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MOLECULAR CHARACTERIZATION OF SPOT BLOTCH AND BACTERIAL LEAF STREAK RESISTANCE IN BREAD WHEAT

BY

GIRMA AYANA

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Plant Science

South Dakota State University

2018

MOLECULAR CHARACTERIZATION OF SPOT BLOTCH AND BACTERIAL LEAF STREAK RESISTANCE IN BREAD WHEAT

GIRMA AYANA

This thesis is approved as a credible and independent investigation by a candidate for the Master of Science in Plant Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This thesis is dedicated to ALMIGHTY GOD for always being there for me

BIBLIOGRAPHIC SKETCH

The author was born on the 07 July 1981 at Addis Alem *kebele*, Debati town, Benishangul-Gumuz Regional State, Ethiopia. He has persuaded his elementary school education at Addis Alem village from 1987-1993. He attended his junior and high school education at Debati Comprehensive Secondary school from 1993-1999 in Benishangul-Gumuz Region and awarded Ethiopian School Leaving Certificate in 1999. He joined Haramaya University of Ethiopia (the then Alemaya University of Agriculture), in September 1999 and graduated with Bachelor of Science Degree in Plant Sciences in July 2003. From 2007-2010 he attended Haramaya University for coursework and the Albert Ludwig University of Freiburg in Germany for thesis research and get graduated Master of Science in plant pathology in 2010.

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ABBREVIATIONS

ANOVA	Analysis of variance
BLS	Bacterial leaf streak
CIM	Composite interval mapping
GBS	Genotyping by sequencing
GLM	General linear model
GWAS	Genome-wide association study
LD	Linkage disequilibrium
LG	Linkage group
LOD	Logarithm of odds
MAS	Marker-assisted selection
MLM	Mixed linear model
NGS	Next-generation sequencing
PCA	Principal component analysis
QTL	Quantitative trait locus
R2	Coefficients of determination
RCBD	Randomized complete block design
RILs	Recombinant inbred lines
SB	Spot blotch
SNP	Single nucleotide polymorphism

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ABSTRACT

MOLECULAR CHARACTERIZATION OF SPOT BLOTCH AND BACTERIAL LEAF STREAK RESISTANCE IN BREAD WHEAT

GIRMA AYANA

2018

Spot blotch (SB), caused by *Bipolaris sorokiniana* (Sacc.) Shoem, and bacterial leaf streak (BLS), caused by *Xanthomonas translucens pv. undulosa (Smith et al.)*, two important foliar diseases of wheat in the major production regions of the US and the world. Deployment of adequate host resistance against them depends on determining the resistance genes or quantitative trait loci (QTLs) responsible for the trait and identification of genetic markers linked to QTL that facilitate markers assisted breeding. We conducted two independent studies and characterized QTLs for BLS and SB resistance. In the first study, we constructed a genetic linkage map of 1,211 SNPs with 92 F_5 recombinant inbred lines (RILs) developed from a cross between BLS resistant (SD52) and susceptible (SD1001) parental lines. Composite interval mapping (CIM) identified genomic regions (LOD≥2.3) on chromosomes 2B, 6D, 7A, and 7B linked to BLS resistance. The four QTLs for BLS resistance *QBls.sdsu-2B-I* (15.8%), *QBls.sdsu-7A-I* (6.1%) and *QBls.sdsu-7B-I* (10.9%), were derived from SD52 and aggregately explained 32.8% of the phenotypic variation. Also, a disease increasing QTL, *QBls.sdsu-6D-I* was identified from SD1001 accounting for 14.8% of the total variation. In the second study, we identified spot blotch resistance QTLs by evaluating 294 genotypes of hard winter wheat association mapping panel (HWWAMP) against *Bipolaris sorokiniana* (isolate SD40). Ranges of reactions were observed with ten highly resistant, 47 moderately resistant and 241 moderately susceptible

to susceptible genotypes. Genome-wide association study (GWAS) using 15,590 high-quality SNPs we identified six QTLs (p= <0.001) on chromosomes 2D, 3A, 4A, 4B, 5A, 7B that collectively explained 30% the total variation. SNPs highly associated (P-value <0.001) with spot blotch resistance were identified for all six QTLs (*QSb.sdsu-7B-1, QSb.sdsu-5A-1, QSb.sdsu-4A, QSb.sdsu-2D-1, QSb.sdsu-3A* and *QSb.sdsu-4B*). Comparative analysis with barley indicated the resistance locus on wheat chromosomes 2D, 3A, 5A and 7B in our study are syntenic to the previously identified locus in barley for spot blotch resistance on chromosomes 2H, 3H, 5H, and 7H. Genotypes resistant to spot blotch and BLS and highly associated SNP markers identified in our study could be useful germplasm for breeding for spot blotch and BLS resistance in wheat.

Keywords: *Triticum aestivum*, BLS, Spot Blotch, GWAS, gene mapping, quantitative trait locus, recombinant inbred lines, SNP markers.

CHAPTER-I

1. INTRODUCTION

Wheat (*Triticum aestivum* L) is an important cereal crop grown worldwide and remains a vital source for human food (FAOSTAT, 2016). Wheat ranks first among cereals in total production (USDA, 2017). It is cultivated on approximately 225 million hectares of land that yielded 736 metric tons worldwide in the year 2015/16. In the same year, USA produced 63 metric tons of wheat from which 62% was accounted for winter wheat (USDA, 2017). The current productivity of the USA and the world respectively reached 3.4 mt/h and 3.12 mt/h, and increases at a rate of 0.9%/year. However, the projected demand to meet the 9 billion population by 2050 and beyond needs a 2.4% increase in productivity of wheat per year (Ray et al., 2013)

Closing a large gap that exists between the current and projected wheat productivity is still challenging due to a number of environmental and biological factors that continues to threaten wheat productivity and challenge growers (Bebber et al., 2014; Duveiller et al., 2007a). From disease point of view, spot blotch (SB) caused by *Bipolaris sorokiniana* (Sacc.) Shoem (Chowdhury et al., 2013; Dubin and Rajaram, 1996; Duveiller et al., 2005a, 2005b, 2007a; Duveiller and Dubin, 2002; Duveiller and Gilchrist, 1994; Joshi and Chand, 2002; Leng et al., 2016; Sharma et al., 2007b; Singh and Singh, 2007) and bacterial leaf streak (BLS) caused by *Xanthomonas translucens pv. undulosa* (Akhtar et al., 1985; Alizadeh and Rahimian, 1989; Duveiller et al., 1992, 1997b; Maraite et al., 2007; McMullen and Adhikari, 2011; Sands and Fourrest, 1989; Vauterin et al., 1995) are among the destructive disease that affect wheat and several small gains worldwide. Reports from several countries indicated that average yield loss due to spot blotch is estimated to be 15–

20% but can reach 40–70% in susceptible genotypes (Acharya et al., 2011; Duveiller, 1998; Fernandez et al., 2014; Fernandez and Jefferson, 2004; Mehta et al., 1992; 2004; Sharma and Duveiller, 2007). Likewise, BLS usually causes yield losses of less than 10%, but can reach be up to 40% under favorable conditions (Barnes et al., 2012a; Byamukama, 2017; Duveiller et al., 1997a; Duveiller and Maraite, 1993; Sands and Fourrest, 1989).

In susceptible genotypes, wheat spot blotch is characterized by small, dark brown lesions during initial infection and develops to dark brown oval to elongated blotches on the leaf (Chand et al., 2003; Duveiller et al., 2005). The pathogen has extensive distribution, however, is more damaging in warmer and humid environments (Chowdhury et al., 2013; Joshi et al., 2007). In case of BLS, the pathogen infects the plant through stomata or wound openings which latter cause water-soaked lesion which latter looks yellow to dark brown longitudinal streaks between the leaf veins (Duveiller, 1997; Duveiller and Maraite, 1993; Kandel et al., 2012). Wind-driven rain splash, plant to plant contact as well as the occurrence of warm temperature under humid condition are among the major factors that exacerbate the disease (Azad et al., 1988; Duveiller, 1997; Duveiller et al., 1991; Duveiller and Maraite, 1995). Both SB and BLS pathogens are primarily seed born although can be residue born and infects wide range of hosts within wild and cultivated *Poaceae* family (Alizadeh et al., 1995; Bragard et al., 1997; Kumar et al., 2002; Matusinsky et al., 2010; Pandey et al., 2005; Sapkota et al., 2017; Wen et al., 2017; Zillinsky and others, 1983).

Managing plant diseases is always challenging due environmental factors, pathogen evolution and cost of disease control (Duveiller et al., 1997a, 2007a; Mehta, 1998). Further in many bacterial diseases like BLS application of pesticides and antibiotic compounds are

not effective. Besides, the frequent application of pesticide is neither sustainable nor economical (Duveiller, 1994; McMullen and Adhikari, 2011; Mehta, 1998). On the other hand breeding for disease resistance is more promising. However, it has to be a continuous effort under changing climatic conditions and constant evolution in the behavior of the pathogen (Longdon et al., 2015). Our ability to deploy and develop spot blotch and BLS resistant genotype depends on an understanding of the mechanism of resistance present in the host and identification of the resistant gene responsible for the traits. Both SB and BLS resistance have polygenic inheritance mechanisms that took several efforts to understand the genetic basis (Dubin and Rajaram, 1996; Duveiller et al., 1992; Joshi et al., 2004b; Kumar et al., 2015b; Schielzeth and Husby, 2014; Tillman et al., 1996; Xu et al., 2017). For instance, knowledge about the genetics of resistance of several polygenic traits like BLS in wheat is still limited, and hardly few consistent quantitative trait loci (QTLs) have been identified. The genetic variation of a quantitative trait is controlled by the additive effects QTLs and is highly prone to environmental variation and method of their detection are confounded with a number of complex issues (Bhadauria and Popescu, 2017; Corwin and Kliebenstein, 2017; Holland, 2004).

Currently, genetic linkage (also called as bi-parental mapping) (Anderson et al., 1993; Collard et al., 2005) and genome-wide association mapping (GWAS) (Ersoz et al., 2007; Gupta et al., 2005; Korte and Farlow, 2013; Miedaner and Korzun, 2012; Ogura and Busch, 2015) are the two useful methods to detect and characterize the major or minor QTLs responsible for resistance in many crops. To its advantage, however, linkage mapping is limited to the crossing of two or few parents at a time that takes years to even develop the low-resolution map. Additionally, this method only captures few couples of

the available genetic diversity; thus the amount of recombination that occurs during the creation of mapping population places a limit on the mapping resolution. On the other hand, GWAS, also known as "linkage disequilibrium mapping," exploits all of the historical recombination and mutation events that have occurred for past long years. Association mapping is useful to uncover association between inter-individual genetic variants, mostly single nucleotide polymorphisms (SNPs), that shows the strongest association to the traits of interest. Previously, spot botch resistance QTLs were identified on chromosome 2A, 2B, 2D, 3B, 5B, 6D, and 7D via genetic linkage mapping methods (Adhikari et al., 2012b; Ban et al., 2016; Gurung et al., 2014; Joshi et al., 2004b; Kumar et al., 2009b, 2010, 2015a, 2016, 2017; Lillemo et al., 2013; Lu et al., 2016; Zhu et al., 2014; Zhuang et al., 2013). Similarly, some QTLs for BLS resistance were detected on wheat chromosomes of 1A, 4A, 4B, 6B, and 7D (Adhikari et al., 2012b; Duveiller et al., 1997a; Kandel et al., 2015)

The usefulness of the above methods in detecting the QTL, however, depends on the number of the mapping populations as well as the nature of markers used for the construction of the high-quality genetic map. Currently, with the discovery of sequencing technologies, SNPs markers generated by point mutation, are gaining more importance as they occur in genome in much frequency than previously discovered markers (Semagn et al., 2006; Tiwari et al., 2014) and thus facilitating high density mapping and genome-wide association studies (GWAS) (Agarwal et al., 2008; Korte and Farlow, 2013; Miedaner and Korzun, 2012; Thomson, 2014; Wang et al., 2014b). Therefore, in this study, we characterized spot blotch and BLS resistance in bread wheat by identification of QTLs and linked markers that would facilitate marker-assisted breeding for spot blotch and BLS resistance in wheat.

Objectives and scope of the thesis

In this thesis, we characterized the genetic basis spot blotch and bacterial leaf streak resistance in bread wheat.

The first part of the study (Chapter III) was entitled to ‘High-density linkage mapping of bacterial leaf streak (BLS) resistance in bread wheat.’ The general objective of this parts of research was to conduct molecular genetic characterization of BLS resistance in bread wheat. The specific objectives were i) to locate putative QTLs associated with BLS resistance and ii) to identify useful SNP markers linked to QTLs for marker-assisted selection.

The second study (Chapter IV) was entitled to ‘Genome-wide association study for spot blotch resistance in hard winter wheat”. The general objective of this experiment was to conduct genetic characterization of spot blotch resistance in hard winter wheat association mapping panel (HWWAMP). The specific objects of this study were i) to identify winter wheat genotypes carrying resistance genes against *Bipolaris sorokiniana,* and ii) to locate putative QTLs and identify SNP markers associate with SB resistance for marker-assisted selection.

Earlier in Chapter II, we reviewed literature covering relevant topics about the research. The first section of this chapter covers about wheat history, biology, resistance breeding, and general methods gene mapping. The second section covers characteristics of the studied diseases. In chapter III and IV, we covered the research introduction, materials, methods, results and discussion made for each of the main investigation.

CHAPTER-II

2. LITERATURES REVIEW

Introduction

This chapter briefly reviewed literature covering relevant topics on origin and history of wheat, wheat biology, resistance breeding and methods of gene mapping in the first section. The second section address description of spot blotch (SB) and bacterial leaf streak (BLS) that affects wheat worldwide. The disease distribution, importance, the pathogen life cycle, the symptoms and the available management methods are covered.

2.1. History, Taxonomy, and Genome of Wheat

The first cultivation of wheat approximately started about 10,000 years ago, and it was recognized to be originated from the fertile crescent of the Middle East (Dubcovsky and Dvorak, 2007; Heun et al., 1997; Zohary and Hopf, 1993). Genetically, wheat is organized into seven chromosomes (1x = 7) and the earliest cultivated forms were diploid (2n = 2x = 14, genome AA) (einkorn), then polyplodized into tetraploid (2n = 4x = 28, genome AABB) (emmer) and latter continued polyploidization process that led to hexaploid wheat (2n = 6x = 42, genome AABBDD) (common wheat) (Avni et al., 2017; Kimber and Sears, 1987; Sax and Sax, 1924; Watkins and others, 1930.

Bread wheat also is known as common wheat is a member of the tribe *Triticeae* in the family *Poaceae.* It is an autogamous allohexaploid (6x) species whose chromosome are subdivided into three closely related (homoeologous) groups, A, B, and D genomes. The evolution of hexaploid wheat occurred through wide-hybridization of diploid grass species *Aegilops tauschii* (2n=2x=14, DD) with the cultivated tetraploid durum wheat *T.turgidum*

(2n=4x=28; AABB). Following an amphidiploidy event, a new species, *T. aestivum*, arose with a genome complement of AABBDD (Dubcovsky and Dvorak, 2007; McFadden and Sears, 1946; Mirzaghaderi and Mason, 2017). The origin of both A and B genomes were recognized as *T. urartu tumanian ex gandylian (2n = 2x = 14, AA)*(Dvo\vrák et al., 1993) and several S genome species in genus *Aegilops sect. sitopsis* (Feldman and Levy, 2015; Giorgi et al., 2003), respectively.

2.2. World Wheat Production and Its Importance

Wheat ranks first among cereals in total production (USDA, 2017). It is cultivated on approximately 225 million hectares of land that yielded 736 million metric tons worldwide in the year 2015/16. Nation China is the world second largest wheat producing country preceded by the European Union in 2015/16. India is the third largest wheat producing nation followed by Russia and United States of America (USDA, 2017).

Wheat Production in the USA

Wheat production in North America has begun since the Europeans came across the vast areas of the West and Northwest (Bell, 1987). Currently, wheat ranks third among U.S. field crops regarding acreage and production of corn and soybeans. In 2016/17, U.S. growers produced a total of 62 million metric tons of winter, durum and other spring wheat on 17,761,840 ha of cropland. The first ten leading USA wheat producing states from 2014 to 2016 are Kansas, followed by North Dakota, Montana, Washington, Oklahoma, South Dakota, Colorado, Idaho, Texas and Minnesota (USDA, 2017).

In the USA, the wheat is classified according to growth habit, the color of the kernel and texture of the ripened grain (Briggle and Reitz, 1963). Hard wheat consisted four

major subclasses such as hard red spring, hard red winter, hard white and durum (amber color). Soft wheat is further sub-classified into soft red winter and soft white. Hard wheat is used primarily for making bread and rolls whereas soft wheat is used to make cakes, cookies, pastries, and crackers. Bread wheat includes both hard and soft types. Durum wheat is used to make pasta products such as spaghetti and macaroni because of the unique coarse nature of its ground kernel (Briggle and Reitz, 1963; Clark et al., 1935).

Table 1. Area, yield, and production of wheat in the USA during 2014 to 2016.

	Year	Winter wheat	Durum wheat	Other spring wheat	Total
Area harvested (ha)	2014	13,071,080	544,710	5,155,750	18,771.550
	2015	13,054,090	767,290	5,237,090	19,058.470
	2016	12,230,540	957,090	4,574,210	17,761,840
Production (metric ton)	2014	37,481,680	1,471,160	16,194,280	55,147,120
	2015	37,290,410	2,244,850	16,304,290	55,839,540
	2016	45,491,650	2,833,570	14,533,830	62,859,050
Yield (metric ton ha^{-1})	2014	2.87	2.7	3.14	2.94
	2015	2.86	2.93	3.11	2.93
	2016	3.72	2.96	3.18	3.54

2.3. Wheat Biology and Physical-Climatic Requirement

T. aestivum L. as described by (Lersten, 1987), is a mid-tall annual or winter annual growth habit. Wheat growth passes numerous stages such as seedling, vegetative and floral

state. The vegetative state of the plant is characterized by the formation of tillers bearing axillary leafy culms. Culms comprise five to seven nodes with three to four foliage leaves. The terminal floral spike in each culm consists of perfect and cleistogamous flowers. Spikes are made up of two rows of spikelet, and each spikelet is separated by short internodes that consist three to four florets. Each floret is enclosed by lemma and palea that contain three stamens with large anthers and smaller pistil (Setter and Carlton, 2000).

Winter wheat needs a certain period of cold temperature (vernalization) before it will produce grain whereas spring wheat does not (Briggle and Curtis, 1987). For comfortable growth wheat needs annual 12 to 15 inches of water, 50 to 100 cm annual average rainfall, and temperatures as warm as 21° to 24° C and soil PH ranges in 5.5 – 6.5. Irrigation serves the best if rainfall falls below 50 cm. For good yield, soft red winter wheat in the USA should be planted within the two-week period following the Hessian fly-free date, which ranges from September 20 to October 10 (Grogan et al., 2016).

2.4. Major Factors Affecting Wheat Production

Closing a large gap between potential yields that have been accomplished in experimental fields versus those attained in farmers' fields still changing. This is true because wheat yields are dependent on interactions of socio-economical, biological, technological and ecological factors. The dynamic form of the currently observed and projected climate change conditions might also impact wheat production variability (Asseng et al., 2015, 2017; Ray et al., 2015). Among biological factors diseases are known to reduce grain yield and continue to remain economically important. Some of the diseases are already established while others are becoming more important due to the factors that lead to their emergences. Closing the yield gap reduced by a disease needs research to

improve wheat yields through the development of resistant varieties, biotechnology techniques, hybrid wheat, understanding host physiology and their interaction with damaging pests (Tester and Langridge, 2010).

2.5. Breeding for Resistance

The antagonistic relationship between pathogens and their host existed since the dawn of host-pathogen coevolution. Few pathogens have been reported to attack wider ranges of plants hence called polyphagous while a large proportion of them, however, have a narrow host range restricted to a few closely related plant species; they are specialized, monophagous pathogens or specialists (Agrios, 1988). In the never-ending arms race between plant and pathogen, developing sustainable methods of managing the disease is very crucial. Host resistance, method entirely depends on genetics, is considered to be the most effective and economical method to protect crops from diseases caused by pests including bacteria and fungi (Bradshaw, 2016; Rejeb et al., 2014; Van Loon, 1997).

2.5.1. The Mendel approach

Soon after Model's work was discovered, in 1905, Biffen reported that disease resistance might be inherited in Mendelian manner. Latter search for disease resistance genes was continued and H. H. Flor in the early 1940s while working with flax and flax rust (Flor, 1955, 1956), discovered the gene-for-gene relationship that underlines interaction between host and pathogen. Gene for gene theory mainly relied on single dominant R gene in the host interacting with specific protein codified by single *avr* gene in the pathogen. The host resistance only occurs when the host and the pathogen have dominant alleles, and in this case, the host shows hypersensitive reaction due to

programmed cell death. Plant resistance (R) proteins recognize pathogen avirulence (Avr) determinants that trigger signal transduction cascades and lead to rapid defense activation (Keen, 1990; Staskawicz et al., 1995). The host is becoming susceptible (+) in two conditions such as i) when host lakes product to recognize and defend itself from pathogen due to the possession of reconceive allele (rr) or ii) the pathogen produces a new kind of elicitor or product that cannot be detected by host R gene and the pathogen genetics is confirmed as receive *avr* gene (aa). The second is very common when the pathogen breaks the existing host resistance due to mutation. The fact that Flor and his group worked on the fungus which lost its avr gene led them to define incompatibility as a basic ability to parasitize and reverse way around.

Host Genotype

Pathogen Genotype	RR	Rr	rr
AA	-	-	+
Aa	-	-	+
aa	+	+	+

'+' indicates compatible and '-indicates incompatible interaction.

First *Avr* gene was cloned from *Pseudomonas syringae* (Staskawicz *et al*., 1990) and first R gene (Hm1) was cloned by (Johal and Briggs, 1992). Later, several host proteins related pathogen virulence targets were discovered (Axtell et al., 2003; Rooney et al., 2005) and the interaction of one of the known R gene NBS-LRR protein domains (DeYoung and Innes, 2006).

2.5.2. Types of disease resistance

Traits in diseases can be either quantitative or qualitative. Quantitative traits are controlled by few to many genes that can interact with the environment and each other, whereas qualitative resistance is controlled by single gene (Poland et al., 2009)

Quantitative resistance

Quantitative disease resistance (QDR) also named as polygenic, adult plant, horizontal, partial disease resistance, has been observed within many crop plants but is not as well understood as qualitative disease resistance (Corwin and Kliebenstein, 2017). Polygenic inheritance refers to a single character that is controlled by more than two genes (Bhadauria and Popescu, 2017). The inheritance of this kind of traits is caused by segregation of many gene pairs, and they are influenced by a lot of minor environmental effects. In quantitative trait individuals within the host population will exhibit a normally distributed continuous range of reaction to disease and the genetic interaction between host and pathogen cannot be characterized by the gene-for-gene relationship an exception to Mendel low. Miner gene resistance is considered to be long-lasting, stable, and nonspecific to races of the pathogen (Bhadauria and Popescu, 2017).

From the plant breeders' standpoint, selection for quantitatively inherited resistance is usually more difficult than selection for qualitative resistance (Bhadauria and Popescu, 2017). This is because the resistance is not absolute and the expression of the trait is largely affected by environment. Similarly, pathogen population is not characterized by different races, but instead, individuals within the population may vary in their degree of aggressiveness or virulence. Because of environmental influence of genetic transmission

from parent to offspring, heritability measurement is one of the important parameters when dealing with the quantitative trait (Bhadauria and Popescu, 2017; French et al., 2016; Krattinger and Keller, 2016).

Qualitative disease resistance

A single gene influences the pattern of inheritance for a qualitative disease resistance, and this kind of trait fits into discrete categories. Unlike qualitative trait, major gene resistance is usually pathogen race-specific that involves a gene-for-gene interaction, and the environment has less influence on the gene expression (French et al., 2016; Krattinger and Keller, 2016). Even though it provides short lasting resistance, it is simple for breeding because of its simple inheritance and ease in measurement (Bhadauria and Popescu, 2017).

2.5.3. Durability of cultivar resistance

Durable disease resistance a cultivar is defined as resistance that has remained effective for long-lasting within an environment favoring the disease (Brown, 2015). Achieving durability was understood within competing views of both 'mechanism of resistance (e.g., horizontal versus vertical resistance) and resistance deployment strategies (e.g., pyramids versus mixtures)' (Mundt, 2014; Quenouille et al., 2014), although implications of this for durability are too vast and still not well understood. According to Johnson and others (1981), the durability of resistance to a pathogen is not due solely to the genetic basis of host plant resistance, but that it also depends on pathogen genetics, host diversity, environment, cropping system. Past several studies indicated that quantitative resistance is more durable than major gene resistance on average (Mundt, 2014; Quenouille

et al., 2014). However, from the practical point of view breeders were hypothesized different strategies for achieving durable resistance (Pilet-Nayel et al., 2017). One of the proposals was pyramiding of the several distinct major genes into the same cultivar (Pilet-Nayel et al., 2017). However, the achievement of strategy depends on the number of resistance genes that are available to control a given pathogen species. The other strategy was to combine the major resistance gene in a quantitatively resistant genetic background (Palloix et al., 2009; Pilet-Nayel et al., 2017; Quenouille et al., 2014)

2.6. Molecular Markers

In genetics, a molecular marker also known as genetic markers is defined as a fragment of DNA that is linked to a certain location within the genome. The advanced application of molecular markers becoming a huge attention in modern plant breeding (Agarwal et al., 2008). The discovery of molecular markers allows plant breeders to locate genes of interest such as pest resistance, desirable agronomic and yield characteristics in wheat (Dreisigacker et al., 2016). Application of genetic markers assisted selection drastically reduced long year time frame required by conventional breeding to achieve the same goal.

Usefulness of molecular markers was evolved with the innovation of PCR and high throughput sequencing technologies (Semagn et al., 2006). Earlier, low-throughput, hybridization-based markers such as restriction fragment length polymorphisms (RFLPs), and medium-throughput PCR-based markers such as random amplification of polymorphic DNA (RAPD) amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) have been used as markers of choice for fingerprinting and characterization of genotypes of different crop species without requiring prior genomic information (Semagn

et al., 2006). However, application of low throughput techniques was very slow and cumbersome that for instance, RFLP analysis, requires a large amount of sample DNA, probe labeling, DNA fragmentation, electrophoresis, blotting, hybridization, washing, and band imaging could take several weeks to complete. After 2000, molecular markers such as simple sequence repeat (SSR) or microsatellite markers, and single nucleotide polymorphism (SNP) markers are becoming useful in wheat breeding.

Currently, with the discovery of sequencing technologies, SNPs markers generated by point mutation, are gaining more importance as they occur in the genome in much frequency than previously discovered markers (Thomson, 2014; Wang et al., 2014b). SNP markers have become extremely popular in genetics due to their genome-wide abundance, construction of saturated genetic map and their ability to capture variation in several parents within the short time (Korte and Farlow, 2013; Miedaner and Korzun, 2012).

2.7. Next-Generation Sequencing (NGS)

Next-generation sequencing (NGS), also known as high-throughput sequencing, is a term that includes all modern sequencing technologies including Illumina (Solexa) sequencing, Roche 454 sequencing, Ion torrent: Proton / PGM sequencing, SOLiD sequencing. NGS revolutionized genomic studies because of its low cost and better accuracy (Mardis, 2008; Pareek et al., 2011).

All NGS involves protocol for template DNA preparation, library preparation, ligating adapter to randomly sheared DNA fragments, genome sequencing and finally sequence analysis (Metzker, 2010). The partial genome representation library preparation in NGS platform can be either based on the (i) complexity reduced representation libraries

constructed by using restriction enzymes, or (ii) sequence capture libraries without involving restriction digestion. The first one is simple, quick, extremely specific, highly reproducible, avoid repetitive regions and addresses genome regions that are difficult to be reached by sequence capture approaches. The most common Restriction enzymes methods in the first one includes reduced-representation libraries (Gore et al., 2009), restriction-site associated DNA sequencing (RAD-seq) (Rowe et al., 2011), complexity reduction of polymorphic sequences (CRoPS) (Mammadov et al., 2010), sequence based polymorphic marker technology (SBP) (Sahu et al., 2012), low coverage multiplexed shotgun genotyping (MSG) (Andolfatto et al., 2011), and genotyping by sequencing (GBS) (Elshire et al., 2011).

2.8. Genotyping by Sequencing (GBS)

GBS is first coined in Buckler lab (Elshire et al., 2011) as a highly multiplexed system for constructing reduced representation libraries for the Illumina NGS platform and latter similar protocols were developed taking advantage of NGS (Poland and Rife, 2012) Poland and Rife, 2012. It is increasingly preferred because it bypasses the entire marker assay development stage by concurrently detect and score SNPs (Deschamps et al., 2012).

GBS protocol as explained by (Wallace and Mitchell, 2017) starts with proper sample preparation that includes DNA extraction, quantification, and restriction digest with GBS enzymes and adapter ligation and ligate clean up. Following ligation, each sample DNA are pooled together, for instance, sample number could be 96 for GBS 96-plex protocol, and PCR amplification with primers specific to each adapter. Finally, the DNA sequence in a FASTQ file is downloaded and subject to SNP calling. The SNP calling involves collapsing reads, assembling contigs, aligning reads, summarizing the alignment

and finding the SNPs in the aligned reads or by comparing to the available reference genome for the species.

2.9. Types of Gene Mapping

2.9.1. Linkage mapping

A linkage mapping is a is a process of locating the position of known genes or genetic markers pertinent to each other in terms of recombination frequency, rather than a specific physical distance along each chromosome (Collard et al., 2005; Hyten and Lee, 2016; Somers et al., 2004). It shows the relative positions of genetic markers along a chromosome that is determined by the recombination frequency during crossover of homologous chromosomes. Both Mendelian gene systems and quantitative trait loci are evaluated. Method of linkage map involves determining the recombination frequency of permanent or temporary population developed from crosses of two or few pure lines, genotyping using polymorphic markers, construction of linkage map and analysis of the association between genetic markers and key genes determining the traits (Collard et al., 2005).

The initial step in linkage mapping involves the development of a bi-parental population derived from two or more individuals showing phenotypic variation for a trait of interest (Anderson et al., 1993). In our experiment, CIMMYT lines were initially screened against bacterial leaf streak from 2014-2016. Among the lines, five genotypes that are showing resistance to the disease was crossed with the susceptible check line to develop F1 and continued selfing. Various linkage population types are commonly used in plant genetics, such as F2:3, recombinant inbred lines (RILs), double-haploids (DH),

introgression lines (ILs) or backcross inbred lines (BILs) (Collard et al., 2005; Mohan et al., 1997). For plant traits with low heritability and insufficient seeds, it is suggested to use the average phenotypic values of F3 population than F2. Due to segregation of the population, called as mortal population, at early generation as well as limited recombination that happened, the power of QTL mapping may be less reliable and the breeding use of the population its self is very limited when compared to recombinant inbreed lines (RILs) (Lander and Botstein, 1989; Zeng, 1993).

Models of QTL mapping

Detection of associations between molecular markers and traits of interest in linkage mapping includes three commonly used models such as single marker analysis, simple interval mapping (SIM), multiple interval mapping (MIM), and composite interval mapping (CIM) (Churchill, 2016; Collard et al., 2005). All the three methods can be conducted using a variety of statistical analyses, including t-tests, ANOVA, regression, maximum likelihood estimations, and log likelihood ratios to test each whether each genotypic classes differ in phenotype for a given molecular marker or among molecular markers.

Single marker analysis (SMA), is an easy method of performing QTL analysis. SMA is mostly needed when analyzing QTLs for unlinked marker data or when the analysis does not require a complete linkage map (Collard et al., 2005). In SMA model, the further a marker is from a QTL, the higher effects underestimated due to the possibility of recombination between the markers and the increase in the number of single marker comparisons that increases the false positive (type I error) rate. SMA cannot tell whether the markers are associated with one or more QTLs and do not give separate estimates of

QTL effect and location. Even if, these limitations SMA may be overcome by using a large number of molecular markers application and reliability of SMA is very limited (Churchill, 2016; Collard et al., 2005; Staub et al., 1996).

Interval mapping is a method of estimating the position/location of a putative QTL associated with the trait of interest in the genome in an interval of two flanking markers (Kao et al., 1999; Lander and Botstein, 1989). Maximum likelihood and simple regression procedures are employed for good approximations of QTL within the flanking marker. Interval mapping is more powerful than SMA, and it has two approaches namely simple interval mapping and composite interval mapping. Simple interval mapping analyzes linkage disequilibrium between a putative QTL and its flanking markers. SIM is not an interval test as the name indicates, and even when there is no QTL within an interval, the likelihood profile on the interval can still exceed the threshold if there is a QTL nearby. The disadvantage of simple interval mapping is that it tests for the presence of a QTL between the intervals of two marker loci regardless of any factor outside of the interval that affects the outcome (Collard et al., 2005; Staub et al., 1996).

Composite interval mapping improves the interval mapping approach by minimizing the confounding effects of other QTLs located outside of flanking markers (Collard et al., 2005; Wang and others, 2009). The QTL effects out of the ranges of the two markers are regarded as background variation or simply "noise." The sets of markers that are used to reduce the background noise to refine in composite interval mapping are regarded as co-factor or control markers. The cofactor markers include both the linked markers and unlinked markers that are significantly linked to the gene of interest and may be located anywhere in the genome. Analysis of CIM incorporates effects of the ‘cofactors'

into the model to reduce confounding effects of other QTLs. For this reason, CIM models have advantages over the SIM and SMA in accommodating multiple QTL and is more powerful in QTL mapping.

2.9.2. Association mapping

Linkage disequilibrium (LD) is the non-random co-segregation of alleles at two or more loci (Ersoz et al., 2007). Two or more loci are co-segregated or linked if the proportion of recombinant gametes are smaller than 50% of the population. LD throughout the genome reflects the population history of mutation, recombination, genetic drift and natural selection. Non-overlapping sets of loci in strong LD forms grouping pattern called a `haplotype block' that the pedigrees show excess transmission of the same allele to progenies (Gupta et al., 2005).

Association mapping (AM), also known as "linkage disequilibrium mapping," is a method of mapping quantitative trait loci (QTLs) to uncover the association between phenotypes and genotypes (Gupta et al., 2005). Linkage disequilibrium mapping exploits all of the historical recombination and mutation events that have occurred in the population in the past. The major goal of AM is to identify inter-individual genetic variants, mostly single nucleotide polymorphisms, which show the strongest association to traits of interest either because they are a causal gene or correlated to the causal gene. AM can be used to find genetic variants in a known genomic regions that are already identified in the case of candidate gene approach, or it examines the representative variants (SNPs) across the whole genome in the case of genome-wide association scan (Ersoz et al., 2007).

AM has advantages and drawbacks when compared to linkage mapping (Korte and Farlow, 2013). Unlike linkage mapping AM not limited to few parents that only represent a portion of the available genetic diversity. Thus it increases the chance of mining several alleles simultaneously that enhances the power of finding several minor genes that determines polygenic traits. Another advantage of association mapping is that it reduces costs and time of creating recombinant lines as in the case of linkage mapping and it gives an advantage of using past phenotypic data as the population is theoretically immortal. Unless used precautiously, a problem with association mapping occurs due to kinship that that likely exists among germplasm and population structure that can increase false discovery rates (Ersoz et al., 2007; Korte and Farlow, 2013).

Models of association analyses

Compared to the MLM, the GLM identifies a greater number of MTAs. However, the GLM has a high risk of false-positive detection. Indeed, with the GLM, too many associations appear that are not detected with the MLM, while most of the MLM MTAs are confirmed by the GLM. In GLM model, which only accounts for population structure as a covariate, the additive variance and error variance cannot be separated because GLM uses maximum compression (compression = n) with all taxa as a single group. Unlike GLM, however, MLM (Zhang et al., 2010b) takes account of population structure and kinship in association analysis to reduce type I error instigated due to relatedness and population structure. MLM uses individual kinship rather than group kinship. Unlike regular MLM, cMLM uses cluster approach where different individuals are compressed together into clusters, and the model uses average kinship among groups. Furthermore, ECMLM (Li et al., 2014) improves cMLM by exploiting multiple ways of clustering and

methods of driving group kinship in addition to using group average (Tang et al., 2016). On the other hand, unlike MLM and its extend methods, FaST-LMM-Select (Listgarten et al., 2013) and SUPER (Wang et al., 2014a) uses the bin approach. In bin approaches, association analysis was first made using simple methods such as GLM and then followed by groupings of all the genetic markers into bines where each bine was represented by the most significant markers out of the group.

2.10. Wheat Spot Blotch

2.10.1. Naming and taxonomy

Bipolaris sorokiniana (teleomorph *Cochliobolus sativus*) is the causal agent of common root rot (Wildermuth et al., 1997), leaf spot disease, embryo blank point, seedling blight, head blight and leaf spot blotch in several *poacae* including wheat (Chowdhury et al., 2013; Dubin and Rajaram, 1996; Duveiller et al., 2005, 2007; Duveiller and Dubin, 2002; Duveiller and Gilchrist, 1994; Gurung et al., 2009; Hudec et al., 2008; Joshi and Chand, 2002; Zillinsky and others, 1983) The genus *Bipolaris* belongs to *Ascomycota, Dothideomycetes, Pleosporales, Pleosporaceae*. The genus name *Bipolaris* (1959) is more frequently in literature than the old name Cochliobolus (1934). The pathogen was formally named with several synonyms during anamorph stage such as *Helminthosporium sorokinianum* Sacc. 1891, *Helminthosporium sativum Pammel*, C.M. King & Bakke 1910, *Drechslera sorokiniana* (Sacc.) Subram. & B.L. Jain 1966 (Maraite et al., 1998).

Species in *Bipolaris* were formerly described in the genus *Helminthosporium*. However, the genera of *Helminthosporium* latter taxonomically revised several times and currently segregated into three anamorphic genera such as *Bipolaris*, *Drechslera*, and

Exserohilum; each of them has the teleomorphic stage (sexual stage) named as *Cochliobolus, Pyrenophora*, and *Setosphaeria*, respectively (Shoemaker, 1959). Thus, one of the species called *Cochliobolus.sativus* form is extremely rare, and thus it is the anamorphic stage named as *B.sorokiniana* which causes infection. In *Bipolaris*, the conidia look fucoid, straight, or curved conidia and germinating by one germ tube from each end (bipolar germination). The character of conidia germination was one of the major reason after the naming and grouping into new genera as proposed by Shoemaker (1959).

2.10.2. Symptom and host range

B. sorokinina can affect a wide range of hosts within wild and cultivated *Poaceae*, although rye is less susceptible and oats are seldom infected (Bakonyi et al., 1997; Kumar et al., 2002a; Matusinsky et al., 2010; O'Boyle et al., 2014; Pandey et al., 2005; Shandikov and Eakin, 2013; Zillinsky and others, 1983) Even though symptom of the leaf depends on cultivar and environment, it is generally characterized by small, dark brown lesions that extends to1-2 mm long without chlorotic margin during initial infection (Chand et al., 2003; Duveiller et al., 2005; Duveiller and Garcia Altamirano, 2000). Infected seedlings develop dark brown necrotic lesions on roots, crowns, and leaves where resistant genotypes show symptom of a very small necrotic dark spot while the susceptible plants develop a distinct oval to elongated light to dark brown blotches that extend and merge quickly and kills the leaves (Chand et al., 2003; Duveiller et al., 2005). Fruiting structures develop readily under humid conditions and are easily observed on old lesions. In addition to leaf, the fungus also causes common root rot (Hudec et al., 2008), foot rot, black point on grains (dark staining of the embryo) disease of wheat (Chand et al., 2003; Duveiller et al., 2005; Duveiller and Garcia Altamirano, 2000; Duveiller and Gilchrist, 1994).

2.10.3. Distribution and yield loss

Spot blotch (SB) is one of the destructive fungal diseases that affect wheat and several small gains worldwide and the disease occurs virtually everywhere wheat is grown (Chowdhury et al., 2013; Dubin and Rajaram, 1996; Duveiller et al., 2005, 2007; Duveiller and Dubin, 2002; Duveiller and Gilchrist, 1994;Joshi and Chand, 2002; Sharma et al., 2007a). Wheat spot blotch pathogen has extensive distribution but is more damaging in warmer and humid regions (Chowdhury et al., 2013).

Reviews from several countries indicted that average yield loss due to spot blotch is estimated to be 15–20% but can reach 40–70% in susceptible genotypes (Acharya et al., 2011; Duveiller, 1998; Duveiller and Sharma, 2009; Fernandez et al., 1998, 2014; Fernandez and Jefferson, 2004; Gurung et al., 2012; Lemerle et al., 1996; Mehta et al., 1992; Sharma et al., 2004, 2007a; Sharma and Duveiller, 2007; Siddique et al., 2006). In Latin America, up to 100 % yield loss under the most severe conditions was reported (Mehta, 1998).

2.10.4. Etiology and epidemiology

Spot blotch is seed transmitted disease, and its conidia survive in the soil debris. Primary inoculum includes mycelium from infected seed, conidia in the soil, or conidia on the kernel surface (Neupane et al., 2010; Pandey et al., 2005). The infection starts when conidia in stable or from seed contacts with the plant owing conducive environment are available. Similar to several seed-transmitted diseases, spot blotch more severely damages lower leaves and progresses from the lower to upper parts of the plants. The disease favorably grows in moderate to warm a temperature that ranges from18˚C to 32˚C, and the

maximum infection occurs when the temperature is about 28 ˚C. In a field condition the disease is sever when the plant exposes to high temperature and long humid or fogy hours (Acharya et al., 2011; Chowdhury et al., 2013; Duveiller et al., 2005; Nema and Joshi, 1973; Neupane et al., 2010; Rosyara et al., 2008, 2010; Sharma et al., 2007b; Sharma and Duveiller, 2004).

2.10.5. Disease cycle

Fungal plant pathogens can be classified as biotrophs, necrotrophs, and ranges between the two (Glazebrook, 2005). Biotrophs require a living host to survive and establish a long-term feeding relationship with the living cells of their hosts. They can have very complex nutrient requirements but do not kill host plants. Conversely, necrotrophs fungi derive their energy by rapidly invading and killing plant cells and then live saprotrophically on the dead remains. Unlike biotrophs, necrotrophs can survive as competitive saprotrophs, affects wide ranges hosts, seldom systemic, and can be controlled by quantitative resistance than qualitative genes. Defense against biotrophic pathogens is largely due to programmed cell death in the host. *Bipolaris sorokiniana* is hemibiotroph and is known to produce a number of toxins. Prehelminthosporol is the most active and abundant phytotoxin produced by B.*sorokiniana* (Apoga et al., 2002) and interferes with proteins of the plasma membrane involved with nutrient and ion uptake and protection of the cell against physical stress (Olbe et al., 1995).

Once the inoculum reaches the plant tissue, the conidia form adhesion to tissue by excretion of the mucilaginous substratum. With the favorable environment, conidia germinate between 4 and 6 hours after inoculation (Han et al., 2010). The fungus then penetrates the cell by forming an outgrowth penetration peg developed from a specialized

structure called an appressorium or directly through stomata. The infection peg pierces through the cuticle, epidermal and parenchyma cells in leaf tissue; and the outer and inner cortex of root tissue. Invasion of the tissue can occur intercellular as well as intracellularly (Carlson et al., 1991; Han et al., 2010). Once it is established pathogen produces itself in the multiple cycles to causes multiple infections within the same season **(Figure 1).**

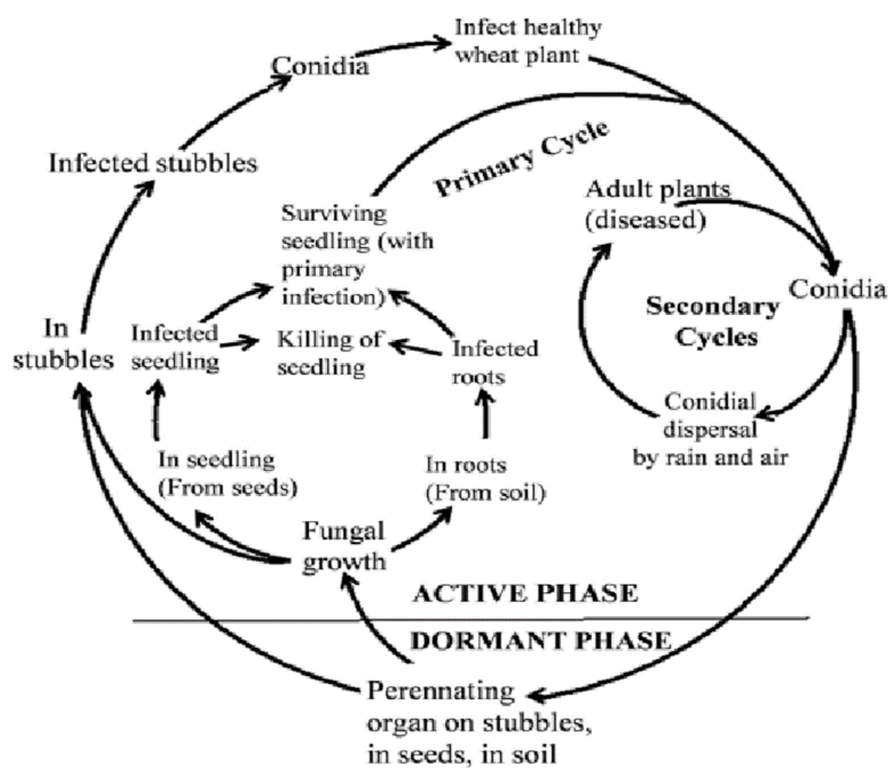


Figure 1. Life cycle of spot blotch (Acharya et al., 2011)

2.10.6. Spot blotch management

Disease incidents of wheat caused by *B. sorokiniana* can be controlled in a number of ways such as cultural, physical, fungicide and host resistance. However, out of all options, developing wheat cultivars resistant to spot blotch is the most economical and sustainable disease management strategy (Ban et al., 2016; Bartoš et al., 2002; Chowdhury

et al., 2013; Crute and Pink, 1996; Duveiller et al., 2007b; Gupta et al.; Khan et al., 2010; Kumar et al., 2017; Vasistha et al., 2016). Genetic assessment of spot blotch lines of wheat (*Triticum aestivum* L.) for spot blotch resistance and yield traits were conducted in several studies (Hetzler et al., 1991; Joshi et al., 2007; Kumar et al., 2017; Leng et al., 2016; Mujeeb-Kazi et al., 2001; Osman et al., 2016) So far, sources and origins SB resistant gens were reported to exist in Latin America, China and wild relatives of wheat or alien species (Van Ginkel and Rajaram, 1998). Existence of genotypes carrying high level of SB resistance gene were reportedly found in Indian national collection (Kumar et al., 2016; Vasistha et al., 2016), CIMMYT germplasms and derivatives of CYMMIT primary synthetic bread wheat (Khan and Chowdhury, 2011; Mikhailova et al., 2004; Singh, 2016; Zhu et al., 2014b), and Chinese germplasms (Ojha *et al*, 2016). Additionally, the existence of resistance sources was reported in the derivatives of modern European winter wheat cultivars and breeding lines (Liatukas and Ruzgas, 2012), multi-resistant cultivars from Nepal (Mahto et al., 2011) and few Brazilian varieties (Mehta et al., 1992). The development outstanding lines in Mayoor and Chirya series against SB was reported (Chowdhury et al., 2013). The previous result indicated the existence of underutilized or unknown sources of winter wheat that possessed historically accumulated noble spot blotch resistance genes.

QTL associated with spot blotch

Wheat spot blotch has quantitative inheritance (Joshi *et al*., 2004; Dubin and Rajaram, 1996; Kumar *et al*., 2015). Several studies were reported related to enhancing spot blotch resistance in wheat by marker-aided backcross breeding (Jaiswal et al., 2017; Vasistha et al., 2016). From genetic linkage and association mapping QTLs were identified

on chromosome 2A, 2B, 2D, 3B , 5B and 6D, 7D via genetic linkage mapping methods (Adhikari, 2014; Adhikari et al., 2012; Gonzalez-Hernandez et al., 2009; Gurung et al., 2014; Kumar et al., 2009, 2010, 2015a, 2016, 2017; Nair et al., 2015; Neupane et al., 2007; Sharma et al., 2007a; Singh et al., 2016; Zhu et al., 2014; Zhuang et al., 2013). So far, three major QTLs such as Sb1 (Lillemo et al., 2013), Sb2 (Kumar et al., 2015a) , and Sb3 on (Lu et al., 2016), on chromosome 7B, 5A, 3B were thoroughly described, respectively.

2.11. Wheat Bacterial Leaf Streak

2.11.1. Pathogen taxonomy and character

Xanthomonas spp. are known as destructive plant bacterial pathogens affecting a variety of important crop plants (Borkar and Yumlembam, 2016; Sundin et al., 2016; Vidaver and Lambrecht, 2004). The genus *Xanthomonas* belongs to Phylum: *Proteobacteria*, Class: *Gammaproteobacteria*, Order: *Xanthomonadales*, and Family: *Xanthomonadaceae*. The *Xanthomonas translucens* species are further classified into at least five pathovars depending on the infection aggressiveness and types of plant they affect. Based on the name of plant pathogenic bacteria included in International Society of Plant Pathology's (ISPP) (Bull et al., 2010; Vauterin et al., 2000; Young et al., 2001) The name *X. translucens PV. undulosa* is primary pathovar causes BLS on wheat and *X. translucens* pv.*translucence* is considered the primary pathovar adapted to barley (Curland et al., 2017).

Like many other *X. translucens pv. undulosa* (*Xtu*) is a gram-negative rod-shaped bacteria, characterized by their typical pale to deep yellow appearance caused by the

pigment called xanthomonadin. Optimal growth conditions include a temperature of 28 °C and a pH value between 5.5 and 6.5 on culture media (Iqbal et al., 2014)(Duveiller, 1989).

2.11.2. Symptom and host range

The BLS pathogen can affect wheat leaves, stems, inflorescences, and seeds (Duveiller, 1994). Typical symptoms on the leaves begin around the edges or midrib, as small, light-brown to translucent water-soaked streaks that are confined by the veins. On the susceptible plants, the streak continues growing parallel to the vain and appears as translucent dark-brown water-soaking lesions. Streaks may coalesce to form large lesions and even kill entire leaves (Duveiller et al., 1997a). After the disease emerges, the sign of the bacteria appears on the leaf initially as honey-like exudates giving a milky slime which later turns into a yellowish resinous granule (Duveiller, 1997; Duveiller and Maraite, 1993; Kandel et al., 2012). The symptom is named as 'black chaff' when it affects the heads, and it appears as black streaks or dark heads on glumes and lemmas. There is also a reported relationship between melanesia associated to stem rust resistance gene Sr2 named as a pseudo-black chaff black symptom that mimics black chaff caused by bacteria (Duveiller et al., 1997a; Duveiller and Maraite, 1993; McIntosh and Yamazaki, 2008) The pathogen mainly affects barley, rye, triticale, wheat and many other wild kinds of grass as major host, and oat as minor host (Alizadeh et al., 1995; Bragard et al., 1997; Sapkota et al., 2017; Wen et al., 2017)

2.11.3. Distribution and importance

The damage of bacterial leaf streak has a wide geographical distribution, and It has been reported in several countries in Asia, Europe, Africa, Australia and South America

(Akhtar et al., 1985; Alizadeh and Rahimian, 1989; Duveiller et al., 1992, 1997b; Maraite et al., 2007; McMullen and Adhikari, 2011; Sands and Fourrest, 1989; Vauterin et al., 1995). It has also been reported in Canada, Mexico and United States in most wheat growing areas, The primary problem in the US in the lower mid-south. In South America, it occurs in Argentina, Bolivia, parts of Brazil, Paraguay, Peru and Uruguay (Duveiller and Maraite, 1993)(Duveiller, 1989; Mehta, 1990; Mohan et al., 1985). During 2008, an average of 80 percent BLS incidence was observed in wheat fields of North Dakota, Minnesota and South Dakota (Barnes et al., 2012; Byamukama, 2017).

So far there is very little quantitative information available on losses caused by BLS. The pathogen causes sporadic but widespread damage. However, from the on-station and farmers field losses assessment made in few countries including USA indicated that average yield lose up to 40 percent was reported depending on the stage of infection (Barnes et al., 2012; Byamukama, 2017; Duveiller and Maraite, 1993; Stromberg et al., 1999; Tubajika et al., 1998, 1999). It was reported that yield loss is a linear function of the percent infected flag leaf area (Tubajika et al., 1998).

2.11.4. Pathogenic and genetic diversity

The pathogenic and genetic diversity of *Xtu* was not well characterized. However, from pathogenicity study conducted on 12 wheat cultivars and 226 strains of the pathogen at five location of North Dakota indicated that Strains varied greatly in aggressiveness, and wheat cultivars also showed differential responses to several strains (Barnes et al., 2012; Bragard et al., 1997).

2.11.5. Epidemiology and biology

Xtu is seed born pathogen and seed are the most important of primary inoculum and distribution sources (Duveiller, 1997; Duveiller and Maraite, 1993; Gitaitis and Walcott, 2007; Kandel et al., 2012). However, low levels of seed contamination, i.e., less than 1000 colony forming unit per gram of seed or 108 CFU/leaf will not result in field disease (McMullen and Adhikari, 2011; Schaad, 1988). Even though it is common in several *Xanthomonas* species, there is no evidence indicating that BLS pathogen follows Quorum sensing mechanism, i.e., behaving based on population density to initiate an attack on the host or defend themselves from external stimuli (Helman and Chernin, 2015). Overall, the pathogen poorly survives in the soil, and the free bacteria cannot survive more than 14 days (Cunfer, 1988). Even within it is primary sources the bacterium will die in 63 to 81 months in seed storage (Gitaitis and Walcott, 2007). Bacterial leaf streak outbreak occurs with optimum temperatures above 26°C. It was reported that pathogen multiplication in leaf tissue is mainly a function of temperature rather than moisture (Duveiller and Maraite, 1995). It was reported that outbreak of an epidemic of BLS is relatively occurring during late in the growing season (Duveiller and Maraite, 1993)(Duveiller and Maraite, 1995).

2.11.6. Lifecycle

The primary infection of BLS mainly starts when bacterium from seed or debris are released during germination (Byamukama, 2017). Bacteria enter through the stomata, lenticels and any wounds from which they multiply in large masses in the parenchyma for further leaf colonization and invasion. Spread of the bacteria as secondary infection within

a crop can occur through plant-to-plant contact, visiting insects, irrigation or water splash from the rain that can be flashed distances as short as 4 to 5 m.

2.11.7. BLS management

Like many other bacterial diseases, application of pesticides and antibiotic compounds is not effective against BLS (Duveiller, 1994; McMullen and Adhikari, 2011). However, standard cultural control methods such as crop rotation, tillage, alternative host control, avoiding susceptible cultivars, use of clean seed, hot water seed treatment would be effective in lowering the inoculum and disease levels (Forster et al., 1988; Gitaitis and Walcott, 2007; Stromberg et al., 1999). Until to date, host plant resistance is the only and most effective methods for controlling BLS in wheat (Adhikari et al., 2012a; Duveiller et al., 1992; Duveiller and Maraite, 1993; Sharma et al., 2017). So far, wide range of wheat (Adhikari et al., 2012b; Duveiller, 1989; Duveiller et al., 1992; Kandel et al., 2015; Maraite et al., 2007b; Tillman et al., 1996) and few triticale (Sapkota et al., 2017; Wen et al., 2017) genotypes exhibited genetic resistance to the pathogen.

BLS resistance gene in wheat

Host plant resistance to *X. translucens* appears to be quantitative and polygenic (Duveiller et al., 1992; Schielzeth and Husby, 2014; Tillman et al., 1996; Xu et al., 2017). QTL conferring resistance to BLS in five wheat lines (Turaco, Alondra, Angostura, Mochis and Pavon) reported being controlled by five genes for which the names Bls1/bls1, Bls2/bls2, Bls3/bls3, Bls4/ bls4, and Bls5/bls5 have been proposed (Duveiller et al., 1997a). Similarly, from 566 spring wheat landraces (Adhikari et al., 2012a) able to discover five novel genomic regions significantly associated with BLS resistance on chromosomes

1A, 4A, 4B, 6B, and 7D with the help of molecular markers. Using an identity by descent (IBD) mapping approach, (Kandel et al., 2015) detected two significant QTLs on spring wheat chromosomes 2A and 6B.

CHAPTER-III

3. High-Density Linkage Mapping of Bacterial Leaf Streak (BLS) Resistance in Bread Wheat

Abstract

Bacterial leaf streak (BLS) caused by *Xanthomonas translucens* pv.*undulosa* has become a serious disease of wheat (*Triticum aestivum* L) in the US Great Plains. To date, developing wheat cultivars resistant to BLS is the most sustainable method for BLS disease management. However, the genetic basis of BLS resistance that is found in few wheat lines largely uncharacterized. Herein we dissected the genetic basis of BLS resistance in wheat and identified of molecular markers linked to Quantitative trait loci (QTL) for BLS resistance. We employed genotyping-by-sequencing (GBS) for single nucleotide polymorphism SNP discovery, and simultaneous genotyping of all 92 F5 recombinant inbred lines (RILs) developed from crosses between SD52 BLS resistant parent and the susceptible parent SD1001. A genetic linkage map was developed from 1,211 SNPs and assembled in 36 linkage groups spanning 2,418 cM. We identified four major QTLs (LOD ≥2.3) for BLS resistance by composite interval mapping on chromosomes 2B, 6D, 7A, and 7B. The QTLs for BLS resistance *QBls.sdsu-2B-I* (15.5%)*, QBls.sdsu-7A-I* (14.1%) and *QBls.sdsu-7B-I* (6.1%), were derived from SD52 and aggregately explained a total of 32.8% of the variation. In addition, a disease increasing QTL, *QBls.sdsu-6D-I* was identified from SD1001 accounting for 14.8% of the total variation. The tightly linked SNP markers will facilitate marker-assisted selection for BLS resistance in wheat

3.1. Introduction

Bacterial leaf streak (BLS), caused by the rod-shaped, gram-negative bacterial pathogen *Xanthomonas translucens* pv. *undulosa* (*Xtu*), is a common disease in many wheat-growing regions worldwide (Akhtar et al., 1985; Alizadeh and Rahimian, 1989; Duveiller et al., 1992, 1997b; Maraite et al., 2007; McMullen and Adhikari, 2011; Sands and Fourrest, 1989; Vauterin et al., 1995). The risk of a BLS epidemic is high when the natural inoculum is abundant in soil or seed, and the plant is exposed to warm temperature under high humid condition. BLS usually causes sporadic yield loss that can reach up to 40% under favorable conditions (Byamukama, 2017; Duveiller and Maraite, 1993; Sands and Fourrest, 1989). Recently, BLS incidence above 80 % was recorded in wheat fields of ND, MN and SD in USA (Barnes et al., 2012; Byamukama, 2017). In addition to reduction in photosynthetic areas, the loss in yield and quality can result from the infection that causes shriveled kernel, reduction in seed protein content, seed weight and number of kernels per spike (Duveiller and Maraite, 1993; Stromberg et al., 1999; Tubajika et al., 1998, 1999). This pathogen largely seed and residue born and can also survive in freezing to warm climates. It infects the plant through stomata or wound openings which latter cause water-soaked lesion that looks yellow to dark brown longitudinal streaks between the leaf veins (Duveiller, 1997; Duveiller and Maraite, 1993; Kandel et al., 2012). Wind-driven rain splash, plant to plant contact as well as the occurrence of warm temperature under humid condition are among the major factors that may exacerbate the disease (Azad et al., 1988; Duveiller, 1997; Duveiller et al., 1991; Duveiller and Maraite, 1995). In addition to leaf, the infected stems and glume show longitudinal brown lesions and black chaff, respectively (Duveiller et al., 1997a).

With BLS, application of foliar products such as fungicides and antibiotic compounds alone have been proven not to have a significant effect (Duveiller, 1994; McMullen and Adhikari, 2011). Current BLS control measures focus on developing resistant varieties (Adhikari et al., 2011), the most sustainable and economically feasible method that depends on host-pathogen genetic interaction (Adhikari et al., 2012a; Duveiller, 1989; Duveiller et al., 1992; Kandel et al., 2015; Maraite et al., 2007; Tillman et al., 1996). BLS resistance reported being partial and with polygenic nature of inheritance. In recent years, some efforts have made towards genetic characterization and breeding of BLS resistance (Adhikari et al., 2012a; Duveiller et al., 1997a; Kandel et al., 2015). So far, only a few QTLs (Adhikari et al., 2012b, Kandel et al., 2015) conferring resistance to BLS are known in addition to five genes proposed by (Duveiller et al., 1997a). Adhikari et al., (2012b) genotyped 566 spring wheat landraces with DArT markers and reported five genomic regions significantly associated with resistance to BLS on chromosomes 1A, 4A, 4B, 6B, and 7D. Using an identity by descent (IBD) mapping approach, Kandel et al., (2015) also detected two significant QTLs on chromosomes 2A and 6B in spring.

Breeding for BLS resistance is still challenging due lack of precise knowledge of the inheritance mechanism of a quantitative trait that took several efforts to understand the genetic basis (Duveiller et al., 1992; Schielzeth and Husby, 2014; Tillman et al., 1996). Further, evaluation of wheat genotypes for BLS resistance by phenotyping alone is still very labor intensive and challenging in field conditions, and therefore its integration with marker-assisted selection can enhance the efficiency of the breeding programs in deploying BLS resistance in wheat (Mammadov et al., 2012). Currently, with the routine availability

of sequencing technologies, SNPs marker are becoming the marker of choice when compared to previously discovered once due to its amenability to high throughput genotyping platforms and genome-wide marker abundance suitable for the high-density genetic map (Mammadov et al., 2012; Wang et al., 2014b). We screened more than 500 genotypes from many breeding programs including CIMMYT and identified a few moderately resistant genotypes including SD52 (CNO79//PF70354/MUS/3/PASTOR/4/BAV92*2/5/HAR311).

In the present study, our objectives were to characterize BLS resistance in SD52 using 92 recombinant inbred lines (RILs), identify putative QTLs associated to BLS resistance, and to identify useful SNP markers linked to QTLs for marker-assisted selection. This work would not only to develop tools that are useful for marker-assisted selection (MAS) but also lays the foundation for fine mapping, map-based cloning, and deployment of BLS resistance in wheat.

3.2. Materials and Methods

3.2.1. Plant material and experimental design

Recombinant inbred line (RIL) populations generated by crossing SD52 (BLS resistant: CNO79//PF70354/MUS/3/PASTOR/4/BAV92*2/5/HAR311) × SD1001 (BLS susceptible: PFAU/MILAN//TROST) comprising of 92 individuals using single seed descent method (Knott and Kumar, 1975). A total of 92 F_5 RILs including the two parents were evaluated for response to BLS and planted in nursery containers (Stuewe and Sons, Inc., Corvallis, OR, USA) filled with professional growing mix (R360 metro mix, Sun Gro Horticulture Canada Ltd.). In each cone, four seeds were planted, and soluble fertilizer

(Miracle-Grow products Inc., Marysville, OH, USA) was applied every two weeks after planting. The two parents, SD52, and SD1001 were repeated six times in each experiment, and they were used as a resistant and susceptible check, respectively. The experiment was repeated three times, and the cone with seedlings of each genotype was arranged in a randomized complete block design (RCBD). Each cone in the rack was considered as an experimental unit, genotype as treatments, plants within a cone as sample replication and the fully extended third leaf on each plant was taken as the sampling unit.

3.2.2. Inoculation and disease assessment

A highly virulent strain of *X. translucens* pv.*undulosa* identified in South Dakota was provided by Dr. Shaukat Ali, SDSU, USA. The bacterium was cultured for 36 hours on 1 L concentration of modified King's B medium (20 g Agar, 15 g Glucose, 20 g Peptone protease, 1.5 g Magnesium phosphate, 1.5 g Potassium sulfate) on 100mmX15mm Petri dish (Fisher Scientific, USA). The inoculum was suspended in water, and the density was adjusted to approximately 3×10^8 colony forming units per ml using a portable Turbidimeter (Model 21907, Biolog Inc.USA).After 36-hours of growth, the inoculum was calibrated as follows. First, the inoculum was diluted in distilled water and counted using hemocytometer. Then, after serial dilution to 3 $x10^8$, the 20ml bacterium density was measured using the Turbidimeter. We obtained OD value of 40 as an indicator of the right concentration for our experiments.

Approximately five µL of inoculum was infiltrated into a fully expanded third- leaf of each plant using a needleless disposable syringe without wounding (Adhikari et al., 2012b; Mirlohi and Milus, 1994). The infiltrated areas were marked by a permanent marker to follow the progress of the disease beyond infiltration. Finally, inoculated plants were

kept in a moist chamber and misted regularly at an interval of 1 min every 30 min for 12 hours to maintain a humid environment for disease development. After 12–h the plants were transferred to growth chamber where the daily temperature was set to 26°C for 14 h, 19°C for ten h, and relative humidity maintained at 80% with a supplement of mist humidifier (SinFiltro, Kaz USA, Inc., www.vickshumidifi.com). The disease was assessed 14 days after inoculation according to rating scale (Adhikari et al., 2011, 2012a; Milus and Chalkley, 1994), in which 0 equals no visible symptoms, 1 equals chlorosis without water-soaked lesions, 2 equals water soaking less than 10%, 3 equals water soaking 10 to 30%, 4 equals water soaking 31 to 70%, 5 equals water soaking 71 to 100%, and 6 equals water soaking extending beyond infiltrated areas. Disease score of disease scores of 0 to 2 was considered resistant (Adhikari et al., 2012a, 2012b; Kandel et al., 2015; Tillman et al., 1996) whereas disease scores greater than 2 were regarded as susceptible. The genetic variance and broad sense heritability was calculated from ANOVA analysis (Holland et al., 2003)

3.2.3. DNA isolation and Ion Proton sequencing of GBS libraries

The RILs and the parent were grown in a greenhouse, and a sample of 100 mg fresh young leaf tissue was collected at 3-leaf stage. DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Zhang et al., 2010a). The resulting genomic DNA was quantified using a Quant-iT™ PicoGreen® dsDNA assay kit following the manufacturer's instructions (LifeTechnologies, Carlsbad, CA). The DNA concentration was normalized to 20 ng/µL per sample for subsequent genotyping. The genotyping was conducted at USDA Central Small Grain Genotyping Lab, Manhattan KS, USA. GBS libraries were constructed after complexity reduction using *Pst-I* and *Msp-I*

enzymes, and barcoding of individuals digested DNA samples of 92 RILs. One GBS library was developed by pooling of 95 barcoded samples (a pool of 92 samples, two parents, and one blank) following the protocol described by (Poland and Rife, 2012). The library was constructed using Ion Torrent library kits for subsequent template preparation. High-throughput automated template preparation and chip loading were done using the Ion Chef System. Next-Generation sequencing was conducted using Ion Proton™ Sequencer according to manufacture protocol (Life Technologies Inc, Thermo Fisher Inc, city USA).

GBS SNP calling

Raw sequence reads were processed for SNPs discovery using TASSEL reference based GBS pipelines (Bradbury et al., 2007). The SNPs were called after alignment against the wheat reference genome (IWGSC.org) with error tolerance rate at 0.01, 0.01/0.5 minimum/maximum minor allele frequencies (MAF), and 0/1 minimum/maximum call rates. SNPs with up to 20%, missing data points were retained for subsequent data analysis. The monomorphic SNPs among the parents of the RIL population were further filtered. Lastly the RILs genotypes with higher than 20% missing markers, markers or showing greater than 10% heterozygosity were removed to reduce the false positive results.

3.2.4. Linkage map construction and QTL analysis

Carthagene version 1.3 in LINUX environment (Mirlohi and Milus, 1994) was used to estimate locus orders, identify linkage groups, and transform estimated recombination frequencies to centimorgans (cM) using Kosambi function (Kosambi, 2016). Before grouping, markers that significantly deviated from the Mendelian expected ratios (p-value ≤ 0.05) were eliminated based on chi-square goodness-of-fit test. A LOD score cut-off of 6.0 at maximum recombination fraction 0.30 was used for assigning markers into

different linkage groups (LGs). Recombination frequencies of markers on the same LG were converted into map distances (cM) through the maximum likelihood (ML) algorithm. The visualized linkage maps and QTL based on composite interval mapping models (CIM) were performed using R/QTL version 3.3.3 (R Core Team, 2016). A minimum LOD threshold above which significant additive QTLs detected was calculated using 10,000 permutation tests (Doerge and Churchill, 1996) with a type 1 error of 0.05. The LOD support interval for all QTLs was a 0.01cM unit, and the P-value inclusion threshold was 0.001.

3.3. Results

3.3.1 Reaction of RIL population against BLS pathogen

A wide range of variation in reactions to bacterial leaf streak (BLS) was observed among 92 RILs. Frequency distribution of the RILs against the mean BLS disease score is presented in Figure 2 and Appendix Table 1. The reactions of RILs showed a significantly different ($p \leq$ 2e-16) response to BLS infiltration, whereas no significant difference observed between replication and between experiments (result not presented). The two parents showed significant differences in response to BLS infiltration (P ≤ 3.94e-15). The observed reaction score among RILs ranged from 2-6, whereas the as resistant and susceptible parent were scored as 2 and 6 respectively (Figure 2 and Appendix Table 1). Out of the 94 lines including the parents tested, 17(18%) of them had disease score of less than 2.0, whereas the other 77 (81%) of the lines had disease reaction ranging from 2 to 6. The frequency distribution of disease scores of the lines was nearly normally distributed, and the RILs population reactions were variable suggesting the trait might be regulated by several genes (Figure 2).

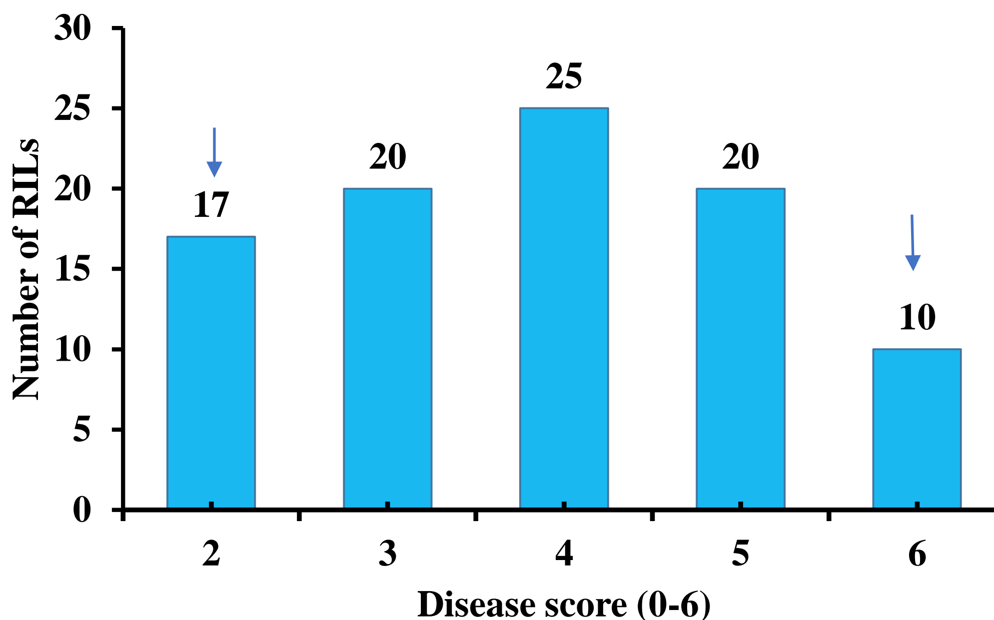


Figure 1. Frequency distribution of response to BLS in 92 recombinant inbred lines (RILs) from the cross SD52/SD1001. Arrows show the mean value of resistant (SD52) and susceptible parents (SD1001). Disease reactions on infiltrated areas (0.2 to 0.4cm2) on flag leaves were assessed 14 days after infiltration using a 0 to 6 rating scale (Adhikari et al., 2011, 2012a; Milus and Chalkley, 1994).The reaction was assessed in three experiments each having four plants unit as replication. None of the genotypes showed a score of 0 and 1.

The genetic variance and heritability of the BLS trait within all the RILs were 1.86 and 0.88 respectively under greenhouse conditions (Table 2). The repeatability of the experiment among parents and RILs are similar to the heritability due to low experiment error. The genetic variance and heritability of BLS from both parents alone were 3.76 and 0.97, respectively (Table 2).

Table 2. Genetic variance and heritability (H2) of both parents and their 92 progenies.

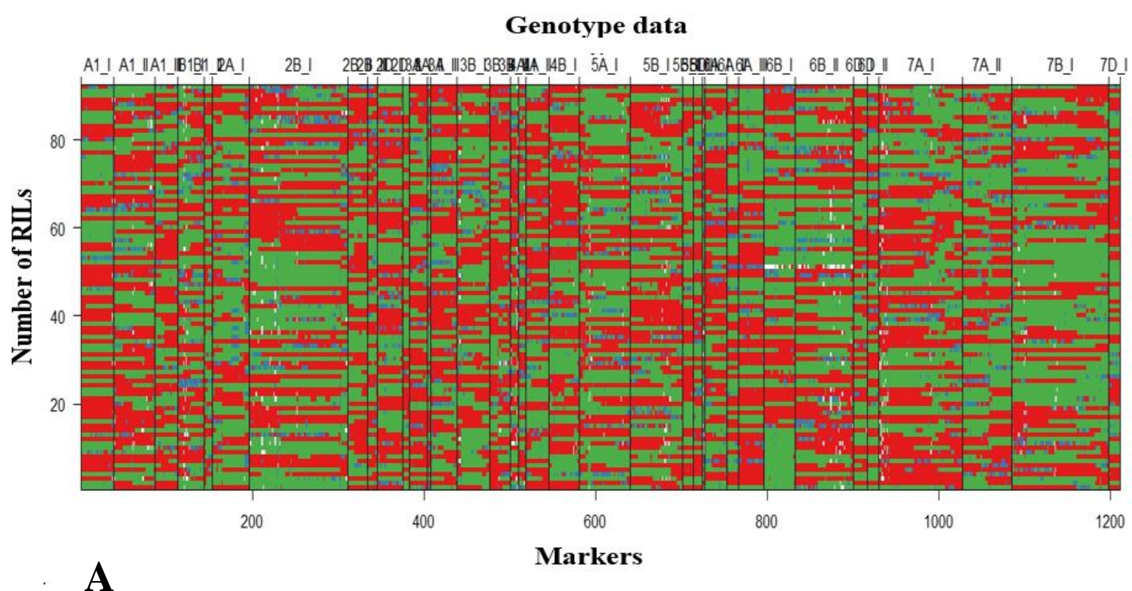
Genotype	Min score	Max score	Mean score	Genetic variance	Heritability (H^2) %
SD52 & SD1001	2	6	4.08	3.76	0.97
RILs (F_5)	1.8	6	3.83	1.86	0.88

3.3.2. SNP discovery and construction of genetic linkage map

We obtained 192,467 unique SNP markers with reference pipeline using IWGSC wheat genome assembly version 1.0 distribution on all 21 wheat chromosomes (Appendix Figure 1, Appendix Figure 2). The number of SNP markers polymorphic between the two parents of the mapping population had seen selected through the successive filtering process. A total of 52,382 out of the 192,467 SNP markers were found non-polymorphic between the two parents. Unexpectedly, some abnormal recombination sites (recombinant hotspots) on chromosomes 2B and 6B with recombination rates higher than 50% were removed (Figure 3A & 3B). After filtering marker with less than 20% missing data, minor allele frequency (MAF<0.05), segregation distortion (p<1%) a total of 1,211 high-quality SNPs were used in subsequent mapping analysis (Figure 3C). Considering all the genotypes, the proportion of AA, AB, and BB allele are 45.7 %, 5.8%, and 48.5%, respectively (Appendix Table 1).

A genetic linkage map was constructed with 1,211 high-quality SNP markers which represented 36 linkage groups across 19 wheat chromosomes, with 5 chromosomes represented by three linkage groups (Table 3 and Figure 3C). Enough number of

polymorphic markers with less than 20% missing data and meeting other filtering were not found on chromosomes 2D, 5D, 6D, and 7D. Largely the marker coverage on D genome was lower as compared to A and B genomes (Table 3, Figure 3C). The total genetic length of total linkage groups covers 2,418 cM for the whole genome with an average marker interval of 2.0 cM. The length of each linkage group (LG) ranged from 1.1cM on 2D_II to 233.3 cM on 7A_I with an average length of 123.24 cM (Table 2, Figure 4C). The number of SNP markers assigned to the A, B and D chromosomes were 531, 636 and 84, respectively. The number of markers in each linkage groups varied from 3 on 5D_I to 83 on 2B_I with an average number of 34 markers per each group. The marker density (marker/cM) ranged from 0.38 to 4.78, whereas the inter-marker distance ranged from 0.35 cM to 6.38 cM. The distribution of markers on each chromosome was not uniform, and a number of markers were lowest on the D genome (Table 3). Whereas the chromosome-wise, the average distance between SNP markers was smaller on A (0.82 cM) and B (0.52 cM) genome when compared to D genome.



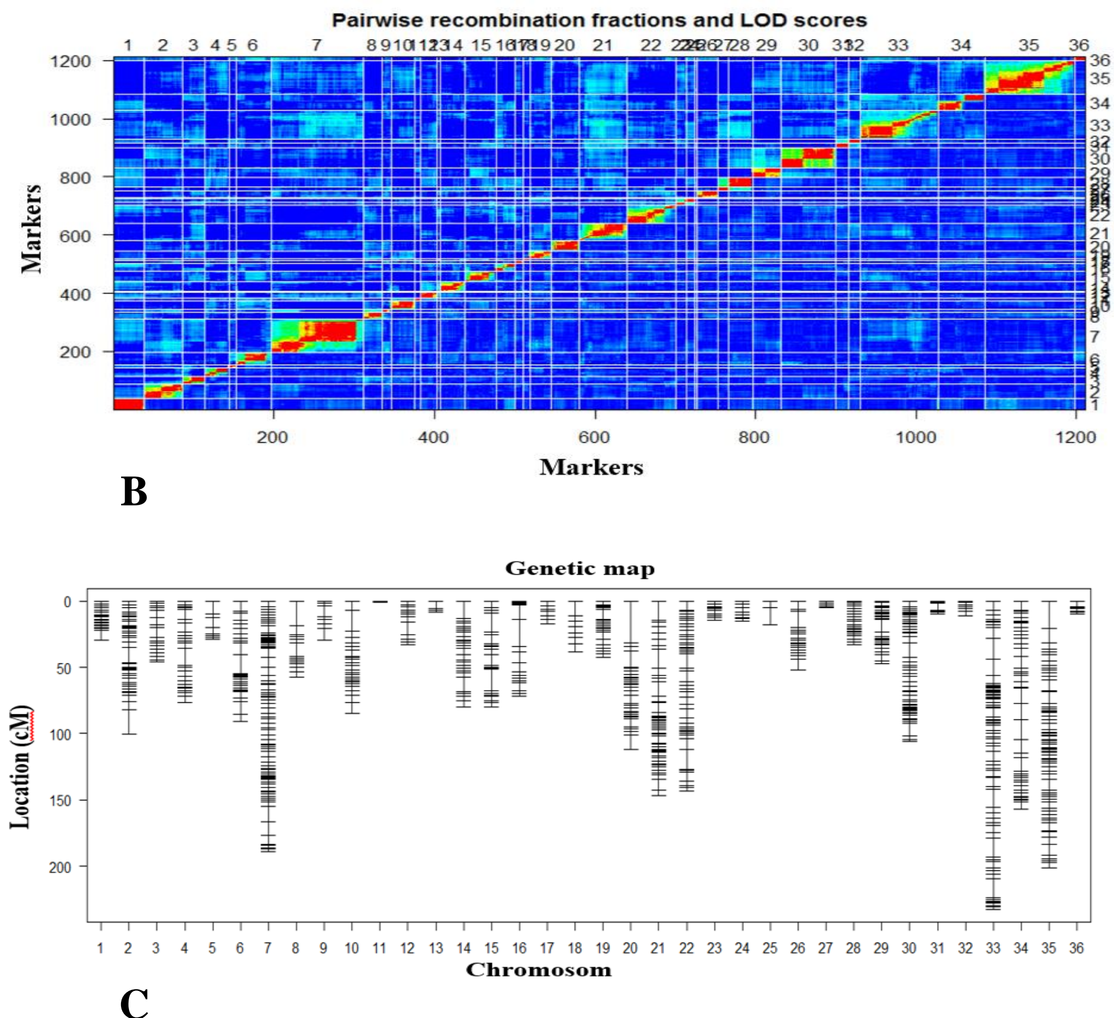


Figure 2. Map showing (A) recombination map (B) heat map and (C) linkage map constructed from 92 RILs and 1,211 SNP markers. White pixels in recombination map indicate missing data (A). Pairwise LOD linkage is displayed below the diagonal of the heat map, and the pairwise estimated recombination fractions are displayed above the diagonal. On heat map, blue color indicates a weak linkage, i.e., low recombination between markers commonly called as ‘cool spot, and red color indicated strong linkage and/or regions of recombination between markers also known as “hot spot” (B).

Table 3. A number of SNP markers and map length and marker density used for mapping QTL for wheat BLS resistance.

Linkage group	Number of Markers	Map length		Average distance between markers		Average no. markers per distance	
		cM	Mbp	cM	Mbp	cM	Mbp
1A_I	38	30.8	12.4	0.80	0.30	1.20	3.00
1A_II	48	100.6	77.0	2.10	1.60	0.48	0.62
1A_III	27	44.9	24.0	1.66	0.89	0.60	1.13
1B_I	10	28.5	531.3	2.85	53.13	0.35	0.02
1B_II	30	76.3	18.0	2.54	0.60	0.39	1.67
2A_I	43	90.5	64.0	2.10	1.49	0.48	0.67
2B_I	115	189.8	669.0	1.65	5.82	0.61	0.17
2B_II	23	57.5	17.1	2.50	0.74	0.40	1.35
2B_III	11	29.2	18.4	2.65	1.67	0.38	0.60
2D_I	30	84.8	51.0	2.83	1.70	0.35	0.59
2D_II	7	1.1	0.70	0.16	0.10	6.36	10.00
3A_I	21	32.6	8.10	1.55	0.39	0.64	2.59
3A_II	4	8.2	9.20	2.05	2.30	0.49	0.43
3A_III	31	79.7	116.0	2.57	3.74	0.39	0.27
3B_I	38	80.0	461.1	2.11	12.13	0.48	0.08
3B_II	24	71.9	50.9	3.00	2.12	0.33	0.47
3B_III	10	17.3	66.1	1.73	6.61	0.58	0.15
4A_I	8	38.2	13.6	4.78	1.70	0.21	0.59
4A_II	27	42.5	32.0	1.57	1.19	0.64	0.84
4B_I	35	111.7	459.6	3.19	13.13	0.31	0.08
5A_I	60	146.5	533.8	2.44	8.90	0.41	0.11
5B_I	61	143	479.0	2.34	7.85	0.43	0.13
5B_II	12	14.1	9.0	1.18	0.75	0.85	1.33

5B_III	11	14.8	6.0	1.35	0.55	0.74	1.83
5D_I	3	17.6	5.0	5.87	1.67	0.17	0.60
6A_I	26	51.7	14.7	1.99	0.57	0.50	1.77
6A_II	13	5.0	1.0	0.38	0.08	2.60	13.00
6A_III	30	33.2	9.0	1.11	0.30	0.90	3.33
6B_I	36	47.5	15.3	1.32	0.43	0.76	2.35
6B_II	68	106.3	622.8	1.56	9.16	0.64	0.11
6D_I	16	9.9	6.1	0.62	0.38	1.62	2.62
6D_II	14	11.3	0.9	0.81	0.06	1.24	15.56
7A_I	97	233.2	622.0	2.40	6.41	0.42	0.16
7A_II	58	156.9	61.3	2.71	1.06	0.37	0.95
7B	112	201.5	67.0	1.80	0.60	0.56	1.67
7D	14	9.7	3.1	0.69	0.22	1.44	4.52
A genome	531	1094.5	1403.5	2.1	2.3	0.5	2.1
B genome	596	1189.4	3490.6	2.0	7.7	0.5	0.8
D genome	84	134.4	66.8	1.6	0.7	0.6	5.6
Whole genome	1211	2418.3	4960.9	2.0	4.0	0.5	0.3

3.3.3 QTLs for BLS resistance

After assessing the homogeneity of data among experiments with Bartlett's test the phenotypic data for BLS response from all experiment were pooled and means of the experiments were used for QTL analysis.

Four QTLs on chromosomes 2B, 6D, 7A, and 7B for resistance against BLS (Table 4, Figure 4) were identified with composite interval mapping (CIM) performed with R-QTL (REF). Four SNPs with significant LOD scores linked to four QTLs (*QBls.sdsu-2B-*

I, *QBls.sdsu-6D-I*, *QBls.sdsu-7A-I* and *QBls.sdsu-7B-I*) explained a total phenotypic variation (R^2) of 47% (Table 3). The source of QTLs on 7A, 7B, and 2B was resistant parent SD52, whereas QTL on 6D was derived from susceptible parent SD1001. The two BLS resistance QTLs *QBls.sdsu-2B-I* (S2B_654732199) and *QBls.sdsu-7B-I* (S7B_620991434) were most consistent and most significant SNPs explained a variation of 15.8% and 10.9 % of the phenotypic variation (Figure 4, Table 3). The *QBls.sdsu-2B-I*, *QBls.sdsu-7A-1, QBls.*sdsu-7B-I were flanked to 4.6 cM (4.3 Mb), 1.1 cM (0.5 Mb), 2.8 cM (8.3 Mb) respectively (Table 4). On the hand the disease increasing QTL *QBls.sdsu-*6D-1 was flanked by marker interval S6D_19567942 to S6D_20468157 (1.6 cM) was stably detected in all the experiments, explaining 14.1 % of the phenotypic variances. This QTL had a positive additive effect (0.529) derived from SD1000 at logarithm of odds (LOD) 3.85 (Figure 3 Table 4). The most significant SNP (S6D_19567889) segregated with *QBls.sdsu-6D-1* and physically 0.9 Mb from the QTL region (Table 4, Figure 3).

1,211 markers.

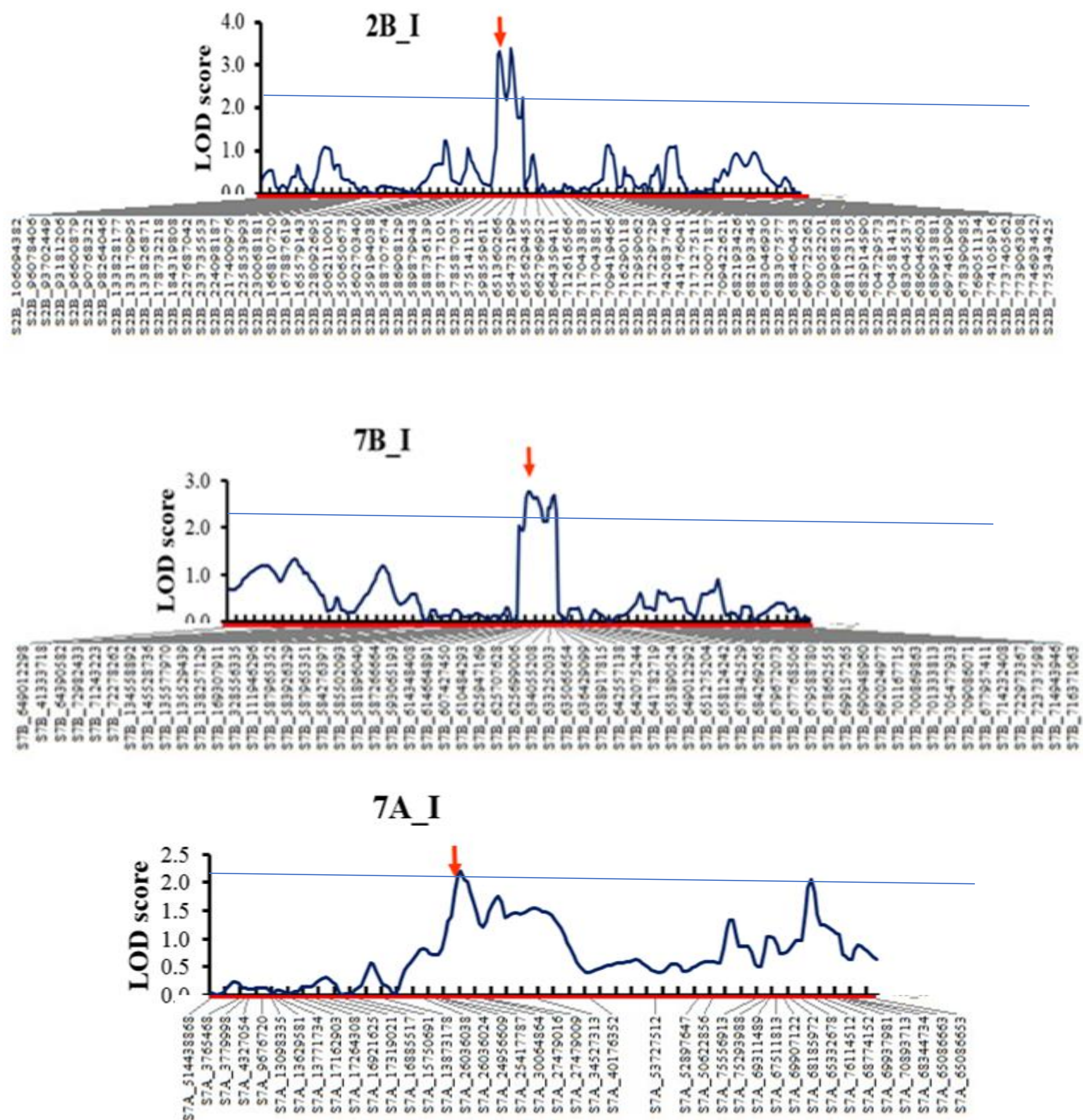


Figure 3. Scatter plot of the genetic map and LOD score of loci on wheat chromosome 2B, 7A, and 7B.Marker names are shown on the left side and distance in centimorgan (cM) shown in left side of each linkage group. The physical position of each marker in hexaploid wheat reference genome IWGSC-WGA1.0 are represented by the numbers in marker names.

Table 4. Quantitative trait loci (QTL) for bacterial leaf streak resistance and significant SNPs.

Marker	QTL	Allele	Chr	Position on LD	LOD[a]	Marker interval			PVE[c]	Additive effect [d]
						Marker [b]	cM	Mbp		
S2B_654732199	*QBls.sdsu-2B-1*	T/C	2B_I	85.5	4.79	S2B_651360266/ S2B_655629455	4.6	4.3	15.8	(-)0.4861
S6D_19567889	*QBls.sdsu-6D-1*	C/A	6D_II	3.9	3.85	S6D_18898228 / S6D_20468354	1.6	1.6	14.1	(+)0.4748
S7A_24956609	*QBls.sdsu-7A-1*	G/C	7A_II	55.0	2.39	S7A_24956609/ S7A_25417787	1.1	0.5	6.1	(-)0.1208
S7B_620991434	*QBls.sdsu-7B-1*	T/A	7B_I	108.1	2.75	S7B_625699006/ S7B_634055208	2.8	8.4	10.9	(-)0.3403

[a] A LOD threshold 2.3 was used for declaration of QTL, based on 10,000 permutations at a significance level of 0.01.

[b] Marker interval distance was calculated at 0.001 LOD to unite from the peak position. The numbers within the names of marker indicted the physical distance (Mb) on the chromosome.

[c] Phenotypic variance explained by QTL.

[d] Positive “additive effect” indicated BLS decreasing allele derived from SD1001; negative “additive effect” indicated BLS increasing alleles derived from SD52

3.4. Discussion

3.4.1 Inheritance of BLS resistance

The genetic resistance is the most sustainable and effective method to manage bacterial diseases including BLS (Adhikari et al., 2012a, 2012b; Duveiller, 1989; Duveiller et al., 1992; Kandel et al., 2012, 2015; Maraite et al., 2007; Tillman et al., 1996). Analyses of variance among RILs from the cross SD52/SD1001 revealed significant differences among the progeny for their reaction to BLS, indicating that their reaction was genotype-dependent. Further, the reaction of RILs population to the BLS pathogen showed a continuous unimodal distribution that implying quantitative inheritance of BLS resistance. The genetic basis of resistance to BLS has been described as multiple genes with quantitative effects with low to medium heritability in wheat, rice and several other crops (Adhikari et al., 2012a; Duveiller et al., 1992; Johnson et al., 1987; Kandel et al., 2012; Maraite et al., 2007; Tillman et al., 1996). A generation with less or better phenotypic performance outside the performance value of the parental are transgressive segregates (Rieseberg et al., 1999). This kind of phenomena is very common in quantitative traits determined by additive genes that are already homozygous at loci that control the traits. The parents used in our studies belong to two extremes of resistance. The mid-parent values for BLS traits were nearly the same as the mean of all the RILs population. However, we did not observe any RILs showing reaction beyond the extremes of the two parents suggesting insufficient evidence to detect the transgressive segregation that might be due to the smaller population size in our study. Broad-sense heritability was estimated for both parents and RILs populations. The estimates were high for all the population because of the controlled environments.

3.4.2 A high-density linkage map and genome coverage

High-density genetic linkage maps are essential for QTL fine mapping. GBS can produce enough information for powerful QTL mapping in bi-parental populations. However, the sequencing coverage required to generate a dense marker across the whole genome remains uneven and affected by GC bias, duplicated positions, trans-chromosomal rearrangement (Yang e al., 2017; Liu et al., 2014). Nevertheless, GBS is rapid and cheaper method for generating a large number of de novo SNPs for high-density genetic mapping (Poland et al., 2012). Moreover using high-quality SNPs with <20% missing data without imputation also proposed although it may likely lead to some essential SNPs being overlooked (Yang e al., 2017; Liu et al., 2014). In this study, we constructed a high-density and high-resolution genetic linkage map with 1,211 single locus SNP markers covering 2,514.8 cM of the whole genome. The map length of the linkage map is comparable with most of the previous maps of wheat which ranged from 2371.40 to 3213.2 cM (Paillard et al., 2003; Wu et al., 2015). The SNPs that were identified were used to construct a high-density map at an average marker density 0.5 cM which is comparable to those reported earlier (Anderson et al., 2017; Verma et al., 2015). However, due to difficulties in detecting polymorphic markers in D chromosomes, the linkage map did not cover the entire wheat genome. Lower coverage of markers on D genome has been well reported in different studies (Adhikari et al., 2012a; Akhunov et al., 2009, 2010). Recent origin of wheat (6-8000 years) (Giles and Brown, 2006; Gill et al., 1991) with only one or two events of tetraploid wheat hybridization with *Aegilops tauschii* lead to the origin of hexaploid wheat

and lead to lower diversity in D genome of wheat as compared to A and B genome (Chowdhury et al., 2013; Gill et al., 1991; Li et al., 2015; Mujeeb-Kazi et al., 1996)

3.4.3 Identification QTL for BLS resistance

The high-density linkage map developed in this study enabled us to identify QTLs with more accuracy and high resolution. A total of four: *QBls.sdsu-2B-1, QBls.sdsu-7A-1, QBls.sdsu-7B-1* and *QBls.sdsu-6D-1* QTLs for BLS were detected which explained phenotypic variance ranging from 6.1% to 15.8% with a total of 47 % of the phenotypic variation explained. It is probable that only the largest effects were identified, and many small QTLs were not significant. There QTLs (*QBls.sdsu-2B-1, QBls.sdsu-7A-1, QBls.sdsu-7B-1*), induced resistance response against BLS whereas one QTL on 6D (*QBls.sdsu-6D-1)* contributes negatively to the BLS resistance that needs attention while clustering favorable multiple alleles during gene pyramiding.

In our study, we identified four novel significant genomic regions linked to BLS resistance were identified. Adhikari et al., (2012b) studied 566 spring wheat landraces reported five genomic regions significantly associated with resistance to BLS on chromosomes 1A, 4A, 4B, 6B, and 7D. Whereas Kandel et al., (2015) using identity by descent (IBD) mapping approach detected two significant QTLs on spring wheat chromosomes 2A and 6B. In our study we did not identify any of the QTLs reported in previous studies (Adhikari et al., 2012b, Kandel et al., 2015). Additionally, in the past, five genes named as *Bls1-BLs5* conferring resistance in five wheat lines (Turaco, Alondra, Angostura, Mochis and Pavon) were reported (Duveiller et al., 1992). However, in the absence of any genetic mapping, their chromosome location cannot be compared.

3.4.4. Implications of high-density SNP markers for disease resistance breeding

With availability of next-generation sequencing and SNP genotyping technologies (He et al., 2014) SNP based markers have become extremely popular in genetics due to their genome-wide abundance, construction of saturated genetic map and their ability to capture variation in several parents within a short time (Korte and Farlow, 2013; Miedaner and Korzun, 2012).

BLS resistance is determined by several minor genes with small individual effects at given locus (Duveiller et al., 1992; Schielzeth and Husby, 2014; Tillman et al., 1996; Xu et al., 2017). Developing high quality of molecular can assist in assembling multiple desirable alleles from multiple parents through gene pyramiding into a single genotype can improve BLS resistance. We identified significant SNPs linked to the four genomic regions associated with BLS resistance. The SNPs flanking the QTLs can be used to develop Kompetitive Allele Specific PCR (KASP) for marker-assisted selection (MAS) as evidenced in several crops (Semagn et al., 2014).

3.5. Summary

Bacterial leaf streak (BLS) caused by *Xanthomonas translucens pv.undulosa* is an emerging disease of wheat (*Triticum aestivum*) in several areas. Our objectives in the present study were to identify and characterize quantitative trait loci (QTL) associated with BLS resistance in the wheat bi-parental mapping population. In total, 92 F_5 generation RILs mapping population developed from crosses of SD52 × SD1001 were evaluated in a greenhouse in three experiments. The total of 92 RILs comprising their parents was genotyped using Ion Proton system, and 1,211 high-quality single nucleotide

polymorphisms (SNP) markers were extracted for construction of linkage map. A Map with 36-linkage groups covered 2514.15 cM across the whole genome of wheat was constructed. Composite interval mapping models (CIM) identified four genomic regions on chromosomes 2B, 6D, 7A and 7B affecting BLS resistance in the RIL population. The three disease reducing QTLs designated as *QBls.sdsu-2B-1*, *QBls.sdsu-7A-1* and *QBls.sdsu-7B-1* were derived from SD52 and aggregately explained 32.8% of the variation, whereas the fourth QTL (*QBls.sdsu-6D-1*) was derived from SD1001. We identify SNP marker linked to all four QTLs to facilitate marker-assisted selection for BLS resistance in wheat.

3.6. Conclusions

In the present study, we identified SNP markers linked to four genomic regions on chromosomes 2B, 6D, 7A and 7B responsible for BLS resistance. We propose the pyramiding of multiple QTLs that have similar effects. The SNP markers identified in this study can have a direct relevance for marker-assisted breeding for BLS resistance in wheat and map-based cloning.

Acknowledgements

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CHAPTER-IV

4. Genome-Wide Association Study for Spot Blotch Resistance in Hard Winter Wheat

Abstract

Spot blotch caused by *Cochliobolus sativus* (anamorph: *Bipolaris sorokiniana*) is an economically important disease of wheat worldwide. Under a severe epidemic condition, the disease can cause yield losses up to 70%. The previous approach of identifying spot blotch resistant QTL was only limited to a portion of the available genetic diversity, low capacity to detect polygenic genes and less marker density. In this study, we carried out a genome-wide association study (GWAS) of spot blotch resistance in hard winter wheat association mapping panel (HWWAMP) of 294 genotypes. The HWWAMP *was* evaluated for response to *Bipolaris sorokiniana* (isolate SD40), and range of reactions was observed with 10 highly resistant, 47 moderately resistant and 241 moderately susceptible to susceptible genotypes. Through GWAS using 15,590 high-quality SNPs and 294 genotypes we identified six QTLs (p= <0.001) on chromosomes 2D, 3A, 4A, 4B, 5A, 7B that collectively explained 30% of the total variation. We identified three QTLs *QSb.sdsu-7B.1* (SNP: TA005844-0160, r^2 = 6%), *QSb.sdsu-5A.1* (SNP: Kukri_rep_c104877_2166, r^2 = 6%) and *QSb.sdsu-2D.1* (SNP: Kukri_c31121_1460, 4%) which are located in genomic regions associated with SB resistance in earlier studies. Our study validates these QTLs and provides SNP markers for markers assisted selection. In addition three novel QTLs *QSb.sdsu-4A*.1 (SNP: IWA8475, r^2 = 5.5%), *QSb.sdsu-4B.1* (SNP: Excalibur_rep_c79414_306, r^2 = 4 %) and *QSb.sdsu-3A.1* (SNP: IAAV2383, r^2 = 4 %) were identified in this study. Comparative analysis with barley indicated the resistance

locus on wheat chromosomes 2D, 3A, 5A and 7B in this study are syntenic to the previously identified locus for barley spot blotch resistance on chromosomes 2H, 3H, 5H, and 7H. The ten genotypes highly resistant spot blotch and the linked SNP markers identified in our study could be very useful resources for breeding for spot blotch resistance in wheat. Further our gene annotation analysis on the candidate regions identified several NBS-LRR and related protein families across multiple QTLs, and these could be used for fine mapping and better characterization of spot blotch resistance in wheat.

4.1. Introduction

Wheat (*Triticum aestivum* L) is an important cereal crop grown worldwide and remains a vital source for human food (FAOSTAT, 2016). Despite this, however, its production is continuously challenged by a number of environmental and biological factors (Duveiller et al., 2005, 2007a). Spot blotch (SB) caused by *Bipolaris sorokiniana* (Sacc.) Shoem syn. *Drechslera sorokiniana* (Sacc.) Subrm and Jain (syn. *Helminthosporium sativum*, teleomorph *Cochliobolus sativus*) is one of the destructive fungal disease that affects wheat and several small gains worldwide (Chowdhury et al., 2013; Dubin and Rajaram, 1996; Duveiller et al., 2005, 2007a; Duveiller and Dubin, 2002; Duveiller and Gilchrist, 1994; Gurung et al., 2009; Joshi and Chand, 2002; Kumar et al., 2015b; Sharma et al., 2007b; Singh and Singh, 2007). It has a wide range of hosts within wild and cultivated *Poaceae* (Kumar et al., 2002; O'Boyle et al., 2014; Pandey et al., 2005; Shandikov and Eakin, 2013). In susceptible lines, SB symptom is characterized by small, dark brown lesions that extends to1-2 mm long without chlorotic margin during initial infection (Chand et al., 2003; Duveiller et al., 2005). Later, the leaf is killed when the light brown to dark brown color oval to elongated blotches extend and merge very quickly. In addition to leaves, the fungus causes common root rot (Wildermuth et al., 1997), seedling blight and seed rot or black point on embryo (Duveiller and Gilchrist, 1994; Hudec et al., 2008; Kumar et al., 2002; Zillinsky and others, 1983). Average yield loss 15–20% due to SB has been reported from several countries, but under suitable climatic conditions the losses in yield can reach 40–70% in susceptible genotypes in addition to the reduction in seed quality (Acharya et al., 2011; Duveiller, 1998; Fernandez et al., 1998, 2014; Fernandez and Jefferson, 2004; Gurung et al., 2012; Lemerle et al., 1996; Mehta et al.,

1992; Sharma et al., 2007b, 2004; Sharma and Duveiller, 2007; Siddique et al., 2006; Wang et al., 2002).

Breeding for resistance is one the most economical and sustainable component of integrated disease management (Ban et al., 2016; Bartoš et al., 2002; Chowdhury et al., 2013; Crute and Pink, 1996; Duveiller et al., 2007b; Gupta et al.; Khan et al., 2010; Kumar et al., 2017; Vasistha et al., 2016). However, our ability to deploy and develop spot blotch resistant genotype depends on an understanding of the mechanism of resistance present in the host and identification of the resistant gene responsible for the traits. Given the challenges in large-scale germplasm screening (Kumar et al., 2017; Leng et al., 2016; Osman et al., 2016), development of molecular markers linked to disease resistance genes can facilitate marker-assisted selection (MAS) and increase the efficiency of breeding for disease resistance in wheat (Collard et al., 2005; Gupta et al., 2010; Miedaner and Korzun, 2012; Müller et al., 2017).

With the availability of large number of molecular markers (Korte and Farlow, 2013; Miedaner and Korzun, 2012) more efficient mapping techniques like genome-wide association studies (GWAS) have become popular for analyzing an unlimited number of traits in genetically identical material across a wide range of environments (Kushwaha et al., 2017). GWAS has been used to characterize disease resistance in many crop species: blast resistance gene in rice (Raboin et al., 2016), maize (Xiao et al., 2017), spot blotch resistance in wild barley (Roy et al., 2010), resistance to multiple leaf spot diseases of spring wheat (Gurung et al., 2014), resistance to bacterial leaf streak and spot blotch in spring wheat (Adhikari et al., 2012b), *Fusarium* head blight resistance in wheat (Arruda et al., 2016), tan spot resistance in European winter wheat (Kollers et al., 2014), mapping for

resistance to leaf and stripe rust in winter-habit hexaploid wheat landraces (Sun et al., 2015).

Complex quantitative inheritance (Dubin and Rajaram, 1996; Joshi et al., 2004b; Kumar et al., 2015b) of SB resistance in wheat has slowed the progress in breeding for SB resistance. Many studies, using methods of both bi-parental mapping (Anderson et al., 1993; Collard et al., 2005) and association mapping (AM) (Ersoz et al., 2007; Gupta et al., 2005; Korte and Farlow, 2013; Miedaner and Korzun, 2012; Ogura and Busch, 2015) have reported several spot blotch resistance QTLs on chromosome 2A, 2B, 2D, 3B , 5B and 6D, 7D (Adhikari et al., 2012; Gonzalez-Hernandez et al., 2009; Kumar et al., 2009b, 2010a, 2015a, 2016, 2017 ; Nair et al., 2015; Neupane et al., 2007; Sharma et al., 2007a; Zhuang et al., 2013). However, only three major QTLs designated as *Sb1* on 7D (Lillemo et al., 2013), *Sb2* on 5B (Kumar et al., 2015a), and *Sb3* on 3B (Lu et al., 2016) are well described. Most of these studies have been focused on hard spring wheat, and relatively few studies characterized SB resistance in hard winter wheat germplasm.

Our ability to deploy and develop spot blotch resistant winter wheat cultivars depends on identification of resistant QTL responsible for the traits. Therefore, the objectives of this study were i) to identify winter wheat genotypes carrying resistance genes against *Bipolaris sorokiniana* and ii) to locate putative QTL and identify SNP markers useful for marker-assisted selection. This work will contribute towards the development of genome-wide breeding strategies and marker-assisted selection (MAS) for spot blotch resistance in wheat.

4.2. Materials and Methods

4.2.1. Plant materials

A set of 294 a hard winter wheat association mapping panel (HWWAMP) of 300 winter wheat accessions (Appendix Table 3) developed under the USDA TCAP project (Guttieri, 2014). The genotypes were comprised of landraces, advanced breeding lines and varieties released since 1940's representing USA Great Plains areas. The AM panels were designed to include genotypes that represent the existing germplasm of the wheat growing regions of the United States such as Texas, Colorado, Kansas, Nebraska, North Dakota, South Dakota, Oklahoma, Montana and Michigan (Grogan et al., 2016; Guttieri, 2014).

4.2.2. Evaluating HWWAMP for spot blotch

The screening of 294 genotypes and the susceptible check Glenlea and resistant check Salamouni against *Bipolaris sorokiniana* were conducted at the South Dakota State University young brother seed technology greenhouse complex in Brookings, SD, U.S.A. The experiment was conducted in a randomized complete block design (RCBD) and repeated three times. Three seeds of each genotype were planted in a single root trainer containers (Ray Leach "Cone-tainer"™ Single Cell System) and rearranged within each tray (Stuewe & Sons, Tangent, Oregon, USA). Plants were grown in a greenhouse at 24°C /16°C (day/night) with 14-hour photoperiod and 32 relative humidity. Soluble fertilizer was added every two weeks days after planting and watering were scheduled every two days.

One of the *Bipolaris sorokiniana* isolate, SD40, was used as inoculum in all experiments. SD40 is predominantly found in Great Plains of USA and routinely used to

screen breeding materials (provided by Dr. Shaukat Ali, SDSU). The isolate was originally derived from a single spore and methods of its isolation and cultivation is well described by (Kumar et al., 2007). This fungus is easily identifiable based on the color morphology and number of nuclei in lab condition. The stored conidia's of the fungus were streaked on per liter V8-PDA (agar: 10g/l, Defco PDA: 10g/l, Calcium carbonate 1.5g/l, V8 fruit juice: 150ml/l,) plates with a sterile rode. The isolates were grown at room temperature under continuous darkness and harvested five days later when the pink colony in the plate began to darken. The spore in the plate was scraped, mixed with distilled water and transferred to beaker covered by cheesecloth to filter out debris and congested spores with mycelium.

All the seedlings were sprayed with spore suspension calibrated at 3500 spore/ml in the sterile distilled water on a fully expanded third leaf of the plants. A 100 µl/L Tween-20 (polyoxyethylene-20-sorbitan monolaurate (Sigma-Aldrich, St. Louis, MO, USA) was added to spore suspension as a dispersing agent. Approximately, 0.5ml of inoculum suspension was applied to each plant using the sprayer. After inoculation, seedlings were incubated separately in darkness for 12 h at 20°C in mist chamber near 100% RH for 20 min to establish a layer of free moisture on the leaf surfaces.After that, the humidifiers were set to release the mist for 2 minutes every 30 minutes to maintain a humid environment for disease development. Subsequently, plants were transferred to greenhouse complex where the temperature was regulated at 24°C /16°C (day/night) until the disease was rated ten days after inoculation.

The infection responses (IRs) of each genotype against the pathogen was assessed based on the five-class (1–5) rating scale used by (Lamari and Bernier, 1989). IRs were

based on the type (presence of necrosis and chlorosis) and relative size of lesions observed on the third leaves of wheat seedlings. The third fully expanded leaf during inoculation was rated as follows: 1 small dark brown to black spot without any surroundings chlorosis or necrosis (resistant), 2 small dark brown to black spot with very little necrosis or chloric (resistant), 3 small dark brown to black spot completely surrounded by distinct chlorosis or tan necrosis ring, lesions generally not coalescing (moderately resistant to moderately susceptible), 4 small dark brown to black spot completely surrounded by distinct chlorotic or tan necrosis zone and some of the lesions coalescing (moderately susceptible), 5 the dark brown or black centers may not be distinguishable, most lesions consists of coalescing chlorotic or tan necrotic zones (susceptible). The infection type corresponding to each score in our experiment is shown in Figure 5.

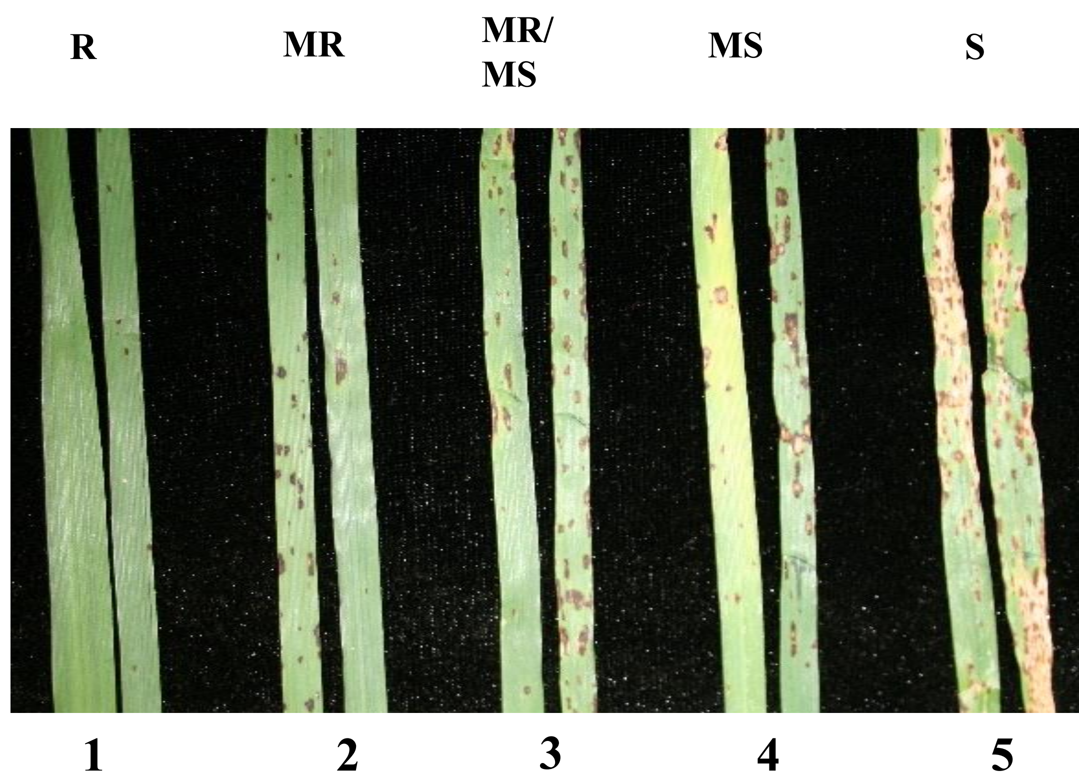


Figure 4. Pictorial representation of Spot blotch rating scale (1-5) in wheat

ANOVA

Distribution of the phenotypic data for spot blotch was visualized using the histogram. The Shapiro-Wilk test was conducted in R to check the normality of the untransformed and transformed data based on square root method. The homogeneity of the variance across experiments was cheeked using Bartlett's test. Data from the different experiment were combined, and the total mean of the experiments was used for analysis of GWAS if the experiment is homogenous. The phenotype data were analyzed using linear mixed model (LMM) approaches with a randomized group based jackknife technique using R version 3.3.3 (R Core Team, 2016). Board sense heritability (H^2) was estimated by dividing genetic variance over the combined sum of error variance and genetic variance.

4.2.3. Imputation and filtering of SNP markers

The genotype data were obtained from the wheat T3 Toolbox a public repository (http://triticeaetoolbox.org). The genotyping was conducted using Illumina iSelect 90K under the USDA-TCAP (Guttieri, 2014; Guttieri et al., 2017). To avoid spurious marker-trait associations, SNP markers with MAF < 0.05 and missing data >10% were excluded from further analyses. The genetic and physical positions of SNP markers from the wheat 90 K array were obtained from the consensus map with 46,977 SNPs developed using a combination of 8 mapping populations (Wang et al., 2014) and the International Wheat Genome Sequencing Consortium website (http://www.wheatinitiative.org) (Jia et al.). After filtering the high-quality polymorphic SNPs, markers were imputed using TASSEL Version 5.0 software (Bradbury et al., 2007).

4.2.4. Population structure and kinship

In order to avoid the distortion of population structure and linkage disequilibrium (LD), SNP markers were first thinned into 0.0005cM apart to retain only markers with high pairwise correlation using TASSEL v5 software (Bradbury et al., 2007). After keeping informative SNPs in the analysis and eliminating redundant information, we analyzed the genetic stratification, i.e., population structure (Q) within the HWWAMP with STRUCTURE v2.3.4 (Pritchard et al., 2000) using a model-based clustering method. STRUCTURE runs were performed for each specified K values (number of subpopulations, from 2 to 6) using the default setting of the admixture model for the ancestry of individuals and correlated allele frequencies. Burn-in period and a number of Markov Chain Monte Carlo (MCMC) iterations under Linux environment were set to 20,000 and 50,000, respectively (Evanno et al., 2005). The best fit number of clusters was calculated according to Evanno et al., (2005) using STRUCTURE HARVESTER (Duncan et al., 2017). The likely number of population structure was chosen from principal coordinates (PCO) plot, i.e., K vs. ΔK where the rate of change in the log probability between successive K values was the highest.

4.2.5. Linkage disequilibrium estimation

Linkage disequilibrium (LD) is defined as the non-random association of alleles at different loci in a given population is represented by the square of the correlation coefficient (r^2) between markers. Markers which were in perfect LD (r^2=1) with another markers' were removed before the LD analysis. The r^2 between intra- and inter-chromosomal SNP markers were estimated using TASSEL v5 (Bradbury et al., 2007). LD Estimates expressed as r^2 and based on a sliding window of 100 markers throughout the

genome, were calculated and plotted against genetic distance. From the unlinked loci, two markers were considered significant when LD P<0.001. We plotted the intra-chromosomal r^2 values against the genetic distance using excel to see how rapidly the LD decay occurs. The distance at which the smooth curve intercepts the critical r^2 was drawn using logarithmic trended smooth lines as described by (Hao et al., 2012). A critical value of r^2 (basal LD) was estimated using 95% percentile of non-synthetic (inter-chromosomal) r^2 distribution below which relationship between two pairs of loci are assumed not to be caused by physical linkage (Laido et al., 2014). The distance at which the LD decays to 0.7 cM was considered as the critical distance up to which a QTL region can extend (Zhao et al., 2005).

4.2.6. Genome-wide association analysis

Genomic regions associated with spot blotch resistance were identified using TASSEL v.5.0 (Bradbury et al., 2007) and the new enhanced version of genome association and prediction integrated tool (GAPIT) (Tang et al., 2016) in R version 3.3.3 (R Core Team, 2016). Enhanced version of GAPT implements computationally powerful statistical approaches such as general linear model (GLM), mixed linear model (MLM) (Zhang et al., 2010b), compressed mixed linear model (CMLM) (Li et al., 2014), enhanced compressed mixed linear model (ECMLM) (Li et al., 2014), factored spectrally transformed linear mixed models (FaST-LMM Select) (Listgarten et al., 2013) and SUPER (Wang et al., 2014a). In GLMs, marker data, disease data and the PCA matrix were integrated as covariates to correct for the effects of population substructure. Unlike GLM, MLM accounts for both population structure and individual kinship as a covariate to reduce type-I error.

We selected the MLM method for our data by comparing the statistical power they have and type I error they produce (result not presented). The mixed linear model for GWAS were represented by $y = X\beta + Qv + Ku + e$, where y is the vector of the phenotypic values, β is fixed effects due to marker, v is fixed effects due to population structures, e is the vector of residuals, and u is a vector of random effects due to the portion of breeding values not accounted by the marker. X, Q, and K represent matrices from the marker, population structure estimated from the structure or principal component analysis and kinship, respectively. The variance of u is derived as, $Var(u) = 2KV_g$, where K represents the relative kinship matrix inferred from genotypes of the HWWAMP based on the proportion of shared alleles and Vg is the additive portion of the genetic variance.

4.2.7. In silico annotation of SNPs and syntenic regions

The sequences of the significant markers associated with SB was extracted from the Infinium iSELECT 90K (Cavanagh, 2013) and were blastn against the CS wheat RefSeq (IWGSC 2017, (https://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies). The search was limited to the top hit with an E-value cut off of 1E-50 with an identity higher than 75%. Being allohexaploid species ($2n = 6x = 42$) wheat chromosomes are found in homeologous status (A, B, D) which shared similarities. Therefore, we removed SNPs that were mapped to multiple chromosomes.

We further identified the target region for each of the QTLs on pseudomolecule that co-localized with the significant markers contained in each LD block. Next, the sequence segments were blastn searched against the wheat coding DNA sequence (CDS) and followed by tbalstn against the wheat protein (Duncan et al., 2017). Out of several lists of an annotated protein family, those related to previously described disease resistance

protein families were further identified by a blastn search against Pfam database (Pfam 31.0, https://xfam.wordpress.com/2017). We then compared the candidate regions with barley for comparative analysis of spot blotch resistance genes across related species and produced synteny representation using CIRCOS (Krzywinski et al., 2009).

4.3. Results

4.3.1 Phenotypic variations in seedling infection response

The seedling infection responses of 294 hard red winter wheat genotypes against *B. sorokiniana* are shown in Figure 6 and Appendix Table 1. Seedling showed a range of infection types within wheat genotypes when inoculated with *B. sorokiniana*. Based on a 1-5 scoring system on an inoculated fully extended third leaves (Figure 5), we considered all accessions with a score below or equal to two (corresponding to incompatible reactions) as resistant, and the remainder as susceptible. The resistant check Salamouni and susceptible check Glenlea exhibited mean disease score of 2 and 5, respectively as expected (Figure 6)

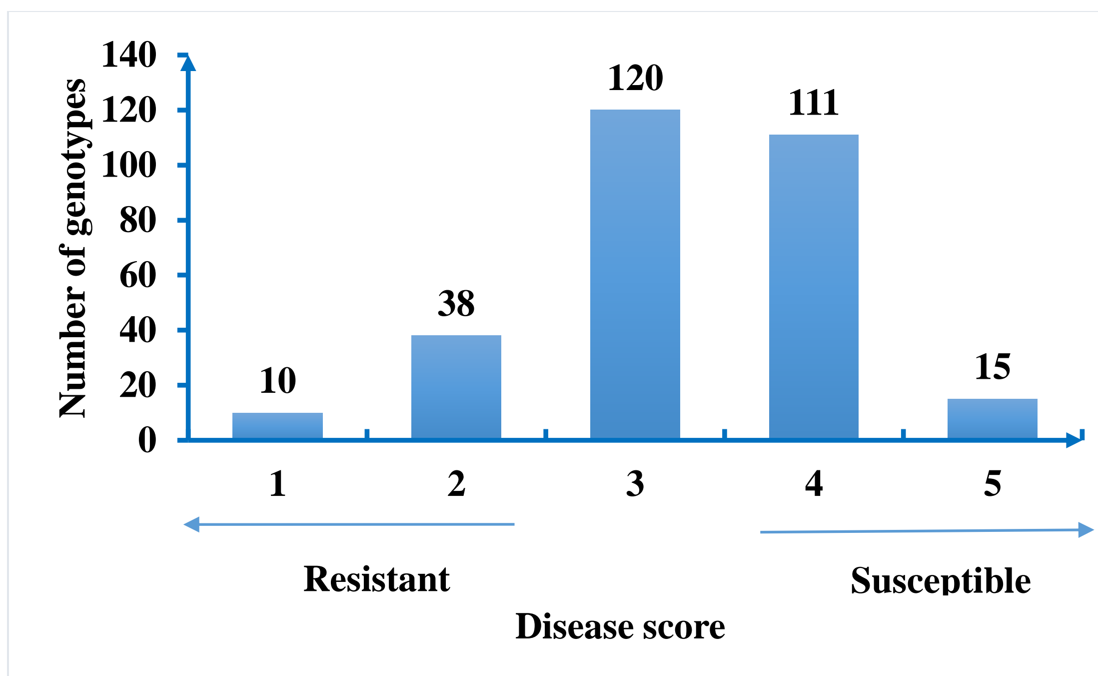


Figure 5. Frequency distribution of the mean spot blotch infection response of 294 HWWAMP genotypes. The *x*-axis exhibits 1-5 scores of mean infection response each genotype. The *y*-axis represents the number of genotypes (also number on the bar) exhibited the infection response. Salamouni and Glenlea were the resistant and the susceptible checks of the experiment, respectively.

Out of 294 genotypes, a total of 48 were resistant whereas 240 genotypes were appeared to be in the susceptible categories in all the experiments. Out of the 48 resistant genotypes, ten of them showed highly resistant and 38 resistant response to the pathogen and could be potential sources for spot blotch resistance (Table 5). In addition, 120 accessions showed either moderately resistant or moderately susceptible response whereas 111 genotypes showed a susceptible and 15 a highly susceptible response to spot blotch of wheat (Figure 6).

Table 5. List of HWWAMP that showed highly resistant reactions against *B. sorokiniana*

Genotype	Pedigree	Source	Year	SB Score	SB reaction
Duster	W0405D/NE78488//W7469C/TX81V6187	OK	2006	1	R
Colt	Agate sib (NE69441)// (Tx65A1503-1) 391-56-D8 / Kaw	NE	1983	1	R
Custer	F-29-76/TAM-105//Chisholm	OK	1994	1	R
Intrada	Rio Blanco / TAM 200	OK	2000	1	R
MT0495	MT9640/NB1133	MT	.	1	R
NE99495	ALLIANCE/KARL 92	NE	.	1	R
OK04525	FFR525W/Hickok//Coronado	OK	.	1	R
OK05122	KS94U337/NE93427	OK	.	1	R
OK05723W	SWM866442/Betty	OK	.	1	R
Venango	HBE1066-105/HBF0551-137	KS		1	R

Cultivars and breeding lines that exhibited a highly resistant response to the spot blotch pathogens were: Duster, Colt, Custer, Intrada, MT0495, NE99495, OK04525, OK05122, OK05723W, and Venango (Table 5). Colt was one of the top cultivars that exhibited a reproducible and highly resistant reaction to the pathogen. It is among the first semi-dwarf wheat ever released in Nebraska in 1983, and it had Agate sib (NE69441)//

(Tx65A1503-1) 391-56-D8 / Kaw pedigree in its background (Table 5). Another cultivar Duster, the most popular variety in the Oklahoma state since 2006 was found to be highly resistant that could be a source of spot blotch resistance. It has a pedigree background of W0405D/NE78488//W7469C/TX81V6187. Similarly, Custer had been a top performer in Oklahoma in 1987s, and some of its parents (F-29-76/TAM-105//Chisholm) were among the HWWAMP accessions evaluated against the pathogen. Both F-29-76 and TAM-105 showed susceptible reaction whereas Chisholm exhibited resistant reaction that is suggesting that Chisholm served as sources of resistance in Custer. Of the 168 winter wheat cultivar released in the US since the green revolution of 1970's and evaluated in our study only 16% of the genotypes showed a resistant and moderately resistant response (Appendix Table 2). A similar proportion of breeding lines evaluated were also resistant (Appendix Table 2).

Repeatability and Heritability

The variation among data sets of three different experiments and among the genotypes was analyzed using linear mixed model (LMM) approaches with a randomized group based jackknife resampling technique. At $\alpha=0.05$, there were highly significant differences among the predicted genotypic effects (P=1.33E-14) when compared to the population mean (μ) (Table 2). On the other hand, the variations among the disease score of different experiments were not significant as expected. There was good repeatability (0.96) among the experiments, and the broad sense heritability (H^2) for response to spot blotch among the genotypes was 0.8 (Table 6).

Table 6. Linear mixed model analysis of spot blotch scores recorded from 294 genotypes from the hard winter wheat association mapping panel.

Effects	**Estimate**	**SE**	**P-value**	**2.5%LL**	**97.5%UL**
Genotype	0.8040	0.0077	1.33E-14	0.7768	0.8311
Experiment	0.0011	0.0009	6.24E-01	-0.0020	0.0042
Genotypes*Experiment	0.0975	0.0042	8.88E-09	0.0828	0.1121
Residual error	0.0975	0.0042	8.88E-09	0.0828	0.1121
Proportion of effects					
V(Genotype)/VP	0.5797	0.0114	9.17E-12	0.5392	0.6201
V(Experiment)/VP	0.0008	0.0006	6.25E-01	-0.0014	0.0031
V(Genotype*Experiment)/VP	0.0702	0.0026	2.51E-09	0.0611	0.0794
V(e)/VP	0.0702	0.0026	2.51E-09	0.0611	0.0794
Heritability & repeatability					
Broad-sense heritability, H^2 (%)	80.4				
Repeatability (%)	96.1				

VP, total variance; SE, slandered error; LL=lower limit; UL, upper limit

4.3.2 Genotypic data

We obtained genotypic data for 294 HWWAMP constituting 21,555 SNPs from the T3 wheat database (https://triticeaetoolbox.org/wheat). We removed 5,487 markers by filtering markers with a minor allele frequency (MAF) less than 5%. Further, 458 markers with unknown position or chromosome were also removed. Finally, 15,590 high-quality SNP markers across 294 accessions of HWWAMP were used for GWAS (Table 7, Appendix Figure 4).

Table 7. Summary of number, minor allele frequency (MAF) and density of markers used for spot blotch association analysis in 294 wheat genotypes.

Chromosomes		Number of markers	Chromosome length (cM)	Minor allele frequency	Average number of marker/cM	Average distance between markers in cM
Genome A	1	1036	161.335	0.2419	6.4	0.16
	2	956	179.116	0.1998	5.3	0.19
	3	773	188.378	0.2442	4.1	0.24
	4	744	164.13	0.1889	4.5	0.22
	5	790	148.304	0.2333	5.3	0.19
	6	958	164.089	0.2093	5.8	0.17
	7	954	244.155	0.2417	3.9	0.26
1A-7A	1	887†	178.501†	0.2227†	5.0†	0.20†
Genome B	1	1254	173.624	0.2415	7.2	0.14
	2	1162	185.666	0.2485	6.3	0.16
	3	1117	149.634	0.2727	7.5	0.13
	4	451	119.446	0.2591	3.8	0.26
	5	1554	219.773	0.2100	7.1	0.14
	6	1237	127.049	0.2620	9.7	0.10
	7	855	178.856	0.2259	4.8	0.21
1B-7B		1090 †	164.864†	0.2457†	6.6†	0.16‡
Genome D	1	438	192.274	0.1939	2.3	0.44
	2	581	138.737	0.1688	4.2	0.24
	3	240	164.672	0.2176	1.5	0.69
	4	52	170.428	0.2205	0.3	3.28
	5	159	207.329	0.2580	0.8	1.30
	6	146	160.498	0.2517	0.9	1.10
	7	133	223.524	0.2662	0.6	1.68
1D-7D		250†	179.637†	0.2252†	1.5†	1.25†
Total		742	174.334	0.2312	4.4	0.54

†, Mean values of genomes A, B, and D

4.3.2.1 Markers distribution and allele frequency

The map position of 15,590 SNP loci obtained after filtering was obtained from Wang et al., (2014). The 15,590 SNPs are covering 3,661 cM on all 21 wheat chromosomes giving an average interval distance of 0.54 cM (Table 7). The number of SNP markers assigned to the A, B and D chromosomes were 6211, 7630 and 1749, respectively. The individual chromosomes had a genetic distance ranging from 127 to 244 cM. An average number of markers per cM on genome A (4.97) and B (6.71) were relatively higher when compared to D genome (1.3) (Table 6). Chromosome 4D (52) and 7D (133) harbored the lowest number of informative markers. Each locus was characterized by the presence of major and minor allele with a frequency between 0.05-0.96, and 0.04 to 0.50, respectively (Table 7).

4.3.2.2 Linkage disequilibrium

Linkage disequilibrium (LD) is the non-random co-segregation of alleles at two or more loci on the same chromosome or between loci on different chromosomes. Out of 15,990 markers used for association mapping, only 1842 markers were used for LD analysis taking out non-informative markers. Among a total of 91,307 locus pairs detected, 13,076 locus pairs (14.3%) were in linkage disequilibrium at the $P<0.001$ of which 7,744 locus pairs (8.5%) were found at $r2>0.1$ & $P<0.001$ (Appendix Table 3).

However, the distance at which linkage disequilibrium starts decaying depends on meiotic events and/or genetic drifts happened in the population. We estimated the LD decay distances in the whole genome and within each genome of winter wheat association panel using logarithmic trended smooth lines developed from scatter plots of syntenic r^2

vs. the genetic distance (cM) between pairs of two-locus (Figure 7). We estimated distance point where LD value (r^2) decreases below 0.1 or half strength of D′ (D′ = 0.5) based on the curve of the nonlinear logarithmic trend line. The LD (r^2>0.1) decay distance of about 4.5 cM was estimated for the whole genome. Similarly, LD decay distances for A, B, and D genomes were approximately 3.4 cM, 3.6 cM, and 14.2 cM, respectively (Figure 7).

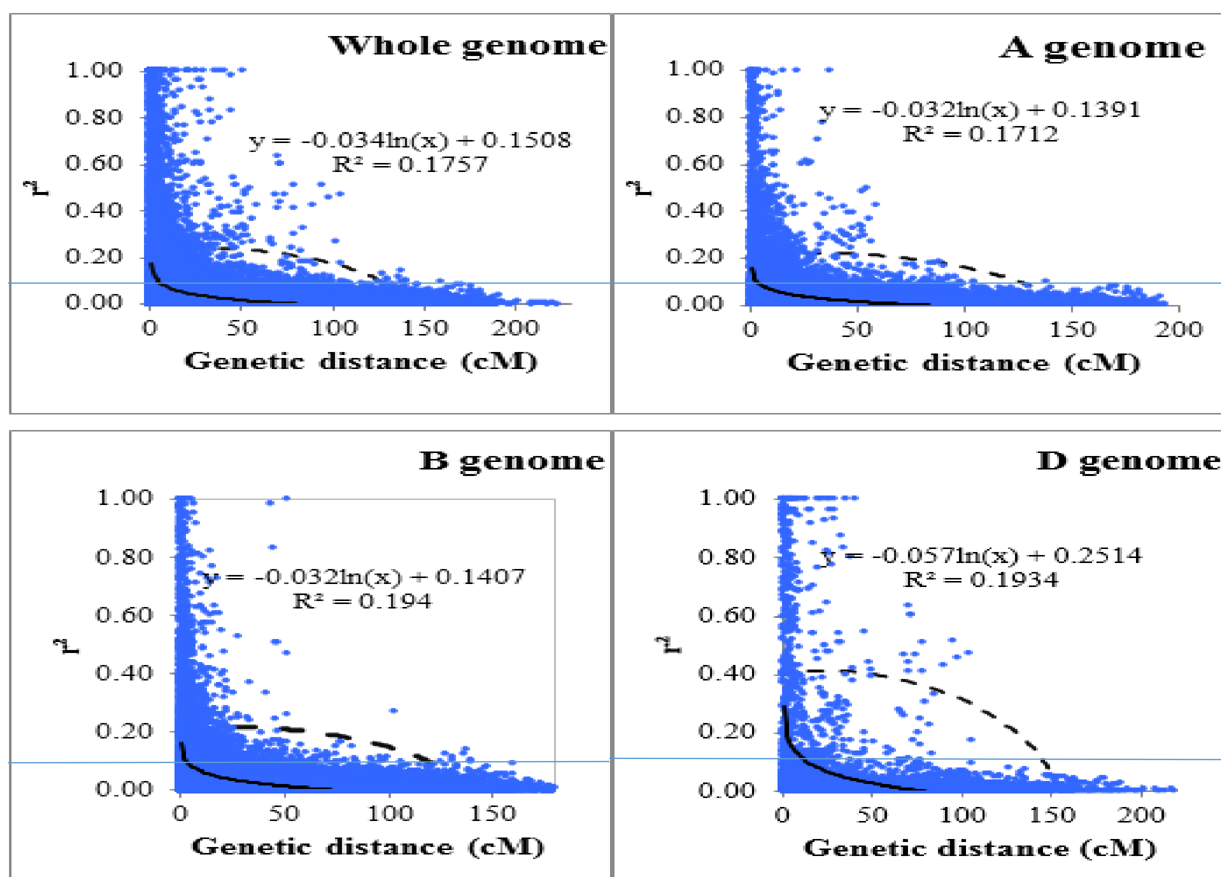


Figure 6. LD decay plots are displaying r^2 vs. genetic distance (cM) in 294 hard red winter wheat association mapping population. The LD was made from the intra-chromosomal pairs of markers in 100 cM sliding windows for the whole, A, B, D genomes of hard winter wheat. The horizontal line indicates the 95% percentile of the distribution of the unlinked r^2, which gives the critical value

of r^2. Logarithmic trended smooth curve indicates the relationship between *r2* and genetic distance between two loci. The upper broken curve indicates a distribution within which 95 percentile of the markers pairs found.

4.3.2.3 Population stratification and kinship assessment

In order to avoid the distortion due to population structure, SNP markers were first thinned to retain only 1,842 markers that were at least 0.0005cM apart in TASSEL 5. A STRUCTURE analysis indicated the presence of four subpopulations (K1-K4) in the HWWAMP that each cluster contained 45, 36, 99, and 113 genotypes, respectively (Figure 8, Appendix Figure 6). Average distances (expected heterozygosity) between individuals within each cluster (K1-K4)) were 0.06, 0.17, 0.27, and 0.23, respectively. Similarly, the net nucleotide distance among clusters, i.e., the average probability that a pair of alleles were different among K1 vs. K2, K3, K4; K2 vs. K3, K4; and K3 vs. K3 were 0.28, 0.19, 0.16; 0.24, 0.21; and 0.10, respectively. Therefore, we used a number of clusters that fitted best and selected first top-four principal component analysis (PCA) that contributed to large variances among the populations for association analysis (Fig. 4). The mixed linear model (MLM) with principal component analysis (PCA) to account for structure in the HWWAMP was used for GWAS analysis. The quintile–quintile (Q–Q) plots for the test statistics using MLM a GLM models shown in figure 9 indicated that absence of inflation of statistics or overall systematic bias caused by the population stratification when MLM model was used than GLM. We further divided the individuals based on their inferred ancestry and made a principal component analysis. Individual genotypes were considered admixed when the cumulative shared ancestry across the clusters was above 40% or they retain greater than 60% ancestry within their cluster. Hence, from PCA analysis proportion

and cumulative variances of the first four PCA were 0.43, 0.29, 0.28, 0.00 and 0.43, 0.72, 1.00, 1.00, respectively (Appendix Figure 7). Similar to population structure, a matrix of kinships among individual genotypes was calculated. The heat map calculated using the classical equation from Vanden (2006) (Appendix Figure 5) showed high kinship relationship among individuals (Appendix Figure 5).

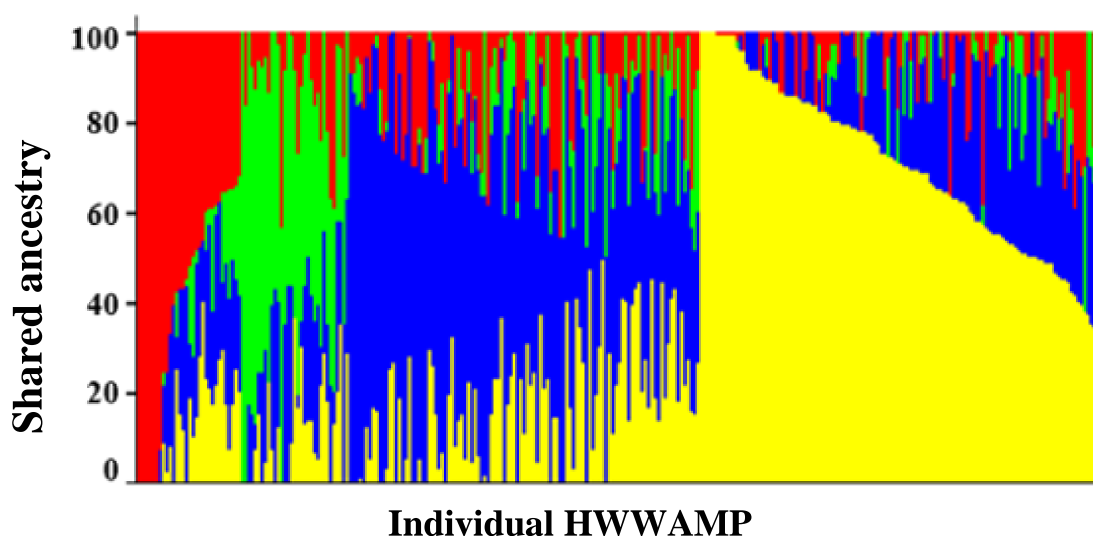


Figure 7. A number of population structure within HWWAMP based on STRUCTURE analysis.The genotype of each line on the figure is represented by a colored line where the red, green, blue, yellow colors indicated population structure K1-K4, respectively.

4.3.3 Model comparison for marker-trait association

Six statistical models such as MLM, CMLM, ECMLM, FaST-LMM, and SUPER were compared to select the one which reduces the type-I error and increase the power of SNP discovery (Result not presented). Our analysis indicated that MLM, CMLM, and ECMLM similarly reduced the type-I error and increased power when compared to others. The analysis showed no differences in the number of significant SNP discovered using all

the models except GLM model and FAST. However, due to its power and robustness, we selected MLM (Zhang et al., 2010b) with optimum compression as the model of choice for subsequent analysis. The Q-Q plot developed from the MLM and GLM models reveals that the observed and expected data points fitted in MLM model when compared to GLM (Figure 9).

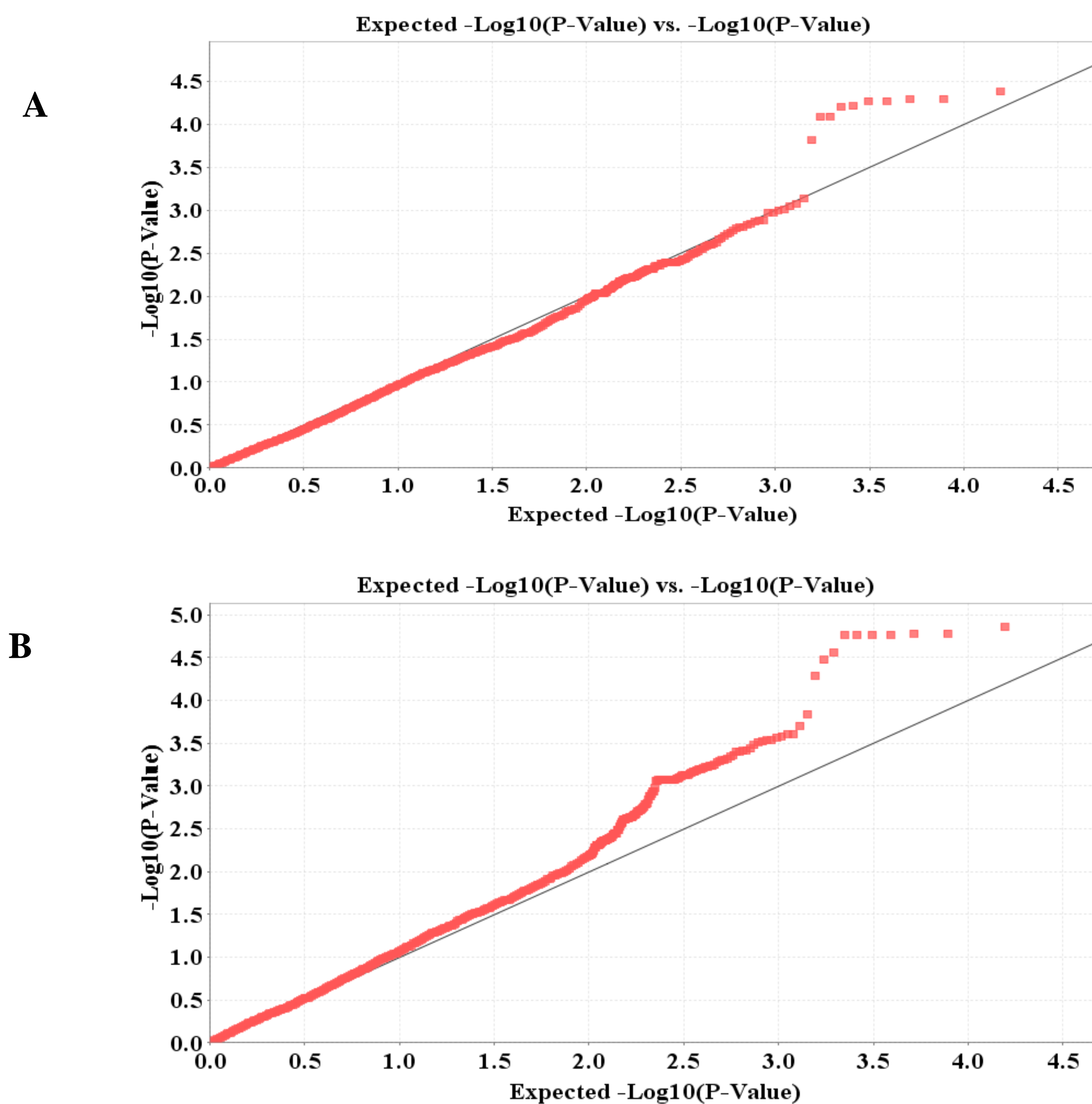


Figure 8. QQ plot (A) based on MLM and B) GLM using TASSEL v.5.0.

The number of significant markers associated with the spot blotch response with the GLM is much greater than with the MLM (Table 8). Indeed, at P<0.01 there was 117 and 198 significant makers trait association that was identified by MLM and GLM models, respectively of which 70 markers that were shared between the two models. However, at P<0.001, a total of 13 highly significant markers were identified by MLM model, and all the markers were included within those 26 markers identified by GLM model (Table 8).

Table 8. Comparative analysis of a number of significant SNP markers associated with spot blotch response detected by MLM and GLM models in HWWAMP.

Number of sig. Markers	MLM Model		GLM Model	
	P<0.01	P<0.001	P<0.01	P<0.001
Total significant markers	117	13	198	26
Shared marker	77	13	77	13
Unique significant marker	40	0	121	13
% shared marker	66	100	39	50
% Unique significant marker	34	0	51	50

4.3.4 Markers associated with the spot blotch QTLs

GWAS analysis identified several genomic regions linked to SB resistance including some of those identified in earlier studies (Joshi et al., 2004a; Kumar et al., 2015a; Lillemo et al., 2013; Lu et al., 2016). We identified six genomic regions showing highly significant (P<0.001) marker associated with SB resistance on chromosome 7B, 5A, 4A, 3B, 3A, and 2D (Table 9, Figure 10). The most significant SNPs explained 30 % of the total variation (Table 9). QTLs for spot blotch resistance have been reported in regions

similar to the QTLs identified in our study on chromosomes 5A (*QSb.sdsu-5A.1*), 7B (*QSb.sdsu-7B.1*) and 2D (*QSb.sdsu-2D.1*). However, we also identified three novel regions that contribute to spot blotch resistance. The QTLs, QSb.sdsu-4A.1, *Q.Sb.sdsu-4B.1* and *QSb.sdsu-3A.1*, (P<0.001) explained 6%, 4% and 4% phenotypic variation, respectively (Table 9). We identified several co-localized SNPs markers were associated with the same QTL helping in delimiting the QTL region and further characterize haplotype block by groups of significant SNP markers in LD (Appendix Table 4).

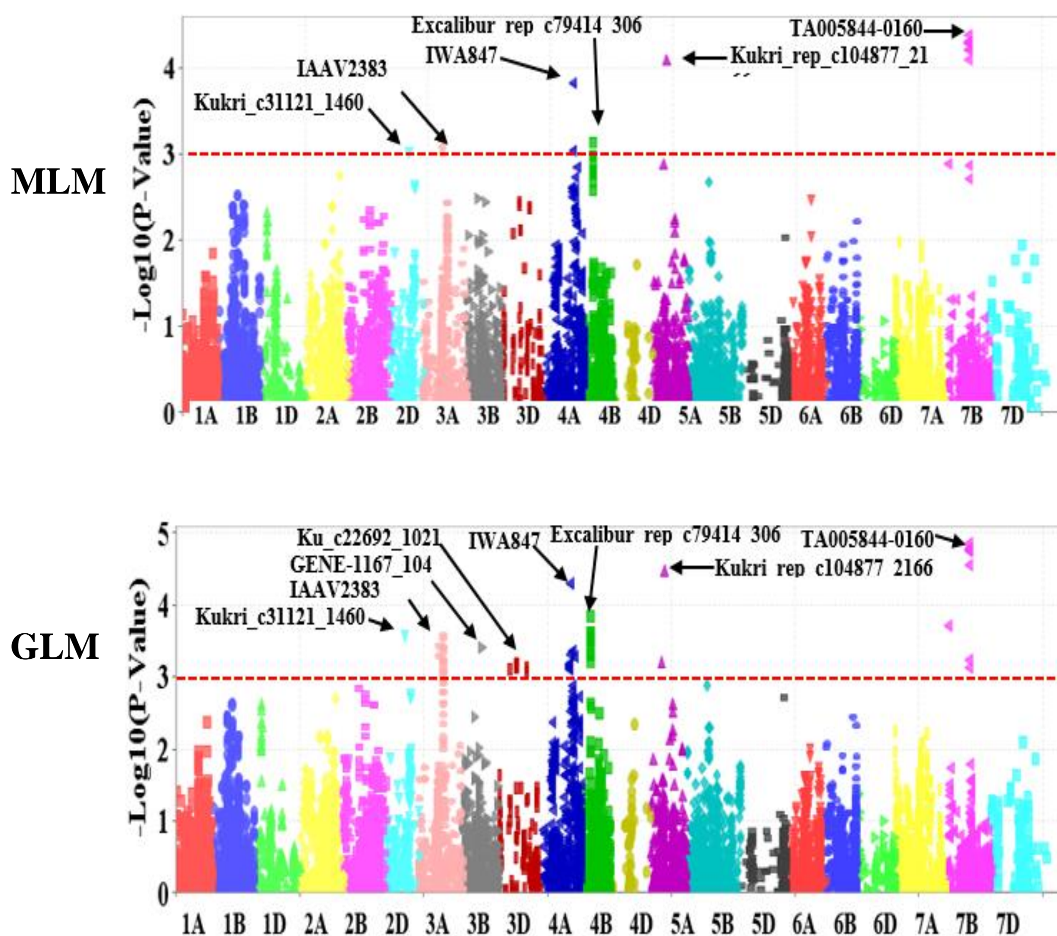


Figure 9. Manhattan plot developed using models of mixed linear model (MLM) and B) general linear model (GLM) in TASSEL v.5

The SNP marker Kukri_rep_c104877_2166 which was about 59.1 cM (480285174 bp) on chromosome 5A had the highest association (P=4.02E-05 and R^2=5.9%). Likewise, group of 7B markers such as Excalibur_c5700_670, Kukri_c21628_1215, Tdurum_contig9966_646, Kukri_c22495_552, Excalibur_c5700_527, Excalibur_c58742_144, TA005844-0160, Excalibur_c5700_705, BS00075332_51, and Tdurum_contig90495_232 found at about 86.1 cM and explained similar phenotypic version (R^2 =6.3%) (Table 9, Appendix Table 4, 5 & 6).

Table 9. Summary of SNP markers linked to significant SB resistance QTLs detected from genome-wide association analysis of 294 winter wheat genotypes.

No.	QTL (SNP Marker)	Chr	Alleles	Position (cM)	p-value	R^2 %	Additive effect	source
1	*QSb.sdsu-7B-1* (TA005844-0160)	7B	CC/TT	86.4	3.1E-05	6.3	-0.46	R
2	*QSb.sdsu-5A-1* (Kukri_rep_c104877_2166)	5A	TT/GG	59.1	3.3E-05	6.2	+0.66	S
3	*QSb.sdsu-4A.1* (IWA8475)	4A	GG/TT	118.7	8.7E-05	5.5	+0.38	S
4	*QSb.sdsu-2D.1* (Kukri_c31121_1460)	2D	CC/TT	80.2	4.8E-04	4.3	-0.45	R
5	*QSb.sdsu-4B.1* (Excalibur_rep_c79414_306)	4B	GG/AA	36.8	7.3E-04	4.1	-0.44	R
6	*QSb.sdsu-3A.1* (Excalibur_c46082_440)	3A	CC/AA	90.6	9.0E-04	4.0	-0.37	R

R=SD52; S=SD1001

3.4.5 QTL effects

QTLs with negative additive effect shows how much disease was decreased by alleles coming from the resistant genotypes and on the other way around (Table 10). The combination of individual QTL effects produces total resistance effects that the genotype exhibited. The cultivar, Colt, for instance, was one of the most resistant genotypes that harbored three QTLs with desirable alleles on chromosome 2D, 4A, and 7B that reduced the disease 1.36 when compared to the mean of the HRWWAMP hence exhibited the highest resistance (Table 10). Similarly, OK05723 and Duster had desirable allele on chromosome 4A, and 7B hat gave higher protection against SB. Each QTL had one desirable and the other undesirable allele that needs a selection of desirable alleles to develop ideal genotype. *QSb.sdsu-2D-1, QSb.sdsu-5A-1, QSb.sdsu-4A, QSb.sdsu-4B, QSb.sdsu-3A* and *QSb.sdsu-7B-1* QTLs could give the highest protection against the disease if a genotype harbored combination of the following desirable allies: TT, GG, TT, AA, AA, and TT, respectively (Appendix Table 6).

Table 10. Genotypes harbored multiple diseases increasing QTLs.

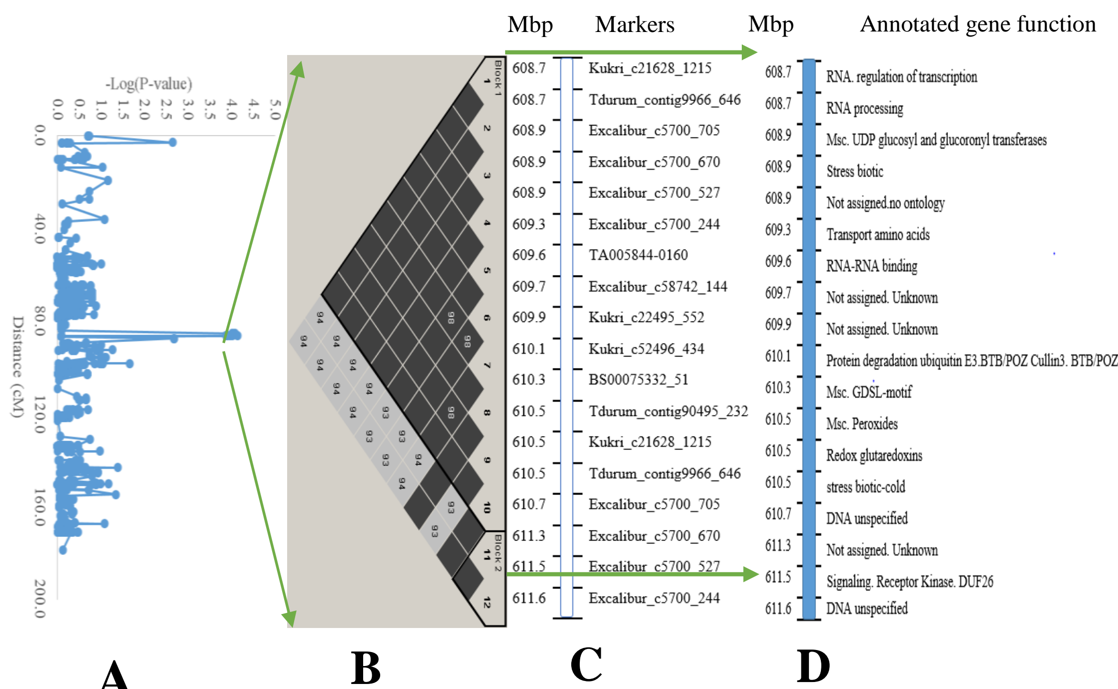
Wheat genotypes	Disease Score	Disease Reactions	Chromosome with disease reducing QTLs	Additive effect	Chromosome with disease increasing QTLs	Additive effect
COLT	1	R	2D, 4A,7B	-1.36	5A,	0.66477
CUSTER	1	R	4A	-0.44	-	-
OK05723W	1	R	4A, 7B	-0.90	4B,3A	0.75293
VENANGO	1	R	7B	-0.46	-	-
Duster	1	R	4A, 7B	-0.90	5A	0.66477

4.3.5 In-silico functional annotation of the candidate region

Plant reactions to diseases are very complex, and involves the activation of sets of genes, encoding for different proteins. We annotated the coding sequences in the candidate regions to identify candidate protein that may be involved in pathogen related response (PR) genes. Regions of chromosome sequences spanning through sets of SNP markers that showed $P<0.05$ up-and downstream of the highest significant markers were taken for analysis (Figure 11A & B). After blast to reference wheat genome 2.07Mb, 6.58Mb, 1.31Mb, 10.03Mb, 3.00Mb of sequence segments on 2D, 4A, 4B, 5A and 7B were taken. Of the 722 proteins annotated we found a number of 21, 39, 10, 28, 18 disease-related proteins in candidate regions on chromosome 2D, 4A, 4B, 5A, and 7B, respectively. These protein domains harbor domains involved in plant recognition of pathogens through protein binding such as nucleotide-binding site-leucine-rich repeat (NBS-LRR) and TIR-NBS class protein. Some QTL regions also harbor protein having serine/threonine kinase activity, protein kinase, and ATP binding activity. The protein description, functions, ID and other related information, are presented (Additional file: Appendix Table 5).

We further studied QTLs *QSb.sdsu-7B-1 and QSbsdsu-2D-1* in detail as they explained the maximum variation spot blotch resistance in our study and were flanked in 3.00Mb and 2.07Mb regions respectively (Figure 9). Figure 11 showed Manhattan plot showing the genetic distance (cM) of significant makers in each chromosome. Within the selected region of the up-and-downstream of the highest significant markers, two haplotype blocks on each of the 7BS and 2D were identified. From segment DNA size of 2.1Mb and 2.6. within the first haplotype block on 2DS and 7BS lists of annotated proteins from Pfam related to the disease defense and their position on physical chromosomes were identified.

i) *QSb.sdsu-7B-1* on chromosome 7B



ii) *QSb.sdsu-2D-1* on chromosome 2D

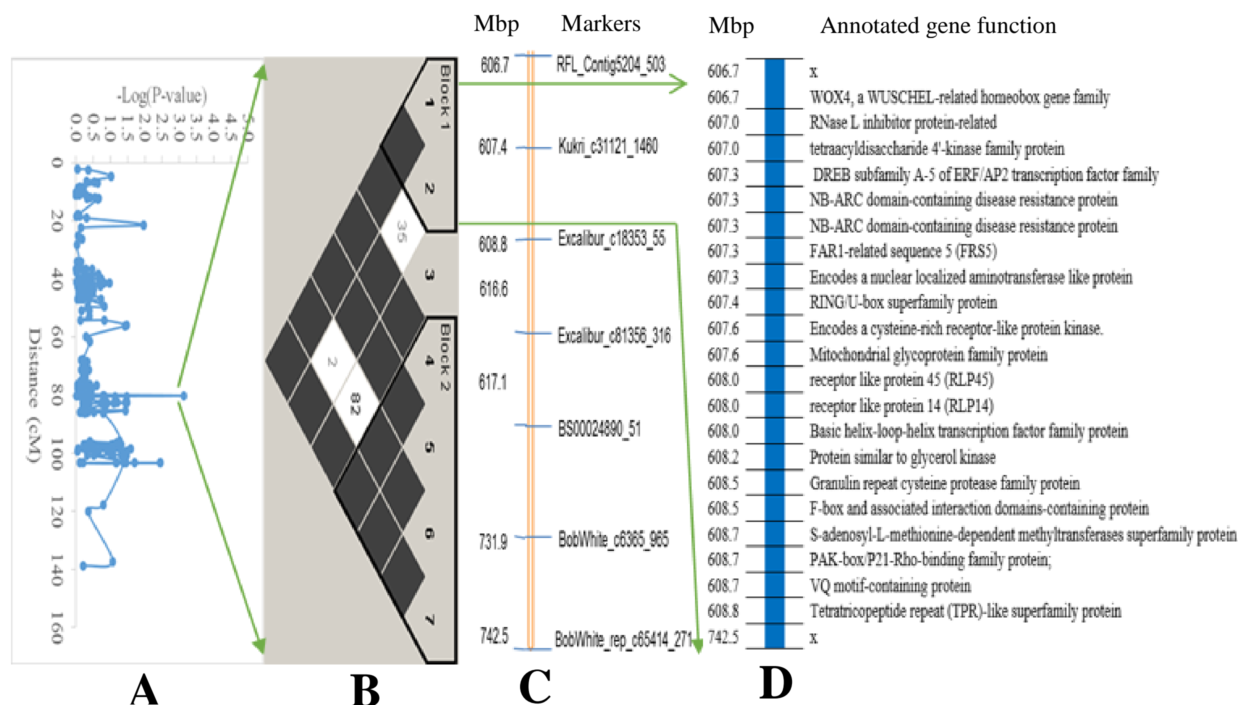


Figure 10. Gene annotation of QTLs identified on chromosome 7B and 2D for spot blotch resistance in hard winter wheat. The far left image is a Manhattan plot

indicating the level of marker association with the trait and putative QTL regions. The central image contains a visualization of linkage disequilibrium (black is a D' value of 100%, another color is a D' value of less than100%). Significant haplotype blocks are outlined in black. The image right to LD plot is a physical map of markers (significant markers co segregated together are found in some haplotype blocks.) The far right image is a physical map of candidate gene function on 7B and 2D chromosomes segment spanning from 608.7 Mb to 611.7 Mb and .606.9 Mb and 608.8 Mb, respectively.

Proteins related to cell death and response to oxidative stress such as peroxidase superfamily protein, oxidoreductase, and zinc-binding dehydrogenase family protein were observed on *QSb.sdsu-7B-1*. In addition, *QSb.sdsu-2D-1* also harbors RING/U-box superfamily protein that expresses in response to the fungal pathogen. PAK-box/P21-Rho-binding family protein was also found near to RING/U-box protein in some QTL regions. Further, protein family reported as transporters (EamA-like transporter family, transmembrane proteins 14C,), endopeptidase inhibitors (Serine protease inhibitor or SERPIN family protein for serine-type, cysteine-type inhibitor, and Protease inhibitor), kinase activity (Protein kinase superfamily protein, protein similar to glycerol kinase.), detoxification (including an ABC transporter C and cytochrome P450 enzymes) were also found across the QTLs.

Proteins with unknown function or indirectly involved in disease resistance were: senescence and dehydration-associated protein, ATP-binding cassette (ABC) transporters, Chl I gene (CHLI2) a subunit of magnesium chelatase required for chlorophyll

biosynthesis, glycosyl hydrolase family 47 protein, mitochondrial glycoprotein family protein, serine protease inhibitor (SERPIN) family, auxin-responsive family protein, auxin signaling F-box 2 (AFB2), pentatricopeptide repeat (PPR) superfamily protein, O-Glycosyl hydrolases family 17 proteins. In addition, some of the gene models identified either have no known function or may not be involved in plant defense to pathogens.

4.3.6 Synteny of SB resistance gene between wheat and barley

Shared synteny is one of the most reliable criteria for establishing the orthology of genomic regions in different species. We performed a comparative analysis of wheat and barley for the candidate regions of six QTLs identified in our study. Synteny analysis indicated that four QTLs (*QSb.sdsu-3A.1, QSb.sdsu-5A.1,QSb.sdsu-7B.1, and QSb.sdsu-2D.1*) on 2D, 3A, 5A and 7B carrying SB resistance QTLs corresponds to 2H, 3H, 5H and 7H chromosomes of barley (Figure 12). Spot blotch resistance genes have been reported in these syntenic regions in barley (Zhou and Steffenson; 2013; Roy et al., 2010, Wang et al., 2017) validating the QTLs identified in our study.

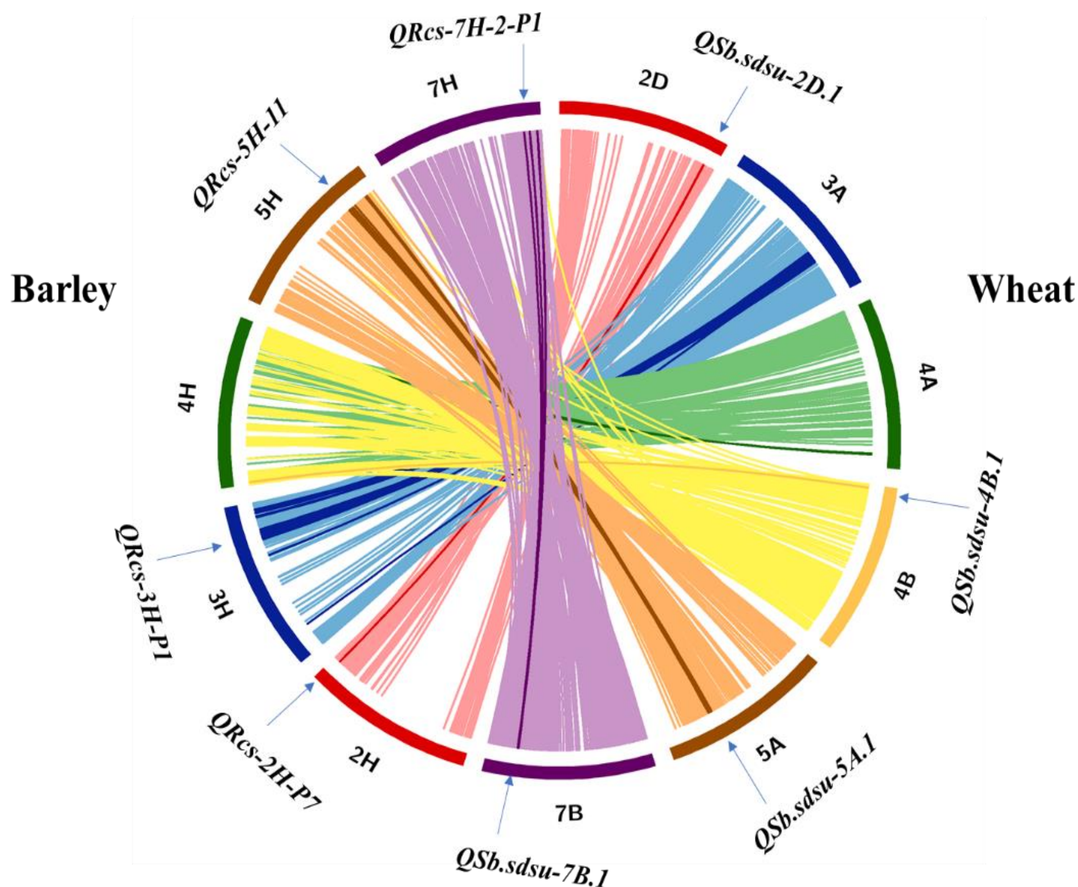


Figure 11. Circular genome data visualization of synteny between wheat and barley chromosomes harboring spot blotch resistance genes. The map was made using CIRCOS (Circular Genome Data Visualization). Each color indicates different chromosomes. Each arc string shows the marker related to the QTLs.

4.4. Discussion

4.4.1 Phenotypic variability for resistance against spot blotch of wheat

The current distribution and the existing damages of spot blotch are presumed to be increasing (Chowdhury et al., 2013; Thapa, 2013). Developing spot blotch resistant wheat cultivar is likely most economical and durable strategy for minimizing loss due to spot

blotch when compared to the overall usage of fungicide (Duveiller and Sharma, 2009) Duveiller, 1994; McMullen and Adhikari, 2011). The breeding programs targeting SB could focus on two perspectives: utilization of the existing resources and search for new genetic basis (Van Ginkel and Rajaram, 1998). Several sources of SB resistance have been reported in Indian national collection (Kumar et al., 2017), CIMMYT germplasms and derivatives of CYMMIT primary synthetic bread wheat (Mikhailova et al., 2004; Singh, 2016; Zhu et al., 2014b), multi-resistant cultivars from Nepal (Mahto et al., 2011) and few Brazilian varieties (Mehta, 1998). However, most of these studies are conducted in spring wheat. In winter wheat modern European winter wheat cultivars and breeding lines (Liatukas and Ruzgas, 2012) have been evaluated, but the US germplasm has not been extensively exploited. In the present study, 294 HWW genotypes showed a variable reaction against spot blotch caused by SD40 virulent isolates of *B. sorokiniana*, indicating the existence of genetic variability among hosts. We identified ten genotypes (Table2) the highly resistant to spot blotch that can be utilized for developing spot blotch resistance in winter wheat cultivars. Several past studies also showed that the resistance reaction of wheat to spot blotch had a positive and negative range of correlation between *in vivo* and *in vitro* conditions (Liatukas and Ruzgas, 2012; Rosyara et al., 2009; Thapa, 2013). In our study, we observed 96 % repeatability of the experiments and the 80% heritability of spot blotch trait confirms the reliability of the spot blotch resistance evaluation under greenhouse conditions.

4.4.2 SNP Markers distribution and LD decay

The distribution and density of informative markers reflect the overall genetic richness and diversity of the wheat genomes. The D genome (2.3-4.2) had the least average

marker density (markers/cM) as compared to A (3.9-6.4) and B (3.8-7.2) genomes. Further the total markers used for association mapping from D genome (11.2%) was very small when compared to A (39.8%) and B (48.9%) genomes suggestings a lower genetic diversity, and lower level of effective recombination in the D genome (Barnes et al., 2012; Nielsen et al., 2014).

Linkage disequilibrium (LD), an integral part of association mapping, is an important parameter and estimation of LD decay distances can help to determine the power of association mapping. We estimated a critical value of *r2* (basal LD=0.1) from 95th percentile distribution of the inter-chromosomal LD (Francki et al., 2006; Zhang et al., 2014) below which we assumed the absence of syntenic relationship within pairs of loci and hence they physically present on different chromosomes. Our result also demonstrated that D genome had high LD decay distance (14cM) when compared to A and B genomes. Whole genome LD decay distance we observed in HWWAMP was similar to whole genome LD decay observed of Chinese winter wheat using SSR marker (Barnes et al., 2012; Chao et al., 2010). However, a lower rate of average LD decay (higher distance) was observed in European hexaploid wheat (23 cM) (Nielsen et al., 2014) and US Elite hard red winter wheat 10cM (r2>0.1)(Zhang et al., 2010a). When comparing the wheat landraces to modern cultivar, the LD decay distance (5cM) was lower in landraces than modern winter wheat cultivars (5-10cM) (Hao et al., 2011) suggesting a possible reduction in diversity the modern Chinese wheat cultivars. The LD decay analysis in HWWAMP showed variation among genomes and within the genomes itself, indicating variability in recombination hot spot, differences in selection pressure imposed on alleles of wheat genome and an evidence of the recombination events in past breeding history.

Like many other bacterial diseases, application of pesticides and antibiotic compounds is not effective against BLS (Duveiller, 1994; McMullen and Adhikari, 2011). However, standard cultural control methods such as crop rotation, tillage, alternative host control, avoiding susceptible cultivars, use of clean seed, hot water seed treatment would be effective in lowering the inoculum and disease levels (Forster et al., 1988; Gitaitis and Walcott, 2007; Stromberg et al., 1999). Until to date, host plant resistance is the only and most effective methods for controlling BLS in wheat (Adhikari et al., 2012a; Duveiller et al., 1992; Duveiller and Maraite, 1993; Sharma et al., 2017). So far, wide range of wheat (Adhikari et al., 2012b; Duveiller, 1989; Duveiller et al., 1992; Kandel et al., 2015; Maraite et al., 2007b; Tillman et al., 1996) and few triticale (Sapkota et al., 2017; Wen et al., 2017) genotypes exhibited genetic resistance to the pathogen.

4.4.4 QTLs for Spot blotch resistance

We identified six genomic locations (2D, 3A, 3B, 4A, 5A, and7B) associated with spot blotch resistance in HWWAMP and the linked SNPs (TA005844-0160, Sb.sdsu-5A-1, Kukri_rep_c104877_2166, IWA8475, Kukri_c31121_1460, Excalibur_rep_c79414_306, and Excalibur_c46082_440) explained about 30% of the variation. We compared the MTA with MLM and GLM, and observed GLM analysis showed low power and higher risk of false-positive detection (result not presented). In GLM model, which only accounts for population structure as a covariate, the additive variance and error variance cannot be separated because GLM uses maximum compression (compression = n) with all taxa as a single group. Unlike GLM, however, MLM (Zhang et al., 2010b) takes account of population structure and individual kinship in association analysis to reduce type I error instigated due to relatedness and population structure. We

observed similar results with MLM, cMLM that cluster approach and ECMLM (Li et al., 2014) which improves cMLM by exploiting multiple ways of clustering and methods of driving group kinship in addition to using group average (Tang et al., 2016).

We identified three novel QTLs for spot blotch resistance (*QSb.sdsu-4A, QSb.sdsu-3A,* and *QSb.sdsu-4B*) in addition to three regions on chromosomes 2D, 5A, 7B reported earlier to be significantly associated with spot blotch resistance. Previously, Gurung et al., (2014) reported significant QTLs associated with SB on chromosomes 1B, 5A, 5B, 6B, and 7B, whereas Gurung et al., (2014) detected on 1A, 3B, 7B, and 7D, and Lillemo et al. (2013) detected QTLs on chromosome 5B, 7A, and 7D. Similarly, spot blotch resistance QTLs were reported on wheat chromosomes 1B,3B, and 5A (Zhu et al., 2014); 2AL, 2BS, 5BL and 6DL (Kumar et al., 2009); 2BS, 2DS, 3BS, 7BS and 7DS (Kumar et al., 2010); 1A, 3B, 7B, and 7D (Adhikari, 2014); and 7B and 7D on (Ban et al., 2016). With dense marker coverage, we did not only validate the QTLs *QSb.sdsu-2D.1, QSb.sdsu-5A.1* and A, *QSb.sdsu-7B.1* on chromosomes 2D, 5A, 7B but also provide highly significant associated SNP markers that could be used for marker-assisted selection (MAS) for SB resistance.

So far, only three major QTLs Sb1 on chromosome 7D (Joshi et al., 2004a; Lillemo et al., 2013), Sb2 on 5B (Kumar et al., 2015a), and Sb3 on 3B (Lu et al., 2016) contributing to spot blotch resistance have been characterized with simple sequence repeat (SSR) marker and bulked sergeant analysis (BSA). The three QTL were found in the interval of flanking markers: gwm1220-swm1055.2, Xgwm639/Xgwm1043, Xbarc133/Xbarc147, respectively. In our analysis, none of the three QTLs were significant at P <0.001 but all these QTLs were showed a peak and were significant P<0.005 suggesting the presence of

these QTLs in the HWWAMP. Each of the three QTLs was explaining 3% (Appendix table) variation respectively which was lower than what was reported in previous studies (Joshi et al., 2004a, 2004b, Kumar et al., 2015a, 2015b; Lillemo et al., 2013; Lu et al., 2016). In addition, we also identified significantly associated SNPs for Sb1, Sb2, and Sb3 that could be used for MAS (appendix table).

4.4.5 Functional annotations of candidate regions

Plants defense system can be categorized into ever existing constitutive defense system that is triggered by pathogen-associated molecular patterns (PAMPs) or the temporarily induced system that targeted to defend an attacked area of the plant. The genes encoding the specificity determinants of effector-triggered immunity are known as resistance (R) genes (DeYoung and Innes, 2006). The production of PR proteins in response to pathogens are the primary mechanisms in induced plant's self-defense system. Numerous PR proteins have been characterized in recent years, and right now they are at least classified into 17 protein families and several pathogenesis-related proteins that do not constitute a superfamily of proteins (Dangl and Jones, 2001; Sels et al., 2008).

The regions of SB QTLs identified in our study harbored many genes. However, not all genes are equally important in the regulation of quantitative traits and many diseases like spot blotch. The candidate genes that were commonly found in across multiple QTLs reported being more likely the one that determines the trait (Swamy et al., 2011). In our study, we found NBS-LRR protein family containing N-terminal nucleotide-binding site (NBS) and C-terminal leucine-rich repeat (LRRs) in many of the annotated QTL regions. The NBS-LRR is the most common R-genes by which highly conserved NBS domains can bind and hydrolyze ATP or GTP, whereas the LRR motif is typically involved in protein-

protein interactions and is responsible for recognition specificity (Wan et al., 2012; Zhang et al., 2016).

A protein rated to glycerol kinase process was observed in several QTLs, and it takes part in glycerol-3-phosphate (G3P) metabolism acting as a novel regulator of plant defense signaling. (Venugopal et al., 2009). Peroxidase (POX) superfamily protein plays a role in self-defense (Hiraga et al., 2001) by catalyzing oxidoreduction between H_2O_2 and various reductants were one of a classical enzyme that was observed in the *QSb.sdsu-B.1* region on chromosome 7B. The other protein was Cysteine-rich receptor-like kinases (CRKs) that play essential roles in stomatal conductance and accelerated senescence that is correlated with accumulation of reactive oxygen species, higher foliar ethylene and salicylic acid (Burdiak Pawełand Rusaczonek et al., 2015). In our study, we also observed thaumatin-like proteins (TLPs) that were reported to be induced in the presence of pathogenic fungi, and they are referred to as pathogenesis-related proteins 5 (PR-5) (Brandazza et al., 2004; Kitajima and Sato, 1999; Liu et al., 2010).

Cysteine protease is one of the well-studied proteolytic enzymes in plants and pathogen by which plats counter utilize the same proteolytic machinery to halt pathogen invasions (Niño et al., 2014). The plant function with the most PUB associations has been related to pathogen defense and the hypersensitive response (HR). Similarly, RING/ ubiquitin-box superfamily protein plays important roles in plant development, including programmed cell death in Arabidopsis (Shirsekar et al., 2010). In some QTL region, we found PAK-box/P21-Rho-binding family protein that increases Rho family GTPase-dependent immunity in plants and animals (Kawano *et al.,* 2014). The regulatory mechanism of the zinc finger proteins was also reported in resistance mechanism through

its active involvement in sequence-specific binding to DNA/RNA and contribution in protein-protein recognitions (Gupta et al., 2012). The disease-related genes identified in the candidate regions can help in the development of a new marker for further characterization of the SB resistance QTLs in wheat.

4.4.7 Implications of GWAS for SB resistance breeding in wheat

The ultimate goal of characterizing SB resistance genes is to find closely linked markers for assisting in the selection and further understand the underlying network of genes and their interaction to achieve resistance response. This comprehensive understanding will help in developing durable disease resistant cultivars. We identified groups of SNP markers associated with six QTLs that had different levels of effects. Most of the genotypes used in our study encompass multiple putative resistant alleles that include wheat lines that were harboring highest level kind of responses (Table 5, 10 & Appendix Table 6). Past inheritance studies on resistance to spot blotch suggested polygenic types of resistance that appears to be based on many minor genes with small individual effects a (Dubin and Rajaram, 1996; Gurung et al., 2014; Joshi et al., 2004b; Kumar et al., 2015b) Backcross of two parents harboring *Qsb.bhu-2A* on chromosome 2A and Qsb.bhu-5B on chromosome 5B in suitable parent achieved higher resistance in susceptible cultivar HUW 234 in India (Vasistha et al., 2016). This indicated that assembling multiple desirable genes from multiple parents into a single genotype through the use of marker-assisted gene pyramiding for disease resistance could help to develop improved wheat varieties. We identify that winter wheat cv. Custer (OKS) carries the SB resistance QTLs on chromosomes 2D, 3A, 4A, and 7B. The significant SNP markers can be used to develop

Kompetitive Allele Specific PCR (KASP) assay and used for marker-assisted selection (MAS) is well evidenced in several crops.

4.5. Summary

Spot blotch (SB), caused by *Cochliobolus sativus* (anamorph: *Bipolaris sorokiniana*), is a worldwide economically important disease of wheat. Under the favorable condition, SB is known to cause yield loss up 70%. Breeding for resistance is one the most economical and sustainable component of integrated disease management. The objectives of this research were to identify winter wheat genotypes carrying resistance genes against the SB pathogen and to identify SNP linked to QTLs for marker-assisted selection. We studied the reaction of 294 winter wheat genotypes including the two checks were evaluated against *Bipolaris sorokiniana* isolate SD40. Off the total, ten highly resistant to resistant, 47 moderately resistant and 241 moderately susceptible to susceptible genotypes were identified.

Genome-wide association studies were conducted to detect significant SB QTLs with significantly associated SNP. Six QTLs cumulatively explaining 30% the total variation were detected across chromosome 2D, 3A, 4A, 4B, 5A, and 7B. *QSb.sdsu-7B.1* (TA005844-0160) on chromosome 7B and *QSb.sdsu-5A.1* (Kukri_rep_c104877_2166) on chromosome 5A each explained the highest phenotypic variation (6%). The synteny analysis indicated that the resistance locus on wheat chromosomes 2D, 3A, 5A and 7B corresponds to the previously identified Spot blotch QTLs on 2H, 3H, 5H and 7H chromosomes of barley. Our gene annotation analysis indicated that NBS-LRR protein family containing N-terminal nucleotide-binding site (NBS) and C-terminal leucine-rich repeat (LRRs) was commonly found across multiple QTLs.

4.6. Conclusion

We identified ten genotypes highly resistant to spot blotch and six genomic regions associated with spot blotch resistance along with tightly linked SNPs. Genotypes that are providing multiple spot blotch resistance QTLs could be used for the future breeding program.

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6. APPENDIX

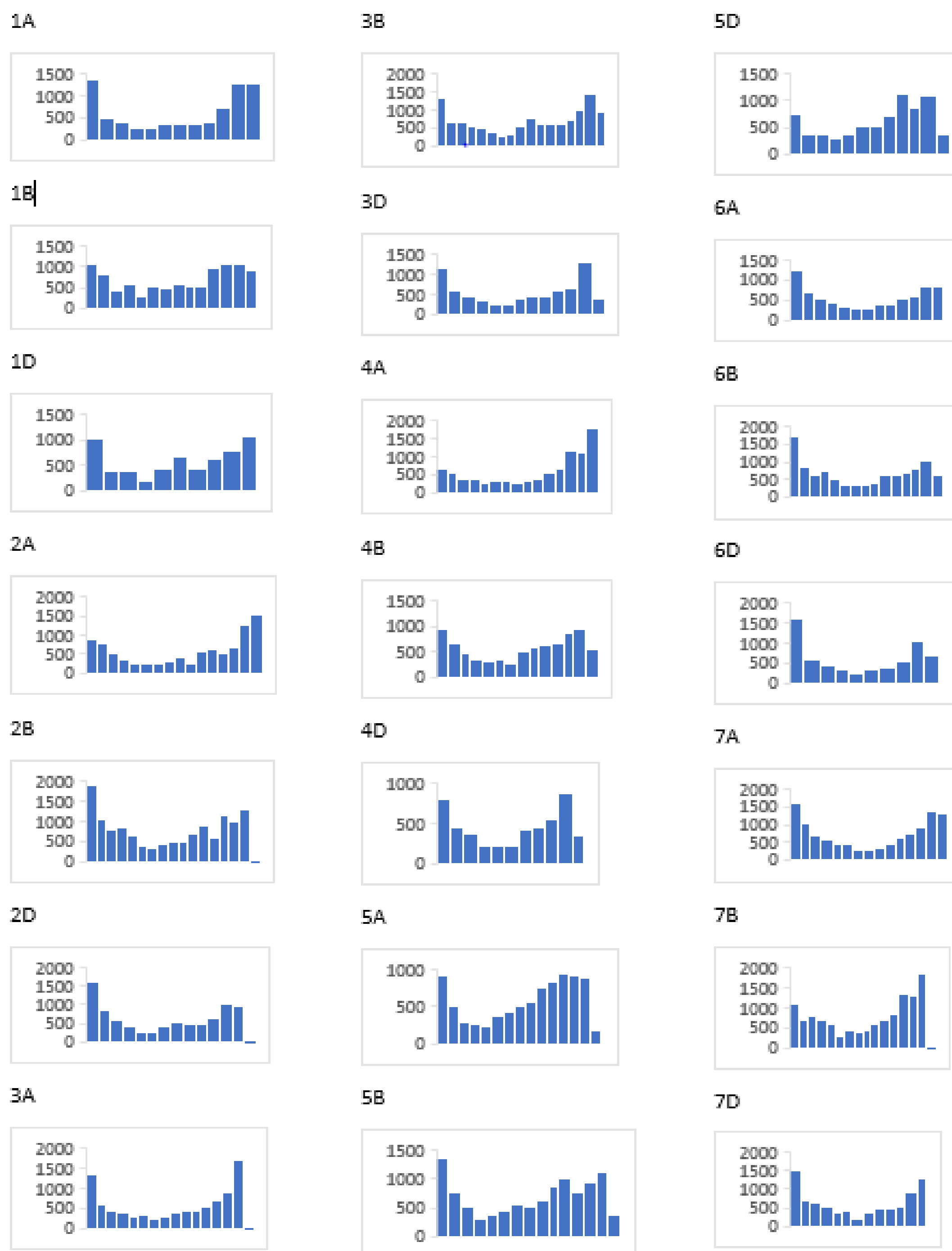
Appendix Table 1. Mean BLS sore from three experiments and allele frequency of 92 RILs population.

Name of RILs	Ex1	Ex2	Ex3	Mean	A allele (%)	B allele (%)	% Missing data
SD17-P1-12-F5RIL-9	4	4	4	4	28.9	58.8	3.5
SD17-P1-14-F5RIL-10	2	2	2	2	36.4	50.2	3.9
SD17-P1-16-F5RIL-12	5	6	5	5	32.4	54.7	6.5
SD17-P1-17-F5RIL-13	4	5	5	5	38.5	46.8	0.8
SD17-P1-1-F5RIL-1	4	4	5	4	42.0	49.1	0.8
SD17-P1-21-F5RIL-15	2	2	2	2	45.7	41.5	2.9
SD17-P1-22-F5RIL-98	6	6	6	6	31.5	55.2	1.1
SD17-P1-24-F5RIL-99	6	6	6	6	40.8	45.9	0.3
SD17-P1-25-F5RIL-17	4	5	4	4	35.1	50.5	2.4
SD17-P1-26-F5RIL-18	4	4	4	4	36.9	53.5	2.2
SD17-P1-27-F5RIL-19	5	4	5	5	34.0	50.0	5.0
SD17-P1-28-F5RIL-100	2	2	2	2	40.4	48.3	1.0
SD17-P1-30-F5RIL-20	6	6	6	6	41.8	42.3	1.8
SD17-P1-31-F5RIL-21	2	2	2	2	30.4	55.1	4.1
SD17-P1-32-F5RIL-22	5	5	5	5	36.5	46.9	2.8
SD17-P1-33-F5RIL-23	3	6	2	4	35.1	53.0	0.2
SD17-P1-34-F5RIL-24	5	5	5	5	37.3	51.1	0.1
SD17-P1-35-F5RIL-25	3	3	4	3	38.1	49.5	2.8
SD17-P1-36-F5RIL-26	4	5	4	4	34.5	50.5	4.6
SD17-P1-37-F5RIL-102	3	3	2	3	37.0	47.6	0.7
SD17-P1-38-F5RIL-27	5	4	5	5	37.9	52.2	2.2

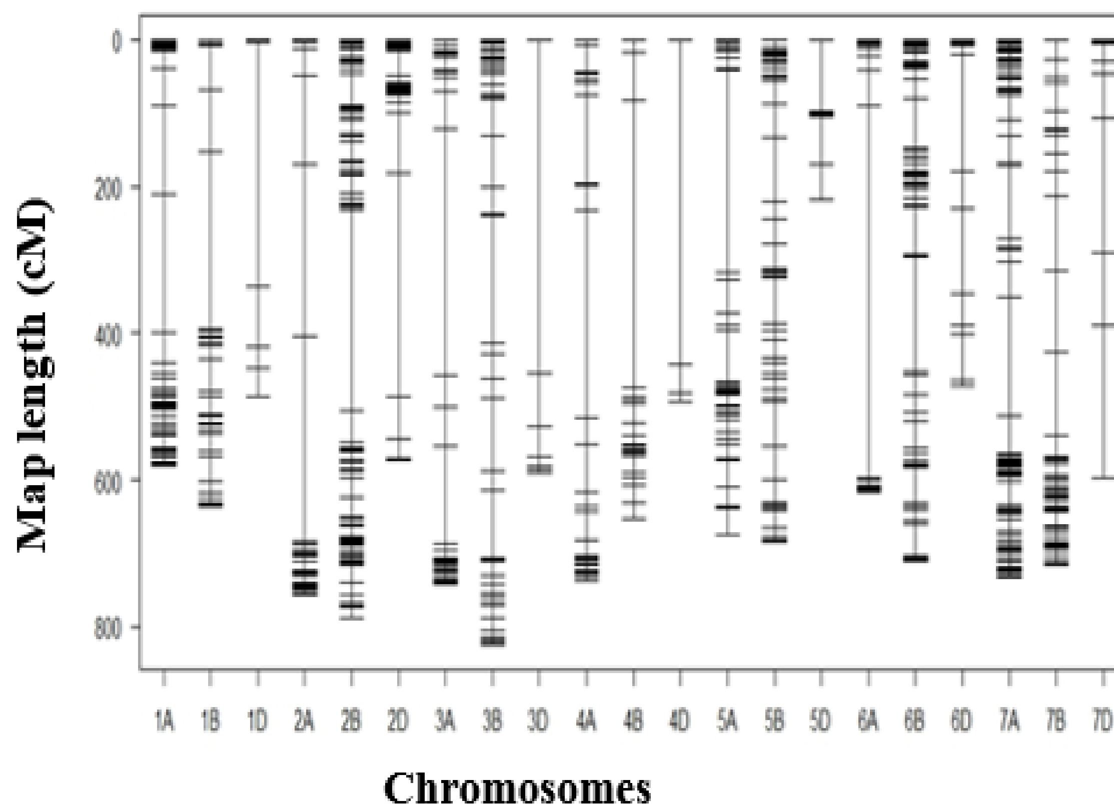
SD17-P1-39-F5RIL-28	3	4	3	3	34.6	54.7	1.3
SD17-P1-3-F5RIL-2	4	4	4	4	40.9	47.1	3.0
SD17-P1-41-F5RIL-30	2	2	2	2	28.5	62.6	0.5
SD17-P1-42-F5RIL-103	4	4	3	4	33.3	50.3	2.4
SD17-P1-43-F5RIL-104	5	5	4	5	41.4	45.9	0.0
SD17-P1-44-F5RIL-31	6	6	6	6	33.3	53.2	0.9
SD17-P1-45-F5RIL-32	5	5	5	5	38.8	48.2	0.7
SD17-P1-49-F5RIL-36	3	3	4	3	34.9	51.7	4.7
SD17-P1-4-F5RIL-3	4	3	4	4	36.5	51.4	3.0
SD17-P1-51-F5RIL-37	2	2	2	2	33.8	55.8	3.0
SD17-P1-52-F5RIL-38	4	4	4	4	30.9	54.8	0.3
SD17-P1-54-F5RIL-40	6	5	4	5	34.1	53.0	0.2
SD17-P1-58-F5RIL-44	5	5	6	5	43.4	42.6	1.1
SD17-P1-59-F5RIL-105	4	4	4	4	32.2	54.8	0.5
SD17-P1-5-F5RIL-4	6	6	6	6	47.8	41.5	3.3
SD17-P1-60-F5RIL-106	2	2	2	2	39.2	45.3	0.9
SD17-P1-61-F5RIL-107	3	2	2	2	26.4	62.7	1.0
SD17-P1-63-F5RIL-45	3	3	2	3	40.6	44.2	1.3
SD17-P1-64-F5RIL-46	3	2	2	2	32.3	56.4	0.4
SD17-P1-65-F5RIL-47	5	5	5	5	38.7	47.6	0.6
SD17-P1-66-F5RIL-109	2	2	2	2	28.0	57.8	1.0
SD17-P1-67-F5RIL-48	6	5	3	5	30.0	53.6	2.8
SD17-P1-70-F5RIL-110	4	3	3	3	40.7	45.8	0.7
SD17-P1-71-F5RIL-51	3	3	4	3	35.1	53.0	1.8
SD17-P1-72-F5RIL-111	6	6	6	6	43.8	43.4	0.8
SD17-P1-74-F5RIL-53	3	3	3	3	32.4	57.0	2.2
SD17-P1-75-F5RIL-54	2	3	2	2	26.8	61.6	0.5

SD17-P1-77-F5RIL-55	5	5	4	5	32.2	51.5	2.9
SD17-P1-78-F5RIL-113	5	5	5	5	30.7	55.1	0.8
SD17-P1-7-F5RIL-6	2	2	2	2	32.1	52.0	3.4
SD17-P1-80-F5RIL-114	2	2	2	2	30.2	56.6	0.8
SD17-P1-83-F5RIL-56	4	4	4	4	34.9	54.1	0.2
SD17-P1-84-F5RIL-115	2	2	2	2	26.7	61.6	0.7
SD17-P1-85-F5RIL-116	2	4	5	4	41.3	44.3	1.1
SD17-P1-86-F5RIL-117	4	4	4	4	33.7	56.6	0.7
SD17-P1-88-F5RIL-118	3	4	4	4	31.4	54.3	3.8
SD17-P1-89-F5RIL-119	4	4	3	4	37.9	48.7	0.3
SD17-P1-8-F5RIL-7	4	5	3	4	34.0	52.4	0.2
SD17-P1-90-F5RIL-58	6	6	6	6	42.2	46.5	1.8
SD17-P1-91-F5RIL-120	3	3	3	3	31.9	53.7	0.6
SD17-P1-92-F5RIL-121	2	3	3	3	50.3	35.5	2.6
SD17-P1-94-F5RIL-123	4	5	4	4	33.4	55.1	0.6
SD17-P1-95-F5RIL-124	2	4	3	3	36.9	50.1	0.3
SD17-P1-99-F5RIL-125	5	5	6	5	37.6	49.1	0.8
SD17-P2-100-F5RIL-85	2	2	2	2	26.8	56.0	3.5
SD17-P2-101-F5RIL-126	4	4	5	4	42.8	45.4	0.2
SD17-P2-105-F5RIL-129	4	5	2	4	38.7	46.8	0.3
SD17-P2-107-F5RIL-130	2	3	2	3	36.0	50.9	2.9
SD17-P2-108-F5RIL-87	5	6	6	6	39.4	47.2	0.0
SD17-P2-110-F5RIL-131	2	3	2	3	33.1	54.6	0.8
SD17-P2-111-F5RIL-132	2	2	4	3	19.0	66.3	0.8
SD17-P2-112-F5RIL-133	3	4	3	3	30.6	54.1	0.9
SD17-P2-114-F5RIL-134	2	3	2	3	32.4	56.5	0.2
SD17-P2-115-F5RIL-135	6	5	5	5	29.0	56.1	0.6

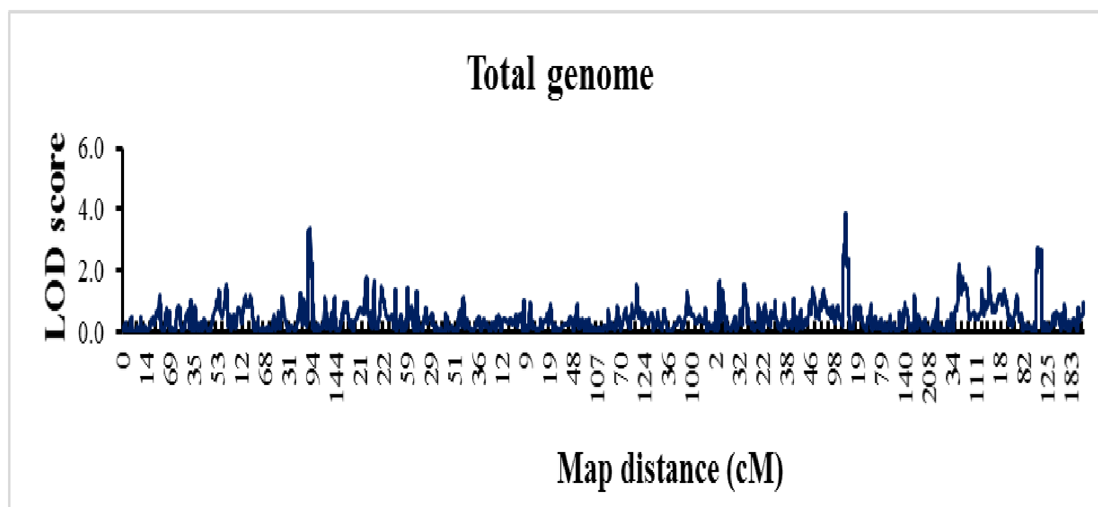
SD17-P2-116-F5RIL-90	3	3	4	4	31.6	54.6	1.1
SD17-P2-117-F5RIL-91	2	4	3	3	42.7	42.1	2.8
SD17-P2-74-F5RIL-60	6	5	6	6	29.8	56.8	1.9
SD17-P2-76-F5RIL-62	2	2	2	2	27.5	56.9	0.9
SD17-P2-77-F5RIL-63	6	6	6	6	32.9	55.4	0.5
SD17-P2-78-F5RIL-64	3	3	4	4	28.2	59.7	0.1
SD17-P2-79-F5RIL-65	3	3	2	3	41.6	44.5	2.5
SD17-P2-80-F5RIL-66	2	3	3	3	31.8	59.0	1.4
SD17-P2-81-F5RIL-67	5	4	3	4	35.8	49.1	4.1
SD17-P2-82-F5RIL-68	6	6	6	6	39.1	46.5	1.4
SD17-P2-83-F5RIL-69	6	6	6	6	41.7	43.0	4.1
SD17-P2-84-F5RIL-70	2	2	2	2	34.9	53.4	0.4
SD17-P2-88-F5RIL-74	6	6	6	6	42.6	44.7	0.8
SD17-P2-90-F5RIL-76	4	4	3	4	38.3	47.2	3.3
SD17-P2-91-F5RIL-77	3	4	4	4	33.6	56.1	0.8
SD17-P2-92-F5RIL-78	3	3	3	3	32.6	52.0	0.7
SD17-P2-97-F5RIL-83	6	5	4	5	38.9	46.7	2.2



Appendix Figure 1. Distribution of 192,467 GBS SNPs on 21 wheat chromosomes.



Appendix Figure 2. Physical map of markers used for mapping of BLS resistance.



Appendix Figure 3. Scatter plot of the genetic map and LOD score of 1211 markets used to map QTLs associated to BLS.

Appendix Table 2. Mean score of wheat spot botch from three experiments.

Name	Mean of Disease				Name	Mean of Disease			
	Ex 1	Ex 2	Ex 3	Mean		Ex 1	Ex 2	Ex 3	Mean
2145	3	3	3	3	CO03W054	4	4	4	4
2180	3	4	4	4	CO04025	4	4	3	3
2174-05	3	3	3	3	CO04393	3	4	3	3
ABOVE	2	3	3	3	CO04499	3	4	4	4
AGATE	2	2	2	2	CO04W320	4	5	4	4
AKRON	1	2	2	2	CO050337-2	4	3	4	4
ALICE	3	3	3	3	CO07W245	3	2	3	2
ALLIANCE	1	2	2	2	CO940610	3	3	3	3
ANTELOPE	4	4	4	4	COLT	1	2	1	1
ANTON	4	4	4	4	COMANCHE	5	4	4	5
ARAPAHOE	4	4	4	4	COSSACK	4	5	5	5
ARLIN	2	2	2	2	COUGAR	2	3	3	3
AVALANCHE	3	3	3	3	CREST	1	2	2	2
BAKERS_WHITE	4	3	3	3	CRIMSON	4	4	4	4
BENNETT	2	2	2	2	CULVER	3	4	4	4
BIG_SKY	3	3	3	3	CUSTER	2	2	2	2
BILL_BROWN	3	3	3	3	CUTTER	4	4	4	4
BILLINGS	2	2	2	2	DANBY	4	4	4	4
BISON	4	3	4	4	DARRELL	4	4	4	4
BOND_CL	4	4	3	4	DAWN	2	1	2	2
BRONZE	3	3	3	3	DECADE	3	3	3	3
BUCKSKIN	3	3	2	3	DENALI	3	3	3	3
BURCHETT	2	3	3	3	DODGE	1	2	2	2
BYRD	4	3	3	3	DUKE	4	4	4	4

CAMELOT	2	2	2	2	DUMAS	4	4	5	4
CAPROCK	2	3	3	3	DUSTER	1	2	2	1
CARSON	2	2	2	2	E2041	2	3	2	2
CENTERFIELD	3	2	3	3	EAGLE	2	3	2	2
CENTURA	4	4	4	4	ENHANCER	4	4	4	4
CENTURK78	3	3	3	3	EXPEDITION	4	4	4	4
CENTURY	2	2	2	2	FULLER	5	5	4	5
CHENEY	3	3	3	3	G1878	3	3	3	3
CHEYENNE	3	3	3	3	GAGE	3	3	3	3
CHISHOLM	2	2	2	2	GALLAGHER	3	4	4	4
CO03064	3	4	4	4	GARRISON	2	2	2	2
CO03W043	4	4	4	4	GENOU	2	3	3	3
GENT	4	3	4	4	LAMAR	3	2	3	3
GOODSTREAK	4	3	3	3	LANCER	3	3	3	3
GUYMON	2	1	2	2	LARNED	3	4	4	4
HAIL	4	4	5	4	LINDON	3	4	4	3
HALLAM	5	4	4	4	LONGHORN	5	5	4	5
HALT	4	5	4	4	MACE	3	4	4	4
HARDING	5	4	4	4	MCGILL	4	3	3	3
HARRY	5	4	4	4	MILLENNIUM	4	4	4	4
HATCHER	4	4	5	4	MIT	4	4	4	4
HEYNE	3	3	3	3	MT0495	1	2	2	2
HG-9	5	4	5	5	MT06103	3	3	3	3
HOMESTEAD	3	3	3	3	MT85200	3	4	4	4
HONDO	4	4	5	4	MT9513	3	3	3	3
HUME	3	3	3	3	MT9904	2	2	2	2
HV906-865	4	4	5	4	MT9982	2	2	2	2
HV9W03-1379R	3	3	4	3	MTS0531	2	3	3	3

HV9W03-1551WP	3	3	3	3	NE02558	4	4	3	4
HV9W03-1596R	4	4	4	4	NE04490	5	4	5	5
HV9W05-1280R	4	4	4	4	NE05430	5	4	4	4
HV9W06-504	1	2	2	2	NE05496	2	3	3	3
INFINITY_CL	4	4	4	4	NE05548	4	3	3	3
INTRADA	2	1	2	2	NE06545	3	3	3	3
JAGALENE	5	5	4	5	NE06607	2	3	2	2
JAGGER	3	3	3	3	NE99495	2	2	2	2
JERRY	4	4	4	4	NEKOTA	4	4	4	4
JUDEE	5	4	4	4	NELL	3	3	3	3
JUDITH	3	4	4	4	NEOSHO	4	3	3	3
JULES	3	3	3	3	NEWTON	3	3	4	3
KARL_92	2	3	3	3	NI06736	2	3	3	3
KAW61	2	2	2	2	NI06737	4	3	3	3
KEOTA	3	3	3	3	NI07703	3	2	2	3
KHARKOF	5	4	5	5	NI08707	3	3	3	3
KIOWA	3	4	4	4	NI08708	3	4	4	4
KIRWIN	3	4	4	4	NIOBRARA	4	4	4	4
KS00F5-20-3	4	4	3	4	NORKAN	4	3	3	3
LAKIN	4	4	4	4	NORRIS	4	4	5	4
NUFRONTIER	2	2	2	2	OK1067274	3	3	3	3
NUHORIZON	3	4	4	3	OK1068002	4	3	3	4
NUPLAINS	4	4	4	4	OK1068009	4	5	5	5
NUSKY	4	3	4	3	OK1068026	3	3	3	3
NW03666	4	5	5	5	OK1068112	4	4	4	4
OGALLALA	4	4	5	4	OK1070267	3	3	3	3
OK_RISING	1	1	1	1	OK1070275	3	3	3	3
OK02405	3	3	2	3	ONAGA	3	4	4	3

OK04111	3	4	3	3	OVERLAND	4	3	4	4
OK04415	4	3	4	4	OVERLEY	4	3	4	4
OK04505	3	3	3	3	PARKER	3	4	4	4
OK04507	4	3	3	3	PARKER76	2	3	3	3
OK04525	2	1	2	2	PETE	2	2	2	2
OK05108	3	4	3	3	PLATTE	5	5	4	4
OK05122	1	1	2	1	POSTROCK	3	3	3	3
OK05134	4	4	4	4	PRAIRIE_RED	4	4	3	4
OK05204	2	2	2	2	PRONGHORN	5	4	4	4
OK05303	4	4	4	4	PROWERS	4	4	5	4
OK05312	3	3	3	3	RAWHIDE	5	4	4	4
OK05511	3	3	3	3	REDLAND	5	4	5	5
OK05526	3	2	3	3	RIPPER	3	4	3	3
OK05711W	2	2	2	2	RITA	2	3	3	3
OK05723W	1	2	1	1	ROBIDOUX	4	4	5	4
OK05830	2	2	2	2	RONL	3	3	3	3
OK06114	4	3	4	4	ROSE	3	3	3	3
OK06210	4	3	3	3	ROSEBUD	3	3	4	3
OK06318	4	4	4	4	SAGE	3	2	3	3
OK06319	3	4	3	3	SANDY	4	4	4	4
OK06336	4	4	4	4	SANTA_FE	2	2	2	2
OK07231	2	3	3	3	SCOUT66	4	5	4	4
OK07S117	3	3	3	3	SD00111-9	4	4	4	4
OK08328	3	4	4	4	SD01058	2	3	3	3
OK09634	2	3	2	2	SD01237	3	3	3	3
OK101	1	2	1	1	SD05118	3	3	3	3
OK10119	4	4	4	4	SD05210	3	3	3	3
OK102	2	3	2	2	SETTLER_CL	5	4	4	4
OK1067071	4	3	4	4	SHAWNEE	2	3	3	2

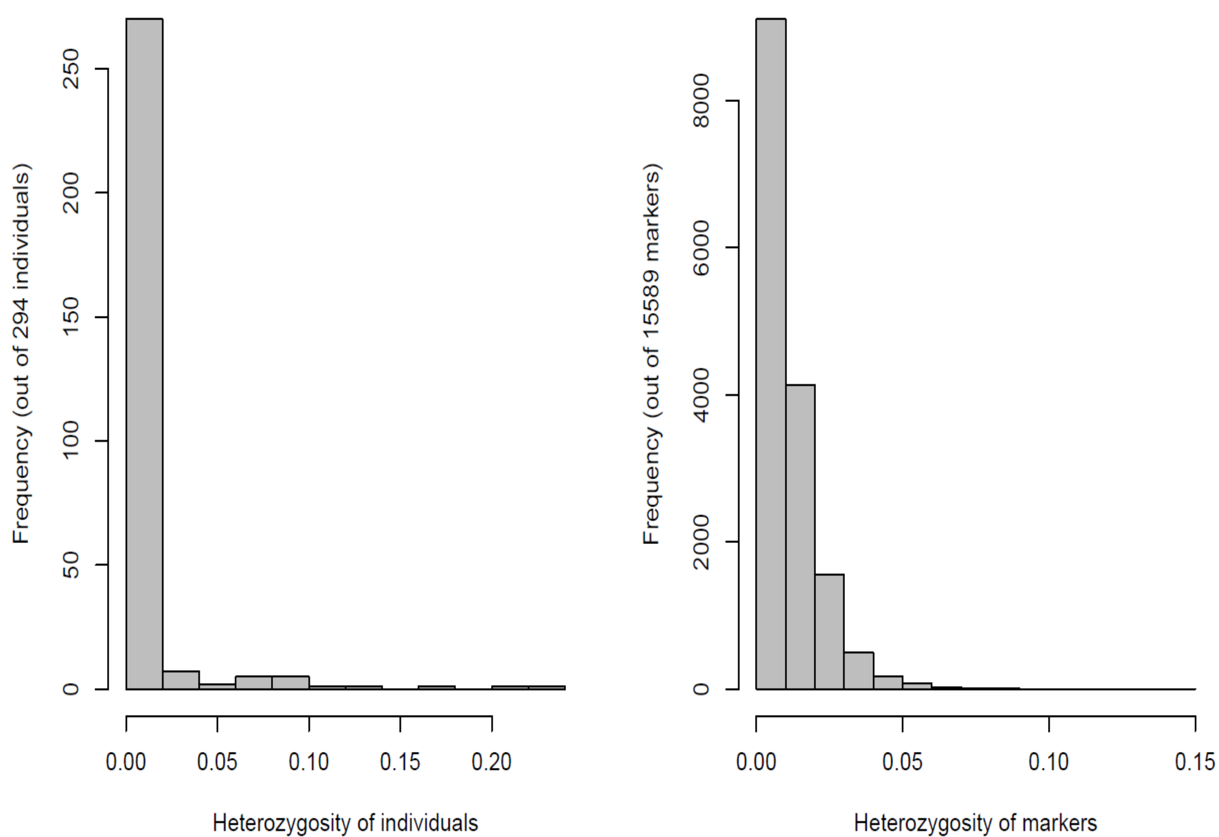
SHOCKER	4	4	3	4	TX03A0563	2	3	3	3
SIOUXLAND	4	4	4	4	TX04A001246	4	3	3	3
SMOKYHILL	4	4	4	4	TX04M410211	3	4	3	4
SPARTAN	5	4	4	4	TX04V075080	2	3	3	3
STANTON	3	3	4	3	TX05A001188	4	3	3	3
STURDY	3	4	5	4	TX05A001822	4	4	4	4
STURDY_2K	4	5	4	4	TX05V7259	3	3	3	3
TAM105	3	3	3	3	TX05V7269	4	4	4	4
TAM107	3	3	3	3	TX06A001132	4	4	3	4
TAM107-R7	5	4	4	4	TX06A001263	3	2	3	3
TAM109	2	3	3	3	TX06A001281	2	2	3	2
TAM110	3	3	3	3	TX06A001386	3	3	3	3
TAM111	3	3	3	3	TX06V7266	2	2	3	2
TAM112	3	2	3	3	TX07A001279	4	4	4	4
TAM200	3	4	3	3	TX07A001318	3	3	3	3
TAM202	4	4	4	4	TX07A001420	2	3	3	2
TAM203	4	4	4	4	TX86A5606	4	5	5	4
TAM302	3	4	4	4	TX86A6880	3	2	3	3
TAM303	4	3	4	3	TX86A8072	2	3	2	3
TAM304	4	3	3	4	TX96D1073	4	4	4	4
TAM400	2	3	4	3	TX99A0153-1	3	4	4	3
TAM401	3	2	5	3	TX99U8618	4	4	4	4
TAMW-101	4	4	4	4	VENANGO	2	2	2	2
TANDEM	4	4	3	4	VISTA	4	3	4	4
TARKIO	5	4	5	5	VONA	3	4	4	4
TASCOSA	3	3	3	3	W04-417	4	4	4	4
THUNDER_CL	5	4	4	4	WAHOO	4	4	4	4
THUNDERBOLT	4	3	4	4	WARRIOR	5	5	5	5
TREGO	3	2	3	3	WB411W	3	2	3	3

TRISON	2	2	2	2	WENDY	5	4	4	4
TRIUMPH64	2	3	2	2	WESLEY	3	3	3	3
TURKEY_NEBSEL	3	3	3	3	WICHITA	5	4	4	5
TX00V1131	4	4	3	4	WINDSTAR	4	4	4	4
TX01A5936	3	4	4	4	WINOKA	2	3	3	2
TX01M5009-28	1	2	2	2	YELLOWSTONE	2	3	3	3
TX01V5134RC-3	2	3	3	3	YUMA	2	2	2	2
TX02A0252	2	4	3	3	YUMAR	1	2	2	2
TX03A0148	3	4	4	4					

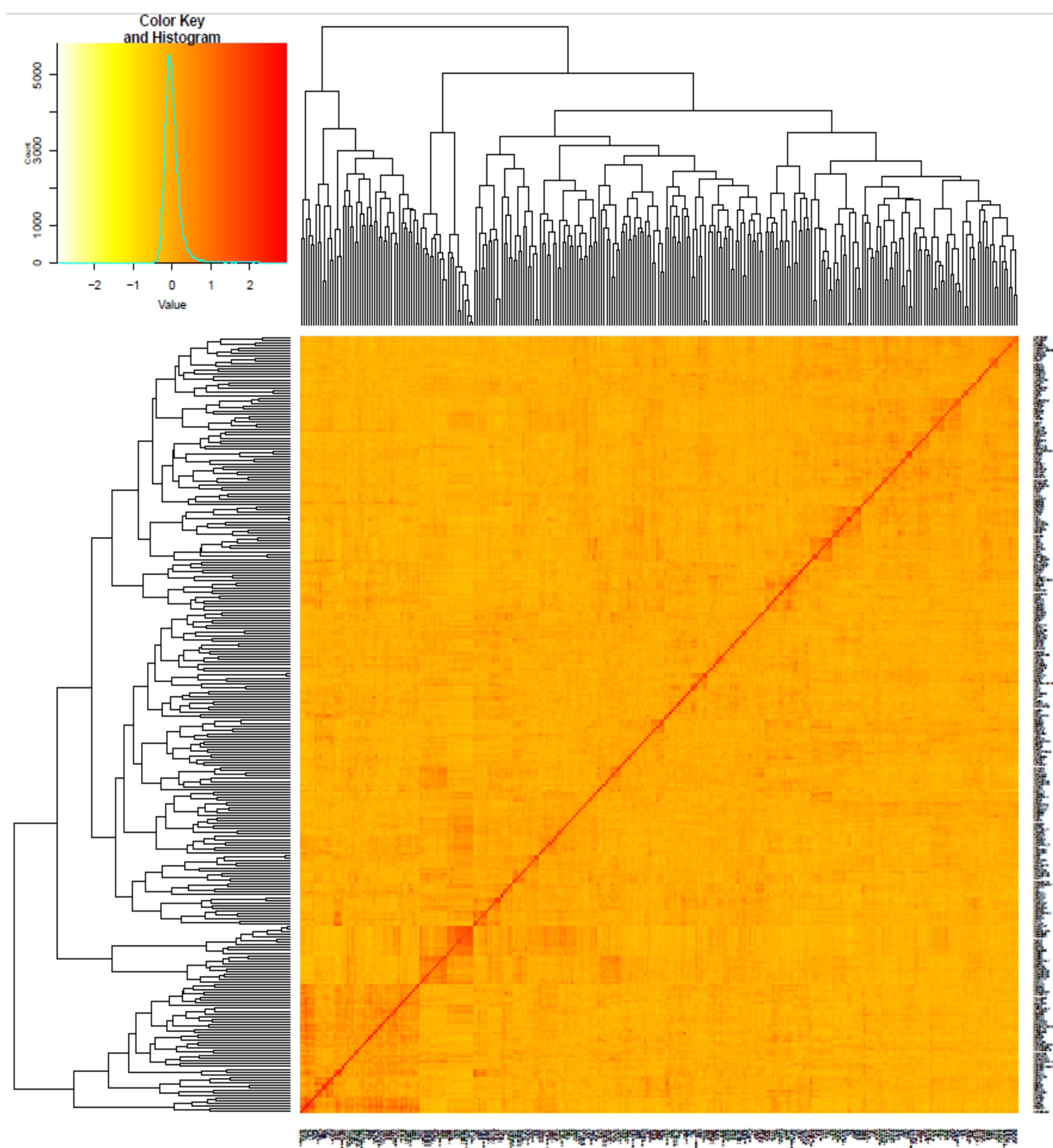
Appendix Table 3. Syntenic and non-syntenic LD for the whole, A, B, D genomes of hard winter wheat.

Dataset	Number of markers	Total marker pairs	Mean of r2 for all pairs	Total unlinked pairs	Significant pairs (P<0.001)		
					Total	Linked (r^2 >0.1)	Unlinked (r^2 <0.1)
Whole genome	1842	91307	0.03	85955	13076 (14.3)	5332 (5.8)	7744 (8.5)
A	739	39216	0.027	37065	5225 (13.3)	2151 (5.5)	3074 (7.8)
B	782	43995	0.028	41491	6489 (14.7)	2504 (5.7)	3985 (9.1)
D	321	8096	0.05	7399	1382 (17.1)	697 (8.6)	685 (8.5)

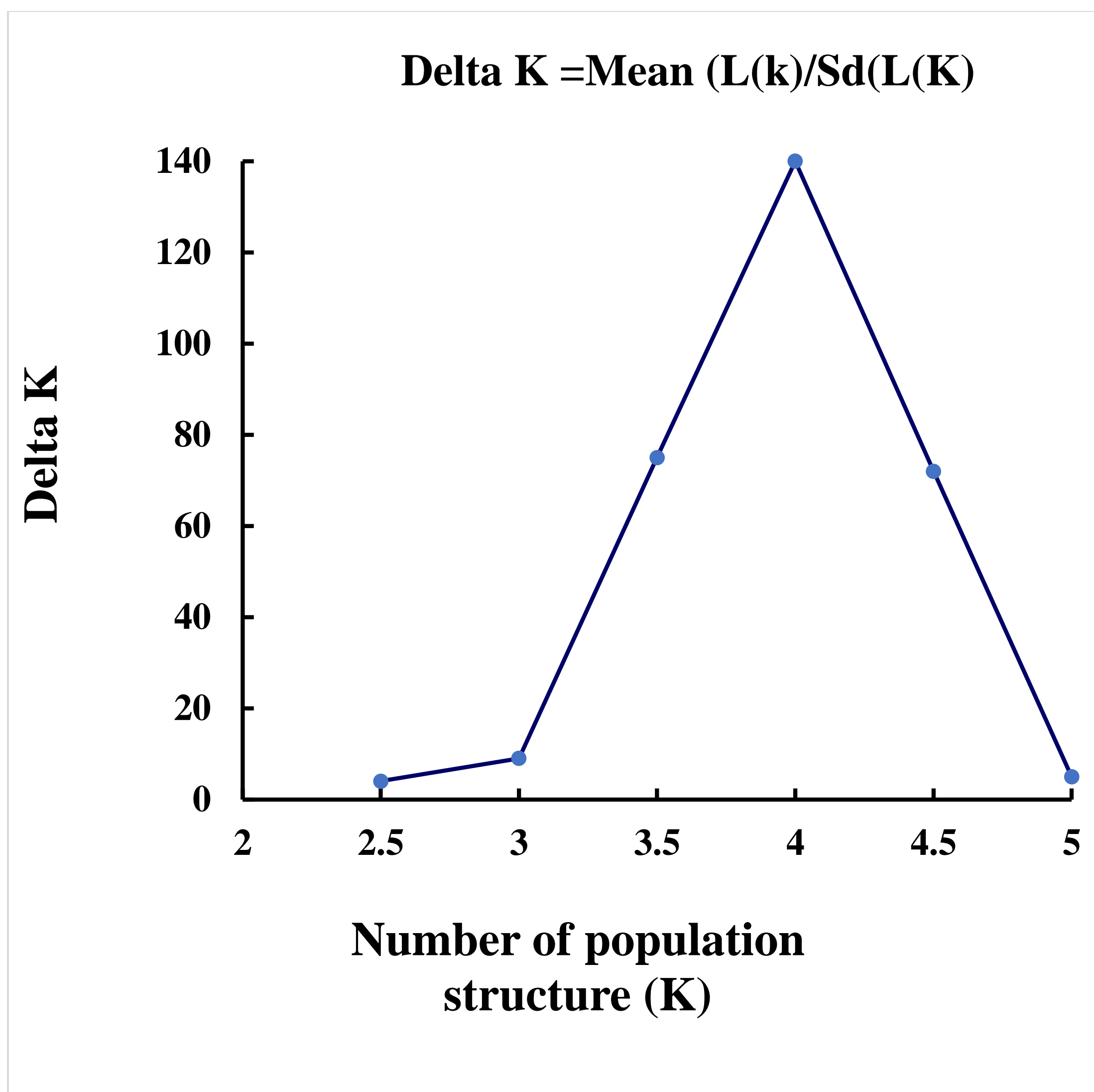
Number of brackets are parentage of markers out of total markers



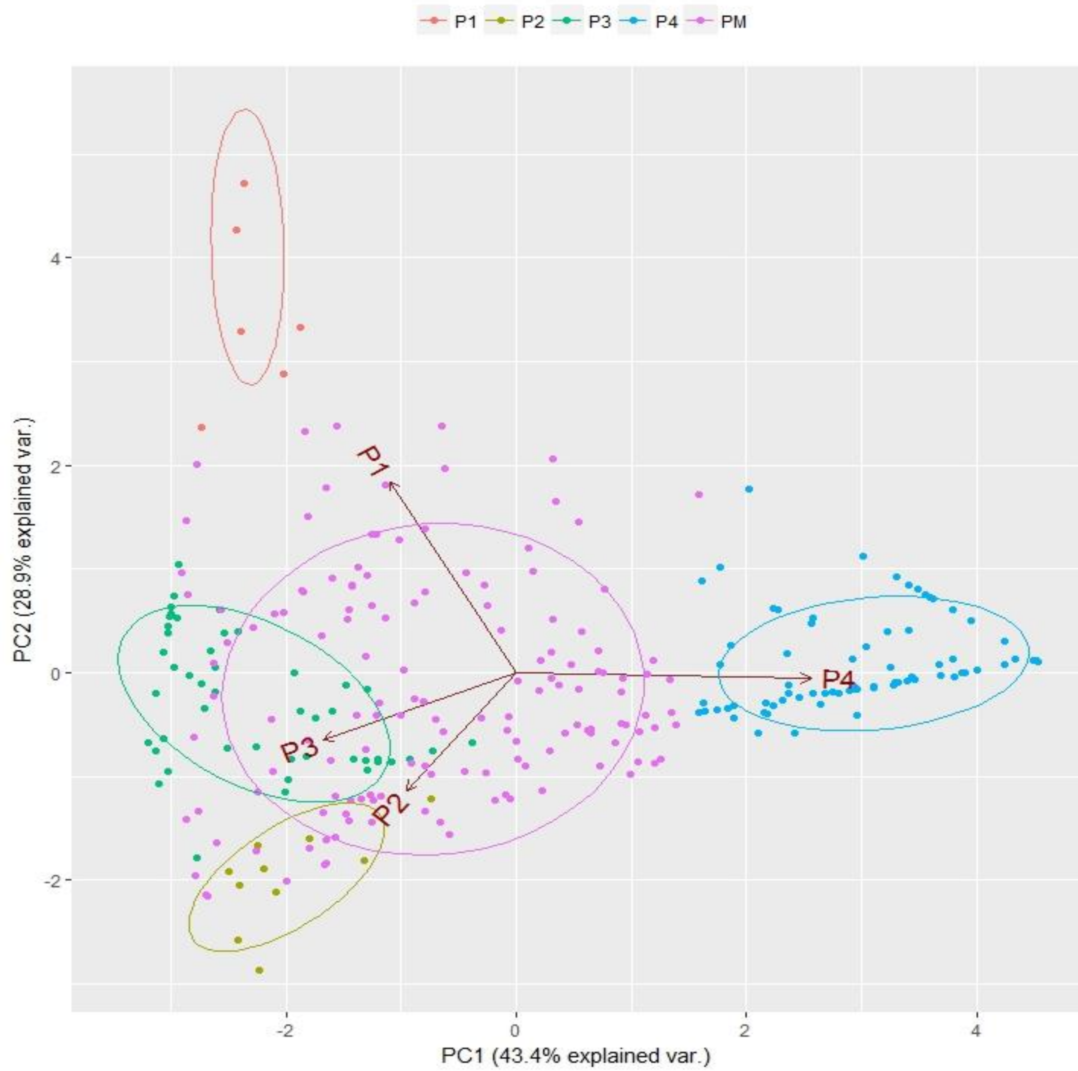
Appendix Figure 4. Frequency distribution of heterozygosity of individuals and markers used for spot blotch GWAS.



Appendix Figure 5. Heat map of 294 HRWWAP and 1590 markers used for AM.



Appendix Figure 6. A principal coordinate plot of K vs. Delta K for analysis of population structure in HWWAMP.



Appendix Figure 7. Principal component analysis of HWWAMP population based on population genetic admixture. P1-P4 and Pm represented population 1,2,3,4 and admixed populations, restively

Appendix Table 4. markers associated with winter wheat spot blotch resistance identified through association mapping in the 294 winter wheat Diversity Collection.

Markers	Chr	Pos	P-value	R^2	A allele	B allele	Additive effects	Genotypes with allele type	
								A	B
TA005844-0160	7B	86.4	4.20E-05	0.06	C	T	-0.46	146	140
Excalibur_c5700_705	7B	86.4	5.05E-05	0.06	A	G	0.45	142	147
Excalibur_c58742_144	7B	86.4	5.11E-05	0.06	A	C	-0.45	147	142
Kukri_c21628_1215	7B	85.3	5.35E-05	0.06	A	G	-0.45	127	163
Excalibur_c5700_527	7B	86.4	5.35E-05	0.06	C	T	-0.45	127	163
Excalibur_c5700_670	7B	85.3	6.14E-05	0.06	G	T	-0.44	127	167
Kukri_c22495_552	7B	86.4	6.26E-05	0.06	A	C	-0.44	131	163
Tdurum_contig9966_646	7B	86.1	8.09E-05	0.05	A	G	-0.43	128	166
Kukri_rep_c104877_2166	5A	59.1	8.27E-05	0.05	G	T	0.66	257	35

IWA8475	4A	118.7	1.55E-04	0.05	G	T	-0.44	204	90
Excalibur_rep_c79414_306	4B	36.8	7.30E-04	0.04	A	G	0.38	121	169
IAAV2383	3A	90.6	8.39E-04	0.04	A	G	0.37	126	163
BS00064369_51	4A	118.7	9.10E-04	0.04	A	C	-0.39	209	84
Kukri_c31121_1460	2D	80.1	9.87E-04	0.04	C	T	-0.45	54	238
Excalibur_c46082_440	3A	90.5	9.97E-04	0.04	C	T	-0.37	166	123
GENE-4933_489	4B	36.8	1.06E-03	0.04	A	G	-0.37	172	122
Tdurum_contig50625_2342	4B	36.8	1.06E-03	0.04	A	C	0.37	122	172
BobWhite_c20735_255	7B	3.0	1.30E-03	0.04	A	G	0.40	77	209
GENE-3572_70	5A	46.7	1.33E-03	0.04	C	T	0.45	55	233
Tdurum_contig90495_232	7B	87.4	1.35E-03	0.04	A	G	0.35	161	129
Excalibur_c11302_186	4A	135.2	1.43E-03	0.04	C	T	-0.72	17	268
IACX7746	4B	36.8	1.47E-03	0.04	A	G	-0.36	166	118
GENE-4933_1085	4B	36.8	1.56E-03	0.04	A	G	0.36	121	166
GENE-4933_1095	4B	36.8	1.59E-03	0.04	A	G	-0.36	167	119

BS00009480_51	4B	36.8	1.63E-03	0.04	A	G	-0.36	169	118
BS00009426_51	4B	36.8	1.73E-03	0.03	C	T	-0.35	171	123
Excalibur_c64568_149	2A	145.3	1.81E-03	0.03	C	T	-0.66	18	272
BS00022998_51	4A	127.1	1.89E-03	0.03	C	T	-0.47	46	242
BS00075332_51	7B	87.4	1.97E-03	0.03	G	T	0.34	163	126
IWA6895	5B	83.0	2.13E-03	0.03	C	T	-0.48	40	248
IACX8647	4B	36.8	2.17E-03	0.03	A	G	0.35	119	169
RAC875_c4851_1600	2D	103.3	2.37E-03	0.03	C	T	-0.62	21	273
Excalibur_c24600_733	4A	114.5	2.45E-03	0.03	C	T	0.36	80	210
Ra_c16330_1197	4A	114.5	2.53E-03	0.03	A	G	-0.36	212	82
BS00039641_51	4A	125.9	2.56E-03	0.03	A	G	0.46	244	47
IAAV9128	2D	103.3	2.59E-03	0.03	A	G	0.61	271	21
BS00010115_51	4B	36.8	2.68E-03	0.03	C	T	-0.34	169	123
Excalibur_c14217_1260	4A	114.5	2.81E-03	0.03	C	T	-0.36	209	80
BS00000577_51	4A	127.1	2.83E-03	0.03	C	T	0.44	241	49

IWA7040	1B	84.4	3.05E-03	0.03	A	C	-0.41	214	78
IWA4940	1B	84.4	3.11E-03	0.03	A	G	0.41	77	214
TA004912-0408	4A	114.5	3.20E-03	0.03	C	T	-0.35	213	80
GENE-0689_791	4A	127.1	3.20E-03	0.03	A	G	0.44	239	49
BS00009440_51	3B	51.1	3.30E-03	0.03	A	G	-0.33	94	200
Excalibur_c25898_434	6A	99.4	3.46E-03	0.03	C	T	0.60	274	20
Kukri_c43208_335	3D	67.2	3.61E-03	0.03	A	G	-0.40	230	61
GENE-1167_104	3B	80.1	3.70E-03	0.03	A	G	0.48	36	255
Kukri_c11709_874	3A	109.9	3.71E-03	0.03	C	T	-0.52	255	31
BS00049637_51	3A	109.9	3.77E-03	0.03	C	T	0.52	31	260
IWA5749	1B	108.4	3.91E-03	0.03	C	T	-0.34	102	192
BobWhite_c20621_541	1B	108.4	3.91E-03	0.03	C	T	-0.34	102	192
IWA4209	3D	67.2	4.03E-03	0.03	A	G	0.39	64	230
IWA5574	2A	113.3	4.04E-03	0.03	C	T	0.38	72	218
IWA8179	3D	107.9	4.06E-03	0.03	A	G	-0.40	229	62

IACX6337	2A	113.3	4.07E-03	0.03	A	G	-0.38	222	72
IACX4411	1B	108.0	4.08E-03	0.03	A	G	0.35	190	99
IWA5449	2A	113.3	4.09E-03	0.03	C	T	-0.38	219	72
Kukri_c27100_823	2A	113.3	4.09E-03	0.03	C	T	0.38	72	219
GENE-0689_776	4A	127.1	4.09E-03	0.03	C	T	-0.42	50	242
IWA3864	4A	127.1	4.09E-03	0.03	A	G	-0.42	50	242
Kukri_rep_c111517_289	1B	64.9	4.15E-03	0.03	C	T	0.47	257	35
Tdurum_contig29769_202	1B	64.5	4.18E-03	0.03	A	G	-0.47	35	258
Excalibur_c51643_145	1B	64.9	4.18E-03	0.03	C	T	-0.47	35	258
BS00084703_51	4A	127.1	4.37E-03	0.03	A	G	0.43	242	48
BobWhite_c17386_221	3D	107.9	4.43E-03	0.03	C	T	-0.39	230	62
Excalibur_rep_c106174_390	1B	64.9	4.47E-03	0.03	C	T	0.46	252	35
Kukri_c25281_99	2B	102.5	4.49E-03	0.03	A	G	0.33	199	90
BS00111091_51	4A	127.1	4.62E-03	0.03	A	G	-0.42	50	241
Excalibur_c15692_532	1D	33.0	4.79E-03	0.03	G	T	0.31	140	150

CAP7_c1241_128	1B	64.9	4.88E-03	0.03	A	G	-0.46	35	259
Excalibur_c27675_912	1B	64.9	4.88E-03	0.03	A	G	0.46	259	35
Excalibur_c32608_500	1B	64.9	4.88E-03	0.03	A	G	-0.46	35	259
Kukri_rep_c106406_265	1B	64.9	4.88E-03	0.03	C	T	-0.46	35	259
BS00099982_51	4A	127.1	4.91E-03	0.03	G	T	0.41	244	50
Excalibur_c15222_313	4A	132.9	4.98E-03	0.03	C	T	0.45	246	39
BS00022104_51	1B	64.9	5.04E-03	0.03	A	G	-0.46	35	257

Appendix Table 5. Chromosome location and nucleotide sequences of significant marks associated with SB.

Chromosome	Markers	location	Marker sequence
2D	Kukri_c31121_1460	607423370-607423456	GGTAAAGGGGTGTCCTTCTACAGATTGTTCTGCGGCAATAGCATGTCAGA**[C/T]**GCTGCTCGACTCTTTCCCAGTGATTCAAGTGTGACCGTAGCAGGAAGCGG
3A	IAAV2383	556462254-556462146	AGGTGGATCGCCGTGGGCGCTGCTCTTCTCCTCTAGGGTCTTCTCGTCGACCCCTCCGGCTCAACAGTCCGCCGGAGATGTGCCCGCCCCATCCGCGGTG**[A/G]**ACCACAAG
4A	IWA8475	692383211-692383111	TTCATCTTTGGACTGAGTTTCCCATGAAGAGGTGGATTATTGGATTGCCT[G/T]GTGACTCGGCTGTACTATTTTGTTAAATCGTTTGTTTCACCTACGGTTTC
4B	Excalibur_rep_c79414_306	14118264-14118164	TTCGAAAGAGCGTTGAAGCAGAGCCTCGAGAGGGTGCGGATCAGCGCTAG**[A/G]**TGGATCGACAGCATCAAGAGCGAGCCCAGCCTTGCGCAAACGGTGCAGCA

5A	Kukri_rep_c104877_2166	480285174-480285274	GAAACATGGCAGTTTCTGATGTGAAGGCTGTCATGTTGGAAATGAACACA**[G/T]**CAGATAGCGTTCAAACACAAGATCTCAAGTCGGCATCTGAAGACAGGAGT
7B	TA005844-0160	608913624-608913673	CTTCCCACGCATGAAACTGTACAATTTGTTACACGGATGCCAATATCCAT**[C/T]**CCT

Nucleotide in the brackets are SNPs

Appendix Table 6. Additive effects of alleles on resistant genotypes based on significant markets associated with SB.

SNP Markers	Additive effect	Alleles		Chrom	Wheat Genotypes				
		A	B		COLT	CUSTER	OK05723W	VENANGO	Duster
Kukri_c31121_1460	-0.45484	C	T	2D	CC	TT	TT	TT	TT
Kukri_rep_c104877_2166	0.66477	G	T	5A	GG	TG	TT	TT	GG
IWA8475	-0.4438	G	T	4A	GG	GG	GG	TT	GG
Excalibur_rep_c79414_306	0.38024	A	G	4B	GG	GG	AA	GG	GG
IAAV2383	0.37269	A	G	3A	GG	GG	AA	GG	GG
TA005844-0160	-4.581E-01	C	T	7B	CC	TT	CC	CC	CC

Allele A indicates additive effect; allele B indicates no effect (zero effect). -, disease reducing alleles, +, disease increasing allele