. 2018). In MAS, linked DNA markers are used for the selection of disease resistance genes rapidly thus enabling in-depth phenotyping of selected lines for pathogen infection screening under field conditions. DNA based markers such as amplified fragment length polymorphism (AFLP), cleaved amplified polymorphic sequence (CAPS), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), sequence characterised amplified region (SCAR), simple sequence repeat (SSR), single nucleotide polymorphism (SNP), and sequence-tagged site (STS) have been commonly used in mapping and characterisation of disease resistance genes in wheat (Collard et al. 2005; Bariana et al. 2007). Traditionally, markers linked with the target resistance gene are identified through its co-segregation with the resistance trait in a bi-parental mapping population. Tightly linked markers are later validated through screening of various breeding lines or cultivars carrying the targeted trait.

Molecular markers are now abundant due to advancements in sequencing technologies and with the availability of a whole-genome reference sequence for wheat and its relatives. This has also paved the way for the development of next generation genotyping methods to assist in large-scale screening of single nucleotide polymorphisms (SNPs) such as genotyping by sequencing, 90K SNP and DArTseq technologies (Elshire et al. 2011; Kilian et al. 2012; Wang et al. 2014). These high-density SNP markers provide closely linked markers for marker assisted breeding and

for rapid characterisation of the rust resistant locus. Reference genome and sequencing technologies have also facilitated the cloning of more resistance genes, thereby delivering perfect markers for MAS. Despite these advances, cloning new wheat disease resistance genes remains tedious due to the highly repetitive DNA content and the polyploid nature of the wheat genome. Conventional map-based positional cloning requires a variety of resources such as a high-density genetic map, markers tightly linked to resistance locus, BAC libraries, mutant populations for candidate gene identification and an efficient genetic transformation technology for validation of potential candidate genes. In recent years, the availability of genome sequences of closely related grass species such as barley (IBGSC et al. 2012), brachypodium (The International Brachypodium Initiative 2010), rice (Kawahara et al. 2013) and the reference genome sequences of hexaploid wheat Chinese Spring (IWGSC 2018), the progenitors of A genome *T. urartu* (Ling et al. 2013), B genome *Ae. speltoides* (IWGSC) and D genome *Ae. tauschii* (Luo et al. 2017) have minimised the duration and the complexity of the map-based cloning technique. They also paved the way for the development of modern cloning techniques such as targeted chromosome-based cloning via long-range assembly (TACCA), mutagenesis with resistance gene enrichment sequencing (MutRenSeq) and association genetics with resistance gene enrichment sequencing (AgRenSeq) (Arora et al. 2019; Periyannan 2018).

Although resistance genes are rapidly identified, the wheat rusts, on the other hand, evolve even more rapidly resulting in new races to overcome these defence genes. This highlights the importance of deploying wheat cultivars with multiple resistance genes that will not be readily overcome by single mutations, thereby achieving long lasting durable resistance (Ayliffe et al. 2008; Mundt 2014). Stacks can be made of different types, either of several *R* genes, broad-spectrum *APR*s, or ideally a combination of both. The key to achieving this is to have numerous resistance genes and their linked DNA markers readily available. Therefore, to assist the process my PhD project is designed to identify linked markers and characterise new source of resistance genes from an *Ae. tauschii* accession.

1.1 Project objectives

Approximately 406 accessions of *Ae. tauschii* from geographical regions between Turkey and China were screened for triple (leaf, stem and stripe) rust resistance at the Plant Breeding Institute, University of Sydney, Cobbitty (Evans Lagudah, unpublished data). From the pool of resistant accessions, an accession of *Ae. tauschii,* CPI110672 collected from Turkmenistan was resolved to be resistant to all three rust diseases of wheat.

This project aimed to genetically characterise the triple rust resistance trait, and fine map the locus (or loci) conferring the resistance. Markers specific to the target locus were developed through comparative genomics of the whole genome sequence of CPI110672 with the reference genome sequences of Chinese Spring and *Ae. tauschii* AL8/78. Subsequently, a physical map was developed to identify candidate genes conferring the rust resistance.

1.2 Thesis outline

Chapter 2 consists of a comprehensive review of the literature describing each of the three rust diseases of wheat, and provides background information to understand the genetics of rust resistance trait in wheat, as well as the recent advances in disease resistance gene cloning and marker assisted breeding in wheat.

Chapter 3 discusses the genetic characterisation of the triple rust resistance of accession CPI110672 through analysis of an $F_{2:3}$ CPI110672xCPI110717 mapping population.

Chapter 4 describes the fine scale mapping and candidate gene identification of the stem rust resistance locus. It also includes the generation of genetic stocks with multiple stem rust resistance genes derived specifically from *Ae. tauschii* accessions, namely CPI110672 (resistance characterised from this study), CPI110799, which carries *Sr33* (Periyannan et al. 2013), and AUS18911 carrying *Sr45* (Steuernagel et al. 2016).

Chapter 5 describes the fine mapping and physical map generation of the leaf rust resistance locus through comparative genomic analysis using the reference

genome sequences of Chinese Spring (IWGSC 2018), *Ae. tauschii* AL8/78 (Luo et al. 2017), and CPI110672 (OWWC).

Chapter 6 deals with the fine mapping and candidate gene isolation of the stripe rust resistance gene from CPI110672. This chapter describes the physical map of the locus generated using pan-genomes involving the reference genome sequences of Chinese Spring (IWGSC 2018), synthetic hexaploid W7984 (Chapman et al. 2015), *Ae. tauschii* AL8/78 (Luo et al. 2017), and CPI110672 (OWWC).

In the final chapter (Chapter 7), the main findings of the thesis are summarised and discussed along with the implications for future rust resistance breeding programs. The prospective approach for the cloning of disease resistance genes using advancement in sequencing technologies and genomics, and rapid breeding of resistant cultivars through generation advancements using speed breeding and marker assisted selection are also discussed. Additionally, the importance of multiple gene stacks for durable rust resistance is discussed in this chapter.

Chapter 2 - Literature Review

2.1 Wheat rust diseases

Rust diseases are caused by important fungal pathogens that pose a constant threat to wheat production worldwide. Rust-causing pathogens are biotrophic and produce large dikaryotic urediniospores or asexual spores that can be transported by wind over thousands of kilometres, therefore, spread across continents or oceans (Kolmer 2005). Three types of rust pathogens affect wheat crops, namely; leaf rust, stem rust and stripe rust, each of which are caused by a different *Puccinia* spp. These diseases are named according to the plant tissues they primarily affect and also based on their visual symptoms (Figure 2.1). They are capable of infecting all aerial plant organs such as leaves, stem, and heads and produce pustules that contain numerous yellow to reddish-brown coloured spores giving an appearance similar to 'iron rust'. Rust diseases are often managed by host-mediated genetic resistance which is often a gene-for-gene interaction. The host plant's resistance molecule detects and resists fungal infection through the perception of the pathogen's virulence molecule (Ellis et al. 2000). Therefore, the emergence of new virulent races is frequent as the pathogens may undergo single step mutations to overcome resistance, thereby causing a significant challenge for delivering durable resistance (Marsalis and Goldberg 2006).

Figure 2.1 Rust infection on susceptible wheat plants. a: Stem rust, b: Leaf rust, c: Stripe rust at CSIRO Agriculture and Food glasshouse facility, Canberra.

2.2 Life cycle of wheat rust

Wheat rust pathogens are heteroecious fungi and require two taxonomically unrelated hosts to complete their life cycle as they undergo asexual reproduction on the primary host, wheat, and sexual reproduction on alternative host plants such as barberry and *Thalictrum* sp. (Figure 2.2). Sexual reproduction on the alternate host enables the rust pathogens to evolve new races through genetic recombination while the dikaryotic urediniospores on the primary host multiply asexually to persist longer through autoinfection. During favourable conditions, urediniospores are produced continuously at 7 to 10-day intervals after infection. However, in the late stage of infection on the primary host, teliospores are produced by the fusion of two nuclei to form a diploid nucleus. Then it undergoes meiosis to produce four haploid basidiospores (two types) that can only infect the alternate host. The alternative host for stem rust and stripe rust is *Berberis vulgaris* (barberry), whereas for leaf rust *Thalictrum speciosissimum* L. also known as Meadow Rue is required to complete the sexual life cycle (Leonard and Szabo 2005; Bolton et al. 2008; Chen et al. 2014). The haploid basidiospores infect the upper surface of the leaf of the alternate host and give rise to the pycnial structure to produce two types of haploid pycniospores. These pycniospores undergo plasmogamy to restore the dikaryotic nuclear condition which then leads to the development of dikaryotic aecium on the under surface of the leaf. The mature aecium produces aeciospores disperse by wind to infect primary hosts, thereby following uredinial infection to complete its cycle (Bolton et al. 2008; Kolmer 2013). In regions where the alternate host is absent or eradicated, the rust pathogen persists in the uredinial stage on volunteer wheat plants that exist outside the growing season and serve as a green bridge (Leonard and Szabo 2005).

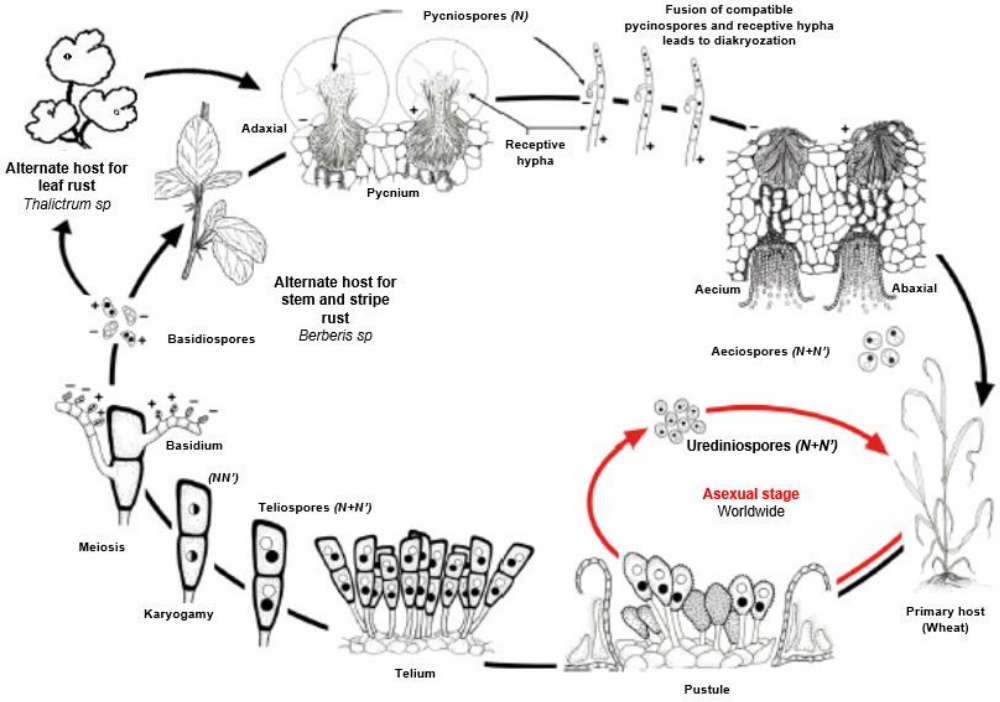

Figure 2.2 The life cycle of wheat rust fungi (modified from Schwessinger 2017; Riaz 2018).

2.2.1 Stem rust

Stem rust (black rust) disease caused by *Puccinia graminis* Pers. f. sp. *tritici* is one of the most feared diseases of bread and durum wheat and has caused severe yield losses since wheat domestication (Singh et al. 2015). Historic evidence of stem rust epidemics exist in the Bible and the Roman festival Robigalia was believed to have originated to protect the wheat crops from rusts through prayer and sacrifice (Leonard and Szabo 2005). *Puccinia graminis* spores are reddish-brown in appearance and occur in diamond-shaped pustules seen primarily on stems and leaves, however, during severe epidemics pustules may also develop on leaf sheaths and glumes (Figure 2.1). Warm and humid conditions with temperatures between 15°C and 30°C provide the ideal environment for stem rust (Marsalis and Goldberg 2006). Severe infection weakens the wheat stem restricting nutrient flow, and during the booting stage leads to poor grain quality and yield loss. Severe infection on a healthy wheat crop can diminish yield even three weeks before maturity and harvest (Leonard and Szabo 2005).

Stem rust epidemics have been managed since 1950 through the deployment of resistant wheat cultivars (McIntosh 1998). Until the early 20th century, occasional outbreaks were reported worldwide due to the evolution of stem rust races with virulence to the deployed resistance genes. Later, the situation became serious due to the emergence of the Ug99 race in Uganda in 1999 that had gained virulence to the widely deployed gene *Sr31* used nearly for 30 years (Pretorius et al. 2000). Subsequently, more than 80% of the commercial wheat cultivars were susceptible to Ug99. Additionally, a number of derivative races related to Ug99 lineage evolved and spread rapidly across Africa and the Middle-East. At present, there are at least 13 races belonging to the Ug99 race group that and have been detected in southern and eastern African countries (Babiker et al. 2015). In its migratory route, the fungus reached the Middle East countries and is feared to reach the top two major wheat producing nations (India and China) in Asia shortly (Singh et al. 2006; Singh et al. 2015; Meyer et al. 2017). In 2016, devastating stem rust epidemics were recorded in Sicily, Italy where stem rust had not been an issue since 1951 (Bhattacharya 2017). Unexpectedly, the new race detected in Europe was different from Ug99 and is a serious concern due to its ability to overcome resistance genes effective against Ug99. Similarly, a race called Digalu (TKTTF), which is also unrelated to the Ug99 group, was detected in Turkey. Apart from its presence in Ethiopia in 2014, lineages of Digalu were also detected in European countries such as Germany, Sweden, Denmark and the UK in 2013, where nearly 80% of the cultivars are susceptible (Lewis et al. 2018).

In Australia, the first incidence of stem rust was recorded in 1925 when race 126 invaded as an exotic introduction. Since then, three additional incursions have taken place, including race 21 in 1954 and races 194 and 326 in 1969. Race 21 is believed to have evolved on the alternate host *B. vulgaris* in Tasmania. The precursor of race 21 and the two other groups 194 and 326 were predicted to originate from central Africa and dispersed through high altitude winds (Park 2015). Consequently, a major stem rust epidemic occurred in 1974 in the southern states causing severe crop damage (Watson 1981). Due to the previous record of exotic incursion of stem rust from Africa, Ug99 is also feared to reach Australia by direct long-distance spore transfer from East Africa, in addition to potential migration through South East Asian nations (Park 2009; Singh et al. 2011).

2.2.2 Leaf rust

Puccinia triticina Eriks. causes leaf rust (brown rust) and is one of the more commonly occurring wheat diseases. Moist and warmer temperature conditions (15°C - 22°C) favours leaf rust epidemics. Leaf rust produces orange/brown raised circular or oval shaped pustules that produce urediniospores (Figure 2.1) (Bolton et al. 2008). Urediniospores are dispersed by wind over thousands of kilometres from the source to infect host plants hence epidemics of wheat leaf rust are frequent on a continental scale (Kolmer 2005). It also adapts well to varying climatic conditions and has a high level of pathogenic variation making it the most widespread of the three rust diseases of wheat (Kolmer 2005; Bolton et al. 2008).

Since 1926 leaf rust pathogenic variations are regularly surveyed in Australia; however, the origin and evolution of races before 1980 are not well understood. Surveys of leaf rust since 1980 provided evidence on five independent exotic incursions such as pathotype 53-1,(6),(7),10,11 (1981); 104-2,3,(6),(7),11 (1984); 76-1,3,5,10,12 (1996); 10-1,3,9,10,12 (2006); 76-3,5,9,10+Lr37 (2006) and one somatic hybridization event between pathotype 53 and 104 in 1990 in New South Wales, Australia. All of these races are the basis for the emergence of new lineages by sequential gain of virulence (Park 2015). A widespread leaf rust epidemic in Western Australia in 1992 caused yield losses up to 37% in susceptible cultivars and an average loss of 15% in many fields (McIntosh et al. 1995). On a global scale, the pathogen has gained virulence to genes, *Lr9, Lr11, Lr17, Lr18, Lr24*, and *Lr26* which are deployed widely in commercial wheat cultivars (Kolmer 2005).

2.2.3 Stripe rust

Stripe rust (yellow rust) caused by *Puccinia striiformis* f. sp. *tritici* has become a serious concern for wheat producers in the past 15 years. Currently, about 88% of world wheat cultivars are susceptible to the prevailing stripe rust races (Schwessinger 2017). *Puccinia striiformis* prefer cool and moist conditions with temperatures between 8 and 15°C. Stripe rust possesses a striking visual phenotype characterised by the presence of light yellow pustules arranged into stripes as they infect along the veins on the leaves and heads (Figure 2.1). The high rate of sexual reproduction of stripe rust on the alternate host barberry observed in the Himalayan and neighbouring regions suggests that it could be a potential centre of origin for new races. Such new

races possess high genetic diversity with the ability to adapt to different climates as observed by cross-continental colonisation via long-distance wind dispersal or by human means of migration (Ali et al. 2014).

In recent years, extremely virulent forms of stripe rust emerged in parts of Australia, America, Europe, South East Asia, East Africa and the Middle East due to exotic incursions (Beddow et al. 2015). Initial migration of stripe rust was in early 1900 from Europe to North America. In Australia, the stripe rust was first noticed in 1979 and believed due to an accidental introduction from Europe through contaminated clothes. Later it resulted in the emergence of 15 races that gained virulence for *YrA, Yr5, Yr6, Yr7*, and *Yr8* resistance genes (Wellings 2007). Similarly, the second introduction of stripe rust into Western Australia was in 2002 possibly from East Africa blown in by the high-altitude air currents. The race group was distinct from the previous incursion and quickly replaced the older races through its ability to overcome *Yr17* and *Yr27* resistances (Wellings 2007). During the same period, highly aggressive and temperature adaptive stripe rust races were detected in the Middle East and are related to the strains in East Africa (Beddow et al. 2015; Schwessinger 2017). In 2011, a new race group called Warrior was identified in Europe and was predicted to originate from the Himalayan region through sexual recombination on barberry (Hubbard et al. 2015; Hovmoller et al. 2017).

2.3 Surveillance for rust pathogenicity

Increased emergence of highly virulent rust races and their rapid spread across wide geographical regions cause severe disease outbreaks on a continental scale. Rust races are normally classified into different race groups based on their virulence profiles on a set of differential wheat lines carrying unique resistance genes. Most countries, where wheat is grown as a primary crop, have established rust survey programs to alert the outbreaks of new races (Park and Cuddy 2015; Ali et al. 2017). In Australia, Professor W. L. Waterhouse at the University of Sydney initiated the annual rust surveillance system for stem and leaf rust in 1921, while a stripe rust survey commenced after the first incursion in 1979. In the late 1970s, a nation-wide cereal rust survey program was initiated with the headquarters at Plant Breeding Institute, University of Sydney, named the Australian Cereal Rust Control Program (ACRCP) (Park 2008; Park 2015). The annual survey for all three rusts is carried out

using both Australian and international wheat differential sets. To date, at least 30 leaf, 21 stem and 16 stripe rust races have been detected (Cuddy 2016; Park 2016a; Park 2016b; Park 2016c).

In the survey, trap plots or breeders nurseries containing resistant cultivars, promising lines with suspected new genes or gene combinations and differentials are used annually to monitor the rusts. However, genotypes with varying growth habits hindered their efficient use and an idea was later proposed to use near-isogenic lines (NILs) carrying single *R* genes. An Australian spring wheat variety Avocet, which is a semi-dwarf and day length insensitive was selected for both greenhouse and filed screening for stripe rust virulence studies.

More recently, PCR based markers such as simple sequence repeats (SSR) markers designed targeting the repeat polymorphisms between races were also used for race grouping (Ali et al. 2011). Subsequently, the advancements in next generation sequencing and genotyping platforms offer new diagnostic tools for surveillance based on single nucleotide polymorphisms (SNPs) as validated in stem rust profiling (Figueroa et al. 2016). The new approaches enabled assessment of genetic diversity among stripe rust races and also assisted in the detection of the recent stem rust races that have re-emerged in Europe after 60 years. Next generation sequencing also facilitated the characterisation of the stem rust races in the UK as a close lineage to the Ethiopian Digalu race (Hubbard et al. 2015; Lewis et al. 2018). Hence these surveillance methods provide vital information about the distribution of races to undertake timely measures to prevent rust disease epidemics (Park et al. 2011).

2.4 Management of wheat rust diseases

Protecting wheat crops against the rusts is crucial for securing global food production. Management strategies for rust disease control can be broadly classified as cultural practices, chemical control and biological control.

2.4.1 Cultural practices

Prevention of wheat rust diseases through cultural method includes strict agronomic practices such as early sowing, growing early maturing cultivars, destruction of wheat stubble and eradication of volunteer plants that act as a “green bridge” to carry over the rust inoculum between growing season (Zadoks and

Bouwman 1985). Although cultural practices have the advantage of reducing environmental pollution, it is less effective when there are severe rust epidemics or windblown spores from different locations (Roelfs et al. 1992).

2.4.2 Chemical control

Application of fungicides is an instant measure to minimise wheat yield losses when there is a sudden outbreak due to accidental entry of new races or due to sudden mutational changes with a gain of virulence to deployed resistance. Nearly US$2.5 million was used to control stripe rust epidemics in Washington State, USA. In Australia, fungicide expenditure increased from AUD$8 million in 1983 to over AUD$350 million in 2008 (Chen 2005; Murray and Brennan 2009b). There are three major groups of fungicides used, C3 quinone outside inhibitor fungicides, G1 demethylase inhibitors and C2 succinate dehydrogenase inhibitors used against rust diseases (Oliver 2014). Continuous application of chemical fungicides also has adverse environmental impacts and imposes selection pressure for pathogens to develop fungicide resistance. Hence genetic resistance is considered the most sustainable method for managing rust diseases (Roelfs et al. 1992).

2.4.3 Genetic resistance

This is the preferred, environmentally safe and economical way to control rust. In Australia, use of genetic resistance against stem rust and stripe rust saved $431 and $438 million, respectively for the wheat industry during 1998 to 2008 (Murray and Brennan 2009a). Resistance to wheat rust diseases is derived mostly from the main host species, commonly referred as host resistance, where in recent years they have also been identified from pathogen non adaptable host or secondary host species commonly known as non-host resistance (NHR)(Gill et al. 2015). Host resistance is divided into two types, all stage resistance (*ASR*) or seedling resistance (*R* genes) and adult plant resistance (*APR* genes). The *ASR* genes generally function from the seedling stage to maturity, whereas *APR* genes typically confer resistance only in the later stages of plant development. So far over 73 leaf rust *(Lr)*, 62 stem rust (*Sr*), and 69 stripe rust (*Yr*) resistance genes have been identified (Park 2016c; McIntosh et al. 2017). Despite this, rust races evolve rapidly to overcome host resistance, thereby rendering resistance genes ineffective if deployed singly.

2.4.3.1. All stage resistance genes

Most of the genes for resistance to wheat rusts are all stage resistance genes. They are also referred to as race-specific, major gene, gene-for-gene, seedling, and qualitative resistance. All stage resistance is often controlled by a single or pair of major genes (*R* genes) and usually confers a high level of resistance characterised by localised programmed cell death or hypersensitive response (HR). So far in wheat, most of the cloned *R* genes encode a typical monocot-specific N-terminal 'coiled-coil (CC) domain', 'nucleotide binding site (NBS)' and a C-terminal 'leucine-rich repeat (LRR)' domain-containing proteins. The only exception is *Yr15* for stripe rust resistance which encodes wheat tandem kinase 1 protein (WTK1) (Fahima et al. 2018). The N-terminal of the *NBS-LRR* genes present in most dicot species encodes a Toll-interleukin-1-receptor (TIR) domain but some also have CC domains (Ayliffe and Lagudah 2004). The *R* genes function in a gene-for-gene manner where they confer resistance only when interacting with a corresponding avirulence *(Avr)* effector carried by selective pathogen races, hence, they are also referred to as race-specific resistance (Ellis et al. 2014). Mutation of *Avr* gene leads to loss of recognition by the *R* gene enabling the pathogen to overcome the host resistance. In general, the host resistance response of the various all stage resistance are scored according to the Stakman scale on a 0 - 4 numerical scale where 0 to 2 are deemed resistant and 3 and 4 are susceptible phenotypes (see section 2.7.2) (Stakman et al. 1962). *R* genes are not durable if deployed alone as the evolution of new races can occur by a single step mutation to overcome resistance (Ellis et al. 2014).

2.4.3.2. Adult plant resistance genes

Adult plant resistance (*APR*) genes as the name indicates are effective at the later stages of the crop growth and also known as slow rusting, partial resistance, minor gene resistance and quantitative resistance. Most *APR* genes are not race-specific and can provide broad spectrum resistance but a small number of race-specific *APR* genes for leaf and stripe rust exists (Kolmer 1997). Despite their minor effect, multiple *APR* genes confer near immunity when combined effectively (Singh et al. 2014). Stem rust resistance gene *Sr2* and multi-pathogen resistance gene *Lr34/Sr57/Yr18/Pm38* are the best-known *APR* genes and have provided durable resistance for more than 100 years (Johnson 1984). Currently, there are four cloned

wheat *APR* genes that belong to different gene families, such as NBS-LRR (*Lr22a,* Thind et al. 2017), Wheat Kinase Start1 (WKS1) gene (*Yr36*, Fu et al. 2009), an ABC transporter gene (*Lr34*, Krattinger et al. 2009) and a hexose transporter (*Lr67*, Moore et al. 2015).

2.4.3.3. Non-host resistance to rust disease

Unlike host resistance, the majority of non-host resistance (NHR) is race non-specific, and they are derived from plant species that are not a preferred host for the targeted pathogen. NHR functions in multiple ways, such as defence signalling factors, leaf surface topology, cell wall and cuticle barriers, induced defences like HR, and lignin accumulation that limits pathogen infection or colonisation in general (reviewed by Gill et al. 2015). Sometimes the pathogens perhaps cross these barriers converting non-host species as their newly preferred host (Ayliffe et al. 2011). A few examples of NHR are the *CcRpp1* gene from *Cajanus cajan* (pigeon pea) that provides resistance to *Phakopsora pachyrizi* (Asian soybean rust). Similarly, *Rps6* from barley confers resistance to *P. striiformis* f. sp. *tritici* (Dawson et al. 2016; Li et al. 2016b). Likewise, NHR genes to wheat stem rust are identified in various crop species such as *Brachypodium distachyon* and several rice accessions (Ayliffe et al. 2011; reviewed by Periyannan et al. 2017). Therefore transferring *R* genes providing NHR from the closely related species could also broaden the available gene pool to control the biotrophic pathogens of wheat (Dracatos et al. 2018).

2.4.4 Management through biocontrol agents

Endophytic bacteria have been touted as biocontrol agents that can potentially limit the growth of other organisms like rust. Such endophytic bacteria were used as a biocontrol agents for various rust diseases such as *Uromyces phaseoli* (bean rust), *Puccinia pelargonii-zonalis* (geranium rust), and *Hemileia vastatrix* (coffee rust) and *P. striiformis* (wheat stripe rust) have been studied (Li et al. 2013b; Pang et al. 2016). Strains of endophytic bacteria such as *Bacillus subtilis* and *Pseudomonas putida* have been previously used for the control of stripe rust (Li et al. 2013b; Pang et al. 2016). However, widespread use or field application of these agents to wheat rust diseases is limited.

2.5 Sources of genetic rust resistance

The Triticeae botanical tribe of grasses includes crops such as *Hordeum vulgare* (barley; 2n), *Secale cereale* (rye; 2n), *T. turgidum* (durum wheat 4n), *T. aestivum* (bread wheat; 6n) and *Triticosecale* (Triticale; 8n) and accounts for one-third of global cereal production; however, hexaploid wheat (AABBDD) accounts for covers over 90% of the cultivated Triticeae worldwide. Hexaploid wheat evolved from two hybridisation events. The first occurred between *Triticum urartu* (AA) and an unknown grass species closely related to *Aegilops speltoides* (BB) to form a tetraploid wheat *Triticum turgidum* that subsequently hybridised with *Aegilops tauschii* (DD) species to form the hexaploid *T. aestivum* species (Figure 2.3) (Feuillet et al. 2008). The cultivated hexaploid bread wheat is predicted to have evolved ~9000 years ago in the Fertile Crescent (Feuillet et al. 2008). During hybridisation, several agronomic traits especially adaptation to various geographic conditions, high yielding capacity and genetic resistance to diseases were also transferred to hexaploid wheat. However, domestication and intensive modern breeding of wheat for better agronomic traits have reduced genetic diversity in elite germplasm, which renders crops more vulnerable to disease epidemics (Feuillet et al. 2008). In contrast, relatives of cultivated wheat such as progenitors or landraces still possess greater genetic variability useful for crop improvement, especially disease resistance traits due to little exposure to the predominant pathogen races.

Broadly, the relatives of cultivated wheat are classified into three gene pools (Friebe et al. 1996; Feuillet et al. 2008):

i) Primary gene pool which refers to landraces, early domesticated lines and wild relatives (such as *T. turgidum, T. monococcum, T. boeoticum, T. uratu, Ae. speltoides* and *Ae. tauschii*) of *T. aestivum* that have genomes identical to the cultivated species. Hence these species can hybridise and recombine with the cultivated species.
ii) Secondary gene pool containing polyploid *Triticum* and *Aegilops* species (such as *T. timopheevii* and the diploid S genome belonging to *Aegilops*) that shares at least one genome similar to the cultivated type.

iii) The tertiary gene pool of distantly related species that does not contain any genome related to wheat. Species of *Secale, Thinopyrum* and *Hordeum* belong to this group.

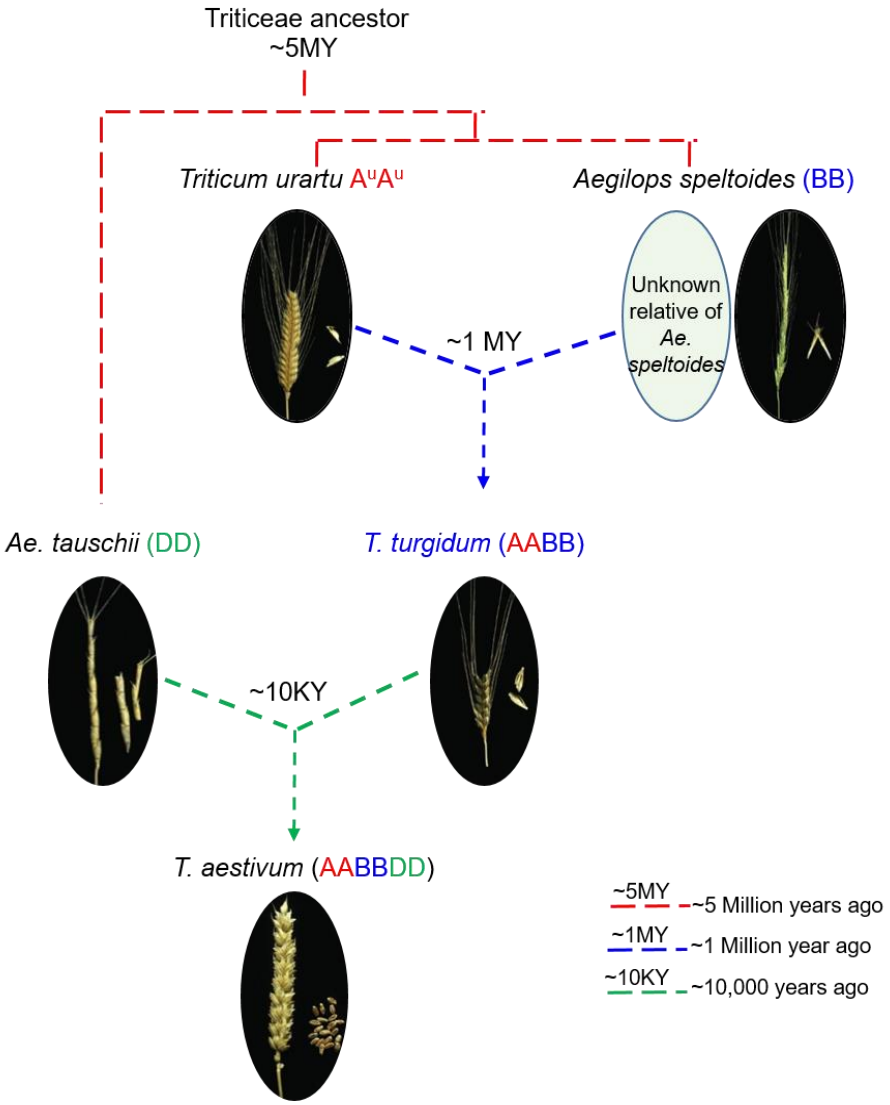

Figure 2.3 Origin of wheat (modified from Feuillet et al. 2008).
Evolution of bread wheat from the two different hybridisation events. The first hybridisation event occurred between *T. urartu* (A genome) and an unknown relative of *Ae. speltoides* (B genome) about 5 million years ago to evolve allotetraploid emmer wheat (*T. turgidum*; AABB). Subsequent hybridisation between *T. turgidum* and *Ae. tauschii* (D genome) originated the bread wheat (*T. aestivum*, AABBDD) at approximately about 10,000 years ago (Feuillet et al. 2008; Rasheed et al. 2018).

Historically, resistance gene pools are often enriched by exploring germplasm collections of progenitors, wild relatives and landraces (McIntosh et al. 1995; Rouse et al. 2011b; Rouse et al. 2011a; Riaz et al. 2017; Winfield et al. 2018; Riaz et al. 2018). Among all the wild relatives, *Ae. tauschii,* the D genome progenitor of hexaploid wheat, possesses higher genetic diversity compared to bread wheat cultivars and landraces (Reif et al. 2005).

2.5.1 Wheat D genome progenitor Aegilops tauschii: a resource for unexplored traits

Aegilops tauschii Coss. (Syn. *Ae. squarrosa* L.; *Triticum tauschii),* a self-pollinating wild grass belonging to the Triticeae family, is the immediate progenitor for the hexaploid wheat's D genome. It has a broad distribution range from Turkey to China and Pakistan. Based on geographical distribution, the accessions of *Ae. tauschii* consist of two lineages, L1 and L2, and each with two sublineages named L1W, L1E and L2W, L2E, respectively (Borner et al. 2015). Accessions within the L2E descent were postulated to be the primary source for the formation of hexaploid wheat. Due to high homology between the D genome of *Ae. tauschii* and hexaploid wheat, natural introgression of genes from *Ae. tauschii* to hexaploid wheat is possible through conventional breeding. Furthermore, *Ae. tauschii* accessions are highly divergent from landraces and bread wheat and they are the source of numerous important agronomic traits of cultivated wheat such as yield, resistance to various pests and diseases, and tolerance to abiotic factors such as aluminium toxicity (Borner et al. 2015).

Aegilops tauschii germplasm is a valuable resource for resistance against rust, powdery mildew, wheat blast, septoria blotch, spot blotch, fusarium head blight, and the tan spot (Arora et al. 2019). Many potential genes from *Ae. tauschii* were identified for rust resistance (Friesen et al. 2008; Rouse et al. 2011a; Olson et al. 2013a; Kalia et al. 2017); however, only a few of these (Table 2.1) have been catalogued and introgressed into commercial cultivars. There are two main approaches for the introgression of traits from *Ae. tauschii* into hexaploid wheat. The most common method involves transfer using synthetic hexaploid wheat generated by crossing *T. turgidum* and the targeted *Ae. tauschii.* The second is direct introgression where the *Ae tauschii* accession is crossed with the bread wheat and subsequently backcrossed to recover a stable bread wheat derivative (Gill and Raupp 1987; Olson et al. 2013a).

Notable introgressions include the transfer of traits for bread making quality and Ug99 stem rust resistance (Rouse et al. 2011a; Borner et al. 2015).

Table 2.1 List of rust resistance genes identified from *Aegilops tauschii* (cloned *R* genes are highlighted in bold)

Type of rust	Rust resistance genes	Reference
Leaf rust	***Lr21, Lr22a****, Lr32, Lr39, Lr42, Lr43*	(Kerber 1987; Cox et al. 1994; Raupp et al. 2001; Huang et al. 2003; Thind et al. 2017)
Stem rust	***Sr33, Sr45, Sr46, SrTA1662****, SrTA10187*	(Olson et al. 2013b; Periyannan et al. 2013; Periyannan et al. 2014; Wiersma et al. 2016)
Stripe rust	*Yr28, YrAs2388*	(Singh et al. 2000; Huang et al. 2011)

2.6 Breeding wheat for rust resistance

In Australia, wheat stem rust has posed a significant threat since settlement. A major outbreak of stem rust in 1888/89 resulted in a disastrous wheat harvest that led to an emphasis on breeding for stem rust resistance in 1892 by William James Farrer. Farrer initiated the first breeding program and provided considerable knowledge to breeders for generating rust resistant wheat cultivars. He developed the rust and drought resistant wheat variety called “Federation” in Australia. Despite the success of rust resistant varieties in the early 20th century, stem rust still caused severe losses (Waterhouse 1929) leading to the development of numerous stem rust resistant cultivars such as Hofed and Fedweb by W.L. Waterhouse in the 1920s and subsequent years (Spennemann 2001; Bariana et al. 2007). However, with the recent outbreak of virulent races in Africa, Europe and UK, stem rust remains as an ever-present threat to global wheat cultivation. Therefore, continuous pathogen monitoring and breeding practices are mandatory to manage wheat rust diseases.

To improve wheat crops for better protection against rapidly evolving rust races, breeders are eager to incorporate novel genetic resistances into commercial cultivars. Therefore, the essential prerequisite is to explore historic germplasm collections of wild relatives, landraces and other related species that have rich diversity and least

exposed to the prevailing pathogen races. But the transfer of resistance from these related sources into the breeding material is challenging requiring crossing and repeated selection. However, with the recent advancements in climate controlled growth chambers and marker technologies, a number of improved and rapid breeding program have been implemented as described below.

2.6.1 Conventional breeding

Conventional breeding for rust resistance relies on visual identification of resistant plants among the pool of genotypes tested. A conventional wheat breeding program commonly involves the pedigree, bulk, single seed descent or backcrossing methods. Often one or more of these approaches are combined for cultivar development. In CIMMYT breeding programs, pedigree selection was a primary focus until 1985 after which the modified pedigree or bulk (further modified as selected bulk) began to be used.

The pedigree method involves selection of individuals from a segregating population derived from the cross between known parents, with selection based on phenotype, genotype, or a combination of both. All individuals are assigned an identity to trace back information to a later generation. Despite the labour-intensive process, this method of breeding gives the most genetic information for a line (Baenziger and Depauw 2009). In the case of the selected bulk method, one spike from each of the selected F_2 plants is bulked to generate a single F_3 lot. Then a subset of seeds from the pooled lot is advanced to the next generation and the process continues until F_5 or F_6 and then pedigree selection begins (Velu and Singh 2013). In case of single seed descent (SSD), a single seed from each F_3 plant is advanced until F_6. Therefore, when compared to bulk methods, the SSD retains more genetic variation of original populations in the advanced generations due to an equal number of seeds advanced to the next generation containing more recombination events (Ortiz et al. 2007). Subsequently, selection for *APR* relies heavily on phenotyping under different environmental conditions as such traits can be influenced by environmental factors. Although phenotyping in the field is a resource-intensive process, it provides opportunities for selection of resistance against multiple pathogen races. In most wheat growing countries there is single growing per year and therefore a duration of 10-12 years is required to develop a new variety.

Backcrossing facilitates the targeted transfer of a desirable trait into a recurrent parent or an elite breeding line whilst retaining the other characteristics of the receiving line. It mainly relies on the selection of the desired trait in the F_1 and in the subsequent backcross generations. Usually, it requires at least six backcrosses as a short-term strategy to develop rust resistant cultivars (Baenziger and Depauw 2009).

2.6.2 Shuttle breeding

In an effort to reduce the time required to develop a cultivar with improved agronomic traits, the Nobel Peace Prize laureate, Dr Norman E. Borlaug initiated a program called shuttle breeding in 1968 which enables an extra generation per year. This program uses two distinct locations (Ciudad Obregon, Sonora, 27.5°N and Toluca, State of Mexico, 19°N) to breed and evaluate wheat lines. Ciudad Obregon is a dry irrigated site located 39 m above sea level in the Yaqui Valley of northwest Mexico, while the second site Toluca is a cool, humid highland located 2640 m above sea level near Mexico City. Borlaug and co-workers initiated the use of these two sites to grow two successive generations per year to speed the breeding process to develop new varieties. At Toluca, the growing season is between May and October, and the materials selected soon after the season are shuttled to Obregon for growing between November and April. Screening for leaf and stem rust resistance are carried out at Obregon while screening for stripe rust, *Septoria tritici* and Fusarium head blight are performed at Toluca. Through the shuttle breeding approach, the time taken to develop wheat cultivar is reduced from 10-12 years to 6-8. Following the success of national shuttle breeding for rust resistance, an international shuttle breeding program targeting hot spot regions in Africa for stem rust resistance was later initiated after the outbreak of Ug99 (Ortiz et al. 2007).

2.6.3 Double haploid technology

Double haploid (DH) technology was developed to generate homozygous lines rapidly. It uses haploid tissues, and chromosome doubling (using colchicine) to produce plants and thereby can cut three to four years from the process of homozygous line development. This technology has been successfully used in crops such as barley, rapeseed (*Brassica napus* L.), maize (*Zea mays* L.), and bread wheat (Li et al. 2013a). The two primary methods are based on anther culture and wheat × maize intercrossing (Laurie and Bennett 1988; Guzy-Wrobelska and Szarejko 2003).

The DH method is highly advantageous for winter wheat where vernalisation requirements further extends the generation time. However, in CIMMYT, the DH generation was found to be expensive compared to the selective bulk method as it costs about US $30 per line. Additionally, shuttle breeding revealed a higher genetic gain than DH and therefore, CIMMYT breeding programs continued with shuttle breeding strategies (Li et al. 2013a). Furthermore, the production of double haploids from F_1 crosses is insufficient to break undesirable linkage due to one recombination event (Ortiz et al. 2007).

2.6.4 Speed breeding

Development of genetically stable lines in a wheat breeding program normally requires 6-7 years. To shorten the time, 'speed breeding' was developed which applies an extended photoperiod in a controlled environment to accelerate plant growth and generation. Speed breeding utilises growth conditions of 22-hour day length at 22°C and 2 hours of darkness at 17°C. Over the past decade, speed breeding has transitioned from the use of sodium vapour lamps to use of light emitting diode (LED) lights to reduce operating costs (Ghosh et al. 2018). To date, speed breeding has been tested on various crops such as spring hexaploid wheat (*T. aestivum*), durum wheat (*T. durum*), barley (*H. vulgare*) and the model grass *B. distachyon.* In wheat, depending on the cultivar, anthesis (flowering time) occurred between 35 and 39 days, which was shortened to 22±2 days compared to normal growth conditions. Effectively, viable seeds are harvested 14 days post anthesis. Using this approach, 4 to 6 generations of wheat are grown per year (Watson et al. 2018). While conventional shuttle breeding takes up to 5 years to generate homozygous lines, SSD in combination with speed breeding takes only one and a half year time. Alternatively, selection of visible traits such as disease resistance in early segregating populations (F_2 or F_3) can be achieved simultaneously. Subsequently, rapid phenotyping methods to detect adult plant response for triple rusts (i.e. stripe, leaf and stem) and yellow spot are developed using the speed breeding approach (Hickey et al. 2012; Dinglasan et al. 2016; Riaz et al. 2016; Riaz and Hickey 2017).

2.6.5 Marker assisted breeding

In recent years, the emphasis on the selection of traits of interest is based on markers. A marker may be classified as morphological or molecular (Collard et al.

2005). Selection based on visible traits such as pseudo black chaff, leaf discolouration and leaf tip necrosis that are linked to disease resistance are known as morphological markers. In contrast, molecular markers represent the DNA sequence variation that are linked to the targeted traits. With the availability of numerous reference genome sequence, identifying such variations among parents are rapid. Subsequently rapid cloning of the genes responsible for rust resistance enables the generation of trait-specific or perfect markers. These markers are crucial for the accurate selection of traits at the earliest generation (F_2) in the breeding program, thereby limiting the population size that needs to be advanced further. Additionally, molecular markers are crucial when two or more genes having an identical resistance pattern are to be selected simultaneously as required in gene pyramiding (Hiebert et al. 2010). For instance, stem rust resistant stacks with two or three gene combinations (involving *Sr31, Sr24, Sr26* or *SrR*) were effectively generated via marker assisted breeding (Mago et al. 2011b). Similarly, marker assisted pyramiding also enabled the combination of leaf rust resistance genes (*Lr24*, *Lr28* and *Lr9*) as reported by Charpe et al. (2012).

2.6.6 Transgenic approaches

Although commercial use of transgenic wheat is still under debate, transgenic approaches ensure rapid varietal generation through the direct transformation of cloned *R* genes into elite wheat lines. It also allows rapid stacking of multiple rust resistance genes provided numerous cloned resistance genes are readily available. An efficient wheat transformation system is currently available to facilitate the transformation of single or multiple *R* genes using cassettes (Richardson et al. 2014; Ishida et al. 2015). This cassette approach of combining *R* genes in a single locus overcomes the difficulties of conventional gene pyramiding where targeted *R* genes segregate in subsequent crossings scattered throughout the genome (Luo et al. 2018). Besides, the multigene cassette is also advantageous for transferring genes that are at homeologous positions (e.g., *Sr33, Sr50*) or from alien segments (e.g., *Sr26, Sr31, Sr50*) that do not recombine normally. It also enables transfer of resistance from non-host species that differ largely with cultivated wheat (Ellis et al. 2014; Singh et al. 2015).

2.7 Mapping genomic regions controlling rust resistance

Identifying the genomic region conferring resistance to rust is crucial to develop tightly linked markers for the effective selection of resistance. Mapping of rust resistance loci are carried out using two approaches, one uses a traditional bi-parental mapping population, and a new alternative is association genetics or genome-wide association studies (GWAS) (Yu et al. 2006). An initial step involves accurate phenotyping of the individuals of a population for the trait of interest followed by genotyping with markers such as single nucleotide polymorphism (SNP) markers. Finally, based on the marker-trait linkage analysis, the chromosomal location and the markers linked with the targeted *R* genes are identified.

2.7.1 Mapping populations

The first step in mapping any trait is to generate a good mapping population. This can be generated by crossing two individuals varying for the targeted trait, and thereby the progeny segregate for the trait due to different recombination events. Adequate genetic diversity must exist among parents to determine the DNA sequence polymorphism that is closely linked with the traits. There are different types of mapping populations such as; F_2 population, F_2 derived F_3 population ($F_{2:3}$), double haploid (DH), backcross population, recombinant inbred lines (RIL) or near-isogenic lines (NIL) (Singh and Singh 2015).

The crossing of two distinct lines produces the first filial generation known as F_1 which is heterozygous for all loci. The segregating F_2 population is derived from the F_1 either by selfing or random crossing. An F_2 population would be ideal for the preliminary mapping of traits. However precise mapping of major traits are usually done in F_2 derived F_3 ($F_{2:3}$) populations where the testing of each F_3 family clearly distinguishes the segregating lines. The phenotypic score is then combined with the marker score for the construction of linkage maps for the rust resistance locus (Singh and Singh 2015). Several rust resistance genes were characterised using $F_{2:3}$ populations, including *Lr34, Lr39, Lr15, Lr65, Sr22,* and *Yr61* (Raupp et al. 2001; Mohler et al. 2012; Dholakia et al. 2013; Zhou et al. 2014).

The F_1 hybrids can also be used to generate the double haploid lines. As the anthers from the F_1 hybrid are generated after the meiosis, they represent the

segregating haploid gametes. Therefore, the haploid plants regenerated from anther culture can be doubled by Colchicine treatment to generate homozygous lines (Jones et al. 1997; Ortiz et al. 2007; Humphreys and Knox 2016). On the other hand, F_1 hybrids can also be used to generate a backcross (BC) population where they are crossed with the recurrent parent to attain a BC_1 population. Subsequently the BC line is again crossed with a recurrent parent to produce a BC_2 or selfed to attain a BC_1F_2. Backcrossing generates recurrent parent like lines with reduced donor segment. In each round of backcrossing, the proportion of the donor segment is reduced by 50% and in each generation lines must be selected for the desired trait before subsequent backcrossing event. Crossing up to seven generations can result in the generation of near-isogenic lines (NIL) of the recurrent parent but carrying the target trait from the donor (Singh and Singh 2015). A NIL population is vital for the functional analysis of genes and highly advantageous for the crops that are difficult to transform. Several rust resistance genes have been studied using NIL populations *Lr2a, Lr3, Lr9, Lr34/Yr18, Lr57, Yr1, Yr5, Yr6, Yr7, Yr8, Yr9, Yr10, Yr15, Yr17, Yr18, Yr24, Yr26, Yr27* and *YrSP* (Hulbert et al. 2007; Wellings et al. 2009; Wang et al. 2013; Yadav et al. 2016).

Recombinant inbred lines (RILs) are homozygous lines produced from the selfing of F_2 individuals to F_6 to F_8 using single seed descent. One seed from each F_2 individual is forwarded to F_3 and single seed from each F_3 to F_4 until F_6 or F_8 generations where the lines will be homozygous at all loci. At the F_6 generation, near homozygosity can be reached. RIL populations can be propagated indefinitely without further recombination change in genotypes as they are homozygous and hence the RILs remain a permanent population. However, the process can take up to 5 years depending on the species. In wheat, speed breeding can decrease RIL line production to two years. Wheat rust resistance genes, *Lr13, Lr46, Lr67, Sr22, Sr33, Sr45,* and *Yr46* were characterised using RIL populations (Sambasivam et al. 2008; Herrera-Foessel et al. 2011; Kolmer 2015; Zhang et al. 2016).

Finally, lines carrying mutations on the targeted gene locus are screened in a mutant population for the interesting traits that remains essential for identifying candidate genes during gene cloning (Periyannan 2018). Mutant lines are commonly generated using chemical agents such as ethyl methane sulphonate (EMS), sodium

azide or alternatively – gamma irradiation using a ^{60}Co source. The treated seeds are advanced to the M_2 generation and screened for susceptibility. Similarly, Targeting Induced Local Lesions IN Genomes (TILLING) population was also used to identify candidate genes. Chemical agents such as EMS or sodium azide are used for high-frequency point mutations, which are genotyped later using high-throughput sequencing and marker techniques. It was first developed in *Arabidopsis thaliana* as an alternative method to insertional mutation (McCallum et al. 2000). In wheat, TILLING population sizes ranging from 1500 to 20000 are reported (Kurowska et al. 2011). TILLING populations have been successfully used in cloning of head blight disease resistance *Fhb1*, powdery mildew resistance *Pm3* alleles and stripe rust resistance gene *Yr36* (Fu et al. 2009; Rawat et al. 2016; Savadi et al. 2018).

2.7.2 Phenotyping rust resistance

To dissect the genetics of rust resistance either by bi-parental population or GWAS, an efficient and accurate assessment of the rust resistance phenotype is essential to associate markers with the trait (Velu and Singh 2013). Measuring all stage rust resistant phenotypes are mostly carried out under controlled environmental conditions while natural field evaluations are required to score adult plant resistance. However, to accelerate *APR* screening, a rapid phenotyping method was devised recently with the help of speed breeding (Hickey et al. 2012; Riaz et al. 2016; Riaz and Hickey 2017). For the evaluation of all stage resistance, germinated seeds are grown for 10 to 12 days under 16-hour photoperiod at 22°C followed by rust infection. For leaf and stem rust, the inoculated plants are incubated at high humidity and then returned to normal humidity for 12 days. For stripe rust, plants are incubated in darkness at 10°C with high relative humidity for 2 days followed by 16-hour photoperiod at 17°C for 12-14 days. Scoring all-stage resistance to rust disease is done using a widely used scale developed by Stakman et al. (1962). The scale ranges from 0 – 4, where the infection types 0 to 1 are classified as resistant, 1+ to 2 are deemed moderately or intermediate resistant, while 3 to 4 are susceptible (Figure 2.4).

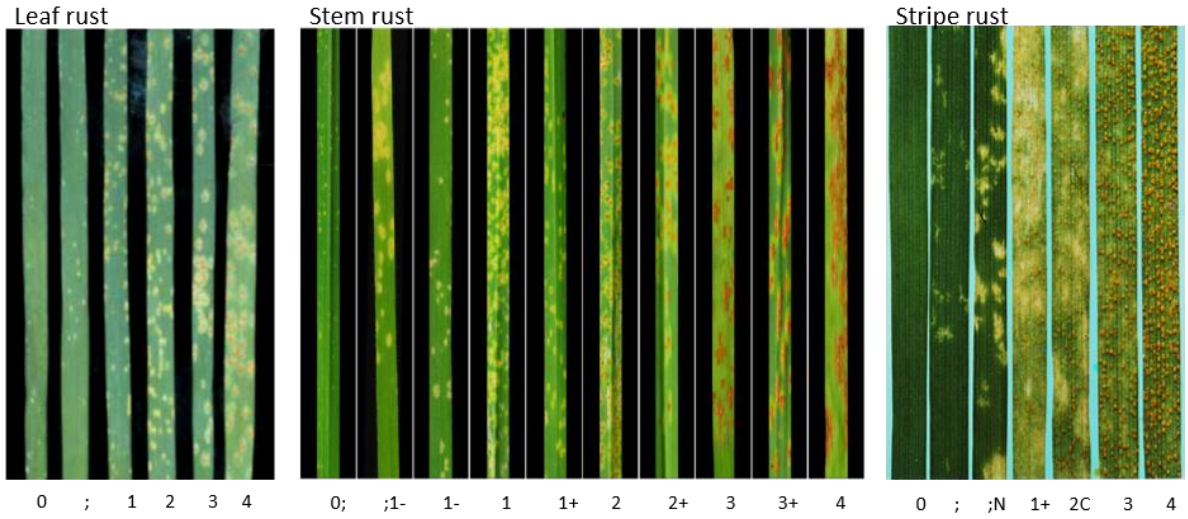

Figure 2.4 Stakman scale for phenotyping all stage rust resistance

Stakman scale for phenotyping all stage triple rust resistance adapted from (McIntosh et al. 1995; Bolton et al. 2008; Arora et al. 2019). *0 or **;** : Immune or nearly immune, 1 or 2: Resistant, 3 or 4 : Susceptible, N or C: Necrotic or Chlorotic, Minus (−): Small pustules, Double minus (=): Very small pustules, Plus (+) signs: Large pustules*

New sensor-based detection methods may also be used to assess disease severity where physiological changes associated with biotic stresses are measured. Various types of optical sensors such as RGB-imaging, multi and hyperspectral reflectance sensors, thermal sensors, and fluorescence imaging that measure temperature, reflectance and fluorescence are used for the sensor-based phenotyping (Mahlein 2016). Diseases such as Fusarium head blight, stripe rust, leaf rust and powdery mildew have been assessed using spectral sensors and fluorescence imaging (Mahlein 2016; Pretorius et al. 2017b). Quantitative assays for scoring rust fungi infection have also been developed in which the fungal chitin content of the infected plants are measured using the fluorophore (alexa488 or fluorescein isothiocyanate) conjugated to wheat germ agglutinin (WGA). As the WGA has high affinity to chitin it indirectly quantifies resistance by measuring the fungal biomass (Ayliffe et al. 2013).

2.7.3 Genotyping for rust resistance

Genotyping is an essential part to identify the marker trait association for mapping of any target rust resistance locus. Until the emergence of molecular markers, genotyping were relied on various phenotypic characters that are termed as morphological markers. Morphological markers linked to rust resistance are usually associated to adult plant resistance genes such as Pseudo-black chaff and seedling chlorosis associated with *Sr2* (Kota et al. 2006), leaf tip necrosis (LTN; *Lr34, Lr46* and *Lr67*) (Singh 1992; Rosewarne et al. 2006; Herrera-Foessel et al. 2014), brown chaff colour and white-chaffed associated to *Yr10* (Bariana et al. 2007). With the emergence of PCR and biotechnological advances development of markers targeting genetic variations have become established. DNA sequence variation among parents of a population or in the diversity panel are the potential targets to develop molecular markers. These variations include insertions and deletions (InDels), variation in repetitive sequences, loss or gain of restriction sites, and single nucleotide polymorphisms (SNPs). In general, molecular markers are divided into DNA markers and biochemical markers. Biochemical markers or protein markers can also be used as polymorphic markers as isozymes and seed storage proteins linked with useful traits were used as molecular markers. However, no biochemical markers associated with rust resistance have been widely used (Xynias et al. 2007; Jiang 2013).

DNA markers are further divided into three groups, hybridisation-based DNA markers, PCR-based DNA markers, and DNA chip-based DNA markers (Collard et al. 2005; He et al. 2014b).

2.7.3.1 Hybridization based markers

Restriction fragment length polymorphism (RFLP) were the first molecular marker identified (Botstein et al. 1980). Detection of polymorphisms using hybridisation-based markers relied on the restriction digestion of genomic DNA and hybridisation with a radioactively labelled probe. These markers are extensively polymorphic, reliable and were used extensively before the introduction of PCR based markers. These markers were used for the construction of linkage maps in *T. aestivum* and *Ae. tauschii* (Helentjaris et al. 1986; Chao et al. 1989; Lagudah et al. 1991). However, hybridisation-markers are limited by cost, time, DNA quantity and use of radioactive or toxic chemicals (He et al. 2014b). Several rust resistance genes like *Lr1,*

Lr24, Lr34, Lr35, Lr57, Sr2, Sr22, Yr15, Yr28 and *Yr40* were mapped using RFLP markers (Paull et al. 1994; Nelson et al. 1995; Schachermayr et al. 1995; Sun et al. 1997; Seyfarth et al. 1999; Singh et al. 2000; Ling et al. 2004; Kuraparthy et al. 2007; Herrera-Foessel et al. 2012).

2.7.3.2 PCR based markers

Since the invention of PCR, the development of various PCR based second generation markers became popular and widely used. These markers include simple sequence repeat (SSR), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence characterised amplified region (SCAR), cleaved amplified polymorphic DNA (CAPS) and sequence tagged site (STS). These PCR based markers are robustly reducing the time and cost for genotyping (He et al. 2014b).

2.7.3.2.1 Microsatellites or simple sequence repeats (SSRs)

Simple sequence repeats of a few base pairs are ubiquitous in eukaryotic genomes. The number and type of repeat units present in a species are variable between individuals. Primers designed specifically to target these differences are known as SSR markers. They are widely used in plant genetic studies due to high levels of polymorphism, low cost and automated techniques (Hayden et al. 2006). SSR markers linked with *Lr19, Lr22a, Lr24, Lr34/Yr18, Lr39, Lr42, Sr2, Sr6, Sr22, Sr36, Sr35, Sr40, Yr5, Yr10, Yr36, YrCH42* and *YrZH84* rust resistance genes were available for marker assisted breeding (Schachermayr et al. 1995; Raupp et al. 2001; Bariana et al. 2002; Spielmeyer et al. 2003; Khan et al. 2005; Spielmeyer et al. 2005; Uauy et al. 2005; Bossolini et al. 2006; Gupta et al. 2006; Li et al. 2006; Hiebert et al. 2007; Wu et al. 2009; Olson et al. 2010; Sun et al. 2010; Tsilo et al. 2010; Zhang et al. 2010b).

2.7.3.2.2 Random amplified polymorphic DNA (RAPD)

RAPD markers represent DNA fragments amplified using single short synthetic primers of random sequence. These primers act as both forward and reverse which can amplify random regions throughout the genome. The amplified fragments are usually polymorphic (Williams et al. 1990). Rust resistance genes *Lr9, Lr19, Lr24, Lr34, Sr22, Yr10, Yr15* and *Yr17* were mapped using RAPD markers (Schachermayr et al. 1995; William et al. 1997; Sun et al. 1997; Suenaga et al. 2003; Khan et al. 2005;

Gupta et al. 2006; Liu et al. 2014). Low reproducibility, low levels of polymorphism, the requirement for high-quality DNA and high standardized experimental procedures are the major drawback of RAPD markers (Kesawat and Kumar 2009).

2.7.3.2.3 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) is the combination of RAPD and RFLP where the restriction site difference between any two lines were detected. Specific double strand adapters are ligated with the digested DNA fragment and primers specific to the adapter sequence and with three varying nucleotide-based at the 3' end are used to amplify a large number of restriction fragments. In the PCR reaction, primers specifically bind to the fragments containing matching ends and the selective amplification of different sized fragments is detected as polymorphic differences (Vos et al. 1995). Markers linked with rust resistance genes *Lr3, Lr26, Lr46, Sr2, Sr30, Sr31, Sr39, Yr7, Yr9* and *Yr29* were identified based on AFLP analysis (Bariana et al. 2001; Spielmeyer et al. 2003; Mago et al. 2005; Dieguez et al. 2006; Rosewarne et al. 2006; Mago et al. 2009).

2.7.3.2.4 Sequence characterised amplified regions

Sequence characterised amplified regions (SCAR) markers are derived by cloning and sequencing the amplified fragments of RAPD markers that appeared to be diagnostic for a specific trait. They use longer primers for specific amplification of a locus which makes it reproducible. SCAR markers exhibit several advantages in mapping studies and map-based cloning of potential traits (Kesawat and Kumar 2009). SCAR markers have been developed for rust resistance genes like *Lr9, Lr19, Lr26, Lr35, Lr59, Sr31, Sr39* and *Yr9* (Cherukuri et al. 2003; Gupta et al. 2005; Das et al. 2006; Gul'tyaeva et al. 2009; Marais et al. 2010; Kadkhodaei et al. 2012).

2.7.3.2.5 Sequence tagged site

Sequence tagged site (STS) markers are readily amplified by PCR whose location is uniquely mapped in the genome. STS is highly advantageous than all the other markers as the loci are unique, mostly co-dominant and reproducible by simple PCR. The amplified DNA segment of the STS marker may have a repetitive element provided the primer annealing ends are unique product and result a specific amplicon. To broadly represent the STS markers can also include all the other type of markers that are uniquely mapped in the genome (Olson et al. 1989). STS marker linked with

Lr9, Lr24 and *Lr35*, *Sr22* rust resistance genes were developed from the RAPD fragments and RFLP probes respectively (Schachermayr et al. 1994; Schachermayr et al. 1995; Seyfarth et al. 1999; Neu et al. 2002; Periyannan et al. 2011) whereas the STS markers linked to *Lr19, Lr26, Lr28, Lr37, SrR, Sr24, Sr26, Sr31, Sr38, Sr39, Yr5* and *Yr9* were converted from AFLP fragments (Naik et al. 1998; Prins et al. 2001; Mago et al. 2002; Mago et al. 2005; Smith et al. 2007; Mago et al. 2009). STS markers linked with *Lr34*, *Sr13*, *Sr25* and *Sr26* were developed from EST's (Lagudah et al. 2006; Liu et al. 2010; Simons et al. 2011).

2.7.3.3 SNP based markers

Advances in high throughput genome sequencing/next generation sequencing have reduced costs associated with sequencing-based genotyping (Elshire et al. 2011). Genotyping by sequencing (GBS) is a cost-efficient next generation genotyping method to identify variation in single nucleotide among complex genomes (Elshire et al. 2011; Kilian et al. 2012; Wang et al. 2014). SNPs are highly abundant and distributed throughout the genome in all plants and are usually detected by array-based detection methods. In wheat, over 90% of genetic variations are due to single nucleotide polymorphisms (SNPs), hence GBS is a logical alternative to array-based detection methods, and an attractive tool for genetic mapping, marker-assisted breeding and map-based cloning. GBS is a restriction based genome complexity reduction system that utilises a two enzyme protocol for reducing genome complexity in wheat and barley (Poland et al. 2012a). At CIMMYT, over 40,000 accessions were genotyped by GBS platform for the Seeds of Discovery initiative (http://seedsofdiscovery.org/). High density SNP data is mainly used in modern day mapping and QTL identification by GWAS. Potential SNPs are selected based on their distribution across the genome to design high density SNP genotyping assays (Ganal et al. 2011; Zhao et al. 2011; Cook et al. 2012; Cavanagh et al. 2013; Jia et al. 2013a; Song et al. 2013). Recently, accessions of the D genome progenitors were sequenced and evaluated using SNP markers developed in various GBS studies for establishing diversity panels to identify stem rust resistance genes in the panel (Arora et al. 2019).

Development of genotyping by sequencing methodologies contributed to the wheat genome SNP database for marker development. As a result, conversions of such SNPs to markers in plant breeding programs has increased significantly. Over

the last decade, SNP based markers increased the marker density for high throughput genotyping. SNPs are identified either through *in silico* comparison of available sequence for different varieties or from analysis of PCR fragments amplified from different individuals variable for the trait of interest. Several genotyping methods are available for SNP analysis includes electrophoresis (fluorescence), FRET (fluorescence resonance energy transfer), fluorescence polarisation, arrays (fluorescence), mass spectrometry, luminescence techniques (Skinnes et al. 2010). Among them, fluorescence array-based detection of polymorphism in a PCR product is the most common for SNP analysis.

2.7.3.3.1 Kompetitive allele specific PCR marker

Kompetitive Allele Specific PCR (KASP) is a fluorescence-based genotyping assay developed to detect single nucleotide polymorphisms (SNPs). KASP markers are rapid, accurate, reproducible, cost-effective, require a relatively low quantity of DNA (10-25 ng) (Semagn et al. 2014). KASP marker primers for the iSelect 90K SNP chip and Axiom 820Kchip were developed with reference to the IWGSC RefSeq v1.0 and are publicly available at http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/KASP_primers_for_iSelect.php or http://polymarker.tgac.ac.uk/. KASP markers can be used for a wide range of applications such as QC analysis, QTL mapping, MARS, large-scale allele mining, association mapping and genomic selection (Semagn et al. 2014). Currently, KASP markers associated with *Lr13, Lr21, Lr34/Yr18, Lr67, Sr2* and *Sr11* are available to use in breeding programs (Neelam et al. 2013; Newcomb et al. 2013; Forrest et al. 2014; Nirmala et al. 2016; Zhang et al. 2016).

2.7.3.3.2 Cleaved amplified polymorphic sequence

Cleaved amplified polymorphic sequence (CAPS) are the amplified PCR fragments from two individual lines that differ in few SNP changes which corresponds to restriction sites. Such SNPs can be potentially converted into a CAPS marker. Therefore digestion of the PCR products with a restriction enzyme can be used to produce different sized fragments representing the polymorphism (Akopyanz et al. 1992; Konieczny and Ausubel 1993). CAPS markers linked with rust resistance genes *Lr1*, *Lr47, Lr34/Yr18/Pm38, Sr35* and *Sr2* are currently available (Tyrka et al. 2004; Lagudah et al. 2006; Lagudah et al. 2009; Mago et al. 2011a).

2.7.4 Identification of marker-trait association for rust resistance

The fundamental objective for the analysis of marker-trait association is to gain information about the linkage disequilibrium (LD) of a population. Linkage disequilibrium is the occurrence of linked genes in non-random proportions in the individuals of the mapping population. However, the LD varies depending on the target loci and the species. Therefore, to understand the LD of a target loci, an adequate density of markers around the target loci is essential (Kraakman 2004). With the recent high throughput sequencing technologies, the availability of high density markers across genomes paved the way for rapid identification of marker-trait association. High density markers can be used for linkage analysis in two ways. Firstly, by genotyping the individuals or by bulk segregant analysis of a bi-parental mapping population and secondly, genotyping diverse individuals of diversity panels and carrying out association studies (Rode et al. 2012).

2.7.4.1 Bulk segregant analysis

Traditionally Bulk segregant analysis (BSA) involves identification of linked markers through screening of two contrasting DNA pools (such as rust resistant and rust susceptible phenotypes). DNA pools representing each of the contrasting phenotypes are generated by mixing randomly selected homozygous lines of a segregating population (Michelmore et al. 1991). The recently developed SNP chip array such as 90K SNP and the diversity array sequence technology (DArTseq) markers are routinely used for bulk segregant analysis. These mapped SNPs serve as a valuable resource to unveil the genetic architecture for various complex traits in wheat. Similarly, DArTseq was developed to substitute the array-based diversity array technology (DArT) for the development of high density genetic markers for any genomic regions that remain unknown. It uses genotyping by sequencing platform for discovering novel plant SNPs and performing genotyping studies. The major advantage of this technique is the simultaneous screening of several thousand different loci in a single cost-effective and reproducible assay. DArT markers linked with *Lr34/Yr18/Pm38, Lr46/Yr29/Pm39*, *Sr2, Sr6, Sr9h, Sr9g, Sr25* and *Yr7* resistance are available for marker assisted selection (Lillemo et al. 2008; Tsilo et al. 2010; Yu et al. 2010; Rouse et al. 2014).

2.7.4.2 Genome wide association studies

Genome wide association studies (GWAS) are an excellent alternative technique to the traditional bi-parental mapping. It applies to natural and diverse population structures with pre-existing recombination events accumulated over many generations. Therefore, it does not necessarily require a single gene line or a bi-parental segregating population to map the trait locus. However, it can be applied to a segregating population provided the entire population is genotyped. GWAS utilises the linkage disequilibrium (LD) for the marker-trait association. Therefore individuals of the genetically diverse population are tested with markers and using the LD between markers and trait, markers linked to the traits are easily identified with its map location determined using the available reference genome (Togninalli et al. 2018). Due to its self-pollinating nature, wheat exhibits a considerable high level of LD, thereby substantially reducing the number of markers required for marker-trait association studies (Breseghello and Sorrells 2006). However, false positive results due to population structure and limited power to detect the allele variants present in low frequencies can be potential disadvantages of GWAS (Zegeye et al. 2014). Despite limited drawbacks, GWAS has been widely used in mapping QTLs for several traits in various plant species. In wheat, various agronomic traits (Breseghello and Sorrells 2006; Reif et al. 2011; Dodig et al. 2012) and resistance to diseases such as Fusarium head blight, bacterial leaf streak and rusts have been successfully mapped by GWAS (Gurung et al. 2014; Yu et al. 2014; Zanke et al. 2015; Arruda et al. 2016). Quantitative trait loci for both seedling and adult plant resistance to leaf, stem and stripe rust have been reported in many studies (Ling et al. 2013; Zegeye et al. 2014; Jordan et al. 2015; Kertho et al. 2015; Maccaferri et al. 2015; Gao et al. 2016; Li et al. 2016a; Kankwatsa et al. 2017; Turner et al. 2017; Arora et al. 2019; Riaz et al. 2018).

2.7.5 Linkage map

Inheritance of a genomic region from one generation to the next can easily be tracked using polymorphic molecular markers. Association of such polymorphic markers and traits are calculated based on the recombination frequency between them that are shown in a schematic representation called a genetic map or linkage map. Therefore, a linkage map reveals the genetic distance between two markers or markers and a trait. Based on the genetic distance of molecular markers, they are

categorised into certain linkage groups. However, the recombination frequencies are not uniform throughout a chromosome, for example, heterochromatic regions like centromeres have reduced recombination frequency. Linkage maps for the rust resistance genes are constructed based on the phenotyping and genotyping data from segregating populations. The linkage between markers is usually calculated using the logarithm of odds (LOD) value (Risch 1992). The large segregating population can increase the density of markers thereby increasing the resolution of the genetic map. In recent years, the genotyping by sequencing platform (such as DArTseq) identified abundant SNPs and enriched useful SNP markers. In wheat, several consensus genetic maps were generated for all the chromosomes using different types of molecular markers such as SSR, 90K SNP, and DArT platforms (Somers et al. 2004; Akbari et al. 2006; Wen et al. 2017). Linkage maps can be constructed using computer programs such as Map-maker/EXP (Lander et al. 1987), JoinMap (Stam 1993), MapManager QTX (Manly et al. 2001), and MapDisto (Heffelfinger et al. 2017).

2.7.6 Physical map

Representation of molecular markers and genes in the chromosome at a fine scale level is called a physical map. The distance is measured in base pairs (bp) and the physical map serves as a platform for cloning genes through chromosome walking. Physical maps were traditionally generated by mapping BAC library clones or by screening markers from a reference genome. Currently, the genome sequence of many species is readily available thus the relative distance of each marker in the genetic map can be physically ordered.

2.7.6.1 Bacterial Artificial Chromosome (BAC) library

Hexaploid and tetraploid wheat genomes are homologous with their corresponding genomes in wild diploid relatives (Keller et al. 2005). The large genome size of hexaploid wheat, of which over 80% is repetitive sequence, makes it difficult to map and characterise genes. Gill et al. (1991) demonstrated the possibility of using diploid subspecies to characterise genes present in hexaploid wheat. This approach reduces the complexity of multiple genomes as sequence duplications are often found between homeologous chromosomes. Further, it became convenient with BAC library construction where the genomic fragments of a targeted species were cloned into bacteria using artificial plasmids. Advancement in cytogenetics and genomic analysis,

also facilitated generation of chromosome specific BAC libraries (Simkova et al. 2011; Luo et al. 2013). Physical maps and tightly linked markers for rust resistance genes *Lr1, Lr10, Lr21, Lr34/Yr18, Sr2, Sr33, Sr50* and *Yr36* were generated using BAC libraries of diploid, tetraploid and hexaploid wheats (Feuillet et al. 2003; Huang et al. 2003; Kota et al. 2006; Cloutier et al. 2007; Fu et al. 2009; Krattinger et al. 2009; Mago et al. 2011a; Periyannan et al. 2013; Mago et al. 2015).

2.7.6.2 Comparative genomics using reference genomes of wheat and its relatives

Breakthroughs in genome sequencing and assembly have enabled the generation of reference genomes for crops and their close relatives. Due to the complexity of the wheat genome, it took over a decade to generate the whole genome sequences of hexaploid wheat and its diploid progenitors. Until recently, mapping of disease resistance genes or other useful traits in wheat were assisted by comparative genomics where the synteny of the wheat genome with rice and *Brachypodium* were exploited. As rice, wheat, *Brachypodium* and several species of *Poaceae* family originate from a common ancestor, a high degree of co-linearity occurs among members of these grass family (Wolfe et al. 1989; Foote et al. 2004; Keller et al. 2005; The International Brachypodium Initiative 2010; Zhang et al. 2010a). Co-linearity between wheat and rice chromosomes was first observed based on RFLP patterns (Ahn et al. 1993; Kurata et al. 1994; Van Deynze et al. 1995). l Later characterisation of *Ph1* and *Lr34* loci revealed the conserved region among rice, wheat and *Brachypodium* (Griffiths et al. 2006; Bossolini et al. 2007). This relationship between the *Poaceae* was well characterised and that led to the development of tightly linked markers for the rust resistance genes *Lr10, Lr34/Yr18, Sr2, Sr31/Lr26/Yr9, Sr35* and *Yr36* (Feuillet et al. 2003; Mago et al. 2005; Bossolini et al. 2006; Kota et al. 2006; Lagudah et al. 2006; Fu et al. 2009; Zhang et al. 2010b). Due to the revolution of next generation sequencing technologies such as Illumina, PacBio etc., the whole genome sequences of bread wheat and the diploid progenitors such as *T. urartu (A genome), Ae. speltoides (B genome)* and *Ae. tauschii (D genome)* were made available (Brenchley et al. 2012; Ling et al. 2013; Luo et al. 2017; IWGSC 2018). Therefore, comparative genomics of wheat with its progenitors stands at the forefront of gene identification. However, increased understanding of the variation of gene presence or absence among different cultivars plays an important role in agronomic trait inheritance. Despite the availability of the reference genome sequence of the wheat

cultivar Chinese Spring, it is still uncertain how well it will address the differences in traits among cultivars. Moreover, a draft wheat pan-genome assembly of various elite wheat cultivars was developed recently to address the issue (Montenegro et al. 2017; http://www.10wheatgenomes.com/).

2.8 Rust resistance gene cloning

Cloning of rust resistance genes (*R* and *APR* genes) is essential for functional studies of rust resistance and to develop diagnostic markers for marker assisted breeding. It will also enable gene stacking via genetic transformation or targeted integration (Ayliffe et al. 2008). Several new methods of cloning and precise candidate gene identification are emerging due to technological advances.

2.8.1 Identification of candidate genes conferring rust resistance

Isolation of rust resistance genes through the generation of physical maps using bi-parental segregating populations is the routine approach. Through fine-mapping, a physical map of the resistance gene locus is generated by screening the BAC or Yeast Artificial Chromosome (YAC) library of the resistant parent or closely related lines. Overlapping contigs from the BAC clones mapped at the gene locus were predicted for a list of possible candidate genes. Subsequently, generation of a loss of function mutant population from the resistant parent enables screening of the candidate genes to predict the gene responsible for the resistance function. Candidate genes associated with all-stage resistance genes such as *Lr1, Lr10, Lr21, Sr13, Sr21, Sr33, Sr35, Sr46,* and *Yr10,* which encode NBS-LRR, and *Yr15,* a tandem kinase gene were identified using the map based approach (Feuillet et al. 2003; Huang et al. 2003; Cloutier et al. 2007; Periyannan et al. 2013; Saintenac et al. 2013; Liu et al. 2014; Zhang et al. 2017; Arora et al. 2019; Chen et al. 2018; Fahima et al. 2018; Keller et al. 2018; Periyannan 2018).

Since most of the all stage disease resistance genes cloned from wheat encode *CC-NBS-LRR* genes, recently a new cloning technique based on resistance gene enrichment and sequencing (RenSeq) was developed. In combination with the loss of function mutants, this technique enables rapid detection of resistance genes where the gene is identified through the comparison of *CC-NBS-LRR* gene sequences between the wild type and the mutants (Steuernagel et al. 2016; Periyannan 2018).

The resistance genes *Sr22,* and *Sr45* were identified using this approach, referred to as MutRenSeq (Steuernagel et al. 2016). A chromosome flow sorting method called targeted chromosome-based cloning via long-range assembly (TACCA) was also identified for the isolation of rust resistance genes from hexaploid wheat. This method involves flow sorting of the targeted chromosome, sequencing and assembling to generate a high-quality chromosome specific sequence for candidate gene prediction (Thind et al. 2017).

More recently a technique combining the association genetics with RenSeq (AgRenSeq) was developed for rapid resistance gene cloning (Arora et al. 2019) . An advantage of this new technique is it does not require bi-parental mapping or induced mutant populations. It utilises natural variations that exist among the germplasm collections and would also enable cloning of more than one resistance gene simultaneously (Arora et al. 2019). However, these new approaches are narrowed to one specific resistance gene family, the *CC-NBS-LRR*s. Furthermore, success depends on the presence of a related probe sequence in the bait library used for the capture and enrichment.

2.8.2 Validation of candidate resistance genes by a complementation test

To determine the function of candidate genes, it is essential to perform a complementation test either by developing transgenic plants harbouring the candidate gene or knocking out the target gene by silencing. So far, in the majority of cases in rust resistance gene cloning, the candidate genes were validated through a transgenic complementation test. Several methods to generate transgenic plants are available and on average yield up to 5% transformation efficiency (Richardson et al. 2014). However the protocol developed by Ishida et al. (2015) can reportedly generate over 50% success when the embryos of the wheat cultivar Fielder are used as explants. Moreover, it was shown to be efficient in various other cultivars of wheat in Australia where the transformation efficiencies ranged from 1.5 to 51% (Richardson et al. 2014). Rust resistance gene candidates isolated for *Sr22, Sr33, Sr45, Sr46, Sr50, Lr34, Lr67* were validated by this transformation method (Krattinger et al. 2009; Periyannan et al. 2013; Moore et al. 2015; Mago et al. 2015; Steuernagel et al. 2016; Arora et al. 2019). Secondly, functional analysis of candidate genes can also be evaluated by virus-induced gene silencing technology (VIGS). A fragment of plant DNA from the target

candidate gene is cloned into the virus vector to form a recombinant virus. When the host plant is infected with the recombinant virus, it activates the post-translational gene silencing by degrading the homologous plant RNA related to the cloned plant DNA fragment. Modified barley stripe mosaic virus (BSMV) has been used successfully as a system ot silence genes in hexaploid wheat. It has been tested effectively for *Sr33* and *Lr21* functional analysis (Periyannan et al. 2013; Lee et al. 2015)

2.9 Conclusion

Reduction in wheat genetic diversity due to domestication and intensive modern breeding render this staple crop vulnerable to disease. Therefore, a constant supply of new sources of resistance is essential to maintain genetic diversity for rust resistance. Wheat ancestors such as progenitors and landraces hold higher natural genetic diversity and resistance to diverse rust races. However, identification and introgression of these resistances from wild relatives are challenging due to the time consuming process of selection and inbreeding. Recent breakthrough technologies such as genotyping by sequencing, reference genome sequence of wheat and wild relatives, rapid resistance gene cloning methods and speed breeding accelerates the identification and introgression of the new resistance genes into the commercial cultivars through marker assisted selection and transgenic approaches.

Chapter 3 - Dissecting the genetics of triple rust resistance in *Aegilops tauschii* accession CPI110672

3.1 Abstract

Global wheat production is under threat due to the constant evolution of highly virulent forms of *Puccinia* spp. causing rust diseases. A primary concern over these new rust forms is their ability to overcome genetic resistance present in commercial wheat cultivars. Therefore, to sustain wheat cultivation and to prevent yield losses due to rust epidemics, a constant supply of unexplored resistance is required to replace defeated genes. Wild relatives of cultivated wheat are a valuable resource for such resistance; specifically the diploid progenitors that share genomes of cultivated wheat. In this study, accession CPI110672 of *Aegilops tauschii*, the D-genome progenitor of bread wheat, was deemed highly valuable because of its ability to resist to all three wheat rusts (leaf, stem and stripe). To determine whether resistance to all three forms of rust was due to a single locus or to multiple loci, we conducted a genetic analysis using a segregating F_2 mapping population derived from the cross between CPI110672 and a rust susceptible accession CPI110717. Through rust infection screening, we identified multiple genes conferring resistance to the three rusts. Furthermore, bulk segregant analysis using 90K Infinium SNP chip marker analysis revealed the chromosome position and molecular markers tightly linked to the rust resistance genes.

3.2 Introduction

Global wheat production is challenged by rapidly evolving and migrating rust pathogens. For instance, the highly aggressive wheat stem rust (*P. graminis* f. sp. *tritici* Erikss & Henn.; *Pgt*) race Uganda 99 (Ug99), emerged in East Africa with virulence to a majority of rust resistance genes present in common wheat. Furthermore, it evolved into 13 lineages and has spread to 13 countries (Pretorius et al. 2000; Babiker et al. 2015; Singh et al. 2015). Subsequently, the emergence of new stem rust (Digalu and Sicilian) races and highly virulent and temperature tolerant stripe rust races in major wheat growing regions such as the USA, Europe, Asia and Australia in recent years has become a serious potential limitation to global wheat production (Ali et al. 2014; Schwessinger 2017; Lewis et al. 2018).

To counteract pathogen attack, wheat like other plants has acquired genetic mechanisms to resist infection and colonisation by rust pathogens. Host-mediated genetic resistance, which is eco-friendly and sustainable, has been widely deployed as the best strategy to control rust and other economically important diseases. Over 200 rust resistance genes have been identified and many have been successfully deployed in wheat breeding for rust resistance (Murray and Brennan 2009a; McIntosh et al. 2017). However, they are not durable as they have been rendered ineffective because the pathogen has acquired virulence. For instance, cultivars carrying resistance genes *Sr24* (Jin et al. 2008)*, Sr31* (Pretorius et al. 2000), *Sr36* (Jin et al. 2009), *Sr38, Lr24, Lr37, Yr17* (Park 2008) and *Yr27* (Duveiller et al. 2007; Wellings 2007) were overcome by virulent races. This highlights the need to continuously explore germplasm collections, such as wild relatives and landraces for identification of novel resistance genes.

Among the wild relatives, progenitor species, such as *Aegilops tauschii* (DD), the D genome donor of bread wheat (AABBDD), has been identified as a valuable source of resistance to rust and other biotic and abiotic stresses (Feuillet et al. 2008; Borner et al. 2015). Due to its close relatedness with wheat, the D genome of *Ae. tauschii* recombines with the corresponding genome of hexaploid wheat. Thus, useful traits from this diploid species are readily integrated into cultivated bread wheat. These traits are transferred either through the direct crossing of *Ae. tauschii* with hexaploid wheat or through the generation of synthetic wheat (AABBDD) by crossing *Ae. tauschii* (DD) with the tetraploids (AABB). Using these approaches, several traits including rust resistance have been successfully introgressed (Gill and Raupp 1987; Olson et al. 2013b).

To mine new sources of rust resistance for Australian and global wheat breeding, a collection of 406 *Ae. tauschii* accessions (Periyannan et al. 2013) maintained at CSIRO, Canberra, were screened for stripe, stem and leaf rust resistance at The University of Sydney Plant Breeding Institute. An accession, identified as CPI110672, originally collected from Turkmenistan was deemed promising as it displayed resistance to all three rusts (stripe, stem and leaf) infecting wheat (Lagudah & McIntosh, unpublished data). Therefore, to understand the genetics behind triple rust resistance and to effectively integrate them in current wheat breeding

germplasm, we undertook to: a) identify the chromosome location for the triple rust resistance, and b) to generate a genetic map for the cloning of the targeted resistance genes.

3.3 Materials and Methods

3.3.1 Plant Material

Aegilops tauschii acc. CPI110672 is maintained at CSIRO, Canberra, as part of the National Small Grains Collection. An F_2 mapping population was derived by crossing CPI110672 with the triple rust (stem, leaf and stripe) susceptible *Ae. tauschii* acc. CPI110717.

3.3.2 Rust Phenotyping

Seedling resistance phenotyping was carried out for all three rusts in a controlled plant growth facility at the Plant Breeding Institute, Cobbitty, Australia as described in Bariana and Mcintosh (1993). Fifteen plants each for resistant and susceptible parental lines (CPI110672 and CPI110717) and 123 $F_{2:3}$ families were grown for three weeks and inoculated with leaf (26-1, 3 [PBI culture no 316]), stem (34-0 [48]) and stripe (104 E137A+ [372]) rust cultures for determining disease response. The Australian leaf, stem and stripe rust races that were used to screen the *Ae. tauschii* germplasm (Evans Lagudah unpublished data) were used to screen the mapping population. Nomenclature for the Australian rust races were classified at Plant Breeding Institute, The University of Sydney, Australia (McIntosh et al. 1995). Virulenece and avirulence profile for rust races used in this study are given in the supplementary file 1. Plants inoculated with leaf and stem rust were maintained at 25°C, while the stripe rust infected population was incubated at 10°C in the dark for 48 hours prior to 17°C, 16/8 hours of day/night regime for the rust development. Phenotyping was repeated twice for the whole mapping population against all three rusts. The $F_{2:3}$ lines failed to show rust infection in any of two experiments were repeated further to confirm the phenotype. Rust infection was scored on the first leaf at 12-14 days post infection (dpi) using the 0-4 Stakman scale (Stakman et al. 1962). Infection types 0 and ; were considered immune and 1 to 2+ were considered resistant to intermediate resistant, while scores of 3 to 4 were considered susceptible. Progeny of the F_2 mapping population had an IT of ; for resistant (R) and 3 for susceptible (S)

responses. A chi-square (χ^2) test was performed to determine the segregation pattern of rust phenotypes in the F_2 population.

3.3.3 Bulk Segregant Analysis (BSA)

Genomic DNA was isolated from the 123 F_2 plants, as described by Lagudah et al. (1991). Bulk segregant analysis (BSA) was performed to identify molecular markers linked to the three rust resistance traits, as described in Michelmore et al. (1991). Two DNA bulks, one for rust resistant lines and the other for the susceptible phenotype for each rust type, were made using genomic DNA from 10 resistant and 10 susceptible F_2 lines. DNA of the two bulks and the two parental lines were subjected to the 90K Infinium Single Nucleotide Polymorphism (SNP) chip (Wheat90k_ConsAkhunovKSU_15033654_A) platform at the AgriBio Centre, LaTrobe University, Victoria (Wang et al. 2014). The SNP markers linked to each trait from the 90K SNP chip were identified by service providers using NormTheta (x-axis coordinate) and NormR (y-axis coordinate) values from the SNP cluster plots. The sequences of the putative trait linked to the SNP markers were mapped using BLASTN tool against the reference genome sequence of Chinese Spring to identify the chromosome location (IWGSC 2013).

3.3.4 Identification of markers flanking the resistance locus

The SNP markers linked with resistance were converted to Kompetitive Allele Specific PCR (KASP) markers for further genotyping of the individual lines of the mapping population (Table 3.1). The KASP markers were designed using FAM (GAAGGTGACCAAGTTCATGCT) or HEX (GAAGGTCGGAGTCAACGGATT) tags as described in He et al. (2014). The KASP reaction was performed in an 8 µl reaction mixture consisting of 25 ng of genomic DNA, 0.11 µl of assay mix (consisting of an equal volume of 100 µM allele specific forward primers and double the volume of 100 µM common reverse primer) and 4 µl of the KASP master mix. The polymerase chain reaction (PCR) was carried out in the BioRad CFX96 real-time PCR system using the following program: initial denaturation at 94°C for 15 minutes, then denaturation at 94°C for 20 seconds followed by annealing at 65°C for 1 minute. The program was repeated nine times with decrease in annealing temperature of 0.8°C per cycle followed by denaturation at 94°C for 20 seconds, annealing at 57°C for 1 minute and

incubation at 25°C for 5 minutes for an additional 35 cycles. The KASP products were read using the inbuilt reader in the BioRad CFX96 real-time PCR machine. Resistant and susceptible alleles were discriminated using the BioRad CFX 3.1 manager software.

In addition to KASP markers, microsatellite markers that mapped to the predicted chromosome region harbouring rust resistance were also used for genotyping (Singh et al. 2004; Somers et al. 2004; Sourdile 2009). Amplification of microsatellite markers was performed in a 20 µl PCR reaction containing 100-200 ng of gDNA, 1X GoTaq Flexi green buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 200 nM of both forward and reverse primers and 1U of *Taq* polymerase (M829B, Promega, USA). PCR reaction was carried out in a BioRad thermal cycler using a touch down program as follows: denaturation at 94°C for 30 seconds; annealing at 65°C for 30 seconds, decreasing by 1°C per cycle; extension at 72°C for 1 minute followed by repeating these steps for 14 cycles; after enrichment, the program continued for 29 cycles to follow: 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 40 seconds. All markers developed in this study were serially named using the term *Csq* (CSIRO and QAAFI, the University of Queensland). The scaffolds corresponding to 4DS of Chinese Spring v0.4 were evaluated to identify additional markers flanking the stripe rust locus (IWGSC; https://urgi.versailles.inra.fr/blast_iwgsc/blast.php).

3.3.5 Construction of a linkage map

Genetic linkage maps were constructed using MapDisto 2.0 Beta 86 (Heffelfinger et al. 2017) with default functional settings, including logarithm of odds (LOD) 3.0, recombination frequency threshold (r max) of 0.30 and map exportation using R/qtl. The phenotypic rust data from the $F_{2:3}$ mapping families of CPI110672 X CPI110717 were used along with the SNP marker scores to construct the linkage map. The linkage maps were constructed using the Kosambi mapping function of MapDisto (Kosambi 1943; Heffelfinger et al. 2017).

3.4 Results

3.4.1 Stem, leaf and stripe rust resistance in *Ae. tauschii* CPI110672

Accession CPI110672 was resistant to all three rusts, displaying infection type (IT) 0;- for leaf, ;1 for stem and ; for stripe rust resistance, while a susceptible response of 3C for leaf and stem rust, and 3+ against stripe rust was observed for CPI110717. Progeny of the F_2 mapping population segregated in a 3:1 ratio (R90: S33, χ^2=0.220, *P*=0.639 against leaf rust and R101: S22, χ^2= 3.320, *P*=0.068 against stripe rust) indicating monogenic inheritance. We temporarily designated the genes *Yr672* and *Lr672*. For stem rust, the population segregated for two genes, as three distinct resistant infection types (belonging to R1, R2, R1+R2) were identified. One group (R1+R2) had IT ;1 which was similar to the resistant parent CPI110672. The other two groups, R1 and R2 had intermediate ITs of 2 and 2+C, which indicated the segregation of two independent *R* genes, temporarily designated *Sr672a* and *Sr672b* (Figure 3.1; Table 3.1). The stem rust phenotypic scores are putatively separated for both loci to conduct linkage analysis (Supplementary file 2).

3.4.2 Identification of trait linked SNP markers and its chromosome location

From the BSA analysis we identified 54 putatively leaf rust resistance trait-linked SNP markers. Mapping the SNP markers on the flow sorted wheat chromosome (FSWC) contigs of Chinese Spring survey sequence mapped 43 of the 54 SNPs to chromosome group 2 (Chr2). Of the 43, 22 SNP ids were specifically mapped to 2DS, which strongly suggests that leaf rust resistance was on the short arm of chromosome 2D of CPI110672 (Table 3.2). Since the mapping population was segregating for two stem rust resistance genes, three independent DNA pools consisting of resistance phenotype R1, phenotype R1+R2, and phenotype R2 was carried out for BSA. The SNPs associated with the R1 bulk showed strong linkage with group 2 (Chr2) and weak linkage with group 5 (Chr5) chromosomes, while SNPs for R1+R2 showed weak linkage to chromosomes 2, 5 and 7 when the R2 group alone had weak to moderate linkage to chromosome groups 2 and 7. The association of most of the SNPs with the group 7 chromosomes were flip-phased (FP), where the resistant bulk was similar to the susceptible parent, and the susceptible bulk was similar to the resistant parent (Table 3.3). Therefore, chromosome 7 specific SNPs are unlikely linked to both stem

rust loci. Since strong linkage of SNPs from Chr2 to the phenotype R1 indicates that Chr2 may hold the R1 locus, while R2 may be located in Chr5. For the bulked DNA corresponding to stripe rust resistance, there were 13 putative trait linked SNPs identified for stripe rust resistance. Further analysis using the Chinese Spring survey sequence mapped 11 of these SNP ids to chromosomes group 4 (Chr4) of which five were specific to the short arm of the 4D chromosome (Table 3.4).

Further conversion of triple rust linked SNPs corresponding to group Chr2D, 4D and 5D as KASP genotyping markers identified four markers (SNP marker Ids IWB64398, IWB18330, IWB25534, and IWB15863; renamed *Csq4, Csq5, Csq6,* and *Csq7*) on Chr2DS, one marker (IWB4781 renamed *Csq1*) on 4DS, and five markers (SNP ids IWB18365, IWB15609, IWB18733, IWB19445, IWB49837) from 5DL as polymorphic between parents with co-dominant allelic patterns (Table 3.5; Supplementary file 3). The markers were then used to genotype the entire mapping population. Additionally, microsatellite markers from 2DS (*Xgdm35*) and 4DS (*Xwmc720, Xwmc52, Xgpw4087* and *Xgpw5072*) chromosome arms which had distinct amplification patterns for the parental lines were also genotyped (Supplementary file 2). One of the SSR markers, *Xwmc285* (4DS), did not have a polymorphic pattern among parents, and was subsequently expelled from the genetic analysis. The 4DS scaffolds 19341 and 72468 of Chinese Spring v0.4 identified two markers *Csq2* and *Csq3* polymorphic between the parents (Table 3.5; Supplementary file 3).

3.4.3 Linkage analysis and flanking marker identification

Preliminary genetic maps were constructed for leaf, stem and stripe rust resistance loci using the data from rust infection and marker (SNP and microsatellite) screenings. Three linkage groups were identified where the stripe rust resistance locus *Yr672* in the 4DS chromosome arm was flanked by markers *Csq1* distally at 12.8 cM and *Csq3* proximally at 0.4 cM distance. All the polymorphic 4DS specific SSR markers were also mapped to the same linkage group, however, far from the stripe rust locus (Figure 3.2). In the second linkage group, leaf rust and stem rust (*Sr672a)* resistance locus were mapped in the same group. The SNP markers *Csq4* and *Csq5* were found flanking the *Lr672* gene locus at 0.8 cM (distally) and 11.5 cM (proximally), on the short arm of 2D (Figure 3.2). Similarly, the stem rust resistance gene *Sr672a* was also

mapped to the same chromosome arm (2DS), where markers *Csq6* and *Csq7* were found flanking the gene at 4.8 cM (distal) and 6.5 cM (proximal). The markers specific to chromosome 5DL formed a separate linkage group, however, it did not include the second stem rust resistance locus and therefore *Sr672b* remains unmapped.

3.5 Discussion

Accessions of the D-genome diploid grass *Ae. tauschii* are a valuable reservoir for numerous key agronomic traits including resistance to the rapidly evolving wheat rust diseases. For instance, accession CPI110672 from Turkmenistan was identified as a potential source for resistance to leaf, stem and stripe rust races infecting wheat. Furthermore, CPI110672 had strong resistance against a range of Australian stripe rust races belonging to both the pre-2002 and WA lineages. As these two lineages predominated in Australia since the first incursion of the pre-2002 stripe rust races in 1979, this new resistance is a valuable resource for breeding stripe rust resistant wheat cultivars. Stripe rust is a global threat to wheat cultivation worldwide as stripe rust races with adaptation to warm temperature, virulent on many *R* genes were frequent in major wheat growing regions of Europe, America, and Southeast Asia (Park 2016c; Schwessinger 2017). Hence, the stripe rust resistance identified in this study is valuable beyond Australia if it is effective against worldwide stripe rust races. This is possible as the two lineages prevalent in Australia were exotic introductions from Europe. However, prior testing of this gene against worldwide stripe rust races is required as recently evolved races were also diverse on a local scale (Schwessinger 2017). CPI110672 was also resistant against diverse races of wheat stem rust. As in addition to its resistance against Australian stem rust races, Rouse et al. (2011) identified TA1675 (accession CPI110672 in the US *Ae. tauschii* collection, https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1556592) as a potential source of resistance against globally important races such as TTKSK (Ug99), TRTTF, TTTTF, QTHJC, RKQQC and TPMKC, which are maintained at the Cereal Disease Laboratory, USA. Along with resistance to stripe and stem rust, *Ae. tauschii* CPI110672 was also resistant to wheat leaf rust races in Australia. Due to widespread epidemics and the prevalence leaf rust races in all wheat growing regions in Australia, the *Lr672* can be a useful resource for the breeding programs.

Given the resistance of CPI110672 to multiple *Puccinia* spp. (causes of leaf, stem and stripe rusts), we were interested to map the resistance as there are few known seedling resistance genes other than the *Yr17/Sr38/Lr37* loci that recognise all three wheat rust pathogens (Bariana and McIntosh 1993). Though the recently cloned pleiotropic rust resistance genes such as *Lr34* and *Lr67* possess multiple defence mechanisms, nonetheless they are functional only when the wheat plant reaches the adult plant stage (Krattinger et al. 2009; Moore et al. 2015). However, in the genetic analysis of the F_2 population derived from the cross between CPI110672 and CPI110717, the three rust resistance genes segregated independent of each other, which was also confirmed later through the bulk segregant analysis using the 90K SNP platform and the screening of chromosome specific molecular markers.

In our study, the stripe rust resistance gene *Yr672* mapped to the short arm of chromosome 4D, where *YrAS2388* / *Yr28* were positioned earlier (Singh et al. 2000; Huang et al. 2011; Liu et al. 2013). Hence it is possible that *Yr672* could be a related or an allelic form of either *YrAS2388* or *Yr28*. *YrAS2388* and *Yr28* are not allelic to each other as they had a different virulence pattern to a stripe rust race SY11-4 in China (Huang et al. 2011). It is also most likely *Yr672* is not allelic to *Yr28* as there was widespread virulence for *Yr28* in both Australia and other wheat growing regions (Sharma-Poudyal et al. 2013). While *Yr672* is resistant to most of the stripe rust forms in Australia, its effect against other stripe rust strains is yet to be tested. However, the allelic test of AS2388 and CPI110672 is essential to identify whether they are the same or different. Cloning of *Yr672* could clarify the allelic relationship among *Yr672, Yr28* and *YrAS2388.* The majority of the previous seedling rust resistance genes identified, such as *Lr1* (Qiu et al. 2007), *Lr10* (Feuillet et al. 2003; Loutre et al. 2009), *Lr21* (Huang et al. 2003), *Sr13* (Zhang et al. 2017), *Sr21* (Chen et al. 2018), *Sr22, Sr45* (Steuernagel et al. 2016), *Sr33* (Periyannan et al. 2013), *Sr46* (Arora et al. 2019), *Sr50* (Mago et al. 2015), *Yr5, Yr7* (Marchal et al. 2018), and *Yr10* (Liu et al. 2014) in wheat belong to the CC-NS-LRR (*CNL)* family. Therefore, it is highly possible that CPI110672 sequences related to either a single *CNL* gene or two or more *CNL* genes together may be responsible for *Yr672* resistance. Identification of *Yr672* will also facilitate the isolation of *YrAS2388* / *Yr28* genes and will provide insights into the evolutionary relationship of closely related rust resistance genes in wheat.

Similar to *Yr672,* we also mapped the leaf rust resistance gene (*Lr672*) of CPI110672 and positioned the gene to the distal region of 2DS. Since *Lr672* is mapped to the 2DS chromosome, verifying its allelic relationship with previously mapped genes such as *Lr22a, Lr39, Lr41* and *LrT* is essential for use in Australian wheat breeding programs. Based on earlier reports, *Lr22a* is an adult plant resistance gene, while *Lr39* and *Lr41* are allelic and linked closely to the SSR marker *Xgdm35* (Singh et al. 2004; Thind et al. 2017). Through 90K SNP analysis, we identified tightly linked markers that are much closer to the gene than marker *Xgdm35* reported earlier (Figure 3.2). Based on the marker position it is also possible that *Lr672* could be allelic or closely related to the recently mapped gene *LrT* that confers broad spectrum resistance against the majority of leaf rust races in India (Saluja et al. 2018). While the stripe and leaf rust resistance traits appeared to be due to single gene inheritance, the stem rust resistance present in CPI110672 was conferred by two genes, which appeared to act as additive resistance genes. In the marker analysis, one of the stem rust resistance genes *(Sr672a)* mapped to the same position as *Sr46* (Yu et al. 2015), while the position of the second locus was unclear due to the few single gene lines used for the initial marker screening. Therefore, *Sr672a* could be an allele of *Sr46* or a new gene located close to the *Sr46* locus. Stem rust resistance gene *Sr46* confers an intermediate resistance (Yu et al. 2015), and in our study the *Sr672a* phenotype was intermediate and in line with *Sr46,* while *Sr672b* confers weak resistance. Despite the weak resistance of *Sr672b*, the presence of both *Sr672a* and *Sr672b* had an additive response. Therefore, identification and characterisation of both stem rust resistance genes are essential to understand the additive mechanism for durable rust resistance. Furthermore, one or both may turn out to be a potential member to stack with another stem rust resistance pair for enhanced resistance in the event of new virulent races. Generating a mapping population segregating only for *Sr672b* is essential to fine map and characterise this locus.

In summary, the stripe rust resistance gene mapped to Chr4DS and was closely linked to the markers *Csq1* and *Csq3* while the leaf rust and stem rust loci *(Sr672a)* mapped to the short arm of chromosome 2D. Leaf rust was flanked by *Csq4* and *Csq5* whereas stem rust resistance was flanked by *Csq6* and *Csq7* SNP markers. This genetic analysis will greatly assist in understanding the candidate gene for triple rust resistance in CPI110672. Follow up studies on the cloning of these genes will facilitate

identification of gene-specific markers and enable mining of novel alleles to enrich the rust resistance gene repository for rapid breeding of rust resistant wheat cultivars.

Table 3.1 Screening of mapping population against stem rust race 34-0 [48]

CPI110672 x CPI110717 mapping population	Phenotype						
	R1	R1+S	R2	R2+S	R1+R2	R+R2+S	S
No of F_2 lines	32	14	7	23	17	21	9

Table 3.2 List of SNP marker IDs associated with the leaf rust resistant bulk

S. No	SNP ID*	SNP	Normalised Theta					Difference B1-B2	Significant Difference	BlastN against FSWC contigs
			Parent-R	BulkP1	R/S Mix	BulkP2	Parent-S			
1	IWB24809	T/C	0.98	0.99	0.98	0.94	0.37	.	.	2AS_5203038
2	IWB32192	T/G	0.98	0.99	0.98	0.91	0.68	.	.	2AS_5212673
3	IWB60803	A/G	0.37	0.40	0.52	0.79	0.85	0.3906	Yes	2AS_5212673
4	IWB24139	A/G	0.90	0.90	0.88	0.63	0.67	0.2638	Yes	2AS_5282792
5	IWB22939	A/G	0.98	0.98	0.98	0.95	0.71	.	.	2BS_3198868
6	IWB39620	T/C	0.98	0.93	0.84	0.75	0.74	0.1795	Yes	2BS_5191334
7	IWB992	A/G	0.98	0.98	0.57	0.06	0.05	0.9231	Yes	2DS_2984967
8	IWB18330	T/C	0.99	0.85	0.51	0.15	0.02	0.6972	Yes	2DS_4110827
9	IWB10239	T/C	0.58	0.54	0.40	0.04	0.03	0.5001	Yes	2DS_5296568
10	IWB25534	A/G	0.01	0.09	0.30	0.66	0.73	0.5681	Yes	2DS_5327373
11	IWB16414	T/C	0.41	0.54	0.78	0.96	0.99	0.4134	Yes	2DS_5354408
12	IWB64398	A/G	0.06	0.04	0.54	0.98	0.98	0.9413	Yes	2DS_5356170
13	IWB41562	T/G	0.59	0.46	0.34	0.01	0.01	0.4533	Yes	2DS_5367886
14	IWB56340	A/G	0.40	0.41	0.32	0.19	0.14	0.2161	Yes	2DS_5388700
15	IWB36873	A/G	0.98	0.88	0.72	0.67	0.63	0.2111	Yes	2DS_5389115
16	IWB10033	T/C	0.54	0.43	0.25	0.22	0.22	0.2132	Yes	2DS_5389158
17	IWB46392	T/C	0.96	0.97	0.98	0.98	0.84	.	.	2DS_603055

18	IWB29379	T/C	0.68	0.70	0.50	0.36	0.35	0.3457	Yes	3B_10761320
19	IWB9843	T/G	0.68	0.67	0.62	0.51	0.50	0.1510	Yes	3B_10763714
20	IWB497	A/G	0.73	0.79	0.88	0.97	0.99	0.1874	Yes	4DS_2324374
21	IWB50063	T/C	0.74	0.74	0.67	0.53	0.51	0.2114	Yes	6AS_1033593
22	IWB57969	T/C	0.75	0.76	0.64	0.59	0.57	0.1724	Yes	7DS_2495396
23	IWA1562	A/G	0.99	0.99	0.99	0.89	0.55	.	.	2AS_5265661
24	IWB11273	A/G	0.91	0.98	0.96	0.96	0.95	.	.	2BS_5168374
25	IWB16802	A/G	0.47	0.43	0.28	0.09	0.03	0.3398	Yes	2DS_5389168
26	IWB23116	T/C	0.03	0.03	0.03	0.07	0.78	.	.	2AS_5203038
27	IWB5374	A/G	0.04	0.03	0.08	0.26	0.34	0.2354	Yes	2BS_5174443
28	IWB41604	T/C	0.02	0.03	0.06	0.41	0.48	0.3822	Yes	2BS_5224109
29	IWB29559	A/C	0.99	0.99	0.99	0.97	0.40	.	.	2BS_3928128
30	IWB31559	A/G	0.01	0.01	0.01	0.02	0.91	.	.	2BS_3928128
31	IWB65489	A/C	0.99	0.99	0.98	0.97	0.76	.	.	2AS_5303427
32	IWB65490	A/G	0.96	0.97	0.96	0.96	0.49	.	.	2BS_3928128
33	IWB49446	A/G	0.53	0.50	0.34	0.10	0.03	0.3937	Yes	2DS_5369824
34	IWB9754	A/G	0.68	0.73	0.83	0.99	0.98	0.2580	Yes	2BS_5177680
35	IWB15227	A/G	0.97	0.98	0.51	0.06	0.06	0.9206	Yes	2DS_5328231
36	IWB58948	A/G	0.02	0.03	0.02	0.60	0.74	0.5756	Yes	2DS_5336208
37	IWB56957	A/C	0.03	0.23	0.59	0.92	0.99	0.6943	Yes	2DS_1373910
38	IWB8539	T/G	0.67	0.71	0.62	0.60	0.55	.	.	2BS_5157588
39	IWB50940	A/G	0.96	0.92	0.66	0.46	0.51	0.4575	Yes	2BS_5245086

40	IWB49760	A/G	0.98	0.99	0.98	0.94	0.72	.	.	2AS_5203038
41	IWB73347	A/G	1.00	0.99	0.98	0.99	0.77	.	.	2BS_5157821
42	IWB44405	A/G	0.01	0.01	0.01	0.06	0.57	.	.	2BS_3928128
43	IWB34642	T/C	0.99	0.99	0.99	0.93	0.80	.	.	2DS_5353061
44	IWB40238	T/G	0.46	0.46	0.52	0.61	0.63	0.1493	Yes	2DS_5316996
45	IWB65506	T/C	0.52	0.53	0.41	0.28	0.18	0.2552	Yes	4BS_4887637
46	IWB15863	A/C	0.91	0.85	0.63	0.25	0.07	0.5922	Yes	2DS_3544596
47	IWB17148	T/C	0.71	0.64	0.34	0.09	0.02	0.5511	Yes	2DS_5381838
48	IWB47710	T/G	0.48	0.57	0.69	0.90	0.98	0.3304	Yes	6BS_3000178
49	IWB11364	T/C	0.37	0.44	0.23	0.09	0.08	0.3509	Yes	2BS_2292931
50	IWB56203	T/C	0.31	0.32	0.18	0.01	0.00	0.3186	Yes	3AL_4335741
51	IWB71041	A/G	1.00	0.99	0.99	0.98	0.71	.	.	5AS_1546940
52	IWB4063	T/C	0.30	0.25	0.44	0.63	0.82	0.3751	Yes	5DL_4606156
53	IWB50369	T/C	0.99	0.87	0.67	0.58	0.55	0.2882	Yes	1DL_2263654
54	IWB51034	T/C	0.98	0.98	0.97	0.79	0.43	0.1887	Yes	2DS_5346789

* Probes for the SNP ids that are converted into KASP markers are given in the supplementary file 3

Table 3.3 List of SNP marker IDs associated with the stem rust resistant bulk

S.No	SNP ID*	SNP	Normalised Theta					Difference B1-B2	Significant Difference	BlastN against FSWC contigs
			Parent-R	BulkP1	R/S Mix	BulkP2	Parent-S			
1	IWB74476	T/C	0.48	0.03	0.03	0.04	0.02	.	.	2AL_6410547
2	IWB334	T/C	0.05	0.17	0.44	0.57	0.60	0.3979	Yes	2AL_6419321
3	IWB45421	A/G	0.04	0.87	0.58	0.32	0.97	0.5491	Yes	2AL_6421780
4	IWB59787	T/C	0.62	0.58	0.40	0.31	0.26	0.2665	Yes	2AL_6434667
5	IWB71923	A/C	0.97	0.97	0.97	0.74	0.76	0.2335	Yes	2AS_5188336
6	IWB23116	T/C	0.03	0.03	0.03	0.36	0.78	0.3328	Yes	2AS_5203038
7	IWB24809	T/C	0.98	0.98	0.98	0.31	0.37	0.6658	Yes	2AS_5203038
8	IWB49760	A/G	0.98	0.98	0.98	0.76	0.72	0.2176	Yes	2AS_5203038
9	IWB32192	T/G	0.98	0.99	0.99	0.76	0.68	0.2307	Yes	2AS_5212673
10	IWB60803	A/G	0.37	0.42	0.53	0.83	0.85	0.4088	Yes	2AS_5212673
11	IWA1562	A/G	0.99	0.99	0.99	0.66	0.55	0.3280	Yes	2AS_5265661
12	IWB65489	A/C	0.99	1.00	0.98	0.78	0.76	0.2134	Yes	2AS_5303427
13	IWB73343	A/G	0.42	0.22	0.29	0.37	0.18	0.1533	Yes	2BL_8091863
14	IWB22939	A/G	0.98	0.99	0.98	0.90	0.71	.	.	2BS_3198868
15	IWB29559	A/C	0.99	1.00	1.00	0.73	0.40	0.2612	Yes	2BS_3928128
16	IWB31559	A/G	0.01	0.01	0.01	0.73	0.91	0.7238	Yes	2BS_3928128
17	IWB44405	A/G	0.01	0.01	0.00	0.32	0.57	0.3096	Yes	2BS_3928128

18	IWB65490	A/G	0.96	0.97	0.98	0.63	0.49	0.3385	Yes	2BS_3928128
19	IWB69712	T/C	0.75	0.96	0.99	0.99	0.98	.	.	2BS_5156201
20	IWB52990	T/C	0.23	0.26	0.19	0.07	0.03	0.1877	Yes	2BS_5157245
21	IWB8539	T/G	0.67	0.66	0.62	0.59	0.55	.	.	2BS_5157588
22	IWB73347	A/G	1.00	0.98	0.98	0.79	0.77	0.1882	Yes	2BS_5157821
23	IWB5374	A/G	0.04	0.08	0.07	0.39	0.34	0.3151	Yes	2BS_5174443
24	IWB39620	T/C	0.98	0.92	0.82	0.72	0.74	0.2090	Yes	2BS_5191334
25	IWB47952	T/C	0.36	0.80	0.54	0.43	0.95	0.3786	Yes	2DL_9827916
26	IWB18068	T/C	0.98	0.28	0.70	0.87	0.03	0.5877	Yes	2DL_9893879
27	IWB17689	T/C	0.97	0.19	0.51	0.77	0.00	0.5767	Yes	2DL_9909801
28	IWB15863	A/C	0.91	0.74	0.59	0.07	0.07	0.6749	Yes	2DS_3544596
29	IWB40238	T/G	0.46	0.46	0.52	0.64	0.63	0.1777	Yes	2DS_5316996
30	IWB25534	A/G	0.01	0.13	0.31	0.69	0.73	0.5560	Yes	2DS_5327373
31	IWB58948	A/G	0.02	0.03	0.02	0.04	0.74	.	.	2DS_5336208
32	IWB16414	T/C	0.41	0.60	0.76	0.96	0.99	0.3597	Yes	2DS_5354408
33	IWB49446	A/G	0.53	0.45	0.35	0.04	0.03	0.4148	Yes	2DS_5369824
34	IWB16988	A/G	0.24	0.32	0.45	0.97	0.97	0.6556	Yes	2DS_5380847
35	IWB17148	T/C	0.71	0.44	0.29	0.02	0.02	0.4167	Yes	2DS_5381838
36	IWB56340	A/G	0.40	0.37	0.32	0.12	0.14	0.2548	Yes	2DS_5388700
37	IWB16802	A/G	0.47	0.34	0.23	0.02	0.03	0.3179	Yes	2DS_5389168
38	IWB46392	T/C	0.96	0.98	0.96	0.78	0.84	0.2016	Yes	2DS_603055
39	IWB48061	T/C	0.01	0.09	0.21	0.36	0.49	0.2776	Yes	5AL_1044410

40	IWB68338	A/G	0.89	0.90	0.87	0.77	0.70	0.1320	Yes	5AL_2678834
41	IWB28556	A/G	0.13	0.18	0.27	0.41	0.48	0.2267	Yes	5AL_2691246
42	IWA2199	A/G	0.58	0.57	0.62	0.74	0.74	0.1676	Yes	5AL_2770640
43	IWB71040	A/G	0.99	0.99	0.99	0.75	0.60	0.2345	Yes	5AS_1546940
44	IWB71041	A/G	1.00	0.99	0.99	0.70	0.71	0.2921	Yes	5AS_1546940
45	IWB73925	T/C	0.24	0.27	0.70	0.82	0.81	0.5493	Yes	5BL_10851786
46	IWB8582	A/G	0.96	0.92	0.84	0.77	0.75	0.1520	Yes	5BL_10859217
47	IWB22610	T/G	0.47	0.26	0.33	0.43	0.19	0.1722	Yes	5BL_10861508
48	IWA3394	A/G	0.56	0.56	0.69	0.84	0.98	0.2807	Yes	5BL_10874005
49	IWB59195	A/G	0.91	0.80	0.59	0.46	0.44	0.3346	Yes	5BL_10903003
50	IWB44512	T/C	0.79	0.95	0.97	0.98	0.98	.	.	5BL_10903355
51	IWB44078	A/G	0.65	0.69	0.79	0.90	0.99	0.2178	Yes	5BL_10919329
52	IWB47744	T/C	0.05	0.24	0.62	0.93	0.99	0.6913	Yes	5BL_10924382
53	IWB45749	A/G	0.96	0.87	0.75	0.59	0.55	0.2720	Yes	5BL_10925653
54	IWB44163	T/C	0.14	0.38	0.29	0.22	0.40	0.1611	Yes	5BS_2288166
55	IWB31883	T/C	1.00	0.99	0.98	0.97	0.66	.	.	5DL_1794748
56	IWB18910	A/G	0.02	0.17	0.47	0.86	0.99	0.6829	Yes	5DL_2948982
57	IWB65355	A/G	0.92	0.84	0.67	0.51	0.50	0.3312	Yes	5DL_3414494
58	IWB22335	T/G	0.67	0.67	0.52	0.24	0.03	0.4332	Yes	5DL_4472075
59	IWB57785	T/C	0.94	0.85	0.67	0.46	0.32	0.3878	Yes	5DL_4493857
60	IWB3201	T/C	0.52	0.54	0.58	0.63	0.77	.	.	5DL_4497827
61	IWB26663	A/G	0.79	0.76	0.84	0.93	0.98	0.1670	Yes	5DL_4497827

62	IWB18365	T/C	0.99	0.82	0.48	0.12	0.01	0.7028	Yes	5DL_4498179
63	IWB49837	A/C	0.02	0.27	0.49	0.80	0.97	0.5271	Yes	5DL_4500667
64	IWB19449	T/C	0.97	0.90	0.78	0.60	0.49	0.3075	Yes	5DL_4516288
65	IWB3297	A/G	0.98	0.87	0.70	0.53	0.45	0.3431	Yes	5DL_4524187
66	IWB47729	T/C	0.33	0.10	0.05	0.02	0.03	.	.	5DL_4529699
67	IWB24821	A/C	0.02	0.02	0.02	0.17	0.60	0.1430	Yes	5DL_4532552
68	IWB17513	T/C	0.67	0.06	0.03	0.03	0.02	.	.	5DL_4532636
69	IWB17539	A/G	0.99	0.83	0.61	0.39	0.33	0.4432	Yes	5DL_4533396
70	IWB26748	A/G	0.73	0.97	0.99	0.98	0.97	.	.	5DL_4538822
71	IWB15609	T/G	0.99	0.87	0.64	0.26	0.03	0.6044	Yes	5DL_4550688
72	IWB15768	A/G	1.00	0.91	0.75	0.59	0.48	0.3189	Yes	5DL_4552571
73	IWB16645	A/G	0.99	0.93	0.84	0.76	0.72	0.1742	Yes	5DL_4552571
74	IWB62442	A/G	0.63	0.65	0.82	0.92	0.98	0.2714	Yes	5DL_4557702
75	IWB23819	A/C	0.45	0.52	0.61	0.85	0.99	0.3287	Yes	5DL_4558558
76	IWB30440	T/C	0.55	0.58	0.68	0.86	0.97	0.2831	Yes	5DL_4558558
77	IWB57795	T/C	0.25	0.25	0.21	0.10	0.02	0.1534	Yes	5DL_4558558
78	IWB10111	T/C	0.97	0.99	0.98	0.94	0.68	.	.	5DL_4560046
79	IWB39544	A/G	0.96	0.96	0.96	0.95	0.60	.	.	5DL_4560046
80	IWB51546	T/C	0.02	0.09	0.18	0.23	0.20	0.1354	Yes	5DL_4560046
81	IWB63147	A/C	0.98	0.87	0.57	0.12	0.03	0.7558	Yes	5DL_4560695
82	IWB12614	T/C	0.56	0.94	0.98	0.99	0.99	.	.	5DL_4563397
83	IWB17208	A/C	0.01	0.01	0.02	0.03	0.70	.	.	5DL_4580722

84	IWB61247	T/C	0.51	0.56	0.70	0.91	0.98	0.3588	Yes	5DL_4586582
85	IWB18733	A/G	0.99	0.87	0.62	0.16	0.00	0.7041	Yes	5DL_4587649
86	IWB6189	A/G	0.02	0.03	0.03	0.05	0.49	.	.	5DL_4595698
87	IWB29359	A/G	0.28	0.40	0.60	0.86	0.98	0.4581	Yes	5DL_4597791
88	IWB19445	A/G	1.00	0.86	0.56	0.13	0.01	0.7344	Yes	5DL_4598647
89	IWB4550	T/G	0.98	0.91	0.74	0.50	0.37	0.4187	Yes	5DL_4599776
90	IWB18530	T/C	0.98	0.79	0.51	0.34	0.29	0.4464	Yes	5DL_4603774
91	IWB14707	T/C	0.44	0.11	0.08	0.10	0.10	.	.	5DL_4604070
92	IWB18666	T/G	0.73	0.68	0.52	0.19	0.08	0.4912	Yes	5DL_4605760
93	IWB4063	T/C	0.30	0.33	0.40	0.57	0.82	0.2487	Yes	5DL_4606156
94	IWB16701	A/G	0.98	0.91	0.70	0.50	0.41	0.4138	Yes	5DL_4606443
95	IWB15587	A/C	0.44	0.35	0.21	0.09	0.03	0.2628	Yes	5DL_912206
96	IWB66303	T/C	0.70	0.38	0.52	0.60	0.35	0.2238	Yes	7AL_4537078
97	IWB5207	A/C	0.94	0.89	0.93	0.94	0.55	.	.	7AS_3781307
98	IWB51562	A/G	0.56	0.29	0.41	0.48	0.25	0.1863	Yes	7AS_4204121
99	IWB44980	T/G	0.78	0.01	0.01	0.02	0.01	.	.	7AS_4224499
100	IWB26025	T/C	0.93	0.99	0.98	0.97	0.98	.	.	7AS_4236605
101	IWB68134	T/C	0.48	0.19	0.37	0.47	0.03	0.2831	Yes	7AS_4246003
102	IWB43413	A/G	0.98	0.97	0.99	0.98	0.76	.	.	7AS_4248100
103	IWB14567	A/G	0.45	0.98	0.98	0.98	0.99	.	.	7AS_4249298
104	IWB73493	A/C	0.79	0.98	0.99	0.98	0.99	.	.	7AS_4252764
105	IWB72344	A/G	0.59	0.43	0.49	0.56	0.41	0.1300	Yes	7DS_305702

106	IWB16798	A/C	0.02	0.28	0.12	0.06	0.41	0.2215	Yes	7DS_3857389
107	IWB18676	A/G	0.98	0.61	0.80	0.92	0.50	0.3105	Yes	7DS_3878726
108	IWB3122	T/C	0.98	0.17	0.51	0.80	0.04	0.6274	Yes	7DS_3878759
109	IWB48862	T/C	0.63	0.77	0.69	0.65	0.67	.	.	7DS_3887946
110	IWB17016	T/G	0.46	0.97	0.97	0.93	0.97	.	.	7DS_3895557
111	IWB17584	T/C	0.46	0.15	0.31	0.44	0.02	0.2926	Yes	7DS_3896488
112	IWB49667	T/C	0.66	0.03	0.03	0.11	0.01	.	.	7DS_3901971
113	IWB48288	T/C	0.97	0.16	0.54	0.83	0.03	0.6701	Yes	7DS_3940481
114	IWB43343	A/G	0.57	0.85	0.67	0.59	0.97	0.2601	Yes	7DS_3961055
115	IWB18319	T/G	0.71	0.02	0.03	0.04	0.01	.	.	7DS_3965856
116	IWB59774	T/C	0.91	0.52	0.70	0.87	0.46	0.3485	Yes	7DS_3966065
117	IWB17072	A/C	0.99	0.71	0.83	0.92	0.71	0.2055	Yes	7DS_3967219
118	IWB19354	T/C	0.51	0.06	0.05	0.06	0.05	.	.	7DS_461939

* Probes for the SNP ids that are converted into KASP markers are given in the supplementary file 3

Table 3.4 List of SNP marker IDs associated with the stripe rust resistant bulk

S.No	SNP ID*	SNP	Normalised Theta					Difference B1-B2	Significant Difference	BlastN against FSWC contigs
			Parent1	BulkP1	R/S Mix	BulkP2	Parent2			
1	IWB22215	A/G	0.02	0.02	0.02	0.80	0.59	0.7820	Yes	4DS_2325102
2	IWB66287	T/C	0.65	0.78	0.66	0.04	0.04	0.7378	Yes	5AS_1504654
3	IWB73506	T/G	0.05	0.06	0.08	0.69	0.03	0.6329	Yes	4BS_4948853
4	IWB1450	T/C	0.99	0.91	0.79	0.62	0.47	0.2925	Yes	4AL_7077850
5	IWB55192	T/G	0.99	0.99	0.99	0.44	0.73	0.5493	Yes	4DS_2327026
6	IWB46784	A/G	0.98	0.98	0.98	0.80	0.85	0.1847	Yes	4BS_4913787
7	IWB61485	T/C	0.03	0.02	0.02	0.70	0.40	0.6818	Yes	4BS_4913787
8	IWB15346	A/G	0.98	0.97	0.98	0.43	0.44	0.5424	Yes	4DS_2327026
9	IWB55585	T/C	0.05	0.04	0.04	0.58	0.46	0.5369	Yes	4DS_2327026
10	IWB71826	A/G	0.96	0.88	0.75	0.66	0.64	0.2259	Yes	5BL_10841528
11	IWB28274	A/G	0.09	0.25	0.48	0.75	0.98	0.4982	Yes	4BS_4947956
12	IWB60203	T/G	0.15	0.29	0.50	0.68	0.95	0.3869	Yes	4AL_7135265
13	IWB47181	A/G	0.92	0.78	0.50	0.28	0.09	0.5011	Yes	4DS_2316344

* Probes for the SNP ids that are converted into KASP markers are given in the supplementary file 3

Table 3.5 List of KASP and STS markers developed in this study to map*Yr672, Lr672* and *Sr672a*

Marker name	Type	Primer sequence	Chr	Remarks
Csq1	KASP	F1: CATCCCACGCAGCG	4DS	G/A
		F2: CATCCCACGCAGCA		
		R: GCCAACTGAATTCTTCTGTTC		
Csq2	STS	F: ACAATCGTATCCATCATTGGTG	4DS	R:502
		R: GCAGCTGGAGTAGGTTCTTGG		S:299
Csq3	STS	F: AATTTAGCTGCCGGTTTACA	4DS	R:718
		R: GCTGAGCGAATCAACAAGGTTG		S:194
Csq4	KASP	F1: CGGCGAGCTCGGAT	2DS	T/C
		F2: CGGCGAGCTCGGAC		
		R: GTTGTGCTGAAGGACTCTATACAG		
Csq5	KASP	F1: GATGATAGTGAACCAGGTGAC	2DS	C/T
		F2: GATGATAGTGAACCAGGTGAT		
		R: CCATAGTCAGGACTAGGTCG		
Csq6	KASP	F1: GTCGTTCATGAGCTCAGGT	2DS	T/C
		F2: GTCGTTCATGAGCTCAGGC		
		R: CCTGAAGCGGTTGCC		
Csq7	KASP	F1: GCCTGAATATATCGACGCA	2DS	A/C
		F2: GCCTGAATATATCGACGCC		
		R: CGACATTTCAGCACTTCTAAAG		

KASP markers excluding the FAM and HEX tag sequence

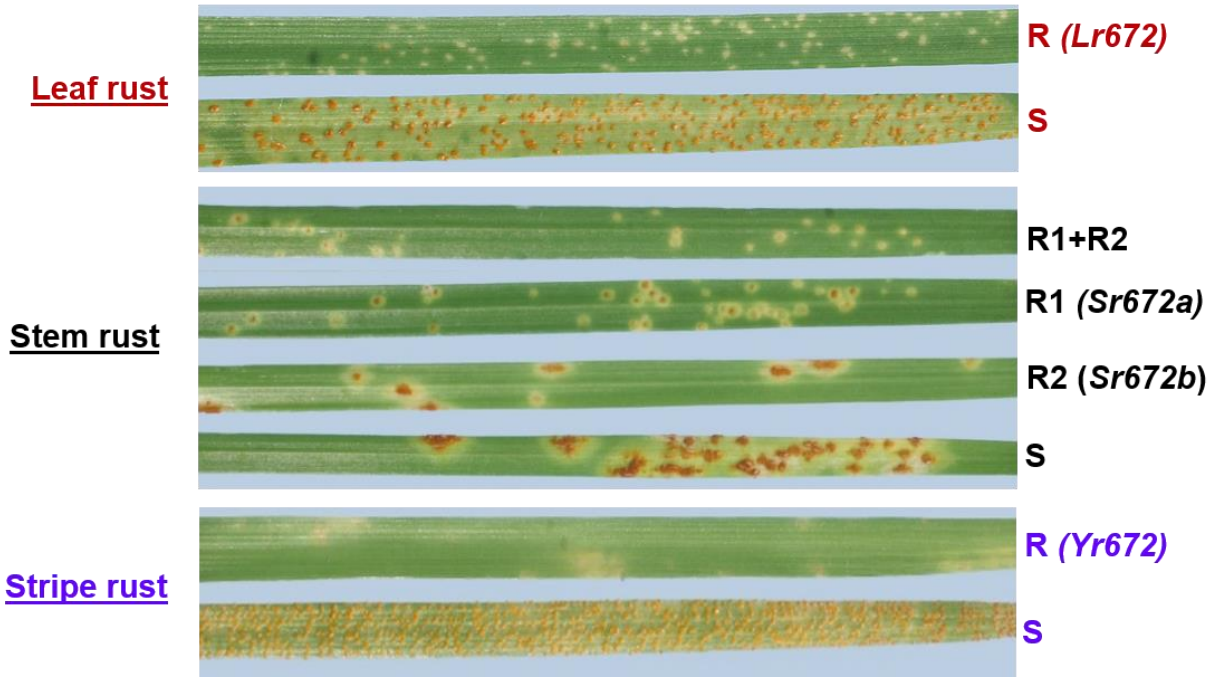

Figure 3.1 Rust resistance profile of the lines from CPI110672 x CPI110717 mapping population

a) Leaf rust phenotype against the race 26-1,3 (316); b) Stem rust phenotype against the race 34-0 (48); c) Stripe rust phenotype against the race 104 E137A-(372). *R* Resistant; *S* Susceptible; *R1+R2* stem rust resistance phenotype segregating for two genes *(Sr672a* and *Sr672b)*

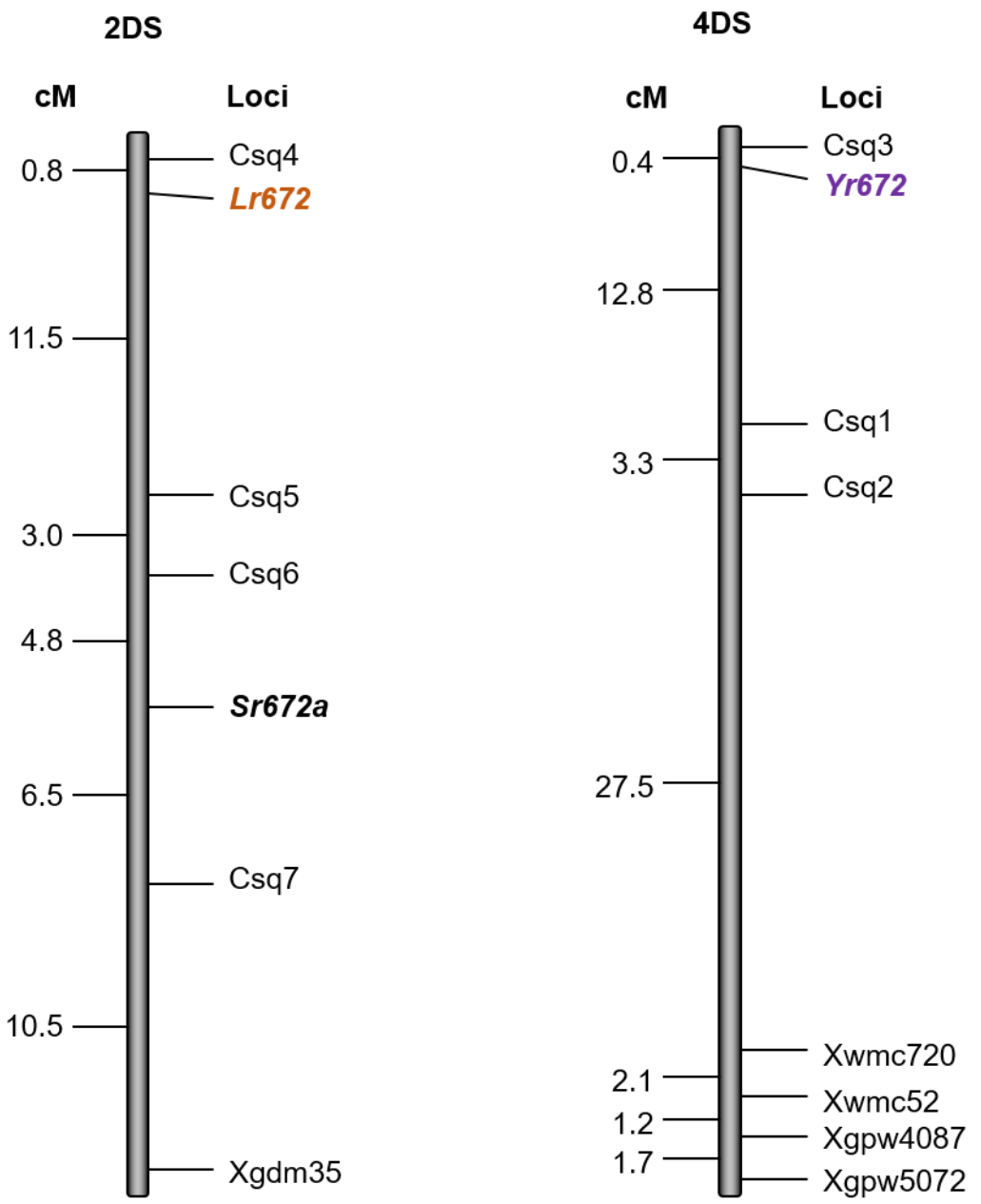

Figure 3.2 Linkage map of *Lr672, Sr672a* and *Yr672* traits with the SNP markers derived from 90K SNP analysis.

Left; the linkage group consists of markers from 2DS flanking the leaf and one of the stem rust loci. *Right;* linkage group consist of markers from 4DS and the stripe rust locus linked.

Chapter 4 - Identification and characterisation of a stem rust resistance gene from *Aegilops tauschii*

4.1 Abstract

Stem rust caused by *Puccinia graminis* Pers. f. sp. *tritici* Erikss & Henn. (*Pgt*) is a devastating fungal disease of wheat. Frequent emergence of new aggressive races of stem rust such as the Ug99, Sicilian and Digalu groups of races pose a threat to global wheat production. An accession of *Aegilops tauschii,* CPI110672, effective against Australian *Pgt* races, has two stem rust resistance genes identified as *Sr672a* and *Sr672b.* In this study, the candidate gene *Sr672a* was identified to be an allele of the previously cloned stem rust resistance gene, *Sr46.* BlastP homology analysis revealed *Sr672a* and *Sr46* differ by one amino acid, hence we have designated the *Sr672a* gene as *Sr46b* whose functional analysis by the complementation test further confirmed the stem rust resistance. To deploy *Sr46b* in wheat breeding programs, we generated a hexaploid backcross population by crossing synthetic hexaploid wheat (SHW) line Lan672 (that contains an identical D genome from the accession CPI110672) with the cultivar Westonia. Screening of the backcross (BC_1F_3) lines carrying *Sr46b* was done using gene-specific molecular markers. Homozygous backcross (BC_1F_3) lines carrying *Sr46b* were confirmed through stem rust resistance screening. Subsequently, the marker-assisted speed breeding facility was utilised to stack *Sr46b* with *Sr33* and *Sr45*, two recently cloned stem rust resistance genes from *Ae. tauschii.* Finally, to map the second stem rust resistance gene, *Sr672b*, we generated a single gene segregating mapping population.

4.2 Introduction

Stem rust, caused by the fungus *Puccinia graminis* Pers. f. sp. *tritici* Erikss & Henn. (*Pgt*), is one of the most destructive diseases of wheat, with the potential to cause 100% grain loss during severe epidemics. In Australia, incidence of stem rust was observed as early as the beginning of 19th century (Waterhouse 1929) and breeding for rust resistance was initiated in the early 20th century. Despite the success in breeding wheat for rust resistance, losses continue as the pathogen often undergoes mutation to overcome the deployed resistances. Globally, stem rust is generally managed through the use of genetic resistance in common wheat varieties. The

emergence of the highly virulent stem rust race “Ug99” also known as “TTKSK" in Uganda poses challenges to this stratergy (Pretorius et al. 2000; Singh et al. 2011). By overcoming one of the widely deployed resistance genes, *Sr31,* Ug99 poses a serious threat to global wheat cultivation (Pretorius et al. 2000). Variants of TTKSK emerged in subsequent years, with added virulence to *Sr24* and *Sr36* genes (Jin et al. 2008; Jin et al. 2009). Ug99 and its 13 variants spread rapidly to adjacent countries reaching as far as Iran and South Africa (Babiker et al. 2015; Singh et al. 2015). On its migratory route, the fungus threatens to reach the top two wheat producing nations of Asia: India and China (Singh et al. 2006; Singh et al. 2015; Meyer et al. 2017). Following the evolution of Ug99, a similar devastating stem rust race (referred as the Sicilian race) was also detected in 2016 in Sicily. Stem rust was not an issue for a long period in Europe as the last incidence of serious stem rust epidemics were noted only in 1951 (Bhattacharya 2017). Similarly, the “Digalu” race (TKTTF) originated in Turkey and spread across continents reaching Ethiopia in 2014. Its close lineages were also detected in European countries such as Germany, Sweden, Denmark and the UK in 2013, where 80 percent of the cultivars were susceptible (Lewis et al. 2018).

To date, ~62 stem rust resistance (*Sr*) genes have been mapped, however the majority are ineffective against prevailing races. Wild relatives of wheat are an excellent source for new and broad-spectrum resistance as they possess higher genetic diversity than cultivated lines. However, transfer of resistance from these relatives to cultivated wheat is often tedious, due to limited recombination between chromosomes of the alien species and wheat. Introgression from accessions of *Ae. tauschii* the D genome progenitor of bread wheat, are less complicated as the D genome is homologous to the hexaploid wheat D genome. *Aegilops tauschii* has been a valuable source for several traits, including the stem rust resistance genes *Sr33, Sr45, Sr46, SrTA1662, SrTA10171*, and *SrTA10187* (Olson et al. 2013a; Olson et al. 2013b; Periyannan et al. 2013; Periyannan et al. 2014; Yu et al. 2015). Introgression of rust resistance genes from *Ae. tauschii* is achieved either by direct hybridisation of hexaploid wheat with a diploid accession, or by a two-step process of developing a synthetic hexaploid wheat by crossing tetraploid wheat with *Ae. tauschii,* followed by chromosome doubling and backcrossing with elite hexaploid wheat lines (Gill and Raupp 1987; Olson et al. 2013b).

In our study (as presented in Chapter 3), over 400 accessions of *Ae. tauschii* were screened and several were identified as potential sources for stem rust resistance genes. One particular accession, CPI110672 (originally from Turkmenistan), exhibited strong stem rust resistance to the two Australian stem rust races used for screening (Evans Lagudah unpublished data). Through genetic analysis, it was determined that stem rust resistance in the accession CPI110672 was conferred by two genes *Sr672a* and *Sr672b*. In the 90K SNP and preliminary marker-trait analyse, one of the stem rust resistance genes was mapped to the short arm of chromosome 2D. As a follow-up study, this chapter aims to isolate *Sr672a* from accession CPI110672, study its relation to a previously mapped stem rust resistance gene (*Sr46)* on 2DS, and combine the gene with two recently cloned *Sr* genes (*Sr33* and *Sr45)* of *Ae. tauschii*.

4.3 Materials and methods

4.3.1 Plant and Genetic material

Genomic DNA of the F_2 mapping population (CPI110672 x CPI110717) from Chapter 3 was used in this study. A second mapping population consist of a single gene (*Sr672b)*-segregating F_3-family derived from a single F_2 line (No. 46) that segregated for stem rust resistance, but negative to the *Sr672a* specific marker (Supplementary file 2). The synthetic hexaploid wheat (SHW) Lan672, derived from a cross between Langdon and *Ae. tauschii* acc. CPI110672, was used to transfer the stem rust resistance genes into the commercial cultivar Westonia. A line carrying both *Sr33* and *Sr45,* derived from a cross between *Sr33*-Wt6-12 and *Sr45*-Kulin was used to stack *Sr33* and *Sr45* with *Sr672a.*

4.3.2 Phenotyping rust resistance

Rust resistance phenotyping was carried out on four distinct genotypic groups of plants: the mapping population ($F_{3:4}$ derived from a single F_2 line of CPI110672 x CPI10717 segregating for *Sr672b*), selected homozygous lines from the backcross population, transgenic lines carrying *Sr672a,* and the F_2 lines derived from the cross between *Sr672a* (homozygous backcross line) and lines carrying *Sr33/Sr45.* Fifteen plants each from the $F_{3:4}$ mapping population, homozygous lines from the backcross population, F_2 lines from the *Sr672a* x *Sr33/45* lines, and T_1 progenies of the *Sr672a*

transgenic lines were used for phenotyping. Rust phenotyping experiments were repeated at least twice to observe the consistence in the stem rust phenotype. Phenotyping was carried out in the automated plant growth facility at CSIRO. Seedlings were grown under a 16-hour photoperiod and 23/17°C day/night temperature for two weeks and inoculated with the stem rust race (98-1,2,3,5,6) to determine the disease resistance response. Initially, inoculated plants were incubated in a closed container for 48 hours to maintain humidity for rust spore germination and infection. Later they were subjected to the growth conditions above for rust development. Rust infection was scored on the first leaf at 12-14 days post infection (dpi) using the 0-4 Stakman scale (Stakman et al. 1962). Stem rust infection types: 0 and ; were considered as immune response, 1 to 2+ or were considered as resistant to intermediate resistant, while scores of ≥3 were considered susceptible. A chi-square (χ^2) test was performed to determine the segregation pattern of rust phenotypes in the $F_{3:4}$ population.

4.3.3 Identification of the candidate gene for *Sr672a*

Markers flanking the *Sr672a* stem rust resistance gene as described in Chapter 3, were positioned on the reference genome sequences of the hexaploid wheat Chinese Spring (CS) IWGSC RefSeq v1.0 (referred hereafter as IWGSC RefSeq v1.0) (IWGSC 2018) and the diploid progenitor *Ae. tauschii* AL8/78 (Luo et al. 2017). The list of annotated *NBS-LRR* genes mapped between the flanking markers in the reference genome sequences were identified. To verify the allelic relationship of *Sr672a* and *Sr46,* we screened the CPI110672 x CPI110717 F_2 mapping population with gene-specific primers (S46ConsFA-GCTGAGATTGTGCTTCTTCTAG and S46PREVR-CATTGTACGCTCTGTCCAATA) (Arora et al. 2019) using standard PCR conditions performed in a 20 µl PCR reaction containing 100 ng of gDNA, 1X GoTaq flexi green buffer, 1.5mM $MgCl_2$, 200 µM dNTP, 200 nM each of forward and reverse primers and a unit of *Taq* polymerase (M829B, Promega, USA). PCR was carried out in a BioRad thermal cycler using the program as follows: after the initial denaturation at 95°C for 3 minutes: denaturation at 94°C for 30 seconds; annealing at 61°C for 30 seconds; extension at 72°C for 2 minutes repeated for 34 cycles. Furthermore, we isolated the sequences of *Sr46* from CPI110672 (a candidate gene for *Sr672a)* and evaluated their homology at the amino acid level using the NCBI BlastP program.

4.3.4 Complementation test for *Sr46b* sequence from CPI110672

The full length *Sr46b* sequence from CPI110672 was amplified using primers GENS46F ACCTTATTCCATCTTCAGAGACCG and GENS46R AATCTGCTAGTGCTTTGCTTACAG and the Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc.) using the manufacturer's recommended conditions with a 60°C annealing and 2 minutes and 30 seconds extension time for 34 cycles. The fragment was cloned into the binary vector VecBarII, a derivative plasmid of pWBvec8 that carries the selectable marker *Bar* gene driven by the CaMv35S promotor and Nos terminator in the T-DNA. The construct was transformed into the *Agrobacterium tumefaciens* strain GV3101 for further transformation into the susceptible wheat cultivar, Fielder (Richardson et al. 2014; Ishida et al. 2015). The independent T_0 transgenic event lines were tested for stem rust resistance against the Australian *Pgt* race 98-1,2,3,5,6. To assist the molecular screening of *Sr46b*, we also developed a gene-specific marker (Sr46F1 GTTGGGAAGTGATGATGTGGAACGG and Sr46R1 CCTCAACTCCTCCGGACAGTGATGA) with a shorter amplicon as compared to the *Sr46* gene specific marker of Arora et al (2019) for robust molecular screening. The primer amplifies the fragment under the standard GoTaq PCR program mentioned above with 61°C annealing temperature and 30 seconds extension for 30 cycles. To identify the *Sr46b* homozygous transgenic lines, six T_1 progenies (four resistant and two susceptible) for each independent T_0 line (PC154-22, PC154-28, PC154-29, PC154-35, PC154-36, PC154-37 and PC154-38) were selected for further advancement. T_2 seeds were harvested and DNA was isolated from 12 T_2 seeds from each of the progenies, using the Nimbus robot system as described by Ellis et al. (2005) and screened for homozygosity using the Sr46F1/R1 primer pair. An additional 12 T_2 seeds from the identified homozygous lines were screened for confirmation. To evaluate the stem rust response against the stem rust race collected from Sicily, Italy (Sicilian race), we screened five homozygous T_2 lines carrying *Sr46b* and a negative line at the Global Rust Reference Centre (GRRC), Denmark. We also included homozygous lines of *Sr46a* to compare the stem rust phenotype with *Sr46b* as they are allelic.

4.3.5 Introgression of *Sr46b* into hexaploid wheat

To deploy *Sr46b* into commercial cultivars, we developed a backcross population by crossing the SHW Lan672 with Westonia. The F_3 families of the backcross population were screened to identify lines homozygous at the *Sr46b* locus using the Sr46F1/R1 marker, followed by rust phenotyping. One seed each from 100 BC_1F_3 families were used for DNA isolation as described in the previous section (Ellis et al. 2005) and screened with the Sr46F1/R1 primer pair. For lines positive for Sr46F1/R1, DNA from twelve seeds were isolated and genotyped to identify homozygous lines. Lines identified as homozygous for Sr46F1/R1 marker were confirmed by genotyping twelve additional seeds. Since the tetraploid wheat Langdon is a component of SHW-Lan672 and it carries *Sr13* (Zhang et al. 2017), the homozygous lines carrying *Sr46b* were screened for *Sr13*-specific CAPS markers, Sr13F/R, and digested with *Hha*I enzyme as described in Zhang et al. (2017). All the homozygous lines identified were screened for stem rust resistance against Australian *Pgt* race 98-1,2,3,5,6.

4.3.6 Marker assisted pyramiding of *Sr46b* under speed breeding

To pyramid *Sr46b* with other stem rust resistance genes identified from *Ae. tauschii*, the BC_1F_3 homozygous line carrying *Sr46b* and a line carrying both *Sr33* and *Sr45* derived from the cross between *Sr33*-Wt6-12 and *Sr45*-Kulin were crossed under speed breeding conditions (23/17°C for 22/2 hours day/night) (Watson et al. 2018). Genomic DNA of the endosperm segment of F_2 seeds was isolated as described above (Ellis et al. 2005). The F_2 DNA was screened for combinations of different gene stacks using marker assisted selection. The remaining embryo segment of select F_2 lines was germinated and tested for the rust resistance.

4.4 Results

4.4.1 Unveiling the allelic relationship: *Sr672a* is an allele of *Sr46*

We anchored the *Sr672a* flanking markers *Csq6* and *Csq7* sequences (see Chapter 3, Figure 3.2) physically to the reference genome sequences of hexaploid wheat cv. Chinese Spring IWGSC RefSeq v1.0 and *Ae. tauschii* AL8/78 v4.0 (Luo et al. 2017; IWGSC 2018). This resulted in an interval of about a 2.9 Mb in Chinese Spring and a 2.6 Mb in *Ae. tauschii* AL8/78 sequence. The targeted interval in both genomes had only one annotated *CC-NBS-LRR* (*CNL*) gene with reference number

TraesCS2D01G016400 and AET2Gv20030800 (Figure 4.1; Table 4.1). The deduced amino acid sequences of the gene in both were verified to have a full CC-NBS-LRR (*CNL*) domain architecture (Figure 4.1). Therefore, we considered this *CNL* gene as a candidate for *Sr672a.* Further verification of the *CNL* gene sequences of Chinese Spring and AL8/78 with the recently cloned gene *Sr46* (Arora et al. 2019) determined they are homologous sequences. Thus screening of the *Sr46* gene specific marker in the $F_{2:3}$ CPI110672 x CPI110717 mapping population amplified the 2.47 kb fragment specific to *Sr46,* while co-segregated with the group 1 (*Sr672a*) resistance phenotype. This suggests that the stem rust resistance gene *Sr672a* mapped on 2DS of this study is probably *Sr46* or its allele.

The full length (2775 bp) of the *Sr46*-related sequence, including the 5' and 3' untranslated regions (UTR), was isolated from CPI110672. BlastP homology analysis of the predicted amino acid sequences resulted in one amino acid difference between the Sr672a candidate gene sequence and Sr46 at position 763, where a lysine was present in Sr672a instead of asparagine (Sr46) (Figure 4.2). Therefore, we designated the cloned *Sr672a* gene from CPI110672 as *Sr46b* to represent its status as an allelic variant of *Sr46*. Further complementation tests of the *Sr46b* sequence through *Agrobacterium* mediated transformation into the wheat cultivar Fielder (transgenic material batch id PC-154) conferred stem rust resistance to the Australian *Pgt* race 98-1,2,3,5,6 (Figure 4.3). For selection of *Sr46b*, we also developed a gene specific marker (Sr46F1/R1) which amplified a smaller amplicon (520 bp) as compared to the *Sr46* specific marker reported in Arora et al (2019). The new marker was validated using *Ae. tauschii* accession CPI110672 and the SHW Lan672 (Figure 4.4). Screening of six progenies from each of the seven independent transgenic lines with the new markers identified up to three homozygous resistant progenies for PC154-22, PC154-28, PC154-29, PC154-35 and PC154-36, and at least one homozygous negative line for PC154-29, PC154-35 and PC154-36. Rust resistance screening for five *Sr46b* homozygous resistant progenies against the Sicilian *Pgt* race conferred weak resistance and were similar to Fielder lines with *Sr46a*, however, the later showed more chlorotic area around the infection sites (Figure 4.5).

4.4.2 Marker assisted breeding of *Sr46b* in hexaploid wheat

We generated about 165 BC_1F_3 families from the cross between SHW-Lan672 and Westonia. Initial screening of 100 F_3 lines for the *Sr46* marker identified 16 positive lines. In the subsequent progeny test, three lines (BC_1F_3-81, 94 and 96) were identified as homozygous for the *Sr46b* locus. Since *Sr13* is a component of Langdon, additional screening for the *Sr13* marker in the homozygous lines identified the lines BC_1F_3-94, and 96 as homozygous for the *Sr13* locus while BC_1F_3-81 was negative for the marker. In the rust infection test, line BC_1F_3-81, carrying only *Sr46b,* showed an IT 2+ phenotype while the lines BC_1F_3-94 and 96 carrying both *Sr13*+*Sr46b* had a score of 1,2-C. Parental line SHW-Lan672 scored 1-, while a hexaploid line with *Sr13* gene alone had an intermediate (2-) phenotype and the negative control, Westonia had a 3+ phenotype (Figure 4.6).

4.4.3 Rapid stacking of *Sr46b* with *Sr33* and *Sr45*

In order to test the additive effect of *Sr46b*, multiple gene combination lines in the Westonia background were developed. The homozygous backcross line BC_1F_3-94 was crossed with a line carrying *Sr33/45* under speed breeding conditions. The F_2 seeds carrying multiple resistance genes were generated and screened with rust against *Pgt* race 98-1,2,3,5,6 in over six months. In our experiment, the parental lines were ready for crossing at 40 days after planting (Table 4.2). The F_1 seeds were harvested after 65 days and germinated for generation advancement using speed breeding. The F_2 seeds were harvested, dried, and cut in half to genotype the endosperm segment to identify multiple gene combinations using gene specific markers, and the embryo germinated for phenotyping between 140 and 150 days (Table 4.2).

Using marker screening, we identified lines with single, two and three gene combinations of *Sr33*, *Sr45* and *Sr46b*, as well as lines without any of these genes. The single gene line *Sr33* conferred intermediate resistance to *Pgt* (2-) and *Sr45* strong resistance(;1-) while the *Sr46b* showed 2+ phenotype as expected (Figure 4.7). Screening the gene combinations *Sr33/Sr45, Sr33/Sr46b* and *Sr45/Sr46b* revealed strong resistance (;C). Despite the intermediate phenotype of *Sr33* and *Sr46b* individually, strong resistance phenotypes by both together confirmed the additive

nature of *Sr33* and *Sr46b*. In addition, line carrying the three gene combination (*Sr33/Sr45/Sr46b*) conferred strong resistance (Figure 4.7).

4.4.4 Mapping *Sr672b* in the CPI110672 x CPI110717 mapping population

Progeny of the $F_{3:4}$ mapping population developed for the second stem rust resistance gene were tested for stem rust had an intermediate resistant reaction of IT 2+ and a susceptible reaction of IT 3. The mapping population segregated for the single gene, *Sr672b* (Figure 4.8).

4.5 Discussion

The *Ae. tauschii* accession CPI110672 was resistant to the Australian *Pgt* races 34-0 and 98-1,2,3,5,6. Through genetic analysis, the genes *Sr672a* and *Sr672b* were identified to confer the stem rust resistance. Gene *Sr672a* was mapped to the short arm of chromosome 2D and the markers *Csq6* and *Csq7* flank the gene locus (refer to Chapter 3). Traditionally, map-based, positional cloning of genes involves generation of a physical map for the gene locus through screening, and then mapping and sequencing of BAC clones (Feuillet et al. 2003; Krattinger et al. 2009; Periyannan et al. 2013; Moore et al. 2015; Arora et al. 2019). However, in this study, we took advantage of the recent version of the reference genome sequence of hexaploid wheat IWGSC RefSeq v1.0 (IWGSC 2018) and the D genome diploid *Ae. tauschii* AL8/78 v4.0 (Luo et al. 2017) to rapidly anchor the flanking markers and gain insight on potential candidate genes within the interval. The recent versions of reference sequences also include annotation of genes.

In the 3 Mb interval, there was only a single *CNL* gene in both CS and AL8/78 reference sequences. Since the map location of *Sr672a* is on 2DS, it prompted us to evaluate the allelic relationship with *Sr46,* which was previously identified from the same region of *Ae. tauschii* accessions CIae 25 and AUS 18913 (Yu et al. 2015; Arora et al. 2019). The full gene sequence of *Sr46* was anchored to IWGSC RefSeq v1.0 and AL8/78 v4.0, where it aligned to the annotated *CNL* gene predicted earlier in both the reference genomes. Further verification from the USDA-ARS National Plant Germplasm System, shows that CPI110672 is different from AUS 18913 and CIae 25, whilst the latter two accessions were the same (Yu et al. 2015). A recent analysis of a panel of *Ae. tauschii* accessions with 317 SNP markers also confirms that CPI110672

and AUS18913 were genetically different (Arora et al. 2019). Regardless of the difference between these accessions, screening of the gene specific marker confirmed the presence of *Sr46* in CPI110672. Full-length amplification confirmed *Sr672a* as an allele of *Sr46*, hence it was designated *Sr46b.* Sr46a and Sr46b differ by one amino acid i.e. N763K where the amino acid asparagine in Sr46a is replaced by lysine in Sr46b. Asparagine is polar and lysine is charged and both are located in the LRR domain of the gene. Often, the LRR domain is postulated to be involved in recognition of pathogen effector molecules (DeYoung and Innes 2006; Dodds and Rathjen 2010). Allelic variants of a single resistance gene can confer different race specificities, which was the case in flax rust (Ellis et al. 1997). Therefore, we opted to evaluate whether the difference in amino acid alters the pathogen recognition and resistance function. The transgenic plants harbouring *Sr46b* conferred stem rust resistance against the Australian *Pgt* race and failed to show a notable difference in comparison with *Sr46a* mediated resistance. Hence, the change in amino acid N763K at the LRR domain did not alter the stem rust resistance function (against the Australian *Pgt* race). Further testing of *Sr46a* and *Sr46b* against multiple rust races is needed to validate the significance of N763K in pathogen recognition. Therefore, we tested the *Sr46a* and *Sr46b* transgenic lines against the new devastating stem race identified in Sicily (Bhattacharya 2017). Both *Sr46a* and *Sr46b* transgenic lines conferred very weak resistance to the Sicilian race. In this case, we observed a small difference in phenotype where *Sr46a* showed higher chlorotic phenotype than *Sr46b*.

During the evaluation of the USDA-ARS National Plant Germplasm System, we also noted that CPI110672 is a synonymous accession of TA1675. Accession TA1675 was reported to confer resistance to different stem rust races in the USA, including Ug99 (pathotype TTKSK), which is the most devastating race in East Africa and the Middle East. Screening of accession TA1675 conferred a strong to intermediate (;,1,2) phenotype, whereas AUS18913 conferred intermediate (IT 2) and weak (IT 3) phenotypes against the races TTTTF, TRTTF, QTHJC, and RKQQC tested in the USA (Rouse et al. 2011a). Such a diverse stem rust resistance phenotype by two *Sr46* carrying accessions to races in the USA suggests *Sr46b* is likely conferring additive rust resistance, when present with *Sr672b* in the diploid background. It is also evident from our mapping population using CPI110672 where three different phenotypes for resistance were identified and lines with *Sr46b+Sr672b* phenotype showed stronger

resistance. Due to the additive nature of *Sr46b* and *Sr672b*, it is of interest to conduct genetic studies to map the location of *Sr672b.* We generated a mapping population from an F_2 line segregating for *Sr672b* and negative for the *Sr46* marker. Furthermore, we phenotyped the population segregating for *Sr672b* stem rust resistance, which will be useful to conduct genetic analysis and fine mapping to isolate *Sr672b*.

Resistance conferred by *Sr46b* to various stem rust races in Australia, and around the world, including the Ug99, indicates that it could be a useful gene for the wheat breeding program to deploy singly or in combination with other genes for rust resistance. Since *Ae. tauschii* is the D genome source of hexaploid wheat, it readily recombines with hexaploid wheat and paves the way for the easy transfer of useful genes. As previously mentioned, the stem rust resistance genes *Sr33, Sr45, Sr46, SrTA1662, SrTA10187,* and *SrTA10171* were previously transferred from *Ae. tauschii* into hexaploid wheat (Rouse et al. 2011a; Olson et al. 2013a; Olson et al. 2013b; Periyannan et al. 2013; Periyannan et al. 2014). In our study, using marker Sr46F1/R1, we also transferred the *Sr46b* into the commercial cultivar Westonia. Due to the high homology between *Sr46* and its related allele from the wheat genome, the previous marker S46ConsFA-S46PREVR used for *Sr46* was tedious as it amplified a fragment of 2.5 kb (Arora et al. 2019). To simplify, we designed a new marker to yield a shorter amplicon of 520 bp to select *Sr46* in breeding programs. Despite highly homologous sequence, the lack of non-specific amplification in *Sr46* negative lines indicated the specificity and robustness of the new *Sr46* marker, which later aided in the screening of homozygous backcross lines. Surprisingly, we observed a difference in rust phenotype among the homozygous lines as we later realised that the tetraploid line, Langdon, used to develop the synthetic hexaploid SHW-Lan672 also carried *Sr13*. *Sr13* confers intermediate resistance to the majority of stem rust races worldwide (Zhang et al. 2017), but shows good additivity with *Sr46b* as seen in our experiment where the backcross lines with both the genes revealed strong resistance. Previously, stem rust resistance gene *Sr33* was identified to provide an additive response in combination with adult plant resistance gene *Sr2* (Ayliffe et al. 2013).

Screening of a diversity panel with gene specific markers of *Sr33, Sr45* and *Sr46* confirmed that some accessions of *Ae. tauschii* that show strong resistance have multiple *Sr* genes. For example, the presence of *Sr46b* and *Sr672b* in CPI110672 and

Sr33 and *Sr46b* in CPI110799 confirmed their additive response, as compared to lines with single *Sr* genes. However, their additive relationship in hexaploid wheat is still untested (Evans Lagudah, personal communication). So, we decided to evaluate the additivity of *Sr46b* with other stem rust resistance genes (*Sr33* and *Sr45*) for *Ae. tauschii* in the hexaploid background. Genes *Sr33* and *Sr46* confer intermediate resistance in hexaploid wheat, while *Sr45* confers strong resistance (Periyannan et al. 2013; Steuernagel et al. 2016; Arora et al. 2019). We anticipate stacking *Sr33* or *Sr46* with *Sr45* will result in a stronger rust resistance due to the presence of *Sr45* which will mask the effect of *Sr33* and *Sr46.* However, we stacked the gene combinations *Sr33* and *Sr46b* with *Sr45* to make sure they do not inhibit with each other. Evaluating the response of the *Sr33/Sr46b* stacking lines are crucial to study the additivity of both genes. We opted for marker assisted speed breeding, which facilitates rapid generation advancement and provides opportunities for simultaneous rust phenotyping during rapid stacking (Watson et al. 2018). As reported earlier, the parental lines grown under speed breeding conditions were healthy and ready for crossing in 40 days. By 65 days, we were able to harvest the mature F_1 hybrid seed. It also facilitated further generation advancement in 140 days, and subsequently, the F_2 plants were screened by gene specific markers and phenotyped for stem rust resistance in about 180 days. Phenotyping the multiple gene stack lines (*Sr33/Sr45, Sr33/Sr46b, Sr45/Sr46b* and *Sr33/Sr45/Sr46b*) showed stronger resistance than the single gene lines. Thus confirming the resistance due to *Sr33* and *Sr46b* was additive in nature when combined with each other, or together with *Sr45.* Quantification of fungal biomass in the multigene combination lines will quantify the reduction of fungal growth by the additive reaction. This signifies the potential to deploy these genes in combination for durable stem rust resistance in breeding programs and marker-assisted speed breeding facilitates rapid stacking.

In summary, an allele of *Sr46 (Sr46b)* from *Ae. tauschii* accession CPI110672 (syn TA1675) was identified in this study. Cloning of the full length *Sr46b* sequence and *Agrobacterium* mediated transformation into hexaploid wheat cultivar Fielder conferred stem rust resistance. It indicated that the change in one amino acid at the LRR domain of *Sr46b* did not alter the rust resistance phenotype as tested against the Australian *Pgt* race used. The use of *Sr46b* in CPI110672 provided an additive response with *Sr672b* and *Sr33*, and indicated its potential in wheat breeding

programs for durable stem rust resistance. Employing marker-assisted speed breeding enabled the stacking of three stem rust resistance genes and we assessed their efficiency for durable resistance in 180 days, a quarter of the time required by conventional methods.

Table 4.1 Physical map of *Sr46b* sequence in chromosome 2D of CS IWGSC RefSeq v1.0 and AL8/78 v4.0

Chinese Spring IWGSC RefSeq v1.0	Start (bp)	End (bp)	*Ae. tauschii* AL8/78 v4.0	Start (bp)	End (bp)
Csq6	6425488	6425588	Csq6	5684448	5684545
Only one annotated *CNL* gene between the flanking markers - TraesCS2D01G016400 (homologous sequence of *Sr46*)	7970131	7972905	Only one annotated *CNL* gene within the flanking markers - AET2Gv20030800 (homologous sequence of *Sr46*)	6773826	6776600
Csq7	9344557	9344806	Csq7	8290367	8290616

Table 4.2 Timeline required for applying marker assisted speed breeding to pyramid *Sr33, Sr45* and *Sr46b*

Stage	Activity	Day
Germination	Sow parental seeds	1
Anthesis	Cross BC_1F_3-94 x Sr33/45	40
Maturity	Harvest F_1 seeds and dry in an oven for 3 days	65
Germination	Cold treatment for 48 hours and room temperature at dark until sprouting	68
	Sow F_1 seed for speed breeding condition	72
Maturity	Harvest of F_2 seeds; oven dry for 3 days	140
Molecular analysis	Isolate DNA from endosperm segment (half seed) of F_2 seed and screen for gene specific markers	143
Germination	Cold treatment of embryo segment of selected seed for 48 hours	148
	Germinate embryos for speed breeding	152
Two leaf stage	Infect stem rust race on PCR positive multiple gene combination lines	167
Three leaf stage	Phenotype stem rust resistance of F_2 seedlings	182

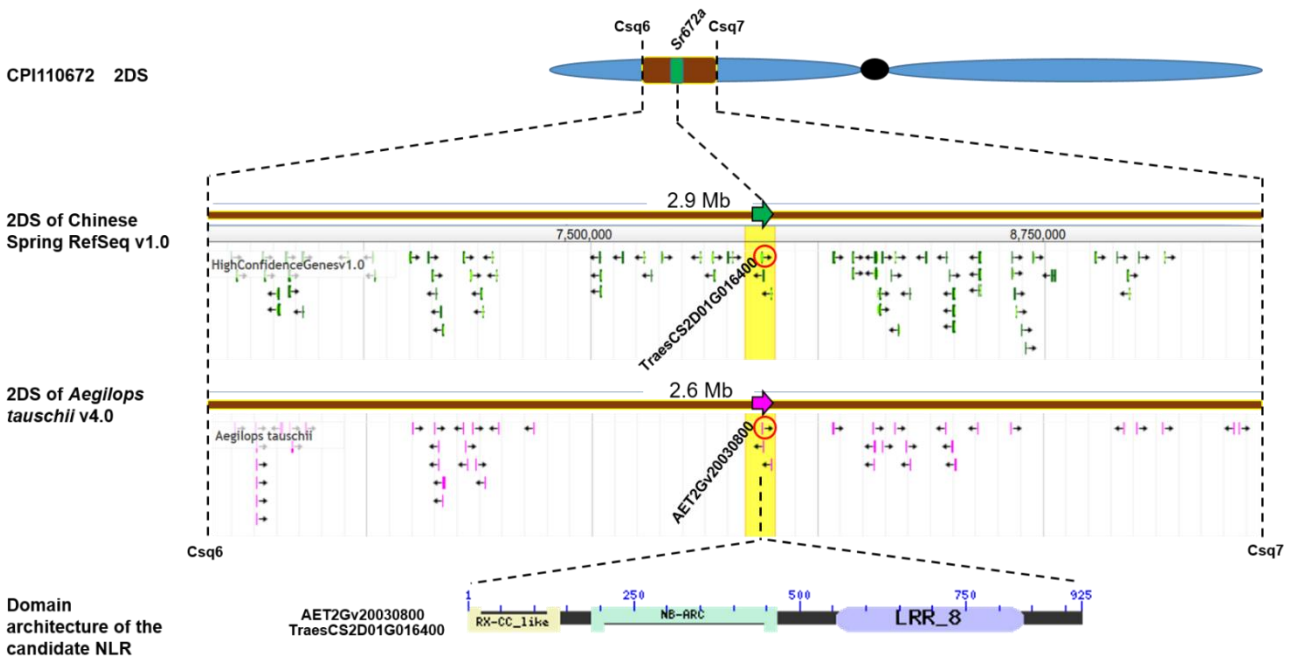

Figure 4.1 Physical map and candidate gene identification for *Sr672a*

CPI110672 2DS genetic map containing *Sr672a* flanked by *Csq6* and *Csq7*. The region between the flanking markers in Chinese Spring (CS) IWGSC-RefSeq v1.0 and *Ae. tauschii* AL8/78 v4.0. The bold green and pink coloured forward arrows represent the position of the only annotated *CC-NBS-LRR* (*CNL*) gene within the flanking markers. The actual annotated candidate *CNL* gene in CS and AL8/78 is circled in red. Small forward and reverse oriented green and pink arrows represent the number of other annotated genes in CS and AL8/78, respectively. The predicted domain architecture of only annotated *CNL* gene from CS and AL8/78 containing RX-CC like domain, an NB-ARC domain and an LRR_8 domain.

```
Sr46a   721  WGMKGIYCEQLCKSLVQMQFLSNLNVNASDENEVLALNALPANLQKLSLSGRLPEGALLA  780
             WGMKGIYCEQLCKSLVQMQFLSNLNVNASDENEVLALNALPA LQKLSLSGRLPEGALLA
Sr46b   721  WGMKGIYCEQLCKSLVQMQFLSNLNVNASDENEVLALNALPAKLQKLSLSGRLPEGALLA  780
```

Figure 4.2 BlastP analysis of the predicted amino acid sequence of Sr46a and Sr46b

Analysis of amino acid sequences of Sr46a and Sr46b illustrating one amino acid change at N763K in red.

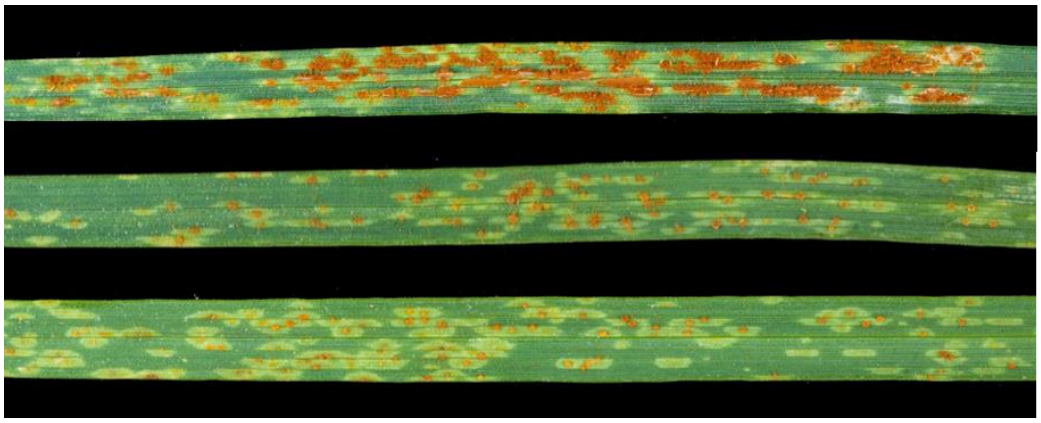

Figure 4.3 Complementation test for *Sr46b* in the hexaploid cultivar Fielder

The two representative transgenic lines PC154-35 and PC154-36 were tested for rust resistance to Australian *Pgt* race 98-1,2,3,5,6 resulting in infection types 2+ and 2-, while the cultivar Fielder displayed the susceptible infection type.

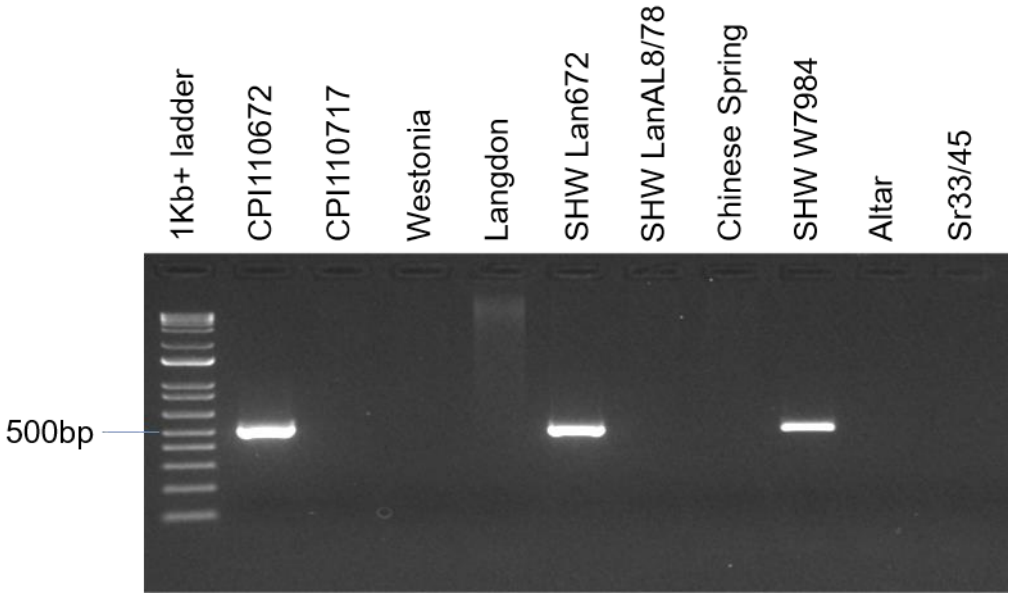

Figure 4.4 Validation of a new *Sr46* gene-specific marker

A new gene specific marker SR46F1/R1 with a 520 bp amplicon size amplified only from lines carrying *Sr46*. CPI110672, the parental line for *Sr46b*; SHW Lan672, the synthetic hexaploid wheat derived from CPI110672; SHW W7984 derived from WX219 (TA2465) also carries *Sr46* (Lagudah unpublished data). Other lines did not carry *Sr46* thus did not produce any amplification. The absence of non-specific amplification in other samples implies the marker anneals accurately at the target position.

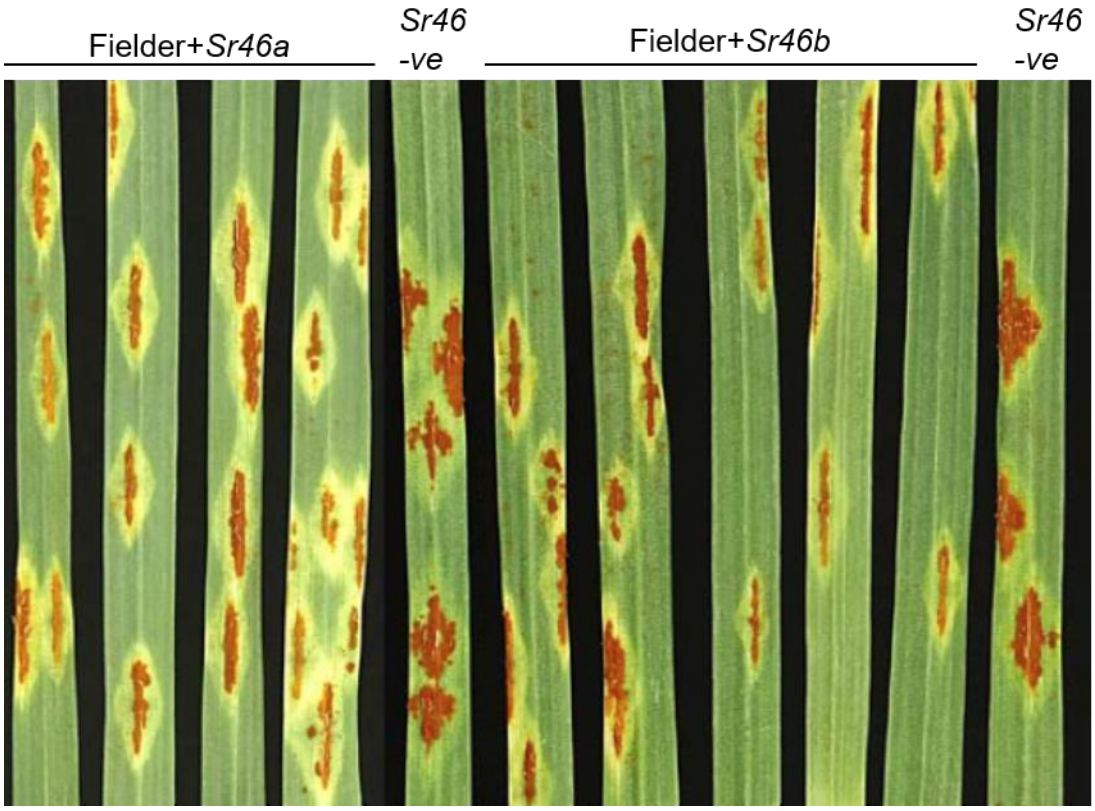

Figure 4.5 Phenotyping *Fielder+Sr46a* and *Fielder+Sr46b* lines inoculation with the Sicilian stem rust race at 14 dpi

Fielder+Sr46a, four independent T_2 homozygous lines; *Fielder+Sr46b,* five independent T_2 homozygous lines; *-ve,* corresponding homozygous negative lines. Both Fielder+*Sr46a* and Fielder+*Sr46b* (PC154-22, PC154-28, PC154-29, PC154-35 and PC154-36) displayed the 3,3- phenotype to Sicilian race TTRTF. Fielder+*Sr46a* is more chlorotic than Fielder+*Sr46b*. The *Sr46* negative lines display phenotypic score 4 on the Stakman scale.

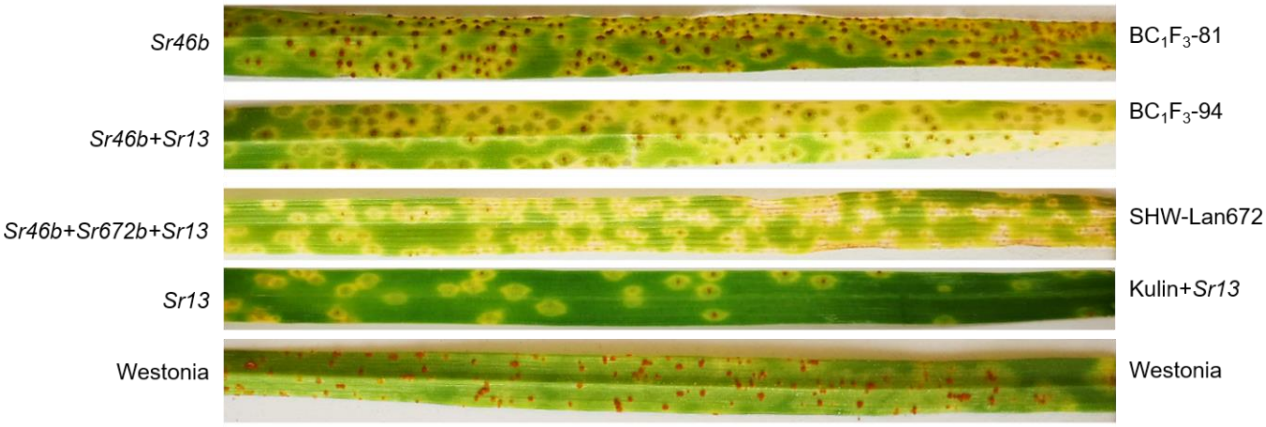

Figure 4.6 Phenotyping *Sr46b* homozygous BC_1F_3 lines against Australian *Pgt* race 98-1,2,3,5,6 at 14 dpi

Screening of *Sr46b* homozygous lines for stem rust resistance and scored using the Stakman scale (Stakman et al. 1962), BC_1F_3-81 (carrying only *Sr46b*) showed IT 2+ while the line BC_1F_3-94 *(Sr13+Sr46b)* showed IT 1,2-C. The SHW Lan672 *(Sr13+Sr46b+Sr672b)* displaying IT 1- phenotype. The hexaploid control for *Sr13* (Kulin+*Sr13*) showing IT 2- while Westonia displaying susceptible phenotype 3+

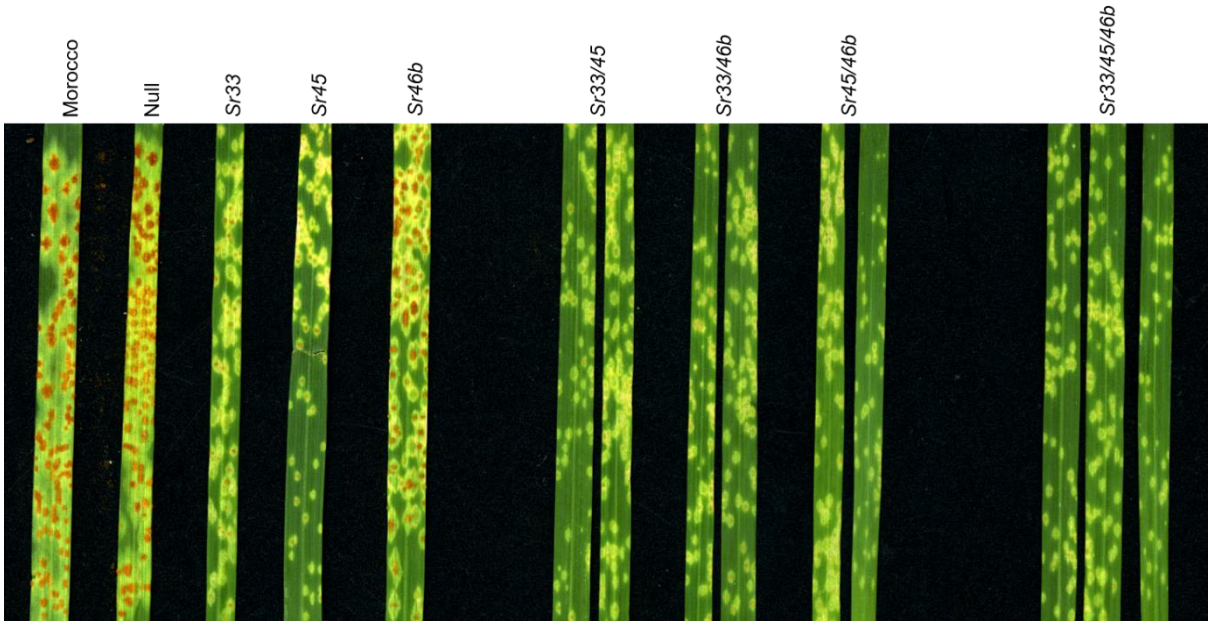

Figure 4.7 Phenotyping gene stacking lines for stem rust resistance against *Pgt* race 98-1,2,3,5,6 at 14 dpi

Morocco, Susceptible control displaying phenotypic score 4 on the Stakman scale; *Null*, the negative line for all the three (*Sr33, Sr45* and *Sr46b*) gene specific markers displaying susceptible phenotype. *Sr33, Sr45* and *Sr46b* are the single gene carrying lines in the population showing 2-, ;1-, and 2+ respectively. The multigene combination carrying lines (*Sr33/Sr45, Sr33/Sr46b, Sr45/Sr46b* and *Sr33/Sr45/Sr46b*) showing strong (;,;1=) resistance phenotypes.

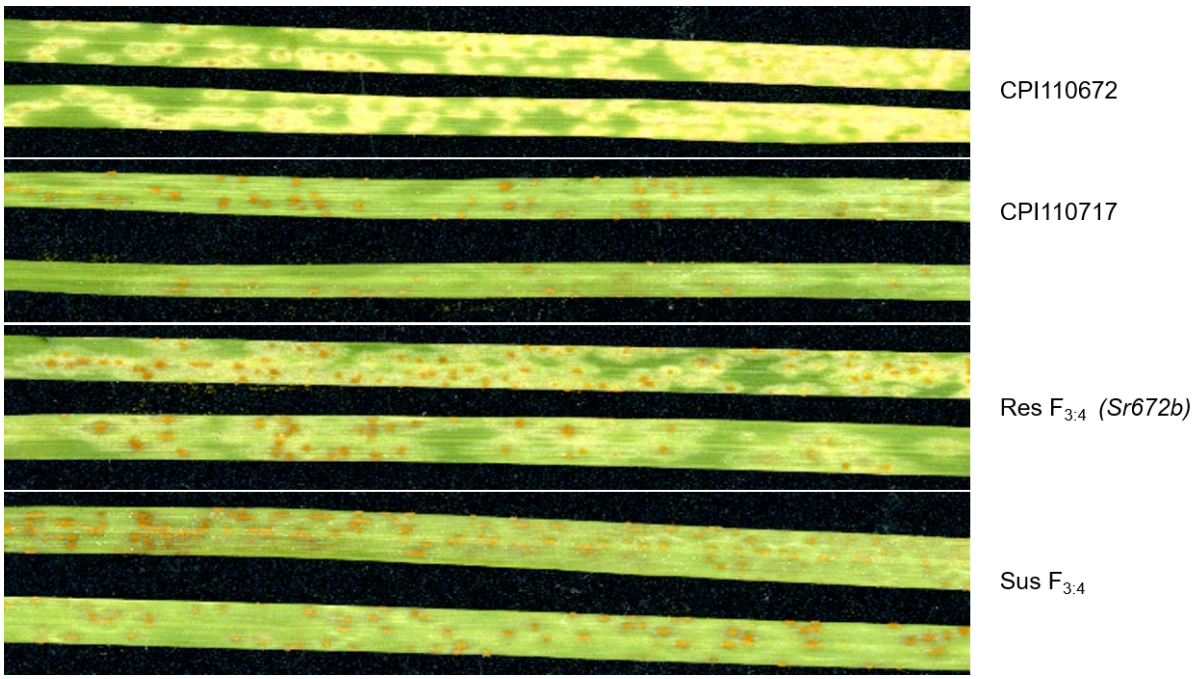

Figure 4.8 Phenotyping the segregating population generated from a single *Sr46b* negative F_2 line but segregating for *Sr672b* at 14 dpi

Sr672b $F_{3:4}$ mapping population for rust resistance to Australian *Pgt* race 98-1,2,3,5,6. CPI110672, Resistant Parent carrying two stem rust resistance genes (*Sr46b*+*Sr672b*) showing IT ;1-. CPI110717, Susceptible parent with IT 3+. Resistant $F_{3:4}$ line for *Sr672b* showing 2+C and Susceptible $F_{3:4}$ line with 3+ phenotype.

Chapter 5 - Fine mapping leaf rust resistance in the *Aegilops tauschii* accession CPI110672

5.1 Abstract

Leaf rust, one of the three wheat rust diseases, is caused by the fungal pathogen *Puccinia triticina* (Pt). In this study, we fine mapped the leaf rust resistance gene *Lr672* located on the short arm of chromosome 2D of *Aegilops tauschii* accession CPI110672. Based on the US National Small Grains Collection information, CPI110672 is synonymous with an *Ae. tauschii* accession TA1675 where this leaf rust resistance was earlier named as *Lr39*. Hence the resistance gene *Lr672* from CPI110672 is hereafter referred as *Lr39*. To identify the candidate genes for *Lr39*, we developed a physical map for the gene locus using the CPI110672 genome and the reference sequences of Chinese Spring (IWGSC RefSeq v1.0) and *Ae. tauschii* (AL8/78 v4.0). As most of the earlier known rust resistance genes belong to *CC-NBS-LRR* (*CNL*) genes, CPI110672 contigs mapped to the *Lr39* locus were analysed specially for *CNL* related sequences. Markers developed from the candidate gene sequences were mapped using the CPI110672 x CPI110717 $F_{2:3}$ mapping population. As the markers tested co-segregate with leaf rust resistance they accelerate deployment of *Lr39* resistance into commercial wheat through marker assisted selection.

5.2 Introduction

Leaf rust, caused by the biotrophic fungal pathogen, *Puccinia triticina* Erikss, is a common fungal disease affecting wheat (*Triticum aestivum* L. 2n = 6x = 42, AABBDD) worldwide (Huerta-Espino et al. 2011). The leaf rust pathogen can adapt to a wide range of climatic conditions, thus is prevalent in diverse wheat growing regions. In Australia, leaf rust is prevalent in both the eastern and western wheat belts, and nearly 30 races of leaf rust corresponding to six lineages have been described (Riaz 2018). In most cases, leaf rust strains in Australia were exotic incursions that later acquired mutations to diversify into new races. Despite various strategies, the use of genetic resistance remains the most economically feasible and sustainable method to manage leaf rust.

Similar to other rust diseases, genetic resistance to wheat leaf rust is also of two types, seedling or all-stage resistance and adult plant resistance. To date, about

73 leaf rust resistance genes have been catalogued from wheat and its relatives (Park 2016c; McIntosh et al. 2017). However, many of the all-stage resistance (*R*) genes deployed in elite cultivars have been overcome by leaf rust through mutations. For instance, the gene *Lr24* provided resistance in Australian wheat belts for nearly 20 years, but has since been overcome by the pathotype 104–1,2,3,(6),(7),11,13 (Park et al. 2002). Hence, to counteract the threat from emerging pathogen races, a continuous supply of new sources of resistance are essential.

Recently, accessions of *Aegilops tauschii,* the D genome progenitor of bread wheat, were identified as a valuable resource for resistance to leaf rust (Arora et al. 2019). However, in the six leaf rust resistance genes (*Lr21, Lr22a, Lr32, Lr39, Lr42,* and *Lr43*) identified from *Ae. tauschii,* only *Lr21* and *Lr22a* are cloned, for which perfect markers are available for their rapid selection in resistance breeding (Huang et al. 2003; Thind et al. 2017). With the evolution of new races, these genes are inadequate to enable the deployment of multi-gene combinations to prevent resistance breakdown. Furthermore, the availability of tightly linked or perfect markers for additional genes will enable the rapid introgression of these genes and their selection in the absence of virulent rust races.

In this chapter, we aim to fine map and identify candidate genes for the leaf rust resistance gene *Lr672* (refer to Chapter 3) by a comparative genomic approach using the reference genome sequence of *Ae. tauschii* AL8/78 v4.0 and the hexaploid wheat Chinese Spring IWGSC RefSeq v1.0 (Luo et al. 2017; IWGSC 2018) (https://urgi.versailles.inra.fr/blast_iwgsc/blast.php ; http://aegilops.wheat.ucdavis.edu/ATGSP/blast.php). The genomic sequence and the assemblies of CPI110672 and CPI110717 were sourced from the open wild wheat consortium (OWWC; Arora et al. 2019) .

5.3 Materials and Methods

5.3.1 Evaluation of *Lr672* relationship to *Lr39*

The geographic survey details of CPI110672 was verified with the US-National Plant Germplasm System (https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1556592). Genetic diversity information was further verified with the recent genetic analysis of 150 non-redundant *Ae. tauschii* accessions (Arora et al. 2019).

5.3.2 Genetic material and genome sequence

Genomic DNA of the F_2 mapping population (CPI110672 x CPI110717) from Chapter 3 was used to genotype the newly developed markers for linkage analysis. The whole genomes of parental accessions CPI110672 (BW_01188), CPI110672 synonymous accessions TA1675 (BW_01115), AE213 (BW_01024) and CPI110717 (BW_01187) were sequenced by the open wild wheat consortium (OWWC; http://www.openwildwheat.org) and for the AgRenSeq study by Arora et al (2019). The *de novo* assembly of BW_01115 representing CPI110672 and BW_01187 (CPI110717) assembled using CLC Assembly Cell were obtained from John Innes Centre, Norwich, UK. The raw sequences of the CPI110672- synonymous accession AE213 (BW_01024) were retrieved from OWWC and *de novo* assembly was performed using CLC workbench.

5.3.3 Fine mapping *Lr39*

To identify tightly linked markers, genomic regions corresponding to the SNP markers *Csq4* and *Csq5* (refer to Chapter 3) were identified in the *Ae. tauschii* accession AL8/78 draft genome sequences (http://probes.pw.usda.gov/WheatDMarker/phpblast/blast.php) and hexaploid reference sequence CS IWGSC RefSeq v0.4 from IWGSC (https://urgi.versailles.inra.fr/blast_iwgsc/blast.php) using the BlastN program. Scaffolds that corresponded to the interval within the flanking markers were shortlisted for additional marker identification. The DNA sequences of the selected scaffolds were masked for repeat sequences using the REPEATMASKER program (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) (Smit et al. 2017) and the low copy sequences were used for marker development. Additionally, contigs of the CPI110672 and CPI110717 assembly were compared for the identification of polymorphic markers between the two parental genotypes (Table 5.1; Supplementary file 3). Amplification of newly developed markers was performed in a 20 µl PCR reaction containing 100 ng of genomic DNA, 1X GoTaq Flexi green buffer, 1.5mM $MgCl_2$, 200 µM dNTP, 200 nM each of forward and reverse primers and one unit of *Taq* polymerase (M829B, Promega, USA). PCR was carried out in BioRad thermal cycler using a touch down program as follows: denaturation at 94°C for 30 seconds; annealing at 65°C for 30 seconds and a decrement of 1°C per cycle; extension at 72°C for 1 minute 20 seconds followed by repeating the steps for 14 cycles; after the

enrichment the program continued for 29 cycles to follow: 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 40 seconds. A linkage map was constructed for the high density markers using MapDisto 2.0 Beta 86 (Heffelfinger et al. 2017) with default functional settings such as logarithm of the odds (LOD) 3.0, recombination frequency threshold (r max) of 0.30 and map exportation using R/qtl.

5.3.4 Comparative genomics for the construction of *Lr39* physical map

Markers closely linked to the leaf rust resistance locus werw mapped on the reference genome sequences of CS IWGSC RefSeq v1.0 and AL8/78 v4.0 to identify their physical position using URGI BlastN program (Chinese Spring https://urgi.versailles.inra.fr/blast_iwgsc/blast.php; AL8/78 http://aegilops.wheat.ucdavis.edu/ATGSP/blast.php). Low copy DNA sequences from the contigs, mapped within the flanking marker interval, were shortlisted for candidate gene analysis.

5.3.5 *CC-NBS-LRR (CNL)* genes as candidates for *Lr39* resistance

As most previously known rust resistance genes in wheat are *CNL* genes, we specifically targeted the screening of this specific gene family from Chinse Spring, AL8/78 and the CPI110672 (BW_01115 and BW_01024) genomes for *Lr39*. Initially, we listed all the annotated *CNL* related sequences from the flanking marker delimited sequences of Chinese Spring and AL8/78. Next, using the Blast tool in CLC workbench we identified their homologous members from CPI110672 and analysed them for linkage with leaf rust resistance.

Alternatively, to ensure the complete coverage of all *CNL* genes from the *Lr39* region of CPI110672, we also analysed the CPI110672 genome sequences (BW_01115 and BW_01024) for *CNL* related sequences using NLR-parser v2 (github/steuernb/MutRenSeq; Steuernagel et al. 2015). The genetic identity of accessions BW_01115 and BW_01024 were verified using the predicted complete *CNL* sequences by performing BLastN analysis in CLC workbench. Contigs containing *CNL* were first mapped to the reference sequences of hexaploid wheat CS IWGSC RefSeq v1.0 (IWGSC 2018; Steuernagel et al. 2018) and *Ae. tauschii* AL8/78 v4.0 (Luo et al. 2017) to identify contigs specific to Chr group 2. Next, *CNL* related sequences within the shortlisted contigs and carrying all the essential motifs of a *CNL* gene were specifically targeted for further analysis. Markers specific to these *CNL*

candidates were genotyped using the CPI110672 x CPI110717 F_2 mapping population to predict the candidate linked with *Lr39* resistance.

5.4 Results

5.4.1 Genetic relationship of *Lr672* and *Lr39*

Evaluation of information from the US National Plant Germplasm System identified the synonymous names for accession CPI110672, which is maintained at various institutes worldwide with unique institute-specific identifiers. CPI110672 is the Commonwealth Plant Introduction (CPI) number designated at the CSIRO, Canberra, Australia. Similarly, PI603236 (National Small Grains Collection), TA1675 (Wheat Genetics Resource Center), 01C215044 (Research Institute for Plant Production), AE213 (IPK Gatersleben), AUS24014 (Australian Winter Cereals Collection), G3427 (University of California), ICAG400669 or IG47218 (Int. Center for Agricultural Research in the Dry Areas), WIR249 (N.I. Vavilov Research Institute of Plant Industry), WX766 (International Maize & Wheat Improvement Center) are identifiers designated to this accession by different institutes. As part of the Open Wild Wheat Consortium (OWWC) at John Innes Centre, UK, CPI110672 was designated BW_01188 while the synonymous accessions TA1675 and AE213 were designated BW_01115 and BW_01024, respectively. Based on the previous studies, the accession TA1675 was one of the donor lines for the leaf rust resistance gene *Lr39/Lr41* (Raupp et al. 2001; Singh et al. 2004). Therefore, we designate the *Lr672* leaf rust resistance locus mapped in this study as *Lr39*.

5.4.2 High-resolution mapping of *Lr39*

The leaf rust resistance locus *Lr39 (Lr672)* was mapped to chromosome 2DS of CPI110672 flanked by *Csq4* and *Csq5* markers (refer to Chapter 3). Anchoring the flanking markers to the reference sequence of Chinese Spring IWGSC v0.4 and AL8/78 (Jia et al. 2013b; Luo et al. 2013) identified scaffolds within the interval. The scaffolds were analysed for low copy sequence to generate different types of markers that are polymorphic between the two parental lines. PCR amplified fragments that did not show size polymorphism in the gel were sequenced to identify single nucleotide (SNP) level polymorphisms. Through this process, we identified CAPS, SSR and STS markers namely *Csq8* (CAPS), *Csq10* (SSR), *Csq12* (STS), *Csq13* (STS), *Csq14* (CAPS), *Csq23* (STS) and *Csq24* (STS) (Table 5.1). Homology analysis of the

genome sequence contigs of parental lines CPI110672 and CPI110717 identified more markers *Csq20, Csq21* and *Csq22* that were validated by genotyping the parental lines and an artificial heterozygote (mixing equal concentrations of DNA). Codominant markers are later used to genotype the CPI110672 x CPI110717 F_2 mapping population (Supplementary file 2). Linkage map construction revealed marker *Csq21* and *Csq22* as closely linked flanking markers for the leaf rust resistance locus located 0.4 cM from the gene (Figure 5.1).

5.4.3 Generation of physical map for the *Lr39* locus

The genetic map developed in this study was compared with the Chinese Spring (IWGSC RefSeq v1.0) and AL8/78 v4.0 reference sequences to construct a physical map for the *Lr39* locus. All flanking markers were anchored on both reference sequences and their physical positions were determined. All markers were identified on chromosome (Chr) arm 2DS of Chinese Spring while most of the markers located on the super scaffold_652 with a few markers located in scaffolds belonging to Chr2 in AL8/78 reference sequence (Table 5.2). The physical interval of the Chinese Spring Chr2D region between the flanking markers was about 1.26 Mb, while in AL8/78 it was 1.28 Mb (Figure 5.2). Subsequently, the high confidence genes annotated in the flanking marker delimited region were analysed for candidate genes.

5.4.4 *CNL* genes as candidates for *Lr39* resistance

In the Chinese Spring reference genome, the physical map region that corresponds to the *Lr39* locus of CPI110672 were predicted to carry five *CNL* related genes *(TraesCS2D01G001000, TraesCS2D01G001100, TraesCS2D01G001200, TraesCS2D01G001600* and *TraesCS2D01G001800)* referred to here as *CS2DCNL1, CS2DCNL2, CS2DCNL3, CS2DCNL4* and *CS2DCNL5,* respectively. In the case of AL8/78 there were four *CNL* genes *(AET0Gv20007400, AET0Gv20009100, AET0Gv20010000* and *AET0Gv20010200)* named here as *At2DCNL1, At2DCNL2, At2DCNL3* and *At2DCNL4,* respectively.

In the second analysis, the genome sequence assemblies of CPI110672 (BW_01115) annotated for the *CNL* gene using NLR parser v2 (Steuernagel et al. 2015) identified 1571 *CNL* type sequences. Of these, 331 predicted sequences were identified to encode the essential motifs needed to produce a complete *CNL* protein, while 1240 sequences lacked one or more of them and were categorised as partial.

We mapped all the *CNL* sequences to the CS IWGSC RefSeq v1.0 and AL8/78 v4.0 reference genomes and identified the physical positions for 320 of the 331 complete *CNL* sequences. The physical position of the remaining 11 complete *CNL* sequences are unknown thus may be unique to the CPI110672 genome (Table 5.3; Supplementary file 4).

Subsequently, five *CNL* containing contigs (contig_121758_2, contig_233763_1, contig_196332_1, contig_196350_1 and contig_89858_1) mapped to the delimited region for *Lr39* in both CS and AL8/78 reference sequences and we referred to hereafter as *6722DCNL1, 6722DCNL2, 6722DCNL3, 6722DCNL4* and *6722DCNL5,* respectively (Table 5.4). However, only one (*6722DCNL5*) of the five *CNL* sequences is predicted to encode complete *CNL* motifs and the remaining four are predicted to encode partial *CNL* motifs. The genes *6722DCNL1* and *6722DCNL3* are homologues of the previously identified *CNL* sequences in both CS (*CS2DCNL1* and *CS2DCNL4*) and AL8/78 (*At2DCNL1* and *At2DCNL2)*. Gene *6722DCNL2* is homologous to the annotated *CS2DCNL3,* and to an unannotated region in thesuper_scaffold_652 of AL8/78. In the case of *6722DCNL4*, and *6722DCNL5,* they aligned to *At2DCNL3* and *At2DCNL4,* respectively in AL8/78 and did not have significant homology in Chinese Spring sequences. Homology analysis of all five predicted CPI110672 *CNL* sequences with the CPI110717 contigs identified diversity among parental accessions. Four of five predicted *CNL* sequences (*6722DCNL1, 6722DCNL3, 6722DCNL4* and *6722DCNL5)* were present only in the resistant parent CPI110672. Despite the absence of the complete *CNL* sequence *6722DCNL5* in the susceptible parent CPI110717, a homologous sequence closely linked to the candidate was identified in both parents for marker development.

Targeting the diversity among parental accessions identified markers closely linked to these *CNL* genes. We developed three dominant markers specific to the three partial *CNL*s (2D93, 2D94 and 2D95) and co-dominant markers *Csq25* and *Csq29* closely linked to *6722DCNL2* and *6722DCNL5,* respectively. Based on the sequence diversity among parental contigs, we developed additional co-dominant markers (*Csq26, Csq27, Csq28* and *Csq30*) linking these candidates (Table 5.1; Supplementary file 3). Genotyping the CPI110672 x CPI110717 F_2 mapping population with the new markers and construction of a linkage map revealed all these

markers co-segregated with the *Lr39* locus (Figure 5.1). The physical map was subsequently updated (Figure 5.2, Table 5.3).

Since *6722DCNL5* is the only candidate predicted to contain the complete *CC-NBS-LRR* architecture in the *Lr39* locus, we evaluated it critically. The predicted genomic sequences for the *6722DCNL5* are 99% identical to annotated region *At2DCNL4* of AL8/78 while the entire region is absent in the Chinese Spring and the susceptible parent CPI110717. Furthermore, the deduced amino acid sequences from the FGENESH (Solovyev et al. 2006) predicted coding sequences of *6722DCNL5* and *At2DCNL4* also confirmed that they are 99% identical, with one amino acid difference. However, evaluation of the 5' regulatory sequences (approximately 2 kb upstream of the predicted start codon) of *6722DCNL5* and *At2DCNL4* revealed significant sequence diversity including approximately a 150 bp deletion likely in the promoter region (approximately between 750 and 900 bp upstream of the putative start codon). Therefore we hypothesise this could be a potential candidate gene for *Lr39* resistance. Further studies on isolation and characterisation of *6722DCNL5* are essential to validate the effect of *6722DCNL5* on leaf rust resistance.

Additionally, we attempted to verify the genetic identity of TA1675 with the AE213 (BW_01024). Homology analysis of all (331) complete *CNL* sequences of BW_01115 showed 317 *CNLs* had 100% coverage length homology to the BW_01024 contigs. The remaining 14 complete *CNLs* showed over 99% identity to BW_01024 sequences thus confirming the genetic identity of CPI110672 and AE213 (Supplementary file 5). Comparatively, BW_01115 showed higher complete *CNL* types than the BW_01024 and vice versa in the case of partial *CNL* sequences. This may be due to the difference in sequencing coverage and the assembly platforms.

5.5 Discussion

The accession CPI110672 was originally collected from Turkmenistan by N.I. Vavilov at All-Russian Scientific Research Institute of Plant Genetic Resources, St. Petersburg, Russian Federation and was shared worldwide for transferring various agronomic traits, particularly disease resistance, into elite wheat varieties (Norris 1998). Geographic survey data at the US National Plant Germplasm System (https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1556592) and other studies identified synonymous names for CPI110672 (PI603236, TA1675,

01C215044, AE213, AUS24014, G3427, ICAG400669, IG47218, WIR249, WX766, BW_01024, BW_01115 and BW_01188) maintained at different research organisations around the world (Norris 1998; Jones et al. 2013; Arora et al. 2019). A recent genetic analysis for 195 *Ae. tauschii* accessions included BW_01024, BW_01115 and BW_01188, where BW_01188 and BW_01115 were found to be identical, while BW_01024 is distinct (Arora et al. 2019). It probably due to more unresolved SNP allelic discrimination among BW_01188/ BW_01115 and BW_01024 accessions in their study. Therefore, the accessions BW_01115 and BW_01024 were considered non-redundant and included for the whole genome shotgun sequences by the OWWC. Our verification of the SNP alleles revealed genetic identity among the three accessions BW_01024, BW_01115, and BW_01188 (Arora et al. 2019). Besides, blast homology of predicted CNL sequences from BW_01115 identified 100% homology in the accession BW_01024. Therefore, we confirm CPI110672 (BW_1115), TA1675 (BW_01188), and AE213 (BW_01024) are genetically the same accession.

The leaf rust resistance gene *Lr672* identified in this study was mapped to the short arm of chromosome 2D. So far chromosome 2DS of *Ae. tauschii* has contributed *Lr22a, Lr39, Lr41* and *LrT* genes that were previously introgressed into hexaploid wheat (Singh et al. 2004; Hiebert et al. 2007; Saluja et al. 2017). The leaf rust resistance gene *Lr22a* is an adult plant resistance gene that was cloned recently and is from a different locus than *Lr39* (Thind et al. 2017). The leaf rust resistance genes *Lr39, Lr41* and *LrT* are deemed to be located at the same locus. The *Lr39* gene was mapped to the short arm of chromosome 2D, where it is positioned distal to the marker *Xgdm35* (Singh et al. 2004). It was later identified to be located distal to the SSR marker *Xbarc124,* which is closer than *Xgdm35* (Sun et al. 2009). *Lr39* and *Lr41* were reported to be allelic, but their relationship with *LrT* is unknown (Singh et al. 2004; Saluja et al. 2018). On the genetic map, *LrT* is positioned distal to the markers *Xbarc124* and *Xgdm35,* similar to the position of *Lr41* suggesting *LrT* could be allelic to *Lr39/Lr41* (Sun et al. 2009; Saluja et al. 2018). *LrT* has been reported to confer resistance to at least 10 leaf rust races in India and there is no virulence recorded so far (Saluja et al. 2018). Since the leaf rust resistance gene identified in this study is also located distal to the markers *Xbarc124* and *Xgdm35* (Table 5.3), evaluation of geographic survey details of the accession CPI110672 revealed it is synonymous to

the accession TA1675. *Lr39* has been identified and introgressed independently from at least five *Ae. tauschii* accessions, of which TA1675 was one (Raupp et al. 2001; Singh et al. 2004). Thus, we confirm the leaf rust resistance *(Lr672)* mapped in this study is *Lr39.* The *Lr39* gene has been widely deployed in commercial wheat cultivars in North America, however, leaf rust races virulent on *Lr39* have been observed in recent years (Kolmer 2013). Although it is not widely deployed in Australia, virulence on *Lr39* has been observed (Bariana et al. 2007). In Australia, the leaf rust races are highly diverse, and to date nearly 30 new races have been observed, however many of them have only occurred once (Riaz 2018). Despite the prevalence of virulence to *Lr39*, isolation and cloning would be crucial to understanding the allelic relationship between *LrT*, marker assisted deployment, possibility of pyramiding with other genes, gene editing to enhance resistance and to identify the corresponding avirulence genes from the virulent leaf rust races.

Comparative genomic analysis using the hexaploid wheat reference sequence CS IWGSC RefSeq v1.0 predicted the 5.35 Mb physical region that corresponds to the *Lr39* locus and has 10 NLR (*CNL*) genes mapped distal to the marker *Xbarc124* (Steuernagel et al. 2018). The markers identified in our study, *Csq21* and *Csq22* flanked the *Lr39* locus, particularly *Csq22* linked closer than the *Xbarc124.* As the proximal marker *Csq22* is recombining in the CPI110672 x CPI110717 $F_{2:3}$ population, it eliminated at least five of 10 previously predicted *CNL* loci from the Chinese Spring reference. The 1.3 Mb region of Chinese Spring and AL8/78 contains five (*CS2DCNLs*) and four (*At2DCNLs*) *CNL* sequences, respectively. Annotation of the CPI110672 genome assembly for *CNL* sequences predicted five *CNL* containing contigs mapped to the targeted *Lr39* region that are homologous to *CS2DCNLs* and *At2DCNLs* genes. However, only three *CNLs* are present in all three (CS, AL8/78 and CPI110672) genomes analysed. Similarly, comparison of the *CS2DCNLs* and *At2DCNLs* of CS and AL8/78 reference sequences with each other also revealed differences suggesting genetic diversity in the 2D chromosome probably associated with unusual recombination altering the copy numbers of the *CNLs* (Thind et al. 2018). A recent study where a high-quality genome assembly of the chromosome 2D of the hexaploid wheat line CH Campala compared with the Chinese Spring sequence revealed more than 100 kb InDel difference in the short arm of chromosome 2D (Thind et al. 2018). Similarly, the 2D chromosome of the AL8/78 v4.0 reference sequences

also seemed to have an unusual breakpoint because the majority of the *Lr39* co-segregating markers and the *CNL* contigs were mapped to the superscaffold_652 covering nearly 1.2Mb sequences. The inconsistent *CNL* homology among the three genomes and the presence of unmapped superscaffold_652 of AL8/78 in the target region indicates complexity in chromosome 2D and the possibility of a unique *CNL* region.

The markers developed in this study specifically for the candidate *CNLs* from the CPI110672 genome co-segregated with the *Lr39* locus. The flanking markers identified in this study reduced the target genomic region from over 5 Mb to less than 1.3 Mb and were limited to only one full *CNL* candidate gene. However, the candidate gene is 99% identical to the annotated *CNL* gene in the superscaffold_652 of AL8/78, while absent in the susceptible parent CPI110717 and in the hexaploid reference genome sequences. Due to high homology of the coding region of the candidate *CNL* between CPI110672 and AL8/78, we evaluated the 5' regulatory sequences where we identified differences. *Cis*- regulatory elements (CREs), typically non-coding DNA sequences such as promoters, enhancers, insulators and silencers are also key components for regulation of gene expression. Divergence in the CREs particularly in promoter and enhancers are common among species (Wittkopp and Kalay 2011). Such divergence in CREs alters transcription factor binding, thereby limiting gene expression (Bilas et al. 2016). Point mutations and deletions are the most common form of polymorphism in CREs among species. Multiple *cis*-elements were reported to regulate *OsWRKY13* in response to pathogen invasion in rice (Cai et al. 2008). Similarly, the pathogenesis-related genes (*PR1* and *PR2*) in *Arabidopsis thaliana* are also regulated by various positive and negative CREs. So far no such report is available for CREs regulating wheat *PR* genes during rust infection (Zhang et al. 2018). Therefore, cloning the candidate gene with its corresponding regulatory sequence from both the accessions, CPI110672 and AL8/78 will help unravel this mystery. The distribution of *Lr39* co-segregating markers covering the 1.2 Mb region will also serve as a valuable tool for screening *Ae. tauschii* germplasm for the *Lr39* haploblock. These markers will also be useful in wheat breeding programs to deploy the *Lr39* in more commercial cultivars either singly or in gene stacks.

Table 5.1 List of CAPS, SSR, and STS markers developed to fine map *Lr39*

Marker name	Primer sequence	Amplicon (bp)	Remarks
Csq8	AATCACATGATCCCTGCCCT ACAATTAAGGTGTTGTCTGC	Refer Suppl. file 2	*Hpa*I or *Hpy*CH4IV
Csq10	TGCCCAATAACATGCAACACA TCCATGTAGGAAGCCAAAGG	R:220 S:198	--
Csq12	CAGCTGCCATGAGTCTCTCG TGCTGATTTGAGATGCACTG	R:751 S: 917	--
Csq13	AATTGGAGTGCTGCCAAGGA GGATGCTGATAGAATGTCTCA	R:1056 S:866	--
Csq14	ACTGAACTGCCGTCCTTTGT ACTGAAAGAGCCCATTCGCT	R:638,300,240 S:938,240	*Hpy*CH4IV
Csq20	TGACGTGTTGGAGATTCGTGC CGTCTATGGCCACCTGGAGA	R:248 S:274	--
Csq21	ACATCTTGGCCTCGCATATC GAGTTTTGGGCGGCTGATCTCCCG	R:343 S:217,130	*Hpy*CH4IV
Csq22	GCAAAGCGGATTACGTCCCCGAG CAGTGCGGTCAGCGGAATCG	R:1050 S:459	--
Csq23	GATATTGGTCAGGTCAAGGCCA TGATCTGATCTGATCCAAGTCAG	R:293 S:283	--
Csq24	CAATTCATGCGTGTGGAACG TCTGAATCATGACGCACCGA	R:358 S:197	--
Csq25	TACCTTAATAAACCAAGGTTC GCATAGCAACCCACATTGCA	R:342 S:360	--
Csq26	AGAGAAACTTGGCACACTGC AAGTATTCACATCAGCCACT	R:466 S:413	--
Csq27	TGGTGTCTTGCATCGGCTTG AAGCCCGACAGAAGTGGCACG	R:348 S:389	--
Csq28	CCCCATCCTGAGAGCAAAGT TGTCATGCCGTCAGCCAAGC	R:252 S:294	--
Csq29	GAGATAGAGGCATGACCAGC CACTGTACGATTAGCCAGGGACC	R:792 S:327	--
Csq30	GAGGACCAAGCAACCACACG GATAGACACACGATGTAACC	R:153,46 S:203	*Sph*I
2D93	AACACCATCCACCATCTCTGG AATTTCTCTAGCCTCATTTTGT	482	Resistant specific
2D94	AGACGATTGGTGCAGACAGC GGTCGTAGATCCTGGCAGCT	1107	Resistant specific
2D96	ACAACTCAGGTGGTGCAGGTCCCG CCAATCTCCACATGGCAGCTT	473	Resistant specific

Table 5.2 Physical position of the *Lr39* genetic map in Chinese Spring IWGSC RefSeq v1.0 and AL8/78 v4.0. Flanking markers are highlighted green

Chinese Spring IWGSC RefSeq v1.0				***Ae. tauschii* AL8/78 v4.0**			
Locus	**Chromosome 2D**		**Description**	**Locus**	**Start (bp)**	**End (bp)**	**Description / Location**
	Start (bp)	**End (bp)**					
Csq4	276619	276519	--	*Csq4*	87498	87598	Super scaffold_652
Csq10	371006	371205	--	*Csq10*	376344	376573	Super scaffold_652
Csq20	385111	385384	--	*Csq20*	387921	388168	Super scaffold_652
Csq21	398698	398356	Flanking marker	*Csq21*	398865	399204	Super scaffold_652
2D93	475976	476457	Co-segregating dominant	*2D93*	503434	503915	AET0Gv20007400
TraesCS2D01G001000	460961	479644	NBS-LRR resistance-like protein	AET0Gv20007400.13	485227	506926	uncharacterised
TraesCS2D01G001100	508313	509233	Disease resistance protein (TIR-NBS-LRR class) family	--	--	--	--
TraesCS2D01G001200	514511	516352	Disease resistance protein (TIR-NBS-LRR class) family	Not annotated	538426	542069	Super scaffold_652
Csq25	519238	518907	Co-segregating marker	*Csq25*	1360729	1361079	Super scaffold_652
Csq26	676790	677202	Co-segregating marker	*Csq26*	1250724	1251136	Super scaffold_652
Csq27	682076	684242	Co-segregating marker	*Csq27*	1243655	1245868	Super scaffold_652
Csq8	693300	694200	Co-segregating marker	*Csq8*	1226974	1227846	Super scaffold_652
Csq28	1512836	1513087	Co-segregating marker	*Csq28*	1491685	1491970	Super scaffold_652
TraesCS2D01G001600	1522616	1526387	Disease resistance protein (TIR-NBS-LRR class) family	AET0Gv20009100	1396595	1400503	NBS-LRR
2D94	1524405	1525521	Co-segregating dominant	*2D94*	1398714	1399830	AET0Gv20009100

--	--	--	--	AET0Gv20010000	1503089	1506106	LRR containing protein
2D96	1523424	1523902	Co-segregating dominant	*2D96*	1503909	1504381	AET0Gv20010000
--	--	--	--	AET0Gv20010200	1544137	1549027	CC-NBS-LRR
TraesCS2D01G001800	1601514	1610005	Disease resistance protein RPM1	--	--	--	--
Csq29	1605966	1606518	Co-segregating marker	*Csq29*	1546698	1547489	Super scaffold_652
Csq30	1639288	1639102	Co-segregating marker	*Csq30*	94775	94979	Chr2D
Csq22	1667499	1668548	Flanking marker	*Csq22*	139762	140218	Chr2D
Csq12	1990194	1989498	--	*Csq12*	1149382	1150078	Chr2D
Csq13	2469361	2468398	--	*Csq13*	1615941	1616904	Chr2D
Csq23	2507307	2507025	--	*Csq23*	1653274	1653556	Chr2D
Csq24	15635552	15635711	ChrUn	*Csq24*	1730794	1731113	Chr2D
Csq14	2681697	2682435		*Csq14*	1751891	1752629	Chr2D
Xbarc124	5227524	5228450	Flanking marker (Sun et al. 2009)	*Xbarc124*	4441837	4442769	Chr2D
Csq5	5234313	5234562	SNP-Id:IWB64398	*Csq5*	4448441	4448690	Chr2D
Csq6	6425588	6425488	SNP-Id:IWB25534	*Csq6*	5684448	5684545	Chr2D
Csq7	9344557	9344806	SNP-Id:IWB15863	*Csq7*	8290367	8290616	Chr2D
Xgdm35	13754096	13754194	Flanking marker (Singh et al. 2004)	*Xgdm5*	13512122	13512290	Chr2D

Table 5.3 Predicted *CC-NBS-LRR* genes in the CPI110672 genome assembly

Genome assembly	No of contigs ≥1000 bp	No of *CNLs* annotated ≥1500 bp		No. of *CNL*s mapped on CS-IWGSC RefSeq v1.0		No. of *CNL*s mapped on AL8/78 v4.0		No of unmapped *CNL*	
		Complete	Partial	Complete	Partial	Complete	Partial	Complete	Partial
BW_01115	246,758	331	1240	288	1065	287	1119	11	40
BW_01024	183,465	252	1463	161	1026	217	1174	20	204

Table 5.4 List of *CNL* encoding CPI110672 contigs mapped to the delimited region in CS and AL8/78

CNL contig	Designated name	Complete /partial	Homologues in reference genomes	
			Chinese Spring	*Ae. tauschii* AL8/78
Contig_121758_2	*6722DCNL1*	Partial	TraesCS2D01G001000	AET0Gv20007400.13
Contig_233763_1	*6722DCNL2*	Partial	TraesCS2D01G001200	Unannotated region
Contig_196332_1	*6722DCNL3*	Partial	TraesCS2D01G001600	AET0Gv20009100
Contig_196350_1	*6722DCNL4*	Partial	--	AET0Gv20010000
Contig_89858_1	*6722DCNL5*	Complete	--	AET0Gv20010200

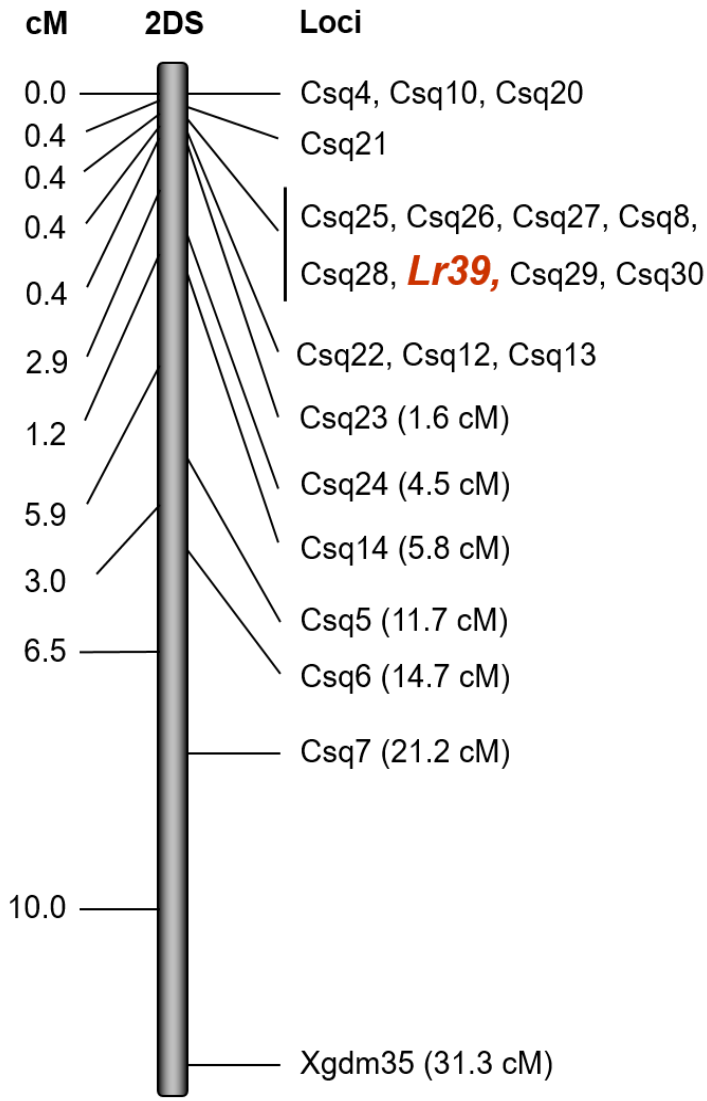

Figure 5.1 Genetic map of the *Lr39* locus on 2DS chromosome of CPI110672

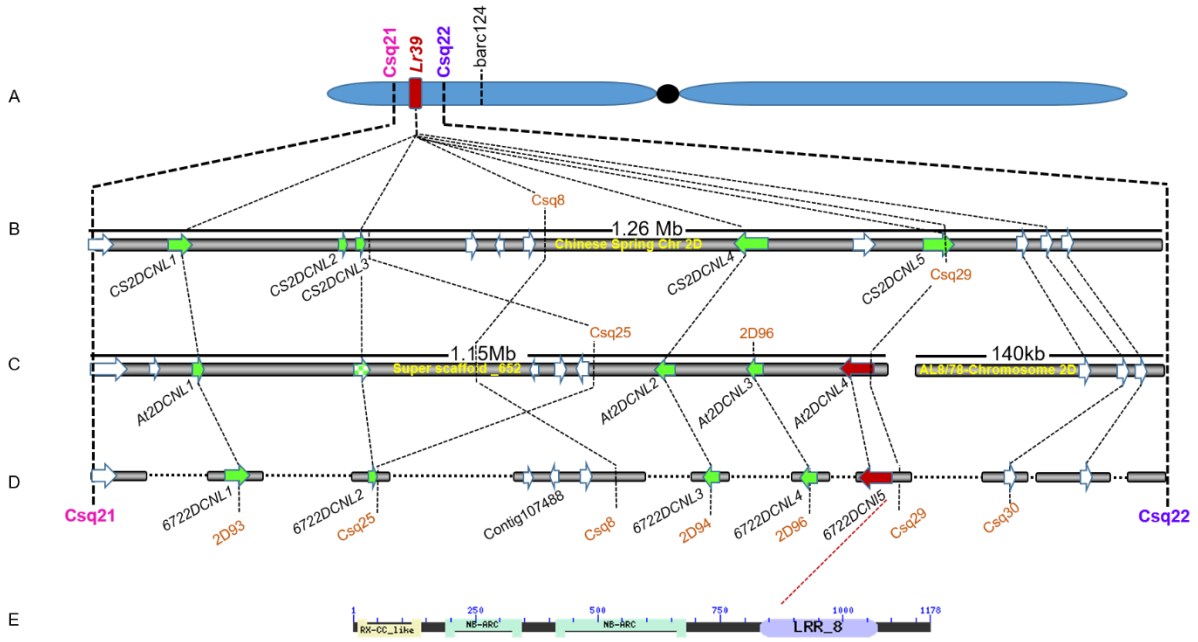

Figure 5.2 Physical map of *Lr39*

(A) CPI110672 linkage map for *Lr39* on chromosome 2DS chromosome. (B) The collinear region of *T. aestivum cv.* Chinese Spring (IWGSC RefSeq v1.0) is within the flanking markers, (C) Collinear region of *Ae. tauschii* accession AL8/78 v4.0 is identified within the flanking markers. (D) CPI110672 contigs containing the predicted *CNL* genes mapped to delimited region Chinese Spring and AL8/78 flanked by *Csq21* and *Csq22* markers. Arrows indicate the annotated genes present in the shortlisted region. Green arrows are the annotated *CNL* genes present within the flanking marker interval. Brown arrow indicates the complete *CNL* candidate gene. (E) Predicted domain architecture for the candidate *CNL* gene.

Chapter 6 - Identification and cloning of candidate genes for stripe rust resistance in the *Aegilops tauschii* accession CPI110672

6.1 Abstract

Puccinia striiformis f. sp. *tritici* (*Pst*) is one of the most important fungal pathogens of wheat, causing stripe rust disease. In this study, a stripe rust resistance gene from *Aegilops tauschii* accession CPI110672 designated *Yr672* was fine mapped through whole genome sequence analysis in comparison with reference genome sequences of hexaploid wheat (Chinese Spring CS) and a D-genome diploid grass (*Ae. tauschii* accession AL8/78). Comparative genomics of genome sequence assemblies yielded closely linked flanking markers *Csq15* and *Csq11* located 0.4 cM distal and 0.8cM proximal to the *Yr672* locus. Anchoring the markers in the reference genome sequences resulted approximately 500 kb in IWGSC RefSeq v1.0 and 400 kb in AL8/78 v4.0 between the flanking markers. Prediction of *CC-NBS-LRR* (*CNL*) gene sequence in the CPI110672 genome assembly and mapping on to the reference sequences identified one candidate gene within the targeted region. Markers designed specifically for the candidate *CNL* gene co-segregated with the stripe rust resistance phenotype. Introgression of *Yr672* into the commercial cultivar Westonia through marker-assisted selection is confirmed through a stripe rust resistance test. Further, the candidate *CNL* gene was validated for its stripe rust resistance function through a transgenic complementation test.

6.2 Introduction

Stripe or Yellow rust caused by the obligate biotrophic fungus *Puccinia striiformis* Westend. f. sp. *tritici* Erikss. (*Pst*) is one of the most important diseases of bread wheat (*Triticum aestivum* L. 2n = 6x = 42, AABBDD). Worldwide, yield loss associated with stripe rust epidemics increases every year due to the emergence of new races. In Australia, stripe rust has become an important disease since its first incursion in 1979 (Pst79 lineage), which caused severe yield losses in the eastern wheat belt (Wellings 2007). Similarly, an incursion of a more aggressive race in Western Australia (WA) in 2002 (commonly referred as the WA pathotype) caused severe yield losses and rapidly spread across Australia and reached the eastern region within a year (Wellings 2007). Currently, the variants of WA races are predominant in Australian wheat

growing regions, overtaking several rust resistance genes and causing annual yield loss of about $127 million (Murray and Brennan 2009a).

The major epidemics of stripe rust in Australia were all due to exotic incursions, particularly from Europe and East Africa (Wellings 2007). Therefore, the emergence of new races of stripe rust threaten wheat cultivation in Australia through exotic introductions, as demonstrated by the previous incidences. Detection of a stripe rust race in 2011 in Europe that has overcome the predominant winter wheat cultivar "Warrior" poses a serious threat on a global scale due to its tolerance of warm temperatures (Hubbard et al. 2015; Hovmoller et al. 2017). Very recently, a new stripe rust race was identified in the state of Victoria, Australia that has overcome one of the most common stripe rust resistance gene (*Yr33*) present in Australian cultivars (Cuddy and Hollway 2018). So far, about 69 stripe or yellow rust resistance (*Yr*) genes have been provisionally designated (Park 2016c; McIntosh et al. 2017). However, many of them are deemed ineffective due to the frequent emergence of new races. Therefore, exploring new sources of genetic resistance to counteract new stripe rust pathogen races is essential to prevent stripe rust epidemics.

Aegilops tauschii Coss. (2n = 2x = 14), the D genome progenitor contributes one-third of the bread wheat genome and possesses high genetic diversity. Accessions of *Ae. tauschii* are grouped into two subspecies - ssp. *tauschii* and ssp. *strangulata*. The subspecies *tauschii* is distributed between Turkey and China, while the ssp. *strangulata* is distributed in two regions, the Caspian Sea of Iran and Transcaucasia (Liu et al. 2013). Accessions of *Ae. tauschii* have been studied for new sources of stripe rust resistance, and it was determined that the accessions belonging to ssp. *strangulata* hold better resistance than ssp. *tauschii* accessions (Liu et al. 2013). Two stripe rust resistance genes, *Yr28* (accession WX219) and *YrAs2388* (accession AS2388) have been mapped to the short arm of chromosome 4D of *Ae. tauschii* (Singh et al. 2000; Huang et al. 2011). Both accessions originated from Iran, however, the allelic relationship between them remains unknown. To prevent future stripe rust epidemics, additional resistance genes beyond these two are needed to enable deployment of multi-resistance gene combinations.

Screening of over 400 *Ae. tauschii* accessions for stripe rust resistance identified potential accessions (Evans Lagudah, unpublished data). An *Ae. tauschii*

ssp. *strangulata* accession originating from Turkmenistan, CPI110672, possesses resistance to stripe rust races belonging to both Pst79 and WA lineages. Using an $F_{2:3}$ mapping population derived from a CPI110672 x CPI110717 cross, *Yr672* was mapped to chromosome 4DS and is flanked by markers *Csq3* and *Csq1* (refer to chapter 3). In this study, we aimed to fine map the region carrying *Yr672* to identify and validate candidate genes for the stripe rust resistance observed.

6.3 Materials and Methods

6.3.1 Plant and genetic material

Genomic DNA of the F_2 mapping population (CPI110672 x CPI110717) from Chapter 3 was used to develop closely linked flanking markers. Genomic DNA from the leaves of the genetic materials was isolated using a method described by Lagudah et al. (1991). The synthetic hexaploid wheat (SHW) LAN672, derived from a cross between Langdon and *Ae. tauschii* acc. CPI110672, was used to transfer the stripe rust resistance genes into the commercial hexaploid wheat cultivar Westonia. Genomic DNA for marker-assisted selection was isolated from seeds using the Nimbus robotic system, as described in Ellis et al. (2005). Total RNA was isolated for the transcript analysis using the Promega MaxWell® RSC Plant RNA Kit (Catalogue No: 1500), as per the manufacturer's instructions using the Promega MaxWell RSC robotic system.

6.3.2 Pathogen materials for rust phenotyping

Freshly revived spores of the Australian *Pst* races belonging to Pst79 lineage (104E137A-) and WA lineage (134E16A+) (supplementary file 1) obtained from The University of Sydney, Plant Breeding Institute, Cobbitty and maintained at CSIRO Agriculture and Food were used to screen the plant materials for seedling stripe rust resistance. Rust resistance phenotyping was carried out for screening transgenic lines and selected backcross lines (BC_1F_3) to deploy *Yr672* into a commercial cultivar performed at the controlled environment plant growth facility at CSIRO, Canberra. Approximately 15 seeds were sown to test the selected backcross lines and T_1 Transgenic lines against both stripe rust races and the experiments were repeated twice. The independent T_0 transgenic lines were tested as single plant against the Pst79 stripe rust race. Seedlings were grown at 23/17°C with 16/8 hours day/night cycle until the two-leaf stage and inoculated with the stripe rust races to determine the

disease response. Inoculated plants were maintained in a closed container to maximise humidity at 10°C in the dark for 48 hours and then moved to 18/12°C with 16/8 hours day/night regime for rust development. Rust infection was scored on the first leaf between 15 and 20 days post infection (dpi) using the 0-4 Stakman scale (Stakman et al. 1962). Rust infection types, ; and 0 were considered as immune, and 1 and 2 were considered resistant, while scores of ≥3 were considered susceptible as per the Stakman scale.

6.3.3 Fine mapping *Yr672* by comparative reference genomics

To identify additional markers, genomic regions that correspond to the closely linked SNP markers for the *Yr672* locus were identified in the *Ae. tauschii* acc. AL8/78 draft genome sequences (http://probes.pw.usda.gov/WheatDMarker/phpblast/blast.php) and hexaploid reference sequence Chinese Spring (CS) v0.4 from IWGSC (https://urgi.versailles.inra.fr/blast_iwgsc/blast.php). Scaffolds that correspond to the gene interval were shortlisted for identification of additional markers. DNA sequences of the selected scaffolds were masked for repeat sequences using the REPEATMASKER program (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) (Smit et al. 2017). The low copy sequences were selected for identification of closely linked flanking markers (Table 6.1; Supplementary file 3). Amplification of newly developed markers was performed in a 20 µl PCR reaction containing 100 ng of gDNA, 1X GoTaq Flexi green buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 200 nM each of forward and reverse primers and a unit of *Taq* polymerase (M829B, Promega, USA). PCR was carried out in BioRad thermal cycler using a touch down program as follows: denaturation at 94°C for 30 seconds; annealing at 65°C for 30 seconds with decrease in 1°C per cycle; extension at 72°C for 80 seconds followed by repeating the steps for 14 cycles; after the enrichment the program continued for 29 cycles as follows: 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 40 seconds. A linkage map was constructed for the high density markers using MapDisto 2.0 Beta 86 (Heffelfinger et al. 2017) with default functional settings such as logarithm of odds (LOD) 3.0, recombination frequency threshold (r max) of 0.30 and map exportation using R/qtl.

6.3.4 Physical map and candidate gene identification for *Yr672*

The physical position of closely linked flanking markers on the recently updated reference sequences of CS IWGSC RefSeq v1.0 and AL8/78 v4.0 were identified using the URGI BlastN program (https://urgi.versailles.inra.fr/blast_iwgsc/blast.php). We manually anchored the physical position of the closely linked markers to chromosome 4D of the CS-RefSeq v1.0 and AL8/78v4.0 reference sequence in the JBrowse genome browser (Chinese Spring https://urgi.versailles.inra.fr/jbrowseiwgsc/gmod_jbrowse/?data=myData/IWGSC_RefSeq_v1.0 ; AL8/78 http://aegilops.wheat.ucdavis.edu/jbrowse/index.html?data=Aet%2Fdata%2F&loc). Annotated high and low copy genes within the flanking marker sequence interval in both the reference sequences were shortlisted for candidate gene prediction.

6.3.5 Identification of Chr4D specific *CNL* genes in the CPI110672 genome

The predicted *CNL* sequences of more than 1.5 kb were shortlisted and then mapped to the reference sequences of hexaploid wheat CS IWGSC RefSeq v1.0 (IWGSC 2018; Steuernagel et al. 2018) and *Ae. tauschii* AL8/78 v4.0 (Luo et al. 2017) using the BLAST tool in the CLC Workbench. The full *CNL* containing contigs that mapped to the group 4 chromosome of CS and AL8/78 were evaluated to identify the *CNL* candidates that mapped within the flanking markers. Markers specific to candidates were developed to genotype the CPI110672 x CPI110717 F_2 mapping population and verify linkage with the *Yr672* locus.

6.3.6 Evaluation of allelic relationship of *Yr672* with *Yr28*

The full length coding sequences of the *Yr672* candidate gene (designated hereafter as *Yr672CNL*) was compared with its homologous candidate in Chinese Spring and AL8/78 reference sequences. The unique genetic variations corresponding to the *Yr672CNL* gene were targeted to develop gene specific diagnostic markers and a breeder friendly KASP genotyping marker. To study the allelic relationship of *Yr672* with *Yr28*, the synthetic hexaploid line carrying *Yr28* (SHW-W7984) was screened with the *Yr672* gene specific markers. The full length genomic DNA sequence of *Yr672* was subjected to homology analysis using the whole genome shotgun sequence of the SHW-W7984 genome (Chapman et al. 2015;

https://wheatis.tgac.ac.uk/grassroots-portal/blast). We also performed the fast minimum evolution tree using the NCBI distance tree viewer function for the phylogenetic analysis of the Yr672 amino acid sequence and the Top 100 blast hits to identify closely related members across monocots.

6.3.7 Deployment of *Yr672* using marker assisted selection

To deploy *Yr672* into commercial cultivars, we developed a backcross population derived from crossing Westonia with SHW-Lan672, with the F_1 backcrossed again to Westonia. The F_3 progenies of the backcross population were screened for homozygous lines using the gene specific marker, followed by rust phenotyping. One seed each from 48 BC_1F_3 families were used for DNA isolation using the Nimbus robot system and screened with the *Yr672* specific markers. For the BC_1F_3 lines that were positive for the marker, an additional twelve seeds were isolated and genotyped to identify homozygous lines. The homozygous backcross lines were phenotyped for stripe rust resistance against the stripe rust races 104E137A- and 134E16A+. The relative expression of *Yr672* in the homozygous backcross lines was quantified as described previously. SHW-W7984, SHW-Lan672 and CPI110672 were used as positive controls.

6.3.8 Cloning and validation of a candidate *CNL* gene for stripe rust resistance

The full length *Yr672CNL* candidate sequence from CPI110672 together with the 5' and 3' untranslated regions (UTR), to include the native promotor and terminator sequences, was amplified using primers Ctg2605F2-Ctg2605R13. Amplification of the desired length fragment was carried out in a 50 µl reaction mixture containing 5x Phusion buffer GC, 100 ng of DNA, 200 µM dNTP, 200 nM each of forward, and reverse primers and Phusion High-Fidelity DNA Polymerase (New England Biolabs Inc.) under the manufacturer's recommended conditions with 66°C annealing and a 5 minute extension and repeated for 34 PCR cycles. The fragment was cloned into the binary vector pVecBarIII, a derivative plasmid of pWBvec8 carrying the selectable marker *Bar* gene driven by the CaMv35S promotor and *Nos* terminator in the T-DNA. The plant transformation construct *(pVecBarIII-Yr672)* was transformed into the *Agrobacterium tumefaciens* strain GV3101 for further transformation into wheat cultivar Fielder (transgenic batch id PC222), AvocetS (PC250) and Sonora (PC251)

(Richardson et al. 2014; Ishida et al. 2015). The T_0 transgenic lines were screened for the *Yr672CNL* specific marker and tested for stripe rust resistance against the Australian *Pst* race 104E137A-.

We quantified the expression of *Yr672* in the T_0 transgenic lines post stripe rust inoculation. Leaf samples were collected five days post infection for the total RNA isolated using the method described above. Complementary DNA (cDNA) was synthesized using 1 µg of total RNA, an oligo(dT)20 primer and the Superscript III reverse transcriptase enzyme (Invitrogen) by following the manufacturers protocol. Quantitative PCR was carried out for the *Yr672* transcript using Ctg2605F7-R6 primers on a Bio-Rad CFX96 Real-Time PCR Detection System using *iTaq* universal SYBR Green supermix (Bio-Rad). FAM/SYBER green channel was used for data acquisition. Relative gene expression of *Yr672* was calculated in relation to the housekeeping genes Tublin and TaCON using the gene expression function in the Bio-Rad CFX96 software, as per user instructions.

To further validate, the PC222-*Yr672* transgenic lines were advanced to the T_1 generation. Twenty four T_1 seeds for each T_0 lines were cut in half to use the endosperm segment for DNA isolation and marker screening. The remaining embryo segment was germinated for phenotyping rust resistance against two Australian *Pst* races 104E137A- and 134E16A+ to verify the rust resistance. We also evaluated the relative gene expression of *Yr672* in T_1 progenies with three biological and technical replicates as described above.

6.4 Results

6.4.1 High-resolution mapping of *Yr672*

The genetic map of *Yr672* developed in Chapter 3 was compared with the 4DS physical and genetic maps of AL8/78 and Chinese Spring v0.4 reference sequences (Jia et al. 2013b; Luo et al. 2013). In the blast search, scaffolds containing the closely linked markers *Csq1* and *Csq3* were found in both AL8/78 and Chinese Spring, and they were used as the starting point to develop additional closely linked markers (Table 6.1). Four Cleaved Amplified Polymorphic Sequences (CAPS) markers namely *Csq9* (Chinese Spring scaffold 107989 of IWGSC WGA v0.4), *Csq11* (scaffold 27727), *Csq17* (scaffold 107989) and *Csq19* (scaffold 90543) and three Sequence Tagged

Site (STS) markers namely *Csq15* (scaffold 72468), *Csq16* (scaffold 27727) and *Csq18* (scaffold 18957) were the additional polymorphic markers derived from the reference sequence of Chinese Spring. Subsequently, comparing with the *Ae. tauschii* AL8/78 reference genome, homologous sequences related to all the markers except *Csq16* were identified in the AL8/78 scaffolds. Genotyping the markers in the CPI110672 x CPI110717 $F_{2:3}$ mapping population and construction of a genetic map revealed a high resolution map for the stripe rust resistance locus (Supplementary file 2 & 3). The markers *Csq15* and *Csq16* did not recombine with each other and were located at 0.4 cM distal position to the stripe rust resistance locus, while *Csq9, Csq11, Csq17* and *Csq19* were mapped proximal to the locus. The CAPS marker *Csq11* was the closest on the proximal side, located 0.8 cM from the locus (Figure 6.1).

6.4.2 Physical map and candidate gene identification

To develop a physical map of the *Yr672* locus we used the recent version of CS IWGSC RefSeq v1.0 and *Ae. tauschii* AL8/78 v4.0 and anchored the flanking markers *Csq15* and *Csq11* on both of the reference sequences. The flanking markers delimit to 510 kb and 417 kb genomic regions in CS IWGSC RefSeq v1.0 and AL8/78 v4.0, respectively (Figure 6.2). We evaluated the list of annotated gene in both the reference sequences to identify *CNL* type genes within the delimited region. The 510 kb region of CS IWGSC RefSeq v1.0 contains three annotated *CNL* type genes *TraesCS4D01G004000, TraesCS4D01G004100,* and *TraesCS4D01G004200*, hereinafter designated *CSCNL1*, *CSCNL2* and *CSCNL3*, while the AL8/78 v4.0 contained only one *CNL* type gene (*AET4Gv20006200*), orthologous to *CSCNL2* of Chinese Spring. Genotyping the marker specific to the *CNL* candidate genes in the parental accessions resulted in a marker specific to *CSCNL1* which was amplified only from the susceptible parent CPI110717. In contrast, markers specific to *CSCNL2* amplified only in the resistant parent, CPI110672 and none of the markers specific to *CSCNL3* amplified in either parental accessions. Therefore, it indicated the resistant parent is likely to have an ortholog of *CSCNL2*. Genotyping the CPI110672 x CPI110717 $F_{2:3}$ mapping population with the marker specific to *CSCNL2* amplified all the resistant and heterozygous lines and therefore co-segregated with the stripe rust resistance phenotype (Supplementary file 2). Hence, we suggest *CSCNL2* is a candidate gene for *Yr672* stripe rust resistance.

6.4.3 Annotation of the 4D specific *CC-NBS-LRR* gene in the CPI110672 genome

Annotation and mapping of *CNL* genes from the CPI110672 genome identified 10 complete *CNL* genes containing contigs that mapped specifically to chromosome 4D (Supplementary file 4). Of these, we identified one, contig_2605_2 with a *CNL* sequence (designated as *Yr672CNL*) that mapped within the *Yr672* region. BlastN analysis of the *Yr672CNL* identified it as an ortholog of *CSCNL2.* Therefore, it further confirms *Yr672CNL* as a candidate gene for *Yr672.* The *CSCNL2* candidate specific marker was also specific to *Yr672CNL* and thus co-segregated with the stripe rust phenotype in the CPI110672 x CPI110717 $F_{2:3}$ mapping population.

6.4.4 Gene specific markers and *Yr672* allelic evaluation

Homology analysis of the coding sequence of *Yr672CNL* with Chinese Spring and AL8/78 identified orthologs with 94% identity in both the reference genomes, indicating differences between resistant and susceptible alleles. To identify markers specific to the resistant allele we converted the polymorphic region of the *CSCNL2* candidate specific marker into a co-dominant CAPS marker. The *Yr672* susceptible alleles from Chinese Spring and AL8/78 encompassed a *Dra*I restriction site within the 830 bp 6510F5-R5 specific PCR product. Therefore, to discriminate between the alleles, restriction digestion of PCR products from the susceptible allele produces two fragments of 430 bp and 400 bp while the resistant allele remained undigested. We also developed a resistant allele-specific dominant (Ctg2605F7/R7) and a breeder-friendly SNP based KASP (KASPYr672) marker for allelic discrimination (Figure 6.3). PCR screening of gene specific markers in the SHW-W7984 genomic DNA identified the resistant specific allele for all three primers indicating the presence of *Yr672* in SHW-W7984. To further confirm, BlastN analysis of the *Yr672CNL* sequence in the SHW-W7984 whole genome shotgun sequence identified a 100% homologous region in scaffold 1489396.

Phylogenetic analysis of the Yr672 candidate amino acid sequence with its top 100 homologous sequences identified closely syntenic NBS-LRR-like members (Figure 6.4). Other than *Ae. tauschii*, *Brachypodium distachyon* and *T. aestivum* also carried orthologs related to *Yr672CNL*. In addition, NBS-LRR-like protein from other

monocot species such as *Oryza sativa, Zea mays, Sorghum bicolor* and *Panicum hallii* are also predicted to be syntenic with the Yr672 candidate amino acid sequence.

6.4.5 Introgression of *Yr672* into hexaploid wheat

Genotyping 48 BC_1F_3 lines of the backcross population using candidate gene specific markers identified the homozygous *Yr672* positive (BC_1F_3-23, BC_1F_3-26) and negative lines (BC_1F_3-28). Phenotyping the homozygous lines BC_1F_3-23, BC_1F_3-26 along with SHW-W7984 against stripe rust races conferred resistance. The diploid parent CPI110672, BC_1F_3-23, BC_1F_3-26 homozygous lines and SHW-W7984 conferred strong resistance against the Pst79 race (104E137A-), while the BC_1F_3-28 line was susceptible. The SHW-Lan672 parental line was not resistant while in contrast, the recurrent parent Westonia was resistant (Figure 6.5). On the other hand, the WA pathotype (134E16A+) showed an intermediate phenotype on the diploid parent CPI110672, SHW-W7984 and homozygous BC_1F_3-23, BC_1F_3-26 lines. The SHW-Lan672, Westonia and BC_1F_3-28 had susceptible phenotypes. The resistant phenotype of the two (BC_1F_3-23, BC_1F_3-26) homozygous lines tested and SHW-W7984 were identical for both the races tested which strengthen our claim *Yr672* and *Yr28* could either be the same gene or located close together.

6.4.6 Validation of *Yr672* by wheat transformation

The full length (3649 bp) coding sequence of the *Yr672CNL* gene along with about 1.7 kb of 5’ and 800 bp of 3’UTR, was amplified from the genomic DNA of accession CPI110672. The full length amplified gene sequence of *Yr672CNL* contained two introns within the coding sequence and therefore part of the plant transformation construct (*pVecBarIII-Yr672*) (Figure 6.2e). The *pVecBarIII-Yr672* construct was transformed into wheat cultivars Fielder, AvocetS and Sonora using *Agrobacterium* strain GV3101. Thirteen independent events carrying *Yr672CNL* in Fielder (PC222-1 to PC222-28), five in AvocetS (PC250-1 to PC250-5) and one in Sonora (P251-1) were recovered for analysis. Screening of PC222, PC250 and PC251 T_0 lines conferred stripe rust resistance (Figure 6.6a). Among the selected T_0 lines tested, PC222-1, PC222-7, PC222-12, PC250-2, PC250-3, and PC251-1 were resistant, while the PC222-3, PC250-4, and Fielder had intermediate resistant phenotypes. The T_0 lines PC250-1, AvocetS and Sonora had susceptible phenotypes. PCR screening

confirmed all the resistant lines were positive for the *Yr672* gene specific marker. Screening the intermediate resistant lines revealed PC222-3 and Fielder were negative, while PC250-4 was positive. The rust susceptible line PC250-1 was positive for the PCR marker, while the control lines AvocetS and Sonora were PCR negative.

Quantification of the *Yr672* transcript in relation to TaCON and tubulin controls revealed the level of *Yr672* expression in all T_0 transgenic lines infected with rust (Figure 6.6b). The *Yr672* transcript in the diploid parent CPI110672 was at least three to four-fold higher than the transgenic lines. Among the transgenic lines, PC222-7 showed the highest level of expression while PC222-12, PC250-1 were the lowest. The relative expression of *Yr672* was correlated with the rust resistant phenotype as line PC222-7 expressed the highest level of *Yr672* and exhibited strong resistance, while the relatively low expression of PC222-12 and PC250-1 plants exhibited susceptible phenotypes. The intermediate level of expression of transgenic lines PC250-2 and PC250-3 in the AvocetS background also exhibited resistance phenotype (Figure 6.6a). No transcript was observed for the PCR negative line (PC222-3) and control lines Fielder, AvocetS and Sonora (Figure 6.6b).

For further validation, we genotyped 24 T_1 seeds of each of the PC222 lines with a gene specific marker (Ctg2605F7-R7) and advanced these to the T_1 generation. The T_1 lines from PC222-7 and PC222-12 did not have any negative lines in the marker screening thus it may have more than one copy integration (Table 6.3). We split the 24 T_1 seeds into two groups and phenotyped against Pst79 (104E137A-) and WA (134E16A+) stripe rust races. Screening against Pst79 race revealed strong stripe rust resistance, while the WA race conferred low to intermediate resistance (Figure 6.7 a&b). Evaluation of *Yr672* transcript in rust-infected T_1 lines (three biological replications each) revealed that the majority expressed at a very low level relative to the diploid (CPI110672) and hexaploid controls (SHW-Lan672, W7984, BC_1F_3-23 and BC_1F_3-26) (Figure 6.7c). The expression of *Yr672* in the T_1 lines correlated with the rust resistant phenotype. The transgenic line PC222-7 showed a stronger phenotype against both the stripe rust races tested in the T_1 generation. Although the lowest expressing line PC222-12 had the resistant phenotype in T_0, and was correlated with the *Yr672* transcript level, it showed a susceptible phenotype in the T_1 generation to both Pst79 and WA races (Figure 6.7).

6.5 Discussion

We mapped a stripe rust resistance gene, temporarily designated *Yr672* to the short arm of chromosome 4D in accession CPI110672. So far chromosome 4DS of *Ae. tauschii* contributes two known stripe rust resistance genes, *Yr28* (WX219 syn. TA2465) (Singh et al. 2000) and *YrAs2388* (AS2388 syn. PI 511384) (Huang et al. 2011). Both of these genes were reported to be located on the distal end of the chromosome 4DS, and are likely collinear to the *Yr672* locus. We evaluated the geographic survey details in the US-National Plant Germplasm System (https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1556592) and the recent genetic analysis of 150 non-redundant *Ae. tauschii* accessions (Arora et al. 2019). The accessions WX219 and AS2388 originated from Iran, while accession CPI110672 originated from Turkmenistan. Apart from the place of origin, no further information about accession AS2388 was available, while genetic analysis confirmed the accessions CPI110672 and WX219 to be genetically different (Arora et al. 2019). The stripe rust resistance gene *YrAS2388* identified from AS2388 was mapped distal to the marker *Xwmc285* (Huang et al. 2011). In our study, *Xwmc285* was not polymorphic, hence we designed a new marker *Csq17* at the *Xwmc285* locus; *Yr672* also mapped distal to *Csq17.*

In order to identify the candidate gene for *Yr672,* we identified the physical position of all markers in the recently updated Chinese Spring IWGSC RefSeq v1.0 and *Ae. tauschii* AL8/78 reference sequences. From the physical position, we narrowed down the size of the target *Yr672* genomic region to about 510 kb in Chinese Spring and about 417 kb in AL8/78 sequences. Within the targeted genomic region, Chinese Spring harbours three *CNL* sequences while AL8/78 contains one *CNL* gene. In fact, it is the only *CNL* gene annotated in the AL8/78 reference sequence present distal to the flanking marker *Xwmc285.* Furthermore, annotation of the *CNL* gene in the whole genome assembly of parental line CPI110672 (sequence obtained from the open wild wheat consortium, John Innes Centre, Norwich, UK) identified 10 complete *CNLs* on chromosome 4D. Evaluation of their physical position based on Chinese Spring and AL8/78, only one *CNL* (*Yr672CNL*) in contig_2605 mapped distal to the flanking marker *Csq11.* Similar to AL8/78, it is the only *CNL* containing the CPI110672 contig positioned distal to the marker *Xwmc285,* which is present in both reference

sequences. Therefore, *Yr672* and *YrAs2388* are most likely the same genes. However, allelic studies are essential to validate their relationship. Homology analysis of *Yr672* in the wheat pan-genome database (https://wheatis.tgac.ac.uk/grassroots-portal/blast) containing the whole-genome shotgun sequence of the synthetic hexaploid wheat SHW-W7984 identified 100% identical sequences in scaffold 1489396 (Chapman et al. 2015). The SHW-W7984 is the parent for *Yr28.* Markers specific to *Yr672CNL* also amplified in SHW-W7984 and in an *Ae. tauschii* accession CPI110800 (TA2465 or WX219). Hence, in addition to our claim that *Yr672* and *YrAs2388* are the same gene, we also postulate *Yr672* and *Yr28* as being either the same, or tightly linked genes.

Through marker-assisted backcross selection, *Yr672* was transferred into commercial cultivar followed by rust phenotyping against both Pst79 and WA races. The *Yr672* lines conferred resistance against both races. Surprisingly SHW-Lan672 displayed a susceptible phenotype. This may have been due to the presence of suppressor elements in the A or B genomes as reported for *YrAs2388* deployment into hexaploid wheat, where a loss of resistance was observed for *YrAs2388* in the synthetic hexaploid wheat but not in the diploid (He et al. 2007; Huang et al. 2011). This further supports our argument that *Yr672* and *YrAs2388* are allelic. This might have hindered the introgression of stripe rust resistance genes from *Ae. tauschii* to hexaploid wheat in past decades. Unlike *Yr672* and *YrAs2388*, the synthetic hexaploid SHW-W7984 *(Yr28)* is still resistant which may be due to a different tetraploid line used for generating SHW-W7984 (Singh et al. 2000). However, the resistance provided by *Yr672* backcross lines derived from SHW-Lan672x Westonia is likely due to independent segregation of suppressor elements and *Yr672*.

To validate *Yr672CNL*, we transformed the gene sequence into the cultivars Fielder, AvocetS and Sonora and tested against the Pst79 race. The diploid parent CPI110672 confers strong resistance against the Pst79 race and only intermediate resistance against WA race. Therefore, we opted to test the transgenic plants against the Pst79 race. The cultivar Fielder is robust for wheat genetic transformation (Richardson et al. 2014; Ishida et al. 2015); however, it has background stripe rust resistance genes *Yr6* and *Yr20* conferring intermediate resistance against Pst79 (Chen et al. 1995; Cuddy 2016). Despite the presence of *Yr6* and *Yr20* in Fielder, we

observed more stripe rust pustules in the *Yr672* negative lines than in the transgenic lines with the *Yr672CNL* candidate gene in both T_0 and T_1 generations against Pst79 race. Strong resistance by one of the *Yr672* transgenic Fielder lines and lack of segregation of the same line indicates the presence of multiple copies of *Yr672*. Similar effects were also reported for the *Sr21* stem rust resistance gene (Chen et al. 2018). Nevertheless, we transformed AvocetS and Sonora but they were less compatible for the *Agrobacterium*-mediated genetic transformation. Despite this, we recovered transgenic lines that provide resistance to Pst79. More independent lines, particularly in the AvocetS background are needed to draw a strong conclusion.

By confirming the stripe rust resistance function of the candidate gene identified, we demonstrate the ability to clone resistance genes using the recently available reference genome sequences without the need of loss-of-function mutants. Therefore, along with these reference genomes, availability of wheat pan-genome databases in the near future pave the way for rapid dissection of potential traits. Subsequently, use of gene specific markers in combination with speed breeding technology will accelerate the deployment of *Yr672* into commercial cultivars. Additionally, cloning of *Yr672* also offered a potential tool to identify the *AvrYr672* gene for a better understanding of stripe rust-wheat interactions.

Table 6.1 List of primers used for fine mapping and cloning of *Yr672*

Marker name	Primer sequence	Type	Remarks
Csq9	GAAGCACTTCCCGTCAACAAG	CAPS	HpaII
	ACTGACAACACGCTGTAACA		
Csq11	GCAGGTCAACTTATCTCTTCCTC	CAPS	*Hpy*CH4IV
	AGAGCACATTGATTCATGGAGA		
Csq15	TGTTTGTAGATCTCATGGCCTCA	STS	--
	AAGCAATGTTGCGAGTAAAGGT		
Csq16	CTCACAGTCCCCGAAGCAGA	STS	--
	GTGAACTCTGGCCATTTGCA		
Csq17	TCACAGAAATGGAACCTTAGG	CAPS	*Hpy*CH4IV
	TCCCCCGAGAATGACTCAGG		
Csq18	GCTTGGATCGCTGTGCGAAG	STS	--
	AAGGGTGGGGAGGCGGTTGC		
Csq19	AGGCCGTTTTTGGGCAGAGACA	CAPS	*Eco*RV
	ACGAAGATGCCGAGCGCTTCAC		
6510F5-R5	ACACAAGATTTCACCGATGTTG	CAPS	*Dra*I
	TGACATTTGAGATACTGACCAC		(Gene specific co-dominant marker)
Ctg2605F7-	CCGAGCTCTTACAAATTTGACTTC	Diagnostic	Gene specific
R7	CCTCCATTCCGTGAAATTCCAACA	Marker	
Ctg2605F2-	*TAGCAGCTCCACCTACCGCA*	Full length	6179 bp
R13	GCTGGGCAAATTCAGAAAATCTCCG	cloning	
Ctg2605F7-	CCGAGCTCTTACAAATTTGACTTC	Resistant allele specific	*Yr672* transcript analysis
R6	CTACCACCACATTTTAAAGCAAGTAC		
KASPYr672	F1: TATGCTAGAAGATATTGGG	SNP	Res allele: G
	F2: TATGCTAGAAGATATTGGC		Sus allele: C
	R: TGACATTTGAGATACTGACCAC		

The forward primers F1 and F2 are tagged with FAM (GAAGGTGACCAAGTTCATGC) and HEX (GAAGGTCGGAGTCAACGGAT) sequences at 5'end respectively.

Table 6.2 Physical position of *Yr672* locus in Chinese Spring RefSeq v1.0 and AL8/78 v4.0 (highlighted in green are closest flanking markers and highlighted in blue is the predicted candidate gene for *Yr672*)

Chinese Spring RefSeq v1.0				***Ae. tauschii* AL8/78 v 4.0**			
Start (bp)	**End (bp)**	**Marker/Gene Id**	**Description**	**Start (bp)**	**End (bp)**	**Gene Id**	**Description**
1306213	1306372	Csq3	Flanking marker	946333	946526	Csq3	Flanking marker
1374786	1375139	Csq15	Flanking marker	1027168	1027491	Csq15	Flanking marker
1640803	1645983	TraesCS4D01G003800	Kinase family protein	1416655	1420267	AET4Gv20005900	U-box domain containing protein
1666001	1668358	TraesCS4D01G003900	Receptor-like protein kinase	--	--	--	--
1666459	1666827	Csq16	Flanking marker	Marker absent in AL8/78			
--	--	--	--	1432254	1434601	AET4Gv20006000	Wall associate receptor kinase like protein
--	--	--	--	1435935	1436208	AET4Gv20006100 *(Low confidenece)*	ATP synthase CF0 B subunit
1704075	1708862	TraesCS4D01G004000	NBS-LRR like resistance protein	2114280	2118129	--	--
1820770	1826513	TraesCS4D01G004100	NBS-LRR like resistance protein	1436596	1442505	AET4Gv20006200	Disease resistance RPP13 like protein
--	--	--	--	1436838	1438003	AET4Gv20006300 *(Low confidence)*	Disease resistance RPP13 like protein
1871232	1878072	TraesCS4D01G004200	NBS-LRR-like resistance protein	1437995	1442049	No annotated gene at this locus in AL8/78, however 88 % identity AET4Gv20006200	

1881346	1882081	Csq11	Flanking marker	1443748	1444482	Csq11	Flanking marker
2982720	2983153	Csq18		1572787	1583143	Csq18	In the genetic map of 672x717, these markers mapped proximal to Csq9
1992994	1993287	wmc285		1599976	1600259	wmc285	
1996045	1996314	Csq17		1603007	1603276	Csq17	
2112052	2112441	Csq9		1698919	1699308	Csq9	
3343804	3344162	Csq19		2114153	2114511	Csq19	Tightly linked to Yr28 Assoc. seq.
3347022	3343573	TraesCS4D01G006000	NBS-LRR-like resistance protein	2114870	2117371	no gene predicted	*Yr28* Assoc seq; NCBI: KX181569.1
3396333	3396719	Csq1	SNP Id IWB47181 Flanking marker	--	--	--	No hit found in AL8/78 v 4.0

Table 6.3 Segregation ration of PC222 T_1 transgenic lines

T_0 line	No. of T_1 seeds genotyped	Segregation ratio			Chi-square test	P value
		R	S	Ratio		
PC222-1	24	14	10	7:5	3.56	0.0592
PC222-2	24	18	6	3:1	0.00	1
PC222-3	24	0	24	0:4	72.00	0.0001
PC222-7	24	24	0	4:0	8.00	0.0047
PC222-12	24	24	0	4:0	8.00	0.0047
PC222-16	24	22	2	11:1	3.56	0.0592
PC222-20	24	18	6	3:1	0.00	1
PC222-21	24	15	9	5:3	2.00	0.1573
PC222-22	24	15	9	5:3	2.00	0.1573
PC222-23	24	20	4	5:1	0.89	0.3455
PC222-25	24	17	7	17:7	0.22	0.639
PC222-26	24	18	6	3:1	0.00	1
PC222-27	19	19	0	4:0	6.33	0.0119
PC222-28	24	16	8	2:1	0.89	0.3455

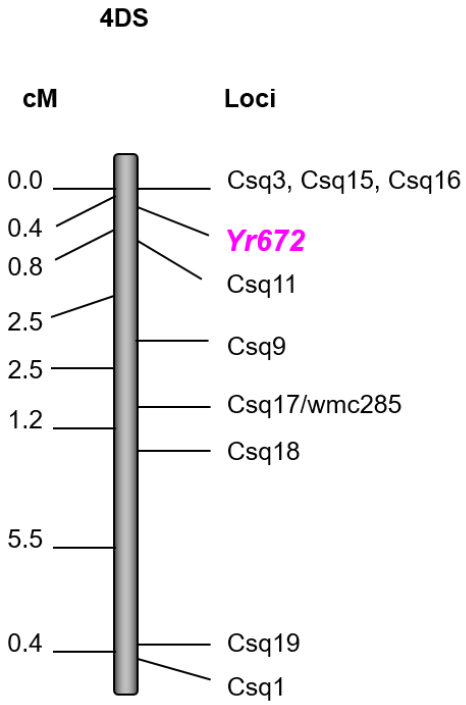

Figure 6.1 Genetic map of *Yr672 l*ocus on 4DS chromosome

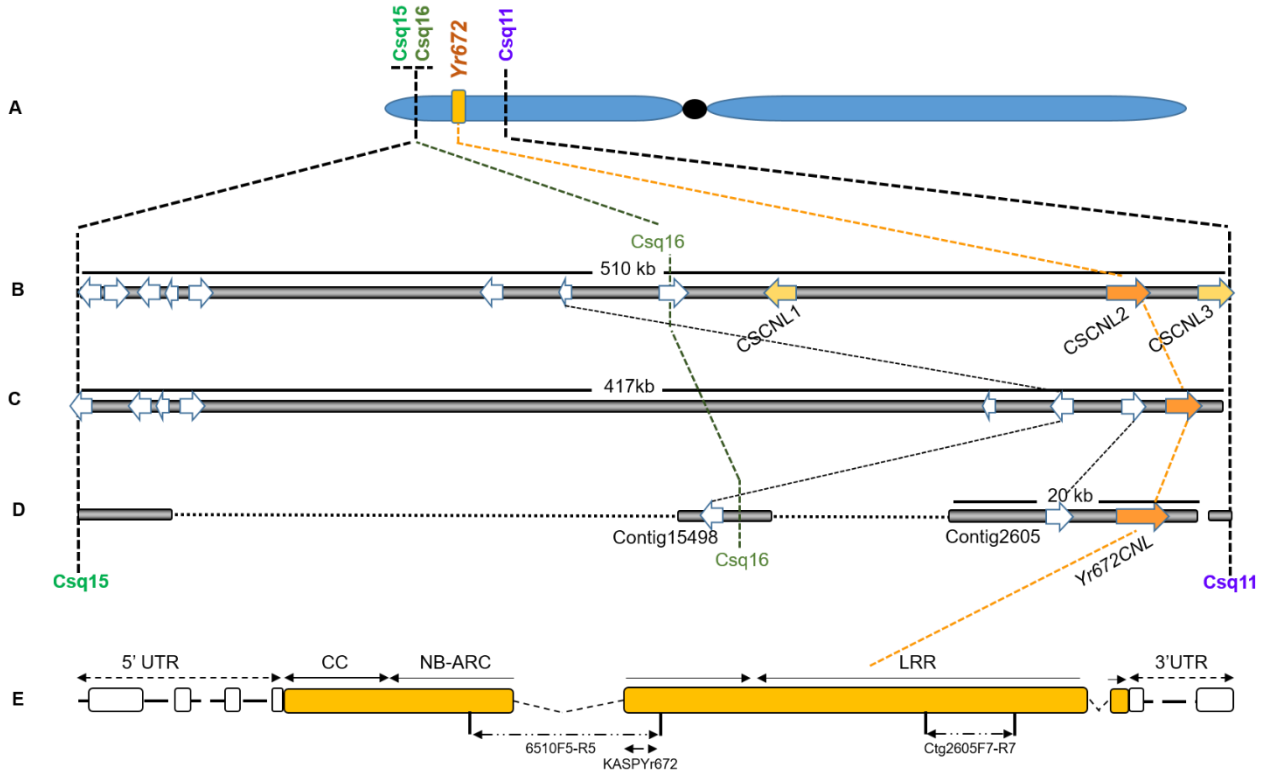

Figure 6.2 Physical map and positional cloning of *Yr672*

(A) *Yr672* linkage map on chromosome 4DS. (B) The identified collinear region of *T. aestivum cv.* Chinese Spring (IWGSC-RefSeq v1.0) within the flanking markers (C) Identified collinear region of *Ae. tauschii* accession AL8/78 v4.0 within the flanking markers. (D) CPI110672 contigs containing predicted *CNL* gene mapped to a shortlisted region of Chinese Spring and AL8/78. Arrows indicate the annotated genes present in the shortlisted region. Yellow and orange coloured arrows are the annotated *CNL* genes. (E) Gene architecture of the *Yr672CNL* candidate gene isolated from CPI110672 contig_2605.

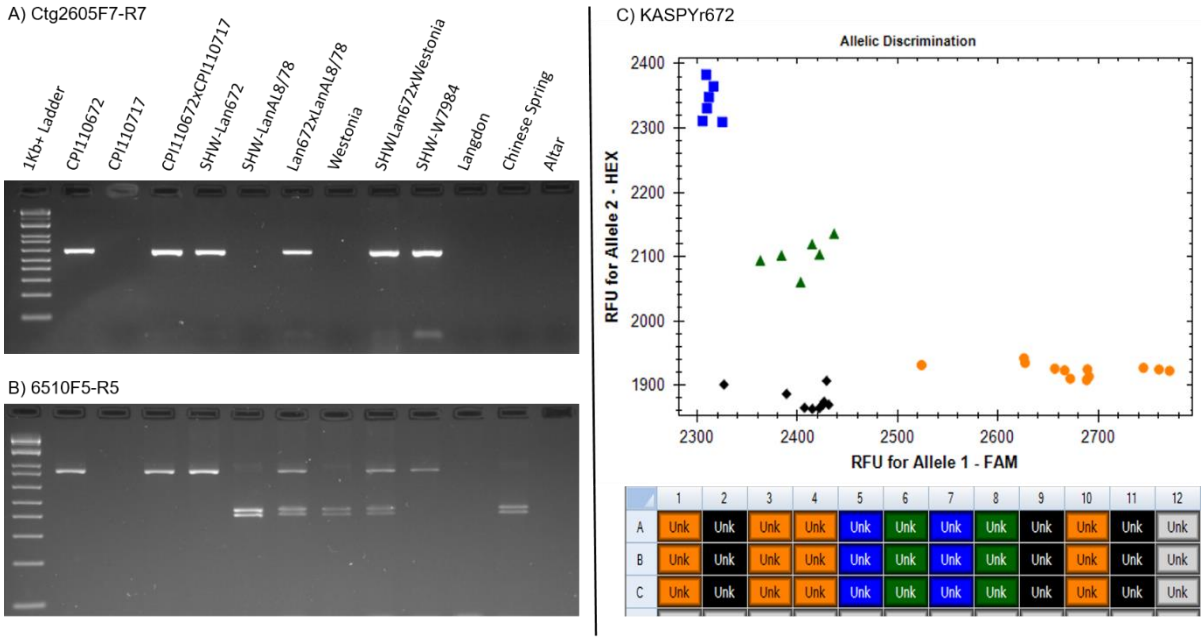

Figure 6.3 Diagnostic gene specific marker for *Yr672*

A) PCR based dominant gene specific marker amplifies 650 bp product from the LRR region. B) PCR followed by restriction digestion based CAPS marker amplifies 800bp from the NB-ARC region covering intron. Digestion of PCR product with *Dra*I results the polymorphism between resistant and susceptible allele. C) SNP based KASP marker in the NBS region showing co-dominant expression. The orange colour dots in the graph representing the resistant allele from DNA samples of CPI110672, artificial heterozygous (mixing equal concentration of CPI110672 and CPI110717 DNA), SHW-Lan672, SHW-W7984. Blue colour indicates the susceptible allele detected in SHW-AL8/78 and Westonia samples. Green colour represents the heterozygous genotype for the two artificial heterozygous samples generated by pooling equal concentration of SHWLan672 x SHWAL8/78 and SHWLan672 x Westonia. Black colour indicates the null allele in CPI110717, Langdon and water control. All the samples for KASP marker validation were replicated thrice.

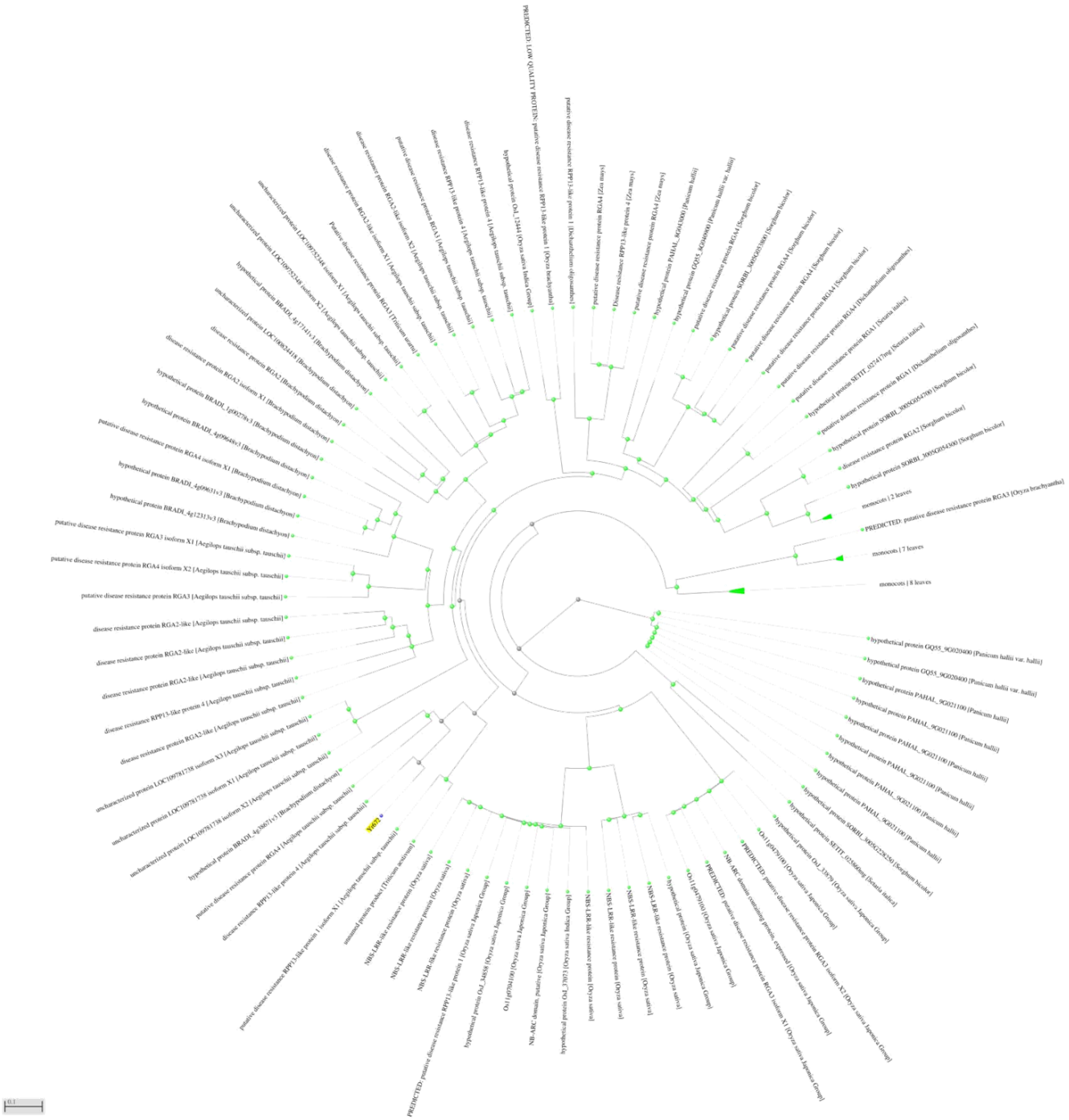

Figure 6.4 Phylogenetic analysis of *Yr672*

Phylogenetic analysis Yr672 amino acid sequence aligned to top 100 hits in the pairwise alignment. Fast Minimum Evolution tree method in the NCBI Tree Viewer function 1.17.3 was used for the tree construction.

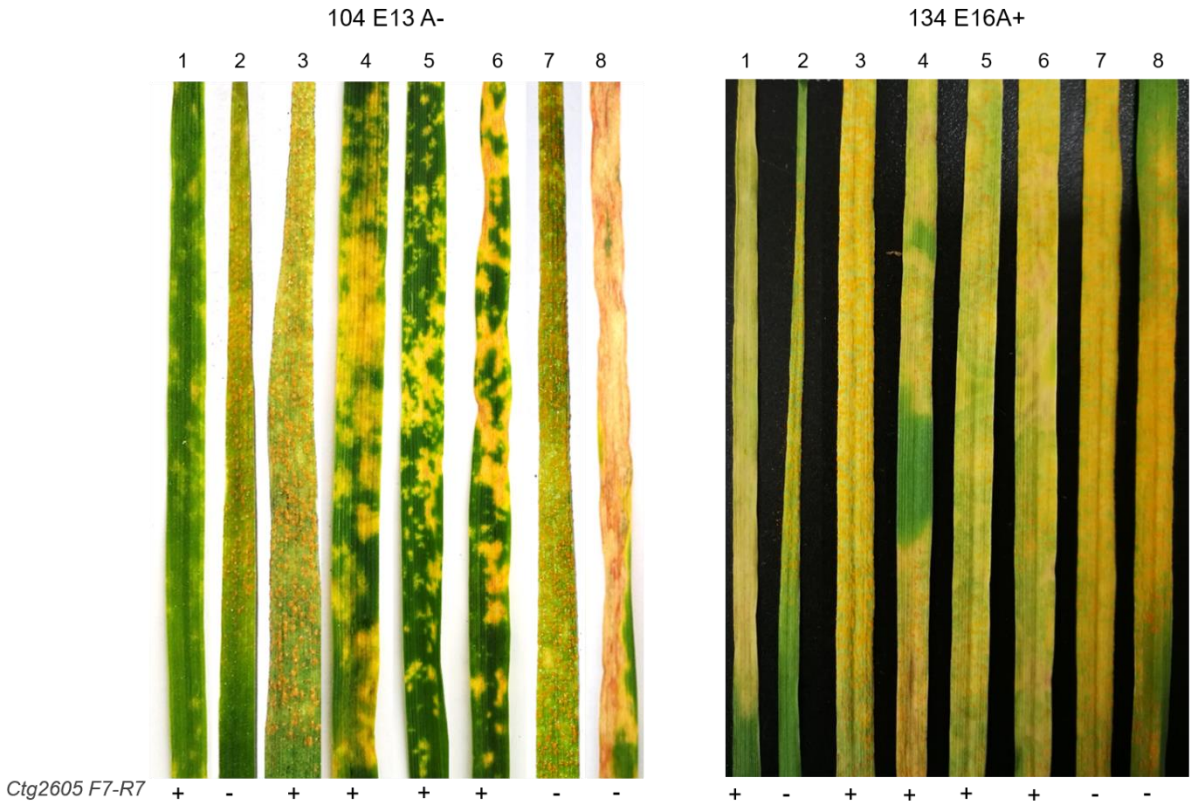

Figure 6.5 Phenotyping the backcross (BC_1F_3) lines for stripe rust resistance *104E13A*- and *134E16A*+, Stripe rust races; Plant details: *1,* CPI110672; *2,* CPI110717; *3,* SHW-Lan672; *4,* SHW-W7984; *5,* BC_1F_3-23; *6,* BC_1F_3-26; *7,* BC_1F_3-28; *8,* Westonia. *Ctg2605F7-R7, Yr672* gene specific marker used for screening the BC_1F_3 lines; + or - sign indicates the presence or absence of *Yr672* gene in the tested lines.

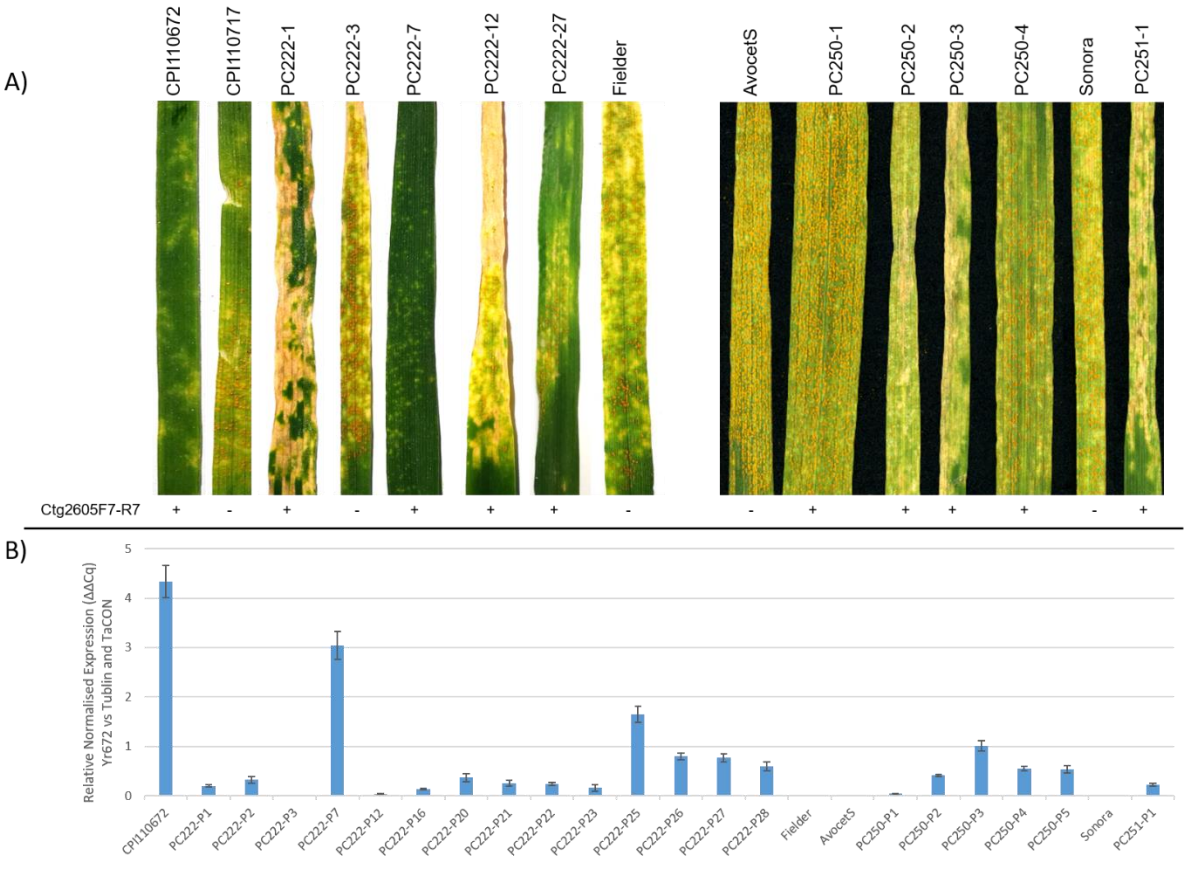

Figure 6.6 Screening of *Yr672* transgenic plants for stripe rust resistance against 104E137A-

A) *Fielder, AvocetS* and *Sonora* are transgenic control; *CPI110672*, Resistant *Ae. tauschii* accession; *CPI110717,* Susceptible *Ae. tauschii* accession; *PC222-1,7,12,27, PC2501,2,3,4 and PC251-1* are *Yr672* positive for Ctg2605F7-R7 marker transgenic lines; *PC222-3, Yr672* negative transgenic lines. B) Quantitative real-time PCR for all the T_0 transgenic lines from the leaf samples collected 5 days post rust infection. Y-axis represents the expression of *Yr672* transcripts in relative to the housekeeping genes TaCON and Tublin.

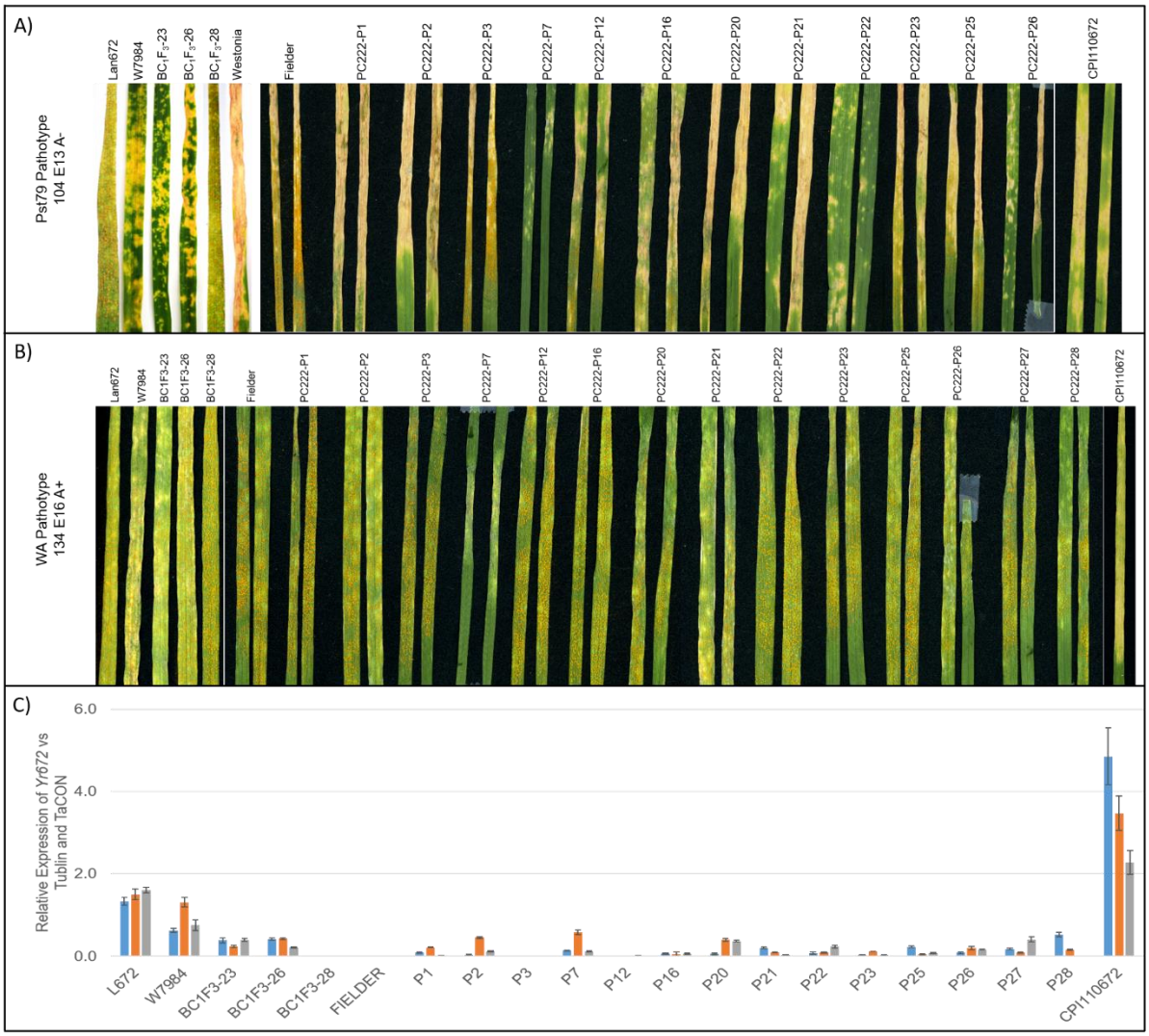

Figure 6.7 Screening of T_1 progenies of *Yr672* transgenic fielder (PC-222 lines) A) Screening of *Yr672* Fielder T_1 progenies against the Pst79 pathotype 104E13A-; B) Screening of *Yr672* Fielder T_1 progenies against the WA pathotype 134E16A+; C) Quantitative real-time PCR for all the T_1 transgenic lines from the leaf samples collected 5 days post rust infection. Y-axis represents the expression of *Yr672* transcripts in relative to the housekeeping genes TaCON and Tublin. Blue, Orange and grey colour bar represents the biological replication for each line. Data collected for SHW-Lan672, SHW-W7984, BC1F3-23, 26, 28 and CPW110672 were presented here as a representative control.

Chapter 7 - General Discussion

Wheat is grown on more land than any other crop in the world and is a staple food for more than one-third of the global population. The demand for wheat production is increasing rapidly as is the global human population. Climate change, declining cultivated land area and water, and biotic stresses constrain wheat production and pose a great threat to meeting global demand (Ray et al. 2013). Breeders need to overcome these challenges by developing high yielding, biotic and abiotic stress resilient varieties to fulfil these needs. Due to the complex genetic nature of wheat, developing an elite breeding line to defend against various challenges is a tedious and often time-consuming process. A conventional breeding approach can take 10 to 12 years to develop and commercialise a wheat variety (Lammerts Van Bueren et al. 2011). Progressive efforts have been made to advance wheat breeding programs to improve various traits, particularly for disease resistance. However, the constant emergence of new virulent forms of diseases, especially wheat rust pose an ongoing threat. Adverse climatic conditions and breeding strategies with narrow genetic gains induce rust fungi to evolve and adapt to new varieties. Thus wheat rusts are an ever-present threat.

7.1 Key advances in the wheat breeding for trait improvement

As part of the approach to improve the wheat breeding programs, initiatives such as shuttle breeding, double haploid technology, speed breeding, marker assisted breeding and transgenic technologies enables the rapid development of elite wheat lines (Ortiz et al. 2007; Collard and Mackill 2008; Richardson et al. 2014; Ishida et al. 2015; Humphreys and Knox 2016; Ghosh et al. 2018; Luo et al. 2018; Watson et al. 2018; Wulff and Dhugga 2018). However, unlocking traits in the complex wheat genome is essential to effectively use these breeding strategies. Constant advances in the field of molecular biology have led to the development of molecular markers to unveil these potential traits. Markers linked with traits can be used as a tagging tool to aid in marker assisted breeding. Despite the development of markers, the complexity of the wheat genome with over 85% repetitive DNA sequence makes the process of isolating genes responsible for traits tedious. Recent advances in the genome sequence technologies have reduced genome complexity and provided a high density of markers across the entire genome. Additionally, high throughput genotyping

technologies such as SNP arrays, genotyping by sequencing and genome wide association studies enable rapid screening of marker-trait associations (Elshire et al. 2011; Kilian et al. 2012; Poland et al. 2012b; Wang et al. 2014). Such next generation sequencing technologies have also paved the way for decoding the complex genomes of polyploid wheat and its wild relatives. For instance, the International Wheat Genome Sequencing Consortium (IWGSC) recently released a high-quality reference genome sequence (IWGSC RefSeq v1.0) for the hexaploid Chinese Spring (IWGSC 2018). The reference genome sequence was annotated with over 107,000 genes along with 4.7 million molecular markers physically mapped across all chromosomes. Similarly, a complete reference genome of *Aegilops tauschii* AL8/78 v4.0 has also become available for public use (Luo et al. 2017; Zimin et al. 2017b). These reference sequences provide markers for accurate positioning of any potential traits.

A major challenge for modern day breeders is the lack of genetic diversity in elite germplasm. Bread wheat originated from a hybridisation event between a tetraploid wheat *Triticum turgidum* and a diploid progenitor *Aegilops tauschii*; however, the intensive breeding of tetraploid wheat during domestication and the inadequate number of hybridisation events with *Ae. tauschii* created a bottleneck in the genetic diversity of bread wheat (Rasheed et al. 2018). In recent years, the germplasm of wild relatives were widely exploited to enrich the rust resistance gene pools for commercial breeding (Rouse et al. 2011a; Liu et al. 2013; Kalia et al. 2017). Direct recombination between chromosome segments of wheat and its wild relatives encouraged breeders to facilitate artificial hybridisation for introgression of novel genes for genetic diversity (Thind et al. 2018). But along with the key traits, artificial hybridisation events also transferred undesirable traits in some cases. Various gene isolation methods using high throughput sequencing technologies such as whole genome sequencing, flow-sorted chromosome sequencing, and exome capture methods have emerged to overcome linkage drag issues (Arora et al. 2019; Periyannan 2018). Gene isolation methods can also deliver perfect markers for rapid introgression and selection of lines with short alien segments and opportunities to develop robust cultivars through transgenic breeding approaches.

Despite the availability of high-quality reference genome sequences, genetic information to unveil unique traits that are present only in old landraces and wild

relatives are insufficient. Efforts are in place to sequence diverse wheat cultivars and landraces to develop high-quality wheat pangenomes (Montenegro et al. 2017; http://www.10wheatgenomes.com/). Similarly, the open wild wheat consortium (OWWC) has also been initiated to untap the diversity present in the diverse accessions of the D genome progenitor, *Ae. tauschii* (http://www.openwildwheat.org). Breeders and researchers are now using new breeding strategies for rapid variety development, which remains essential to combat the contineous emergence of new aggressive new rust races.

7.2 Overview of the research findings

This study aimed to unlock the genetic architecture of triple rust resistance in an accession of *Ae. tauschii* CPI110672. The accession was identified to provide resistance to all the three wheat rust diseases, namely leaf, stem and stripe rust (Evans Lagudah unpublished data). Our priority was to verify whether the triple rust resistance is pleiotropic or conferred by multiple genes. Pleiotropic resistance is a common phenomenon in adult plant resistance where a single gene confers resistance to multiple diseases. For instance, the rust resistance genes *Lr34/Yr18/Sr57/Pm38/Sb1, Lr46/Yr29/Sr58/Pm39, Lr67/Yr46/Sr55/Pm46* and *Sr2/Yr30/Lr27* provide partial resistance to multiple diseases at the adult plant stage (Krattinger et al. 2009; Lagudah et al. 2009; Mago et al. 2011c; Kolmer et al. 2015; Moore et al. 2015). This phenomenon is very rare in the case of all stage resistance, although an alien segment introgressed from *T. ventricosum* conferred *Lr37/Sr38/Yr17* resistance. It has is yet to be verified whether a single gene expresses all the resistance, or two or more tightly linked genes mapped at the locus are responsible (Bariana and Mcintosh 1993).

7.2.1 Mapping genomic regions controlling triple rust resistance

Extensive genetic studies were conducted by phenotyping a mapping population challenged with leaf, stem and stripe rust races. The phenotypic results revealed that the resistance to each rust segregated independently (monogenic inheritance for leaf and stripe rust, and digenic segregation for stem rust resistance). It also revealed at least four independent genes were responsible for providing the triple rust resistance. To identify the genomic location of these resistance genes, we utilised the high throughput genotyping platform 90K SNP. Advances in next generation sequencing

technology led to the discovery of high density single nucleotide polymorphisms (SNP) in the wheat genome and facilitated the development of SNP based arrays for high throughput genotyping (Wang et al. 2014). Currently, SNP based genotyping is the most preferred and robust system for studies such as genome wide association, marker-trait linkage and marker assisted breeding applications (He et al. 2014a). Genotyping trait specific bulks (creating resistant and susceptible bulks from at least 10 lines each for each phenotype) of the mapping population with 90K SNP markers yielded closely associated SNPs and thereby enabled the precise identification of the chromosomal location of the three genes. The SNPs closely associated with each *R* gene were converted into Kompetitive Allele Specific PCR (KASP) markers to genotype the whole mapping population. KASP is a fluorescence-based homogenous genotyping methodology based on allele specific primer extension and fluorescence resonance energy transfer (Semagn et al. 2014). Genotyping the mapping population with KASP markers and construction of a linkage map using the MapDisto 2.0 (Heffelfinger et al. 2017) resulted in the identification of flanking markers for the resistance traits. The leaf rust resistance locus and one of the stem rust resistance loci were designated *Lr39* and *Sr46b,* respectively and was mapped on 2DS, while the stripe rust locus was designated *Yr672* on 4DS.

7.2.2 Fine mapping and cloning of rust resistance genes

Mapping and cloning of genes controlling desirable traits in complex genomes is a tedious process. The polyploid nature of the wheat genome posed challenges to isolate genes of interest. Disease resistance genes in wheat were traditionally isolated based on the map-based cloning approaches involving the development of large mapping populations, mutant populations, preparation of BAC libraries for the target parent and construction of physical maps for target loci (Keller et al. 2018; Periyannan 2018). Continuous technological advancements particularly high throughput sequencing techniques facilitated the development of the reference genome sequences of hexaploid wheat and its A and D genome progenitors (Jia et al. 2013b; Ling et al. 2013; Luo et al. 2013; IWGSC 2014; Zimin et al. 2017a; Zimin et al. 2017b; Clavijo et al. 2017; IWGSC 2018). Availability of these genomic resources greatly reduced complication by providing high density molecular markers to fine map the region of interest. In this study, the reference sequences of hexaploid wheat, the D genome diploid *Ae. tauschii* AL8/78 and the sequenced genomes of parental

accessions obtained from the Open Wild Wheat Consortium (OWWC, JIC, UK) were used for high-density marker development and fine mapping.

High density markers significantly delimited the region of physical map construction and facilitate the rapid identification of candidate genes for the target resistance. The markers developed in this study narrowed the target region to five-fold less than those previously reported for the leaf rust (*Lr39*) locus (Sun et al. 2009). It also facilitated the identification of candidate genes and markers co-segregating with the leaf rust resistance locus. Comparative genomic analysis also identified tightly linked flanking markers for the stripe rust resistance locus and yielded a candidate gene that conferred stripe rust resistance in the transgenic complementation test. Similarly, anchoring the flanking markers of the stem rust resistance locus on the reference genomes facilitated the identification of a candidate gene that conferred stem rust resistance. Therefore, the availability of reference genome sequences greatly simplified the process involved in fine mapping and candidate gene identification in this study. In a future perspective, the sequencing of more germplasm to create a pangenome will accelerate marker and candidate gene identification at a much faster rate than the present scenario.

7.2.3 Development of perfect markers to use in breeding programs

Cloning of a disease resistance gene will undoubtedly hasten the deployment of resistance in commercial cultivars, enable the study of the plant-pathogen interaction, assist in the identification of the corresponding avirulence effector molecule (*Avr* gene), and present opportunities to develop gene stacks. Among them, the prime need to clone genes is to identify perfect markers for rapid selection of resistance in conventional breeding. In past decades, without much success in gene cloning, detection of even tightly linked markers was a protracted process due to lack of dense polymorphic markers across the wheat genome and high-throughput genotyping facilities. With the introduction of low cost and next generation genome sequencing along with reference genomes, easy to use genotyping approaches were introduced to overcome these challenges and to rapidly clone disease resistance genes (Elshire et al. 2011; Kilian et al. 2012; Wang et al. 2014; Periyannan 2018). Sequence diversity such as InDels and SNPs between paralogous and orthologous gene members are the potential sites to develop gene specific markers.. In this study, diagnostic markers

specific to *Sr46b* (STS) and *Yr672* (STS, CAPS and KASP markers) resistance were developed.

7.2.4 Maker assisted deployment of rust resistance genes

Introgression of desirable traits in commercial wheat cultivars is a time-consuming process. Combining strategies such as marker assisted selection and speed breeding could accelerate elite line development. The markers developed in this study were utilised to introgress the resistance genes *Sr46b* and *Yr672* into a commercial cultivar using marker assisted backcross selection. It facilitated identification of homozygous lines without extensive phenotyping of the large population. Phenotyping such a limited number of selected lines is more economical and time efficient. Testing the *Yr672* gene against two major Australian stripe rust races (Pst79 and WA) confirmed rust resistance to both races. Backcross lines with *Yr672* exhibited different resistance phenotypes to both stripe rust races. Resistance gene *Yr672* was most effective against Pst79, while it showed intermediate resistance to WA. This suggests *Yr672* possesses different specificity against these stripe rust races. Cloning of *Yr672* gene will be crucial to isolate the effector molecule from both the races to unlock the molecular mechanism involved in the host-pathogen interaction that provides the resistance specificity against different races.

We coupled marker assisted selection and speed breeding to rapidly stack multiple stem rust resistance genes to test the additive nature of different gene combination lines. Stacking of multiple rust resistance genes is suggested to provide durable and sustainable rust resistance (Ellis et al. 2007); however, stacking through conventional approaches could be challenging and time-consuming (Todorovska et al. 2009). A significant advance in crop breeding programs is the development of a speed breeding protocol to advance generations rapidly under an extended photoperiod. It can facilitate 5-6 generation of wheat per year (Ghosh et al. 2018; Watson et al. 2018). In this study, we successfully demonstrated the combination of speed breeding and marker assisted selection to rapidly stack three stem rust resistance genes (*Sr33, Sr45* and *Sr46b*). Speed breeding technology also enables simultaneous selection for multiple disease resistance that can include rust (Dinglasan et al. 2016; Riaz et al. 2016; Riaz and Hickey 2017; Watson et al. 2018). Marker assisted speed breeding facilitated the development of lines carrying two or three genes, and screened for rust

resistance within 180 days. Phenotyping multiple gene combination lines for stem rust resistance identified the additive effect of *Sr46b* when diploid in combination with other stem rust resistance genes. Use of marker assisted speed breeding highlights the possibility of generating lines with various gene stacks through biparental crossing and rapid testing of the additive responses. It can potentially be an alternate strategy to transgenic technology for which commercial testing and cultivation is restricted for a variety of crops in many countries.

7.3 Conclusion and future directions

This study characterised triple rust resistance from an accession of *Ae. tauschii* CPI110672. The use of high throughput technologies such as 90K SNP genotyping and comparative reference genomics facilitated the fine mapping and rapid isolation of candidate genes conferring rust resistance. The newly cloned resistance genes *Sr46b* and *Yr672* and a candidate gene for *Lr39* paved the way to the development of perfect markers to accelerate the generation of rust resistant commercial cultivars. In this study, we cloned the first stripe rust resistance gene from *Ae. tauschii*. This study also demonstrated rapid stacking of rust resistance genes through marker assisted speed breeding. Furthermore, it also identified the additive nature of stem rust resistance gene *Sr46b,* thus enabling it as a potential candidate for gene pyramiding.

Advances in wheat genomics will facilitate rapid gene isolation in wheat and its relatives (Wulff and Moscou 2014). So far, only 25 disease resistance genes have been cloned from wheat and its progenitors in the past fifteen years (Keller et al. 2018). It was hindered due to the complex polyploid nature of the wheat genome and the lack of reference genome sequences. Now genomes of wheat and wheat relatives are being decoded, pinpointing the precise location of genes and markers in the genomes. Cloning of *Sr46b* and *Yr672* enriches the cloned gene pool and gene specific markers for marker assisted selection. Further fine mapping and candidate gene identification for *Lr39* will facilitate its cloning and functional characterisation. More resistance genes will become available in near future to enrich gene pool to address the race specific resistance. Therefore, it will provide opportunities for rapid resistance gene introgression into commercial cultivars, evaluating gene stack combinations and effective deployment. Furthermore, an enriched genepool will be a crucial resource for identifying avirulence factors from diverse rust races. Such avirulence factors could

potentially provide a resource to develop an array of diagnostic tools to forecast pathogen emergence and select suitable gene deployment strategies for rust management (Keller et al. 2018; Periyannan 2018). A gene pool with abundant resistance genes is certainly a key resource for gene editing to alter or enhance rust resistance.

In summary, reference genomes, together with high throughput sequencing and speed breeding will form the basis for many objectives in next generation breeding programs. It includes high density genotyping, genome wide association studies, rapid cloning of resistance genes, enriching the gene and marker pools, rapid transfer of target traits into commercial cultivars, gene editing opportunities, gene stacking for durable resistance, and potential identification of corresponding avirulence factors. These resources are potential components in the development of an early warning system for wheat rust management, thus promoting global food security.

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Appendices

Supplementary file 1

Wheat Rust	Races	Virulence/Avirulence profiles
Leaf rust	26-1, 3	*Lr13/Lr12,Lr17,Lr22a,Lr23,Lr24,Lr27,Lr31*
Stem rust	34-0	*Not available*
	98-1,2,3,5,6	*Sr5,Sr6,Sr8a,Sr9b,Sr9g,Sr11,Sr17 /Sr15,Sr22,Sr24, Sr26,Sr27,SrSatu,Sr30,Sr31,Sr36,Sr38*
	TTRTF	*Sr5,Sr6,Sr7b,Sr8a,Sr9a,Sr9b,Sr9d,Sr9e,Sr9g,Sr10,Sr11,Sr17,Sr21,Sr36,Sr38,SrMcN,SrTmp/Sr24,Sr30,Sr31*
Stripe rust	104 E137A+	*Yr2,Yr3,Yr4,Yr25,YrA / Yr1,Yr5,Yr6,Yr7,Yr8,Yr9, Yr10,Yr15,Yr17,Yr27,Yr32,Yr33,YrJ,YrT*
	104E137A-	*Yr2,Yr3,Yr4,Yr25 / Yr1,Yr5,Yr6,Yr7,Yr8,Yr9,Yr10, Yr15,Yr17,Yr27,Yr32,Yr33,YrA,YrJ,YrT*
	134E16A+	*Yr2,Yr6,Yr7,Yr8,Yr9,Yr25,YrA / Yr1,Yr3,Yr4,Yr5, Yr10,Yr15,Yr17,Yr27,Yr32,Yr33,YrJ,YrT*

Supplementary file 2

Phenotypic and genotyping data of CPI110672 x CPI110717 $F_{2:3}$ mapping families

Loci	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Lr672	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Sr672a*	S	R	H	S	S	R	S	H	R	S	S	H	S	H	H	R	R	R	H	H	H
Sr672b*	S	S	H	S	R	S	H	H	S	S	S	-	R	S	-	-	S	S	-	H	H
Yr672	S	R	H	H	H	R	H	H	H	H	H	S	H	S	R	H	R	R	H	H	H
Csq1	S	R	H	H	H	R	S	H	H	H	H	S	H	R	R	H	H	R	R	H	H
Csq2	H	R	H	H	-	R	S	H	H	H	H	S	H	R	R	H	S	R	R	H	H
Csq3	S	R	H	H	H	R	H	H	H	H	H	S	H	S	R	H	R	R	H	H	H
Csq4	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq5	S	R	H	S	S	R	S	H	R	S	H	H	S	H	H	R	R	R	H	H	H
Csq6	H	R	H	S	S	R	S	H	R	S	H	H	S	H	H	R	R	R	H	H	H
Csq7	S	H	H	S	S	R	S	H	R	S	-	H	S	H	H	R	R	R	H	H	H
Csq8	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq9	S	R	H	H	H	R	S	H	H	H	H	S	H	S	R	H	R	R	H	H	H
Csq10	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq11	S	R	H	H	H	R	H	H	H	H	H	S	H	S	R	H	R	R	H	H	H
Csq12	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq13	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq14	S	R	H	S	S	R	S	H	R	S	R	H	S	H	S	R	H	H	H	H	H
Csq15	S	R	H	H	H	R	H	H	H	H	H	S	H	S	R	H	R	R	H	H	H
Csq16	S	R	H	H	H	R	H	H	H	H	H	S	H	S	R	H	R	R	H	H	H
Csq17	S	R	H	H	H	R	S	H	H	H	H	S	H	H	R	H	H	R	H	H	H
Csq18	S	R	H	H	H	R	S	H	H	H	H	S	H	H	R	H	H	R	H	H	H
Csq19	S	R	H	H	H	R	S	H	H	H	H	S	H	R	R	H	H	R	R	H	H
Csq20	S	R	H	S	S	R	S	H	H	S	-	H	S	H	S	R	H	H	H	H	H
Csq21	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq22	S	R	H	S	S	R	S	-	H	S	R	H	S	H	S	-	H	H	H	H	H
Csq23	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq24	S	R	H	S	S	R	S	H	R	S	R	H	S	H	S	R	H	H	H	H	H
Csq25	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq26	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq27	S	R	H	S	S	R	S	H	H	S	R	H	S	H	S	R	H	H	H	H	H
Csq28	S	R	H	S	S	R	S	H	H	S	-	H	S	H	S	R	H	H	H	H	H
Csq29	S	R	H	S	S	R	S	H	H	S	-	H	S	H	S	R	H	H	H	H	H
Csq30	S	R	H	S	S	R	S	H	H	S	-	H	S	H	S	R	H	H	H	H	H
SNP S4	S	S	H	S	H	H	R	R	H	S	S	H	H	R	H	H	H	S	S	H	H
SNP S7	S	S	H	S	S	R	H	H	H	S	S	R	H	R	H	H	H	H	S	H	H
SNP S9	S	S	H	S	H	H	H	R	H	S	S	R	H	R	H	H	H	S	S	H	H
SNP S10	S	S	H	S	H	H	R	R	H	S	S	R	H	R	H	H	H	S	S	H	H
SNP S11	S	S	H	S	H	H	R	R	H	S	S	R	H	R	H	H	H	S	S	H	H
Xgdm35	S	H	H	S	S	R	S	H	R	R	S	H	H	H	R	R	R	R	H	H	H
Xwmc52	H	R	H	H	H	H	S	R	R	H	R	S	H	R	H	R	S	R	R	H	H
Xwmc720	H	R	H	H	H	R	S	R	R	H	R	S	H	R	H	R	S	R	R	H	H
Xgpw4087	H	R	H	H	H	H	S	R	R	H	R	S	H	R	H	R	S	R	R	H	H
Xgpw5072	H	R	H	H	H	H	S	R	R	H	R	S	H	R	H	R	S	R	R	H	H
Dominant Markers																					
S46ConsFA/ S46PREVR	-	+	+	-	-	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+
Sr46F1/R1	-	+	+	-	-	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+
2D93	-	+	+	-	-	+	-	+	+	-	+	+	-	+	-	+	+	+	+	+	+
2D94	-	+	+	-	-	+	-	+	+	-	-	+	-	+	-	+	+	+	+	+	+
2D96	-	+	+	-	-	+	-	+	+	-	-	+	-	+	-	+	+	+	+	+	+
6510F5/R5	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+
Ctg2605F7/R7	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+
* The stem rust score are putatively separated based on the recommendation by Prof Robert McIntosh																					

Loci	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Lr672	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Sr672a*	-	H	R	S	S	H	R	H	H	H	H	R	R	H	R	H	R	S	H
Sr672b*	-	H	S	R	S	S	S	S	S	S	H	S	S	S	S	H	S	H	-
Yr672	H	R	H	R	R	H	H	H	H	H	R	R	H	H	R	R	R	H	H
Csq1	H	R	H	R	H	H	H	H	R	R	R	H	H	R	R	H	R	H	H
Csq2	H	R	R	R	H	H	H	H	R	R	R	H	H	R	R	H	R	H	H
Csq3	H	R	H	R	R	H	H	H	H	H	R	R	H	H	R	R	R	H	H
Csq4	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq5	R	H	R	S	H	H	R	H	H	H	H	H	H	H	R	H	R	S	H
Csq6	R	H	R	S	S	H	R	H	H	H	H	H	H	H	R	H	R	S	H
Csq7	H	H	R	S	S	H	R	H	H	H	H	H	H	S	H	H	R	S	S
Csq8	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq9	H	R	H	R	R	H	H	H	H	H	R	R	H	H	R	H	R	H	H
Csq10	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq11	H	R	H	R	R	H	H	H	H	H	R	R	H	H	R	R	R	H	H
Csq12	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq13	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq14	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	H	R	S	H
Csq15	H	R	H	R	R	H	H	H	H	H	R	R	H	H	R	R	R	H	H
Csq16	H	R	H	R	R	H	H	H	H	H	R	R	H	H	R	R	R	H	H
Csq17	H	R	H	R	H	H	H	H	H	H	R	R	H	H	R	H	R	H	H
Csq18	H	R	H	R	H	H	H	H	H	R	R	R	H	H	R	H	R	H	H
Csq19	H	R	H	R	H	H	H	H	R	R	R	H	H	R	R	H	R	H	H
Csq20	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq21	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq22	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq23	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq24	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq25	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq26	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq27	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq28	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq29	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
Csq30	R	H	R	S	S	R	R	H	S	H	H	H	H	H	R	R	R	S	H
SNP S4	H	H	H	H	R	H	R	S	R	H	R	S	H	S	R	H	R	H	R
SNP S7	H	H	H	H	H	H	R	S	R	H	R	S	H	S	R	H	R	H	R
SNP S9	H	H	H	H	H	H	R	S	R	H	R	S	H	S	R	H	R	H	R
SNP S10	H	H	H	H	H	H	R	S	R	H	R	S	H	S	R	H	R	H	R
SNP S11	H	H	H	H	R	H	R	S	R	H	R	S	H	S	R	H	R	H	R
Xgdm35	H	H	H	S	H	H	H	H	H	H	H	H	H	S	H	H	R	H	S
Xwmc52	R	R	H	H	S	R	S	H	R	R	R	H	R	H	R	S	R	R	R
Xwmc720	R	R	H	H	S	R	H	H	R	R	R	H	R	R	R	S	R	R	R
Xgpw4087	R	R	H	H	S	R	S	H	-	R	R	H	R	H	R	S	R	R	R
Xgpw5072	R	R	S	H	S	R	S	H	H	R	R	H	R	H	R	S	R	R	R
Dominant Markers																			
S46ConsFA/ S46PREVR	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Sr46F1/R1	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
2D93	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+
2D94	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+
2D96	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+
6510F5/R5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ctg2605F7/R7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Loci	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Lr672	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Sr672a*	R	R	H	H	S	S	S	S	H	H	S	S	R	H	H	R	S	H	H	S
Sr672b*	S	S	H	S	H	H	S	H	-	-	H	H	S	-	S	S	S	-	H	H
Yr672	H	R	R	S	H	H	H	S	R	S	R	R	S	R	H	S	R	R	R	S
Csq1	H	R	R	H	H	H	H	S	R	S	R	R	H	R	H	S	R	H	R	H
Csq2	H	R	R	H	H	H	H	S	R	S	R	R	H	R	H	H	R	H	R	H
Csq3	H	R	R	S	H	H	H	S	R	S	R	R	S	R	H	S	R	R	R	S
Csq4	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq5	R	H	H	H	S	S	S	S	H	H	S	S	R	H	H	-	S	H	H	S
Csq6	R	R	H	H	S	S	S	S	H	H	-	S	R	H	H	R	S	H	H	S
Csq7	R	R	R	H	S	S	S	S	H	H	S	S	R	H	H	R	S	H	H	S
Csq8	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq9	H	R	R	S	H	H	H	S	R	S	R	R	H	R	H	S	R	R	R	S
Csq10	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq11	H	R	R	S	H	H	H	S	R	S	R	R	S	R	H	S	R	R	R	S
Csq12	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq13	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq14	R	H	H	H	S	S	H	S	H	H	S	S	R	H	H	R	S	H	H	S
Csq15	H	R	R	S	H	H	H	S	R	S	R	R	S	R	H	S	R	R	R	S
Csq16	H	R	R	S	H	H	H	S	R	S	R	R	S	R	H	S	R	R	R	S
Csq17	H	R	R	S	H	H	H	S	R	S	R	R	H	R	H	S	R	R	R	H
Csq18	H	R	R	H	H	-	H	S	R	S	R	R	H	R	H	S	R	R	R	H
Csq19	H	R	R	H	H	H	H	S	R	S	R	R	H	R	H	S	R	H	R	H
Csq20	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq21	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq22	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	-	S	H	H	S
Csq23	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq24	R	H	H	H	S	S	H	S	H	H	S	S	R	H	H	R	S	H	H	S
Csq25	R	H	H	H	S	-	H	S	H	H	-	S	H	H	H	R	S	H	H	S
Csq26	R	H	H	H	S	-	H	S	H	H	-	S	H	H	H	R	S	H	H	S
Csq27	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq28	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq29	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
Csq30	R	H	H	H	S	S	H	S	H	H	S	S	H	H	H	R	S	H	H	S
SNP S4	H	H	H	H	H	H	S	H	H	H	R	H	S	S	S	H	H	H	S	S
SNP S7	H	H	H	H	H	R	H	H	H	H	R	S	S	S	S	H	H	S	H	S
SNP S9	H	H	H	H	H	H	S	H	H	H	R	H	S	S	S	H	H	H	H	S
SNP S10	H	H	H	H	H	H	S	H	H	H	R	H	S	S	S	H	H	H	S	S
SNP S11	H	H	H	H	H	H	S	H	H	H	R	H	S	S	S	H	H	H	S	S
Xgdm35	R	R	R	H	S	S	S	S	R	H	S	S	H	H	H	R	S	S	H	S
Xwmc52	H	R	R	R	H	S	R	R	R	H	R	H	H	S	H	R	R	H	H	R
Xwmc720	H	R	R	R	H	S	R	R	R	R	R	H	H	S	H	R	R	H	H	R
Xgpw4087	H	R	R	R	H	S	R	R	R	H	R	H	H	H	H	R	R	H	H	R
Xgpw5072	H	R	R	R	H	S	R	R	R	H	R	H	H	S	H	R	R	H	H	R
S46ConsFA/ S46PREVR	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	-
Sr46F1/R1	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	-
2D93	+	+	+	+	-	-	+	-	+	+	-	-	+	+	+	+	-	+	+	-
2D94	+	+	+	+	-	-	+	-	+	+	-	-	+	+	+	+	-	+	+	-
2D96	+	+	+	+	-	-	+	-	+	+	-	-	+	+	+	+	-	+	+	-
6510F5/R5	+	+	+	-	+	+	+	-	+	-	+	+	-	+	+	-	+	+	+	-
Ctg2605F7/R7	+	+	+	-	+	+	+	-	+	-	+	+	-	+	+	-	+	+	+	-

Loci	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Lr672	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Sr672a*	S	R	R	S	R	H	R	H	H	H	S	S	S	H	S	R	S	H	H	S
Sr672b*	R	S	S	H	S	S	S	-	H	S	H	H	H	S	H	S	H	H	S	H
Yr672	R	R	H	S	R	R	H	R	H	R	S	R	H	R	R	R	R	R	H	R
Csq1	R	R	H	S	R	R	H	R	R	R	H	H	H	R	R	R	R	R	R	R
Csq2	R	R	H	H	R	R	H	R	R	R	H	H	H	R	R	R	R	R	R	H
Csq3	R	R	H	S	R	R	H	R	H	R	S	R	H	R	R	R	R	R	H	R
Csq4	S	R	H	S	H	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq5	S	R	H	S	R	H	R	H	H	H	H	S	S	H	S	R	S	H	H	S
Csq6	S	R	H	S	R	H	R	H	H	H	S	S	S	H	S	R	S	H	H	S
Csq7	S	R	H	S	R	H	H	H	H	H	S	S	S	H	S	R	S	H	H	S
Csq8	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq9	R	R	H	S	R	R	H	R	H	R	S	R	H	R	R	R	R	R	H	R
Csq10	S	R	H	S	H	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq11	R	R	H	S	R	R	H	R	H	R	S	R	H	R	R	R	R	R	H	R
Csq12	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq13	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq14	S	R	H	S	R	H	R	H	H	H	H	S	S	H	H	R	S	H	H	S
Csq15	R	R	H	S	R	R	H	R	H	R	S	R	H	R	R	R	R	R	H	R
Csq16	R	R	H	S	R	R	H	R	H	R	S	R	H	R	R	R	R	R	H	R
Csq17	R	R	H	S	R	R	H	R	R	R	S	R	H	R	R	R	R	R	R	R
Csq18	R	R	H	S	R	R	H	R	R	R	S	R	H	R	R	R	R	R	R	R
Csq19	R	R	H	S	R	R	H	R	R	R	H	H	H	R	R	R	R	R	R	R
Csq20	S	R	H	S	H	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq21	S	R	H	S	H	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq22	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq23	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq24	S	R	H	S	R	H	R	H	H	S	H	S	S	H	H	R	H	H	H	S
Csq25	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq26	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq27	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq28	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq29	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
Csq30	S	R	H	S	R	H	R	R	H	S	H	S	S	H	H	R	H	H	H	S
SNP S4	S	H	R	S	R	H	H	R	H	H	R	S	H	-	S	R	H	S	H	H
SNP S7	S	H	R	S	R	H	H	R	H	H	R	S	H	H	S	R	H	S	H	H
SNP S9	S	H	R	S	R	H	H	R	H	H	R	S	H	H	S	R	H	S	H	H
SNP S10	S	H	R	S	R	H	H	R	H	H	R	S	H	H	S	R	H	S	H	H
SNP S11	S	H	R	S	R	H	H	R	H	H	R	S	H	H	S	R	H	S	H	H
Xgdm35	S	H	H	S	R	S	H	H	S	H	S	H	S	H	S	H	S	H	H	S
Xwmc52	S	H	H	R	R	H	R	R	R	R	R	H	S	H	R	H	R	H	R	H
Xwmc720	S	H	H	R	R	H	R	R	R	R	R	H	S	H	R	H	R	H	R	H
Xgpw4087	S	H	H	R	R	H	R	R	R	R	R	H	S	H	R	H	R	H	R	H
Xgpw5072	S	H	H	R	R	H	R	R	R	R	R	H	S	H	R	H	R	H	R	H
S46ConsFA/ S46PREVR	-	+	+	-	+	+	+	+	+	+	-	-	-	+	-	+	-	+	+	-
Sr46F1/R1	-	+	+	-	+	+	+	+	+	+	-	-	-	+	-	+	-	+	+	-
2D93	-	+	+	-	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	-
2D94	-	+	+	-	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	-
2D96	-	+	+	-	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	-
6510F5/R5	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Ctg2605F7/R7	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+

Loci	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
Lr672	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
Sr672a*	H	H	H	H	-	H	R	S	H	-	S	R	S	S	S	H	H	H	R	S
Sr672b*	H	H	H	-	-	H	S	S	H	-	-	S	R	S	-	-	H	S	S	-
Yr672	H	S	H	H	R	R	H	H	H	H	S	H	S	H	R	H	R	S	H	H
Csq1	H	S	H	H	R	R	H	R	R	H	S	R	S	H	R	R	R	S	H	R
Csq2	H	S	H	H	R	R	H	R	R	H	S	R	S	H	R	R	R	S	H	R
Csq3	H	S	H	H	R	R	H	H	H	H	S	H	S	H	R	H	R	S	H	H
Csq4	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	S	H	H	H	S
Csq5	H	H	H	S	H	H	R	S	H	R	H	R	S	H	S	H	H	H	H	S
Csq6	H	H	H	S	H	H	R	S	H	R	-	R	S	H	S	H	H	H	R	S
Csq7	H	H	H	H	H	H	R	S	H	R	S	R	S	S	S	H	H	H	R	S
Csq8	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
Csq9	H	S	H	H	R	R	H	H	R	H	S	H	S	H	R	R	R	S	H	H
Csq10	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	S	H	H	H	S
Csq11	H	S	H	H	R	R	H	H	H	H	S	H	S	H	R	H	R	S	H	H
Csq12	S	H	S	H	H	H	H	S	H	R	S	R	S	R	S	H	H	H	H	S
Csq13	S	H	S	H	H	H	H	S	H	R	S	R	S	R	S	H	H	H	-	S
Csq14	H	H	H	H	H	H	R	S	H	R	S	R	S	R	S	H	H	H	H	S
Csq15	H	S	H	H	R	R	H	H	H	H	S	H	S	H	R	H	R	S	H	H
Csq16	H	S	H	H	R	R	H	H	H	H	S	H	S	H	R	H	R	S	H	H
Csq17	H	S	H	H	R	R	H	H	R	H	S	H	S	H	R	R	R	S	H	H
Csq18	H	S	H	H	R	R	H	H	R	H	S	H	S	H	R	R	R	S	H	H
Csq19	H	S	H	H	R	R	H	R	R	H	S	R	S	H	R	R	R	S	H	H
Csq20	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	S	H	H	H	S
Csq21	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
Csq22	S	H	S	H	H	H	H	S	H	R	S	R	S	R	S	H	H	H	H	S
Csq23	S	H	S	H	H	H	H	S	H	R	S	R	S	R	S	H	H	H	H	S
Csq24	H	H	H	H	H	H	R	S	H	R	S	R	S	R	S	H	H	H	H	S
Csq25	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
Csq26	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
Csq27	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
Csq28	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
Csq29	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
Csq30	S	H	S	H	H	H	H	H	H	R	S	R	S	R	S	H	H	H	H	S
SNP S4	H	H	H	S	R	H	R	H	S	R	R	H	H	H	H	S	S	R	S	H
SNP S7	H	H	H	S	R	H	R	-	S	R	R	H	S	H	H	H	S	H	S	H
SNP S9	H	H	H	S	R	H	R	H	S	R	R	H	H	H	H	S	S	R	S	H
SNP S10	H	H	H	S	R	H	R	H	S	R	R	H	H	H	H	S	S	R	S	H
SNP S11	H	H	H	S	R	H	R	H	S	R	R	H	H	H	H	S	S	R	S	H
Xgdm35	H	H	H	H	H	H	R	S	H	R	S	R	S	S	S	R	H	H	R	S
Xwmc52	H	R	H	H	R	R	H	R	R	H	R	R	S	H	R	R	R	H	H	R
Xwmc720	H	R	H	H	R	R	H	R	R	H	R	R	S	H	R	R	R	H	H	R
Xgpw4087	H	R	H	H	R	R	H	H	R	H	R	R	S	H	R	R	R	H	H	R
Xgpw5072	H	R	H	H	R	R	H	H	R	H	R	R	S	H	R	R	R	H	H	R
S46ConsFA/ S46PREVR	+	+	+	+		+	+	-	+		-	+	-	-	-	+	+	+	+	-
Sr46F1/R1	+	+	+	+		+	+	-	+		-	+	-	-	-	+	+	+	+	-
2D93	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	-
2D94	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	-
2D96	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	-
6510F5/R5	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+
Ctg2605F7/R7	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+

Loci	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116
Lr672	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Sr672a*	S	H	S	R	H	S	S	H	R	H	R	R	S	R	H	R
Sr672b*	H	-	H	S	-	H	H	S	S	-	S	S	R	S	-	S
Yr672	S	H	S	H	S	H	R	H	R	H	S	H	H	R	S	S
Csq1	S	H	S	H	S	H	H	H	R	H	S	H	R	R	H	R
Csq2	S	H	S	H	H	H	H	H	R	H	S	H	R	R	H	R
Csq3	S	H	S	H	S	H	R	H	R	H	S	H	H	R	S	S
Csq4	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq5	S	H	S	H	H	S	S	R	H	H	R	R	S	R	H	R
Csq6	S	H	S	R	H	S	S	R	H	-	R	R	S	R	H	R
Csq7	H	H	S	R	H	S	S	R	H	H	R	H	S	R	H	H
Csq8	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq9	S	H	S	H	S	H	R	H	R	H	S	H	H	R	S	H
Csq10	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq11	S	H	S	H	S	H	R	H	R	H	S	H	H	R	S	R
Csq12	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq13	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq14	S	H	S	H	H	S	H	R	H	H	R	H	S	R	H	R
Csq15	S	H	S	H	S	H	R	H	R	H	S	H	H	R	S	S
Csq16	S	H	S	H	S	H	R	H	R	H	S	H	H	R	S	S
Csq17	S	H	S	H	S	H	R	H	R	H	S	H	H	R	S	H
Csq18	S	H	S	H	S	H	R	H	R	H	S	H	H	R	S	R
Csq19	S	H	S	H	S	H	H	H	R	H	S	H	R	R	H	R
Csq20	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq21	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq22	S	H	H	H	-	S	H	R	H	H	R	H	S	R	H	H
Csq23	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	R
Csq24	S	H	S	-	H	S	H	R	H	H	R	H	S	R	H	R
Csq25	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq26	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq27	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq28	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq29	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
Csq30	S	H	H	H	H	S	H	R	H	H	R	H	S	R	H	H
SNP S4	S	R	R	H	R	H	R	H	H	R	H	H	H	H	H	H
SNP S7	S	R	R	H	R	H	R	H	H	R	H	H	H	H	H	R
SNP S9	S	R	R	H	R	H	R	H	H	R	H	H	H	H	H	H
SNP S10	S	R	R	H	R	H	R	H	H	R	H	H	H	H	H	H
SNP S11	S	R	R	H	R	H	R	H	H	R	H	H	H	H	H	H
Xgdm35	H	R	R	R	H	S	S	R	H	H	R	H	S	H	H	R
Xwmc52	R	H	H	H	H	R	H	H	H	S	R	H	H	R	R	R
Xwmc720	R	H	H	H	H	R	H	H	R	S	R	H	H	R	R	R
Xgpw4087	R	H	H	H	S	R	H	H	H	S	R	H	H	R	R	R
Xgpw5072	R	H	H	H	S	R	H	H	H	S	R	H	H	H	R	R
S46ConsFA/ S46PREVR	-	+	-	+	+	-	-	+	+	+	+	+	-	+	+	+
Sr46F1/R1	-	+	-	+	+	-	-	+	+	+	+	+	-	+	+	+
2D93	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+
2D94	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+
2D96	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+
6510F5/R5	-	+	-	+	-	+	+	+	+	+	-	+	+	+	-	-
Ctg2605F7/R7	-	+	-	+	-	+	+	+	+	+	-	+	+	+	-	-

Loci	117	118	119	120	121	122	123
Lr672	H	H	H	H	H	R	R
Sr672a*	S	H	R	S	H	R	R
Sr672b*	H	H	S	R	-	S	S
Yr672	R	R	H	H	R	R	H
Csq1	R	R	H	H	R	R	H
Csq2	R	R	H	H	H	R	H
Csq3	R	R	H	R	R	R	H
Csq4	H	H	H	H	H	R	R
Csq5	H	H	H	H	H	R	R
Csq6	S	H	H	H	H	R	R
Csq7	S	H	H	S	H	R	R
Csq8	H	H	H	H	H	R	R
Csq9	R	R	H	H	R	R	H
Csq10	H	H	H	H	H	R	R
Csq11	R	R	H	H	R	R	H
Csq12	H	H	H	H	H	R	R
Csq13	H	H	H	H	H	R	R
Csq14	H	H	H	H	H	R	R
Csq15	R	R	H	R	R	R	H
Csq16	R	R	H	R	R	R	H
Csq17	R	R	H	H	R	R	H
Csq18	R	R	H	H	R	R	H
Csq19	R	R	H	H	R	R	H
Csq20	H	H	H	H	H	R	R
Csq21	H	H	H	H	H	R	R
Csq22	H	H	H	H	H	R	R
Csq23	H	H	H	H	H	R	R
Csq24	H	H	H	H	H	R	R
Csq25	H	H	H	H	H	R	R
Csq26	H	H	H	H	H	R	R
Csq27	H	H	H	H	H	R	R
Csq28	H	H	H	H	H	R	R
Csq29	H	H	H	H	H	R	R
Csq30	H	H	H	H	H	R	R
SNP S4	H	H	R	R	R	R	H
SNP S7	H	H	R	R	R	R	H
SNP S9	H	H	R	R	R	R	H
SNP S10	H	H	R	R	R	R	H
SNP S11	H	H	R	R	R	R	H
Xgdm35	S	H	H	S	R	H	R
Xwmc52	H	H	H	H	H	R	H
Xwmc720	H	H	H	H	H	R	H
Xgpw4087	H	H	H	H	H	R	H
Xgpw5072	H	H	S	H	H	R	H
S46ConsFA/ S46PREVR	-	+	+	-	+	+	+
Sr46F1/R1	-	+	+	-	+	+	+
2D93	+	+	+	+	+	+	+
2D94	+	+	+	+	+	+	+
2D96	+	+	+	+	+	+	+
6510F5/R5	+	+	+	+	+	+	+
Ctg2605F7/R7	+	+	+	+	+	+	+

Supplementary file 3

Sequence of *Csq* markers developed in this study

Csq1 SNP Id IWB47181

CATGGCAGGGCGTTCTCGCTGGCCACTTGTATCCCGGCATCCCACGCAGC[A/G]TTCAGCACCTGCCATACCAAGCCCTCTG
GATCTGCCAAGGCCTCGGGGTA

Csq2

CPI110672

ACAATCGTATCCATCATTGGTGATGAACTCGATCGAGAAGCATGTCGGCACGTCGGCATTCTAGCCTCGAGTCAGAGTCCAAC
CATATCACTCACTAGTCACTAGTCCAATAAATCGGCGGTGTATTTTTCAGCAGGGAATCAAACAGTGACTTTTCTCGATCTCC
ACTAGCAGCGACTTGCCCGTCTCCATCGGGATTTAGTACCAGTCCAAAAAATCGGCGCCGCAGCTCACAGAGATCCAGAATTC
ATTTTGCGTGGACACAGTTTTTTTGATACTTCAGCAGTTGAGAGTAACAGAATTTCCACCTGTGTGTGTGTGTGTGTGATG
CATGGACAGTACGAGAGCCCAGCATTTCACGGTATGTCGGTATATCAATATAAGTCAGCGTATAGCATTCTTGGTGCCGACTT
CTTGCGACGACTGGGTGCACCTACATATATAAGAAGACTCCACGGATCAGTTTCACAGGCACAAAACCAAGAACCTACTCCAG
CTGC

CPI110717

CAATCGTATCCATCATTGGTGATGAACTCGATCGAGAAGCATGTCGGCACGTCGGCATTCTAGCCTCGAGTCAGAGTCCAACC
ATATCACTCACTAGTCACTAGTCCAATAAATCGGCGGTGTATTTTTCAGCAGGGAATCAAACAGTGACTTTTCTCGATCTCCA
CTAGCAGCGACTTGTCCGTCTCCATCAGAATTTAGTACCAGTCCAAAAAATCGGCGCCGCAGCTCACAGAGATCCAGAATTCA
TTTTGGGTGGACACAGTTTTTTTGATACTTCAGCAGTGAGAGGATCCACG

Csq3

CPI110672

AATTTAGCTGCCGGTTTACATTTCCATCAAATCAAATGGTTGTATATATATGTGTGTTCTAGCAGATGAGTCCTTAATGGGGT
TTATGTGAGTGTGGCTACCAACAGTATTTTTAGAATAAACTTAAGACTTACTTTTGCACTGGTATATAATTTTTTACGAAAAC
AAAACCAACGTTGACTTCATAGCGAAAAAGGCAAAATTTATTTGCTATTATTGGTCACTATTCACACTATTTTGGTCAAAAAA
AATTCTCACTTACAATGGAAGGTCAAGTTTATTTCAAAAATATTTTCACTTACAATGGCAGGTCAAGTTTATTTCAAAAATAT
TTTAAGAGTTTTTTGACTTTATTGGATTACTAAAAAGATTTACATATAGGGTGTAGATGTCCCATGTCCACGTCCCACAAACA
TCGGATATAAAATAGTCGCAAAAGACATGCATAGGCCATGTCGACTTGTTTCAATGTATCTGTTCACTCTCATGGTCATTCTG
TAAATCTGAATCCACTCATCGTGCTCGTGTACTGGAAACTAAGCAACAAATCAGATCAACTATAACCCCAATCAATGGCCGAC
CAACTGAATTAATGTCATGGCATGGGCCGGCCCAGGTCAACGGCCCAAAGCCACGAGGTGTAACCCAAGACTGGCAGCGACTA
GAGACAGAGACGAGAGCTAAGAGCTGGCTATTCAACCTTGTTGATTCGCTCAGC

CPI110717

AGTTAAATTTAGCTGCCGGTTTACATTTCCATCAAATCAAATGGTTGTATATATATGTGTTCTAGCAGATGAGTCCTTAATCA
GTTTATGTGAGTGTGGCTACCAACAGTATTTTTAGAATAAACTTAAGACTTACTTTTGCACTGGTATATAATTTTTTGCACTG
GCTATTCAACCTTGTTGATTCGCTCAGC

Csq4 SNP Id IWB64398

TGGAGCAGACCGTTTATCCCCGACCGCTGGCACCGGTCCCGGACGTTGTC[A/G]TCCGAGCTCGCCGCGAGGGCCACGATCA
GCGGCGGCCAGCTGTTGCTCCT

Csq5 SNP Id IWB18330

GACTCGCTGGACGACCATAGTCAGGACTAGGTCGTGACCTTCGGTACATTGGGCTGACAGAACGGCGATATGAACTGTCATCC
GGCCTTCCATAGCCACCGCCTCTACTTGGCCTGTCATACTT[A/G]TCACCTGGTTCACTATCATCCCTGAAGGCATACTCAA
CAGAAATCACTCTGTCCAGTAACTTCCTGCAGAAGGATGAGAATTATTCAGGCATATTTTTACACTGTAAGACTGTTTTCAAA
CAATC

Csq6 SNP Id IWB25534

CTTCTTGATGATGGCTTCACCTTCCTAGTATGGTTGGGTAGGATGCTCCA[A/G]CCTGAGCTCATGAACGACATTCTTGGAG
TCAGCCTCTCAAACTTCCCCGA

Csq7 SNP Id IWB15863

CTGCTCTTAGAATATTCATTGTATGAACTACATTGATTGCTAGATACACACCCATTATAGCAGTGACATATATGATTTGTCAA
TTTGCATGCAGTGGGTACGTACCGCCTGAATATATCGACGC[A/C]GCTATAATCTCTATTAAGTTTGACATATACAGTTTGG
GCGTGGTAATCATAAAGTTAATGACAGGACCAATGGGCTACTTTAGAAGTGCTGAAATGTCGCCTGAACAATTCATAGAACTT
GTAAG

Csq8

CPI110672

CGCCCGTTAACGTGCTACAAACATGTCCAAACCTCGGAGAAAGGACGGCCATACAACAGTCTCCACAACAGCCCTCGAACCAA
CAAGTCACGGCTAGTAGAGAGGAGGTACTCTTTAGCTACAGCTCCAAGAAGCATCGTATCCTTTGATCCCCATCGAACCAAGG
CTTCGCTAGCCCATCCAGCAAGCCGAACGCCGGGGAAGGGATCCGCACAGCGCCAACAGAGAGCAAGCTGAAGCCGTGCTGCA
TCATCAGTAGCAGACCATGGCCGCTGGATCCGAGAGCCCACCGATCGACCTACAACGTCACCCTGGAAGCGATCACACATGAT
GAACATTTTTGAATTACAAAATATATGATGAACGATTCTTTAATACCTTGCGAATTGCATGATGAACATTTTACAACGCAATG
AGCATTTTTAAAATAATAGATGAACATTTCGAAAATTACAAGATGATTTTTTAATATGTGGTTAATATTTTTAATATACCATA
AACATGTTAAAAATACACAATTACACACAATAACATTTTGAATACAAACTTCACATCTTTGAAATGCATGATTGACATTTTTA
ATACACATTCAACATTACTAAAATACACAATGAATATTTTTTATACATGATAATATATTTTTCATACACGGTCAACACTATTC
AAATACAGGATGAACTATTCATGAAAAGAACATAACCATGCATTTACAAAATGTTCACGTAGTTGAAGAAAAAATTGGTCGCA
TTTTCTGAAAATTGTTCATACATTTCAAAAAATGTTCATAATATTTTGAAAAACTGTCCATGCAATGTTAGAAAAAGATGCAT
GACACTAAAAAATATATTCACAACACTAAGGCAATGTCCCAA

CPI110717

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GAACCAACAAGTCACGGCATAGTAGAGAGGAGGTACTCTTTAGCTACAGCTCCAAGAAGCATCGTATCCTTTGATCCCCATCG
AACCAAGGCTTCGCTAGCCCATCCAGCAAGCCGAACGCCGGGGAAGGGATCCGCACAGCGCCAACAGAGAGCAAGCCGAAGCC
GTGCTGCATCATCAGTAGCAGACCATGGCCGCTGGATCTGAGAGCCCACCGATCGACCTACAACGTCACCCTGGAAGCGGTCA
CACATGATGAACATTTTTGAATTACATGATGAGTTAACTTTAAATATATGATGAACGATTCTTTAATACCTTGCGAATTGCAT
GATGAACATTTTACAACGCAATGAGCATTTTTAAAATAATAGATGAACATTTCGAAAATTACAAGATGATTTTTTAATATGTG
GTTAATATTTTTAATATACCATAAACATGTTAAAAATACACAATTACACACAATAACATTTTGAATACAAACTTCACATCTTT
GAAATGCATGATTGACATTTTTAATACACATTCAACATTACTAAAATACACAATGAATATTTTTTATACATGATAATATATTT
TTCATACACGGTCAACACTATTCAAATACAGGATGAACTATTCATGAAAAGAACATAACCATGCATTTACAAAATGTTCATGT
AGTTGAAGAAAAAATTGGTCGCATTTTCTGAAAATTGTTCATACATTTCAAAAAATGTTCATAATATTTTGAAAAACTGTCCA
TGCAATGTTAGAAAAAGATGCATGACACTAAAAAATATATTCACAACACTAAGGCAATGTCCCAA

AL8/78

CGCCCGTTAACGTGCTACAAACATGTCCAAACCTCGGAGAAAGGACGGCCATACAACAGTCTCCACAACAGCCCTCGAACCAA
CAAGTCACGGCCTAGTAGAGAGGAGGTACTCTTTAGCTACAGCTCCAAGAAGCATCGTATCCTTTGATCCCCATCGAACCAAG
GCTTCGCTAGCCCATCCAGCAAGCCGAACGCCGGGGAAGGGATCCGCACAGCGCCAACAGAGAGCAAGCTGAAGCCGTGCTGC
ATCATCAGTAGCAGACCATGGCCGCTGGATCCGAGAGCCCACCGATCGACCTACAACGTCACCCTGGAAGCGATCACACATGA
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AATACACATTCAACATTACTAAAATACACAATGAATATTTTTTATACATGATAATATATTTTTCATACACGGTCAACACTATT
CAAATACAGGATGAACTATTCATGAAAAGAACATAACCATGCATTTACAAAATGTTCATGTAGTTGAAGAAAAAATTGGTTGT
ATTTTCTGAAAATTGTTCATACATTTCAAAAAATGTTCATAATATTTTGAAAAACTGTCCATGCAATGTTAGAAAAAAATGCA
TGACACTAAAAAATATATTCACAACACTAAGGCAATGTTCCAA

Csq9

CPI110672

TGGAAGGAAAATCACATACTTCCTGCCTGTTCCCAAGGCAGAGCCGGTCACCCCGCTCACGACGACCGCCTAGTCTAAGAGAC
AGGCTTGTGGAAATCACCGATACCAAGTGTGTTTCGCTTACCCATATCATCATTCATGTTACTTCCTTTGCATCAACCATTTC
ATGATTTGCTTTATTTGATTAGGTATGTTTTAACTATTTGTTGCTTCTCTTGCCACTCCCTGGCCAGCGCCAGCCCTGGGGAA
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AGCCCTCTAGTGCTCCTGTTCTTGAGCTGAATAAAAGCCATGTTACAGCGTGGTTGTCAGT

CPI110717

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TGTTACTTCCTTTGCATCAACCATTTCATGATTTGCTTTATTTGATTAGGTATGTTTTAACTATTTGTTGCTTCTCTTGCCAC
TCCCTGGCCAGCGCCAGCCCTGGGGAAGCCATTCCTCGACAGATGGTGTTGGTGCTACCGGTGGAAGATTTGTGTTGATTGCT
TACTGTCGCGTGTTTGACTGTCTGTGCAGCCCTCTAGTGCTCCTGTTCTTGAGCTGAATAAAAGCCATGTTACAGCGTGTTGT
CAGT

Csq10

CPI110672

TGCCCAATAACATGCAACACAAACACACAATTGTAGAATTGCTTAAAAACCACACACACACACACACACACACACACACAC
ACACACAATAGTAGAACATAAACACACTATCTTACAAATGACAACACCCCCCTCACACACAATTGTAGAGTGAGAAAGGGGGT
GCTCACCTTCTCTGCCATGCTTTCGATGTGGCCTCCTTTGGCTTCCTACATGGA

CPI110717

TGCCCAATAACATGCAACACAAACACACAATTGTAGAATTGCTTAAAAACCACACACACACACACACACAATAGTAGAACATAAA
CACACTATCTTACAAATGACAACACCCCCCTCACACACAATTGTAGAGTGAGAAAGGGGGTGCTCACCTTCTCTGCCATGCTT
TCAATGTGGCCTCCTTTGGCTTCCTACATGGA

Csq11

CPI110672

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AGCCTCTCAGAGAAAAGTTTTGTGATAGGAGTGCTTCGAGTATATGGTCGTGATGAAGAGCGAGATCAAGTGATTGAGATTGG
GTTTGATGATTGGACGTAACAACGTACGGGATCAACACATCAAGCAATTGGGTCTCTCACTTATCAGGGCTGGTACGTCGATC
GACATGGCCTGTTGGAAAAAGGCAGCAGCTCCAGGCACGAGTGTTGTTGTATATACACCACAACTCCTAAGAGACTAAAAGGA
GAGAGTAGCAAGAGTTTTTCTGCCAAGGTGACTAATGACTTCTTTTCCAGTCTTGCCGATCATTGGCATTGGCGGGGTGGTAA
AGACTACCTTGGCCCAATACATCTACAATGATAGAAGGGTTAAAAGAGAGTTTGACATGGGAGCGTGGGTTTGTGTTACCGAT
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CCATTAGTTATGCCAACAATCAGATCTTCATAGCAAAACCTATAATAGCTTCCCCATTCTTCTCCATGAACAATGGTGCTCTA
AA

CPI110717

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CGATTATTTTGATGAGAAAAGAATATCATCCCAAAATTTTAGCTTTCCAAGAGTTCACCTTATGGCGGATGATAGTTTGATCA
TACACCATTAGTTATGCCAACACTCAGATCTTCATAGCAAAACCTATAATAGCTTCCCCATTTTTCTCCATGA

Csq12

CPI110672

CAGCTGCCATGAGTCTCTCGGGGCGCTCTGCCCTCTGCCATTCGCGGCACAGGCTATGCCCGACCACCTCCTCCAACGGAGGC
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ACCTCAAACGCTCCTCTATGAGTCAAATAAAAAAAAGAATATTCTGAATTTTTCGGCAACGAATATTGTTGAATGTTCTACAT
ACACTTAAATTGCCATGAAGAAAAATACATTCCTAATGCTCTGGGATAAAAAGGAAACAAAAGCAATGCTCCAAAAAGACAAA
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GATCACGGAACCTCTACCTATTCAGAAAAGGAAACATAATAAAGAAGAACAACAGTTTTCATCTCAACAGTGCATCTCAAATC
AGCA

CPI110717

CAGCTGCCATGAGTCTCTCGGGGCGCTCTGCCCTCTGCCATTCGCGGCACAGGCTATGCCCGACCACCTCCTCCAACGGAGGC
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ACCTCAAACGCTCCTCTATGAGTCAAATAAAAAAAAGAATATTCTGAATTTTTCGGCAACGAATATTGTTGAATGTTCTACAT
ACACTTAAATTGCCATGAAGAAAAACACATTCCTAATGCTCTGGGATAAAAAGGAAACAAAAGCAATGCTCCAAAAAGACAAA
TGTGATTTCGTGAAGAAAAAAAAAAATTGCACACAAGAAGTAAACTAAGCAATGTTTGTTATAAAAGAATGTCTGAATTTTTTG
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ACTTGTTTATTCTTTATTTTGCTGCCCATATAGAGGAGCATTTGAGCTCAAGATTAGGATGGCATTTTATTTTCGCACTCGGG
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GATCACGAAACCTCTACCTATTCAGAAAAGGAAACATAATAAAGAAGAACAACAGTTGTCATCTCAACAGTGCATCTCAAATC
AGCA

Csq13

CPI110672

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Csq14

CPI110672

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Csq15

CPI110672

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Csq16

CPI110672

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GCCAGAGTTCAC

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Csq17

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Csq18

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Csq19

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Csq20

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Csq21

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Csq22

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Csq23

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AL8/78

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Csq24

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AL8/78

CAATTCATGCGTGTGGAACGTTTTTTTTTTTACTTTTTTACTGGCTGTTTAGCACGCAGGCCGTTTTCTCGCACG
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Csq25

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CPI110717

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Csq26

CPI110672

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Csq27

CPI110672

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CPI110717

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Csq28

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ACA

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Csq29

CPI110672

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Csq30

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KASPYr672

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2D93

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2D94

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2D96

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Xgdm35

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AGG

Supplementary file 4

Mapping of annotated complete *CNL* sequence from CPI110672 (BW_01115) in the Chinese Spring IWGSC RefSeq v1.0 and *Ae. tauschii* AL8/78 v4.0 reference sequences

NLR Parser results of CPI110672 genome											Chinese Spring RefSeq v1.0					*Ae. tauschii* AL8/78 v4.0				
Contig Name	Contig Siz	start	end	NLR S	clas	complet	strand	2-NLR	MotifList		Chr	% ident	align le	start	end	Chr	% identity	align length	start	end
contig_72305_3	8378	596	5289	4693	CNL	complete	reverse	TRUE	7,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,1		chr1D	98.297	4698	2719730	2715042	chr1D	98.297	4698	941502	936813
contig_180100_5	27170	2041	26758	24717	CNL	complete	reverse	TRUE	20,9,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,17,16,1,6,4,5,10,3,12,2,8,7,9,9,12							chr1D	98.63	21387	1860148	1881414
contig_7473_1	14501	7595	10628	3033	CNL	complete	forward	FALSE	16,1,6,4,5,3,2,8,7,9,19,9,11		chr1B	88.179	2318	1846845	1844540	chr1D	99.835	3033	1898615	1901647
contig_7473_2	12708	5322	8953	3631	CNL	complete	reverse	FALSE	18,17,14,1,6,6,4,5,10,3,12,2,8,7,11,9,9		chr1A	93.321	2785	2478135	2475370	chr1D	99.245	3445	1911169	1914613
contig_303843_1	9658	6263	9526	3263	CNL	complete	forward	FALSE	12,17,16,1,6,4,5,3,12,2,8,7,9,11,11,11		chr1B	92.132	2936	6488134	6485213	chr1D	88.896	2936	5594285	5597191
contig_714972_1	9919	30	7543	7513	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,9,11,20,19		chr1D	99.69	7414	3422408	3429820	chr1D	99.847	4578	5895670	5900246
contig_306400_1	7537	5661	7533	1872	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,2,8,7,9,11		chr1A	92.646	1863	5505927	5504065	chr1D	93.727	1865	6441512	6439648
contig_472144_2	15226	9488	11963	2475	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,2,8,7,9,9,9,11		chr1D	99.758	2475	7056412	7058883	chr1D	99.919	2475	7842881	7845355
contig_639_1	7849	1875	4410	2535	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,2,8,7,9,9,9,11,11		chr1D	99.921	2535	7065458	7067992	chr1D	99.882	2535	7851931	7854465
contig_387722_1	5911	766	3301	2535	CNL	complete	forward	TRUE	17,16,1,6,4,5,10,3,2,8,7,9,9,9,1,11,11		chr1D	99.053	2535	7422619	7420085	chr1D	99.763	2535	8129306	8126772
contig_7922_3	6977	4174	5884	1710	CNL	complete	reverse	FALSE	16,10,3,12,2,8,7,9,9,9,11,11		chr1D	98.772	1710	7822983	7821292	chr1D	96.835	1264	8640708	8639473
contig_261734_1	3812	179	2696	2517	CNL	complete	reverse	FALSE	17,16,14,1,6,4,10,3,2,8,7,9,9,9,11,11		chr1D	90.787	2529	7871208	7868680	chr1D	90.351	2539	8703863	8701328
contig_225459_1	5117	119	2767	2648	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,2,8,7,11,9,9,9,11,11,15		chr1B	86.717	2665	10017451	10014797	chr1D	85.102	2685	8704000	8701328
contig_272254_2	13699	816	11230	10414	CNL	complete	forward	FALSE	8,6,14,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11		chr1D	98	11121	8183115	8194236	chr1D	98	10956	9509844	9520800
contig_7562_1	14543	818	13665	12847	CNL	complete	forward	TRUE	4,9,12,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11,11		chr1D	98.256	10548	8237773	8227243	chr1D	99.992	12847	9554790	9541945
contig_181721_1	28288	2868	23750	20020	CNL	complete	forward	FALSE	12,17,16,6,4,5,1,6,4,5,3,12,2,8,7,11,11,9,9,2,13		chr1D	99.83	15927	8603856	8619774	chr1D	99.985	20022	9879890	9899911
contig_406427_1	6892	2589	6577	3988	CNL	complete	reverse	FALSE	17,16,6,4,5,1,6,4,4,5,10,3,12,2,8,7,11,11,9		chr1D	98.748	2237	9089086	9091322	chr1D	100	3988	10409527	10413514
contig_381466_1	8896	837	8648	7811	CNL	complete	forward	FALSE	17,16,2,14,1,4,5,10,3,12,2,8,7,11,9,9,9		chr1D	99.77	7811	9478788	9470978	chr1D	99.834	7811	10787458	10779648
contig_470919_1	5612	2703	5513	2810	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9							chr1D	99.964	2810	11793106	11790297
contig_112972_1	14867	4394	11545	7151	CNL	complete	forward	FALSE	17,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,11,20,20		chr1D	98.63	3,060	11175049	11178108	chr1D	98	5928	12505362	12511290
contig_658749_1	13012	7247	11369	4122	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,12,9,11,20,13		chr1D	94.339	2844	11178212	11181043	chr1D	93.322	2381	12709690	12707310
contig_455881_1	15951	4589	13748	9159	CNL	complete	reverse	FALSE	15,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,9,11		chr1D	97.894	6457	12535951	12529536	chr1D	99.924	9160	13523215	13514057
contig_223213_1	21879	5170	20147	14977	CNL	complete	reverse	FALSE	17,12,15,17,16,1,6,4,5,3,12,2,8,7,11,11,11,11,11,11,11,11,11,11,11,11,11,2,11,18							chr1D	99.92	14985	20763377	20748394
contig_624641_2	9620	3691	9379	5688	CNL	complete	forward	FALSE	6,17,16,1,6,4,5,3,12,2,8,7,11,11,11,19,11,11,11,11,11,11		chr1D	99.974	3886	19806858	19810743	chr1D	99.596	5693	21235480	21241172
contig_228652_2	5513	327	3957	3630	CNL	complete	forward	FALSE	17,16,6,4,5,1,4,5,3,2,8,11,9		chr1D	99.669	3630	38907086	38910715	chr1D	100	3630	41492844	41496473
contig_668577_2	11426	3789	9920	6131	CNL	complete	reverse	FALSE	17,16,1,6,4,5,3,12,2,8,7,7,19,11,11,11,11,11,11,11,11,11,11		chr1D	99.87	6132	79136318	79142449	chr1D	99.853	6138	81387650	81393787
contig_284144_1	8121	2936	7471	4535	CNL	complete	reverse	FALSE	17,16,1,6,4,5,3,12,2,8,7,11,19,11,11,11,11,11,11,11,11		chr1D	99.89	4535	86209589	86205056	chr1D	99.956	4535	88471027	88466493
contig_340163_1	10053	4971	7674	2703	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,11,9,19,11,11,11,11		chr1D	99.926	2703	205478015	205475313	chr1D	99.963	2703	211164244	211161542
contig_263240_1	8816	5113	7134	2021	CNL	complete	reverse	FALSE	16,1,6,4,5,3,12,2,7,11,9,9		chr1D	99.019	2039	248928478	248930516	chr1D	98.923	2043	255076007	255078049
contig_12598_1	5238	607	4552	3945	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,11,11,11,11,11,11,11,7		chr1D	99.417	3948	352195631	352191685	chr1D	99.468	3948	357685073	357681127

contig_299564_1	10189	1688	7384	5696	CNL	complete	forward	FALSE	4,17,16,14,1,6,4,5,10,3,12,2,8,7,9,11,20,5	chr1D	99.596	5696	363120157	363125843	chr1D	99.702	5697	368949900	368955588
contig_650375_1	23366	4351	22386	18035	CNL	complete	forward	TRUE	9,8,14,17,16,1,6,4,5,10,3,12,2,8,7,9,9,11,11,11,1,11,11,11,11,11,18,7	chr1D	99.956	13788	405057485	405071272	chr1D	99.363	18061	412079722	412097729
contig_52536_1	14091	1340	13016	11676	CNL	complete	reverse	TRUE	7,19,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9	chr1D	99.896	11532	415403881	415392350	chr1D	99.649	11677	422664040	422652383
contig_187720_1	41742	4861	39143	34282	CNL	complete	forward	TRUE	7,17,7,18,19,17,1,6,4,5,10,3,12,2,8,7,9,9,9,11,9,11,5,10	chr1D	99.27	15,133	416840500	416855632	chr1D	99	16833	424332246	424349079
contig_228769_1	24951	2308	5404	3096	CNL	complete	forward	FALSE	16,1,4,5,3,12,2,8,7,9,9,11,11,11,11,11,11,11,11,11,11,11,11	chr1D	99.839	3096	431518596	431521691	chr1D	99.935	3096	438456345	438459440
contig_86217_1	14765	1056	14385	13329	CNL	complete	forward	FALSE	13,17,16,1,6,4,5,10,3,2,7,9,9,11,11,11,11,11,11,11,11,11,11,19,11,11,11,11,11,11,11,11,1,11,11,11,11,11,2	chr1D	98.753	10102	435265961	435255878	chr1D	99.835	13333	441680568	441667242
contig_101275_4	12468	3581	8743	5162	CNL	complete	reverse	TRUE	11,16,1,6,4,5,3,12,2,8,7,11,11,11,11	chr1D	98.879	5174	455755534	455750362	chr1D	98.123	5168	462831362	462826211
contig_360374_1	21148	6472	20607	14135	CNL	complete	forward	FALSE	18,16,1,6,4,5,3,12,2,7,9,11,9,11,11,11,11,2						chr1D	99.83	14136	462864508	462878640
contig_360405_3	13792	3437	11554	8117	CNL	complete	reverse	FALSE	10,16,1,6,4,5,3,12,2,7,11,9,11,11,11,20,11	chr1D	95.881	6240	455800903	455794695	chr1D	99.667	8118	462916638	462908527
contig_166921_2	10320	1802	6317	4515	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,9,9,11,11,11,11,19,16	chr1D	99.532	4058	458142930	458146986	chr1D	99.319	4404	465174167	465178569
contig_210299_1	24176	3714	17139	13425	CNL	complete	forward	TRUE	16,1,6,4,5,10,3,2,8,7,9,9,1,11,11,9,11,16,1,6,4,5,10,3,2,8,7,9,9,11,11,11						chr1D	99.926	13426	466028646	466015223
contig_133855_1	22885	3900	12481	8581	CNL	complete	reverse	TRUE	4,5,10,3,12,2,8,7,11,9,4,11,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,11	chr1D	97.1	5380	460848319	460853681	chr1D	97.323	5380	467890079	467895442
contig_244253_2	14669	293	11785	11492	CNL	complete	forward	FALSE	8,17,16,1,6,4,5,10,3,2,9,7,9,9,11,11,11,11,11,11,11,19,11,11,11,11,18,11,11,11,11,12	chr1D	99.332	7783	465410692	465402914	chr1D	99.101	5784	472685016	472679237
contig_196242_1	19961	6370	18602	12232	CNL	complete	reverse	FALSE	18,2,17,16,1,6,4,5,10,3,12,2,8,7,11,9,11,3	chr1D	98.95	8856	473411596	473402779	chr1D	98.671	6924	480653614	480646715
contig_56225_4	9196	198	7448	7250	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,2,8,7,11,11,11,19,11,11,11,11,11,11,11,11,18	chr1D	99.6	7251	486116226	486123472	chr1D	99.876	7250	493390990	493398237
contig_391516_1	25281	2028	22334	20306	CNL	complete	forward	TRUE	7,9,9,11,11,11,11,9,11,11,9,11,11,11,11,11,11,11,11,11,17,16,1,6,16,4,5,3,12,2,18,7,9,9,11,11,11,11,11,11,11,11,11,9,11,9,11,11,11,11,11,7,8	chr1D	98.992	18151	487280519	487298652	chr1D	98.925	18332	494427726	494446039
contig_758179_1	14435	418	13871	13453	CNL	complete	reverse	FALSE	5,17,16,14,1,6,4,5,10,3,12,2,8,7,9,9,9,11,20						chr1D	99.978	13454	495018099	495031552
contig_163830_3	6552	2372	5680	3308	CNL	complete	reverse	FALSE	20,17,16,14,1,6,4,5,10,3,12,2,8,7,9,11	chr1A	91.428	3278	586516633	586519881	chr1D	95.751	3271	495088388	495091653
contig_30439_1	7686	214	3861	3647	CNL	complete	forward	FALSE	3,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11	chr1D	99.909	2192	494860447	494858256	chr1D	99.909	2192	501756986	501754795
contig_123233_2	10688	2095	8498	6403	CNL	complete	reverse	FALSE	17,16,4,5,1,6,4,5,10,10,3,12,2,8,7,11,9	chr1D	99.821	3352	494940865	494937514	chr1D	99.821	3352	501830496	501827145
contig_101293_1	12309	497	7645	7071	CNL	complete	forward	FALSE	15,17,16,1,6,4,5,3,12,2,8,7,11,9,9,7						chr2D	99.845	7079	287306	280229
contig_235672_1	9263	856	7976	7120	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,9,11,11,17	chr2D	95.464	7033	7977174	7970188	chr2D	95.707	7034	6780873	6773883
contig_239763_3	6239	2129	5096	2967	CNL	complete	reverse	FALSE	17,16,1,10,5,10,3,2,8,7,9,11,9	chr2D	98.921	2967	10870612	10867649	chr2D	99.697	2967	10075246	10072283
contig_13839_1	9306	3378	7495	4117	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,9,11,15,20	chr2D	94.838	4146	11609494	11613594	chr2D	99.976	4118	10867807	10871924
contig_95556_1	8139	740	4632	3892	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,15	chr2A	97.852	2421	17002434	17000014	chr2D	91.857	2370	15027789	15025458
contig_243024_3	5803	1632	4017	2385	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,9,11	chr2D	99.287	2385	16344595	16346979	chr2D	99.287	2385	16879242	16881626
contig_243024_4	14693	1035	8992	7957	CNL	complete	forward	TRUE	9,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11	chr2D	99.698	7957	16349857	16357811	chr2D	99.698	7957	16884504	16892458
contig_433906_2	5719	382	2670	2288	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,3,12,2,8,7,11,9,9	chr2D		1291	16493839	16495130	chr2D	99	1291	17026382	17027673
contig_107172_3	4624	1034	3620	2586	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,11,11,5	chr2D	100	2586	17790112	17792697	chr2D	100	2586	18330738	18333323
contig_143731_1	13516	687	4365	7709	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,20	chr2D	95.52	2,601	22956866	22959466	chr2D	99	5568	24215919	24221487
contig_52175_1	19681	320	11308	10988	CNL	complete	forward	FALSE	18,17,3,14,14,5,12,2,8,7,9,11	chr2D	99.703	5721	23483290	23489009	chr2D	99.973	10991	24515235	24504245
contig_234162_4	9790	6760	9783	3023	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,11,9,9,11,11,8	chr2D	99.438	3024	27588204	27585181	chr2D	99.405	3024	28617430	28614407
contig_319663_1	12771	2897	8923	6026	CNL	complete	reverse	FALSE	16,12,1,6,4,5,3,12,2,8,7,11,9,9,3,19	chr2D	99.851	6027	38202165	38196139	chr2D	99.359	3276	39722161	39718886
contig_354069_2	19809	664	18931	18267	CNL	complete	forward	TRUE	20,17,16,6,4,1,6,4,3,2,8,11,11,9,1,6,12,10,5	chr2D	99.803	18278	155457251	155438974	chr2D	99.962	18270	157046531	157028262
contig_62277_3	13930	1048	10773	9725	CNL	complete	forward	TRUE	4,9,17,16,1,6,4,5,10,3,2,8,7,9,9,9,11,11	chr2D	99.897	9728	157129431	157139157	chr2D	99.969	9725	158763021	158772744

contig_107359_1	14439	1296	12291	10995	CNL	complete	forward	FALSE	17,17,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,10	chr2D	99.875	6393	399356535	399350143	chr2D	99.927	10995	398258191	398247197
contig_394684_1	20320	1532	16757	15225	CNL	complete	reverse	FALSE	14,17,16,17,1,6,4,5,10,3,12,2,8,7,9,9,19,11,11,11,11,11,11,11,3,20	chr2D	99.573	15237	461535156	461550380	chr2D	99.967	15225	460920276	460935500
contig_336948_2	7773	2460	4985	2525	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11,11,8	chr2D	99.96	2525	532157742	532155218	chr2D	100	2525	530840104	530837580
contig_544808_1	6633	566	6580	6014	CNL	complete	reverse	TRUE	9,11,20,18,14,1,6,15,4,5,10,3,12,2,8,7,9,11,11,11,11	chr2D	99.538	4547	571446103	571441561	chr2D	99.693	4564	568157492	568152931
contig_119402_1	17551	3776	9999	6223	CNL	complete	reverse	FALSE	20,16,1,6,4,5,3,12,2,8,7,19,11,11,11,11,11,11,11,19	chr2D	98.04	6175	577114454	577108282	chr2D	92.909	4992	575318395	575313450
contig_313743_2	7261	1996	6371	4375	CNL	complete	forward	FALSE	16,20,1,6,4,5,3,12,2,8,7,16,19,11,11,11,11,11,11,11	chr2B	93.323	2681	693336545	693339212	chr2D	98.22	2696	575346145	575348835
contig_758452_1	8000	2755	7243	4488	CNL	complete	reverse	FALSE	17,16,1,6,4,5,3,12,2,8,7,16,19,11,11,11,11,11,11						chr2D	99.933	4488	575436352	575431865
contig_85441_2	9442	44	6367	6323	CNL	complete	reverse	TRUE	12,10,9,16,1,6,4,5,10,3,12,2,8,7,9,9,9,9,9,11,11,12	chr2D	99.131	6329	580196189	580189861	chr2D	99.255	4966	578436971	578432006
contig_287675_1	7569	202	7080	6878	CNL	complete	forward	FALSE	17,16,3,1,6,4,5,3,12,2,8,7,9,11,11,9,19,11,7	chr2D	98.97	4756	592622887	592618138	chr2D	100	6878	591112606	591105729
contig_3358_1	20453	2542	19988	17446	CNL	complete	reverse	FALSE	2,12,7,16,1,6,4,5,10,3,12,2,17,7,11,4,20,13,10,5	chr2D	99	15751	616939833	616955584	chr2D	99	15796	615701193	615716989
contig_164387_1	11205	230	7985	7755	CNL	complete	forward	FALSE	12,17,16,1,6,4,5,10,3,12,2,8,7,11,10,11	chr2D	98.26	5001	621969391	621964448	chr2D	99.92	5002	620941166	620936165
contig_248846_1	15950	2079	13590	11511	CNL	complete	forward	TRUE	18,11,12,16,1,6,4,5,10,3,2,8,7,9,11,20	chr2D	99.604	8588	625144089	625152658	chr2D	99.604	8588	623661945	623670513
contig_520166_1	7976	4777	7232	2455	CNL	complete	reverse	FALSE	17,16,14,1,6,5,4,5,10,3,12,2,8,7,11,9,7	chr2D	99.878	2455	646651229	646653683	chr2D	99.878	2455	640317152	640314698
contig_486132_1	5844	1014	3142	2128	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,11	chr2D	99.765	2128	646573941	646571816	chr2D	100	2128	640396282	640398409
contig_669599_1	6637	71	6233	6162	CNL	complete	forward	FALSE	20,17,16,14,1,6,4,5,10,3,12,2,8,7,9,11,4	chr2D	99.919	6162	646508776	646502615	chr2D	99.951	6162	640457375	640463536
contig_85454_1	13064	3769	7906	4137	CNL	complete	reverse	FALSE	7,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,11	chr2D	92.048	2930	646502259	646505168	chr2D	91.98	2930	640463892	640460983
contig_59695_5	14659	9411	11495	13899	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,11	chr2D	99.856	13900	646486586	646500485	chr2D	99.957	13899	640479565	640465667
contig_544805_1	10011	645	5477	4832	CNL	complete	reverse	FALSE	12,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9	chr2D	99.974	3834	639360928	639364760	chr2D	99.566	4834	646679516	646674690
contig_577345_1	5060	211	3990	3779	CNL	complete	reverse	FALSE	7,16,1,6,4,5,3,12,2,8,7,19,11,11,9,9,11,11,11,11,11	chr2D	100	3779	637337740	637341518	chr2D	99.417	2402	650062292	650059891
contig_181147_1	11619	4865	9078	4213	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,11,9,11,11,11	chr2D	99.929	4214	635876005	635871792	chr2D	99.715	4214	651248646	651252859
contig_211128_1	25751	2495	21807	19312	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,11,19,20	chr2D	99.345	9773	584530786	584540549	Chr2D				
contig_263740_1	7135	1138	5386	4248	CNL	complete	reverse	FALSE	17,16,1,6,4,5,3,12,2,8,7,11,9,9,4	chr2D			Repetative		chr2D	Repetative			
contig_565421_1	8881	2276	8575	6299	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,11,19	chr2D			Repetative		chr2D	Repetative			
contig_381440_1	33813	10919	33695	22776	CNL	complete	reverse	FALSE	10,17,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11,11,11,12	chr3D	99	10690	848918	859608	chr3D		86920	3400840	3487760
contig_832030_1	4886	692	4165	3473	CNL	complete	reverse	FALSE	5,17,16,1,6,4,5,10,3,12,2,8,7,9,15,11,13,3	chr3D	98.993	3474	10635384	10638837	chr3D	99.482	3474	10775593	10779065
contig_520925_1	12244	6309	11155	4846	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,10,3,2,8,7,5,9,11	chr3D	99.34	4847	12243293	12238453	chr3D	99.087	4052	12319205	12315168
contig_70368_1	24359	4921	20170	15249	CNL	complete	reverse	FALSE	5,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,9,9,19,11,3,12	chr3D	99.687	12784	13167337	13180117	chr3D	99.849	15268	13052123	13067390
contig_372865_1	25527	8767	25111	16344	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,17,9,9,9,11,11,11,13,3	chr3D	99.829	16344	13427799	13411467	chr3D	99.878	16350	13328047	13311699
contig_49760_1	19928	6748	19153	12405	CNL	complete	reverse	TRUE	4,20,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,3,6,1	chr3D	99.686	12405	15037787	15025407	chr3D	99.653	12405	14948711	14936331
contig_478434_2	4876	694	4056	3362	CNL	complete	forward	TRUE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,12,17,1						chr3D	99.911	3362	35720303	35716942
contig_181378_1	11826	1507	4335	2828	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,2,8,7,9,9,11,9,19,11,19,11,11	chr3D	95.963	2849	33335559	33332711	chr3D	99.823	2830	36404588	36401759
contig_247356_2	8795	4190	6860	2670	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,2,8,7,9,9,11,9,19,11,11	chr3D	100	2670	33356880	33354211	chr3D	100	2670	36428330	36425661
contig_379307_2	8035	3974	6760	2786	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,2,8,7,11,9,9,11,9,9,19,11,11,11	chr3D	100	2786	33374702	33371917	chr3D	98.237	2780	36445804	36443028
contig_761150_1	7074	3745	6844	3099	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,4	chr3D	98.58	3099	36835694	36838792	chr3D	98.548	3099	38384151	38387249
contig_219487_1	12661	270	11578	11308	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,13,15	chr3D	99.425	11308	41677753	41666453	chr3D	99.752	11308	43292400	43281099

contig_377839_1	8662	350	6412	6062	CNL	complete	forward	FALSE	10,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,11,20	chr1D	91.95	3528	11287938	11291422	chr3D	98.902	5100	147893321	147898381
contig_284791_1	25500	70	23596	23526	CNL	complete	forward	TRUE	5,19,11,16,1,6,4,5,10,3,2,8,7,9,8,9,11,11,11,9,11,20,8,16,1,6,4,5,10,3,2,8,7,9,9,11,11,9,11,11,20,9	chr3D	99.864	23528	428237998	428261523	chr3D	99.991	23525	435460391	435483915
contig_81593_1	14137	44	10823	10779	CNL	complete	reverse	TRUE	17,16,1,6,4,5,10,3,12,2,8,7,9,9,11,19,11,11,11,11,11,11,1,4,19	chr3D	99.917	10781	439660543	439671321	chr3D	99.889	10783	447082207	447092987
contig_624682_1	13304	1978	8343	6365	CNL	complete	forward	TRUE	2,11,17,16,6,4,5,1,6,4,1,3,2,8,11,9	chr3D	99.874	6365	533934791	533941155	chr3D	99.859	6368	542764870	542771237
contig_52431_3	14348	2805	9369	6564	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,17,19,11,11,11,11,11,18	chr3D	99.97	6564	537911669	537918232	chr3D	99.939	6564	546644739	546651302
contig_438300_2	9441	5383	8543	3160	CNL	complete	forward	FALSE	17,16,1,6,4,10,3,12,2,8,7,9,9,11	chr3D	99.778	3160	544412184	544415343	chr3D	99.937	3162	553103456	553106617
contig_60807_2	36946	3861	36288	32427	CNL	complete	forward	TRUE	2,19,12,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11,17,16,1,6,4,5,10,3,12,2,8,7,9,9,9,9,11,11,9	chr3D	99.76	23360	550642221	550665555	chr3D	99.654	24017	559171647	559195636
contig_762743_1	5125	210	3188	2978	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,11,20	chr3D	98.765	2996	565186017	565183023	chr3D	99.765	2978	574330806	574327829
contig_62390_2	12656	2284	6680	4396	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,19	chr3D	99.419	3786	569529411	569533191	chr3D	99.841	4396	579577298	579581693
contig_910243_1	6567	17	3871	3854	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,2,8,7,9,9,9,11	chr3D	97.91	3876	571135260	571139121	chr3D	99.896	3858	581410091	581413948
contig_178575_1	15779	2839	13630	10791	CNL	complete	forward	FALSE	16,17,16,18,1,6,4,5,10,3,2,8,7,9,9,9,11,11,11,11,11,10,11,11,11,9,19,11,11,11,11,11,11,11,11,11	chr3D	98.595	10319	571506008	571495731	chr3D	99.901	8055	581947295	581939242
contig_185503_2	18638	766	18345	17579	CNL	complete	forward	FALSE	17,16,3,1,6,4,5,10,3,2,7,9,9,11,11,11,11,11,11,11,9,11,11,11,11,11,11,11,11,9,18,3,17,16	chr3D	99.812	17579	571568135	571550575	chr3D	99.92	17581	582023819	582006242
contig_155598_2	7492	3545	5921	2376	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9	chr3D	99.537	2377	575481072	575478696	chr3D	99.832	2376	585886222	585888597
contig_134231_1	12320	3641	10077	6436	CNL	complete	forward	FALSE	10,17,16,1,6,4,5,10,3,12,2,8,7,11,11,11,11,11,11,11,11,11,11,11,11,11,11,10	chr3D	99.171	6271	586950004	586943749	chr3D	99.984	6436	598317823	598311389
contig_245500_1	7928	525	7047	6522	CNL	complete	reverse	FALSE	13,17,16,1,6,4,5,10,3,2,8,5,7,11,9,3	chr5B	96.577	3885	596068128	596064262	chr3D	99.601	6524	614862857	614869372
contig_261034_1	30585	2940	28973	26033	CNL	complete	forward	TRUE	17,16,1,6,4,5,3,12,2,8,7,9,11,11,19,11,11,11,11,11,6,17,16,1,6,4,5,3,12,2,8,7,9,11,11,19,11,4,11,11,11,11,11,11,11,11,11,11,1,11,10,17,16,6,4,5,1,6,4,5,10,3,12,2,8,7,11,9,11,11,11,11						chr3D	99.931	26037	617413866	617439902
contig_248572_1	23383	1242	17985	16743	CNL	complete	reverse	TRUE	1,6,4,5,10,3,12,2,8,7,9,9,11,9,17,16,6,4,5,18,12,1,6,4,5,10,3,12,2,8,7,9,9,11						chr3D	99.172	16782	617605558	617622320
contig_3374_2	17776	2095	9372	7277	CNL	complete	reverse	FALSE	12,17,16,14,1,6,4,5,3,12,2,8,7,11,9,9,9,7,11	chr3D	99.519	4782	612856790	612861566	chr3D	99.368	7277	624790444	624797702
contig_559688_1	10057	689	8288	7599	CNL	complete	reverse	TRUE	3,5,4,5,3,12,2,8,7,9,16,1,6,4,5,3,12,2,2,8,7,11,11,11,11,9,11,11,11,15						chr3D	100	7599	627074007	627066409
contig_222961_1	17394	3422	15214	11792	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,2,8,17,16,14,6,4,5,10,3,12,2,8,7,11,9,11,9	chr3D			Repetative		chr3D	Repetative			
contig_2605_2	20671	8798	13602	4804	CNL	complete	reverse	FALSE	3,17,16,1,6,4,5,10,3,2,8,7,9,9,11,9,19,11,11	chr4B	94.193	3582	3735646	3732076	chr4D	94.776	4728	1441369	1436655
contig_408212_1	9828	2233	8252	6019	CNL	complete	reverse	FALSE	3,17,16,1,6,4,5,10,3,12,2,8,7,9,19,11,11,11,11,11,11,11,11,11,11,11	chr4D	99.934	6019	3343687	3349704	chr4D	99.95	6019	2114036	2120054
contig_506713_2	10982	1518	10799	9281	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11,9,12,9,19,2	chr4D	99.892	9282	64538219	64547500	chr4D	99.828	9286	68033710	68042995
contig_420996_1	10050	4397	8603	4206	CNL	complete	reverse	FALSE	17,16,6,4,5,1,6,4,10,10,3,2,8,11,9	chr4D	99.715	4216	64725937	64730152	chr4D	99.938	3210	68215493	68218701
contig_248758_2	13691	4781	7314	2533	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,11,11,11	chr4D	99.842	2533	98945269	98947801	chr4D	99.961	2533	102645243	102647775
contig_69006_1	14626	3710	10806	7096	CNL	complete	reverse	FALSE	7,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11	chr4D	99.831	7099	123098188	123091092	chr4D	99.93	7096	126454296	126447203
contig_266215_1	8788	3040	7632	4592	CNL	complete	reverse	FALSE	14,6,17,16,14,1,6,12,4,5,10,3,12,2,8,7,11,9,9	chr4D	99.695	4593	480974340	480969748	chr4D	98.674	4600	487447051	487442456
contig_78481_1	20525	4056	12563	8507	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,13,10,3,12,2,8,7,9,11,20,9,14	chr4D	97.441	4376	502760440	502756107	chr4D	99.929	8507	510432007	510423501
contig_17162_1	31459	5576	18090	12514	CNL	complete	forward	FALSE	6,17,16,1,6,4,5,3,12,2,8,7,9,9,11,9,11,11,11,11,1	chrUn	99.003	7723	126945018	126937298	chr4D	99.776	12518	513410916	513423422
contig_328463_4	11805	6026	9599	3573	CNL	complete	forward	FALSE	16,1,6,4,5,3,12,2,8,7,11,9,19,11,11,11	chr4D	99.972	3573	509854875	509851303	chr4D	99.664	3571	517235760	517232190

contig_577010_1	15487	9616	13878	4262	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9	chr5D	99.296	4264	14392169	14396426	chr5D	99.977	4262	14914861	14919122
contig_101763_2	18962	1124	18655	17531	CNL	complete	reverse	TRUE	1,6,4,5,10,3,2,8,9,9,9,6,2,16,1,6,4,5,10,3,12,2,8,7,9,9,9,9,11						chr5D	99.59	17551	27195000	27212531
contig_109673_2	7314	661	3598	2937	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,2,8,7,9,9,9,11	chr5D	100	2937	24570827	24573763	chr5D	99.558	2938	27701199	27704133
contig_507642_1	14347	152	11455	11303	CNL	complete	forward	FALSE	12,2,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,19,11,11,11,11,11,11,11,11,11	chr5D	99.032	6508	28454316	28447829	chr5D	99.637	11310	31510559	31499271
contig_172783_1	6079	245	4737	4492	CNL	complete	forward	TRUE	19,16,1,6,4,5,10,3,12,2,7,19,11,11,11,11,11,11,11,11	chr5A	93.997	2532	33178984	33181500	chr5D	99.955	4492	46998826	47003315
contig_630851_1	9046	296	4976	4680	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,4,19,11,11,11,11,11,11,11,6	chr5D	99.915	4680	43748101	43752780	chr5D	100	4680	47351398	47356077
contig_368660_1	9631	27	6248	6221	CNL	complete	reverse	FALSE	1,4,10,3,17,16,1,6,4,5,10,3,2,8,7,9,11,11,9,11,3	chr5D	99.92	6221	43964084	43957864	chr5D	99.791	6221	47598241	47592021
contig_38219_1	17553	1531	17442	15911	CNL	complete	reverse	TRUE	17,16,5,1,6,4,5,3,12,2,8,7,9,11,20,6,4,5,10,3,17,16,14,1,6,4,5,10,6	chr5D	91.3	3485	70151454	70154939	chr5D	92	3486	73662885	73666371
contig_175045_1	9934	1920	5108	3188	CNL	complete	reverse	TRUE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,1	chr5D	99.969	3189	91626533	91629721	chr5D	99.937	3189	95198427	95201614
contig_148274_1	11122	3241	10617	7376	CNL	complete	reverse	FALSE	17,16,6,4,5,1,6,4,5,3,12,2,8,7,11,11,9,11	chr5D	99.57	5580	92117971	92112392	chr5D	99.635	7397	95736862	95729470
contig_313671_1	11251	601	9966	9365	CNL	complete	forward	TRUE	4,19,17,16,2,11,1,4,5,3,2,8,7,9,9,11,11,9,19,11,18	chr5D	99.818	9365	287793673	287784311	chr5D	99.861	9366	294915528	294906167
contig_184979_2	10316	683	6791	6108	CNL	complete	reverse	FALSE	16,1,6,4,5,3,2,8,9,9,11,11,11,11,11,11	chr5D	99.509	6108	287804913	287798826	chr5D	99.935	6108	294926781	294920674
contig_326814_1	10769	4140	7548	3408	CNL	complete	forward	FALSE	16,1,4,5,10,3,2,8,7,9,9,9,11,11,11,11,11,11	chr5D	99.853	3408	291928317	291924910	chr5D	99.824	3408	299063154	299059752
contig_321192_1	13640	984	5637	4653	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,2,7,9,9,9,11,11,11,11,11,11,11,9,11,11,11,16,11	chr5D	99.893	4653	292146929	292142277	chr5D	99.979	4653	299273204	299268552
contig_5217_1	16568	518	14293	13775	CNL	complete	reverse	TRUE	20,5,19,16,1,5,3,12,2,8,7,9,9,9,11,11,11	chr5D	99.768	10365	302340480	302330127	chr5D	99.773	10586	309488924	309478348
contig_95582_1	9117	1856	4698	2842	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,2,8,7,9,11,9,9	chr5D	99.05	2842	345497347	345494529	chr5D	99.578	2842	352713850	352711021
contig_211029_1	13241	392	10561	10169	CNL	complete	forward	FALSE	3,17,16,1,6,4,5,10,3,12,2,8,7,11,9,11,2	chr5D	99.773	10146	372049160	372039025	chr5D	99.99	10169	378782674	378772506
contig_564489_1	6996	1785	5165	3380	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,2,8,7,9,11,9	chr5D	88.783	2933	399162211	399159302	chr5D	100	3380	406575309	406571930
contig_158959_1	17308	3730	15848	12118	CNL	complete	forward	FALSE	8,16,1,6,4,5,10,3,2,8,7,9,19,9,11,7	chr5D	99.772	7462	399219724	399212265	chr5D	99.959	12121	406769052	406756932
contig_254135_1	13794	8917	12894	3977	CNL	complete	forward	FALSE	7,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,17	chr5D	99.799	3977	419027782	419031756	chr5D	99.849	3977	427131326	427135302
contig_62392_3	10809	3331	6206	2875	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,9,11	chr5D	99.965	2875	432030760	432033633	chr5D	97.391	2875	439855989	439858861
contig_716170_1	5297	23	5143	5120	CNL	complete	forward	FALSE	16,14,1,6,4,5,10,3,12,2,8,7,9,9,11,20,20,9	chr5D	99.473	5120	443355418	443360532	chr5D	99.453	5120	451361233	451366346
contig_92520_3	7865	1531	6866	5335	CNL	complete	reverse	FALSE	15,16,14,1,6,4,5,10,3,12,2,8,7,11,9,11	chr5D	99.906	5335	459065633	459060300	chr5D	99.963	5335	467859344	467854010
contig_350432_1	15306	9326	15182	5856	CNL	complete	reverse	TRUE	10,19,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11,11,11,11,11	chr5D	99.829	5856	464455175	464449321	chr5D	99.898	5856	473466058	473460203
contig_138557_2	5631	1072	4033	2961	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,8,7,11,9,9,19	chr5D	99.662	2961	470465676	470468636	chr5D	99.359	2807	479429666	479432472
contig_329713_2	6185	984	3375	2391	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,9,9,11,11	chr5D	99.665	2391	482142253	482144643	chr5D	99.958	2391	491899528	491901918
contig_79949_4	11254	1398	6563	5165	CNL	complete	forward	TRUE	17,16,1,6,4,5,10,3,2,8,7,9,9,11,9,19,11,11,11,4,1	chr5D	97.43	5175	486254158	486249015	chr5D	97.469	5175	496080762	496075619
contig_213209_2	17248	6731	15026	8295	CNL	complete	reverse	TRUE	19,15,16,1,6,4,5,10,3,12,2,8,7,9,19,11,11,11,11,11	chr5D	99.395	7268	497824830	497832088	chr5D	99.918	7299	508043581	508050878
contig_115137_1	10970	2335	7944	5609	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,2,7,9,9,11,11,11,11,11,11,11,11,11,9,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11	chr5B	87.838	2919	695482545	695485439	chr5D	100	5609	524479401	524473793
contig_261449_2	14599	1369	12557	11188	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,9,11,9,7,10	chr5D	99.955	11188	525579823	525591010	chr5D	99.991	11188	537262294	537273481
contig_111719_4	13310	4079	13170	9091	CNL	complete	forward	FALSE	17,16,1,4,5,3,2,7,9,11,11,11,11,11,11,11,11	chr5D	99.956	9092	528745830	528754921	chr5D	98.083	6470	540547493	540553928
contig_651597_1	7519	1133	6048	4915	CNL	complete	reverse	TRUE	17,16,7,2,1,4,5,3,10,2,7,9,11,9,11,11,19,11,11,11,11,11,1	chr5D	100	4915	528767184	528762270	chr5D	98.812	3283	540567206	540563925
contig_5219_1	15374	1722	7494	5772	CNL	complete	forward	FALSE	17,17,16,1,6,4,5,3,2,7,9,11,11,11,9,11,11,11,9,11,11,11,11	chr5D	99.965	5772	528801505	528807276	chr5D	99.365	4097	540606687	540610783
contig_172782_1	15293	1964	12433	10469	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,8,7,19,11,11,12,11,11,11,11,18	chr5D	99.57	10473	537641438	537630968	chr5D	99.99	10469	549758677	549748209
contig_166487_1	12212	1825	10723	8898	CNL	complete	forward	FALSE	16,1,4,5,10,3,12,2,2,8,7,11,9,9,2,11,17	chr5D	99.261	8929	542664430	542655510	chr5D	99.227	8929	554617766	554608846

contig_115139_3	13360	555	9507	8952	CNL	complete	forward	TRUE	11,17,16,1,6,4,5,10,3,2,7,9,9,11,11,11,11,11,11,11,11,11,19,11,11,11,11,11,15						chr5D	99.654	8957	563748915	563757870
contig_236972_1	6502	842	6049	5207	CNL	complete	forward	FALSE	16,1,6,5,10,3,1,6,4,5,3,12,2,7,11,9	chr5D	99.712	5208	551875917	551881124	chr5D	99.539	5209	563797675	563802883
contig_563857_1	10168	5952	8809	2857	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,11	chr5D	99.266	2861	555929054	555931914	chr5D	99.301	2861	567687462	567690322
contig_191357_1	14828	7734	10953	3219	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,9,11,11,11,8	chr5D	99.907	3219	564230116	564226898	chr5D	100	3219	570916363	570919581
contig_174155_1	18160	3824	14375	10551	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,18	chr5D	99.073	10573	563979607	563969047	chr5D	99.991	10551	571158366	571168916
contig_267269_1	10499	4448	8357	3909	CNL	complete	reverse	FALSE	16,1,6,4,5,3,12,2,7,11,9,11,11,11,11,11,11	chr5D	92.12	3769	561641359	561645104	chr5D	90.3	3505	576213635	576210162
contig_748344_2	7420	2805	5362	2557	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9	chr6D	86.994	1384	2323972	2322603	chr6D	99.726	2557	2000409	2002964
contig_342741_3	12940	5275	11936	6661	CNL	complete	forward	FALSE	17,16,14,6,4,5,3,6,4,1,6,4,3,2,8,11,9,9	chr6D	99.505	6661	4404137	4410797	chr6D	99.85	6661	3907794	3901134
contig_174166_1	21854	8958	21649	12691	CNL	complete	forward	FALSE	17,15,17,16,6,4,5,1,6,4,5,10,3,12,2,8,7,11,11,9,11						chr6D	98.286	7528	10741017	10748518
contig_176661_2	18536	656	16481	15825	CNL	complete	forward	TRUE	18,17,16,6,4,5,19,1,6,4,5,10,3,12,2,8,7,11,9,9,11,11						chr6D	98.608	14013	10776298	10790297
contig_654864_1	9122	716	7221	6505	CNL	complete	forward	FALSE	2,18,16,6,1,6,4,5,3,12,2,8,7,9,9,19,11,11,11,11,11,11,11	chr6D	100	6505	12345184	12338680	chr6D	99.217	3575	11828756	11825185
contig_176636_1	14805	5626	11663	6037	CNL	complete	reverse	FALSE	18,1,6,4,5,3,12,2,8,7,9,9,11,11	chr6D	100	6037	12470299	12464263	chr6D	99.917	6037	11899907	11893871
contig_446333_1	14908	5069	14706	9637	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,11,9,19,11,11,14	chrUn	99.526	6746	95707573	95714316	chr6D	99.392	6746	29236928	29243662
contig_633453_1	10139	940	6796	5856	CNL	complete	reverse	TRUE	15,17,16,1,6,4,5,10,3,12,2,8,7,9,11,9,1,19,11,11	chr6B					chr6D	99	5888	29299647	29305535
contig_524632_1	7328	2031	6240	4209	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,2,8,7,9,9,11,9,19,11,11,11,11	chr6A	93.546	2355	27009133	27006781	chr6D	88.752	2116	29482252	29480141
contig_177584_1	14384	2247	13015	10768	CNL	complete	forward	FALSE	12,17,16,1,6,4,5,10,3,12,2,8,7,9,9,11,9,11,11,11,3	chrUn	96.488	5865	96221033	96215224	chr6D	96.437	5866	29546209	29540399
contig_753148_1	4784	135	4566	4431	CNL	complete	forward	FALSE	5,17,16,1,6,4,5,10,1,3,12,2,8,9,9,11,9,19,11	chrUn	98.031	3454	96290042	96286612	chr6D	98.147	3454	29619232	29615802
contig_207354_3	9890	4865	9214	4349	CNL	complete	reverse	FALSE	20,17,16,1,6,4,5,10,3,12,2,8,7,9,9,11,9,19,11,9,11,11	chrUn	98.184	4351	96296608	96300955	chr6D	98.069	4350	29625664	29630012
contig_42208_4	17478	4018	11194	7176	CNL	complete	forward	FALSE	17,16,1,6,4,5,3,12,2,8,7,11,19,11,11,11,11,11,11,11,11,11,9,11,11,11	chr6D	99.708	7180	28302501	28295324	chr6D	99.208	7201	33796487	33789287
contig_98599_3	9178	884	3618	2734	CNL	complete	forward	FALSE	17,16,1,4,5,10,3,12,2,8,7,11,9	chrUn	99.488	2734	112613340	112616073	chr6D	99.963	2734	47202230	47204963
contig_204537_1	9209	1344	8713	7369	CNL	complete	forward	FALSE	10,18,16,1,6,4,5,3,12,2,8,7,11,9,11,11,11,11,11,11,11,11,11,11,11,11	chr6A	96.981	3677	49105987	49102326	chr6D	99.932	7370	52695542	52688173
contig_157595_1	16663	3521	10362	6841	CNL	complete	forward	FALSE	16,1,6,4,5,3,12,2,8,7,9,11,11,11,11,11,11,20	chr6B	96.263	3987	130302075	130306049	chr6D	99.854	6846	82208787	82215632
contig_282763_1	4384	565	3732	3167	CNL	complete	reverse	FALSE	16,1,6,4,5,3,12,2,8,7,9,19,11,11,11,11,11,11,11,11	chr2D	99.968	3167	636772491	636775657	chr6D	86.399	1794	82210578	82208787
contig_140875_2	17498	1404	10222	8818	CNL	complete	forward	FALSE	5,17,16,1,6,4,5,10,3,2,8,7,9,9,11,9,11,11,11,11,1,11,11,11,11,11,19,11,11,11,11,18	chr6D	99.751	8822	67506670	67515483	chr6D	99.615	8832	92176415	92185238
contig_48260_1	8615	99	3577	3478	CNL	complete	reverse	FALSE	16,1,6,5,10,3,12,2,8,7,11,11,11,11	chr6D	99.914	3478	83166237	83162760	chr6D	100	3478	107729683	107726206
contig_368314_2	10280	833	4076	3243	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,19,11,11,11,11,11	chr6D	99.322	3243	426486796	426490038	chr6D	100	3243	450702357	450705599
contig_752952_1	13824	4369	13758	9389	CNL	complete	forward	FALSE	18,12,17,16,1,6,4,5,10,3,12,2,8,7,11,9	chr6D	99.565	6431	440636290	440642699	chr6D	99.818	8256	464368020	464376273
contig_104561_1	5721	2626	4741	2115	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,19,9,11,11,11,11,11	chr6D	99.953	2115	454589099	454586985	chr6D	99.953	2115	477291892	477289778
contig_118559_1	19029	1335	18800	17465	CNL	complete	forward	TRUE	20,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,15,12,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,2						chr6D	99.828	17471	480894830	480877362
contig_118560_1	9894	48	7397	7349	CNL	complete	forward	TRUE	9,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,2,2,8,7,11,9,9						chr6D	100	7349	480916265	480908917

contig_138067_1	11529	304	11493	11189	CNL	complete	forward	TRUE	17,9,9,11,9,11,11,11,17,16,1,6,4,5,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11,11,11,11	chr6D	93.97	5,597	462858079	462863675	chr6D	95	9027	485305052	485314079
contig_183911_3	10842	5542	8964	3422	CNL	complete	reverse	FALSE	18,17,16,1,6,4,5,10,3,12,2,8,7,11,11,9	chr6D	96.591	3432	464174982	464178400	chr6D	100	3422	486532778	486536199
contig_468342_1	6313	4064	6218	2154	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,11,11	chr6D	99.954	2154	464346203	464348356	chr6D	100	2154	486705243	486707396
contig_35472_3	14220	333	12425	12092	CNL	complete	reverse	TRUE	19,12,5,17,16,1,6,4,5,10,3,12,2,8,7,9,9,11						chr6D	99.926	8088	486733674	486741755
contig_70569_1	15064	4407	13604	9197	CNL	complete	reverse	FALSE	17,16,6,4,1,6,4,5,10,3,12,2,8,7,11,11,9,9	chr6A	96.858	7702	613991522	613983858	chr6D	98.687	6320	490420082	490413771
contig_492057_2	10377	3464	7385	3921	CNL	complete	reverse	FALSE	17,16,8,1,6,4,5,3,12,2,7,11,11,11,11,11,11,11	chr6D	99.745	3921	468875907	468871987	chr6D	99.924	3923	490934754	490930832
contig_42789_2	9237	1870	6306	4436	CNL	complete	reverse	FALSE	4,16,5,1,6,4,5,3,12,2,7,11,20,11,11,11,11	chr6D	98.399	4436	468914421	468909998	chr6D	100	4436	490975969	490971534
contig_79332_2	8811	4044	7386	3342	CNL	complete	reverse	FALSE	16,1,6,4,5,3,12,2,7,11,11,11,11,11,11						chr6D	99.97	3342	491040148	491036807
contig_492056_3	3187	65	2699	2634	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,9,2,7,9,11,11	chr6D	99.62	2634	469053421	469050788	chr6D	99.962	2634	491072677	491070044
contig_462070_1	8755	506	7528	7022	CNL	complete	reverse	TRUE	16,14,1,6,4,5,3,12,2,8,7,9,9,11,17,16,1,6						chr6D	99.857	7006	491685529	491692534
contig_197287_1	15499	2552	8445	5893	CNL	complete	reverse	FALSE	10,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,5						chr6D	99.931	4358	492056191	492060548
contig_323708_1	14885	2301	12314	10013	CNL	complete	reverse	FALSE	17,16,1,6,12,4,5,10,3,12,2,8,7,9,9,11,19,8						chr6D	99.96	10014	492128972	492138984
contig_28390_1	12813	2129	12385	10256	CNL	complete	reverse	FALSE	4,10,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9						chr6D	99.932	10259	492202366	492212624
contig_453208_2	5368	993	4967	3974	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,11,9,6						chr6D	99.95	3975	492217189	492221163
contig_197286_2	8369	1586	5604	4018	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,11,6	chr6B	89.622	2168	715965877	715968028	chr6D	99.975	4018	492274116	492278133
contig_206540_2	11376	2599	11316	8717	CNL	complete	reverse	TRUE	3,11,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9	chr6D	96.815	5023	469991436	469996448	chr6D	99.03	5566	492348150	492353703
contig_843356_1	6669	47	4477	4430	CNL	complete	reverse	FALSE	7,3,17,16,1,6,4,5,10,3,2,8,7,9,9,11,9,19	chr6D	98.172	4430	470099695	470095329	chr6D	99.323	4432	492417818	492413411
contig_166203_1	10944	2860	10440	7580	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,10,3,2,8,7,9,9,9,19,11,11,11,2	chr6D	99.226	3876	470296671	470292800	chr6D	99.921	7582	492512060	492504479
contig_298790_2	11654	4085	11577	7492	CNL	complete	reverse	TRUE	8,9,11,11,11,11,11,11,11,11,11,11,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11,11,11	chr4,7			Repetative		Chr7	Repetative			
contig_147152_1	23674	1753	22069	20316	CNL	complete	reverse	TRUE	18,11,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11,11,11,2						chr7D	99.951	20319	923018	943335
contig_369457_1	10397	432	4379	3947	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,9,11,11,11,11,11,11,11,15	chr4A	93.251	3067	744042432	744039382	chr7D	100	3947	1017768	1021714
contig_131163_1	4694	595	4548	3953	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11	chrUn	99.848	3954	89024952	89020999	chr7D	99.823	3954	1400286	1404239
contig_310761_1	13326	5984	8986	3002	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11	chr7D	99.767	3002	1711489	1708488	chr7D	99.767	3002	1686333	1683332
contig_132666_1	10193	2349	5972	3623	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,11	chr7D	88.686	2139	3785911	3788013	chr7D	88.277	2141	2717105	2719210
contig_61359_1	11549	87	11066	10979	CNL	complete	forward	FALSE	20,16,1,6,4,5,10,3,12,2,8,7,9,11,6,20,4,9	chr7D	99.663	10980	2613732	2602756	chr7D	99.745	10979	2721984	2711006
contig_45259_1	16695	8018	15630	7612	CNL	complete	forward	FALSE	17,16,1,6,4,3,2,7,9,9,11,11,11,20	chr7D	99.691	4212	2865752	2869963	chr7D	99.561	5015	2976246	2981259
contig_351089_1	32579	681	25912	25231	CNL	complete	forward	FALSE	10,5,16,6,17,16,14,1,6,4,5,3,12,2,8,7,11,9,9	chr7D	99.123	12878	4024765	4011905	chr7D	99.114	12863	3670824	3657977
contig_39457_1	18088	3193	10936	7743	CNL	complete	forward	TRUE	16,9,1,6,4,5,10,3,2,8,7,9,9,11,11,9,11,7,4,20	chr7D	99.961	5088	5448887	5443801	chr7D	85.188	4017	5168819	5164855
contig_204319_1	9609	659	5790	5131	CNL	complete	forward	FALSE	12,8,16,1,6,4,5,3,2,8,7,9,9,11,11,11	chr7D	97.722	5137	5639461	5634325	chr7D	85.783	3728	5169001	5165301
contig_498457_3	11141	2732	8048	5316	CNL	complete	forward	TRUE	2,16,9,1,6,4,5,10,3,2,8,7,9,9,11,11,11,11,11	chr7D	99.906	5316	5488446	5483131	chr7D	99.755	5316	5203348	5198033
contig_388416_1	18095	5302	15184	9882	CNL	complete	forward	TRUE	11,11,11,11,11,11,11,9,16,10,16,1,6,4,5,10,3,12,2,8,7,9,9,11,11,9,11	chr7D	99.803	7121	5652761	5645643	chr7D	99.383	9890	5316853	5306965
contig_81897_1	14057	144	8571	8427	CNL	complete	forward	TRUE	11,11,11,11,11,11,15,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11	chr7D	99.124	8448	7543406	7551852	chr7D	100	8427	7291588	7300014
contig_81897_2	16592	2626	7442	4816	CNL	complete	forward	FALSE	14,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11,11,11,11	chr7D	98.358	4628	7559981	7564607	chr7D	100	4816	7308125	7312940
contig_222494_1	12228	630	9076	8446	CNL	complete	reverse	FALSE	17,16,1,6,4,5,3,12,2,8,7,9,11,11,11,11,11,11,11,11,8,6,6	chr7D	99.799	8446	8093847	8085403	chr7D	100	8446	7345007	7336562
contig_237059_1	18415	5927	10790	4863	CNL	complete	reverse	FALSE	16,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11,11,11,20	chr7D	99.779	4518	8181030	8176514	chr7D	99.978	4561	7429354	7424794

contig_212132_1	7587	86	7186	7100	CNL	complete	reverse	FALSE	7,16,1,6,4,5,3,12,2,8,7,9,10,11,11,5	chr7D	99.901	7100	8185821	8192919	chr7D	99.972	7100	7434144	7441243
contig_237058_1	24008	3925	22591	18666	CNL	complete	reverse	TRUE	11,8,17,16,1,6,4,5,10,3,12,8,7,9,11,11,11,11,11,17,16,1,6,4,5,10,3,12,2,8,7,11,11,11,2,11,11,11,11,11,11,9,8,2,10	chr7D	99.689	9658	8228631	8218976	chr7D	99.973	18670	7485536	7466867
contig_116376_3	5088	1390	4762	3372	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,17,9,11,11,12,11,11,11,11,11,11,11,11,11	chr7D	99.733	3372	8255947	8252576	chr7D	99.703	3372	7656252	7652881
contig_150494_3	19846	467	15695	15228	CNL	complete	forward	TRUE	18,7,16,1,6,4,5,10,3,12,2,8,7,9,11,1	chr7D	99.613	15234	8342270	8357476	chr7D	99.832	13729	7713720	7727446
contig_554121_2	9140	1111	3804	2693	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,11,6	chr7D	99.22	2693	9305392	9308081	chr7D	99.443	2693	8462718	8465410
contig_88019_1	13580	2005	8752	6747	CNL	complete	forward	TRUE	6,20,11,17,16,14,1,6,4,5,10,3,12,2,8,7,11,11,3	chr7D	99.259	4586	10034899	10039482	chr7D	99.436	6205	8765089	8771273
contig_158704_3	26642	1646	14883	13237	CNL	complete	reverse	TRUE	13,1,6,4,5,10,3,2,8,7,9,17,16,14,1,6,4,5,10,3,12,2,8,7,11,10,9,9,11,11,2	chr7D	98.477	9649	11671800	11662208	chr7D	98.518	9649	9877388	9867795
contig_233404_2	5703	1981	4828	2847	CNL	complete	reverse	FALSE	17,16,20,1,6,4,5,10,3,12,2,8,7,11,9,9	chr7D	99.93	2847	16223016	16220170	chr7D	99.824	2847	15448484	15445638
contig_246286_3	8765	2335	7925	5590	CNL	complete	reverse	FALSE	17,16,1,6,4,5,3,12,2,8,7,9,9,11,3	chr7D	92.897	3196	16258186	16254993	chr7D	92.929	3196	15483958	15480765
contig_344932_1	28792	1854	28768	26914	CNL	complete	reverse	TRUE	5,3,12,2,8,7,9,9,11,12,6,17,16,1,6,4,5,3,12,2,8,7,9,11,7,18						chr7D	99.754	22801	17560856	17583631
contig_293580_2	3901	799	3019	2220	CNL	complete	reverse	FALSE	16,1,5,10,3,12,2,8,7,9,9,11	chr7A	91.464	1523	17465685	17467180	chr7D	90.93	1753	17629089	17630833
contig_344931_1	8645	4165	7017	2852	CNL	complete	reverse	FALSE	17,16,15,1,6,4,5,3,12,2,8,7,11,9,11	chr7D	98.319	2856	18400989	18403842	chr7D	99.965	2852	17856362	17859213
contig_3636_2	8827	6142	8707	2565	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11	chr7D	94.893	2565	20310934	20313494	chr7D	98.558	2566	19968593	19971157
contig_65792_3	21383	2363	19048	16685	CNL	complete	forward	TRUE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9,16,1,6,4,5,10,3,12,2,8,7,9,9,9,9,11,11	chr7D					chr7D	98	15325	19991051	20006376
contig_31100_3	14133	8545	13569	5024	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11,12,4	chr7D	99.813	3751	20404562	20408311	chr7D	99.813	3751	20075726	20079475
contig_34787_3	8833	3143	5533	2390	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,9,9,11,11	chr7A	86.876	2385	20983236	20980863	chr7D	85.029	2378	20078116	20075741
contig_1439_1	21505	6673	17885	11212	CNL	complete	reverse	TRUE	16,19,1,6,4,5,10,10,3,12,2,8,7,9,9,9,11,11,2,5	chr7D	98.52	4,313	20425554	20421242	chr7D	98	9529	20092172	20101701
contig_34788_1	23157	6244	11472	5228	CNL	complete	forward	FALSE	2,1,17,16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11	chr7D	99.448	4714	20440384	20445097	chr7D	99.512	4715	20111050	20115764
contig_318637_1	9129	3415	5803	2388	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,11,11	chr7D	99.832	2388	20460678	20463065	chr7D	99.414	2388	20139821	20142208
contig_329712_1	9264	4058	8065	4007	CNL	complete	reverse	FALSE	10,16,1,6,4,5,10,3,12,2,8,7,9,9,11,11	chr7D	98.852	4007	20488266	20484267	chr7D	98.927	4007	20160679	20156679
contig_329714_1	8378	1611	5877	4266	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11,5	chr7D	98.816	3631	20501198	20504828	chr7D	99.035	3628	20180101	20183728
contig_60289_1	16769	5767	11658	5891	CNL	complete	forward	FALSE	10,17,16,1,6,4,5,10,3,12,2,8,7,9,9,11,11	chr7D	99.88	4152	20546024	20550174	chr7D	99.898	5893	20222767	20228658
contig_34786_5	10171	1156	4572	3416	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,11,8	chr7D	99.971	3416	20578827	20575412	chr7D	99.971	3416	20257394	20253979
contig_34786_4	9705	2248	7236	4988	CNL	complete	reverse	TRUE	11,16,1,6,4,5,10,3,12,2,8,7,9,9,11,11,11	chr7D	99.644	4772	20586243	20581473	chr7D	99.639	4989	20266041	20261053
contig_104538_2	18259	4555	17059	12504	CNL	complete	reverse	FALSE	2,17,16,1,6,4,5,10,3,12,2,8,7,9,9,11,11						chr7D	99.944	12505	20557215	20544711
contig_13003_2	21745	2762	12612	9850	CNL	complete	reverse	FALSE	18,17,16,1,6,4,5,3,2,8,7,11,11,11,11,6,11,11,11,11,11,12,11	chr7D	99.811	8997	36204134	36213126	chr7D	99.949	9852	35233942	35224093
contig_132909_2	9317	1538	8998	7460	CNL	complete	reverse	FALSE	17,16,1,4,5,3,2,8,7,9,9,9,11,12,11,11	chr7D	99.705	7466	43036812	43029347	chr7D	99.759	7464	42012992	42005529
contig_383227_2	13225	3267	8049	4782	CNL	complete	forward	FALSE	10,17,16,1,6,4,5,10,3,12,2,8,7,11,9,9	chr7D	99.833	4782	53346486	53341705	chr7D	99.979	4782	51884103	51879322
contig_163591_1	18099	7405	17140	9735	CNL	complete	forward	FALSE	17,16,6,4,5,1,6,4,5,10,3,12,2,8,7,11,11,9,9,11,18	chr7D	99.938	9735	58486928	58496662	chr7D	99.979	9735	57340417	57350151
contig_41729_3	14822	1626	8143	6517	CNL	complete	reverse	FALSE	17,16,14,1,6,8,4,5,10,3,12,2,8,7,11,9,9,20,10,10	chr7D	99.541	6539	63937165	63943703	chr7D	99.618	6539	62615252	62621790
contig_28857_1	6239	1844	5970	4126	CNL	complete	forward	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,9,11,9,11,11	chr7D	99.54	4126	71632229	71628105	chr7D	99.371	4134	73163891	73168024
contig_515065_2	4602	617	4579	3962	CNL	complete	forward	FALSE	10,17,16,1,4,5,10,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11	chr7D	98.865	3966	76636696	76632739	chr7D	98.866	3967	77632464	77628509
contig_628832_1	11421	3305	8892	5587	CNL	complete	forward	FALSE	17,16,1,6,12,4,5,10,3,12,2,8,7,11,9,9	chr7D	99.364	5192	83339663	83344851	chr7D	99.642	5590	84450850	84456438
contig_538702_2	7630	1100	6578	5478	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,2,8,7,9,9,11,11,11,11,11,11,1,11,9,11,11,11,11,11,11,11,11,11,4	chr7D	99.708	5478	83597623	83592147	chr7D	99.957	4704	84700265	84695562
contig_481660_2	6343	833	3252	2419	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,9	chr7D	99.793	2419	160433800	160436217	chr7D	99.876	2419	161394002	161396420

contig_307277_2	8209	124	7215	7091	CNL	complete	reverse	FALSE	17,16,14,1,12,4,5,10,3,12,2,8,7,9,11,20,5,3	chr7D	99.873	7094	188296206	188289117	chr7D	99.901	7094	189590442	189583350
contig_283544_1	16862	380	16787	16407	CNL	complete	reverse	FALSE	2,15,3,7,14,16,1,6,4,5,10,3,12,2,8,7,11,10						chr7D	99.403	16417	530035357	530018980
contig_128611_1	10702	2383	10252	7869	CNL	complete	reverse	FALSE	6,3,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9	chr7D	99.53	7869	530926428	530918584	chr7D	99.898	7876	532099004	532091129
contig_25336_1	11141	6129	8217	2088	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,9,11	chr7D	99.952	2088	535411260	535413347	chr7D	100	2088	536521366	536523453
contig_331113_1	13121	4760	10066	5306	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,9,11,19	chr7D	99.868	5308	549299735	549294428	chr7D	99.925	5306	550626898	550621593
contig_659243_1	4305	28	2081	2053	CNL	complete	reverse	FALSE	16,1,1,6,4,5,10,3,12,2,8,7,9,11,11	chr7D	99.659	2053	550074754	550072702	chr7D	100	2053	551395739	551393687
contig_372072_1	15074	1259	3649	2390	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,14,9,9,11,11	chr7D	98.453	2391	555196134	555193744	chr7D	100	2390	555853619	555856008
contig_58112_1	9163	3667	7719	4052	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,2,8,7,9,9,11,19,11,11,11	chr7D	99.852	4052	555051408	555047357	chr7D	100	4052	556004719	556008770
contig_32700_1	10755	4215	9812	5597	CNL	complete	reverse	FALSE	17,16,1,6,2,4,5,10,3,2,8,7,11,9,2	chr7D	99.885	4335	571972248	571967914	chr7D	100	5597	573152445	573146849
contig_207353_1	22466	14604	18659	4055	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,9,3,2,8,7,9,9,11,19,9,11	chr7D	99.975	4055	579759762	579763816	chr7D	99.951	4055	584599857	584603911
contig_639775_1	6830	1446	4581	3135	CNL	complete	reverse	FALSE	16,1,6,4,5,3,12,2,8,7,11,9,11,11,11,11,11,11	chr7D	99.841	3135	588020075	588023208	chr7D	99.872	3135	593514999	593518133
contig_140260_1	9515	242	5016	4774	CNL	complete	reverse	FALSE	16,1,6,6,4,5,10,3,2,8,7,19,9,11,18,7	chr7B	86.485	2649	697604667	697607307	chr7D	99.937	4774	614508120	614512892
contig_54023_2	15673	5214	8802	3588	CNL	complete	reverse	FALSE	17,16,1,6,4,5,3,12,2,8,7,9,11,11,11,11,11,15,11,11,11,11,11,11,11,11	chr7D	99.805	3588	618894221	618890634	chr7D	99.833	3588	623332778	623329191
contig_253757_3	16849	3212	16223	13011	CNL	complete	forward	FALSE	17,17,16,1,6,4,5,3,1,17,16,1,6,4,5,3,12,2,8,7,11,11,11,11,11,11,11,11,11,11,11,11,11,11,11,15,2	chr7D	99.562	11645	619161621	619149978	chr7D	99.43	9651	623605696	623596048
contig_672674_1	8005	1536	5901	4365	CNL	complete	reverse	FALSE	12,17,16,1,6,4,5,10,18,3,2,8,7,9,9,11,19,11,11,11	chr7D	99.707	3411	621263904	621260495	chr7D	99.702	4365	625448133	625452497
contig_142525_2	8383	3026	6974	3948	CNL	complete	reverse	FALSE	17,16,6,4,7,1,11,6,4,3,2,8,11,11,9	chr7D	99.949	3948	621297766	621293819	chr7D	99.949	3948	626013042	626009095
contig_520221_4	5043	610	3529	2919	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,9,11,9,19,11,11,11	chr7D	99.966	2919	635582928	635580010	chr7D	99.931	2919	639813550	639816468
contig_606273_1	6031	2636	5711	3075	CNL	complete	forward	FALSE	10,17,16,1,6,17,4,5,3,12,2,8,7,9,11,9,9	chr7D	99.805	3077	634112652	634109578	chr7D	99.805	3077	641600418	641603492
contig_232968_1	4823	1258	4000	2742	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,9,11						chr7D	100	2742	641973266	641976007
contig_430281_1	5647	2824	5333	2509	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,9,11	chr7B	93.514	2513	750151126	750148619	chr7D	100	2509	642124393	642126901
contig_369485_1	13862	2394	10123	7729	CNL	complete	forward	FALSE	17,16,1,6,16,4,5,10,3,2,8,7,9,9,11,19,6,11,11,10	chr7D	92.832	3976	632098208	632102143	chr7D	92.832	3976	644103653	644099718
contig_89858_1	14014	606	11131	10525	CNL	complete	forward	TRUE	12,19,17,16,6,4,1,6,4,5,3,2,8,11,9,1						Super sca	99	10638	1545178	1555816
contig_55998_2	12890	4575	10869	6294	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,11,15,2,10	chr1A									
contig_403270_1	10079	15	8083	8068	CNL	complete	reverse	TRUE	9,19,19,9,2,17,16,1,6,4,5,3,12,2,8,7,9,11,11	chr1D	99.467	5821	77378	83198					
contig_102211_1	11028	132	6687	6555	CNL	complete	reverse	FALSE	4,17,16,1,6,4,5,3,12,2,8,7,9,11,11,11	chr1D	97.358	5450	3972513	3977921					
contig_827697_1	10733	2230	10577	8347	CNL	complete	forward	TRUE	17,16,1,6,4,5,3,12,2,8,7,9,11,11,11,11,11,11,11,11,11,11,11,11,11,9,17,16,1,6,4,5	chr1D	99.162	6804	4879755	4872957					
contig_23660_1	22479	880	12236	11356	CNL	complete	forward	TRUE	19,13,17,16,6,1,6,4,5,3,12,2,8,7,9,11,11,11,1,11,11,11,11,11,11,11,11,11,11	chr1D	99.281	7375	5138502	5131134					
contig_694703_1	7657	578	6844	6266	CNL	complete	forward	FALSE	12,17,16,1,6,4,5,10,2,3,2,8,7,9,9,11,9,19,11,1,11,20	chr1D	99.221	3724	332683217	332686939					
contig_5983_2	27368	1575	22941	21366	CNL	complete	reverse	FALSE	17,16,6,4,5,1,6,4,5,10,3,12,2,8,7,11,11,9,9,11,10,11	chr1D									
contig_9437_2	8933	126	5666	5540	CNL	complete	reverse	FALSE	17,16,14,18,1,6,2,7,2,4,5,10,3,12,2,8,7,11,9,9,3	chr2D	98.453	4525	29182320	29177800					
contig_329766_1	17011	6111	13448	7337	CNL	complete	forward	TRUE	7,16,19,1,6,4,5,10,3,12,2,8,7,9,9,9,11,11	chr2D	99.428	7339	598809386	598816724					

contig_442049_1	8934	3301	6990	3689	CNL	complete	forward	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,9,20,19,11,11,11,1 1,11,9,11,11,3	chr2D	100	3689	636652678	636648990					
contig_83009_1	13740	1707	5110	3403	CNL	complete	forward	FALSE	10,16,1,6,4,5,10,3,12,2,8,7,15,11,11,11,11,11 ,11,11,11,11	chr2D	100	3403	636738199	636734797					
contig_212831_1	5673	290	3781	3491	CNL	complete	reverse	FALSE	7,18,16,7,1,6,4,5,3,12,2,8,7,15,9,9,11,11,11,9 ,11	chr2D	99.971	3491	636792264	636795754					
contig_157596_2	11123	1304	9773	8469	CNL	complete	forward	TRUE	16,1,6,5,3,12,2,8,7,15,19,4,11,11,11,11,11,3, 7,16,1,6,4,5,3,12,2,8,7,11	chr2D	100	8469	636919931	636911463					
contig_15156_4	17641	330	16251	15921	CNL	complete	forward	FALSE	3,16,1,6,4,3,2,8,7,5,11,6	chr2D	99.843	10184	638323201	638333377					
contig_11862_1	36030	1651	34459	32808	CNL	complete	reverse	TRUE	2,15,15,11,1,17,11,17,16,14,1,6,4,5,10,3,12,2 ,8,7,11,9,9	chr2D	99.991	32808	639088905	639121712					
contig_20914_2	15008	2423	9842	7419	CNL	complete	reverse	FALSE	12,16,1,6,4,5,10,3,12,2,8,7,7,11,11,11,11,11, 11,18,11	chr3D	98.638	6608	11815990	11822590					
contig_112073_1	8416	2010	6813	4803	CNL	complete	forward	FALSE	3,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,1 1,11,11,11,11,11	chr3D	99.604	4803	600153204	600158004					
contig_360210_1	14541	7215	11829	4614	CNL	complete	reverse	FALSE	8,7,16,1,6,4,5,10,3,2,8,7,19,9,11	chr4A	97.12	4584	729276902	729281457					
contig_536947_2	14093	7037	12950	5913	CNL	complete	reverse	FALSE	16,1,6,16,6,4,5,10,3,12,2,8,7,9,12,9,11,16,6	chr5A									
contig_5108_2	24812	671	19398	18727	CNL	complete	forward	FALSE	17,16,15,1,6,4,5,10,3,12,2,8,7,11,9,9,11,6,4,5 ,10,3,12,2,8,7,9,9,18,11	chr5D	96	11042	555649750	555660792					
contig_263444_1	13186	4846	7661	2815	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9	chr6D	99.259	2834	2322603	2325436					
contig_55269_1	6659	2130	5790	3660	CNL	complete	reverse	FALSE	17,16,14,20,1,6,11,4,5,10,3,12,2,8,7,11,11,20 ,15	chr6D	98.86	3683	7280965	7284647					
contig_176635_1	12955	769	12755	11986	CNL	complete	forward	TRUE	11,13,17,16,4,5,1,6,4,5,10,3,12,2,8,7,11,11,9, 5,10,3,12,2,8,7,11,11,9,11	chr6D	99.445	8106	11083130	11091232					
contig_59461_1	7747	2528	7699	5171	CNL	complete	forward	TRUE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,17,16,14, 1,6	chr6D	99.865	5171	75874009	75868839					
contig_220952_2	7943	787	4049	3262	CNL	complete	forward	FALSE	17,17,16,12,4,5,10,3,12,2,8,7,1,9,11,11	chr6D	99.939	3262	75887208	75883947					
contig_52059_1	10085	3584	9803	6219	CNL	complete	forward	FALSE	17,16,1,6,4,5,10,3,12,2,8,7,11,9,11	chr6D	91.949	3577	469966600	469963069					
contig_409266_1	8885	2406	6311	3905	CNL	complete	reverse	FALSE	17,16,1,6,4,5,12,10,3,12,2,8,7,11,9,11	chr6D	99.949	3906	472132350	472136255					
contig_478807_1	5660	2507	5086	2579	CNL	complete	forward	FALSE	16,1,6,4,5,3,12,2,7,9,19,11,11	chr7A	87.253	2581	726770086	726767521					
contig_58110_1	20091	5267	9081	3814	CNL	complete	forward	FALSE	17,15,16,1,6,4,5,10,3,2,8,7,19,9,9,11,11,11	chr7B	92.766	3304	744339302	744336061					
contig_109646_2	13191	3257	8989	5732	CNL	complete	reverse	TRUE	9,17,16,1,6,4,5,10,3,12,2,8,7,9,11,11,11,11,1 1,11,11,11,11,11,11,11,9	chr7D	99.93	5730	1254215	1259944					
contig_55144_1	11778	2	10565	10563	CNL	complete	reverse	FALSE	7,4,16,1,6,4,5,10,3,2,8,7,9,9,11,11,11,7,11	chr7D	99.835	5441	5784094	5789531					
contig_143229_1	10943	4065	10777	6712	CNL	complete	reverse	FALSE	16,1,6,4,5,10,3,12,2,8,7,9,11,9,9,11,11,11,11, 9,3	chr7D	97.948	6628	19932032	19925423					
contig_85429_1	16298	1549	14219	12670	CNL	complete	reverse	FALSE	14,6,17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9	chr7D	98.265	6859	631463271	631470116					
contig_941_1	24342	6005	19197	13192	CNL	complete	forward	FALSE	14,2,17,16,1,6,4,5,3,12,2,8,7,9,11,9,12,4,5,3, 12,2,8,7,9,9,19,2,15										
contig_55853_1	14241	4676	11464	6788	CNL	complete	reverse	FALSE	16,1,6,4,5,3,12,2,7,9,9,11,11,11,11,2,19										
contig_58111_2	21580	3458	19964	16506	CNL	complete	reverse	FALSE	7,17,16,1,6,4,5,10,3,2,8,7,9,9,11,5,19,11,11,1 1,11,20,5										
contig_714079_1	7875	2945	6923	3978	CNL	complete	forward	TRUE	17,16,6,4,5,1,6,4,3,2,8,11,9,1										
contig_97608_1	21339	1535	20570	19035	CNL	complete	forward	TRUE	17,2,4,16,1,6,4,5,3,12,2,8,7,20,9,11,11,13,9,1 7,1,6,4,5,10,3,12,2,8,11,9										
contig_120140_1	16213	770	13395	11002	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,3,12,2,8,7,11,9,9,20,2										
contig_207362_1	14161	9299	11662	2363	CNL	complete	reverse	FALSE	17,16,1,6,4,5,10,3,2,7,11,9,9										
contig_210896_1	12609	8695	11931	3236	CNL	complete	reverse	FALSE	17,16,1,6,4,5,3,2,8,7,9,9,4,11										
contig_219860_1	6716	2512	5500	2988	CNL	complete	forward	FALSE	16,1,6,4,5,3,12,2,8,7,9,9,11,11,11,11										
contig_351658_1	4386	147	2885	2738	CNL	complete	forward	FALSE	16,14,1,6,4,5,10,3,12,2,8,7,11,9,18										
contig_392849_2	8222	145	6359	6214	CNL	complete	reverse	FALSE	17,16,14,1,6,4,5,10,3,12,2,8,7,11,9,9,14										

Supplementary file 5

Blast analysis of predicted complete *CNL* sequences of CPI110672 (BW_01115) vs AE213 (BW_01024)

Blast analysis of extracted complete CNL sequences of CPI110672 (BW_01115) vs AE213 (BW_01024)								
CPI110672 CNL		AE213 genome assembly						
Query	Lowest E-value	Accession (E-value)	Greatest identity	Accession (identity %)	Greatest HSP lengt	Accession (HSP length)	Greatest bit score	Accession (bit score)
contig_101275_4:3581-8743	0	contig_59274	100	contig_59274	5162	contig_59274	9,310.28	contig_59274
contig_101293_1:4325-11396	0	contig_73070	100	contig_73070	4904	contig_73070	8,845.01	contig_73070
contig_101763_2:1124-18655	0	contig_128969	100	contig_128969	5002	contig_128969	9,021.74	contig_128969
contig_102211_1:132-6687	0	contig_175650	100	contig_116969	3374	contig_175650	5,424.02	contig_175650
contig_104538_2:4555-17059	0	contig_105777	100	contig_31910	8299	contig_105777	14,862.80	contig_105777
contig_104561_1:2626-4741	0	contig_39570	100	contig_39570	2115	contig_39570	3,815.41	contig_39570
contig_107172_3:1034-3620	0	contig_37046	100	contig_37046	2586	contig_37046	4,664.80	contig_37046
contig_107359_1:1296-12291	0	contig_95187	100	contig_95187	6331	contig_95187	11,418.40	contig_95187
contig_109646_2:3257-8989	0	contig_119858	100	contig_119858	5732	contig_119858	10,338.20	contig_119858
contig_109673_2:661-3598	0	contig_39870	100	contig_39870	2937	contig_39870	5,297.78	contig_39870
contig_112972_1:4394-11545	0	contig_95583	100	contig_112149	4860	contig_95583	8,760.25	contig_95583
contig_115137_1:2335-7944	0	contig_63611	100	contig_63611	5609	contig_63611	10,116.40	contig_63611
contig_115139_3:555-9507	0	contig_56686	100	contig_56686	4467	contig_56686	8,056.93	contig_56686
contig_116376_3:1390-4762	0	contig_37438	100	contig_37438	3372	contig_37438	6,082.25	contig_37438
contig_118559_1:1335-18800	0	contig_24758	100	contig_24758	11174	contig_24758	20,152.10	contig_24758
contig_118560_1:48-7397	0	contig_78259	100	contig_51382	4053	contig_78259	7,265.26	contig_78259
contig_11862_1:1651-34459	0	contig_119556	100	contig_119556	13898	contig_119556	25,064.50	contig_119556
contig_119402_1:3776-9999	0	contig_55970	100	contig_55970	6223	contig_55970	11,223.70	contig_55970
contig_120140_1:2062-13064	0	contig_38579	100	contig_120594	6412	contig_38579	11,523.00	contig_38579
contig_123233_2:2095-8498	0	contig_30919	100	contig_30919	3324	contig_30919	5,995.68	contig_30919
contig_12598_1:607-4552	0	contig_19955	100	contig_19955	3674	contig_19955	6,626.86	contig_19955
contig_128611_1:2383-10252	0	contig_92097	100	contig_92097	5059	contig_92097	9,124.53	contig_92097
contig_13003_2:2762-12612	0	contig_78554	100	contig_78554	9850	contig_78554	17,764.50	contig_78554
contig_131163_1:595-4548	0	contig_36618	100	contig_36618	3587	contig_36618	6,469.97	contig_36618
contig_132666_1:2349-5972	0	contig_36899	100	contig_36899	2169	contig_60796	3,379.00	contig_36899
contig_132909_2:1538-8998	0	contig_34774	100	contig_33136	3473	contig_34774	6,258.98	contig_34774
contig_133855_1:3900-12481	0	contig_127799	100	contig_127799	8238	contig_127799	14,857.40	contig_127799
contig_134231_1:3641-10077	0	contig_153406	100	contig_153406	3610	contig_153406	6,511.45	contig_153406
contig_138067_1:304-11493	0	contig_102413	100	contig_102413	6559	contig_102413	11,829.60	contig_102413
contig_13839_1:3378-7495	0	contig_114863	100	contig_114863	4117	contig_114863	7,425.76	contig_114863
contig_140260_1:242-5016	0	contig_98807	100	contig_98807	2492	contig_98807	4,495.28	contig_98807
contig_140875_2:1404-10222	0	contig_50128	100	contig_50128	8818	contig_50128	15,903.40	contig_50128
contig_142525_2:3026-6974	0	contig_8174	100	contig_8174	3948	contig_8174	7,120.99	contig_8174
contig_143229_1:4065-10777	0	contig_32328	100	contig_21576	3621	contig_32328	6,468.17	contig_32328
contig_143731_1:5678-13387	0	contig_176721	100	contig_176721	3939	contig_176721	7,104.76	contig_176721
contig_1439_1:6673-17885	0	contig_46890	100	contig_103770	10179	contig_46890	18,330.70	contig_46890
contig_147152_1:1753-22069	0	contig_42612	100	contig_42612	10587	contig_42612	19,093.60	contig_42612
contig_148274_1:3241-10617	0	contig_106431	100	contig_106431	5547	contig_106431	10,004.60	contig_106431
contig_150494_3:467-15695	0	contig_49460	100	contig_49460	15228	contig_49460	27,463.00	contig_49460
contig_15156_4:330-16251	0	contig_87415	100	contig_34093	10310	contig_87415	18,588.60	contig_87415
contig_155598_2:3545-5921	0	contig_87719	100	contig_87719	2376	contig_87719	4,286.09	contig_87719
contig_157595_1:3521-10362	0	contig_77715	100	contig_77715	6841	contig_77715	12,338.10	contig_77715
contig_157596_2:1304-9773	0	contig_86124	100	contig_86124	3968	contig_86124	7,157.05	contig_86124
contig_158704_3:1646-14883	0	contig_73811	100	contig_73811	7497	contig_73811	13,521.10	contig_73811
contig_158959_1:3730-15848	0	contig_42649	100	contig_42649	12118	contig_42649	21,854.50	contig_42649
contig_163591_1:7405-17140	0	contig_47360	100	contig_47360	9735	contig_47360	17,557.10	contig_47360
contig_163830_3:2372-5680	0	contig_135378	100	contig_135378	3091	contig_135378	5,575.50	contig_135378
contig_164387_1:230-7985	0	contig_112710	100	contig_112710	5655	contig_112710	10,199.30	contig_112710
contig_166203_1:2860-10440	0	contig_154743	100	contig_154743	4477	contig_154743	8,074.97	contig_154743
contig_166487_1:1825-10723	0	contig_56447	100	contig_56447	8898	contig_56447	16,047.70	contig_56447
contig_166921_2:1802-6317	0	contig_83088	100	contig_83088	4515	contig_83088	8,143.50	contig_83088
contig_17162_1:5576-18090	0	contig_22259	100	contig_22259	6717	contig_22259	12,114.50	contig_22259
contig_172782_1:1964-12433	0	contig_59478	100	contig_59478	7884	contig_59478	14,219.00	contig_59478
contig_172783_1:245-4737	0	contig_110121	100	contig_110121	4492	contig_110121	8,102.02	contig_110121
contig_174155_1:3824-14375	0	contig_78212	100	contig_78212	7662	contig_78212	13,793.50	contig_78212
contig_174166_1:8958-21649	0	contig_101666	100	contig_101666	5249	contig_101666	9,467.17	contig_101666
contig_175045_1:1920-5108	0	contig_152295	100	contig_152295	3188	contig_152295	5,750.43	contig_152295
contig_176635_1:769-12755	0	contig_43575	100	contig_43575	10858	contig_43575	19,582.30	contig_43575
contig_176636_1:5626-11663	0	contig_60341	100	contig_60341	5002	contig_60341	9,021.74	contig_60341
contig_176661_2:656-16481	0	contig_116284	100	contig_116284	7823	contig_116284	14,109.00	contig_116284
contig_177584_1:2247-13015	0	contig_72382	100	contig_72382	5875	contig_61797	10,552.80	contig_72382

contig_180100_5:2041-26758	0	contig_9565	100	contig_14922	12191	contig_9565	21,899.60	contig_9565
contig_181147_1:4865-9078	0	contig_17507	100	contig_17507	4213	contig_17507	7,598.88	contig_17507
contig_181378_1:1507-4335	0	contig_56301	100	contig_56301	2850	contig_22736	3,582.78	contig_56301
contig_181721_1:123-20143	0	contig_89726	100	contig_89726	9220	contig_89726	16,628.30	contig_89726
contig_183911_3:5542-8964	0	contig_51307	100	contig_170667	2131	contig_51307	3,838.86	contig_51307
contig_184979_2:683-6791	0	contig_77987	100	contig_77987	6108	contig_77987	11,016.30	contig_77987
contig_185503_2:766-18345	0	contig_31812	100	contig_31812	5957	contig_31812	10,744.00	contig_31812
contig_187720_1:4861-39143	0	contig_63758	100	contig_137006	15447	contig_63758	27,655.90	contig_63758
contig_191357_1:7734-10953	0	contig_29919	100	contig_29919	3219	contig_29919	5,806.33	contig_29919
contig_196242_1:6370-18602	0	contig_35365	100	contig_123833	8271	contig_35365	14,830.40	contig_35365
contig_197286_2:1586-5604	0	contig_34998	100	contig_34998	3776	contig_34998	6,810.81	contig_34998
contig_197287_1:2552-8445	0	contig_41830	100	contig_41830	4891	contig_41830	8,821.56	contig_41830
contig_204319_1:659-5790	0	contig_132092	100	contig_132092	3768	contig_83410	5,344.67	contig_132092
contig_206540_2:2599-11316	0	contig_52027	100	contig_52027	8257	contig_52027	14,891.70	contig_52027
contig_207353_1:14604-18659	0	contig_120955	100	contig_120955	4055	contig_120955	7,313.95	contig_120955
contig_207354_3:4865-9214	0	contig_65178	100	contig_65178	2750	contig_65178	4,960.55	contig_65178
contig_207362_1:9299-11662	0	contig_36488	100	contig_36488	1477	contig_36488	2,664.86	contig_36488
contig_210896_1:8695-11931	0	contig_137015	100	contig_137015	3236	contig_137015	5,836.99	contig_137015
contig_211029_1:392-10561	0	contig_137929	100	contig_137929	5126	contig_137929	9,245.35	contig_137929
contig_211128_1:2495-21807	0	contig_14334	100	contig_140191	12417	contig_14334	22,381.10	contig_14334
contig_212132_1:86-7186	0	contig_101935	100	contig_101935	6298	contig_101935	11,358.90	contig_101935
contig_212831_1:290-3781	0	contig_144483	100	contig_144483	3491	contig_144483	6,296.85	contig_144483
contig_213209_2:6731-15026	0	contig_69930	100	contig_69930	6204	contig_69930	11,189.40	contig_69930
contig_219487_1:270-11578	0	contig_4637	100	contig_4637	11308	contig_4637	20,393.80	contig_4637
contig_219860_1:2512-5500	0	contig_19653	100	contig_19653	2988	contig_19653	5,389.75	contig_19653
contig_220952_2:787-4049	0	contig_150526	100	contig_150526	3262	contig_150526	5,883.88	contig_150526
contig_222494_1:630-9076	0	contig_56043	100	contig_56043	8430	contig_56043	15,169.40	contig_56043
contig_222961_1:3422-15214	0	contig_11275	100	contig_180258	10846	contig_11275	18,954.70	contig_11275
contig_223213_1:5170-20147	0	contig_87241	100	contig_87241	8173	contig_87241	14,740.20	contig_87241
contig_225459_1:119-2767	0	contig_60897	100	contig_60897	2668	contig_30406	4,776.61	contig_60897
contig_228652_2:327-3957	0	contig_21	100	contig_21	3630	contig_21	6,547.52	contig_21
contig_232968_1:1258-4000	0	contig_30176	100	contig_30176	2742	contig_30176	4,946.12	contig_30176
contig_233404_2:1981-4828	0	contig_56386	100	contig_56386	2847	contig_56386	5,135.48	contig_56386
contig_234162_4:6760-9783	0	contig_103214	100	contig_103214	2903	contig_103214	5,236.47	contig_103214
contig_235672_1:856-7976	0	contig_67076	100	contig_67076	6435	contig_67076	11,606.00	contig_67076
contig_23660_1:880-12236	0	contig_102606	100	contig_102606	4692	contig_102606	8,462.69	contig_102606
contig_236972_1:842-6049	0	contig_16858	100	contig_26861	2575	contig_16477	3,382.60	contig_16858
contig_237058_1:3925-22591	0	contig_38939	100	contig_138176	9818	contig_38939	17,692.30	contig_38939
contig_239763_3:2129-5096	0	contig_8477	100	contig_56059	2967	contig_8477	5,346.47	contig_8477
contig_243024_3:1632-4017	0	contig_156474	100	contig_156474	2385	contig_156474	4,302.32	contig_156474
contig_243024_4:1035-8992	0	contig_157036	100	contig_157036	5746	contig_157036	10,363.40	contig_157036
contig_244253_2:293-11785	0	contig_116963	100	contig_116963	8989	contig_116963	16,211.80	contig_116963
contig_245500_1:525-7047	0	contig_67218	100	contig_67218	6522	contig_67218	11,762.90	contig_67218
contig_246286_3:2335-7925	0	contig_59199	100	contig_59199	5590	contig_59199	10,082.10	contig_59199
contig_247356_2:4190-6860	0	contig_22736	100	contig_22736	2670	contig_22736	4,816.28	contig_22736
contig_248572_1:1242-17985	0	contig_80632	100	contig_80632	11885	contig_80632	21,434.30	contig_80632
contig_248758_2:4781-7314	0	contig_11291	100	contig_11291	2533	contig_11291	4,569.22	contig_11291
contig_248846_1:2079-13590	0	contig_143057	100	contig_143057	9024	contig_143057	16,274.90	contig_143057
contig_25336_1:6129-8217	0	contig_64388	100	contig_64388	2088	contig_64388	3,766.72	contig_64388
contig_253757_3:3212-16223	0	contig_59075	100	contig_59075	11465	contig_59075	20,676.90	contig_59075
contig_254135_1:8917-12894	0	contig_129128	100	contig_129128	3977	contig_129128	7,173.28	contig_129128
contig_2605_2:8798-13602	0	contig_6893	100	contig_6893	4804	contig_6893	8,664.67	contig_6893
contig_261034_1:2940-28973	0	contig_112613	100	contig_80633	17978	contig_112613	32,353.70	contig_112613
contig_261449_2:1369-12557	0	contig_86528	100	contig_86528	5730	contig_86528	10,334.60	contig_86528
contig_263240_1:5113-7134	0	contig_81675	100	contig_81675	1405	contig_81675	2,535.02	contig_81675
contig_263444_1:4846-7661	0	contig_55045	100	contig_55045	2815	contig_55045	5,077.77	contig_55045
contig_263740_1:1138-5386	0	contig_85274	100	contig_85274	4248	contig_85274	7,662.00	contig_85274
contig_266215_1:3040-7632	0	contig_135667	100	contig_135667	4592	contig_135667	8,282.36	contig_135667
contig_267269_1:4448-8357	0	contig_16477	100	contig_26855	2689	contig_16477	3,494.41	contig_16477
contig_272254_2:816-11230	0	contig_172949	100	contig_172949	5398	contig_172949	9,735.87	contig_172949
contig_282763_1:565-3732	0	contig_85691	100	contig_85691	3169	contig_77715	5,712.56	contig_85691
contig_283544_1:380-16787	0	contig_4121	100	contig_4121	8792	contig_4121	15,856.50	contig_4121
contig_28390_1:2129-12385	0	contig_9878	100	contig_9878	5219	contig_9878	9,413.07	contig_9878
contig_284144_1:2936-7471	0	contig_105817	100	contig_105817	4535	contig_105817	8,179.56	contig_105817
contig_284791_1:70-23596	0	contig_7490	100	contig_7490	9064	contig_7490	16,347.00	contig_7490
contig_287675_1:202-7080	0	contig_5703	100	contig_5703	6520	contig_5703	11,759.30	contig_5703
contig_28857_1:1844-5970	0	contig_138337	100	contig_138337	4126	contig_138337	7,441.99	contig_138337
contig_293580_2:799-3019	0	contig_64607	100	contig_64607	2220	contig_64607	4,004.77	contig_64607
contig_299564_1:1688-7384	0	contig_54714	100	contig_54714	3624	contig_54714	6,536.69	contig_54714

contig_303843_1:6263-9526	0	contig_65142	100	contig_65142	2694	contig_48374	3,443.92	contig_65142
contig_30439_1:214-3861	0	contig_158695	100	contig_158695	3599	contig_158695	6,491.61	contig_158695
contig_306400_1:5661-7533	0	contig_83890	100	contig_31307	1931	contig_59075	2,379.93	contig_83890
contig_307277_2:124-7215	0	contig_100906	100	contig_100906	7091	contig_100906	12,789.00	contig_100906
contig_310761_1:5984-8986	0	contig_119857	100	contig_119857	3002	contig_119857	5,415.00	contig_119857
contig_31100_3:8545-13569	0	contig_65198	100	contig_65198	5024	contig_65198	9,061.41	contig_65198
contig_313671_1:601-9966	0	contig_57000	100	contig_143774	6083	contig_57000	10,940.50	contig_57000
contig_313743_2:1996-6371	0	contig_79581	100	contig_79581	4380	contig_32229	7,891.02	contig_79581
contig_318637_1:3415-5803	0	contig_90362	100	contig_90362	2420	contig_2224	4,307.73	contig_90362
contig_319663_1:2897-8923	0	contig_128040	100	contig_128040	6026	contig_128040	10,868.40	contig_128040
contig_321192_1:984-5637	0	contig_53188	100	contig_53188	4653	contig_53188	8,392.36	contig_53188
contig_323708_1:2301-12314	0	contig_3920	100	contig_135910	7616	contig_3920	13,730.30	contig_3920
contig_326814_1:4140-7548	0	contig_29110	100	contig_29110	3408	contig_29110	6,147.17	contig_29110
contig_32700_1:4215-9812	0	contig_69709	100	contig_69709	4202	contig_69709	7,579.04	contig_69709
contig_328463_4:6026-9599	0	contig_16480	100	contig_104528	3308	contig_16480	4,991.21	contig_16480
contig_329712_1:4058-8065	0	contig_4422	100	contig_4422	4007	contig_4422	7,227.39	contig_4422
contig_329713_2:984-3375	0	contig_39153	100	contig_39153	2420	contig_2224	4,313.14	contig_39153
contig_329714_1:1611-5877	0	contig_85289	100	contig_85289	4266	contig_85289	7,694.46	contig_85289
contig_329766_1:6111-13448	0	contig_76591	100	contig_76591	5043	contig_76591	9,095.68	contig_76591
contig_331113_1:4760-10066	0	contig_176852	100	contig_176852	2556	contig_36601	2,825.36	contig_176852
contig_3358_1:2542-19988	0	contig_108200	100	contig_108200	13982	contig_108200	25,216.00	contig_108200
contig_336948_2:2460-4985	0	contig_65195	100	contig_65195	2525	contig_65195	4,554.79	contig_65195
contig_3374_2:2095-9372	0	contig_40370	100	contig_40370	5841	contig_40370	10,534.80	contig_40370
contig_340163_1:4971-7674	0	contig_123267	100	contig_123267	2703	contig_123267	4,875.79	contig_123267
contig_342741_3:5275-11936	0	contig_183426	100	contig_183426	3872	contig_183426	6,983.93	contig_183426
contig_344931_1:4165-7017	0	contig_92098	100	contig_92098	2852	contig_92098	5,144.49	contig_92098
contig_344932_1:1854-28768	0	contig_13329	100	contig_13329	20988	contig_13329	37,850.40	contig_13329
contig_34786_4:2248-7236	0	contig_11711	100	contig_11711	4988	contig_11711	8,996.49	contig_11711
contig_34786_5:1156-4572	0	contig_91469	100	contig_91469	3416	contig_91469	6,161.59	contig_91469
contig_34787_3:3143-5533	0	contig_65198	100	contig_163255	2405	contig_2224	2,659.45	contig_65198
contig_34788_1:6244-11472	0	contig_81238	100	contig_81238	5228	contig_81238	9,429.30	contig_81238
contig_350432_1:9326-15182	0	contig_27267	100	contig_115949	4641	contig_27267	8,231.86	contig_27267
contig_351089_1:681-25912	0	contig_56334	100	contig_21051	14901	contig_56334	26,813.80	contig_56334
contig_351658_1:147-2885	0	contig_112050	100	contig_112050	2755	contig_145658	4,938.91	contig_112050
contig_354069_2:664-18931	0	contig_30617	100	contig_72873	9821	contig_30617	17,688.70	contig_30617
contig_35472_3:333-12425	0	contig_41152	100	contig_41152	12092	contig_41152	21,807.60	contig_41152
contig_360210_1:7215-11829	0	contig_9721	100	contig_9721	4614	contig_9721	8,322.03	contig_9721
contig_360374_1:6472-20607	0	contig_17305	100	contig_17305	8972	contig_17305	16,181.10	contig_17305
contig_360405_3:3437-11554	0	contig_33161	100	contig_95429	6107	contig_33161	9,102.89	contig_33161
contig_3636_2:6142-8707	0	contig_45802	100	contig_45802	2596	contig_2224	4,626.93	contig_45802
contig_368314_2:833-4076	0	contig_666	100	contig_666	3243	contig_666	5,849.61	contig_666
contig_368660_1:27-6248	0	contig_94546	100	contig_94546	3456	contig_94546	6,233.73	contig_94546
contig_369457_1:432-4379	0	contig_84555	100	contig_84555	3047	contig_84555	5,496.15	contig_84555
contig_369485_1:2394-10123	0	contig_59028	100	contig_59028	4256	contig_32328	5,375.33	contig_59028
contig_372072_1:1259-3649	0	contig_14034	100	contig_14034	2420	contig_2224	3,608.02	contig_14034
contig_372865_1:8767-25111	0	contig_42365	100	contig_42365	12362	contig_42365	22,294.50	contig_42365
contig_377839_1:350-6412	0	contig_168771	100	contig_168771	5126	contig_168771	9,245.35	contig_168771
contig_379307_2:3974-6760	0	contig_22737	100	contig_22737	2790	contig_22736	4,996.62	contig_22737
contig_381440_1:10919-33695	0	contig_65086	100	contig_182152	16102	contig_65086	29,033.70	contig_65086
contig_381466_1:837-8648	0	contig_57627	100	contig_57627	3666	contig_100591	6,511.45	contig_57627
contig_38219_1:1531-17442	0	contig_45352	100	contig_45352	7631	contig_45352	13,762.80	contig_45352
contig_383227_2:3267-8049	0	contig_75783	100	contig_75783	4782	contig_75783	8,625.00	contig_75783
contig_387722_1:766-3301	0	contig_118282	100	contig_118282	2534	contig_30406	3,117.51	contig_118282
contig_388416_1:5302-15184	0	contig_83410	100	contig_83410	9882	contig_83410	17,822.20	contig_83410
contig_391516_1:2028-22334	0	contig_77548	100	contig_77548	9376	contig_77548	16,909.70	contig_77548
contig_392849_2:145-6359	0	contig_64230	100	contig_152534	3839	contig_64230	6,897.37	contig_64230
contig_39457_1:3193-10936	0	contig_170045	100	contig_1568	4428	contig_83410	4,787.43	contig_170045
contig_394684_1:1532-16757	0	contig_167897	100	contig_167897	5613	contig_167897	10,123.60	contig_167897
contig_403270_1:15-8083	0	contig_83890	100	contig_97170	6377	contig_83890	11,496.00	contig_83890
contig_406427_1:2589-6577	0	contig_84062	100	contig_84062	3988	contig_84062	7,193.12	contig_84062
contig_409266_1:2406-6311	0	contig_67906	100	contig_67906	3905	contig_67906	7,043.44	contig_67906
contig_41729_3:1626-8143	0	contig_97822	100	contig_115810	3855	contig_97822	6,942.45	contig_97822
contig_420996_1:4397-8603	0	contig_30628	100	contig_30626	4212	contig_30628	7,566.42	contig_30628
contig_42208_4:4018-11194	0	contig_774	100	contig_774	7176	contig_774	12,942.30	contig_774
contig_42789_2:1870-6306	0	contig_31714	100	contig_31714	4012	contig_38211	5,232.86	contig_31714
contig_430281_1:2824-5333	0	contig_37154	100	contig_37154	2509	contig_37154	4,525.94	contig_37154
contig_433906_2:382-2670	0	contig_113020	100	contig_113020	2288	contig_113020	4,127.39	contig_113020
contig_438300_2:5383-8543	0	contig_35414	100	contig_35414	3160	contig_35414	5,699.93	contig_35414
contig_442049_1:3301-6990	0	contig_123607	100	contig_123607	3171	contig_77715	4,625.12	contig_123607
contig_45259_1:8018-15630	0	contig_30756	100	contig_30756	4047	contig_30756	7,299.52	contig_30756
contig_453208_2:993-4967	0	contig_166716	100	contig_166716	3974	contig_166716	7,167.87	contig_166716
contig_455881_1:4589-13748	0	contig_31622	100	contig_31622	2908	contig_84869	4,702.67	contig_31622

contig_462070_1:506-7528	0	contig_170247	100	contig_170247	3874	contig_170247	6,987.54	contig_170247
contig_468342_1:4064-6218	0	contig_163329	100	contig_171581	2135	contig_41152	3,133.74	contig_163329
contig_470919_1:2703-5513	0	contig_45988	100	contig_45988	2810	contig_45988	5,068.75	contig_45988
contig_472144_2:9488-11963	0	contig_30406	100	contig_48195	2449	contig_30406	2,861.43	contig_30406
contig_478434_2:694-4056	0	contig_180731	100	contig_180731	2000	contig_3920	3,142.75	contig_180731
contig_478807_1:2507-5086	0	contig_99220	100	contig_99220	2595	contig_19653	4,652.17	contig_99220
contig_481660_2:833-3252	0	contig_23914	100	contig_23914	2419	contig_23914	4,363.64	contig_23914
contig_48260_1:99-3577	0	contig_81893	100	contig_81893	3361	contig_32229	4,064.28	contig_81893
contig_486132_1:1014-3142	0	contig_90866	100	contig_157485	2121	contig_90866	2,132.87	contig_90866
contig_492056_3:65-2699	0	contig_38211	100	contig_38211	2634	contig_38211	4,751.36	contig_38211
contig_492057_2:3464-7385	0	contig_31163	100	contig_31163	3936	contig_38211	7,072.30	contig_31163
contig_49760_1:6748-19153	0	contig_79692	100	contig_79692	7271	contig_79692	13,113.60	contig_79692
contig_498457_3:2732-8048	0	contig_129137	100	contig_129137	3407	contig_83410	5,097.61	contig_129137
contig_506713_2:1518-10799	0	contig_39080	100	contig_39080	9281	contig_39080	16,738.40	contig_39080
contig_507642_1:152-11455	0	contig_57788	100	contig_57788	6946	contig_57788	12,527.50	contig_57788
contig_5108_2:671-19398	0	contig_9969	100	contig_9969	18727	contig_9969	33,773.00	contig_9969
contig_515065_2:617-4579	0	contig_37031	100	contig_37031	3962	contig_37031	7,146.23	contig_37031
contig_520166_1:4777-7232	0	contig_90866	100	contig_90866	2455	contig_90866	4,428.56	contig_90866
contig_520221_4:610-3529	0	contig_135151	100	contig_135151	2919	contig_135151	5,265.32	contig_135151
contig_52059_1:3584-9803	0	contig_183971	100	contig_183971	2540	contig_183971	4,581.84	contig_183971
contig_520925_1:6309-11155	0	contig_86656	100	contig_86656	2799	contig_86656	5,048.92	contig_86656
contig_5217_1:518-14293	0	contig_56678	100	contig_56678	10084	contig_56678	18,186.50	contig_56678
contig_52175_1:320-11308	0	contig_30061	100	contig_30061	5758	contig_30061	10,385.10	contig_30061
contig_5219_1:1722-7494	0	contig_111915	100	contig_111915	2041	contig_111915	3,681.96	contig_111915
contig_52431_3:2805-9369	0	contig_149026	100	contig_149026	3332	contig_149026	6,010.11	contig_149026
contig_524632_1:2031-6240	0	contig_62257	100	contig_62257	2161	contig_61797	3,207.68	contig_62257
contig_52536_1:1340-13016	0	contig_48131	100	contig_48131	7473	contig_48131	13,477.90	contig_48131
contig_536947_2:7037-12950	0	contig_74912	100	contig_74912	5913	contig_74912	10,664.60	contig_74912
contig_538702_2:1100-6578	0	contig_29358	100	contig_29358	5478	contig_29358	9,880.14	contig_29358
contig_54023_2:5214-8802	0	contig_118209	100	contig_118209	3588	contig_118209	6,471.77	contig_118209
contig_544805_1:645-5477	0	contig_127725	100	contig_127725	4493	contig_127725	8,103.82	contig_127725
contig_544808_1:566-6580	0	contig_32857	100	contig_32857	4571	contig_32857	8,244.49	contig_32857
contig_55144_1:2-10565	0	contig_89799	100	contig_158146	4337	contig_89799	7,737.74	contig_89799
contig_55269_1:2130-5790	0	contig_979	100	contig_979	3660	contig_979	6,601.62	contig_979
contig_554121_2:1111-3804	0	contig_175954	100	contig_175954	2613	contig_49460	4,287.89	contig_175954
contig_55853_1:4676-11464	0	contig_26856	100	contig_26856	6788	contig_26856	12,242.60	contig_26856
contig_559688_1:689-8288	0	contig_52644	100	contig_52644	7599	contig_52644	13,705.10	contig_52644
contig_55998_2:4575-10869	0	contig_40219	100	contig_40219	5311	contig_40219	9,578.98	contig_40219
contig_56225_4:198-7448	0	contig_40981	100	contig_40981	5493	contig_40981	9,907.19	contig_40981
contig_563857_1:5952-8809	0	contig_40203	100	contig_40203	2857	contig_40203	5,153.51	contig_40203
contig_564489_1:1785-5165	0	contig_31289	100	contig_31289	3380	contig_31289	6,096.67	contig_31289
contig_565421_1:2276-8575	0	contig_128755	100	contig_128755	6299	contig_128755	11,360.70	contig_128755
contig_577010_1:9616-13878	0	contig_129079	100	contig_129079	4262	contig_129079	7,687.24	contig_129079
contig_577345_1:211-3990	0	contig_118849	100	contig_118849	3762	contig_118849	6,785.56	contig_118849
contig_58110_1:5267-9081	0	contig_179697	100	contig_179697	2997	contig_179697	5,405.98	contig_179697
contig_58111_2:3458-19964	0	contig_30518	100	contig_30518	11466	contig_30518	20,678.70	contig_30518
contig_58112_1:3667-7719	0	contig_57592	100	contig_57592	4052	contig_57592	7,308.54	contig_57592
contig_59461_1:2528-7699	0	contig_122325	100	contig_122325	5171	contig_122325	9,326.51	contig_122325
contig_59695_5:360-14259	0	contig_18533	100	contig_18533	8464	contig_18533	15,265.00	contig_18533
contig_5983_2:1575-22941	0	contig_72294	100	contig_72294	9055	contig_72294	16,330.80	contig_72294
contig_60289_1:5767-11658	0	contig_65193	100	contig_65193	5891	contig_65193	10,624.90	contig_65193
contig_606273_1:2636-5711	0	contig_132308	100	contig_132308	3075	contig_132308	5,546.65	contig_132308
contig_60807_2:3861-36288	0	contig_5121	100	contig_5121	16740	contig_5121	29,839.80	contig_5121
contig_61359_1:87-11066	0	contig_60794	100	contig_60794	9829	contig_60794	17,726.60	contig_60794
contig_62277_3:1048-10773	0	contig_125909	100	contig_125909	4393	contig_125909	7,923.49	contig_125909
contig_62390_2:2284-6680	0	contig_137060	100	contig_137060	4396	contig_137060	7,928.90	contig_137060
contig_62392_3:3331-6206	0	contig_26506	100	contig_26506	2875	contig_26506	5,185.97	contig_26506
contig_624641_2:3691-9379	0	contig_145864	100	contig_145864	3472	contig_87241	4,765.79	contig_145864
contig_624682_1:1978-8343	0	contig_100792	100	contig_100792	5105	contig_100792	9,207.48	contig_100792
contig_628832_1:3305-8892	0	contig_92637	100	contig_92637	2082	contig_92637	3,755.90	contig_92637
contig_630851_1:296-4976	0	contig_121226	100	contig_121226	4680	contig_121226	8,441.05	contig_121226
contig_633453_1:940-6796	0	contig_29684	100	contig_29684	5856	contig_29684	10,561.80	contig_29684
contig_639_1:1875-4410	0	contig_30406	100	contig_30406	2535	contig_30406	4,572.83	contig_30406
contig_639775_1:1446-4581	0	contig_92880	100	contig_92880	3135	contig_92880	5,654.85	contig_92880
contig_650375_1:4351-22386	0	contig_30911	100	contig_144146	12719	contig_30911	22,932.90	contig_30911
contig_651597_1:1133-6048	0	contig_109904	100	contig_109904	4915	contig_109904	8,864.84	contig_109904
contig_654864_1:716-7221	0	contig_57759	100	contig_57759	5631	contig_57759	10,156.10	contig_57759
contig_65792_3:2363-19048	0	contig_2224	100	contig_10791	14238	contig_2224	25,318.80	contig_2224
contig_658749_1:7247-11369	0	contig_37986	100	contig_179615	4083	contig_37986	6,466.36	contig_37986
contig_659243_1:28-2081	0	contig_167330	100	contig_167330	1861	contig_167330	3,357.36	contig_167330
contig_668577_2:3789-9920	0	contig_32229	100	contig_32229	6131	contig_32229	11,057.70	contig_32229
contig_669599_1:71-6233	0	contig_142057	100	contig_142057	3738	contig_142057	6,742.28	contig_142057
contig_672674_1:1536-5901	0	contig_98418	100	contig_98418	4365	contig_98418	7,872.99	contig_98418
contig_69006_1:3710-10806	0	contig_86755	100	contig_86755	5409	contig_86755	9,755.71	contig_86755
contig_694703_1:578-6844	0	contig_69691	100	contig_69691	6064	contig_69691	10,936.90	contig_69691

contig_70368_1:4921-20170	0	contig_57591	100	contig_57591	10357	contig_57591	18,678.80	contig_57591
contig_70569_1:4407-13604	0	contig_61304	100	contig_61304	9197	contig_61304	16,586.90	contig_61304
contig_714079_1:2945-6923	0	contig_30619	100	contig_30619	3978	contig_30619	7,175.09	contig_30619
contig_714972_1:30-7543	0	contig_141587	100	contig_141587	6813	contig_141587	12,287.60	contig_141587
contig_716170_1:23-5143	0	contig_41478	100	contig_181310	5118	contig_41478	9,194.86	contig_41478
contig_72305_3:596-5289	0	contig_31906	100	contig_31906	4308	contig_31906	7,770.20	contig_31906
contig_7473_1:7595-10628	0	contig_151614	100	contig_151614	3033	contig_151614	5,470.90	contig_151614
contig_7473_2:5322-8953	0	contig_25999	100	contig_25999	3631	contig_25999	6,549.32	contig_25999
contig_748344_2:2805-5362	0	contig_38408	100	contig_38408	2557	contig_38408	4,612.50	contig_38408
contig_752952_1:4369-13758	0	contig_84937	100	contig_84937	4869	contig_84937	8,781.89	contig_84937
contig_753148_1:135-4566	0	contig_64281	100	contig_65178	4194	contig_64281	7,559.20	contig_64281
contig_7562_1:818-13665	0	contig_28969	100	contig_28969	5147	contig_28969	9,283.23	contig_28969
contig_758179_1:418-13871	0	contig_30004	100	contig_30004	12157	contig_30004	21,924.80	contig_30004
contig_758452_1:2755-7243	0	contig_131426	100	contig_131426	3858	contig_79581	4,199.53	contig_131426
contig_761150_1:3745-6844	0	contig_102787	100	contig_102787	3099	contig_102787	5,589.93	contig_102787
contig_762743_1:210-3188	0	contig_54713	100	contig_54713	2978	contig_54713	5,371.72	contig_54713
contig_78481_1:4056-12563	0	contig_47610	100	contig_47610	4533	contig_47610	8,175.96	contig_47610
contig_7922_3:4174-5884	0	contig_42892	100	contig_42892	1710	contig_42892	3,085.05	contig_42892
contig_79332_2:4044-7386	0	contig_1688	100	contig_1688	3342	contig_1688	6,028.14	contig_1688
contig_79949_4:1398-6563	0	contig_66321	100	contig_66321	5165	contig_66321	9,315.69	contig_66321
contig_81593_1:44-10823	0	contig_145021	100	contig_145021	6701	contig_145021	12,085.70	contig_145021
contig_81897_1:144-8571	0	contig_33261	100	contig_33261	7945	contig_33261	14,329.10	contig_33261
contig_81897_2:2626-7442	0	contig_25066	100	contig_25066	4314	contig_25066	7,781.02	contig_25066
contig_827697_1:2230-10577	0	contig_48374	100	contig_48374	8137	contig_48374	14,675.30	contig_48374
contig_83009_1:1707-5110	0	contig_51115	100	contig_51115	3484	contig_77715	6,138.15	contig_51115
contig_832030_1:692-4165	0	contig_183053	100	contig_183053	1966	contig_77877	2,466.49	contig_183053
contig_843356_1:47-4477	0	contig_94751	100	contig_94751	2269	contig_94751	4,093.13	contig_94751
contig_85429_1:1549-14219	0	contig_145658	100	contig_23621	10092	contig_145658	17,070.20	contig_145658
contig_85441_2:44-6367	0	contig_92617	100	contig_92617	6187	contig_92617	11,158.70	contig_92617
contig_85454_1:3769-7906	0	contig_74015	100	contig_104331	3605	contig_74015	6,405.05	contig_74015
contig_86217_1:1056-14385	0	contig_40849	100	contig_40849	7938	contig_40849	14,316.40	contig_40849
contig_88019_1:2005-8752	0	contig_12714	100	contig_12714	6078	contig_12714	10,962.20	contig_12714
contig_89858_1:606-11131	0	contig_16392	100	contig_16392	9396	contig_16392	16,945.70	contig_16392
contig_910243_1:17-3871	0	contig_87765	100	contig_87765	2861	contig_87765	5,160.72	contig_87765
contig_92520_3:1531-6866	0	contig_24134	100	contig_35715	2696	contig_24134	4,857.76	contig_24134
contig_941_1:5973-24307	0	contig_87684	100	contig_87684	13358	contig_87684	24,090.70	contig_87684
contig_95556_1:740-4632	0	contig_116502	100	contig_116502	3892	contig_116502	7,020.00	contig_116502
contig_95582_1:1856-4698	0	contig_31763	100	contig_31763	2842	contig_31763	5,126.46	contig_31763
contig_97608_1:1535-20570	0	contig_12457	100	contig_183725	6583	contig_12457	11,501.40	contig_12457
contig_98599_3:884-3618	0	contig_164485	100	contig_164485	2734	contig_164485	4,931.70	contig_164485
contig_178575_1:2839-13630	0	contig_38344	99.99	contig_38344	10791	contig_38344	19,456.00	contig_38344
contig_204537_1:1344-8713	0	contig_29258	99.99	contig_29258	7369	contig_29258	13,284.90	contig_29258
contig_298790_2:4085-11577	0	contig_103306	99.99	contig_103306	7492	contig_103306	13,506.70	contig_103306
contig_446333_1:5069-14706	0	contig_50875	99.99	contig_50875	8754	contig_50875	15,782.60	contig_50875
contig_112073_1:2010-6813	0	contig_1197	99.98	contig_1197	4803	contig_1197	8,657.46	contig_1197
contig_210299_1:3714-17139	0	contig_97927	99.96	contig_97927	13420	contig_97927	24,188.10	contig_97927
contig_138557_2:1072-4033	0	contig_148179	99.93	contig_148179	2961	contig_148179	5,332.04	contig_148179
contig_261734_1:179-2696	0	contig_60898	99.92	contig_60898	2535	contig_30406	4,531.35	contig_60898
contig_228769_1:2308-5404	0	contig_1026	99.58	contig_1026	3204	contig_92880	5,512.38	contig_1026
contig_111719_4:4079-13170	0	contig_25483	99.48	contig_25483	9044	contig_25483	16,220.80	contig_25483
contig_20914_2:2423-9842	0	contig_82088	99.34	contig_82088	6975	contig_82088	12,372.40	contig_82088
contig_237059_1:5927-10790	0	contig_18568	99.3	contig_18568	4884	contig_18568	8,680.90	contig_18568
contig_408212_1:2233-8252	0	contig_4285	99.2	contig_4285	5971	contig_4285	10,677.20	contig_4285
contig_9437_2:126-5666	0	contig_1972	99.13	contig_1972	4132	contig_1972	7,346.41	contig_1972