

Genome-Wide Association Study For Disease Traits In

Wheat And Its Wild Relatives

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Abstract

Wheat is the most widely grown crop in the world and as such, is an essential source of energy and nutrition. The challenges that breeders presently face is to increase production to feed the rising population of the world, while also accounting for climate change, pollution, water and environmental stresses. As genetic uniformity of modern cultivars has increased vulnerability to pests and diseases, the wild relatives of wheat offer a rich source of genetic diversity and stress tolerance traits, that can be harnessed and transferred in to modern wheat.

In this study, we used array-based genotyping to explore genetic diversity in 385 domesticated and non-domesticated lines of wheat and their wild relatives. Genetic characterization using the wheat 90K array, and subsequent filtering and validation mapped 9,570 single nucleotide polymorphic markers onto the wheat reference genome. Phylogenetic analyses illustrated four major clades, clearly separating the wild species from the domesticated, and the ancient Triticum turgidum species from modern T. turgidum cultivars.

Using this diverse germplasm, a genome-wide association study (GWAS) was performed for leaf rust, the most widespread rust disease of wheat. Identification of novel sources of resistance is necessary to maintain disease resistance and stay ahead in the plant-pathogen evolutionary arms race. GWAS was conducted using eight statistical models for infection types against six leaf rust isolates and leaf rust severity rated in field trials for 3-4 years at 2-3 locations in Canada. Functional annotation of genes containing significant quantitative trait nucleotides (QTNs) identified 96 disease-related nucleotide associated with leaf rust resistance. A total of 21 QTNs were in haplotype blocks or within flanking markers of at least 16 known leaf rust (Lr) resistance genes. The remaining significant QTNs were considered loci that putatively harbor new Lr resistance genes. Future efforts to validate these loci will help understand their role in disease resistance and promote their utility for marker-assisted selection in pre-breeding.

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Résumé

Le blé est la plante la plus cultivée au monde, et représente une source essentielle d'énergie et d'aliment. Les cultivateurs doivent relever de gros défis en vue de nourrir une population toujours croissante, tels qu'une augmentation de la production tout en tenant compte des changements climatiques, de la pollution, de la sécheresse et des stress environnementaux. Comme l'uniformité génétique dans les cultivars modernes a causé une vulnérabilité contre les pestes et les maladies, ce sont les espèces sauvages apparentées au blé qui offrent une source riche en diversité génétique et en caractères de tolérance aux stress. Cette diversité retrouvée chez les espèces sauvages peut être ciblée et transférée dans le blé moderne.

Dans cette étude, nous utilisons du génotypage basé sur des bio-puces pour explorer la diversité génétique de 385 lignées de blé domestiqué et non-domestiqué ainsi que leurs espèces sauvages apparentées. La caractérisation génétique avec la bio-puce 90K du blé, suivi d'un filtrage et d'une validation, a cartographié 9570 marqueurs de polymorphisme à nucléotide unique (SNP) sur le génome de référence du blé. Des analyses phylogénétiques illustrent quatre clades majeurs qui démontrent clairement la démarcation qui sépare les cultivars modernes des cultivars anciens de T. turgidum, ainsi que les espèces domestiquées des espèces sauvages.

Avec ce matériel génétique aussi diversifié, une étude d'association pan-génomique (GWAS) a été menée pour identifier où se situe les gènes de résistance à la rouille brune, la rouille la plus répandue chez le blé. L'identification de nouvelles sources de résistance à cette maladie est primordiale afin de garder cette résistance et demeurer à la fine-pointe de la guerre entre plante et pathogène. Cette étude d'association pan-génomique a été produite avec l'utilisation de huit modèles statistiques pour les types d'infection contre six isolats de rouille brune et la mesure de sévérité telle qu'évaluée en champ pendant 3-4 années, à 2-3 sites au Canada. L'annotation fonctionnelle des gènes qui contiennent des nucléotides associés aux caractères quantitatifs (QTNs) significatifs a identifié 96 loci associés à la résistance à la rouille brune. En

tout, 21 QTNs se retrouvent à l'intérieur des blocs d'haplotype ou à proximité des marqueurs qui accompagnent 16 gènes connus de résistance à la rouille brune. Les QTNs significatifs qui restent sont considérés comme des loci qui pourraient contenir des nouveaux gènes de résistance contre la rouille. Des études futures envisagent de valider ces loci afin de mieux comprendre leurs rôles dans la résistance à la maladie, et à promouvoir leur utilité pour la sélection assistée par marqueurs dans des programmes d'amélioration génétique du blé.

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Dedications

This thesis is dedicated my 16-year-old self who was too afraid to dream big

You are your only limit

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Acronyms

1 General introduction

1.1 Agriculture of wheat

Wheat is a staple food crop cultivated worldwide for its grain products. Its economic importance and contribution to the diets of humans and livestock cannot be overlooked. It is in fact the second most cultivated food crop in the world, feeding approximately one-third of its population (FAO, 2018). In 2018, the global wheat production was 734 million tonnes, with China, India, Russia and the United States of America collectively providing 41% of the world's total wheat (FAO, 2018). In terms of nutrition, wheat accounts for 20% of the total human calories consumption, and is the highest source of protein than any other cereal crop, contributing to 20-21% of our needs (CGIAR, 2017). Apart from this, wheat is also a rich source of vitamins, dietary fibres and phytochemicals.

In recent years, wheat production has not met global demands, leading to price instability and food insecurity. By 2050, the world's population is predicted to rise to 9 billion and the demand of wheat is expected to increase by 60%; annual wheat yield gains must rise from the current sub-1% to at least 1.6% (Godfray et al., 2010; Tadesse et al., 2016). This, coupled with the challenges imposed by water scarcity, climate change, and constant outbreak of new pathogens, has delayed progress and hampered food security (Asseng et al., 2015; Chaves et al., 2013; Rasheed et al., 2018). Wheat producing countries must increase yield, tolerance to abiotic stresses, pathogens and pests, as well as improve agronomic practises to meet the rising demands of wheat production.

1.2 Evolution of wheat

Common or bread wheat (Triticum aestivum) is a hexaploid species (AABBDD genome, 2n=6x=42 chromosomes) that originated approximately 10,000 years ago as a result of two ancestral polyploidization events (Figure 1.1). Hybridization between Triticum urartu (AA,

2n=2x=14), commonly named red wild einkorn, and an unknown species (BB, 2n=2x=14), closely related to Aegilops speltoides $(SS, 2n=2x=14)$ formed the allotetraploid T. turgidum ssp. dicoccoides (AABB, 2n=4x=28), commonly known as wild emmer (Blake et al., 1999; Dvorak et al., 1993; Dvorak and Zhang, 1990). Wild emmer was cultivated for several hundred years before being domesticated as emmer wheat (T. turgidum ssp. dicoccum), which eventually evolved into durum or pasta wheat (T. turgidum durum) (Dubcovsky and Dvorak, 2007). The second polyploidization event occurred between domesticated emmer wheat and the diploid wild species Ae. tauschii (DD, 2n=2x=14), commonly known as Tausch's goatgrass, to give rise to T. aestivum or bread wheat (McFadden and Sears, 1946). Through the course of wheat evolution, farmers have been selecting favorable traits such as non-brittle rachis and non-hulled glumes by repeatedly sowing and harvesting wheats that displayed these domestication traits which had been acquired through natural mutations; the non-brittle rachis phenotype prevents the shattering of seeds, and non-hulled glumes refers to the free-threshing ability of the grains.

Figure 1.1 Overview of the evolution of wheat.

Timeline shows the estimated time points in number of years ago (YA) of the polyploidization events.

1.3 Wheat species

1.3.1 Cultivated species

Wheat belongs to the genus Triticum and the tribe Triticeae. The genus Triticum comprises several wheat species, of which bread wheat $(T.$ aestivum) and pasta wheat $(T.$ turgidum durum) are most widely cultivated commercially for food. Domesticated wheats are large-seeded, nonshattering and free-threshing.

Common or bread wheat, which accounts for 95% of all consumed wheat, is widely used for the production of baked products. The remaining 5% is T. turgidum durum which is used to make pasta, couscous and semolina flour. Other domesticated, but lesser-grown hexaploid

wheats, include compact (T. aestivum compactum), spelt (T. aestivum spelta), shot (T. aestivum sphaerococcum), makha (T. aestivum macha) and Vavilov's (T. vavilovii) wheats, which, except for the latter, are considered subspecies of bread wheat $(T.$ aestivum). These subspecies differ in their morphology and geographical distribution (Table 1.1). For example, compact wheat, still grown in some parts of South America, in southern Europe and Southwestern Asia, has a more compact ear (or head) compared to common wheat, and spelt wheat, popular in the past and now considered a speciality crop, is hulled as opposed to free-threshing bread wheat. Similarly, aside from durum wheat, other lesser cultivated subspecies of tetraploid T . turgidum include persian (T . turgidum carthlicum), khorasan (T. turgidum turanicum), polish (T. turgidum polonicum) and rivet (T. turgidum turgidum) wheats (Table 1.1).

Species name	Common name	Spike ¹	Geographical distribution ²
Hexaploid (AABBDD)			
T. aestivum ssp. aestivum	Bread wheat	FT, NB	Widely cultivated
T. aestivum ssp. compactum	Compact wheat	FT, NB	S America, S Europe and SW Asia
T. aestivum ssp. sphaerococcum	Shot wheat	FT, NB	Middle East; Asia
T. aestivum ssp. spelta	Spelt wheat	H, PB	Iran, Europe
T. aestivum ssp. macha	Makha wheat	FT, NB	Georgia
T. vavilovii	Vavilov's wheat	H, PB	Armenia
Tetraploid (AABB)			
T. turgidum ssp. carthlicum	Persian wheat	FT, NB	Middle East, Armenia, Georgia
T. turgidum ssp. turanicum	Khorasan wheat	FT, NB	Iran, Iraq
T. turgidum ssp. polonicum	Polish wheat	FT, NB	S. Europe, Middle East, S Asia
T. turgidum ssp. turgidum	Rivet wheat	FT, NB	Middle East
T. turgidum ssp. durum	Durum wheat	FT, NB	Europe, Asia, N America
T. turgidum ssp. dicoccum ᇊᅮ Eroo throphing: U Uullod:	Emmer wheat ND. DD. Nan hrittar	H, B Dortiolly	widely cultivated in ancient times brittle: D. Drittle: C. Coutborn: CINI

Table 1.1 Cultivated wheat species, their spike morphology and geographical distribution

FT, Free-threshing; H, Hulled; NB, Non-brittle; PB, Partially brittle; B, Brittle; S, Southern; SW, Southwestern; N, Northern

¹Spike morphology information obtained from (Dvorak, 2001)

²Geographical distribution obtained from the "Plants For A Future" Database (www.pfaf.org)

The planting and harvesting seasons of wheat depend on the variety of the cultivar. In

Canada, spring wheats are planted in the spring and harvested in late summer or early fall, while

winter wheats are planted in the fall and harvested in the summer. Winter wheats, grown particularly in the northern hemisphere, must undergo a vernalization period when they are exposed to cold temperatures for several weeks in order to trigger flowering or heading, i.e., emergence of the ears. In many areas of the world, facultative varieties requiring shorter vernalization time and minimal cold exposure can be grown in either spring or winter. In countries which experience mild winters, such as those of South Asia and the Middle East, spring wheats, which do not require a vernalization period, can also be planted in the winter and harvested in late spring of the next year.

1.3.2 Wild relatives

The tribe Triticeae can be classified into 20-27 genera, of which the cultivated or edible species belong to the genera Triticum (wheat), Hordeum (Barley), Secale (Rye) and Aegilops (ancient food grains) (Kellogg, 2015; Soreng et al., 2015). The phylogenetic relationships between species within these genera are of interest because they constitute a gene pool of favorable traits for the improvement of modern wheat.

1.3.2.1 Progenitor species

Modern hexaploid wheat (2n= 6x= 42, AABBDD) was derived from three diploid wild species, that also originally diverged from a common ancestor. The A, B and D genome progenitors or their closely related species include the diploid T. urartu (A), T. monococcum (A^m) , Ae. tauschii (D) and Ae. speltoides (S), and the non-domesticated forms of tetraploid wheat T. turgidum ssp. dicoccon and dicoccoides (AB) (Figure 1.1). T. urartu, the A genome donor, was never domesticated and is sparsely distributed across the Fertile Crescent (Brunazzi et al., 2018). However, its sister species, T. monococcum, available in domesticated (einkorn wheat) and wild forms, was a significant source of food thousands of years ago, before it was replaced by polyploid wheat. Ae. speltoides, the closest known relative to the B genome donor of wheat, is one of the five species

of the Sitopsis section of the Aegilops that possess an S-type of genome (van Slageren, 1994). Ae. tauschii the D genome donor, also known as Tausch's goatgrass, is one of the most well studied progenitors, and has been associated with important traits for adaptation and stress tolerance (Kishii, 2019).

Cultivated wheats have long been recognized to be able to naturally hybridize with their wild relatives, depending on species sympatry, concurrent flowering, and sexual compatibility (van Slageren, 1994). Wild species carrying the A, B and D genomes, along with domesticated polyploid wheats, are regarded as the primary sources of genes for improvement of modern wheat. Interspecific crossing of modern wheat with species from the primary gene pool occur as a consequence of direct hybridization, and, in this case, the progeny is not hindered by linkage drag (the undesirable effects of genes linked to the introgressed region) because crossing over is uninhibited (Mujeeb-Kazi and Rajaram, 2002).

1.3.2.2 Non-progenitor species

The non-progenitor wild relatives encompass all species of the tribe Triticeae that were not directly involved in the early evolution and domestication of wheat. However, the most studied relationships are those that belong to the Aegilops and Triticum genera (Figure 1.2).

The genus Aegilops comprises 23 species, of which 11 are diploids and 12 are allopolyploids. It can be divided into five sections (van Slageren, 1994):

(1) Aegilops: diploid and allopolyploid species with the U genomes e.g. Ae. umbellulata

(2) **Cylindropyrum**: diploid and allopolyploid species with the C and DC genomes e.g. Ae. markgrafii

(3) Vertebrata: diploid and allopolyploid species with the D genomes e.g. Ae. tauschii, Ae. crassa

(4) Comopyrum: diploid species with the M and N genomes e.g. Ae. comosa, Ae. uniaristata

(5) Sitopsis: diploid species with the S genomes e.g. Ae. bicornis, Ae. longissima

Figure 1.2 Spike morphology of some of the Aegilops species. Photographs were taken by the Cloutier lab of the Ottawa Research and Development Centre.

Triticum species can be distinguished from Aegilops species by the presence of a welldeveloped keel on the glumes, creating a sharp outline around the glumes (Kilian et al., 2011). Apart from the previously mentioned T. monococcum, T. urartu, T. turgidum, T. aestivum and T. vavilovii, the genus Triticum also includes T. timopheevii and T. zhukovskyi. Hexaploid T. zhukovskyi originated through a natural interspecific hybridization between tetraploid T. timopheevii and diploid T. monococcum (Dvorak, 2001).

Natural hybridization between wheat and its wild relatives has been observed. Most hybrids were sterile, but some seeds were observed (Zaharieva and Monneveux, 2006). Artificial interspecific crossing between wheat and its wild relatives can be deployed to transfer useful traits into cultivated wheats. Species that share at least one genome with the A, B or D genomes of wheat e.g. Ae. cylindrica (DC) and T. zhukovskyi (GAA^m) belong to the secondary gene pool of wheat, while those which do not share any genome with wheat belong to the tertiary gene pool.

Introgression via the secondary and tertiary gene pools require special breeding protocols and manipulation strategies to recover fertile and viable seeds. In addition, the progeny often carry undesirable traits that can be difficult to breed-out as a consequence of linkage drag (Mujeeb-Kazi and Rajaram, 2002). Extensive crossing and backcrossing to elite varieties are often required prior to incorporation into breeding program.

1.4 The wheat genome

Bread wheat has a large and highly repetitive genome. Each of the A, B and D sub-genomes have seven pairs of homologous chromosomes, for a total of 42 chromosomes. The wheat genome also consists of homoeologous chromosomes, where each set of homoeologous chromosomes is made up of chromosomes from each sub-genome (Figure 1.3). Homoeologous chromosomes are considered duplicated chromosomes that are derived from different parental species, brought together in the same genome by allopolyploidization (Glover et al., 2016). Homoeologous chromosomes have largely similar gene content, but differ in their repetitive DNA which is mostly transposable elements. Therefore, as most genes have homoeo-alleles, there is functional redundancy. Chromosome pairing, however, is restricted to homologues during meiosis and the genome behaves as a diploid (Riley and Chapman, 1958). This can be altered by mutating the Ph1 locus, that controls strict homologous chromosome pairing, to allow the creation of hybrids form wide crosses (Sears, 1976). As wheat is a self-pollinating (autogamous) plant, genotypes are largely homozygous, hence the homologs tend to have the same alleles at most loci (Dixon et al., 2018).

The International Wheat Genome Sequencing Consortium (IWGSC) recently released a fully annotated reference genome for bread wheat variety Chinese Spring, consisting of an assembly of 14.5Gb comprising 107,891 predicted high-confidence protein coding genes (IWGSC, 2018). The hexaploid genome comprises an estimated 17 billion nucleotides, i.e., five times the size of the human genome, of which more than 85% is repetitive (IWGSC, 2018). The

repetitive DNA is largely made up of major classes of transposable elements (TEs) i.e. 3,968,974 copies of TEs belonging to 505 families (IWGSC, 2018). The B sub-genome has the greatest assembly size (5.2Gb), followed by the A (4.9Gb) and the D (3.9Gb), however the distribution of high-confidence genes was relatively equal across all sub-genomes (35,345, 35,643 and 34,212, respectively) (IWGSC, 2018). Tetraploid and diploid wheat genome sizes are approximately 2/3 and 1/3 that of bread wheat. Until now, the genomes of durum wheat, (Maccaferri et al., 2019), wild emmer wheat (Avni et al., 2017), Ae. tauschii (Luo et al., 2017) and T. urartu (Ling et al., 2013) have also been sequenced. Overall, access to this sequence level information allows plant breeders to implement new strategies to improve wheat through genomics-assisted breeding.

Figure 1.3 Structural organization of the hexaploid bread wheat chromosomes. The size of the chromosomes are drawn to scale, based on the physical length (in base pairs) of each chromosome (IWGSC, 2018). The figure is adapted with permission from Haldar (2019).

1.4.1 Genetic diversity bottlenecks

The wheat genome has been subjected to a series of genetic diversity bottlenecks caused by polyploid speciation, domestication and, natural and artificial selections. The bottleneck of speciation is a founder effect where the genomes of a small number of individuals contributed to the formation of the newly formed polyploid species (Dubcovsky and Dvorak, 2007). For example,

as only a few Ae. tauschii participated in the origin of T . aestivum, the genetic diversity of the D sub-genome in modern wheat is only 15% of the Ae. tauschii gene pool diversity (Dvorak et al., 1998). Similarly, nucleotide diversity in the A and B genomes of T. aestivum was 31% that of wild emmer wheat (Haudry et al., 2007). In addition to natural selection caused by the diverse environments where these species grew, and long before genomic tools were available, farmers have been selecting for favorable traits by repeatedly sowing and harvesting wheats with desirable phenotypes. Modern day breeders now employ advanced husbandry techniques, coupled with genomic resources and biotechnological tools to artificially enhance important agronomic traits such as grain yield and quality, and resistance to biotic (disease) and abiotic stresses. These events of domestication and, environmental and artificial selections have further reduced genetic diversity, a fact that has in some cases and is forecasted to lead to improvement plateaus (Tanksley and McCouch, 1997).

1.4.2 New sources of genetic diversity

Modern wheat breeders have realized the importance of expanding diversity for successful crop improvement and are starting to use synthetic wheats, landraces and wild relatives in their breeding program. Synthetic hexaploid wheats (SHWs) are derived from recreating crosses between Ae. tauschii and T. turgidum, which allow breeders to explore novel genes in the tetraploid and diploid progenitors of wheat. They offer low reproduction barrier, producing fertile hybrids that may potentially carry alleles that are not present in modern wheat (Cox, 1997). Similarly, wheat landraces, evolved locally through a mixture of natural and artificial selections, are a source of putatively lost genetic diversity that can provide new genes or alleles for wheat improvement (Lopes et al., 2015).

 Wild relatives of wheat, although lack adaptation traits for agriculture, have retained greater genetic diversity for many traits, such as abiotic and biotic stress tolerance, as they were

not subjected to domestication and artificial selection by breeders (Ceoloni et al., 2017). These relatives have evolved through separate paths allowing them to maintain certain genes or alleles that may no longer be present in cultivated bread or pasta wheats (Marcussen et al., 2014). For example, genes responsible for leaf rust resistance were identified in Ae. tauschii (Huang et al., 2003) and stem rust resistance in Ae. sharonensis (Nevo et al., 1986). Apart from these, numerous other genes from the wild gene pool have been identified for resistance to stripe rust (Helguera et al., 2003), powdery mildew (Weidner et al., 2012), Fusarium head blight (FHB) (Fedak et al., 2007), leaf rust (McCallum et al., 2012) and stem rust (Olivera et al., 2018). Resistance against pests such as greenbug and hessian fly (Gill and Raupp, 1987) and abiotic stresses such as freezing (Iriki et al., 2001), salinity (Colmer et al., 2006) and drought (Nevo and Chen, 2010) were also identified from wild relatives.

The traditional approach for introgressing the desired gene of interest into adapted germplasm is by interspecific crossing the wild relative with a high-crossability line. This is followed by crossing to a $ph1b$ mutant line to break linkage drag and subsequent crossing and backcrossing to a recurrent parent to recover a desirable phenotype (Sears, 1976). However, this methodology requires special breeding protocols and is often impeded by sterility in the progeny and/or linkage drag resulting from the low recombination surrounding the introgressed alien fragment(s). Modern genome-assisted breeding strategies, such as gene cassettes and genome editing, have recently been proposed to overcome some of the disadvantages of the crossing method, facilitating the transfer of desirable genes/alleles from any germplasm in the secondary and tertiary gene pool (Keller et al., 2016; Wulff and Dhugga, 2018). Hence, breeders are showing an ever growing interest in crop wild relatives as a resource to harness beneficial traits. This can be achieved utilizing molecular markers such as single nucleotide polymorphism (SNP) genotyping and genome-wide association studies (GWAS).

1.5 SNP genotyping

SNPs are the most common type of genetic variation in plants and animals. A SNP is a substitution of a single DNA building block, called a nucleotide that occurs at specific positions in the genome. SNPs present within a gene or nearby a gene regulatory region may alter the gene's function and cause an altered phenotypic trait. They serve as useful molecular markers for investigating trait associations, drug response and creating genetic maps.

Molecular markers have gained increasing attention over the past couple of decades, due to their applications in crop genetics. Genotyping uses molecular markers such as SNP, simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP), to determine sequence variations at specific positions within a genome. SNP genotyping specifically identifies a set of SNPs or alternative alleles in an individual, allowing comparisons across individuals. It can be performed using three general allele discrimination methods: hybridization, primer extension and enzyme cleavage. SNP genotyping offers advantages over SSR and RFLP genotyping because SNPs are abundant, well-distributed across the genomes, cost-effective and offered in a range of sophisticated yet speedy genotyping platforms (Thomson, 2014).

1.5.1 SNP genotyping arrays

High-density SNP arrays, an application of the hybridization-based SNP genotyping method, have become increasingly popular. These SNP arrays follow the same principle as DNA microarrays, i.e., hybridization of single-stranded DNA fragments to thousands of allele-specific nucleotide probes. The ability of such arrays to interrogate variations between whole genomes simultaneously makes them an excellent tool for trait-association studies, gene discovery and linkage analysis. Over the past decade, several fixed SNP genotyping arrays have been developed for numerous crops, ranging from a 1,000 (1K) SNP array for pear to the high-density rice array with a million SNPs (Rasheed et al., 2017). Higher density arrays are accompanied with

higher genotyping costs per sample, whereas low-density array suffer from ascertainment bias and failure to capture rare alleles (Albrechtsen et al., 2010).

In wheat, the Illumina's Infinium and Affimetrix's Axiom array technologies enable the simultaneous genotyping of 9,000 to 819,571 SNP markers from seven custom SNP arrays (Rasheed et al., 2017). Illumina's Wheat 9K and 15K SNP arrays are a subset of their Wheat 90K SNP array, and Affimetrix's Wheat 35K SNP array and Wheat 55K SNP array are a subset of the Wheat 820K and Wheat 660K SNP arrays, respectively (Sun et al., 2020). The Infinium and Axiom arrays are based on two different technologies and each of their respective arrays are developed from distinctive sets of germplasm, catering to research objective and marker-assisted breeding programs. For example, the Wheat Breeders' 35K Axiom array provides breeder-oriented informative markers in bread wheat (Allen et al., 2017). On the other hand the 90K and 660K SNP arrays include markers from hexaploid and tetraploid wheat, emmer wheat and Aegilops tauschii, with the addition of some landraces, SHWs and wild relatives in the 820K SNP array, thereby offering a greater genetic diversity for different types of studies (Sun et al., 2020; Wang et al., 2014; Winfield et al., 2016).

1.6 Genome-wide association studies

Genome-wide association studies is a methodology which surveys hundreds and thousands of genetic variants across the genomes of many individuals to identify valuable marker-trait associations (MTAs). The study is performed using statistical models that requires at least two sets of data as input: genome-wide markers to account for genetic variation in the population and phenotypic scores of the population for the trait of interest (Figure 1.4). GWAS have typically been performed using genome-wide SNP markers, but association can also be identified using insertion/deletion and structural variants (Misra et al., 2017). For crops, the phenotypic trait can be any measurable trait, ranging from agronomic traits to biotic and abiotic stress tolerances.

Figure 1.4 Workflow of genome-wide association studies and potential breeding applications. PCR; polymerase chain reaction, GBS, genotyping-by-sequencing; QTL, qualitative trait loci. Figure created with BioRender.com.

1.6.1 Basic principles of association analysis

Genome-wide association analysis has shown to be advantageous over linkage mapping experiments that also identify functional genetic variants (loci, alleles) linked to a trait of interest. They are both based on the ability of recombination to break up the genome into fragments that can be correlated with phenotypic trait of interest. The main difference, however, is the control over recombination (Myles et al., 2009). In linkage mapping individuals are crossed to create a mapping population. Here, relatedness is known and recombination breakpoints are few as there has not been enough time to shuffle the genome into small fragments. Phenotypic diversity may also be limited to that of the parental population. On the other hand, in GWAS, genotypic and phenotypic data is often collected from a diverse population, thereby taking advantage of all evolutionary historical recombination events that have occurred (Myles et al., 2009). The number of quantitative trait loci (QTL) or MTAs that can be mapped for a given trait are not limited to what

segregates between the parents of the cross, but rather by the number of real QTL underlying the trait (based on how well the mapping population captures genetic diversity available in nature) (Zhu et al., 2008). In addition association genetics is especially useful with organisms that are difficult to cross and develop mapping populations of, for example, when working with species from the secondary and tertiary gene pool of wheat.

Linkage disequilibrium, the non-random association between two markers or loci, also plays an important role in association genetics (Slatkin, 2008). Genotyped genetic markers that are in strong linkage disequilibrium (LD) with the functional variant(s) can be identified. This is useful when not all functional variant(s) were among the genotyped markers. Markers in LD can become proxies for the functional variant as their genotypes are highly correlated with the genotypes of the functional variant (Myles et al., 2009). Similarly, markers in high LD can also serve as proxies for each other; this is used to determine and optimize the number of nonredundant genetic markers required for association analysis. Linkage disequilibrium varies along the genome and is affected by population structure and size, recombination rates, mutation and inbreeding, among others (Flint-Garcia et al., 2003; Soto-Cerda and Cloutier, 2011). Similarly, for GWAS, the choice of germplasm, genotypic and phenotypic data quality, the use of appropriate statistical models and control for population structure are key for identifying reliable MTAs.

1.6.2 Statistical models

Over the past two decades, due to increased availability of cost-effective next-generation sequencing (NGS) and genotyping technologies, such as SNP arrays, MTAs identified in humans and plants through GWAS exponentially increased (Claussnitzer et al., 2020; Rasheed et al., 2017). GWAS have been historically based on single-locus models that performed onedimensional genome scans, measuring the effect of one SNP marker at a time. Examples of such models include the general (GLM) (Price et al., 2006) and mixed (MLM) linear models (Yu et al., 2006). These models, however, have inherent limitations; they fail to capture complex traits which

are controlled by multiple loci, particularly those of small effects (Segura et al., 2012). Another problem with single-locus models is the multiple test corrections required for the critical value of a significance test. Here, Bonferroni correction is usually applied, but this type of correction is known to be too conservative; many important small effect loci may not pass the stringent correction (Segura et al., 2012; Zhang et al., 2018c).

To overcome these drawbacks, multi-locus GWAS methods such as restricted two-stage multi-locus GWAS (RTM-GWAS) (He et al., 2017) and multi-locus random-SNP-effect mixed linear model (mrMLM) (Wang et al., 2016) have been developed. Here, QTL or MTAs, are identified by multi-dimensional genome scans, measuring the effects of all SNPs simultaneously. These models follow a two-step algorithm: in the first step, a single-locus GWAS is applied, such as MLM, but a less stringent correction is used to select potential markers. In the second step, all potential markers associated with the trait are evaluated by a distinct multi-locus model. For example, in the first stage of mrMLM, the SNP-effect is treated as random and single-locus MLM GWAS is performed, makers selected from this step are simultaneously evaluated in a second model using an empirical Bayes approach (Wang et al., 2016). In the case of RTM, the model first groups SNPs into linkage disequilibrium blocks (SNPLDBs) and then uses a two-stage analysis for QTL identification, where markers are preselected by a single-locus model followed by multilocus multi-allele model stepwise regression (He et al., 2017). These models do not require Bonferroni correction, have a higher power for QTN detection and were found superior to singlelocus models in identifying small-effect loci for complex associations (Cui et al., 2018; Zhang et al., 2018b).

Candidate genes identified and validated from GWAS can be transferred into adapted germplasm through conventional backcross breeding using gene-specific markers or by modern approaches such as gene pyramiding (gene cassettes) and gene editing (Ahmad et al., 2020; Wulff and Moscou, 2014; Zhang et al., 2016) (Figure 1.4).

1.7 Diseases of wheat

Reduced genetic diversity, climate change, and decreased availability of suitable farmland has led to an unprecedented breakout of pathogens that continually threaten local and global wheat production. Biotic factors affecting wheat production include pathogens (fungi, bacteria, viruses), pests (nematodes, insects, mites) and weeds. Of the pathogens, fungi cause the greatest global losses in wheat, whereas bacterial and viral diseases are usually less problematic (Juroszek and von Tiedemann, 2013; Oerke, 2006). Diseases affecting the seed include FHB, common bunt, loose smut and Stagonospora nodorum blotch; these directly affect safety of the grain as well as its quality, size, yield, color and odor (Duveiller et al., 2012). Similarly, diseases affecting the leaves include rust (leaf, stem and stripe), powdery mildew, Septoria blotch and tan spot and, those affecting stems and roots include stem rust, common root rot and crown and foot rots (Wolf et al., 2011). These diseases can be further classified based on their symptoms, geographical distribution, economic importance and development conditions (humidity, temperature, etc.).

Disease management strategies include cultural, chemical and genetic control practices. Examples of cultural control include crop rotation, tillage and removal or eradication of alternative hosts, while chemical control involves usage of fungicides and pesticides. While chemical control may be costly and raise environmental and health concerns, genetic control to disease is considered more effective, sustainable and environmentally-friendly. The latter involves transferring disease resistance genes into wheat cultivars through conventional and modern breeding strategies as discussed above.

Systematic efforts have led to the identification of several hundreds of disease resistance genes in wheat, however, only a few of them have been isolated and cloned (McIntosh et al., 2017). To date, 32 disease resistance genes have been cloned in wheat, 24 of which are for rust diseases, while the remaining are for powdery mildew, Septoria and Stagonospora nodorum blotch, tan spot and FHB (Keller et al., 2016; Zhang et al., 2020). Cloning of a resistance gene

reveals its nucleotide sequence, exact chromosomal position and molecular function and enables its transfer for breeding through transgenesis, genome editing, or gene-specific marker-assisted crossing and selection.

1.7.1 Disease response mechanisms

Plant disease resistance is driven by complex mechanisms involving several layers of defense relying on pathogen detection, signal transduction and defence response. Detection of pathogen and damage associated molecular patterns (PAMPs and DAMPs) at the cell membrane leads to pattern-triggered immune (PTI) response and detection of pathogen effectors by intracellular receptors with nucleotide-binding domains and leucine-rich repeats (NLR) leads to effectortriggered immune response (ETI) (Figure 1.5) (Andersen et al., 2018). PRRs, WAKs, and NLRs activate a number of signalling cascades including mitogen-activated protein kinase cascades, G-proteins calcium ion signaling, hormone production and transcription factor activity that regulate expression of gene associated with defense response. PTI is often, but not always, achieved without the death of the affected plant cells, while ETI is often associated with programmed cell death of the affected cell, also known as hypersensitive response (HR) (Kanyuka and Rudd, 2019). Other defense responses include cell wall modifications, closure of stomata, or the production of anti-pathogen proteins, inhibits pathogen reproduction and further infection (Andersen et al., 2018).

 The majority of plant disease resistance genes encode immune receptors that detect pathogen effectors. In the case of wheat, 23 (72%) of the 32 cloned wheat resistance genes encode NLR receptor proteins that either directly bind to pathogen effectors or indirectly recognize modifications triggered by the effector proteins, activating multiple defense signalling cascades (DeYoung and Innes, 2006; Keller et al., 2016; Zhang et al., 2020). Proteins encoded by other

wheat disease resistance genes include WAKs, protein kinases, transporter proteins and poreforming toxin-like protein (Keller et al., 2016).

Figure 1.5 A simplified overview of the pathogen response mechanism in plants.

PRRs: pattern recognition receptors, WAKs: wall-associated kinases, PAMPS: pathogenassociated molecular patterns, DAMPs: damage-associated molecular patterns, NLR: nucleotidebinding domains with leucine-rich repeats, PTI: pattern-triggered Immunity, ETI: effector-triggered Immunity. Figure created with BioRender.com.

1.8 The scope and purpose of this study

In recent years, a number of GWAS have identified QTL associated with agronomic traits and

disease resistance using elite cultivars and landraces of bread and durum wheats. However,

these studies were based on traditional single-locus GWAS models whose inherent limitations

are now widely recognized. As multi-locus association methodologies are recent, few have been

reported in wheat and, the potential for multi-locus GWAS covering a diverse range of cultivated

and wild wheat remains largely untested.

This study aims to use SNP array genotyping to explore the phylogenetic relationships within a diverse panel of various Triticum and Aegilops species and to investigate the genetic basis of disease resistance using SNP array genotyping and multi-locus GWAS. The outcomes of this research will guide decisions for the transfer of useful genetic traits for wheat improvement, a field of research referred to as pre-breeding.

1.8.1 Hypotheses

Genomic regions associated with quantitative phenotypic traits can be identified in a diverse panel of wheat, progenitors and wild relatives using SNP data generated by the wheat 90K array.

SNP data generated by the 90K array can be used to infer phylogenetic relationships between of diverse panel of wheat, progenitors and wild relatives.

1.8.2 Specific objectives

The specific objectives of this study include:

- 1. To genotype a diverse panel of cultivated wheat, progenitors and wild relatives;
- 2. To obtain a reliable SNP dataset by appropriate SNP filtering and validation;
- 3. To enhance our understanding of the evolutionary relationships between the Triticum and Aegilops species represented in the collection;
- 4. To perform association analysis for disease resistance traits using multiple GWAS models;
- 5. To annotate QTL identified for gene function using the wheat reference genome annotation;
- 6. To cross-check QTL identified against known genes previously reported in the literature;
- 7. To identify and map novel QTL in the germplasm;

The challenges and limitations of this study include ascertainment bias, genotyping complexities due to polyploidy, and lack of scientific literature available for some of the wild relatives included in the germplasm collection. Apart from Ae. tauschii, the majority of the accessions included in the development of the high-density 90K array were cultivars of bread and durum wheats (Wang et al., 2014). Ascertainment bias may exist when calling genotypes of the various wild relatives and progenitor species, resulting in failure to capture rare alleles and incorrect genotype calling. Genotyping is also complicated by the inherent complexities associated with the polyploid nature of wheat. Depending on the sequence similarities between probes and targets, a SNP probe may hybridize to the homoeologous and/or paralogous loci of the target, thereby affecting the allele-specific fluorescent signal ratios, and further complicating genotype clustering (Rasheed et al., 2017; Wang et al., 2014). Moreover, the majority of the GWAS studies reported in wheat are for cultivated varieties and their closely related progenitor species, and only a few such association and diversity analysis studies exist for its wild relatives. As 19 different species of wild wheat relatives are included in this study, research ambiguities and literature gaps are anticipated to constitute additional challenges for efficient and accurate germplasm data analysis.

2 Identification of new leaf rust resistance loci in wheat and wild relatives by array-based SNP genotyping and association genetics

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Authors' contributions:

FF performed the data analysis and wrote the manuscript; FY provided bioinformatics and statistics guidance; BDM, SC and CP performed leaf rust severity phenotyping; BDM performed race-specific phenotyping; CM and CH produced the wheat 90K array data; GF co-developed the original experiment and provided some of the key germplasm; SC designed the experiment and co-wrote the manuscript.

2.1 Abstract

Leaf rust caused by Puccinia triticina is the most widespread rust disease of wheat. As pathogen populations are constantly evolving, identification of novel sources of resistance is necessary to maintain disease resistance and stay ahead of this plant-pathogen evolutionary arms race. The wild genepool of wheat is a rich source of genetic diversity, accounting for 44% of the Lr genes identified. Here we performed a genome-wide association study (GWAS) on a diverse germplasm of 385 accessions, including 27 different Triticum and Aegilops species. Genetic characterization using the wheat 90K array and subsequent filtering identified a set of 20,501 single nucleotide polymorphic (SNP) markers. Of those, 9,570 were validated using exome capture and mapped onto the Chinese Spring reference sequence v1.0. Phylogenetic analyses defined four major clades, clearly separating the wild species from the T. aestivum and T. turgidum species. GWAS was conducted using eight statistical models for infection types against six leaf rust isolates and leaf rust severity rated in field trials for 3-4 years at 2-3 locations in Canada. Functional annotation of genes containing significant quantitative trait nucleotides (QTNs) identified 96 disease-related loci associated with leaf rust resistance. A total of 21 QTNs were in haplotype blocks or within flanking markers of at least 16 known Lr genes. The remaining significant QTNs were considered loci that putatively harbor new Lr resistance genes. Isolation of these candidate genes will contribute to elucidate their role in leaf rust resistance and promote their usefulness in markerassisted selection and introgression.

Keywords: wheat, leaf rust, GWAS, QTN, genotyping array, single nucleotide polymorphism
2.2 Introduction

Triticum aestivum, commonly known as bread wheat, is an allohexaploid (AABBDD) species, created through the sequential hybridization of three grass species: T. urartu (AA), a species (BB) closely related to Aegilops speltoides (SS) and Aegilops tauschii (DD) (McFadden and Sears, 1946). Genetic diversity bottlenecks such as polyploidization, domestication, and natural and artificial selections have reduced diversity in modern wheat, and consequently increased its vulnerability to diseases, pests and environmental stresses (Tanksley and McCouch, 1997).

Leaf rust caused by Puccinia triticina is the most prevalent wheat rust disease, causing tremendous annual yield losses. Puccinia triticina attacks the foliage, covering its surface and thus causing loss of photosynthates, dehydration and early defoliation. Genetic resistance combatting yield losses can be categorized into seedling resistance and adult plant resistance (APR). Typically, seedling resistance is controlled by single major effect genes that confer hypersensitive and other responses, causing necrosis and preventing the pathogen from spreading (Dyck and Kerber, 1985). APR occurs at a post-seedling stage and confers either a race-specific or a quantitative race non-specific response (Dyck and Kerber, 1985; Samborski, 1985).

To date, 66 leaf rust resistance (Lr) genes have been characterized, six of which, namely Lr1 (Cloutier et al., 2007), Lr10 (Feuillet et al., 2003), Lr21 (Huang et al., 2003), Lr22a (Thind et al., 2017), Lr34 (Krattinger et al., 2009) and Lr67 (Moore et al., 2015), have been isolated. The majority of the Lr genes described to date confer seedling-type resistance. Well-known APR genes include the race-specific Lr12 (Dyck et al., 1966) and Lr13 (Dyck et al., 1966) and the race non-specific Lr34 (Dyck, 1977) and Lr67 (Hiebert et al., 2010). Of the 66 Lr genes designated to date, 37 were identified in T . aestivum and T . turgidum and 29 originated from progenitors and other wild relative species such as Ae. tauschii, Ae. speltoides, Ae. neglecta and Ae. peregrina, among others (McCallum et al., 2012; USDA, 2017).

The traditional approach for introgressing Lr genes into adapted germplasm is by interspecific crossing a donor line to an adapted line followed by backcrossing. Although many Lr genes have been described, few are utilized by present-day breeders because they have either been overcome by virulence changes in the pathogen populations, are not in an adapted background or suffer from linkage drag. Modern approaches, such as gene cassettes and genome editing may overcome some of the disadvantages of the crossing method and have been proposed to provide long-lasting broad spectrum resistance (Arora et al., 2019; Keller et al., 2016). However, commercialization of transgenic wheat has not received broad acceptance and introgression via crossing remains commonly used.

Identification of novel sources of resistance in the cultivated and the wild genepools of wheat is expected to contribute to broadening and maintaining the genetic base of leaf rust resistance. Array-based SNP genotyping platforms provide fast and cost-effective access to genetic variation in a diverse germplasm. In wheat, the Illumina's Infinium iSelect and Affimetrix's Axiom array technologies enable simultaneous genotyping of 9,000 to 819,571 SNP markers (Cavanagh et al., 2013; Winfield et al., 2016). Genome-wide association studies (GWAS) associate such genotypic data to phenotypic data to identify significant marker-trait associations. To date, numerous quantitative trait loci (QTL) associated with leaf rust resistance in elite cultivars and landraces of bread and durum wheats have been discovered (Aoun et al., 2016; Gao et al., 2016; Riaz et al., 2018). These QTL were identified based on traditional single-locus GWAS models whose inherent limitations, such as failure to capture complex traits controlled by multiple loci, are now widely recognized (Segura et al., 2012). Multi-locus GWAS models overcome these drawbacks by performing a multi-dimensional genome scan, and measuring the effects of multiple SNPs simultaneously to identify small-effect loci for complex traits (Wen et al., 2018). As multilocus association methodologies are recent, few have been reported in wheat and the potential

for multi-locus GWAS covering a diverse range of cultivated wheat and wild relatives remains largely untested.

Here, we performed a GWAS for leaf rust severity and reaction types against six P. triticina isolates in a highly diverse germplasm of 385 accessions. The wheat 90K array was used to capture the genetic variation in cultivated wheats, progenitor species, synthetic hexaploid wheats (SHWs) and wild relatives (Wang et al., 2014). We used one single-locus and seven multi-locus models to identify quantitative trait nucleotides (QTNs) which were mapped on the reference genome, thus validating known loci and identifying new loci to be mined for novel candidate leaf rust resistance genes.

2.3 Materials and methods

2.3.1 Plant materials

A diverse collection of 385 accessions, encompassing 27 different species of cultivated wheats, SHWs, progenitor species and wild relatives were used in this study (Appendix 1A). The geographical origin of these accessions traverse 51 different countries (Appendix 1B). The AB and ABD genomes are represented by 170 accessions representing T. vavilovii and several subspecies of T. turgidum and T. aestivum as well as 65 primary SHWs. The A, B and D genome progenitors (or their closely related species) and the non-domesticated forms of tetraploid wheat comprised 93 accessions of T. urartu (A), T. monococcum (A^m) , Ae. tauschii (D), Ae. speltoides (S), as well as T. turgidum ssp. dicoccon and dicoccoides (AB). Another 47 accessions belonged to the following Aegilops species: Ae. bicornis (S^b), Ae. longissima (S^I), Ae. searsii (S^s), Ae. sharonensis (S^{sh}), Ae. markgrafii (C), Ae. comosa (M), Ae. umbellulata (U), Ae. geniculate (MU), Ae. peregrina (SU), Ae. triuncialis (UC/CU), Ae. columnaris (UM), Ae. cylindrica (DC), Ae. crassa (DM/DDM), Ae. juvenalis (DMU), Ae. biuncialis and Ae. neglecta (UM/UMN). The collection also contained six accessions of T. timopheevii (A^tG), five of T. zhukovskyi (GAA^m), and one of

Haynaldia villosa (V), a related grass species. Overall, the germplasm consisted of 75 diploid, 136 tetraploid, 165 hexaploid and nine accessions that could be either tetraploid or hexaploid. The species names and genome symbols are according to (Kimber and Tsunewaki, 1988).

2.3.2 Seed increase

Seeds were planted and grown under controlled conditions at the Ottawa Research and Development Centre (RDC), Agriculture and Agri-Food Canada (AAFC) (Ottawa, Canada). Depending on their growth habit, the seeds were divided into a spring and a winter panel of 213 and 164 accessions, respectively . For the spring panel, the growth conditions were 20°C/16 hours light, and 16°C/8 hours dark. The winter panel was grown under the same conditions for approximately three weeks, i.e., the 4-5 leaf stage, at which time they were transferred to a vernalization cabinet (constant 2°C/12 hours photoperiod) for ten weeks to trigger meristem differentiation prior to being returned to the original growing conditions. Seeds harvested from all accessions were used for the greenhouse and field experiments described below.

2.3.3 Leaf rust race-specific response

Consecutive inoculations with six P. triticina isolates were performed for 360 of the 385 accessions of the panels (Appendix 2). The removed 25 accession consisted of those with >90% missing data or accessions removed from the collection due to ambiguities in their species classification. All tests were performed under controlled greenhouse conditions at the Morden RDC, AAFC (Morden, Canada). Briefly, test lines and the Thatcher and Emerson check lines were sown into fibre trays at a rate of approximately 5 seeds per clump and 3cm between clumps, which were inoculated with individual P . triticina isolates at the two-leaf stage (McCallum and Seto-Goh, 2006). The isolates tested were 12-3 MBDS, 128-1 MBRJ, 74-2 MGBJ, 11-180-1 TDBG, 06-1-1 TDBG, and 77-2 TJBJ, which represent the prevalent leaf rust race groups across Canada (McCallum et al., 2016). For simplicity, these will be referred to as MBDS, MBRJ, MGBJ,

TDBG1, TDBG2, and TJBJ, respectively. Infection type (IT) was rated 12 days post-inoculation using a 0–4 scale (Stakman et al., 1962), where ";" = hypersensitive flecks, "0" = no uredinia (reddish pustule like structure of hyphae and spores) or macroscopic sign of infection, "1" = small uredinia with necrosis, " 2 " = small to medium uredinia with chlorosis, " 3 " = medium uredinia without chlorosis or necrosis, "4" = large uredinia without chlorosis or necrosis. IT ";" and "0" to "2" were considered resistant, while "3" and "4" were considered susceptible (Long and Kolmer, 1989). The "+" or "-" IT qualifiers indicate larger or smaller than average uredinia, respectively. The "=" IT qualifier represents the lower size limit of the uredinia for the IT (Long and Kolmer, 1989). Plants with randomly distributed uredinia of variable sizes, or mesothetic (intermediate) response, were considered resistant and were rated with an "X" IT (Roelfs and Martens, 1988).

For downstream analysis, the IT scores were converted into a 1-9 linear scale, where $"0/0;1;" = 1; "1=1;1-" = 2; "1-1/1/1+" = 3; "12/1-2-" = 4; "2-1/22+" = 5; "X" = 6; "3-1/3/3+" = 7; "3+4/34"$ $= 8$, and "4" = 9 (Appendix 2). Scores 1-6 correspond to resistant reaction types while 7-9 are characteristic of susceptible reaction types, i.e., with medium to large uredinia.

2.3.4 Field leaf rust severity

Phenotyping of rust severity was performed in separate field trials for the spring and winter panels. The spring panel included 213 accessions, of which 20 were SHWs, while the remaining were subspecies of T. aestivum and T. turgidum. Trials for the spring panel were carried out in Morden, Manitoba, Canada (2016-2019), Ottawa, Ontario, Canada (2017-2019) and Saskatoon, Saskatchewan, Canada (2019). The winter panel comprised 164 diverse Aegilops and Triticum species, including 115 progenitors and wild relatives, 45 SHWs, and four winter wheat cultivars. Screening for the winter panel was performed in Morden (2017-2019) and in Ottawa (2017 & 2019).

For each panel, year and location, a completely randomized design with two replicates was used, except for the 2016 Morden field trial where a single replicate was used due to the limited seed availability in the first year. For the spring panel, 65 seeds/accession were planted in 1m-long rows with 20cm between rows. A mixture of P. triticina isolates was inoculated onto spreader rows of susceptible lines Thatcher, Morocco, and Little Club, planted every six test rows in Morden and every ten in Saskatoon. The mixture of isolates comprised more than 50 different virulence phenotypes representing the P. triticina population in western Canada identified during the annual virulence survey. In Ottawa, infection relied on natural inoculum but used the same interspersed spreader-row design as Morden with the Morocco spreader. Cultivars Thatcher, Roblin and Eurostar were used as checks and five plots of each were randomly distributed across each replicate.

For the winter panel, ten seeds/accession were planted indoors in early March at both Morden and Ottawa RDCs. At the 3-5 leaf stage, the plants were transferred into vernalization chambers as described above. Approximately ten days after planting the spreader-rows, the vernalized plantlets were transplanted as hills in the field. The cultivar Emerson served as a check. At peak infection and prior to senescence, the flag leaves were rated for leaf rust severity using a modified Cobb's scale (Peterson et al., 1948).

Leaf rust severity ratings across locations and years were modelled using the R package Lme4 (Bates et al., 2014) with the following mixed linear model (MLM) equation:

$$
Y_{ijk} = \mu + G_i + L_j + Y_k + GL_{ij} + GLY_{ijk} + e_{ijk}
$$

where Y_{ijk} is the leaf rust severity measured, μ is the overall mean, G_i is the effect resulting from the i^{th} genotype, L_j is the effect resulting from the j^{th} location, Y_k is the effect resulting from the k^{th} year, GL_{ij} and GLY_{ijk} are the effects resulting from genotype \times location and genotype \times location x year interactions, respectively, and e_{ijk} is the residual error (effect resulting from

experimental error). In this model, location was considered a fixed effect, while year, genotype and interaction were considered random. The *ImerTest* package was used to generate an ANOVA-like table for the random effects and calculate P-values from the Satterthwaite's t-tests for the fixed effect (Kuznetsova et al., 2017). Best linear unbiased predictors (BLUP) estimates, also known as conditional means, were extracted for the random effects to account for environmental deviations and provide more precise estimates of phenotypic values (Appendix 3) (Mi et al., 2011; Wang et al., 2017).

2.3.5 Genotyping and SNP filtering

Young leaf tissue (75-100mg) from the germplasm grown in growth chambers was sampled at the 4-5 leaf stage. The DNA was extracted using the DNeasy Plant kit (Qiagen, Valencia, CA, USA) and quantified using the Quant-it PicoGreen kit (Thermo Fisher, Waltham, MA, USA). Genotyping was performed using the wheat 90K array (Illumina, San Diego, CA, USA) on the iScan instrument (Wang et al., 2014).

Genotype calling was performed for the entire collection using the default genotyping module, and separately for the different ploidy levels using the polyploidy module in GenomeStudio software v2.0.4 (Illumina). The tetraploid and hexaploid sets also included the nine accessions of unknown ploidy. The SNP markers with >20% missing data, <5% minor allele frequency (MAF), and >5% heterozygosity were removed. For the polyploid module, markers with >3 clusters were also removed (Hourcade et al., 2019).

2.3.6 SNP validation

To validate the filtered SNP dataset, we used an exome dataset obtained from 136 accessions of the panels. First, the position of the SNP markers in the protein coding regions of the Chinese Spring (CS) reference genome v1.0 was obtained by mapping the SNP probe sequences of the wheat Infinium array to the exome sequence of all the high-confidence annotated genes of the

CS reference sequence and their 5Kb upstream and downstream sequences (IWGSC, 2018; Wang et al., 2014). The SNP probes were aligned to the indexed exome reference sequence using the MEM-BWA algorithm (v0.7.12, http://bio-bwa.sourceforge.net/). Samtools (v1.3, http://samtools.sourceforge.net/) was used to generate and sort the BAM file alignment. The positions of the mapped SNPs were extracted using BBMap (v.38.43 https://sourceforge.net/projects/bbmap/). The mapped SNP probes were filtered using R to remove the misaligned probes and their coordinates were converted to their actual positions on the CS reference genome v1.0 (IWGSC, 2018).

Upon mapping of the SNP markers, the genotyping dataset was re-filtered with the following updated criteria: markers with <80% of X_i missing data, <5% MAF, and >5% heterozygosity were removed, where χ_i is the proportion of each sub-genome represented in the germplasm. This less stringent criterion ensures retention of SNPs from underrepresented subgenomes. The positions of the filtered SNPs from the wheat 90K array was compared to the variant call results of the exome-sequence data obtained from 136 of the 385 accessions (Appendix 1A). The exome sequencing data were obtained using the Nimblegen SeqCap EZ wheat exome design (120426 Wheat WEC D02, https://sequencing.roche.com/en/productssolutions/by-category/target-enrichment/shareddesigns.html). Raw reads were mapped to the same exome reference genome using the BWA-Samtools pipeline, and variant calling was performed using Bcftools (v1.3, https://samtools.github.io/bcftools/bcftools.html). SNPs common between the filtered wheat 90K array and the exome capture datasets were identified using Bcftools.

2.3.7 Phylogenetic relationships, population structure and kinship

To illustrate evolutionary relationships between the species in the collection, the filtered set of SNPs was used to perform a phylogenetic analysis. A maximum likelihood (ML) tree was generated with 1,000 bootstrap iterations using the default parameters of MEGA-CC (Nearest-Neighbor-Interchange heuristic and Tamura-Nei models) (Kumar et al., 2012). The tree was graphically displayed using iTol v3 (Letunic and Bork, 2016). PCAs were performed using the filtered set of SNPs for each ploidy level and the results were displayed using the R package Scatterplot3d (Ligges and Maechler, 2003).

Population structure analyses were carried out using the R packages LEA (Frichot and François, 2015) and PCAdapt (Luu et al., 2017), as well as the software Admixture v1.3 (Alexander et al., 2009). Both LEA and PCAdapt estimate structure using PCA-based methods. The proportion of variance explained by each PC was graphically illustrated in the form of scree plots. The "knee" in the scree plot (Cattell's rule) was used to determine the number of subpopulations. Admixture is an ML-based approach which uses cross-validation to approximate the K number of sub-populations (Alexander and Lange, 2011). Cross-validation errors for K=2-30 were graphically illustrated using R and the value of K was selected using the rule described above. The approximate number of sub-populations was selected based on the congruity between the plots. The SNMF approach in LEA was used to visualize ancestry proportions in the Q matrix through structure plots. The kinship coefficient matrix was generated using Tassel v5.0 (Bradbury et al., 2007).

2.3.8 Genome-wide association analysis

GWAS was conducted for race-specific response and leaf rust severity rated in the field. For the race-specific response, the converted IT scores for each isolate were considered as individual

traits. For leaf rust severity, genotypic and location-specific BLUP estimates were used as phenotypic inputs, where the former summarizes the severity ratings across all locations and years, and the latter represents the severity ratings separately for each location.

GWAS was performed using one single-locus and seven multi-locus models. The MLM in Tassel v5.0 (Bradbury et al., 2007) was used for single-locus association analysis. Here, population structure and kinship were accounted for using K principle components and the Tasselgenerated kinship matrix. The P-values were adjusted using the false discovery rate (FDR) (Benjamini and Hochberg, 1995). QTNs with FDR (False discovery rate) -adjusted P-values<0.05 were considered significant.

Of the seven multi-locus models, the six from the R package *mrMLM* (Wen et al., 2018) were mrMLM (Wang et al., 2016), FASTmrMLM (Tamba and Zhang, 2018), FASTmrEMMA (Wen et al., 2018), pLARmEB (Wang et al., 2016), pKWmEB (Ren et al., 2018) and ISIS EM-BLASSO (Tamba et al., 2017). The Q matrix generated by Admixture and the Tassel-generated kinship matrix were used to account for population structure and kinship. The seventh multi-locus model, RTM-GWAS, first grouped SNPs into linkage disequilibrium blocks (SNPLDBs) and then utilized a restricted two-stage multi-locus analysis for QTL identification (He et al., 2017). Here, population structure was accounted for by the RTM-generated covariate matrix and kinship and by the Tassel-generated kinship matrix. As with the single-locus MLM, the P-values of QTNs from all the multi-locus models used the same FDR-adjusted threshold. Allelic effect of QTNs was determined using the Kruskal–Wallis statistics to test the phenotypic variation of the associated traits between homozygous alleles.

2.3.9 In silico annotation of significant markers

Because only markers that aligned to the exome sequence of the CS reference genome v1.0 were used for the association analyses, all significant QTNs were within or close to high-

confidence annotated genes. Transcript IDs of the genes containing the significant QTNs were used to extract the protein products using EnsemblPlants (https://plants.ensembl.org/Triticum_aestivum/Info/Index) (IWGSC, 2018; Kersey et al., 2016). For significant SNPLDBs detected by RTM-GWAS that contained multiple SNPs, annotation was carried out for the first and last SNP marker of each SNPLDB.

2.3.10 Positioning Lr genes and QTNs onto the wheat

reference sequence

Sequences coding for the six previously cloned Lr genes were retrieved from GenBank and mapped against the CS reference sequence v1.0 (IWGSC, 2018) using default BLASTn parameters (Expect threshold = 10, Word size = 15, Match/Mismatch scores = $2, -3$) on the Graingenes website (https://wheat.pw.usda.gov/cgi-bin/segserve/blast_wheat.cgi). Through the same exercise, sequences of flanking or co-segregating markers were also mapped onto the reference genome so that a total of 55 of the 66 Lr genes were positioned (Appendix 4). A physical map of previously cloned or mapped Lr genes was constructed using the R package KaryoploteR (Gel and Serra, 2017). Linkage between the QTNs detected and known Lr genes, or their markers, was determined using haplotype block analysis. The SNP dataset was split into haplotype blocks using the R package *gpart* (Kim et al., 2019) and pairwise linkage disequilibrium between the SNPs was calculated using Tassel v5.0 (Bradbury et al., 2007). Known Lr genes and QTNs within the same haplotype block were considered linked, while the relationship between those in neighbouring blocks was determined by comparing D' statistics between the blocks.

2.4 Results

2.4.1 Race-specific resistance

IT response against six P. triticina isolates (MBDS, MBRJ, MGBJ, TDBG1, TDBG2, and TJBJ) was evaluated in the greenhouse for 360 accessions. Of these, 156, 171, 173, 177, 209 and 206 accessions were resistant (IT rating < 3, linear score < 7) to isolates MBDS, MBRJ, MGBJ, TJBJ, TDBG1 and TDBG2, respectively (Appendix 2 and 5). The resistant accessions included 85-131 SHW and cultivated species, 44-56 progenitors and 25-32 wild relatives. Overall, a total of 102 accessions were resistant to all six isolates, and another 153 to at least five isolates.

2.4.2 Field resistance

Phenotypic variation across the different environments was modelled. For both spring and winter panels, the year effect explained the smallest proportion of the variance with 2.1% and 0.31% for each panel, respectively, while the largest proportion was accounted for by the genotype effect with 43.0% and 60.2%, respectively (Appendix 6). The P-values from Satterthwaite's t-tests were <0.005 for all location effects and the genotype-location interaction explained 16.7% and 21.1% of the variation in the spring and winter panels, respectively. The genotypic and location-specific BLUP estimates were extracted from the models and compared to raw aggregate genotypic and location-specific mean values. A linear relationship was observed between the raw mean values and BLUP estimates (Appendix 7 (a,b)). However, due to the inherent nature of BLUP estimation to shrink outliers to the mean, the interquartile ranges (Q3-Q1) of location-specific BLUP estimates were smaller than the raw mean values (Appendix 7 (c-f)).

In the spring panel, 73 accessions were rated resistant (average severity <10%) and 70 were moderately resistant (11-30% average severity) (**Appendix 8 (a)**). The majority of the moderately resistant to resistant accessions belonged the subspecies of T. turgidum (Appendix 3 (a)). In the winter panel, respectively 90 and 38 accessions were rated resistant and moderately

resistant; these included 52 progenitors, 36 wild relatives and 12 SHWs (Appendix 8 (b), Appendix 3 (b)). These distributions, however standardized, were also reflected in the genotypic BLUP estimates calculated for each panel (Appendix 8 (c,d)).

2.4.3 SNP filtering, mapping and validation

A total of 27,418 SNPs from the 385 accessions had a call rate >80%, of these, 20,501 had a MAF >5% and a maximum heterozygosity <5%. Genotype calling and filtering performed separately for the three ploidy levels yielded 34,614 SNPs in the hexaploid, 24,142 in the tetraploid and 15,364 in the diploid datasets. Of the 43,804 SNPs in the hexaploid and tetraploid accessions, 14,952 SNPs (34.13%) were shared (Figure 2.1a). A total of 7,243 SNPs (47.2%) in the diploid dataset were shared with the hexaploid, while only 3,877 SNPs were shared between the tetraploid and diploid datasets.

Mapping was performed to locate the position of the 81,587 SNPs of the wheat Infinium assay on the exome sequence of the CS reference genome v1.0 (IWGSC, 2018). A total of 52,550 SNP marker sequences were successfully mapped, of which 43,013 were retained after filtering out the misaligned probes (Appendix 9). Exome-capture sequencing and subsequent variant calling of 136 accessions identified a total of 142,181,095 SNPs, of which 27,852 were part of the 43,013 mapped SNPs from the array. Re-filtering of the genotyping dataset from the complete germplasm (call rate >80% of χ_i , MAF>5%, and heterozygosity <5%) positioned 12,627 SNPs on the exome reference genome, including 9,570 that were also called using exome capture. The chromosomal distribution of these 9,570 filtered and mapped SNP loci is illustrated (Figure 2.1b).

Figure 2.1 Filtered single nucleotide polymorphism (SNP) markers. (a) Shared SNPs between the hexaploid, tetraploid and diploid datasets. (b) Distribution of 9,570 filtered and validated SNPs across the chromosomes of the CS reference genome v1.0 (IWGSC, 2018).

2.4.4 Phylogenetic relationships and principal component

analysis

A ML phylogenetic tree was constructed to determine the relationships between the species in the collection (**Figure 2.2**). Four main clades were observed. The first consisted of all the Aegilops and non-domesticated Triticum species, where accessions clustered based on their shared subgenomes. The second and largest clade comprised accessions with the ABD genome: SHWs, T. *vavilovii* and T . aestivum subspecies. The other two clades were primarily a mixture of T . turgidum subspecies and SHWs. Ancient tetraploid species T. turgidum ssp. dicoccum (emmer wheat) and the non-domesticated T. turgidum ssp. dicoccoides formed one clade, while modern cultivated species, such as T. turgidum ssp. durum and T. turgidum ssp. carthlicum, formed the other. SHWs were distributed between these clades based on their tetraploid parent species. All T. aestivum ssp. spelta clustered with the ancient tetraploid species.

Figure 2.2 Phylogenetic trees illustrating the relationships between species. The tree was generated using the maximum likelihood approach with 1,000 bootstrap iterations. Both rooted (left) and unrooted (right) trees are shown. The size of the internal node symbols reflects the bootstrap confidence level and the leaf node labels correspond to the identification number and genome of each accession. The species are color-coded as indicated in the legend.

PCA was performed to assess the genetic variation at different ploidy levels. In the hexaploid dataset, the first three PCs explained 29.0% of the variation (Figure 1.3a). Three to four main clusters were observed: the ABD genome species, T. aestivum and T. vavilovii, formed one cluster, Ae. crassa (DM/DDM) and Ae. juvenalis (DMU) formed a second closely related cluster, while Ae. neglecta (UM/UMN) and T. zhukovskyi (GAA^m) clustered into two distinct groups. Similarly, in the tetraploid dataset, the first three PCs explained 28.3% of the variation (Figure 2.3b). Here, T. turgidum subspecies clustered into three groups, while T. timopheevii (A^tG), Ae. crassa and Ae. cylindrica (DC) clustered into individual groups. Accessions belonging to species with the U or M sub-genome (Ae. geniculata, Ae. peregrina, Ae. triuncialis, Ae. biuncialis, Ae. columnaris and Ae. neglecta) clustered together. In the diploid dataset, the first three PCs explained 39.9% of the variation. Ae. tauschii (D) accessions clustered into two groups, Ae. speltoides (S) and T. monococcum (A^m) clustered separately, and nine other species, each

represented by few accessions, all clustered as individual groups (Figure 2.3c). Eight accessions did not cluster with other individuals of their respective species. They were assumed to have been mis-labeled and were removed from the datasets (Appendix 10).

Figure 2.3 Scatter plots of the first three principal components at each ploidy level. Accessions in the (a) hexaploid, (b) tetraploid, and (c) diploid datasets are colored based on their species. The percentages of the variance explained by each principal component are in brackets on the axes.

2.4.5 Genome-wide association analysis

GWAS was performed using IT scores against six P. triticina isolates and the leaf rust severity measured in multiple field environments. The population structure was estimated using three tools and the optimal number of sub-populations was selected based on agreement between methods. For IT scores, K=8 was selected (Appendix 11 (a)) and ancestry proportions from Admixture

were used to survey the population structure. Here, the T . aestivum and T . turgidum subspecies were divided into two and four populations, respectively, while the wild relatives formed two separate populations. For the spring panel rated in the field, K=8 was also suggested (Appendix 11 (b)), where groupings on a subspecies level were observed for the T . aestivum, T . turgidum and SHWs of this panel. For the winter panel, K=6 was selected (Appendix 11 (c)). Here, the SHWs were grouped into three populations, T. timopheevii and T. zhukovskyi grouped together, while the remaining wild relatives formed two separate populations. The LEA structure plots for the three population structure analyses are shown in Appendix 12.

GWAS was conducted using one single-locus and seven multi-locus models, all of which accounted for kinship and population structure. For IT response, the single-locus MLM identified five QTNs for which the proportion of variance explained (r^2) ranged from 6-12% (Appendix 13). Of these, four QTNs were identified for response against the isolate MBDS. The six multi-locus models from *mrMLM* identified a total of 116 unique QTNs across the genome, of which 32 were identified by more than one model and 23 were associated to more than one isolate (Appendix 13). Of note, markers Tdurum contig18471 456 and IAAV6025 associated with MBDS and Kukri_c12869_154 associated with TDBG1 had r^2 values >27%, while r^2 values ranged from 1-23% for the remaining QTNs. RTM, the seventh multi-locus model, grouped the SNPs into 7,607 SNPLDBs and identified 15 QTL with r^2 of 4-15%, including eight that had previously been detected by other multi-locus models (Appendix 13). Of the five QTNs identified by single-locus GWAS, four were identified by at least one of the seven multi-locus models.

Table 2.1 Number of QTNs or SNPLDBs identified by each statistical model Table shows quantitative trait nucleotides (QTNs) and significant SNP linkage disequilibrium blocks (SNPLDBs) identified for both infection type (IT) and leaf rust (LR) severity.

Values in parentheses indicate the number of non-redundant loci i.e. loci identified by at least one statistical model

GWAS for leaf rust severity was conducted separately for the spring and winter panels. MLM identified five significant QTNs (r^2 =18-24%), all of which were associated with leaf rust severity in Morden and located in the D sub-genome (Appendix 14). In the spring panel, *mrMLM* identified 85 unique QTNs (r^2 =1-22%) associated with leaf rust severity, of which 30 were identified by more than one model and 57 were location-specific (Appendix 14). In the winter panel, 38 QTNs were identified including 10 by more than one model and one at both Morden and Ottawa locations (Appendix 14). Marker wsnp_Ex_c6548_11355524 on 5B explained the highest proportion of the variance (40%), while r^2 of the remaining QTNs ranged from 2-24%. RTM identified 37 QTL in the two panels, including seven that were also identified by other multilocus models (Appendix 14). Overall, five QTNs associated with leaf rust severity were also associated with race-specific IT response against at least one isolate. The number of QTNs identified by each model, for both, IT response and leaf rust severity, are shown in Table 2.1. For each phenotypic dataset, the multi-locus model mrMLM identified the highest number of QTNs, while the single-locus model MLM identified the fewest.

2.4.6 Functional annotation

The transcript IDs of the genes within 5Kb of one or more QTN were extracted along with their functional annotation. Between 79-85% of the QTNs associated with race-specific IT response and leaf rust severity were annotated for gene function. These included genes involved in signaling pathways, metabolism, transport and DNA replication and repair. The complete annotation list of the QTNs within or nearby genes is compiled in Appendix 15.

A total of 46 loci associated with IT scores were within 5Kb of genes coding for known plant disease resistance proteins, including seven CC-NBS-LRR, nine F-box-like domaincontaining proteins, seven proteins with kinase domains and three alcohol dehydrogenase domains among others (Appendix 15). Similarly, for leaf rust severity rated in the field, a total of 50 loci (37 in the spring panel and 13 in the winter panel), coded for proteins related to disease resistance: 11 CC-NBS-LRRs, eight kinases, four zinc finger-types and, three LRRs, among others (Appendix 15). Other disease-resistance-related proteins identified include ethylene receptor, alpha/beta hydrolase fold, ABC transporter and, WRKY and WD40 domain proteins. A combined total of 53 QTNs located within plant disease resistance genes explained more than 5% of the phenotypic variation observed (Table 2.2). For each of these, Kruskal–Wallis tests were performed to assay the difference in phenotypic values corresponding to the homozygous alleles. Results showed significant allele-phenotype differences (P-value<0.05) for 35 of the 53 QTNs (Table 2.2), where favorable alleles were detected in the domesticated T . aestivum and T . turgidum species, as well as the wild relative species (Appendix 16). Phenotypic variation for 11 of these significant QTNs present within CC-NBS-LRR, ABC-transporter and sereine/threonine protein kinase domains are illustrated (Figure 2.4). Of these, two QTNs represent nonsynonymous substitutions, five synonymous substitutions, while the remaining were intronic or present upstream of the gene coding sequence. Such QTNs located within known classes of

resistance genes were considered high-confidence as their function and allelic-variation may add to their potential causation or correlation with leaf rust resistance.

Figure 2.4 Boxplots showing significant allelic effects for a subset of the QTNs.

QTNs (quantitative trait nucleotides) associated with race-specific IT response. (b) QTNs associated with leaf rust severity in the winter panel. (c) QTNs associated with leaf rust severity in the spring panel. Labels at the top of each boxplot show the associated trait, i.e., the isolate for (a) and the location if the QTN was location-specific or overall, when severity was significant in all locations. For each QTN, figure also shows its annotated gene and its position relative to the gene's exons/introns and its amino acid change (synonymous vs. non-synonymous). Amino acid changes are represented by standard one-letter amino acid codes. Locations are Morden (MDN), Ottawa (OTT) and Saskatoon (SK). The level of significance was determined through Kruskal– Wallis tests and significance levels "*", "**", "***" and "****" correspond to P-value ≤ 0.05 , 0.01, 0.001 and 0.0001, respectively.

2.4.7 Comparing associated loci with previously reported Lr

genes

To identify novel putative disease resistance loci, the physical positions of the QTNs identified were compared to the positions of the 66 previously reported Lr genes (**Appendix 4**). All QTNs and Lr genes, except for Lr10, Lr14 (a,b), Lr25, Lr26, Lr29, Lr30, Lr36, Lr44, Lr56, Lr59 and Lr66, were physically mapped on the CS reference genome v1.0 (IWGSC, 2018). The position of these mapped Lr genes and the IT and leaf rust severity QTNs identified herein by at least two models are illustrated (Figure 2.5).

Of the Lr genes mapped using both proximal and distal flanking markers, markers for Lr12, Lr13, Lr15, Lr19, Lr27, Lr28, Lr49, Lr64 and Lr75 co-located with 13 of the QTNs identified (Appendix 17). These include seven QTNs associated with leaf rust severity and six with IT response. Haplotype block analysis was used to evaluate the relationships between the QTNs detected and the Lr genes mapped using gene sequences or single genetic markers. A total of 2113 haplotype blocks ($D' \ge 0.5$) were obtained, with an average block size of 4.9MB. Two QTNs, BS00094333_51, associated with leaf rust severity, and D_GDS7LZN02F1Q5F_180, with IT caused by isolates TDBG1, MGBJ and TJBJ, were in the same haplotype blocks as the cloned genes Lr1 and Lr34, respectively, while three co-located in the same blocks as genetic markers of Lr16, Lr32 and Lr73 (Appendix 17). Apart from this, another three QTNs were in neighboring blocks of the markers linked to Lr18 and Lr54. Pairwise linkage analysis between these blocks resulted in mean D' statistics ranging from 0.44 to 0.66. Overall, Kruskal–Wallis tests identified significant allele-phenotype differences (P-value<0.05) for 14 of the 21 QTNs mapping near positions of known Lr genes (Table 2.2, Appendix 17).

Chapter 2

Figure 2.5 Physical map of known Lr genes and QTNs identified.

Map shows the position of known leaf rust resistance genes (Lr) and quantitative trait nucleotides (QTNs) associated with leaf rust severity (LRS) and infection type (IT) against six leaf rust races. The positions of the previously cloned Lr genes are indicated by a single vertical line on the chromosome. Regions shaded in grey indicate linkage disequilibrium blocks., while those shaded in blue indicate the location of the proximal and distal markers of mapped $\mathcal{L}r$ genes. Lr genes previously mapped with a single marker are indicated with an asterix (*). For simplicity, QTNs associated with race-specific IT are shown in orange and those associated with LRS in the spring and winter panels are color-coded in yellow and blue, respectively. Solid dot (●) QTNs indicate association through a multi-locus model while star $(*)$ QTNs were identified with the single-locus model MLM. Centromeres are denoted with a "c" symbol. Only QTNs identified by more than one model are shown.

Table 2.2 Chromosomal location and functional annotation of significant loci. For both, race-specific infection type and leaf rust severity, only quantitative trait nucleotides (QTNs) or linkage disequilibrium blocks (LDBs) located within disease resistance-related proteins explaining greater than 5% of the phenotypic variation are shown.

[†]Lr genes present within the same or neighbouring haplotype block, or Lr genes mapped using flanking markers

Chr, Chromosome; MDN, Morden; OTT, Ottawa; SK, Saskatoon; R allele, allele for resistance; KW, Kruskal–Wallis test significance level where "ns","*", "**", "***" and "****" correspond to not-significant and P-value ≤ 0.05, 0.01, 0.001 and 0.0001, respectively

2.5 Discussion

P. triticina populations are constantly evolving, as exemplified by the presence of more than 70 races detected in North America each year (Ellis et al., 2014). This can quickly render the deployed Lr genes ineffective. Identification of novel sources of disease resistance is necessary to stay ahead in this plant-pathogen evolutionary arms race and to maintain disease resistance in crops. The ability to detect novel Lr genes through marker-based association studies depends greatly on the phenotypic and genetic variation present in the germplasm. The majority of the GWAS in wheat are based on elite cultivars, breeding lines or landraces sourced from breeding programs, genebanks or private seed collections, mainly because introgression into adapted germplasm is easier and faster from the primary genepool as compared to more distant germplasm (Gao et al., 2016; Riaz et al., 2018). These collections, although geographically adapted, often provide limited genetic diversity due to the domestication and selective breeding bottlenecks. Conversely, ancestors and wild relatives of wheat lack adaptation traits for agriculture, but are a rich source of genetic variation, accounting for 44% of the Lr genes identified to date (McCallum et al., 2012; USDA, 2017). In the past century, research to identify and transfer resistance genes from wild relatives was laborious, lengthy and focused on one gene at the time. Recent development in genotyping technologies and the release of the wheat reference genome are enabling high throughput identification of new resistance genes regardless of the genepool, and thus accelerating their gene cloning (Arora et al., 2019; IWGSC, 2018). Here, we described an efficient method to identify new Lr gene loci and candidate genes from many Triticum and Aegilops species using an array-based SNP genotyping platform and eight GWAS models. Through this approach, we identified a total of 50 and 46 disease-related QTNs associated with field leaf rust severity and IT response against six P. triticina isolates, respectively, several of which near known Lr genes and others linked to putatively new ones. The QTNs identified in this

study provide the framework for investigating novel and effective Lr genes from this diverse germplasm and for cloning known Lr genes.

2.5.1 Genetic diversity

Bread wheat, an allohexaploid species, comprises an estimated 17 billion nucleotides, more than 85% of which is repetitive DNA (IWGSC, 2018). Array-based SNP genotyping platforms provide a quick and cost-effective opportunity to survey whole genomes of a large number of samples. We used the wheat 90K array to genotype a diverse collection of 385 accessions. A total of 34.1% of the SNPs were shared between the hexaploid and tetraploid datasets, similar to a previous report of 33.9% (Wang et al., 2014). The high percentage of shared SNPs is indicative of the extensive gene flow from the tetraploid ancestors to hexaploid wheat (Dvorak et al., 2006). Because nearly half of the diploid accessions were Ae. tauschii, the D genome donor of hexaploid wheat, the total of 7,243 (47.2%) of shared SNPs between the diploid and hexaploid datasets also agrees with the gene flow between these species.

The SNPs markers used to develop the wheat 90K array were generated from RNA-Seq data of T. aestivum, T. turgidum and Ae. tauschii (Wang et al., 2014). A total of 43,013 of the SNP markers from the array were physically mapped to the CS exome sequences, again similar to the 46,977 that were genetically mapped using eight T. aestivum mapping populations (Wang et al., 2014). Mapping against the CS exome sequence and subsequent comparison with exome capture data identified $9,570$ SNPs, from which the B (45.1%) , A (36.6%) and D (17.3%) subgenome distribution compared to several previous reports (Daba et al., 2018; Pont et al., 2019; Wang et al., 2014).

2.5.2 Structure analysis

Relationships between the 27 species in the collection were explored using phylogenetic tree analysis. Four major clades were observed, clearly separating the wild species from the T.

aestivum and T. turgidum subspecies. T. aestivum ssp. spelta, hypothesized to have emerged from hybridization between T. aestivum and T. turgidum ssp. dicoccum (Blatter et al., 2004; Pont et al., 2019), was observed to cluster among the T. aestivum subspecies, separately from all T. turgidum ssp. dicoccum and dicoccoides. While some SHWs clustered with T. aestivum, the majority of them were distributed among their tetraploid donor. A population structure analysis showed similar clustering of SHWs on the basis of the origin and growth habit of the tetraploid parent (Bhatta et al., 2018).

The major clade of wild relatives was separately analysed to highlight the relationships between the species (**Appendix 18**). With the exception of Ae. sharonensis, clustering of the Aegilops species of the Sitopsis section was consistent with previous studies, where Ae. speltoides ssp. speltoides and Ae. speltoides ssp. ligustica, formed one clade and Ae. longissima, Ae. bicornis and Ae. searsii formed the other (Bahrman et al., 1988; Miki et al., 2019; Sasanuma et al., 1996). The majority of the Triticum species with an A genome also grouped together, where accessions of T. zhukovskyi were clustered closer to their tetraploid ancestor T. timopheevii (Dvorak et al., 1993). The unique amphiploid EKC22_RL5347 resulting from a cross between Ae. speltoides (S) and T. monococcum (A^m) also clustered with the A-genome species. The close relationship between Ae. crassa (DM or DDM) and Ae. juvenalis (DMU) species was also expected because they both share a D and an M genomes (Baum et al., 2012; Edet et al., 2018). Both genomes of Ae. triuncialis (UC or CU) are nearly identical to the diploid genomes of Ae. umbellulata (U) and Ae. markgrafii (C) (Badaeva et al., 2004) and, unsurprisingly, the Ae. triuncialis cluster located between the U and C genomes diploid accessions. With the exception of Ae. juvenalis (DMU), which clustered with its D genome progenitor, polyploid species carrying a U genome were closely related to one another and to Ae. umbellulata despite having different non-U genomes (Badaeva et al., 2004; Kilian et al., 2011). Some of the wild relative species were sparsely represented in our collection, somewhat limiting our ability to establish clear relationships

between the various genomes. Increasing the number of accessions representing these species is expected to create a more refined picture of their relationships, leading to a better understanding of the evolution of these genomes and species.

The relationships observed in the phylogenetic tree were also observed by PCA. Overall, clustering patterns hinted at possible ascertainment biases; species of the A, B or D sub-genomes segregated more clearly, with few to no outliers, compared to other species. As the 90K array consisted of SNPs previously discovered in cultivars of polyploid wheat, and its D genome progenitor Ae. tauschii, genotype calling may be limited to common alleles identified in the initial SNP discovery process (Albrechtsen et al., 2010; Rasheed et al., 2017). Although the genotyping data may not be sufficient to uncover novel ancestral relationships, it was nonetheless effective in revealing genetic variations at the species level and corroborating previously observed relationships (Badaeva et al., 2004; Bahrman et al., 1988).

2.5.3 Detection of previously reported Lr genes

A total of 13 QTNs identified were present within the mapped flanking markers of nine catalogued Lr genes. The QTN Excalibur c21395 291 mapped between psr119 and mag3092, two markers tightly linked to Lr28 (McIntosh et al., 1982; Younas Sohail et al., 2014). Similarly, the QTN Excalibur rep c68362 62, mapped 1.6Mb upstream, in a neighboring haplotype block of IWB41960, a marker tightly linked to the resistant gene Lr18 (Carpenter et al., 2018; Dyck and Samborski, 1968). Both Lr18 and Lr28 loci QTNs were present within CC-NBS-LRR genes and showed significant allele-specific phenotypic differences, making them candidate genes.

Five QTNs were found to be in the same haplotype blocks as the cloned genes Lr1 and Lr34 and the genetic markers for Lr16 (wmc764), Lr32 (wmc43) and Lr73 (wPt-4453) (Cloutier et al., 2007; Krattinger et al., 2009; McCartney et al., 2005; Park et al., 2014; Thomas, 2010). The QTNs close to Lr1 and Lr32 were present within CC-NBS-LRR and serine/threonine kinase

domains, while the others were located within a 3-ketoacyl-CoA synthase domain or within genes of unknown function. These QTNs identified had within-block D' statistics ranging from 0.54 to 0.85, where higher values suggest high linkage disequilibrium and similar association with phenotypic traits between pairs of SNPs the same block (Cuyabano et al., 2014). Moreover, four of these five QTNs showed significant allele-specific phenotypic variation.

Overall, the lack of cloned genes or tightly linked markers restrict the ability to pinpoint the precise physical position of some Lr genes. QTNs linked to or within flanking markers of known Lr genes may serve as novel markers for gene cloning, however fine-mapping, allelism tests, transformation genome editing (e.g. CRISPR) experiments must be performed to ascertain their identities.

2.5.4 Identification of novel sources of leaf rust resistance

The most prevalent class of known resistance genes encode intracellular immune receptors with NBS-LRR domains, many of which also possess a coiled-coil (CC) N-terminal motifs. These genes play an important role in pathogen recognition and initiation of downstream signaling cascades. In the wheat genome, as many as 661 to 1,560 full-length NBS-LRR genes have been reported, higher than any other plant species (Gu et al., 2015; Steuernagel et al., 2020). Four of the six Lr genes cloned to date encode CC-NBS-LRR proteins (Cloutier et al., 2007; Feuillet et al., 2003; Huang et al., 2003; Thind et al., 2017).

GWAS for race-specific IT response and leaf rust severity identified a total of 18 QTNs within genes encoding complete CC-NBS-LRR domains. Of these, 11 explained greater than 5% of the phenotypic variation, while the remaining were small-effect loci. As discussed above, QTNs close to Lr1, Lr18, Lr28, and Lr54 were in CC-NBS-LRR genes but the remaining were located where no known Lr genes have been mapped to date. For IT response, the most prominent QTNs within CC-NBS-LRR genes included D_contig18780_204 and Kukri_c19466_627, where for the

former, all species in the U-genome group, SHWs and Ae. tauschii var. strangulata expressed the favorable allele, while for the latter, resistant accessions included wild relatives in the Dgenome group and some SHWs, among others.

The highest number of QTNs within genes encoding CC-NBS-LRR proteins was identified in the spring panel rated for leaf rust severity. Of note is BS00065623 51 on the distal end of 7DS, where different subspecies of T. aestivum, such as T. aestivum ssp. spelta, was associated with the low-severity A allele, while most T , aestivum ssp. aestivum and all SHWs associated with the high-severity G allele. While the spring panel was made up of subspecies of T. aestivum, T. turgidum and SHWs, the winter panel was predominantly a collection of SHWs and wild relative species. The most notable Lr severity associated QTN identified in the winter panel may be Ex c6145 2193. This QTN, present within a CC-NBS-LRR gene, was located on the distal end of the short arm of chromosome 1D. Here, all the wild relatives including Ae. crassa, Ae. juvenalis and Ae. cylindrica among others, associated with the low-severity T allele, but the high-severity C allele was only detected in some Ae. tauschii and SHW accessions. Candidate CC-NBS-LRR genes identified here, in the primary as well as the wild gene pool, are valuable sources of genetic resistance.

Plant disease resistance is driven by complex mechanisms involving several layers of defense. Not surprisingly, the classes of known disease resistance genes have expanded greatly in the past few years. For leaf rust, in addition to Lr34 encoding an ABC transporter (Krattinger et al., 2009), the cloned Lr67 gene codes for a hexose transporter (Moore et al., 2015). Other pathogen resistance genes cloned in wheat encode serine/threonine protein kinases and wallassociated kinases (Cao et al., 2011; Shi et al., 2016). Identification of these diverse resistance proteins supports the possibility of uncovering novel classes of disease resistance genes. Consequently, in addition to those in CC-NBS-LRR genes, we identified a number of QTNs present in genes coding for other known resistance proteins in wheat and other plant species. A

key QTN identified herein was Excalibur rep c67475 1759 on 7B. It was located within a pleiotropic drug resistance-type ABC transporter protein, which is known to be involved in the secretion of fungal defense-related metabolites, including resistance to DON accumulation in wheat Fusarium head blight infection (Jasiński et al., 2001; Shang, 2009). Similarly, QTN Kukri c39321 112 on 6B was associated with IT responses against three isolates (TDBG2, MBDS, and TJBJ). It was found within a gene encoding a ZTL-type beta-propeller/F-box domain protein known to regulate plant flowering time and provide resistance against yellow rust in wheat and powdery mildew in barley (Bozkurt et al., 2007; Dagdas et al., 2009; Kim et al., 2005). Here, species with the favorable allele included modern T. turgidum cultivars, Ae. speltoides, Ae. sharonensis and T. timopheevii, among others. These loci, located in novel genomic regions, are also recognized as putative candidate leaf rust resistance genes, and some may potentially confer resistance against multiple leaf rust isolates.

Overall, twice as many QTNs were identified in the spring panel as compared to the winter panel. This imbalance may be due to the difference in the number of accessions in each panel or the nature of the germplasm within each one where the spring panel comprised mostly the species used to design the wheat 90K array, thereby providing higher quality genotyping. In addition, the potential for identifying novel disease resistance genes is also dependent on the mapping of the QTNs to the T. aestivum reference genome. As the reference only represents the A, B and D genomes of a single genotype, it may limit, but not prevent, our ability to identify rare resistance genes unique to the contrasting genomes of the wild relatives.

2.6 Conclusion

The GWAS described herein highlights the multi-genic and complex nature of pathogen disease resistance where multiple markers were associated with different field environments and pathogen races. We identified several QTNs located near known Lr resistance genes providing, at the very least, novel markers for the cloning of these genes. Some of them were located within

known resistance gene classes such as CC-NBS-LRR. As such, these become prime candidates for direct investigations. This study also identified novel leaf rust resistance loci from the domesticated T. aestivum and T. turgidum species that can be capitalized upon quickly but also others from wild relative species that may be harnessed to add to the leaf rust resistance repertoire of wheat. Once cloned, the novel Lr genes can be transferred into adapted germplasm using modern genome-assisted breeding strategies, such as gene cassettes and genome editing (Wang et al., 2018; Wulff and Moscou, 2014). Gene cassettes allow multiple cloned disease resistance genes to be transformed simultaneously into a single genome to provide durable and broad-spectrum resistance, because the closely linked genes will not segregate, will be easy to select for, and will essentially have the advantages of gene pyramiding (Arora et al., 2019; Kolmer et al., 2009). Gene-specific markers can also be developed to facilitate the transfer of these genes through conventional breeding. The recently introduced CRISPR-Cas9 system in wheat (Liang et al., 2017; Zhang et al., 2016) offers many advantages. It can facilitate the investigation of candidate genes in any germplasm, bypassing the laborious fine-mapping experiments and enabling their functional analyses. We believe that gene editing could also be capitalized upon to "transfer" resistance genes from wild relatives through the allelic conversion of the orthologous domesticated alleles, providing that sufficient sequence similarity exists between the wheat and the wild relative alleles. This "long-shot" strategy would eliminate the need for the long, laborious and difficult introgression via crossing, and eradicate its associated linkage drag drawbacks. In conclusion, we described a powerful approach to identify QTN markers and candidate genes for leaf rust resistance through combining a broad germplasm including cultivated species and wild relatives, array-based genotyping, field severity and IT phenotyping and, through the use of several GWAS models.

3 General discussion and conclusion

Wheat is arguably the most important crop in the world. For millennia it has been a symbol of harvest, prosperity and fertility. Before the advent of bread wheat, ancient wheats were cultivated in the Fertile Crescent (modern day Middle East) and spread from there towards Europe, Asia and onwards (Shewry, 2009). Wheat is now the most widely grown crop in the world, an essential source of energy and nutrients, and a major source of revenue to wheat producing and trading countries. The challenges that breeders currently face are to increase production to accommodate population growth and the increased consumption per capita, while also accounting for climate change, pollution, water scarcity and biotic and abiotic stresses (Curtis and Halford, 2014).

For various plant and animal species, access to a fully annotated reference genome has promoted the systematic development of approaches to study and select for important traits (Hickey et al., 2017). The genome of rice was sequenced in 2002 (Chen et al., 2002), soybean in 2008 (Schmutz et al., 2010) and maize in 2009 (Schnable et al., 2009). Wheat, while being one of the most important crops, has lagged behind in terms of genomic advances. The International Wheat Genome Sequencing Consortium (IWGSC) has been trying to decode the genome of wheat since 2005 and finally, after 14 years and a few incomplete drafts, in mid-2018, it released a fully sequenced and annotated reference genome of the Chinese Spring (CS) variety of bread wheat (IWGSC, 2018). This lag was largely due to the colossal size and highly repetitive nature of the polyploid genome. Approximately 113,653 (63%) of the total annotated genes in CS were present as homoeologous genes, i.e., were present as copies across the A, B and D subgenomes. In addition, 15% of these had paralogous copies in at least one sub-genome, i.e., genes copied from tandem or segmental trans-duplication (IWGSC, 2018). This complicated the genome assembly process in terms of chromosome assignment and gene order. Nonetheless, availability of an annotated CS reference genome constitute a revolutionary milestone for wheat research,

giving researchers access to sequence-level information and facilitating the isolation of important genes, while also spurring the implementation of new strategies for genomics-assisted breeding.

The research project presented herein took advantage of this opportune time, leveraging positional and functional information from the newly available reference genome to identify novel genomic regions involved in leaf rust resistance. This reference sequence has served as a backbone to anchor all previously known disease resistance genes onto a single annotated reference, while also allowing comparison and validation of newly identified loci.

3.1 Highlights of the study

In this study, an array-based single nucleotide polymorphism (SNP) genotyping was used to characterize a diverse set of species and perform a genome-wide association study (GWAS) to map candidate leaf rust resistance genes. The germplasm consisted of 385 accessions, encompassing 27 different species of cultivated wheats, synthetic hexaploid wheats (SHWs) and wild relatives belonging to the primary, secondary and tertiary gene pools of wheat. Genetic diversity was captured using the wheat 90K array (Wang et al., 2014) and GWAS was conducted using eight statistical models for reaction ratings against six leaf rust isolates and for leaf rust severity rated in field in multiple year and location environments.

Genotyping using the 90K array allowed comparison of genetic diversity across different ploidy and species levels. Upon initial filtering, 20,501 SNP markers were retained from the complete germplasm. Using the sequences of the 81,587 SNP markers in the 90K array (Wang et al., 2014), one of the first physical map to position these markers on the protein coding regions of the CS reference genome was created. Knowledge of the chromosomal position of the makers provided the information necessary to perform SNP re-filtering to accommodate the differential genotype calls in the sub-genomes. For example, only 59% of all accessions carry the D subgenome; an otherwise 80% call filter would have removed genome-specific SNPs will lower call

rates. This filtering resulted in 12,627 SNP markers, of which 9,570 were validated through exome-capture variant calling of a subset of 136 accessions.

Phylogenetic and population structure analysis illustrated the relationships between the Aegilops and Triticum species in the collection. Apart from eight accessions, the majority clustered based on their shared sub-genomes, species divergence and/or pedigree information. Ancient tetraploid species T. turgidum ssp. dicoccum (emmer wheat) and the non-domesticated T. turgidum ssp. dicoccoides formed one clade, while modern cultivated species, such as T. turgidum ssp. durum and T. turgidum ssp. carthlicum, formed the other. These modern tetraploids wheats differentiated from wild emmer wheat due to selection pressures imposed by local environments and cultural practices across a larger distribution of areas (Bozzini et al., 2012). These recently differentiated species form a taxonomic subgroup separated from T. turgidum ssp. dicoccum and dicoccoides, as was observed through other phylogenetic studies of the T. turgidum subspecies (Laidò et al., 2013; Maccaferri et al., 2019; Marcotuli et al., 2016). Previous studies have shown a decrease in nucleotide diversity from wild emmer wheat (π=2.28), to domesticated emmer (π =1.17) and to durum wheat (π =0.87) (Akhunov et al., 2007).

Synthetic hexaploid wheats (SHWs) created by crossing tetraploid T. turgidum with diploid Ae. tauschii, were distributed between the two tetraploid clades based on the genetic characterization of their tetraploid parent. For example, 12 SHW accessions created by crossing the durum wheat cultivar 'Langdon' with different Ae. tauschii accessions, clustered with 'Langdon' in the modern tetraploids clade. Similarly, multiple SHWs created by crossing wild emmer wheat accessions PI113961 and PI355465 with Ae. tauschii, clustered with their tetraploid parents in the ancient tetraploid clade. Similar studies using SSR and AFLP markers have reported the genetic diversity of SHWs to clearly reflect the sub-species, geographical origin and morphological traits of their tetraploid parent, possibly due to the fact that it contributed to twothird of their genome (Dreisigacker et al., 2008; Lage et al., 2003; Szabo-Hever et al., 2018).
All wild Aegilops and Triticum species clustered in one clade; however sub-clades were formed based on related species. In agreement with previous studies, genome-based grouping was observed for Aegilops species with the D, S, U, and C sub-genomes, and Triticum species with the A sub-genome (Badaeva et al., 2004; Bahrman et al., 1988; Baum et al., 2012; Dvorak et al., 1993). Clustering patterns observed in the phylogenetic tree were also reflected in principal component analyses and ancestry mix distributions of population structure. These analyses clearly illustrate the effectiveness of the 90K array filtered genotyping data set in differentiating the species and sub-species of the collection.

Using the filtered genotyping data, a GWAS was performed for leaf rust, the most widespread rust disease of wheat caused by the fungal pathogen *Puccinia triticina*. Two types of phenotypic data were collected, leaf rust infection type (IT) measured against six leaf rust isolates and multi-environment field leaf rust severity rated separately for the spring and winter panels. One single-locus and seven multi-locus statistical models were used, identifying 281 quantitative trait loci (QTL), some of which were unique to specific models but 53-68%, depending on the trait, were identified by more than one model. Multi-locus models, previously shown to have higher detection power because of their less stringent criteria for multiple test corrections, outperformed the single-locus model in identifying small-effect loci for complex associations (Cui et al., 2018; Segura et al., 2012; Zhang et al., 2018b). Indeed, in this study, the single locus model, MLM, identified the fewest QTL, with a maximum of five for race-specific IT, while multi-locus models identified up to 54 QTL for this same trait. The proportion of phenotypic variance explained, however, was higher for the single-locus model (average r^2 =8-19%) compared to multi-locus models (average r^2 =5-7%) which identified a large number of small-effect QTL. At the same time, of the total eleven QTL identified using MLM, six were also identified by multi-locus GWAS; comparison between five of these six QTL showed that the r^2 percentage for single-locus models was estimated to be 2-4 folds than that obtained from the multi-locus models.

Protein function of genes within 5kb of the QTL identified were extracted using the CS reference genome annotation v1.1. Between 79-85% of the QTL associated with race-specific IT and leaf rust severity were successfully annotated. Of these, a subset of 35 coding for known plant disease resistance proteins each explained greater than 5% of the phenotypic variation and showed significant allele-phenotype differences (P-value<0.05). The majority of the plant disease resistance genes classified encode immune receptors that detect pathogen effectors. In the case of wheat, 23 (72%) of the cloned disease resistance genes encode NLR receptor proteins (CC-NBS-LRR domains) (DeYoung and Innes, 2006; Keller et al., 2016; Zhang et al., 2020). Proteins encoded by other wheat disease resistance genes include protein kinases and transporter proteins (Keller et al., 2016). In this study, GWAS identified a total of 18 QTNs within genes encoding complete CC-NBS-LRR domains, while several other QTL encoded serine/threonine protein kinases, ABC transporter proteins and F-box domain containing proteins, among others. The favorable alleles associated with low severity and IT were present in the domesticated T. aestivum and T. turgidum species, as well as in the wild relatives of wheat.

Using gene sequences and sequences of flanking or co-segregating markers, the physical positions of 55 of the 66 previously reported Lr genes was determined on the CS reference genome v1.1 (McCallum et al., 2012; USDA, 2017). A total of 13 QTL identified were present within the mapped flanking markers of nine catalogued Lr genes, and an additional seven were within the same or neighboring haplotype block as six other Lr genes. The remaining significant QTLs, especially those which (1) were identified by multiple model, (2) encode disease related proteins and/or (3) explain high phenotypic variance, were considered loci that putatively harbor new Lr resistance genes. Examples of such loci in novel positions include Kukri_c19466_627 on 7D present within a CC-NBS-LRR domain (r^2 =18.8) and Excalibur_rep_c67475_1759 present within a ABC-transporter domain (r^2 =5-6%).

3.2 Practical and scientific implications of the results

In this study, not only were the markers from the 90K wheat array were mapped on the wheat reference genome, but also a compressive physical map of 55 of the 66 known Lr genes was created. This reference map will allow comparison and validation of newly identified loci from this and future association genetics studies. One of the strengths of this study was the diverse germplasm; through phylogenetic and clustering analyses, light was shed on to relationship between different Aegilops and Triticum species, their subspecies and synthetic crosses. Our analyses identified relationships hypothesized in the past from genetic diversity studies conducted using SNP, SSR and RFLP markers, and identified caveats to consider in future investigations. For example, the amphiploid resulting from a cross between Ae. speltoides (S) and T. monococcum (A^m) clustered with its A-genome progenitor, away from all the Ae. speltoides in the collection.

The QTNs identified by GWAS provide the framework for investigating novel Lr genes from this diverse germplasm and for cloning known Lr genes. Identification of novel sources of disease resistance is important to stay abreast of the plant-pathogen evolutionary arms race and to maintain disease resistance in crops. P. triticina populations are constantly evolving, which can quickly render the deployed Lr genes ineffective. For example, Lr14a and Lr10, once popular sources of resistance, are no longer effective against leaf rust races in Canada, while some genes such as Lr13, Lr30 and Lr34 still remain effective when used in combination with other Lr genes, a strategy referred to as gene pyramiding (McCallum et al., 2016).

Lr genes which bestow seedling-type resistance usually confer a race-specific response controlled by single major-effect genes, while adult plant resistance (APR) genes can also confer quantitative race non-specific responses. Of all the cloned Lr genes, seedling-type genes encode CC-NBS-LRR domains, while APR genes encode transport proteins. Candidate genes identified here, that were either associated with race-specific IT or leaf rust severity conferred by a mixture

of pathogen races, especially those which encode known classes of resistance proteins, are the prime targets for direct investigations. Functional validation of these loci can be performed using transformation (Cheng et al., 1997), CRISPR/Cas9 (Zhang et al., 2016), genomic selection strategies (Bassi et al., 2016), screening through KASP (Kompetitive Allele Specific PCR) assays (Rasheed et al., 2016) and mapping using bi-parental populations to select a donor parental line (Zhang et al., 2018a).

As wild relatives of wheat account for nearly half of the Lr genes identified to date, the cultivated and the wild gene pools in this study presents an excellent avenue for the identification of new sources of resistance to broaden and maintain the genetic base of leaf rust resistance. Novel leaf rust resistance loci such as BS00065623 51 and Excalibur c21395 291 from the domesticated T. aestivum and T. turgidum species (Appendix 16, Table 2.2) can be capitalized upon quickly by standard breeding methods like hybridization, backcrossing and selection, using adapted germplasm. Resistant loci in Ae. tauschii can be transferred into hexaploid wheat through the creation of SHW lines or through direct hybridization with T. aestivum, which would be both followed by embryo rescue and backcrossing to T . aestivum to recover semi-adapted resistant genotypes (Cox et al., 2017). Lastly, resistant alleles such as Ex_c6145_2193 and D contig28902 391, identified in the secondary and tertiary gene pools (Appendix 16, Table 2.2), can be transferred by interspecific crossing of the wild relative with an adapted cultivar followed by backcrossing to the recurrent parent (Kishii, 2019; Mujeeb-Kazi and Rajaram, 2002). This technique requires special breeding protocols and is plagued by sterility in the progeny and linkage drag resulting from the low recombination surrounding the introgressed alien fragment. On the other hand, modern genome-assisted breeding strategies, such as gene cassettes and CRISPR-Cas9 genome editing, offer an interesting alternative to bypass long and laborious introgression via crossing, allowing gene transfer and quick validation from any germplasm (Wulff and Moscou, 2014; Zhang et al., 2016).

Overall, novel candidate genes associated with leaf rust resistance were identified and several markers located near known Lr resistance genes were mapped. The outcomes of this research is expected to guide pre-breeding decisions for the transfer of useful Lr genes for wheat improvement. The genotyping data generated and the association analysis pipeline developed herein can also be further applied to perform GWAS and genomic selection strategies for the other disease-related and agronomic traits that were rated by our collaborators for this germplasm.

3.3 Limitations and suggested future studies

While this study provides insights into the population structure of various species and identifies candidate genes associated with leaf rust resistance, there are some limitations and gaps that must be acknowledged and taken into consideration.

SNP arrays, like the 90K wheat array, are made of a fixed set of SNPs identified from a discrete set of individuals. Inherent design biases from initial SNP discovery and array development hinder the capture of rare alleles during genotype calling, especially from the disparate germplasm used in this study. Genotyping was also complicated by the inherent complexities associated with the presence of homoeologous chromosomes in wheat. For some markers, genotype calls that were not specific to unique sub-genomes were observed, e.g., positive genotype calls for SNP markers aligning to the D sub-genome in species that did not carry a D sub-genome. This is because some SNP probes can not only hybridize to their target locus, but also to their homoeologous and/or paralogous loci, thereby affecting the allele-specific fluorescent signal ratios, complicating genotype calling and possibly resulting in false positives. Although, validation using exome-capture data and genome-specific filtering helped validate overall genotyping quality, it is likely that markers with some false positive calls were retained post-filtering and one must be mindful of error propagation when interpreting GWAS results from this subset of markers. To some extent, this limitation can be overcome by increasing the number

and diversity of individuals/SNPs used for array development and by combining fixed arrays with de novo NGS-based platforms, such as the exome capture used herein for cross-validation, genotyping by sequencing (GBS), and restriction site-associated DNA sequencing (RAD-seq), however, such approaches can be costly and carry their own complexities.

To perform GWAS, eight different statistical models were used, identifying a large number of QTL, some of which were unique while others were recurrent. Functional annotation and allelic tests using Kruskal–Wallis statistics were performed to shortlist effective candidate genes, however, there is still a need for further functional validation of the loci identified. A drawback of association analyses is that the statistical models used can identify many false positives (Ioannidis et al., 2009). Controlling for population structure and kinship, using more stringent criteria for multiple test corrections and performing cross-validation using multiple models are all practical ways to minimize this problem. The outcomes of GWAS in this study can be further studied by pinpointing the position of the QTL relative to the gene's exons/introns and its amino acid change (synonymous vs. non-synonymous). These QTL in high linkage disequilibrium with the functional variants as can become proxies or genetic markers as their genotypes are highly correlated with the genotypes of the functional variant (Myles et al., 2009). The biological role of candidate genes identified must also be further explored, i.e., their interaction networks, molecular pathways and contributions to disease resistance. For associated loci linked to or within flanking markers of known Lr genes additional fine-mapping, allelism tests, transformation or genome editing (e.g., CRISPR) experiments must be performed to ascertain their functional role(s). Moreover, reproducibility of the GWAS results can be further assessed using additional phenotypic or genotypic data. Strategies such as genomic selection can also be applied. Genomic selection uses genotypic and phenotypic data of a training population to predict the breeding value of each marker or QTN in a test population (Lorenz et al., 2011).

Other limitations of the study include unequal sample representation and missing phenotypic data. Our germplasm included 27 different species, but some of the wild relative species, such as Ae. markgrafii, Ae. triuncialis, and Ae. comosa, were sparsely represented. Increasing the number of accessions representing these species is expected to create a more refined picture of their relationships. This will also allow more in-depth analyses such as comparison of shared SNPs, allelic frequencies and nucleotide diversity (π) between the different species and historical groups (landraces, old cultivars and modern varieties). Association analysis studies are only as good as the weakest link which is often the phenotypic data. For some years/locations, the amount of missing data in the winter panel was substantial because some lines matured prior to the establishment of the infection for example. Although, using conditional means in the form of best linear unbiased predictors (BLUPs) provided extrapolated values, decreasing the amount of missing data can potentially improve this estimation.

3.4 Conclusions

Wheat is the most widely grown and consumed crop in the world. Changes in wheat production and utility over the past couple of centuries has clearly shown how farmers have progressively improved yield and quality through agronomic and genetic advances ̶ conventional crop breeding combined with modern genomic and biotechnological tools now allows breeders to maximize genetic gains and improve grain quality and yield. However the broad use of single uniform varieties could lead to fast resistance breakdown. The wild relatives of wheat and other forms of non-adapted germplasm are a rich source of genetic diversity and stress tolerance traits. Prebreeding identifies desirable genes from these non-adapted germplasm to transfer useful traits to adapted germplasm that breeders can use to produce new varieties.

Array-based genotyping was used to explore genetic diversity in various domesticated and non-domesticated species of wheat and their wild relatives. Phylogenetic analyses illustrated

four major clades, clearly separating the wild species from the domesticated and ancient T. turgidum species from modern T . turgidum cultivars. The clusters observed showed genetic differentiation between the various species and their sub-species, and reproduced relationships previously observed using genetic markers. Multiple statistical models combined this genotyping data to conduct a genome-wide association study for leaf rust resistance using scores for field leaf rust severity and IT against six leaf rust isolates. Several loci associated with race-specific IT and severity were identified, some of which were located near known leaf rust resistance genes and others linked to putatively new ones. A subset of 35 loci coding for known disease resistance proteins explained a substantial proportion of the phenotypic variation and showed significant allele-phenotype differences (r^2 >5%, P-value<0.05). These were considered high-confidence candidate genes. Future efforts to validate these loci will help understand their role in disease resistance and promote their utility for marker-assisted selection in pre-breeding.

Overall, a powerful approach that combines a broad germplasm, array-based genotyping, multi-environment trait data and several GWAS models was described to identify candidate genes underlying leaf rust resistance in wheat and that could be applied to other traits such as other biotic and abiotic stress-related traits as well as agronomic traits in wheat.

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5 Appendix

Appendix 1 List of accessions included in this study

Appendix 1A For each accession table shows their respective genome, ploidy, growth habit, origin, and seed source.

The species names and genome symbols are according to Kimber and Tsunewaki (Kimber and Tsunewaki, 1988)
¹ Entry numbers followed by a "*" symbol represents accessions that were a part of the 136 accession used for exome-²2X, Diploid; 4X, Tetraploid; 6X, Hexaploid

³GH, Growth habit; S, Spring; W, Winter

Appendix 1B Geographical distribution of 217 of the 385 accessions in the collection.

Number of accessions originating in each country is represented by a gradient color scale, where the color red indicates the lowest number of accessions (one) and blue the highest number of accessions (48).

Appendix 2 Infection type scores of accessions rated for response against leaf rust isolates

Accessions were scored using the Stakman scale (Stakman et al., 1962) and the linear conversion using a 1-9 scale is shown in brackets.

Entry Accession MBDS MBRJ MGBJ TDBG1 TDBG2 TJBJ 813 EKC166 RL5012 2- (5) 22+ (5) 2- (5) 1+ (3) 2- (5) 2- (5) 814 EKC171 RL5722 NA NA 3- (7) 3+ (7) 3+ (7) 3+ (7) 815 EKC186_RL5847 3+ (7) 3+ (7) 23- (5) 3+ (7) 2- (5) 2- (5) 818 IPK-AE1 3 (7) 3+ (7) 3 (7) 3+ (7) 3+ (7) 3+ (7) 819 IPK-AE8 3 (7) 3 (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 820 IPK-AE30 3 (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 821 IPK-AE91 3+ (7) 3+ (7) 3+ (7) X (6) X (6) 3+ (7) 822 IPK-AE111 NA ; (1) NA ; (1) ; (1) ; (1) 823 IPK-AE113 1- (2) NA ;1- (2) 2- (5) NA 827 IPK-AE162 23 (5) 3 (7) 3+ (7) 3 (7) 3+ (7) 3+ (7) 828 IPK-AE182 3+ (7) NA 34 (8) X (6) X (6) 3+ (7) 830 IPK-AE316 NA ; (1) ; (1) ; (1) ; (1) ;/3+ (NA) 831 IPK-AE346 1- (2) 13 (3) ; 1- (2) 1- (2) 1- (2) ; 1- (2) 835 IPK-AE467 3- (7) 3 (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 838 IPK-AE714 23- (5) 2- (5) NA 2+ (5) 2+ (5) NA 841 IPK-AE825 11+ (3) 1- (2) 1- (2) ; (1) NA ;1- (2) 842 IPK-AE899 23 (5) 23 (5) 3 (7) 23 (5) 3+ (7) 3+ (7) 843 IPK-AE906 ;1- (2) 11+ (3) 2- (5) 2- (5) 2- (5) 2- (5) 847 IPK-AE1580 12 (4) ;1- (2) 2- (5) 2- (5) 3+ (7) 2+ (5) 848 IPK-AE1607 1- (2) ; (1) ; (1) ; (1) ; (1) ; (1) ; (1) 849 IPK-TRI677 ; (1) 0 (1) ; (1) ; (1) ; (1) ; (1) ; (1) 852 IPK-TRI3365 11- (3) 11- (3) 2+ (5) 2+ (5) 3 (7) 2- (5) 853 IPK-TRI4349 0; (1) ; (1) ; (1) ; (1) ; (1) ; (1) ; (1) 855 IPK-TRI4630 3+ (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 3+4 (8) 856 IPK-TRI4653 11- (3) 11- (3) 2- (5) 2- (5) 2- (5) 1- (2) 857 IPK-TRI5416 0; (1) 0; (1) 0; (1) ; (1) ; (1) ; (1) 858 IPK-TRI7258 ; (1) NA ; (1) ; (1) ; (1) ; (1) 860 IPK-TRI7270 ; (1) ; (1) ; (1) ; (1) 861 IPK-TRI7272 3+ (7) NA 3+ (7) ; (1) 3+ (7) 3+ (7) 863 IPK-TRI7315 3+ (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 3+4 (8) 864 IPK-TRI11555 3+4 (8) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 865 IPK-TRI11556 3+ (7) 3+4 (8) 3+ (7) 3+ (7) 3+4 (8) 3+ (7) 866 IPK-TRI12094 0 (1) 0; (1) ; (1) NA ; (1) ; (1) 867 IPK-TRI13604 0; (1) 0; (1) ; (1) ; (1) ; (1) ; (1) ; (1) 868 IPK-TRI13606 0; (1) ; (1) ; (1) ; (1) ; (1) ; (1) ; (1) 871 Emerson-1 3+ (7) 1- (2) X (6) 3+ (7) 3+ (7) 3- (7) 872 17CAN-SYNT-01A 1-(2) ; (1) ; 1- (2) 3 (7) 1- (2) ; 1- (2) 873 17CAN-SYNT-02B 22+ (5) ;1- (2) ;1- (2) 3 (7) ;1- (2) ;1= (2) 874 17CAN-SYNT-03A 3 (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 3+ (7) 875 17CAN-SYNT-04A 3+ (7) 23 (5) 3+ (7) 3 (7) 3+ (7) 3 (7) 876 17CAN-SYNT-05A 3+ (7) 3+ (7) 1+ (3) 2+ (5) 3+ (7) 3+ (7)

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877 17CAN-SYNT-06A ;1= (2) ;1- (2) ;1- (2) ;1- (2) ;1= (2) ;1= (2)

The isolates tested were 12-3 MBDS, 128-1 MBRJ, 74-2 MGBJ, 11-180-1 TDBG, 06-1-1 TDBG, and 77-2 TJBJ. For simplicity, these were referred to as MBDS, MBRJ, MGBJ, TDBG1, TDBG2, and TJBJ, respectively.

IT scores were converted into a 1-9 linear scale using the following scale: " $0/0; 1; 1 = 1; 1 = 1; 1 - 1 = 1$ " 2, "1-/1/1+" = 3, ";12/1-2-" = 4, "2-/2/2+" = 5, "X" = 6, "3-/3/3+" = 7, "3+4/34" = 8, and "4" = 9

Appendix 3 BLUP estimates and raw average score for leaf rust severity

Appendix 3A Overall and location-specific best linear unbiased predictors (BLUP) for leaf rust severity of the spring diversity panel (left of the vertical separator line). Table also shows raw average severity and infection type across all years and locations (right of the vertical separator line).

MDN, Morden; OTT, Ottawa; SK, Saskatoon; Overall, all locations and years ¹R, resistant; MR, moderately resistant; I, Intermediate; MS, moderately susceptible; S, susceptible.

Appendix 3B Overall and location-specific best linear unbiased predictors (BLUP) for leaf rust severity of the winter diversity panel (left of the vertical separator line). Table also shows raw average severity and infection type across all years and locations (right of the vertical separator line).

MDN, Morden; OTT, Ottawa; Overall, all locations and years

R, resistant; MR, moderately resistant; I, Intermediate; MS, moderately susceptible; S, susceptible.

Appendix 4 Gene and marker position of known leaf rust resistance genes.

Table shows names of catalogued wheat leaf rust resistance (Lr) genes, their source and position on the Chinese Spring reference genome (IWGSC, 2018) mapped using gene sequences or flanking and linked markers.

 † Lr genes that could not be mapped onto the reference genome.

P, proximal; D, distal; Co, co-segregating; GS, gene sequence; QTL, quantitative trait loci

Appendix 5 Genotype distribution of infection type response against leaf rust isolates

Each bar represents the distribution of scores for a specific leaf rust isolate. Scores from the Stakman scale were converted to a linear 1-9 scale.

Appendix 6 Linear mixed model for leaf rust severity across years and locations.

Linear mixed model fit by REML. t-tests use Satterthwaite's method: Formula: Severity ~ Location + (1|Genotype) + (1|Year) + (1|Genotype:Location) + (1|Genotype:Year:Location)

Spring Panel: Number of observations: 2818, groups: Genotype:Year:Location, 1569; Genotype:Location, 640; Genotype, 216; Year, 4

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Winter Panel: Number of obs: 978, groups: Genotype:Year:Location, 558; Genotype:Location, 310; Genotype, 167; Year, 3

Random effects:

Fixed effects:

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Appendix 7 Comparison between raw means and BLUP estimates for leaf rust severity.

Scatter plots show mean values (y-axis) vs. BLUP estimates (x-axis) for overall severity in the spring (a) and winter (b) diversity panels. Boxplots show the interquartile range and median severity calculated using aggregate means (c,e) and BLUP estimates (d,f) for Morden (MDN), Ottawa (OTT) and Saskatoon (SK) in the spring diversity panel, and MDN and OTT in the winter diversity panel.

Appendix 8 Distribution of severity scores in the spring and winter panels.

Raw mean values of the spring (a) and winter (b) diversity panels and genotypic best linear unbiased predictor (BLUP) estimates of the spring (c) and winter (d) diversity panels are illustrated. Genotypic BLUP estimates are conditional means which summarize phenotypic variation across all locations and years.

Appendix 9 Chromosomal distribution of markers from the wheat 90K array

A total of 43,804 markers were mapped. For each chromosome, the density plot shows the number of SNPs within one million base (Mb) window size.

Appendix 10 Accessions that did not cluster with other individuals of the same species

Appendix 11 Scree plots for estimating the K number of sub-populations

K number of sub-populations was estimated using the SNP datasets for the race-specific infection type (a) and field leaf rust severity of the spring (b) and winter (c) diversity panels. PCAdapt and LEA, are both PCA-based methods, where PCAdapt plots the number of PCs vs. proportion of variance explained, and LEA plots the Tracy–Widom statistic vs. K. Admixture detects population structure through trends in cross-validation error vs. K.

Appendix 12 Structure plots illustrating the ancestry mix of the subpopulations

Ancestry mix shown for K=8 for the race-specific infection type (a), K=8 for leaf rust severity of the spring (b) and K=6 for the winter (c) diversity panels.

Appendix 13 Significant QTNs and LDBs for IT response against six leaf rust isolates

QTNs (Quantitative trait nucleotides) and LDBs (linkage disequilibrium blocks) for IT (infection type) response were identified by association mapping using one single-locus and seven multi-locus models.

Appendix 14 Significant QTNs and LDBs for leaf rust severity in spring and winter panels

QTNs (Quantitative trait nucleotides) and LDBs (linkage disequilibrium blocks) for leaf rust severity in the spring and winter diversity panels response were identified by association mapping using one single-locus and seven multi-locus models.

2 (%)

¹ MDN, Mordern; OTT, Ottawa; SK, Saskatoon
² For significant SNP LDBs identified by RTM, only the SNP index are shown.

Appendix 15 Functional annotation of genes located within 5KB of associated loci

Table shows quantitative trait nucleotides (QTNs) or peak single nucleotide polymorphism (SNP) markers of linkage disequilibrium blocks (LDBs) associated with infection type response against six leaf rust isolates and leaf rust (LR) severity of the spring and winter diversity panels.

¹ MDN, Morden; OTT, Ottawa; SK, Saskatoon; All, all locations

2 M1, pLARmEB; M2, FASTmrEMMA ; M3, RTM; M4, ISIS-EM-BLASSO; M5, FASTmrMLM; M6, pLARmEB; M7, mrMLM; M8, MLM

Appendix 16 Genotypes for QTNs showing significant allele-specific phenotype differences

Appendix 16A Heatmap showing genotype calls for quantitative trait nucleotides (QTNs) that displayed significant allele-specific phenotype differences for infection type against six leaf rust isolates. Rows represent the entry number and genome symbol of the accessions in the germplasm and columns represent the QTNs. Genotypes AA and BB represent homozygous alleles associated with low and high infection type scores, respectively. Genotype AB and NA represent heterozygous alleles and no genotype calls, respectively. Different colors in the left panel represent the different groups of species in the germplasm.

Appendix 16B Heatmap showing genotype calls for quantitative trait nucleotides (QTNs) that displayed significant allele-specific phenotype differences for leaf rust severity in the spring diversity panel. Rows represent the entry number and genome symbol of the accessions in the germplasm and columns represent the QTNs. Genotypes AA and BB represent homozygous alleles associated with low and high leaf rust severity scores, respectively. Genotype AB and NA represent heterozygous alleles and no genotype calls, respectively. Different colors in the left panel represent the different groups of species in the germplasm.

Appendix 16C Heatmap showing genotype calls for quantitative trait nucleotides (QTNs) that displayed significant allele-specific phenotype differences for leaf rust severity in the winter diversity panel. Rows represent the entry number and genome symbol of the accessions in the germplasm and columns represent the QTNs. Genotypes AA and BB represent homozygous alleles associated with low and high leaf rust severity scores, respectively. Genotype AB and NA represent heterozygous alleles and no genotype calls, respectively. Different colors in the left panel represent the different groups of species in the germplasm.

Appendix 17 QTNs located within flanking markers of Lr resistance genes

Appendix 17A Quantitative trait nucleotides (QTNs) located within flanking markers of leaf rust (Lr) resistance genes positioned onto the Chinese Spring reference genome sequence v1.0 (IWGSC 2018).

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¹IT, infection type; LRS, leaf rust severity; SP, spring panel; WP, winter panel; MDN, Morden; OTT, Ottawa; SK, Saskatoon ²M1, mrMLM; M2, pLARmEB; M3, ISIS EM-BLASSO; M4, pKWmEB; M4; M5, FASTmrMLM; M6, FASTmrMLM; M7, MLM; M8, RTM

³ KW, Kruskal–Wallis statistical test; significance levels "ns", "*", "**", "***" and "****" correspond to not significant and P-values ≤ 0.05, 0.01, 0.001 and 0.0001, respectively

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Appendix 17B Quantitative trait nucleotides (QTNs) located within the same linkage disequilibrium block (LDB) as leaf rust (Lr) resistance genes positioned onto the Chinese Spring reference genome sequence v1.0 (IWGSC 2018).

 1 Intra-block D' were calculated by taking the average of all pairwise D' in a haplotype block

 2 IT, infection type; LRS, leaf rust severity; SP, spring panel; SK, Saskatoon; AL, all locations

3 M1, pLARmEB; M2, FASTmrMLM; M3, FASTmrEMMA; M4, ISIS EM-BLASSO; M5, pKWmEB; M6, MLM; M7, RTM

 4 KW, Kruskal–Wallis statistical test; significance levels "ns","*", "***", "***" and "****" correspond to not significant and P-values ≤ 0.05, 0.01, 0.001 and 0.0001, respectively

Appendix 17C Quantitative trait nucleotides (QTNs) located within a neighboring linkage disequilibrium block (LDB) of one containing a leaf rust (Lr) resistance gene positioned onto the Chinese Spring reference genome sequence v1.0 (IWGSC 2018).

¹ Neighboring linkage disequilibrium blocks containing the QTN and mapped Lr genes

 2 Inter-block D' were calculated by taking the average of all pairwise D' between the two haplotype blocks

³ IT, infection type; LRS, leaf rust severity; SP, spring panel; SK, Saskatoon

4 M1, pKWmEB; M2, ISIS EM-BLASSO; M3, pLARmEB

 5 KW, Kruskal–Wallis statistical test; significance levels "ns","*" and "**" correspond to not significant and P-values ≤ 0.05 and 0.01, respectively

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Appendix 18 Phylogenetic tree illustrating the relationships between the wild species.

The leaf node labels are the entry number and genome symbol of the Aegilops, Triticum and Haynaldia accessions in the germplasm.