GENOMIC MAPPING OF LEAF RUST AND STEM RUST RESISTANCE LOCI IN DURUM WHEAT AND USE OF RAD-GENOTYPE BY SEQUENCING FOR THE STUDY OF POPULATION GENETICS IN PUCCINIA TRITICINA

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Leaf rust, caused by *Puccinia triticina* Erikss. (*Pt*), and stem rust, caused by *Puccinia graminis* f. sp. *tritici* Erikss. and E. Henn (*Pgt*), are among the most devastating diseases of durum wheat (*Triticum turgidum* L. var. *durum*). This study focused on the identification of *Lr* and *Sr* loci using association mapping (AM) and bi-parental population mapping. From the AM conducted on the USDA-National Small Grain Collection (NSGC), 37 loci associated with leaf rust response were identified, of which 14 were previously uncharacterized. Inheritance study and bulked segregant analysis on bi-parental populations developed from eight leaf rust resistance accessions from the USDA-NSGC showed that five of these accessions carry single dominant *Lr* genes on chromosomes 2B, 4A, 6BS, and 6BL. The other three accessions have *Lr* genes with more complex inheritance. All eight accessions carry different genes than those already mapped in durum cultivars except one accession with *Lr61*. Linkage mapping in two of the bi-parental populations showed that the gene in PI 209274 (*LrCA*) was mapped to 6BS between SNPs *IWA3298* and *IWB39456*, while the gene in PI 192051 *(LrPort*) was mapped to 4AL, flanked by *IWA4254* and *IWA8341*. Resistance to *Pgt*-race TTKSK was also observed in PI 534304 and PI 192051. PI 534304 was found to carry *Sr13* on chromosome 6AL, while PI 192051 carries a novel *Sr* gene (*SrPort*) mapped to 7AS flanked by *IW8390* and *IWA1805*. The genotype PI 192051 has an additional QTL (*QSr.ndsu-5B*) to *Pgt* races in a field trial in Ethiopia in 2016. The *QSr.ndsu-5B* was mapped to 5BL and delimited by *IWA6992* and *IWA2181*. The study of virulence diversity in *Pt* isolates collected from several countries identified seven races among 51 isolates collected from durum wheat and 21 races among 40 isolates collected from common wheat. The phylogeny study on 30 *Pt* isolates based on the Restriction-Associated DNA (RAD)-Genotype by Sequencing (GBS), clustered the isolates into eight clades, with higher

diversity in the SNP genotypes in common wheat isolates compared to that in durum wheat isolates. RAD-GBS is identified as a suitable and informative genotyping technique to study the population genetics of *Pt*.

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DEDICATION

To my beloved parents Ibrahim Aoun and Mahbouba Aoun and to my brothers and sisters

Mouna, Mohamed, Marwa, Maha, Makrem, and Mehdi who always inspire me.

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GENERAL INTRODUCTION

Wheat is the most widely grown cereal crop (Snape and Pánková 2007) in more than 122 countries (FAOSTAT 2015). It accounts for over 35% of the world food sources and provides 20% of proteins and calories to humans (Braun et al. 2010; Hawkesford et al. 2013). Common wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L. var *durum* (Desf.) Husn.) are the two main commercial types of wheat. Wheat (common wheat and durum wheat) is grown on approximately 215 million hectares annually, with an estimated production of 700 million metric tons (Singh et al. 2011a; North Dakota Wheat Commission 2016). Durum wheat occupies around 17 million hectares with a production of 38.5 million metric tons. Durum wheat is an important crop, used for making a range of products such as pasta, couscous and flat bread. Durum wheat is produced in the Mediterranean countries including Southern Europe (Italy, France, Spain, and Greece), West Asia (Turkey), and North Africa (Tunisia, Morocco, and Algeria), in North and South America (Canada, USA, Mexico, Chile, and Argentina), and in Ethiopia (Ordoñez and Kolmer 2007b; Habash et al. 2009; Goyeau et al. 2012). Half of the durum producing areas are localized in developing countries, where durum represents a major staple food. Durum wheat is more adapted to drought, and marginal soils compared to common wheat. Therefore, improvement of durum production should be a priority to ensure food security.

The US produces more than 2 million tons of durum wheat annually mainly from North Dakota and Montana. These areas are characterized by long warm days, cooler summer nights, moderate rainfalls, and dry harvest period which provide the necessary environmental conditions for durum wheat to thrive. North Dakota is the leader in durum production in the US with approximately 650,000 hectares grown annually. North Dakota contributes more than 50% of the

total US durum production, valued at over $300 million per year (NASS 2016). Other states producing durum in the US are California and Arizona.

Durum wheat production is facing both abiotic (mainly climate change and drought) and biotic stresses that continue to reduce the potential of the crop. Leaf rust and stem rust are major biotic constraints challenging the durum production globally. For instance, over the last 15 years, susceptibility to leaf rust has increased in several durum wheat producing countries, due to the emergence of highly virulent races on durums (Singh et al. 2004; Martinez et al. 2005; Goyeau et al. 2006; Ordoñez and Kolmer 2007a; Huerta-Espino et al. 2009; Goyeau et al. 2012). A highly virulent race (BBG/BN) on durum wheat appeared in Mexico in 2001 and overcame the resistance of widely grown cultivar Altar C84 (Singh et al. 2004). Similar race in virulence phenotype designated as BBBQJ appeared later in California (2009) and recently in Kansas (2013) (Kolmer 2013, 2015a). This race may spread, via the "*Puccinia* pathway" to ND where over 50 % of the US durum is produced. Similarly, stem rust pathogen races that appeared in East Africa are another major threat to the global durum production. These races include race TTKSK and its variants (Ug99) and other race lineages such as JRCQC, TRTTF, RRTTF, and TKTTF (Pretorius et al. 2000; Olivera et al. 2012; Olivera et al. 2015; Singh et al. 2015).

Genetic resistance is the best rust management strategy as it is economical and environmentally friendly. However, the genetic resistance to rust in durum wheat is not well studied and only few genes have been catalogued in this crop. On the other hand, there is a continuous virulence evolution of rust pathogens to deployed resistance genes. Therefore, it is important to identify new leaf rust and stem rust resistance genes to broaden genetic diversity of rust resistance in durum wheat. However, in most breeding programs, there is a bottleneck of genetic diversity as a result of decades of breeding selection for specific traits. This significantly

has limited the number of resistance sources available to breeding programs (Tanksley et al. 1997). Several studies reported that wheat landraces are reliable genetic resources to identify new or underutilized resistance genes to several diseases including wheat rusts because landraces have rich genetic ancestry and exposed to less selection pressure compared to breeding lines and cultivars (Akhunov et al. 2009; Newton et al. 2010; Bux et al. 2012; Newcomb et al. 2013; Zurn et al. 2014; Kertho et al. 2015). Even though incorporation of resistance genes from landraces is time consuming through traditional breeding, the process can be shortened using marker assisted selection (MAS).

The USDA-ARS National Small Grains Collection (NSGC) at Aberdeen, ID, maintains a global collection of over 142,000 diverse accessions of small grains. (http://www.ars.usda.gov/main/docs.htm?docid=2884). The NSGC includes 8,325 durum wheat accessions of which 5,700 are landraces, while the rest are breeding lines and cultivars. These durum accessions were collected from 80 countries. A core subset of 782 accessions was randomly selected, from different geographical regions (Chao et al. 2016). A sub-set of 496 randomly selected accessions were genotyped using the Illumina iSelect 9K wheat array (Cavanagh et al. 2013) through the Triticeae Coordinated Agricultural Project. This diverse collection with high number of landraces is suitable for identifying new sources of resistance to several diseases including rusts.

The objectives of this dissertation were to 1) identify sources of resistance to leaf rust in durum wheat USDA-NSGC and SNP markers associated with leaf rust response using genome wide association mapping; 2) characterize the inheritance and genomic location of leaf rust and stem rust resistance genes using biparental populations in which the resistant parents were selected from the durum wheat USDA-NSGC; 3) investigate the virulence diversity of *P.triticina*

isolates collected from several countries and assess the application of RAD- GBS for the study of genetic diversity of *P. triticina* populations.

CHAPTER I. LITERATURE REVIEW

Evolution of durum wheat

Durum wheat belongs to grass family (Poaceae) which evolved 50-70 million years ago. This family includes three sub families namely, Pooideae, Panicoideae, and Ehrhartoideae. Durum wheat belongs to the sub-family of Pooideae and the tribe of Triticeae (Huang et al. 2002; Levy and Feldman 2002). The Triticeae includes other important food crops such as common wheat, barley, oats, and rye (Löve 1984). Sorghum, maize, and sugarcane fall in the sub family of Panicoideae while Brachypodium and rice are categorized in the sub family of Ehrhartoideae (Kellogg and Buell 2009).

Despite variation in the ploidy levels of all these grasses in the family of Poaceae (from 2x to 10x), the comparative mapping showed conserved syntenic relationships between genomes of different grass species (Moore et al. 1995). This suggests that all the grass species most likely evolved from a common ancestor through genome duplication and chromosome fusion (Bolot et al. 2009). The evolutionary relationship between the different grasses species helps in understanding the genome evolution and also aids in the assembly of genome sequences of different members of the grass families (Luo et al. 2007).

Different species, of different ploidy levels, make up the genus *Triticum*. *Triticum monococum* L. (einkorn wheat: 2n=14, AA genome) and *Triticum urartu* Tumanian ex Gandilyan (2n=14, AA genome) are diploid members while the *Triticum turgidum* L. (2n=28, AABB genome), *Triticum timopheevii* (Zhuk.) Zhuk. (2n=28, AAGG genome) are classified as species with tetraploid ploidy level. The *Triticum* with hexaploid ploidy levels are *Triticum aestivum* L. (2n=42, AABBDD genome) and *Triticum zhukovskyi* Menabde & Ericz (2n=42, AAAAGG genome). The *Triticum urartu* exists only as wild form, whereas the two hexaploid

species *T. aestivum* and *T. zhukovskyi* exist only as cultivated form. The other three species, *T. monococcum*, *T. turgidum* and *T. timopheevii* have both wild and cultivated forms (Matsuoka 2011).

Around 300,000-500,000 years ago, spontaneous hybridization occurred between the wild diploid wheat *T. urartu* (A genome) with the B genome donor that was likely derived from *Aegilops speltoides* (2n=2x=14, SS genome). This hybridization was followed by chromosome doubling which resulted in the allotetraploid wild emmer (*T. turgidum* ssp. *dicoccoides*, 2n=4x=28, AABB genome) (Huang et al. 2002; Levy and Feldman 2002; Petersen et al. 2006; Marcussen et al. 2014).

About 12,000 years ago, hunter-gatherers began to cultivate wild emmer and initiated selection for desired traits like non-shattering grains or non-brittle rachis, thus gradually creating a cultivated emmer (*T. dicoccum*, 2n=4x=28, genome AABB). About 8,500 years ago, natural mutation changed the ears of emmer to a more easily threshable type which later evolved into the free-threshing ears of durum wheat (Dvorak et al. 2006). The common wheat or bread wheat, called also hexaploid wheat (*T. aestivum*, 2n=6x=42, AABBBDD genome) was most probably the result of natural hybridization about 9,000 years ago between cultivated emmer (AABB) and the diploid goat grass *Aegilops tauchii* (2n=2x=14, DD genome), followed by chromosome doubling (Levy and Feldman 2002; Salamini et al. 2002; Matsuoka 2011). Phylogenetic analyses of wheat genome sequences showed that the wheat genome resulted not only from hybridization and allopolyploidization of A, B, and D genomes but also from the hybridizations that had occurred between the ancestors of the three genomes (Marcussen et al. 2014). It was discovered that the A and B genomes diverged from a common ancestor around 7 million years ago.

Hybridization between A and B genome lineages ~5.5 million years ago created the D genome lineage through homoploid hybrid speciation (Marcussen et al. 2014).

The genome of durum wheat contains 14 pairs of chromosomes, 7 pairs for each of the genomes A and B. Durum wheat is characterized by extensive homoeologous alleles between the chromosomes in the two genomes (Nachit et al. 2001). The genome sizes differ for the members of the grass family, from 450 Mb for rice to 16,000 Mb for hexaploid wheat (Arumuganathan and Earle 1991). The genome size of durum wheat is estimated to be 11,660 Mb (Bennett and Leitch 2010) which is around 26 fold larger than the rice genome. The variation of the genome sizes between the members of the grass family is in part due to differences in ploidy level and the amount of repetitive DNA present in each species (Keller et al. 2005).

Origin and domestication of durum wheat

Archaeological, morphological, cytological, and genetic studies have showed that all species in *Triticum* originated from the Fertile Crescent of the Near East. This region presently includes the eastern Mediterranean, Turkey, Iraq, Iran, and the regions of the Transcaucasus (Mastuoka 2011). Ethiopia is considered one of the centers of genetic diversity for tetraploid wheat (Vavilov 1951), where landraces of tetraploid emmer wheat have been grown for thousands of years. Currently, in Ethiopia, common wheat and durum wheat are cultivated in close proximity.

The genetic analysis of wheat domestication was studied by the use of a mapping population developed from wild emmer wheat and the cultivated tetraploid durum wheat cultivar, ‘Langdon’ (Peng et al. 2003; Peleg et al. 2011). Peng et al (2003) pointed out that for almost all the studied domestication traits (brittle rachis, heading date, plant height, grain size, and yield), the number of QTL mapped on the A genome was higher or equal to those mapped

on the B genome, suggesting that the A genome of wheat most likely played higher role in domestication evolution than the B genome.

Genetic improvement of traits such as tough rachis, loose glumes, thick stems, high number of grains per spike, rapid and uniform germination, and large seed size are probably the most important signs of domestication (Ayal et al. 2005). The wild emmer wheat possessed tough glumes and fragile rachis that made them susceptible to shattering. Upon domestication, genetic modifications resulted in non-shattering phenotypes, which allowed early farmers to harvest the grains more efficiently (Faris and Gill 2002; Ayal et al. 2005). The final and probably the most important sign of domestication was the free-threshing ears (Dvorak et al. 2006). However, all these domestication events have altered the genetic diversity of tetraploid wheat (Thuillet et al. 2005). It is therefore necessary to pursue the domestication process to determine the functional and regulatory genes that were eliminated from the durum wheat during the domestication process. Although wild emmer wheat possesses agronomically deleterious features, it also carries beneficial traits such as resistance to biotic and abiotic stresses, high protein content, high tillering capacity and yield, short stature, and early maturity (Cakmak et al. 2004; Uauy et al. 2006).

Dissemination of domesticated durum wheat

After the domestication event, the cultivation of tetraploid emmer wheat (*T. diccocum*) extended through the Mesopotamian plain and expanded to India, and westward through Anatolia to the Mediterranean coastal region (~8000 years ago), to Balkans and Danube (~7000 years ago), and Europe (~7000 years ago). Cultivated tetraploid wheat reached the United Kingdom and Scandinavia by ~ 5000 years ago, and then was introduced to Central Asia and China by about ~3000 years ago via Iran. Cultivated emmer wheat was introduced to Africa via

Egypt (Mastuoka 2011). Currently, the most widely cultivated tetraploid wheat is durum wheat which was derived from cultivated emmer wheat in the Eastern Mediterranean region (Mastuoka 2011).

In 1521, durum wheat seeds were introduced to Mexico by Spanish missionaries. Later in the 1600s, the explorers, traders, settlers, and soldiers transported seeds of durum wheat to California. During the same time, the immigrants from Europe carried with them durum seeds and cultivated durum in the eastern US. The Mennonites who immigrated from Southern Russia and settled in the Central Great Plains were the first to introduce durum wheat to the United States (Damania 2013). Although durum wheat was introduced to the US at various time points, it had failed to become a commercial crop because of poor adaptation to humid conditions of the East and also lack of market for the produce (Ball 1930). This was further complicated by the lack of proper equipment to process and mill the hard grain of durum wheat (Paulsen and Shroyer 2008).

Commercialization of durum wheat in the USA took effect when it was reintroduced to the farmers after 1900 when the United States Department of Agriculture collected and evaluated many varieties from the around world. The superior yield and resistance to stem rust of durum wheat compared to the hexaploid hard red spring wheat resulted in a rapid gain of durum production (Olmsted and Rhode 2011). The increased durum production in the Northern Great Plains coincided with a failure of production in Europe in 1911, which allowed the farmers to sell durum wheat at premium prices over hard red spring wheat (Isern 2000). A durum breeding program was established at North Dakota State University in 1929, and it remains the only public durum breeding program in the United States.

The market price of durum wheat is always higher compared to other wheat classes. For example, the US prices of durum wheat in 2015 and 2016 were 6.50–9.16 $/bu compared to 4.61-5.18 $/bu for the hard red spring wheat (NASS 2016). However, the world durum production is challenged by a number of abiotic and biotic constraints including fungal diseases such as Fusarium head blight, rusts, septoria leaf blotch, root rot, tan spot, ergot, and powdery mildew.

Wheat rusts

Taxonomy and disease cycle

The leaf rust caused by *Puccinia triticina* Erikss., stripe rust caused by *Puccinia striiformis* Westend. f.sp. *tritici* Erikss., and stem rust caused by *Puccinia graminis* f.sp. *tritici* Erikss. & E. Henn. have historically been diseases of great importance on wheat. These three diseases significantly influenced the development of human civilization (McIntosh et al. 1995). The influence of rust on earlier civilization was more anchored on religious connotation with limited knowledge on the biology, ecology, and epidemiology of rust diseases. Progressively over generations, the understanding of rust diseases has grown in folds beyond religious believes (Chaves et al. 2008). The three rust diseases continue to affect wheat production in many regions of the world. Because urediniospores are wind-borne, they can be dispersed widely with great potential to devastate large acreage of wheat at a continental scale over short period of time (Roelfs et al. 1992).

Rust pathogens are obligate biotroph thriving on living plant tissue to survive and complete their life cycle (Hovmoller et al. 2011). Rust fungi can infect wheat at all plant growth stages by extracting nutrients from mesophyll cells, resulting in increased respiration and transpiration causing decline in the rate of photosynthesis. Low growth vigor, reduced yield, and

lighter kernel weight are among the characteristics of plants infected by rust diseases (Mathre 1985). Despite the great progress made for rust management, rusts are still the most important wheat diseases (Mathre 1985; Huerta-Espino 1992).

Rust fungi belong to the class of Basidiomycetes, known by septate hyphae, dolipore septa connecting the hyphal cells, and the production of basidiospores (Gäumann 1928). Further down the taxonomy ladder, rust fungi are in the order of Uredinales (Gäumann 1928; Aurthur 1934; Savile 1984), producing five spore stages, namely, urediniospores, teliospores, basidiospores, pycniospores, and aeciospores. The rust fungi affecting cereal plants belong to the family of Pucciniacea. The members of this family are known to have stalked teliospores (Aurthur 1934). Seventeen genera with distinct morphological differences make up the family of Pucciniacea. Cereal rust pathogens are in the genus *Puccinia* characterized by septate teliospore with two cells (Aurthur 1934). The classification of species within *Puccinia* are based on the host and symptoms on the plant (Aurthur 1934; Savile 1984).

Wheat rust pathogens are polycyclic, macrocyclic, and heteroecious with five spore stages in their life cycle. The pathogens complete their sexual and asexual part of their life cycle on two taxonomically unrelated host species (Peterson 1974; Roelfs et al. 1992; Leonard and Szabo 2005; Jin et al. 2010). The repeating asexual cycles occur on the primary hosts with the production of dikaryotic (n+n) urediniospores in a structure called uredinia (Peterson 1974; Roelfs 1985; Roelfs et al. 1992; Anikster et al. 2005; Leonard and Szabo 2005). Once wheat growing season comes to an end, melanized and thick wall teliospores are formed in a structure called telia. Teliospores are at the beginning dikaryotic (n+n), then shortly change to diploid (2n) through a process called karyogamy. The teliospores overwinter in warmer climates then germinate in the spring and undergo meiosis to produce four haploid (n) basidiospores (Roelfs

1985; Bolton et al. 2008). In the presence of alternate host (mainly *Berberis* spp. for *P. striiformis* f.sp. *tritici* and *P. graminis* f.sp. *tritici*; and *Thalictrum* spp. for *P. triticina*), the wheat rust pathogens go through the sexual cycle. The ejected basidiospores infect the alternate host to form pycnium on the upper side of the alternate host leaf. The haploid pycniospores fertilizes the opposite mating type which is the receptive haploid hyphae. Subsequently, the dikaryotic hyphae (n+n) germinates and form acia on the lower side of the alternate host leaf. The resulting dikaryotic aeciospores infect the primary host and the life cycle starts over again (Roelfs 1985; Bolton et al. 2008).

Leaf rust

Leaf rust importance and symptoms

Leaf rust is the most widely distributed and common disease of the three types of wheat rusts and continues to be a threat to wheat production in many countries (Knott 1989). Symptomatically, the leaf rust appears as round lesions, harboring orange to brown urediniospores that are scattered over leaf blades and sometimes on leaf sheaths (Roelfs et al. 1992; McCallum et al. 2007). Due to wide geographical distribution and frequent disease occurrence, leaf rust causes significant losses (Huerta-Espino et al. 2011). It was reported that in every 1% increase in leaf rust severity, the yield reduces by 1% (Khan et al. 1997). Yield losses due to leaf rust in susceptible wheat cultivars is around 5–15% (Roelfs 1988) or higher, depending on environmental conditions and the stage of plant growth when the initial leaf rust infections occur (Chu et al. 2009). Yield reductions are higher if the infections occur on the flag leaf (Chester 1946). Leaf rust is globally distributed with diverse races that are continuously evolving to form new virulence phenotypes (Kolmer 2005) which makes the management of leaf rust challenging.

Puccinia triticina hosts, biology, and epidemiology

Puccinia triticina is thought to have originated from southwest Asia, somewhere in the Fertile Crescent (Arthur 1929). The primary telial hosts of *P. triticina* are: common hexaploid wheat, tetraploid durum wheat, wild emmer wheat, cultivated emmer wheat, Triticale (X *Triticosecale)*, common goatgrass (*Aegilops cylindrical*), and *Ae. speltoides* (Bolton et al. 2008). Interestingly, the isolates infesting durum wheat and *Ae. speltoides* are host specific and differ from those infecting common wheat, suggesting that these two groups may be two different formae speciales (Yehuda et al. 2004; Goyeau et al. 2006; Bolton et al. 2008).

The alternate host of *P. triticina* is mainly *Thalictrum speciosissimum* L. (meadow rue) (Mains and Jackson 1921), a species native to Spain and Portugal. In addition, sexual reproduction on *Thalictrum* spp. was observed in Northeast Kazakhstan (J.A. Kolmer, unpublished) and in Siberia on *Isopyrum fumarioides* (Chester 1946). However, the knowledge on their contribution in the disease epidemiology and virulence phenotypes is rather limited. In most of the other wheat growing regions around the world, the alternate hosts are geographically isolated or resistant to leaf rust. Consequently, the sexual recombination does not contribute significantly to the pathogen virulence diversity (Bolton et al. 2008; Kolmer 2013). Therefore, *P. triticina* is thought to reproduce asexually through production of dikaryotic urediniospores, cycling on wheat and its relatives.

Puccinia triticina is a well-adapted pathogen to different climates (Kolmer 1996). Spore germination, spore movement, and leaf rust infection severity can change from one season to another, depending on prevailing environmental conditions. Temperatures of 10–25°C and presence of adequate moisture on the leaf surface are conducive for infections (Anikster 1986). Depending on prevailing environmental conditions, the uredinial cycle is repeated every 8 to 20

days (Chester 1946). Favorable conditions induce urediniospore germination. This will occasion the germ tube to elongate perpendicularly to the epidermal cells upon sensing the leaf topography (thigmotropism) until a stoma is encountered. Thereafter, an appressorium is formed followed by a penetration peg (Dickinson 1969; Bolton et al. 2008). In the case of a susceptible host, a compatible interaction between the host and the pathogen takes place leading to formation of haustoria. Haustoria will be used to channel nutrient from the plant living cells to the pathogen. The uptake of nutrients from infected cells enables the infection to spread locally throughout the leaf tissue. In the case of resistant host, an incompatible interaction occurs, and the haustorium development is aborted or the rate of haustorium formation is slowed down (Roelfs et al. 1992).

In the United States, *P. triticina* infects winter wheat and volunteer wheat in the Southern states in the fall then the urediniospores overwinter on the crop. This is known as a "green bridge" (Roelfs et al. 1992). In the following spring season, when the environmental conditions become favorable, *P. triticina* continuously produces huge number of urediniospores that will then be blown Northwards. This is referred to as "*Puccinia* pathway". The wind transported urediniospores infect spring wheat crops in the southern and northern Great Plains of the United States, leading to significant yield losses as result of infections (Kolmer 2013).

Population genetics of Puccinia triticina

The study of the genetic diversity in *P. triticina* populations is very critical to the success of wheat breeding programs. Knowledge on virulence/avirulence diversity will enable breeders to select and deploy suitable sources of leaf rust resistance in wheat cultivars (Kolmer 1996; McCallum et al. 2010). The key factors influencing diversity of clonal *P. triticina* populations are mutation, genetic drift, host selection, and gene flow. Parasexual recombination (somatic

recombination) of *P. triticina* in the field has been reported in Australia (Park et al. 1999). Mutation is the primary source of variation in *P. triticina* populations resulting in the appearance of new virulence phenotypes (or races) against deployed resistance genes after few years of release of resistant wheat cultivars (Ordoñez and Kolmer 2007a).

The variation in *P. triticina* isolates was traditionally assessed using a set of wheat cultivars with each carrying unknown resistance genes. Later on, a differential set of 'Thatcher' near isogenic lines (NILs) each differing for a single leaf rust resistance (*Lr*) gene was developed by Dr. Peter Dyck. Currently an international differential set containing five sets of Thatcher near isogenic lines carrying different *Lr* genes are used for the isolate phenotyping. The first set of NILs are known to carry *Lr1* (isogenic line RL6003), *Lr2a* (RL6000), *Lr2c* (RL6047), and *Lr3a* (RL6002) genes; the second one are lines with *Lr9* (RL6010), *Lr16* (RL6005), *Lr24* (RL 6064), and *Lr26* (6078) genes; the third set includes lines with genes *Lr3ka* (RL6007), *Lr11* (RL6053), *Lr17* (RL6008), and *Lr30* (RL6049); the fourth set includes NILs with genes *LrB* (RL6047), *Lr10* (RL6004), *Lr14a* (RL6013), and *Lr18* (RL6009) while the fifth set comprises of NILs known to carry *Lr3bg* (RL6042), *Lr14b* (RL6006), *Lr20* (RL 6092), and *Lr28* (RL6079).

Based on the combination of high and low infection types (ITs) on the 20 aforementioned Thatcher NILs, five-letter designation are assigned to the isolate following the nomenclature of Long and Kolmer (1989). Virulence surveys are conducted annually in the US, Canada, and Australia as well as in other countries to monitor the races present in a particular location and detect the possible appearance of new virulence phenotypes. Generally, *Puccinia triticina* population is highly diverse for virulence toward existing *Lr* genes in wheat. In North America, over 50 virulence phenotypes are collected from common wheat annually (McCallum et al. 2007; Kolmer 2013; Hughes and Kolmer 2016).

Genotypic assessment of the diversity of *P. triticina* populations have been done using several types of molecular markers. The markers give insights into the origin and spread of *P. triticina* genotypes. The Random Amplified Polymorphic DNAs (RAPD) markres were the first markers used in studying the genetic variation of *P. triticina* populations (Kolmer and Liu 2000; Park et al. 2000). However, RAPD marker could not effectively differentiate between *P. triticina* isolates with different virulence phenotypes (Kolmer et al. 1995). Thereafter Amplified Fragment Length Polymorphisms (AFLPs), were used and they provided improved separation between isolates compared to RAPD markers (Kolmer 2001). Both The AFLPs and RAPDs are dominant markers. Thus, these markers provide only the phenotypes of the *P. triticina* isolates because the urediniospores are dikaryotic (n+n). This led to the subsequent use of codominant Simple Sequence Repeats (SSRs) also known as microsatellites to genotype *P. triticina* isolates (Duan et al. 2003). The SSRs are easier to score, provide more repeatable results, and can distinguish multiple alleles at single loci. As such SSR markers are considered to provide more accurate genotypic data that can be used to make comprehensive assumptions on the evolution and connectivity within and among *P. triticina* populations worldwide. Currently 23 polymorphic SSRs (Duan et al. 2003; Szabo and Kolmer 2007) are in use for genetic diversity studies of *P. triticina* populations. Strong correlation between SSR genotypes and virulence phenotypes were observed in *P. triticina* clonal population (Ordoñez and Kolmer 2007b, 2009; Ordoñez et al. 2010; Kolmer et al. 2011, 2013; Kolmer 2015b; Kolmer and Acevedo 2016).

Ordoñez and Kolmer (2009) identified six distinct clusters in North American *P. triticina* population based on SSRs. Isolates within the same SSR group were related for virulence phenotypes to several *Lr* genes present in Thatcher NILs. Isolates collected from common wheat in South American countries like Argentina, Brazil, Chile, and Uruguay were similar for

SSR genotypes to those collected from North America (Ordoñez et al. 2010). This suggests common ancestor and intercontinental migration of *P. triticina* races.

The worldwide clonal populations of *P. triticina* are characterized with high levels of heterozygosity compared to expected heterozygosity levels (under random mating), higher linkage disequilibrium between SSRs, and presence of strong correlation between virulence phenotypes and SSR genotypes. This opposes what we expect in a sexually reproducing population where observed heterozygosity levels are similar to the expected levels, low linkage disequilibrium between SSR markers was observed in addition to no significant correlation between virulence phenotypes and SSR genotypes (Kolmer 2013).

Characteristics of P. triticina populations in durum wheat

Susceptibility of durum wheat to leaf rust became a significant problem over the last 15 years because of the emergence of new highly virulent races on durum wheat cultivars (Singh et al. 2004; Goyeau et al. 2006; Huerta-Espino et al. 2009; Goyeau et al. 2012). In 2001, *P. triticina* race BBG/BN appeared in northwestern Mexico and overcame the resistance of widely adapted CIMMYT durum wheat cultivars, causing yield losses estimated at US32$ million during 2001–2003 crop seasons (Singh et al. 2004). The race nomenclature of BBG/BN was based on avirulence/virulence profile on *Lr* genes present in five sets. Set 1-to-3 were as described by Long and Kolmer (1989), Set 4 included lines with genes *Lr3bg*, *Lr13*, *Lr15*, and *Lr18* while set5 contained lines with genes *Lr10*, *Lr19*, *Lr23*, and *Lr27+31* (Singh 1991). This race overcame the resistance conferred by *LrAltar*, later designated as *Lr72* (Herrera-Foessel et al. 2014). A similar race in virulence phenotype to the Mexican race, BBG/BN, was collected first in California, and more recently on the hard red winter wheat cultivar ‘Overley’ in Kansas (Kolmer 2015a). This race was designated as BBBQJ. Race BBBQJ is virulent to *Lr39/41* that is found in many hard

red winter wheat cultivars grown in the southern Great Plains. This race could migrate northward to the durum producing region of North Dakota and Canada (Kolmer 2015a). Susceptibility of durum wheat to leaf rust was also detected in the entire Mediterranean basin, Chile, Ethiopia, and USA (Singh et al. 2004; Martinez et al. 2005; Goyeau et al. 2006; Ordoñez and Kolmer 2007a, b; Goyeau et al. 2012; Kolmer and Acevedo 2016).

Several studies indicated that *P. triticina* populations collected on durum wheat cultivars are different in virulence phenotypes and SSR genotypes from isolates infecting common wheat (Huerta-Espino and Roelfs 1992; Ordoñez and Kolmer 2007a, b). The majority of *P. triticina* isolates collected from durum wheat worldwide belong to the physiological race BBB-- based on the nomenclature system of Long and Kolmer (1989). These isolates exhibit avirulence on most of the *Lr* genes present in common wheat (Singh 1991; Huerta-Espino and Roelfs 1992; Ordoñez and Kolmer 2007a, b; Kolmer and Acevedo 2016). This is different from the races of common wheat type isolates that are virulent on several *Lr* genes. In addition, there was limited variation of virulence phenotypes and SSR genotypes among durum wheat specific races. For instance, virulence exhibited by *P. triticina* isolates on durum wheat in Mexico were very similar to virulence phenotypes and SSR genotypes of isolates occurring on durum wheat in Argentina, France, Mexico, Spain, and Southern United States (California), suggesting a common ancestor (Ordoñez and Kolmer 2007a, b). However, a distinct virulence phenotype and SSR genotype, collected on tetraploid wheat (Emmer and durum) was observed in Ethiopia. Unlike all other isolates found on durum and common wheat type isolates worldwide, these Ethiopian tetraploid type isolates are avirulent on the common wheat Thatcher (designated as race EEEEE) (Huerta-Espino and Roelfs 1992; Ordoñez and Kolmer 2007a, b; Liu et al. 2014; Kolmer and Acevedo 2016). Ethiopia being the center of diversity of tetraploid wheat is known for diverse genetic

pool of wheat (Vavilov 1951). This may have helped in selection and maintenance of EEEEE isolates (Kolmer and Acevedo 2016).

Leaf rust resistance in durum wheat

Genetic resistance is the most sustainable, cost effective, and environmentlly friendly management strategy to wheat leaf rust. The seedling resistance and adult plant resistance (APR) are the two main groups of leaf rust resistance. Seedling resistance genes confer resistance at all stages (at seedling and adult plant stages). However, these genes are generally race specific and are vulnerable to the rapidly evolving leaf rust pathogens (Lagudah 2011). Adult plant resistance genes cannot be detected at seedling stage and are expressed optimally at adult plant stage. The APR gene could further be divided into race-specific APR genes and slow rusting race non-specific resistance genes.

Race-specific APR genes are characterized by low infection type accompanied by hypersensitive response (Kolmer 2013). Slow rusting race non-specific APR genes are characterized by partial resistance to many races, lack of hypersensitive response and are considered to be more durable (Singh et al. 2011b). Race non-specific resistance is associated with extended latency period, increased number of aborted colonies as result of necrosis, and reduced size of uredinium (Caldwell 1968; Lagudah 2010; Soleiman et al. 2013). The genes *Lr34*, *Lr46*, *Lr67*, and *Lr68* are examples of slow rusting genes in common wheat (Singh et al. 1998; Hiebert et al. 2010; Herrera-Foessel et al. 2012).

Currently, 77 resistance loci have been designated and mapped to specific chromosomes in wheat (McIntosh et al. 2014; Bansal et al. 2016). Only few of these catalogued *Lr* genes were mapped in durum wheat. After the leaf rust epidemic in Mexico, a number of major race-specific *Lr* genes were mapped in the CIMMYT durum wheat cultivars. These genes included the

complementary gene pair *Lr27+31* mapped in the cultivar Jupare C2001 (Singh and McIntosh 1984a; Singh and McIntosh 1984b; Singh et al. 1993; Herrera-Foessel et al. 2005), *Lr3a* mapped in the cultivar Storlom (Herrera-Foessel et al. 2007b) and a linked gene designated as *LrCamayo*, mapped in the cultivar Camayo (Herrera-Foessel et al. 2007b), *Lr14a* found in the cultivars Llareta INIA and Somateria (Herrera-Foessel et al. 2008a), *Lr61* found in the cultivar Guayacan INIA (Herrera-Foessel et al. 2008b), and *Lr72* mapped in the cultivar Altar C84 and Atil C2000 (Herrera-Foessel et al. 2014).

Other genes in durum include *Lr23* that was mapped in the durum cultivar Gaza and thought to be frequently present in durum (McIntosh and Dyck 1975; Nelson et al. 1997). In addition, some genes mapped in common wheat have been postulated in durum wheat, including *Lr10* (Aguilar-Rincon et al. 2001), *Lr13* (Singh et al. 1992), *Lr16* and *Lr17a* (Zhang and Knott 1990). It is possible that genes *Lr53* (Marais et al. 2003) and *Lr64* (Kolmer 2008), originated in wild emmer wheat may occur in durum wheat.

Unfortunately, virulent races to most of the identified genes in durum are present. For instance, virulence to *Lr23* and *Lr10* is common among the current durum wheat specific races (Huerta-Espino and Roelfs 1992; Ordóñez and Kolmer 2007a). Additionally, virulence to almost all the *Lr* genes that were identified after the detection of race BBG/BN in 2001 occurred just few years after their deployment. For instance, race BBG/BN and its variants are virulent to *Lr72* (Singh et al. 2004; Huerta-Espino et al. 2011). However, *Lr72* is thought to protect many of the durums against the common wheat type races (Herrera-Foessel et al. 2014). The durum type races BBG/BP and CBG/BP identified in Mexico showed virulence to *Lr27+Lr31* with latter race showing additional virulence to *Lr3a* (Huerta-Espino et al. 2009a, b). The old Mexican durum wheat specific race BBB/BN with an additional virulence to *Lr61* (BBB/BN_ *Lr61*vir)

was collected in Mexico in 2010 (J. Huerta Espino, unpublished). Similarly virulence to *Lr14a* in races collected in France, Spain, Chile, Argentina, Tunisia, and Ethiopia have been reported (Ordoñez and Kolmer 2007a; Goyeau et al. 2012 ; Gharbi et al. 2013; Soleiman et al. 2016). With this trend of leaf rust spread and virulence, durum wheat programs are urgently in need of new *Lr* genes.

Adult plant resistance (APR) genes have also been identified in durum. For example, Zhang and Knott (1993) identified a dominant and a recessive gene conferring APR to race15 in Canadian cultivars Lloyd and Pelissier, respectively. Loladze el al (2014) reported that the cultivar Gaza carries both seedling resistance gene and APR gene to race BBG/BP. Little is known about the genetic basis of slow-rusting resistance in durum wheat. Singh et al. (1993) studied adult plant resistance to an old Mexican race BBB/BN in five CIMMYT durum wheat genotypes 'Kingfisher', 'Diver', 'Mexicali 75', 'Somorguho' and 'Yavaros 79'. At least two to three genes with additive effect were identified with one of the gene being common to all the tested genotypes. With the increased susceptibility of durum wheat in many countries, more research on slow rusting genes was carried out (Herrera-Foessel et al. 2007a; 2008c). For instance, Herrera-Foessel et al (2008c) assessed the genetic basis of slow rusting genes in eight CIMMYT durum lines using the Mexican race BBG/BN. The results showed that the lines 'Playero', 'Planeta', and 'Trile' carry at least three independent *Lr* genes with additive effect, while the slow-rusting resistance in the lines 'Piquero', 'Amic', 'Bergand', 'Tagua', and 'Knipa' was conferred by at least two genes with additive effects. Intercrosses of the eight resistant lines showed transgressive segregation, indicating that some of these genes were not allelic. Since slow rusting genes may be more durable, the goal in most of the durum programs is to develop

durum wheat cultivars carrying slow rusting genes similar to what was accomplished in common wheat breeding programs (Singh et al. 2000).

Wheat stem rust

Importance, symptoms, and hosts

Stem rust is one of the most devastating diseases of common wheat, durum wheat, and barley (*Hordeum vulgare* L.). Severe stem rust infections cause plant to lodge and reduces photosynthetic rate resulting in several yield losses reaching up to 100% (Roelfs 1985a,1992b; Marsalis and Goldberg 2006). Stem rust infections are favored by hot days (25-30°C), mild nights (15-20°C), and humid conditions (Roelfs et al. 1992b; Schumann and Leonard 2000). Eight–ten days after inoculation, diamond shaped brick red lesions (pustules or uredinia) appear. This causes the breakage of the infected host epidermal cells. The uredinia appear mainly on the stem and leaf sheaths, but can also be seen on the leaves and spikes, awns, glumes, and even grains (Leonard and Szabo 2005). In the case where stem pustules appear on the leaves, uredinia generally penetrate to break through the leaf and sporulate on both surfaces.

Puccinia graminis contains seven *formae speciales* (Johnson 1961; Anikster 1984; Niks 1986). However, *Puccinia graminis* f.sp. *tritici* (*Pgt*) is the most economically important and is known to have a broad range of hosts (Roelfs 1985a; Leonard and Szabo 2005). The primary hosts are common wheat, durum wheat, barley, Triticale, and wild wheat relatives (Singh et al. 2011b). The most common alternate host for wheat stem rust pathogen is barberry (*Berberis vulgaris* L.). *Mahonia* species, and barberry and *Mahonia* hybrids are other alternate hosts (Roelfs 1982). It is thought that *Pgt* originated from the Middle East (Roelfs 1985a; Peterson 2001); however, due to the wide distribution of wheat, this pathogen has been historically found in all wheat producing regions of the world (Jones and Clifford 1983; Zurn 2015).

Historical and contemporary situation of stem rust

Stem rust has been and continue to cause major problems on wheat. For example, around 700 A.D, the Romans, used to celebrate the festival of Robigalia each year to appease the rust god Rubigus to protect their crop (Chester 1946; Peterson 2001). Periodic emergence of virulent *Pgt* races causing severe yield losses have been reported around the world. For instance, prior to the appearance of the stem rust pathogen race group Ug99 in Africa in 1998, stem rust epidemics occurred in North and South America, Africa, Australia, New Zealand, Middle East, Europe, and Asia (Saari and Prescott 1985; Zurn 2015).

In the United States, in the late 19th and early 20th century, severe wheat stem rust epidemics resulted in high yield losses (Roelfs 1985b). The epidemics of 1916, was considered the worst outbreak that resulted in reduction of wheat production by approximately 60% mainly in the states of Minnesota, North Dakota and South Dakota (Peterson 2013). Because of this epidemic, the first ever massive barberry eradication program was implemented from 1918 to 1977 across 18 states (Roelfs 1982; Campbell and Long 2001; Peterson 2013). The goal of the program was to delay disease onset, reduce the amount of initial inoculum, and stop the stem rust pathogen from going through its sexual reproduction on the alternate host barberry, thus reducing the virulence variation (Roelfs 1982). Despite the implementation of this program, there was occurrence of stem rust epidemics in the US in 1935, 1937, 1953, and 1954 (Roelfs 1985b) due to favorable climate, appearance of new races, and planting of susceptible cultivars (Roelfs 1978). Sexual recombination results in higher number of races compared to asexual populations. For instance, Roelfs and Groth (1980) reported that one race per 4.3 isolates was found among sexual population compared to only one race identified in a collection of 148 isolates among a clonal population. The barberry eradication in the US helped in extending the durability of

effective resistance genes (Roelfs 1982; Schumann and Leonard 2000). Sexual recombination still occurs in the Pacific Northwest, however, the Rocky Mountains provided barriers and helped to geographically isolate this sexual population from wheat growing areas in the Midwest (Jin 2011). Barberry has been recently observed in abandoned farms in a number of Midwestern states, for example MN, suggesting that eradication of this alternate host was not totally successful. However, there has been no major stem rust epidemic in the US since 1950s partly attributed to eradication of Barberry. Currently, mutation is the main source of genetic variation in the asexual population of *Pgt* in the USA. Currently *Pgt* overwinters on winter wheat in southern Great Plains and Mexico (Kolmer et al. 2007) and migrate northward in the spring season (Kolmer 2001; Zurn 2015).

Other countries have equally experienced major stem epidemics. In China, outbreaks occurred in the1940's and 1950's because of conducive environmental conditions (Roelfs and Martens 1987). Similarly, severe cases of stem rust epidemics were reported in Kenya (1972 and 1978), Tunisia (1975), Zambia (1976), Zimbabwe (1978), and Ethiopia (1979, 1993, and 1994) (Saari and Prescott 1985; Shank 1994; Admassu and Fekadu 2005; Olivera et al. 2012, 2015; Zurn 2015). In response to these outbreaks several resistant genes were deployed and that significantly reduced the impact of stem rust on wheat globally. But in 1998, *Pgt* isolates (Ug99 lineage) with virulence to widely used stem rust resistance (*Sr*) genes *Sr31* and *Sr38* was reported in Uganda in 1998 (Pretorius et al. 2000). The race Ug99 was designated as TTKS (Wanyera et al. 2006) following the North American nomenclature system (Roelfs and Martens 1988). After adding a fifth set of differentials in the nomenclature system, race Ug99 was re-designated as TTKSK (Jin et al. 2008). The Ug99 race group was spread to other countries in Africa: Kenya, Ethiopia, Eritrea, Tanzania, Zimbabwe, Mozambique, Rwanda, South Africa,

Sudan, and Egypt. This race lineage was also detected in Yemen and Iran. This causes a real threat to the wheat production areas in the Middle East and Asia (Jin et al. 2008; Nazari et al. 2009; Pretorius et al. 2010; Singh et al. 2011a; Hale et al. 2013; Szabo et al. 2014; Mukoyi et al. 2015; Wolday et al. 2015; Patpour et al. 2016). Since 1999, stepwise mutation has allowed the Ug99 lineage to gain virulence on additional deployed *Sr* genes. For example, virulence on genes *Sr24*, *Sr36*, *Sr9h*, and *SrTmp* were detected in 2006, 2007, 2012 and 2014, respectively (Jin et al. 2008, 2009; Singh et al. 2011a; Rouse et al. 2014; Pretorius et al. 2016). This *Pgt* lineage is a real threat to global wheat production, because of its striking virulence combination, making 90% of the world wheat cultivars vulnerable to this race lineage (Singh et al. 2008).

Stem rust resistance in durum wheat

Durum wheat exhibit higher percentage of resistance to Ug99 compared to common wheat (Singh et al. 20111a). Currently, over 60 *Sr* genes have been characterized in wheat (McIntosh et al. 2014; Rahmatov et al. 2016) and around 29 are effective against Ug99 lineage races (Niu et al. 2014; Yu et al. 2014; Yu et al. 2015). Around 50% of these effective *Sr* genes in wheat were introgressed from wild wheat relatives (Rouse et al. 2014). Only three *Sr* genes; *Sr33*, *Sr35*, and *Sr57* have been cloned (Krattinger et al. 2009; Periyannan et al. 2013; Saintenac et al. 2013). In durum wheat, few *Sr* genes and QTL were mapped compared to those mapped in common wheat. The reported *Sr* genes in tetraploid wheat are *Sr2*, *Sr8*, *Sr9*, *Sr11*, *Sr12*, *Sr13*, *Sr14*, *Sr17*, *Sr36*, *Sr37*, *Sr40*, *Sr47*, *Srdp-2*, *SrGH*, *SrM*, *SrPl*, *SrPt*, and *SrTt-3* (McIntosh et al. 1995; Hare 1997; Faris et al. 2008; McIntosh et al. 2011; Klindworth et al. 2012; Toor et al. 2013; Singh et al. 2015; Yu et al. 2015). The seedling resistance genes *Sr8a*, *Sr8b*, *Sr9e*, *Sr9g*, *Sr12*, *Sr13*, *Sr17*, and *Sr23* have been postulated in tetraploid wheat landraces from the Watkins collection (Toor et al. 2013). However, virulent *Pgt* races on most of these genes including *Sr8*,

Sr9, *Sr11*, *Sr12*, *Sr1*3, *Sr14*, *Sr17*, and *Sr36* were reported (Toor et al. 2013; Singh et al. 2015), while *Sr37* and *Sr40* originated from *T. timopheevi* and *T. araraticum*, respectively are not deployed in new cultivars due to the linkage with undesirable traits in the translocations (McIntosh et al. 1995). The only APR genes reported so far in durum wheat are *Sr2* and *SrGH* (Mcfadden 1930; Hare 1997; Toor et al. 2013). The common *Sr* genes in commercial durum wheat cultivars are *Sr8b*, *Sr9e* and *Sr13*, which are found even singly or in different combinations in durum cultivars (Bhavani et al. 2008; Qamar et al. 2009; Toor et al. 2013).

The stem rust resistance to the Ug99 race group found in most North American cultivars is mainly due to *Sr13*, originated from the wild emmer wheat Khapli (Jin et al. 2007; Klindworth et al. 2007). A single mutation in *Pgt* races with virulence to *Sr13*, could breakdown the resistance in many of these durum cultivars (Singh et al. 2011a). There is more focus on developing resistance towards Ug99 lineage. However, recent races not part of the Ug99 race group have been collected in Ethiopia with virulence on many durum lines. These new races namely TRTTF and JRCQC have combined virulence on *Sr13* and *Sr9e* which are major components of stem rust resistance in North American and CIMMYT durum cultivars and germplasm (Olivera et al. 2012; Singh et al. 2015). Low percentage of resistance (5.2%) to these races was reported in a very diverse collection of 996 tetraploid wheat accessions tested at Debre Zeit, Ethiopia (Olivera et al. 2012). In addition, these races are virulent to *SrTmp*, and *Sr1A.1R* (Singh et al. 2011a). Similar race to TRTTF was also collected in Yemen and Pakistan (Mirza et al. 2010).

Another race was recently detected in Ethiopia named as TKTTF or ‘Digalu’ race is causing a problem on durum and common wheat (Olivera et al. 2015). This race appeared in Ethiopia after the introduction of the cultivar Digalu, which was introduced to Ethiopia after the

stripe rust epidemics in the country in 2010. This cultivar occupied over 500,000 ha in 2013, due to its high yield and resistance to stripe rust and stem rust (Ug99 race group). It was postulated that the *Sr* gene in Digalu is *SrTmp* which is affective to most Ug99 lineage races. However, the narrow genetic basis of stem rust resistance in Digalu, selected for virulent races to *SrTmp* which are JRCQC, TRTTF, RRTTF, and TKTTF. These races are phylogenetically different from Ug99-lineage races (Olivera et al. 2015). Phylogenetic study showed that the recently observed race TKTTF in Ethiopia did not appear from recent mutations of existing races. This suggests a recent introduction from other regions or the race TKTTF was already present in the country at low frequency and was selected for by the cultivar Digalu (Olivera et al. 2015). Races RRTTF and TKTTF are widely distributed across East Africa, South Asia, and the Middle East (Singh et al. 2015). Efforts to monitor and find new sources of resistance to the Ug99 and the other lineage groups are very important for the global wheat production.

Molecular marker evolution and genotyping

A molecular marker is a specific segment of DNA with defined genomic position of any given species. Scientists have used markers to study human, animal, plant, and microbe genomes. Some of the key areas of marker applications includes; genetic and physical mapping, MAS, map based cloning, genome evolution, and phylogenic analysis (Rafalski et al. 1996; Gupta et al. 1999). The development of markers in hexaploid common wheat and tetraploid durum wheat has been slower compared to other crops such as rice and maize. This is due to large complex wheat genome, higher ploidy level, and higher level of repetitive DNA.

The choice of marker system depends on the reliability, cost, and level of polymorphism and available technology platforms (Collard and Mackill 2008). Various types of molecular markers have been developed over the last 35 years including: (1) hybridization markers, such as

Restriction Fragment Length Polymorphisms (RFLPs); (2) Polymerase Chain Reaction (PCR) markers such as RAPDs, AFLPs, Sequence Tagged Sites (STS), Expressed Sequence Tags (ESTs), Inter-Simple Sequence Repeat Amplification (ISA), Cleaved Amplified Polymorphic Sequences (CAPS), and SSRs or Microsatellites; (3) plant retrotransposon-based markers; (4) DNA chip and sequencing based DNA markers such as Single Nucleotide Polymorphism markers (SNPs) (Gupta et al. 1999; Landjeva et al. 2007); (5) Diversity Array technology (DArT) (Jaccoud et al. 2001).

RFLPs were the first generation of markers applied initially to human genetic studies (Botstein et al. 1980) and later adopted for mapping of crop species including wheat (Chao et al. 1989; Blanco et al. 1998). RFLPs are codominant markers detected using labeled DNA (probe) which is hybridized to genomic DNA digested with a specific restriction enzyme. RFLPs have been used to identify several resistance genes in wheat such as powdery mildew resistance genes like *Pm1-Pm4* in wheat (Ma et al. 1994) and rust resistance genes like *Lr9* (Schachermayr et al. 1994) and *Lr34/Yr18/Pm38/Sb1/Bdv1* (Lagudah et al. 2006). However, this marker system is laborious, radioactive, and do not provide high level of polymorphism in wheat (Chao et al. 1989). Thereafter, other markers like AFLP, RAPD, and SSR markers with higher level of polymorphism were developed. RAPD markers (Williams et al. 1990) are based on the use of arbitrary sequences of deca-nucleaotide primers. This marker can easily be used in the lab, and is inexpensive, however RAPD is a dominant marker and has low repeatability. Thus, the use of RAPD markers to develop wheat genetic maps was not successful.

AFLP is based on selective amplification of restrictions fragments (Vos et al. 1995). The use of AFLPs allows for generation of large number of fragments in a PCR reaction. This made AFLP a suitable marker in many organisms including wheat. AFLPs have been used in

combination with other marker types to generate high density maps of wheat (Lotti et al. 2000; Peng et al. 2000). AFLPs were used to map a number of disease resistance genes such as *Pm22* (Singrün et al. 2003), *Pm24* (Huang and Röder 2003), *Yr31* (Singh et al. 2003), and *Lr46*/*Yr29/Pm39* (William et al. 2003), However, AFLPs are dominant markers, technically challenging, and time consuming. The conversion of RFLPs, RAPDs, and AFLPs into STS or SCAR markers solved some of the problems and allowed to provide useful markers for several wheat genes including disease resistance genes such as *Lr19* (Prins et al. 2001; Cherukuri et al. 2003); *Yr17* (Robert et al. 1999) *Sr2* (Johnston et al. 1998) and *Pm21* (Liu et al. 1999).

Microsatellites or SSRs (Wang et al. 1994) rely on a variable number of usually 2-4 bp nucleotide repeats which are abundant in the genome. SSRs are codominant, reproducible, and highly polymorphic, thus the use of SSRs has dominated the mapping of wheat (Röder et al. 1995; Prasad et al. 2000). By 2007, around 2,500 SSRs, distributed over the wheat genomes A, B, and D were used in MAS (Ganal and Röder 2007). In hexaploid wheat, Somers et al. (2004) developed an SSR-based consensus map that has been widely used. A number of important genes/quantitative trait loci (QTL) in wheat have been mapped using SSRs (Cheong et al. 2004; Chen et al. 2005; McCartney et al. 2005; Yang et al. 2005). However, SSRs are expensive to discover, time consuming, and labor intensive.

SNP markers detect DNA sequence variation based on a single nucleotide (A, T, C or G) difference between individuals (Wang et al. 1998). The popularity of SNP markers was due to the availability of advanced and low cost of automated genotyping techniques and powerful computational and statistical tools. Even though SNPs are less polymorphic compared to SSR as they are biallelic, SNPs are abundant across the whole genome, accurate, codominant, and amenable to high throughput technologies. SNPs are preferred marker system and are

extensively being used for genetic studies. Genotyping platforms such as 9K and 90K Illumina Infinium iSelect SNP arrays have been developed to genotype the complex wheat genome (Cavanagh et al. 2013; Wang et al. 2014).

Genotyping-by-sequencing (GBS) approach is the latest application of next-generation sequencing technology for the discovery of SNPs across the entire genome in diverse organisms (Elshire et al. 2011). The sequencing cost per sample using this technique is relatively low because only the subsets of the genome targeted by methylation sensitive restriction enzymes are sequenced. DNA-barcoded adapters are then ligated to the flanking restriction regions which allows the multiplexing of several individuals in a single sequencing run (Mascher et al. 2013). The low cost of GBS makes it a suitable approach for saturating genetic maps with SNPs, in addition to application to other areas like genomic selection, diversity studies, and phylogenetic analysis of a large number of individuals in germplasm collections or natural populations. Poland et al. (2012) developed two-enzyme GBS protocol to genotype complex plant genomes of barley (~5.5 Gb) and hexaploid wheat (~16-Gb). GBS is based on optical sequencing using Illumina GAII and HiSeq platforms. Semiconductor devices for non-optical (based on magnitude of the pH change) genome sequencing with Ion Torrent/ Proton Torrent personal genomics machine (PGM) have been developed recently by Rothberg et al (2011). A comparison between Illumina and Ion Torrent semiconductor sequencing technology platforms showed that GBS performed on Ion Torrent platform did not produce enough reads for barley. However, GBS adapted for Ion torrent was successful in genotyping organisms with smaller genome size such as plant pathogens (Leboldus et al. 2015; Gao et al. 2016).

DArT is based on microarray hybridizations to capture DNA variations such as SNPs and insertions/deletions (InDels). DArT enables whole-genome profiling by scoring presence/

absence of DNA fragments in a genomic representation (Jaccoud et al. 2001). DArT was first developed for rice, then applied to several other plant species including wheat and barley (Jaccoud et al. 2001; Wenzl et al. 2004; Akbari et al. 2006). Akbari et al. (2006) developed the first hexaploid wheat map using 788 polymorphic DArt markers on double haploid population and a collection of 62 wheat cultivars. The map length was 2,383 cM with fairly well distributed markers over the A and B genomes but insufficient marker coverage on the D genome was obtained.

The molecular markers developed for common hexaploid wheat were applied for genetic mapping of tetraploid durum wheat as both of them shared the A and B genomes (Blanco et al. 1998; Mantovani et al. 2008). However, marker polymorphism information content in durum wheat germplasm differ from that in common wheat (Maccaferri et al. 2003, 2005, 2015). Recently, a high-density, SNP-based consensus map of tetraploid wheat was developed by Maccaferri et al (2015).

Bi-parental population mapping

Mapping populations

A bi-parental mapping population is developed by crossing two individuals of a species that are polymorphic for traits of interest. Different types of mapping populations may be generated from heterozygous F_1 hybrid individuals including F_2 population, double haploid lines (DHLs), backcross (BC) population, and recombinant inbred lines (RILs) (Sehgal et al. 2016).

F_2 population is easy and fast to develop by self-pollinating F_1 plants. The derived F_2 plants segregate for the traits that distinguish the parents of the cross. However, the F_2 plants result from a single meiotic event (one recombination event). Therefore, mapping of the gene/QTL responsible for the phenotype of interest in an F_2 population may generate low

mapping resolution if the size of F_2 population is low. F_2 population is genetically 'mortal' population, thus the phenotype is based on a single plant basis with no replication. This is adequate only for qualitative traits where genotype × environment is not significant (Liu 2006; Gurung 2011).

Backcross population is where the F_1 plants are repeatedly backcrossed to one of the parents (recurrent or recipient parent) creating BC_nF_n. This allows for the study of the gene of the donor parent in the background of recipient parent. Repeated backcrossing to the recurrent parent will reconstitute the genome of the recipient parent without losing the desired gene (s). In each round of backcross, the genome of donor parent is reduced by half, therefore only tightly linked DNA segments are kept with the gene (s) of interest, resulting in creating NILs. Backcross populations and NILs are used to map the desired gene (s) (Liu 2006; Gurung 2011; Sehgal et al. 2016).

Population of RILs is developed by selfing individual plants of F_2 population using single seed descent method. Homozygous lines (true-breeding lines) are created after several generations of selfing or inbreeding. Long time is needed to develop RIL population (usually six to eight generations). However, more recombination events (meiosis) occur in a population of RILs compared to that of F_2 population. Thus, there is high chance of getting higher mapping resolution of the gene/QTL in RIL population. Population of RILs is an 'immortal' or permanent mapping population because the seeds of homozygous RILs can be multiplied with no genetic change. Therefore, replicated trials can be performed across different environments and genes/QTL for various traits (quantitative or qualitative) can be mapped using the same population (Liu 2006; Gurung 2011; Sehgal et al. 2016).

Doubled haploid line (DHLs) population is developed from F_1 hybrid. Production of DHLs in wheat includes two main steps which are haploid induction through anther culture or by chromosome selective elimination using hybridization with maize, followed by chromosome doubling using colchicine. Recently, DHLs in common wheat and durum wheat are mainly produced using crosses with maize (Niu et al. 2014). Double haploids are perfect for mapping populations because every locus is homozygous and the population is developed in very short time (one generation). Similar to RIL population, DHL population is immortal allowing for replicated trials (Liu 2006; Gurung 2011; Sehgal et al. 2016).

Linkage mapping in durum wheat

Linkage map is constructed using genotyping data on any type of bi-parental populations before conducting gene/QTL mapping. A linkage map determines the genetic distances between markers and how they are ordered in relation to each other. The map construction is based on recombination frequency in a segregating population. The higher the frequency of recombination (higher crossover events) between two markers, the further they are on a chromosome and vice versa (Sehgal et al. 2016). The observed recombination frequency in a segregating population is converted to an estimated recombination frequency in cM using two possible mapping functions: The Haldane's mapping function (Haldane 1919) or Kosambi's mapping function (Kosambi, 1944). Kosambi's mapping function is more accurate as it accounts for interference between crossover events, while Haldane's mapping function assumes no interference. Linkage between markers is calculated using a statistics called odds ratio which is the ratio of linkage versus no linkage. The logarithm of odds (LOD) (Risch 1992) is used to determine how linked markers are grouped together into linkage groups (Sehgal et al. 2016). The first linkage maps in tetraploid wheat were constructed in 1998 using mainly RFLP markers. A total of 65 RILs derived from a

cross between durum wheat cultivar ‘Messapia’ and wild emmer wheat accession ‘MG4343’ were used for this mapping. The total length of the linkage map was 1,352 cM with an average of 6.3 cM distance between loci covering all 14 chromosomes (Blanco et al. 1998). Subsequently other linkage maps were developed using SSR markers (Korzun et al. 1999). Additional more extensive genetic maps were created integrating several types of molecular markers including RFLP, SSR, AFLP, and DArT markers (Lotti et al. 2000; Nachit et al. 2001; Elouafi and Nachit 2004; Pozniak et al. 2007; Mantovani et al. 2008; Peleg et al. 2008; Maccaferri et al. 2008). Two consensus maps have been developed in durum wheat based on SSR and DArT markers (Marone et al. 2012; Maccaferri et al. 2015).

Recently, SNPs became more useful due to the genotyping cost effectiveness and their abundance across the entire genome (Gupta et al. 2008). AFLP-based complexity reduction combined with pyrosequencing technology (CRoPS) were applied and this allowed for the discovery of more than 2500 SNPs in durum wheat (Trebbi et al. 2011). Further, van Poecke et al. (2013) saturated the maps with additional SNPs with final marker density of 0.8 cM/marker. High-density tetraploid wheat consensus map was created, joining genetic maps from 13 independent biparental populations from durum wheat, cultivated emmer wheat, and wild emmer wheat. Ten of these populations used to develop the consensus map were genotyped with Illumina 9K and 90K wheat SNP arrays (Cavanagh et al. 2013; Wang et al. 2014). The consensus map had a total of 30,144 loci (including mainly 26,626 SNPs and 791 SSRs) covering 2,631 cM of all 14 durum wheat chromosomes with an average distance between markers of 0.087 cM (Maccaferri et al. 2015). The availability of the consensus map will greatly help in mapping of new genes/QTL, facilitate cloning of economically important genes, and assist breeding programs with MAS. Several of these linkage maps have been used to identify

genes/QTL for disease resistance and agronomically important traits in durum wheat (Elouafi and Nachit 2004; Uauy et al. 2006; Pozniak et al. 2007; Patil et al. 2008; Chu et al. 2010; Haile et al. 2012; Buerstmayr et al. 2013; Bansal et al. 2014).

Bulked segregant analysis

Bulked segregant analysis (BSA) is a rapid method used to identify markers linked to a single gene for a qualitative trait (Michelmore et al. 1991). The BSA is done by creating two DNA bulks with one sample containing DNA from homozygous dominant individuals and the second from homozygous recessive individuals. The two bulk samples together with the two parents of cross are genotyped for polymorphism. The two genetic bulks are equally random for all markers in the genome except those linked with the gene of interest controlling the trait we bulked upon (Michelmore et al. 1991).

Although the BSA is mainly used to identify markers linked to qualitative trait such as disease resistance (Chague et al. 1999; Cao et al. 2001; Shen et al. 2003; Herrera-Foessel et al. 2008b; Medini et al. 2014), this technique has also been used to map QTL for quantitative traits such as abiotic stress tolerance and grain yield (Quarrie et al. 1999; Altinkut and Gozukirmizi 2003; Ma et al. 2005; Shashidhar et al. 2005; Kanagaraj et al. 2010; Venuprasad et al. 2011).

Gene and QTL mapping

Traits can be grouped into two categories, qualitative and quantitative. A qualitative trait such as resistance to several plant diseases is usually controlled by a single gene or few genes. For a qualitative trait, the segregating population shows discrete phenotype distribution. A quantitative trait such as plant yield is polygenic controlled by several small effects loci, referred to as QTL. The quantitative trait shows continuous phenotypic variation. In mapping of a qualitative trait controlled by a single gene, the phenotype value is used to calculate the genetic

distances between the gene and the marker (Lander et al. 1987), however mapping of QTL is much more complex (Liu 2006).

There are three widely used approaches to detect QTL. Single-marker regression is the basic method used to identify a QTL in the vicinity of a marker. It tests for independent associations between markers and phenotypic values. It looks at each individual markers and performs essentially one-way analysis of variance. However, this method is limited by the confounding effect of one QTL by others which also influence the studied trait. In addition, QTL with minor effect and tight marker linkages cannot be distinguished from QTL with major effect and loose marker linkages. To overcome these limitations, new statistical approaches were developed. Interval mapping QTL analysis allows for testing of a model for the presence of a QTL between each marker interval (Lander and Bostein 1989; Haley and Knott 1992). Some problems are associated with this approach. For instance, the test statistic of an interval can be affected by other nearby QTL on the chromosome. Linked QTL that fall outside the interval may cause biased estimates of the position and level of effect of any QTL within the interval (Zeng 1993). Zeng (1994) proposed composite interval mapping (CIM) as solution to overcome the challenges of interval mapping. The CIM is based on multiple cycles of regression where loci detected previously are removed from the next analysis. With CIM, markers outside the interval are considered as cofactors. Removing the effects of these cofactors, improved the estimation of effect and location of a QTL within the interval. This method allows to detect QTL with minor effects. Identifying tightly linked or co-segregating markers to the gene/QTL facilitate their cloning and enhance the chance of using them for MAS. Increasing the size of the mapping population and the number of markers results in a better mapping resolution of the gene/QTL of interest (Liu 2006).

Association mapping

Association mapping (AM) also called linkage disequilibrium (LD) mapping is a powerful approach to determine marker-trait associations in a population. The AM approach is based on LD which is the non-random association of alleles at different loci (Weir 1979). AM has been used extensively in human and animals (DeWan et al. 2006; Karlsson et al. 2007) and in several crop species such as wheat, barley, soybean, and maize (Rostoks et al. 2006; Ersoz et al. 2007; Zhu et al. 2008). The LD approach has been used as an alternative to linkage mapping (Yu et al. 2006; Zhu et al. 2008). In contrast to linkage mapping, AM is usually applied on a contemporary germplasm collection which saves time and cost of developing segregating populations. Unlike linkage mapping, AM allows for determining marker-trait associations for multiple traits simultaneously using the same germplasm collection. This technique also allows for maping of genes/QTL at a higher resolution compared to linkage mapping. For instance, since the progenies in a bi-parental population (recombinant inbred lines or double haploids) are only few generations away from both parents, the number of recombination events are limited compared to those found in any AM panel. The latter has more recombination events taking place throughout the evolutionary history of the germplasm. Therefore, in a linkage mapping even distant markers can co-segregate with gene/QTL of interest, because LD decays slower. Thus, the gene/QTL obtained via linkage mapping could extend over several centiMorgans (cM) and this makes mapping resolution lower than that obtained through AM (Tomassini 2007; Nordborg and Weigel 2008; Zhu et al. 2008; Neumann et al. 2011; Dugo 2013). For example, a 390-fold higher maker resolution was obtained for *Stagonospora nodorum* blotch resistance gene (*QSng.sfr-3BS*) using AM in 44 varieties of winter wheat compared to QTL mapping in 240 RILs (Tomassini et al. 2007).

The main factors influencing the LD are mutation and recombination. Mutation creates LD between two loci, while recombination breaks the LD blocks, resulting in faster decay of LD in an AM population (Nachman 2002; Rafalski 2002). The most used measure of LD is r^2 with r representing the correlation coefficient between pairs of loci (Weiss and Clark 2002). The extent of LD in a species does not only determine the resolution of the map but also the density of markers required for the AM. For example, in several self- pollinated species like *Arabidopsis thaliana* and barley, LD extends over long physical distance (Nordborg et al. 2002; Malysheva-Otto et al. 2006). Therefore, lower number of markers evenly spaced on the genome are enough to perform AM. On the other hand, in the out-crossing species such as corn, the LD extends over very short physical distance (Remington et al. 2001), thus higher density of markers across genome is required for AM. In both animals and plants, there are unequal distribution of recombination hotspots across different regions on the chromosomes (Lichten and Goldman 1995; Mézard 2006). Therefore, the LD extent will not only differ between species but also between different chromosomes within a species and even between different regions on a single chromosome (Nachman 2002; Rafalski and Morgante 2004).

Population stratification is usually observed in most germplasm collections. This stratification is caused by several factors including breeding history, selection, founder effects, or genetic drift. The population stratification in an AM panel usually increases the chance of false positives of marker-trait associations (Slatkin 1991; Lander and Schork 1994). To overcome this limitation, the structure matrix (Q matrix) and the Kinship or relatedness matrix (K-matrix) are used as covariate in the model to account for these spurious associations. In addition, highly related individuals are easily assigned to related populations which results in increased number of subpopulations (Falush et al. 2003). Therefore, it was suggested that removing highly related

individuals in an AM panel reduces the overestimation of the number of subpopulations, which increases the power of AM (Liu et al. 2003; Breseghello and Sorrells 2006; Dugo 2013).

Despite the power of AM, some limitations do exist. For instance, rare alleles occurring at a low frequency in the panel increases LD between unlinked markers, resulting in increased chance of false positives detection (Wilson et al. 2004; Somers et al. 2007). To overcome this problem, removal of markers with very low minor allele frequency (usually <5%) is usually recommended in AM. However, removal of markers with low minor allele frequency, decreases the power of AM to detect rare alleles. Another important drawback of AM is the difficulty to identify QTL with small phenotypic variations (low heritability and strong genotype by environment interaction) using AM compared to QTL mapping (Chao et al. 2016). To overcome some of the AM limitations, new methods combining the advantages of both linkage mapping and AM have been developed in recent years. These new methods involve the use of multiparent advanced generation intercross (MAGIC) populations (Kover et al. 2009; Huang et al. 2012; Bandillo et al. 2013; Mackay et al. 2014; Sannemann et al. 2015) and nested association mapping populations (Yu et al. 2008; McMullen et al. 2009; Kump et al. 2011).

Association mapping can be used in two different approaches: genome wide association (GWA) mapping or candidate gene analysis, depending on the extent of LD (Thornsberry et al. 2001; Kraakman et al. 2004; Rostoks et al. 2006; Hall et al. 2010; Pasam et al. 2012). The candidate gene approach allows for identification of associations between candidate genes for a particular trait where genome wide LD is limited (Hall et al. 2010). However, this approach is based on prior information on the gene location, gene function in biochemical or regulatory pathways. This may lead to failure to detect other unknown non coding loci that may be important for the observed phenotype (Zhu et al. 2008). The candidate gene analysis has led to

identification of a number of genes (Zheng et al. 2008; Ramsay et al. 2011) such as the major QTL *Vgt1* controlling flowering time in maize (Salvi et al. 2007).

The GWA mapping is the main application of AM. This approach allows to scan the entire genome for significant marker-trait associations (Remington et al. 2001; Hall et al. 2010). The first GWA mapping in plants was performed on wild beet (*Beta vulgaris* ssp. *maritima*) to find markers associated with bolting (Hansen et al. 2001). Subsequently the GWA mapping were then used on several crop species including maize, rice, sorghum, wheat, and barley (Huang et al. 2012; Kump et al. 2011; Morris et al. 2012; Pasam et al. 2012; Li et al. 2013; Gao et al 2016; Kertho et al. 2015;). In durum wheat, the GWA mapping has been used for several agronomic traits such as height, root architecture, yellow pigment, drought, salinity, and yield (Reimer et al. 2008; Maccaferri et al. 2010; Cane et al. 2014; Hu et al. 2015; Turki et al. 2015). In addition, the GWA mapping identified marker associations with disease traits in durum wheat such as Fusarium head blight and rusts (Maccaferri et al. 2010b; Ghavami et al. 2011; Letta et al. 2014; Arruda, et al. 2016).

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CHAPTER II. GENOME-WIDE ASSOCIATION MAPPING OF LEAF RUST RESPONSE IN A DURUM WHEAT WORLDWIDE GERMPLASM COLLECTION [1]

Abstract

Leaf rust (caused by *Puccinia triticina* Erikss. [*Pt*]) is increasingly impacting durum wheat (*Triticum turgidum* L. var. *durum*) production with the recent appearance of races with virulence to widely grown cultivars in many durum producing areas worldwide. A highly virulent *P. triticina* race on durum wheat was recently detected in Kansas. This race may spread to the northern Great Plains, where most of the US durum wheat is produced. The objective of this study was to identify sources of resistance to several races from the United States and Mexico at seedling stage in the greenhouse and at adult-plant stage in field experiments. Genome-wide association study (GWAS) was used to identify single-nucleotide polymorphism (SNP) markers associated with leaf rust response in a worldwide durum wheat collection of 496 accessions. Thirteen accessions were resistant across all experiments. Association mapping revealed 88 significant SNPs associated with leaf rust response. Of these, 33 SNPs were located on chromosomes 2A and 2B, and 55 SNPs were distributed across all other chromosomes except for 1B and 7B. Twenty markers were associated with leaf rust response at seedling stage, while 68 markers were associated with leaf rust response at adult-plant stage. The current study

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[1]The material in this chapter is reprinted with permission from Crop Science Society of America. Aoun, M., M. Breiland, M. Kathryn Turner, A. Loladze, S. Chao, S. S. Xu, K. Ammar, J. A. Anderson, J. A. Kolmer, and M. Acevedo. 2016. Genome-Wide Association Mapping of Leaf Rust Response in a Durum Wheat Worldwide Germplasm Collection. Plant Genome 9. doi:10.3835/plantgenome2016.01.0008. Meriem Aoun's role in this manuscript was the conduction of the experiment and analysis of the data. In addition, Meriem Aoun wrote the manuscript and performed corrections suggested by the co-authors and the journal's reviewers.

identified a total of 14 previously uncharacterized loci associated with leaf rust response in durum wheat. The discovery of these loci through association mapping (AM) is a significant step in identifying useful sources of resistance that can be used to broaden the relatively narrow leaf rust resistance spectrum in durum wheat germplasm.

Introduction

Durum wheat (2n = 4x = 28) is an important cereal crop grown in many parts of the world, especially in the Mediterranean basin where ~50% of global production and 75% of the growing area are located (Elias and Manthey 2005). The world durum wheat production was estimated at 33.5 million metric tons in 2014. Durum wheat is an important crop that is concentrated in localized areas, often in developing countries, where it represents a large portion of total wheat planted as well as a major staple food used for pasta, couscous, and flat bread. Moreover, because of its adaptability to arid climate conditions, marginal soils, and relatively low water requirements, improvement of durum wheat production should be an agricultural and economic priority to ensure food security in these regions. Despite the broad adaptability of durum wheat, its production is often limited by different fungal diseases including rusts, septoria leaf blotch, fusarium head blight, and root rot (Nachit 2000; Nsarellah et al. 2000; Singh et al. 2005).

Leaf rust is a significant disease affecting wheat production worldwide. Durum wheat has been traditionally considered more resistant to *Pt* than bread wheat (*T. aestivum* L.; 2n = 6x = 42) in many regions worldwide. However, races of the leaf rust pathogen, virulent to widely grown durum cultivars in several production areas, are increasingly impacting durum production (Singh et al. 2004; Goyeau et al. 2006; Huerta-Espino et al. 2009). A *Pt* race, BBG/BN, in northwestern Mexico that appeared in 2001, with virulence to *Lr72*, overcame the resistance of

widely adapted durum wheat cultivars from the CIMMYT breeding program that had been effective in Mexico for >25 yr (Singh et al. 2004; Herrera-Foessel et al. 2014). Increased susceptibility of durum wheat to leaf rust has also been observed in the Mediterranean basin (Martinez et al. 2005; Goyeau et al. 2012), the Middle East (Ordonez and Kolmer 2007a), and Chile (Singh et al. 2004). In the United States, a *Pt* race, designated BBBQD using the North American *Pt* nomenclature system (Long and Kolmer 1989) and possessing a similar virulence pattern to previously identified Mexican races, was collected from California durum fields in 2009. In 2013, an isolate of the same race was found in Kansas. The occurrence of this race in these regions increases the likelihood of its spread to the northern Great Plains (Kolmer 2015a) and most importantly, to North Dakota where 58% of the total US durum wheat is produced (USDA–National Agricultural Statistics Service 2015).

A number of studies have determined that the *Pt* populations predominant on bread wheat differ from those found on durum wheat. The *Pt* isolates from bread wheat are often avirulent on durum wheat (Singh 1991; Huerta-Espino and Roelfs 1992; Ordonez and Kolmer 2007a). In contrast, *Pt* isolates collected from durum wheat are avirulent to all but the most susceptible bread wheat cultivars (J.A. Kolmer, unpublished data, 2013). Huerta-Espino and Roelfs (1992) determined that the majority of the *Pt* collections from durum wheat were race BBB, indicating that they were avirulent to all resistance genes carried by the first three sets of international leaf rust differentials (Long and Kolmer 1989). Isolates collected from durum wheat in several countries share highly similar virulence patterns on ‘Thatcher’ near-isogenic lines, suggesting a common origin (Ordonez and Kolmer 2007a; Ordonez and Kolmer 2007b). However, some *Pt* isolates collected from durum wheat in Ethiopia have a distinct virulence phenotype with avirulence to commonly susceptible bread wheat hosts including Thatcher. These isolates also

grouped separately for simple-sequence repeat (SSR) genotypes (Kolmer and Acevedo 2016) and DNA sequence compared with other isolates collected on durum and bread wheat in Europe, South America, and Mexico (Liu et al. 2014).

Although a limited amount of information is available about the genetic basis of resistance to *Pt* in durum wheat, a few leaf rust resistance (*Lr*) genes have been reported. After the leaf rust epidemics in Mexico in 2001, extensive screening of the CIMMYT durum germplasm, resulted in the identification of effective leaf rust resistance genes including: the complementary gene pair *Lr27+31* located on chromosome arms 3BS and 4BS, respectively (Singh and McIntosh 1984a, b; Singh et al. 1993); *Lr14a* on 7BL (Herrera-Foessel et al. 2008b); and *Lr3a* on 6BL (Herrera-Foessel et al. 2005). These genes are also present in common wheat but are highly ineffective against *Pt* races usually found on common wheat. Other effective resistance genes that seem to be present only in durum wheat cultivars are *LrCamayo*, which presumably links to *Lr3*a (Herrera-Foessel et al. 2007), and *Lr61* on 6BS (Herrera-Foessel et al. 2008a). Other major *Lr* genes mapped or postulated in durum wheat include *Lr10* (Aguilar-Rincon et al. 2001), *Lr23* (Watson and Luig 1961; McIntosh and Dyck 1975; Nelson et al. 1997), *Lr33* (Dyck et al. 1987; Dyck 1994), *Lr47* (Dubcovsky et al. 1998), *Lr52* (Singh et al. 2010), and *Lr64* (Dyck 1994; McIntosh et al. 2009). However, virulence in durum specific races is common on *Lr10*, *Lr23*, and *Lr33* (Huerta-Espino and Roelfs 1992; Singh et al. 2005; Ordonez and Kolmer 2007a).

In addition, the adult plant resistance slow rusting gene *Lr46* has been reported in CIMMYT durum wheat (Herrera-Foessel et al. 2011). The extensive use of single-race-specific resistance genes caused rapid selection of new virulent *Pt* races only a few years after their deployment in commercial cultivars. For instance, in 2008, *Pt* races were detected in Mexico that

carried virulence to *Lr3* and *Lr27+Lr31* (Huerta-Espino et al. 2009). Similarly, a variant of the old Mexican race BBB/BN, designated as BBB/BN_*Lr61*Vir (Herrera-Foessel et al. 2014), has virulence to *Lr61*. Both *Lr14a* and *LrCamayo* are still effective against the current Mexican races, however virulence on *Lr14a* has been reported in France (Goyeau et al. 2006), Tunisia (Gharbi et al. 2013), Morocco, and Spain (J.A. Kolmer and M. Acevedo, unpublished data, 2015). The mapping of plant disease resistance genes and quantitative trait loci (QTL) has been traditionally performed through biparental mapping populations. Recently, as a result of the progress in high-throughput genotyping technologies and the improvement of statistical programs, GWAS or AM has been used as an alternative approach to biparental mapping (Yu et al. 2006; Zhu et al. 2008). Association mapping is based on revealing correlations between phenotype and genotype in a germplasm collection (Zondervan and Lon 2004). The GWAS takes advantage of the linkage disequilibrium (LD) between alleles to identify molecular markers significantly associated with a trait response. The GWAS uses the recombination events that occur throughout the evolutionary history of a germplasm. This allows the breakup of the LD blocks within the genome and results in a faster decay of the LD in the AM panels than in recombinant inbred lines and double haploid populations, in which only the allelic diversity that segregates between the parents can be assessed. Therefore, GWAS can identify associated loci with the trait response at a much higher mapping resolution than biparental mapping (Rafalski 2002; Nordborg and Weigel 2008; Zhu et al. 2008; Neumann et al. 2011).

In durum wheat, AM has been used to dissect the genetic basis of important agronomic traits including grain yield, yellow pigment, root architecture, plant height, and drought and salinity tolerance (Reimer et al. 2008; Maccaferri et al. 2010a; Cane et al. 2014; Hu et al. 2015; Turki et al. 2015). Moreover, GWAS has been used to identify markers associated with disease

resistance to Fusarium head blight, leaf rust, and stem rust (Maccaferri et al. 2010b; Ghavami et al. 2011; Letta et al. 2014). However, the previous AM analysis for leaf rust response only included elite germplasm (cultivars and advanced lines) that was genotyped using only 225 SSR markers. Wheat landrace germplasm collections may carry new genes for resistance to diseases, including leaf rust, stem rust, and stripe rust, since the use of landraces in the modern breeding programs has been relatively rare (Reif et al. 2005; Bonman et al. 2007; Newton et al. 2010; Bux et al. 2012; Gurung et al. 2014). Recently, a high-density, SNP-based consensus map of tetraploid wheat was developed by Maccaferri et al (2015), which will increase the effectiveness of GWAS and QTL meta-analyses.

The current study describes the first durum wheat leaf rust GWAS using a highly diverse germplasm panel comprised of a worldwide collection of landraces, cultivars, and breeding lines genotyped using the Illumina iSelect 9K wheat array. This study provides relevant insight into the genetic basis underlying resistance in durum wheat to North American *Pt* races.

Materials and methods

Genetic material

A durum wheat collection of 496 accessions from the USDA–Agricultural Research Service (ARS)–National Small Grain Collection (NSGC) was evaluated for leaf rust resistance in this study. These accessions were originally sourced from 67 countries including accessions from Africa (96), Asia (172), Australia (7), North America (42), South America (34), Europe (140), and unknown origin (5). The collection includes 234 landraces, 55 cultivars, 128 cultivated lines, 77 breeding lines, and 2 of unknown accession type. Seeds used in this project were obtained from single plant selections increased in a nursery at the USDA–ARS–NSGC and Potato Germplasm Research Unit, Aberdeen, ID (Supplemental Table S1).

Phenotyping at seedling stage

The disease screenings at the seedling stage of plant development were conducted at three locations: (i) North Dakota (ND) Agricultural Experiment Station Greenhouse Complex, Fargo, ND, USA; (ii) the USDA–ARS Cereal Disease Laboratory (CDL) in Saint Paul, MN, USA; and (iii) the CIMMYT, Mexico (El Batán, State of Mexico). The durum wheat collection was evaluated for response to *Pt* races BBBQD (California isolate), BBBQJ (Mexican isolate, BBG/BP), BBBDB (USA, Race 1), MBDSD (North Dakota isolate), MCDSS (North Dakota isolate), and a mixture of bread-wheat-type races predominant in the United States (MHDSB, MFPSB, MLDSB, TBBGG, TFBJQ, and TFBGQ). The virulence–avirulence profile of the rust races was based on infection types (ITs) on seedlings of Thatcher wheat differentials that are near isogenic for single-resistance genes following the race nomenclature of Long and Kolmer (1989) (Table 2.1).

Table 2.1. Virulence–avirulence profile of *Puccinia triticina* races.

Race	Virulent on genes	Avirulent on genes
BBBQD [a]	*Lr10*, *39*, *B*	*Lr1*, *2a*, *2c*, *3*, *3ka*, *9*, *11*, *14a*, *16*, *17*, *18*, *21*, *24*, *26*, *28*, *30*, *42*
BBBDB [b]	*Lr14a*	*Lr1*, *2a*, *2c*, *3*, *3ka*, *9*, *11*, *16*, *17*, *18*, *21*, *24*, *26*, *28*, *30*, *39*, *42*, *B*
MBDSD [a]	*Lr1*, *3*, *10*, *14a*, *17*, *39*, *B*	*Lr2a*, *2c*, *3ka*, *9*, *11*, *16*, *18*, *21*, *24*, *26*, *30*, *28*, *42*
MHDSB [b]	*Lr1*, *3*, *10*, *14a*, *16*, *17*, *26*, *B*	*Lr2a*, *2c*, *3a*, *3ka*, *9*, *11*, *18*, *21*, *24*, *28*, *39*, *42*
MFPSB [b]	*Lr1*, *3a*, *3ka*, *10*, *14a*, *17*, *24*, *26*, *B*	*Lr2a*, *2c*, *9*, *11*, *16*, *18*, *21*, *28*, *30*, *39*, *42*
MLDSB [b]	*Lr1*, *3a*, *9*, *10*, *14a*, *17*, *B*	*Lr2a*, *2c*, *3ka*, *11*, *16*, *18*, *21*, *24*, *26*, *30*, *28*, *39*, *42*
TBBGJ [b]	*Lr2a*, *2c*, *3*, *10*, *28*, *39*	*Lr3ka*, *9*, *11*, *14a*, *16*, *17*, *18*, *21*, *24*, *26*, *30*, *42*, *B*
TFBJQ [b]	*Lr3*, *2a*, *2c*, *10*, *14a*, *21*, *24*, *26*, *28*	*Lr3ka*, *9*, *11*, *16*, *17*, *18*, *30*, *39*, *42*, *B*
TFBGQ [b]	*Lr2a*, *2c*, *3*, *10*, *21*, *24*, *26*, *28*	*Lr3ka*, *9*, *11*, *14a*, *16*, *17*, *18*, *30*, *39*, *42*, *B*
MCDSS [a]	*Lr1*, *3*, *10*, *14a*, *17*, *21*, *26*, *28*, *39*, *B*	*Lr2a*, *2c*, *3ka*, *9*, *11*, *16*, *18*, *24*, *30*, *42*
BBBQJ [ac]	*Lr10*, *Lr14b*, *20*, *B*	*Lr1*, *2a*, *2c*, *3*, *3ka*, *3bg*, *9*, *11*, *14a*, *16*, *17*, *18*, *24*, *26*, *28*, *30*

[a] Race collected from durum wheat.
[b] Race collected from common wheat.
[c] The fifth letter in the race nomenclature was based on reactions of lines carrying genes *Lr3bg*, *Lr14b*, *Lr20*, and *Lr28*, while for the rest of the isolates the fifth letter was based on the reactions of lines carrying genes *Lr21*, *Lr28*, *Lr39*, and *Lr42*.

The isolates BBBQD, BBBQJ, MBDSD, and MCDSS were collected from durum wheat. Races MBDSD and MCDSS are known to be virulent on bread wheat as well. All other isolates were collected from bread wheat germplasm. The North Dakota experiment was performed in a randomized complete block design with two replicates for race BBBQD and three replicates for races MBDSD and MCDSS. A second experiment was conducted for race BBBQD. The seedlings were grown in the greenhouse as previously described by Kertho et al (2015). The susceptible bread wheat cultivar 'Little Club' and susceptible durum wheat 'RL6089' were included in each tray as susceptible checks. Two replicates of Thatcher near-isogenic line differentials were planted alongside each experiment to confirm the purity of the races. Urediniospores of each race were increased by inoculating them onto the seedlings of susceptible wheat host (RL6089 or 'Morocco') treated with 30 mL of water solution of maleic hydrazide (3 g L^{-1}) per small pot at coleoptile emergence. After sporulation, the urediniospores were collected and kept at 4°C until needed.

Seedlings at the two-leaf stage, usually 12 days after planting, were sprayed with fresh urediniospores suspended in Soltrol- 170 mineral oil (Phillips Petroleum) at a concentration of 10^6 spores mL^{-1} using an inoculator pressurized by an air pump. The inoculated seedlings were left to air-dry and later placed in a dark dew chamber at 20°C overnight. The humidifiers were set for 20 s every 4 min to maintain 100% leaf wetness. Thereafter, the plants were transferred to the greenhouse maintained at 22 and 18°C (day vs. night) with 16-hour photoperiod until evaluation of ITs.

The experiment performed at the CDL with races BBBDB, BBBQD, and the race mixture (MHDSB, MFPSB, MLDSB, TBBGJ, TFBJQ, and TFBGQ) were conducted in augmented design with one replicate. Five to seven seeds of each durum wheat accession were

planted in 50-cell trays containing vermiculite. The susceptible checks Little Club and RL6089 were included two times in each 50 cell tray. Urediniospore increase, inoculation, incubation, and greenhouse conditions were as previously described by Kolmer and Hughes (2013). The screening using race BBBQJ was conducted at CIMMYT in augmented design and the experiment was repeated twice. The seedlings were grown as previously described by Loladze et al (2014). Seven to 10 seedlings from each accession were grown at 22°C in the greenhouse in pots with four accessions planted per pot with a soil mix consisting of one part peat moss, one part sand, and one part black soil. Pots were fertilized twice with a urea fertilizer (5 g per 10 L of water) 4 to 5 days after planting and 2 to 3 days after inoculation. Seedlings were inoculated with a suspension of urediniospores and light mineral oil (Soltrol 170; 1 mg of urediniospores per 1 mL of oil) using a hydrocarbon propellant pressure pack. The oil was allowed to evaporate from the leaves for at least 30 min before incubating in dark at 20°C for 18 h. Following incubation, plants were transferred to the greenhouse at 23°C with 16 h photoperiod.

Leaf rust ITs were assessed 12 days postinoculation on the second leaves of seedlings using a 0-to-4 scale (Long and Kolmer 1989, McIntosh et al. 1995) where IT 0 = no visible symptom, ; = hypersensitive flecks, 1 = small uredinia with necrosis, 2 = small- to medium-size uredinia surrounded by chlorosis, 3 = medium-size uredinia with no chlorosis or necrosis, and 4 = large uredinia with no necrosis or chlorosis. Larger and smaller uredinia than expected for each IT were designated with + and −, respectively. Symbols ‘C’ and ‘N’ are used to indicate more than usual degrees of chlorosis and necrosis, respectively. Heterogeneous IT evenly distributed over the leaf surface was designated as X (mesothetic reaction). Accessions with ITs of 0 to 2+ and X were considered resistant, while accessions with 3 and 4 scores were considered susceptible (McIntosh et al. 1995; Long and Kolmer 1989).

To account for multiple ITs in a single plant at seedling stage, the 0-to-4 scale for leaf rust was converted to a linearized 0-to-9 scale using the weighted mean of the most and the least predominant IT on the same leaf surface (Zhang et al. 2014). Ratings of 0 to 6 were classified as resistant IT and 7 to 9 were considered as susceptible IT.

Analysis of variance (ANOVA) on the linearized 0-to-9 ratings was performed using SAS software 9.3 (SAS Institute 2011) to test for the homogeneity of variances for experiments and replicates. The homogeneity of variance test resulted in combining IT data from the two North Dakota experiments on race BBBQD and the mean was used for the GWAS, while the CDL experiment for the same race was analyzed separately. For all other experiments (the rest of the races) with two or three replicates, the mean across replicates for each accession was used.

Evaluations of adult plants

The accessions were field tested in 2012 at St. Paul (MN_ StP_F) and Crookston (MN_Cr_F) in Minnesota and at CIMMYT Centro Experimental de Norman E. Borlaug station in Ciudad Obregón, Sonora (MX_Ob_F) and at CIMMYT Headquarters, El Batán experimental station (MX_EB_F) in 2013.

In MN_StP_F and MN_Cr_F, 50 to 60 seeds of each accession were planted in a 3-m row and 30 cm apart. Rows of the susceptible wheat cultivars, Thatcher, Little Club, and Morocco were planted perpendicular to the durum wheat entries. The spreader rows were inoculated with a mixture of six *Pt* races (MHDSB, MFPSB, MLDSB, TBBGJ, TFGJQ, and TFBGQ) from bread wheat that were present in the Great Plains region. The common spring wheat cultivars Thatcher, ‘Tom’, ‘Verde’, and ‘Knudson’ were planted every 100 entries as checks. Field inoculation was conducted as described by Kolmer (2015b).

In both Mexican field locations, the accessions were planted in single 1.2-m-long rows with a leaf rust-susceptible cultivar ‘Banamichi C2004’ planted as a rust spreader. Susceptible durum wheat lines (Atil*2/Local Red; Sooty_9/Rascon_37; Jupare C 2001; Bergand/Amic//Playero/Piquero; and ATIL/3/Bergand/Amic//Playero/Piquero), resistant durum wheat lines (Cirno C 2008; Sooty_9/ Rascon_37//Llareta INIA; and Sooty_9/Rascon_37//Guayacan INIA), and the bread wheat Thatcher were included as checks in the experiment every 50 entries. All accessions and the susceptible spreader rows were inoculated with leaf rust race BBBQJ, the predominant durum-specific race in Mexico carrying virulence to *LrB*, *Lr10*, *Lr14b*, *Lr11*, *Lr20*, *Lr23*, *Lr27+31*, and *Lr72* as described by Loladze et al (2014). The suspension of the urediniospores in light mineral oil (Soltrol 170; 2 mg of urediniospores per 1 mL of oil) was applied using a hand sprayer at least three times at tillering stage of plant development.

The germplasm at all locations was evaluated when at least 70 to 80% of the flag leaf area of the susceptible checks was covered by uredinia. The accessions were evaluated using the modified Cobb scale (Peterson et al. 1948) ~2 months after planting. The scoring was based on both the disease severity (the percentage of tissue infected) and the plant response to infection. Plant response was recorded as resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S) reactions (McIntosh et al. 1995). In some cases, infection responses were a combination between any two categories on the same leaf with most predominant infection response first followed by the least predominant one. For instance, MSMR referred to overlapping of MS and MR categories, where MS was observed more frequently than MR response. All accessions with R, RMR, MR, and MRMS infection responses were considered

resistant. In addition, accessions with MSMR and MS infection response but severity lower than 20% were also considered resistant.

For AM analysis, disease severity and infection response data were combined in a single value as the coefficient of infection, which was calculated by multiplying the severity and a constant for host response where immune = 0.0, R = 0.2, MR = 0.4, MS = 0.8, S = 1.0, RMR = 0.3, MRMS = 0.5, MSMR = 0.6, and MSS = 0.9 (modified Yu et al. 2011).

Single-nucleotide polymorphism marker genotyping and analysis

The durum wheat collection was genotyped through the Triticeae Coordinated Agricultural Project (TCAP) using the Illumina iSelect 9K wheat array (Cavanagh et al. 2013) at the USDA–ARS genotyping laboratory in Fargo, ND. A total of 5490 high-quality polymorphic SNPs were originally selected. Marker data are available through the USDA–NIFA-funded TCAP (http://www.triticeaecap.org) and only 0.7% of SNP data points were missing. A total of 3569 SNP markers (i.e., 1.37 marker per cM), which were in common with those included in the tetraploid wheat consensus map of ~2600 cM (Maccaferri et al. 2015), were used in the GWAS(Supplemental Table S2). Markers with minor allele frequency (MAF) of <5.0% were eliminated to reduce the chance of detecting false positives. In addition, markers that had >10% missing data were discarded from further analysis. The genetic position of the SNP markers was estimated based on the tetraploid wheat consensus map (Maccaferri et al. 2015). Redundant accessions sharing exactly the same SNP genotypes were also excluded, which resulted in eliminating 64 accessions from the analysis.

Linkage disequilibrium for all pairwise comparisons between intrachromosomal SNPs was computed and the genome-wide LD decay was estimated using JMP Genomics 6.1 software (SAS Institute, 2012). The LD was computed as the squared correlation coefficient (R^2) for each

of the marker pairs. The genome-wide LD decay was estimated by plotting LD estimate (R^2) from all 14 chromosomes against the corresponding pairwise genetic distances (cM). Smoothing spline Fit (lambda = 114,551.3) was applied to the figure of LD decay.

Association analysis

The population structure (**Q** matrix) (Price et al. 2006) was assessed through principal component (PC) analysis. The familial relatedness was estimated using an identity by-state matrix (**K** matrix) (Zhao et al. 2007). Both **K** and **Q** matrices were generated using JMP Genomics 6.1. Four regression models were used to analyze marker–trait association using JMP Genomics 6.1. They included (i) naive model that did not account for kinship and population structure, (ii) kinship, (iii) kinship plus population structure (first two PCs that collectively explained 21.8%), and (iv) kinship plus population structure (first three PCs that collectively explained 26.6%). The **K** and the **Q** matrices were included in the regression equation to ensure that only genetically significant associations were detected from the GWAS and were not spurious associations resulting from population structure or familial relatedness. The general formula of the mixed linear model used for the GWAS follows the regression equation $\mathbf{y} = \mathbf{Xb} + \mathbf{Qu} + \mathbf{I\mu} + \mathbf{e}$, where **y** is a vector of phenotypic values, **X** is a vector of SNP marker genotypes, **b** is a vector of fixed effects as a result of the genotype, **Q** is matrix of principle component vectors estimating population structure, **u** = vector of fixed effects resulting from population structure, **I** is an identity matrix, **μ** is a vector of random effects that estimates the probability of coancestry between genotypes, and **e** is a vector of residuals. The variances of μ and *e* effects are based on these assumptions; $\mathrm{Var}(u) = 2\mathbf{K}V\mathrm{g}$ and $\mathrm{Var}(e) = V\mathrm{R}$, where **K** is the kinship matrix deduced from genotypes based on the proportion of shared allele values, $V\mathrm{g}$ is the genetic variance, and $V\mathrm{R}$ is the residual variance (Yu et al. 2006; Zegeye et al. 2014). Each SNP marker was then

fitted into the regression equation to generate a *P*-value. Marker–trait associations were considered significant at a $P \leq 0.001$.

For each regression model, the SNP markers were ranked from smallest to largest *P*-values. The best model for each leaf rust race–field location was chosen based on the mean squared difference (MSD) between observed and expected *P*-values (Mamidi et al. 2011) because of the uniform distribution of random marker *P*-values (Yu et al. 2006). The MSD was calculated using the following formula: $\text{MSD} = \{\sum_{i=1}^{n}[p\text{i} - (i/n)]^2\}/n$

where *n* is the number of markers, *i* is the rank number that is from 1 to *n*, and *pi* is the probability of the *i*th-ranked *P*-value. Significant markers associated with response to leaf rust were selected only from the model with the lowest MSD value. The *P*-values of the selected model were later adjusted by calculating the corresponding positive false discovery rate (pFDR) (Benjamini and Yekutieli 2001) using JMP genomics 6.1. Marker–trait associations were finally considered significant at a $\text{pFDR} \leq 0.1$. Furthermore, we performed a stepwise regression using JMP genomics 6.1 on the significant SNPs of each trait. This allowed determining the minimum number of SNPs independently associated with leaf rust response (Gurung et al. 2014). The selected SNPs from the stepwise regression explain similar phenotypic variation as that described by all the significant SNPs considered together for each trait (Mamidi et al. 2014).

Results

Phenotypic data analysis

A total of 496 durum wheat accessions were evaluated for response to different *Pt* races at seedling stage in the greenhouse and at adult-plant stage in the field. Most of the accessions were phenotyped in the experiments conducted in the United States. In Mexico, 364 were evaluated with race BBBQJ at the seedline stage in the greenhouse, while 371 and 383

accessions were screened at adult-plant stage in MX_EB_F and MX_Ob_F, respectively (Table 2.2; Supplemental Table S1).

Table 2.2. Number of leaf rust resistant accessions in each trial.

Trait	BBBQD (ND) [a]	BBBQD (CDL) [b]	BBBDB	MBDSD	MCDSS	BBBQJ	Race mix [c]	MN_StP_F [c]	MN_Cr_F [c]	MX_Ob_F [c]	MX_EB_F [c]
Accessions screened	489	489	494	496	495	364	464	453	432	383	371
Resistant landraces	10	31	146	59	80	39	120	174	144	97	64
Resistant cultivars or cultivated lines	9	20	139	59	67	22	101	156	155	66	49
Resistant breeding lines	4	8	66	33	40	14	59	74	70	42	34
Resistant accessions, unknown type	1	1	2	0	1	2	1	2	2	1	1
Total resistant accessions	24	60	353	151	188	77	281	406	371	206	148
Percentage of resistance	4.91	12.27	71.46	30.44	37.98	21.15	60.56	89.62	85.88	53.79	39.89

[a] ND, North Dakota.

[b] CDL, Cereal Disease Laboratory, Saint Paul, MN.

[c] Race mix, MHDSB, MFPSB, MLDSB, TBBGJ, TFBJQ, and TFBGQ; MN_StP_F, races in Minnesota St. Paul field; MN_Cr_F, races in Minnesota Crookston field; MX_Ob_F, races in Mexico Ciudad Obregón field; MX_EB_F, races in Mexico El Batán field.

Phenotypic data at seedling stage were homogeneous based on Levene's test (Levene 1960) on ITs for each *Pt* race except for BBBQD. Therefore, phenotypic data of replicates and experiments were pooled for each race and arithmetic means were calculated and used for AM. The North Dakota and CDL experiments for race BBBQD (BBBQD (ND) and BBBQD (CDL), respectively) were analyzed separately in the GWAS because of the non-homogeneity of variance (Supplemental Table S1). The discrepancies between experiments with race BBBQD could be due to different experimental conditions at the CDL and North Dakota sites and differing interpretation of ITs.

The percentage of accessions with resistance to races collected from durum wheat was low. For instance, resistance within the 496 accessions to BBBQD (ND), BBBQD (CDL), BBBQJ, MBDSD, and MCDSS was 4.91% (24 accessions), 12.27% (60 accessions), 20.98% (77 accessions), 30.44% (151 accessions), and 37.98% (188 accessions), respectively. As expected, the percentage of accessions resistant to most *Pt* races collected from bread wheat was high. For instance, resistance to BBBDB was 71.46% (353 accessions) and 60.56% (281 accessions) for the race mixture (Table 2.2).

A high percentage of resistance was observed among the 496 accessions when evaluated at adult plant stage in both Minnesota trials that were inoculated with bread wheat specific races. At MN_Cr_F, 85.90% (371 accessions) of the durum germplasm was resistant, while 89.60% (406 accessions) of the collection screened at MN_StP_F was classified as resistant. On the contrary, there was much lower proportion of resistant accessions at both Mexico locations, where races virulent to durum wheat were used for inoculation and present as natural inoculum in the field. The percentage of resistant germplasm was estimated at 53.79% (206 accessions) and 39.89% (148 accessions) in MX_Ob_F and MX_EB_F, respectively (Table 2.2).

Across most experiments, resistant accessions were mainly landraces (Table 2.2). The only exception was the experiment in MN_Cr_F where 41.78 and 38.81% of resistant accessions were cultivars and landraces, respectively. Thirteen accessions comprised of eight landraces, two breeding lines, two cultivated lines, and one cultivar were resistant across all experiments. These accessions were collected from Australia (PI 209274), Portugal (PI 192051 and PI 193920), Ethiopia (PI 534304, PI 387263, CItr 14623, and PI 195693), Lebanon (PI 342647 and PI 519832), Malta (PI 278379), Yemen (PI 244061), Jordon (PI 223155), and Argentina (PI 324928) (Table 2.3).

Table 2.3. Durum wheat accessions resistant to leaf rust across all trials.

Accession	Origin	Type	MBDSD [a]	BBBQD (ND) [ab]	BBBQD (CDL) [ab]	BBBDB [a]	Race mix [ac]	MCDSS [a]	BBBQJ [a]	MX_Ob_F [d]	MX_EB_F [d]	MN_StP_F [d]	MN_Cr_F [d]
PI 209274	Australia	Breeding line	1	1	0	0	0	1	0	0R	0	TR	TR
PI 193920	Portugal	Landrace	2	2	0	0	0	2	0	0R	0	TR	TR
PI 244061	Yemen	Landrace	2	2	0	0	1	1	–	–	–	TR	5R
PI 324928	Argentina	Breeding line	2	2	0	0	0	1	–	5MR	0	TR	TR
PI 192051	Portugal	Landrace	3	2	0	0	1	1	1	0R	0	TR	TR
PI 519832	Lebanon	Cultivar	4	4	0	0	0	1	0	0R	0	TR	TR
PI 195693	Ethiopia	Landrace	2	3	1	0	0	0	1	5MR	10MR	TR	TR
PI 342647	Lebanon	Cultivated line	2	2	1	0	1	1	0	5MR	0	20RMR	5R
PI 387263	Ethiopia	Landrace	4	2	1	0	0	5	1	10MR	5MR	5R	10R
PI 223155	Jordan	Cultivated line	5	4	1	1	2	5	–	–	–	10RMR	–
PI 278379	Malta	Landrace	3	2	2	0	2	1	5	0R	0	TR	TR
PI 534304	Ethiopia	Landrace	3	2	2	0	0	4	1	10MR	0	20RMR	–
CItr 14623	Ethiopia	Landrace	6	–	2	0	2	3	2	5MR	0	TR	TR

[a] Linearized disease rating for leaf rust at seedling stage as described by Zhang et al. 2014.

[b] ND, North Dakota; CDL, Cereal Disease Laboratory, Saint Paul, MN.

[c] Race mix: seedling test with inoculum composed of races MHDSB, MFPSB, MLDSB, TBBGJ, TFBJQ, and TFBGQ.

[d] Adult plant response to leaf rust. MN_StP_F, races in Minnesota St. Paul field; MN_C_F, races in Minnesota Crookston field; MX_Ob_F, races in Mexico Ciudad Obregón field; MX_EB_F, races in Mexico El Batán field. Plant response was recorded as resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S) reactions (McIntosh et al., 1995); Rust severity indicated with trace (T).

Marker properties and linkage disequilibrium analysis

Markers with MAF <5.0% and missing data points >10.0% were discarded. Therefore, 3067 SNP markers were kept for further analysis and were ordered according to the scaled map positions of the SNP marker-based tetraploid wheat consensus map (Maccaferri et al. 2015). These SNPs were distributed across all chromosomes in the A and B genomes, 1549 markers (50.5%) and 1518 markers (49.5%), respectively. The LD decayed to 0.2 within 2.63 cM, on average (Figure 2.1). Significant associated SNPs between which the pairwise LD (R^2) ≥ 0.7 were considered a single association or locus.

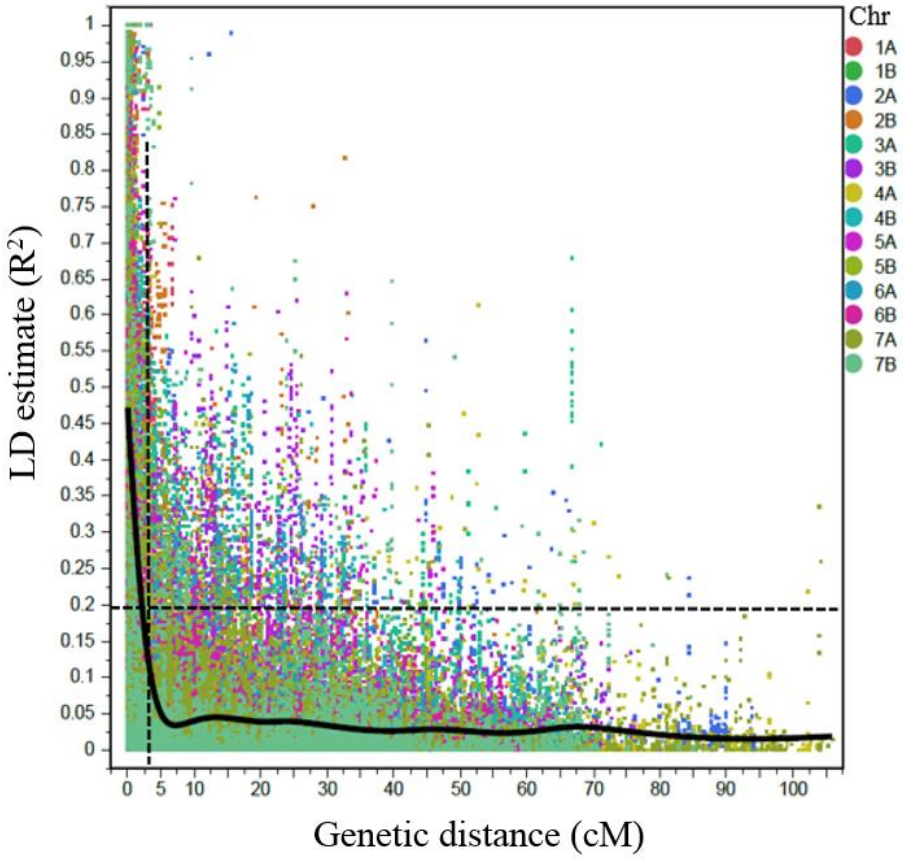

Figure 2.1. Scatter plot showing the linkage disequilibrium (LD) decay across the chromosomes (Chr) for 432 durum wheat accessions. The genetic distance in cM is plotted against the LD estimate (R^2) for pairs of SNPs.

Population structure, kinship analysis, and regression model selection for association mapping

Population structure was inferred using principal component analysis (PCA). The PCA shows that two, three, and 10 PCs explain a cumulative 21.2, 26.1, and 41.5% of the genotype variation, respectively. The first three PCs clustered the collection into three subpopulations (Figure 2.2).

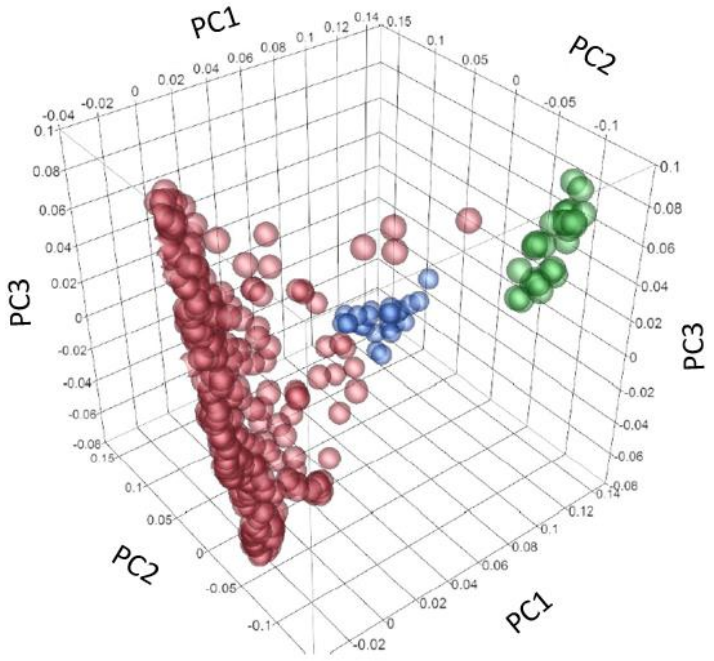

Figure 2.2. Principal component (PC) analysis obtained from 3067 polymorphic SNPs, indicating population structure in 432 durum wheat accessions. PC1, PC2 and PC3 explain 15.5, 5.7, and 4.9% of the variation, respectively. The colors represent clusters: red = cluster 1; blue = cluster 2; green = cluster 3.

Subpopulation 1 (in red color) contains the largest number of accessions (376), which are mainly from Europe (121 accessions), Asia (123 accessions), North America (45 accessions), and South America (27 accessions). Subpopulation 2 (blue color) was the smallest with only 27 accessions mainly from Africa (7 accessions), Asia (7 accessions), and Europe (8 accessions).

Subpopulation 3 (green color) includes 29 accessions mostly from Africa (21 accessions, 18 from Ethiopia and three from Eritrea). Thus, clustering of these accessions was not based on the geographic location except for Subpopulation 3. In Subpopulation 1, 59% were cultivars and breeding lines, while in Subpopulations 2 and 3, 63.0 and 76.0% were landraces, respectively (Figure 2.2).

The familial relatedness was estimated using an identity-by-state matrix (**K** matrix). Kinship between accessions was calculated and a heat map of the marker-based **K** matrix is illustrated in Appendix A Figure A1. The durum collection had intermediate familial relationships as some hotspots with related lines were observed on the heat map. Accounting for the population structure and familial relationship between individuals in the AM analysis reduces the number of false-positive associated markers. Based on MSD values of the four regression models tested, no single model fits best for all traits. For instance, the kinship model was used for MX_ Ob_F, MX_EB_F, MN_Cr_F, and MN_StP_F, while 2PCs+kinship was the model used for BBBQD (ND) and BBBQD (CDL). Model 3PCs+Kinship fits best for MBDSD, MCDSS, BBBDB, and race mixture (Table 2.4).

Table 2.4. Model mean square difference for each test.

Trait	Naïve	Kinship	2PCs + Kinship [a]	3PCs + Kinship [a]
MX_EB_F [b]	6.19×10^{-2}	**1.25×10^{-4}** [c]	1.29×10^{-4}	2.95×10^{-4}
MX_Ob_F [b]	9.06×10^{-2}	**1.41×10^{-4}**	3.91×10^{-4}	7.28×10^{-4}
BBBQJ	3.79×10^{-2}	2.31×10^{-4}	1.62×10^{-4}	**1.37×10^{-4}**
MBDSD	6.40×10^{-2}	7.20×10^{-5}	3.69×10^{-5}	**1.59×10^{-5}**
MCDSS	6.73×10^{-2}	2.88×10^{-4}	2.06×10^{-4}	**1.44×10^{-4}**
BBBQD (ND)[d]	1.92×10^{-3}	7.49×10^{-5}	**4.90×10^{-5}**	7.69×10^{-5}
BBBQD (CDL)[d]	8.42×10^{-3}	7.16×10^{-4}	**5.73×10^{-4}**	6.44×10^{-4}
BBBDB	6.31×10^{-2}	8.80×10^{-4}	4.82×10^{-4}	**4.95×10^{-4}**
Race mix [b]	7.42×10^{-2}	5.05×10^{-4}	5.40×10^{-4}	**1.29×10^{-4}**
MN_StP_F [b]	1.22×10^{-1}	**1.76×10^{-4}**	2.63×10^{-4}	2.64×10^{-4}
MN_Cr_F [b]	1.08×10^{-1}	**1.98×10^{-4}**	3.60×10^{-4}	3.15×10^{-4}

[a] 2PC, population structure matrix (Q matrix) based on the first two principal components; 3PC, population structure matrix (Q matrix) based on the first three principal components.

[b] Race mix, seedling test with inoculum composed of races MHDSB, MFPSB, MLDSB, TBBGJ, TFBJQ, and TFBGQ; MN_StP_F, races in Minnesota St. Paul field; MN_Cr_F, races in Minnesota Crookston field; MX_Ob_F, races in Mexico Ciudad Obregón field; MX_EB_F, races in Mexico El Batán field.

[c] Numbers in bold indicate lowest MSD and best model for each trait. The best model was used to investigate single-nucleotide polymorphism–leaf rust response associations.

[d] ND, North Dakota; CDL, Cereal Disease Laboratory, Saint Paul, MN.

Marker-trait associations for leaf rust response

Association mapping based on the IT to different *Pt* isolates at seedling stage in the greenhouse and at adult-plant stage in the field revealed 88 significant SNP markers. The leaf rust response–SNP associations were distributed across all chromosomes except for 1B and 7B. Twenty markers were associated with leaf rust response at seedling stage, while 68 markers were associated with leaf rust response at adult-plant stage. Of these 88 markers, 33 were located on chromosomes 2A and 2B. Based on the pairwise LD, the 88 SNPs represented 37 different loci (Table 2.5; Supplemental Table S3, S4). Evaluation of the 88 SNPs in all resistant accessions allowed verification of the association of the SNP markers with disease response and identification of alleles associated with the resistance to *Pt* (Supplemental Table S4).

Table 2.5. Significant markers associated with response to each *Puccinia triticina* race at seedling stage in the greenhouse and race mixture at adult-plant stage in the field.

Trait [a]	Marker	Chromosome	Major allele	Minor allele	MAF [b]	cM [c]	-$\log_{10}$ (*P* value)	R^2 [d]	pFDR [e]
BBBQJ	***IWA1818*** [f]	**4BL**	**C**	**T**	**0.09**	**70.9**	**4.67**	**6.19E-02**	**6.60E-02**
BBBQJ	***IWA3023***	**6AL**	**A**	**G**	**0.45**	**88.2**	**4.22**	**5.21E-02**	**7.98E-02**
BBBQJ	*IWA3024*	6AL	A	G	0.44	88.2	4.11	5.04E-02	7.98E-02
MBDSD	***IWA7547***	**2AL**	**T**	**C**	**0.07**	**137.1**	**6.81**	**6.28E-02**	**4.65E-04**
MBDSD	*IWA757*	5BS	A	G	0.38	2.7	5.04	4.51E-02	1.37E-02
MCDSS	***IWA7547***	**2AL**	**T**	**C**	**0.07**	**137.1**	**5.54**	**5.04E-02**	**8.81E-03**
MCDSS	*IWA757*	5BS	A	G	0.38	2.7	4.03	3.53E-02	1.42E-01
BBBQD (ND)	*IWA6089*	2AL	A	G	0.08	136.9	3.9	3.49E-02	4.20E-02
BBBQD (ND)	***IWA7547***	**2AL**	**T**	**C**	**0.07**	**137.1**	**5.05**	**4.61E-02**	**6.65E-03**
BBBQD (ND)	*IWA5449*	2AL	A	G	0.09	137.1	3.64	3.33E-02	6.31E-02
BBBQD (ND)	*IWA1765*	2BL	T	C	0.13	148.0	4.75	4.46E-02	1.07E-02
BBBQD (ND)	*IWA2459*	2BL	A	G	0.13	148.0	4.2	3.75E-02	3.15E-02
BBBQD (ND)	***IWA7955***	**2BL**	**A**	**C**	**0.12**	**148.0**	**4.03**	**3.57E-02**	**3.54E-02**
BBBQD (ND)	*IWA1076*	2BL	A	G	0.22	151.0	3.26	2.80E-02	1.19E-01
BBBQD (ND)	*IWA4130*	2BL	A	G	0.22	151.1	3.26	2.80E-02	1.19E-01
BBBQD (ND)	***IWA6471***	**3B**	**C**	**T**	**0.30**	**4.2**	**3.65**	**3.21E-02**	**6.31E-02**
BBBQD (ND)	*IWA5968*	4AL	T	C	0.21	130.6	3.48	3.05E-02	8.29E-02
BBBQD (ND)	*IWA4069*	5A	A	G	0.13	27.2	5.92	5.45E-02	1.43E-03
BBBQD (ND)	*IWA5615*	5A	T	G	0.13	27.2	5.86	5.41E-02	1.43E-03
BBBQD (ND)	*IWA3811*	5AS	T	C	0.13	27.2	5.84	5.39E-02	1.43E-03
BBBQD (ND)	*IWA1062*	5AS	T	C	0.13	27.2	4.03	3.59E-02	3.54E-02
BBBQD (CDL)	*IWA7547*	2AL	T	C	0.07	137.1	4.75	4.29E-02	5.48E-02

Table 2.5. Significant markers associated with response to each *Puccinia triticina* race at seedling stage in the greenhouse and race mixture at adult-plant stage in the field (continued).

Trait [a]	Marker	Chromosome	Major allele	Minor allele	MAF [b]	cM [c]	-$\log_{10}$ (*P* value)	R^2 [d]	pFDR [e]
BBBQD (CDL)	***IWA3741***	**2BL**	**A**	**C**	**0.19**	**137.9**	**3.62**	**3.15E-02**	**1.05E-01**
BBBQD (CDL)	***IWA1765***	**2BL**	**T**	**C**	**0.13**	**148.0**	**3.8**	**3.48E-02**	**8.10E-02**
BBBQD (CDL)	*IWA5615*	5A	T	G	0.13	27.2	4.24	3.78E-02	5.95E-02
BBBQD (CDL)	*IWA4069*	5A	A	G	0.13	27.2	4.11	3.64E-02	5.97E-02
BBBQD (CDL)	*IWA3811*	5AS	T	C	0.13	27.2	4.28	3.81E-02	5.95E-02
BBBQD (CDL)	***IWA3023***	**6AL**	**A**	**G**	**0.45**	**88.2**	**3.9**	**3.48E-02**	**7.68E-02**
BBBQD (CDL)	*IWA3024*	6AL	A	G	0.44	88.2	3.55	3.12E-02	1.07E-01
BBBQD (CDL)	***IWA8438***	**6AL**	**G**	**A**	**0.28**	**120.5**	**3.45**	**2.98E-02**	**1.21E-01**
BBBDB	***IWA757***	**5BS**	**A**	**G**	**0.38**	**2.7**	**4.17**	**3.66E-02**	**2.05E-01**
Race mix	***IWA7547***	**2AL**	**T**	**C**	**0.07**	**137.1**	**5.09**	**4.90E-02**	**2.41E-02**
Race mix	*IWA757*	5BS	A	G	0.38	2.7	4.72	4.49E-02	2.81E-02
MN_StP_F	***IWA2656***	**1AS**	**C**	**T**	**0.18**	**44.3**	**3.76**	**3.53E-02**	**8.98E-02**
MN_StP_F	***IWA7421***	**1AS**	**A**	**G**	**0.18**	**44.6**	**3.43**	**3.31E-02**	**1.21E-01**
MN_StP_F	***IWA601***	**1AL**	**A**	**G**	**0.23**	**87.3**	**3.93**	**3.95E-02**	**8.98E-02**
MN_StP_F	*IWA2696*	2AS	T	C	0.10	38.0	6.09	6.03E-02	2.47E-03
MN_StP_F	***IWA1536***	**3AL**	**G**	**A**	**0.10**	**83.4**	**3.77**	**3.67E-02**	**8.98E-02**
MN_StP_F	*IWA3512*	3AL	G	A	0.11	83.4	3.52	3.39E-02	1.21E-01
MN_StP_F	*IWA1700*	3AL	T	C	0.10	83.4	3.4	3.37E-02	1.21E-01
MN_StP_F	*IWA3999*	3AL	A	G	0.09	83.4	3.28	3.03E-02	1.33E-01
MN_StP_F	*IWA1701*	3AL	A	G	0.09	83.5	3.28	3.03E-02	1.33E-01
MN_StP_F	***IWA1570***	**4AL**	**G**	**T**	**0.17**	**59.9**	**3.43**	**3.20E-02**	**1.21E-01**
MN_StP_F	*IWA6999*	6AS	G	A	0.10	5.9	5.48	5.38E-02	5.12E-03

Table 2.5. Significant markers associated with response to each *Puccinia triticina* race at seedling stage in the greenhouse and race mixture at adult-plant stage in the field (continued).

Trait [a]	Marker	Chromosome	Major allele	Minor allele	MAF [b]	cM [c]	-$\log_{10}$ (*P* value)	R^2 [d]	pFDR [e]
MN_StP_F	***IWA1749***	**6AS**	**C**	**T**	**0.11**	**5.9**	**5.1**	**4.99E-02**	**8.13E-03**
MN_Cr_F	*IWA2696*	2AS	T	C	0.10	38.0	4.68	4.67E-02	5.48E-02
MN_Cr_F	***IWA3999***	**3AL**	**A**	**G**	**0.09**	**83.4**	**3.72**	**3.61E-02**	**1.10E-01**
MN_Cr_F	*IWA1700*	3AL	T	C	0.10	83.4	3.51	3.60E-02	1.23E-01
MN_Cr_F	*IWA1536*	3AL	G	A	0.10	83.4	3.48	3.46E-02	1.23E-01
MN_Cr_F	*IWA3512*	3AL	G	A	0.11	83.4	3.22	3.16E-02	1.35E-01
MN_Cr_F	*IWA1701*	3AL	A	G	0.09	83.5	3.72	3.61E-02	1.10E-01
MN_Cr_F	*IWA4110*	3AL	C	T	0.10	83.5	3.37	3.23E-02	1.25E-01
MN_Cr_F	***IWA5178***	**3B**	**G**	**A**	**0.07**	**100.2**	**3.21**	**3.16E-02**	**1.35E-01**
MN_Cr_F	*IWA1704*	3B	A	G	0.07	100.2	3.2	3.15E-02	1.35E-01
MN_Cr_F	*IWA4784*	4AL	A	G	0.14	71.2	4.43	4.43E-02	5.48E-02
MN_Cr_F	***IWA4786***	**4AL**	**C**	**T**	**0.15**	**71.2**	**3.77**	**3.71E-02**	**1.10E-01**
MN_Cr_F	*IWA4785*	4AL	C	T	0.15	71.2	3.65	3.54E-02	1.10E-01
MN_Cr_F	***IWA4618***	**4BL**	**A**	**G**	**0.05**	**123.3**	**3.41**	**3.27E-02**	**1.25E-01**
MN_Cr_F	***IWA4860***	**5AL**	**C**	**T**	**0.10**	**138.6**	**3.25**	**3.10E-02**	**1.35E-01**
MX_EB_F	*IWA1387*	1AS	G	A	0.08	27.9	3.7	4.46E-02	1.17E-01
MX_EB_F	***IWA7151***	**1AS**	**C**	**T**	**0.13**	**31.9**	**3.09**	**3.65E-02**	**1.17E-01**
MX_EB_F	*IWA7548*	2A	T	C	0.10	107.7	3.36	4.07E-02	1.17E-01
MX_EB_F	***IWA1242***	**2AS**	**G**	**A**	**0.13**	**35.6**	**3**	**3.37E-02**	**1.29E-01**
MX_EB_F	***IWA2831***	**2AS**	**G**	**A**	**0.09**	**107.6**	**3.07**	**3.46E-02**	**1.17E-01**
MX_EB_F	*IWA1370*	2AS	C	T	0.11	107.7	3.42	4.14E-02	1.17E-01
MX_EB_F	*IWA11*	2AS	G	A	0.10	107.7	3.07	3.50E-02	1.17E-01

Table 2.5. Significant markers associated with response to each *Puccinia triticina* race at seedling stage in the greenhouse and race mixture at adult-plant stage in the field (continued).

Trait [a]	Marker	Chromosome	Major allele	Minor allele	MAF [b]	cM [c]	-$\log_{10}$ (*P* value)	R^2 [d]	pFDR [e]
MX_EB_F	*IWA7683*	2AS	G	A	0.10	107.7	3.07	3.46E-02	1.17E-01
MX_EB_F	*IWA8016*	2AS	C	T	0.10	107.7	3.07	3.46E-02	1.17E-01
MX_EB_F	*IWA6991*	2AL	C	A	0.11	107.7	3.39	4.10E-02	1.17E-01
MX_EB_F	*IWA3946*	2AL	G	A	0.10	107.7	3.33	4.04E-02	1.17E-01
MX_EB_F	*IWA877*	2AL	G	A	0.10	107.7	3.14	3.56E-02	1.17E-01
MX_EB_F	*IWA2654*	2AL	G	A	0.10	107.7	3.07	3.46E-02	1.17E-01
MX_EB_F	*IWA6874*	2AL	A	G	0.10	107.7	3.07	3.46E-02	1.17E-01
MX_EB_F	*IWA8210*	2AL	A	G	0.10	107.7	3.07	3.46E-02	1.17E-01
MX_EB_F	*IWA3512*	3AL	G	A	0.11	83.4	2.95	3.44E-02	1.36E-01
MX_EB_F	***IWA6914***	**3AL**	**A**	**G**	**0.11**	**83.7**	**3.17**	**3.60E-02**	**1.17E-01**
MX_EB_F	***IWA5775***	**3B**	**C**	**A**	**0.13**	**101.6**	**3.06**	**3.62E-02**	**1.17E-01**
MX_EB_F	***IWA6126***	**5AL**	**A**	**G**	**0.27**	**84.7**	**3.19**	**3.63E-02**	**1.17E-01**
MX_EB_F	*IWA7742*	5AL	T	C	0.08	90.6	3.29	3.77E-02	1.17E-01
MX_EB_F	***IWA4805***	**5AL**	**T**	**G**	**0.37**	**147.4**	**3.74**	**4.35E-02**	**1.17E-01**
MX_EB_F	***IWA6904***	**6BL**	**C**	**T**	**0.05**	**89.6**	**4.4**	**5.45E-02**	**1.06E-01**
MX_EB_F	*IWA657*	6BL	T	C	0.05	89.7	3.73	4.32E-02	1.17E-01
MX_EB_F	*IWA477*	7AL	T	C	0.06	133.7	3.64	4.21E-02	1.17E-01
MX_EB_F	*IWA5797*	7AL	T	C	0.06	206.4	4.16	5.05E-02	1.06E-01
MX_Ob_F	*IWA1387*	1AS	G	A	0.08	27.9	3.37	3.88E-02	5.06E-02
MX_Ob_F	*IWA7151*	1AS	C	T	0.13	31.9	2.95	3.34E-02	1.15E-01
MX_Ob_F	*IWA6003*	2BL	G	A	0.09	94.2	3.81	4.45E-02	2.19E-02
MX_Ob_F	***IWA2183***	**2BL**	**T**	**G**	**0.09**	**94.2**	**3.93**	**4.43E-02**	**1.95E-02**

Table 2.5. Significant markers associated with response to each *Puccinia triticina* race at seedling stage in the greenhouse and race mixture at adult-plant stage in the field (continued).

Trait [a]	Marker	Chromosome	Major allele	Minor allele	MAF [b]	cM [c]	-$\log_{10}$ (*P* value)	R^2 [d]	pFDR [e]
MX_Ob_F	*IWA2665*	2BL	G	A	0.09	94.2	3.93	4.43E-02	1.95E-02
MX_Ob_F	*IWA3889*	2BL	T	C	0.08	95.2	3.93	4.43E-02	1.95E-02
MX_Ob_F	*IWA3452*	2BL	A	C	0.08	95.2	4.09	4.67E-02	1.85E-02
MX_Ob_F	*IWA3453*	2BL	G	A	0.08	95.2	4.09	4.67E-02	1.85E-02
MX_Ob_F	*IWA5260*	2BL	T	G	0.08	95.2	4.09	4.67E-02	1.85E-02
MX_Ob_F	*IWA4965*	2BL	T	C	0.08	94.2	4.12	4.73E-02	1.85E-02
MX_Ob_F	*IWA5610*	2BL	T	C	0.09	94.2	4.53	5.28E-02	1.85E-02
MX_Ob_F	*IWA4541*	2BL	G	A	0.07	100.9	3.87	4.45E-02	2.11E-02
MX_Ob_F	*IWA8624*	3AS	C	T	0.10	51.1	5.12	5.94E-02	1.13E-02
MX_Ob_F	*IWA2662*	3B	A	G	0.15	61.6	2.96	3.20E-02	1.15E-01
MX_Ob_F	*IWA3788*	3B	T	C	0.15	61.6	2.96	3.20E-02	1.15E-01
MX_Ob_F	*IWA7512*	3B	A	G	0.08	100.7	3.58	4.00E-02	3.61E-02
MX_Ob_F	*IWA4653*	3B	A	G	0.11	100.9	3.26	3.58E-02	6.28E-02
MX_Ob_F	*IWA5710*	3B	C	A	0.10	100.9	4.03	4.56E-02	1.85E-02
MX_Ob_F	*IWA7692*	3B	G	A	0.10	100.9	4.03	4.56E-02	1.85E-02
MX_Ob_F	*IWA8290*	3B	G	A	0.10	100.9	4.03	4.56E-02	1.85E-02
MX_Ob_F	***IWA4218***	**3B**	**C**	**T**	**0.08**	**100.9**	**4.81**	**5.69E-02**	**1.56E-02**
MX_Ob_F	*IWA2087*	4BL	A	G	0.06	124.4	3.83	4.31E-02	2.19E-02
MX_Ob_F	*IWA2469*	4BL	C	T	0.06	123.3	4.18	4.74E-02	1.85E-02
MX_Ob_F	***IWA1798***	**4BL**	**T**	**C**	**0.05**	**123.3**	**4.21**	**4.83E-02**	**1.85E-02**
MX_Ob_F	*IWA4618*	4BL	A	G	0.05	123.3	4.27	4.86E-02	1.85E-02
MX_Ob_F	*IWA2470*	4BL	T	C	0.05	123.3	5.4	6.68E-02	1.13E-02

Table 2.5. Significant markers associated with response to each *Puccinia triticina* race at seedling stage in the greenhouse and race mixture at adult-plant stage in the field (continued).

Trait [a]	Marker	Chromosome	Major allele	Minor allele	MAF [b]	cM [c]	-$\log_{10}$ (*P* value)	R^2 [d]	pFDR [e]
MX_Ob_F	*IWA6904*	6BL	C	T	0.05	89.6	4.3	5.12E-02	1.85E-02
MX_Ob_F	*IWA657*	6BL	T	C	0.05	89.7	3.45	3.81E-02	4.48E-02
MX_Ob_F	*IWA2513*	7AS	T	C	0.08	68.9	3.54	3.95E-02	3.74E-02

[a] ND, North Dakota; CDL, Cereal Disease Laboratory, Saint Paul, MN. Race mix, seedling test with inoculum composed of races MHDSB, MFPSB, MLDSB, TBBGJ, TFBJQ, and TFBGQ; MN_StP_F: races in Minnesota St. Paul field; MN_Cr_F: races in Minnesota Crookston field; MX_Ob_F, races in Mexico Ciudad Obregón field; MX_EB_F: races in Mexico El Batán field.
[b] MAF, minor allele frequency.
[c] cM, marker position on consensus map.
[d] R^2, proportion of phenotypic variation explained by the individual marker.
[e] pFDR, positive false discovery rate.
[f] Markers in bold were maintained after stepwise regression.

At seedling stage, 14 SNPs on chromosomes 2A, 2B, 3B, 4A, and 5A were associated with response to race BBBQD (ND). These markers represent six different loci. Three of the 14 markers fit into a stepwise regression, accounting for 11.9% of the phenotypic variation. The experiment at the CDL with the same race (BBBQD) revealed a total of nine associated SNPs located on 2A, 2B, 5A, and 6A, representing six loci. Four of the nine markers fit into a stepwise regression and accounted for 11.9% of the phenotypic variation. The experiments with BBBQD at the North Dakota and CDL sites shared five associated SNPs corresponding to three loci located on 2A, 2B, and 5A.

Two markers, *IWA7547* and *IWA757*, on chromosomes 2A and 5B, respectively, were associated with response to *Pt* races MCDSS and MBDSD and to the *Pt* race mixture at seedling stage. The marker *IWA7547* was the only one generated from the stepwise regression model in races MCDSS, MBDSD, and *Pt* race mixture.

Only one significant marker, *IWA757* (located on chromosome 5B), was found associated with response to race BBBDB. This marker was considered significantly associated despite a pFDR of 0.2 (pFDR $\leq$ 0.1 cutoff used for significant associated markers across all experiments) because it was also associated with response at seedling stage to races MBDSD, MCDSS, BBBQD (ND), BBBQD (CDL), and the race mixture.

Three markers on chromosomes 4B and 6A were associated with response to BBBQJ representing two loci on chromosomes 4B and 6A. Two markers fit into a stepwise regression and accounted for 7.8% of the phenotypic variation.

At the adult-plant stage in the field experiments, 12 markers on chromosomes 1A, 2A, 3A, 4A, and 6A were significantly associated with response to *Pt* races in MN_StP_F,

representing six loci. Furthermore, six of the 12 markers fit into a stepwise regression and accounted for 52.7% of the phenotypic variation.

Fourteen markers on chromosomes 2A, 3A, 3B, 4A, and 5A were significantly associated with response to *Pt* races in MN_Cr_F. These SNPs represent six loci. Five of the 14 significant markers fit into a stepwise regression and accounted for 27.1% of the phenotypic variation. Six markers, representing two loci, on chromosomes 2A and 3A were associated with response to *Pt* races at both locations in Minnesota.

Similarly, 25 markers were significantly associated with response to *Pt* races in the MX_EB_F trial and were located on chromosomes 1A, 2A, 3A, 3B, 5A, 6B, and 7A. Twelve out of these 25 SNPs represent different loci. Eight of the 25 significant markers fit into a stepwise regression and together accounted for 48.0% of the phenotypic variation.

At the MX_Ob_F trial, 29 markers were associated with leaf rust response. These markers, located on chromosomes 1A, 2B, 3A, 3B, 4B, 6B, and 7A, represented 11 loci. Three of the 29 markers fit into a stepwise regression and explained 19.0% of the phenotypic variation.

Two markers, *IWA1387* and *IWA7151*, on chromosome 1A and two markers, *IWA757* and *IWA6904*, on chromosome 6B were detected in response to *Pt* races across the Mexican locations. Moreover, markers *IWA3512* on 3A and *IWA4618* on 4B were associated with resistance or susceptibility to the races in field trials in Minnesota and Mexico (Table 2.5; Figure 2.3).

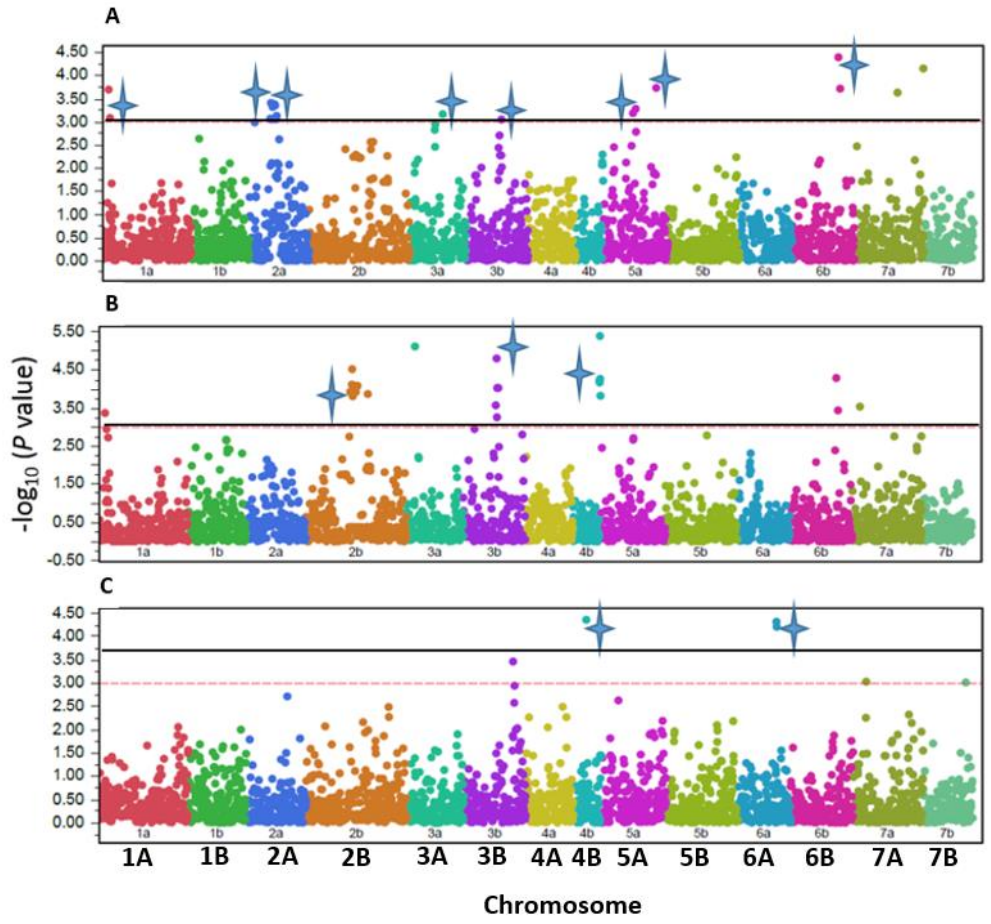

Figure 2.3. Manhattan plots showing *P*-values for single-nucleotide polymorphism (SNP) markers associated with response to leaf rust at seedling and adult plant stages. (A) Races in Mexico El Batán field; (B) races in Mexico Ciudad Obregón field; (C) BBBQJ; (D) MBDSD; (E) MCDSS; (F) BBBDB; (G) race mixture (MHDSB, MFPSB, MLDSB, TBBGJ, TFBJQ, and TFBGQ); (H) races in Minnesota St. Paul field; (I) races in Minnesota Crookston field; (J) BBBQD (North Dakota); (K) BBBQD (Cereal Disease Laboratory). The horizontal dotted red line indicates significant threshold at *P*-value = 0.001. The black horizontal line indicates significant threshold at positive false discovery rate (pFDR) = 0.1. The SNPs included in stepwise regression are shown in blue stars.

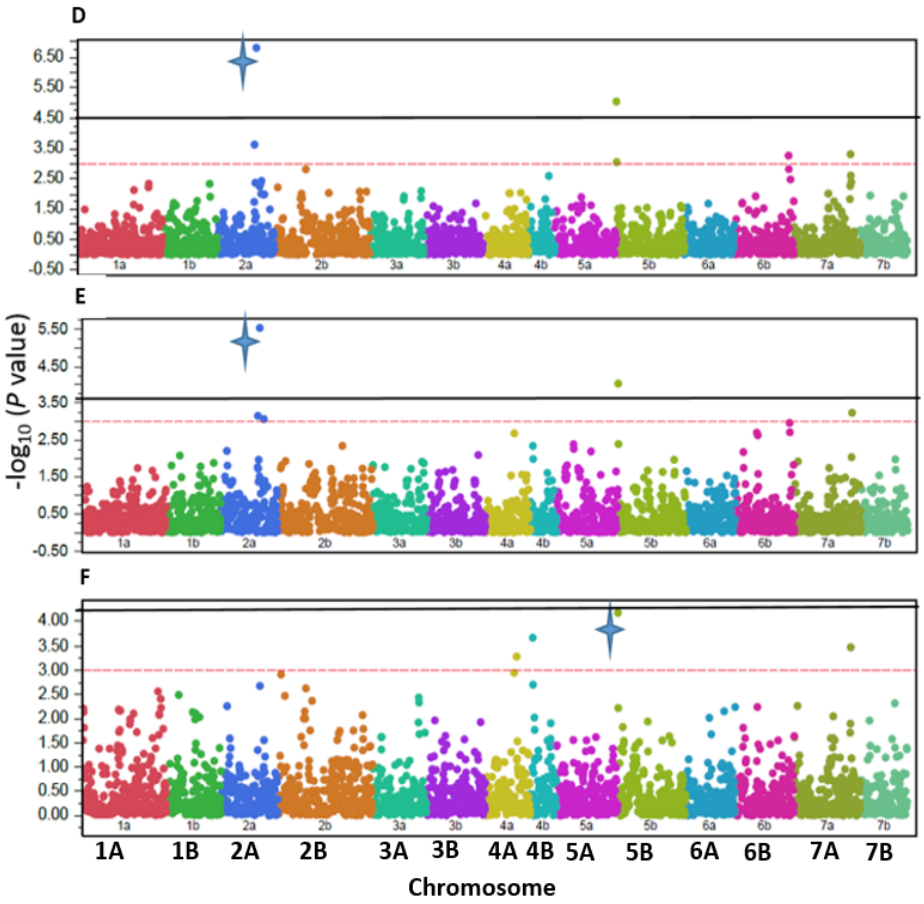

Figure 2.3. Manhattan plots showing *P*-values for single-nucleotide polymorphism (SNP) markers associated with response to leaf rust at seedling and adult plant stages (continued).

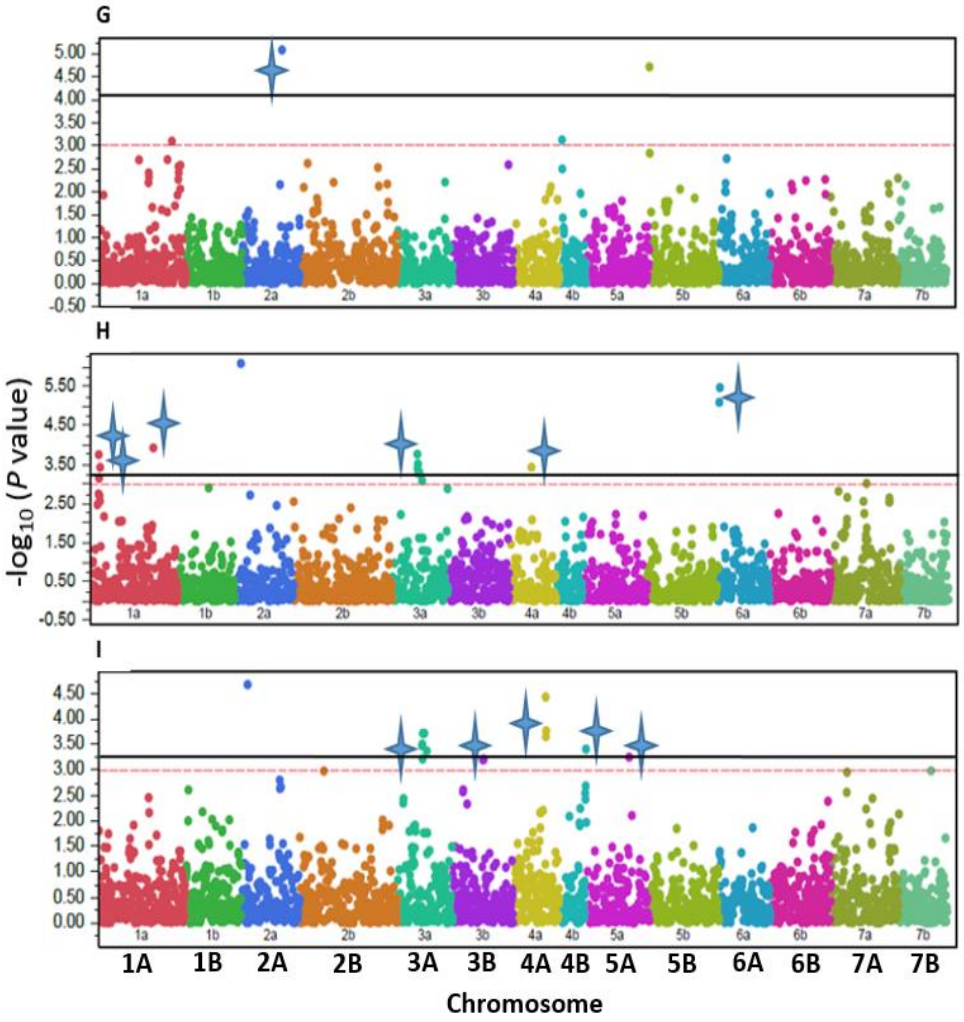

Figure 2.3. Manhattan plots showing *P*-values for single-nucleotide polymorphism (SNP) markers associated with response to leaf rust at seedling and adult plant stages (continued).

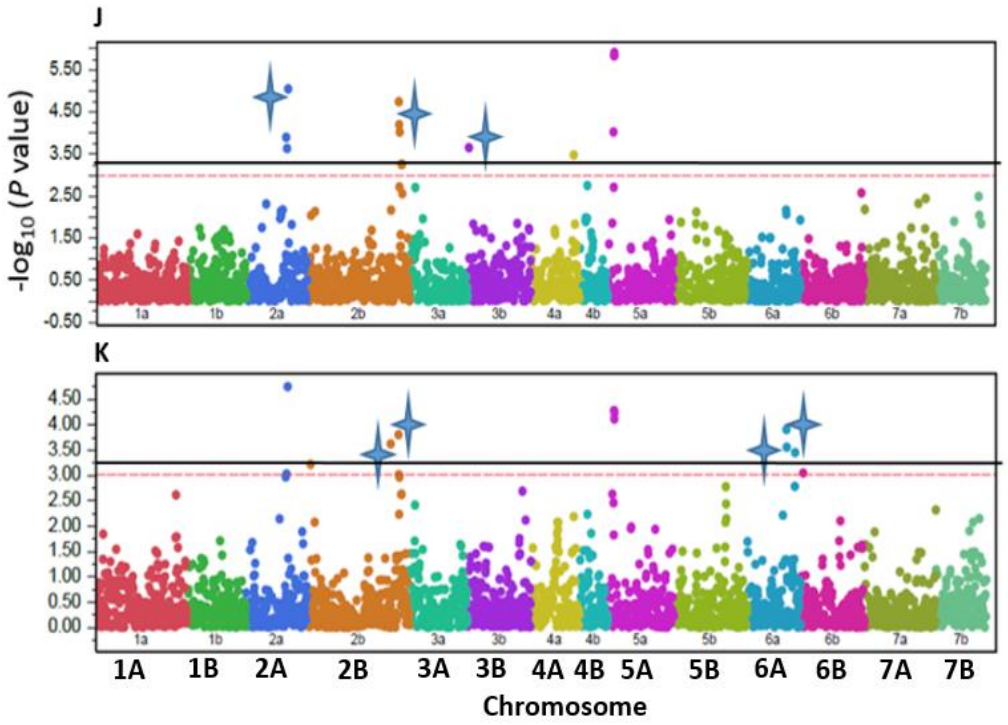

Figure 2.3. Manhattan plots showing *P*-values for single-nucleotide polymorphism (SNP) markers associated with response to leaf rust at seedling and adult plant stages (continued).

A total of 19 SNPs, representing 11 loci, were significantly associated with leaf rust response in two or more tests. Marker *IWA7547* located on chromosome 2AL at 137.1 cM position based on the consensus map of Maccaferri et al (2015) was associated with response to all the *Pt* races tested at seedling stage except BBBQJ (Table 2.6).

Table 2.6. Significant markers associated with leaf rust for two or more *Puccinia triticina* races or race mixture.

Trait [a]	Locus	Marker	Chromosome	MAF [b]	cM [c]
MX_Ob_F + MX_EB_F	*Lr.locus-1A1*	*IWA1387*	1AS	0.08	27.9
MX_Ob_F + MX_EB_F	*Lr.locus-1A2*	*IWA7151*	1AS	0.13	31.9
MN_StP_F + MN_Cr_F	*Lr.locus-2A2*	*IWA2696*	2AS	0.10	38.0
MBDSD + MCDSS + BBBQD (ND) +BBBQD (CDL) + Race mix	*Lr.locus-2A4*	*IWA7547*	2AL	0.07	137.1
BBBQD (ND) + BBBQD (CDL)	*Lr.locus-2B5*	*IWA1765*	2BL	0.13	148.0
MN_StP_F + MN_Cr_F	*Lr.locus-3A2*	*IWA1536*	3AL	0.10	83.4
MN_StP_F + MN_Cr_F	*Lr.locus-3A2*	*IWA1700*	3AL	0.10	83.4
MX_EB_F + MN_StP_F + MN_Cr_F	*Lr.locus-3A2*	*IWA3512*	3AL	0.11	83.4
MN_StP_F + MN_Cr_F	*Lr.locus-3A2*	*IWA3999*	3AL	0.09	83.4
MN_StP_F + MN_Cr_F	*Lr.locus-3A2*	*IWA1701*	3AL	0.09	83.5
MX_Ob_F + MN_Cr_F	*Lr.locus-4B3*	*IWA4618*	4BL	0.05	123.3
BBBQD (ND) + BBBQD (CDL)	*Lr.locus-5A1*	*IWA3811*	5AS	0.13	27.2
BBBQD (ND) + BBBQD (CDL)	*Lr.locus-5A1*	*IWA4069*	5A	0.13	27.2
BBBQD (ND) + BBBQD (CDL)	*Lr.locus-5A1*	*IWA5615*	5A	0.13	27.2
MBDSD + MCDSS + BBBDB+ Race mix	*Lr.locus-5B1*	*IWA757*	5BS	0.38	2.7
BBBQJ + BBBQD (CDL)	*Lr.locus-6A2*	*IWA3023*	6AL	0.45	88.2
BBBQJ + BBBQD (CDL)	*Lr.locus-6A2*	*IWA3024*	6AL	0.44	88.2
MX_Ob_F + MX_EB_F	*Lr.locus-6B*	*IWA6904*	6BL	0.05	89.6
MX_Ob_F + MX_EB_F	*Lr.locus-6B*	*IWA657*	6BL	0.05	89.7

[a] Race mix, seedling test with inoculum composed of races MHDSB, MFPSB, MLDSB, TBBGJ, TFBJQ, and TFBGQ; MN_StP_F: races in Minnesota St. Paul field; MN_Cr_F: races in Minnesota Crookston field; MX_Ob_F, races in Mexico Ciudad Obregón field; MX_EB_F: races in Mexico El Batán field.
[c] MAF, minor allele frequency.
[d] cM, marker position on consensus map.

Discussion

In this study, we identified durum wheat accessions carrying race-specific and broad-spectrum resistance to leaf rust. The GWAS identified 88 significant SNPs associated with leaf rust response. These markers represent 37 loci distributed across all chromosomes except 1B and 7B. Chromosomes 1B and 7B are known to carry adult-plant leaf rust resistance genes in bread wheat including *Lr46* (1BL) and *Lr68* (7BL) (Singh et al. 1998; Herrera-Foessel et al. 2012).

Some of the identified loci in this study appeared to be located in genomic regions of previously characterized *Lr* genes and QTL in earlier AM studies and biparental populations in durum and bread wheat. However, several other loci are previously unknown to be associated with leaf rust response. These findings will be valuable to both durum and bread wheat breeding programs.

Difference in frequency of resistance to races collected from bread wheat and durum wheat were evident in the germplasm collection. The majority of accessions were resistant to races collected from bread wheat, while most were susceptible to races collected from durum wheat, which agrees with previous studies (Singh 1991; Huerta- Espino and Roelfs 1992; Ordonez and Kolmer 2007a). Unexpectedly, low levels of resistance to the isolates collected from durum wheat fields in North Dakota in 2012 (race MBDSD and MCDSS) were observed. Isolates of these races are also commonly found on bread wheat in North Dakota and other regions in the United States (Kolmer and Hughes 2014).

Of all the evaluated genotypes, 13 accessions were resistant across all experiments. The SNP genotype showed that none of these accessions were duplicates. However, genetic mapping and allelism tests are needed to verify whether these accessions have different or the same resistance genes. Accession data in the NSGC records indicates that seven of these resistant accessions were originally collected from Mediterranean countries and the Fertile Crescent, while four accessions were from Ethiopia. This is not surprising, as these countries belongs to the center of origin and diversity of durum wheat (Demissie and Habtemariam 1991; Tesemma and Belay 1991; Salamini et al. 2002). The coevolution of the leaf rust pathogen and wheat in these areas is believed to exert selection pressure, resulting in fitness advantages and maintenance of diverse disease resistance sources in wheat (Newton et al. 2010).

Further screening at seedling stage with races collected from durum wheat from Argentina (Arg. 9.3: BBBQD), France (FRA4.3: BBBQD), Ethiopia (E11D2-1: MCDSB; E114-1: BBBQD; and E125-1: EEEEE with avirulence on Thatcher), Arizona-USA (09AZ103A: BBBQB), Mexico (LCJ/BN and BBB/BN_*Lr61*vir), and Italy (PSB7: FGBQ) and at adult-plant stage in Ethiopian and Moroccan fields in 2014 showed that eight out of the 13 previously mentioned accessions were resistant across all experiments. Thus, these accessions can be used to diversify leaf rust resistance in breeding programs globally. The remaining five accessions were susceptible to only few isolates. For instance, PI 278379 was susceptible only to Ethiopian isolates (E125-1 and E114-1), PI 519832 was susceptible to a French isolate (FRA 4.3) and an Ethiopian isolate (E114-1), PI 387263 was susceptible to only one Ethiopian isolate (E125-1), PI 324928 was susceptible to only Mexican races (LCJ/BN and BBB/BN_*Lr61*vir), and CItr 14623 was susceptible to only an Ethiopian isolate (E125-1) (Appendix B Table B1).

Out of the 37 loci associated with leaf rust resistance or susceptibility in durum wheat germplasm in this study, 11 were associated with response to at least two *Pt* races and experiments. Loci associated with both seedling and adult-plant response were not observed at the cutoff used, $P \leq 0.001$ and pFDR ≤ 0.1. This probably is due to the difference in the races used for screening of the durum accessions at seedling stage in the greenhouse and at adult stage in the field in addition to the presence of natural inoculum in field trials. However, increasing the pFDR to 0.3 showed SNPs (136.9–137.1 cM) associated with response to races in MN_Cr_F, MN_StP_F, and MX_EB_F were also significantly associated with response to US races (BBBQD, MBDSD, MCDSS, and race mixture) tested at seedling stage. Similarly, marker *IWA4187* (176.2 cM) on 7A was associated with response to MBDSD and races in MN_StP_F.

Twenty-six of the discovered loci in this project were race or assay specific, while the other 11 loci appear to be more stable. This suggests that breeders may need to pyramid multiple loci to achieve broad-spectrum and stable leaf rust resistance.

Comparison to previously mapped *Lr* genes

Chromosomes 3A, 5A, and 6A have not been previously shown to carry known *Lr* genes in either bread wheat or durum wheat (McIntosh et al. 2014). However, some *Lr* genes from the ancestors of wheat were reported on chromosomes 3A and 6A. This includes *Lr63* (from *T. monococcum* L.) and *Lr66* (*Aegilops speltoides* Tausch), both on 3A, and *Lr64* [from *T. dicoccoides* (Korn. ex Asch. & Graebn.) Schweinf.] on 6A (Kolmer et al. 2010; Marais et al. 2010; Kolmer 2008). The *Lr* genes introgressed from alien species were not considered in this comparison because of the low chance of their presence in the current durum wheat collection used. In addition, distantly located known *Lr* genes from the associated loci in this study were regarded as different.

Two loci on chromosome 2BL, *Lr.locus-2B2* (95.2 cM) and *Lr.locus-2B3* (100.9 cM), associated with response to leaf rust in MX_Ob_F were in close proximity to the adult plant resistance gene *Lr*35. However, this gene has been introgressed from *A. speltoides* (Seyfarth et al. 1999). On chromosome 4AS, the gene *Lr30* was located close to the identified *Lr.locus-4A1* (59.9 cM); however, the mapping information of *Lr30* was not sufficient to make inferences (Figure 2.4).

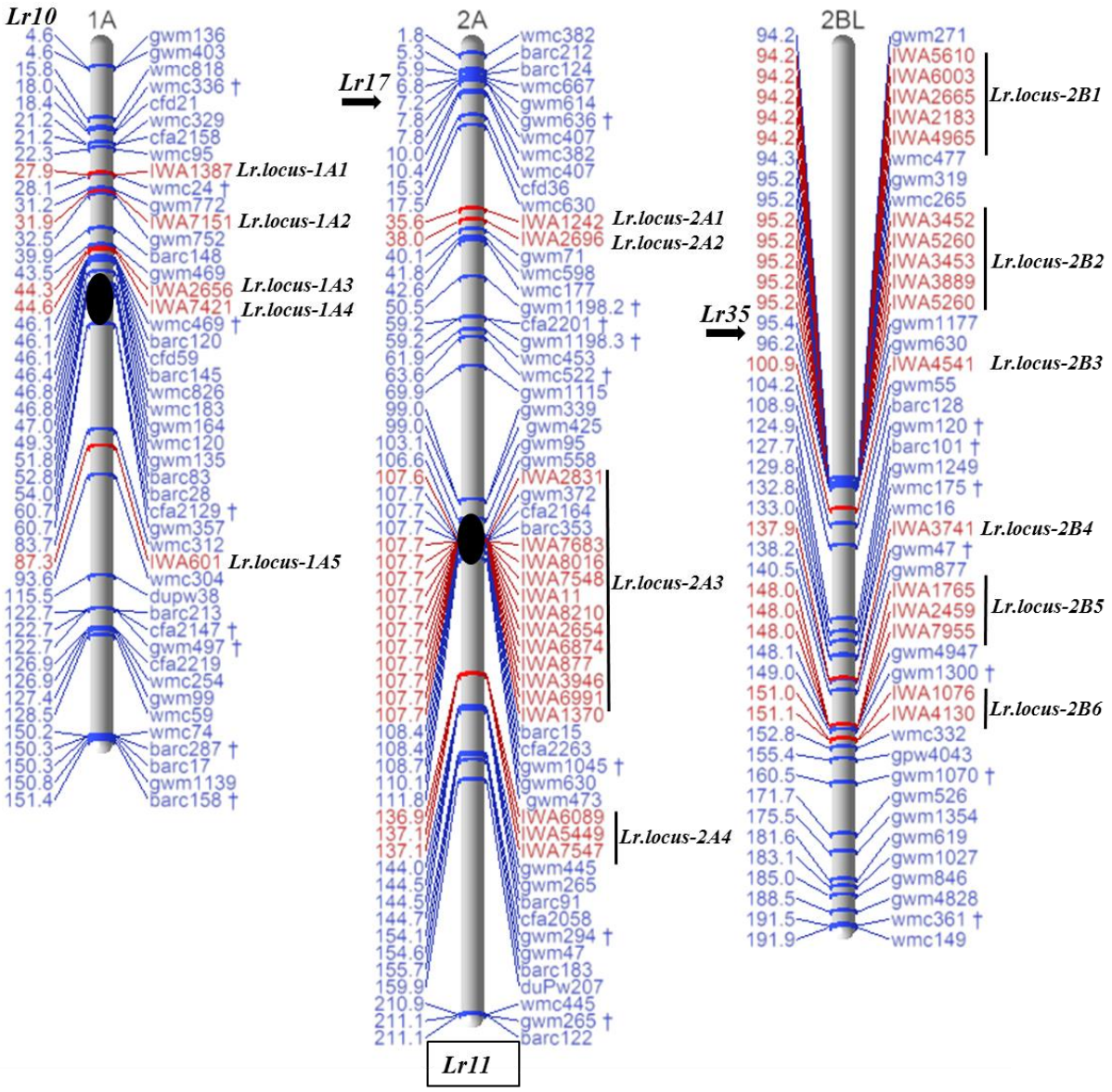

Figure 2.4. Chromosome locations of significant single-nucleotide polymorphism (SNP)–leaf rust associations in this study relative to mapped known *Lr* genes. Marker order and locations (left side of chromosome bars) are as reported by Maccaferri et al (2015). The association mapping results are reported for *Puccinia triticina* races used at seedling stage in the greenhouse and at artificially inoculated field trials performed in the United States and Mexico. Markers in red are the significant SNP–leaf rust association observed in this study. † Simple-sequence repeat (SSR) marker associated with leaf rust response in durum wheat in Maccaferri et al. study (2010b). The genetic locations of *Lr* genes are indicated with arrows. The *Lr* genes assigned to chromosomes but not yet mapped are in a box at the bottom of each chromosome. Centromere position is indicated with a black circle on the chromosome bar. Not all SSR in the tetraploid consensus map (Maccaferri et al. 2015) are presented in this figure, a more saturated genetic map is presented in Supplemental Table S3.

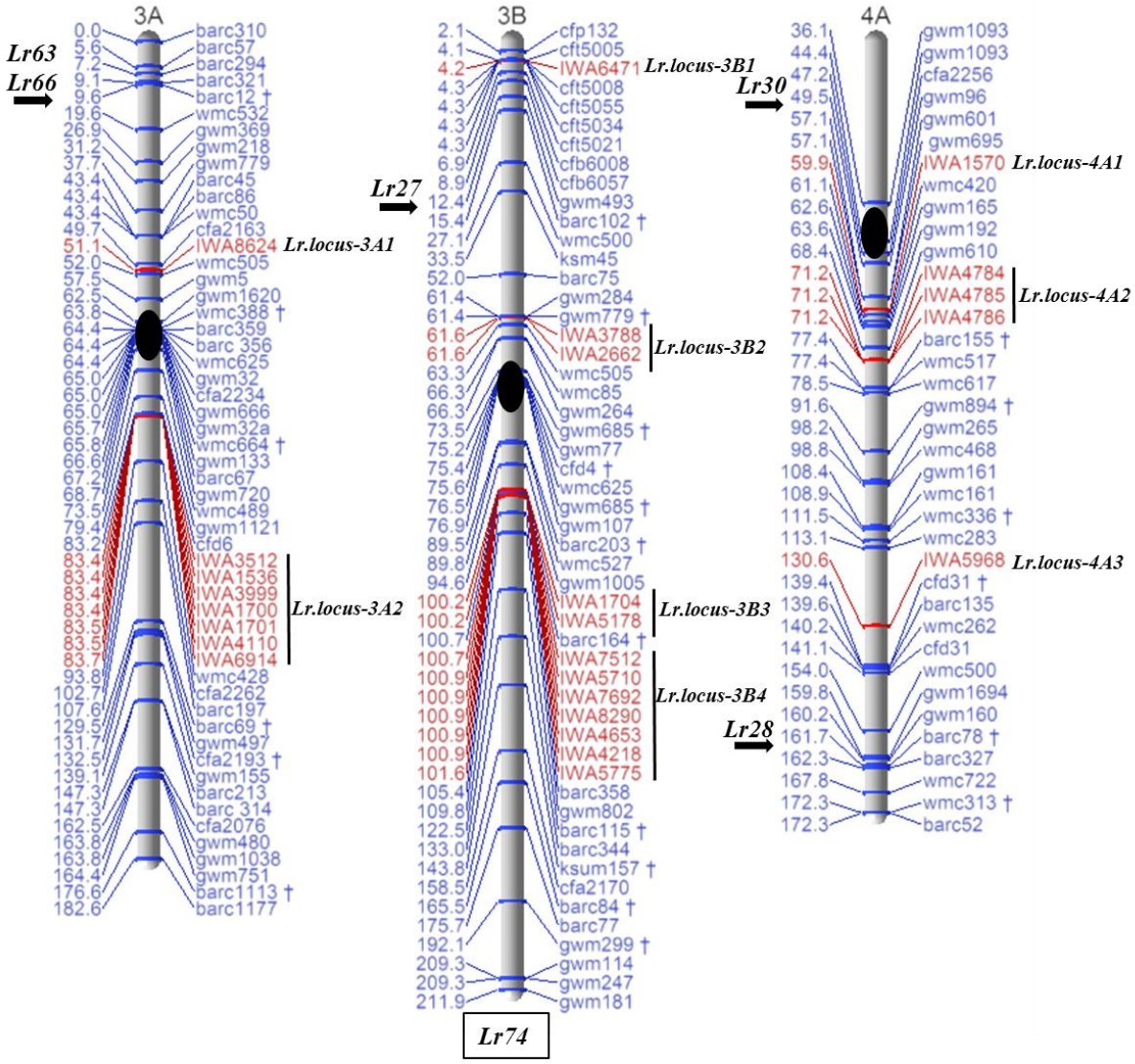

Figure 2.4. Chromosome locations of significant single-nucleotide polymorphism (SNP)–leaf rust associations in this study relative to mapped known *Lr* genes (continued).

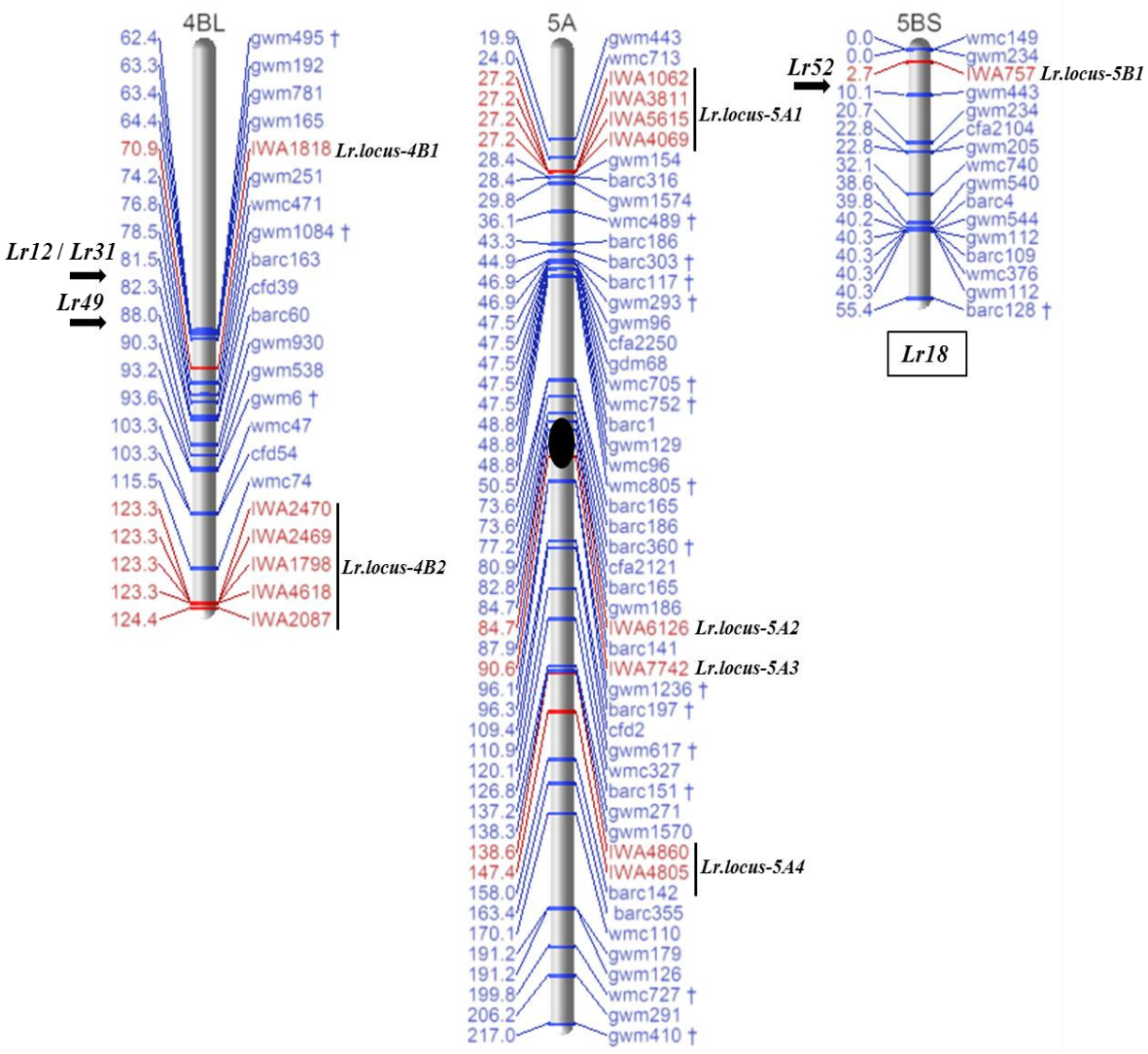

Figure 2.4. Chromosome locations of significant single-nucleotide polymorphism (SNP)–leaf rust associations in this study relative to mapped known *Lr* genes (continued).

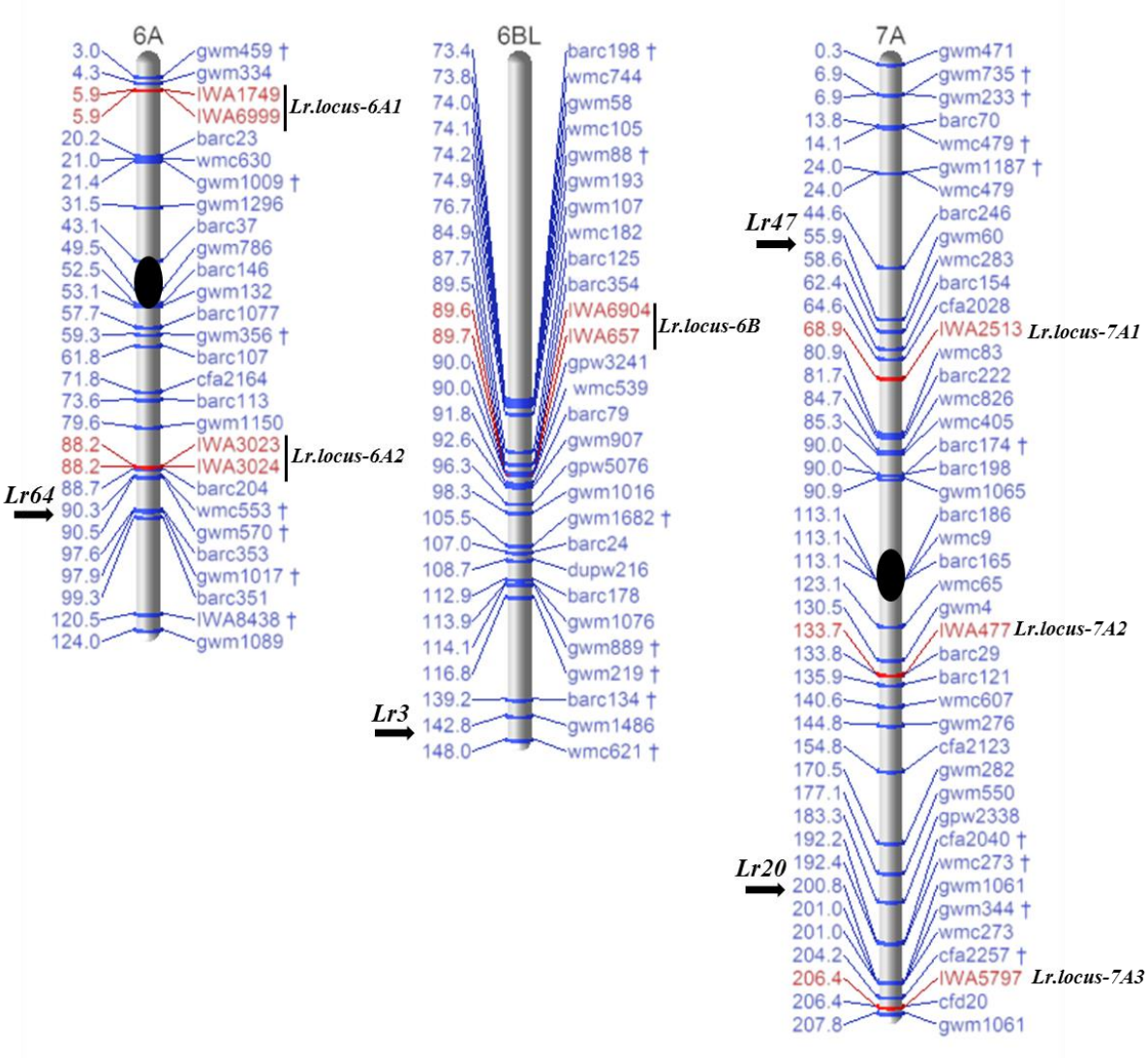

Figure 2.4. Chromosome locations of significant single-nucleotide polymorphism (SNP)–leaf rust associations in this study relative to mapped known *Lr* genes (continued).

On chromosome 4BL, *Lr.locus-4B1* (70.9 cM) was mapped in the vicinity of *Lr12*, *Lr31*, and *Lr49*. The *Lr.locus-4B1* was associated with response to race BBBQJ tested as seedling stage, which distinguishes them from the adult-plant resistance genes *Lr12* and *Lr49* (Dyck 1991; Bansal et al. 2008). Likewise, *Lr.locus-5B1* (2.7 cM) on 5BS was in close proximity to *Lr52*, which is closely linked to *gwm443* and *gwm234* and is also thought to confer leaf rust resistance in the Australian durum cultivar Wollaroi (Singh et al. 2010) and in accessions of the Watkins wheat collection (Dyck and Jedel 1989). In addition, the locus *Lr.locus-6A2* (88.2

cM) on chromosome 6A was located within the genomic region close to *Lr64* (Kolmer 2008; Kolmer et al. 2010; Marais et al. 2010). On chromosome 7AL, *Lr.locus-7A3* (206.4 cM) appeared near the genomic region where the gene *Lr20* (Watson and Luig 1966; Sears and Briggle 1969; Neu et al. 2002) was mapped (Figure 2.4).

Associated SNPs within the genomic region of *Lr14a* (7BL), which has been widely used, especially in the CIMMYT durum wheat breeding programs, were not detected in the present study. This most probably is due to the rare frequency of *Lr14a* in the current germplasm collection as estimated based on the screening with the *Lr14a* diagnostic markers (*gwm344* and *gwm146*) (data not shown). This agrees with the statement that GWAS has limited power to detect alleles that occur at low frequency in the germplasm (Myles et al. 2009; Brachi et al. 2011).

In summary, from this comparison, four of the currently identified loci are probably associated with *Lr31*, *Lr52*, *Lr64*, and *Lr20*. However, allelism tests are necessary to determine the relationship between these loci and the above-mentioned *Lr* genes.

Comparison to previous durum wheat–leaf rust association mapping study

The recently published tetraploid wheat consensus map (Maccaferri et al. 2015) containing different types of markers provided an opportunity to compare our GWAS results based on SNP markers with the only available AM of leaf rust response in durum wheat that was based on SSR markers (Maccaferri et al. 2010b) (Figure 2.4).

***Chromosome 1AS*:** The loci *Lr.locus-1A1* (27.9 cM) and *Lr.locus-1A2* (31.9 cM) were located close to *wmc24* (28.1 cM). The latter was strongly associated with response to a durum specific race from Italy and a bread wheat isolate from Poland. Two more loci, *Lr.locus-1A3* (44.3 cM) and *Lr.locus-1A4* (44.6 cM), were mapped close to *wmc469* (46.1 cM). Marker

wmc469 was previously reported to be associated with response to European and Mexican isolates tested at both adult plant and seedling stages.

Chromosome 2AL**:** *Lr.locus-2A3* (107.6–107.7 cM) was located close to SSR marker *gwm1045* (108.9 cM), which was shown to be associated with response to durum-wheat-type races from Italy both at adult-plant and seedling stages.

Chromosome 2BL: *Lr.locus-2B4* (137.9 cM) was located close to *gwm47* (138.2 cM). Two more loci, *Lr.locus-2B5* (148 cM) and *Lr.locus- 2B6* (151 cM), were proximal to *gwm1300* (149 cM). Both SSR markers (*gwm47* and *gwm1300*) were associated with leaf rust response at seedling stage to both bread wheat- and Triticale-specific isolates from Poland.

Chromosome 3B: *Lr.locus-3B2* (61.6 cM) was in the same genomic location as the SSR marker *gwm779* (61.4 cM), which was associated with response at seedling stage to isolates collected from Poland, Italy, and the United Kingdom. Two other loci, *Lr.locus-3B3* (100.2 cM) and *Lr.locus-3B4* (100.7–101.6 cM), on the same chromosome, were close to *barc164* (100.7 cM). The latter was associated with response at seedling stage to the durum-specific race BBBGJ collected from Italy.

Chromosome 4AL: *Lr.locus-4A2* (71.2 cM) was closely mapped to *barc155* (77.4 cM), which was previously associated mainly with response at seedling stage to BBBGJ from Italy.

Chromosome 4BL: *Lr.locus-4B2* (70.9 cM) was in the vicinity of *gwm1084* (78.5 cM). This SSR was associated with reaction at seedling stage to a Triticale isolate (Poland) and two isolates from bread wheat that were collected from Poland and the United Kingdom.

Chromosome 5AL: *Lr.locus-5A3* (90.6 cM) was in close proximity to *gwm1236* (96.1 cM) and *barc197* (96.3 cM). The marker *gwm1236* was previously associated with leaf rust response at seedling stage to bread-wheat-type isolates collected from Poland and the United

Kingdom, while *barc197* was associated with response at adult stage to races in Italian field trials.

Chromosome 6A: *Lr.locus-6A1* (5.9 cM) was in close proximity to SSR marker *gwm459* (3.0 cM), which was associated with response at seedling stage to some bread-wheat-type isolates that originated from Poland and the United Kingdom. One more locus, *Lr.locus-6A2* (88.2 cM), was proximal to *wmc553* (90.3 cM) and *gwm570* (90.5 cM). The *wmc553* was associated with response at seedling stage to two bread-wheat-type races from the United Kingdom, while *gwm570* was related to response at seedling stage to a bread-wheat-type isolate from the United Kingdom and a Triticale isolate from Poland.

Chromosome 7AL: *Lr.locus-7A3* (206.4 cM) was proximal to *gwm344* (205.7 cM) and *cfa2257* (204.2 cM). The SSR marker *gwm344* was associated with response at seedling stage to some tested bread wheat isolates from the United Kingdom, a Triticale isolate from Poland, and at adult-plant stage in field trials in Italy. The marker *cfa2257* was related to response at seedling stage to bread wheat isolates from Poland and the United Kingdom and a Triticale isolate from Poland.

Based on this comparison between the results of the current GWAS and the previous one performed by Maccaferri et al (2010b), 17 associations were mapped in similar positions in both studies. Of these, 12 loci were associated with adult-plant response, while five loci were associated with seedling-stage response to leaf rust in the current study.

Comparison to recent bread wheat–leaf rust association mapping studies

To investigate possible cross-relationship between associated loci with leaf rust response found in durum and bread wheat, a comparison with GWAS on bread wheat germplasm was performed. Based on the genomic position of the SNPs in the tetraploid consensus map, eight

identified loci in this study were previously reported in two AM analyses in bread wheat (Kertho et al. 2015; Gao et al. 2016).

For instance, the GWAS by Kertho et al (2015) on winter wheat germplasm reported *IWA3160* (50.1 cM) on 1A to be associated with response to the bread-wheat-type race TBDJ. This locus was proximal to the significant loci *Lr.locus-1A3* (44.3 cM) and *Lr.locus-1A4* (44.6 cM) for bread-wheat-type races in MN_StP_F. Similarly, *IWA7429* (107.7 cM) on 2A and *IWA5526* (135.1 cM) on 7A that were found to be in association with response to the bread wheat- type race TDBG in winter wheat were mapped close to *Lr.locus-2A3* (107.6–107.7 cM) and *Lr.locus-7A2* (133.7 cM), respectively. The marker *IWA6244* (66.1 cM) on 3B that was significant for bread-wheat-type race MCDL was closely mapped to *Lr.locus-3B2* (61.6 cM). The loci, *Lr.locus-2A3*, *Lr.locus-7A2*, and *Lr.locus-3B2* in the current work were associated with durum-type races in field experiments (MX_EB_F and MX_Ob_F).

In addition, a comparison with the GWAS on spring wheat germplasm by Gao et al. (2016) was conducted. This revealed that *IWB74350*/*IWB73424* (2.4 cM) on 3B, *IWB25253/IWB57347* (72.1–73 cM) on 4A, and *IWB43173/IWB45939* (5.9 cM) on 6A, which were significant for North American bread-wheat-type races in spring wheat, were in close proximity to the *Lr.locus-3B1* (4.2 cM), *Lr.locus-4A2* (71.2 cM), and *Lr.locus-6A1* (5.9 cM), respectively. The latter loci were associated with response to bread-wheat-type races in field trials (MN_ Cr_F and MN_StP_F) in the present study. The results of this comparison suggest that some similarities between durum and bread wheat leaf rust resistance do exist.

Even though, some bread-wheat-type races (BBBDB, race mixture, and races in MN_StP_F and MN_Cr_F) used by Gao et al (2016) were the same as those in the current work, there were very few similarities between reported loci. In addition, the race BBBQD (isolate

collected from California) was also used by Gao et al (2016); however, no common associations were observed with the present study. This shows that leaf rust resistance in durum and bread wheat are under the control of different genetic loci.

Comparison with quantitative trait loci meta-analysis for leaf rust resistance in durum and bread wheat

The identified SNPs in this work were also compared with the recent QTL meta-analysis study that was performed using 20 biparental mapping populations and 33 different parental lines of durum and bread wheat (Soriano and Royo 2015). The comparison showed that nine identified loci in this study were previously reported in the QTL meta-analysis. For instance, two loci on 1A, *Lr.locus-1A1* (27.9 cM) and *Lr.locus-1A2* (31.9 cM), were in the genomic region of *wmc95* (22.3 cM), which is a marker linked to resistant QTL in the bread wheat cultivars Apache and Sujata (Azzimonti et al. 2014; Lan et al. 2015). Another locus on 2A, *Lr.locus-2A3* (107.6 cM), was positioned close to *gwm339* (99.0 cM), which is one of the flaking marker of a QTL identified in the durum cultivar Creso (Marone et al. 2009). Likewise, *Lr.locus-2B1* (94.2 cM) and *Lr.locus-2B2* (95.2 cM) on 2B were closely mapped to *gwm55* (94.0 cM) that is linked to QTL in bread wheat cultivars W-7984, Kariega, Avocet, and Carberry (Faris et al. 1999; Prins et al. 2011; Singh et al. 2014). The comparison also revealed that *Lr.locus- 3B2* (61.6 cM) on 3B was located close to *gwm566* (59.0 cM), which is in the genomic region of QTL in the bread wheat cultivars TA4152-6 and Francolin#1 (Chu et al. 2009; Lan et al. 2014). Three loci, *Lr.locus-2B4*, *Lr.locus-4B1*, *Lr.locus-5A1*, that were associated with response to leaf rust at seedling stage in this study, were in the genomic regions of QTL in Apache and Cresso on 2B; Avocet, Creso, and Forno on 4B; and Oberkulmer on 5A; respectively (Messmer et al. 2000;

Schnurbusch et al. 2004; William et al. 2006; Marone et al. 2009; Azzimonti et al. 2014). However, these previously identified QTL were associated with adult plant resistance.

In summary, the comparison between the 37 loci identified in the current study, characterized *Lr* genes, loci identified in previous GWAS, and QTL meta-analysis in biparental mapping populations in both durum and bread wheat revealed that 22 of the generated loci in the present work were previously reported. Consequently, 14 loci (*Lr.locus-1A5*, *Lr.locus-2A1*, *Lr.locus-2A2*, *Lr.locus-2A4*, *Lr.locus-2B3*, *Lr.locus-3A1*, *Lr.locus-3A2*, *Lr.locus-4A1*, *Lr.locus-4A3*, *Lr.locus-5A1*, *Lr.locus-5A2*, *Lr.locus-5A4*, *Lr.locus-6B*, and *Lr.locus-7A1*) may be novel. Five of those (*Lr.locus-2A2*, *Lr.locus-2A4*, *Lr.locus-3A2*, *Lr.locus-5A1*, and *Lr.locus-6B*) are of special importance, as they are associated with leaf rust response to more than one *Pt* race. The identification of these loci through GWAS is a significant step in characterization of genes that may be used to widen the genetic diversity of leaf rust resistance in durum wheat germplasm.

Conclusions

This study identified durum wheat accessions with both race-specific and broad-spectrum resistance. The majority of durum accessions were resistant to races collected from bread wheat, while most were susceptible to races collected from durum wheat. Thirteen accessions showed resistance to all races tested at seedling stage and to a race mixture in field experiments in both the United States and Mexico. Of these, eight accessions (PI 209274, PI 192051, PI 244061, PI 223155, PI 534304, PI 193920, PI 342647, and PI 195693) were also resistant at seedling stage to additional nine isolates, collected from Mexico, Argentina, France, Ethiopia, and Italy, and at adult stage in field trials in Ethiopia and Morocco. These broad-spectrum resistant accessions could be a good leaf rust resistance source to introgress into locally adapted germplasm in breeding programs globally.

The GWAS revealed 88 SNPs representing 37 loci associated with leaf rust response across all durum wheat chromosomes except 1B and 7B. The comparison of their genomic regions with the known *Lr* genes, previous AM studies, QTL mapping in biparental populations in durum and bread wheat revealed 14 previously uncharacterized loci, of which, five were associated with leaf rust response to two or more *Pt* races tested at seedling stage or race mixture in field trials. The marker *IWA754* (137.1 cM) on *Lr.locus-2A4* was associated with response to all *Pt* races tested at seedling stage with the exception of BBBQJ. To validate the loci revealed by the GWAS study, biparental populations have been developed from selected accessions showing wide-spectrum leaf rust resistance. Furthermore, this study will facilitate the development of tightly linked markers for marker assisted selection.

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CHAPTER III. INHERITANCE AND BULKED SEGREGANT ANALYSIS OF LEAF RUST AND STEM RUST RESISTANCE GENES IN EIGHT DURUM WHEAT GENOTYPES

Abstract

Leaf rust, caused by *Puccinia triticina* (*Pt*) and stem rust caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) are important diseases of durum wheat. Our goal was to determine the inheritance and genomic locations of leaf rust resistance (*Lr*) genes to *Pt*-race BBBQJ and stem rust resistance (*S*r) genes to *Pgt*-race TTKSK in durum accessions. Eight leaf rust resistant genotypes were used to develop bi-parental populations. Two of these accessions, PI 192051 and PI 534304 were also resistant to *Pgt*-race TTKSK. The resulting progenies were phenotyped for leaf rust and stem rust response at seedling stage. The *Lr* and *Sr* genes were mapped in six populations using SNP markers and bulked segregant analysis. Five leaf rust resistant genotypes carry single dominant genes on chromosomes 2B, 4A, 6BS, and 6BL. In the remaining accessions, the segregation did not fit into expected segregation ratio of a single dominant gene. Further mapping using KASP assays, showed that the single dominant *Lr* gene in PI 209274 was flanked by *IWA3298* and *IWB39456* on 6BS. Seven genotypes carry different *Lr* genes to those previously characterized in durum wheat cultivars. The stem rust resistance to *Pgt* race TTKSK in PI 534304 is most likely conferred by *Sr13*, while the *Sr* gene in PI 192051 appears to be new.

Introduction

Durum wheat (*Triticum turgidum* L. var. *durum* (Desf.), an allotetraploid (2n=4x=28), is economically an important cereal crop used primarily for pasta production. Durum wheat is grown mainly in the Mediterranean countries, Canada, Mexico, United States, and Ethiopia (Vavilov 1951; Ordoñez and Kolmer 2007b; Habash et al. 2009; Goyeau et al. 2012). North

Dakota (ND) is the largest durum-producing state in USA accounting for more than 50% of the total US production, which is worth more than $300 million per year (NASS 2016).

Wheat rust diseases have historically been a major constrain for wheat production, severely reducing yield and kernel quality. Durum wheat has been traditionally considered more resistant to leaf rust (caused by *Puccinia triticina* Erikss.) compared to common wheat (*T. aestivum* L.; 2n= 6x=42). However, in recent years, *Puccinia triticina* (*Pt*) races highly virulent on resistant durum wheat cultivars are increasingly impacting the durum production worldwide (Singh et al. 2004; Goyeau et al. 2006; Huerta-Espino et al. 2009). For instance, *Pt* race BBG/BN and its variants, with virulence to *Lr72,* overcame the resistance of the adapted CIMMYT durum wheat cultivars in northwestern Mexico, which resulted in severe yield losses (Singh et al. 2004; Huerta-Espino et al. 2011). Similarly, increased susceptibility of durum wheat cultivars to leaf rust occurred in other durum producing areas including the Mediterranean basin, the Middle East, and Chile (Singh et al. 2004; Martinez et al. 2005; Ordoñez and Kolmer 2007a; Goyeau et al. 2012). In the United States, a race with similar virulence phenotype and SSR genotype to the previously identified BBG/BN Mexican race, was collected on durum in California in 2009 (Kolmer 2013). This race was designated as BBBQJ following the *Pt* nomenclature system of Long and Kolmer (1989). The same race was later collected in 2013 on the hard red winter wheat cultivar ‘Overley’ in Kansas (Kolmer 2015). This race is also virulent to *Lr39/41* that is present in many hard red winter wheat cultivars grown in the Southern Great Plains. This race could become established in the winter wheat crop and then migrate northward to the durum producing region of ND (Kolmer 2015).

Typically, the *Pt* isolates virulent on durum wheat cultivars are different in their virulence phenotypes from the common wheat-type isolates as these are avirulent to many of the *Lr* genes

present in common wheat (Goyeau et al. 2006; Ordoñez and Kolmer 2007a). The *Pt* isolates collected from common wheat are generally avirulent on durum wheat (Singh 1991; Huerta-Espino and Roelfs 1992; Ordoñez and Kolmer 2007a). Currently, few *Lr* genes have been mapped in durum wheat. Characterized *Lr* genes in durum and other tetraploid wheat include *Lr3a* (Herrera-Foessel et al. 2005), *Lr10* (Aguilar-Rincon et al. 2001), *Lr14a* (Herrera-Foessel et al. 2008b), *Lr23* (McIntosh and Dyck 1975; Nelson et al. 1997), the complementary gene pair *Lr27+31* (Singh and McIntosh 1984a; Singh and McIntosh 1984b; Singh et al. 1993), *Lr33* (Dyck et al. 1987; Dyck 1994), *Lr46* (Herrera-Foessel et al. 2011), *Lr47* (Dubcovsky et al. 1998), *Lr52* (Singh et al. 2010), *Lr61* (Herrera-Foessel et al. 2008a), *Lr64* (Dyck 1994; McIntosh et al. 2009), *Lr72* (Herrera-Foessel et al. 2014a), and *LrCamayo* (Herrera-Foessel et al. 2007). However, races with virulence to most of these *Lr* genes are currently present. For instance, virulence to *Lr10*, *Lr23*, and *Lr33* is common in durum type *Pt* races (Huerta-Espino and Roelfs 1992; Singh et al. 2005; Ordóñez and Kolmer 2007a). In addition, *Pt* race BBG/BN and its variants are virulent to *Lr72* (Singh et al. 2004; Huerta-Espino et al. 2011). A *Pt* race virulent to *Lr27+Lr31* and *Lr3a* was detected in Mexico in 2008 (Huerta-Espino et al. 2009). Similarly, a race of *Pt* that was collected in Mexico in 2010 is virulent to *Lr61* (Herrera-Foessel et al. 2014b). The gene *Lr14a* is not effective against the common races currently present in France, Spain, Chile, Argentina, Morocco, and Tunisia (Ordoñez and Kolmer 2007a; Goyeau et al. 2012 ; Gharbi et al. 2013; Soleiman et al. 2016; Kolmer and Acevedo, unpublished). Therefore, the identification of new *Lr* genes is crucial to mitigate the durum wheat yield loss caused by leaf rust.

Stem rust caused by *Puccinia graminis* f. sp. *tritici* Erikss. & E. Henn. (*Pgt*) is one of the most destructive diseases of common wheat and durum wheat that can result in a complete loss

of the crop under high disease severity (McIntosh and Brown 1997; Singh et al. 2011). The race TTKSK (Ug99) was first detected in Uganda in 1998 (Pretorius et al. 2000). This race spread to Kenya in 2001 and to Ethiopia by 2003. It was later detected in Sudan, Yemen, Iran, South Africa, and Egypt (Jin et al. 2008; Nazari et al. 2009; Pretorius et al. 2010; http://rusttracker.cimmyt.org/). Currently, more than 60 *Sr* genes have been identified in wheat (McIntosh et al. 2013, 2014; Rahmatov et al. 2016) and approximately 29 are effective to races of the Ug99 lineage (Yu et al. 2014; Niu et al. 2014; Yu et al. 2015). However, the resistance levels conferred by these *Sr* genes differ. For instance, only few of these effective *Sr* genes have broad spectrum of resistance to the current Ug99 lineage races. In addition, many of these *Sr* genes were transferred to wheat from wild relatives, thus reducing the linkage drag associated with the alien translocations carrying the genes is required before the use of these resistance sources in breeding lines (Singh et al. 2011).

In durum wheat, the mapped *Sr* genes and quantitative trait loci (QTL) associated with stem rust resistance are limited compared to those mapped in common wheat. The resistance to race TTKSK in durum wheat, particularly in the North American cultivars, is mainly due to the presence of *Sr13* originating from the emmer wheat (*T. turgidum* L. ssp. *dicoccum*) Khapli (Jin et al. 2007; Klindworth et al. 2007). However, in recent years *Pgt* races, different from the Ug99 lineage group (TRTTF and JRCQC), have been identified in Ethiopia with combined virulence on *Sr13* and *Sr9e* (Olivera et al. 2012, 2015). Additionally, race TKTTF (‘Digalu’ race) is highly virulent on both durum and common wheat (Olivera et al. 2015). Therefore, widening the global genetic diversity of stem rust resistance in durum wheat germplasm is urgently required for more durable resistance.

Whereas quantitative adult plant resistance (APR), often based on several minor alleles/genes (Gustafson and Shaner 1982) is a very important objective in breeding programs, pyramiding several qualitative resistance genes that are usually identified at seedling stage is another approach to achieve durable resistance. Seedling tests allow for screening many lines in short period of time and small space compared to adult-plant tests in field trials (Letta et al. 2014).

The bi-parental mapping populations has been the standard approach used to identify the chromosomal locations of plant disease resistance loci. Bulked segregant analysis (BSA) is a quick and relatively inexpensive method to efficiently identify molecular markers associated with a trait response. The procedure consisted of comparing two pooled DNA samples of individuals from a segregating population originating from a single cross. Within each bulk, the individuals are identical for the trait or gene of interest but are segregating randomly for all other genes. The two bulks that are contrasting for a trait such as resistance and susceptibility to a disease are analyzed to find molecular markers that differentiate them. The markers that are polymorphic between the pools will therefore be linked genetically to the locus that is associated with the trait used to make the bulk (Michelmore et al. 1991).

The objective of the current study was to determine the inheritance of leaf rust (*Pt*-race BBBQJ) and stem rust (*Pgt*-race TTKSK) resistance at seedling stage in eight durum wheat genotypes selected from the USDA-National Small Grains Collection (NSGC), Aberdeen, ID. Genomic regions of the *Lr* and *Sr* genes were mapped in six biparental populations using a high density SNP markers and the BSA approach.

Materials and methods

Biparental crosses and characterization of leaf rust resistance inheritance

Eight resistant genotypes were selected from the USDA–NSGC for their low infection types to *Pt* race BBBQJ to develop biparental crosses (Table 3.1).

Table 3.1. Origin, type, and reaction to leaf rust and stem rust of the parental genotypes used in the crosses.

Parents of the crosses	Type	Origin	IT to BBBQJ[a]	IT to TTKSK[b]
PI 534304	Landrace	Ethiopia	;1-	2
PI 192051	Landrace	Portugal	0;	2-
PI 313096	Landrace	Cyprus	;1-	.
PI 387263	Landrace	Ethiopia	;1	.
PI 209274	Breeding line	Australia	;1	.
PI 278379	Landrace	Malta	;1+	.
PI 244061	Landrace	Yemen	;1	.
PI 195693	Landrace	Ethiopia	;	.
Rusty[c]	Line	North Dakota (USA)	3+	3+
Divide[c]	Cultivar	North Dakota (USA)	3	.

[a] Infection types of the parental genotypes to *P. triticina* race BBBQJ.
[b] Infection types of the parental genotypes to *P. graminis* f. sp. *tritici* race TTKSK.
[c] Susceptible parents of the crosses.

These genotypes are PI 534304, PI 313096, PI 387263, PI 209274, PI 278379, PI 244061, PI 192051, and PI 195693. These genotypes were previously reported to carry resistance to several *Pt* races at seedling stage in the greenhouse and at adult-plant stage in the field in several locations worldwide (Aoun et al. 2016). These resistant parental lines were originally collected from Ethiopia, Portugal, Cyprus, Australia, Malta, and Yemen (Table 3.1). All of these genotypes are landraces except for PI 209274 which is a breeding line. The susceptible parents of the crosses were Rusty and /or Divide. Divide was released in 2005 by North Dakota State University (NDSU) and it occupies currently around 30% of the total durum wheat acreage in ND (NASS 2015). The rust susceptible line, Rusty (Reg. no. GS-155, PI 639869), was released

in 2004 by the USDA-ARS Northern Crops Science Laboratory, Fargo, ND and NDSU (Klindworth et al. 2006).

Crosses between resistant and susceptible parents were made at the North Dakota (ND) Agricultural Experiment Station Greenhouse Complex, Fargo, ND, USA during the summer of 2013. In all the bi-parental populations, Rusty and Divide were the female parents of the crosses and the resistant genotypes were the pollen donors. Biparental crosses were advanced using the single seed descent method to F_6 generation except the biparental crosses involving the resistant genotypes PI 192051, PI 244061, and PI 195693 that were advanced to F_3 generation. The biparental populations were screened at seedling stage with *Pt*-race BBBQJ during winter months (December-February) in the biosafety level two facility at the Agricultural Experiment Station Greenhouse Complex in Fargo, ND, in generations F_1, F_2, F_3, and F_6.

The single pustule isolate CA1.2 of race BBBQJ was originally isolated from a sample collected from durum wheat fields in California (USA). Its virulence/avirulence phenotype was given based on infection types (ITs) at seedling stage on the international differential sets of 'Thatcher' wheat near-isogenic lines, with each line carrying single *Lr* resistance gene (Long and Kolmer 1989).

The inheritance of the gene (s) was determined in each of the biparental crosses. For the crosses that were evaluated at F_1, five to six seeds were evaluated for response to race BBBQJ. For the crosses that were tested at the F_2 stage, 118 to 342 plants were evaluated for disease response. At the F_3 generation approximately 18 to 30 seedlings from each F_3 family (101–255 families) were screened. The F_6 recombinants inbred lines (RILs) from each tested population were evaluated in a randomized complete block design with three replications with five to eight seeds from each RIL per replicate. For all tests, the seedlings were grown in the greenhouse as

described by Kertho et al. (2015). The resistant and susceptible parents of each cross, the susceptible durum wheat genotype 'RL6089', and the susceptible common wheat cultivar Thatcher were included in each tray as checks. Two replicates of differentials of Thatcher near-isogenic lines were planted alongside each experiment to confirm the purity of the race BBBQJ. Urediniospore increase, inoculation, incubation, and greenhouse conditions were as previously described by Aoun et al. (2016).

Leaf rust ITs were assessed, on the second leaf stage, 12 days after inoculation using 0-to-4 scale (Long and Kolmer 1989; McIntosh et al. 1995) where IT 0 = no disease symptom, ; = hypersensitive flecks, 1 = small uredinia surrounded by necrosis, 2 = small- to medium-size uredinia surrounded by chlorosis, 3 = medium-size uredinia with no chlorosis or necrosis, and 4 = large uredinia with no chlorosis or necrosis. The mesothetic reaction (X reaction) is a mixture of fleck and higher infection types evenly distributed on the leaf surface. The seedlings showing ITs of 0 – 2+ and X were considered resistant, while the plants showings ITs of 3 and 4 were considered susceptible (Long and Kolmer 1989; McIntosh et al. 1995).

Based on the ITs, the F_2 plants were classified as resistant (R) or susceptible (S). The F_3 families and the RILs were classified as homozygous resistant (HR), segregating (Seg), and homozygous susceptible (HS). The number of genes that were involved in the inheritance of leaf rust resistance were estimated based on segregation ratios and the χ^2 goodness of fit test. The segregating F_6 derived RILs were excluded when computing the *P* values of the χ^2 test as only approximately 3% of the RILs were expected to be segregating.

Characterization of stem rust resistance inheritance in two biparental crosses

Two of the biparental populations that were described above, Rusty X PI 534304 and Rusty X PI 192051, were also screened with the *Pgt*-race TTKSK (isolate 04KEN156/04) at

seedling stage at F_3 generation. The genotype PI 192051 was previously reported to be resistant to race TTKSK by Olivera et al (2012), while PI 534304 was identified to be resistant to race TTKSK in the current study. Rusty was the susceptible parent to race *Pgt*-TTKSK (Table 3.1). The virulence/avirulence profile of *Pgt* race TTKSK is *Sr24, 36, Tmp/ Sr5, 6, 7b, 8a, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 21, 30, 31, 38, McN*.

The disease screenings were conducted in the biosafety level three facility at the USDA-ARS Cereal Disease Laboratory, St. Paul, MN. Twenty plants of each F_3 family were inoculated approximately 10 days after planting with *Pgt* race TTKSK. Urediniospores, stored at -80 °C, were heat shocked at 45 °C for 15 min, then rehydrated at room temperature under a relative humidity of 80 % created with a KOH solution (Rowell 1984). The plants were inoculated as previously described by Rouse et al. (2012). Thereafter, the plants were transferred to the greenhouse and maintained at 18 ± 2°C with 16-hour photoperiod until evaluation of disease. Stem rust ITs were assessed 14 days after inoculation using 0–4 Stakman scale (Stakman et al. 1962). Seedlings showing ITs of 0–2+ were considered resistant and those with ITs of 3–4 were considered susceptible.

Based on the ITs, the F_3 families were classified HR, Seg, and HS. The segregation ratios were analyzed using the χ^2 goodness of fit test. This allowed for the estimation of the number of genes involved in the inheritance of stem rust resistance. The number of families evaluated for Rusty X PI 534304 and Rusty X PI 192051 were 131 and 118, respectively.

Bulked segregant analysis

Based on the inheritance study, six biparental populations that carry single *Lr* or *Sr* dominant resistance gene were chosen for bulked segregant analysis (BSA). Leaf tissues were collected from the F_2 plants from each population before advancing them to next generations.

The genomic regions associated with response to *Pt*- race BBBQJ was identified in five of these biparental populations (Divide X PI 313096, Rusty X PI 387263, Rusty X PI 209274, Divide X PI 244061, and Rusty X PI 192051). In three of these five biparental crosses (Rusty X PI 209274, Divide X PI 244061, and Rusty X PI 192051), the BSA was performed using DNA extracted from 10–22 HR and 10–22 HS F_2 plants. The homozygous F_2 plants were identified by phenotyping $F_{2:3}$ seedlings. For the remaining two populations, the BSA was done using DNA extracted from F_6-RILs.

The biparental cross Rusty X PI 534304 was used to locate the genomic region associated with response to *Pgt*-race TTKSK. The DNA of 16 HR and 16 HS RILs were used in the BSA. Since this population was screened with race TTKSK only at F_3 generation, The HR and HS F_6-RILs were identified for BSA based on the phenotype of the corresponding F_3 families.

The DNA of HR and HS plants was extracted using a CTAB extraction method described by Riede and Anderson (1996) and modified by Liu et al. (2006). Additional modifications of lyophilizing and grinding the leaf tissue were as described by Rouse et al. (2012). The DNA was then diluted to 50 ng/µl, and pooled in equal volumes to obtain resistant and susceptible bulks as described by Michelmore et al (1991). The HR and HS bulks and parents in each of the crosses were genotyped using the Illumina's iSelect 9K SNP array (Cavanagh et al. 2013) at the USDA-ARS Small Grain Genotyping Laboratory in Fargo, North Dakota, USA. The data generated were scored using Illumina Genome Studio software. Durum wheat cultivars that carry previously mapped *Lr* genes: Creso (*Lr14c*), Guayacan INIA (*Lr61*), Llareta INIA (*Lr14a*), Jupare C2001 (*Lr27+31*), Storlom (*Lr3a*), and Altar84 (*Lr72*) as well as the universal susceptible durum cultivar RL6089 were included in the genotyping in order to evaluate any similarities in the genetic regions of interest that were determined using BSA.

Response of the resistant genotypes to *P. triticina* races virulent to known *Lr* genes mapped in durum wheat cultivars

In order to verify whether the resistant genotypes that were used to develop the biparental crosses carry previously characterized *Lr* genes in durum wheat cultivars, *Pt* races with virulence to *Lr3a, Lr14a, Lr27+31, Lr61*, and *Lr72* were used to phenotype the parents of the crosses. Twelve durum cultivars were also included in this test including Alred as a susceptible check, the susceptible parents of the crosses (Rusty and Divide), Llareta INIA carrying *Lr14a* (Herrera-Foessel et al. 2008a), Camayo carrying *LrCamayo* (Herrera-Foessel et al. 2007), Jupare C2001 carrying *Lr27+31* (Singh and McIntosh 1984a, 1984b; Singh et al. 1993), Creso carrying *Lr14c* (Marone et al. 2009), Guayacan INIA carrying *Lr61* (Herrera-Foessel et al. 2008b), Capelli, Mindum, Russello, and Mexicali75. The *Pt* races used were BBBSJ, CBBQS, and BBB/BN_*Lr61* vir. Race BBB/BN_*Lr61* vir is avirulent on *Lr72*, which is widely present in CIMMYT's durum germplasm (Herrera-Foessel et al. 2013) and virulent to *Lr10*, *Lr23*, and *Lr61*. The race BBBSJ was collected from durum in Spain in 2014 and it is virulent to *LrB, Lr10*, *Lr14a, Lr14b*, *Lr20, Lr23*, and *Lr72*. The race CBBQS (also called CBG/BP based on the CIMMYT differential sets) was collected from durum fields in Mexico in 2008 and it carries virulence on *LrB*, *Lr3a*, *Lr3bg*, *Lr10, Lr14b*, *Lr23, Lr27+31*, and *Lr72* (Huerta-Espino et al. 2009; J. Huerta-Espino, personal communication).

Mapping of *Lr* gene in PI 209274

A total of 130 F6-RILs were phenotyped using *Pt* race BBBQJ and DNA was extracted from the RILs using the CTAB extraction protocol as described above. Based on the BSA results, defining the genomic location of the *Lr* gene in PI 209274, subsequent 11 simple-sequence repeat (SSR) and 34 kompetitive allele specific PCR (KASP) markers were used to

genotype the susceptible parent (Rusty) and the resistant parent (PI 209274). The markers were developed using the tetraploid wheat consensus map (Maccaferri et al. 2015). Only the polymorphic KASP and SSR markers were used to genotype the RILs.

For the SSR markers, the polymerase chain reactions (PCRs) were accomplished in 25µL volumes. Each reaction contains 1µl of 10µM of the forward primer, 1µl of 10µM of the reverse primer, 2.5 µl of 2.5 mM dNTPs, 5µl of 5X Green Go Taq Flexi buffer, 2.5µL of 25 mM $MgCl_2$, 0.15µl of 5 units/uL GoTaq Flexi DNA (Promega, USA), 10.85µl of H_2O, and 2µL of 30 ng/µl of DNA. The PCRs were performed in a thermal cyclers programed to denature the DNA at 94°C for 5 min, followed by 35 cycles of a 30s of 94°C denaturation step, 30s of annealing step (depending on the annealing temperatures of the respective SSR markers), and 45s of 72°C extension step. The program was then finished with a final 7 min of extension step at 72°C and a 4°C permanent hold. The PCR products were separated on 3% agarose gels and DNA was visualized under UV light after staining with gel red nucleic acid gel stain (Biotium).

For the KASP markers, the primer sequences were obtained from polymaker website (http://polymarker.tgac.ac.uk/). For each KASP marker, three primers were used in PCRs. Two of them are allele-specific forward primers which results in bi-allelic discrimination and one common reverse primer (Ramirez-Gonzalez et al. 2014, 2015). Oligos, carrying standard FAM or HEX compatible tails (FAM tail: 5′GAAGGTGACCAAGTTCATGCT3′; HEX tail: 5′GAAGGTCGGAGTCAACGGATT3′) were added to the forward primer sequences with the target SNP at the 3′ end (Ramirez-Gonzalez et al. 2014). The PCRs were in 10 µL volumes and prepared as described by the manufacturer (LGC, UK). Each reaction contained 0.25µl of 10µM of each of the forward primers, 0.5µl of 10µM of the reverse primer, 5µl of KASP 2X master mix (LGC, UK), 1µl of H_2O and 3µl of 30 ng/µl DNA. PCRs were placed in multiplate of 96-

well unskirted PCR plates MLP-9601 and sealed with an optical plate seal (BIO-RAD, UK). The PCRs were performed in a thermal cycler BIO-RAD CFX-96 real time system programed as follows: Hot-start activation at 94°C for 15 min followed by 10 touchdown cycles of denaturation at 94°C for 20s and annealing/elongation (61-55°C) for 60s with a drop of 0.6 °C per cycle. This was followed by 26 cycles of denaturation step at 94°C for 20s and annealing/elongation step at 55°C for 60s. The PCR plate was read at 37°C and fluorescent end-point genotyping was carried. Data analysis was performed using genotype cluster analysis software BIO-RAD CFX Manager 3.1 using the allelic discrimination option. If genotype clusters were not clearly defined after the initial KASP thermal cycle, the plate was thermally cycled for an additional three cycles of denaturation step at 94°C for 20s and annealing/elongation step at 57°C for 60s and the PCR plate was read again at 37°C. The latter cycling and reading was in some cases repeated until distinct genotyping clusters were obtained.

For linkage mapping, the phenotypic response of the RILs (0-4 scale) was converted into binary data. Then, the phenotypic and genotypic data were combined to generate a linkage map using MapDisto.2.0 (Lorieux 2012), with a LOD of 7.0. The Kosambi mapping function was used to calculate genetic distance between markers (Kosambi 1944).

Results

The inheritance of leaf rust resistance

The number of genes conferring resistance against *Pt*-race BBBQJ in the eight durum wheat genotypes were determined by evaluating the ITs at seedling stage of F_1 plants and the segregation ratios of F_2, F_3, and F_6 progenies (Table 3.2).

Table 3.2. Characterization of leaf rust resistance (*P. triticina* race BBBQJ) inheritance at seedling stage in eight resistant durum genotypes based on infection types of F_1 plants and segregation ratios at F_2, F_3, and F_6.

	F_1	F_2 segregation ratios			F_3 segregation ratios			F_6 segregation ratios		
Populations		Segregation R:S (n)[a]	Expected ratio R : S	*P* value for χ^2	Segregation HR:Seg:HS (n)[b]	Expected ratio HR : Seg : HS	*P* value for χ^2	Segregation HR : Seg : HS (n)[c]	Expected ratio HR : HS	*P* value for χ^2
Rusty X PI 534304	1+	.[d]	.	.	17:79:33	1:2:1/1:8:7	0.005*/1.7E-05*	114:2:61	1:1/3:1	<1E-05*/0.03*
Rusty X PI 192051	;1	.	.	.	37:89:44	1:2:1	0.33	.	.	.
Divide X PI 313096	1+	.	.	.	62:144:49	1:2:1	0.06	57:3:38	1:1	0.05
Rusty X PI387263	1+	.	.	.	18:58:30	1:2 :1	0.16	76:7:57	1:1	0.10
Rusty X PI 209274	1+	253:89	3:1	0.66	39:78:37	1:2:1	0.78	62: 8:60	1:1	0.86
Rusty X PI 278379	.	47:166	1:3 / 3:13[e]	0.32/ 0.22	4: 48:50	1:8:7	0.43	22:6:65	1:3	0.95
Divide X PI 278379	3	31:172	3:13	0.20	.	.	.	.	.	.
Divide X PI 244061	1+	231:80	3:1	0.77	19:69:29	1:2:1	0.06	.	.	.
Rusty X PI 195693	.	36:82	1:3	0.38	.	.	.	.	.	.
Divide X PI 195693	.	88:125	7:9	0.48	18:52:31	1:8:7	0.18	.	.	.

[a] Number of resistant (R) and susceptible (S) F_2 progenies.

[b] Number of homozygous resistant (HR), segregating (Seg), and homozygous susceptible (HS) F_3 families.

[c] Number of homozygous resistant (R), segregating (Seg), and homozygous susceptible (HS) recombinant inbred lines at F_6 generation.

[d] Population was not evaluated at this generation.

[e] Observed segregation ratios could fit into two possible expected segregation ratios 1R:3S or 3R:13S.

(*) *P* value indicate that the observed segregation ratio is significantly different from the expected segregation ratio at 95% level of confidence.

In six of the crosses (Rusty X PI 192051, Divide X PI 244061, Rusty X PI 387263, Rusty X PI 209274, Rusty X PI 534304, and Divide X PI 313096), the F_1 plants showed resistant ITs to *Pt*-race BBBQJ suggesting that the resistance was dominant. The F_1 plants of the cross Divide X PI 278379 were susceptible to BBBQJ, indicating that the resistance was recessive (Table 3.2).

Evaluation of 170 F_3 families derived from the cross Rusty X PI 192051 showed a segregation ratio of 1HR: 2 Seg: 1HS (*P* value = 0.33), suggesting that the *Pt*- race BBBQJ resistance in PI 192051 is conferred by a single dominant gene. Similarly, evaluation of 255 F_3 families and 98 F_6-RILs of the cross Divide X PI 313096 segregated as 1HR: 2Seg: 1HS (*P* value=0.06) and 1HR: 1HS (*P* value= 0.05), respectively which also fits the expected Mendelian ratios for a single gene. Therefore, the *Lr* gene in PI 313096 is conferred by a single dominant gene (Table 3.2).

The segregation ratios of 311 F_2 plants generated from the cross Divide X PI 244061 was 3R: 1S (*P* value=0.77). Further screening of 117 F_3 families of the same cross showed a segregation of 1HR: 2 Seg: 1HS (*P* value=0.06) which suggests that a single dominant resistance gene confers resistance to *Pt*- BBBQJ in PI 244061 (Table 3.2).

In the cross of Rusty X PI 387263, the 106 F3 families and 140 RILs evaluated segregated as 1 HR: 2 Seg: 1 HS (*P* value=0.16) and 1HR: 1HS (*P* value = 0.10), respectively. This indicated that a single dominant resistance gene controls the resistance to *Pt*-race BBBQJ in PI 387263 (Table 3.2).

The F_2 population (342 plants) of the cross Rusty X PI 209274 segregated as 3R: 1S (*P* value=0.66) while segregation ratio of 154 F_3 families was 1 HR: 2 Seg: 1 HS (*P* value=0.78) and the F_6-RILs segregated as 1HR: 1HS (*P* value= 0.86), suggesting a single dominant gene conferring the observed resistance in PI 209274 (Table 3.2).

All five F_1 plants derived from the cross Rusty X PI 534304 showed resistant IT, indicating that the resistance to *Pt*-race BBBQJ is dominant. The subsequent screening of 129 F_3 and 177 F_6 resulted in segregation of 17 HR: 79 Seg: 33 HS and 144 HR: 2 Seg: 61 HS, respectively which did not fit a Mendelian inheritance for one or two genes, based on *P* value the χ^2 test (< 0.05) at 95% level of confidence (Table 3.2).

The segregation pattern of cross Rusty X PI 278379 showed that F_2 segregation ratios could fit two possible models. One of the models was 1R: 3S ratio (*P* value = 0.32) which suggests the presence of a single recessive gene controlling resistance to *Pt*-race BBBQJ. The observed segregation at F_2 also fits into 3R:13S (*P* value = 0.22) which indicates the involvement of two genes, with one suppressing the expression of the other. The same segregation ratio (3R:13S; *P* value = 0.20) was obtained by crossing the same resistant parent PI 278379 with the susceptible parent Divide. Further evaluation of the population Rusty X PI 278379 showed a segregation ratios of 1 HR: 8 Seg: 7 HS (*P* value=0.43) and 1HR: 3 HS (*P* value= 0.95) of F_3 families and F_6-RILs, respectively. These results suggest that two genes may be involved in this cross (Table 3.2).

Two populations were developed for the resistant genotype PI 195693. Evaluation of each population suggested different modes of inheritance. The segregation ratio of 118 F_2 plants of the cross Rusty X PI 195693 was 1R:3S (*P* value = 0.38), indicating that the resistance was conferred by a single recessive gene. However, the F_2 plants (213 individuals) of the cross Divide X PI 195693 segregated as 7R:9S (*P* value = 0.48), indicating the presence of two recessive genes. Further screening of the F_3 lines of the cross involving Divide and PI 195693 were distributed in accordance with a 1HR: 8Seg: 7HS ratio, indicating the presence of two genes (Table 3.2).

Stem rust resistance inheritance

The inheritance of *Sr* gene (s) to *Pgt* race TTKSK in the two populations Rusty X PI 534304 and Rusty X PI 192051 was determined based on the evaluation of F_3 progenies. The 131 F_3 families of the bi-parental cross Rusty X PI 534304 segregated into 1 HR: 2 Seg: 1HS (*P* value= 0.51) which suggested that PI 534304 carries a single *Sr* gene controlling the resistance to TTKSK. The segregation observed in the cross Rusty X PI 192051 was as 31 HR: 70 Seg: 17 HS that did not fit segregation for a single gene based on the *P* value of the χ^2 test (*P* value= 0.02) (Table 3.3).

Table 3.3. Characterization of stem rust resistance (*P. graminis* f. sp. *tritici* race TTKSK) inheritance at seedling stage in two resistant durum lines based on segregation ratios of F_3 progenies.

	Rusty X PI 534304	Rusty X PI 192051
Homozygote resistant	27	31
Segregating	69	70
Homozygote susceptible	35	17
Expected segregation ratio	1 HR : 2 Het : 1HS [a]	1 HR : 2 Het : 1HS
P value for χ2	0.51	0.02*

[a] HR: homozygous resistant, Seg: segregating, and HS: Homozygous susceptible.
(*) *P* value indicates that the observed segregation ratio is significantly different from the expected segregation ratio at 95% level of confidence.

Bulked segregant analysis

Genomic regions associated with *Lr* and *Sr* resistance genes were identified via BSA in six bi-parental populations in which the resistance appeared to be conferred by single dominant resistance genes. Five of these crosses were used to map the chromosomal regions associated with *Lr* resistance to *Pt*-race BBBQJ, while one cross was used to identify the region associated with the *Sr* gene conferring resistance to *Pgt*-race TTKSK (Table 3.4).

Table 3.4. Generation, trait, number of plants in homozygous resistant and homozygous susceptible bulks of the bi-parental crosses used in the bulked segregant analysis (BSA), and results of BSA.

Populations	Generation	Trait	Pathogen race	HS bulk (n)[a]	HR bulk (n)[b]	Chromosome	Number of associated SNPs with rust response [c]	Possible gene
Divide X PI 313096	F_6	Leaf rust	BBBQJ	20	20	6BS	6	*Lr61*
Rusty X PI 387263	F_6	Leaf rust	BBBQJ	22	22	6BL	5	Likely novel
Rusty X PI 209274	F_2	Leaf rust	BBBQJ	10	10	6BS	10	Likely novel
Divide X PI 244061	F_2	Leaf rust	BBBQJ	10	10	2B	33	Likely novel
Rusty X PI 192051	F_2	Leaf rust	BBBQJ	22	22	4A	59	Likely novel
Rusty X PI 534304	F_6	Stem rust	TTKSK	16	16	6AL	32	*Sr13*

[a] Number of homozygous susceptible F_2 plants or RILs included in the homozygous susceptible bulk.
[b] Number of homozygous resistant F_2 plants or RILs included in the homozygous resistant bulk.
[c] Markers linked with rust response in these populations are presented in Appendix C Table C1-C6.

Divide X PI 244061 population: Thirty-three SNPs located on chromosome 2B appeared to be associated with leaf rust response in the cross involving Divide X PI 244061. The positions of the SNP markers were based on the hexaploid consensus map (Cavanagh et al. 2013). Based on the BLASTn of the SNP sequences against the Chinese Spring chromosome survey sequences (https://urgi.versailles.inra.fr/blast/?dbgroup=wheat_all&program=blastn), six markers were found on 2BL, while the rest of the markers were on 2BS (Table 3.4, Appendix C Table C1).

Rusty X PI 192051 population: On chromosome 4A, 59 SNPs were found associated with leaf rust response in the cross Rusty X PI 192051. Based on the BLASTn search of the SNP sequences against the Chinese Spring chromosome survey sequences, 18 SNPs were on 4AL, while the remaining 41 markers were on 4AS (Table 3.4, Appendix C Table C2).

Rusty X PI 209274 and Divide X PI 313096 populations: The leaf rust resistance in the cross Rusty X PI 209274 was associated with ten SNPs on chromosome 6BS. The resistant parent PI 209274, the HR bulk, and the durum wheat cultivar Guayacan INIA which carries *Lr61* share the same alleles for three of the markers (*IWA3991*, *IWA5058*, and *IWA52*). Rusty (the susceptible parent of the cross), HS bulk, and Guayacan INIA share the same allele for the other seven SNPs (Table 3.4, Appendix C Table C3).

Six SNPs on 6BS were associated with leaf rust response in the population Divide X PI 313096. The resistant genotype PI 313096, the HR bulk, and Guayacan INIA share common alleles for the markers *IWA1495*, *IWA1254*, and *IWA666.* Guayacan INIA, HS bulk, and the susceptible parent Divide share common alleles for the SNPs *IWA4997* and *IWA4612*. Even though, the *Lr* gene (s) in PI 209274 and PI 313096 were both located on 6BS, the BSA did not reveal any common SNPs linked with response to *Pt*-race BBBQJ between the two populations.

However, two genomic locations on 2.6 cM and 14.5 cM (based on the hexaploid consensus map of Cavanagh et al. 2013) were identified in both of these durum populations (Table 3.4, Appendix C Table C4).

Rusty X PI 387263 population: Five SNPs associated with leaf rust response were detected on chromosome 6BL in the cross Rusty X PI 387263. The resistant parent PI 387263, HR bulk, and the durum cultivar Storlom share the same allele for *IWA3464*, while for the other four markers, Storlom, HS bulk, and Rusty share the same alleles (Table 3.4, Appendix C Table C5). Storlom carries *Lr3a* which has been previously mapped to 6BL (Herrera-Foessel et al. 2005).

Rusty X PI 534304 population: Thirty-two SNPs on 6AL were associated with stem rust response to race *Pgt*-TTKSK in the cross Rusty X PI 534304 (Table 3.4, Appendix C Table C6).

Response of the parental genotypes to *P. triticina* races virulent to known *Lr* genes in durum

The resistant parents to *Pt*-race BBBQJ that were used to develop the bi-parental populations alongside other durum cultivars were screened using *Pt* races BBBSJ, CBBQS, and BBB/BN_*Lr61*vir. The ITs indicated that race BBBSJ which carries virulence to *LrB, Lr10, Lr14a, Lr14b, Lr23, Lr20,* and *Lr72* was avirulent to all the resistant parents of the crosses and on the durum wheat cultivars Camayo and Juapare C2001. Race CBBQS, virulent to *LrB, Lr3a, Lr3bg, Lr10, Lr14b, Lr23, Lr27+31,* and *Lr72* was avirulent to the eight resistant parental genotypes used in the crosses and to cultivars Creso, Camayo and Llareta INIA. Race BBB/BN_*Lr61* vir which carries virulence on *Lr10, Lr23,* and *Lr61* was avirulent to all the eight genotypes and cultivars except PI 313096, Alred, and Guayacan INIA. This suggests that the

resistance in the eight genotypes used to develop the bi-parental populations is conferred by a different or additional genes to the previously characterized *Lr* genes in durum cultivars including *Lr3a, Lr14a*, *Lr27+31*, *Lr61*, and *Lr72*, except PI 313096 which is most likely carrying *Lr61* (Table 3.5).

Table 3.5. Infection types of the parental genotypes of the crosses and durum wheat cultivars to *P. triticina* races BBBSJ, CBBQS, and BBB/BN_*Lr61*vir at seedling stage.

Entries	BBBSJ [a]	CBBQS [b]	BBB/BN _*Lr61*vir [c]
PI 534304	0;	;	;1-
PI 192051	;	;1	;
PI 313096	0;	0;	3+
PI 387263	;	;1	;1-
PI 209274	;1+	.	X
PI 278379	;2+ C	2+C	;1+
PI 244061	;	;	.
PI 195693	;	;1	;1
Rusty	3	3	.
Divide	2+3	3	.
Alred	3	4	3+
Llareta INIA	3	;13-	X
Camayo	;1-	;1	;1
Jupare C 2001	;1	3	;1
Capelli	2+3	3+	.
Mindum	3	3	.
Russello	3	3	.
Mexicali 75	3	3	.
Creso	3	;1	;1
Guayacan INIA	.	.	3

[a] *P. triticina* race virulent to *Lr B, Lr10*, *Lr14a, Lr14b, Lr20*, *Lr23,* and *Lr72.*
[b] *P. triticina* race virulent to *LrB*, *Lr3a*, *Lr3bg*, *Lr10*, *Lr14b*, *Lr23, Lr27+31*, and *Lr72.*
[c] *P. triticina* race virulent to *Lr10*, *Lr23*, and *Lr61.*

Mapping of leaf rust resistance gene in PI 209274

The population Rusty X PI 209274 was selected for linkage mapping using 130 F_6 RILs. This is because the identified SNPs that were associated with leaf rust response in this population using BSA were spanning smaller genomic region of 21.9 cM compared to other populations (Table 3.4, Appendix C Table C3). Thus, fewer markers would be used for mapping the *Lr* gene

in PI 209274. Linkage mapping for the remaining populations in which the gene is likely to be novel (Table 3.4) will be conducted in future work.

The SNP markers identified in the biparental cross Rusty X PI 209274 using the BSA were used to develop KASP markers as described by Ramirez-Gonzalez et al (2014). Three KASP markers corresponding to the SNPs *IWA7070*, *IWA3298*, and *IWA4290* gave clear polymorphism between the resistant parent (PI 209274) and the susceptible parent (Rusty). Therefore, these KASP markers were used initially to genotype the RILs of this bi-parental cross. The mapping of the *Lr* gene associated with leaf rust response to *Pt*-race BBBQJ in PI 209274 showed that the gene was initially flanked by the KASP markers *IWA3298* and *IWA7070*. Therefore, additional KASP and SSR markers located between these two markers, based on the tetraploid consensus map (Maccaferri et al. 2015), were used to genotype the parents of the cross. Six KASP and one SSR (*dupw217*) markers that were polymorphic between the parents were then used to genotype the F6-RILs. The mapping identified two flanking markers (*IWA3298* and *IWB39456*) that delineated the *Lr* gene to race BBBQJ (collected from California) here designated as *LrCA* (Figure 3.1).

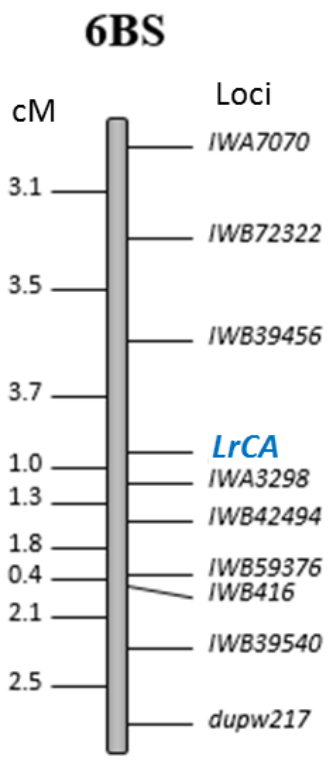

Figure 3.1. Distance in centimorgans between simple-sequence repeat (SSR) and kompetitive allele specific PCR (KASP) markers linked to the leaf rust (*P. triticina* race BBBQJ) resistance gene (*LrCA*) on chromosome 6BS using phenotypic and genotypic data of the recombinant inbred lines of the cross Rusty X PI 209274 at F_6 generation.

The distance between the flanking markers was 4.7 cM. The KASP marker *IWA3298* was the most closely linked to *LrCA* at a distance of 1.0 cM while *IWB39456* was located at 3.7 cM distal to *LrCA.* The rest of markers were located further away from the gene with most of them distal to the gene (Figure 3.1). All linked markers with *LrCA* (Figure 3.1) in this durum population conformed to the expected ratio of 1:1 at 95% level of confidence (*P* value of χ^2 test = 0.13 to 0.84 for the KASP markers and *P* value = 0.05 for the SSR marker *dupw217*).

Discussion

Race BBBQJ, a highly virulent *Pt* phenotype on tetraploid wheat was recently found in the southern Great Plain region of the United States (Kolmer 2015). Therefore, the spread of this race to North Dakota which is the major durum-producing region of the US is possible. Since

most the ND durum cultivars are susceptible to this race and few effective *Lr* genes are available to the durum wheat breeding programs globally, we aimed to identify new *Lr* genes. In the present study, the resistance to *Pt*-race BBBQJ was conferred by single dominant genes in five of the durum populations among the eight studied genotypes. The BSA showed that the genomic locations of the genes in these five genotypes were on chromosomes 2B, 4A, 6BS, and 6BL. This was a fast and relatively inexpensive method to identify that the resistance in these five populations was conferred by at least four different genes. This method allowed to assess the genetic diversity of resistance in these genotypes and to identify new *Lr* genes that can be used to broaden the genetic diversity of leaf rust resistance in durum wheat. Apart from being resistant to BBBQJ, the eight genotypes used to develop these populations have showed a broad spectrum of resistance to several *Pt* races collected worldwide at seedling stage in the greenhouse and at adult-plant stage in field trials (Aoun et al. 2016). In addition, based on our results from the current study, these genotypes are resistant to *Pt* races virulent to commonly used *Lr* genes in durum breeding programs including *Lr3a, Lr14a, Lr27+31*, *Lr61*, and *L72*, suggesting that new or underutilized *Lr* genes may be present in theses genotypes. The genotypes utilized were collected from different countries and seven out of eight were landraces. Wheat landraces are known to carry new resistance genes to several diseases, including rust, since the use of landraces in the modern breeding programs is not frequent (Reif et al. 2005; Bonman et al. 2007; Newton et al. 2010; Bux et al. 2012; Gurung et al. 2014).

Our study shows that PI 192051 carries a *Lr* gene located in 4A. No previously characterized *Lr* genes have been mapped to 4A except *Lr30,* originated from *T. aestivum* cultivar TC*6/Terenzio (Dyck and Kerber 1981). However, not enough mapping information is available for *Lr30* to make inferences. Therefore, developing tightly linked genetic markers

associated with the *Lr* gene in PI 192051 is very important to introgress this gene into durum wheat breeding programs. Five SNPs on 4A were previously identified using GWAS to be associated with leaf rust response in the USDA- NSGC from which PI 192051 was selected (Aoun et al. 2016). Of these five markers, *IWA5968* was associated with leaf rust response at seedling stage to the California race BBBQJ, which is the same isolate used in the current study. The remaining four SNPs on 4A, were associated with leaf rust response at adult stage to common wheat type races in field trials in Saint Paul and Crookston, MN, USA. Similarly, in the association mapping study of Maccaferri et al. (2010), the SSR marker *barc155* on 4A was associated with leaf rust response at seedling stage to *Pt*-durum type race BBBGJ.

The *Lr* gene in PI 244061 was mapped to chromosome 2B. Several previously mapped *Lr* genes on this chromosome have been reported including *Lr23* on 2BS (Watson and Luig 1961; McIntosh and Dyck 1975; Nelson et al. 1997). However, PI 244061 was resistant to races BBBSJ and CBBQS which are virulent to *Lr23*. Virulence to *Lr23* is common in *Pt* races isolated from durum wheat (Huerta-Espino and Roelfs 1992; Singh et al. 2005; Ordóñez and Kolmer 2007a). Other genes on 2BS include *Lr13* (Singh et al. 1992) and *Lr16* (Zhang and Knott 1990) that have been postulated in durum. However, *Lr13* is an adult plant resistance, which makes it unlikely to be the gene of interest in PI 244061. McCartney et al (2005) reported a number of SSR markers including *wmc764*, *gwm210*, and *wmc661* to be excellent candidate markers for *Lr16* that can be used for marker assisted selection (MAS). However, genotyping of PI 244061 with the marker *wmc661* showed that this genotype likely does not carry *Lr16*. In addition, *Lr16* was tagged with SSR markers on the distal end of chromosome 2BS (between 5.5–12.4 cM) while the SNPs identified in the current durum population were located closer to the centromere of 2B (between 53.0–108.2 cM), based on the tetraploid consensus map

(Maccaferri et al. 2015). In addition, in a previous study, PI 244061 was resistant to a mixture of six *Pt* races from North America, collected from common wheat including race MHDSD that is virulent to *Lr16* (Aoun et al. 2016). Therefore, the *Lr* gene in PI 244061 is unlikely to be *Lr16.* Another seedling resistance gene on 2BS, designated as *Lr73,* was mapped in the common wheat line 'Morocco' (Park et al. 2013). However, Morocco is highly susceptible to race BBBQJ, suggesting that *Lr73* is not the gene of interest in PI 244061. The adult plant resistance *Lr35* (Kerber and Dyck 1990; Gold et al. 1999; Knox et al. 2000) originating from *Aegilops speltoides*, was mapped to chromosome 2B, which distinguished it from the seedling resistance gene in PI 244061. Therefore, the resistance in PI 244061 is most probably a new *Lr* gene. Seventeen SNPs on 2BL were associated with leaf rust response based on GWAS using the USDA- NSGC from which PI 244061 was obtained (Aoun et al. 2016). Of these, eight SNPs, located on 149.4 cM, based on Cavanagh et al. (2013) consensus map, were associated with leaf rust response at adult-plant stage in a field experiment in Ciudad Obregón, Mexico. On the same position, five SNPs were found to be linked with response to *Pt*-race BBBQJ, based on the BSA in the current study.

The *Lr* gene (s) in PI 209274 and PI 313096 were both located on 6BS. The *Lr61* is the only characterized gene on 6BS in durum cultivars to date and was previously mapped in the CIMMYT cultivar Guayacan INIA (Herrera-Foessel et al. 2008a). The genotype PI 313096 was susceptible to *Pt*-race BBB/BN_*Lr61*vir suggesting that the resistance in PI 313096 is most likely *Lr61*, while PI 209274 was resistant to this race, indicating that the single dominant *Lr* gene in PI 209274 is different from *Lr61*. Although, PI 209274 and Guayacan INIA have common alleles for markers *IWA3991, IWA5058,* and *IWA52*, the mapped *Lr* gene in PI 209274 showed that none of these markers were tightly linked to *LrCA*, suggesting that *LrCA* is most

likely different from *Lr61*. Other *Lr* genes mapped on 6BS in wheat include *Lr36* originated from *Aegilops speltoides* (Dvorak and Knott 1990), *Lr53* originated from *T. turgidum ssp dicoccoides* (Marais et al. 2005; Dadkhodaie et al. 2011), and *Lr59* originated from *Aegilops peregrina* (Marias et al. 2008; Mostafa Pirseyedi et al. 2015). The genes *Lr36*, *Lr53*, and *Lr59* were transferred to hexaploid wheat from wild relatives which makes them unlikely to be the *Lr* gene in PI 209274.

The *Lr* gene (s) in the population Rusty X PI 387263 was located on 6BL. A genomic region on 6BL was previously identified using GWAS performed on the USDA-NSGC of durum wheat accessions. The identified locus in this GWAS was represented by markers *IWA6904* and *IWA657* that were associated with leaf rust response at adult stage in field trials in Ciudad Obregón and El Batán, Mexico (Aoun et al. 2016). Herrera-Foessel et al. (2007) identified two genes *Lr3a* and *LrCamayo* on chromosome 6BL that were effective against *Pt*-race BBG/BN collected in Mexico (Herrera-Foessel et al. 2007). The gene *Lr3a* that co-segregated with *Xmwg798* (Sacco et al. 1998) was confirmed to be present in the durum cultivar Storlom (Herrera-Foessel et al. 2007). In the present study, PI 387263 is resistant to the *Pt* race CBBQS which is virulent to *Lr3a,* indicating that the resistance gene in PI 387263 is different from *Lr3a*. Further screening of Camayo and PI 387263 with *Pt*-isolate (Eth-63-1, race EEEEE avirulent on Thatcher) collected from durum wheat in Ethiopia showed virulence on PI 387263 but not on Camayo (M. Aoun, unpublished). This suggests that the resistance in PI 387263 is conferred by a different gene from *LrCamayo*.

The genotype PI 195693 showed resistance to BBBSJ, CBBQS, and BBB/BN_*Lr61*vir. Therefore, the resistance in PI 195693 is conferred by a different or additional gene to *LrB*, *Lr3a*, *Lr3bg*, *Lr10*, *Lr14a*, *Lr14b*, *Lr20*, *Lr23*, *Lr27+31*, *Lr61* and *Lr72*. The segregation in F_2 of

1R:3S in the cross Rusty X PI 195693 (one recessive gene) and 7R:9S in the cross Divide X PI 195693 (two recessive genes) could be due to the difference in the genetic background of the susceptible parents Divide and Rusty. Even though the segregation ratio of 1HR: 8Seg: 7HS at F_3 in Divide X PI 195693 could confirm the presence of two recessive genes, the same ratio could also suggest the involvement of two complementary dominant genes. Similar segregation patterns at seedling stage (susceptible F_1, 7R: 9S at F_2, and 1HR: 8Seg: 7HS at F_3) was observed previously in the cross Atil C200 X Hualita to the Mexican *Pt* race BBG/BN (Herrera-Foessel et al. 2005). However, Herrera-Foessel et al. (2005) reported that the resistance in the cross involving Atil C200 and Hualita was due to the presence of two dominant complementary genes rather than two recessive genes since the F_1 plants were resistant in the field. Only one single case of complementary genes with dominant interaction conditioning leaf rust resistance has been reported in durum wheat. The durum wheat cultivars Jupare C2001 and Banamichi C2004 carry the complementary genes *Lr27+31* on chromosomes 3BS and 4BL, respectively (Herrera-Foessel et al. 2005; Herrera-Foessel et al. 2014b) which were originally characterized in common wheat (Singh and McIntosh 1984a, 1984b; Singh et al. 1993).

The F_1 plants of the cross Divide X PI 278379, were susceptible to *Pt*-race BBBQJ, indicating the presence of recessive resistance (dominant susceptibility) to leaf rust. The segregation of 3R:13S in the F_2 in Rusty X PI 278379 and Divide X PI 278379 populations and the distribution of 1HR: 8Seg: 7HS in the F_3 families and 1HR: 3HS in the F_6 RILs of Rusty X PI 278379 could mean the involvement of one dominant resistance gene with one suppressor gene. A possible scenario for this ratio might be due to the presence of a dominant resistance gene in PI 278379 that is suppressed by a suppressor gene from the susceptible parent (Rusty or Divide). Cases of suppressor genes of rust resistance have been reported in wheat-rust pathosystem. For

instance, a suppressor gene of *Lr23* designated as *SuLr23* on chromosome arm 2DS that was derived from *A. tauschii* was identified in synthetic hexaploid wheat (Nelson et al. 1997). In addition, suppressors of *Lr* genes have been identified in the A and B genomes in durum wheat (Assefa and Fehrman 2000). Knott (2000) also characterized suppressors of stem rust resistance genes in the A and B genomes in the durum cultivar 'Medea'.

The resistance to race BBBQJ in the population Rusty X PI 534304 is dominant, while the segregation ratios at F_3 and F_6 did not fit into expected segregation ratios for one or two genes. The same population was used to map *Sr* gene to *Pgt*-race TTKSK. The resistance to race TTKSK in PI 534304 is conferred by a single resistance gene that is located on 6AL. Chromosome 6AL is also known to carry *Sr13* (Jin et al. 2007, Klindworth et al. 2007), which is commonly found in durum wheat cultivars. However, a diagnostic marker of *Sr13* is currently not available. The ITs of PI 534304 and the segregating population to *Pgt*-race TTKSK were similar to that of *Sr13.* Therefore, the *Sr* resistance in PI 534304 is most probably *Sr13*. Unfortunately, this *Sr* gene is not effective against the recent races in Ethiopia including TRTTF and JRCQC (Olivera et al. 2012).

The stem rust resistance to race TTKSK in the cross Rusty X PI 192051 did not follow segregation ratio of a single gene. The resistance in PI 192051 was not only effective against *Pgt*-race TTKSK, but also against *Pgt*-race JRCQC with virulence to *Sr13* and *Sr9e* which are commonly present in durum wheat cultivars (Olivera et al. 2012). The genotype PI 192051 was also highly resistant in field trials in Debre Zeit Ethiopia in 2009 (Olivera et al. 2012), 2014 and 2016 (unpublished data). Thus, PI 192051 is an effective source of resistance not only to race Ug99 but also other *Pgt*-races recently observed in Ethiopia which are phylogenetically different from Ug99-lineage races. These *Pgt*-races are JRCQC, TRTTF, RRTTF, and TKTTF ('Digalu'

race) (Olivera et al. 2015). Gene/QTL mapping of both *Lr* and *Sr* genes in PI 192051 should be prioritized in follow-up studies as it seems to carry previously uncharacterized genes in durum cultivars.

Conclusion

The objective of the current study was to identify new leaf rust and stem rust resistance genes that can be useful to broaden the narrow rust resistance spectrum in durum wheat by characterizing the resistance present in eight durum genotypes that were selected from the USDA-NSGC. The inheritance study revealed that five of the crosses, Rusty X PI 192051, Divide X PI 244061, Rusty X PI387263, Rusty X PI 209274, and Divide X PI 313096, carry single dominant *Lr* genes to *Pt*-race BBBQJ. The *Lr* genes in these five crosses were located on chromosomes 2B, 4A, 6BS, and 6BL. The *Lr* gene in PI 313096 is most likely *Lr61*. The new *LrCA* in PI 209274 is flanked by KASP markers *IWA3298* and *IWB39456* to a 4.7 cM region. In the remaining crosses (Rusty X PI 534304, Rusty X PI 278379, Rusty X PI 195693, and Divide X PI 195693), the inheritance of *Lr* genes was more complex involving recessive resistance, two genes, or deviated from Mendelian inheritance. The leaf rust resistance in seven genotypes used to develop the bi-parental populations was conferred at least in part by gene (s) different from previously mapped genes in durum cultivars including *Lr3a, Lr10, Lr14a*, *Lr23, Lr27+31*, *Lr61,* and *Lr72*.

The eight resistant genotypes to BBBQJ have resistance to additional *Pt* races tested at both seedling stage in the greenhouse and adult stage in field trials. Therefore, more research is needed to verify whether the resistance to different races in each of these genotypes is conferred by the same or different genes. Two of the eight genotypes were also resistant to *Pgt* -race TTKSK. The resistance in PI 534304 was conferred by a single dominant gene on 6AL, which is

most likely *Sr13*. The stem resistance in PI 192051 appeared to be different from previously reported *Sr* genes in durum cultivars.

Characterizing the identified resistance genes in the current study and developing diagnostic markers is needed to bring new sources of rust resistance to durum wheat breeding programs. Additionally, investigating the presence of possible adult plant resistance with minor effects in these genotypes is important to achieve durable rust resistance.

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CHAPTER IV. MAPPING OF NEW LEAF AND STEM RUST RESISTANCE GENES IN THE PORTUGUESE DURUM WHEAT LANDRACE PI 192051

Abstract

Leaf rust caused by *Puccinia triticina* Erikss. (*Pt*) and stem rust caused by *Puccinia graminis* f. sp. *tritic*i Erikss. and E. Henn (*Pgt*) pose serious challenge to the production of durum wheat. The objective of this study was to map leaf rust resistance (*Lr*) gene (s) and stem rust resistance gene (s) (*Sr*) in the Portuguese durum wheat landrace PI 192051. Four *Pt*-isolates, representing different virulence phenotypes, and *Pgt*-race TTKSK were used to evaluate F_6 recombinant inbred lines (RILs) derived from Rusty (susceptible) X PI 192051 (resistant) at seedling stage. The RILs were further screened at adult-plant stage in a field stem rust nursery in Ethiopia. The RILs were genotyped using the Illumina's iSelect 9K SNP wheat array. The linkage mapping showed that the *Lr* gene in PI 192051, designated as *LrPort* and conferring resistance to the four *Pt*-isolates was located within a 3.9 cM region on chromosome 4AL and flanked by SNPs *IWA4254* and *IWA8341*. The *Sr* gene in PI 192051 effective against *Pgt*-race TTKSK, currently designated as *SrPort* was mapped within a 3.6 cM region on 7AS flanked by SNPs *IW8390* and *IWA1805*. No previously characterized *Lr* or *Sr* genes were reported in these regions in durum nor common wheat. The QTL analysis identified *QSr.ndsu-5B* conferring resistance in PI 192051 to *Pgt*-races in a field trial in Ethiopia. *QSr.ndsu-5B* mapped to 5BL within a 4.8 cM region. The *QSr.ndsu-5B* is delimited by SNPs *IWA6992* and *IWA2181*. These findings will enrich the genetic basis of resistance to leaf rust and stem rust in durum wheat.

Introduction

Leaf rust has become a serious threat to durum wheat (*Triticum turgidum* L. var. *durum;* 2n = 4x = 28) production. The occurrence of the disease is widespread throughout durum growing areas including Mexico, USA, India, Ethiopia, and the entire Mediterranean basin (Singh et al. 2004; Goyeau et al. 2006; Kolmer 2013, 2015; Mishra et al. 2015; Kolmer and Acevedo 2016). This recent problem on durum wheat was attributed to the emergence of new pathotypes with virulence to previously resistant durum cultivars (Singh et al. 2004; Goyeau et al. 2012). Interestingly, these emerging pathotypes are different from those prevalent on hexaploid common wheat (*T. aestivum* L.; 2n = 6x = 42) as they exhibit avirulence on most of the leaf rust resistance (*Lr*) genes found in common wheat (Huerta-Espino and Roelfs 1992; Ordoñez and Kolmer 2007a). This difference has been further supported by phylogenetic analysis using SSR markers that clearly separated durum type isolates from common wheat type isolates (Ordoñez and Kolmer 2007b).

The *Pt* populations collected in durum wheat fields from several countries shared similar phenotypes on 'Thatcher' near-isogenic lines and similar SSR genotypes which suggests a common origin (Ordoñez and Kolmer 2007a, 2007b). However, some isolates collected in Ethiopia with preferential virulence on durum wheat have shown a different virulence phenotype with avirulence on Thatcher (Huerta-Espino and Roelfs 1992; Ordoñez and Kolmer 2007a, 2007b; Kolmer and Acevedo 2016). They also have distinct SSR genotypes from isolates collected worldwide. These unique Ethiopian *Pt* isolates, designated as race EEEEE, have most likely been selected and maintained in the *Pt* population in Ethiopia due to the diverse host population in the country (Kolmer and Acevedo 2016), which is considered a center of diversity for tetraploid wheat (Vavilov 1951).

The genetic basis of leaf rust resistance has not been extensively studied and only a few *Lr* genes have been reported in durum wheat. The detection of highly virulent races in Mexico and subsequent large scale screening of CIMMYT cultivars led to the identification of sources of resistance including *Lr14a* (Herrera-Foessel et al. 2008), *Lr3a* (Herrera-Foesselet al. 2007), and *Lr27+Lr31* (Huerta-Espino et al. 2009). These *Lr* genes were previously identified in common hexaploid wheat but are not effective against the common wheat type races (McIntosh et al. 1995). Three additional mapped *Lr* genes, *Lr61, Lr72,* and *LrCamayo,* were mapped only in durum wheat (Herrera-Foessel et al. 2007, 2008, 2014).

The majority of the durum cultivars growing globally seem to carry single race specific resistance genes. This resulted in selection for *Pt* pathotypes with virulence on those genes. For instance, *Lr3a*, *Lr27+Lr31*, *Lr61* and *Lr72* succumbed a few years after their employment in durum wheat cultivars in Mexico (Huerta-Espino et al. 2009a, 2009b, 2011; Herrera-Foessel et al. 2014). Both *Lr14a* and *LrCamayo* are still effective against the current durum-type *Pt* races in Mexico. However, virulence to *Lr14a* in durum-type isolates has been reported in Argentina, Chile, Spain, France, and Tunisia (Ordoñez and Kolmer 2007a; Goyeau et al. 2012 ; Gharbi et al. 2013; Soleiman et al. 2016).

Stem rust, caused by *Puccinia graminis* f. sp. *tritici* Erikss. and E. Henn (*Pgt*), poses a significant threat to world common wheat and durum wheat production. The *Pgt* race TTKSK (Ug99) and its rapidly and continuously evolving lineage are virulent to several wheat stem rust resistance (*Sr*) genes (Jin et al. 2007; Singh et al. 2011). Currently, more than 60 *Sr* genes have been characterized in wheat (McIntosh et al. 2013, 2014; Rahmatov et al. 2016). Around 29 genes continue to be effective against races of the Ug99 lineage (Niu et al. 2014; Yu et al. 2014, 2015).

Extensive research has been done to map *Sr* genes in common wheat and its wild relatives, however only few studies have been done to identify new *Sr* genes in durum wheat through association mapping and linkage mapping. Generally, higher percentage of resistance to *Pgt*-race TTKSK is observed in durum wheat germplasm compared to that observed in common wheat (Jin et al. 2007; Pozniak et al. 2008). The resistance present in durum wheat against *Pgt*-race TTKSK is mainly due to the presence of *Sr13,* especially in the North American cultivars. The gene *Sr13* was first identified in the emmer wheat (*T. turgidum,* L. ssp. *dicoccum*) Khapli (Jin et al. 2007, Klindworth et al. 2007). Recently, *Pgt*-races virulent to *Sr1*3 and *Sr9e,* designated as TRTTF and JRCQC emerged in Ethiopia (Olivera et al. 2012, 2015). It was reported that low percentage of resistance (5.2%) to these races was observed in a highly diverse collection of 996 tetraploid wheat accessions (Olivera et al. 2012).

Landraces generally carry new sources of resistance that can be used to enrich the narrow resistance spectrum in adapted cultivars. However, the use of landraces is usually limited in most breeding programs (Bonman et al. 2007; Newton et al. 2010; Bux et al. 2012; Gurung et al. 2014) due to the presence of associated negative agronomic effects. This has created a diversity-bottleneck resulting in limited sources of disease resistance in wheat breeding programs.

Screening of worldwide durum wheat collection maintained by the USDA-National Small Grains Collection (NSGC) at Aberdeen, Idaho for leaf and stem rust resistance showed that the Portuguese landrace PI 192051 was highly resistant to several *Pt* races (Aoun et al. 2016a). This landrace also exhibited high level of resistance to *Pgt*-races TTKSK and JRCQC in Ethiopia (Olivera et al. 2012; Chao et al. 2016). Subsequent inheritance study and bulked segregant analysis (BSA) of progenies derived from the cross ‘Rusty’ (susceptible) X PI 192051

(resistant) showed the presence of a single dominant *Lr* gene in PI 192051 to *Pt*-race BBBQJ, collected from California, USA (Kolmer 2013). Based on BSA, the *Lr* gene conferring resistance to *Pt*-race BBBQJ in PI 192051 was mapped to chromosome 4A. This gene is localized on a genomic region previously unknown to carry characterized *Lr* gene(s) in durum wheat. The phenotyping of F_3 families of this population with *Pgt*-race TTKSK at seedling stage showed deviation from inheritance of single gene (Aoun et al. 2016b).

The objective of the present study was to map and develop tightly linked SNP markers to *Lr* and *Sr* genes in PI 192051 using F_6 recombinant inbred lines (RILs) to facilitate the transfer of these resistance genes into durum wheat cultivars.

Materials and methods

Population development

The population used for this study was developed by crossing the female parent Rusty to the pollen donor PI 192051 (Aoun et al. 2016b). Rusty is the susceptible genotype while PI 192051 is the resistant genotype to both leaf rust and stem rust. The Landrace PI 192051 was collected from Lisboa, Portugal where it is known by the identifier 'Amarelo de Barba Branca' and it was included in NSGC in 1950. The bi-parental population was advanced via single seed descent at North Dakota (ND) Agricultural Experiment Station Greenhouse Complex, Fargo, ND, USA. A total of 180 F_6-RILs were derived from this bi-parental cross.

Leaf rust evaluation

In a previous study, it was reported that the F_1 plants of the cross Rusty X PI 192051 was resistant to *Pt*-isolate BBBQJ collected from durum wheat in California. The segregation of the derived F_3 families followed the expected pattern of a single resistance gene. Therefore, it was concluded that the *Lr* gene in PI 192051 was conferred by a single dominant gene (Aoun et al.

2016b). In the present study, the F_6-RILs plants were screened at seedling stage with four highly virulent *Pt*-isolates on durum wheat during winter season (December-February) in the biosafety level two facility at the Agricultural Experiment Station Greenhouse Complex in Fargo, ND. The *Pt* isolates were collected from Ethiopia, Morocco, Tunisia, and USA (California) and named as Eth-50-4, Mor-38-2, Tun-20-4, and USA-CA1.2, respectively. The virulence/avirulence phenotypes of these isolates were determined based on the infection types at seedling stage on 20 Thatcher' near-isogenic lines (NILs) as described by Long and Kolmer (1989). The isolates USA-CA1.2 and Mor-38-2 had the same race phenotype BBBQJ, while isolates Tun-20-4 and Eth-50-4 had the race BBBSJ and EEEEE, respectively (Table 4.1).

Table 4.1. Origin, host, and avirulence/virulence responses on Thatcher differentials of four isolates of *P. triticina* (*Pt*) isolates and one race of *P. graminis* f. sp. *tritici* (*Pgt*) used on the biparental population Rusty X PI 192051 at seedling stage.

Isolate	Country	Host	Race	Virulent on genes	Avirulent on genes
***Pt* races**					
USA-CA1.2	USA	*Triticum turgidum*	BBBQJ	*Lr10, 14b, 20, B*	*Lr1, 2a, 2c, 3, 3ka, 3bg, 9, 11, 14a, 16, 17, 18, 24, 26, 28, 30*
Mor-38-2	Morocco	*Triticum turgidum*	BBBQJ	*Lr10, 14b, 20, B*	*Lr1, 2a, 2c, 3, 3ka, 3bg, 9, 11, 14a, 16, 17, 18, 24, 26, 28, 30*
Tun-20-4	Tunisia	*Triticum turgidum*	BBBSJ	*Lr10, 14a, 14b, 20, B*	*Lr1, 2a, 2c, 3, 3ka, 3bg, 9, 11, 16, 17, 18, 24, 26, 28, 30*
Eth-50-4	Ethiopia	*Triticum diccocum*	EEEEE	–[a]	*Lr1, 2a, 2c, 3, 3ka, 3bg, 9, 10, 11, 14a, 14b, 16, 17, 18,* 20, *24, 26, 28, 30, B*
***Pgt* race**					
04KEN156/04	Kenya	*Triticum aestivum*	TTKSK	*Sr5, 6, 7b, 8a, 9a, 9b, 9d, 9e, 9g, 10, 11, 17, 21, 30, 31, 38, McN*	*Sr24, 36, Tmp*

[a] The isolate Eth-50-4 is avirulent on Thatcher

The F_6-RILs were evaluated for leaf rust in a randomized complete block design (RCBD) with two replicates. In each replication 8-10 plants per RIL were screened for disease response. The parents of the cross, the common wheat cultivar Thatcher and the susceptible, durum wheat line 'RL6089' were included in each 50 cell tray as susceptible checks. In each experiment two replicates of Thatcher NILs differentials were planted to confirm virulence phenotype of *Pt*-races. The seedlings were grown under same greenhouse conditions as described by Kertho et al (2015). The inoculum increase, inoculation process, and greenhouse conditions under which the inoculated plants were grown until disease screening, were as described by Aoun et al (2016a).

Leaf rust infection types (ITs) were evaluated 12–14 days after inoculation on the second leaf stage, using a 0-to-4 scale (Long and Kolmer 1989; McIntosh et al. 1995). Seedlings showing ITs of 0 – 2+ and 'X' (a mixture of fleck and higher ITs evenly distributed on the leaf surface) were considered resistant, while seedlings showing ITs of 3–4 were considered susceptible (Long and Kolmer 1989; McIntosh et al. 1995). The RILs that showed only resistant plants across the replicates were considered homozygous resistant (HR), while the RILs that showed only susceptible plants across the replicates were considered homozygous susceptible (HS). In the case of segregation, the RILs were classified as segregating (Seg). The $\chi 2$ test for goodness-of-fit was used to assess the deviation of observed segregation from theoretically expected segregations of F_6-RILs. The segregating F_6-RILs were excluded when calculating the *P*-value of the $\chi 2$ test.

Stem rust evaluation

The F_6-RILs of the population Rusty X PI 192051 were phenotyped with *Pgt*-race TTKSK (isolate 04KEN156/04) at seedling stages (10-12 days after planting) in the biosafety level three facility at the USDA-ARS Cereal Disease Laboratory, St. Paul, MN. The RILs were

planted in a RCBD with two replications. Five seedlings per RIL were evaluated in each replicate. The urediniospores stored at -80°C were heat shocked at 45°C for 15 min, then rehydrated at 80 % relative humidity created with a KOH solution for 2–4 h under room temperature condition (Rowell 1984). The spores were then suspended in mineral oil (Sotrol 170, Phillips Petroleum, Borger, TX, USA), then sprayed onto the primary leaves of the seedlings. The inoculated seedlings were placed in a humidity chamber in the dark for 14–18h, then retained under florescent light for 3–4h to enhance spore germination. After that, the plants were kept in the greenhouse until disease screening as described by Rouse et al (2012).

Disease reactions of the F_6-RILs and the parents of the cross were assessed 10–12 days after inoculation using the 0–4 scale as described by Stakman et al (1962). Plants showing ITs of 0 – 2+ were considered resistant and those with IT of 3– 4 were considered susceptible. The classification of RILs into HR, HS, and Seg and $\chi 2$ tests were done as described above in leaf rust evaluation.

The evaluation of population under field condition was carried out at the international stem rust nursery at the Ethiopia Institute for Agricultural Research center in Debre Zeit, Ethiopia (EIAR-DZ). This nursery has been classified as an international durum wheat screening site for stem rust as part of the Borlaug Global Rust Initiative. The station is located at 1900 m above sea level. The geographic coordinates are 8° 44’ N latitude and 38° 85’ E longitude. This center represents a hotspot of wheat stem rust during the two cropping seasons in Ethiopia (July–November and January–May) (Letta et al. 2013).

A total of 138 F_5-RILs of the population Rusty X PI 192051 were phenotyped for stem rust response in the field in EIAR-DZ. The F_5-RILs were planted in hill plots with 20–30 seeds per RIL. Rusty, PI 192051, the susceptible common wheat Thatcher, and the susceptible durum

wheat RL6089 were planted every 30 entries as checks. The plants were grown during the off-season of 2016 (January-May). Stem rust spreaders of susceptible wheat cultivars were artificially inoculated 2-3 times starting from stem elongation stage. Races TTKSK (Ug99) and JRCQC were used for the artificial inoculation. Natural inoculum includes other races such as other Ug99 lineage races, TKTTF, TRTTF, RRTTF that are known to be present in the region (Olivera et al. 2012, 2015). The disease was assessed at the soft-dough stage of plant development, following a modified Cobb scale that accounted for both disease severity and infection reaction (Peterson et al. 1948; Roelfs et al. 1992). The plants were classified, based on the host response into resistant (R), moderately resistant (MR), intermediate (M), moderately susceptible (MS), and susceptible (S) as described by Roelfs et al (1992). A combination of two categories of host response on the same plant is possible. Plants with infection reaction of R, MR or M were considered resistant, while plants with reaction MS or S and disease severity higher than 20% were considered susceptible. For data analysis, the phenotypic response at seedling stage of the RILs was converted into binary data, while the disease screening data at adult plant stage was converted into coefficient of infection (CI). The CI was obtained by multiplying the severity and a constant for host response, where immune = 0.0, R = 0.2, MR = 0.4, MS = 0.8, S = 1.0, RMR = 0.3, M= 0.6 and MSS = 0.9 (modified Yu et al. 2011).

SNP genotyping and linkage mapping

Leaf tissue from approximately five plants from each of the parental genotypes and 180 F_6-RILs were collected, lyophilized, and ground as described by Rouse et al (2012). The DNA was extracted using a CTAB protocol described by Riede and Anderson (1996) and modified by Liu et al (2006). The DNA was diluted to 50 ng/µl and genotyped at the USDA-ARS Small

Grain Genotyping Lab in Fargo, ND using the Illumina's iSelect 9K SNP wheat array (Cavanagh et al. 2013). The Illumina Genome Studio software was then used to score the genotypic data. The polymorphic markers were used to construct linkage groups, based on a LOD score of 4.0 using MapDisto 2.0 (Lorieux 2012). Genetic distances between markers were calculated using Kosambi mapping function (Kosambi 1944). Adjustment were made by removing all redundant markers and then linkage maps were reconstructed using MapDisto. The new map was then used to identify SNPs associated with response to leaf rust and stem rust using QGene 4.0 (Joehanes and Nelson 2008). The QTL significantly associated with leaf rust and stem rust resistance were identified using composite interval mapping (Zeng 1994) as described by Faris et al (2014). A permutation test, involving 1000 permutations, produced a LOD threshold of 3.2 for an experiment-wise significance level of 0.05.

Results

Phenotypic evaluation

Evaluation of 161–180 F_6-RILs, derived from the cross Rusty X PI 192051, to the four *Pt* isolates used in this study showed a segregation ratio of 1HR: 1HS (*P* value $\geq$ 0.05). This confirms that the leaf rust resistance in PI 192051 is conferred by single gene (s) (Table 4.2).

Table 4.2. Number of homozygous resistant, homozygous susceptible, and heterozygous recombinant inbred lines (RILs) of the cross Rusty X PI 192051 at seedling stage to four *P. triticina* isolates, *P. graminis* f. sp. *tritici* race TTKSK, and to stem rust under field conditions in Ethiopia.

Trait	Pathogen race	Homozygous resistant (HR)	Segregating (Seg)	Homozygous susceptible (HS)	Total	*P* value for χ^2 1HR:1HS
Seedling evaluation						
Leaf rust	BBBQJ_California	100	5	75	180	0.06 Ns
Leaf rust	BBBQJ_Morocco	80	4	95	179	0.257 Ns
Leaf rust	EEEEE_Ethiopia	98	6	73	177	0.06 Ns
Leaf rust	BBBSJ_Tunisia	71	2	88	161	0.177 Ns
Stem rust	TTKSK	110	2	57	169	4.10E-05 *
Field evaluation						
Ethiopia _2016	TTKSK, JRCQC, and Natural inoculum	59	0	79	138	0.088 Ns

Ns: observed ratio of homozygous resistant (HR) and homozygous susceptible (HS) RILs is not significantly different than the ratio 1HR: 1HS; *: Observed ratio of HR and HS RILs is significantly different from the ratio 1HR: 1HS at 95% level of confidence.

For all the *Pt*-isolates, the lowest observed IT (';') in the F_6-RILs was similar to that of the resistant parent PI 192051, while the highest observed IT ('3+') in the F_6-RILs was similar to that of the susceptible parent Rusty.

The segregation of 169 F_6-RILs screened with *Pgt*-race TTKSK at seedling stage was 110 HR: 2Seg: 57HS, which did not fit expected segregation ratio for a single gene (*P* value =4.10E-05) (Table 4.2). The type '2-' reaction was the lowest observed IT to *Pgt*-race TTKSK which was similar to that of the resistant parent PI 192051, while '3+' was the highest observed IT in the F_6-RILs, similar to that of the susceptible parent Rusty. For the stem rust field experiment in Debre Zeit-Ethiopia, 138 F_5-RILs were evaluated. The lowest infection response observed in the RILs was '5M' while the highest was '30SMS'. The observed median infection responses for the resistant parent PI 192051 and the susceptible parent Rusty were '5RMR' and '25MSS, respectively. Based on the stem rust infection responses of the F_5-RILs in the Ethiopian

field experiment, the F_5-RILs could be grouped into 59 HR and 79 HS (segregation ratio 1HR:1HS, *P* value=0.088), suggesting the presence of a single stem rust resistance gene.

Mapping of *Lr* gene (s)

The Illumina's iSelect 9K SNP wheat array generated 1139 polymorphic SNPs between the parental genotypes Rusty and PI 192051. Fifteen linkage groups were created with a total map size of 1774.71cM. Before removing the redundant markers, the number of markers per linkage group varied from 16-271 SNPs.

The *Lr* gene (s) in PI 192051 conferring resistance to the four *Pt* isolates mapped to chromosome 4A. Ninety-two SNPs were mapped on the linkage group that corresponds to chromosome 4A. When the redundant markers (mapped on the same genomic positions) were removed from the linkage group 4A, the map length was 114.8 cM. Collinearity of this map was conserved with the hexaploid wheat consensus map of Cavanagh et al (2013) with the exception of rearrangements in two cases: *IWA5363*/*IWA603* and *IWA8341*/*IWA5123* (Figure 4.1).

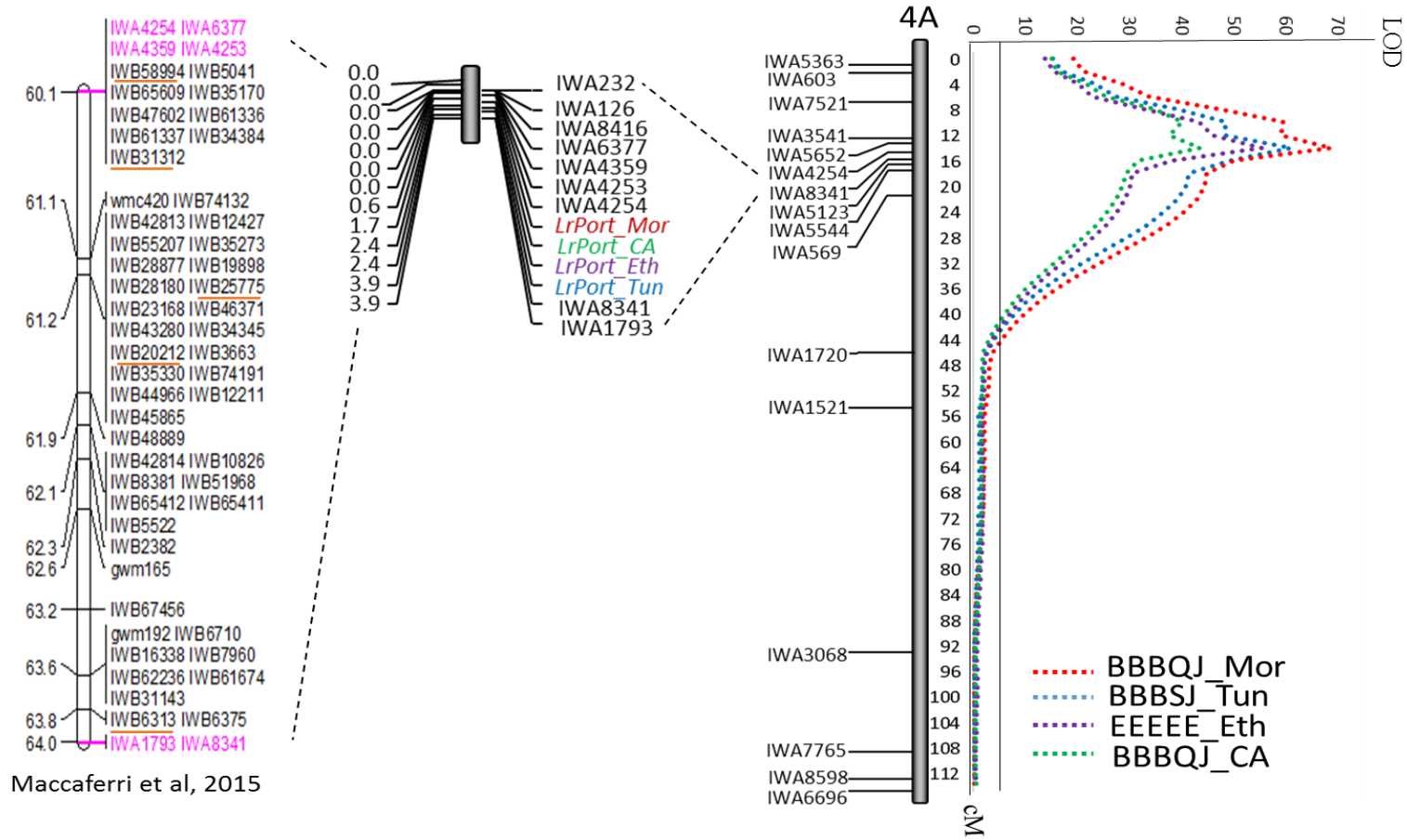

Figure 4.1. Mapping of leaf rust resistance gene *LrPort* in PI 192051 to *P. triticina* races BBBQJ_CA, BBBQJ_Mor, BBBSJ_Tun, and EEEEE_Eth at seedling stage. Markers located between the flanking SNPs of *LrPort* based on the tetraploid consensus map of Maccaferri et al (2015) were presented in this figure with the five *IWB*- SNPs (underlined) showing polymorphism between the parents Rusty and PI 192051.

The four *Lr* genes, here designated as *LrPort_Mor*, *LrPort_CA*, *LrPort_Eth*, and *LrPort_Tun*, were mapped to a 3.9 cM region delimited by *IWA4254* and *IWA8341* on 4AL very close to the centromere. The flanking markers co-segregated with other SNPs as shown in Figure 4.1. Further QTL analysis showed that *LrPort_Mor*, *LrPort_CA*, *LrPort_Eth*, and *LrPort_Tun* had LOD values of 69, 44, 56, and 62, respectively. The four genes *LrPort_Mor*, *LrPort_CA*, *LrPort_Eth*, and *LrPort_Tun* identified in this study accounted for 85%, 72%, 83%, and 85% of the observed disease variations, respectively.

All the four genes appeared to be mapped close to each other with *LrPort_Tun* and *LrPort_Eth* mapped at the same genomic position towards marker *IWA8341*, while *LrPort_CA*, and *LrPort_Mor* were positioned at 0.7 cM and 1.8 cM, proximal to both *LrPort_Tun* and *LrPort_Eth*, respectively (Figure 4.1). Thus, it is most likely that the leaf rust resistance to these four *Pt*- isolates is conferred by the same *Lr* gene, temporarily designated as *LrPort*. These small deviations in the mapping positions of the identified *Lr* genes could be explained by different experimental conditions per isolate, different number of RILs evaluated per isolate, and differing interpretation of ITs.

Based on the tetraploid consensus map of Maccaferri et al (2015), 47 SNPs (*IWB*–) and three SSRs (*wmc*– and *gwm*–) were found between the markers flanking *LrPort.* Further genotyping of the parents Rusty and PI 192051 using the llumina's iSelect 90K SNP wheat array (Wang et al. 2014) showed that five of the 47 SNPs (*IWB58994*, *IWB31312*, *IWB25775*, *IWB20212*, and *IWB6313*) were polymorphic between the parents (Figure 4.1). These five markers could be used to further saturate the map.

Mapping of *Sr* genes

Eventhough, the segregation of F_6-RILs of Rusty X PI 192051 did not fit expected segregation ratio for a single gene conferring resistance to *Pgt*-race TTKSK at seedling stage, the *Sr* gene in PI 192051 was mapped on a single location on chromosome arm 7AS. Sixty-nine SNPs were mapped on the linkage group that corresponds to chromosome 7A. The linkage map of 7A, generated after the elimination of the redundant markers, had a total length of 117.02 cM. Collinearity of this map was conserved with the hexaploid wheat consensus map of Cavanagh et al (2013) with the exception of rearrangements of markers in two cases: *IWA1031*/*IWA4621/IWA6670* and *IWA502*/*IWA6576*. In the current map, we were also able to map markers *IWA3903* and *IWA471* on 7A, which were unmapped in the hexaploid consensus map of Cavanagh et al (2013) (Figure 4.2).

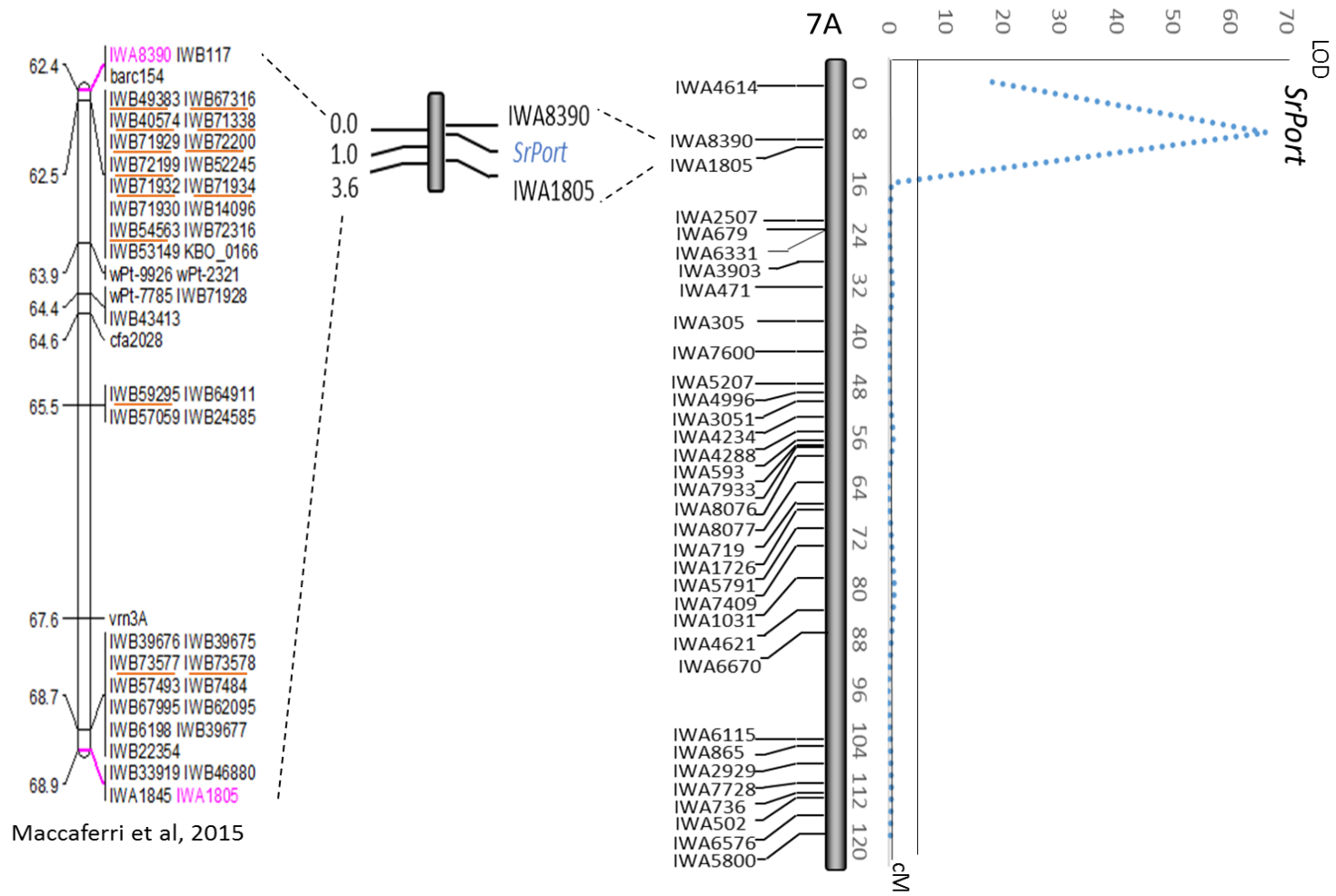

Figure 4.2. Mapping of stem rust resistance gene *SrPort* in PI 192051 to *P. graminis* f. sp. *tritici* race TTKSK at seedling stage. Markers located between the flanking SNPs of *SrPort* based on the tetraploid consensus map of Maccaferri et al (2015) were presented in this figure, with 13 *IWB*- SNPs (underlined) showing polymorphism between the parents Rusty and PI 192051.

The *Sr* gene in PI 192051, temporarily designated as *SrPort*, was mapped to a 3.6 cM region delimited between *IWA8390* and *IWA1805* (Figure 4.2). Marker *IWA8390* is 1 cM distal to *SrPort*, while *IWA1805* was 2.6 cM proximal to *SrPort*. Based on the QTL analysis, a very high LOD score of 65 for *SrPort* was observed. The gene *SrPort* explained approximately 84% of the observed disease variations. The SNP marker *IWA8390* was the closest to the peak position of *SrPort* (Figure 4.2).

There are, 35 SNPs (*IWB*– and *KBO_0166*), two SSRs (*barc154* and *cfa2028*), three DArT (*wPt-*), and one STS (*vrn3A*) within the flanking region of *SrPort* based on the tetraploid consensus map (Figure 4.2). Based on this study, 13 of the 35 SNPs are polymorphic between the parental genotypes (*IWB49383*, *IWB67316*, *IWB40574*, *IWB71338*, *IWB71929*, *IWB72200*, *IWB72199*, *IWB71932*, *IWB71934*, *IWB54563*, *IWB59295*, *IWB73577*, and *IWB73578*) (Figure 4.2). Thus, they could be used in the development of more tightly linked markers to *SrPort*.

A QTL on chromosome arm 5BL was identified to be associated with stem rust resistance at adult-plant stage for the Ethiopian field experiment data in 2016. One hundred and six SNPs were mapped on chromosome 5B. After removing the redundant markers, the map of chromosome 5B had a total length of 151.44 cM. Collinearity of this map was conserved with the hexaploid wheat consensus map of Cavanagh et al (2013) with the exception of three cases where micro-rearrangements of markers between *IWA894*-to-*IWA4494*, *IWA6468*-to-*IWA1374*, and *IWA8097*-to-*IWA6024* were observed (Figure 4.3).

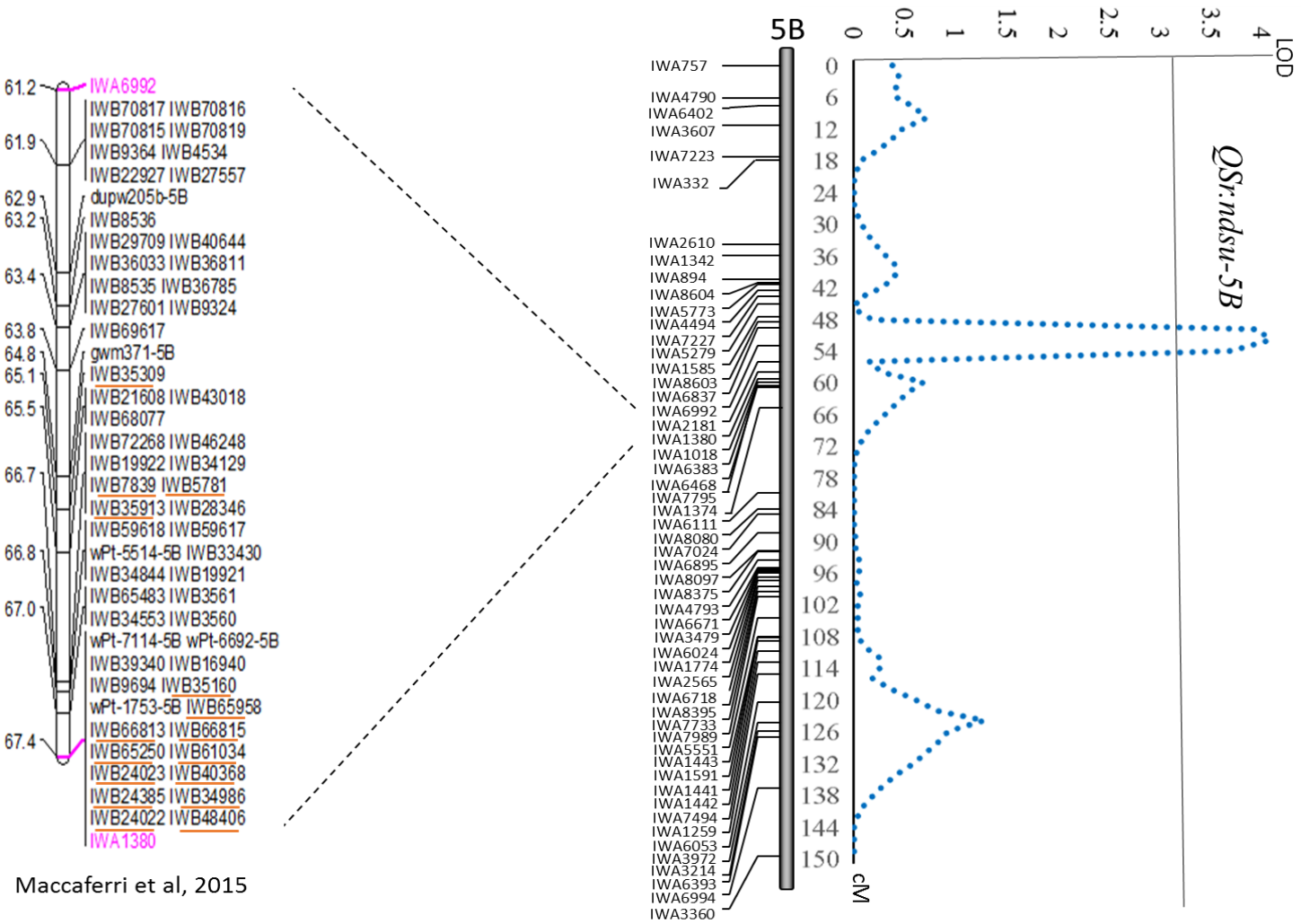

Figure 4.3. Mapping of stem rust resistance gene *QSr.ndsu-5B* in PI 192051 to *P. graminis* f. sp. *tritici* races at adult plant stage in Ethiopia under filed conditions. Markers located between the flanking SNPs of *QSr.ndsu-5B* based on the tetraploid consensus map of Maccaferri et al (2015) were presented in this figure with 16 *IWB*- SNPs (underlined) showing polymorphism between the parents Rusty and PI 192051.

The QTL from PI 192051 associated with resistance to stem rust in Ethiopia, designated temporary as *QSr.ndsu-5B*, was mapped to a 4.8 cM region on 5BL and was flanked by *IWA6992* (50.14 cM) and *IWA2181* (54.94 cM). The QTL analysis showed that *QSr.ndsu-5B* had a LOD value of 4.1 and explained 17.01% of the observed disease variations. The SNP *IWA6992* was the closest to the peak position of *QSr.ndsu-5B* (Figure 4.3). Prior to removing the redundant markers, two SNPs *IWA2182* and *IWA8343* co-segregated with the flanking marker *IWA2181*. No significant QTL on 7AS were identified to be associated with stem rust resistance in Ethiopia. This suggests that the genetic basis of resistance in PI 192051 to *Pgt*-TTKSK at seedling stage differs from the stem rust resistance at adult-plant stage observed in the Ethiopian field experiment where inoculation was made using *Pgt*-races TTKSK and JRCQC in addition to the natural inoculum present in the nursery. Further validations of *QSr.ndsu-5B* in future seasons in Ethiopia will be performed.

Fifty-four SNPs (*IWB*–), two SSR (*gwm371-5B* and *dupw205b-5B*), and four DArt (*wPt-*) were found within the genomic region of *QSr.ndsu-5B* between the flanking markers based on the tetraploid consensus map. Of the 54 SNPs, 16 were polymorphic between the parents based on the 90K SNP genotyping (*IWB35309*, *IWB7839*, *IWB5781*, *IWB35913*, *IWB35160*, *IWB65958*, *IWB66813*, *IWB66815*, *IWB65250*, *IWB61034*, *IWB24023*, *IWB40368*, *IWB24385*, *IWB34986*, *IWB24022*, and *IWB48406*) (Figure 4.3). These markers could provide more saturated mapping of *QSr.ndsu-5B.*

Discussion

In the current study, durum wheat landrace PI 192051 showed a wide range of leaf rust resistance to the durum type-*Pt*-isolates collected in Ethiopia, Morocco, USA, and Tunisia. This

genotype also carries resistance to stem rust not only to race TTKSK (Ug99), but also to other durum-specific Ethiopian *Pgt*–races with combined virulence on *Sr13* and *Sr9e*.

The gene *LrPort* conferring resistance at seedling stage to the *Pt* isolates used in this study was mapped to chromosome 4AL. This finding confirmed the BSA, done in earlier generations of the population Rusty X PI 192051 using the *Pt*-race BBBQJ collected from California, USA (Aoun et al. 2016b), which is one of the isolate used in this study. Interestingly, the only previously reported *Lr* gene on chromosome 4A is *Lr30* which originated from *T. aestivum* common wheat line Terenzio (Dyck and Kerber 1981). However, there is no sufficient mapping information available for *Lr30* to make concrete comparisons between the mapped positions of *LrPort* and *Lr30*. It was reported that *Lr30* is rarely found in wheat. For instance, the *Lr* gene postulation done on a world common wheat collection of 275 accessions showed that only two accessions from North America possibly carry *Lr30* (Dakouri et al. 2013). The evaluation of PI 192051 with the North American *Pt*- race TNRJJ that is virulent on Thatcher NIL carrying *Lr30* showed that PI 192051 is highly resistant (IT= ‘;’) (M. Aoun, unpublished). However, PI 192051 could have *Lr72* or another durum gene that gives resistance to common wheat races. Finding a new *Lr* gene in this landrace is not surprising as wheat landraces usually carry novel sources of resistance to several diseases including rust (Bux et al. 2012; Bansal et al. 2013; Kertho et al. 2015; Aoun et al. 2016a, 2016b).

Moreover, associated markers with leaf rust resistance on 4AL were observed in an association mapping study using the USDA-NSGC from which PI 192051 was selected (Aoun et al. 2016a). The AM revealed five SNP associations with leaf rust response on 4AL. One of them is *IWA1570* (143.0 cM) found within the mapped region of *LrPort* (141.4 cM–144.11 cM), based on the 9K wheat consensus map (Cavanagh et al. 2013).

In a previous study, PI 192051 showed resistance to several other *Pt*- isolates collected worldwide at seedling stage and at adult-plant stage in field trials in USA, Mexico, Morocco, and Ethiopia (Aoun et al. 2016a). Therefore, the RILs of the population Rusty X PI 192051 will be evaluated in field trials in several geographical locations to determine whether the seedling resistance gene *LrPort* is the same gene conferring resistance to *Pt* races at adult-plant stage in different locations. The presence of possible adult-plant resistance (APR) genes to leaf rust in PI 192051 will also be investigated. One of the *Pt*-isolates used in this study, Tun-20-4 (race BBBSJ) is virulent to the widely used *Lr* gene in durum wheat *Lr14a*, therefore *LrPort* can be introgressed into durum wheat cultivars in the regions where virulence to *Lr14a* is prevalent (Ordóñez and Kolmer. 2007a; Goyeau et al. 2006; Gharbi et al. 2013; Soleiman et al. 2016). In addition, in a previous study, PI 192051 showed resistance to *Pt*- races virulent to other *Lr* genes mapped in durum wheat such as *Lr3a*, *Lr27+31*, *Lr61*, and *Lr72* (Aoun et al. 2016b).

The landrace PI 192051 also carries *SrPort* conferring resistance to *Pgt*-race TTKSK at seedling stage on chromosome 7AS. Since catalogued *Sr* genes on 7AS have not been identified before in tetraploid or hexaploid wheat, *SrPort* is most likely a novel *Sr* gene. A number of QTL on 7A, associated with stem rust resistance at both seedling and adult-plant stages have been previously identified via AM and linkage mapping in durum wheat. For instance, AM showed that *IWA7200* was associated with stem rust response in the USDA-NSGC of durum wheat (Chao et al. 2016). The SNP *IWA7200* (42.0 cM) on 7AS is within the mapping position of the *SrPort* (41.04 – 42.5 cM), based on the 9K wheat consensus map (Cavanagh et al. 2013). In another AM, a significant DArt marker on 7AS, *wPt-6668*, was associated with response to *Pgt*-race TRTTF at seedling stage in a world durum panel of 183 cultivars and breeding lines (Letta et al. 2014). In the same durum collection, the AM revealed that *wPt-2799* and *wPt-7885* on 7AS

were associated with response to race TTKSK and a mixture of durum specific *Pgt*-races in field trials in Ethiopia (Letta et al. 2013). Additionally, two QTL for stem rust resistance in Ethiopia were mapped to 7A in the tetraploid bi- parental population Kristal X Sebatel. The cultivar Sebatel is the resistant parent, while Kristal is the susceptible parent to stem rust. One of the QTL on 7AS (*QSr.1PK-7A.1*) in Sebatel was flanked by SSR markers *gwm974* and *gwm631*, while the second QTL (*QSr.1PK-7A.2*) is most likely *Sr22* on 7AL (Haile et al. 2012). Since the flanking markers of *QSr.1PK-7A.1* are not mapped in the tetraploid consensus map, comparison between the genomic locations of *SrPort* and *QSr.1PK-7A.1* was not possible.

In the present study, *QSr.ndsu-5B* on 5BL conferred resistance to stem rust at adult-plant stage in Debre Zeit-Ethiopia. Interestingly, the resistance gene at adult plant stage in Ethiopia was conferred by a different gene from *SrPort* that conferred resistance to the race TTKSK in PI 192051 at seedling stage. This is most likely due to the inoculum present in Ethiopia which included other races in addition to the Ug99 race group such as JRCQC, TRTTF, RRTTF, and TKTTF (Olivera et al. 2012, 2015). Earlier studies reported a number of QTL for stem rust resistance in durum wheat found on 5BL, but none of them was mapped close to *QSr.ndsu-5B,* based on the tetraploid consensus map of Maccaferri et al (2015). For instance, Letta et al (2013) reported the association of *wPt-9300* on 5BL with response to stem rust in durum wheat in Ethiopia, but the position of *wPt-9300* (118.1cM) is mapped far from *QSr.ndsu-5B* (61.2–67.4 cM). In addition, in an AM study conducted on the USDA-NSGC of durum wheat, it was reported that *IWA7585* on 5BL was associated with response to *Pgt*-race TRTTF (Chao et al. 2016). However, *IWA7585* (152.1cM) is distantly located from *QSr.ndsu-5B.* Moreover, the QTL analysis in the population Kristal X Sebatel showed the involvement of *QSr.ipk-5B* on 5BL in the genetic resistance to stem rust in Ethiopia (Haile et al. 2012). The identified QTL in

Sebatel was delimited by *gwm408* (122.9 cM) and *barc142* (141.6) cM which is far from *QSr.ndsu-5B*.

In this study, we identified previously uncharacterized leaf rust and stem rust resistance genes in the Portuguese durum wheat landrace PI 192051. The genes *LrPort* on 4AL and *SrPort* on 7AS were effective against several *Pt*-races and *Pgt*- race TTKSK at seedling stage, respectively. The gene *QSr.ndsu-5B* on 5BL conferred resistance in PI 192051 to stem rust at adult plant stage in Ethiopia, however validation of this QTL in coming seasons is needed. Mapping of *Sr* genes in PI 192051 to recently emerged *Pgt*-races in Ethiopia at seedling stage and at adult plant stage in field trials will also be performed. The closely linked markers are candidates for marker assisted selection to facilitate the introgression of these potentially novel *Lr* and *Sr* genes in durum wheat cultivars. Follow up study is underway to reduce the flaking regions of the *Lr* and *Sr* genes in PI 192051. The possible presence of APR genes in PI 192051 to leaf rust and stem rust will also be investigated.

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CHAPTER V. GENOTYPE BY SEQUENCING FOR THE STUDY OF POPULATION GENETICS IN *PUCCINIA TRITICINA*

Abstract

Leaf rust, caused by *Puccinia triticina* Erikss., is the most widespread wheat rust disease. *P. triticina* population is highly diverse for virulence phenotypes. Information on the virulence and genetic diversity of *P. triticina* is important for understanding the evolution of this pathogen, and thus effective management of wheat leaf rust. The genetic diversity of *P. triticina* populations has been previously assessed using different types of molecular markers, including Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) markers. However, the study of population genetics benefits from more abundant markers distributed across the genome. This study investigated the virulence diversity of *P. triticina* isolates collected from diverse hosts and locations worldwide. We then assessed the application of the Restriction-Associated DNA (RAD)-Genotype By Sequencing (GBS) adapted for the Ion Torrent sequencing platform for the study of population genetics in *P. triticina*. A collection of 102 isolates, collected mainly from tetraploid wheat and common wheat was used. The virulence phenotypes of the isolates were determined on 20 Thatcher wheat near isogenic lines. The genetic diversity was investigated in a subset of 30 genotyped isolates using RAD-GBS. Seven races, BBBQJ, BBBSJ, BBBQK, BCBQJ, BBBSQ, CBBQS, and EEEEE were found among 57 isolates collected from tetraploid wheat, while 21 races were observed among 40 common wheat type isolates. Since the Ethiopian EEEEE isolates were avirulent on Thatcher, nine durum differential genotypes were identified to distinguish between seven EEEEE isolates. Phylogenetic analysis on 30 isolates using 2,125 Single Nucleotide Polymorphism (SNP) markers showed eight clusters. Higher genotypic diversity was observed in common wheat type

isolates compared to that in the tetraploid wheat type isolates. Generally, there was a correlation between virulence phenotypes and SNP genotypes. Phylogeny results suggest that RAD-GBS is promising as a new technique for the study of population genetics in *P. triticina*.

Introduction

Leaf rust is a common fungal disease in all wheat growing regions. This disease is the most prevalent among the three wheat rusts (Chester 1946), reducing the quantity and quality of kernels. The severity of leaf rust is usually the highest in areas where wheat grows under warm to hot climates such as the Great Plains of North America and the steppes of Central Asia. Similarly, rust severity has been reported extensively in regions with warm and humid climate such as the Mediterranean basin, South America, and the coastal regions of North America (Kolmer and Acevedo 2016). The occurrence and distribution of leaf rust on hexaploid common wheat is global while for tetraploid durum wheat, leaf rust is a problem mainly in Mediterranean basin, Middle East, Ethiopia, Mexico, and South America (Ordoñez and Kolmer 2007a).

Puccinia triticina is a macrocyclic-heteroecious pathogen, producing five spore stages. Wheat and wild relatives are the primary hosts where urediniospores, teliospores and basidiospores are produced. The pycniospores and aeciospores are produced on the alternate hosts, mainly *Thalictrum speciosissimum L.* (Mains and Jackson 1921). *Thalictrum speciosissimum* is native to Spain and Portugal, where pycniospores and aeciospores have been observed. In addition, sexual reproduction on *Thalictrum* spp. was observed in Northeast Kazakhstan (J.A. Kolmer, unpublished data) and in Siberia on *Isopyrum fumarioides* (Chester 1946). Since the alternate hosts in many parts of the world are absent or resistant to leaf rust, *P. triticina* reproduces mainly by clonal production of dikaryotic urediniospores.

Despite the very rare contribution of sexual recombination, *P. triticina* population continues to evolve asexually and shows high diversity for virulence to leaf rust resistance (*Lr*) genes in wheat (Roelfs et al. 1992; McCallum et al. 2007; Kolmer 2013). For instance, over 50 virulence phenotypes are detected annually in North America (McCallum et al. 2007; Kolmer 2013; Hughes and Kolmer 2016). Mutation, genetic drift, migration, and host selection are the key causes of the observed diversity in *P. triticina* population, while cases of parasexual recombination in *P. triticina* have also been reported in Australia (Park et al. 1999). Mutation is the primarily source of *P. triticina* diversity, resulting in the rapid appearance of virulence phenotypes on deployed *Lr* genes (Ordoñez and Kolmer 2007a).

In recent years, susceptibility to leaf rust has increased on durum wheat cultivars grown in most of the producing areas around the world due to the emergence of *P. triticina* races highly virulent on durum wheat (Singh et al. 2004; Goyeau et al. 2006; Martinez et al. 2005; Ordoñez and Kolmer 2007a). The majority of the *P. triticina* isolates virulent on durum wheat are avirulent on most of the *Lr* genes identified in common wheat (Goyeau et al. 2006; Ordoñez and Kolmer 2007b). In addition, *P. triticina* isolates collected from durum wheat are less diverse for virulence phenotypes and SSR genotypes compared to those collected from common wheat (Martinez et al. 2005; Goyeau et al. 2012; Ordoñez and Kolmer 2007a, b). *P. triticina* isolates virulent on durum wheat in Mexico (Singh et al. 2004) were very similar in virulence phenotypes and (SSR) genotypes to isolates collected on durum wheat in Spain, France, Mexico, Argentina, and the United States, suggesting a common ancestor (Ordoñez and Kolmer 2007a, b). However, some *P. triticina* isolates collected from Ethiopia on tetraploid wheat had a virulence phenotype and SSR genotype distinct from all other worldwide collections of isolates from both durum and common wheat (Kolmer and Acevedo 2016). These Ethiopian durum specific isolates are

avirulent on the common wheat Thatcher, thus designated as race EEEEE. These isolates have most likely evolved and been maintained in Ethiopia due to the high diversity of tetraploid wheat population grown in the country (Kolmer and Acevedo 2016).

Molecular markers have been used to assess the genetic diversity in *P. triticina* populations. The random amplified polymorphism of DNA (RAPD) were the first markers used to genotype *P. triticina* populations (Kolmer and Liu 2000; Park et al. 2000), followed by AFLPs (Kolmer 2001), and currently by SSRs (Duan et al. 2003). A set of 23 SSRs (Szabo and Kolmer 2007) are used in current studies to assess the genetic diversity of clonal populations of *P. triticina*, showing high correlation between SSR genotypes and virulence phenotypes (Ordoñez and Kolmer 2007b, 2009; Ordoñez et al. 2010; Kolmer et al. 2011, 2013, Kolmer 2015; Kolmer and Acevedo 2016). Two models of mutation are illustrated with SSRs which are the infinite allele model and the stepwise mutation model. In the case of infinite allele model of mutation, the measures of the population differentiation is estimated using F_{ST} (Wright 1951), while R_{ST} (Slatkin 1995) is the measure in the stepwise mutation model. Statistical problems associated with the use of SSRs when estimating the population diversity were discussed by Balloux and Lugon-Moulin (2002).

The study of population genetics requires more abundant molecular markers, distributed throughout the genome. Genotype-by-sequencing (GBS) is a low-cost approach used to generate reduced representation sequencing of the whole genome by targeting subsets of genomic regions (Elshire et al. 2011; Poland et al. 2012a). This technique was used in several studies such as genomic selection, genetic mapping, and genetic diversity in several organisms (Elshire et al. 2011; Poland et al. 2012a, 2012b; Lu et al. 2013). The RAD-GBS technique was used to generate high density SNP marker data for complex plant genomes (Poland et al. 2012a, 2012b). The

RAD-GBS uses restriction enzymes to capture targeted regions of the genome. Then, adaptors are ligated to the flanking regions of the restriction sites to create barcoded libraries which allows the sequencing of several individuals at a time (Mascher et al. 2013). The RAD-GBS is mainly performed based on Illumina GAII and HiSeq platforms (Poland and Rife 2012). However, recently an optimized RAD-GBS protocol, adapted for Ion Torrent platform has become available (Rothberg et al. 2011) and has been used to generate sufficient genotyping density for a number of plant pathogens (Leboldus et al. 2015; Gao et al. 2016).

The objective of the current study was to assess the virulence diversity in a collection of 102 isolates collected from common wheat, durum wheat, Triticale, and cultivated emmer wheat (*Triticum diccocum*). The isolates were collected from Morocco, Tunisia, Spain, Ethiopia, Chile, Pakistan, Mexico, and USA. We also assessed the application of RAD-GBS using the Ion Torrent sequencing platform for the study of population genetics in *P. triticina.*

Materials and methods

Puccinia triticina isolates

Forty-five samples infected with *P. triticina* were received from Morocco, Tunisia, Spain, and Ethiopia. Each sample corresponds to multiple infected leaves with leaf rust, collected from a single plant. Sampling was done randomly in the field. A set of 12 samples was collected in 2014 from research plots of durum wheat, common wheat, and cultivated emmer at the Debre Zeit Agricultural Research Center (DZARC), Ethiopia. A second set of eight samples were collected in 2014 in Spain from common wheat, durum wheat, and Triticale fields at Peralta, Learza (Navarra), Huesca, Coril (Cadiz), and Jerez de la Frontera locations. Six samples were also collected in 2014 in Tunisia from research plots of common wheat, durum wheat, and Triticale at the National Institute of Agronomic Research of Tunisia (INRAT), Beja experiment

station, Grombalia, and Mateur. Additional 19 samples were collected in Morocco in 2014 and 2015 from durum wheat and common wheat fields at Zemamra, Jemaa-Shaam, Rabat, Marchouch, and Tahrir. The samples (infected leaves) were placed in paper envelopes and air dried at room temperature. The samples were then shipped to North Dakota State University, where they were stored at -80°C until processed.

The samples were processed during winter months (December-February) at the North Dakota Agricultural Experiment Station Greenhouse Complex (ND-AESGC), Fargo, ND. Urediniospores from each sample were collected using a cotton swab immersed in Soltrol-170 mineral oil (Phillips Petroleum). The swab containing the collected spores was used to inoculate 7-day-old seedling of 'RL6089' for spore increase. The susceptible seedling of 'RL6089' had been treated with a 0.3% solution of maleic hydrazide to increase the sporulation and prevent secondary leaves from forming. Seven days after inoculation, spores from a single uredinium were collected with a cotton swab which was in turn used to inoculate a seedling of RL6089 for increase of the purified single uredinium isolate. From each sample, 1–4 single-uredinial isolations were derived. Single uredinium isolate was increased on a seedling plant, covered with an isolation box to avoid cross-contamination. Incubation and greenhouse conditions were as described by Aoun et al. (2016).

A total of 86 isolates resulted from the samples collected in Ethiopia, Morocco, Spain, and Tunisia. Seven more single uredinial isolates from Mexico (collected in 2001-2014) were provided by Dr. Julio Huerta-Espino at CIMMYT-Mexico. In addition, Dr. James A. Kolmer at Cereal Disease Laboratory, St. Paul, MN (USDA-CDL, MN) provided two isolates from USA (Race1 is an old North American common wheat type race and was used in this study as control because its whole genome sequence is available; and isolate CA1.2 collected on durum wheat in

California in 2009), five isolates from Chile (collected in 2013), and five isolates from Pakistan (collected in 2013 and 2014). In total, 102 single pustule isolates were assembled for this study with 51 collected from durum wheat, 40 from common wheat, five from Triticale, and six isolates from cultivated emmer wheat (Table 5.1).

Table 5.1. Number of collections, single uredinial isolates collected from different countries on durum wheat, common wheat, emmer wheat, and Triticale.

Country	Number of collections	Number of isolates	Number of isolates per host species			
			Common wheat	Durum wheat	Emmer wheat	Triticale
Ethiopia	12	17	1	10	6	0
Mexico	-[a]	7	2	5	0	0
Morocco	19	32	14	18	0	0
Chile	-[a]	5	3	2	0	0
Pakistan	-[a]	5	5	0	0	0
Spain	8	21	12	5	0	4
Tunisia	6	13	2	10	0	1
USA	-[a]	2	1	1	0	0
Total	45	102	40	51	6	5

[a] Single uredinial isolates were obtained from collaborators.

Virulence phenotypes of *P. triticina* isolates

Urediniospores of each of the 102 single uredinial isolates were increased to generate enough inoculum for phenotyping and DNA extraction. The phenotyping of 81 isolates was conducted by Dr. James A. Kolmer at the USDA-CDL, MN, while the remaining 21 isolates were phenotyped at ND-AESGC, Fargo, ND. Five sets of four Thatcher near isogenic lines carrying different *Lr* genes were used for the isolate phenotyping. The first set included lines with genes, *Lr1* (isogenic line RL6003), *Lr2a* (RL6000), *Lr2c* (RL6047), and *Lr3a* (RL6002); the second set included lines with genes, *Lr9* (RL6010), *Lr16* (RL6005), *Lr24* (RL 6064), and *Lr26* (6078); the third set comprised of lines with genes, *Lr3ka* (RL6007), *Lr11* (RL6053), *Lr17* (RL6008), and *Lr30* (RL6049); the fourth set included lines with genes *LrB* (RL6047), *Lr10* (RL6004), *Lr14a* (RL6013), and *Lr18* (RL6009); and the fifth set of lines carrying genes *Lr3bg*

(RL6042), *Lr14b* (RL6006), *Lr20* (RL 6092), and *Lr28* (RL6079). Thatcher was included as a check. The plants were inoculated at the first leaf stage (7-8 days after planting) with fresh urediniospores suspended in Soltrol-170 mineral oil (Phillips Petroleum) using an inoculator pressurized by an air pump. The inoculated plants were then placed in a mist chamber overnight and later transferred and kept in the greenhouse at 18–25°C and at 16-h photoperiod. The seedlings were evaluated for infection types (ITs) 10–12 days after inoculation. At ND-AESGC, Fargo, ND, the virulence phenotype of these isolates was based on two replicates per isolate. The virulence phenotypes of the isolates, at the USDA-CDL, MN, was based on single tests. However, in the case where disease development or plant development were suboptimal and ITs were difficult to determine, a second test was performed. Seedlings showing ITs of 0–2+ and mesothetic reaction (or 'X' reaction) were classified as resistant while, those showing ITs of 3–4 were classified as susceptible. Based on the ITs on the isogenic lines, five-letter designation was assigned to each isolate following the nomenclature of Long and Kolmer (1989).

Since some of the Ethiopian isolates are avirulent on Thatcher (race EEEEE), we could not assign a proper race code using Thatcher differentials. Therefore, seven Ethiopian EEEEE isolates (six isolates collected from durum wheat and one isolate collected from emmer wheat) were tested on a set of 23 durum wheat accessions and the common wheat Thatcher as resistant control. Twelve of these accessions were selected from the USDA-National Small Grain Collection (NSGC) of durum wheat as they showed resistance to several *P. triticina* isolates (Aoun et al. 2016). These 12 accessions were PI 45442, PI 192051, PI 195693, PI 209274, PI 244061, PI 278379, PI 313096, PI 324928, PI 342647, PI 387263, PI 519832, and PI 534304. The remaining 11 genotypes were durum cultivars Divide, Rusty, Capelli, Mindum, Russello

S.G.7, Alred, Creso with *Lr14c*, Llareta INIA (*Lr14a*), Camayo (*LrCamayo*), Jupare C2001 (*Lr27+Lr31*), and Mexicali 75.

DNA extraction, library preparation, and sequencing

A total of 44 isolates, selected from the 102 isolates, representing most of the virulence phenotypes per country were genotyped. These isolates included two from USA, four from Mexico, five from Chile, five from Pakistan, four from Ethiopia, six from Tunisia, eight from Morocco, and ten from Spain. Twenty-one isolates from common wheat, 17 from durum wheat, four from Triticale, and two from emmer wheat were genotyped (Table 5.2).

Table 5.2. Races of *Puccinia triticina* identified from samples collected from different countries and hosts.

Isolate ID	Country	Location	Host	Variety/line	Race	DNA [b]
CHL03-D2	Chile		*T. aestivum*		MCDSS	X
CHL04-D2	Chile		*T. aestivum*		MBDSS	X
CHL33-D2	Chile		*T. aestivum*		MFPNQ	X
CHL02-D1	Chile		*T. turgidum*		BBBSJ	X
CHL14-1	Chile		*T. turgidum*		BBBQJ	X
52-1 [a]	Ethiopia	DZARC	*T. aestivum*	B21	MBJS-	
45-6 [a]	Ethiopia	DZARC	*T. diccocum*		EEEEE	
47-2 [a]	Ethiopia	DZARC	*T. diccocum*		EEEEE	
48-3 [a]	Ethiopia	DZARC	*T. diccocum*		EEEEE	X
49-1 [a]	Ethiopia	DZARC	*T. diccocum*		EEEEE	
49-2	Ethiopia	DZARC	*T. diccocum*		EEEEE	
50-1 [a]	Ethiopia	DZARC	*T. diccocum*		EEEEE	
50-4 [a]	Ethiopia	DZARC	*T. diccocum*		EEEEE	X
57-2	Ethiopia	DZARC	*T. turgidum*	182/*Sr30*	EEEEE	
58-1 [a]	Ethiopia	DZARC	*T. turgidum*	Rusty	EEEEE	
62-2 [a]	Ethiopia	DZARC	*T. turgidum*	PI 272553 (NSGC)	EEEEE	X [c]
63-1	Ethiopia	DZARC	*T. turgidum*	PI 387346 (NSGC)	EEEEE	
63-2	Ethiopia	DZARC	*T. turgidum*	PI 387346 (NSGC)	EEEEE	
63-3	Ethiopia	DZARC	*T. turgidum*	PI 387346 (NSGC)	EEEEE	X [c]
64-1 [a]	Ethiopia	DZARC	*T. turgidum*	PI 478427 (NSGC)	EEEEE	
64-3 [a]	Ethiopia	DZARC	*T. turgidum*	PI 478427 (NSGC)	EEEEE	
65-2	Ethiopia	DZARC	*T. turgidum*	PI 298547 (NSGC)	EEEEE	
12	Mexico		*T. aestivum*		TBDKT	X
17	Mexico		*T. aestivum*		CBDSS	X [c]
13	Mexico		*T. turgidum*		BBBQJ	
10	Mexico		*T. turgidum*		BCBQJ	
11	Mexico		*T. turgidum*		CBBQS	X
14	Mexico		*T. turgidum*		BBBQK	X
15	Mexico		*T. turgidum*		BBBQJ	
28-1	Morocco	Zemamra	*T. aestivum*		MCDSS	
28-2	Morocco	Zemamra	*T. aestivum*		MCDSS	
29-1	Morocco	Zemamra	*T. aestivum*		MCDSS	
29-2	Morocco	Zemamra	*T. aestivum*		MCDSS	
30-1 [a]	Morocco	Zemamra	*T. aestivum*		BBBQJ	
30-2	Morocco	Zemamra	*T. aestivum*		BBBQJ	
30-3	Morocco	Zemamra	*T. aestivum*		BBBQJ	X
34-2	Morocco	Jemaa-Shaam	*T. aestivum*		MBDSS	X
35-1	Morocco	Jemaa-Shaam	*T. aestivum*		FBBPQ	

Table 5.2. Races of *Puccinia triticina* identified from samples collected from different countries and hosts (continued).

Isolate ID	Country	Location	Host	Variety/line	Race	DNA[b]
41-1	Morocco		*T. aestivum*	Taza (seis ii)	MCDST	X [c]
42-1 [a]	Morocco	Tahrir	*T. aestivum*	Amal	MCDS-	
43-1 [a]	Morocco		*T. aestivum*	Meknes	MCDSS	
43-3	Morocco		*T. aestivum*	Meknes	MCDSS	X [c]
44-1 [a]	Morocco		*T. aestivum*	Seiz	MCDSS	
44-1 [a]	Morocco		*T. aestivum*	Seiz	MCDSS	
25-1	Morocco	Zemamra	*T. turgidum*	MERZAV	BBBQJ	
25-2 [a]	Morocco	Zemamra	*T. turgidum*	MERZAV	BBBQJ	
25-6 [a]	Morocco	Zemamra	*T. turgidum*	MERZAV	BBBQJ	
26-1	Morocco	Zemamra	*T. turgidum*		BBBQJ	X [c]
26-2	Morocco	Zemamra	*T. turgidum*		BBBQJ	
26-3	Morocco	Zemamra	*T. turgidum*		BBBSJ	
27-1	Morocco	Zemamra	*T. turgidum*	CARIOCA	BBBQJ	
27-2 [a]	Morocco	Zemamra	*T. turgidum*	CARIOCA	BBBQJ	X [c]
32-2	Morocco	Jemaa-Shaam	*T. turgidum*		BBBSJ	
33-1 [a]	Morocco	Jemaa-Shaam	*T. turgidum*		BBBSJ	X [c]
36-1 [a]	Morocco	Jemaa-Shaam	*T. turgidum*		BBBSJ	
36-3	Morocco	Jemaa-Shaam	*T. turgidum*		BBBQJ	
37-2 [a]	Morocco	Rabat	*T. turgidum*	RL7075	BBBQJ	
38-1	Morocco	Rabat	*T. turgidum*	Cali (A33File1)	BBBQJ	
38-2	Morocco	Rabat	*T. turgidum*	Cali (A33File1)	BBBQJ	X
38-3	Morocco	Rabat	*T. turgidum*	Cali (A33File1)	BBBQJ	
39-9	Morocco	Marchouch	*T. turgidum*	Kristal/Sebatel #7	BBBQJ	
40-1	Morocco	Marchouch	*T. turgidum*	Kristal	BBBQJ	
13PAK15-1	Pakistan		*T. aestivum*		FHPSQ	X
13PAK17-1	Pakistan		*T. aestivum*		CCPSL	X
14PAK1-2	Pakistan		*T. aestivum*		BBBDH	X
14PAK1-3	Pakistan		*T. aestivum*		BBBDH	X
14PAK1-4	Pakistan		*T. aestivum*		CBBDK	X
6-1	Spain	Jerez de la Frontera	*Triticale*		FBBNQ	X
6-2	Spain	Jerez de la Frontera	*Triticale*		FBBPQ	X
6-3	Spain	Jerez de la Frontera	*Triticale*		CBGNQ	X
6-4	Spain	Jerez de la Frontera	*Triticale*		FBBPQ	
2-1	Spain	Learza (Navarra)	*T. aestivum*	NSA11-8606	MCPSS	X
2-2	Spain	Learza (Navarra)	*T. aestivum*	NSA11-8606	MCPSS	
2-3	Spain	Learza (Navarra)	*T. aestivum*	NSA11-8606	MCPSS	
3-1	Spain	Huesca	*T. aestivum*	L6W14-8082	MCDSQ	
3-2	Spain	Huesca	*T. aestivum*	L6W14-8082	MCDSQ	
7-1	Spain	Huesca	*T. aestivum*	12-15-1515	MCTNQ	X

Table 5.2. Races of *Puccinia triticina* identified from samples collected from different countries and hosts (continued).

Isolate ID	Country	Location	Host	Variety/line	Race	DNA[b]
7-2	Spain	Huesca	*T. aestivum*	12-15-1515	MHTNQ	X
7-3	Spain	Huesca	*T. aestivum*	12-15-1515	MCTNQ	X
8-1	Spain	Peralta/Navarra	*T. aestivum*	125B0254-B	MCPSS	
8-2	Spain	Peralta/Navarra	*T. aestivum*	125B0254-B	MCPSS	
9-1	Spain		*T. aestivum*	Garcia	MHPSQ	
9-2	Spain		*T. aestivum*	Garcia	MCPSQ	X
4-1	Spain	Coril (Cadiz)	*T. turgidum*	Don Jaime *Lr14a*	BBBSJ	X [c]
4-2	Spain	Coril (Cadiz)	*T. turgidum*	Don Jaime *Lr14a*	BBBSJ	
4-3	Spain	Coril (Cadiz)	*T. turgidum*	Don Jaime *Lr14a*	BBBSJ	
5-1	Spain	Coril (Cadiz)	*T. turgidum*	Gallareta *Lr72*	BBBSJ	
5-2	Spain	Coril (Cadiz)	*T. turgidum*	Gallareta *Lr72*	BBBSJ	X [c]
19-1	Tunisia		*Triticale*		FCBPQ	X
18-1	Tunisia	Beja exp. station	*T. aestivum*	UNK	BBBSQ	X
18-2	Tunisia	Beja exp. station	*T. aestivum*	UNK	BBBSJ	
20-1	Tunisia	Grombalia	*T. turgidum*	Maali	BBBSJ	
20-2	Tunisia	Grombalia	*T. turgidum*	Maali	BBBSJ	
20-4	Tunisia	Grombalia	*T. turgidum*	Maali	BBBSJ	X [c]
22-2	Tunisia	Jendouba	*T. turgidum*	Maali	BBBSJ	X [c]
22-3	Tunisia	Beja exp. station	*T. turgidum*	Maali	BBBSJ	
23-1	Tunisia	Mateur	*T. turgidum*	Nasr	BBBSJ	
23-2	Tunisia	Mateur	*T. turgidum*	Nasr	BBBSJ	
23-3	Tunisia	Mateur	*T. turgidum*	Nasr	BBBSJ	
24-1	Tunisia	INRAT	*T. turgidum*		BBBQJ	X [c]
24-2	Tunisia	INRAT	*T. turgidum*		BBBSQ	X [c]
Race1	USA		*T. aestivum*		BBBDJ	X
CA1.2	USA	California	*T. turgidum*		BBBQJ	X

[a] Isolates phenotyped at North Dakota Agricultural Experiment Station Greenhouse Complex, Fargo, ND, while the rest of the isolates were phenotyped at USDA-ARS Cereal Disease Laboratory, St. Paul, MN.
[b] The samples with symbol 'X' were genotyped using RAD-GBS.
[c] isolates removed from analysis because of missing genotypic data points > 10%.

For the 12 isolates, obtained from the USDA-CDL, ~25 mg of urediniospores from each isolate were germinated and the fungal material was lyophilized and ground as described by Ordoñez and Kolmer (2007b). The DNA was extracted using OmniPrep (G-BioSciences) extraction kit according to the manufacturer instructions. For the remaining 32 isolates, ~20-30

mg of urediniospores from each isolate were germinated at room temperature in a glass petri dish containing 500 ml of a germination solution [499 ml of Milli Q water and 1ml of 500 X stock solution (made from 72 µL of Nonyl alcohol, 0.5 µL of Tween 20, 10 mL of ethanol, and 10 mL of distilled water Milli Q water (Barnstead International, Dubuque, IA)] (Nirmala et al. 2011). The urediniospores were left to germinate overnight and the DNA was extracted from the germinated spore-mats using a cetyltrimethylammonium bromide (CTAB) protocol (Riede and Anderson, 1996). The DNA of the 44 isolates was quantified using Qubit®2.0 fluorometer (Invitrogen, life technologies). A concentration of 250 ng total DNA was used per isolate for genotyping.

For library preparation, the DNA from each isolate was digested with *Hha*I enzyme (NEB, Ipswich, MA, USA) for 2 h and 30 min at 37°C. The samples were then digested with a second enzyme *Ape*KI (NEB, Ipswich, MA, USA) for 2 h and 30 min at 75°C. The digestion reactions were extracted using magnetic particles (Agencourt®AMPure® XP, manufactured by Seradyn). The DNA pellet was then washed twice with 75% ethanol and air dried, before resuspension in H_2O. Ligation reactions containing 3µL of 10 X ligase buffer (Promega, Madison, WI, USA), 3 units of T4 DNA ligase (Promega, Madison, WI, USA), 100 µM universal P1-Hha1 adaptor, 4µl H_2O, and 100 µM ApeKI specific barcoded adaptor were added to 20µl of DNA of each isolate. The sequences of adaptors are as described by Leboldus et al (2015). The ligation reaction was left for 16-20 h at 4°C and thereafter inactivated for 20 min at 65°C. The uniformity of DNA concentration of all the reactions was checked on 1% agarose gel before the reactions were combined. Each twenty-two reactions (barcoded isolates) were combined into one sample. Unligated adaptors from the two created samples were removed using PureLinkTM quick gel extraction and PCR purification Combo kit (Invitrogen, Thermo Fisher

Scientific). The two samples containing each 22 isolates were then size selected for 275-bp fragments on 1.5 % agarose cassettes using the Pippin Prep (Sage Science, Beverly, MA, USA) size selection system. The size selected samples were then used to amplify the GBS library as described by Leboldus et al (2015). The quality of PCR reactions were verified on 1 % agarose gel before the two samples were normalized to a working concentration of 70 pM. The library sequencing was performed on an Ion Torrent PGM™ Sequencers (Life Technologies, Grand Island, NY) using two Ion 318™ Microprocessor Chips for the two created samples. The sequencing was performed following Ion Torrent PGM sequencing protocol at the barley pathology laboratory at North Dakota State University, Fargo, ND.

Sequencing data analysis

The generated sequencing reads of each isolate were individually aligned to the *P. triticina* reference genome (race1-BBBD) available on the Broad institute website (http://www.broadinstitute.org/annotation/genome/puccinia_group/Downloads.html). This alignment was done using the Burrows-Wheeler Aligner (BWA) 'mem' algorithm with default settings (Li and Durbin 2009). The SNP calling was conducted using the Genome Analysis Toolkit Unified Genotyper and subsequently filtered using 'vcftools' for a minimum genotype quality of ten and a minimum read depth per SNP per individual of three (DePristo et al. 2011; Danecek et al. 2011). The vcf file was then imported into Golden helix SNP and variation suite (SVS) software to recode the SNP alleles. SNPs and isolates with missing data > 10% were discarded. In addition, marker with minor allele frequency (MAF) <5% were eliminated from further analysis.

Neighbor joining phylogenetic tree and discriminant analysis of principal components based on genotyping data

The generated SNPs were used to create neighbor joining phylogenetic tree using R (version 3.3.1) with the package 'Poppr' (Kamvar et al. 2014). The libraries Ape v3.1-4 (Paradis et al. 2004), Adegenet v1.4-2 (Jombart 2008), and Pegas v 0.6 (Paradis 2010) were also imported. The neighbor joining tree was generated using the following parameters: Nei's distance (Nei 1972, 1978), neighbor-joining (Saitou and Nei 1987), and 5,000 bootstrap replicates with a cutoff of 75%. Discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was also performed in Poppr using the 'dapc' function to visualize the genetic pattern among isolates. The PCA plot was created based on the first three principal components (PCs), displayed using 'scatter' function**.**

Results

Virulence phenotypes

Virulence phenotypes on Thatcher differential lines showed that races of *P. triticina* collected from tetraploid wheat (durum and emmer wheat) differ from those collected from hexaploid common wheat. Isolates with virulence on tetraploid wheat had fewer races compared to isolates found on common wheat (Table 5.2). For instance, in 57 isolates collected from tetraploid wheat, only seven races were observed, while in 40 isolates collected from common wheat, 21 races were identified. Therefore, one race per 1.9 isolates were found among *Pt* common wheat-type isolates compared to one race per 8.14 isolates among *Pt* tetraploid wheat-type isolates. In addition, tetraploid wheat type races showed virulence to fewer number of *Lr* genes (0 –5 *Lr* genes). In contrast, the common wheat type races were virulent to many *Lr* genes.

The races observed on tetraploid wheat were BBBQJ, BBBSJ, BBBQK, BCBQJ, BBBSQ, CBBQS, and EEEEE. Race EEEEE which is avirulent on Thatcher wheat was only collected from Ethiopia on durum wheat (10 isolates) and emmer wheat (six isolates). In Morocco and Chile, races BBBQJ and BBBSJ were among the isolates collected on durum wheat, indicating that virulence to *LrB*, *Lr10*, *Lr14a*, *Lr14b*, and *Lr20* exists in these two countries. In Tunisia, the virulence phenotypes BBBQJ, BBBSJ, and BBBSQ were found among the isolates collected from durum wheat, showing that virulence to *LrB*, *Lr3bg*, *Lr10*, *Lr14a*, *Lr14b*, and *Lr20* is present in Tunisia. In Spain, only BBBSJ was identified on durum wheat with virulence to *LrB*, *Lr10*, *Lr14a*, *Lr14b*, and *Lr20.* Four races BBBQJ, BBBQK, BCBQJ, and CBBQS were observed on durum wheat in Mexico. Three of these races (BBBQK, BCBQJ, and CBBQS) were not present in other countries. These Mexican races were virulent to *LrB*, *Lr3a*, *Lr3bg*, *Lr10*, *Lr14b*, *Lr20*, *Lr26*, and *Lr28.* In the US, the only described race on durum wheat was BBBQJ with virulence to *LrB*, *Lr10*, *Lr14b*, and *Lr20* (Table 5.2).

Twenty-one races were observed in the isolates collected from common wheat across experimental sites. These common wheat type races were virulent to many of the Thatcher differential lines. The isolates from Spain included the phenotypes MCPSS, MCDSQ, MCTNQ, MHTNQ, MHPSQ, and MCPSQ, while races from Pakistan were FHPSQ, CCPSL, CBBDK, and BBBDH. Four races BBBQJ, MCDSS, MBDSS, and MCDST were found in Morocco, while in Chile, the common wheat races were MBDSS, MCDSS, and MFPNQ. The single isolate collected from common wheat in Ethiopia had the virulence phenotype MBJS-. In Mexico, two races CBDSS and TBDKT were observed on common wheat, while in Tunisia, phenotypes BBBSQ and BBBSJ, were found among isolates collected from common wheat. Races BBBQJ, BBBSQ, and BBBSJ are usually isolated from durum wheat, but they also appeared in few

isolates collected from common wheat in Morocco (BBBQJ) and Tunisia (BBBSQ and BBBSJ). Triticale isolates collected from Spain had virulence phenotypes CBGNQ, FBBPQ, and FBBNQ, while race FCBPQ was collected from Triticale in Tunisia (Table 5.2).

Unlike all other isolates collected for this study, appropriate race (s) was not determined for the Ethiopian EEEEE isolates, because these unique isolates were avirulent on Thatcher. Further phenotyping of seven EEEEE isolates, collected from durum and emmer wheat, on a set of 23 durum accessions showed that there were differences between the EEEEE isolates based on their virulence patterns on some genotypes of this durum set. Fourteen durum accessions were resistant to all the seven EEEEE isolates except Alred and Rusty which were susceptible to all isolates, therefore these 14 accessions were not informative thus, could not be retained as differentials. The remaining nine durum accessions, PI 209274, PI 244061, PI 278379, PI 387263, Mindum, Llareta INIA, Camayo, Mexicali 75, and Russello G.S.7 displayed differential reactions to the tested EEEEE isolates, thus retained as differential hosts (Table 5.3).

Table 5.3. Virulence/avirulence profile of seven Ethiopian EEEEE isolates tested on durum wheat accessions.

Line	Type	Country	49-1 [b]: EEEEE	50-4: EEEEE	57-2: EEEEE	63-1: EEEEE	63-3: EEEEE	64-1: EEEEE	65-2: EEEEE
PI45442	Cultivated	South Africa	0;	;13	0;	;	;1	;1-	;1
PI192051	Landrace	Portugal	0;	;1	;1-	0;	0;	0;	0;
PI195693	Landrace	Ethiopia	;1	1+3	;1-	;1	;1	;1-	;1
PI209274 [a]	Breeding line	Australia	3	3	;1+	3	3	3	3
PI244061 [a]	Landrace	Yemen	.	31	0;	;	;	;	0;
PI278379 [a]	Landrace	Malta	1	3	2-	3	3	.	;1
PI313096	Landrace	Cyprus	0	;1	;1	0	0	1-	0
PI324928	Breeding line	Argentina	0;	;13	1-	;1	;	;13-	;1-
PI342647	Cultivated	Lebanon	0;	;1+	;1-	;	0;	;	0
PI387263 [a]	Landrace	Ethiopia	1+	3	;1	3	3	3-	1+
PI519832	Cultivar	Lebanon	0	;1	;1-	1	;1	0;	;1
PI534304	Landrace	Ethiopia	0;	;13	0	;	0;	0;	0;
Divide	Cultivar	USA	2+3-	13	1	;1	1+	2+3	;1
Rusty	Line	USA	3	3	3-	3	.	3	3
Capelli	Cultivar	Italy	;1-	;1+	;1	;1	1	;1-	1
Mindum [a]	Cultivar	Italy	3	;1	1	;1	;1	1	;1-
Creso	Cultivar	Italy	0;	;1-	;1-	;	0;	;1-	0
Llareta INIA [a]	Cultivar	Mexico	3	3+	1	;13	;13	1+	;13
Camayo [a]	Cultivar	Mexico	;1-	3	0;	1	;1	;1-	;1
Jupare C2001	Cultivar	Mexico	;1-	;	1	;1	;1-	;1	;1-
Mexicali 75 [a]	Cultivar	Mexico	3	3	;1	3	3-	3-	2
Alred	Line	Mexico	3	4	3	3	32+	3	32+
Russello S.G.7 [a]	Cultivar	Italy	3	3	2	3	3	3	3
Thatcher	Cultivar	USA	;1	;1-	2+	;1-	1-	;1	2

All the accessions are *T. turgidum* spp *durum* except Thatcher is *T. aestivum* (resistant check)

[a] Accessions that differentiated between the Ethiopian EEEEE isolates

[b] Isolate number.

The *Lr* gene in Llereta INIA and Camayo is *Lr14a* (Herrera-Foessel et al. 2008b) and *LrCamayo* (Herrera-Foessel et al. 2007), respectively while, the *Lr* genes in the other differential genotypes are unknown. Based on the virulence phenotypes on the nine durum differentials, each of the four isolates 50-4, 49-1, 57-2, and 65-2 had a unique virulence/avirulence profile. The remaining three isolates 63-1, 63-3, and 64-1 shared the same virulence/avirulence profile on the durum differentials that was distinct from the phenotypes of other four isolates (Table 5.3). Therefore, based on the nine differentials, five virulence phenotypes were found among the seven EEEEE isolates. This revealed that despite being avirulent on the highly susceptible common wheat cultivar Thatcher, the EEEEE isolates are diverse for virulence on durum wheat genotypes.

Principal component analysis and phylogenic analysis based on SNP genotyping

From the 44 genotyped isolates, 30 were kept for further analysis after filtering missing data points. A total of 2,125 SNPs, appearing on several supercontigs of the *P. triticina* genome were retained. Of the 30 isolates, 18 were collected from common wheat, six from durum wheat, four from Triticale, and two from emmer wheat. These isolates were from diverse origin with eight from Spain, followed by Pakistan and Chile each with five, Mexico and Morocco each had three isolates, while Ethiopia, Tunisia, and USA each contributed two isolates.

The DAPC was used to classify the 30 isolates based on their SNP genotypes using the first three PCs. These three PCs cumulatively accounted for 35.31% of the observed variation with 1st, 2nd, and the 3rd PC explaining 20.50%, 7.90%, and 6.91% of the variation, respectively. (Figure 5.1).

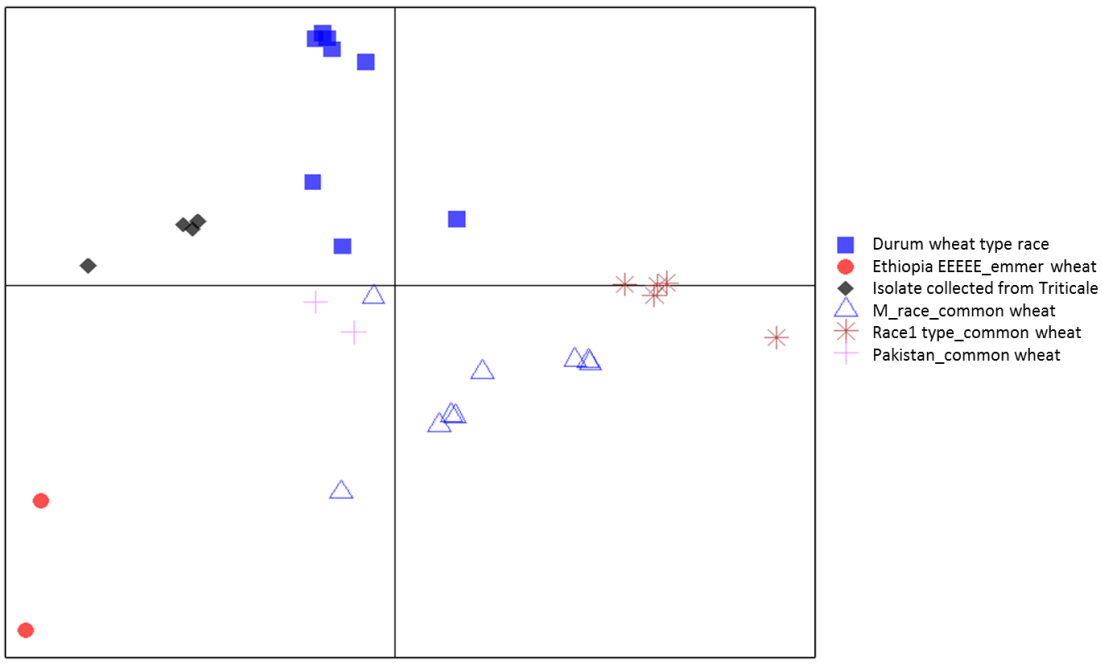

Figure 5.1. Discriminant analysis of principal components of 30 *Puccinia triticina* isolates, collected from durum wheat, emmer wheat, common wheat, and Triticale, grouped using 2,125 SNPs. The analysis was based on the first three principal components (PCs). PC1, PC2, and PC3 explained 20.50%, 7.90%, and 6.91%, of variation, respectively.

As shown in figure 5.1, five clusters were observed with cluster I representing the durum isolates (blue squares) with virulence phenotypes BBBQJ, BBBSQ, CBBQS, and BBBSJ that originated from Morocco, Chile, Tunisia, and Mexico. The isolates MEX_14_BBBQK, USA_CA1.2_BBBQJ, and TUN_18-1_BBBSQ were positioned separately from the rest of the durum isolates. Cluster II comprised the Ethiopian EEEEE isolates (red circles) collected from emmer wheat. The isolates in this particular cluster were located furthest from all other isolates. Cluster III represented by blue triangles included M-races collected from common wheat in Spain and Chile. Cluster IV was represented by race1-type isolates from common wheat (brown stars) originated from USA and Pakistan. Cluster V contained isolates collected from Triticale in Tunisia and Spain (black diamonds). The two isolates collected from common wheat in Pakistan (pink crosses) were grouped away from the majority of the other common wheat type isolates.

Phylogenetic analysis using neighbor joining tree based on Nei's genetic distance grouped the 30 isolates into eight clades with all showing evidence of high bootstrap values. The Nei's distances between isolates ranged from 0.02–0.63. The lowest Nei's genetic distance was observed between isolates CHL_03-D2_MCDSS and CHL_04-D2_MBDSS and between isolates PAK_1-2_BBBDH and PAK_1-3_BBBDH. The highest Nei's genetic distance was found between the isolates TUN_18 _BBBSQ and MEX_12_TBDKT. There was correlation between the SNP genotypes, the virulence phenotypes, and the host from which the isolates were collected from. Durum and emmer wheat isolates were clustered in two well-supported clades with 100% bootstrap values. The durum type phenotypes BBBQJ, BBBSJ, BBBSQ, and CBBQS, were clustered together in clade I, despite coming from different countries. This suggests that these phenotypes have a common origin. Similar to what was observed using DAPC, the isolates USA_CA1.2_ BBBQJ and MEX_14_BBBQK were not clustered with the other durum type isolates in clade I. However, the bootstrap value associated with the MEX_14_BBBQK was lower than the bootstrap cutoff of 75 %. The dissociation of these two isolates from the durum group may have resulted from possible spores or DNA sample contamination. Generally, the isolates within the same clade have similar virulence phenotypes to the *Lr* genes on Thatcher NILs (Figure 5.2).

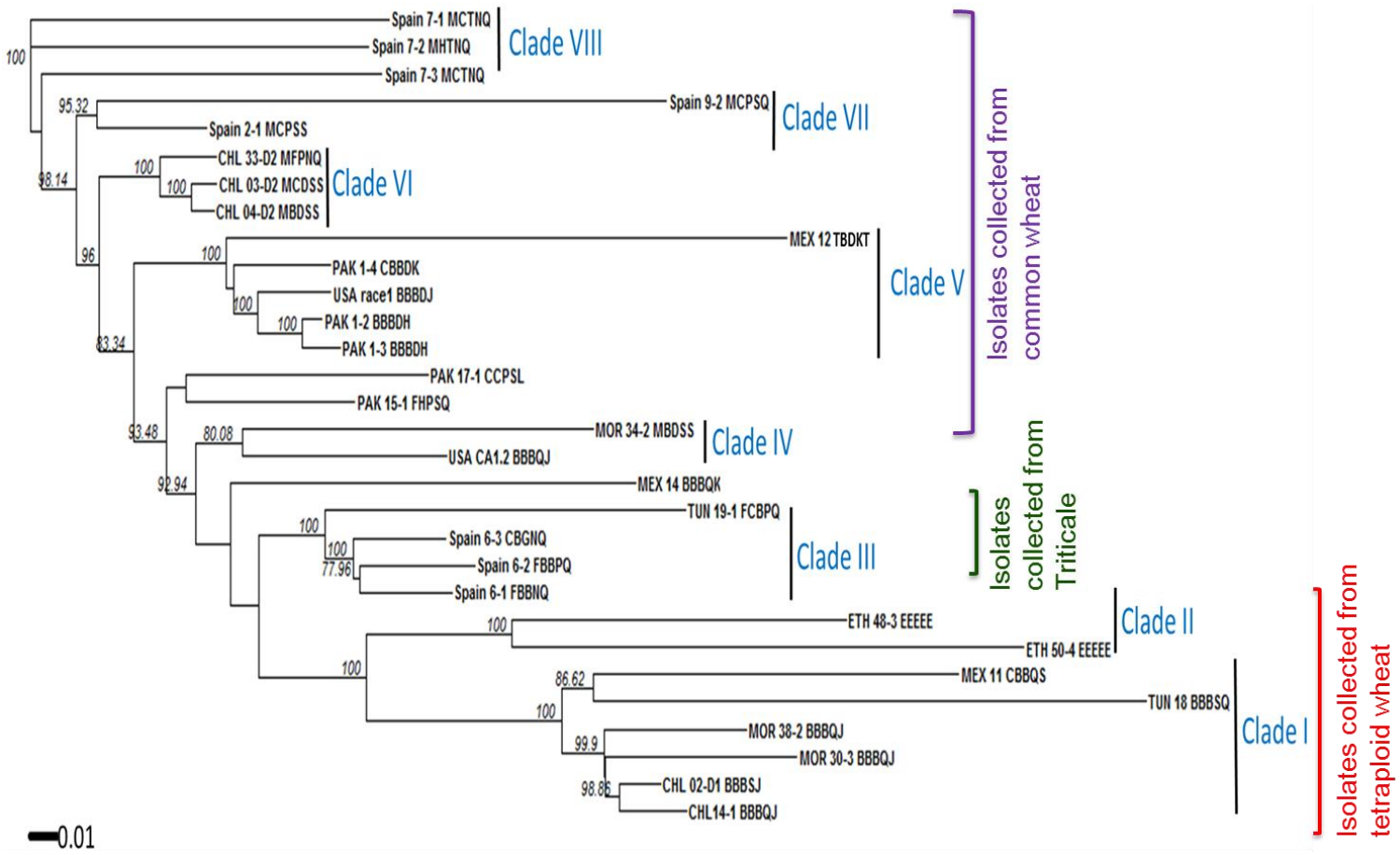

Figure 5.2. Neighbor-joining phylogenetic tree of 30 *Puccinia triticina* isolates, collected on durum wheat, emmer wheat, common wheat, and Triticale, based on Nei's genetic distances that were calculated using 2,125 single nucleotide polymorphism markers. Isolate name was based on country (Spain, USA, CHL: Chile, MEX: Mexico, PAK: Pakistan, MOR: Morocco, TUN: Tunisia, and ETH: Ethiopia), collection number, and race based virulence phenotypes on Thatcher differentials. Numbers along the nodes are bootstrap values >75% in 5,000 replicates.

The two Ethiopian isolates with phenotype EEEEE were placed in Clade II. Even though grouped together, the SNP genotypes of the EEEEE isolates were not identical (Nei's genetic distance = 0.34). The isolates collected from Triticale with virulence phenotypes FBBNQ, FBBPQ, CBGNQ, and FCBPQ from Tunisia and Spain were grouped in Clade III. Although, USA_ CA1.2_ BBBQJ and MOR_34-2_MBDSS were grouped together in Clade IV, the validity of this clustering needs to be ascertained. The Pakistani isolates with virulence phenotypes CCPSL and FHPSQ were grouped together but not supported with a high bootstrap value (< 75%), thus not considered as a clade. The common wheat race1 from USA (BBBDJ), its variants from Pakistan (BBBDH, and CBBDK), and the Mexican race TBDKT were clustered in CladeV. Even though, the common wheat race1 isolates have similar virulence phenotypes to those durum wheat type isolates in clade I, these two groups appeared in different clades. Clades VI, VII, and VIII included common wheat M-races. Isolates from Chile with virulence phenotypes MFPNQ, MCDSS, and MBDSS clustered in clade VI, while, races MCPSS and MCPSQ from Spain were grouped in Clade VII. Clade VIII contained Spanish races MCTNQ and MHTNQ (Figure 5.2). Generally, the isolates within the same clade have similar virulence phenotypes to the *Lr* genes on Thatcher NILs.

Discussion

In the current study, we used the RAD-GBS based on the Ion Torrent platform to genotype *P. triticina* isolates collected from different hosts in several countries and representing several virulence phenotypes. There was relationship between the SNP genotypes and virulence phenotypes, which is expected in clonal populations. This finding is in agreement with several studies, showing high correlation between *P. triticina* virulence phenotypes and SSR genotypes (Ordoñez and Kolmer 2007b, 2009; Ordoñez et al. 2010; Wang et al. 2010a, 2010b; Kolmer et

al. 2011, 2013; Kolmer 2015). The present study also revealed clear phylogenetic separation between isolates collected from different hosts. In addition, there were more phylogenetic clades observed for the common wheat isolates, compared to those observed for tetraploid wheat isolates. This agrees with previous reports that showed different virulence phenotypes between isolates collected from common wheat and those from tetraploid wheat (Singh 1991; Huerta-Espino and Roelfs 1992; Martinez et al. 2005; Ordoñez and Kolmer 2007a, b; Goyeau et al. 2012; Kolmer and Acevedo 2016). In contrast to previous phylogenetic trees based on SSRs or AFLPs, the present phylogenic tree, based on SNPs was supported by high bootstrap values. This indicates that RAD-GBS using Ion Torrent is reliable tool providing sufficient and robust SNPs for accurate estimation of the genetic diversity in *P. triticina* population.

The virulence phenotypes of 102 *P. triticina* isolates showed distinct difference between durum wheat isolates and common wheat isolates. Durum wheat races were avirulent to many *Lr* genes found in Thatcher differentials, indicating that the selection and spread of these races occur only on durum wheat cultivars. The phylogenetic analysis in this study showed that apart from the Ethiopian EEEEE isolates, most of the tetraploid wheat type isolates were related, suggesting a common ancestor. This limited genetic variation of durum wheat specific isolates (except EEEEE isolates), indicates that most of the *Lr* genes deployed in one region could also be used in other regions worldwide (Ordoñez and Kolmer 2007b). For example, the same *Lr* gene in the USDA-NSGC accession PI 192051 conferred resistance against all isolates collected from durum wheat in Tunisia, California, Morocco, and Ethiopia (Aoun et al. unpublished). Seven races we identified on durum wheat were BBBQJ, BBBSJ, BBBQK, BBBSQ, BCBQJ, and CBBQS, thus virulence to nine genes *LrB*, *Lr3a*, *Lr3bg*, *Lr10*, *Lr14a*, *Lr14b*, *Lr20*, *Lr26*, and *Lr28* in Thatcher differential lines were observed. Virulence to some of these *Lr* genes were

reported previously among durum wheat isolates. For instance, virulence to *LrB*, *Lr3a*, *Lr10*, *Lr14a*, *Lr14b*, *Lr20*, and *Lr28* were identified in previous studies (Ordoñez and Kolmer 2007a; Huerta-Espino et al. 2009; Goyeau et al. 2012; Gharbi et al. 2013; Herrera-Foessel et al. 2014b; Kolmer and Acevedo 2016; Soleiman et al. 2016). This is the first study to report virulent races to *Lr3bg* in Tunisia, *Lr14a* in Morocco, and *Lr3bg* and *Lr28* in Mexico. The high virulence diversity observed in fields in Mexico is due to the use of a number of *Lr* genes in the CIMMYT durum cultivars since the outbreak of disease in the country (Herrera-Foessel et al. 2005, 2007, 2008a, 2008b, 2014a) which created a selection pressure on the pathogen.

The isolates collected from common wheat showed high level of diversity in their virulence phenotypes (21 races in 40 isolates) and SNP genotypes. High level of diversity in virulence phenotypes and SSR genotypes among common wheat isolates has been reported in previous studies. For instance, over 50 virulence phenotypes are detected annually in USA and Canada (McCallum et al. 2007; Kolmer 2013; Hughes and Kolmer 2016). In Spain, a virulence survey conducted between 1998-2000 on a collection of 56 isolates identified seven races collected from durum wheat and 28 races collected from common wheat (Martinez et al. 2005). Other studies on common wheat *P. triticina* isolates revealed high level of virulence phenotypes and SSR genotypes with seven groups in Europe (Kolmer et al. 2013), five in North America (Ordoñez and Kolmer 2009), five in South America (Ordoñez et al. 2010), four in Central Asia (Kolmer and Ordoñez 2007), and two groups in the Middle East (Kolmer et al. 2011). This high level of diversity in *P. triticina* population collected from common wheat is mostly due to pathogen mutation in response to the deployment of several *Lr* genes in common wheat cultivars. Therefore, it is very important to characterize the leaf rust resistance in the new released cultivars to identify the possible host selection of new *P. triticina* races (Kolmer and Hughes 2014).

Virulent isolates on tetraploid wheat with avirulence on Thatcher, designated as race EEEEE, were only observed in Ethiopia. The phylogenetic analysis based on SNP genotypes distinguished the EEEEE isolates from all other isolates. Similar observations were made in previous studies based on virulence phenotypes and SSR genotypes (Huerta-Espino and Roelfs 1992; Ordoñez and Kolmer 2007a, 2007b; Kolmer and Acevedo 2016). In this study, we observed small genotypic variation between EEEEE isolates, which was similar to results obtained by whole genome sequencing of a number of EEEEE isolates (J.A Kolmer, unpublished). The genetic uniqueness of the EEEEE isolates suggests that this race may have followed a different evolutionary and origin path from all other *P. triticina* isolates globally. The unique wheat population in Ethiopia may have selected this particular phenotype (Kolmer and Acevedo 2016). Despite the lack of differences in virulence phenotype among EEEEE isolates based on Thatcher differential lines, we identified five virulence phenotypes among seven tested EEEEE isolates based on durum differentials. These identified durum differentials are PI 209274, PI 244061, PI 278379, PI 387263, Mindum, Llareta INIA, Camayo, Mexicali 75, and Russello G.S.7. Therefore, virulence variation between EEEEE isolates do exist on durum wheat genetic background. This differential set could be useful to study the virulence diversity in more Ethiopian EEEEE isolates. Previous initiative to develop differential set adapted for *P. triticina* isolates collected on durum wheat was implemented by Goyeau et al (2012). With the increased susceptibility of durum to leaf rust worldwide, there is need to develop a standardized differential set adequate to assess virulence diversity in durum type *P. triticina* population.

In summary, the current study is the first of its kind to use RAD-GBS to study the population genetics of *P. triticina.* The abundant and robust SNPs that are distributed across the genome provided higher accuracy in estimating the genetic distances between isolates. There

was clear separation between isolates from different hosts with higher genotypic diversity in isolates collected from common wheat compared to that observed in isolates collected from tetraploid wheat. In contrast to common wheat races, virulence to few *Lr* genes (*LrB*, *Lr3a*, *Lr3bg*, *Lr10*, *Lr14a*, *Lr14b*, *Lr20*, *Lr26*, and *Lr28*) was observed among the durum wheat races. The EEEEE isolates collected from tetraploid wheat in Ethiopia showed a unique virulence phenotype and SNP genotype. Since appropriate race designation could not be given to the EEEEE isolates on Thatcher lines, durum differentials were developed to distinguish between them.

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GENERAL CONCLUSIONS

Leaf rust, caused by *Puccinia triticina* Erikss. (*Pt*) and stem rust caused by *Puccinia graminis* f. sp. *tritici* Erikss. and E. Henn (*Pgt*) inflict significant yield losses in durum wheat worldwide. Highly virulent *Pt* races on durum wheat have been observed in recent years in several countries. For example, race BBG/BN detected in Mexico in 2001 caused an estimated yield loss of US$32 million during 2001-2003 seasons. A similar race designated as BBBQJ was found in California (in 2009) and Kansas (in 2013) and is thought to have migrated from Mexico. This may cause a new threat to the US durum production in North Dakota. Similarly, durum wheat is facing a serious challenge from continuously emerging *Pgt* races in East Africa that may cause a threat to the global wheat production. These *Pgt* races include the Ug99 lineage races (TTKSK and its variants), durum specific races (JRCQC, TRTTF, and RRTTF), and TKTTF ('Digalu' race). Host resistance is the most economically viable rust management strategy, however, only few *Lr* and *Sr* genes have been previously identified in durum wheat cultivars. Unfortunately, majority of these genes succumbed to the current *Pt* and *Pgt* populations. Therefore, durum wheat breeding programs are in need of new *Lr* and *Sr* genes. The USDA- National Small Grain Collection (NSGC) is a repository for genetically diverse germplasm that can provide useful agronomic traits to breeding programs.

In this study, 496 accessions from the USDA-NSGC were evaluated for leaf rust resistance. This allowed to identify 13 resistant accessions to several *Pt* races from the US and Mexico, including PI 209274, PI 192051, PI 193920, PI 534304, PI 387263, CItr 14623, PI 195693, PI 342647, PI 519832, PI 278379, PI 244061, PI 223155, and PI 324928. Furthermore, association mapping (AM) identified 88 significant SNPs associated with leaf rust response. Of these, 33 SNPs were located on chromosomes 2A and 2B, while the rest of the SNPs were

distributed across all other chromosomes except for 1B and 7B. Twenty markers were associated with leaf rust response at seedling stage while 68 markers were associated with leaf rust response at adult-plant stage. The AM identified a total of 14 novel loci associated with leaf rust response.

For further validation of the AM results, bi-parental populations were developed by crossing eight leaf rust resistant genotypes (PI 534304, PI 313096, PI 387263, PI 209274, PI 278379, PI 244061, PI 192051, and PI 195693) selected from the USDA-NSGC to the susceptible parents Rusty and/or Divide. The inheritance study in these populations showed that five of these genotypes carry single dominant *Lr* genes to *Pt*-race BBBQJ at seedling stage, while the remaining three genotypes exhibited the presence of resistance genes with more complex inheritance. The eight genotypes were resistant to virulent races on existing *Lr* genes in durum wheat cultivars except PI 313096 that was susceptible to a virulent race on *Lr61*. This suggests that all eight accessions carry new genes except PI 313096 that seems to carry *Lr61*. The bulked segregant analysis (BSA) of the five populations carrying single dominant genes showed that the *Lr* genes in PI 244061, PI 192051, PI 209274/PI 313096, and PI 387263 were mapped to chromosomes 2B, 4A, 6BS, and 6BL, respectively.

Linkage mapping in two bi-parental populations Rusty X PI 209274 and Rusty X PI 192051 was conducted. The *Lr* gene in PI 209274 (*LrCA*) to *Pt*-race BBBQJ at seedling stage was mapped to a 4.7 cM region between markers *IWA3298* and *IWB39456* on 6BS, while the *Lr* gene in PI 192051 (*LrPort*) to four *Pt* isolates was mapped within a 3.9 cM region on chromosome 4AL, flanked by *IWA4254* and *IWA8341*. Bi-parental population mapping confirmed some of the identified loci in AM study including those on chromosomes 2BL, 4AL and 6BL.

In addition to their resistance to leaf rust, the accessions PI 534304 and PI 192051 carry resistance to *Pgt*-race TTKSK at seedling stage. The *Sr* gene in PI 534304 mapped on chromosome 6AL is most likely *Sr13*, while the *Sr* gene in PI 192051 (*SrPort*) seems to be novel and mapped within a 3.6 cM on 7AS flanked by *IWA8390* and *IWA1805*. PI 192051 also carries an additional QTL to *Pgt* races at adult-plant stage in a field trial in Ethiopia. This QTL was designated as *QSr.ndsu-5B* and mapped to 5BL within a 4.8 cM region, delimited by *IWA6992* and *IWA2181*. *SrPort* was not the gene conferring resistance in Ethiopia, which could be explained by the presence of other *Pgt* races in addition to the Ug99 lineage races. Validation of *QSr.ndsu-5B* is needed in coming seasons. The findings of this research enhance the genetic diversity of leaf rust and stem rust resistance in durum wheat. Transferring some of the resistance genes to the North Dakota adapted durum wheat cultivar 'Divide' is ongoing.

Another objective of this study was to investigate the virulence and genetic diversity of 102 *Pt* isolates collected mostly from durum wheat and common wheat in several countries. Seven races BBBQJ, BBBSJ, BBBQK, BCBQJ, BBBSQ, CBBQS, and EEEEE were found among 51 durum wheat type-isolates, while 21 races were observed among 40 common wheat type isolates. Genotyping of a subset of 30 *Pt* isolates which represents the majority of virulence phenotypes using RAD-GBS allowed the identification of 2,125 SNPs. The phylogeny study clustered the 30 isolates into eight clades with common wheat type isolates showing more genotypic diversity compared to durum wheat type isolates. There was correlation between virulence phenotypes and SNP genotypes. The study showed that RAD-GBS is powerful tool for studying the population genetics in *Pt.* This will help for increased understanding of this rapidly evolving pathogen.

This dissertation provided insights for new sources of resistance to leaf rust and stem rust that will assist to overcome the bottleneck of rust resistance diversity in durum wheat cultivars. This study also recommends RAD-GBS as a suitable genotyping technique for the study of population genetics in *Pt.*

APPENDIX A. HEAT MAP DISPLAYING THE FAMILIAL RELATEDNESS BETWEEN DURUM ACCESSIONS FROM THE USDA-NATIONAL SMALL GRAIN COLLECTION

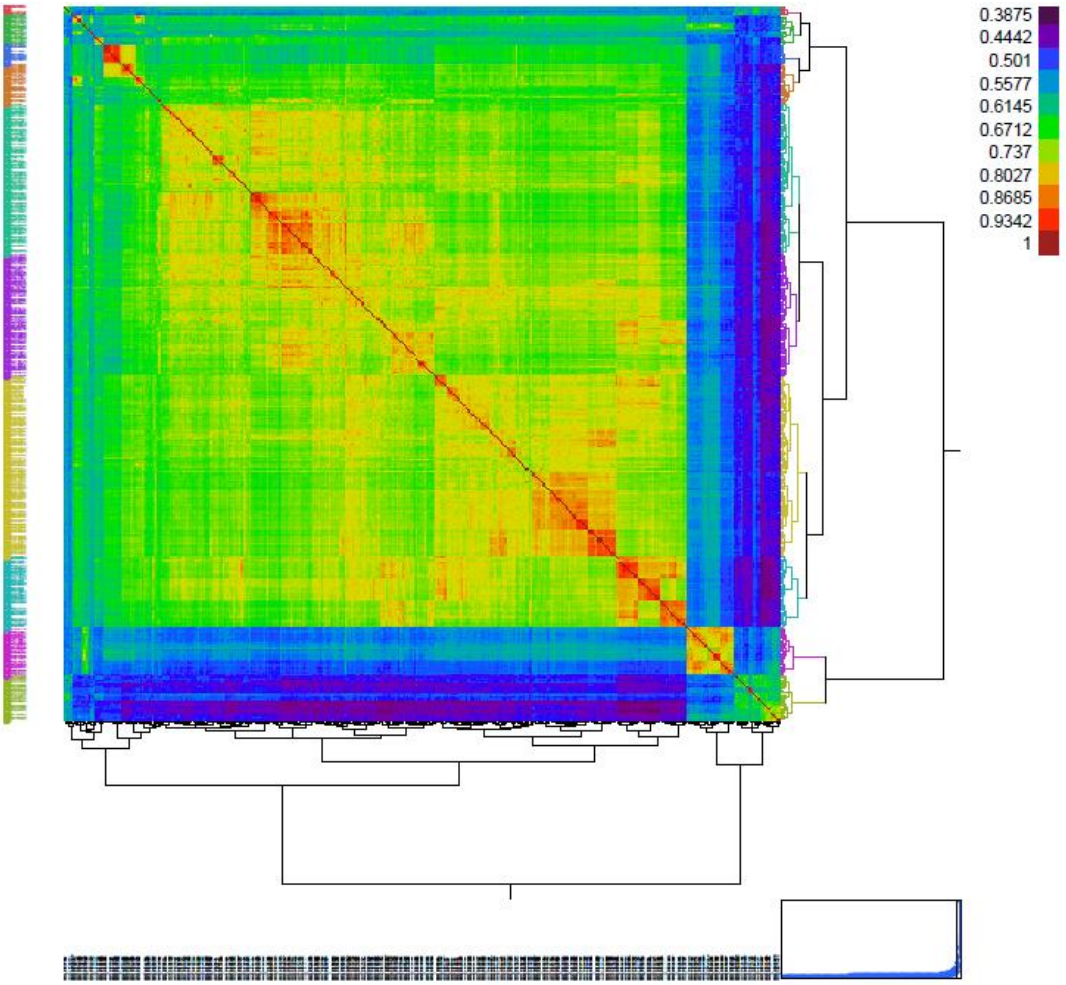

Figure A1. Heat map displaying the relationship matrix among durum accessions.
The red diagonal represents perfect relationship of each accession with itself. The symmetric off-diagonal elements represent relationship measures (Identity by state) for pairs of accessions. The blocks of warmer colors on the diagonal show clusters of closely related accessions.

APPENDIX B. LIST OF THE LEAF RUST RESISTANT DURUM WHEAT GENOTYPES TO SEVERAL *P. TRITICINA* RACES

Table B1. List of leaf rust resistant accessions from USDA-National Small Grain Collection tested with several *P. triticina* races.

Accession	Origin	Type	MBDSD[a]	BBBQD (ND)[a]	BBBQD (CDL)[a]	BBBDB [a]	Race mix [ab]	MCDSS [a]	BBBQJ [a]
PI 209274	Australia	Breeding line	1	1	0	0	0	1	0
PI 193920	Portugal	Landrace	2	2	0	0	0	2	0
PI 244061	Yemen	Landrace	2	2	0	0	1	1	-
PI 324928	Argentina	Breeding line	2	2	0	0	0	1	-
PI 192051	Portugal	Landrace	3	2	0	0	1	1	1
PI 519832	Lebanon	Cultivar	4	4	0	0	0	1	0
PI 195693	Ethiopia	Landrace	2	3	1	0	0	0	1
PI 342647	Lebanon	Cultivated	2	2	1	0	1	1	0
PI 387263	Ethiopia	Landrace	4	2	1	0	0	5	1
PI 223155	Jordan	Cultivated	5	4	1	1	2	5	-
PI 278379	Malta	Landrace	3	2	2	0	2	1	5
PI 534304	Ethiopia	Landrace	3	2	2	0	0	4	1
CItr 14623	Ethiopia	Landrace	6	-	2	0	2	3	2

Table B1. List of leaf rust resistant accessions from USDA-National Small Grain Collection tested with several *P. triticina* races (continued).

Accession	Origin	Type	Arg 9.3: BBBQD[a]	FRA 4.3: BBBQD[a]	E125-1: EEEEE[a]	E11D2-1: MCDSB[a]	E114-1: BBBQD[a]	PSB7: FGBQ[a]	09AZ103A: BBBQB[a]
PI 209274	Australia	Breeding line	0	0	0	1	0	0	1
PI 193920	Portugal	Landrace	1	1	0	0	0	0	0
PI 244061	Yemen	Landrace	1	1	2	1	1	0	5
PI 324928	Argentina	Breeding line	-	4	2	0	4	0	-
PI 192051	Portugal	Landrace	0	0	0	0	1	0	0
PI 519832	Lebanon	Cultivar	-	9	5	1	9	2	0
PI 195693	Ethiopia	Landrace	3	0	3	0	1	2	0
PI 342647	Lebanon	Cultivated	1	1	2	0	1	1	6
PI 387263	Ethiopia	Landrace	2	2	7	1	0	0	6
PI 223155	Jordan	Cultivated	2	2	2	5	2	3	5
PI 278379	Malta	Landrace	2	2	7	2	9	3	6
PI 534304	Ethiopia	Landrace	2	2	1	4	3	4	2
CItr 14623	Ethiopia	Landrace	0	0	9	0	3	2	1

Table B1. List of leaf rust resistant accessions from USDA-National Small Grain Collection tested with several *P. triticina* races (continued).

Accession	Origin	Type	LCJ/BN[a]	BBB/BN_ *Lr61*Vir[a]	MX_Ob_F [c]	MX_EB_F [c]	MN_StP_F [c]	MN_Cr_F [c]	Ethiopia/field (Rep1/Rep2)	Morocco field
PI 209274	Australia	Breeding line	0	6	0R	0	TR	TR	-	-
PI 193920	Portugal	Landrace	0	0	0R	0	TR	TR	30MRMS /5MRMS	30R
PI 244061	Yemen	Landrace	1	-	-	-	TR	5R	0/5RMR	TMR
PI 324928	Argentina	Breeding line	9	9	5MR	0	TR	TR	0 /TMR	-
PI 192051	Portugal	Landrace	0	0	0R	0	TR	TR	0 /10RMR	5R
PI 519832	Lebanon	Cultivar	0	0	0R	0	TR	TR	20MS /0	0
PI 195693	Ethiopia	Landrace	0	0	5MR	10MR	TR	TR	20MS /10MR	15 R MR
PI 342647	Lebanon	Cultivated	0	1	5MR	0	20RMR	5R	20MR /20MRMS	-
PI 387263	Ethiopia	Landrace	0	1	10MR	5MR	5R	10R	30MRMS /30MSS	-
PI 223155	Jordan	Cultivated	-	-	-	-	10RMR	-	10MS/20MRMS	5MR
PI 278379	Malta	Landrace	0	2	0R	0	TR	TR	5MRMS /10MSMR	TMRMS
PI 534304	Ethiopia	Landrace	0	1	10MR	0	20RMR	-	30MS /5RMR	-
CItr 14623	Ethiopia	Landrace	1	1	5MR	0	TR	TR	10MSS/-	TMR

[a] Linearized disease rating for leaf rust at seedling stage as described by Zhang et al. 2014

[b] Race mix, inoculum composed of races MHDSB, MFPSB, MLDSB, TBBGJ, TFBJQ, and TFBGQ.

[c] MN_StP_F: races in Minnesota St. Paul field; MN_Cr_F: races in Minnesota Crookston field; MX_Ob_F, races in Mexico Ciudad Obregón field; MX_EB_F: races in Mexico El Batán field.

APPENDIX C. LINKED MARKERS WITH LEAF RUST AND STEM RUST RESPONSE BASED ON BULKED SEGREGANT ANALYSIS IN SIX BIPARENTAL POPULATIONS

Table C1. Markers linked with leaf rust response to *P. triticina* race BBBQJ based on bulked segregant analysis for the population Divide X PI 244061.

Marker	Marker_org	Chromosome	Position cM [a]	Divide	PI 244061	Homozygous resistant Bulk	Homozygous susceptible Bulk
IWA4421	wsnp_Ex_c6099_10674508	2B	61.5	AA	BB	BB	AA
IWA8083	wsnp_Ra_c8489_14382125	2B	61.5	AA	BB	BB	AA
IWA4420	wsnp_Ex_c6099_10674406	2B	61.5	AA	BB	BB	AA
IWA4554	wsnp_Ex_c66052_64232430	2B	61.6	AA	BB	BB	AA
IWA5392	wsnp_Ex_rep_c67391_65971023	2B	71.8	AA	BB	BB	AA
IWA6069	wsnp_JD_c4621_5757093	2B	71.8	AA	BB	BB	AA
IWA1763	wsnp_Ex_c13351_21042379	2B	72.8	AA	BB	BB	AA
IWA2624	wsnp_Ex_c2153_4043746	2B	72.8	AA	BB	BB	AA
IWA4673	wsnp_Ex_c741_1456698	2B	76.0	AA	BB	BB	AA
IWA4323	wsnp_Ex_c57_116914	2B	110.8	AA	BB	BB	AA
IWA50	wsnp_BE404601B_Ta_2_1	2B	112.3	AA	BB	BB	AA
IWA771	wsnp_CAP11_c5240_2436441	2B	126.3	AA	BB	BB	AA
IWA777	wsnp_CAP11_c5474_2542616	2B	126.3	AA	BB	BB	AA
IWA829	wsnp_CAP11_rep_c4012_1894511	2B	126.3	AA	BB	BB	AA
IWA869	wsnp_CAP11_rep_c4487_2113015	2B	126.3	AA	BB	BB	AA
IWA874	wsnp_CAP11_rep_c5367_2492424	2B	126.3	AA	BB	BB	AA
IWA1102	wsnp_CAP7_c7341_3280398	2B	126.3	AA	BB	BB	AA
IWA1127	wsnp_CAP7_rep_c6301_2835323	2B	126.3	AA	BB	BB	AA
IWA1128	wsnp_CAP7_rep_c7219_3228859	2B	126.3	AA	BB	BB	AA
IWA1129	wsnp_CAP7_rep_c7347_3283296	2B	126.3	AA	BB	BB	AA

Table C1. Markers linked with leaf rust response to *P. triticina* race BBBQJ based on bulked segregant analysis for the population Divide X PI 244061 (continued).

Marker	Marker_org	Chromosome	Position cM [a]	Divide	PI 244061	Homozygous resistant Bulk	Homozygous susceptible Bulk
IWA1130	wsnp_CAP7_rep_c7349_3284058	2B	126.3	AA	BB	BB	AA
IWA1131	wsnp_CAP7_rep_c8024_3598111	2B	126.3	AA	BB	BB	AA
IWA1177	wsnp_CAP8_c2869_1478615	2B	126.3	AA	BB	BB	AA
IWA1188	wsnp_CAP8_c4328_2115116	2B	126.3	AA	BB	BB	AA
IWA1229	wsnp_CAP8_rep_c4147_2032009	2B	126.3	AA	BB	BB	AA
IWA2766	wsnp_Ex_c22946_32163010	2B	126.3	AA	BB	BB	AA
IWA1239	wsnp_CAP8_rep_c6230_2943068	2B	128.4	AA	BB	BB	AA
IWA837	wsnp_CAP11_rep_c4105_1940985	2B	149.0	AA	BB	BB	AA
IWA7204	wsnp_Ku_c60592_62437239	2B	149.0	AA	BB	BB	AA
IWA3236	wsnp_Ex_c29434_38471452	2B	149.0	AA	BB	BB	AA
IWA838	wsnp_CAP11_rep_c4105_1941066	2B	149.4	AA	BB	BB	AA
IWA7103	wsnp_Ku_c47082_53641298	2B	149.4	AA	BB	BB	AA
IWA7449	wsnp_Ku_rep_c68888_68067293	2B	160.1	AA	BB	BB	AA

[a] Positions of markers are based on wheat consensus map of Cavanagh et al. 2013.

Table C2. Markers linked with leaf rust response to *P. triticina* race BBBQJ based on bulked segregant analysis for the population Rusty X PI 192051.

Marker	Marker_org	Chromosome	Position cM [a]	Rusty	PI 192051	Homozygous resistant Bulk	Homozygous susceptible Bulk
IWA482	wsnp_BF474615A_Ta_1_1	4A	133.3	AA	BB	BB	AA
IWA4432	wsnp_Ex_c6139_10740143	4A	134.9	AA	BB	BB	AA
IWA483	wsnp_BF474615A_Ta_1_4	4A	134.9	AA	BB	BB	AA
IWA569	wsnp_BG604678A_Ta_1_3	4A	134.9	AA	BB	BB	AA
IWA4431	wsnp_Ex_c6139_10739829	4A	134.9	AA	BB	BB	AA
IWA4657	wsnp_Ex_c7335_12579818	4A	134.9	AA	BB	BB	AA
IWA568	wsnp_BG604678A_Ta_1_2	4A	134.9	AA	BB	BB	AA
IWA5544	wsnp_Ex_rep_c68569_67411985	4A	135.8	AA	BB	BB	AA
IWA1793	wsnp_Ex_c13615_21393638	4A	137.3	AA	BB	BB	AA
IWA4359	wsnp_Ex_c58286_59646499	4A	141.4	AA	BB	BB	AA
IWA4254	wsnp_Ex_c5492_9691880	4A	141.4	AA	BB	BB	AA
IWA6377	wsnp_Ku_c10224_16965872	4A	141.4	AA	BB	BB	AA
IWA4253	wsnp_Ex_c5492_9691241	4A	141.4	AA	BB	BB	AA
IWA126	wsnp_BE443291A_Ta_2_1	4A	144.1	AA	BB	BB	AA
IWA232	wsnp_BE490599A_Ta_2_1	4A	144.1	AA	BB	BB	AA
IWA8416	wsnp_RFL_Contig3024_2910610	4A	144.1	AA	BB	BB	AA
IWA6944	wsnp_Ku_c34883_44172415	4A	149.5	AA	BB	BB	AA
IWA7522	wsnp_Ku_rep_c77171_75478137	4A	149.5	AA	BB	BB	AA
IWA3792	wsnp_Ex_c4166_7525617	4A	150.0	AA	BB	BB	AA
IWA4771	wsnp_Ex_c8131_13753986	4A	150.0	AA	BB	BB	AA
IWA4772	wsnp_Ex_c8131_13754852	4A	150.0	AA	BB	BB	AA
IWA2292	wsnp_Ex_c18229_27041048	4A	150.0	AA	BB	BB	AA
IWA5498	wsnp_Ex_rep_c68124_66892390	4A	150.0	AA	BB	BB	AA
IWA3581	wsnp_Ex_c3666_6687275	4A	150.0	AA	BB	BB	AA
IWA1919	wsnp_Ex_c14641_22698595	4A	150.0	AA	BB	BB	AA

Table C2. Markers linked with leaf rust response to *P. triticina* race BBBQJ based on bulked segregant analysis for the population Rusty X PI 192051 (continued).

Marker	Marker_org	Chromosome	Position cM (a)	Rusty	PI 192051	Homozygous resistant Bulk	Homozygous susceptible Bulk
IWA3311	wsnp_Ex_c31006_39850673	4A	150.4	AA	BB	BB	AA
IWA5729	wsnp_Ex_rep_c71305_70087742	4A	150.9	AA	BB	BB	AA
IWA1904	wsnp_Ex_c14529_22547438	4A	151.3	AA	BB	BB	AA
IWA7859	wsnp_Ra_c31915_40982091	4A	151.3	AA	BB	BB	AA
IWA2000	wsnp_Ex_c15282_23505878	4A	151.3	AA	BB	BB	AA
IWA3845	wsnp_Ex_c4286_7734046	4A	151.3	AA	BB	BB	AA
IWA7133	wsnp_Ku_c50991_56423564	4A	151.3	AA	BB	BB	AA
IWA7134	wsnp_Ku_c50991_56423610	4A	151.3	AA	BB	BB	AA
IWA1824	wsnp_Ex_c1387_2659020	4A	151.3	AA	BB	BB	AA
IWA6540	wsnp_Ku_c14803_23225628	4A	151.3	AA	BB	BB	AA
IWA7657	wsnp_Ra_c16634_25331368	4A	151.3	AA	BB	BB	AA
IWA2781	wsnp_Ex_c23130_32358433	4A	151.3	AA	BB	BB	AA
IWA3361	wsnp_Ex_c3178_5868813	4A	151.3	AA	BB	BB	AA
IWA6597	wsnp_Ku_c16481_25377573	4A	151.3	AA	BB	BB	AA
IWA7270	wsnp_Ku_c7197_12439299	4A	151.3	AA	BB	BB	AA
IWA8414	wsnp_RFL_Contig30_2884966	4A	151.3	AA	BB	BB	AA
IWA7092	wsnp_Ku_c46057_52907637	4A	152.2	AA	BB	BB	AA
IWA110	wsnp_BE442776A_Ta_2_2	4A	152.6	AA	BB	BB	AA
IWA115	wsnp_BE442961A_Ta_2_1	4A	152.6	AA	BB	BB	AA
IWA3565	wsnp_Ex_c36141_44153175	4A	152.6	AA	BB	BB	AA
IWA5309	wsnp_Ex_rep_c66839_65233112	4A	152.6	AA	BB	BB	AA
IWA5975	wsnp_JD_c27944_22630918	4A	152.6	AA	BB	BB	AA
IWA3542	wsnp_Ex_c35839_43909849	4A	153.0	AA	BB	BB	AA
IWA109	wsnp_BE442776A_Ta_2_1	4A	153.4	AA	BB	BB	AA
IWA5865	wsnp_JD_c15643_15039462	4A	153.4	AA	BB	BB	AA
IWA5652	wsnp_Ex_rep_c69890_68851948	4A	153.9	AA	BB	BB	AA

Table C2. Markers linked with leaf rust response to *P. triticina* race BBBQJ based on bulked segregant analysis for the population Rusty X PI 192051 (continued).

Marker	Marker_org	Chromosome	Position cM [a]	Rusty	PI 192051	Homozygous resistant Bulk	Homozygous susceptible Bulk
IWA6020	wsnp_JD_c36045_26751163	4A	153.9	AA	BB	BB	AA
IWA172	wsnp_BE445427A_Ta_2_1	4A	153.9	AA	BB	BB	AA
IWA3326	wsnp_Ex_c31249_40066886	4A	153.9	AA	BB	BB	AA
IWA7271	wsnp_Ku_c7197_12439730	4A	162.6	AA	BB	BB	AA
IWA7521	wsnp_Ku_rep_c76865_75281903	4A	164.7	AA	BB	BB	AA
IWA5363	wsnp_Ex_rep_c67145_65628860	4A	176.9	AA	BB	BB	AA
IWA603	wsnp_BM138178A_Ta_2_1	4A	177.4	AA	BB	BB	AA
IWA2764	wsnp_Ex_c22913_32130617	4A	187.1	AA	BB	BB	AA

[a] Positions of markers are based on wheat consensus map of Cavanagh et al. 2013.

Table C3. Markers linked with leaf rust response to *P. triticina* race BBBQJ based on bulked segregant analysis for the population Rusty X PI 209274.

Marker	Marker_org	Chromosome	Position cM [a]	Rusty	PI 209274	Homozygous resistant Bulk	Homozygous susceptible Bulk	Guayacan INIA
IWA1493	wsnp_Ex_c1143_2195442	6BS	0.6	AA	BB	BB	AA	AA
IWA7070	wsnp_Ku_c4446_8062906	6BS	0.6	AA	BB	BB	AA	AA
IWA8477	wsnp_RFL_Contig3512_3672726	6BS	0.6	AA	BB	BB	AA	AA
IWA3298	wsnp_Ex_c30813_39686227	6BS	2.6	AA	BB	BB	AA	AA
IWA5857	wsnp_JD_c15167_14703349	6BS	12.5	AA	BB	BB	AA	AA
IWA3991	wsnp_Ex_c4728_8444212	6BS	14.5	AA	BB	BB	AA	BB
IWA5058	wsnp_Ex_rep_c102186_87408828	6BS	20.3	AA	BB	BB	AA	BB
IWA4290	wsnp_Ex_c56091_58346859	6BS	21.8	AA	BB	BB	AA	AA
IWA7725	wsnp_Ra_c20409_29673950	6BS	21.8	AA	BB	BB	AA	AA
IWA52	wsnp_BE404947B_Ta_2_12	6BS	22.5	AA	BB	BB	AA	BB

[a] Positions of markers are based on wheat consensus map of Cavanagh et al. 2013.

Table C4. Markers linked with leaf rust response to *P. triticina* race BBBQJ based on bulked segregant analysis for the population Divide X PI 313096.

Marker	Marker_org	Chromosome	Position cM [a]	Divide	PI 313096	Homozygous resistant Bulk	Homozygous susceptible Bulk	GuayacanINIA
IWA1495	wsnp_Ex_c1143_2196102	6BS	0.0	BB	AA	AA	BB	AA
IWA4997	wsnp_Ex_rep_c101133_86572194	6BS	2.6	BB	AA	AA	BB	BB
IWA1254	wsnp_CD453605B_Ta_2_1	6BS	2.6	BB	AA	AA	BB	AA
IWA666	wsnp_CAP11_c1355_767877	6BS	2.6	BB	AA	AA	BB	AA
IWA2086	wsnp_Ex_c16008_24427927	6BS	10.9	BB	AA	AA	BB	–
IWA4612	wsnp_Ex_c702_1383612	6BS	14.5	BB	AA	AA	BB	BB

[a] Positions of markers are based on wheat consensus map of Cavanagh et al. 2013.

Table C5. Markers linked with leaf rust response to *P. triticina* race BBBQJ based on bulked segregant analysis for the population Rusty X PI 387263.

Marker	Marker_org	Chromosome	Position cM [a]	Rusty	PI 387263	Homozygous resistant Bulk	Homozygous susceptible Bulk	Storlom
IWA1816	wsnp_Ex_c1383_2651887	6BL	121.3	AA	BB	BB	AA	AA
IWA3464	wsnp_Ex_c34123_42489621	6BL	127.5	AA	BB	BB	AA	BB
IWA5204	wsnp_Ex_rep_c66342_64519823	6BL	144.9	AA	BB	BB	AA	AA
IWA6140	wsnp_JD_c6439_7601847	6BL	144.9	AA	BB	BB	AA	AA
IWA1046	wsnp_CAP7_c1735_859744	6BL	151.3	AA	BB	BB	AA	AA

[a] Positions of markers are based on wheat consensus map of Cavanagh et al. 2013.

Table C6. Markers linked with Stem rust response to *Puccinia graminis* f. sp. *tritici* race TTKSK based on bulked segregant analysis for the population F_6 Rusty x PI 534304.

Marker	Marker_org	Chromosome	Position cM (a)	Rusty	PI 534304	Homozygous resistant Bulk	Homozygous susceptible Bulk
IWA7397	wsnp_Ku_rep_c102901_89769309	6A	121.9	BB	AA	AA	BB
IWA4111	wsnp_Ex_c51820_55631329	6A	133.3	BB	AA	AA	BB
IWA3585	wsnp_Ex_c36801_44683992	6A	133.7	BB	AA	AA	BB
IWA4112	wsnp_Ex_c51820_55631560	6A	135.0	BB	AA	AA	BB
IWA6434	wsnp_Ku_c11846_19262918	6A	169.4	BB	AA	AA	BB
IWA7764	wsnp_Ra_c2270_4383252	6A	170.7	BB	AA	AA	BB
IWA3487	wsnp_Ex_c34597_42879693	6A	180.2	BB	AA	AA	BB
IWA3488	wsnp_Ex_c34597_42879718	6A	180.2	BB	AA	AA	BB
IWA214	wsnp_BE489894A_Ta_2_1	6A	183.2	BB	AA	AA	BB
IWA6484	wsnp_Ku_c1318_2624758	6A	184.0	BB	AA	AA	BB
IWA5704	wsnp_Ex_rep_c70675_69579757	6A	186.8	BB	AA	AA	BB
IWA6116	wsnp_JD_c5872_7032077	6A	186.8	BB	AA	AA	BB
IWA5964	wsnp_JD_c26552_21868492	6A	192.9	BB	AA	AA	BB
IWA4691	wsnp_Ex_c749_1472258	6A	202.0	BB	AA	AA	BB
IWA6538	wsnp_Ku_c1468_2913072	6A	202.0	BB	AA	AA	BB
IWA6536	wsnp_Ku_c1468_2912489	6A	202.4	BB	AA	AA	BB
IWA4918	wsnp_Ex_c946_1813956	6A	203.8	BB	AA	AA	BB
IWA6543	wsnp_Ku_c14920_23377027	6A	214.4	BB	AA	AA	BB
IWA7496	wsnp_Ku_rep_c71567_71302046	6A	214.4	BB	AA	AA	BB
IWA7497	wsnp_Ku_rep_c71567_71302229	6A	215.2	BB	AA	AA	BB
IWA7498	wsnp_Ku_rep_c71567_71302766	6A	215.2	BB	AA	AA	BB
IWA7621	wsnp_Ra_c13998_21994095	6A	215.7	BB	AA	AA	BB
IWA1866	wsnp_Ex_c14156_22088518	6A	216.1	BB	AA	AA	BB
IWA8358	wsnp_RFL_Contig2597_2250942	6A	216.1	BB	AA	AA	BB
IWA7495	wsnp_Ku_rep_c71567_71302010	6A	216.1	BB	AA	AA	BB
IWA4699	wsnp_Ex_c7546_12900094	6A	216.5	BB	AA	AA	BB
IWA3203	wsnp_Ex_c28973_38050204	6A	217.7	BB	AA	AA	BB

Table C6. Markers linked with Stem rust response to *Puccinia graminis* f. sp. *tritici* race TTKSK based on bulked segregant analysis for the population F6 Rusty x PI 534304 (continued).

Marker	Marker_org	Chromosome	Position cM (a)	Rusty	PI 534304	Homozygous resistant Bulk	Homozygous susceptible Bulk
IWA3204	wsnp_Ex_c28973_38050405	6A	217.7	BB	AA	AA	BB
IWA3205	wsnp_Ex_c28973_38050756	6A	217.7	BB	AA	AA	BB
IWA1868	wsnp_Ex_c14156_22088799	6A	217.7	BB	AA	AA	BB
IWA4165	wsnp_Ex_c53281_56571602	6A	217.7	BB	AA	AA	BB
IWA1867	wsnp_Ex_c14156_22088738	6A	217.7	BB	AA	AA	BB

[a] Positions of markers are based on wheat consensus map of Cavanagh et al. 2013.