THE ACQUISITION OF USEFUL DISEASE RESISTANCE GENES FOR HARD RED WINTER WHEAT IMPROVEMENT

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The Acquisition of Useful Disease Resistance Genes for Hard Red Winter Wheat Improvement

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ABSTRACT

This study was part of a larger pre-breeding effort to develop new parental materials carrying useful genes for disease resistance and adaptation. Firstly, marker-assisted backcrosses were employed to transfer and pyramid combinations of disease resistance genes (*Fhb1*, *Lr34, Lr53*, *Sr2, Sr26*, *Sr39,* and *Sr50*) and a reduced height gene (*Rht-B1b*) into the variety Norstar. Following the third backcross to Norstar, the various backcrossed progenies were inter-mated to derive progenies having combinations of *Fhb1* and *Rht-B1b* plus the targeted leaf and/or stem rust resistance genes. Five NILs (each carrying *Fhb1* and *Rht-B1b*) that differ for the leaf and stem rust resistance genes they possess were recovered. Secondly, a mapping study using the 9K Illumina Infinium iSelect wheat assay was conducted with a F_2 mapping population developed by crossing *Lr59*-25 (0306/2*CS*ph1b*//CSN1AT1B/3/Thatcher) and Superb. *Lr59* was mapped 0.5cM distally from the co-segregating SNPs IWA1495, IWA6704, IWA2098 and IWA969 on wheat chromosome arm 6BS.

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LIST OF ABBREVIATIONS

%..... Percentage

°F.....Degree Fahrenheit

APR.....Adult Plant Resistance

CAPS.....Cleaved Amplified Polymorphic Sequence

CIMMYT..... International Maize and Wheat Improvement Center

cM..... Centimorgan

DON..... Deoxynivalenol

ESTs.....Expressed Sequence tags

F_2.....Second filial generation

FAOUN..... Food and Agriculture Organization of the United Nations

Fg .....*Fusarium graminearum*

FHB.....Fusarium Head Blight

ha.....Hectare

HR..... Hypersensitive Response

hrs..... hours

HRSW.....Hard Red Spring Wheat

HRWW..... Hard Red Winter Wheat

HWW..... Hard White Wheat

IFPRI.....International Food Policy Research Institute

mRNA..... messenger RNA

NASS..... National Agricultural Statistics Services

ND.....North Dakota

NDSCO..... North Dakota State Climate Office

NDSU.....North Dakota State University

NDWC..... North Dakota Wheat Commission

NIL..... Near Isogenic Lines

PCR..... Polymerase Chain Reaction

Pgt..... *Puccinia graminis* f. sp. *tritici*

ROS.....Reactive Oxygen Species

SCAR.....Sequence Characterized Amplified Region

SNP.....Single Nucleotide Polymorphism

SRWW..... Soft Red Winter Wheat

SSRs..... Simple Sequence Repeats

STS.....Sequence Tagged Site

SWW..... Soft White Wheat

US..... United States

USDA..... United States Department of Agriculture

1. INTRODUCTION

North Dakota (ND) is a major producer of durum and spring wheat, yet the acreage and production of winter wheat is comparatively low. However, growing interest in winter wheat production in recent years has prompted North Dakota State University (NDSU) to implement a winter wheat breeding program in 2011. The first objective of the new program is to develop a productive breeding population with adequate variation for cold-hardiness, yield, disease resistance and processing quality.

The winters in North Dakota are severe, making cold-hardiness a primary breeding objective. The climate is also conducive to the development of diseases such as leaf rust, stem rust, tan spot, Stagonospora nodorum and fusarium head blight (FHB), which necessitates breeding for resistance. So, this project focused on the development of winter-hardy parental germplasm with incorporated disease resistance genes. In view of the difficulty to pyramid cold-hardiness (low heritability) with disease resistance through regular crosses, it was decided to upgrade Norstar for its future use as a breeding parent. Norstar is an old Canadian variety with exceptional cold-hardiness, yet is lacking in disease resistance and is too tall under North Dakota growing conditions (Fowler, 2008). The targeted genes included in this study are a Fusarium head blight resistance gene (*Fhb1*), two leaf rust resistance genes (*Lr34, Lr53*) and four stem rust resistance genes (*Sr2, Sr26, Sr39, Sr50*). An attempt was also made to co-transfer the reduced height gene, *Rht-B1b*, with the disease resistance genes.

In addition to the development of germplasm, this study also aimed to map a recombined alien translocation chromosome carrying the *Lr59* leaf rust resistance gene. New virulent races with the ability to overcome commercially available disease resistance genes, are constantly

evolving. Breeding programs should therefore prioritize the introduction of novel sources of disease resistance in their germplasm (Kolmer et al., 2007).

The major objectives of this study were to:

i) Employ marker-assisted backcrossed based gene pyramiding to transfer and pyramid combinations of resistance and reduced-height genes from spring wheat into the Norstar genetic background.

ii) Develop different set of NILs carrying *Fhb1* and *Rht-B1b* in combination with one to two rust resistance genes.

iii) Linkage map a novel recombined alien translocation carrying the leaf rust resistance gene, *Lr59,* to a wheat chromosome.

2. LITERATURE REVIEW

2.1. Introduction

Wheat (*Triticum aestivum* L.) is one of the major grains of the world, ranking third after maize and rice, which is the staple food of about 35% of the world population (http://faostat3.fao.org/; Tyagi et al., 2014). The total dietary calories consumed from wheat are 16% in developing and 26% in developed countries. It is the most widely adapted crop and can be grown across a diverse range of environmental conditions. Bread, biscuit, chapatti, macaroni, pasta, noodles and dumplings are some of its widely used end products. Even in the Neolithic period, wheat and barley formed the principal grain stocks that became the basis for the successful spread of agriculture and settled societies (Zohary et al., 2012, Brenchley et al., 2012). Therefore, wheat has been crucial in sustaining human societies and contributed to their continued development since its domestication in the old world.

The production of wheat increases significantly each year. In 2012, around the world, about 215.4 million ha of land was planted with wheat producing 670.9 million tons with an average productivity of 3.43 tons/ha (Food and Agricultural Organization of United Nation, 2014). Among all the wheat producers, the European Union is the top producer with a production of 135.9 million tons, followed by China (120.6 million tons) and India (94.9 million tons). Total, worldwide production is forecasted to reach 701.7 million tons in 2014/15 (Food and Agricultural Organization of United Nation, 2013).

2.1.1. Taxonomy of wheat

Wheat is a member of the Poaceae (grass) family that consists of more than 600 genera and over 10,000 species. The Poaceae family encompasses three major subfamilies, the Pooideae, the Panicoideae and the Ehrhartoideae (Bolot et al., 2009; Kellogg and Buell, 2009;

Davidson et al., 2012). Wheat belongs to the sub family Pooideae that also includes the agriculturally important crops oat, rye and barley. The major crops in the sub family Panicoideae are maize, sorghum and sugarcane and in the Ehrhartoideae are rice and Brachypodium. Molecular analyses of the species within the family Poaceae showed the existence of shared sets of genes suggesting that whole genome duplication and chromosome fusions may have occurred 56-70 million years ago (MYA). This whole genome duplication event was followed by evolutionary divergences that lead to the formation of the numerous different species within the grass family. The Triticeae is a tribe within the sub family Pooideae that includes wheat, barley and rye which supposedly diverged from the closely related Brachypodium around 30 MYA (Wicker et al., 2011).

The species of the wheat group (*Aegilops* and *Triticum*) consist of 13 diploid species and 18 allopolyploid species (Feldman and Levy, 2012). Evolutionary studies suggested that some of the diploids may have diverged from a common progenitor some 2.5-4.5 million years ago (Huang et al. 2002; Feldman and Levy, 2012). The evolution of the wheat group of species involved different hybridization and polyploidization occurrences apart from speciation. The first hybridization was between *Triticum urartu* (AuAu) and an unidentified species (BB) similar to *Aegilops speltoides* that occurred around 0.5 to 3 MYA (Berkman et al., 2013) to produce wild emmer or *Triticum turgidum* (AuAuBB). Cultivated emmer wheat subsequently hybridized with *Aegilops tauschii* (DD) in a second event that finally resulted in the allohexaploid genome of *T. aestivum* (AABBDD).

2.1.2. Domestication of wheat

The history of wheat can be traced back to the Neolithic period (~10,000 years ago), a period marking the domestication of tetraploid and hexaploid wheat (Dubcovsky and Dvorak,

2007; Berkman et al., 2013) and suggests that domestication selection was imposed only on wild einkorn and wild emmer wheat. Since hexaploid wheat is the result of recent hybridization and polyploidization events, it has never been subjected to domestication selection nor does it have wild hexaploid wheat progenitors. Accordingly, molecular analyses showed that genes related to domestication occur mainly on the A and B genomes (Gegas et al., 2010; Berkman et al., 2013).

The first domestication selection was done on the diploid wild progenitor, *T. boeoticum,* to result in domesticated diploid einkorn wheat, *T. monococcum* (Peng et al., 2011). However, during subsequent evolution and domestication this species was completely replaced by tetraploid and hexaploid wheat making it a relic in modern day agriculture (Zohary et al., 2012). Domestication selection also occurred at the tetraploid level. Based on biological and archaeological evidence, wild emmer wheat (*T. turgidum spp. dicoccoides*) is considered as the first domesticated tetraploid, AABB wheat (Shewry, 2009). Wild wheat typically had ears that were fragile and shattered once ripe, and glumes that were tightly attached to the grains. These characteristics were restrictive in harvesting and threshing. Domesticated emmer wheat retained some of the primitive features such as a relatively fragile rachis and non-free-threshing habit. Modern day domesticated wheat has non-shattering ears and grains that are easier to thresh. Together with other domesticated cereals, such as einkorn wheat and barley, emmer wheat played a major role in the beginning of agriculture and constituted an important breakthrough in the transformation to an agrarian life style in human history. More advanced forms of tetraploid wheat with free-threshing spikes (for example durum wheat: *T. turgidum* ssp. *turgidum* conv. *durum*) evolved from domesticated emmer wheat (Thanh et al., 2013).

In addition to the rachis fragility, genetic factors that control other domestication related traits have been studied in tetraploid wheat. A study by Thanh et al., (2013) helped to clarify the

genetic modifications that took place during the early stages of domestication prior to the appearance of durum wheat. Durum wheat acquired desirable characteristics such as a soft glume, tough rachis, and free-threshing habit as compared to more primitive emmer wheat. QTL analyses were conducted using durum wheat, which is relatively new among the domesticated tetraploid wheats, as representative of domesticated tetraploid wheat (Thanh et al., 2013). They were able to detect seven domestication trait associated QTL on chromosomes 1B, 2A, 2B, 3A, 3B, 4A, 5B, and 7B of tetraploid wheat. Among these, chromosomes 2A and 3B were found to harbor regions affecting rachis fragility which were selected during domestication prior to the appearance of relatively recent tetraploid forms of *T. turgidum* conv. *durum.* The fragile rachis of the wild forms allows seed dispersion, whereas the firm rachis of cultivated forms allows the harvesting of the kernels after ripening. Among the traits that underwent changes during the early cultivation of tetraploid wheat, those related to rachis fragility, growth pattern and seed production played the most significant and important roles.

An additional trait that distinguishes between the more advanced forms of domesticated tetraploid wheat, e.g. durum wheat, and both the wild and domesticated emmer wheat is kernel threshability. In hexaploid wheat, the glume tenacity gene *Tg*, along with the major domestication locus *Q*, control threshability (Simons et al., 2006). The *Q* gene also influences traits like spike length, glume shape and tenacity, rachis fragility, and plant height. In the non-free-threshing wild and domesticated emmer wheat, kernels were not easily separated from the spikelet whereas in free-threshing durum wheat, the glumes are easily detachable from the kernels (Sood et al., 2009). The pleiotrophic *Q* gene has not only changed brittle rachis, tenacious glumes and non-free threshability; it also played a major role in increasing yield and

yield components (Peng et al., 2011). Subsequently, ongoing selection and, recently, directed breeding has continued to change the wheat plant.

2.1.3. The wheat genome

The genome of wheat is huge and complex. The haploid genome consists of ~16,000 base pairs of DNA, almost 40 times the size of the rice genome. Only 1-5% of the wheat genome represents genes (Lazo et al., 2009, Sidhu et al., 2008). The wheat genome is contained in 21 pairs of chromosomes (2n=6x=42) derived from diploid (A, B and D genome) ancestral species (Berkman, 2013). The homoeologous chromosomes give wheat an extraordinary ability to buffer the loss of complete chromosomes or chromosome segments (Lazo et al., 2009). Although, wheat is an allo-hexaploid species with three homoeologous chromosome sets, it behaves like a diploid with normal disomic inheritance (Sidhu et al., 2008). Pairing among the homoeologous chromosomes is strongly suppressed by the *Ph1* (pairing homoeologous) gene located in the long arm of chromosome 5B.

2.1.4. Wheat in the United States

Wheat is the major crop in the Great Plains and the primary food grain in the United States (Paulsen and Shroyer, 2008). It was introduced into the United States in 1602, 81 years after its introduction in the neighboring country, Mexico (Olmstead and Rhode, 2011). However, until the mid-1850s wheat was not successfully and extensively cultivated throughout the United States. The introduction of the first hard red spring wheat, Red Rife, during the mid-1850s marked the expansion of wheat into Wisconsin, Minnesota, the Dakotas, and Canadian Prairies (Olmstead and Rhode, 2008; 2011). In 1873, a new winter variety, Turkey, entered Kansas which eventually helped broaden the climatic ranges of wheat to colder areas. This variety quickly gained popularity and was disseminated across North America. Eventually, the great

plains of the US became the largest winter wheat growing area and winter wheat got established as the most successful class of wheat grown in the US.

Currently, six different classes of wheat are grown in 50 US states (Kansas Wheat Commission and Kansas Association of Wheat Growers, 2014). These are Hard Red Winter Wheat (HRWW), Hard Red Spring Wheat (HRSW), Hard White Wheat (HWW), Soft White Wheat (SWW), Soft Red Winter Wheat (SRWW) and Durum. The different classes are unique in their milling, baking and other end use product characteristics. HRWW is used for bread making and has moderate protein content (11-12%) as well as good baking and milling qualities. It is mainly grown in the Great Plains and in California. HRSW is spring-seeded bread wheat that is well known for its higher protein content (13-14%) and better milling and baking qualities. North Dakota, South Dakota, Minnesota and Montana are its major growing areas. The newest class of wheat is HW that is well suited for the production of noodles, yeast breads and flat breads. SW and SWW are bread wheat classes with low protein content (10%) that are mainly used for making crackers, cakes, flat breads, noodles and pastries. The hardest of all is Durum that is being predominantly used for macaroni, spaghetti and other pasta products.

The US currently ranks fourth in terms of acreage, production and productivity of wheat with a contribution of 9.2% towards global wheat production (Food and Agricultural Organization of United Nation, 2014). In the 2013/14 crop season, 55.16 million acres of land in the US was planted with wheat producing 2,129.70 million bushels with an average yield of 47.2 bushels per acre (USDA-NASS. 2014). HRWW contributes most to the total US crop. In 2013/14 it was grown on 44% of the wheat-acreage and produced 34.9% of the total wheat production. In the same season, HRSW covered 23.69% of the total harvested acreage and

contributed 23.26% to the overall wheat production. Durum is the least cultivated among all known wheat classes in the US.

2.1.5. Wheat in North Dakota

North Dakota is a rural state that generates the majority of its revenue from agriculture (Enz, 2003; NDWC, 2014).Wheat is the chief commodity in North Dakota and the state contributes 13% towards the total US wheat production (USDA-NASS, 2014). About one fourth of the total land area in ND is covered by wheat, producing nearly one-third of total farm revenue (NDWC, 2014). North Dakota ranks second within the nation in total wheat production, after Kansas. Producers in ND focus mainly on hard red spring wheat, durum, hard red winter wheat and a small portion of white wheat. The state is the nation's leader in hard red spring wheat and durum wheat production. Durum wheat produced in North Dakota accounted for 48% of the national production and hard red spring wheat production accounted for 44% of the total US crop in 2013 (USDA-NASS, 2014).

Winter wheat production in North Dakota is low because of the harsh winter conditions, and accounts for only 3 to 10 percent of the total wheat acreage in the state (NDWC, 2014). Only a small group of winter wheat cultivars can endure the severe winters of ND (Schumann and Leonard, 2000). In 2013, ND ranked 30th in the US in winter wheat production and contributed only 1% to the nation's winter wheat production (USDA-NASS, 2014). Nevertheless, interest in winter wheat has increased in recent years because of the higher return as compared to spring wheat. In 2012, the winter wheat acreage was a record high in North Dakota, covering 700 thousand acres of planted area which was twice much as much as in 2011 (USDA-NASS, 2012,). In 2013, the winter wheat acreage plummeted to 220 thousand acres with only 205 thousand

acres harvested (USDA-NASS, 2014). However, a new record acreage was reached in 2014, amounting to 800 thousand acres planted and 560 thousand acres harvested.

2.1.6. Constraints in wheat production

The growth, yield and quality of wheat are affected by many abiotic (environmental) and biotic stresses. Abiotic or environmental stresses include unfavorable environmental conditions like extreme temperatures, drought, frost, acidity, salinity and deficiency or toxicity of minerals (Sutton, 2009). In the US, the majority of the wheat producing states can be disastrously affected by drought (Hegeman, 2013; Dreibus, 2014). For winter wheat, drought combined with late spring freeze or late fall warmth can reduce production to below average or normal yields (Potter, 2014). Late fall warmth can induce excessive vegetative growth in winter wheat. An abrupt decrease in temperature during the fall and/or late fall warmth can limit the period required for winter wheat to gain cold hardiness and makes them prone to winter kill. Cold tolerance in winter wheat is also affected by dry soil which causes improper development of the secondary roots with few tillers being formed. Underdeveloped plants have reduced ability to withstand bitter cold winter conditions. Dry soil conditions can also cause desiccation in winter wheat, making it prone to winter injury.

Along with suboptimal environmental conditions, biotic stresses act independently or in combination with abiotic stresses to prevent a wheat crop from reaching its full genetic development and production potential. Thirty one to 42 % of wheat crops are assumed to be destroyed by biotic stresses each year (Agrios, 2005; Dubin and Brennan, 2009). Biotic stresses mainly imply damage caused by living organisms, like insects, mites, weeds or pathogens (fungi, bacteria, nematodes, and viruses) (Trueman, 2014). In the US, wheat is attacked by more than 30 different species of insects and mites (Elliot, 2010). The Russian wheat aphid (*Diuraphis noxia*)

and greenbug (*Schizaphis graminum*) are the most prevalent insect pests in the Great Plains that can cause significant yield loss, if uncontrolled. These insects affect the wheat directly by physical damage and the extraction of nutrients while feeding and/or acting as a vector to transfer pathogens

Weed poses another production problem as it competes with wheat for resources like space, radiation, soil moisture, nutrients, and harbors unwanted insects, pathogens and diseases (Alberta Agriculture and Rural Development, 2001). Downy brome, goat grass, and volunteer rye are the most common weeds that pose serious threats to winter wheat in the Great Plains (Elliot, 2010). Significant amounts of yield are compromised if these weeds remain unmanaged. Herbicides combined with scientific cultural practices are used to effectively control the weed menace.

The most damaging among the biotic stresses are those caused by the plant pathogens. Diseases caused by pathogens alone account for 14 % of overall losses attributed to biotic stress (Agrios, 2005; Dubin and Brennan, 2009). Among the pathogens, fungi are the major cause of plant diseases and more than 8,000 plant disease causing species are known (Trueman, 2014). Other disease causing pathogens like bacteria involve only 14 genera, while very few plant pathogenic viruses are known to exist. In wheat, the soil borne fungal pathogen, *Fusarium* spp. and the rust diseases (caused by *Puccinia* spp.) rank among the most damaging (Elliot, 2010).

2.2. Wheat Rust

Cereal rust is the oldest known epidemic disease in wheat and other small grain cereals and is caused by rust fungi belonging to the genus *Puccinia* (USDA-ARS, 2013, Wegulo, 2012, Marsalis and Goldberg, 2006). *Puccinia* ssp. have been infecting cereal crops since old age

agriculture. Evidence of its destruction can be traced back to 384-322 BC in Aristotle's literature on the havoc caused by epidemics of wheat rust (Roelfs et al., 1992). The first detailed and precise report on wheat stem rust was provided by the Italians Fontana and Tozzetti in 1767. Since then numerous research studies and surveys of wheat rust epidemics and their distribution were produced. A huge loss, estimated at 300 million bushels of wheat was caused by stem rust in the US and Canada in 1916 (USDA-ARS, 2013). It was followed by another stem rust epidemic in 1935 that destroyed approximately 135 million bushels of wheat, mainly in the Dakotas and Minnesota. The US wheat crop also experienced losses estimated at 350 million dollars in 1953 to 1954. Consequently, the effective, immediate and durable control of wheat rust became a major objective in the majority of wheat breeding programs.

Taxonomically, *Puccinia* belongs to largest order, Pucciniales under the sub phylum Basidiomycota which consists of 160 genera and 7,000 species (Aime, 2006; Duplessis et al., 2011). The genus *Puccinia* consists of 4,000 species, among which *P. triticina* (leaf rust), *P. graminis* (stem rust), and *P. striiformis* (stripe rust) are the most destructive species (Broad Institute, 2010). They are obligate biotrophs and can only grow in the living tissue of their host (Duplessis et al., 2011). They survive as spores, mainly as teliospores, in the absence of living tissue (Schumann and Leonard, 2000). The rust fungi infiltrate the host plant through specialized structures called haustoria to extract the nutrients from them and to shut down their defense system. Rust fungi possess an extraordinarily ability to mutate, develop and spread rapidly under different environmental conditions (Wegulo, 2012). These characteristics enabled the fungus to readily overcome host defense mechanisms and allowed it to become the most notorious and destructive pathogen of wheat and other cereals crops.

Wheat rust fungi are heteroecious and require two phylogenetically separate hosts and five different spore stages to complete their life cycle (Duplessis et al., 2011; Marsalis and Goldberg, 2006). Among the two hosts, one is considered an economic/primary host (wheat) and another one serves as an alternative/secondary host, which normally is a weed or native plant, (e.g. barberry (*Berberis vulgaris*) in the case of stem rust). An alternative host has not been identified for stripe rust, thus it is believed that its life cycle is modified to complete in a single host.

2.2.1. Stem rust

Stem rust is caused by the fungus *Puccinia graminis* f. sp. *tritici* (Roelfs, 1992). It is also known as black or summer rust because of the profuse production of shiny black teliospores in the uredinium late in the season or under favorable conditions. It requires warm temperatures within the range of 80°F to 100°F as well as dew or rain moistened leaves for infection (McMullen et al., 2008a). In ND, these temperature ranges are mostly experienced late in the season causing the incidence of stem rust at a late stage, close to maturity. Stem rust can be identified as irregular-shaped pustules containing dark-red brown spores that burst through the epidermis. Pustules are primarily noticed on stem and leaf sheaths, however, scattered pustules can also develop in the glumes and awns.

The disease cycle of stem rust starts with the exposure of freshly planted wheat to its spores (Schumann and Leonard, 2000). The source and type of spores depends upon climate as well as on the presence or absence of barberry. Wheat in warm climates of the US mainly gets infected by urediniospores that have been overwintering in the Gulf Coast and Southern Texas on susceptible wheat cultivars or volunteer wheat (Roelfs, 1989). In temperate climates, the infecting spores may be the aeciospores from barberries or urediniospores from far away regions

with moderate winters (Schumann and Leonard, 2000). So, the disease cycle of stem rust occurs both in the presence and absence of the alternative host.

In the presence of barberry, Northern American wheat gets exposed to a continuous and immediate source of inoculum of stem rust. Barberry hosts thin-walled and colorless haploid basidiospores produced from teliospores. Teliospores are the black and thick walled diploid spores that can overwinter in wheat or other grasses during the late growing season. Teliospores undergo meiosis to give four haploid basidiospores that can only infect barberry. Basidiospores in barberry germinate to produce haploid mycelia which forms pycnia within the leaf. Receptive hyphae and pycniospores are produced from pycnia. Sticky honeydew produced in pycniospores attracts insects to allow cross-fertilization of receptive hyphae across pycnia that eventually produce dikaryotic mycelia. A dikaryotic mycelium gradually grows to give rise to a new structure, the aecium, which later releases dikaryotic aeciospores. Germ tubes grow out of the germinating aeciospores that penetrate the plant and allow fungus to grow as a dikaryotic mycelium. In about 1 to 2 weeks, uredinia packed with urediniospores are produced from each mycelium. Urediniospores are dikaryotic and enters the host through the leaf and stem epidermis. Later during the commencement of the harvesting season, teliospores are formed in telia completing the lifecycle. In the absence of barberry, the genetically recombined basidiospores will not find an alternate host to survive on. Thus, barberry not only acts as a source of inoculum but also aids in the creation of genetic variation in the fungal pathogen.

In the absence of barberry, the spores that infect wheat are urediniospores. In the northern regions of the USA such as ND, urediniospores get carried from the southern states through southerly winds to infect the winter and spring wheat. Due to the harsh winter in the northern Great Plains, the stem rust disease cycles ceases after harvesting of the wheat. In the southern

states the milder winters allow for the spores to survive on volunteer wheat plants that later serve as source of inoculum. This remains as the only source of inoculum to initiate a new disease cycle in the south as the short winters do not permit the stem rust teliospores to germinate and infect barberry.

In ND, the problem of stem rust occurring in spring wheat and durum is not severe because of the availability of varieties that are resistant to the prevailing races in the US (McMullen, et al., 2008a). However, some winter wheat cultivars are susceptible to some of the current races. Similarly, even in the absence of an alternative host, mutation and selection on susceptible varieties can allow stem rust races to acquire new virulence against currently effective race-specific resistance genes (Singh et al., 2008). It is furthermore suspected that asexual recombination among the conjugating hyphae of two different races infecting the same host tissue may also result in new virulent races. The recent evolution of a new race of stem rust, TTKS or race UG99, in Uganda, resulted in the breakdown of widely used resistance genes. This poses a new and imminent threat to spring, durum and winter wheat should the new virulence spread to the US. Most of the varieties in ND are not resistant to UG99 and its later variants. Thus, the development of wheat varieties resistant against prevailing and known races that can spread here from other regions should be a quintessential part of any wheat breeding program.

2.2.2. Leaf rust

Leaf rust, also known as brown rust, is the most common and widely spread wheat disease in the US. It is caused by the fungus *Puccinia triticina*, which was previously known as *P. recondita* Roberge ex Desmaz. f. sp. *tritici* (McMullen, et al., 2008a; USDA-ARS, 2013). Leaf rust epidemics can readily develop in the presence of virulent spores, susceptible wheat plants, leaf moisture or dew for about six to eight hours and temperatures of 60°F to 80°F.

Infection will result in the appearance of rusty-red spores from round to oval pustules emerging through the leaf surface. Leaf rust infection can be occasionally also be noticed on glumes and awns (Marsalis and Goldberg, 2006).

In North Dakota, leaf rust is not seen until late May or early June (McMullen, et al., 2008a, Kolmer et al., 2007). The earliest sign of a disease outbreak are noticed on wheat in the southern counties of ND under conditions of high humidity. Spores spread by southerly winds from the southern states of the US to these areas. Once introduced, urediniospores start multiplying rapidly in about a week or 10 days and spread through the rest of the state. Under favorable conditions, leaf rust can become an epidemic by the end of June. As the wheat ripens, spores form telia under the leaf sheaths and blades (Wegulo, 2012). Like in stem rust, teliospores are genetically recombined spores that can infect the alternative host in order to overwinter. However, lack of the alternative host in the US makes urediniospores the only epidemiologically important spore stage for leaf rust.

Leaf rust can lead to a substantial yield loss depending upon the developmental stage of the wheat (Kolmer et al., 2007). Yield loss could reach 30 to 40 percent if severe infection occurs before flowering (McMullen, et al., 2008a). Generally, losses are almost double or more if the flag leaf is heavily infected. Yield reduction is due to the premature loss of leaves that results in a shorter grain filling period and smaller kernel size. These losses are dramatically reduced if infection occurs during or after the dough stage of kernel development.

2.2.3. Stripe rust

Stripe rust (yellow rust) is caused by the fungus *Puccinia striiformis* f. sp. *tritici* (McMullen, et al., 2008a). Infection by this fungus is characterized by parallel lines of yellow-orange spore pustules on the leaf surface. It mostly proliferates in cool and wet conditions within

an optimum temperature range of 50°F to 60°F. The lower temperature requirement for disease development makes stripe rust rare in North Dakota. Warmer temperatures in July reduce the rapid growth and spread of this disease. Infections of stripe rust were noticed in 2004, 2005 and 2012 when spores from Kansas and South Dakota got carried over ND by winds. This project will not deal with stripe rust due to its rare occurrence in ND.

2.2.4. Rust disease management

Wheat rust can be managed and controlled using better cultural practices, fungicides and genetic resistance. Effective cultural practices involve the efficient and proper use of fertilizer and irrigation as well as the removal of volunteer wheat (Schumann and Leonard, 2000, Wegulo, 2012). Abundant use of foliar nitrogen aids the rust fungus in infection and disease development. Similarly, irrigated fields and no-till or minimum till practices can increase the incidence of volunteer wheat that harbors rust urediniospores. Farmers have to optimize their farming practices in terms of row spacing, plant density, fertilization, irrigation and field monitoring to control wheat rust. Eradication of the alternative host is also an important aspect in controlling the rust epidemics. By removing an alternative host, it is possible to reduce the early source of disease inoculum and the genetic variation of rust pathotypes. Still, cultural practices cannot be an effective means of disease control in the presence of high rust pressure.

Fungicides provide an effective measure to control wheat rust in areas with high disease pressure. However, fungicides add cost to wheat production and thus, should be used only if a higher return is guaranteed (Hooker, 1967; Marsalis and Goldberg, 2006, Wegulo, 2012). Profitable return from the use of fungicides is highly likely under conditions favoring rust spore development and spread. Proper timing of fungicide spray can save time, money and effort and ensure good disease control. The decision to use fungicide and timing of its application are

mainly based on winter conditions, the wheat cultivar, wind direction, growth stage of the wheat and weather conditions. Routine scouting of wheat fields can help producers recognize conditions favoring disease outbreak and requiring fungicide spray. Fungicide application will be most profitable under mild winter conditions, when a susceptible wheat cultivar is grown, when southerly winds carrying rust spores prevail, when rust infection occurs at the flag leaf emergence stage and under wet conditions favoring rust development. However, fungicides are curative rather than preventive and may not be highly effective in the case of epidemics. Fungicides are also detrimental to the environment (Murray et al., 2010). Moreover, in the long run, fungicides may become ineffective due to the evolution of a fungicide-resistant rust population.

Genetic resistance is the most widely employed and feasible means of preventing and controlling the rust diseases (Marsalis and Goldberg, 2006; McMullen, et al., 2008a). Use of genetically inherited resistance avoids the need to spray fungicides and to maintain strict cultural practices (Roelfs et al., 1992); as such it is the most economic and environment friendly means to combat the rusts. The growth, development and infection of a particular race of the rust pathogen are limited in the presence of corresponding, effective resistance genes. However, after a period of deployment of a resistance gene in commercially grown varieties, new pathogen races may evolve that are able to overcome the resistance. Thus, a breeding program should continuously monitor the evolution of new pathogen races, acquire and employ new resistance genes to effectively counter the current and evolving pathogens.

2.2.5. Genetic basis of rust resistance

Resistance against a particular rust pathogen implies an ability of the host plant to impede the growth and development of that pathogen at any stage of its lifecycle (Hooker, 1967).

Numerous rust resistance genes in wheat are based on the gene-for-gene host-pathogen interaction; however, resistance genes also exist that are not based on a gene-for-gene mechanism. In the gene-for-gene interaction, genetic resistance refers to the recognition of an *Avr* gene product from the pathogen by a receptor encoded by a resistance (*R*) gene of the host to trigger a rapid and effective (hypersensitive) defense response to prevent disease (Flor, 1971; Catanzariti et al., 2010).

Infection follows a series of physiological and molecular processes. The process initiates with the infecting rust fungi penetrating the host leaf surface and producing intercellular hyphae (Catanzariti et al., 2006; 2007; 2010). Intercellular hyphae grow to form specialized structures called haustoria and begin to infiltrate the mesophyll wall. An extra-haustorial membrane is then formed around the haustorium to separate it from the host cell cytoplasm. The interface between the host cells and haustoria provides a platform for nutrient uptake, molecular signal transduction, modification of the host metabolism and secretion of a suite of pathogen effectors, also known as avirulence proteins. Recognition of these effectors by corresponding plant resistance (*R*) gene encoded proteins will induce an oxidative burst and rapid ion flux leading to cell death, known as the hypersensitive response (HR) (Catanzariti et al., 2010; Morel and Dangl, 1997). The HR arrests the growth and spread of the pathogen within a localized area and kills it directly or by triggering a chain of other processes.

At the molecular level, the gene-for-gene interaction may take one of two forms. In a defense response known as Effector Triggered Immunity (ETI), resistance results from a direct receptor-ligand type of interaction between the R-protein produced by the host and the effector protein produced by the pathogen (Catanzariti et al., 2010). Another type of gene-for-gene interaction is based on the guard hypothesis and involves an indirect interaction between a

pathogen avirulence protein and plant resistance protein (Dangl and Jones, 2001). In this model the pathogen effector acts on a certain (guardee) protein of the host that is guarded by the R (guard) protein of the host. Any interaction of the pathogen effector with the guardee protein will be detected by the guard protein which will then trigger a defense response resulting in programmed cell death or HR.

Apart from the qualitative resistance conferred by a single resistance gene (gene-for-gene, HR, race-specific, vertical or major gene resistance), rust resistance can also be quantitative (non-HR, race non-specific, horizontal or minor gene resistance) involving multiple small effect partial resistance genes or quantitative trait loci (QTL) (Kou and Wang, 2010). Resistance mediated through quantitative resistance (QR) genes is believed to be more durable than race-specific resistance (Kou and Wang, 2010; Miedaner and Korzun, 2012). A combination of quantitative and qualitative resistance genes will combine the durability of quantitative resistance with the high effectiveness of qualitative resistance.

2.2.6. Rust resistance genes employed in the United States

A total of 183 rust resistance genes (72 for leaf rust resistance, 57 for stem rust resistance and 53 for stripe rust resistance) along with some putative QTL have been catalogued so far (McIntosh et al., 2012; 2013). Different suites of the leaf and stem rust resistance genes have been deployed in various classes of wheat in the US (Kolmer et al., 2007). Leaf rust resistance genes *Lr2a, Lr3ka, Lr9, Lr10, Lr11, Lr16, Lr17, Lr18, Lr23, Lr24, Lr26, Lr34,* and *Lr41* are some of the most widely used genes in HRWW and HRSW of the south-eastern states and the Great Plains region of the United States (Kolmer 2003; Kolmer et al., 2007; McVey and Long, 1993; Oelke and Kolmer, 2004). These genes were effective for a long time (Kolmer et al., 2007); however, their wide use has created a strong positive selection pressure for newly formed

virulent races. Positive selection exerted by these resistance genes did open an avenue for the evolution of a diverse pathogen population in the US. In 2004, 50 different races were identified that can potentially mutate to result in an outbreak of a new virulent pathotype.

Similarly for stem rust, various sets of stem rust resistance genes have been deployed in US wheat cultivars at different times. Hard red winter wheat cultivars grown in the Great Plains and soft red winter wheat of the Midwest and south-eastern US mostly carry *Sr6, Sr24, Sr31, Sr36, SrTmp,* and the resistance transferred from the 1AL.1RS translocation. Spring wheat cultivars are mainly resistant with seedling resistance genes *Sr6, Sr9b, Sr11,* and *Sr17* and adult plant resistance gene, *Sr2*. These genes, either singly or in combination, have made most of the wheat cultivars in the US resistant against the prevalent races of stem rust. However, outbreaks of race Ug99 (later re-designated as TTKSK) of stem rust have created an eminent threat to the US wheat production (Singh et al., 2011). The Ug99 virulence was first noticed in February 1999 on wheat cultivars carrying the 1BL-1RS chromosome translocation containing *Sr31* (Pretorius et al., 2000). Since then different Ug99 derivative races were identified showing virulence to various widely used stem rust resistance genes (Singh et al., 2011). Resistance gene *Sr24*, which was initially resistant to TTKSK showed susceptibility to Ug99 derived races TTKST, TTKSP and PTKST (Jin et al., 2008; Singh et al., 2011). Likewise, another commonly used resistance gene, *Sr36,* became susceptible to race TTTSK despite being resistant to all other known variants of Ug99. The results of recent seedling screening tests performed on hard red winter, soft red winter, and hard red spring wheat cultivars grown in the US have shown wide-spread susceptibility to different isolates of Ug99 (Kolmer, 2007; Bernado et al., 2013). Failure to address this issue may again render the US crop vulnerable to stem rust. To avoid this, novel rust

resistance genes should be introduced in the US winter wheat germplasm to enhance and complement the existing rust resistance.

2.2.7. Novel rust resistance genes

Wild, close or distant relatives of *T. aestivum* are the most commonly used sources from which to obtain novel genetic resistance against the prevailing and evolving races of rust (Marais et al., 2003; Dundas et al., 2007). Alien introgressions are mostly successful in wheat because the complex, polyploid wheat genome is capable of buffering new genetic imbalances that may result from this (Qi et al., 2007). Still, introgressed chromatin from alien species may carry undesirable associated genes that impact negatively on yield and quality (Dundas et al., 2007, Qi et al., 2007, Gill et al., 2011, Niu et al., 2011). However, advances in chromosome engineering, genomics and genotyping techniques have made it possible to map alien translocations and to replace undesirable chromatin bordering the resistance locus with wheat chromatin. New rust resistance genes without associated negative effects can thereby be transferred from different species as an effective means to enhance resistance against evolving rust pathotypes. Genes *Lr53, Lr62, Sr26, Sr39* and *Sr50* are a set of novel rust resistance genes that were utilized in this project.

Lr53 is a leaf rust resistance gene transferred from *T. dicoccoides* to common wheat (Marais, 2005). *Lr53* is located on the short arm of wheat chromosome 6B and is closely linked with stripe rust resistance gene *Yr35* at a distance of 3cM (Dadkhodaie et al., 2011). Marais et al., (2005) did not observe any recombination between *Lr53* and *Yr35* but suspected that the two parents of their cross differed structurally within that region, thus accounting for the lack of recombination. *Lr53*/*Yr35* was observed to have preferential pollen transmission (91-96%), while showing a comparatively lower (41-66%) transmission of resistance through the female

(Dadkhodaie et al., 2011; Marais, 2005). *Lr53* can serve as a potential leaf resistance gene in US wheat germplasm as no known virulence of leaf rust races and association with negative traits have been detected for this gene. Also, plants homozygous for *Lr53/Yr35* were found to be fertile and gave plump seed.

Lr62 is another leaf rust resistance gene that has been introgressed to common wheat from its wild relative *Aegilops neglecta* (Marais, 2009; 2010). It is linked with seedling stripe rust resistance gene, *Yr42* conferring resistance to *P. striiformis. Lr62/Yr42* is carried on a large translocation to wheat chromosome 6A. The translocation involves the complete short arm plus a large part of the long arm of an unknown group 6 chromosome of *Ae. neglecta* plus the distal end of wheat chromosome 6AL. This big translocation also showed preferential transmission. At the onset of the study it was not known whether the translocation would have associated negative effects. However, in the course of the study it appeared to be associated with poor spike development under hot conditions and it was decided not to continue using it. Attempts are currently being made to identify more useful, shortened versions of the translocation among recombinants produced in an earlier study (Marais et al., 2010).

Lr59 is a leaf rust resistance gene transferred from the wild species *Aegilops peregrina* (Hackel in J. Fraser) Maire & Weiller (syn. *Ae. variabilis)* to a common wheat variety, Chinese Spring (Marais et al., 2008). The translocation line 0306 (pedigree: *Ae. peregrina*-680/2*CS//5* W84-17) has chromosome arm 1AL replaced with an alien segment on which *Lr59* occurs. Meiotic, monosomic and microsatellite analyses suggested that it is probably a Robertsonian translocation (Marais et al., 2008).

Since the *Glu-A1* locus that occurs on wheat chromosome arm 1AL (Lucas et al, 2013) was most likely replaced by a species-derived storage protein locus, the translocation may have a

detrimental associated effect on quality. Furthermore, the alien chromosome region is unlikely to pair with 1AL of wheat in the presence of *Ph1,* causing all of the alien chromatin to be inherited as a single, large linkage block. This limited the utility of this translocation line in crossbreeding with other common wheat lines (Marais et al., 2008; 2010). Marais et al. (2010) then used the CS*ph1b* mutant to induce homoeologous recombination with 1AL of wheat and derived recombined progeny with reduced amounts of alien chromatin. They identified eight recombinants (designated *Lr59*-10, -21, -25, -29, -36, -101, -144 and -151, respectively) carrying *Lr59* on comparatively smaller translocations. This study will try to map recombinant *Lr59*-25.

Sr26 is a stem rust resistance gene transferred from *Thinopyrum* (*Th*) *ponticum* (Podp.) Barkworth and Dewey (2*n* = 10*x* = 70) [syn. *Agropyron elongatum* (Host) Beauvois and syn. *Lophopyrum ponticum* (Podp.) Löve] to the long arm of wheat chromosome 6A (Knott, 1961; Friebe et al., 1994; Liu et al., 2010). Since 1961, it has been widely used in wheat breeding in Australia but was reported to reduce yield (The et al., 1988; Dundas et al., 2007). More recently, lines with reduced amounts of *Thinopyrum* chromatin were developed from the original *Sr26* translocation (Dundas et al., 2007). Shortened versions of *Sr26* that do not have the associated yield penalty were identified and made available for breeding (Singh et al., 2011). Since *Sr26* is among those few stem rust resistance genes that are effective against all known races of Ug99, line WA1 carrying *Sr26* was obtained from Dr. I. Dundas (University of Adelaide, Glen Osmond, SA 5064, Australia) and included in the study.

Sr39 is a seedling stem rust resistance gene transferred from *Ae. speltoides*; initially into wheat cultivar Marquis (Kerber and Dyck, 1990). A big chunk of *Ae. speltoides* chromosome 2S, carrying *Sr39* and the adult plant, hypersensitive, leaf rust resistance gene *Lr35,* was translocated to wheat chromosome 2B (Friebe et al., 1996; Mago et al., 2009). *Sr39* was found to provide

resistance against all known variants of stem rust races belonging to the Ug99 lineage (Singh et al., 2011). However, the original translocation has not been used in wheat breeding because of associated negative traits. Mago et al. (2009) and Yu et al. (2010) developed lines with reduced *Ae. speltoides* chromatin, however, the remaining chromatin still had a negative effect on plant performance (Niu et al., 2011). In 2011, Niu et al. were able to develop a recombined translocation line retaining *Sr39* with only 3-10% of the initial amount of *Ae. speltoides* chromatin. The present study used the line developed by Niu et al. (2011) for backcrossing and pyramiding.

Sr50 is a stem rust resistance gene that occurs on the 1RS.1DL translocation from 'Imperial' rye (Koebner and Shepherd, 1986; Mago et al., 2002, Anugrahwati et al., 2008). Initially, it was named *SrR* until it was found to be different from *Sr31* which occurs on a 1RS translocation from 'Petkus' rye that carries *Lr26*, *Sr31*, and *Yr9*. Lack of any associated leaf rust and stripe rust resistance suggested that *Sr50* differs from *Sr31*. While the *Sr31* resistance has been overcome by the Ug99 virulence, *Sr50* remained resistant to all known races of the Ug99 lineage. However, the utility of the full length translocation has been reduced by the presence of the rye secalin locus, *Sec-1,* that causes dough stickiness which reduces the mixing and baking quality of bread wheat (Anugrahwati et al., 2008). Allosyndetic pairing induction was used in attempts to produce a recombinant in which the *Sec-1* locus of rye was replaced by the wheat storage protein locus, *Gil-D1*. Initial attempts of Koebner and Shepherd (1986) were unsuccessful as they could only recover secondary recombinants with *Sr50, Gli-D1* and *Sec-1*. Anugrahwati et al. (2008) did recover a tertiary recombinant, T6-1, that lacks *Sec-1* while having *Sr50* and *Gli-D1*. No variety with the recombined translocation has been release till date (Mujeeb-Kazi et. al., 2013). The improved T6-1 translocation has been included in this study.

Use of these novel resistance genes in US wheat germplasm will undisputedly contribute towards breeding for effective resistance against rust. However, to prolong the commercial life of the major resistance genes, it will be necessary to employ them in combinations rather than singly. Furthermore, if possible they should also be pyramided with minor (partial) resistance genes that are less likely to be overcome by pathogen evolution (Kolmer et al., 2007; Singh et al., 2011). *Lr34* and *Sr2* are such race non-specific genes for leaf rust and stem rust, respectively that were utilized in this study.

Lr34 is a pleiotropic, broad spectrum, quantitative, slow rusting and race non-specific leaf rust resistance gene first characterized by Dyck et al. (1966) in Canada (Kolmer et al., 2008; Lagudah et al., 2009; Singh et al., 2011; Risk et al., 2013). *Lr34* confers a moderate level of resistance which in mainly expressed on the flag leaf of adult plants at the grain filling stage (Krattinger et al., 2009). It is located in the short arm of chromosome 7D of wheat and is tightly linked with adult plant stripe rust resistance gene, *Yr18* and powdery mildew resistance gene *Pm38.* It is also linked to *Ltn1* that induces a leaf tip necrosis (LTN) on the flag leaf at the adult stage, depending upon the cultivar background, multigenic background effects and environmental conditions. Cloning of *Lr34* revealed that a pleiotropic drug resistance (PDR)-like ATP binding cassette (ABC) transporter gene singly controls the expression of *Lr34, Yr18, Pm38* and *Ltn1*. *Lr34* is also reported to enhance resistance against stem rust, barley yellow dwarf virus and spot blotch disease (Risk et al., 2013; Lillemo et al., 2013). Being a multiple pathogenic resistance gene, it has been globally deployed to obtain a moderate level of resistance against leaf rust and other associated diseases. After its extensive use in wheat breeding programs over a long period around the globe, no isolates in the US have shown complete virulence to *Lr34* (Kolmer et al., 2007). Instead, it has provided more durable and longer lasting

resistance against rust diseases as compared to any race specific resistance gene (Risk et al., 2013). In contrast to the situation in spring wheat, very few cultivars of winter wheat contain *Lr34* (Kolmer et al., 2007).

Likewise, *Sr2* is an APR, slow rusting, race non-specific gene that induces moderate and non-hypersensitive resistance against a broad spectrum of stem rust races (Hare and McIntosh, 1979; Singh et al., 2011; Mago et al., 2011a; 2011b). It was first transferred from tetraploid emmer wheat, *T. dicoccum* Schronk, into hexaploid wheat in 1920 (Hare and McIntosh, 1979; Mago et al., 2011a). In 1925, *Sr2* was made available in the agronomically sound and rust resistant variety 'Hope' which allowed wheat breeders to easily and widely utilize it in their breeding programs. *Sr2* is located on the short arm of wheat chromosome 3B and is linked to the pseudo black chaff trait that induces an irregular as well as genotype and environmentally dependent black pigmentation on the stem and spikes (Hare and McIntosh, 1979). Varieties with *Sr2* were documented to provide resistance against all known races of Ug99 (Singh et al., 2011). However, *Sr2* was not highly effective in an area with high disease pressure. Under such conditions, adequate protection could only be obtained in combination with race specific resistance genes. Thus, this study aimed to stack the novel rust resistance genes with APR and race non-specific genes, *Lr34* and *Sr2.*

2.3. Fusarium Head Blight

Fusarium Head Blight (FHB) or scab is the most destructive fungal disease of wheat and small grain (McMullen et al., 1997). In North America and the majority of the world, FHB is mainly caused by the fungal pathogen *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch]. FHB occurs in almost any wheat growing area with heavy rainfall, high humidities and moisture during the flowering and grain filling periods of the crop. Wheat

infected with *F. graminearum* (*Fg*) will show premature discoloration in the top, middle and bottom of the head that spread across it over time (Schmale III and Bergstrom, 2003). Warm and humid conditions will induce light pink/salmon colored spores (sporodochia) to amass on the rachises and glumes of infected spikelets. During the late season, the surface of infected spikes gets filled with bluish-black spherical bodies (perithecia) which later aggregates on grain causing it to shrivel and wrinkle. Infection from *Fg* will cause reduction in yield, deterioration of quality, discoloration and shriveling of kernels (tombstone kernels) and contamination of grain with mycotoxin (McMullen et al., 1997). Deoxynivalenol (DON) is the most common mycotoxin (vomitoxin) produced by FHB that poses a serious threat to animal and human health. The level of DON in FHB infected crops are mostly higher than 20ppm, while DON >1ppm in food is regarded as unhealthy for human consumption (Schmale III and Bergstrom, 2003).

Apart from infecting small grains, *Fg* can cause ear, stalk, and root rot of corn. Spores of *Fg* can thus overwinter in the residue of corn along with barley and wheat (Schmale III and Bergstrom, 2003; McMullen et al., 2012). The spores on these residues produce asexual spores (macroconidia) which are rain splashed or wind blown into other plants or plant debris. Spores on the debris develop into their sexual stage (*Gibberella zeae*) under hot, humid and moist conditions and produce sexual spores (ascospores). Wind currents and rain eventually spread the ascospores across the wheat growing areas. Ascospores and macroconidia can both be a source of infection for FHB. Wheat becomes vulnerable to infection by spores from flowering to the hard dough stage of kernel development (McMullen et al., 2008b). Warm temperatures (75°F to 85°F) and extended humidity (48 to 72 hrs) favors infection, while humidity lasting longer than 72 hrs can still permit infection under much cooler conditions.

FHB was first described in England in 1884 but was not considered a serious threat to wheat and barley until the early 20th century (Goswami et al., 2004). Increased infection was then noticed throughout the world. In view of the elevated FHB incidence, the US did a survey in its 40 states in 1917 and identified scab in 34 states with an estimated associated loss of 10.6 million bushels (McMullen et al., 1997). Continued and extensive field surveys in subsequent years recorded the highest yield losses from scab within 1928 to 1937. The incidence of scab appeared to be under control until the 1980s when rainy and cold conditions brought scab epidemics to some states of the US. By 1991, scab was a regular problem in the eastern half of the US. The scab problem got aggravated when the major wheat growing states, ND and Minnesota, suffered an estimated billion dollar loss in a single year (1993). Wet conditions due to severe flooding, increased corn production, cultivation of susceptible hosts, mulching and minimum or no till farming practices that permits debris in field, are believed to be core causes of the re-emergence of scab in North America. Since 1993, FHB continued to cause significant losses in the yield and price of wheat in the US (Nganje et al., 2004). From 1993 to 2001, scab alone accounted for about $2.492 billion in losses, ND being the worst affected state. Scab is now a serious threat to the wheat growers in ND. Taylor and Koo (2013) reported an 8% increment in corn acreage in ND which can potentially reinforce the risk of wheat being infected by scab.

2.3.1. Management of FHB

The management of scab is a formidable task and requires the proper integration of cultural practices, chemical control and genetic resistance (McMullen et al., 1997; 2008b). None of these strategies alone can ensure a disease free environment and reduce the level of DON in those areas with high inoculum pressure (McMullen et al., 2012). Management through cultural

practices includes proper crop rotation, tillage and multiple planting dates. The inclusion of non–susceptible host crops in a rotation cycle, tillage cultivation and the spreading of chaff and residue for speedy decomposition can reduce the opportunity for spores to overwinter. Since scab infection occurs during the flowering to grain filling stages and depends on environmental conditions, planting the crop at multiple dates could help minimize the risk of losing the entire crop (Arthur, 1891; McMullen et al., 2007).

The chances of scab infection can also be reduced by planting healthy seed as well as treating them with seed treatment fungicides. Proper care and selection of stocks after harvesting will ensure healthy and vigorous seeds. While in the field, the use of a systemic fungicide like Triazole in the early flowering stage was reported to suppress disease severity by 50-60%. The chances of ameliorating scab infection using a fungicide can be perfected with better technology and disease forecasting services. However, use of fungicide will add cost to the overall production and reduce profits.

Genetic or host resistance is the most economic and competent method to manage scab and DON levels in wheat (Mackintosh et al., 2007; Rudd et al., 2001). It has been a major focus in wheat breeding programs ever since Arthur (1891) emphasized its importance. Breeding programs that focused on developing FHB resistant cultivars were successful and reduced the susceptible cultivars by 45% within the period 1999 to 2011 (McMullen et al., 2012). Unfortunately, few sources of significant genetic resistance are available; the best of these are Sumai 3 and Ning 7840 (Zhang et al., 2011). Moreover, all known resistance genes provide only partial resistance that is insufficient to withhold the extreme disease pressure (Mackintosh et al., 2007; Buerstmayr et al., 2009). Thus, to attain higher levels of protection it is necessary to

couple resistant cultivars with proper cultural practices and chemical controls rather than using any of these tactics alone.

2.3.2. Basis of FHB resistance

Resistance to FHB is categorized into five different components: Type I (resistance to primary infection), Type II (resistance to disease spread within the spike), Type III (resistance to accumulation of DON), Type IV (resistance to kernel infection) and Type V (resistance to yield loss) (Schroeder and Christensen, 1963; Mesterházy, 1995; Zhang et al., 2011). Type II is the most widely explored and utilized form of resistance in wheat breeding, while Type I has gained subtle importance despite being available in some cultivars (McMullen et al., 2012). Type IV and Type V resistance are poorly understood and rarely utilized in breeding programs (Zhang et al., 2011). The basis of Type I and Type II resistance is briefly described below.

Resistance to FHB involves a cascade of physiological and molecular processes (Walter et al., 2010). For its establishment, FHB requires a suitable host and favorable environmental conditions. Its infection involves a transient biotrophic phase before entering the necrotrophic stage, making *Fg* not a complete necrotroph (Gosawami and Kistler, 2004; Saville et al., 2012). Several receptor proteins like G protein-coupled receptor proteins, transduction beta sub-unit and tetraspanins encoded by the *Fg* genome facilitates the recognition process and appressorium formation (Walter et al., 2010). In the desired environment, *Fg* spores land on a head of wheat, mostly on the middle portion of the spike which harbors spikelets with comparatively elevated water content. Various signals are then transduced through the fungal membrane to their respective target sites in the fungal genome in order to induce pathogenicity and virulence. *Fg* then grows hyphae and secretes a diverse range of hydrolyzing enzymes (e.g. *Fg* lipase gene FGL1) to enter through the wheat cuticle. Hyphae gradually grow into the subcuticular,

substomatal and intercellular regions of spikes and up to the middle lamella and cell wall. *Fg* penetrates these barriers by secreting pectin degrading and cell wall-degrading enzymes. Eventually, hyphae extend into the cell apoplast and trigger a cytological turbulence which leads to cell death. This stage is characterized by the induction of DON, a trichothecene mycotoxin that is involved in the killing of cells by generating hydrogen peroxide, inhibiting the production of plant proteins and damaging components of cells. The amount of DON produced by *Fg* is thus directly correlated to its virulence. *Fg* also maintains its pathogenicity and colonization by neutralizing plant defense proteins or toxins as well as extracting nutrients from the plant to facilitate its own growth and spread.

A resistant plant will try to trigger a defense mechanism against these fungal actions based on the type of resistance in that plant. The morphological and physiological features (waxy cuticle, flowering time/period, kernel density) will act as a first wall of defense or Type I resistance against *Fg* entrance and establishment (Walter et al., 2010). Once the fungal hyphae break the basal barriers, plant chitin-binding proteins and chitinases sense and degrade fungal chitin while plant glucanases and thaumatin-like proteins will detect and degrade fungal glucans. The integrity of the fungal membrane may also be destroyed by non-specific lipid transfer proteins (nsLTPs), thionins and puroindolines. Degeneration of the cuticle or cell membrane of resistant plants induces jasmonic acid (JA) and methyljasmonate (MeJA) through polyunsaturated fatty acid signaling. JA and MeJA are the two key components in signaling for activation of a defense response against necrotrophic pathogens. The two hormones also cascade a signal to alarm distant tissue about the invading pathogen, thereby preparing an entire region of plant cells for the defense response. Along with the disintegration of the fungus, resistant plants tend to repair and fortify their barriers by expanding the cell wall and establishing cell wall

appositions to reduce incoming toxin and outgoing plant nutrients. Since *Fg* produces DON, resistant plants will either alter the toxin or its target by obstructing interaction of the toxin and target or by generating downstream signaling cascades. Wheat will also trigger a defense response against DON by opposing the oxidative stress induced by it. These concerted actions against FHB infection tends to provide both Type I and Type II forms of resistance. Type II resistance will mainly involve the mechanisms that tend to reduce the influx of FHB toxin as well as spread of the fungus through the plant. Reduced vessel size, solid sclerenchyma and cells walls, thicker vascular bundles and smaller internodes will aid in conferring Type II resistance.

2.3.3. Genetic resistance against FHB

FHB resistance is a quantitative trait affected by more than 100 QTL; each QTL conferring a varying level of resistance (Anderson et al., 2001; Buerstmayr et al., 2009). Of all the QTL studied so far, *Qfhs.ndsu-3BS* (renamed *Fhb1*) and *Qfhs.ifa-5A* are the most effective and consistent till date (Anderson et al., 2001; Buerstmayr et al., 2009; Cuthbert et al., 2006; Schweiger et al., 2013). *Fhb1* has been established as a persistent and competent QTL that can potentially reduce the severity of FHB in diverse breeding populations with a concomitant increase in their yield potential (Pumphrey et al., 2007). This project will only utilize *Fhb1* derived from Sumai-3 in its winter wheat germplasm to confer Type-II resistance against scab. The Type II resistance provided by *Fhb1* appears to be additive with respect to resistance provided by other resistance loci (Cuthebert et al., 2006).

Fhb1 was first identified as a major QTL in the Chinese cultivar ‘Sumai-3’ and was mapped in the distal part of chromosome 3BS (Anderson et al., 2001). Considering the robustness and consistence of *Fhb1,* extensive studies were made to map and clone the candidate gene lying within this QTL (Cuthbert et al., 2006; Liu et al., 2008; Schweiger et al., 2013).

Cuthbert et al. (2006) were able to place *Fhb1* as a Mendelian factor within a 1.2 cM interval of the wheat STS3B-80 and STS3BS-142 markers, which later was reduced to a 261Kb region containing seven putative genes (Liu et al., 2008). Despite these mapping and cloning efforts, scientist have still not been able to isolate a functional gene explaining this resistance (Schweiger et al., 2013; Zhuang et al., 2013). However, transcriptomic and biochemical analyses made it possible to link *Fhb1* with resistance to the toxin DON and FHB (Lemmens et al., 2005; Walter et al., 2009). Lemmens et al. (2005) speculated on the role of *Fhb1* in the induction of several glucosyl transferases through a suite of genes at this locus that are capable of recognizing *Fusarium* derived DON so as to detoxify and neutralize it. Enzymatic action of UDP-glucosyltransferase (UGT) is assumed to be responsible for transforming DON into a less toxic DON-3-glucoside. DON is an important element of *Fusarium* spread, so, its detoxification by *Fhb1* ultimately halts the spread of *Fg* throughout the spike and confers a Type II resistance (Schweiger et al., 2013).

Resistance to FHB has also been postulated to be significantly related to the height of the crop (Mesterházy, 1995). Shorter plants were supposed to allow an easy and quick movement from a natural source of inoculum (soil debris) to the leaf and spikes (Mesterházy, 1995; Miedaner and Voss, 2008). However, tall plant stature is undesirable in wheat production while plants with short and stiff straw provide resistance to lodging and provide for better partitioning of assimilates between biomass and grain (Borojevic and Borojevic, 2005; Saville et al., 2012).

Norin-10, a dwarf variety developed in Japan was introduced to the US after 1945 (Hedden, 2003). Later in 1960, the semi-dwarfing (*Rht*) genes, *Rht-B1b* and *Rht-D1b* of Norin-10 were widely employed in commercial wheat varieties of the US and lines of CIMMYT, Mexico. These genes were considered a major component of the ‘Green Revolution’ and were

incorporated into 90% of the world's wheat (Borlaug, 1968; Ganeva et al., 2005). Isolation of the *Rht* genes showed that the dwarf trait is the result of interference in the signal transduction pathway of the growth hormone, gibberellin (GA) (Hedden, 2003). Disruption of this signaling pathway prevents the plant from gaining height even if GA is applied exogenously (Ellis et al., 2002).

The possible resistance tradeoff towards FHB associated with the commercial use of these impressive yield improving *Rht* genes prompted investigation of the relationship between height and FHB resistance. Studies by Draeger et al. (2007) revealed a possible link between *Rht-D1b* and a gene for FHB susceptibility instead of plant height. Srinivasachary et al. (2009) used Mercia and Maris Huntsman near-isogenic lines to study and compare the potential impact of *Rht-B1b* and *Rht-D1b* on FHB severity. Increased Type II resistance to FHB under moderate disease pressure was noticed in the line carrying *Rht-B1b*, while Type I resistance was negatively related to both *Rht-B1b* and *Rht-D1b*. Functional analysis of the *Rht* genes suggested a role for DELLA protein in controlling the expression of Type I and Type II resistance in wheat (Saville et al., 2012). DELLA protein is encoded by *Rht-B1b* and *Rht-D1b* to confer dwarfism by suppressing GA-responsive growth (Peng et al., 1999; Saville et al., 2012). The same protein was found to be involved in resistance to necrotrophic fungi but susceptibility to biotrophic fungi through the suppression of ROS-induced cell death. The presence of a transient biotrophic phase in FHB before the onset of the necrotrophic stage explained an initial susceptibility followed by increased resistance to disease spread in the presence of the *Rht* genes. Association mapping studies by Miedaner et al. (2011) also showed that FHB resistance can be strengthened by *Rht-B1b* - not by its main effect but through its epistatic interaction with non-124-bp alleles at the locus *Xbarc147* on chromosome 3B. This study aimed to utilize *Rht-B1b* so as to develop near-

isogenic material with reduced height in the genetic background of the winter-hardy variety, Norstar. The use of *Rht-B1b* should ensure that there will not be an associated negative effect on FHB resistance.

2.4. Breeding for Disease Resistance

Since the dawn of agriculture, man has been knowingly or unknowingly selecting plants to improve or enhance their performance. Gradually people explored and understood plant diversity and tried to utilize the variation to combine preferred characters in crop plants. Plant breeding has been defined as the art and science of purposely manipulating the genetics of plants so as to attain permanent and heritable changes that are beneficial for mankind (Acquaah, 2007). This particular science grew more precise and directional with the revolutionary work of Gregor Mendel and subsequent advances in the knowledge of heredity factors or genes.

Plant breeding primarily aims to improve yield and quality and deals with the factors that potentially impact them. Disease is one of such factor that requires considerable breeding activities directed towards attaining resistance. Breeding for disease resistance enables disease control in a more durable, cheaper and environmental friendly manner (Roelfs et al., 1992). Backcrossing and gene pyramiding are the most widely employed breeding tools to establish durable disease resistance in plants (Roelfs et al., 1992; Singh and Rajaram, 2002; Acquaah, 2007; Murray et al., 2010).

2.4.1. Backcrossing

The use of backcrossing in small grain breeding was first introduced by Harlan and Pope (1922). Later in the same year, F.N. Briggs also initiated an extensive backcrossing program to add diverse disease resistance to well-established self-pollinated varieties. Since then, it is being used extensively to introgress desired characteristics (a trait, a gene or even an anonymous locus

or chromosome segment) from a donor parent into an adapted or elite recurrent variety (Hospital, 2005). Backcrossing can also be used to develop isogenic lines, i.e. lines that differ for a single gene/allele at a given locus (Acquaah, 2007).

Backcrossing is used to substitute a small number of genes without breaking up existing desirable gene combinations in a successful variety (Briggs and Allard, 1953). Each generation of backcrossing will reduce the genetic contribution of the donor parent by half (Acquaah, 2007). After 'm' backcrosses, the percentage of the donor genome that remains in a backcrossed population is $½^{m+1}$. The success of backcrossing is mainly determined by the availability of a satisfactory recurrent parent, the possibility of maintaining the intensity of a valuable character under transfer and the number of backcrosses. This study utilized Norstar as the recurrent parent. 'Norstar' is a winter hardy variety developed at the Agriculture Canada Res. Stn., Lethbridge, Alberta in 1977 (Grant, 1980). 'Norstar' has superior winter hardiness and acceptable milling and baking quality but has many agronomic deficiencies that detract from its usefulness as a breeding parent (Fowler, 2012). It has been widely utilized as a source of freezing tolerance genes in various Canadian and North American winter wheat breeding programs (Cox et al., 1988; Fowler, 2008). Due to its tall straw, Norstar has a lodging problem. Norstar is also susceptible to many diseases such as stem and leaf rust and FHB. For these reasons, Norstar is not grown in North Dakota. If near-isogenic lines of Norstar can be produced that are of intermediate height, have excellent winter-hardiness and possess resistance to major diseases of winter wheat, it will constitute very valuable breeding material.

2.4.2. Gene pyramiding

Gene pyramiding refers to the incorporation of multiple genes affecting a trait into a single genotype. Pyramiding of several disease resistance genes results in the simultaneous

expression of more than one gene in a variety, which will promote the durability of resistance (Joshi and Nayak, 2010). Simply defined, pyramiding is the process of combining various genes from multiple parents into a single genotype. The objectives of gene pyramiding includes (a) Combining two or more complementary genes to enhance trait performance, (b) Introgression of genes from multiple sources to fix deficits, (c) Increasing the durability of disease resistance, and (d) exploiting the wider genetic basis of released cultivars (Ye and Smith, 2008).

In breeding to combat disease, gene pyramiding could be extremely valuable in conferring durable resistance. Using single race specific or HR genes in new varieties will ensure resistance only for a limited number of years as pathogens tends to mutate and gain virulence (Murray et al., 2010). The strong selection pressure created by the wide use of these resistance genes, coupled with management practices, eventually results in a pathogen mutation to gain virulence (Hovmøller and Justesen, 2007). However, the frequency of a mutation leading to virulence for a specific resistance gene is independent of the mutation rate at each of the other resistance loci; consequently making it very unlikely (product of the respective mutation frequencies) for the pathogen to gain simultaneous virulence for all pyramided genes (Murray et al., 2010). Thus, gene pyramiding allows breeders to develop durable resistance against a particular pathogen. Still, the success of gene pyramiding depends upon the availability of race specific as well as race non-specific resistance genes that are effective against the prevalent races (Kolmer, et al., 2007).

2.4.3. Use of molecular markers

Conventional breeding, backcrossing and gene pyramiding schemes used to rely completely on phenotypic markers/variation. Breeders were limited to work only with traits conferring strong phenotypic variation (Ye and Smith, 2008). In the absence of better selection

tools, breeders were unable to detect recessive alleles in heterozygous plants. Pyramiding of two or more resistance genes in a single background was formidable in the absence of isolates that could distinguish them (Liu et al., 2010). Breeders also could not completely rely on phenotypic markers as they were sometimes highly sensitive to environmental influences and could easily be misleading. Moreover, the phenotypes were not always an effect of the target gene only and may have involved many other linked genes. Linked genes were a major drawback in conventional backcrossing schemes resulting in what is known as linkage drag (Stam and Zeven, 1981; Allard, 1999). The probability of eliminating a linked gene by chance during backcrossing is $(1 – (1\text{-}r)^{(n+1)})$, where 'r' is the recombination fraction and 'n' is the number of backcrosses. Thus, the chances of getting rid of undesired genes tightly linked to the target gene were very small. This problem can be exacerbated by the unavailability of suitable tools to detect the rare recombinants available in a big population (Tanksley *et al.*, 1989). So, even with reliable phenotypic parameters and careful consideration in selection, conventional breeding and gene pyramiding can still end up with poor isogenic lines. However, when supplemented with Marker Assisted Selection (MAS) protocols to accurately detect the presence of the target gene, identify and select recombinants with reduced linkage drag, backcrossing can be done with greater precision and efficiency.

MAS refers to a tool used in breeding programs that utilizes molecular markers for confident and competent selection of desirable traits (Collard and Mackill, 2008). Generally, a molecular marker is a piece of DNA closely linked to the gene(s) with no phenotypic variation associated with it (Griffiths et al., 2000). However, with recent advances in genomics and sequencing, they are not only limited to a flanking region but may also occur within a gene (Poczai et al., 2013). Molecular markers tag a DNA sequence variation in a particular locus or

genomic region that controls the trait of interest (Langridge and Chalmers, 2005). A variety of molecular marker types are available and the choice of markers to use generally depends upon its cost, reliability, technology platform, species transferability, quality and quantity of DNA needed and level of polymorphism detected by that marker (Collard and Mackill, 2008; Kumar et al., 2009). An ideal marker is one that is tightly linked to the target gene, preferably less than 5cM; works well even with a small amount of poor quality DNA; is easy to score; detects a high level of polymorphism; has co-dominant inheritance and is cheap. Advances in molecular tools and technology have made it possible to develop markers that satisfy most of these requirements. Sequence Characterized Amplified Region (SCAR), Sequence Tagged Site (STS), and Single Nucleotide Polymorphism (SNP) markers are the most widely employed markers in MAS. This study used Cleaved Amplified Polymorphic Sequence (CAPS), Expressed Sequence tag (EST), and Simple Sequence Repeats (SSR) or microsatellite, STS and SNP markers.

A STS is a short (200-300 bases long) and unique region of the genome whose location and sequence is readily available (Olson et al., 1989; Doggett, 1992; Wikipedia, 2014). The STS sequence may consist of repetitive elements that are shared across genomes, but this region can be precisely identified by designing primers from conserved and unique flanking sequences. STS markers potentially serves as specific, co-dominant (markers that distinguish heterozygotes from homozygotes), highly reproductive and easy to assay markers (Kumar et al., 2009; Wikipedia, 2014). The STS markers that were utilized in this study were developed as diagnostic tags for four of the target genes and include: Sr39#22r (detects *Sr39*) (Mago et al., 2009), Sr26#43 (detects *Sr26*) (Liu et al., 2010), IB-267 (detects *Sr50*) (Mago et al., 2002), BF and MR1 (co-dominant marker for *Rht-B1b*) (Ellis et al., 2002).

ESTs are based on short sequences of complementary DNA (cDNA) that derive from messenger RNA (mRNA) (Lazo et al., 2004). The EST sequence information can be used to develop a gene-specific marker (Chee et al., 2003). Two EST based markers were used in this study, i.e. BE500705 (a dominant marker for the absence of the *Ae. speltoides* introgressed region on which *Sr39*) occurs and BE518379 (a dominant marker for the absence of the *Th. elongatum* introgressed region on which *Sr26* occurs) (Mago et al., 2009; Liu et al., 2010).

A CAPS is a fragment of DNA that is used to identify a polymorphism at the nucleotide level across a given set of lines (population) for a given gene of interest (Kumar et al., 2009). Unlike, STS and EST derived markers; CAPS markers do not require sequence information. CAPS markers are mostly co-dominant, highly reproducible and easy to use. Marker csSr2 (co-dominant marker for *Sr2*) is the only CAPS marker used in this study (Mago et al., 2011a).

SSR or microsatellite markers are based on tandemly repeated mono- to penta- nucleotide units of DNA. Such sequences are found across the genomes of eukaryotic species (Kumar et al., 2009). Unique flanking regions of such SSRs can be used to develop co-dominant markers specific to particular loci of interest. The marker loci *Umn10* (co-dominant marker for *Fhb1*) (Liu et al., 2007) and *Xgwm533* (co-dominant marker for *Sr2*) (Hayden et al., 2004) are the SSR markers used in this study.

Another class of marker employed in this study was the Single Nucleotide Polymorphism (SNP) marker. SNPs are DNA sequence variants in a population at the level of the single nucleotide base pair (A, T, C or G) (Gupta et al., 2008). SNPs are now the mostly widely utilized marker type with which to obtain an even and dense spread of markers that aids in mapping, MAS as well as the cloning of genes (Kumar et al., 2009; Mammadov et al., 2012). Advances in sequencing, automation technology and computational power have made it possible to discover

abundant SNP loci and utilize them in low-cost, ultra-high-throughput genotyping (Gupta et al., 2008). For wheat, genotyping platforms such as the 9K and 90K Illumina Infinium iSelect SNP assays are available (Cavanagh et al., 2013; Wang et al., 2014). This study will employ the 9K Illumina Infinium iSelect wheat SNP assay.

2.4.4. Marker assisted backcross based gene pyramiding

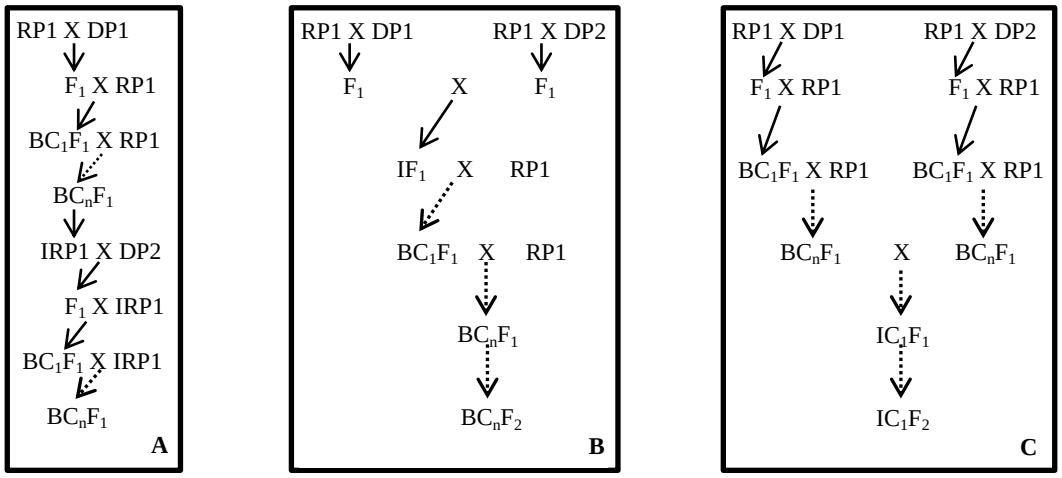

Figure 1. Three schemes of backcross based gene pyramiding: (A) Stepwise transfer; (B) Simultaneous transfer; (C) Simultaneous and stepwise transfer. RP = Recurrent parent; DP = Donor parent; BC = Backcross; IRP = improved recurrent parent. Dotted line represents any number of backcrosses (Modified from Joshi and Nayak, 2010).

Marker assisted backcross based gene pyramiding refers to the use of backrossing techniques to transfer different genes of interest from multiple sources into a common adapted backround using molecular markers. Joshi and Nayak (2010) outlined three strategies to accomplish this. The first strategy (Figure 1; Stepwise transfer) involves crossing the recurrent parent (RP1) and first donor parent (DP1) to produce the F_1 hybrid which is then backcrossed 'n' times (BC_n) to produce the first improved recurrent parent (IRP1). IPR1 is then crossed with the second donor parent (DP2) and the progeny backcrossed 'n' times to give the second improved recurrent parent, with which to initiate the third cycle, etc. This is the less acceptable strategy but

provides for precise pyramiding as it involves one gene at a time. The second strategy (Simultaneous transfer) involves crosses of RP1 with each donor parent to get different F_1 hybrids which are then intercrossed to produce an improved F_1 (IF_1). The IF_1 is then backcrossed a number of times to the recurrent parent with selection of the traits during each cycle. In the end, homozygotes are selected after selfing (Figure 1). This strategy is not well-suited for the simultaneous transfer of several genes because of the risk of losing some of the genes being pyramided. The third strategy (Simultaneous and stepwise transfer) is simply a combination of the first and second strategies. It involves crossing RP1 with many donor parents and then backcrossing them individually up to the BC_n generation. The backcross populations with the individual genes are then intercrossed with each other to get the pyramided lines (Figure 1). This method not only reduces the time needed (compared to method 1) but also ensures that all the target genes are retained. Simultaneous and stepwise transfer was done in this study.

2.5. Linkage Mapping

Linkage mapping, also known as genetic mapping or meiotic mapping is the most widely used approach to identify the position and relative distance between genes (markers or loci) across the chromosomes (Semagn et al., 2006). It takes account of the linkage relationship between two loci and positions them based on the recombination frequency between them (Mohan et al., 1997; Semagn et al, 2006). The recombination frequency is converted into a map distance/unit called centimorgan (cM) using either the Haldane or Kosambi mapping function (Semagn et al., 2006). Haldane's mapping function does not account for crossover interference (Haldane, 1931) while, crossover interference is considered in Kosambi's mapping function (Kosambi, 1944). Kosambi's mapping function is most commonly used as the Haldane mapping

function tends to over-estimate map distance for recombination frequencies higher than 10% (Semagn et al., 2006).

The accuracy with which the linkage map represents the correct gene order depends upon the type and size of a population as well the kinds of markers used in the analysis (Ferreira et al., 2006; Semagn et al., 2006). The population used in linkage mapping studies, i.e. mapping population is derived from two parents that differ for genes/loci of interest. Such a population is generally referred to as a biparental population or biparental mapping population. In self-fertilizing species, F_2, backcross populations (BC), recombinant inbred lines (RILs) and double haploid (DH) lines are the most commonly used biparental mapping populations. F_2 and BC populations are easy and faster to construct but are heterozygous, temporary and do not allow replication, while RILs and DH population require a long time to develop but are homozygous, permanent and allows replication. Several studies showed that an F_2 population can be informative if mapped using co-dominant markers, while RILs were advantageous in the majority of situations (Reiter et al., 1992; Ferreira et al., 2006).

SSRs and SNPs are the most effective and widely used co-dominant markers in mapping studies (Mohan et al., 1997; Song et al., 2005; Ferreira et al., 2006). Liu et al. (2007) mapped stripe rust resistance gene *YrChk* on chromosome 1BL flanked by five SSR markers *Xwmc44*, *Xgwm259*, *Xwmc367*, *Xcfa2292*, and *Xbarc80*. They screened 400 Chike/Taichung-49 F_2 individuals with a set of 400 microsatellites. Mebrate et al. (2007) was able to map leaf rust resistance gene *Lr38* on the proximal end of chromosome arm 6DL by screening 94 F_2 individuals from the cross RL6097/Kusba with a set of 54 SSRs . *Lr38* was flanked by SSR markers *Xwmc773* and *Xbarc273* at a distance of 6.1 and 7.9 cM, respectively. Zurn et al. (2014) mapped stem rust resistance gene *SrWLR* using F_4 and F_5 RILs developed from the cross LMPG-

6/PI 626573 and by assaying them with a set of SSR markers and the 9K Illumina Infinium iSelect wheat assay, respectively. Linkage mapping using SSR markers in the F_4 population located *SrWLR* within an 8.8cM region spanned by SSR loci *Xgwm47* and *Xwmc332*. The SNP assay on the F_5 population reduced the distance between the informative markers and *SrWLR*. *SrWLR* was mapped 1.5cM distally to *Xgwm47* and 0.4cM proximally to co-segregating SNPs IWA6121, IWA6122, IWA7620, IWA8295, and IWA8362. This study will also utilize the 9K Illumina Infinium iSelect wheat assay to map leaf rust resistance gene *Lr59* in a F_2 mapping population.

3. MATERIAL AND METHODS

3.1. Plant Materials

3.1.1. Backcrossing and pyramiding

Eight spring wheat varieties and breeding lines carrying the genes of interest (Table 1) were crossed with the winter hardy variety 'Norstar' to develop eight different F_1 progenies. The F_1 were then backcrossed to the recurrent parent Norstar to give BC_1F_1 seeds which were used in this study.

Table 1. Wheat germplasm used in the study: Designations and donor genes

Genotype and pedigree	Designation	Donor genes
RWG28[c] = BG290/3*Alsen//BG282/3*Alsen	P1	*Fhb1, Lr34, RhtB1b*, *tsn1*, *Qts.fcu-1BS*, *Qts.fcu-3BL*, *snn2*[a]
RWG10[c] = BG282/3*Alsen	P2	*Fhb1, Lr34, RhtB1b, tsn1, Qts.fcu-1BS, snn2*
RWG1[d] = Chinese Spring(CS)//CS*ph1b**2/RL6082	P3	*Lr34, Sr39*
Thatcher-*Lr*53 = S8(*Lr53/Yr35*)/5*Thatcher	P4	*Lr53*
*Lr*62-translocation = S20 = CSDM3B5B/3/CS*5/*Ae. neglecta*-155//CS-S/4/W84-17	P5	*Lr34, Lr62*
Pavon	P6	*Sr2*
WA-1 = *Sr*26 rec 43[e]	P7	*Sr26, RhtB1b*
Recombinant T6-1[f]	P8	*Sr50*
W84-17[b]	C	*Sr2, Rht-B1b*
Chinese Spring (CS)[b]	C	*Lr34*
Norstar	RP	Winter-hardy, Quality

[a]Underlined genes were not considered for transfer in this study
[b]Lines included as checks as they occur in the pedigree of some resistance sources
[c]Obtained from Dr. S. Xu (USDA-ARS, NCSL, Fargo, ND)
[d]Recombined version of the original translocation that was produced by Niu *et al*., 2011.
[e]Recombinant that is without the yield penalty associated with the original translocation (obtained from Dr. Ian Dundas, University of Adelaide, Australia).
[f]1AL.1RS translocation recombinant that lacks the sticky dough trait (obtained from Dr. Ian Dundas, University of Adelaide, Australia).

3.1.2. *Lr59*-mapping population

A F_2 mapping population was developed by making a cross between a line carrying a *Lr59* recombinant chromosome, *Lr59*-25 (= 0306/2*CS*ph1b*//CSN1AT1B/3/Thatcher) and the Canadian spring wheat variety, Superb. The same plants were then phenotypically evaluated using leaf rust pathotype, TDBG and also screened for SNP polymorphisms using the 9K iSelect wheat assay. Following screening, the F_2 plants were transferred to pots and moved to a greenhouse.

3.2. Vernalization of Seeds

In each generation of backcrossing, five to six sets of the recurrent parent were vernalized at 3^oC in a vernalization chamber (Biocold Chamber, Conviron, Winnipeg, Manitoba, Canada) *at* 10-days intervals. The F_1 seeds were vernalized with the 2^{nd} and 3^{rd} sets of the recurrent parent. On the 56^{th} day of vernalization, the majority of the pots were transferred to a greenhouse and slow release fertilizer, Osmocote 15-9-12 (3-4 month formula) and a pinch of a micronutrient mix were applied to each pot. The F_1 from crosses involving *Lr53* and *Lr62* were first transferred to a growth chamber for rust inoculation and selection for resistance before transferring to a green house. The same procedure was followed for all the intercrossed F_1 plants i.e. IC_1F_1 (development of IC_1F_1 and IC_1F_2 is described in section 3.6.). For IC_1F_2 progenies, vernalization was done after collecting leaves for DNA extraction and leaf rust inoculation (progenies segregating for *Lr53*). The mapping population did not require vernalization since it was derived from spring wheat crosses.

3.3. DNA Extraction

DNA extractions and marker analyses were done either in the Plant Sciences department or at the USDA Genotyping Center (Fargo, ND). For analyses performed in the Plant Sciences

department, fresh leaf samples were collected at the 2-3 leaf stage and lyophilized for 48 hrs. Lyophilized leaves were transferred to 2ml Eppendorf tubes containing silicon and 2.5mm metal beads and ground in a Retsch Mixer Mill MM301 (Retsch Inc, Newtown, PA; closed now). DNA was then extracted using the DART DNA extraction protocol (http://www.diversityarrays.com/sites/default/files/resources/DArT_DNA_ isolation.pdf). For some of the samples, DNA was extracted by the USDA Genotyping Center using their own protocol (http://wheat.pw.usda.gov/GenotypingLabs/ fargo.html). The latter samples were collected by cutting 1.5" to 2" fresh leaf samples at the 2-3 leaf stage in 96- deep-well blocks filled with silica gel.

3.4. Marker Assays

Primer sequences and PCR conditions for each primer are shown in Tables 2 and 3, respectively. The genotypes listed in Table 1 were assayed using all the available markers (Table 2; except umn10, Rwg27 and gwm333) to determine whether they produced polymorphisms different from Norstar. The backcrossed and intercrossed individuals were then analyzed with the same set of markers. Each PCR reaction was performed using a 25µl volume (20µl volume for *Sr2*) consisting of 4µl (~100ng) of template DNA, 1X GoTaq® Flexi Buffer (Promega Corporation, Madison, WI), 2mM $MgCl_2$, 0.2mM of PCR Nucleotide Mix (Promega Corporation, Madison, WI), 1 µl of primers (forward and reversed primers mixed) and 1.25u of GoTaq® Flexi DNA Polymerase (Promega Corporation, Madison, WI). PCR amplification was performed in a BIORAD T100™ Thermal Cycler (Life Sciences, Hercules, CA). For *Sr2*, each PCR amplicon was further treated with 5 µl of a mixture containing 2.5ml 10X NEB buffer 4 and 0.5 µl of *Bsp*HI (10U/ µl; NEB) (New England Biolabs, Ipswich, MA) and incubated in a

Table 2. List of markers used in this study: Primer sequences and PCR conditions

Gene	Marker	Forward Sequence	Reverse Sequence	PCR Conditions	Reference
Sr2	*csSr2*	CAAGGGTTGCTAGGATTGGAAAAC	AGATAACTCTTATGATCTTACATTTTTCTG	95°C 2 min : 1 cycle 95°C 30 sec 60°C 40 sec 72°C 50 sec } 30 cycles 72°C 5 min : 1 cycle 15°C 1 min : 1 cycle	Mago et al., 2011a
	gwm533	GTTGCTTTAGGGGAAAAGCC	AAGGCGAATCAAACGGAATA	94°C, 3 min : 1 cycle 94°C, 60 sec 60°C, 60 sec 72°C, 120 sec } 30 cycles 72°C, 10 min : 1 cycle	Hayden et al., 2004
Sr26	*Sr26#43*	AATCGTCCACATTGGCTTCT	CGCAACAAAATCATGCACTA	94°C, 3 min : 1 cycle 94°C, 60 sec 60°C, 60 sec 72°C, 120 sec } 30 cycles 72°C, 10 min : 1 cycle	Liu et al., 2010
	BE518379	AGCCGCGAAATCTACTTTGA	TTAAACGGACAGAGCACACG		
Sr39	*Sr39#22r*	AGAGAAGATAAGCAGTAAACATG	TGCTGTCATGAGAGGAACTCTG	94°C, 5 min : 1 cycle 92°C, 30 sec 58°C, 30 sec 72°C, 40 sec } 30 cycles 72°C, 10 min : 1 cycle	Mago et al., 2009
	BE500705	ATCTGTGGCAGTGTGCTCCT	TCCTGCAAATGCTTGTCGTT	94°C, 3 min : 1 cycle 92°C, 30 sec 56°C, 30 sec 72°C, 40 sec } 30 cycles 72°C, 10 min : 1 cycle	

Table 2: List of markers used in this study: Primer sequences and PCR conditions (continued)

Gene	Marker	Forward Sequence	Reverse Sequence	PCR Conditions	Reference
Sr39	*Xrwgs27*	GCCTTGGTGGATTTTGTGAT	GCGCTTTCAGTACAGGGTTC	95°C, 5 min : 1 cycle 95°C, 40 sec 55°C, 40 sec 36 cycles 72°C, 40 sec 72°C, 10 min : 1 cycle	Niu et al., 2011
Sr50	*IB-267*	GCAAGTAAGCAGCTTGATTTAGC	AATGGATGTCCCGGTGAGTGG	94 °C, 3 min: 1 cycle 94 °C, 30 s 55 °C, 60 s } 30 cycles 72 °C, 60 s 25 °C, 60 s 1 cycle	Mago et al., 2002
Lr34	*csLV34*	GTTGGTTAAGACTGGTGATGG	TGCTTGCTATTGCTGAATAGT	94 °C, 3 min 1 cycle 94 °C, 15 s 58 °C, 15 s } 45 cycles 72 °C, 15 s 72 °C, 5 min 1 cycle	Kolmer et al., 2008; Lagudah et al., 2009
RhtB1b	BF-MR1	GGTAGGGAGGCGAGAGGCGAG	CATCCCCATGGCCATCTCGAGCTA	94 °C, 5 min: 1 cycle 94 °C, 30 s 65 °C, 60 s } 7 touchdown cycles 72 °C, 80 s 94 °C, 15 s 58°C, 15 s } 30 cycles 72 °C, 50 s	Ellis et al., 2002
Fhb1	Umn10	CGTGGTTCCACGTCTTCTTA	TGAAGTTCATGCCACGCATA	94 °C, 3 min: 1 cycle 94 °C, 60 s 60 °C, 60 s } 35 cycles 72 °C, 120s 72 °C, 120s 1 cycle	Liu et al., 2008

BIORAD T100™ Thermal Cycler for 1 hr (Mago et al., 2011a). PCR products were separated in 2 to 3% (w/v) AMRESCO® Agarose SFR™ agarose gel (AMRESCO, Solon, OH) treated with EtBr. DNA was visualized using ProteinSimple AlphaImager® HP System (ProteinSimple, Santa Clara, CA). Some of the backcrossed and intercrossed progenies involving *Fhb1*, *Rht-B1b* and *Lr34* were analyzed by the USDA Genotyping center (Fargo, ND) using their own protocol (http://wheat.pw.usda.gov/Genotyping Labs/fargo.html).

SNP analysis of the *Lr59* mapping population was done using the wheat 9K iSelect genotyping assay on the Illumina BeadStation and iscan instrument following the manufacturer's protocol (Illumina, Inc., San Diego, CA). Analyses were performed at the USDA Genotyping Center (Fargo, ND) (Cavanagh et al., 2013). SNP calling and allele clustering was done using Illumina® GenomeStudio V.2011.1 software (Illumina, Inc., San Diego, CA). Using the software's default algorithm, assays producing three distinct clusters ('AA', 'AB', and 'BB') were identified. Those assays that had no distinct classes due to compressed SNP allele clustering were manually curated to define three distinct clusters Assays with no significant effect after manual curation were left as default and not used for analysis.

3.5. Phenotypic Evaluation

The backcrossed and intercrossed (IC_1F_1) progenies segregating for *Lr53* and *Lr62* were screened by inoculating them with leaf rust pathotype MFPS at 8-10 days after they were transferred from the vernalization chamber to a growth chamber maintained at 24°C and 16hrs light/day. Pathotype MFPS is virulent on the genetic backgrounds of Thatcher-*Lr53* and the *Lr62* translocation but is avirulent to the two respective resistance genes. The inoculation was done by spraying fresh urediniospores of pathotype MFPS mixed in distilled water and a wetting agent (Tween-20) (Marais et al., 2005). The inoculated seedlings were covered with moist plastic bags

and kept at room temperature for 24 hrs under indirect light. The pots were then returned to the growth chamber and scored when the symptoms were fully developed 8-10 days later. The 0-4 rating scale developed by Stakman et al. (1961) was used. Plants with infection types 3-4 were excluded from the studies.

The *Lr59*-mapping population was also phenotypically evaluated using similar procedures. Leaf rust pathotype, TDBG was used to inoculate 8-10 days old seedlings. A single - F_1 plant showing resistance to *Puccinia triticina* pathotype TDBG was used as source of the mapping population. Pathotype TDBG is virulent on CS, Thatcher and Superb but is avirulent on *Lr59.* Ninety three individual F_2 plants were screened phenotypically for their reaction to pathotype TDBG. The resistant and susceptible individuals were identified and transplanted to individual pots. A Chi-square test was conducted to test conformance to a 3:1 segregation ratio.

3.6. Backcrossing and Pyramiding

The simultaneous and stepwise transfer method of marker assisted backcross based pyramiding was used to produce specific gene combinations (Figure 2). The progenies from the crosses with the eight donor parents (Table 1) were backcrossed to Norstar to obtain BC_3F_1 (BC_2F_1 for the Thatcher-*Lr53*/Norstar cross) (Figure 2). The BC_3F_1 progenies developed from RWG10 were excluded from the study following the third backcross as the target gene could not be recovered. Selected BC_3F_1 progenies (BC_2F_1 for the Thatcher-*Lr53*/Norstar cross) of six of the crosses were crossed with the BC_3F_1 developed from the RWG28/Norstar cross (Figure 2). Six IC_1F_1 groups with different gene combinations in the heterozygous state were recovered in the first stage of pyramiding. The IC_1F_1 was then selfed in an attempt to generate IC_1F_2 groups with all the desired gene combinations in the homozygous state.

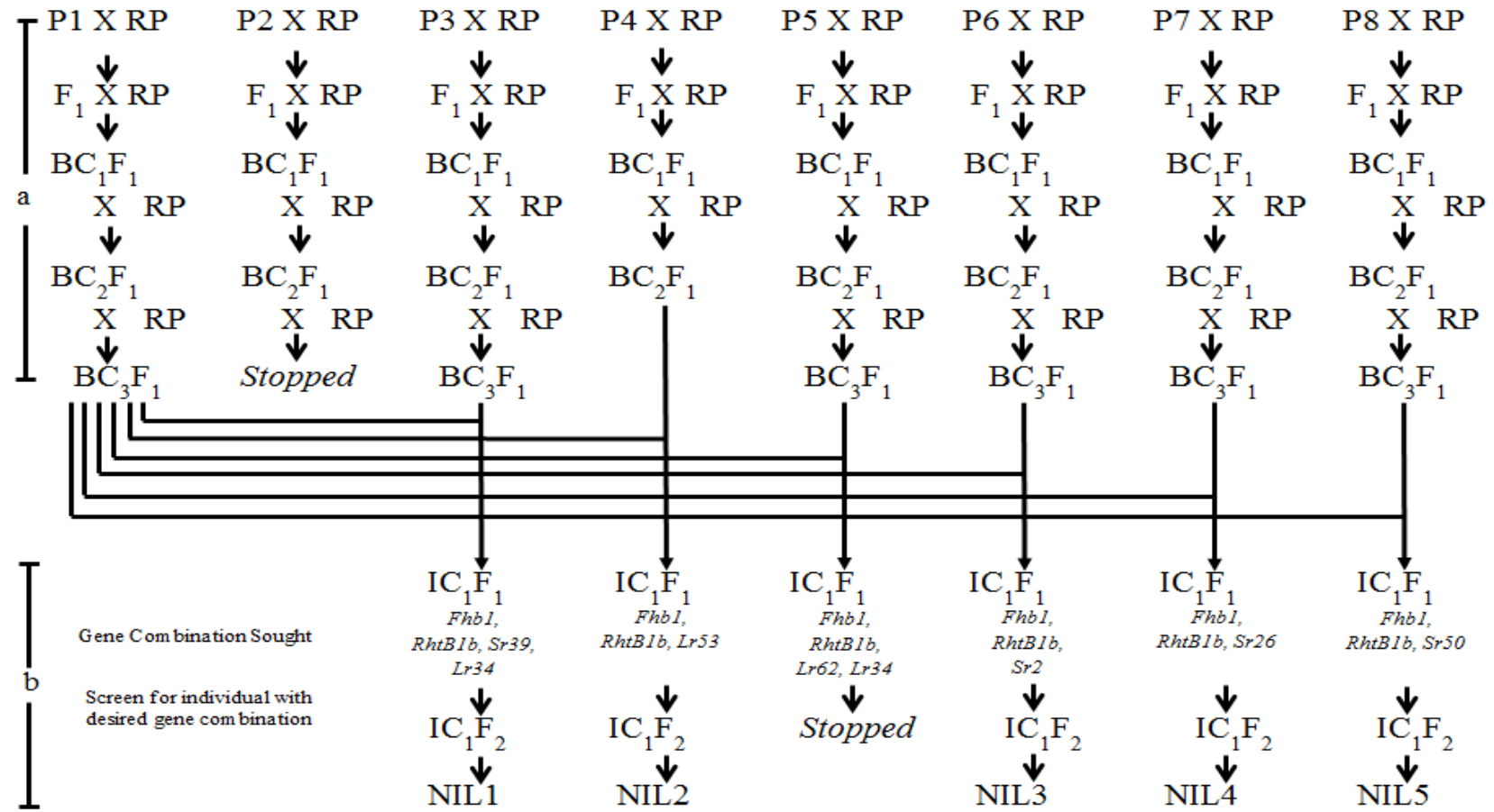

Figure 2. (a) In the backcross phase eight donor parents (P1-P8) were crossed with and backcrossed to Norstar (recurrent parent, RP) to produce BC_3F_1 (BC_2F_1 for P4 X RP) with the target genes. The target genes in the respective spring wheat donor parents were P1 = P2 = *Fhb1*, *Rht-B1b*, *Lr34*; P3 = *Lr34*, *Sr39*; P4 = *Lr53*, P5 = *Lr34*, *Lr62*; P6 = *Sr2*; P7 = *Sr26*, *Rht-B1b*; P8 = *Sr50*. Backcrosses initiated with P2 were discontinued when BC_2F_1 with the target genes could not be recovered. (b) In the pyramiding phase BC_3F_1 derived from the P1 backcross was crossed to each of the remaining final backcross F_1 to produce intercross F_1 (IC_1F_1) from which near-isogenic lines with pyramided gene combinations were derived. The IC_1F_1 derived from P5 was not pursued further when it became apparent that the *Lr62* translocation may be associated with deleterious phenotypic effects.

The IC_1F_2 progenies segregating for *Lr62* were excluded from the study when it became apparent that this translocation may carry associated deleterious genes.

The minimum number of seeds to be used in each stage of the backcrossing and pyramiding schemes was determined based on the theoretical probability of obtaining a desired genotype for a given number of genes (Table 3). Population sizes higher than the minimum required were used in order to improve the odds of recovering genotypes with the desired gene or gene combination.

Table 3. Theoretical probability of recovering a genotype with the desired gene combination

Breeding Stage	No. of Genes	Theoretical Probability[a]	Min. Seeds [b]
Backcross[c]	1	½	2
	2	¼	4
	3	1/8	8
Intercross F_1[c]	3	1/8	8
	4	1/16	16
Intercross F_2[d]	3	1/64	64
	4	1/256	256

[a]- Theoretical probability of recovering a desired genotype for a given number of genes, if two genotypes being crossed have different genes (Acquaah, 2007)

[b] Minimum number of seeds that could possibly include the desired gene combination

[c]- Gene combination recovered in heterozygous condition

[d]- Gene combination recovered in homozygous condition

3.7. Linkage Mapping

The final data obtained after manual curation of the wheat 9K iSelect genotyping assay on genome studio were exported to Excel to conduct the mapping analysis. The markers that were monomorphic and/or had a call frequency less than 95% were excluded from the mapping analysis. Chi square tests at the 95% confidence level were conducted to determine whether the markers conformed to the Mendelian F_2 segregation ratio (1:2:1). Finally, polymorphic markers with Mendelian segregation were used for the linkage analysis.

The informative markers were analyzed with JMP genomics 6.1 (SAS Institute Inc., Cary, NC) software to generate 42 linkage groups using the 'Average' clustering option of the 'Automatic Hierarchical Clustering' linkage grouping method. Multiple hypothesis testing for all segregation ratios were conducted using the False Discovery Rate (FDR) method with p-value set at 5%. The information from the consensus map developed by Cavanagh et al. (2013) was used to assign the linkage groups obtained to the 21 wheat chromosomes. The linkage group that included the phenotypic leaf rust resistance marker was separated and analyzed with JMP genomics v6.0 to generate a map for that linkage group. The 'Map order optimization' algorithm was used to order the markers, while the Kosambi mapping function (Kosambi, 1944) was used to determine the distance between markers.

4. RESULTS

4.1. Marker Assays on Parents

The donor parents used in this study produced polymorphisms different from the recurrent parent, Norstar, for all of the markers used to trace the genes of interest (Figure 3; 4; 5; 6; 7; 8). RWG28 and RWG10 showed polymorphic bands associated with *Lr34*, *Rht-B1b* and the 1BL.1RS rye translocation when analyzed with markers cslv34 (Figure 4), BF-MR1 (Figure 7) and IB-267 (Figure 8), respectively. The marker cslv34 also detected the *Lr34* diagnostic band in RWG1 and CS-*Lr62* (Figure 4). The DNA of donor parent RWG1 generated a PCR amplicon of ~818bp associated with stem rust resistance gene *Sr39* when assayed with STS marker Sr39#22r (Figure 3). Stem rust resistance gene *Sr2* was detected in donor parents Pavon and CS-*Lr62* (Figure 5). Donor parent WA-1 gave the diagnostic bands for the height reducing gene *Rht-B1b* (Figure 7) and stem rust resistance gene *Sr26* (Figure 6). A rye-specific band of ~210bp associated with the presence of the shortened 1DL.1RS (*Sr50*) translocation was amplified in Recombinant T6-1(Figure 8).

For all these markers, Norstar either showed absence of the diagnostic band (dominant markers) or produced a band that was associated with the absence of the desired gene (co-dominant markers). Apart from the set of donor plants, Chinese Spring and W84-17 were also included in the overall marker assay since the two genotypes occur in the pedigrees of some of the resistance sources. Chinese Spring has *Lr34* while W84-17 (has *Sr2*) was initially used in developing the CS-*Lr62* line which retained the *Sr2* locus (Marais et al., 2009).

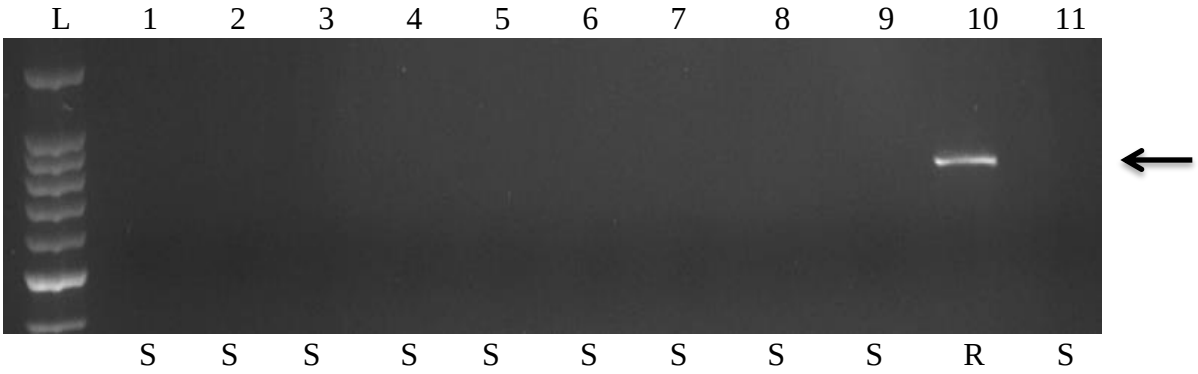

Figure 3. PCR amplification of dominant STS marker Sr39#22r in the different genotypes employed in the study and separation on 2% agarose gels. Lanes: L = 100bp ladder, 1 = RWG28; 2 = Thatcher-*Lr53*; 3 = Norstar; 4 = W84-17; 5 = Pavon; 6 = CS-*Lr62*; 7 = Chinese Spring; 8 = Recombinant T6-1; 9 = WA-1; 10 = RWG1; 11 = RWG10; S = *Sr39* absent; R = *Sr39* present. The arrow indicates the polymorphic band (~818bp) associated with *Sr39* based resistance (Mago et al., 2009; Bernardo et al., 2013).

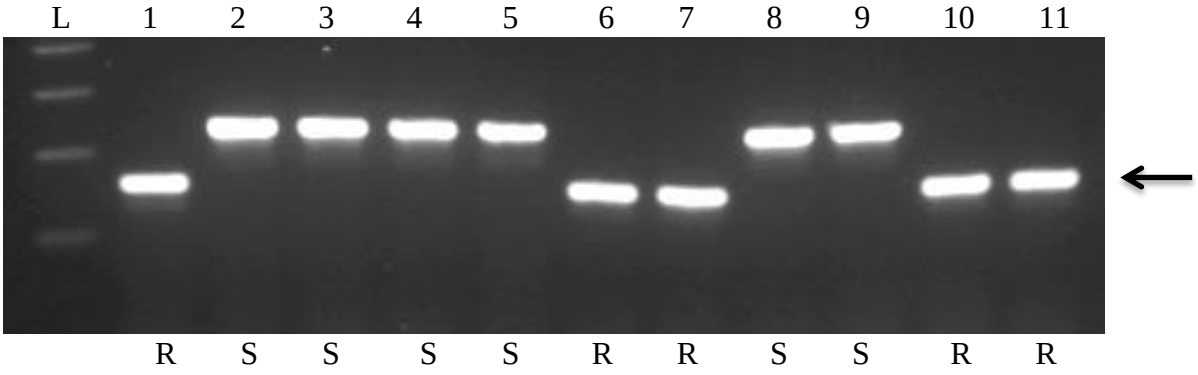

Figure 4. PCR amplification of co-dominant STS marker cslv34 in the different genotypes employed in the study and separation on 2.5% agarose gels. Lanes: L = 100bp ladder; 1 = RWG28 ; 2 = Thatcher-*Lr53*; 3 = Norstar; 4 = W84-17; 5 = Pavon; 6 = CS-*Lr62*; 7 = Chinese Spring; 8 = Recombinant T6-1; 9 = WA-1; 10 = RWG1; 11 = RWG10; S = *Lr34* absent; R = *Lr34* present. The arrow indicates the band (~150bp) associated with *Lr34* based resistance (Kolmer et al., 2008).

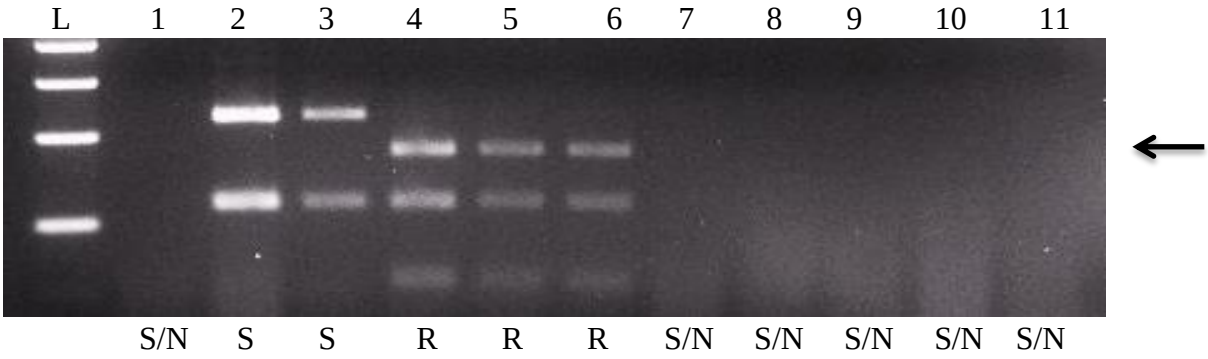

Figure 5. PCR amplification of co-dominant CAPS marker csSr2 in the different genotypes employed in the study and separation on 2.5% agarose gels. Lanes: L = 100bp ladder; 1 = RWG28; 2 = Thatcher-*Lr53*; 3 = Norstar; 4 = W84-17; 5 = Pavon ; 6 = CS-*Lr62*; 7 = Chinese Spring; 8 = Recombinant T6-1; 9 = WA-1; 10 = RWG1; 11 = RWG10; S = *Sr2* absent; R = *Sr2* present; S/N = *Sr2* absence associated with null allele. The arrow indicates a band (~172bp) associated with *Sr2* based resistance (Mago et al., 2011a).

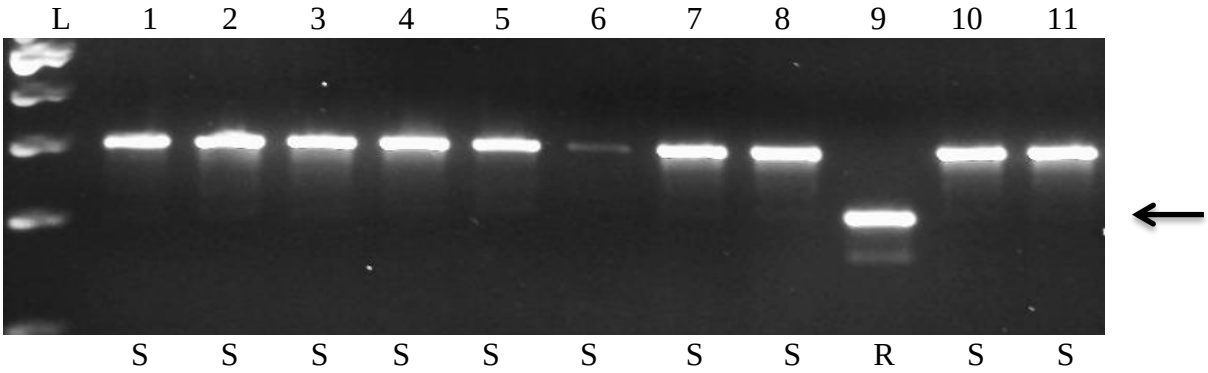

Figure 6. Multiplex PCR amplification of STS marker Sr26#43(207bp) and EST marker BE518379 (303bp) in the different genotypes employed in the study and separation on 3% agarose gels. Lanes: L= 100bp ladder; 1 = RWG28; 2 = Thatcher-*Lr53*; 3 = Norstar; 4 = W84-17; 5 = Pavon; 6 = CS-*Lr62*; 7 = Chinese Spring; 8 = Recombinant T6-1; 9 = WA-1; 10 = RWG1; 11 = RWG10; S = *Sr26* absent; R = *Sr26* present. The arrow indicates a band (207bp) associated with *Sr26* based resistance (Liu et al., 2010).

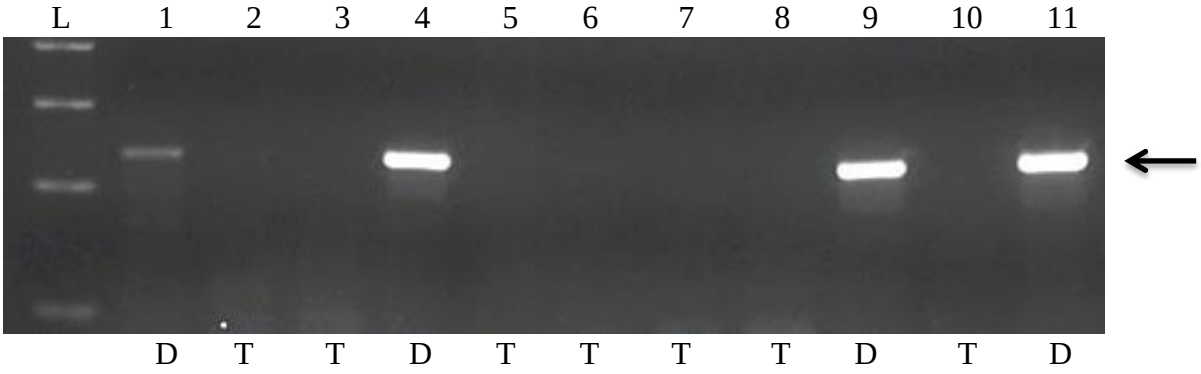

Figure 7. PCR amplification of dominant STS marker BF-MR1 in the different genotypes employed in the study and separation on 2% agarose gels. Lanes: L = 100bp ladder; 1 = RWG28; 2 = Thatcher-*Lr53*; 3 = Norstar; 4 = W84-17; 5 = Pavon; 6 = CS-*Lr62*; 7 = Chinese Spring; 8 = Recombinant T6-1; 9 = WA-1; 10 = RWG1; 11 = RWG10 ; D = *Rht-B1b* present; T = *Rht-B1b* absent. The arrow represents a band (~237bp) associated with *Rht-B1b* (Ellis et al., 2002).

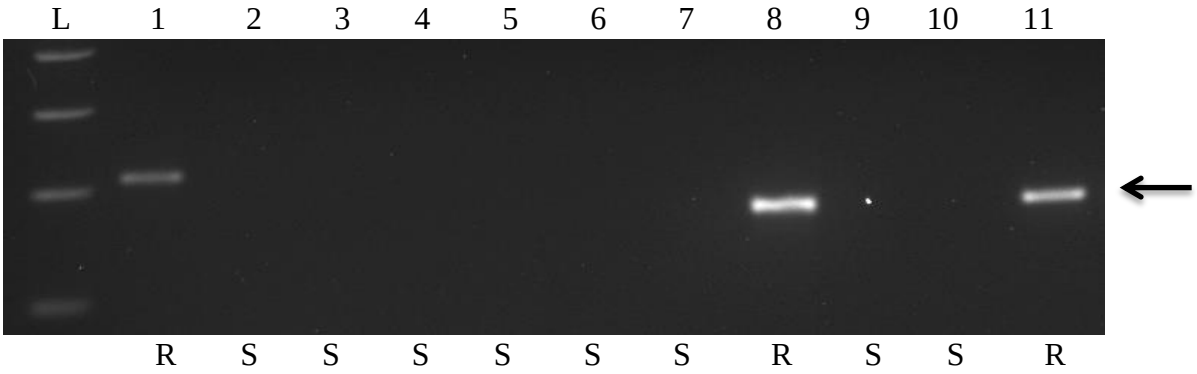

Figure 8. PCR amplification of dominant STS marker IB-267 in the different genotypes employed in the study and separation on 2% agarose gels. Lanes: L = 100bp ladder; 1 = RWG28 (1BL.1RS); 2 = Thatcher-*Lr53*; 3 = Norstar; 4 = W84-17; 5 = Pavon; 6 = CS-*Lr62*; 7 = Chinese Spring; 8 = Recombinant T6-1 (1DL.1RS.1DS); 9 = WA-1; 10- RWG1; 11 = RWG10 (1BL.1RS). The marker detects rye chromatin: S = no rye segment; R = rye segment present. The arrow indicates a band (~210bp) associated with *Sr50* based resistance (Mago et al., 2009; Bernardo et al., 2013).

4.2. Marker Assisted Backcrossing

BC_1F_1 plants from eight different cross combinations were further back-crossed to Norstar. Marker assisted selection was initiated using ten seeds of each of six BC_1F_1 progenies. BC_1F_1 progenies Thatcher-*Lr53*/2*Norstar and CS-*Lr62*/2*Norstar were evaluated phenotypically (Figures 9; 10). Adequate numbers of plants with the desired gene(s) were identified in BC_1F_1 progenies of each cross. However, BC_1F_1 progenies from the RWG28/2*Norstar and RWG10/2*Norstar crosses could not be analyzed for the presence of genes *Fhb1*, *Rht-B1b* and *Lr34* and were randomly backcrossed to Norstar to obtain BC_2F_1 seeds. For the cross RWG10/3*Norstar, an inadequate number of BC_2F_1 seeds were obtained.

Twenty BC_2F_1 of each cross combination, except for BC_2F_1 Thatcher-*Lr53*/3*Norstar and CS-*Lr62*/3*Norstar, were analyzed using available markers to identify plants with the desired gene(s). BC_2F_1 seeds with the target genes could not be recovered from cross RWG10/3*Norstar, while the similar combination involving the same target genes (BC_2F_1 = RWG28/3*Norstar) could only recover *Fhb1* and *Rht-B1b*.

With the exception of one cross, the selected BC_2F_1 individuals were then backcrossed to Norstar to produce BC_3F_1 seeds. A further backcross was not made with BC_2F_1 Thatcher-*Lr53*/3*Norstar. Seventy four BC_3F_1 seeds of RWG28/4*Norstar and 40 BC_3F_1 seeds of each of the remaining crosses (BC_2F_1 seeds in the case of Thatcher-*Lr53*/3*Norstar) were planted for evaluation. Numerous plants with the desired gene(s) were obtained for each cross combination and were used for crosses with BC_3F_1 plants of the RWG28/4*Norstar cross. Of the 17 BC_3F_1 individuals of the RWG28/4*Norstar cross that had *Fhb1* and *Rht-B1b*, six individuals were found to be without the 1BL.1RS rye translocation. Only the latter plants were used in continued crosses as the 1BL.1RS translocation (background-derived) is associated with the undesirable

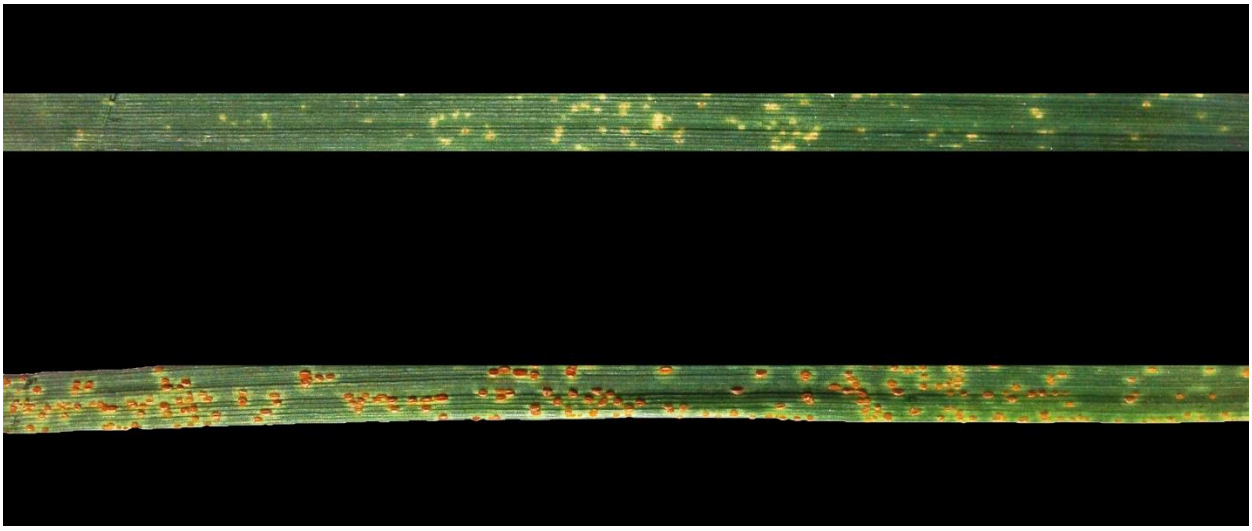

Figure 9. Infection types produced on wheat seedling leaves of genotypes carrying *Lr62* (top leaf) and without *Lr62* (bottom leaf) when inoculated with *Puccinia triticina* pathotype MFPS

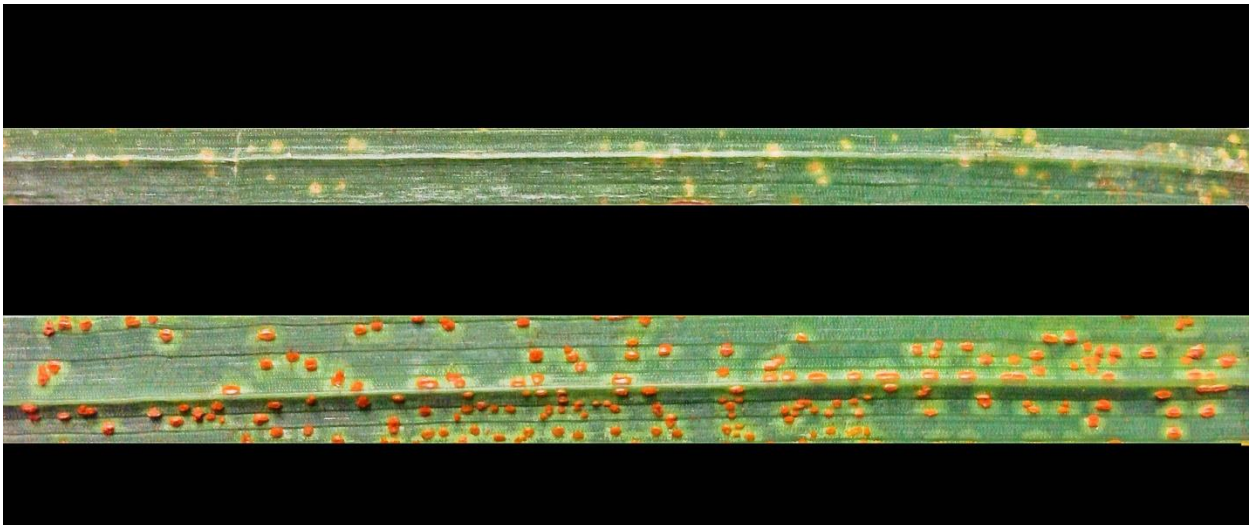

Figure 10. Infection types produced on the bottom sides of wheat seedling leaves from genotypes carrying *Lr53* (top leaf) and without *Lr53* (bottom leaf) when inoculated with *Puccinia triticina* pathotype MFPS

'sticky dough' trait. As it produces the same marker polymorphism as the shortened 1DL.1RS translocation that is without this trait, it can result in false identification of *Sr50*.

The results obtained for the different backcross generations are summarized in Table 4. Table 4 provides the numbers of progeny tested, target genes that were retained after each backcross as well as the numbers involved and the conformance of the progeny ratios to Mendelian expectations. To facilitate further discussion of the results each backcross lineage is assigned a different code (C1 to C8).

4.3. Marker Assisted Gene Pyramiding

Gene pyramiding was initiated by crossing the selected BC_3F_1 plants of backcross RWG28/4*Norstar with selected BC_2F_1 individuals of the Thatcher-*Lr53*/3*Norstar cross and BC_3F_1 progenies of the remaining five backcrosses (Table 5).

The C1/C8 cross (involving *Fhb1, Rht-B1b* and *Sr50*) was made by crossing BC_3F_1 seeds of RWG28/4*Norstar lacking the 1BL.1RS rye translocation with BC_3F_1 seeds of the Recombinant T6-1/4* Norstar cross. Two sets of 100 seeds representing the C1/C3 and C1/C5 crosses and four sets of 50 seeds each from the remaining crosses were evaluated using markers and rust inoculation (individuals from crosses C1/C4 and C1/C5). Adequate numbers of individuals with the desired gene combinations were identified and selfed to obtain first intercross (IC_1) F_2 seeds. However, the IC_1F_2 seeds from cross C1/C5 were excluded from further analysis. This was done because the fairly big *Lr62* translocation appeared to have detrimental effects on the plant phenotype under summer greenhouse conditions, and it was decided to put more emphasis on the recovery of the remaining, more promising gene combinations. The IC_1F_2 seeds from each remaining inter cross were then tested to recover individual plants with the desired gene combination in homozygous condition (Table 5).

Table 4. Numbers of backcross plants with the desired gene/gene combinations that were recovered in each backcross generation

Cross	Designation	Generation	Target genes segregating in selected F_1 plants	No. of seeds planted	Expected [a]	Observed [b]
RWG28 X Norstar	C1	BC_1F_1	*Fhb1, Rht-B1b,Lr34*	10	~1	N/A[c]
			Fhb1,Rht-B1b			N/A
		BC_2F_1	*Fhb1, Rht-B1b,Lr34*	20	~3	0
			Fhb1,Rht-B1b			1
		BC_3F_1	*Fhb1,Rht-B1b*	74	~19	17
RWG10 X Norstar	C2	BC_1F_1	*Fhb1, Rht-B1b,Lr34*	10	~1	N/A
			Fhb1,Rht-B1b			N/A
		BC_2F_1	*Fhb1, Rht-B1b,Lr34*	20	~3	0
			Fhb1,Rht-B1b			0
RWG1 X Norstar	C3	BC_1F_1	*Sr39, Lr34*	10	~3	3
			Sr39			2
		BC_2F_1	*Sr39, Lr34*	20	5	2
			Sr39			11
		BC_3F_1	*Sr39, Lr34*	40	10	5
			Sr39			10
Thatcher-*Lr53* X Norstar	C4	BC_1F_1	*Lr53*	20	10	9
		BC_2F_1	*Lr53*	40	20	14
CS-*Lr62* X Norstar	C5	BC_1F_1	*Lr62, Lr34*	10	~3	2
			Lr62			3
		BC_2F_1	*Lr62, Lr34*	20	5	2
			Lr62			11
		BC_3F_1	*Lr62, Lr34*	40	10	2
			Lr62			13
Pavon X Norstar	C6	BC_1F_1	*Sr2*	8	4	5
		BC_2F_1	*Sr2*	20	10	7
		BC_3F_1	*Sr2*	40	20	20
WA-1 X Norstar	C7	BC_1F_1	*Sr26,Rht-B1b*	10	~3	0
			Sr26,Rht-B1b			6
		BC_2F_1	*Sr26*	20	10	6
		BC_3F_1	*Sr26*	40	20	24
Recombinant T6-1 X Norstar	C8	BC_1F_1	*Sr50*	10	5	9
		BC_2F_1	*Sr50*	20	10	10
		BC_3F_1	*Sr50*	40	20	13

[a]- The number of seeds tested was always more than the theoretical minimum that is expected to include the desired gene/gene combination in the heterozygous state (Acquaah et al., 2007)
[b]- Observed no. of seeds with the sought after gene combination in the heterozygous state. If the best gene combination could not be recovered, the second best gene combination was used for further backcrosses
[c]- Data are not available

Table 5. Numbers of plants with the desired gene/gene combination that were recovered during pyramiding

Cross	Pyramiding stages	Gene/Gene combination[a]	No. of Seeds	Expected[b]	Observed[c]
C1 X C3	IC_1F_1	*Fhb1, Rht-B1b, Sr39, Lr34,*	100	~6	7
		Fhb1,Rht-B1b,Sr39			9
	IC_1F_2	***Fhb1, Rht-B1b, Lr34,*** *Sr39*	375	~3	2
		Fhb1, Rht-B1b, *Sr39*			4
		Fhb1, ***Lr34***, *Sr39,*			5
		Fhb1, *Sr39*			5
C1 X C4	IC_1F_1	*Fhb1, Rht-B1b, Lr53*	50	~6	4
	IC_1F_2	***Fhb1, Rht-B1b, Lr53*****	350	~16	12
		Fhb1, Lr53			13
C1 X C5	IC_1F_1	*Fhb1, Rht-B1b,Lr34,Lr62*	100	~6	1
		Fhb1, Rht-B1b, Lr62			1
C1 X C6	IC_1F_1	*Fhb1, Rht-B1b,Sr2*	50	~6	3
	IC_1F_2	***Fhb1, Rht-B1b***,*Sr2*	201	~1	1
		Fhb1, *Sr2*			0
C1 X C7	IC_1F_1	*Fhb1, Rht-B1b,Sr26*	50	~6	4
	IC_1F_2	***Fhb1, Rht-B1b,Sr26***	253	~4	1
		Fhb1, Rht-B1b,Sr26		~8	7
		Fhb1, Sr26			2
		Fhb1, *Sr26*			18
C1 X C8	IC_1F_1	*Fhb1, Rht-B1b,Sr50*	50	~6	8
	IC_1F_2	***Fhb1, Rht-B1b,Sr50***	347	~16	12
		Fhb1, Sr50			17

[a]- Genes shown in bold were recovered in the homozygous state while those shown in regular script were in the heterozygous state. Genes that are underlined and in bold require progeny testing to determine whether they occur in the heterozygous or homozygous state
[b]-Number of the total seeds tested that were expected to have the targeted genes
[c]-Number of the total seeds tested that turned out to have the target genes.

Fhb1 and *Rht-B1b* were recovered in homozygous condition in a significant number of individuals from each intercross. A homozygote for all of the desired genes was recovered only in the IC_1F_2 of cross C1/C7. For the remaining crosses, genes other than *Fhb1* and *Rht-B1b* were recovered in heterozygous condition in numerous individuals. In the case of cross C1/C3, *Lr34* was recovered in the homozygous state along with *Fhb1* and *Rht-B1b*. Furthermore, several subsets of gene combinations were identified in the IC_1F_2 of each intercross. For each of the

cross combinations, individuals with and without *Rht-B1b* were identified, while among IC_1F_2 of the C1/C3 cross, plants with and without *Lr34* as well as *Rht-B1b* were identified.

4.4. Linkage Mapping

4.4.1. Phenotypic evaluation

The 93 F_2 plants inoculated with pathotype TBDG segregated into 68 resistant and 25 susceptible (Figure 5). The chi-square test on the observed segregation ratio was consistent with monogenic Mendelian segregation (Table 6a). Resistant plants identified among the F_2 were selfed to generate $F_{2:3}$ populations. A minimum of 30 $F_{2:3}$ from each F_2 family were inoculated with TDBG in order to identify homozygotes. The 68 resistant F_2 segregated into 22 homozygous and 43 heterozygous individuals, while three F_2 families were excluded from the analysis for having germination less than 50%. The observed ratio of 22 homozygous resistant: 43 heterozygous resistant: 25 homozygous susceptible plants once again conformed to monogenic inheritance (Table 7). The phenotypic marker information (named *Lr59*) was added to the genotypic data.

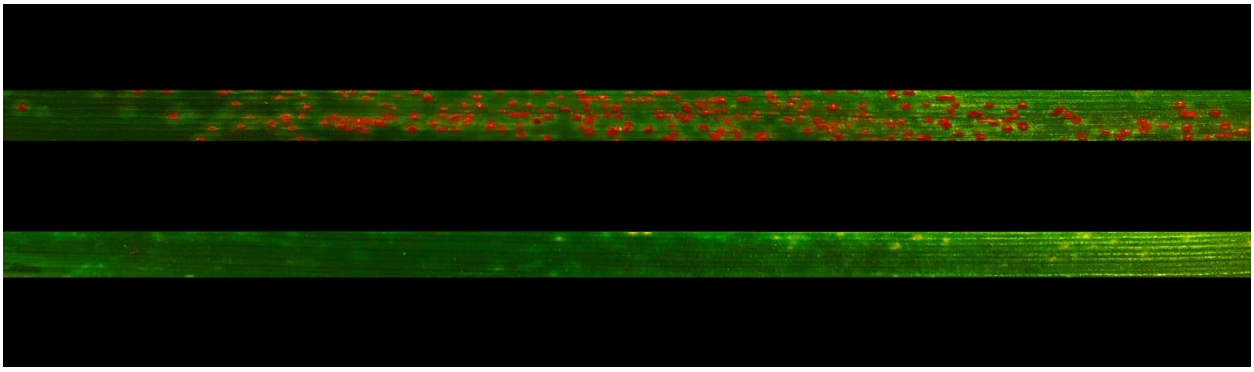

Figure 11. Leaf rust infection types produced by individuals in the mapping population. The top leaf with rust pustules lacks Lr59 and the bottom leaf with flecks carries Lr59.

Table 6. Segregation of the F_2 mapping population for leaf rust resistance gene *Lr59*

Generation	Classes	No. of plants		Chi-square test	
		Observed	Expected	χ^2	P-value
F_2	Resistant	68	70	0.18	>0.68
	Susceptible	25	23		

Table 7. Segregation of the $F_{2:3}$ mapping population for leaf rust resistance gene *Lr59*

Generation	Classes	No. of plants		Chi-square test	
		Observed	Expected	χ^2	P-value
$F_{2:3}$	Homozygous resistant	22	22	0.74	>0.60
	Heterozygous resistant	43	46		
	Homozygous susceptible	25	22		

4.4.2. Mapping analysis

Analysis of the F_2 mapping population using the 9K wheat iSelect assay produced 1755 polymorphic SNPs of which only eight had missing data (less than 2%) for each of the eight affected loci. The polymorphic markers along with the phenotypic marker (*Lr59*) were distributed across 42 linkage groups developed using the defined clustering method in JMP genomic v6.1. The phenotypic marker was assigned to linkage group 10 along with 171 other markers. Information from the consensus map developed by Cavanagh et al. (2013) was used to assign linkage group 10 to a wheat chromosome. Ninety six percent of the markers assigned to linkage group 10 were mapped in chromosome 6B of the consensus map, while 2 SNPs were mapped in chromosome 6A and the remaining four were unmapped (Appendix, Table 1A). This confirmed that linkage group 10 developed in this map was equivalent to chromosome 6B of wheat.

The total length of the linkage map produced for chromosome 6B was 106.5 cM (Figure 11). *Lr59* was mapped in the most distal position on 6BS. The closet SNP loci were the co-segregating SNPs IWA1495, IWA6704, IWA2098 and IWA969 (0.5cM proximally from *Lr59*). An additional 11 SNP loci mapped within an interval 0.5-6.5 cM proximally of *Lr59.*

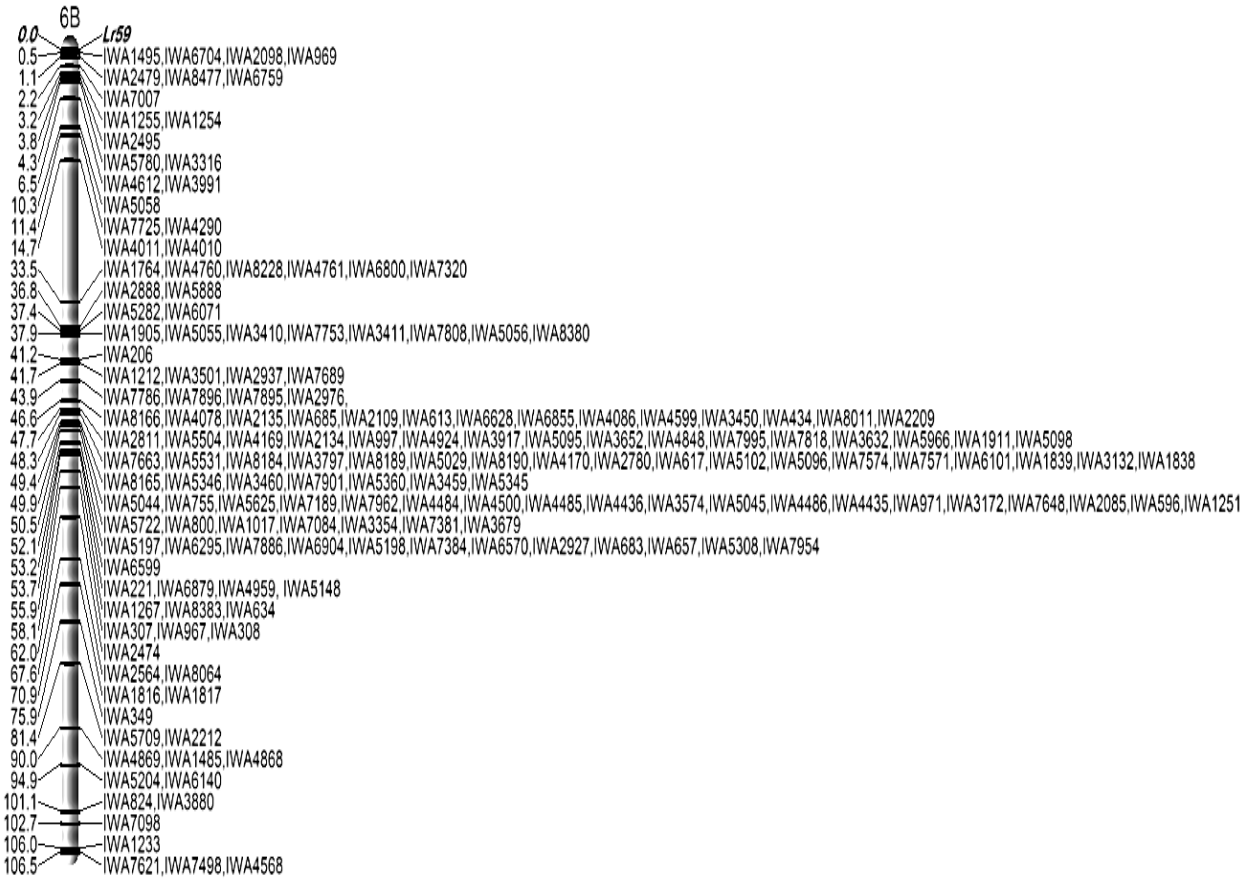

Figure 12. Linkage map of wheat chromosome 6B developed using the 9K Illumina Infinium iSelect wheat assay to genotype a F_2 mapping population developed by crossing a line with the *Lr59*-25 recombined translocation (pedigree = 0306/2*CS*ph1b*//CSN1AT1B/3/Thatcher) with the Canadian spring wheat variety, Superb.

5. DISCUSSION

5.1. Analysis of the Parents

Analyses of the parents using the available markers confirmed the absence of all the targeted genes in the recurrent parent 'Norstar'. The marker assay showed each donor parent to be unique for the genes of interest, except for *Lr34, Rht-B1b* and *Sr2* which occurred in more than one parent. RWG28 and RWG10 were the only two donor parents that had similar genes of interest. Both of them were used in this study with the aim to recover each of *Fhb1*, *Rht-B1b* and *Lr34* in the Norstar background. However, all of the target genes could not be recovered in progenies of RWG10 and its backcrosses were not pursued further. Also, with respect to the backcross progenies from RWG28, *Lr34* could not be recovered following the third backcross and thus, only *Fhb1* and *Rht-B1b* were retained in the reconstituted Norstar background. Apart from the targeted genes, the 1BL.1RS rye translocation was also detected in RWG28 and RWG10 when analyzed with marker IB-267 (Figure 8). The rye translocation in these wheat lines apparently derive from BR34, a parent in the pedigrees of BG282 and BG290, which were used as parents of RWG10 and RWG28, respectively (S.S. Xu, Personal Communication, 2013; Table 1). BG282 and BG290 are random inbred lines derived from a BR34/Grandin cross and BR34 was found to carry the 1BL.1RS translocation (S. S. Xu, Personal Communication, 2013; Simons et al., 2012). This translocation is different from the 1AL.1RS translocation harboring the stem rust resistance gene *Sr50.* While both full length translocations carry the rye secalin locus, *Sec1*, which has a negative effect on the baking quality of wheat, the rye translocation (*Sr50*) in Recombinant T6-1 is reduced in size (the rye region containing *Sec1* was replaced with 1DS chromatin) to remove the sticky dough trait (Anugrahwati et al., 2007). The stem rust

resistance gene *Sr50* in recombinant T6-1 can still be detected using marker IB-267 (Anugrahwati et al., 2007; Figure 8).

Since, the marker IB-267 cannot distinguish the larger rye translocation in RWG28 from the reduced *Sr50* translocation in Recombinant T6-1, it could not be diagnostic in identifying intercrossed individuals from cross C1/C8 that have recovered stem rust resistance gene *Sr50*. However, this drawback was overcome by selecting for cross C1 BC_3F_1 individuals that were positive for *Fhb1* and *Rht-B1b* but lacked the 1BL.1RS rye translocation. Any individuals derived from this cross can now be accurately identified as *Sr50* carriers using marker IB-267.

RWG28 and RWG10 also produced a null allele when analyzed using the CAPS marker csSr2 (Figure 5). A null allele at this marker locus implies absence of the 'Marquis' type allele; Marquis being a susceptible bread wheat variety (Mago et al., 2011a). A null allele will render the co-dominant csSr2 marker a dominant marker. So, use of this marker to characterize intercross progenies that involved backcross derivatives of RWG28 and Pavon may require a progeny test to differentiate *Sr2* homozygotes from *Sr2* heterozygotes. However, having recovered 93.7% Norstar background in the third backcross, it is likely that the 'Marquis' allele has been recovered and that csSr2 will correctly identify *Sr2* homozygotes. Primer pair csSr2 also produced a null allele for RWG10, Chinese Spring, Recombinant T6-1, WA-1 and RWG1. Chinese Spring was used as a check in the panel of parents as it was present in the pedigree of donor parent RWG1 and the CS-*Lr62* translocation. The inclusion of Chinese Spring also helped to validate the csSr2 marker as it was used in the screening panels while designing the csSr2 marker (Mago et al., 2011a).

RWG10, CS-*Lr62* and WA-1 were three other donor parents from which multiple genes were targeted for transfer to Norstar. Both of the genes from RWG1 (*Sr39* and *Lr34*) and CS-

Lr62 (*Lr62* and *Lr34*) were successfully recovered in backcross as well as intercross progenies. However, the C1 X C5 IC_1F_2 was later removed from the study as it carries *Lr62* on a large alien translocation and it appeared that it could potentially affect spike development under hot conditions (G.F. Marais, personal communication, 2014). Along with two leaf rust resistance genes, line CS-*Lr62* was found to also possess stem rust resistance gene *Sr2* (Figure 5). The pedigree of CS-*Lr62* suggested that *Sr2* was obtained from one of its parents, W84-17 (Marais et al., 2009). W84-17 (a semidwarf wheat carrying *Rht-B1b*) in turn has Inia 66 in its pedigree. Inia 66 also carries both *Rht-B1b* and *Sr2*. W84-17 was confirmed to be positive for the presence of *Sr2* (Figure 5) and *Rht-B1b* (Figure 7).

Donor parent WA-1 was initially selected with the aim to transfer *Sr26* and *Rht-B1b* to Norstar. However, *Rht-B1b* could not be recovered along with *Sr26* in its backcross progenies. WA-1 thus contributed only *Sr26.*

5.2. Backcrossing and Pyramiding

The transfer of genes to Norstar was fairly simple because of the availability of diagnostic markers that could easily identify the backcross progenies with a desired gene. Norstar was used as the female in all backcrosses to ensure that the gene(s) identified in each backcross F_1 derived from backcrossing and not from selfing of donor parents. The expected incidence of a desired gene combination was calculated based on the theoretical probability of recovering the desired gene(s) in backcross progenies and used as a guide to determine the numbers of plants to be tested. An adequate number of plants with the desired gene(s) were subsequently recovered in each generation for each backcross.

On average, a BC_3F_1 individual will have recovered 93.75% of the Norstar genetic background while the BC_2F_1 seeds obtained with the *Lr53* backcrosses would have recovered

87.5% of Norstar. Following intercrossing of BC_3 derivatives there will have been no change in the amount of Norstar recovered in the intercrossed progenies. In intercross C1 X C4, however, BC_3F_1 were crossed with BC_2F_1 individuals which will have resulted in an average recovery of 90.625% in the intercross F_1. Thus, it is likely that most of the cold-hardiness characteristics of Norstar would have been recovered among the near-isogenic lines. Since the purpose of this study was not to upgrade Norstar as a variety but to pyramid desirable disease resistance genes in a winter-hardy genetic background and use it as cross parents, it was not necessary to fully recover the Norstar genetic background. Obviously, the lines will have to be evaluated in field trials to confirm that cold hardiness, FHB and rust resistance is being expressed.

Pyramiding of the target genes into single lines was comparatively easy using marker assisted selection. Despite having multiple genes from various parents, pyramiding of the genes was facilitated for three reasons: a) they were qualitative major genes, b) all of them have tightly linked markers or strong phenotypic expression (*Lr53, Lr62*), and c) they reside on different chromosomes (except *Fhb1* and *Sr2*). The presence of tightly linked markers makes gene pyramiding accurate, effective and fast (Ye and Smith, 2008). Qualitative traits governed by major genes that are tightly linked to diagnostic markers can easily be identified with high accuracy. With the target genes residing on different chromosomes and segregating independently, it becomes possible to recover pyramided genes even in comparatively small segregating populations (no need to break unfavorable linkages first).

Among the genes studied, only *Fhb1* and *Sr2* occur on the same chromosome. They are linked in repulsion on chromosome 3B at a distance of 10cM (Flemmig et al., 2011). Repulsion linkage of these genes reduces the chance of recovering them in the homozygous condition in a single F_2 individual from 6.25% (independent segregation) to 0.25%. So, the chance of

recovering single plants simultaneously homozygous for *Fhb1*, *Sr2* and *Rht-B1b* from a trihybrid was 0.06% and would have required testing of at least 1,667 plants (minimum representation). However, only 201 IC_1F_2 seeds were available of cross C1/C6. From this, one plant was recovered that had *Fhb1* and *Rht-B1b* in homozygous condition but, *Sr2* in heterozygous condition. The expected percentage of F_2 plants that would have had *Fhb1* and *RhtB1b* in homozygous state and *Sr2* in heterozygous state is 1.13% (minimum representation = 89). Twenty five percent of the F_3 progeny of the selected monohybrid plant should therefore be homozygous at all three loci.

None of the IC_1F_2 seeds from cross C1/C3 recovered all four target genes, *Fhb1, Rht-B1b, Lr34* and *Sr39,* in homozygous condition. The minimum representation for this cross was 256 plants and therefore 375 plants were evaluated. However, the two best plants had genes *Fhb1, Lr34* and *Rht-B1b* in the homozygous state while *Sr39* was heterozygous. Thus, plants that are homozygous for all four loci should be readily recoverable from the selfed progeny of the two selected F_2.

Among the targeted gene combinations, the combination of *Fhb1, Rht-B1b,* and *Sr26* was the only combination recovered in homozygous state (Table 2). In crosses C1/C4 and C1/C8, it was not possible to distinguish homozygotes and heterozygotes of *Sr50* and *Lr53*, as the molecular marker IB-267 (for *Sr50*) and the phenotypic expression of resistance (for *Lr53*) are dominant. Progeny testing will be required to identify homozygotes among the 12 selected $F_{2:3}$ families that segregate for *Lr53* and the eight $F_{2:3}$ families that segregate for *Sr50*.

Apart from the potential utility of the various NILs in HRWW crosses, the material can also be very valuable for phenotypic studies. *Rht-B1b* has been reported to have a potential role in elevating FHB resistance in wheat (Srinivasachary et al. 2009; Miedaner et al., 2011).

However, early studies done by Hilton et al. (1999) showed that the effect of *Rht-B1b* was dependent upon environment and genetic background and increased FHB severity. The present NILs have recovered at most 93% of Norstar and will therefore not be directly comparable in genetic studies; however, a NIL homozygous for *Fhb1, Rht-B1b* can be crossed with a NIL homozygous for *Fhb1* to produce a monohybrid F_1 that can either be subjected to doubled haploid development or single seed descent inbreeding to generate populations with very similar overall genetic background but differing with respect to *Rht-B1b*. Similarly, lines with and without *Lr34* derived from cross C1/C3 can be used to study *Lr34* phenotypic effects more closely. *Lr34* is a well characterized, non-HR leaf rust resistance gene that is widely utilized, particularly in spring wheat cultivars (Kolmer et al., 2008; Lagudah et al., 2009; Singh et al., 2011). It is also linked with the *Ltn1* gene that imparts leaf tip necrosis. *Lr34* does not occur widely in hard red winter wheat grown in the northern prairies and it has been hypothesized that under low temperature conditions, the gene may have a detrimental effect on winter-survival, however, this possibility has not been tested. (G.F. Marais, Personal Communication, 2013).

Norstar is a winter hardy winter wheat variety with suitable milling and baking quality and had been widely used in Canadian and North American winter wheat breeding programs (Cox et al., 1988; Fowler, 2008). However, it is too tall, tends to lodge and is disease susceptible. This study has attempted to combine the winter-hardiness and good processing quality of Norstar with improved disease resistance in new breeding parents. Norstar was chosen as the backcross parent in view of the low heritability and seemingly complex inheritance of cold-hardiness as well as the complex nature and intermediate heritability of processing quality. The disease resistance genes on the other hand are single genes that can readily be detected using markers or phenotypic evaluation. Although most of the rust resistance genes are in the heterozygous

condition in the selected intercross F_2, a single selfing will allow their recovery in the homozygous condition along with *Fhb1, RhtB1b* and *Lr34*. Small numbers of segregating plants will need to be screened as 25% of the F_3 progenies are expected to be homozygotes.

The set of genes utilized in this study was selected judiciously to address the current and future problems in winter wheat breeding. The most widely grown HRWW varieties in North Dakota are highly susceptible to Fusarium head blight (Ransom et al., 2014). The increasing incidence of FHB and DON in winter wheat affects the profitability of production. Corn acreage in ND has also increased by 8% and can potentially enhance the chance of FHB infection in wheat (Taylor and Koo, 2013). In such a scenario, the incorporation of a FHB resistance gene like *Fhb1* in winter wheat breeding material and its dissemination throughout the breeding population is of utmost importance. However, the lines developed from this study will first need to be validated under field conditions (Pumphrey et al., 2007).

The targeted rust resistance genes include two non-HR genes, i.e. *Lr34* and *Sr2*, as well as widely effective major HR genes (*Lr53, Sr26, Sr39* and *Sr50*). The combination of both non-HR and HR rust resistance genes in gene pyramids can be expected to result in more stable and sustainable resistance. Until now, no winter wheat varieties with the shortened *Sr26* and 1DL.1RS translocations have been released. The availability of *Sr39* and *Lr53* in winter wheat background will similarly be unique and very useful resources in winter wheat breeding. The four stem rust resistance genes are effective against local pathotypes as well as the Ug99 variants and will safeguard varieties should Ug99 pathotypes reach the US. The current five pyramids of either three or four target genes can furthermore easily be intercrossed for one or two more cycles to derive even more formidable gene combinations for breeding. Finally, studies have shown that reduced (semi-dwarf) plant height optimize the performance of winter wheat, so

availability of *Rht-B1b* in Norstar will definitely promote its value as a useful parent (Zhao et al., 2014). Having the characteristic built into a breeding parent that will be used widely will remove the need to discard large numbers of segregating progeny that are simply too tall and prone to lodging.

5.3. Linkage Mapping

The genetic analysis of the mapping population confirmed that the resistance derived from *Ae. peregrina* is conferred by a small translocated region that probably harbors a single dominant gene (*Lr59*). The segregation ratio complied with monogenic Mendelian segregation. However, strong segregation distortion with preferential male transmission of the original Robertsonian *Lr59* translocation was reported by Marais et al. (2008; 2010). Thus, the alien chromatin that was removed during the allosyndetic pairing induction experiment (Marais et al., 2010) must have been the cause of the segregation distortion. If the segregation distortion had been caused by morphological differences between the alien and wheat chromosome regions on 1AL, then the *Lr59*-25 recombinant may have improved utility compared to the original version.

Comparison of the F_2 linkage map developed here with the consensus map (Cavanagh et al., 2013) located *Lr59* in chromosome 6B. The marker order produced for this linkage group was collinear with the 9K consensus map, with few marker order rearrangements (Appendix Table 1A). Zurn et al. (2014) also observed similar minor rearrangements after developing a map using JMP genomics v6.1. Such rearrangements were attributed to the algorithm and methods used in the Mergemap software used to develop a consensus map from multiple linkage maps (Endelman, 2011; Zurn et al., 2014). Compared to the consensus map, higher numbers of co-segregating markers were observed in the linkage map developed for chromosome 6B in this study. This might have contributed to alteration of the marker order. The accuracy of a linkage

map is strongly correlated to the size and type of population used in mapping analysis (Ferreira et al., 2006). The F_2 individuals employed and small size of the mapping population could further explain the minor rearrangements in marker order. However, the marker order was not significantly different from the 6B maps produced by Cavanagh et al. (2013) and Zurn et al., (2014) (Appendix Table 1A).

Leaf rust resistance gene, *Lr59* was mapped very close to the cosegregating SNP markers IWA969, IWA1495, IWA2098 and IWA6704. These markers were compared with the results obtained from SNP analyses on wheat ditelosomic stocks conducted by Cavanagh et al. (2013). The four markers were absent in 6BL ditelosomics and were mapped in the telomeric end of 6BS. Also, no other SNPs were mapped distally to IWA1495 and IWA2098 which confirms that *Lr59* sits at the telomeric end of the short arm of chromosome 6B. These SNPs can therefore be used to develop diagnostic markers as they were tightly linked (0.5cM) to *Lr59*. SNPs can be converted to allele specific KBioscience Competitive Allele-Specific Polymerase chain reaction (KASPar) assay markers (Hiremath et al., 2014; Forrest et al., 2014). KASPar assay is a patented SNP genotyping system developed by KBioscience based on Fluorescent Resonance Energy Transfer (FRET) (http://www.lgcgroup. com/; http://www.ksre.ksu.edu/igenomics/doc1363.ashx). The sequence information required for designing a KASPar assay for each of these SNPs were made readily available by Wang et al. (2014).

The chromosome location of *Lr59*-25 as determined in this mapping study differs from the location of the full length translocation on 1AL (Marais et al. 2008) as well as the presumed location (1AL) of the allosyndetic recombinants derived by Marais et al. (2010). Eight different recombinants were identified that have reduced alien segments. However, no conclusive observations were made regarding the location on *Lr59* in each of the recombinants. Attempts

are now being made to identify the location of *Lr59* in each recombinant using in-situ hybridization, deletion mapping (SSRs) and genetic mapping (SNPs). The difference in chromosome location of *Lr59*-25 as compared to the original translocation can be attributed to the presence of areas in the *Ae. peregrina* alien segment with homoeology to wheat chromosomes 1A and 6B. In the absence of the meiotic pairing control locus, *Ph1*, pairing among related (homoeologous) chromosome segments is possible and could account for the difference in location of the original and recombined *Lr59* translocations.

The continuous and extensive use of major gene resistance facilitates the appearance newly evolved virulent races (Kolmer et al., 2007). This in turn creates a need for further sources of genetic resistance to combat the new virulence. *Lr59* has not been used in US wheat germplasm and thus, is a novel source of leaf rust resistance. It has been reported to provide resistance against all known races of *P. triticina* in Canada and South Africa (Marais et al., 2008). Before it can be used commercially, it is necessary to determine which of the eight recombinants developed by Marais et al. (2010) has retained the least alien chromatin and could be the most useful. Preliminary results (Marais 2014, Personal Communication) have shown that two of the eight recombined translocations occur on chromosome arm 1AL. This study identified the location of recombinant 25 as 6BS. The remaining recombinants are likely to also occur on chromosome arm 6BS but this needs to be confirmed through SSR analyses.

6. REFERENCES

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APPENDIX. COMPARISON OF THE GENETIC MAP DEVELOPED IN THIS STUDY WITH GENETIC MAPS PRODUCED IN PREVIOUS STUDIES BY CAVANAGH ET AL. (2013) AND ZURN ET AL. (2014)

Map Order	Marker Name	Current Study		Cavanagh et al. (2013)		Zurn et al. (2014)[d]	
		Chr[a]	Distance (cM)[b]	Chr	Distance (cM)	Chr	Distance (cM)
1	***Lr59***	6B	0	-[c]	-	-	-
2	IWA969	6B	0.5	6B	4.12	6B1	96.09
3	IWA2098	6B	0.5	6B	0.00	6B1	96.09
4	IWA6704	6B	0.5	6B	1.01	6B1	95.67
5	IWA1495	6B	0.5	6B	0.00	6B1	95.67
6	IWA6759	6B	1.1	6B	1.01	6B1	95.46
7	IWA8477	6B	1.1	6B	1.01	-	-
8	IWA2479	6B	1.1	6B	4.12	6B1	95.46
9	IWA7007	6B	2.2	6A	-	-	-
10	IWA1254	6B	3.2	6B	4.12	-	-
11	IWA1255	6B	3.2	-	-	-	-
12	IWA2495	6B	3.8	-	-	6B1	91.53
13	IWA3316	6B	4.3	6B	11.38	-	-
14	IWA5780	6B	4.3	6B	11.38	-	-
15	IWA3991	6B	6.5	6B	23.19	-	-
16	IWA4612	6B	6.5	6B	23.19	-	-
17	IWA5058	6B	10.3	6B	32.35	-	-
18	IWA4290	6B	11.4	6B	34.70	-	-
19	IWA7725	6B	11.4	6B	34.70	-	-
20	IWA4010	6B	14.7	6B	34.70	-	-
21	IWA4011	6B	14.7	6B	34.70	-	-
22	IWA7320	6B	33.5	6B	49.76	-	-
23	IWA6800	6B	33.5	6A	-	-	-
24	IWA4761	6B	33.5	6B	49.76	-	-
25	IWA8228	6B	33.5	6B	49.76	-	-
26	IWA4760	6B	33.5	6B	49.76	-	-
27	IWA1764	6B	33.5	6B	49.76	-	-
28	IWA5888	6B	36.8	6B	59.85	-	-
29	IWA2888	6B	36.8	6B	61.40	-	-
30	IWA6071	6B	37.4	6B	54.75	6B1	64.82
31	IWA5282	6B	37.4	6B	54.75	-	-
32	IWA8380	6B	37.9	6B	59.25	-	-
33	IWA5056	6B	37.9	6B	60.35	-	-

Order	Marker	Current Study		Cavanagh et al. (2013)		Zurn et al. (2014)[c]	
		Chr[a]	Distance (cM)[b]	Chr	Distance (cM)	Chr	Distance (cM)
34	IWA7808	6B	37.9	6B	72.51	-	-
35	IWA3411	6B	37.9	6B	59.85	-	-
36	IWA7753	6B	37.9	6B	57.91	-	-
37	IWA3410	6B	37.9	6B	59.85	-	-
38	IWA5055	6B	37.9	6B	60.35	-	-
39	IWA1905	6B	37.9	6B	60.90	6B1	61.78
40	IWA206	6B	41.2	6B	77.26	6B1	59.86
41	IWA7689	6B	41.7	6B	77.81	6B1	59.86
42	IWA2937	6B	41.7	6B	78.37	-	-
43	IWA3501	6B	41.7	6B	77.81	6B1	59.86
44	IWA1212	6B	41.7	6B	77.26	6B1	59.86
45	IWA7896	6B	43.9	6B	81.93	6B1	59.44
46	IWA7895	6B	43.9	6B	81.93	6B1	59.44
47	IWA2976	6B	43.9	6B	80.93	6B1	59.23
48	IWA7786	6B	43.9	6B	81.93	6B1	59.44
49	IWA2209	6B	46.1	6B	92.77	6B1	56.07
50	IWA8011	6B	46.1	6B	89.34	6B1	57.34
51	IWA434	6B	46.1	6B	127.55	6B1	56.07
52	IWA3450	6B	46.1	6B	95.85	6B1	56.28
53	IWA4599	6B	46.1	6B	95.35	-	-
54	IWA4086	6B	46.1	6B	127.55	6B1	56.07
55	IWA6855	6B	46.1	6B	127.55	6B1	56.07
56	IWA6628	6B	46.1	6B	127.55	6B1	56.07
57	IWA613	6B	46.1	6B	127.55	6B1	56.07
58	IWA2109	6B	46.1	6B	127.55	-	-
59	IWA685	6B	46.1	6B	127.55	6B1	56.07
60	IWA2135	6B	46.1	6B	94.85	6B1	56.28
61	IWA4078	6B	46.1	6B	94.34	6B1	57.34
62	IWA8166	6B	46.6	6B	127.55	6B1	55.23
63	IWA5098	6B	47.2	6B	117.51	6B1	55.02
64	IWA1911	6B	47.2	6B	124.95	6B1	55.02
65	IWA5966	6B	47.2	6B	124.95	6B1	55.02
66	IWA3632	6B	47.2	6B	117.51	6B1	55.02
67	IWA7818	6B	47.2	6B	117.01	6B1	55.02
68	IWA7995	6B	47.2	6B	119.02	6B1	55.02
69	IWA4848	6B	47.2	6B	127.55	6B1	55.02
70	IWA3652	6B	47.2	6B	122.94	6B1	55.02
71	IWA5095	6B	47.2	6B	117.51	6B1	55.02

Order	Marker	Current Study		Cavanagh et al. (2013)		Zurn et al. (2014)[c]	
		Chr[a]	Distance (cM)[b]	Chr	Distance (cM)	Chr	Distance (cM)
72	IWA3917	6B	47.2	6B	124.95	6B1	55.02
73	IWA4924	6B	47.2	6B	120.86	6B1	55.02
74	IWA997	6B	47.2	6B	154.94	6B1	55.02
75	IWA2134	6B	47.2	6B	127.55	-	-
76	IWA4169	6B	47.2	6B	128.56	6B1	55.02
77	IWA5504	6B	47.2	6B	119.52	6B1	55.02
78	IWA2811	6B	47.7	6B	117.51	6B1	55.02
79	IWA1838	6B	48.3	6B	120.86	6B1	55.02
80	IWA3132	6B	48.3	6B	117.51	6B1	55.02
81	IWA1839	6B	48.3	6B	124.45	6B1	55.02
82	IWA6101	6B	48.3	6B	129.06	6B1	55.02
83	IWA7571	6B	48.3	6B	126.04	6B1	55.02
84	IWA7574	6B	48.3	6B	120.86	6B1	55.02
85	IWA5096	6B	48.3	6B	117.51	6B1	55.02
86	IWA5102	6B	48.3	6B	121.19	6B1	55.02
87	IWA617	6B	48.3	6B	123.45	6B1	55.02
88	IWA2780	6B	48.3	6B	120.52	6B1	55.02
89	IWA4170	6B	48.3	6B	125.53	6B1	55.02
90	IWA8190	6B	48.3	6B	117.51	6B1	55.02
91	IWA5029	6B	48.3	6B	115.00	6B1	55.02
92	IWA8189	6B	48.3	6B	117.51	6B1	55.02
93	IWA3797	6B	48.3	6B	129.06	6B1	55.02
94	IWA8184	6B	48.3	6B	118.02	6B1	55.02
95	IWA5531	6B	48.3	6B	117.51	6B1	55.02
96	IWA7663	6B	48.3	6B	117.51	6B1	55.02
97	IWA5345	6B	49.4	6B	131.83	6B1	53.54
98	IWA3459	6B	49.4	6B	101.91	6B1	53.96
99	IWA5360	6B	49.4	6B	131.83	6B1	53.54
100	IWA7901	6B	49.4	6B	131.83	6B1	53.54
101	IWA3460	6B	49.4	6B	130.21	6B1	53.96
102	IWA5346	6B	49.4	6B	131.83	6B1	53.54
103	IWA8165	6B	49.4	6B	131.83	6B1	53.54
104	IWA1251	6B	49.9	6B	132.19	-	-
105	IWA596	6B	49.9	6B	132.40	-	-
106	IWA2085	6B	49.9	6B	139.77	6B1	51.85
107	IWA7648	6B	49.9	6B	139.27	-	-
108	IWA3172	6B	49.9	6B	139.77	6B1	51.85
109	IWA971	6B	49.9	6B	132.40	-	-

Order	Marker	Current Study		Cavanagh et al. (2013)		Zurn et al. (2014)[c]	
		Chr[a]	Distance (cM)[b]	Chr	Distance (cM)	Chr	Distance (cM)
110	IWA4435	6B	49.9	6B	138.77	6B1	51.85
111	IWA4486	6B	49.9	6B	139.77	6B1	51.65
112	IWA5045	6B	49.9	6B	132.40	-	-
113	IWA3574	6B	49.9	6B	134.74	6B1	51.85
114	IWA4436	6B	49.9	6B	139.77	6B1	51.85
115	IWA4485	6B	49.9	6B	139.77	6B1	51.65
116	IWA4500	6B	49.9	6B	132.19	-	-
117	IWA4484	6B	49.9	6B	133.74	6B1	51.65
118	IWA7962	6B	49.9	6B	132.40	-	-
119	IWA7189	6B	49.9	6B	134.41	6B1	51.65
120	IWA5625	6B	49.9	6B	134.74	6B1	51.85
121	IWA755	6B	49.9	6B	132.40	-	-
122	IWA5044	6B	49.9	6B	132.40	-	-
123	IWA3679	6B	50.5	6B	143.86	6B1	51.44
124	IWA7381	6B	50.5	6B	140.99	-	-
125	IWA3354	6B	50.5	6B	141.11	6B1	51.44
126	IWA7084	6B	50.5	6B	140.99	6B1	51.44
127	IWA1017	6B	50.5	6B	140.99	6B1	51.44
128	IWA800	6B	50.5	6B	141.62	6B1	51.44
129	IWA5722	6B	50.5	6B	142.33	6B1	51.44
130	IWA7954	6B	52.1	6B	151.40	6B1	49.29
131	IWA5308	6B	52.1	6B	151.40	6B1	49.29
132	IWA657	6B	52.1	6B	151.40	6B1	49.29
133	IWA683	6B	52.1	6B	152.03	6B1	49.29
134	IWA2927	6B	52.1	6B	151.40	6B1	49.29
135	IWA6570	6B	52.1	6B	151.53	6B1	49.29
136	IWA7384	6B	52.1	6B	150.95	-	-
137	IWA5198	6B	52.1	6B	151.40	6B1	49.29
138	IWA6904	6B	52.1	6B	151.40	6B1	49.29
139	IWA7886	6B	52.1	6B	151.40	6B1	49.29
140	IWA6295	6B	52.1	6B	151.40	6B1	49.29
141	IWA5197	6B	52.1	6B	151.40	6B1	49.29
142	IWA6599	6B	53.2	6B	154.53	-	-
143	IWA6879	6B	53.7	6B	166.25	-	-
144	IWA4959	6B	53.7	6B	154.94	6B1	46.49
145	IWA5148	6B	53.7	6B	163.31	6B1	46.49
146	IWA221	6B	53.7	6B	161.30	6B1	46.49
147	IWA634	6B	55.9	6B	173.90	-	-

Order	Marker	Current Study		Cavanagh et al. (2013)		Zurn et al. (2014)[c]	
		Chr[a]	Distance (cM)[b]	Chr	Distance (cM)	Chr	Distance (cM)
148	IWA8383	6B	55.9	6B	173.90	-	-
149	IWA1267	6B	55.9	6B	173.90	6B1	44.78
150	IWA308	6B	58.1	-	-	6B1	42.38
151	IWA967	6B	58.1	6B	185.11	6B1	42.38
152	IWA307	6B	58.1	-	-	6B1	42.38
153	IWA2474	6B	62	6B	186.90	-	-
154	IWA8064	6B	67.6	6B	191.07	-	-
155	IWA2564	6B	67.6	6B	191.57	-	-
156	IWA1817	6B	70.9	6B	193.34	6B1	36.76
157	IWA1816	6B	70.9	6B	193.34	6B1	36.76
158	IWA349	6B	75.9	6B	200.92	-	-
159	IWA2212	6B	81.4	6B	219.26	-	-
160	IWA5709	6B	81.4	6B	219.26	-	-
161	IWA4868	6B	90	6B	226.85	6B1	19.52
162	IWA1485	6B	90	6B	226.85	6B1	19.52
163	IWA4869	6B	90	6B	227.35	6B1	19.52
164	IWA6140	6B	94.9	6B	231.03	-	-
165	IWA5204	6B	94.9	6B	231.03	-	-
166	IWA3880	6B	101.1	6B	245.62	-	-
167	IWA824	6B	101.1	6B	235.23	-	-
168	IWA7098	6B	102.7	6B	235.83	-	-
169	IWA1233	6B	106	6B	242.51	-	-
170	IWA4568	6B	106.5	6B	243.25	-	-
171	IWA7498	6B	106.5	6B	235.83	-	-
172	IWA7621	6B	106.5	6B	235.83	-	-

[a]- Location in wheat chromosome

[b]- Distance based on the Kosambi Mapping function

[c]- Marker position and location unknown for respective studies.

[d]- Markers are in reverse order compared to current study and consensus map developed by Cavanagh et al. (2013).