ASSOCIATION MAPPING FOR LEAF TIP NECROSIS AND PSEUDO BLACK CHAFF IN RELATION TO DURABLE RUST RESISTANCE IN WHEAT

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ABSTRACT

The partial rust resistance genes *Lr34* and *Sr2* have been used extensively in wheat improvement as they confer exceptional durability. Interestingly, the resistance of *Lr34* is associated with the expression of leaf tip necrosis (Ltn) and *Sr2* with pseudo black chaff (Pbc). Though previous studies have detected several QTL's linked to these traits, their genetic basis is still intriguing. Hence, association mapping was employed in this study for CIMMYT's Stem Rust Resistance Screening Nursery wheat lines to identify Genotyping by Sequencing (GBS) markers linked to Ltn and Pbc. Phenotyping for these traits was done in Ithaca, U.S.A. (Fall 2011); Njoro, Njoro (Summer and Fall 2012) and Wellington, India (Fall and Winter 2012).Using the Q + K matrix, 21 GBS markers were identified to be significantly associated with Ltn. While some of them were linked to loci where the durable leaf rust resistance genes *Lr34* (7DS), *Lr46* (1BL), *Lr67* (4DL), *Lr68* (7BL) were mapped, significant associations were also detected with loci previously known to interact with *Lr34* (2BL and 5B) and few other loci (3BS, 3D, 5A and 7BS). On the other hand, 15 GBS markers were significantly associated with Pbc. Besides the *Sr2* locus (3BS) which was highly consistent, a locus previously identified to be linked to stem rust resistance (2BL) and other loci (2DS, 4A, 6AS and 7DS) were identified. Thus, this study provides a better insight into the genetic control of Ltn and Pbc. Further efforts to fine map and characterize these loci might aid in determining the underlying mechanism of their association with durable resistance.

BIOGRAPHICAL SKETCH

Philomin Juliana was born on 23rd September, 1989 in Tuticorin, India as a miracle to her parents, Mr. Johnson and Mrs. Daisy Johnson after 8 years. Right from her childhood her educational career was greatly blessed by the Lord and she received the 'General Proficiency meritorious student award' throughout her schooling in Holy Cross Anglo Indian Higher Secondary School. Her great interest in plant sciences then persuaded her to pursue her Bachelor of Technology degree in Plant Biotechnology from Tamil Nadu Agricultural University, Coimbatore, India from 2007-2011. After her graduation, she was directed by the Lord to take up Plant Breeding and Genetics for her Masters at Cornell University, Ithaca. Here, she joined the reputed lab of Dr. Mark Earl Sorrells and focused on durable rust resistance in wheat. Her thesis research was carried out partly in the U.S. and partly in India. Besides, her Masters at Cornell, she also pursues her Master of Technology in Biotechnology Business Management from Tamil Nadu Agricultural University, Coimbatore. While a graduate student at Cornell, Philomin Juliana prepared this thesis as partial fulfillment of the Master of Science degree. It is expected that her degree will be conferred by the College of Agriculture and Life Sciences in May 2013.

Dedicated to my Lord and Savior Jesus Christ

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CHAPTER 1

INTRODUCTION

Wheat (*Triticum aestivum L*.) is one of the major cereal food crops in the world, credited for supplying 21% of the world's calories and 20% of the world's protein (Braun et al. 2010). The significance of wheat globally is evident from the fact that it is cultivated on an area of approximately 220 million hectares which is larger than any other commercial crop. Besides, it is also the second most important food crop with an estimated annual production of approximately 660 million tonnes (FAO Stat. 2012). But, despite this huge production, the continuous spur in population has led to the projection of a 60% increase in the demand for wheat in the developing world by 2050 (Rosegrant and Agcaoili 2010). To meet this demand, efforts should be focused on increasing its yield potential which is constantly threatened by several biotic and abiotic stresses. Among the biotic stresses, the major constraints are the rust diseases, namely the leaf rust, stem rust and stripe rust (Saari and Prescott 1985; Roelfs et al. 1992). These rusts cause huge economic losses and hence, breeding for rust resistance, with a view to increase the yield potential is imperative for sustainable wheat improvement.

The wheat rust pathogens are highly specialized obligate fungi having a complex life cycle (Knott 1989). They belong to the genus *Puccinia* which is of the family Puccinaceae, order Uredinales and class Basidiomycetes (Littlefield 1981). Among the rusts, the leaf rust or brown rust caused by *Puccinia triticina* Eriks. is the most common and the widely distributed rust (Chester 1946; Samborski 1985; Roelfs et al. 1992). The annual losses caused by the leaf rust worldwide are greater than all the other rusts. These losses can be very drastic upon an early onset of the disease before flowering (upto 50% loss), whereas an infection of 60–70% on the

flag leaf at spike emergence and soft dough stage might result in yield losses of 30% and 7% respectively (Huerta-Espino et al. 2011). The temperatures favorable for leaf rust infection lie between 10° and 30°C. The leaf blades are the primary sites of infection but the leaf sheath and the glumes are also affected in highly susceptible cultivars (Roelfs et al. 1992). *Puccinia triticina* depends on both a primary (telial/uredinial) host and an alternative (pycnial/aecial) host to complete its full life cycle thus making it heteroecious in nature. While wheat is the primary host of the fungus, several alternate hosts exist in different geographical locations. These include *Thalictrum speciosissimum*, *Isopyrum fumarioides*, *Clematis* spp. and *Anchusa* spp. (Jackson and Mains 1921; Chester 1946; Sibilia 1960; d'Oliveira and Samborski 1966). Besides, several diverse races of this fungus also said to exist, worldwide (Huerta-Espino 1992).

Another devastating disease of wheat, which is caused by the fungus *Puccinia graminis* (Pers. *f. sp. tritici* Eriks. and E. Henn) is the stem rust or the black rust (Roelfs 1985). This pathogen is very much feared as it has the potential to cause upto 100% losses in some cases (Singh et al. 2002). Stem rust infections require an optimum temperature of 30°C which is warmer compared to the other rusts (Roelfs 1992). Similar to the leaf rust fungus, *Puccinia graminis* is also heteroecious in nature with five distinct spore stages. While the fungus depends on its gramineous hosts to complete its asexual reproduction, the sexual cycle requires alternate hosts like *Berberis* and *Mahonia* (Roelfs 1985; Leonard and Szabo 2005). The mode of spread of the fungi is by airborne asexual spores called urediniospores (Christensen 1942). The typical symptoms of stem rust include elongated blister-like pustules (uredinia) which occur primarily on the leaf sheaths and also on the leaves, stem tissues, awns and glumes (Singh et al. 2008). This infection weakens the stem and makes it prone to lodging and yield loss (Roelfs et al. 1992). While stem rust occurs in warmer temperatures, another major disease that affects wheat

grown in the cool temperate regions is the stripe rust or yellow rust caused by the fungus, *Puccinia striiformis* West. (Zadoks 1961; Stubbs 1985). The average yield losses due to stripe rust range from 10 to 70% depending on various factors leading to the development of disease (Chen 2005). *P. striiformis* differs from the other rust fungi in that it has a hemiform life cycle consisting of only the uredinial and the telial stages (Stubbs 1985; Roelfs 1992).

Given that these rusts pose a serious threat to the wheat growing regions in the world, effective measures to control them have to be devised. Although, fungicides have been a potential panacea (von Meyer et al. 1970; Rowell 1972; Line and Rakotondradona 1980), the fact that they are expensive and not environmentally safe has led to the search for better control methods. While the eradication of alternate hosts like barberry turned out to be successful in some areas (Peterson et al. 2005), the deployment of cultivars with genetic resistance proved to be the best measure (Samborski 1985; Knott 1989; Singh and Rajaram 1992; Line et al. 1995). This genetic resistance was hypothesized to be of two distinct types namely vertical and horizontal (Vanderplank 1963). In a typical vertical resistance, the gene-for-gene interactions between the resistance genes of the host (R-genes) and the avirulence (Avr) genes of the pathogen form the basis of resistance (Flor 1956). As a result of this incompatible interaction, hypersensitive cell death response (HR) is elicited and defense related genes that stop the growth of the pathogen are induced. In a typical hypersensitive response (HR) chlorotic or necrotic flecks appear on the resistant plants (Bowles 1990) which result in the rapid death of the host cells around the infection region thus preventing further colonization of the pathogen (Robinson 1976). In wheat, several rust resistance genes have been catalogued to date which include 71 leaf rust resistance genes, 57 stem rust resistance genes and 53 stripe rust resistance genes (McIntosh et al. 1995, 2008, 2010, 2011; Singh et al. 2012; Krattinger et al. 2012; Xu et al. 2012). Most of

these genes are said to confer vertical or race specific resistance. But, the major problem with this type of resistance is that they are not long lasting (Bjarko and Line 1988; Kolmer 1992; McIntosh et al. 1995) and can be easily overcome by the evolution of new virulent races of the pathogen (Johnson 1981; Samborski 1985; Kolmer et al. 2003; McCallum et al. 2007). As a result, the host resistance sets into boom and bust cycles (Parlevliet 2002). One of the best examples of the emergence of virulent races is the stem rust pathogen race Ug99. This race not only carried virulence to the widely deployed gene *Sr31* but also to most of the resistance genes of wheat origin (Pretorius et al. 2000; Wanyera et al. 2006; Singh et al. 2008). More than 80% of the wheat varieties cultivated worldwide are susceptible to this race which was first identified in Uganda and later spread throughout East Africa, the Middle East and West Asia (Singh et al. 2008). As the further spread and establishment of Ug99 will have drastic consequences in the wheat production worldwide, durable genetic resistance against Ug99 is the need of the hour.

Durability is the ability of a widely-deployed resistance gene to provide an economic level of protection over an extended period of time according to Johnson (1984). The quantitative/partial/slow rusting resistance is the widely preferred mechanism to achieve durability. In this type of resistance, although the infection is not completely stopped, the spread of the disease is delayed (Parlevliet 1975; Singh et al. 2005). It is typically expressed in the adult plant stage (McIntosh et al. 1995) and is characterized by extended latent periods and production of fewer/smaller uredinia (Kolmer 1996). Among the catalogued rust resistance genes, the known race non- specific resistance genes are *Lr34*, *Lr46*, *Lr67*, *Lr68, Sr2* and *Yr36* (McIntosh et al. 2003). Among these, the gene *Lr34* has conferred durable resistance for several decades (Kolmer et al. 2008; Krattinger et al. 2009). It is the only durable rust resistance gene that has been cloned to date (Krattinger et al. 2009) and is a model for unraveling the molecular

biological basis of race-nonspecific resistance. The value of *Lr34* is also enhanced by the fact that it is present in more than 50% of wheat cultivars in the world and no increase in virulence to this gene has been observed so far (Krattinger et al. 2012). This locus also designated as the *Lr34/Yr18/Sr57/Pm38/Sb1/Bdv1* is one of the most valuable disease resistance regions in wheat breeding as it confers effective resistance against several biotrophic fungal pathogens that cause leaf rust, stripe rust, stem rust, powdery mildew, spot blotch and barley yellow dwarf virus (McIntosh 1992; Singh 1992b; Singh 1993; Joshi et al. 2004; Spielmeyer et al. 2005; Vanegas et al. 2008). But what's more interesting is the phenotype of leaf tip necrosis (LTN) which is exhibited by *Lr34* as well as the other durable leaf rust resistance genes - *Lr46*, *Lr67* and *Lr68*. (Singh 1992a, 1993; Rosewarne et al. 2006; Herrera Foessel et al. 2008, 2012). Drawing parallels with these genes is the adult-plant stem rust resistance gene *Sr2*. This gene which also confers effective durable resistance to multiple pathogens is associated with a black pigmentation called pseudo black chaff (PBC) which is suspected to be a result of its pleiotropism (Sheen et al. 1968; Hare and McIntosh 1979; Kota et al. 2006). Thus the intriguing association of these traits with the durable rust resistance genes draws our attention towards them.

The genetic factors controlling leaf tip necrosis and pseudo black chaff are complex and poorly understood. Although many Quantitative Trait Loci (QTL) have been identified for LTN (Messmer et al. 2000; Schnurbusch et al 2004b) and PBC (Bariana et al. 2001) in different bi-parental mapping populations, the number and effect of the QTL's associated with these traits showed great variations. This is attributed to the fact that bi-parental populations sample reduced genetic diversity and hence have poor resolution in detecting QTL. On the other hand, association mapping (AM), is an excellent alternative to traditional linkage mapping as it takes into account greater allelic diversity at a given locus and captures greater diversity using genetic

markers (Flint-Garcia et al. 2003). It has several advantages and is the choicest method for dissecting complex traits (Lander and Schork 1994; Risch and Merikangas 1996; Flint- Garcia et al. 2003). Although first applied in human genetics (Kerem et al. 1989) it has proved to be an useful approach in plants too (Remington et al. 2001; Jannink et al. 2001; Thornsberry et al. 2001; Breseghello and Sorrells 2006a). The first successful application of association mapping in wheat was by Breseghello and Sorrells (2006b) for kernel size and milling quality traits. This was followed by several other studies which successfully exploited its potential (Tommasini et al. 2007; Crossa et al. 2007; Peng et al. 2009; Yu et al. 2011).

The two approaches commonly used in association mapping are the candidate gene approach (Thornsberry et al. 2001) and the whole-genome scan approach (Rafalski 2002). Among these, the candidate gene approach is used for directly testing the effects of the genetic variants of a gene affecting a particular trait. But the limited existing knowledge about LTN and PBC and the genes underlying the QTL intervals identified in previous studies, made this approach not feasible in this study. However, the other approach also known as the genome-wide association study (GWAS) identifies genomic regions throughout the genome that are associated with the trait of interest. As the genetic loci conferring LTN and PBC are also known to be scattered across the genome, the GWAS approach was used to identify Genotyping by Sequencing (GBS) markers that are closely associated with these traits. The GBS technology which has been advocated as the genotyping platform of the future, provides an attractive option for association studies in many ways. Besides considerably increasing the efficiency of selection, the GBS strategy is very economical, aids in the de novo marker discovery and is a robust approach for complexity reduction in large genomes such as wheat (Elshire et al. 2011; Poland et al. 2012). Hence, they were used in association mapping the traits LTN and PBC in this study.

The results of this study would provide an excellent opportunity to gain insights into the poorly understood molecular mechanisms underlying durable disease resistance. Besides, it would also answer the question if it is possible for breeders to select for durable resistance without these undesirable traits.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Association of leaf tip necrosis to partial disease resistance in wheat

2.1.1. What is leaf tip necrosis?

Leaf tip necrosis (LTN) was first observed in association with the slow rust resistance gene, *Lr34* by Dyck (1991). Later, Singh (1992a) also observed LTN on the tips of the flag leaves of the *Lr34* lines that gradually extended along the edges of the leaf. This association was further confirmed in the studies of Messmer et al. (2000), Suenaga et al. (2003), Schnurbusch et al. (2004 a and b), Spielmeyer et al. (2005), Lillemo et al. (2008), Krattinger et al. (2009) and Risk et al. (2012). But, despite the fact that the *Lr34* mediated resistance was invariably associated with the expression of LTN, the mechanism behind the association of these two traits is still intriguing. One possible mechanism hypothesized by Singh and Huerta-Espino (1997) is that the toxic metabolites produced as a result of the resistance conferred by *Lr34* might induce LTN. Whereas, Messmer et al. (2000) hypothesized that LTN might alter the physiology of the flag leaf thus making it less appealing for the pathogen to grow and establish itself. But, it is unclear what senescence product(s)/toxic metabolite(s) result in LTN. However it was observed that apical leaf necrosis is a successful defense mechanism not only in wheat but in many other plants too, thus making it a universal trait of great importance. van den Berg et al. (2007) suggested that the principle underlying this defense mechanism is that the biotrophic pathogens are unable to grow on the necrotic leaf material (thus leading to reduced sporulation efficiency of the pathogen and subsequently reduced disease pressure). But, this mechanism is effective only when the

benefits of reduced disease pressure by early apical necrosis outweigh the cost associated with losing healthy leaf tissue by early necrosis (van den Berg et al. 2008).

The key difference between leaf tip necrosis and host cell necrosis (that results from typical major gene resistance) is that, while LTN occurs spontaneously in resistant *Lr34* lines even before pathogen challenge (Rubiales and Niks 1995; Hulbert et al. 2007), the hypersensitive necrosis results only after the production of haustorium. Navabi et al (2005) observed that wheat lines with LTN displayed an average of 30.5% and 20.8% less leaf and stripe rust severities respectively. In addition to its association with rust resistance, LTN was also associated with resistance to powdery mildew (Lillemo et al. 2008), moderate resistance to wheat spot blotch (Joshi et al. 2004) and tolerance to barley yellow dwarf virus (Singh 1993). This association of LTN in conferring partial resistance to several biotrophic pathogens makes it an interesting and valuable trait for breeders.

2.1.2. Genetic basis of leaf tip necrosis

Leaf tip necrosis was initially thought to be a monogenic trait. The gene causing LTN was first designated as '*Ltn*' by Singh (1992a) who suggested it to be the effect of close linkage or pleiotropism of the *Lr34* gene. But, later another LTN phenotype was observed by Rosewarne et al. (2006) as a pleiotropic effect of the partial rust resistance gene *Lr46*. Consequently, the *Ltn* gene associated with *Lr34* was redesignated as '*Ltn1*' and that associated with *Lr46* was designated as '*Ltn2*' (Rosewarne et al. 2006). This association was also recently confirmed in a study by Lillemo et al (2012). In addition to these two genes, LTN was also observed in lines carrying the partial resistance genes, *Lr67* (Dyck and Samborski 1979, Hiebert et al. 2010b) and *Lr68* (Herrera Fossel et al. 2012). Despite the fact that all these genes were associated with LTN,

the expression level varies for each gene. While, the *Lr67* lines display strong LTN comparable to that exhibited by the *Lr34* lines (Herrera Foessel et al. 2011), the lines carrying *Lr46* and *Lr68* show weaker necrosis (Rosewarne et al. 2006, Herrera Foessel et al. 2012). The involvement of genes other than *Lr34* in contributing to LTN was also indicated in the studies of Singh et al. (2007), Kolmer et al. (2008) and Lagudah et al. (2009) who observed that few lines that carried the susceptible and non LTN csLV34a allele also exhibited LTN. Although, there was a big question whether *Ltn* is closely linked to *Lr34* or if it is a pleiotropic effect of the gene, transgenic studies by Risk et al. (2012) have confirmed the pleiotropism of *Lr34* on LTN.

Besides single genes, several gene combinations are also reported to confer LTN. A study by Messmer et al. (2000) indicated the involvement of several loci for the expression of LTN which varied across different environments and genetic backgrounds. Barcellos et al. (2000) reported an exceptional case of LTN expression in the Brazilian cultivars, Toropi and IAC 13 where the necrosis was expressed on both rust-resistant and susceptible plants only in the F2 generation when two homozygous recessive genes occurred together. Given all the above discrepancies, defining the 'true expression' of LTN associated with the *Lr34*, *Lr46*, *Lr67* and *Lr68* genes vs the 'modified expression' due to the combinations/interaction of genes, genotype x environment (G x E) interactions and several other unknown factors will be challenging and interesting. Moreover, LTN is not a favored trait for breeders, due to the slight yield penalty associated with it in the *Lr34* lines (Drijepondt et al. 1990; Singh and Huerta-Espino 1997) and also it's reduced acceptance by the farmers (Messmer et al. 2000). Hence unraveling the genetic basis of LTN in order to manipulate its expression becomes imperative.

2.1.3. Nature and characterisation of the *Lr34*/*Ltn1* gene region

2.1.3.1. Origin and occurrence of the *Lr34*/*Ltn1* gene region in wheat germplasm

The *Lr34* gene was first described in a wheat line PI58548 by Dyck (1977). It is widely deployed in wheat breeding for almost a century and is present in a large number of wheat cultivars (Dyck and Samborski 1982; Shang et al. 1986; Singh 1993). Wheat cultivars with *Lr34* also occupy more than 26 million ha in developing countries (Marasas et al. 2003) and make significant contributions to yield savings during epidemics (Singh and Huerta Espino 1997). Several origins were suggested for *Lr34* including an Uruguay landrace named Americano 44D (Roelfs 1988), an Argentinian cultivar 38MA (Dyck 1991) and a Brazilian cultivar Frontana (Kolmer et al. 2008). Later the cultivars, Mentana and Ardito that were released by an Italian breeder, Nazareno Strampelli at the beginning of the 20th century (Lagudah et al. 2009) were suggested to be the origin. Finally with the cloning of the *Lr34* gene, Krattinger et al. (2009) observed that the three breeding lineages of *Lr34* had a common resistance haplotype and thus suggested a single origin of *Lr34*.

2.1.3.2. Nature of the resistance conferred by the *Lr34*/*Ltn1* region

The resistance conferred by *Lr34* is similar to that of other genes conferring slow rusting resistance in cereals (Wilcoxon 1981). This was proved by the characterisation of *Lr34* that led to the following observations (i) *Lr34* conditions prehaustorial resistance that is non-hypersensitive (Rubiales and Niks 1995) (ii) *Lr34* resistance leads to a reduction in the haustorium formation and pustule size (Dyck 1977; Singh and Huerta-Espino 2003) (iii) *Lr34* resistance causes high early abortion of the leaf rust germ tubes (Rubiales and Niks 1995; Kemp et al. 1999) (iv) small colony size (Rubiales and Niks 1995; Kloppers and Pretorius 1997)

(v) increase in the latent period (Rubiales and Niks 1995; Singh and Huerta-Espino 2003). Besides these, the expression of resistance at the adult plant stage (Dyck, 1987; Roelfs 1988) is another similarity between *Lr34* and the slow rusting genes. Although the seedling expression of *Lr34* is observed at low temperatures and low light intensity (Dyck and Samborski 1982), it is best expressed in the flag leaves of the adult plants during the critical grain-filling stage (Rubiales and Niks 1995; Risk et al. 2012). In the adult plant stage, the *Lr34* phenotype is typically exhibited by a rust pustule gradient on the flag leaf, which goes along with the presence of LTN (Singh 1992a; Kolmer 1996). While more and larger pustules are found at the leaf base, fewer and smaller pustules appear on the leaf tip which exhibits necrosis (Kolmer et al. 1992). As LTN is usually exhibited at the adult plant stage, there is a good possibility that other effective 'R' genes in the field might mask its expression (Kolmer et al. 2008).

Another feature of *Lr34* that is analogous to partial resistance is its ability to condition additive and enhanced resistance in combinations with other resistance genes (Dyck and Keber 1985; Drijepondt and Pretorius 1989; German and Kolmer 1992; Sawhney 1992). This nature of *Lr34* brought in the concept of the '*Lr34* complex' which is defined as the product of additive interaction involving *Lr34* and two to three additional slow rusting genes (Singh and Rajaram, 1992). While Dyck and Samborski (1982) reported the interaction of *LrT3* and *Lr*33 with *Lr34* to condition enhanced resistance, Roelfs (1988) suggested that gene combinations involving *Lr34* and other adult plant resistance genes like *Lr12* and *Lr13* might be the sources of the most durable resistance to leaf rust. The synergistic action of the *Lr34*res transgene with other leaf rust resistance genes was recently documented by Risk et al. (2012). The combination of *Lr34* with other slow rusting genes like *Lr46* and *Lr68* in the cultivar Parula made it near immune against the pathogens (Herrera-Foessel et al. 2012). Besides, interacting with leaf rust resistance loci,

Lr34 also enhances a wheat adult plant stem rust resistance QTL on chromosome arm 2BL (Kolmer et al. 2011) and interacts with loci contributing adult plant stem rust resistance to the Ug99-complex races (Vanegas et al. 2008; Yu et al. 2012). However, the effect of *Lr34* is quantitative and so combinations of *Lr34* with other rust resistance genes might be required to achieve adequate levels of resistance (Lillemo et al. 2012; Risk et al. 2012).

The gene *Lr34* is highly environment specific requiring optimum combinations of environmental factors for expression (Drijepondt et. al 1990). While, lower temperatures are conducive for the effective expression of *Lr34* resistance (Singh et al. 2007) higher temperatures lead to inadequate resistance (Drijepondt and Pretorius 1989; Rubiales and Niks 1995). Similarly, the expression of LTN also follows this pattern. The *Lr34*res transgenics that were exposed to a cold temperature treatment at the seedling stage exhibited a much stronger LTN compared to the plants that were not treated thus, suggesting the increase in LTN to be a consequence of elevated *Lr34*res levels at low temperatures (Risk et al. 2012). Besides temperature, the location also influences the expression of *Lr34* resistance. While, the *Lr34* resistance is detected by the production of fewer and smaller uredinia in the greenhouse (Drijepondt and Pretorius 1989), it expresses variable pustule size of a typical 'M' reaction in the field (Dyck and Samborski 1982, Dyck 1987).

Another interesting characteristic of the *Lr34* gene is its pleiotropism. *Lr34* is completely linked or pleiotropic to *Yr18* which is a gene for adult-plant resistance to stripe rust (McIntosh 1992; Singh 1992b, Imtiaz et al. 2004). Similar to the *Lr34* complex, the *Yr18* complex involves additive interactions between *Yr18* and few other slow rusting genes thus conferring a good level of adult plant resistance (Ma and Singh 1995; Singh and Huerta-Espino 1997). The *Lr34* locus also confers enhanced resistance to stem rust (Dyck and Samborski 1982; Dyck, 1987; Kolmer

1996). It is proposed that *Lr34* enhances stem rust resistance by its anti-suppressor effect which permits the expression of resistance genes that were previously inhibited (Kerber and Aung 1999; Vanegas et al. 2008). Another gene that is closely linked/pleiotropic to *Lr34* is the gene *Bdv1* for resistance to barley yellow dwarf virus (Singh 1993). Spielmeyer et al. (2005) also observed that *Lr34* resistance cosegregates with powdery mildew resistance and this gene has been named as *Pm38* (Lillemo et al. 2008). Finally, it was also found that the *Lr34* region exhibits resistance to spot blotch (Joshi et al. 2004) and the gene has been designated as *Sb1* (Krattinger et al. 2012). Thus the *Lr34/Ltn1* region is unique and a very valuable source of disease resistance in wheat breeding.

2.1.3.3. Mapping the *Lr34*/*Ltn1* region

Lr34 was mapped to the chromosome 7D by Dyck (1987). Initially, microsatellite markers like gwm295 and the gwm1220 were identified on chromosome 7DS (Suenaga et al. 2003; Schnurbusch et al. 2004a) but had poor diagnostic capability. Later, Schnurbusch et al. (2004b) developed two flanking markers Xsfr.BE493812 and Xsfr.BF473324 using rice BAC clones orthologous to the wheat *Lr34* region. This was followed by the identification of the wEST BE495774, within the *Lr34* QTL region by Rosewarne et al. (2006). The microsatellite marker, Xswm10 (Bossolini et al. 2006) and the Sequence Tagged Site (STS) marker, csLV34 (Lagudah et al. 2006) were more closely linked to the *Lr34* region and provided a much better diagnostic capacity across varied germplasms.The csLV34 marker had two allelic variants: a larger ‘a’ allele associated with the non-*Lr34* or the susceptible haplotype and a smaller ‘b’ allele associated with the *Lr34* or the resistant haplotype (Lagudah et al. 2006). Although widely diagnostic, this marker was not perfectly diagnostic as rare recombinants were occasionally

encountered while using this marker (Lagudah et al. 2006). While, Spielmeyer et al. (2008) positioned the microsatellite marker csLVMS1 about 0.13 cM from *Lr34*, Krattinger et al. (2008) fine mapped the region with markers csLVE17 and csLVA1/SWSNP3 thus positioning *Lr34* in a 0.15 cM interval containing six predicted genes. This was followed by the isolation, cloning and sequencing of the *Lr34* gene by Krattinger et al. (2009). *Lr34* spanned 11,805 base pairs (bp), had 24 exons and was also found to encode a 1401–amino acid protein. Lagudah et al. (2009) developed six gene specific markers (cssfr1–cssfr6) for *Lr34* based on sequence changes in the exon 11 and 12 of the *Lr34*-gene which corresponded to the presence or absence of the resistant and susceptible alleles. In addition to these sequence changes, the cultivar Jagger exhibited a single point mutation that resulted in a truncated protein (Lagudah et al. 2009). This point mutation causing susceptibility was later identified in the exon 22 by Cao et al. (2010) who developed markers to identify this mutation. Following this, Dakouri et al. (2010) developed several novel markers (cam1, cam2, cam8, cam11, cam16, cam23, ISBP1, caSNP4, caIND11, and caSNP12) in order to characterize the three ABC transporter polymorphisms that were previously identified. Among these, caIND11 proved to be the best diagnostic marker for the selection of *Lr34*.

2.1.3.4. Molecular basis of the mechanism of resistance conferred by the *Lr34*/*Ltn1* region

The broad spectrum of resistance conferred by *Lr34* was initially suspected to be Systemic Acquired Resistance (SAR). But this possibility was negated in a study by Hulbert et al. (2007), who reported that typical Pathogenesis Related (PR) proteins were not significantly up-regulated in the mock-inoculated resistant leaves. This was in contrast to SAR which is usually associated with the elevated expression of PR proteins prior to pathogen challenge (van

Loon et al. 2006). Although Induced Systemic Resistance (ISR) (Bostock 2005) could be another mechanism behind *Lr34* resistance, it was not the case, as Hulbert et al. (2007) found that gene expression patterns showed large changes even without the pathogen challenge, which is contrary to the ISR mechanism. Hulbert et al. (2007) also observed that several abiotic stress related transcripts were highly upregulated in the leaf tips than in the leaf bases which explains why the leaf tips are more resistant than the bases. Further studies by Bolton et al. (2008), on transcript profiling of the *Lr34*-mediated leaf rust resistance revealed that a high energetic demand accompanied by the transient recruitment of multiple metabolic pathways was involved in the resistance. Finally, it was concluded that a putative ABC transporter gene belonging to the pleiotropic drug resistance family was responsible for the resistance conferred by *Lr34* (Krattinger et al. 2009).

The pleiotropic drug resistance transporters usually contain two cytosolic nucleotide binding domains (NBDs) and two hydrophobic transmembrane domains (TMDs). Krattinger et al. (2011) predicted six membrane-spanning helices in each TMD whose function is to shuttle specific substrates across the biological membrane. Another ABC transporter, which belongs to the same family as *Lr34* is the PEN3/PDR8 in *Arabidopsis*. This transporter is also said to confer resistance towards non-host pathogens and has a proposed role of translocating toxic compounds affecting fungal growth derived from the glucosinolates into the apoplast. Hence, there is a possibility that the nature of resistance of *Lr34* might be similar to that of PEN3. Transgenic studies of Risk et al. (2012) have proved that this ABC transporter was alone sufficient for the broad spectrum disease resistance. Risk et al (2012) also observed that the putative pleiotropic drug resistance transporter was expressed at higher levels in the flag leaves of the adult plants that exhibited LTN whereas the expression was very low in the seedling stage. They also

suggested that the resistance conferred by the *Lr34*res transgene is not dependent on the induction of PR genes, but pathogen infection might upregulate these genes. On the other hand, Krattinger et al. (2009) observed the high expression of the barley cDNA HvS40 (that is known to be upregulated during leaf senescence) and the presence of non fluorescent chlorophyll catabolites (typically involved in leaf senescence) in the uninfected flag leaf tips of *Lr34* lines. This raises the possibility of the involvement of a senescence-like process in the resistance conferred by *Lr34*.

2.1.3.5. Allelic and functional variability of the *Lr34*/*Ltn1* region

Several alleles which differentiated the resistant and the susceptible lines were identified for the *Lr34* gene. Initially, three polymorphisms constituting two haplotypes for the resistant (*Lr34*res-D) and susceptible (*Lr34*sus-D) cultivars were reported by Krattinger et al (2009). These two haplotypes include (i) the deletion of a phenylalanine residue in *LR*34res-D due to the deletion of three base pairs TTC in the exon 11 of the resistant allele (ii) the conversion of tyrosine to a histidine due to a C/T SNP in exon 12. A third *Lr34* haplotype that involved a point mutation (a G/T SNP in exon 22) resulting in a premature stop codon was identified by Lagudah et al. (2009) in cultivar 'Jagger' which does not exhibit the *Lr34* resistance despite carrying the *Lr34*+ haplotype. Based on these, it is suggested that mutations resulted in the present day *Lr34*res-D allele from its ancient version, the *Lr34*sus-D allele. Besides the 'D' genome, two other *Lr34* homeologs on chromosomes 4A and 7A were identified by Krattinger et al. (2011) indicating gene colinearity. While the homeolog on chromosome 4A was expressed and putatively functional, the copy on 7A was disrupted by the insertion of repetitive elements. *Lr34* orthologs were absent in *Zea mays*, *Brachypodium distachyon* and *Hordeum vulgare*, but the

Oryza sativa and *Sorghum bicolor* had the ortholog with the susceptible allele. This led to the conclusion that the *Lr34*-haplotype found in the resistant wheat cultivars is very unique and might have evolved as a consequence of functional gene diversification in bread wheat (Krattinger et al. 2011).

2.1.4. Nature and characterisation of the *Lr46, Lr67* and *Lr68* regions expressing LTN

The other slow rusting genes involved in conferring LTN like *Lr46*, *Lr67* and *Lr68* share some similarities with the gene *Lr34* (Rosewarne et al. 2006; Hiebert et al. 2010b; Herrera Foessel et al. 2012). Considering the *Lr46* gene, its nature of expressing non–hypersensitive resistance in the adult plant stage, additive effects with other genes in combinations, high abortive penetration, increased latent period and small colony size makes it analogous to the *Lr34* gene (Martinez-Hernandez et al. 2001; Rosewarne et al. 2006). Besides, *Lr46* is also pleiotropic or closely linked to the stripe rust resistant gene *Yr29* (William et al. 2003; Rosewarne et al. 2006), the powdery mildew resistance gene *Pm39* (Lillemo et al. 2007) and resistance to spot blotch (Lilemo et al. 2012). Despite expressing a common resistance phenotype, preliminary molecular studies which analysed the *Lr46* region failed to detect the ABC transporter sequences that were associated with the *Lr34* gene (Lagudah 2011). Hence, it is suggested that the molecular basis of *Lr46* might be different from *Lr34*. Besides, the gene *Lr46* exhibited a strong additive interaction with the gene *Lr*P (later designated as *Lr68*) but showed very little evidence for interaction with the gene *Lr34* (Lagudah 2011). Mapping studies of the *Lr46* gene identified markers Xwmc44, Xwms259 and Xwms140 proximal to the gene on the chromosome 1BL (Martinez-Hernandez et al. 2001).

The gene *Lr67* also exhibits the same slow rusting mechanism as *Lr34* and it was initially suspected to be *Lr34* that has been translocated to a different chromosome (Dyck et al. 1994). But later it was mapped to the chromosome 4DL and designated as *Lr67* (Hiebert et al. 2010b). This gene displays various degrees of expression in the field in different backgrounds (Dyck and Samborski 1979; Herrera-Foessel et al. 2011). Besides conferring resistance to leaf rust, *Lr67* also had an effect on stripe rust (Dyck and Samborski 1979; Dyck et al. 1994; Herrera-Foessel et al. 2011) and enhances resistance to stem rust against the Ug99 race (Herrera-Foessel et al. 2011). This multi pathogen resistance led to the redesignation of the locus as *Lr67*/*Yr46/Sr55*. Molecular mapping of the *Lr67* gene led to the identification of five microsatellite markers on chromosome 4DL that were associated with it namely Xgwm165, Xgwm192, Xcfd71, Xbarc98 and Xcfd23 (Herrera-Foessel et al. 2011). Finally, the slow rusting gene *Lr68* also shares a common defense mechanism as the *Lr34* gene (Herrera-Foessel et al. 2012). The expression of *Lr68* is better at lower temperatures and it works additively in combination with the genes *Lr34* and *Lr46*. Molecular mapping studies of the *Lr68* gene identified markers gwm146, cs7BLN*LR*R and csGS on the chromosome 7BL (Herrera-Foessel et al. 2012).

2.2. Association of pseudo black chaff to durable rust resistance in wheat

2.2.1.What is pseudo black chaff?

Pseudo black chaff (PBC) is a pigmentation which occurs around the glumes and the internodes of the stem (Kuspira et al. 1958), usually associated with the durable stem rust resistance gene, *Sr2* (Hare and McIntosh 1979). While the original black chaff referred to the darkening of the glumes caused by the bacterium *Xanthomonas campestris pv. translucens*

(Bamberg 1936), PBC derives its name from the fact that its symptoms mimic the original one except that it is not caused by the bacterium. The only hypothesis for PBC expression in resistant plants is that, it might form physical or chemical barriers which subsequently delay the infection process (Kota et al. 2006). PBC usually has varying degrees of expression (Singh et al. 2011) and is expressed after anthesis. Although tolerable at low levels, higher expression of PBC might lead to shrunken kernels that reduce yield and makes it undesirable for the farmers (Sheen et al. 1968; Hare and McIntosh 1979). Breeders usually select for moderate levels of PBC while selecting for *Sr2* in order to prevent its over-expression (Brown 1997). Hence, understanding the molecular basis of PBC might enable breeders to manipulate its expression besides maintaining sufficient rust resistance (Kota et al. 2006).

2.2.2. Genetic basis of pseudo black chaff

Initially, PBC was considered to be a disease and an antagonistic relationship was suggested between stem rust resistance and susceptibility to black chaff (Waldron 1929; Sheen et al. 1968). An incomplete linkage between these two traits was suggested as exceptions to complete linkage occurred in some cases (Goulden and Neatby 1929; Pan 1940). Bhowal and Narkhede (1986) also suggested that PBC is incompletely dominant and its expression level can be modified by several genes. Although Mc-Fadden (1939) reported breaking the association, he later concluded that lines which had resistance to both these diseases were the effect of the epistatic effect of another gene over the gene causing PBC. Mishra et al. (2005) also suggested that resistance was not invariably associated with PBC. But, this was refuted by Kota et al. (2006) who were unable to separate these two traits by recombination in a high resolution

population. While some studies suggested a monogenic inheritance of this trait (Sheen et al. 1968; Bariana et al. 2001), digenic inheritance was also suggested (Kaur et al. 2009).

2.2.3. Nature and characterisation of the *Sr2*/*Pbc* rust resistance region

2.2.3.1. Origin and occurrence of *Sr2*/*Pbc* region in wheat germplasm

The gene *Sr2* is one of the most important disease resistance genes (McIntosh et al. 1995) as it has provided durable resistance against several races of the rust pathogens for many decades. This gene originated in the cultivar 'Hope' whose resistance was transferred from *Triticum turgidum* (cv Yaroslav) into hexaploid wheat by McFadden (1930). *Sr2* was detected in several CIMMYT cultivars (Rajaram et al. 1988, Yu et al. 2011) and in several Njoro varieties that exhibited adult plant resistance to Ug99 (Njau et al. 2009).

2.2.3.2. Nature of the resistance conferred by the *Sr2*/*Pbc* region

The resistance conferred by *Sr2* is of a non-hypersensitive type that is best expressed in the adult plant stage (Roelfs 1988). The resistance is expressed after anthesis and characterized by fewer/smaller uredia (Sunderwirth and Roelfs 1980). Although, *Sr2* alone provides inadequate resistance, combinations of *Sr2* with other unknown minor genes also known as the '*Sr2* complex' provide moderate to high levels of resistance (McIntosh 1988; Knott 1989; Bariana et al. 2007). Besides, PBC another phenotypic marker that is also closely associated with *Sr2* is seedling chlorosis. This is usually induced under high-temperatures (Brown 1997). Besides, *Sr2* is also tightly linked or pleiotropic with the leaf rust resistance gene *Lr27*, the stripe

rust resistance gene *Yr30* and a gene for powdery mildew resistance (Singh and McIntosh 1984; Singh et al. 2000; Singh et al. 2005; Mago et al. 2011a).

2.2.3.3. Molecular genetic characterisation of the *Sr2*/*Pbc* gene

The chromosome 3BS was associated with the *Sr2* gene and PBC (Kuspira et al. 1958; Sheen et al. 1968; Hare and McIntosh 1979). Microsatellite markers gwm533 and gwm389 that flanked the *Sr2* locus were identified by Spielmeyer et al. (2003). As Xgwm533, was found to be a compound microsatellite that displayed allelic homoplasy, it was converted to a Sequence-Tagged Microsatellite (STM) marker (Hayden et al. 2004). The *Sr2* interval was then narrowed down to 0.4 cM by Kota et al. (2006) who identified additional markers from the wheat EST-rice synteny. But, due to the poor diagnostic capacity of all these markers, McNeil et al. (2008) identified three other markers (3B042G11, 3B061C22 and 3B028F08) that were tightly linked to the *Sr2* gene and were mapped by analyzing the sequence of BAC clones within this region. This was followed by the identification of four DArT markers that were significantly associated with *Sr2* (Yu et al. 2010). More recently Mago et al. (2011a) developed several EST-derived markers and a Cleaved Amplified Polymorphic Sequence (CAPS) marker, cs*Sr2* (Mago et al. 2011b) associated with the presence or absence of the *Sr2* gene. The cs*Sr2* marker proved to be highly diagnostic for *Sr2*.

2.3. Association mapping

2.3.1. What is association mapping?

Association mapping also known as linkage disequilibrium (LD) mapping is a powerful tool for crop genetic improvement due to its potential to genetically dissect complex traits and

identify significant correlations between phenotypes and the underlying sequence variations. (Thornsberry et al. 2001; Flint-Garcia et al. 2003). The principle underlying association mapping is linkage disequilibrium (Weir 1996; Zondervan and Cardon 2004) which is discussed later. Association mapping provides an excellent alternative to traditional linkage mapping in many ways (Flint-Garcia et al. 2003). First, association mapping helps to overcome the problem of poor resolution, which is one of the major limitations of linkage mapping. The resolution of linkage mapping to detect a QTL is typically 10-20cM (Alpert and Tanksley 1996, Doerge 2002; Holland 2007) which might be inadequate to precisely identify several genes which lie within this vast interval. The poor resolution is mainly because this approach uses structured biparental populations, which have a limited number of recombination events (Flint-Garcia et al. 2003). Association mapping overcomes this impediment by dealing with unstructured populations with no well defined pedigrees in order to harness all the evolutionary recombination events that have occurred at the population level (Risch and Merikangas 1996; Jannink et al. 2001; Nordborg and Tavare 2002; Borevitz and Nordborg 2003). This provides a much finer resolution as only the polymorphisms which are tightly linked to the trait of interest will be significantly associated with it (Remington et al. 2001). Secondly, association mapping offers great advantage in terms of the time taken for a mapping study. While traditional linkage analysis might involve several years in developing a mapping population, association mapping avoids these generations of time-consuming crosses by using the existing diversity and ancestral meiotic events (Morrell et al. 2011). Compared to traditional biparental mapping, while only two alleles of a particular trait could be identified, association mapping also has an advantage of detecting several alleles (Yu and Buckler 2006). Besides these, association mapping also utilizes resources very efficiently by using available phenotypic and genotypic data at no

additional cost (Rafalski 2002). All these advantages make association mapping a very powerful technique beyond doubt.

2.3.2. Linkage disequilibrium

Linkage disequilibrium (LD) which forms the basis of association mapping studies is the non-random association of alleles at different loci within a population (Hedrick 1987). A large, randomly mated population where the loci segregate independently will be in linkage equilibrium in the absence of external forces like selection, mutation, migration etc. On the contrary, linkage disequilibrium occurs when an allele at a particular locus is observed along with a specific allele at another locus more frequently than what is expected if the alleles at the loci combined independently in the population (Falconer and Mackay 1996). Knowledge of the patterns of LD across the genome is indispensable as it enables us to understand the recombination biology of the species and facilitates the design of association studies (Pritchard and Przeworski 2001; Rafalski and Morgante 2004). The genome wide LD is also the major determinant of the number of markers and the mapping resolution of association studies. The decay of LD within a short distance around the candidate gene requires a large number of markers but provides a high mapping resolution. On the other hand, extension of LD over a long distance around the gene of interest will require a relatively smaller number of markers but has low mapping resolution (Weiss et al. 2002; Zhu et al. 2008). While, the regions of the genome that are highly divergent have a tendency to recombine less, it is vice versa in regions of high similarity thus maintaining the structure of a block haplotype (Rafalski and Morgante 2004).

2.3.3. Measures of Linkage disequilibrium

Several measures have been proposed for the estimation of LD. Among them, the most commonly used measure is the digenic 'D' (Lewontin 1964). This quantifies disequilibrium, as the difference between the observed frequency of co-occurrence of an allele of locus 'A' with an allele of another locus 'B' and the expected frequency of their co-occurrence under linkage equilibrium which is represented as $D = P_{AB} - P_A P_B$. LD is said to exist when D differs significantly from zero. The rate of LD decay also depends on the time and recombination (Falconer and Mackay 1996). This is given by the formula, $D_t = (1 - \theta)^t D_0$ where θ denotes the recombination fraction; D_0 and D_t denote the LD at generations 0 and t respectively. Another measure of LD is the standardized measure of D called D' (Lewontin 1964) which is given as,

$$D' = \begin{cases} \frac{D}{\min(p_1 q_2, p_2 q_1)} & D > 0 \\ \frac{D}{\min(p_1 q_1, p_2 q_2)} & D < 0 \end{cases}$$

Here, p_1, q_1, p_2 and q_2 are the allele frequencies at the two loci. When $|D'| = 1$, the two loci are said to be in complete LD and less than four of the possible haplotypes are observed. On the other hand, $|D'| < 1$ indicates the disruption of complete LD by recombination which results in the observation of all the four haplotypes.

Estimation of the r^2 is yet another measure of linkage disequilibrium. This r^2 is the square of the correlation coefficient between the two loci and it measures the proportion of the variance of a response variable that is explained by a predictor variable (Hill and Robertson 1968). The value of r is given by,

$$r = \frac{D}{(p_1 p_2 q_1 q_2)^{1/2}}$$

where 'D' is the disequilibrium and p_1, q_1, p_2 and q_2 are the allele frequencies at the two loci. The value of r^2 will be equal to one if only two haplotypes are present. Extending this measure, Breseghello and Sorrells (2006) made square root transformations of the r^2 estimates and used the 95th percentile of that distribution as a critical value to infer that beyond that value, LD was likely caused by genetic linkage. The final LD measure is the odds ratio (Devlin and Risch 1995). This ratio is given by the probability of an event happening divided by the probability of the event not happening. It ranges from 0 to 1 and is given by,

Odds ratio = $\frac{P_{11}P_{22}}{P_{12}P_{21}}$

where p_{11}, p_{22}, p_{12} and p_{21} are the allele frequencies at different loci.

2.3.4. Factors affecting linkage disequilibrium and association mapping

2.3.4.1. Population structure

Population structure is a major factor that influences association studies. The subgroups present in a population might have an unequal distribution of alleles which can result in spurious associations between a phenotype and markers unlinked to the causative variant (Knowler et al. 1988; Lander and Schork 1994; Ewens and Spielman 1995; Pritchard and Rosenberg 1999). This effect was well observed in a study by Knowler et al. (1988) where the frequency of alleles of the type 2 diabetes haplotype was higher in a population of a certain ancestry and led to spurious associations. This population structure also affects LD to a great extent. Reich and Goldstein (2001) suggested that LD in humans is highly dependent on the population as LD varies significantly among populations of different ancestry. To overcome this problem, Spielman et al. (1993) proposed the transmission/disequilibrium test (TDT). This test is based on the fact that

the probability of transmission of each parental allele to the offspring is 0.5. Deviation from this frequency indicates that the marker allele is in gametic phase disequilibrium and linked to the disease susceptibility allele (Jannink and Walsh 2002). This test which is mostly used in human genetics usually uses family trios comprising of two parents (one of them is a heterozygote) and an affected child. A variation of the TDT test known as the S-TDT (Spielman and Ewens 1998) considers only the genotypes from each discordant sib pair and could be used when the parental DNA is not available.

Two other methods that have been suggested to control population structure arising due to population stratification are Genomic Control (GC) and Structured Association (SA). A typical Genomic Control (GC) approach assumes that the effect of population structuring is equivalent on all the loci that are considered genome-wide (Devlin and Roeder 1999; Devlin et al. 2001; Bacanu et al. 2002; Devlin et al. 2004). In this approach, the influence of population structure on the association test statistic is estimated using a small random set of markers in order to adjust the significance of the association statistic (p value) for population structure. On the other hand, the Structured Association (SA) methodology (Pritchard et al. 2000), considers the marker loci which are unlinked to the candidate genes in the study to get inferences about their subpopulation membership. This approach has also been extended for quantitative trait association study (Q-model) which involves a two stage procedure. First, the probability of membership of the individuals in each subpopulation is estimated using a clustering program such as STRUCTURE (Pritchard et al. 2000b). This is followed by a test of association between the genotypes and the phenotypes and the estimated subpopulation membership is used as a co-variate (Thornsberry et al. 2001; Camus-Kulandaivelu et al. 2006). The sampled individuals are modeled such that they have inherited their genes from '*K'* unstructured subpopulations. In this

case, the null hypothesis of no association within subpopulations is tested against an alternate hypothesis which signifies association (Pritchard et al 2000b). Despite their efficacies in many studies both Genomic Control and Structured Association have their own limitations (Price et al. 2006). The uniform adjustment applied by Genomic Control might appear to be inadequate for markers that have an unusually strong differentiation across ancestral populations At the same time, it might be unnecessary for markers which do not have such a differentiation, thus leading to a loss in power. The problem with Structured Association (SA) is that the number of clusters to which the individuals are assigned is highly sensitive and not clearly defined. To address these limitations, Price et al. (2006) proposed the use of Principal Component Analysis (PCA) to correct for population structure and this has been validated in several studies (Evanno et al. 2005; Camus- Kulandaivelu et al. 2007). In the PCA analysis, the variation observed across all markers is summarized into a smaller number of underlying component variables which indicate the degree of membership of each individual in underlying populations.

2.3.4.2.Evolutionary and population genetic forces

Several other factors like mutation, recombination, selection, mating pattern, random drift and admixture can result in LD. Among these, mutation is a major factor that affects LD. A new mutation that occurs on a chromosome carries a particular allele at a locus nearby, thus creating LD (Flint-Garcia et al. 2003). Recombination is another major factor as it breaks down LD in the chromosomes. While recombination will be high in gene dense regions causing high LD, it is vice versa in gene poor regions (Rafalski and Morgante 2004). Selection at a particular locus, both natural and artificial will increase LD in the surrounding region. This will reduce diversity in that region and lead to significant allelic associations over large distances (Peterson et al.

1995; Rafalski and Morgante 2004). There are two ways by which selection influences LD. The first is the hitchhiking effect in which an entire haplotype which flanks the interested region is either fixed or swept to high frequency in the population (Parsch et al. 2001; Wang et al. 2002). Alternatively, selection against deleterious variants sweeps them away from the population and this can also inflate LD. Secondly, epistasis resulting from the selection for combinations of alleles that are present at different loci on the same or even different chromosomes also affects LD. The next factor that influences LD is the mating pattern. Usually, the breakdown of LD in out crossing species is higher than in selfing species due to the greater chance for recombination (Nordborg 2000; Flint-Garcia et al. 2003; Rafalski and Morgante 2004). Wheat being a self pollinated crop which undergoes inbreeding has a high level of LD (Zhang et al. 2010). Another factor that influences LD is admixture which can result in LD even between unlinked sites (Pritchard and Rosenberg 1999). This admixture arises from factors like adaptation or domestication (Wright and Gaut 2005). The union of previously separate populations into a single panmictic one results in an admixed population, which is a case of a structured population (Jannink and Walsh 2002; Flint-Garcia et al. 2003). In recently admixed populations the LD can extend over several centiMorgans (Wilson and Goldstein 2000). LD is also influenced by the reduction in population size known as the bottleneck effect. Populations that have recently experienced a bottleneck accompanied by extreme genetic drift can create LD (Dunning et al. 2000), as only few allelic combinations are passed on to future generations (Flint-Garcia et al. 2003). Similarly, populations that expand from a small number of founders generate LD as the haplotypes of the founders will occur more frequently than what is expected (Jannink and Walsh 2002).

2.3.5. Association Mapping Populations

The success of an association study is mainly determined by the choice of the germplasm (Flint-Garcia et al. 2003; Breseghello and Sorrells 2006a; Yu and Buckler 2006). The germplasm that is suited for association mapping might include the following: (i) exotic accessions from germplasm bank collections which are expected to have high allelic diversity, low LD and subtle population structure (Maccaferri et al. 2005, Breseghello and Sorrells 2006a) (ii) natural or wild populations (Nordborg et al. 2002; Morrell et al. 2005) (iii) intermated populations where the initial high LD is broken down in several cycles of recombination (Breseghello and Sorrells 2006a) (iv) a regional gene pool (Somers et al. 2007) and (v) elite lines which are expected to have a high LD and population structure (Breseghello and Sorrells 2006a).

2.3.6. Association mapping approaches

There are two major approaches on which association mapping is based on - the candidate gene approach and the genome wide association studies (GWAS). In the candidate-gene approach prior information about the trait of interest from biochemical, genetic or physiology studies is utilized to dissect it (Risch and Merikangas 1996). The first candidate gene based association study involved the analysis of flowering time and the *dwarf8* (*d8*) gene in maize (Thornsberry et al. 2001). The candidate gene approach has an advantage of reducing the amount of genotyping required besides reducing the issues related to multiple testing while using many markers (Flint-Garcia et al. 2003). However, this approach proved to be inadequate as it requires extensive prior knowledge of the trait and some presumption while choosing the candidate genes and deciding the number of markers to be used. This major limitation was also

clearly seen in human genetics where even confirmed disease genes were not detected using this approach (Altshuler et al. 2008). On the other hand, Genome Wide Association Studies (GWAS) exploits whole genome genetic variation in order to find significant associations of the phenotypes with the causative genotypes (Risch and Merikangas 1996). This strategy revolves on the fact that utilization of sufficient markers across the genome will probably result in at least one of them being in LD with the functional alleles. This approach has enormously revolutionized the genetic mapping in humans (Altshuler et al. 2008; Donnelly 2008) and is used considerably in plants too (Nordborg and Weigel 2008). Hence, this approach was used in this study to map the traits LTN and PBC.

CHAPTER 3

MATERIALS AND METHODS

3.1.Plant materials

The germplasm used for association mapping in this study comprises of CIMMYT's Stem Rust Resistance Screening Nursery (SRRSN) lines. The presence of *Lr34*/*Ltn1* and *Sr2/Pbc* in CIMMYT's germplasm is well documented (Dyck 1991, Singh 1992a, Spielmeyer et al. 2003, Singh et al. 2005, Kota et al. 2006), thus making it an ideal for mapping LTN and PBC. The association panel used in this study comprises of three different populations evaluated across three locations.

i) Five hundred and four wheat lines were evaluated for LTN at Cornell's greenhouses in Ithaca during the fall of 2011 (PBC could not be scored under greenhouse conditions as its expression is highly influenced by light). This population was previously developed by crossing 14 parental lines from CIMMYT's 2^{nd} and 5^{th} SRRSN in two rounds of crosses. The first round involved the partial diallel crossing scheme (to produce a set of F1s) followed by the second round, where the crosses were made in such a way that each F1 participated in at least 6 crosses with another F1 that did not share a common parent.

ii) The progenies of the above 504 wheat lines were evaluated for both LTN and PBC at the Njoro Agricultural Research Station, Njoro, Njoro during the main and off seasons, 2012.

iii) Two hundred elite wheat lines from CIMMYT's 2^{nd}, 5^{th} and 6^{th} SRRSN were evaluated for LTN and PBC at the Wheat Research Station, Wellington, India during the summer of 2012.

3.2. Phenotyping

3.2.1. Phenotyping for leaf tip necrosis

Phenotyping for LTN is complicated as it is a quantitative trait, whose expression is highly influenced by Genotype x Environment interactions (Messmer et al. 2000; Schnurbusch et al. 2004b). This requirement of a combination of optimal moisture and cool night temperatures the consistent expression of LTN was also indicated by Wamishe and Milus (2004). Hence, two different methods were initially adopted to phenotype LTN at Cornell's greenhouses to optimize the best method to score this trait. The first method involved measuring the leaf length and the extent of LTN from the leaf tip upto a few centimeters along the edges (Figure 1a). The second method involved image analysis of the leaves using the 'R' statistical package (http://www.r-project.org/). The flag leaves were harvested from the plants at the anthesis stage (Singh 1992a) and photographed. A binary image was obtained from the color image of the necrotic leaf through an operation that selects a subset of the image pixels (yellow in the case of the necrotic region) as foreground pixels and considers the rest of the green leaf region as background pixels (Figure 1b). The necrotic area of the leaf was obtained as a ratio between these two pixel areas. The optimized method was used to phenotype the lines at Njoro, Kenya (main and off seasons, 2012) and Wellington, India (summer, 2012). Out of the total 504 lines that were evaluated in Njoro, only 393 lines could be scored during the main season due to high incidence of stripe rust.

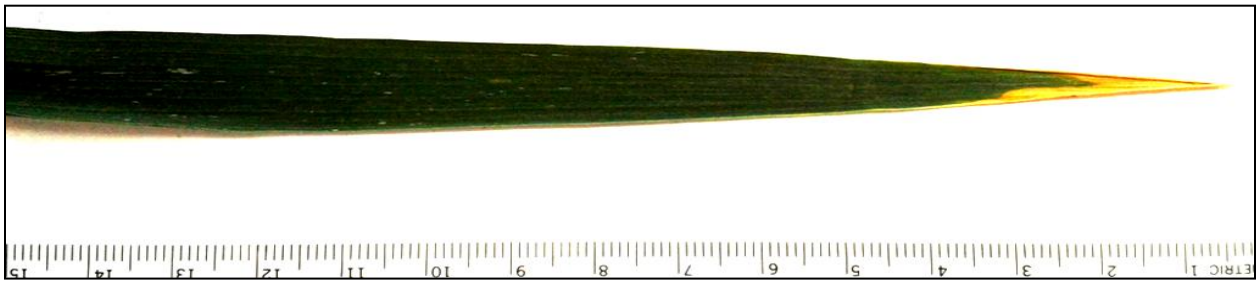


Figure 1a: Phenotyping LTN by quantitative measurement

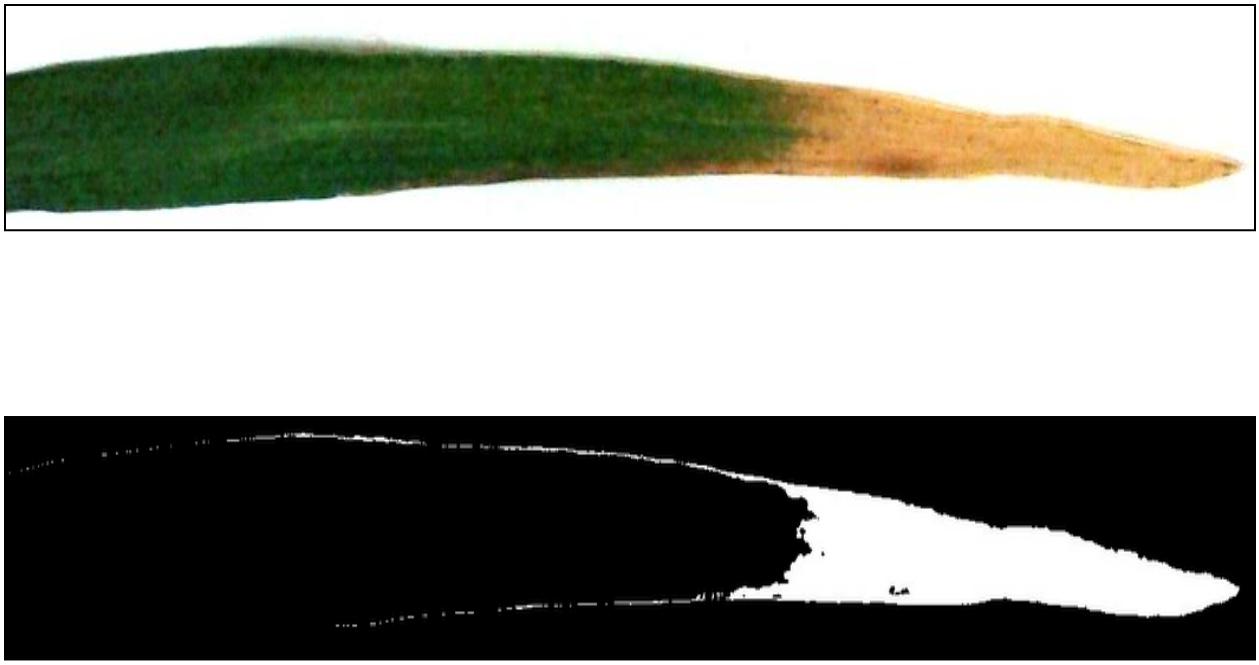


Figure 1b: Phenotyping LTN by image analysis using 'R' statistical package

3.2.2. Phenotyping for pseudo black chaff

Phenotyping for PBC is also difficult as it is known to be induced by certain environmental factors like light and moisture (Broadfoot and Robertson 1933; Hagborg 1936). Besides, its expression is also complicated by the recessive nature of *Sr2* which is the major gene associated with it (McIntosh et al. 1995). PBC was scored at anthesis based on the presence of the black pigmentation around the stem internodes and glumes (Kota et al. 2006; Kaur et al. 2009) on a 0-3 scale where 0= no pigmentation; 1= slight pigmentation; 2= medium pigmentation, 3 = high pigmentation (Figure 3).

Figure 2: Phenotyping for PBC on the glumes and internodes

3.3. Statistical analysis of phenotyping data

The statistical package JMP 10 (SAS Institute, http://www.jmp.com) was used for all the statistical analysis. The distribution of the phenotypic data for LTN and PBC was analyzed. In cases where the distribution was skewed, trait transformations were performed on the data using the boxcox function in 'R' with a lambda value of 0, equivalent to the log transformation. These transformed values were then used to calculate Best Linear Unbiased Predictors (BLUP's) using a random effects model with blocks and families as random effects. The 'r' value or the Pearson's correlation coefficient was also calculated between the different methods of measuring LTN and for the comparison of phenotyping data in different locations/seasons. An Analysis Of Variance (ANOVA) was run using the Fit model option with the families as fixed effects and a probability level of 0.001 was used to declare significance.

3.4. Genotyping

The number of markers required is a key issue that needs to be addressed while designing association mapping studies (Yu et al. 2009). Genotyping an adequate number of markers across the genome is essential such that at least some of them will likely be in LD with the causative alleles (Zhu et al. 2008; Myles et al. 2009). While the effect of LD on deciding the number of markers required is discussed in the previous chapter, dense genome-wide marker coverage usually improves the resolution of mapping considerably (Morrell et al. 2011). Besides coverage, markers are also used to estimate the relationship among individuals based on population structure (Pritchard and Rosenberg 1999, Pritchard 2001) and kinship (Yu et al. 2005). Compared to pedigree based methods markers provide a better quantification of the genetic

variance and covariance underlying the phenotypic variation. This was also shown to be the case using computer simulations under various scenarios (Yu et al. 2009). In addition to the number of markers, the choice of a suitable marker is also important in association studies. While, Single Nucleotide Polymorphisms (SNP's) are widely used in association studies (Rafalski 2002), the time period for identifying polymorphisms and the need for high-throughput methods for their detection is a matter of concern. To circumvent these limitations, Next-Generation Sequencing (NGS) technologies have been recently used in GWAS (Metzker 2010) and they act as a powerful tool for detecting polymorphisms in a short time period. One of the marker systems that exploits the potential of NGS technologies in identifying SNP's is the Genotyping By Sequencing (GBS) approach which provides an attractive option for association mapping studies due to its simplicity, robustness, reproducibility, complexity reduction in large complex genomes, low time and cost per sample (Elshire et al. 2011; Poland et al. 2012).

The GBS strategy involves creating a reduced representation of the genome followed by ligation of adapters, PCR amplification of the pooled library and multiplex sequencing. Although several target enrichment methods are available for complexity reduction, using restriction enzymes has been demonstrated to be superior in terms of its quickness, high specificity and ability to target low-copy genomic regions that cannot be accessed by the sequence capture approaches (Elshire et al. 2011). The original GBS approach developed by Elshire et al. (2011) for maize and barley used a single restriction enzyme (*ApeKI*) and two double stranded adapters namely barcode and common adapters. Whereas, Poland et al. (2012) extended this approach to barley and wheat using a two-enzyme system comprising a rare cutter (*PstI*) and a frequent cutter (*MspI*) along with Y-adapters to generate uniform libraries. This approach proved the robustness of GBS for species with large, complex and polyploid genomes without a reference sequence.

The schematic protocol adopted by the GBS approach is outlined below. The first step involves complexity reduction with restriction enzyme(s) followed by ligation with the adapters and the addition of appropriate primers which contain complementary sequences for amplification. PCR is then performed and the amplified sample pools constitute a sequencing library. These libraries are checked to make sure that they have minimum adapter dimers and single end sequencing of the 86bp reads is done on the Illumina platform (Elshire et al. 2011). The raw sequence data obtained from sequencing is then filtered based on three different criteria (i) the reads that exactly matched the barcodes and the restriction enzyme cut site (ii) non adapter – adapter dimers (iii) absence of 'N's in the first 72 bases. The barcode is then trimmed from the reads that fulfilled the above criteria and 64 base sequence tags are generated. These tags are further filtered based on their Q-score and their occurrence thus generating a reference set of tags. These tags are then tested for their presence/absence in a population according to their segregation. A 'success' is recorded to indicate the co-occurrence of the tag in an individual which carried the SNP allele from its expected parent and a 'failure' is recorded otherwise (Elshire et al. 2011). A single, biallelic GBS marker is then obtained by merging the pairs of tags that meet two criteria

(i) tags that perfectly aligned to the same unique position and strand in the reference genome

(ii) tags that co-segregated with the physically closest SNP. These biallelic GBS markers are then incorporated into a framework map and ordered based on their positions in the reference genome. In the case of heterozygous SNP calls, presumed to be due to sequencing errors, the call is considered as missing data. Using the GBS approach, Poland et al. (2012) identified 20,000 SNP's in wheat and assigned them to their map locations based on the OWB and SynOpDH reference genetic maps besides positioning them in recombination bins.

Considering the complex nature of LTN and PBC, GBS markers were preferred in this study as they might provide a better understanding of the genetic architecture of these traits. The different populations used in this study comprising of two different panels of 504 and 200 wheat lines were previously genotyped using the GBS approach. From the original GBS data set only 3211 markers that had definite map positions were retained. These markers were further filtered such that, those with minor allele frequencies (MAFs) less than 5% and a missing allele frequency of greater than 20% were removed. Finally, 684 polymorphic markers (for the 504 lines evaluated at Ithaca and Njoro) and 1364 polymorphic markers (for the 200 CIMMYT lines at Wellington) were obtained.

3.5. Genotype imputation

One of the serious problems in sequencing approaches is the missing data. Despite the removal of GBS markers that had a missing allele frequency of greater than 20%, there is still some missing data that might reduce the power of the association study. This problem was dealt with genotype imputation methods that have now become an essential tool in Genome Wide Association Scans (Servin and Stephens 2007; Ellinghaus et al. 2009). In fact, imputation has become the cornerstone of modern association studies due to its potential of estimating unobserved genotypes with high accuracy besides enhancing the power of association studies by increasing the chances of finding true associations sites (Marchini et al. 2007; Servin and Stephens 2007; Guan and Stephens 2008; Howie at al. 2009; Li et al. 2009 and 2010). Genotype imputation can be defined as the process of using statistical tools to predict genotypes that are not directly assayed in a sample of individuals (Browning 2008; Li et al. 2009; Marchini and

Howie 2010). The basic principle underlying genotype imputation is that even in the samples of unrelated individuals, the haplotypes of the individuals will be related to each other atleast over short stretches of sequence by being identical by descent (IBD) (Li et al. 2009). The goal of imputation methods is to identify sharing between the underlying haplotypes of the sparsely typed study individuals and the haplotypes of the densely typed genotypes in the reference panel and then impute the missing alleles in study individuals using this sharing (Marchini et al. 2010; Howie et al. 2009).

Several tools are available for performing genotype imputation. While tools like PLINK, TUNA, WHAP and BEAGLE take into account only the genotypes for a small number of nearby markers, tools like IMPUTE, MACH and fast PHASE/BIMBAM consider all the observed genotypes when imputing each missing genotype (Li et al. 2009). Among these, the Markov Chain Haplotyping software (MACH, http://www.sph.umich.edu/csg/abecasis/MACH/) was used for imputation analysis in this study due to its efficiency, robustness and accuracy (Willer et al. 2008; Nothnagel et al. 2009; Pei et al. 2010; Marchini et al. 2010). MACH initially generates a random pair of haplotypes that is compatible with the observed genotypes for each sampled individual and then subjects them to a series of iterations. MACH then uses a Hidden Markov Model (HMM) that generates a new pair of haplotypes in each of these iterations. This haplotype pair is described as an imperfect mosaic of the other haplotypes. After several iterations, the haplotypes sampled in each round are merged to construct a consensus haplotype (Li et al. 2009). When a reference panel of haplotypes is not available, the phase of each individual's genotype data is updated conditional on the current haplotype estimates of all the other samples. On the other hand, when a reference panel of haplotypes is available, it is added to the set of estimated haplotypes.

MACH requires a set of observed genotypes for each study individual in Merlin format with data and pedigree files as input (http://www.sph.umich.edu/csg/abecasis/MACH/tour/). The parameter --rounds specifies the number of iterations of the Markov sampler that should be run and the parameter --states specifies the number of haplotypes that should be considered when updating each individual. In the case of our data set which has only less than 20% missing data, the value of --rounds was set to 100 and the value of --states was set to 200. As the missing data was not distributed evenly among the individuals, the --weighted parameter which favors the use of individuals with more genotype data as templates for haplotyping other individuals was used. The --prefix option was used to specify the file names where the model parameters are stored.

3.6. Tag SNPs selection

The selection of tag SNP's is an essential step in association studies as using such SNPs can drastically increase the power besides reducing the effort and genotyping costs (Gabriel et al. 2002; Thompson et al. 2003; Hu et al. 2004; Liu et al. 2005; Nothnagel et al. 2007; Davidovich et al. 2009). SNP's usually lie in haplotype blocks which are regions with consistent pairwise linkage disequilibrium and no recombination for all the pairs of markers in the region (Gabriel et al. 2002). In this case, the information from some SNPs within each haplotype block may be redundant and using one SNP within the block might be enough to provide information about the presence of other alleles at different loci (Johnson et al. 2001). This process of selecting a smaller number of SNPs or a subset that best explains the haplotype diversity existing within a block is known as 'haplotype tagging' SNPs or htSNPs (Johnson et al. 2001; Daly et al. 2001; Gabriel et al. 2002; Nothnagel et al. 2004). Several methods have been proposed to tag

SNP's based on haplotype blocks (Zhang et al. 2002; Meng et al. 2003). But this approach has been criticized because population history and marker choice can influence block patterns resulting in erroneous findings (Ke et al. 2004; Sun et al. 2004; Nothnagel et al. 2005). Hence, Carlson et al. (2004) suggested a block free method using the coefficient of determination r^2 between the markers based on a minimum cut-off value (typically 0.8) between the tag SNPs and the uncollected ones. de Bakker et al. (2005) also suggested the incorporation of pairwise LD between multiple markers into the analysis to improve the genetic coverage and statistical power. Given the advantages of this approach, the tool Tagger (http://www.broadinstitute.org/mpg/tagger/) which combines the simplicity of pairwise tagging methods along with the efficiency benefits of multimarker haplotype approaches was used to select the tags in this study. Tagger picks tags in two different ways (i) it captures the alleles of interest by single-marker tests at the given r^2 using a greedy pairwise tagging (Carlson et al. 2004) (ii) it does an aggressive search to replace each tag with a specific multimarker predictor based on the remaining tags so as to improve efficiency. The acceptance of this predictor is based on its ability to capture the alleles originally captured by that discarded tag at the given r^2, else that tag would be considered essential (de Bakker et al. 2005). The genotype data in the pedigree linkage format was given as input to Tagger from which linkage disequilibrium patterns were calculated. An r^2 threshold of 0.8 along with the aggressive algorithm of Tagger (which uses both multimarker and pairwise LD) was used to pick the tag SNPs. After excluding the SNPs which had a high correlation coefficient between 0.8 and 1 (indicating high LD), Tagger produced an output set of 674 tag SNPs (for the 504 lines evaluated at Ithaca and Njoro) and 815 tag SNPs (for the 200 CIMMYT lines at Wellington).

3.7. Association analysis for LTN and PBC using Generalized and Mixed Linear Models

Although a variety of software packages are available for Association mapping, TASSEL (Trait Analysis by aSSociation Evolution and Linkage) has been the most commonly used program (Bradbury et al. 2007). TASSEL 4 was used in this study for Linkage disequilibrium analysis, principal component analysis, kinship analysis and also for association mapping. Initially, the linkage disequilibrium (LD) was calculated and then visualized by making a scatter plot of the allele frequency correlations (r^2) between the GBS markers across the chromosomes against the genetic distance (cM) using the statistical package 'R'. Prior to performing the association analysis, the estimation of population structure which is an essential component in the models is imperative. The necessity to consider population structure to reduce spurious associations is a*Lr*eady discussed in the previous chapter. As PCA was considered to be a fast and effective way compared to other methods (Patterson et al. 2006; Zhao et al. 2007), it was used to characterize population structure in the different germplasms represented in this study (2^{nd}, 5^{th} and 6^{th} SRRSN). The default correlation matrix in TASSEL was used to estimate PCA and the covariates were used as the 'Q' matrix for association analysis. The principal components were also plotted in a graph to determine the population membership of each individual. The subpopulations identified in PCA analysis were also confirmed using the software package STRUCTURE 2.1 (http://pritch.bsd.uchicago.edu/structure.html) (Pritchard et al. 2000b). Eight independent STRUCTURE runs were conducted, based on an admixture model and correlated allele frequency. The length of burn-in periods and the number of interactions was set at 10,000.

Both the generalized and the mixed linear models were employed for association mapping in this study. While the Generalized Linear Model (GLM) (McCullagh and Nelder 1989) accounts for only the population structure, the unified mixed model (Yu et al. 2006) considers both the population structure and familial relatedness. First, the GLM, which uses the marker allele frequencies and the population structure (Q matrix) as explanatory variables, was run in TASSEL 4 in order to calculate associations between the GBS markers and the traits LTN and PBC. But, as our population comprises of several families derived from common parents, considering the familial relatedness is also very crucial. Hence, the potential of the unified mixed model which uses both the population structure (Q) and the kinship matrix (K) to significantly decrease the false associations and improve statistical power (Yu et al. 2006; Malosetti et al. 2007; Zhao et al. 2007; Kang et al. 2008) was exploited in this study. The mixed linear model acts as a powerful complement to GLM in association studies as it incorporates both the fixed effects (genetic marker effects and population structure) and the random effects (additive genetic effects from multiple background QTL for the lines). The use of the traditional pedigree-based coancestry matrix for accounting for familial relatedness in mixed models (Henderson 1984), has been successfully replaced by the use of marker based kinship estimates (Eding 2001). Although kinship can be calculated from markers by using software packages like SPAGedi (Hardy and Vekemans 2002), TASSEL was preferred in this study. TASSEL's Kinship function generates a distance matrix where each element d_{ij} of the distance matrix represents the proportion of the SNPs differing between the taxons i and j. A similarity matrix was then obtained from this distance matrix by scaling the values between 0 and 2 and used in the mixed model. Association analysis was then conducted using the Mixed Linear Model (MLM) with the default optimum level of compression to identify GBS markers associated with the traits LTN and PBC. The

method P3D (Zhang et al. 2010) that initially optimizes the variance components and then tests the markers using these estimates was chosen before running MLM. To quantify evidence for the existence of a genetic effect of a marker at a particular locus, an alpha level of 0.001 was used to declare significance.

The interpretation of results in association studies that include multiple statistical tests is an issue of concern. This is because in multiple testing which is carried out at the same significance level, there is a greater probability of obtaining at least one significant result than that significance level (Zaykin et al. 2002). This leads to an increased probability of rejecting the null hypothesis which might lead to spurious results. Hence, the obtained P-values have to be corrected to control the type I error (false positive) rate. One of the most common multiple testing adjustments is the Bonferroni correction (Bonferroni 1935; Holm 1979). It works by making the alpha level more stringent (the alpha value for each comparison is taken as 'α/n' where n is the number of comparisons and 'α' is the value of alpha for the entire set of comparisons) such that it will create fewer errors. This correction strongly controls the family-wise error rate (FWER), which is the probability of rejecting one or more true null hypotheses in a series of tests. This approach can also be used to control the error rate across the genome also known as the Genome Wide Error Rate (GWER, Lander and Kruglyak 1995). But the Bonferroni correction is very conservative and leads to power loss for detection if the polymorphisms are in linkage disequilibrium and or the traits are correlated with one another. An alternative to the FWER corrections is the false discovery rate (FDR, Benjamini and Hochberg 1995, 2000; Benjamini and Yekutieli 2001). The expected proportion of false discoveries among the rejected null hypotheses is known as the false discovery rate. If V denotes the number of mistakenly rejected null hypotheses and R the number of rejected null hypotheses, then the FDR can be

represented as, FDR = $E(\frac{V}{R}|R>0)$ Pr (R>0). The step up procedure of Benjamini and Hochberg (1995) controls the FDR at a level 'q' when $m_o/m < q$ (where 'm' is the total number of null hypotheses tested and 'm_o' is the number of hypotheses that are true). As this approach is effective in controlling false positives, it was used in this study. Initially, the p-values resulting from the 'm' tests were ordered such that P (1) < P(2) < < P (m). Then the condition k = max {i: $p_{(i)} \leq \frac{i}{m}q$} was defined and all H(i) where i = 1, 2, ..., k. were rejected based on the cutoff value 'k' for all the analysis. Only, the markers significant after the FDR adjustment were considered to be the truly significant markers and their map positions were located on the chromosomes.

CHAPTER 4

RESULTS

Genotyping data for association analysis

3.1. Marker statistics

3.1.1. Marker statistics for the lines evaluated at Ithaca /Njoro

Six hundred and eighty four GBS markers were used to map LTN and PBC in the lines evaluated at Ithaca and Njoro. The majority of markers were distributed across the wheat A and B genomes (40 and 55%, respectively) while the D genome had the fewest (5%). The distribution of markers was very uneven. The chromosome 2B had the highest marker coverage followed by chromosomes 5B and 7B. Chromosomes 3D, 4D and 7D had the least number of markers covering them (Figure 3).

3.1.2. Marker statistics for the Wellington population

Eight hundred and fifteen GBS markers were used to map LTN and PBC in the population evaluated at Wellington. The marker distribution followed the same trend as mentioned above, with 43%, 50% and 7% markers covering the A, B and D genomes respectively. While, the highest number of markers was distributed on chromosome 2B followed by chromosomes 7A, 3B and 7B and the least covered chromosomes 4D and 5D (Figure 4).

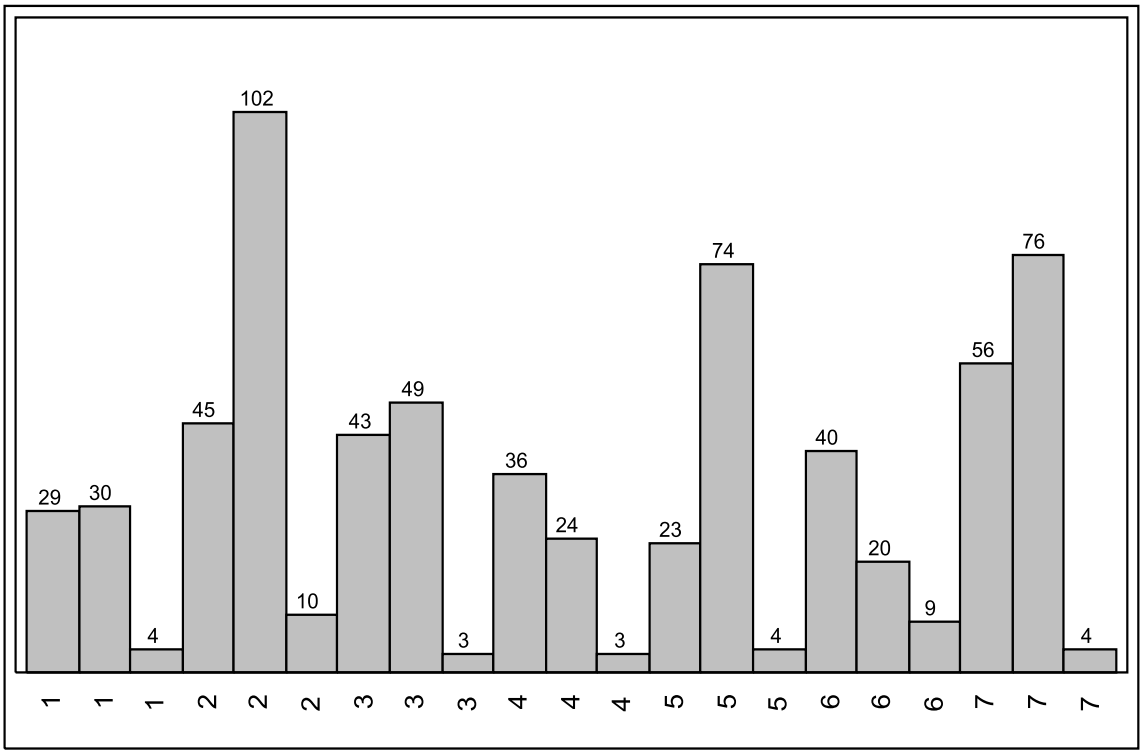


Figure 3 Distribution of markers for the population evaluated at Ithaca /Njoro

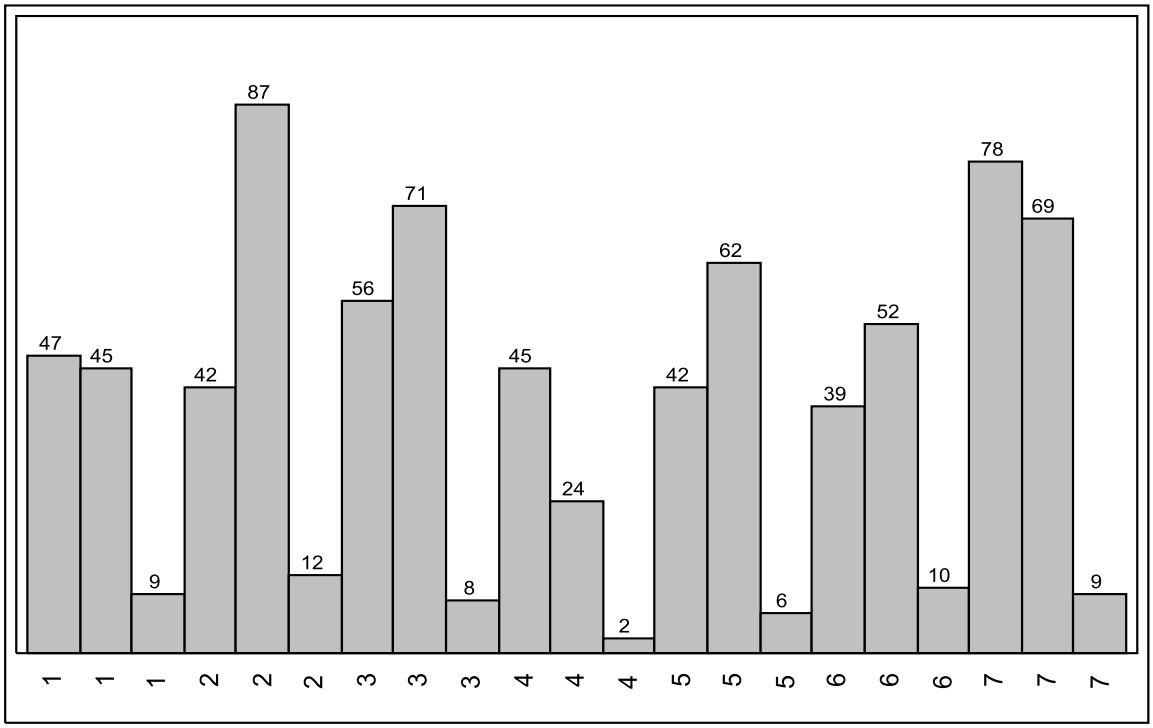


Figure 4 Distribution of markers for the population evaluated at Wellington

3.2.Linkage Disequilibrium analysis

Linkage Disequilibrium (LD) estimates are very important in association studies and are influenced by the type of marker used. High LD is particularly favorable when using a limited number of markers. LD was estimated for both populations used in this study using GBS markers. For the population that was evaluated at Ithaca and Njoro 10,708 (33.07%) of the total 32,376 marker pairs exhibited a significant level of LD ($p < 0.01$). For the population evaluated at Wellington, only 6,798 (17.22%) of the total 39,476 marker pairs exhibited a significant level of LD ($p < 0.01$). The average r^2 for the marker pairs in the population evaluated at Ithaca and Njoro was 0.051 and it was 0.023 for the other population. It was also observed that LD was highly variable throughout the genome and it declined within 20 cM for the two panels (Figures 5 and 6). Other studies have observed similar LD ranges in cM distances in wheat (Breseghello and Sorrells 2006b; Chao et al. 2007; Maccaferri et al. 2005; Somers et al. 2007; Yu et al. 2011). The LD estimates were typically 2 to 3 cM but extended to 40 cM in some regions. In the case of each of the linkage groups, the LD decay followed the same pattern with the exception of the sparsely genotyped D genome where LD decayed within 40cM (Figures 7 to 12).

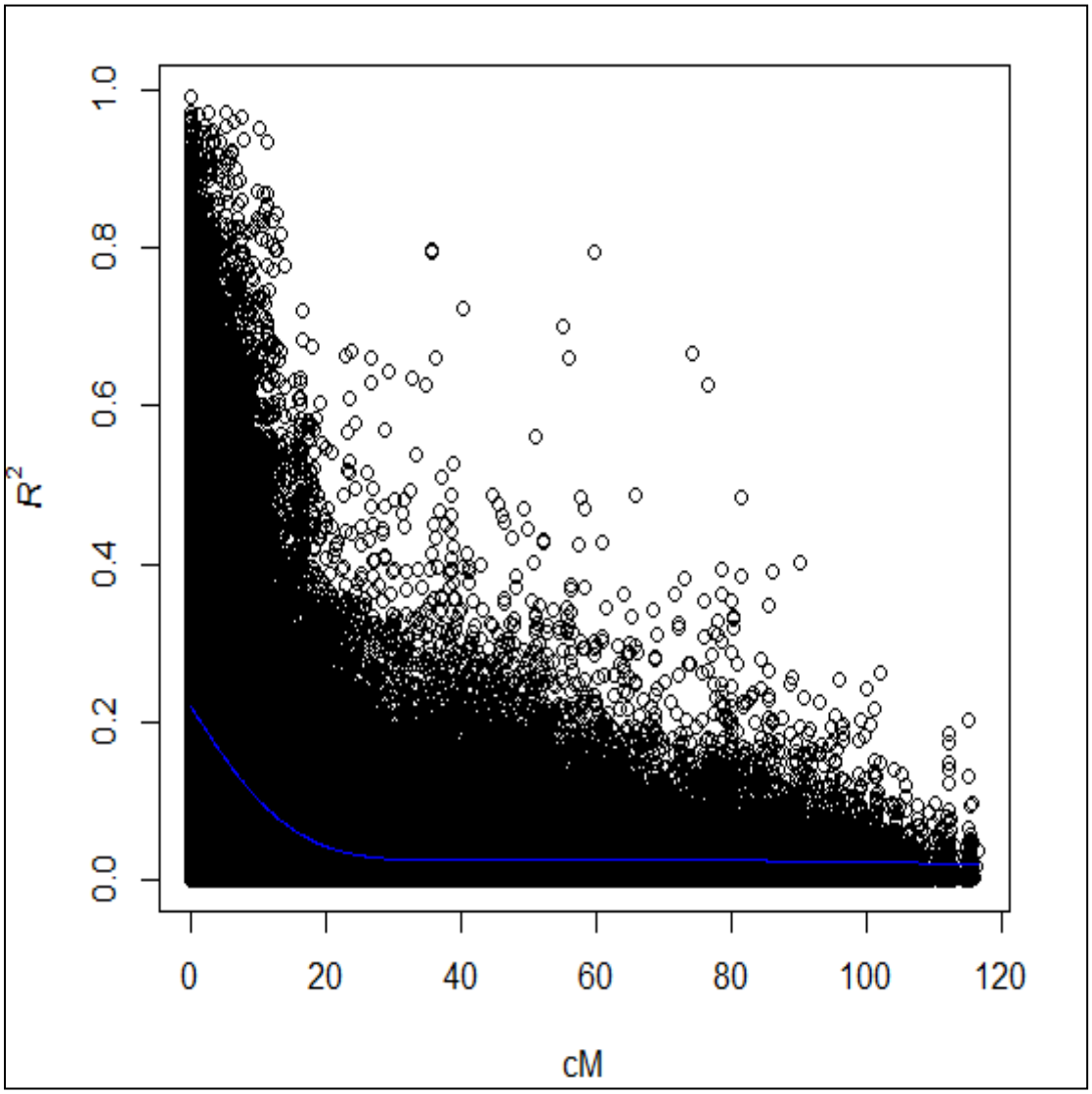


Figure 5 Scatter plot of estimates of R^2 for pairs of GBS markers across chromosomes and genomes showing LD decay, as measured by R^2 against genetic distance (cM) for the population evaluated at Ithaca and Njoro

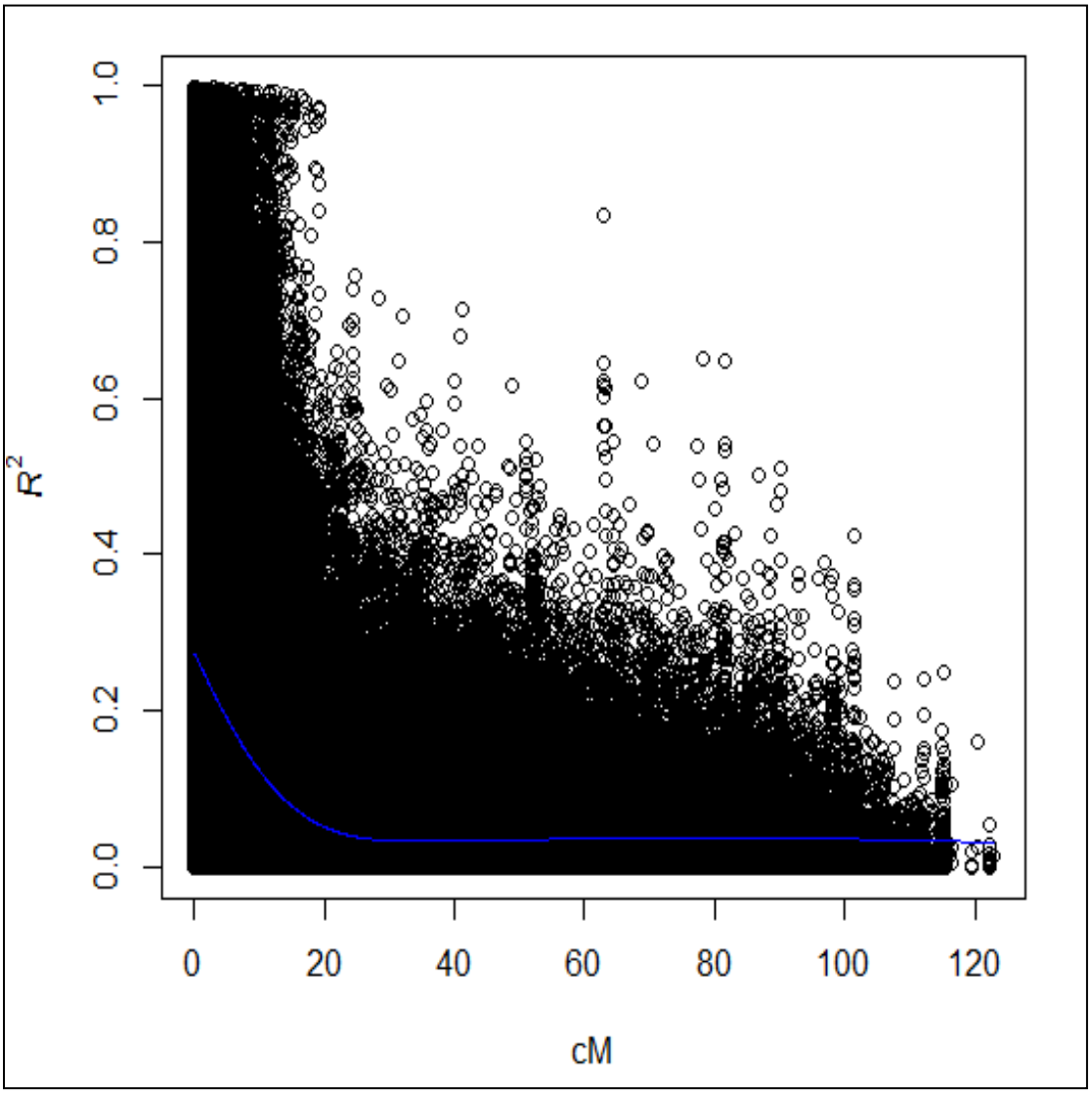


Figure 6 Scatter plot of estimates of R^2 for pairs of GBS markers across the wheat chromosomes and genomes showing LD decay, as measured by R^2 against genetic distance (cM) for the population evaluated in Wellington

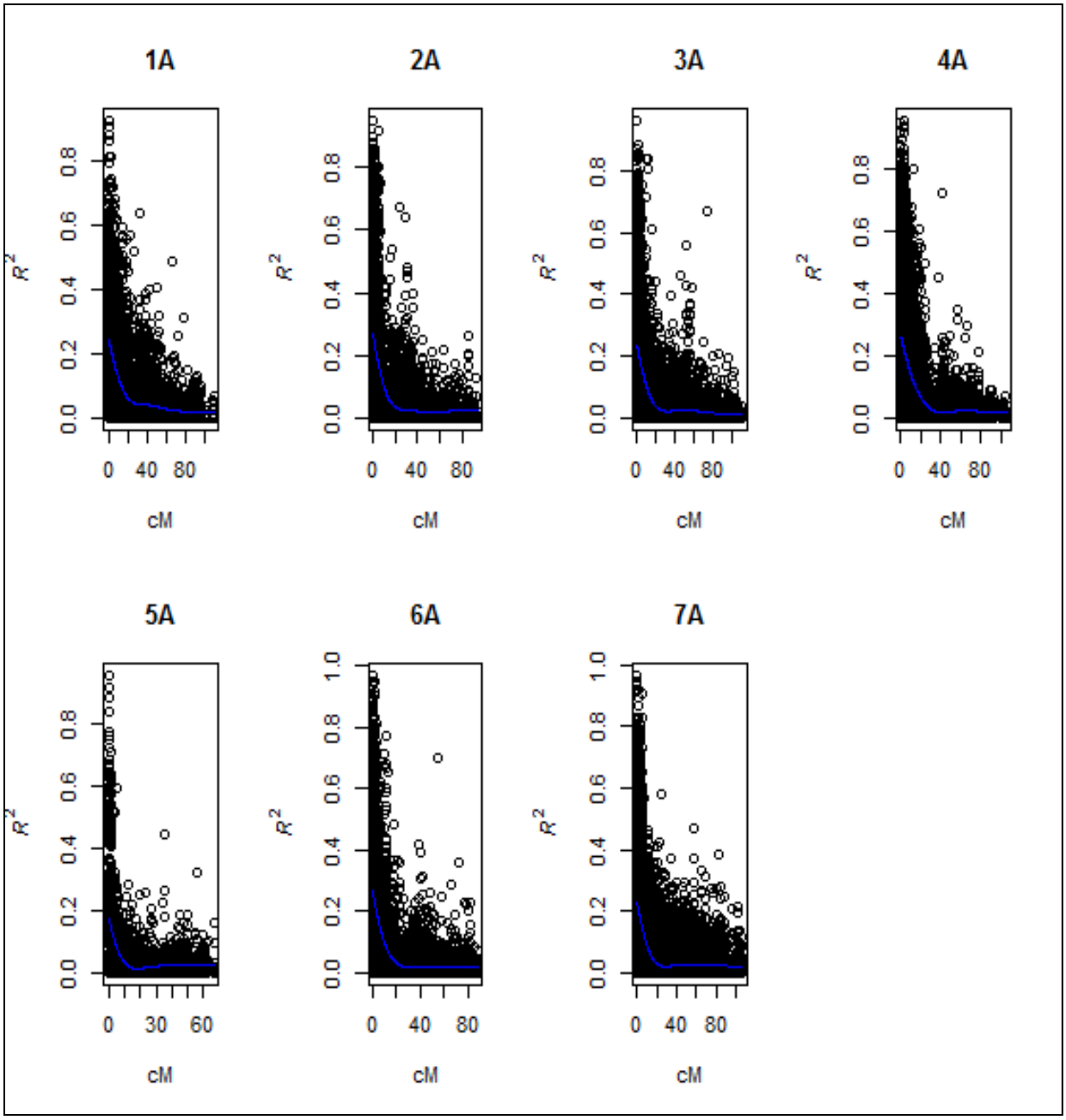


Figure 7 Scatter plot of estimates of R^2 for pairs of GBS markers across the wheat 'A' genome showing LD decay, as measured by R^2 against genetic distance (cM) for the population evaluated at Ithaca and Njoro

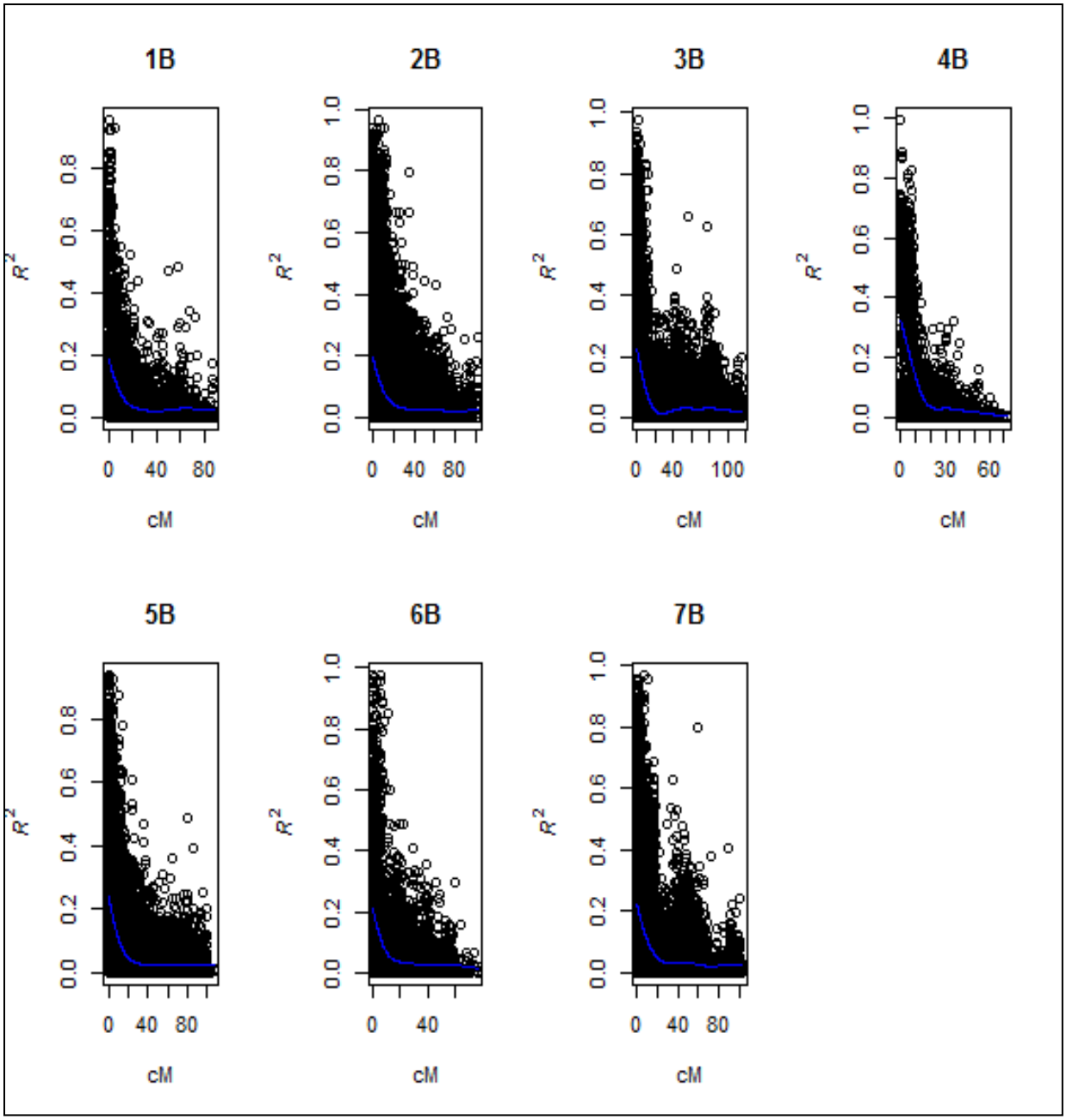


Figure 8 Scatter plot of estimates of R^2 for pairs of GBS markers across the wheat 'B' genome showing LD decay, as measured by R^2 against genetic distance (cM) for the population evaluated at Ithaca and Njoro

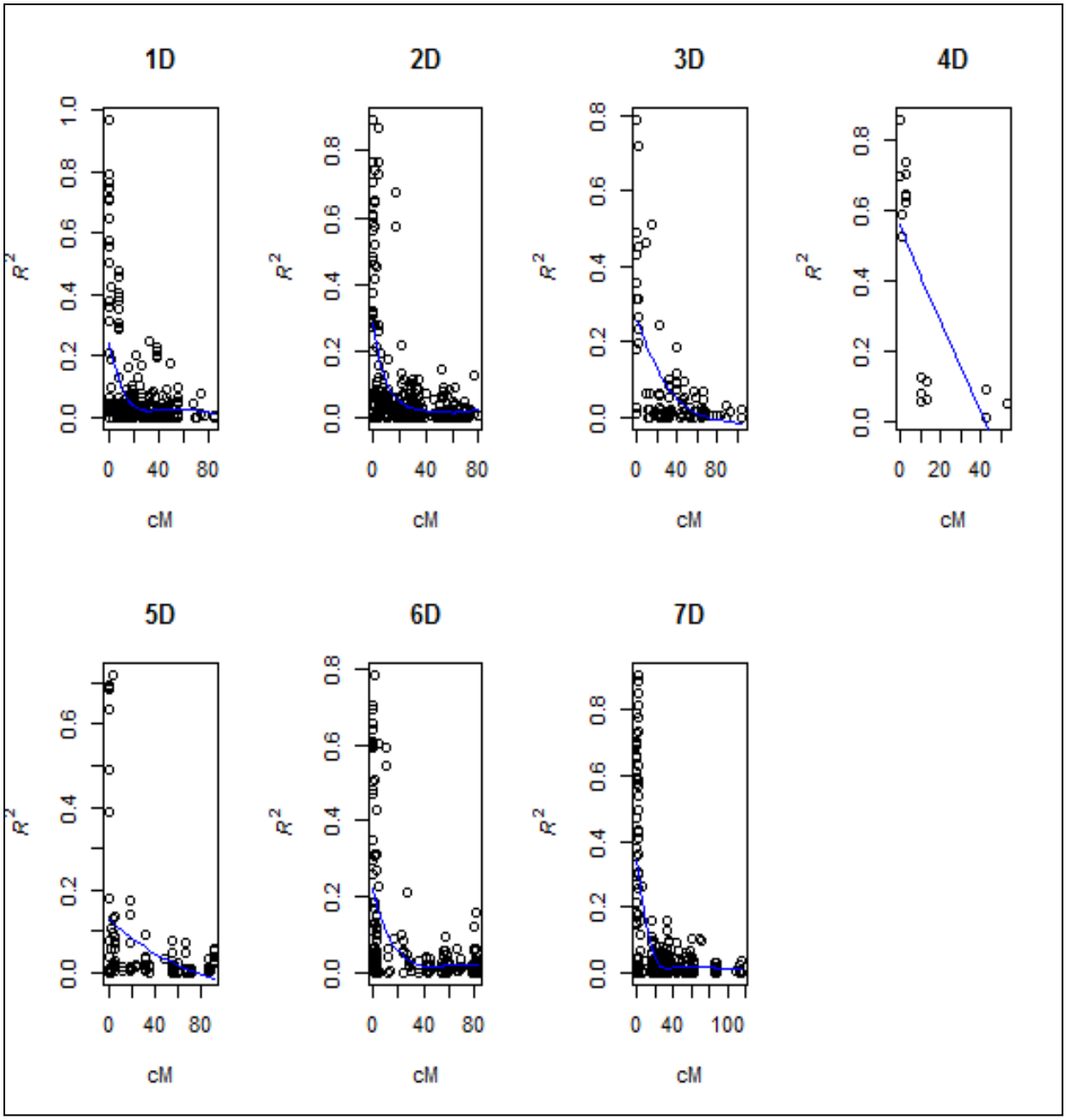


Figure 9 Scatter plot of estimates of R^2 for pairs of GBS markers across the wheat 'D' genome showing LD decay, as measured by R^2 against genetic distance (cM) for the population evaluated at Ithaca and Njoro

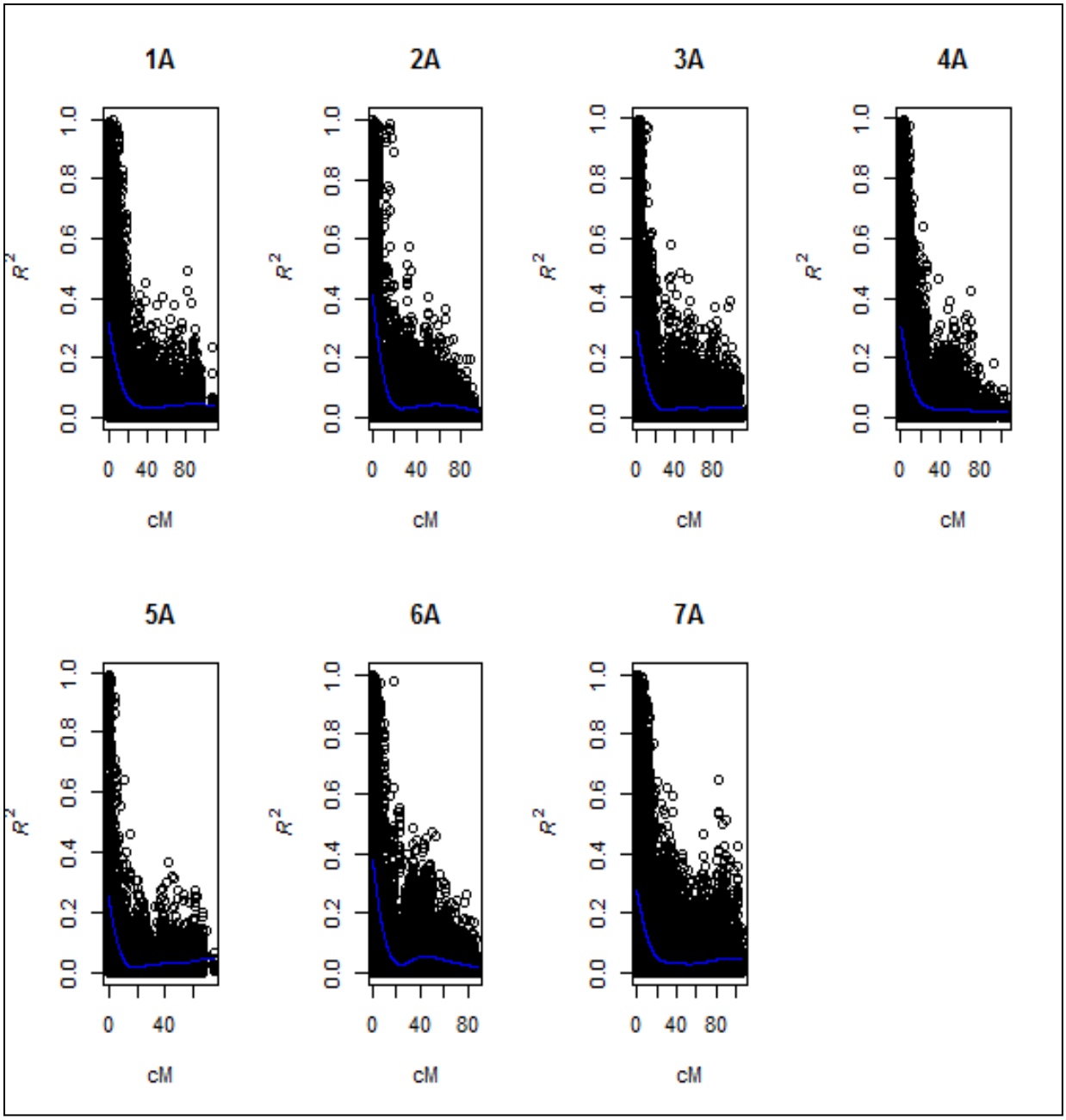


Figure 10 Scatter plot of estimates of R^2 for pairs of GBS markers across the wheat 'A' genome showing LD decay, as measured by R^2 against genetic distance (cM) for the population evaluated in Wellington

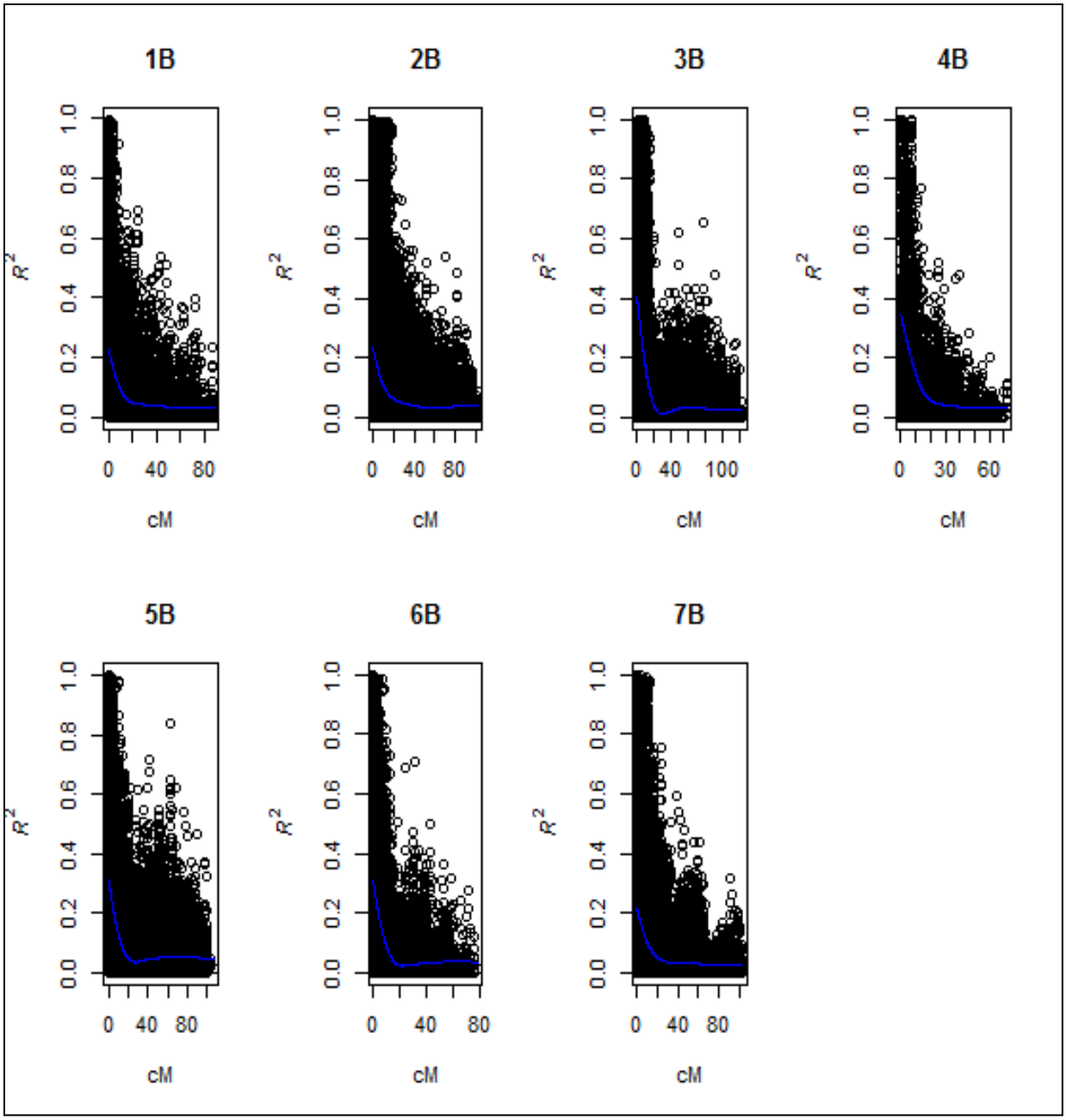


Figure 11 Scatter plot of estimates of R^2 for pairs of GBS markers across the wheat 'B' genome showing LD decay, as measured by R^2 against genetic distance (cM) for the population evaluated in Wellington

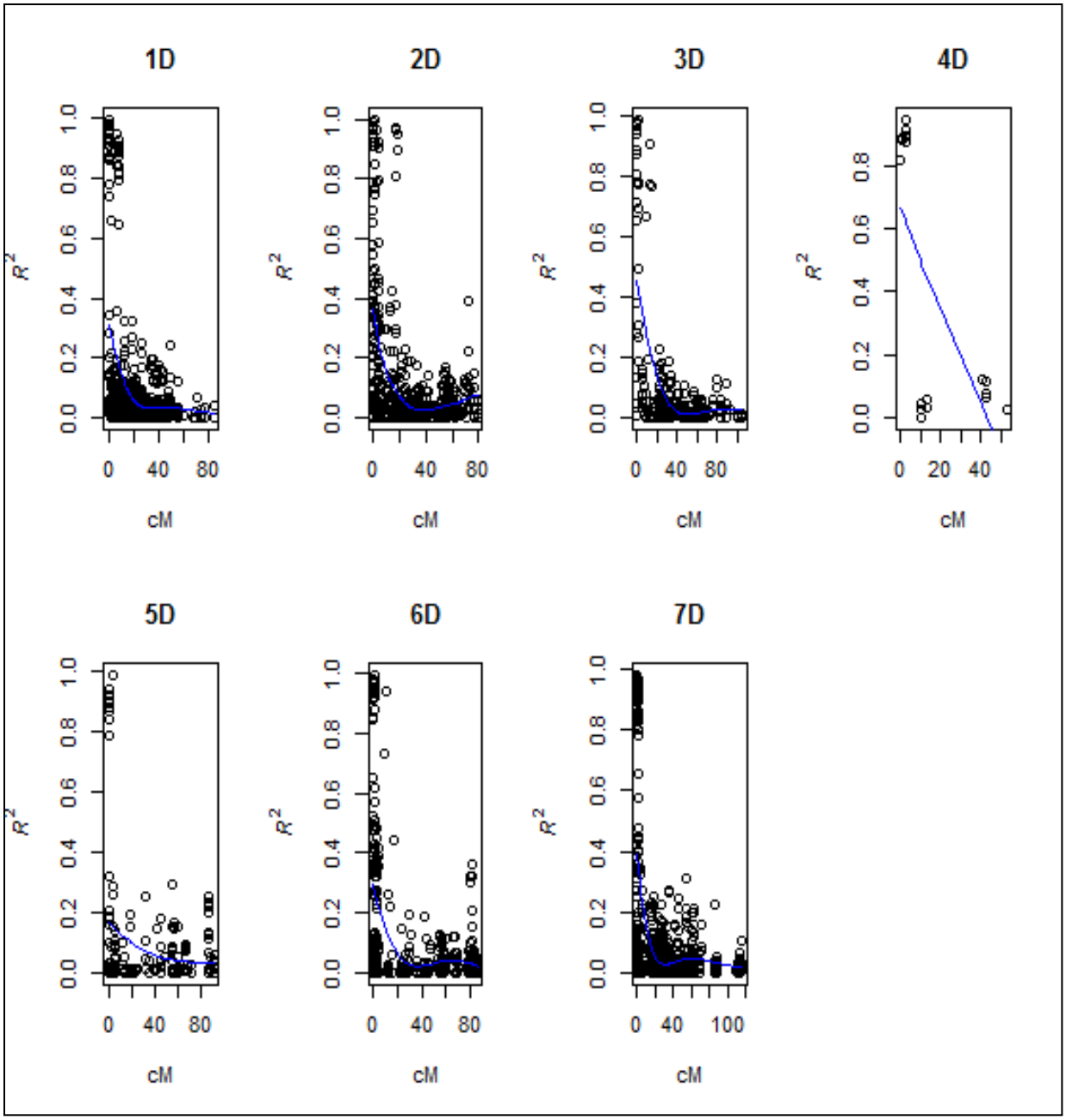


Figure 12 Scatter plot of estimates of R^2 for pairs of GBS markers across the wheat 'D' genome showing LD decay, as measured by R^2 against genetic distance (cM) for the population evaluated in Wellington

3.3.Principal component analysis

Population structure of the different germplasms used in the study was characterized using principal component analysis (PCA). PCA clearly captured the variation among the 86 families (Figure 13a) and 107 families (Figure 13b) comprising the 2 different populations respectively. The plot was color coded based on the families. Three groups were detected in the population evaluated at Ithaca/Njoro and five groups were detected in the population evaluated at Wellington by PCA. In the STRUCTURE analysis, the highest logarithm of the probability of likelihood [LnP(D)] was obtained for K = 3 and K = 5 for the two different populations respectively. Beyond that, the plateauing of LnP(D) values was observed. These consistent results obtained by PCA and STRUCTURE indicated that similar subpopulations were identified in both the analysis.

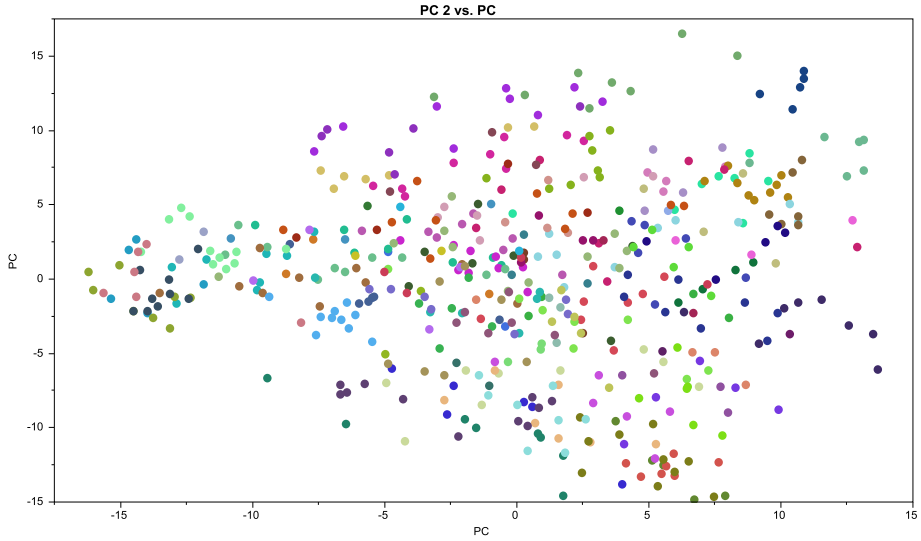


Figure 13a PC1 vs PC2 for the population evaluated at Ithaca and Njoro

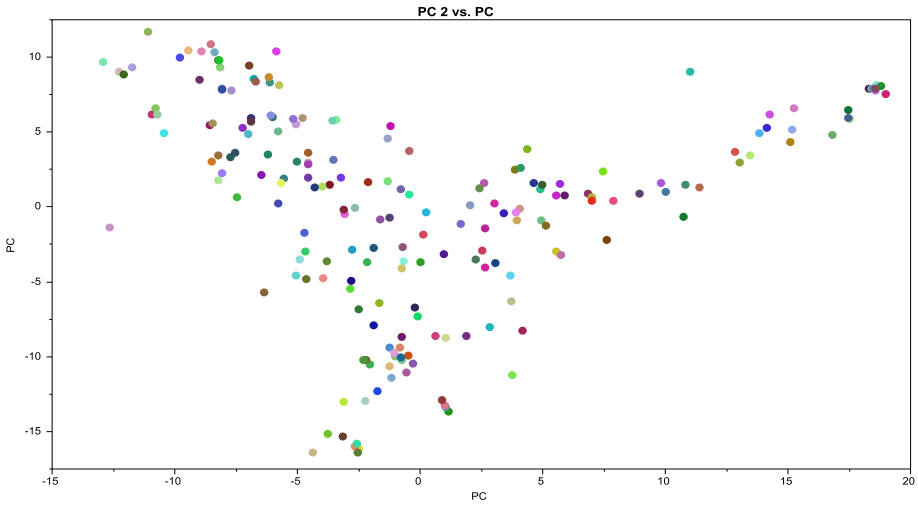


Figure 13b PC1 vs PC2 for the population evaluated at Wellington

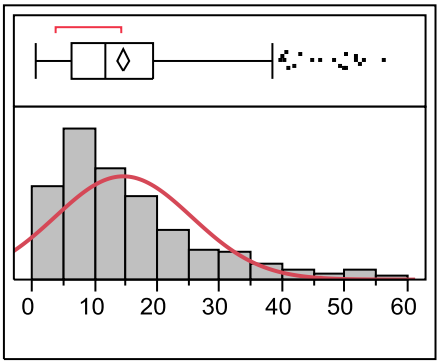
Association mapping for leaf tip necrosis

3.4. Statistical analysis of LTN data

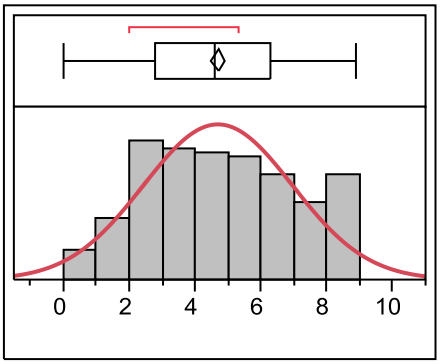
3.4.1. Statistical analysis of LTN data for the lines evaluated at Ithaca

The distribution of the trait values was analyzed (Figure 14). For the lines evaluated at Ithaca, the mean percentage of LTN obtained by image analysis was 14.56% with a standard deviation of 10.95%. The mean length of LTN was 4.69 cm $\pm$ 2.26 cm and the mean leaf length was 18.45 cm $\pm$ 3.57 cm. The ratio of LTN to the leaf length was 26.23% with a standard deviation of 12.61%. In order to find out which of these methods would be suitable to score LTN, the correlation between the percent necrosis that was obtained by image analysis and the ratio of the necrotic length by leaf length was calculated. This was found to be 0.8039 (Figure 15a). The correlation can be attributed to the fact that one method was based on length while the other was based on area. For the sake of convenience, measuring the length of LTN quantitatively was preferred to be the suitable method for scoring this trait. To determine if there is a necessity to calculate the LTN length/leaf length ratio or simply the necrotic length would

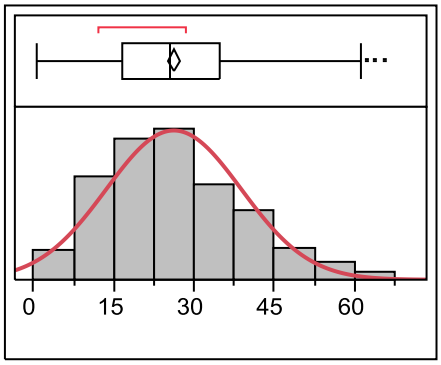
suffice, the correlation between these two was calculated. As this correlation (0.9484) was very high (Figure 15b), measuring the necrotic length (in cm) alone was considered to be sufficient for phenotyping LTN. The results of the Analysis of Variance indicated that the families had a significant effect on the length of LTN (p-value of < 0.0001*) (Table 1). An R^2 value of 0.4025 was obtained indicating that the families contributed to 40.25% of the variability in the length of LTN. The length of LTN was better explained by a model (R^2 value of 0.52) which included the days to heading, leaf length, block and the families. The families (p-value of <.0001*), days to heading (p-value of 0.0024*) and the leaf length (p-value of <.0001*) were highly significant whereas the blocks were significant (p-value of 0.004*) at the 0.005 level (Table 2).



Percent necrosis (Image analysis)



Necrotic length in cms (measured)



Percent necrotic length/leaf length

Figure 14 Distribution of LTN data for the lines evaluated at Ithaca

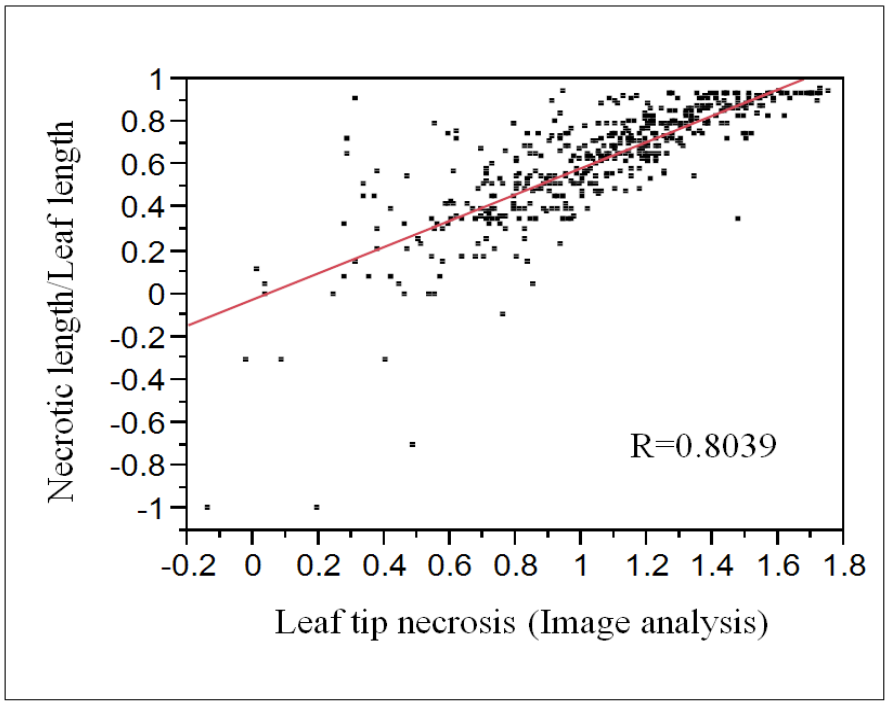


Figure 15a Correlation between the percent necrosis (Image analysis) and the ratio of necrotic length to the leaf length

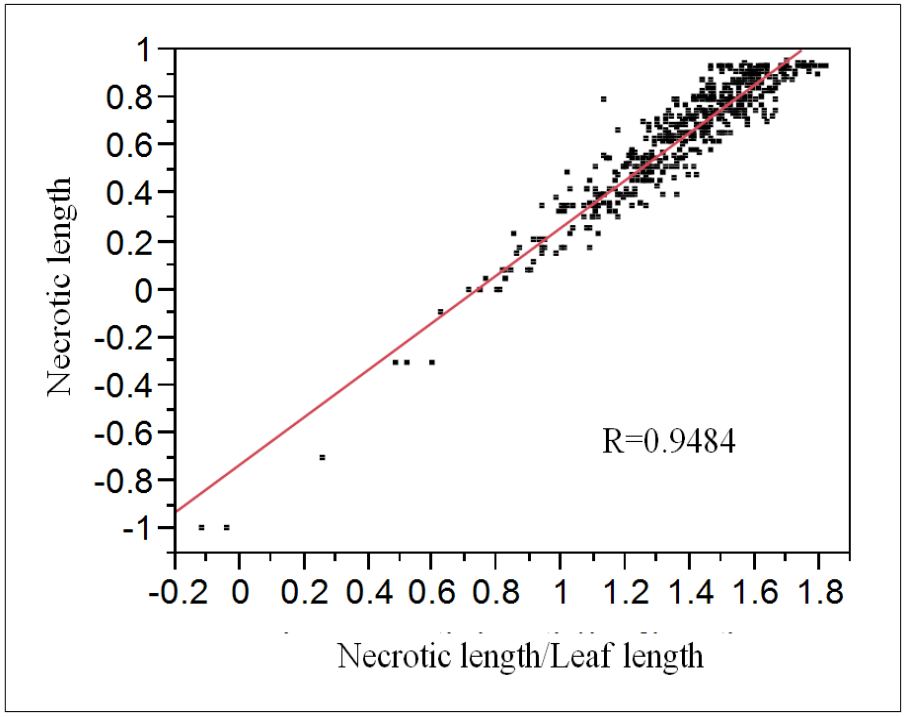


Figure 15b Correlation between necrotic length and necrotic length/leaf length

Table 1 Analysis of Variance (Length of LTN vs family) for the population evaluated in Ithaca

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	86	895.6637	10.4147	2.7117
Error	417	1601.5719	3.8407	**Prob > F**
C. Total	503	2497.2356		<.0001*

Table 2 Analysis of Variance (Length of LTN vs days to heading, leaf length, block and family) for the population evaluated in Ithaca

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	94	1230.1724	13.0869	4.3308
Error	409	1235.9162	3.0218	**Prob > F**
C. Total	503	2466.0886		<.0001*

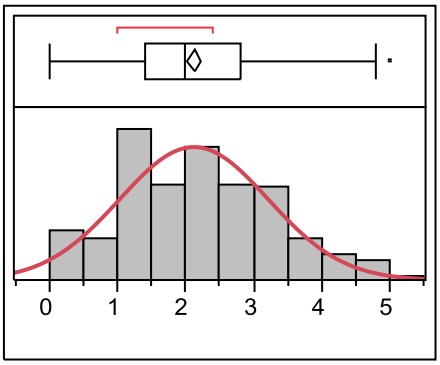
Effect Tests

Source	DF	Sum of Squares	F Ratio	Prob > F
Family	86	687.23959	2.6445	<.0001*
Days to heading	1	110.32428	36.5090	<.0001*
Leaf length	1	142.38016	47.1171	<.0001*
Block	6	58.15025	3.2072	0.0044*

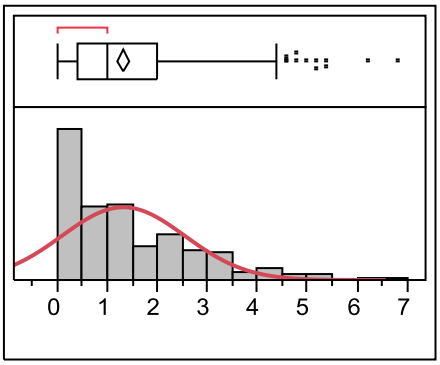
3.4.2. Statistical analysis of LTN data for the lines evaluated at Njoro

The distribution of LTN data for the lines evaluated in Njoro in both the main and off seasons of 2012 was analyzed (Figure 16). The mean length of LTN in the main season was 2.12 $\pm$ 1.09 cm and 1.32 $\pm$ 1.23 cm in the off season. Results of the Analysis of Variance indicated

that families contributed significantly to LTN both in the main and off seasons (p-value of <.0001*) (Tables 3 and 4). The families explained 54.64% of the variability in the length of LTN in the main season and 53.82% of the variability in the length of LTN during the off season.



Necrotic length in cms (main season)



Necrotic length in cms (off season)

Figure 16 The distribution of LTN data for the lines evaluated in Njoro

Table 3 Analysis of Variance (Length of LTN vs family) for the population evaluated in Njoro (main season)

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	83	66.23076	0.797961	4.4857
Error	309	54.96793	0.177890	**Prob > F**
C. Total	392	121.19870		<.0001*

Table 4 Analysis of Variance (Length of LTN vs family) for the population evaluated in Njoro (off season)

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	86	407.09992	4.73372	5.2786
Error	417	373.95726	0.89678	**Prob > F**
C. Total	503	781.05718		<.0001*

3.4.3. Comparison of LTN data across locations, generation and seasons

Correlations among the LTN data evaluated in Ithaca and Njoro (combined for both seasons) were weak with a correlation coefficient r=0.29 (Figure 17) which might be due to the environmental dependence of the trait and also its segregation in the progenies. The variance across locations could not be partitioned *per se* as the lines evaluated at Njoro were also the progenies of the lines at Ithaca. Hence an ANOVA for location*generation was performed and a highly significant difference was observed (Table 6) which might also be attributed to the two reasons mentioned above. In addition, an ANOVA was performed to see if the seasons had an effect in the expression of LTN by using the data obtained in the 2 different Njoro seasons. A low p value of <.0001* indicated that the seasons contributed significantly to the differences in the expression of LTN (Table 5) which confirms the temperature dependence of this trait.

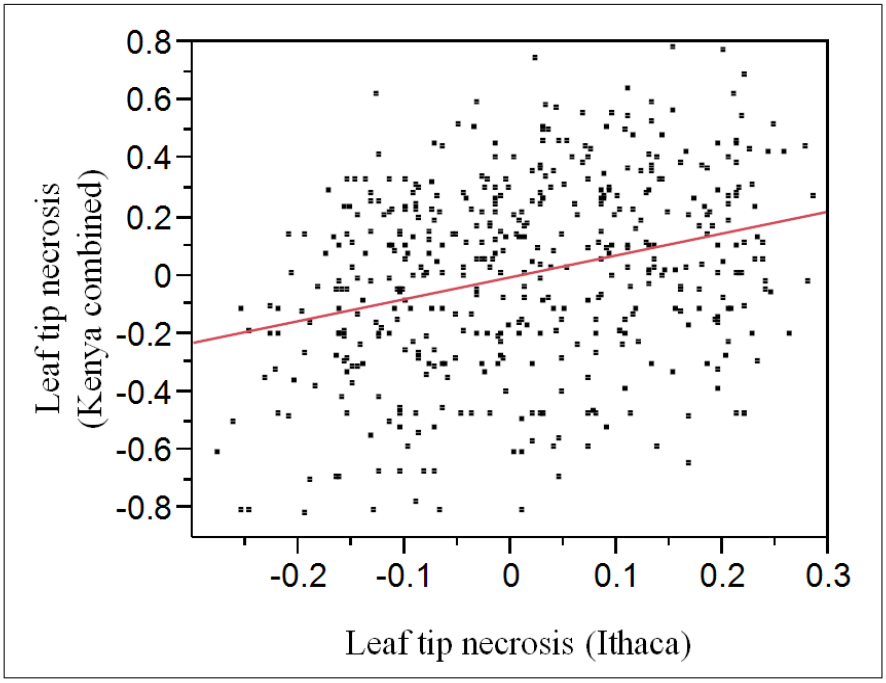


Figure 17 Correlation between the LTN data evaluated at Ithaca and Njoro

Table 5 Analysis of Variance (Length of LTN vs Njoro seasons)

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	278.6966	278.697	339.0283
Error	895	735.7305	0.822	**Prob > F**
C. Total	896	1014.4272		<.0001*

Table 6 Analysis of Variance (Length of LTN vs location*generation)

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	2842.9423	2842.94	1022.148
Error	1400	3893.8744	2.78134	**Prob > F**
C. Total	1401	6736.8167		<.0001*

3.4.4. Statistical analysis of LTN data for the lines evaluated at Wellington

The distribution of LTN data for the 200 lines evaluated at Wellington (summer, 2012) was analyzed (Figure 18) and the mean leaf tip necrotic length was found to be 3.64 $\pm$ 1.58 cm. The families explained 40.68% of the variability in the length of LTN and results of the Analysis of Variance also indicated that families contributed significantly to LTN (p-value of <.0001*) (Table 7).

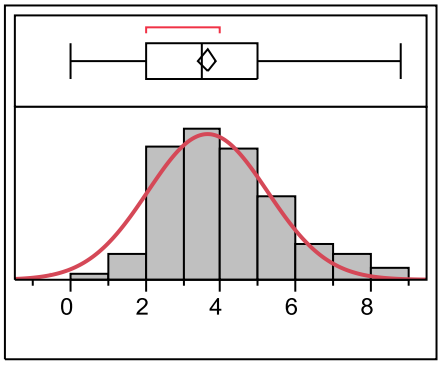


Figure 18 Distribution of LTN data for the population evaluated at Wellington (2012)

Table 7 Analysis of Variance (Length of LTN vs family) for the population evaluated in Wellington

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	107	5.2700167	0.049252	2.4639
Error	93	1.858977	0.019989	**Prob > F**
C. Total	200	7.1289937		<.0001*

3.5.Analysis of marker-trait associations for LTN

Although several markers were significantly associated with LTN in the two different mapping panels, only the markers that were significant both in the GLM and MLM were considered. After making adjustments for False Discovery Rate (FDR, Benjamini and Hochberg 1995) associated with multiple hypothesis testing, 21 significant GBS markers were identified including 7 markers in the population evaluated at Ithaca, 3 markers in the population evaluated at the Njoro main season, 5 markers in the population evaluated at the Njoro off season, 5 markers in the Njoro combined analysis, 7 markers in the population evaluated at Wellington, and 5 markers that were identified in more than one data set Table 8). The cut-off value used for FDR was 0.05 in the case of GLM. The positions of the markers significant in the present study was determined using the Synthetic x Opata map and compared with the positions of the markers detected in previous studies using information from the website, GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml). The inference about the significant markers below is based on their MLM p-values only. Seven GBS markers on five chromosomes were significantly associated with LTN in the population evaluated at Ithaca. The most significant marker among them was GBS_11611 (p = 4.84E-04) on chromosome 7DS followed by markers

GBS_22604 (chromosome 3D), GBS_9433 (chromosome 1BL), markers GBS_371, GBS_1425 and GBS_15635 (chromosome 7BS) and GBS_302 (chromosome 2BL). In the Njoro main season, three GBS markers on three chromosomes were significantly associated with LTN. GBS_11149 (p = 3.58E-04) on chromosome 3BS was the most significant marker followed by markers GBS_9224 (chromosome 2BL) and GBS_23503 (chromosome 5B). In the Njoro off season, five significant markers on two chromosomes included GBS_1203 (p =1.34E-03) on chromosome 7BS (most significant), markers GBS_4088, GBS_23290 and GBS_15572 (chromosome 7BS), and GBS_11149 (chromosome 3BS). In the combined analysis of the Njoro data, five significant markers on two chromosomes were obtained. The most significant marker (p=4.35E-05) was GBS_11149 (on chromosome 3BS) followed by markers GBS_1203 (chromosome 7BS), and markers GBS_15572, GBS_4088, GBS_23290 (chromosome 7BS). In the case of the Wellington population, seven GBS markers on seven chromosomes turned out to be significant. Among these, GBS_18690 (chromosome 7BS), was the most significant marker (p= 1.33 E-03), followed by markers GBS_3202 (chromosome 4DL), GBS_2277 (on chromosome 2BL), GBS_2248 (chromosome 1BL), GBS_22182 (chromosome 5B), GBS_18258 (chromosome 7BL), GBS_2547 (chromosome 5A).

The marker, GBS_11611 was associated with LTN only in the population evaluated at Ithaca and was located on chromosome 7DS (94.3cM). This marker was 0.6 cM and 1.9cM from the *Lr34* associated markers, csLV34 and cssfr5, respectively (Figure 19). While, marker csLV34 is 0.31cM proximal to *Lr34* (Kolmer et al. 2008; Lagudah et al. 2009), cssfr5 is a gene specific marker for *Lr34* (Lagudah et al. 2009). The chromosome location of GBS_11611 indicates that it is closely linked to *Lr34*, the major gene associated with LTN. Previous studies by Messmer et al. (2000), Schnurbusch et al. (2004b) have reported QTL on chromosome 7DS

corresponding to LTN. However, as only 4 markers covered the 7D region in our study more closely linked markers could not be identified. The well documented pleiotropism of *Lr34* on LTN was confirmed in this study. The reason why this region was not detected in the Wellington season can be attributed to two reasons. First, this GBS marker was not in the Wellington data set and secondly it could be due to the fact that LTN is well expressed only at lower temperatures than the Wellington temperatures. The latter reason also applies to the failure to detect this region in Njoro where temperatures might have not favored the expression of leaf tip necrosis.

On chromosome 1BL, two markers, GBS_2248 and GBS_9433, were significant in the Wellington and Ithaca populations, but not in Njoro. These markers were located at 87.4 and 88 cM (Figure 19) and hence should be identifying the same locus. Rosewarne et al. (2006) have previously reported that the *Lr46* region on chromosome 1BL contributed to LTN. The marker Xwmc44 which is 12.5 cM proximal to *Lr46* (Martinez-Hernandez et al. 2006) was used to verify if the GBS markers significant in the present study corresponded to the *Lr46* region. Xwmc44 is also located at a distance of 12cM from the GBS markers significant in the present study. Hence, there is a high probability that these markers, GBS_2248 and GBS_9433 lie in the exact predicted interval for the *Lr46* region and are tightly linked to the gene. This makes them good candidates to be used in selecting for this gene and they can be validated. Although, the *Lr46* gene has not been cloned this confirms previous studies indicating that LTN is pleiotropic to this gene.

On chromosome 4DL, the marker GBS_3202 which is located at 55.8cM (Figure 19) was identified only in the Wellington population. The poor coverage of the 4D chromosome with only 2 markers might be the reason why this region could not be detected in the other datasets. The association of LTN with the gene *Lr67* which is pleiotropic for this trait has a*Lr*eady been

reported (Hiebert et al. 2010b). Hence, the markers Xgwm165 and Xgwm192 which are 0.4 cM proximal to *Lr67* (Herrera-Foessel et al. 2011) were used to determine if the marker identified in the present study also corresponded to this gene. These markers were not in the Synthetic x Opata map and so their approximate location was determined using another marker, Xwmc473. This marker is approximately 5.8cM proximal to Xgwm165 in the Wheat, Consensus SSR, 2004 map (GrainGenes) and is also 12.5cM from GBS_3202 which is significant for LTN in the present study. Hence, GBS_3202 would be approximately 6cM away from the marker tightly linked to the *Lr67* gene and likely corresponds to the *Lr67* region.

On chromosome 7BL, the marker GBS_18258 which is located at 6.8cM (Figure 19) was significant only in the Wellington population. The likely association of *Lr68* with LTN was recently reported by Herrera-Foessel et al (2012) who observed that the QTL for *Lr68* was in the same region as the QTL for LTN previously reported by Messmer et al. (2000) and Schnurbusch et al. (2004b). The marker Xgwm146 which was estimated to be 0.6 cM (Herrera-Foessel et al. 2012) from *Lr68* was used to verify if the significant marker in this study corresponded to this gene region. This marker was not present in the Synthetic x Opata map so the marker, barc182 which is 1.6cM from Xgwm146, (Wheat Synthetic x Opata BARC) was used to find the approximate position of Xgwm146. The marker barc182 is at the same position (6.8cM) as the marker GBS_18258 that was significant in this study and hence GBS_18258 might be just proximal to *Lr68* confirming the association of the slow rusting gene *Lr68* with LTN. Messmer et al (2000) found three QTL on chromosome 7BL that were significant for LTN. But the relative position of their markers flanking the QTL to the marker significant in the present study could not be confirmed as those markers are present only in the Forno x Oberkulmer map. In addition to the long arm of chromosome 7B, a region on the short arm of this chromosome also

affected LTN significantly. The markers that were consistently significant for LTN in all the datasets were located at the same position (68.9cM) on chromosome 7BS. These include GBS markers GBS_1203, GBS_15572, GBS_23290 and GBS_4088 (significant in the Njoro off season); GBS_1203, GBS_15572, GBS_23290 and GBS_4088 (significant in the Njoro combined analysis); GBS_1425, GBS_15635 and GBS_371 (significant in the Ithaca population), GBS_18690 (significant in the Wellington population). Although Messmer et al (2000) detected three *Ltn* QTL on chromosome 7B, all of them were in the long arm. A leaf rust resistance region flanked by the marker gwm573 which also flanks the region significant in the present study has been reported earlier in a study by Schnurbusch et al (2004b). But it is unclear if this resistance locus might affect LTN as there are no previous reports on the durability of this region. However, one study by Li et al. (2012) reported a locus on 7BS that was found to reduce the necrotic length. But this was not the case in this study, based on preliminary investigations.

On chromosome 2BL, the markers significant for LTN were GBS_2277 (Wellington population), GBS_302 (Ithaca population) and GBS_9224 (Njoro main season). These markers span an interval of 3cM from 48.7cM to 50.7cM (Figure 19). Interactions between csLV34 and a DArT marker wPt8460 were identified by Yu et al. (2011). However, wPt-0950 is 1.5cM away from the interacting marker identified by Yu et al. (2011) and wPt-8460 is approximately 14cM away from the markers significant in the present study and hence might not be the same locus. Kolmer et al. (2011) identified another DArT marker wPt4199 that flanked a stem rust QTL on 2BL and was enhanced by *Lr34*. The markers significant in the present study are only 1 to 3cM away from wPt-4199, the marker said to be enhanced by *Lr34* and hence might refer to the same region. The genes *Lr13*, *Lr16*, *Lr23* and *Lr35* are the known major leaf rust resistance genes, mapped on chromosome 2B (McIntosh et al. 2003). The markers linked to these genes were used

to determine if any of them were located in the same region as the markers significant in the present study. The results suggested that the *Lr13* gene was closest to the markers identified. Maccaferri et al. (2010) identified markers barc183 and barc40 flanking the *Lr13* gene. Although the positions of these markers could not be traced in the Synthetic x Opata map, their comparitive positions were located with the marker wmc474. This marker which is approximately 4cM away from barc40 in the Wheat Consensus SSR, 2004, is also within 1-4cM of the markers significant in the present study and hence should be identifying the same region as *Lr13*. Interaction of *Lr13* and *Lr34* has been reported (Roelfs 1988; Kloppers and Pretorius 1997; Oelke and Kolmer 2005). Hence, this study confirms these observations and also raises the possibility that *Lr13* might be a part of the '*Lr34* complex'.

On chromosome 5B, the markers GBS_23503 and GBS_22182 were associated with LTN in the Njoro main season and in Wellington respectively. These markers are found at 66.3cM and 70.3cM (Figure 19). Previous report of a LTN affecting region on 5B was by Messmer et al. (2000) who detected a region flanked by markers Xglk163b - Xpsr426 significant for LTN. But their position cannot be compared to the position of the markers in the present study as those markers are available only in the Forno x Oberkulmer map. However, Yu et al. (2011) also reported that interactions were found between csLV34 and the DArT marker wPt2707 on 5B. Although wPt2707 could not be located in the Synthetic x Opata map, its approximate location was determined using markers wPt9103 and wPt9598 which are only 0.5cM away from wPt2707 in the CIMMYT integrated map. The markers GBS_23503 and GBS_22182 significant in the present study are approximately 2.5cM and 6cM away from markers wPt9103 and wPt9598. Hence this interacting region is also most likely the same loci identified in the present study. As

the location of any well known resistance gene on chromosome 5B (*Lr18, Lr52*) did not coincide with this region, it is assumed to be a region affecting LTN by interacting with the *Lr34* gene.

On chromosome 3BS, the marker GBS_11149 located at 10.2cM was significant in the Njoro main, off seasons and also in the combined analysis (Figure 19). Previously, Messmer et al. (2000) identified two LTN QTL in this region flanked by markers *Lr*k10b - Xpsr1196b and Xpsr907 - Xglk538. Although the positions of these QTL could not be located in the Synthetic x Opata map, their approximate location in the telomeric region of chromosome 3BS indicate that they might be located at the *Sr2* region. Schnurbusch et al. (2004b) have reported the marker Xcfd79b (located at the *Sr2* region) to be associated with LTN. The marker significant in the present study was also located in the *Sr2* region, approximately 3cM away from the DArT marker, wPt8446 that flanked the interval for *Sr2* (Yu et al. 2011). The role of *Sr2* in conferring LTN is unknown but there is a possibility that it might be the leaf rust resistance gene, *Lr27* which is tightly linked to *Sr2*.

On chromosome 3D, the marker GBS_22604 was associated with leaf tip necrosis in the Ithaca population and was located at 72.2cM (Figure 19). A minor leaf tip necrosis QTL on chromosome 3D flanked by the markers gwm645 and gwm383a was also detected by Schnurbusch et al. (2004b). This marker gwm383 is approximately 6cM away from GBS_22604 significant in the present study and hence should be identifying the same region. *Lr32, Lr24* and *Lr38* are the known leaf rust resistance genes on chromosome 3D (McIntosh et al. 2003). Among these, the gene *Lr24/Sr24* is 5cM away from the marker psr1203 (Schachermayr et al. 1995; Mago et al. 2005) and 4cM away from the marker gwm383 (Wheat Composite map, 2004). *Lr*24 was a*Lr*eady observed in association with LTN in the studies of Singh et al. (2007) where Australian lines (Swift and Stretton) with csLV34 ‘a’ allele and the *Lr24* gene expressed LTN.

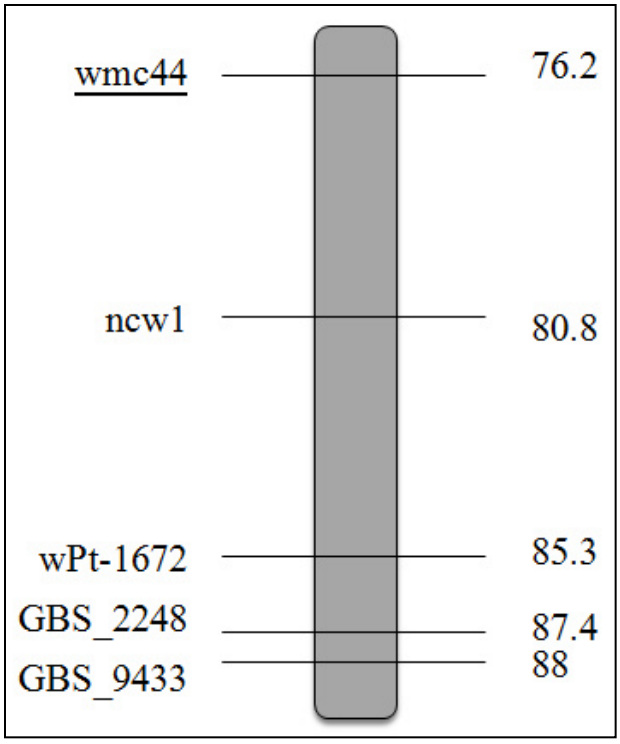
On chromosome 5AL, the marker GBS_2547 was associated with LTN in the Wellington population and is located at 103.4cM (Figure 19). Both Messmer et al (2000) and Schnurbusch et al. (2004b) have detected a minor QTL for LTN on 5AL. The marker gwm595 that flanked the interval for a minor QTL for LTN on 5AL (Schnurbusch et al. 2004b) could not be located on the Synthetic x Opata map. Hence another marker, wmc727 which is 2cM away from gwm595, (Wheat consensus SSR, 2004) was used to locate the comparative position of gwm595. Based on that location, the marker gwm595 corresponds exactly to the same location as GBS_2547 and hence should be identifying the same region.

Table 8 Markers significantly associated with LTN in the different populations

Marker	Chr	Pos_ snp_ start	Pos_ snp_ end	Marker P value (Q)	Marker R^2 (Q)	Marker P value (Q +K)	Marker R^2 (Q + K)	Location
GBS_2248	1BL	87.4	88	1.90E-03	0.04938	6.23E-03	0.04256	Wellington '12
GBS_9433	1BL	88	88	3.10E-03	0.0188	2.00E-03	0.0152	Ithaca '11
GBS_2277	2BL	47.7	53.1	2.06E-02	0.0259	3.85E-03	0.02419	Wellington '12
GBS_302	2BL	48.9	49.2	1.36E-02	0.01108	9.34E-03	0.02063	Ithaca '11
GBS_9224	2BL	50.7	51.8	1.51E-04	0.03224	3.81E-03	0.01535	Njoro '12 main
GBS_11149	3BS	10.2	12.8	1.59E-12	0.10721	3.58E-04	0.01706	Njoro '12 main
GBS_11149	3BS	10.2	12.8	2.00E-06	0.04243	9.31E-03	0.03019	Njoro '12 off
GBS_11149	3BS	10.2	12.8	3.03E-14	0.10128	4.35E-05	0.01308	Njoro '12 cmbd
GBS_22604	3D	72.2	72.2	2.36E-03	0.01992	1.59E-03	0.01585	Ithaca '11

GBS_3202	4DL	55.8	55.8	3.32E-03	0.04548	3.56E-03	0.04387	Wellington ‘12
GBS_2547	5A	103.4	107.9	7.19E-03	0.03826	9.87E-03	0.0321	Wellington ‘12
GBS_23503	5B	66.3	66.3	5.45E-05	0.0297	9.44E-03	0.01843	Njoro ‘12 main
GBS_22182	5B	70.3	70.3	3.62E-04	0.0602	9.76E-03	0.0321	Wellington ‘12
GBS_18258	7BL	6.8	6.8	9.14E-03	0.03508	9.83E-03	0.03175	Wellington ‘12
GBS_1203	7BS	68.9	68.9	6.38E-07	0.0464	1.34E-03	0.0373	Njoro ‘12 off
GBS_1203	7BS	68.9	68.9	6.02E-10	0.0684	5.55E-05	0.0338	Njoro ‘12 cmbd
GBS_1425	7BS	68.9	68.9	1.26E-04	0.0314	8.12E-03	0.0403	Ithaca ‘11
GBS_15572	7BS	68.9	68.9	1.80E-05	0.0346	9.27E-03	0.0733	Njoro ‘12 off
GBS_15572	7BS	68.9	68.9	1.39E-07	0.0501	5.48E-04	0.032	Njoro ‘12 cmbd
GBS_15635	7BS	68.9	68.9	1.06E-05	0.0413	8.33E-03	0.0323	Ithaca ‘11
GBS_18690	7BS	68.9	68.9	1.04E-06	0.1093	1.33E-03	0.0348	Wellington ‘12
GBS_23290	7BS	68.9	68.9	4.24E-05	0.0316	8.74E-03	0.0448	Njoro ‘12 off
GBS_23290	7BS	68.9	68.9	5.31E-06	0.0377	7.10E-03	0.059	Njoro ‘12 cmbd
GBS_371	7BS	68.9	68.9	6.75E-03	0.0158	5.04E-03	0.052	Ithaca ‘11
GBS_4088	7BS	68.9	68.9	2.18E-06	0.0421	4.55E-03	0.0402	Njoro ‘12 off
GBS_4088	7BS	68.9	68.9	8.02E-08	0.052	1.35E-03	0.0321	Njoro ‘12 cmbd
GBS_11611	7DS	94.3	94.3	2.60E-04	0.0195	4.48E-04	0.03286	Ithaca ‘11

*****cmbd – combined data



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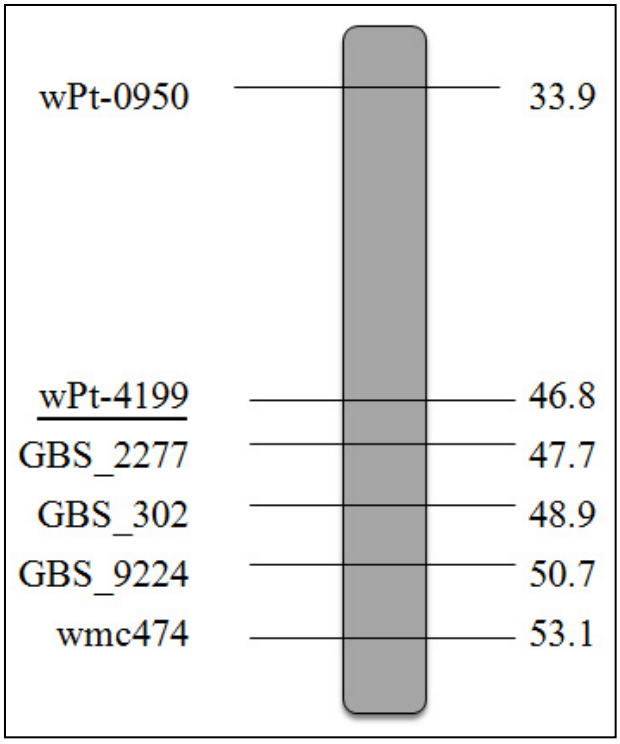
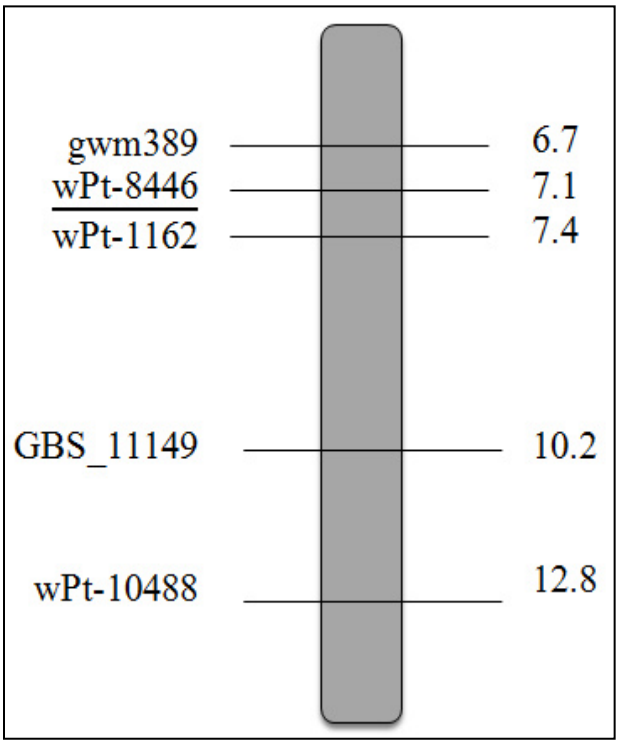


2BL



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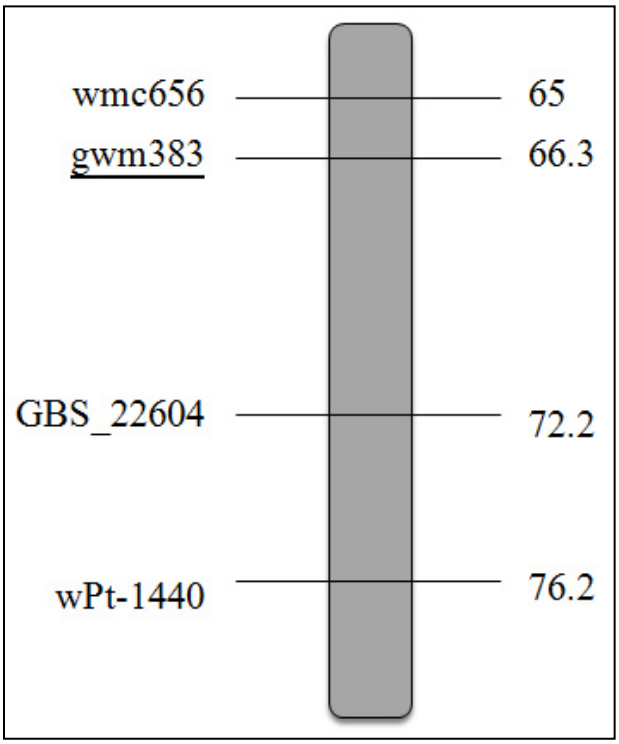

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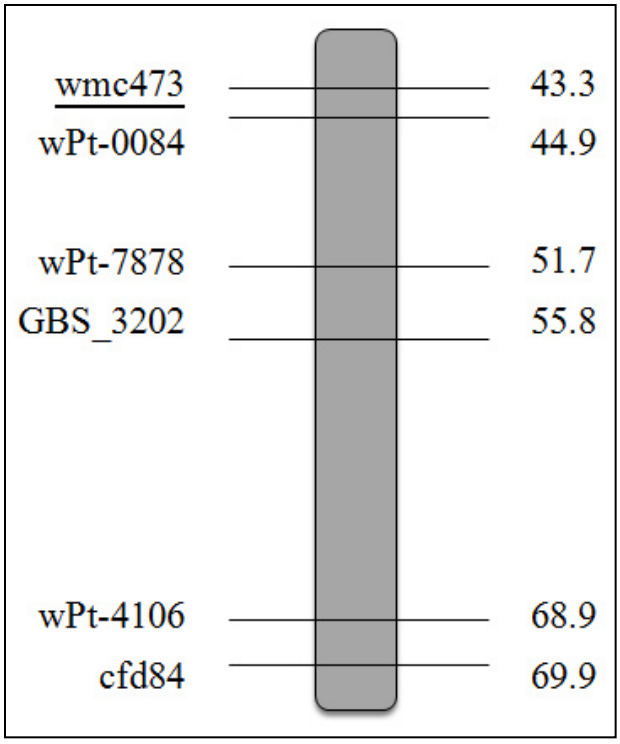
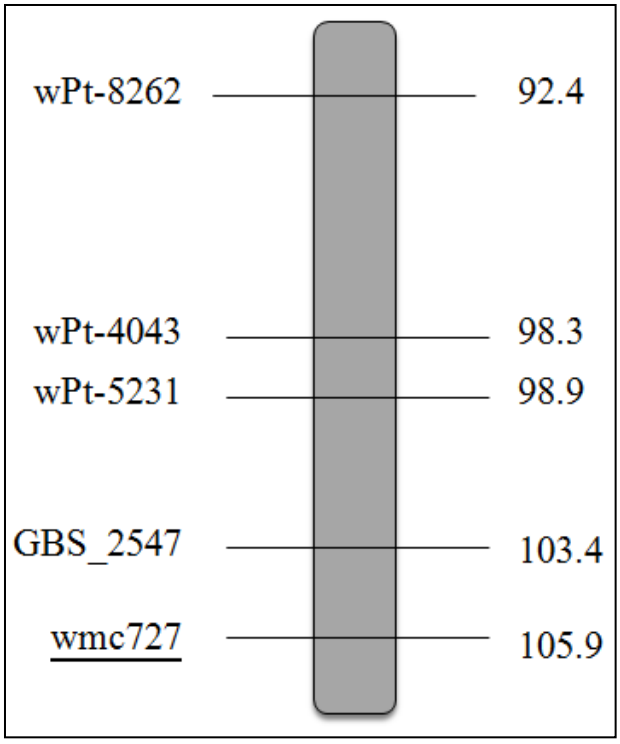

wmc473
43.3
wPt-0084
44.9
wPt-7878
51.7
GBS_3202
55.8
wPt-4106
68.9
cfd84
69.9

4DL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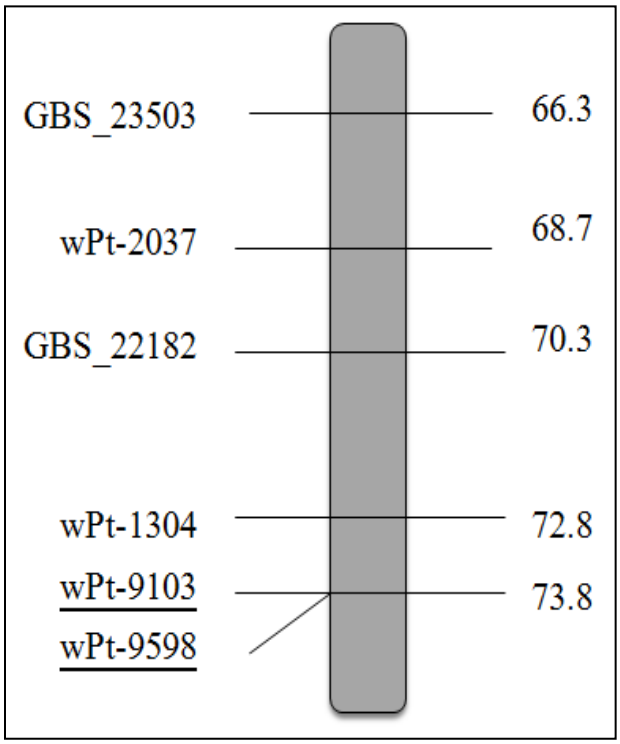
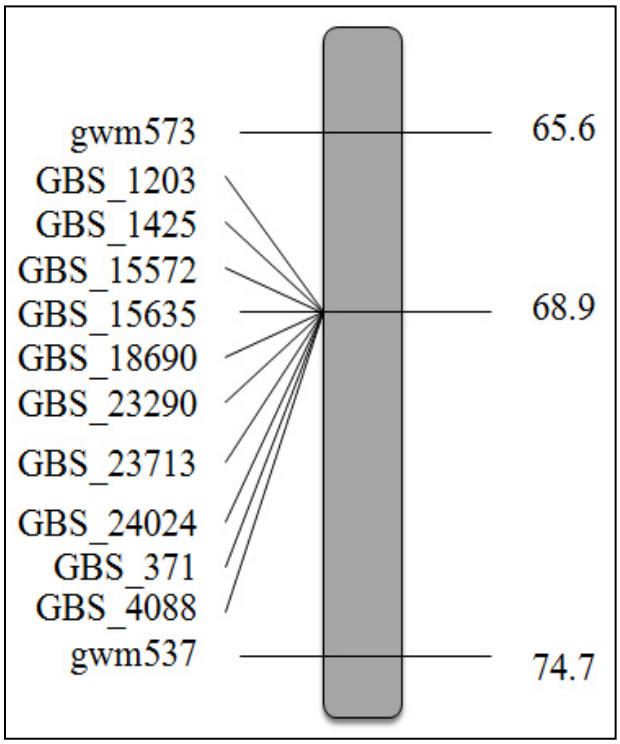
92.4
wPt-4043
98.3
wPt-5231
98.9
GBS_2547
103.4
wmc727
105.9

5A


GBS_23503
66.3
wPt-2037
68.7
GBS_22182
70.3
wPt-1304
72.8
wPt-9103
73.8
wPt-9598

5B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65.6
GBS_1203
GBS_1425
GBS_15572
GBS_15635
68.9
GBS_18690
GBS_23290
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GBS_24024
GBS_371
GBS_4088
gwm537
74.7

7BS

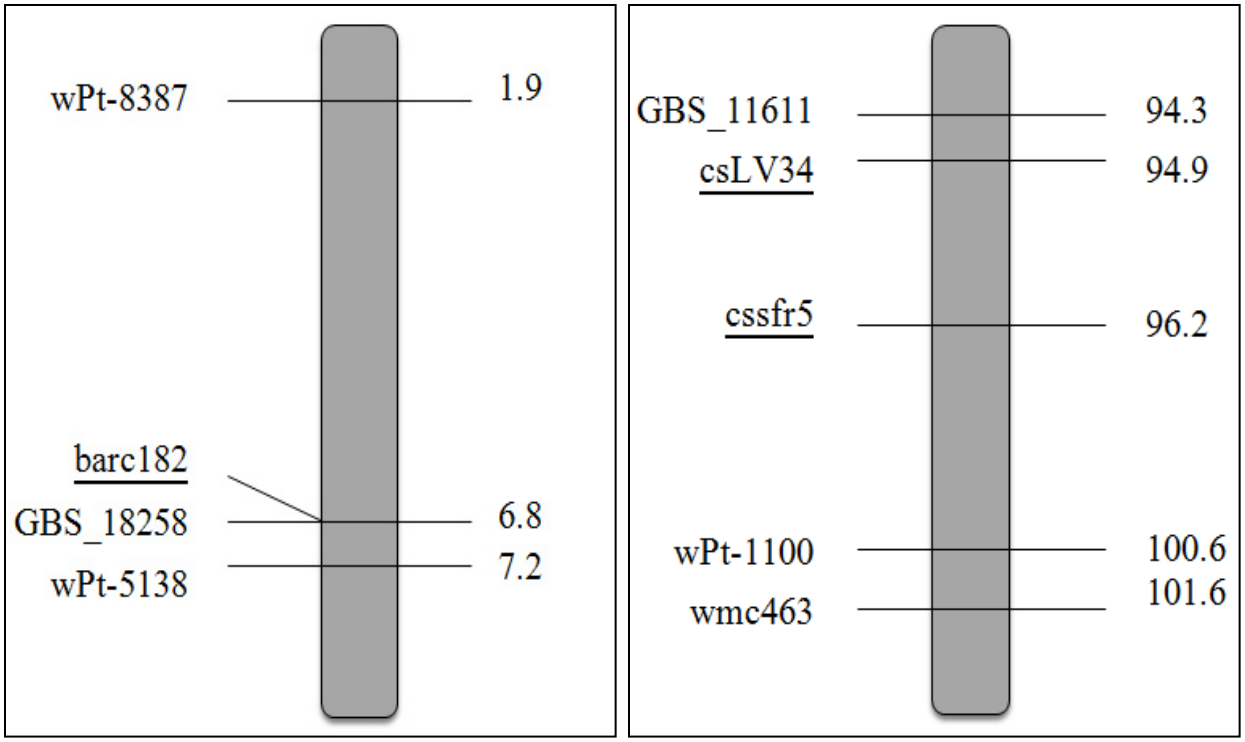


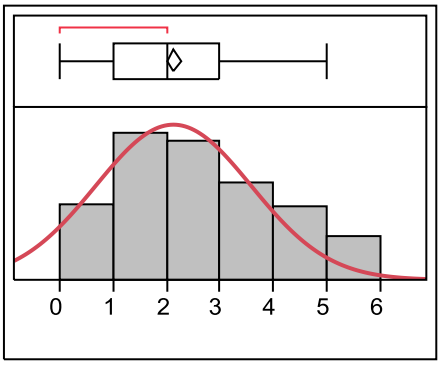
Figure 19 Chromosome positions of significant markers associated with LTN

Association mapping for pseudo black chaff

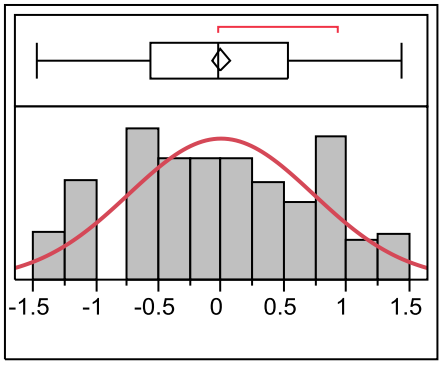
3.6.Statistical analysis of PBC data

3.6.1. Statistical analysis of PBC data for the lines evaluated at Njoro

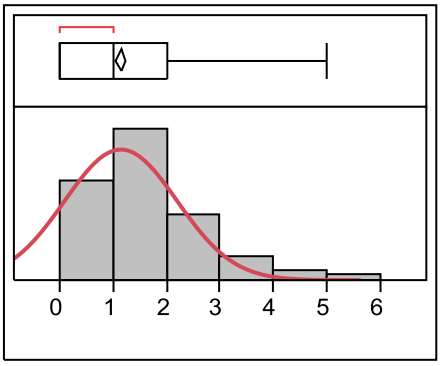
A total of 504 lines were evaluated in Njoro, out of which only 464 lines could be scored for PBC in the main season. The mean PBC score was 2.12 $\pm$ 1.44 in the main season, whereas it was 1.14 $\pm$ 1.04 in the off season. ANOVA indicated that families contributed significantly to PBC in both the seasons (p-value of <.0001*). The families explained 57.29% of the variability in the PBC in the main season and 37.9% of the variability in PBC during the off season.



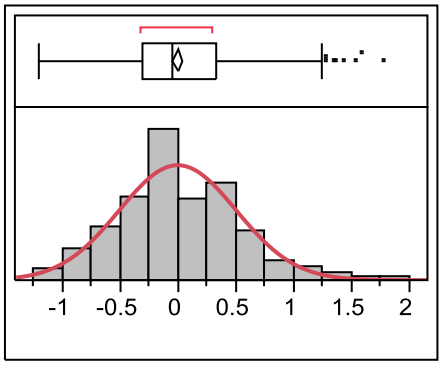
PBC scores (Njoro main season)



Transformed PBC scores (Njoro main season)



PBC scores (Njoro off season)



Transformed PBC scores (Njoro off season)

Figure 20 Distribution of PBC scores for the lines evaluated in Njoro (2012)

Table 9 Analysis of Variance (PBC scores vs family) for the population evaluated in Njoro (main season)

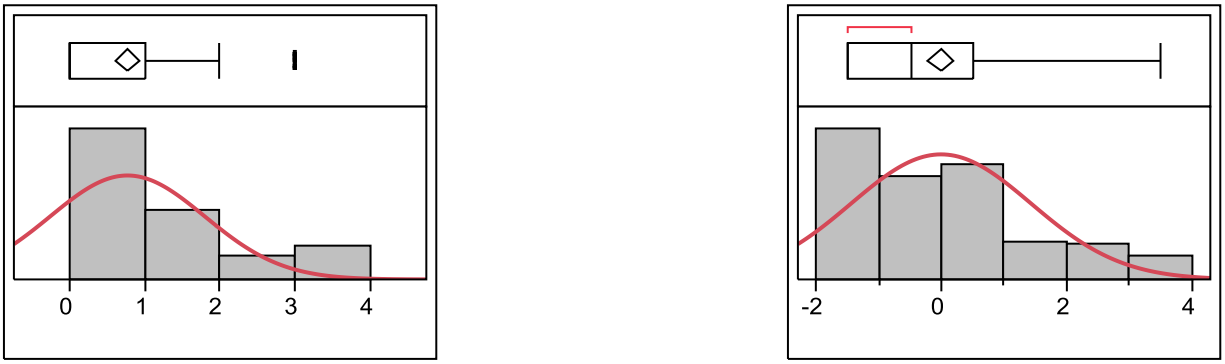
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	77	202.10498	2.62474	5.4850
Error	372	178.01329	0.47853	**Prob > F**
C. Total	463	380.11827		<.0001*

Table 10 Analysis of Variance (PBC scores vs family) for the population evaluated in Njoro (off season)

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	86	163.56420	1.90191	2.9615
Error	417	267.80602	0.64222	**Prob > F**
C. Total	503	431.37022		<.0001*

3.6.2. Statistical analysis of PBC data for the lines evaluated at Wellington

The 200 lines evaluated in Wellington had a mean PBC score of 1.48 ± 1.43. Results of the Analysis of Variance indicated that families contributed significantly to PBC (p-value of <.0001*) explaining 56.68% of the variability in the trait.



PBC scores **Transformed PBC scores**

Figure 21 Distribution of PBC scores for the lines evaluated in Wellington

Table 11 Analysis of Variance (PBC scores vs family) for the population evaluated in Wellington 2012

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	123	197.07873	1.60227	3.5988

Error	68	30.27476	0.44522	**Prob > F**
C. Total	191	227.35350		<.0001*

3.7. Analysis of marker-trait associations for PBC

Several markers were significantly associated with PBC in the two different mapping panels. Only the markers that were significant both in the GLM and MLM were considered. After making adjustments for False Discovery Rate (FDR, Benjamini and Hochberg 1995) associated with multiple hypotheses testing, 15 unique GBS markers were identified including 11 markers in the population evaluated at Njoro, 6 markers in the population evaluated at Wellington, and 2 markers that were identified in both the studies. The cut-off value used for FDR was 0.05 in the case of GLM but it was relaxed to 0.1 in the case of MLM to reduce the number of rejected true positives. The markers significant in the different analyses are given in Table 12. The interpretation for the significant markers below is based on their MLM p-values only. The position of the markers significant in the present study was determined using the Synthetic x Opata map and compared with the positions of the markers detected in previous studies using GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml). Nine GBS markers in three chromosomes were significantly associated with PBC in the Njoro main season. The most significant marker among them was GBS_22809 (p = 1.11E-14) on chromosome 3BS followed by markers GBS_11007, GBS_11008, GBS_2495, GBS_24916, GBS_10791, GBS_11149 (which are also on chromosome 3BS), GBS_11611 (chromosome 7DS) and GBS_12121 (chromosome 2BL). In the Njoro off season, only five significant markers in two chromosomes were obtained. GBS_22809 (p = 1.06E-06) on chromosome 3BS was the most significant marker in this case too, followed by markers GBS_2495, GBS_10791, GBS_24916 (chromosome 3BS)

and marker GBS_23424 (chromosome 2DS). In the combined analysis of the Njoro data, seven significant markers in two chromosomes were obtained. The marker GBS_22809 was once again the most significant (p=4.86E-14), followed by markers GBS_2495, GBS_24916, GBS_10791, GBS_11007, GBS_11008 (chromosome 3BS) and marker GBS_1229 (on chromosome 2DS). On the other hand, six GBS markers on five chromosomes turned out to be significant in the Wellington population. Among these, GBS_11007 was the most significant marker (p=4.07E-07), followed by marker GBS_22809 (chromosome 3BS), marker GBS_2022 (chromosome 4A), GBS_12038 (chromosome 2BL), GBS_5796 (chromosome 2DS) and GBS_25923 (chromosome 6AS).

The most significant markers observed in all the datasets were on the chromosome 3BS at the approximate location of *Sr2* (Figure 22), which confirmed the association of PBC with this gene (Hare and Macintosh 1979). These include GBS markers GBS_22809 (significant in the Njoro main and off seasons, Wellington main season and also in the Njoro combined analysis); GBS_11007 (significant in the Njoro main season, Wellington main season and also in the Njoro combined analysis); GBS_10791, GBS_2495 and GBS_24916 (significant in the Njoro main, off seasons and the combined analysis); GBS_11008 (significant in the Njoro main season and the combined analysis) and GBS_11149 (significant in the Njoro main season alone). These markers span an interval of approximately 10cM (7.1 to 17cM). The DArT marker, wPt8446 that was 11cM away from cs*Sr2* and flanks the *Sr2* locus (Yu et al. 2011) is at the same location (7.1cM) as GBS_22809, the most significant marker in the present study. Thus, the chromosomal locations of these six other GBS markers indicate that they are linked to the gene *Sr2*/*Pbc* and could be used for haplotyping it.

On chromosome 2BL, two GBS markers namely GBS_12038 and GBS_12121 were found to be significant in the Njoro main season and in Wellington respectively. These markers were located in the same chromosomal location at 35.7cM (Figure 22). Previous associations of PBC with DArT markers wPt-5672, wPt-5556 and wPt-7757 on chromosome arm 2BL were reported by Yu et al. (2011). But the region corresponding to these markers in the Synthetic x Opata map was not significant in the present study. Instead, these markers were approximately 5cM away from the stem rust resistance/cs*Sr2* interacting loci (wPt-8460) identified by Yu et al. (2011). Although the exact position of wPt-8460 could not be located in the Synthetic x Opata map, its comparative position was determined using the marker wPt-9736 that is 3cM proximal to wPt-8460 (CIMMYT integrated map) and also located approximately 5cM away from the significant GBS markers in the Synthetic x Opata map. A stem rust resistance locus in a similar location was also reported by Bhavani et al. (2011) and Kolmer et al. (2011) in different populations. Chromosome 2BL has three known stem rust resistance genes namely *Sr9, Sr16, Sr28* (McIntosh et al. 2003). More recently, a putative gene, *SrWeb* which carries resistance to Ug99, (Hiebert et al. 2010a), SrGabo56 (Rouse et al. 2010) and *SrWLR* (Zurn et al. 2012) were identified. The SSR marker wmc332 which is approximately 4cM away from the GBS markers significant in the present study is informative because it flanks the *SrWLR* locus (Zurn et al. 2012); is 12.4 cM from *SrWeb* (Hiebert et al. 2010a), is the closest marker for the gene *Sr28* (Rouse et al. 2010) and is also the approximate position of the gene *Sr9a* (Tsilo et al. 2007). But the position of these loci in relation to the markers significant in the present study could not be confirmed due to the lack of fine mapping information for these genes.

On the chromosome 2DS, three markers were significant, namely GBS_1229 (Njoro combined analysis), GBS_5796 (Wellington) and GBS_23424 (Njoro off season). These markers

span an interval of 4.5cM from 38.3cM to 42.8cM (Figure 22) and must be identifying the same loci. Bariana et al. (2001) observed a region on chromosome 2D that enhanced PBC expression in one season. As the location of this region is unknown, it was not possible to compare it with the present study. Among the known stem rust resistance genes on chromosome 2DS, *Sr6* was located close to the region significant in the present study. This gene has been mapped close to the DArT marker XwPt_0330 within a distance of 6.5 cM (Tsilo et al. 2010). Although this marker was not present in the Synthetic x Opata map, the marker wPt-3728 which is about 1cM proximal to this marker (CIMMYT integrated DArT map) is found in the Synthetic x Opata map, about 8cM away from the markers significant in the present study. Hence, it is likely that these GBS markers correspond to the location of *Sr6* gene region or might correspond to a novel region affecting the expression of PBC.

Chromosome 4A had only one significant marker namely GBS_2022 (Wellington) located at 78cM (Figure 22). Previous studies by Yu et al. (2011) identified two loci on chromosome 4A using DArT markers wPt-5857 and wPt-5825 that were significantly associated with PBC. The marker significant in the present study is 0.3 cM away from the marker wPt-5857 identified by Yu et al (2011) and hence it should be identifying the same region. On chromosome 7D, the marker GBS_11611 was significant for PBC in the Njoro main season. This marker is 0.6 cM away from csLV34 (the marker tightly linked to *Lr34*) and 1.9cM away from cssfr5 (the gene specific marker for *Lr34*) (Figure 22). A QTL named as QPbc.sun-7DS close to *Lr34* was reported to enhance PBC by Kaur et al (2009). The marker GBS_25923 was significant for PBC on the chromosome 6AS at 85cM (Figure 23). Bariana et al. (2001) also identified a PBC enhancing region on chromosome arm 6AS. The microsatellite marker gwm334 which is close to the PBC loci identified by Bariana et al (2001) is close to the markers significant in the present

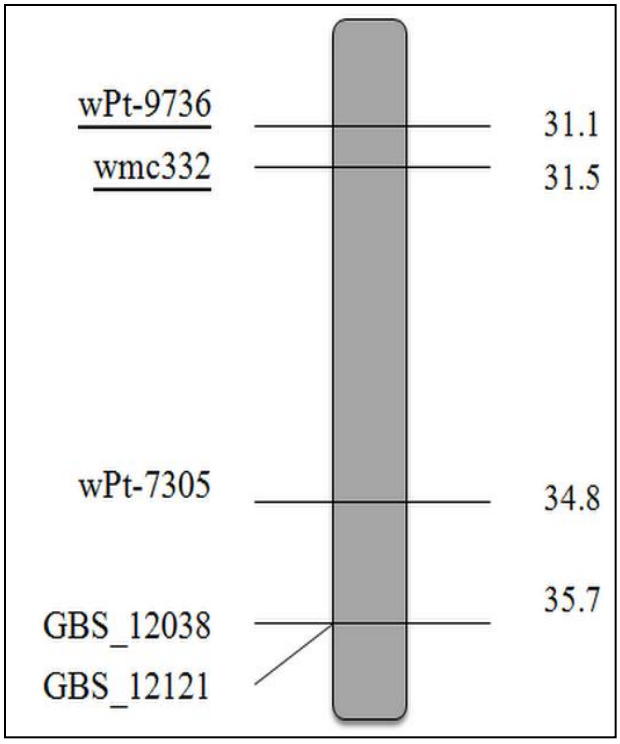
study (unable to find the exact location). But the role of this region in enhancing PBC is unclear as no stem rust resistance genes are located in this region.

Table 12 Markers significantly associated with PBC in the different populations

Marker	Chromosome	Pos_snp_Start	Pos_snp_End	Marker P value (Q)	Marker R2 (Q)	Marker P value (Q+K)	Marker R2 (Q+K)	Location
GBS_12038	2BL	35.7	35.7	6.15E-09	0.15727	9.23E-04	0.03207	Wellington
GBS_12121	2BL	35.7	36	2.99E-03	0.05025	9.62E-04	0.0462	Njoro main
GBS_1229	2DS	38.3	40.2	5.14E-04	0.0236	3.48E-03	0.01712	Njoro cmbd
GBS_5796	2DS	39.2	39.2	3.41E-04	0.06295	1.17E-03	0.05458	Wellington
GBS_23424	2DS	42.8	42.8	2.78E-04	0.02581	8.30E-03	0.01402	Njoro off
GBS_22809	3BS	7.1	7.1	1.04E-33	0.27346	1.11E-14	0.13925	Njoro main
GBS_22809	3BS	7.1	7.1	6.45E-14	0.10527	1.06E-06	0.04874	Njoro off
GBS_22809	3BS	7.1	7.1	2.36E-31	0.23527	4.86E-14	0.11963	Njoro cmbd
GBS_22809	3BS	7.1	7.1	2.54E-21	0.36517	2.70E-06	0.11756	Wellington
GBS_11007	3BS	10.2	12.8	1.11E-23	0.19723	2.63E-07	0.05957	Njoro main
GBS_11007	3BS	10.2	12.8	2.31E-19	0.14799	1.27E-05	0.03863	Njoro cmbd
GBS_11007	3BS	10.2	12.8	1.28E-21	0.36951	4.07E-07	0.13833	Wellington
GBS_11008	3BS	10.2	11.2	2.75E-24	0.20204	3.33E-07	0.05851	Njoro main

GBS_11008	3BS	10.2	11.2	6.63E-18	0.13676	1.57E-04	0.02883	Njoro cmbd
GBS_11149	3BS	10.2	12.8	7.70E-12	0.09727	5.93E-04	0.02608	Njoro main
GBS_10791	3BS	11.5	11.8	3.82E-17	0.14338	4.63E-06	0.04687	Njoro main
GBS_10791	3BS	11.5	11.8	2.70E-09	0.06762	2.21E-03	0.01889	Njoro off
GBS_10791	3BS	11.5	11.8	3.83E-18	0.1386	6.43E-06	0.04133	Njoro cmbd
GBS_2495	3BS	11.5	11.8	1.15E-19	0.16455	5.45E-07	0.05632	Njoro main
GBS_2495	3BS	11.5	11.8	5.97E-11	0.08125	1.31E-04	0.02966	Njoro off
GBS_2495	3BS	11.5	11.8	5.77E-21	0.16019	1.70E-07	0.05593	Njoro cmbd
GBS_24916	3BS	17	23	2.29E-15	0.12817	6.68E-07	0.05541	Njoro main
GBS_24916	3BS	17	23	2.57E-07	0.05117	2.71E-03	0.01813	Njoro off
GBS_24916	3BS	17	23	1.16E-14	0.11125	1.51E-06	0.0471	Njoro cmbd
GBS_2022	4A	78	78	3.81E-10	0.18008	2.15E-05	0.09542	Wellington
GBS_25923	6AS	85	86	2.76E-05	0.0852	3.96E-03	0.04279	Wellington
GBS_11611	7DS	94.3	94.3	2.75E-04	0.02851	8.76E-04	0.02446	Njoro main

*cmbd – combined data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GBS_12121
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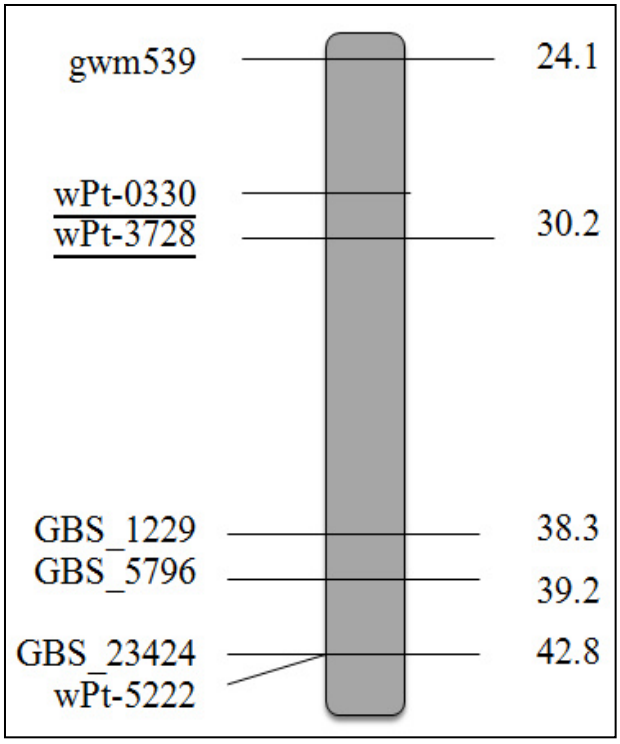
34.8
35.7

2B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wPt-3728
GBS_1229
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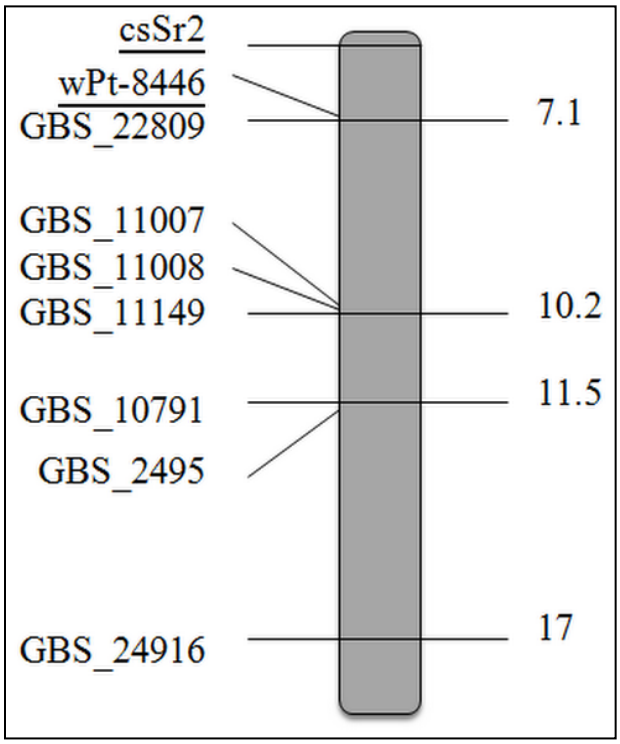
39.2
42.8

2D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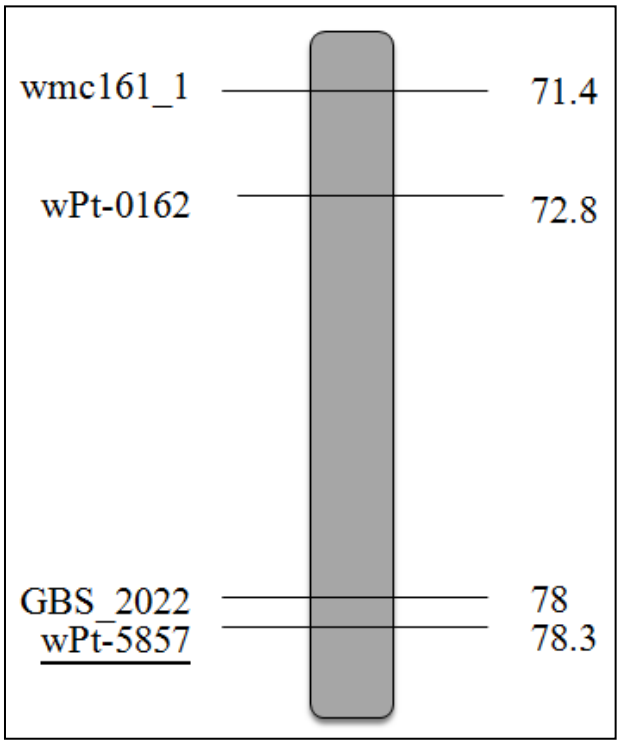
GBS_11007
GBS_11008
GBS_11149
GBS_10791
GBS_2495
GBS_24916
7.1
10.2
11.5
17

3B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78
78.3

4A

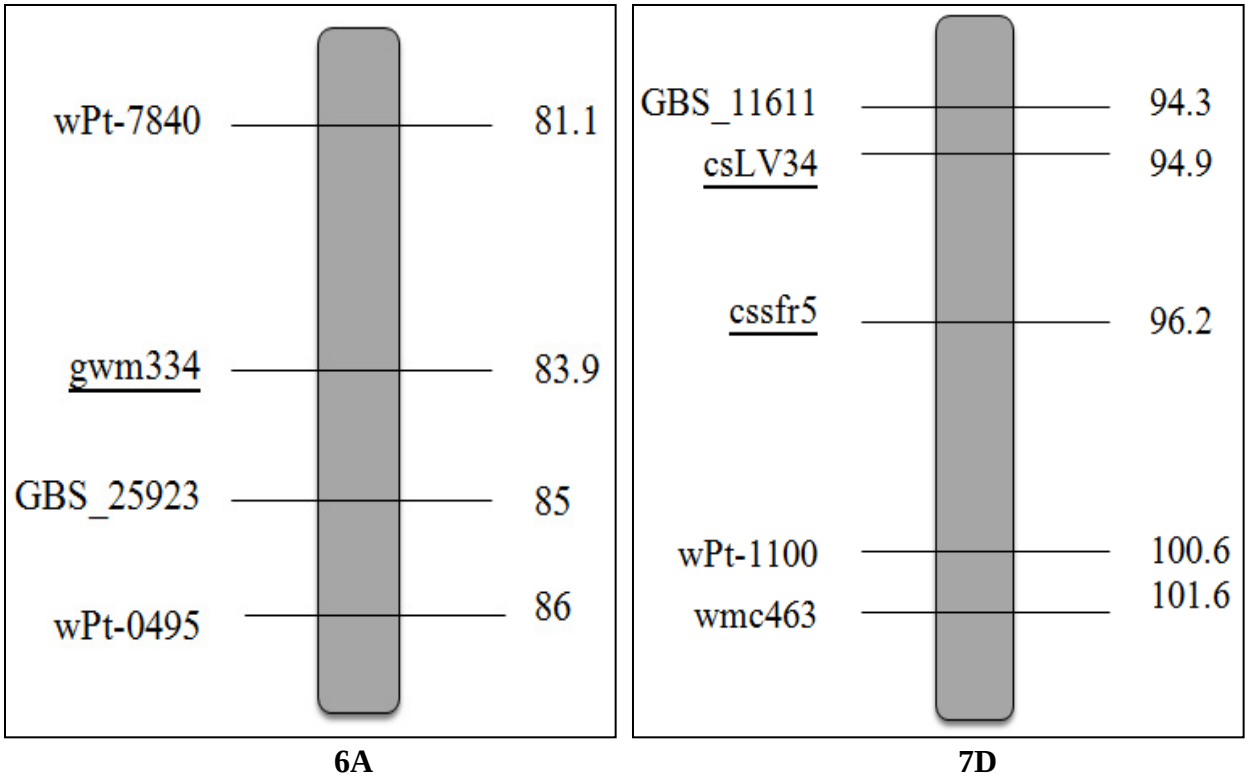

Figure 22 Chromosome positions of significant markers associated with PBC

3.8. Loci associated with both LTN and PBC

The marker GBS_11611 close to the *Lr34* loci on chromosome 7DS was significantly associated with LTN and also with PBC in one environment (Table 13). Similarly, the marker GBS_11149 close to the *Sr2* locus on chromosome 3BS was significantly associated with PBC and also with LTN in the Njoro warmer temperatures (Table 13). This indicates that there exists an association either between these traits or between the genes conferring these traits. But the lack of consistency in the different environments again indicates the high environmental dependence of these traits.

Table 13 Markers significantly associated with both LTN and PBC

Marker	Chr	Pos_ snp_ start	Pos_ snp_ end	Marker P value (Q)	Marker R^2 (Q)	Marker P value (Q +K)	Marker R^2 (Q + K)	Location
GBS_11149	3BS	10.2	12.8	1.59E-12	0.10721	3.58E-04	0.01706	Njoro '12 main LTN
GBS_11149	3BS	10.2	12.8	2.00E-06	0.04243	9.31E-03	0.03019	Njoro '12 off LTN
GBS_11149	3BS	10.2	12.8	3.03E-14	0.10128	4.35E-05	0.01308	Njoro '12 cmbd LTN
GBS_11149	3BS	10.2	12.8	7.70E-12	0.09727	5.93E-04	0.02608	Njoro main PBC
GBS_11611	7DS	94.3	94.3	2.60E-04	0.0195	4.48E-04	0.03286	Ithaca '11 LTN
GBS_11611	7DS	94.3	94.3	2.75E-04	0.02851	8.76E-04	0.02446	Njoro main PBC

*cmbd – combined data

CHAPTER 5

DISCUSSION

5.1. Association mapping for leaf tip necrosis

A total of 21 unique GBS markers scattered across nine chromosomal locations (1BL, 2BL, 3BS, 3D, 4DL, 5A, 5B, 7BL, 7BS, 7DS) were significantly associated with LTN. The loci detected for these traits varied among locations, as reported in previous studies (Messmer et al. 2000, Schnurbusch et al. 2004b), although some of them were significant in more than one analysis. The inconsistency suggests that a more accurate technique is needed for evaluating this trait. The loci that were detected in a previous study by Messmer et al. (2000) but not in the present study included loci on chromosomes 1A, 1BS, 2A, 3A, 4B and 5DL. Similarly, the regions identified by Schnurbusch et al. (2004b) but not in this study included loci on chromosomes 2DL, 4BS and 6AL. Based on this study, the genes/regions associated with the expression of LTN, can be placed into three groups:

(i) Major genes that can express LTN independently and/or in combinations

The association of the slow-rusting, durable leaf rust resistance genes *Lr34*, *Lr46*, *Lr67* and *Lr68* with leaf tip necrosis was confirmed in this study. These genes have been known to confer LTN independently in various studies (Singh 1992a; Rosewarne et al. 2006; Hiebert et al. 2010b; Herrera-Foessel et al. 2012). However, these genes may sometimes act additively with other genes in combinations to confer enhanced LTN. Although *Lr34* was the major gene associated with LTN, a marker close to this region on 7DS was detected only in the population evaluated at Ithaca. Similarly, markers close to the genes *Lr67* and *Lr68* on chromosome 4DL and 7BL

respectively were detected only in the Wellington location. For the *Lr46* gene on chromosome 1BL, significant markers were detected both in Ithaca and Wellington. None of these genes were associated with LTN in the population evaluated in Njoro. This confirms reports that the expression of LTN is enhanced at lower air temperatures (Risk et al. 2012).

(ii) Genetic loci that can additively interact to enhance LTN

Some of the genetic loci detected in this study acted in an additive manner to confer LTN similar to the observations of Messmer et al. (2000). One locus each on chromosomes 2BL and 5B that were significant for LTN were found to be close to the regions that were previously identified to interact with the *Lr34* gene (Kolmer et al. 2011; Yu et al. 2011). The region on 2BL (which was consistently observed in all the environments) is suspected to be the seedling resistance gene, *Lr13* and previous studies have also observed its interaction with *Lr34* in conferring LTN (Singh 1992; Singh 2007). But the inability of the *Lr13* gene to confer LTN independently was also observed in those studies. Another *Lr34* interacting locus on chromosome 5B previously observed by Yu et al. (2011) was detected in this study (Njoro and Wellington populations), although its role is not understood. Interaction between *Lr34* and other seedling rust resistant genes has been suggested to be a mechanism contributing to durable rust resistance (Sawhney 1992; German and Kolmer 1992). Can the genes/ loci detected in the present study be a part of the durable rust resistance '*Lr34* complex', which confers LTN is a question to be answered.

(iii) Other regions whose role in conferring LTN is unknown

The 7BS region that was consistently identified in all the seasons and locations is interesting as its association with LTN has not been commonly observed. Only one study by Li et al. (2012) in a Chinese wheat breeding line has identified the involvement of this region in reducing LTN. Further studies are required to confirm the association of this region with LTN. The other region of interest that has been observed in both the Njoro seasons but not in any other location is the locus close to the *Sr2* gene on chromosome 3BS. Detected in two previous studies earlier (Messmer et al. 2000; Schnurbusch et al. 2004b), this region was detected in this study too, thus confirming its association with LTN. But the fact that this region was associated with LTN only in the warmer temperatures of Njoro raises the question if the genetic basis of LTN might be different in different environments. The only parallelism that exists between the major genes influencing LTN and the gene *Sr2* is that both are durable multi pathogen resistance regions. As the gene *Sr2* has neither been cloned nor is its functionality known, the role of this gene in affecting LTN is not known. The other region with unknown role in conferring LTN is on chromosome 3D (identified in the population evaluated at Ithaca) and is suspected to be the gene *Lr24,* although it could be a novel locus as well. If the assumption that this gene is *Lr24* is true, then the only possible explanation why it might contribute to LTN is that this locus is pleiotropic to a gene conferring stem rust (*Sr24*) which is similar to the other genes conferring LTN (McIntosh et al. 2011). The other region associated with LTN in the Wellington population alone is on chromosome 5AL. As no catalogued rust resistance gene has been previously reported in this region, its role in contributing to LTN is unknown.

5.2. Association mapping for pseudo black chaff

In this study, 15 unique GBS markers scattered across 6 chromosomal locations (2BL, 2DS, 3BS, 4A, 6AS, and 7DS) were significantly associated with PBC. Among these, only the loci on chromosomes 3BS, 2BL and 2DS were consistent in all the analysis. The other regions were significant in only one location. Although, all these regions were detected in previous studies, the only region which was identified in a study by Kaur et al. (2009) but not in the present study was a minor QTL on chromosome 5DL, suggested to be the *Sr30* region. The regions significant in this study can be broadly classified into two:

(i) Major gene conferring PBC

Highly consistent and significant association of PBC to several markers located in the 3BS region, near the *Sr2* gene confirms that it is the major gene conferring PBC. This observation is in accordance with all the studies on PBC so far (Kuspira et al. 1958; Waldron 1929; Mc-Fadden 1939; Pan 1940; Sheen et al. 1968; Hare and McIntosh 1979; Brown 1997; Kota et al. 2006; Singh et al 2011). As the *Sr2* gene has neither been cloned, nor its function known, the mechanism behind its association with PBC is not clear. However, the markers identified in this study can be validated to select for the *Sr2* gene.

(ii) Other loci whose role in the expression of PBC is unknown

The involvement of additional loci in conferring PBC has been reported by Bariana et al. (2001) and Yu et al. (2011). A common underlying mechanism contributing to both PBC and stem rust resistance was also suggested by Yu et al. (2011) with the level of PBC expression

varying with the degree of resistance conferred. In this study too, a major stem rust resistance region on chromosome 2BL was associated with PBC consistently. As this region was close to several stem rust resistance genes including *SrWLR, SrWeb, Sr28* and *Sr9a,* the contributions of these genes to PBC could not be ascertained in this study. One possibility is that this region affecting PBC might be the *Sr9* gene, as it is suspected to be a part of the '*Sr2* complex' and confers high levels of resistance when present together with *Sr2* (McIntosh et al. 1995; Knott 1968). The *Sr9* is a very unique gene as it is multi-allelic and recently one of its alleles was found to confer resistance to Ug99 (Letta et al. 2013). However, further studies are needed to confirm this hypothesis.

The other locus that was consistently detected in all the analyses is the 2DS region. Although, this region could not be precisely assigned to any major stem rust resistance gene, it is putatively thought to be the gene, *Sr6*. The slow rusting nature of this gene was previously suggested by Cox and Wilcoxson (1982), but virulence to this gene has been observed in some regions. Nevertheless, *Sr6* confers a high level of resistance against most stem rust races in North America (Leonard 2001, Tsilo et al. 2010) and was one of the main factors for defeating the race15B epidemics during the 1950s (Kolmer 2001). This broad spectrum resistance makes it analogous to the *Sr2* gene although it has never been observed to confer PBC monogenically in any study. Hence, the region significant in this study might correspond to this gene or a novel locus. A region on chromosome 4A was also significant for PBC only in the Wellington population. Although Yu et al. (2011) identified two loci associated with PBC on this chromosome, only one of them was detected in this study. As this chromosome does not have any catalogued stem rust resistance gene, this locus is only associated with PBC and not rust resistance. The region on chromosome 6AS that was identified in the Wellington population

alone, was approximately at the same location as the PBC QTL identified by Bariana et al. (2001). This region was not associated with any catalogued stem rust resistance gene locus. Finally, a locus on chromosome 7DS close to the *Lr34* region was also associated with the expression of PBC in the Njoro main season. Although the role of *Lr34* in enhancing PBC is unknown, given that *Lr34* is an ABC transporter, it might play a role in transporting the substance which causes the black pigmentation. Further differential expression studies to understand more on the genes conferring this trait is needed.

An interesting observation in this study is that the *Lr34* loci on chromosome 7DS and the *Sr2* loci on chromosome 3BS confer both LTN and PBC under certain environmental conditions. Although the actual role of these loci in conferring LTN and PBC is unknown, there is a possibility that the *Lr27* gene on the *Sr2/Lr27/Yr30* locus might confer LTN and the *Sr57* gene on the *Lr34/Yr18/Sr57/Pm38/Sb1/Bdv1* locus might confer PBC. Further studies are required to examine what degree of resistance could be achieved when both these traits co-occur. In conclusion, the potential of association mapping was successfully applied in this study to identify genetic loci associated with leaf tip necrosis and pseudo black chaff - the two most valuable traits associated with genes conditioning durable rust resistance. Besides, the application of the high throughput Genotyping by Sequencing technology (GBS) to dissect complex traits by association mapping was also successfully demonstrated. The results of this study indicate the oligogenic (despite being monogenic in some cases) nature of these traits and hence it is suggested that it is not possible to eliminate them while breeding for durable rust resistance. Further efforts to clone the durable rust resistance genes might provide a better insight of the genetic control of these traits.

CHAPTER 6

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