# MAKERERE UNIVERSITY

## BREEDING FOR RESISTANCE TO STEM RUST IN BREAD WHEAT

**By**

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**Declaration**

I declare that this study is original and has never been presented for examination for a degree or any award in any University. However, any sources of information are duly acknowledged.

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**Preamble**

This thesis is based on the following scientific papers:

i. Nzuve FM, Bhavani S, Tusiime G, Njau P, Wanyera R (2012). Evaluation of bread wheat for both seedling and adult plant resistance to stem rust. African Journal of Plant Science. 6 (15): 426-432

ii. Nzuve FM, Tusiime G, Bhavani S, Njau P, Wanyera R (2013). Studies of the Genetics of Inheritance of Stem Rust Resistance in bread Wheat. African Journal of Biotechnology. 12 (21): 3153-3159

iii. Manuscript in preparation: Mapping of Adult Plant Resistance in the PBW343/Juchi RIL bread wheat population

**Abstract**

Stem rust disease caused by *Puccinia graminis* f. sp. *tritici* (Eriks and E. Henn) poses the greatest threat to global wheat production due to the emergence of a highly virulent race of the *Puccinia graminis* f. sp. *tritici* Ug99 race and its variants. This study was set out to a) to identify sources of resistance to stem rust in bread wheat b) to determine the genetics of the stem rust resistance genes in bread wheat and c) to identify molecular markers and the genomic regions (quantitative trait loci, QTL) harbouring the stem rust resistance genes in bread wheat. Twenty-five wheat genotypes designated as "KSL"were evaluated for both field and seedling resistance at Kenya Agricultural Research Institute (KARI), Njoro. The most resistant wheat genotypes KSL-2, KSL-3 and KSL-20 also exhibited the pseudo black chaff (PBC) trait suggesting presence of the *Sr2* gene. The presence of trace responses in field tests and resistant infection types during the seedling tests suggested involvement of major genes in conditioning the stem rust resistance. For the inheritance studies, five promising stem rust resistant parents namely KSL-2, KSL-3, KSL-5, KSL-12 and KSL-19 were crossed in a partial diallel to the susceptible parent CACUKE and advanced into $F_2$ derived $F_3$ families. The purpose of the inheritance study was to establish the number and mode of inheritance of the stem rust resistance genes among these parents. The Chi square analysis revealed that the segregation data for KSL-2 consistently followed the 1:2:1 genetic ratio in two seasons implying that one dominant gene conditioned the stem rust resistance in this parent. The parents KSL-3 and KSL-19 consistently followed a 12:3:1 genetic ratio implying that two epistatic genes conditioned the resistance to stem rust. The knowledge of the nature and the number of genes revealed among the wheat parents form a core component of the wheat breeding program in ensuring an efficient breeding strategy. In conclusion, the

resistance genes associated with this resistance could be identified through further dissection of the QTLs to elucidate the exact gene effects and their chromosomal locations. The population could also be advanced further into recombinant inbred lines (RILs) for marker assisted selection (MAS) and quantitative trait loci (QTL) mapping. The QTL mapping of PBW343/Juchi $F_6$ recombinant inbred line (RIL) population for adult plant resistance to stem rust involved the use of Diversity Array genotyping Technology (DArTs). The resistance to stem rust in PBW343/Juchi was conditioned by both epistatic and additive genes and this could be exploited through MAS, gene pyramiding and other breeding strategies to transfer these QTLs into elite wheat backgrounds. In this research, three new DArT markers namely wPt-9493, tPt-9767 and tPt-6872 were identified and these markers could be used in the co-selection and improvement of important traits through MAS.The QTL mapping also revealed eight QTLs flanked by different DArT markers. Positional cloning of genes and QTLs through fine mapping within the gene-rich regions of bread wheat could lead to identification of more Adult plant resistance (APR) QTLs for better detection, mapping and estimation of gene effects. Also, the QTLs could be used to develop reliable markers for marker assisted breeding.

# CHAPTER ONE

## 1.0 General Introduction

### 1.1 Origin and Taxonomy of Wheat

Wheat (*Triticum* species) originated from the Fertile Crescent region of the Near East in Southwestern Asia (Kingfisher, 2004). The domestication of wheat was achieved through continuous cultivation and selection of mutant wheat forms which have limited capacity to propagate in the wild (Smith, 1995). Two common species of wheat include *Triticum aestivum* (bread wheat) and durum wheat *T. turgidum* ssp. Durum. *Triticum aestivum* comprises 95% of wheat grown in the world (Belderok *et al*., 2000).

The wheat genome compri*se*s of three ploidy levels; diploid (2n=2x=14); tetraploid (2n=2x=28); and hexaploid (2n=6x=42 (Pumphrey *et al*., 2009; Hancock, 2004). The tetraploid and hexaploid genomes are allopolyploids since they contain dissimilar genomes. The tetraploid wheat arose from the natural crossing between *Triticum monococcum* (AA) and *Triticum speltoides* (BB). The hexaploid wheat arose from the hybridization of tetraploid wheat (AABB) with wild relative (DD) - a grass *Triticum tauschii* followed by the doubling of chromosome (Belderok *et al*., 2000). Hexaploid wheat behaves as a diploid during meiosis leading to pairing of homologous chromosomes. This is due to the presence of a gene on chromosome 5B called *Ph1* which causes homologous pairing within the same genome.

### 1.2 Importance of wheat

Wheat is used as a major ingredient in food products, a major market commodity which is internationally traded as a cash crop. Wheat is also used for livestock and poultry feed as a by-product of the flour milling industry while wheat straw is used

for livestock bedding. It is grown on about 17% of global crop acreage and 40% of the world population depends on wheat for food (Gupta *et al*., 2008). Globally, wheat is grown on 215 million hectares producing about 630 million tons of grain annually valued at US$ 150 billion (Singh *et al*., 2011). Wheat provides on average, 21% of the total calorific and 20% protein input of the world's population (Reynolds *et al*., 2008; Gupta *et al*., 2008).

The developing countries produce 308 million tons of grain on 116 million hectares of wheat valued at about US$ 75 billion Wheat also provides 16% of total dietary calories in the developing countries (Dixon *et al*., 2009). About 4.5 billion people in 94 developing countries depend on wheat. Demand for wheat in these developing countries is increasing due to extensive urbanization and high population growth. The urban population growth is estimated to increase by 32% by 2050 (Weigand, 2011), thus calling for increased wheat production. It is estimated that current wheat production will have to increase at a rate of 2% per annum so as to meet human needs (Gill *et al*., 2004; Gupta *et al*., 2008). Currently African farmers produce about 10 to 25% of its wheat requirements. This quantity might unfortunately decrease given the adverse effects of climate change which could see 29% wheat grain decreases in production (Singh *et al*., 2008). Thus, to meet this demand, Africa needs to import 40 million tons of wheat annually valued at 18 billion US dollars. This trend has to change given the acute food insecurity, political instability and price shocks being experienced in Africa affecting the nutritional and national security at large (Negassa *et al*., 2012). In Kenya, wheat grows in different agro-ecological zones on 150, 000 ha. Formerly produced in large farms, small scale farmers have now taken up wheat production on smaller plots. However, the consumption of wheat at 750,000 metric

tons per year is higher than the current production of 350,000 metric tons in Kenya (Wanyera, 2008).

### 1.3 Wheat production constraints

Wheat production in the developing countries is constrained by the following; limited access to mechanization, low market preferences, poor infrastructure coupled with increased urbanization and decreased public sector investment in wheat production (Reynolds *et al*., 2008; Maredia and Eicher, 1995). The current climate changes and the natural resource degradation have led to limited water resources (Negassa *et al*., 2012). Biotic factors such as Russian wheat aphid, fusarium and the cereal rusts further limit wheat production in Kenya. The cereal rusts include stripe rust (caused by *Puccinia striiformis*); brown leaf rust (caused by *Puccinia recondita* f. sp. *tritici)* and stem rusts (caused by *Puccinia graminis* f. sp. *Tritici).*

### 1.4 Stem Rust Pathogenic Variability in Kenya

Currently, stem rust disease caused by *Puccinia graminis* f. sp. *tritici* (Eriks and E. Henn) threatens wheat production. In 1998, extraordinary susceptibility was noted among wheat materials grown in Uganda which were known to carry the *Sr31* gene (Bai *et al*., 2009; Ayliffe *et al*., 2008; Pretorius *et al*., 2000). The responsible stem rust race (Ug99) was later designated as TTKSK based on the North American pathotype nomenclature system (Wanyera *et al*., 2004). More stem rust resistant varieties have further succumbed to the Ug99 after evolution of the Ug99 (TTKSK) into more virulent forms TTTSK and TTKST due to added virulences to *Sr36* and *Sr24* respectively (Xu *et al*., 2009; Singh *et al*., 2008). The Ug99 pathogen spores have further spread from Uganda over vast areas causing epidemics in Kenya and Ethiopia (2002), Sudan, Iran and Yemen (2007). The disease is likely to spread further with a possible route proposed as East Africa – Middle East – West Asia – South Asia

(Singh *et al.*, 2006; Ayliffe *et al*., 2008). These new pathogenic races have emerged due to mutations and selection pressure of virulence against the few stem rust resistance genes and also the diversity of the *Puccinia graminis* f. sp. *tritici* over these stem rust resistance genes (Todorovska *et al*., 2009). More favourable conditions like the growing of wheat in different agro ecological zones in Kenya have also provided a green bridge for rust spores (inoculum) throughout the year (Singh *et al*., 2008; Saari and Prescott, 1985). The growing of wheat varieties with narrow genetic base makes most varieties grown in Kenya susceptible to the new races of *Puccinia graminis* (Beteselassie *et al*., 2007). This threatens the current and immediate future of wheat production in eastern Africa.

Along the proposed Ug99 spore path, about 50 million hectares of wheat is produced annually, feeding about one billion of the world's population (Singh *et al*., 2011). Additionally, about 80 to 90% of the global wheat cultivars have succumbed to the stem rust disease (Iqbal *et al*., 2010). Furthermore, stem rust is capable of turning a healthy looking crop into a tangle of black stems and shriveled grains at harvest time leading to yield losses in excess of 70% (Singh *et al*., 2008). Greater yield losses are bound to occur since the fungus intercepts nutrients' flow to the sink (head) and leads to very weak stems which lodge easily. Thus harvesting becomes difficult leading to reduced wheat yields (Leornard and Szabo, 2005; Xue *et al*., 2012). The developing countries will be greatly affected by the losses accruing from the Ug99 and its variants due to lack of jobs, increased rural-urban migration and declined economic growth. In the East African region, heavy yield losses of over 70% have been experienced by the small scale farmers who produce 20% of the wheat consumed in East Africa (Wanyera *et al*., ,2004). This loss can be higher if environmental

conditions favour the disease. For example, in 2007, Wanyera (2008) reported 100% yield losses among farmers in Kenya.

Most of the wheat farmers in the Ug99 spore path are resource poor who cannot afford chemical fungicides, spray equipments or the expertise to use them. Besides, the use of fungicides is not economically feasible in developing countries and is also environmentally unsafe especially if poorly used. Thus, the use of host resistance remains one of the feasible options in combating this imminent threat from Ug99 and its derivative variants. Host resistance breeding has integrated the use of race specific (major, single genes) and race non specific stem rust resistance genes. The success in use of single resistance genes has been hindered by the intense selection pressure due to mutations at a single locus making the resistance ineffective after a short period of time resulting in "boom and bust cycles". Therefore one of the long lasting solutions to increase durability of resistance is through gene combination or even gene pyramiding. The other alternative is to combine several race-non specific resistance genes with additive effects resulting in near immune phenotypes as an option to achieve durability. In addition, the efficiency of durable resistance could greatly be enhanced by integrating the use of molecular markers to help tag the specific stem rust resistance genes in the wheat germplasm and ultimately improve the selection efficiency by the use of Marker Assisted Selection (MAS).

Realizing the threat posed by the Ug99 race group, over 400,000 wheat lines that included accessions from germplasm collections to breeding materials from wheat breeding programs throughout the world were screened for resistance to Ug99 in Kenya and in Ethiopia (Bhavani et al. pers. comm, Singh et al. 2011). The results showed that 85-95% of wheat lines grown globally are susceptible to the Ug99 races.

The results obtained from screening the global germplasm highlighted the risk looming over worldwide wheat production due to the susceptibility of current varieties. Also, based on global wind trajectories (FAO, 2010; Hodson et al. 2010), it is predicted that the races might someday reach the breadbaskets in Asia, Europe, and the Americas. It is essential that resistance genes are identified and used in breeding. Therefore, there is urgent need to identify and characterize new genes for resistance to the Ug99 races and rapid incorporation of these genes in the breeding pipeline to develop improved varieties. Thus this study was conceived to address the Ug99 race and its variants.

### 1.5 Main Objective

Identify more sources of stem rust resistance genes to Ug99 race and its variants for introgression into the local wheat varieties to increase wheat production in Kenya

#### 1.5.1 Specific Objectives

1. To identify sources of resistance to stem rust in bread wheat
2. To determine the genetics of the stem rust resistance genes in bread wheat
3. To identify molecular markers and the genomic regions harbouring the adult plant stem rust resistance genes.

#### 1.5.2 Study Hypotheses

1. There are lines resistant to the *Puccinia graminis Ug99* race
2. The resistance to stem rust is conferred by a single major gene
3. There are quantitative trait loci associated with the stem rust resistance

# CHAPTER TWO

## 2.0 Literature Review

### 2.1 Historical background of cereal rusts

Wheat rust is an ancient disease. During the Neolithic period, wheat rusts were used as signatures of religious beliefs, greatly influencing human civilization. The ancient Romans offered sacrifices to the rust god Robigo or Robigus, to appease them to avert rusts among their wheat crops (Schumann and Leornard, 2000). However, Aristotle and Theophrastus discovered that cereal rusts developed in the presence of warm and wet weather (Leornard and Szabo, 2005). The cereal rusts have caused heavy disease epidemics since time immemorial. High yield losses ranging from 40-50% were reported in the 1950s. Since then, concerted efforts to combat the stem rust have been aimed at the use of host resistance and eradicating barberry; the alternate host of stem rust (Voegele *et al*., 2009).

### 2.2 Taxonomy of the rust fungi

The rust fungi called *Puccinia* species have complex life cycles with many different spore-producing stages. It has about 7000 species in more than 100 genera (Webb and Fellers, 2006). These rust fungi are obligate parasites which need live plant tissues for survival. The fungus has infectious structures with limited secretory activity. They also have carbohydrate and protein rich layers which secrete the fungal and host plasma membranes (Bolton *et al*., 2009). The presence of haustoria enhances their feeding ability leading to prolonged host defence suppression (Leornard and Szabo, 20005). The rusts are heteroecious, requiring two phylogenetically distinct or unrelated host plants to complete their life cycle (Schumann and Leornard, 2000). It has a macrocyclic life cycle involving five spore stages; basidiospores, pycniospores (spermatia), aeciospores, urediniospores (uredospores), and teliospores (Kolmer *et al*.,

2009). Cereal rusts produce numerous infectious spores that are easily disseminated by wind over large areas. The three economically important rust fungi of wheat include leaf rust (also known as brown rust); yellow rust (also known as stripe rust) rust and stem rust (also known as black rust.

### 2.3 Stem Rust Disease in Wheat

This is caused by *Puccinia graminis* f. sp. *tritici* (Eriks and E. Henn). It belongs to the phylum Basidiomycota, class Urediniomycetes, order Uredinales, and family *Pucciniaceae* / uredinaceae, which contains 17 genera and approximately 4121 species, of which the majority are in the genus *Puccinia* (Leonard and Szabo, 2005). The stem rust genus attacks around 365 species of cereals and grasses in 54 genera and exhibits high genetic diversity (Groth *et al*., 1995).

#### 2.3.1 The Stem Rust Life Cycle

The fungus has both sexual and asexual reproduction systems implying the presence of more phenotypes with more distribution (Mago *et al*., 2005). Through asexual reproduction, billions of identical spores are released onto mature wheat (http://www.newscientist.com). The fungus which is an obligate parasite has no resting stage in its cycle and this complicates the management of the stem rust epidemics. The stem rust cycle is macrocyclic with five spore stages. In addition, two hosts are required for a complete life cycle. In the beginning of a growing season, diploid teliospores (dark brown to black) germinate on the host plant or even dead tissues. The teliospores undergo meiosis to produce four or more haploid basidiospores which are later dispersed into the air (Leonard and Szabo, 2005). The basidiospores infect the alternative host through the stomatal cell walls to produce haploid spores, pycnia which are of different mating types. Upon the dispersal of the pycniospores through rain or dew or insects to other plants, fertilization or

plasmogamy occurs leading to formation of a single cell with aecium and aeciospores (Kolmer *et al*., 2009). The dikaryotic aeciospores are deposited on the host plant from which successful infection and colonization leads to formation of uredinium containing urediniospores. The uredinium can repeatedly infect same host plant leading to severe epidemics. The infection leads to formation of pustules (uredium containing urediniospores) on stems, leaves, sheaths, chaff and beards of the wheat plant leading to shrivelling of the kernels and stem lodging (Roelfs, 1985; Wu, 2008). With declining plant nutrients, the uredinia develop into telia which are hardy and can survive harsh conditions until another growing season commences (Staples, 2000).

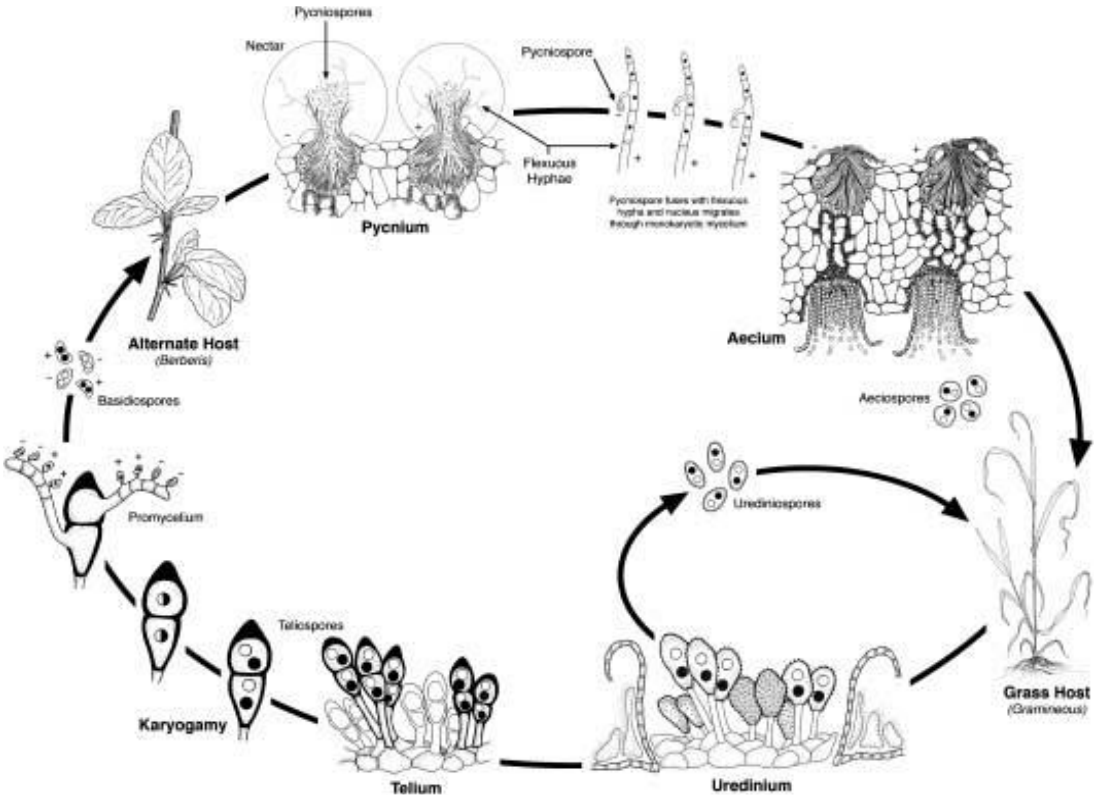


**Figure 2.1 Life cycle of the stem rust fungus, Puccinia graminis**
(Source: Kurt J. Leonard and Les J. Szabo, 2005).

### 2.3.2 Evolution of new pathogen races of stem rust

New pathogen races evolve through mutations, genetic drift, gene flow, reproduction and selection (McDonald and Linde, 2002). Mutations cause changes in the DNA sequences of individual genes forming new alleles of pathogen populations which later turn into virulent races which erode the available genetic resistance (Bariana *et al*., 2007; Rajender *et al*., 2004). With regard to genetic drift, the use of small host populations leads to loss of valuable alleles (Leonova *et al*., 2002). Through gene flow, virulent mutant alleles of pathogens when moved across different field populations increase their effective population size within a specific region as is the case with cereal rusts. Given the efficiency of sexual, asexual and para-sexual recombinations, a pathogen population recombines new virulent alleles well ahead of the breeders thus overcoming the available resistance (Rosewarne *et al*., 2008). Finally, selection is a great force influencing pathogen evolution especially with the practice of wheat monoculture (Webbs and Fellers, 2006).

In the 1920s, the barberry (*Berberis vulgaris*) eradication in the USA reduced the adverse effect of the *Puccinia graminis* f. sp. *tritici* on wheat production. This implied that the emergence of new races of the fungus through sexual stages was interrupted. Thus, mutation remained the most probable cause of evolution enabling wheat breeders to combine race specific resistance genes. In 1954, stem rust wiped out 40 per cent of the crop in North America (Smith *et al*., 2009). Through research and host resistance breeding, the stem rust incidences were suppressed for many years through efforts by Dr. Norman Borlaug. This meant that research efforts were geared towards other constraints. This was until 1998 when high susceptibility to stem rust was noted among previously resistant wheat varieties grown in Uganda (Smith *et al*., 2009). The increase in stem rust severity was attributed to the race Ug99. The race Ug99 broke

down the stem rust resistance gene, *Sr31* and was later designated as TTKS based on North American stem rust nomenclature (Xu *et al*., 2009) and later TTKSK after a fifth set of differentials was added following further characterization (Singh *et al*., 2011). The Ug99 race also has shown virulence to *Sr38* stem rust resistance gene transferred from *Triticum ventricosum*. The Ug99 has continued to evolve into more virulent forms like TTKST and TTTSK showing virulence to stem rust resistant genes *Sr24* and *Sr36* respectively (Singh *et al*., 2008). The Ug99 spores have further spread over vast areas causing epidemics and high yield losses in Kenya and Ethiopia (2002), Sudan, Iran and Yemen (2007) with the possible route of stem rust spread been proposed as East Africa – Middle East – West Asia – South Asia (Ayliffe *et al*., 2008; Singh *et al.*, 2008).

### 2.3.3 The management of stem rust

This involves crop rotation, application of fungicides and host resistance breeding. Crop rotation helps to limit the genetic diversity of the pathogen population and also to minimize the number of urediniospores produced (Bariana *et al*., 2007). The use of fungicides is limited by the fact that most wheat farmers are small holders who are resource constrained and cannot afford chemicals. In addition the chemical fungicides are environmentally unsafe. The use of crop rotation and fungicides cannot match up with the aggressiveness of the Ug99 race and its variants. Thus, breeding for resistance to stem rust and the diversification of the genetic base of the available wheat germplasm are the most amicable solutions to the Ug99 race (s) menace.

Host resistance is defined as the ability of the host to limit the growth or development of the pathogen (Roelfs, 1984; Parlevliet, 1985). Over 50 stem rust resistance (Sr) genes have been identified and characterized against the different races of stem rust

(McIntosh *et al*., 2003). The wheat stem rust resistance genes were designated after their actual locations on the chromosome arms were established (Leornard and Szabo, 2005). Twenty of these stem rust resistance genes were transferred into the *Triticum aestivum* from the wild relatives of wheat by introgression of wheat alien species chromosome translocations through genetic engineering (Klindworth *et al*., 2012). All the designated stem rust resistance genes, except *Sr2,* are race specific and are expressed in both seedling and adult plants (Singh *et al*., 2008).

#### 2.3.3.1 Race specific resistance

The race specific resistance is also referred to as major gene resistance and is qualitative in nature. It functions against certain rust races or biotypes but not against others (Babiker *et al*., 2009; Steffenson *et al*., 2007). It has simple inheritance and exhibits discrete segregation pattern following simple Mendelian genetic ratios. The race specific genes are characterized by dominant or recessive patterns of inheritance which show no crossing over in usual genetic studies. Most of these resistance genes result into hypersensitive responses; the rapid death of the infected cells which aims to restrict the spread of the pathogen to other parts of the plant (Dyck and Kerber, 1985; Singh *et al*., 2008; Jin *et al*., 2007; Lowe *et al*., 2011). This leads to the collapse and death of the infected host cells preventing a compatible host pathogen interaction (Leornard and Szabo, 2005).

The stem rust fungi are host-specific with either compatible or incompatible associations with their host plants in a gene-for-gene manner (Flor, 1955). The gene for gene concept implies that with each host plant resistance gene (R gene); a corresponding gene locus (race specific effectors) is present in the pathogen with alternate alleles conditioning avirulence (Avr) gene and virulence (Flor, 1971). The

stem rust fungi produce elicitor (effector) molecules detected by receptor molecules in wheat. The effectors contain many chemical compounds like oligosaccharides, lipids, peptides and proteins. Race specific effectors are produced only when specific Avr genes are present in a particular pathotype of the pathogen (Flor, 1971). When the plant's receptors detect the pathogen's elicitors, a host defence mechanism is stimulated. This is followed by the death of the infected cells and the pathogen growth is hindered. Any breakdown in resistance leads to the absence of the defence mechanism (McDonald and Linde, 2002). This implies that changes in the elicitor leads to the non recognition by the receptors of host plant thus increasing the frequency of the pathogenic races which eventually cause rust infection and reproduction; a compatible host-pathogen response (Crute and Pink, 1996). In nature, pathogens with high evolutionary ability overcome host resistance. When a host resistance gene with a large effect is spread over a vast area (boom), the pathogen adapts by evolving into a new population which overcomes the subsequent resistance (bust) in the host plant rendering some stem rust resistance genes ineffective (McDonald and Linde, 2002). Given the fact that most wheat varieties in use today have a narrow genetic base, more resistance genes or multigenes should be identified to help slow down the stem rust disease (Jin and Singh, 2006; Babiker *et al*., 2009). Thus, breeders need to continuously replace cultivars and introgress new resistance genes a process deemed necessary to counteract this boom and bust cycle (Crute and Pink, 1996).The race-specific resistance genes could be pyramided in new wheat cultivars to develop stable sources of resistance (Leornard and Szabo, 2005).

#### 2.3.3.2 Race non-specific resistance

It is also referred to as generalized, horizontal, field, adult plant, polygenic or minor gene resistance (Watson, 1970). The race non specific resistance is conditioned by

several genes each having small effects on the phenotype. It does not show genetic interactions between the host and the pathogen genotype (Parlevliet, 1995). In addition, the confounding effects of environment and /or segregation of several loci lead to the continuous variation exhibited in race non specific resistance (Prashant, 2007). The resistance also works against all biotypes greatly reducing the probability of mutation through asexual reproduction since the specific resistance delays the start of an epidemic while the non specific resistance genes retard epidemic progress (Watson, 1970; Roelfs *et al*., 1988). This is because the pathogen would require multiple mutations to acquire virulence against all resistance genes (biotypes) involved in conditioning the resistance thus, the utilization of this type of resistance contributes to durable resistance.

Durable resistance has been defined as resistance which is effective for a long duration over generations under vast environments with disease pressure (Johnson, 1984). It is characterized by slow rusting or partial resistance, and is associated with adult plant resistance as opposed to seedling resistance or hypersensitive reaction (Singh *et. al.,* 2000). The partial resistance is a form of incomplete resistance where the individuals show lesions indicating susceptible infection types (Parlevliet, 1985). It is conditioned by minor genes with small effects and is difficult to detect individually due to the presence of a functionally diversified and heterogeneous class of genes (Kolmer, 1996; Lowe *et al*., 2011). With partial resistance, the host shows susceptibility but the infection frequency, latent period and rate of spore production which are components of partial resistance are greatly reduced. Examples of resistance genes exhibiting partial resistance include the genes Lr34/Yr18/Pm38 and the stripe rust resistance gene Yr36. Rust resistance gene Lr34 exhibits pleiotrophy and is also associated with leaf tip necrosis. The Lr34/Yr18 gene is also associated

with pre-haustorial resistance while the Yr36 inhibits the post-haustorial fungal spread to other plant tissues leading to increased mesophyll cells (Lowe *et al*., 2011). Slow rusting resistance refers to the slow development of rust disease on a plant due to long latent period from infection to sporulation (Johnson, 1984). The slow rusting genes are expressed quantitatively with small genetic effects (Lagudah, 2008). The germplasm with the slow rusting resistance form a thick mycelium and haustoria in parenchyma tissue, which later develop thick walled sclerenchyma tissue. These tissues eventually hinder fungal spread and pustule eruption preventing epiphytotics (Singh *et al*., 2007; Hooker, 1967). This affects the rate of leaf penetration, causing fewer lesions per unit area, smaller lesions, fewer spores per lesion, restriction on rate of growth and sporulation over shorter periods (Naz *et al*., 2008; Watson, 1970). Thus, the existing pathogen populations are greatly stabilized prolonging the resistance. In determining the race non specific resistance, the epidemic expressed would be a measure of amount of disease and the rate of disease increase (Van der Plank, 1963). This goes along with effective disease management where the germplasm being tested for durability are grown in many locations under high disease pressure involving many pathogen races (Johnson, 1984). Through such a criterion, the disease progression would be attributed to the cumulative effect of various minor genes in a genotype and effect of each gene.

#### 2.3.3.2.1 *Sr2* and the pseudo-black chaff

The race non specific gene *Sr2* was transferred from tetraploid wheat, Yaroslav emmer into a common wheat variety called Hope, a cross between Marquis and Yaslov emmer (McIntosh and Brown, 1997). The cultivar Hope contains *Sr2*, Sr7b, Sr9d, Sr17, and Sr18 (Johnson, 1984). The *Sr2* gene is located in the short arm of chromosome 3B. It has been associated with blackening of the ear and nodes at

ripening, known as pseudo-black chaff. It is also a non-race specific gene conferring slow rusting characteristics and is expressed in both seedling and adult plants (Singh *et al*., 2008). This gene is expressed as partial resistance and has been characterized by slow rusting and is additive in nature with high heritability (Ginkel and Rajaram, 1993). It has been effective against *Puccinia graminis* f. sp. *tritici* since 1920 (Ayliffe *et al*., 2008; Liu and Kolmer, 1998) and constitutes non-hypersensitive, partial reaction and has varying disease severities with regard to differences in genetic and environmental backgrounds (McNeil *et al*., 2008). However, the masking effects and the recessive nature of inheritance associated with the *Sr2* makes its phenotyping difficult when present in germplasm backgrounds with other resistance genes (Babiker *et al*., 2009). More so, the expression of this pseudo black chaff at adult stage is characterized by dark pigmentation on the stem internodes and glumes delaying progeny classification and is thought to reduce yields (McNeil *et al*., 2008). Despite all these factors, the use of the *Sr2* gene and other genes of minor effects remain paramount in prolonging the lifespan of available stem rust resistance and ensuring sustainable host resistance management.

### 2.4 Breeding for resistance to stem rust

Common methods used in breeding for stem rust resistance involves pedigree, bulk breeding, single seed descent and backcrossing methods. The basis of pedigree method is that stem rust resistance is a highly heritable character and is genetically manipulated. Thus, selection can be done in early generations and encompasses major gene effects like the hypersensitive response and immunity (Roelfs and Bushnell, 1985). This is accomplished by making multiple crosses between resistant and susceptible parents with the ultimate goal of enhancing the genetic diversity of the germplasm. Individual plant selections are then done during the early generations

among elite genotypes from a heterogeneous population. The space planted $F_2$'s segregate enabling the use of single plant selections by assessing the qualitative characters. This makes it possible to obtain true breeding lines.

The single seed descent method is used to advance generations into inbred lines. Each seed harvested from $F_1$ is planted into $F_2$ and then advanced into subsequent generations. This ensures that elite genotypes are not lost during the segregation. The best parents are then involved in back crosses, top crosses and double crosses for further improvement. When the $F_2$ generation of crosses and parents are analysed, crucial resistance genes could be identified (Hanzalová *et al*., 2009). Eventually, the bulk method is applied to advance the material into further generations with the aim of conserving genetic diversity and exposing highly resistant cultivars (Singh *et al*, 2005).

The backcross method aims at introducing new forms of disease resistance into superior wheat genotypes (Roelfs and Bushnell, 1985). An elite genotype (recurrent parent) is improved by introducing genetic material from a donor parent (Park *et al*, 2009). This involves repeated crossing of the hybrid generation with the recurrent parent and the selection of segregants heterozygous for the desired trait (Parlevliet, 1993). This leads to accumulation of favourable alleles leading to efficiency gains (Koebner and Summers, 2003). Backcross also reduces population size (Bonnett *et al*., 2005) making their handling simpler.

### 2.4.1 Plant breeding techniques

#### 2.4.1.1 Gene pyramiding

Gene pyramiding incorporates many desirable genes into elite genotypes (Ayliffe *et al*., 2008). The pyramids used have involved major genes, minor genes, effective

genes, race or race non specific genes which confer resistance (Pedersen and Leath, 1988). The accumulation of minor genes of additive nature is usually followed by intercrosses and recurrent selection (Singh and Rajaram, 1992). With the introgression of a few genes of large effects, effective monitoring of these genes is imperative through accurate phenotyping (Guoyou and Kevin, 2008). There has been increased preference of multiple adult plant resistance genes as opposed to the R genes in gene pyramiding. This is because it's difficult for new races of the *Puccinia graminis* to overcome the multiple resistance genes since this will require multiple mutations in the pathogen genes (Ayliffe *et al*., 2008). Gene pyramiding has been achieved through screening of germplasm under artificial disease inoculation and selecting parents exhibiting partial resistance. However, gene pyramiding is limited where resistance genes are transferred from species of lower-ploidy into hexaploid bread. This is due to the interaction between resistance genes and suppressor genes in the different genomes (epistasis) (Kolmer, 1996).

#### 2.4.1.2 Regional deployment of genes, multiline cultivars, mixtures of cultivars and gene stacking

The regional deployment of genes, multiline cultivars, mixtures of cultivars and gene stacking has been vital in ensuring effective resistance (Hogenboom, 1993). Gene deployment involving cultivars with complementary sets of resistance genes in combinations has led to durable disease resistance (Young, 1996). This has also ensured yield stability while compensating for disease resistance among the different cultivars. Thus, to restrict pathogen spread, separate maintenance of resistance would help to generate negative gametic disequilibria in pathogen population (Wolfe, 1993).

#### 2.4.1.3 Marker assisted selection

Conventional breeding and marker assisted selection (MAS) have also been combined to enable accurate and efficient selection of the stem rust resistant genes. The

selection at early generations using molecular markers increases the number of favourable alleles in segregating progenies ultimately increasing the efficiency of a breeding program (William *et al*., 2007). The marker assisted selection also aids in gene pyramiding where multiple genes can be tagged with closely linked markers to develop superior genotypes with desired gene combinations. Since stem rust resistance is conditioned by recessive genes, the continuous selfing could help to expose the individuals carrying these alleles. However, MAS removes the need for the extra selfing and progeny testing associated with the backcross introgression techniques (William *et al*., 2007). Pyramiding could also be achieved easily by a single transformation step instead of hybridizations and the backcrosses (McDonald and Linde, 2002). Molecular characterization enables the easy manipulation of genetic resistance and transfer of elite resistance to other species as cassette to produce multilines with superior alleles which disrupt selection hence slowing down pathogen evolution. The ability of the wheat genomes to withstand chromosomal aberrations due to the close relationships of the three genomes (A,B and D) has led to the development of genetic stocks for example the deletion stocks used to make the physical maps of wheat (William *et al*., 2007). Thus, with effective breeding strategies, more stable sources of resistance could be obtained to avert the imminent threat posed by the Ug99 races.

### 2.4.2 The utilization of race specific and race non specific resistance genes in breeding for stem rust resistance

Most wheat varieties have a narrow genetic base and cannot counter the ever mutating pathogen races (Babiker *et al*., 2009). On the other hand, most resistance genes are race specific genes and are short lived with complex inheritance due to their recessive nature (Babiker *et al*., 2009). The breeding efforts are further limited by the presence

of other stem rust resistance genes in the background of most wheat cultivars. Another impediment has been in the utilization of the resistance in wild relatives of wheat due to low fertility after intercrosses and the associated linkage drag with undesirable traits (Anikister *et al*., 2005). For example, in spite of the promising stem rust resistance conferred by *Sr25* and *Sr26*, there are shortfalls in their utilization. The *Sr2*5 and *Sr2*6 were transferred from *Thinopyrum ponticum*. The *Sr25* linked to *Lr19* is associated with the undesirable yellow pigment in flour while *Sr2*6 gene reduces wheat grain yield by up to 9% (Bariana *et al*., 2007). Another race specific gene *Sr35* located on 3AL could offer resistance to Ug99 while in combination but it's linked to the red grain colour affecting wheat flour quality (Babiker *et al*., 2009). The use of race non specific resistance is associated with difficulties during evaluation since distinguishing germplasm under high disease pressure becomes difficult (Bariana *et al*., 2007).

The integration of both race specific and race non specific stem rust resistance genes in the wheat breeding programs will go a long way in ensuring sustainable wheat productivity (Bariana *et al*., 2007). The race specific genes in combination could offer a solution especially in areas with low rust epidemic from the Ug99 race. However, in Ug99 race risk areas, the race specific genes should be used in combination with genes of minor effect. There is a need to ensure reduced asexual changes of a race after gaining genes for virulence. Based on Van der Plank (1968) statement, after combination of genes for virulence in one race, the race loses fitness. Thus, there is need to develop cultivars containing more resistance genes in complementary or in additive manner which are more stable (Watson, 1970). Given that more resistance genes are succumbing to Ug99 and its derivative pathotypes; this calls for good understanding of the chromosomal location and biological effects of important stem

rust resistance genes in order to successfully develop appropriate crosses (Rosewarne *et al*., 2008).Thus, more sources of resistance should be identified from uncharacterized wheat germplasm followed by the elucidation of the genetic basis of that resistance for efficient utilization in breeding programs and also ensure sustainable resistance management strategy (Sharma and Saini, 2011). The proper characterization and introgression of any identified resistance into elite wheat germplasm will enhance the value of commercial varieties (Bariana *et al*., 2007).

More concerted efforts should focus on objective seedling tests and field screening. Seedling tests are crucial in helping breeders to elucidate the genetic composition of wheat lines and also to establish their genetic diversity. During field screening, high disease pressure and the use of segregating populations in early generations could ensure breeding efficiency. Field screening has also integrated a shuttle breeding strategy where wheat germplasm is exposed to different stem rust hot spots enabling the selection of plants for disease resistance and adaptation. It has also enhanced the genetic diversity of elite wheat germplasm while reducing their vulnerability (Singh *et al*., 1992). The shuttle breeding strategy has helped to determine whether disease resistance is stable while enhancing the expression of any fixed traits (Prashant, 2007). This is because after several generations, recessive or additive (minor) genes are eventually accumulated. This has led to exposure of non durable major genes which are artificially inoculated using pathogen races with complementary virulence.

To ensure that maximum gains arise from any breeding effort, the type of genetic resistance should be matched with the appropriate gene management strategy based on the pathogen biology. This will limit the chances of the pathogen population evolving into more virulent forms hence increasing the life of a commercial cultivar

(McDonald and Linde, 2002). With major genes which are easily recognized and utilized, gene deployment in multiline or cultivar mixtures could be carried out during the early generations of a breeding program. With regard to the quantitative resistance where the resistance genes do not uniformly contribute to resistance, different strategies should be used. For example gene pyramiding of the major resistance into a cultivar could ensure durability since multiple mutations to virulence rarely occur in nature. However, in cases where there are mixed pathogen populations and high gene flow, mutations could threaten any available resistance. This is because the recombinations of virulence mutations into many genetic backgrounds could arise leading to pathogens with a higher fitness prone to vast migration. Thus, the use of durable resistance involving genes of minor effects offers the best solution especially in the high Ug99 race risk areas (Singh *et al*., 2011).

### 2.5 Characterization and mapping of stem rust resistance

Most traits of economic importance are under multigenic control and are thus quantitative or complex (Kliebenstein, 2009; Collard *et al*., 2005). The recessiveness of some stem rust resistance genes and their confounding effects in the genetic backgrounds of most cultivars coupled with incomplete penetrance, variable aggressiveness of the pathogen and the variable expression of levels of resistance under different environmental conditions complicates genetic studies through phenotypic evaluations (Tsilo *et al*., 2009; Rajender *et al*., 2004; Gold, 1998). The phenotypic selection exposes deleterious effects and the deleterious linkages associated with pleiotrophy and linkage drag. However, linkage drag limits the transfer of superior alleles especially in traditional breeding (Narasimhamoothy *et al*., 2006). Thus, the understanding of the genetics of traits of interest is crucial in

ensuring breeding efficiency and this could be achieved through integration of closely linked genetic markers.

Closely linked genetic markers facilitate indirect selection of quantitative traits (Bariana *et al*., 2007; Koebner and Summers, 2003). They show differences among organisms and because they are located close to the genes of interest, they are referred to as gene tags. Genetic markers are broadly classified as morphological (classical), biochemical (isozymes) and DNA (molecular) markers. In wheat, an example of a morphological marker is the pseudo black chaff in wheat. Biochemical markers are represented by differences in enzymes after electrophoresis and staining. However, both morphological and biochemical markers are few and are confounded by environmental and growth stages of plants. The DNA markers are abundant and arise from DNA mutations like point mutations, indels, or errors in replication of repeat DNAs (Farooq and Azam, 2002). These markers are also selectively neutral due to their location in non coding regions of DNA. The method of DNA markers' detection include hybridization-based; polymerase chain reaction (PCR)-based and DNA sequence-based. DNA markers must be polymorphic for them to be useful in that they should reveal genetic differences between individuals of the same or different species (Azhaguvel *et al*., 2006). The polymorphism could be codominant or dominant. The codominant markers have the capacity to discriminate between homozygotes and heterozygotes. The different forms of DNA markers are called marker alleles (Collard *et al*., 2005).

Molecular markers help in selecting resistant plants with the aid of linked markers even in the absence of disease (Babiker *et al*., 2009). They also help to accurately select plants with superior traits even at seedling stages in which the genes are not

influenced by environment and the low penetrance and complexity associated with most quantitative traits (Neumann *et al*., 2011). Where the genetic components involved have small effects with regard to their contribution to a phenotype, molecular markers enable their effective fixation in a homozygous state even during the early generation stages of a pedigree based program. At the $F_2$ generations, the frequency of selected alleles is greatly increased thus maintaining a minimum population size with desired traits at more loci (Bonnett *et al*., 2005). This is because unfavourable alleles are reduced by the use of the molecular markers. Thus, the screening for multiple or multigenic traits which are epistatic to one another greatly reduces the linkage drag (Young, 1999; Michelmore, 1995). Molecular markers have been integrated in the utilization of important recessive genes or incompletely dominant genes (Francia *et al*., 2005). These genes are maintained in the early segregating generations through backcrosses without the need for progeny tests (Koebner and Summers, 2003). Subsequently, inbreeding is done to increase the frequency of homozygotes in the population (Bonnett *et al*., 2005). Closely linked markers are also crucial since they offer phenotype neutral selection of the linked genes (Bariana *et al*., 2007). A single resistance gene could be selected easily even in the background of other genes (Nocente *et al*., 2007). The use of molecular markers has enhanced the combination of effective genes against several isolates through gene pyramiding. This coupled with testing with pathogen for confirmation of the presence of resistance gene has enhanced the breeding for stem rust resistance (Michelmore, 1995).

### 2.5.1 Marker assisted selection (MAS)

To achieve maximum gains in plant breeding, the integration of genomic selection and conventional selection remains paramount. Molecular markers have been used to

tag important resistance genes thus improving selection of genotypes which contain combinations of non-race specific genes providing durable resistance (Todorovska *et al*., 2009). The subsequent crossing of these elite parents increases genetic variability when the favourable alleles are fixed at specific genomic regions during each generation (Ribaut and Betran, 1999). Marker assisted selection helps to trace these favorable allele(s) (dominant or recessive) across generations; in order to accumulate favorable alleles. This involves the genotyping of each line in the mapping population relative to the parental genotypes (Buerstmayr *et al*., 2009). From this, the elite individuals among a segregating population are identified. MAS helps in the recovery of the recurrent parent in back cross programmes. It also enhances the selection of traits which are difficult to phenotype by selection for a marker allele from the donor parent at a locus near the target gene. Markers have been crucial in selection of back cross progeny with less amount of donor parent germplasm in the genome outside the target region and also in selection of rare progenies minimizing linkage drag. This has led to the effective utilization of recessive genes even in early generations greatly accelerating the breeding process.

Due to the boom and bust cycle of the rust fungi, increasing the longevity of stem rust resistance would offer an amicable solution. Through traditional breeding, it's difficult to stack two or more genes into a common background. The lack of isolates specific to avirulent/virulent gene combinations also makes the clear classification of resistant genotypes complex (Mago *et al*., 2005). Thus, with MAS, multiple genes related to a single trait could be identified and stacked or combined into wheat lines by tagging them with closely linked markers (William *et al*., 2007). This also diversifies the genetic base of resistance greatly limiting the ability of the pathogen to adapt and form new rust races. Through MAS, major gene resistances have been

introgressed into wheat lines through backcrosses and selecting against race specificity followed by accumulation of the slow rusting genes (Narasimhamoothy *et al*., 2006; Synman *et al*., 2004). Ultimately, gene pyramiding involving combination of resistance genes helps to lower the frequency of pathogen evolution into virulent races through random process of genetic mutation. Thus all stages of plant and pathogen interaction could be noted while identifying the loci which explain any genetic variation (Michelmore, 1995). The MAS technique is also crucial in reducing the duplication of alleles and enables fixation of superior genes (Koebner and Summers, 2003).

### 2.5.2 Bulk segregant analysis (BSA)

Bulk segregant analysis is a method used to identify markers in specific regions of the genome without the need to construct a detailed genetic map (Bonnett *et al*., 2005; Collard *et al*., 2005). It excludes the need to genotype an entire mapping population (Poulsen *et al*., 1995). This is because a few individuals segregating for the trait of interest and with extreme phenotypes for a segregating trait are pooled together and then genotyped (William *et al*., 2007). The polymorphic markers identified are then used to genotype the large population to confirm the genetic linkage between the markers and the target gene (Yang *et al*., 2012).

### 2.5.3 Quantitative trait loci (QTL) mapping

Quantitative trait loci mapping involves identification of quantitative trait loci (QTL). The QTL are regions within the genome with genes associated with a quantitative trait in a segregating population (Collard *et al*., 2005). It is very important in the identification of genes with major and minor effects. Its value also lies in establishing the number and the chromosomal location of genes involved in the inheritance of important traits (Haile *et al*., 2012). QTL mapping is divided into linkage mapping

and association mapping. Linkage mapping deals with the identification of chromosome regions of interest with low marker coverage. The association mapping offers high resolution by use of prior information on organism's genes and /or genome scan with very high marker coverage (Steffenson *et al*., 2007). Association mapping also involves the correlation of the genotype with the phenotype of each line and is based on the fact that linkage disequilibrium is maintained through generation between genetically linked loci (Neumann *et al*., 2011; Bonnett *et al*., 2005).

QTL mapping is limited in wheat due to its large genome size and the repeat sequences. The wheat genome has a narrow genetic base arising from interspecific hybridization, polyploidy and continuous selection done over time due to its domestication in farmers' fields (Warburton *et al*., 2006). The breeding strategies adopted should enhance efficiency of QTL mapping while in tandem with MAS. However, to ensure breeding efficiency in QTL mapping, the integration of both linkage and association mapping systems still remains crucial (Yu *et al*., 2008). This should also incorporate the accurate dissection of the QTLs and mapping of the resistance genes at their genomic locations in order to generate informative QTLs. For efficiency in QTL mapping, the phenotyping should be accurate; repeated over locations and in years; involve appropriate genetic analyses; presence of a wide genetic background and use of advanced generations (Young, 1999). QTL mapping has been utilized in breeding programs through the use of advanced back cross QTL analyses. Crosses are made between adapted wheat and an unadapted wild wheat relative (Young, 1999). The wild QTLs are then isolated to eliminate this wild genetic background. This method has led to the production of near isogenic lines after further back crossing and selfing. Thus, the genetic diversity of wheat is broadened and the superior alleles or genes are utilized in breeding as opposed to their exclusion due to

linkage drag (Warburton *et al*., 2006; Narasimhamoorthy *et al*., 2006). Thus rare recombinants that break unfavourable linkage and the fixation of superior alleles accrue from the use of QTL mapping in wheat breeding. Individuals with cross overs near genes of interest could easily be selected thus removing linkage drag from donor parents. QTL mapping dissects quantitative traits into Mendelian factors in the form of QTLs and also establishes their genomic locations (Yi and Shriner, 2008; William *et al*., 2007; Koebner and Summers, 2003; Young, 1996). The successful mapping depends on the recombination between markers and the QTL (Demuth and Wade, 2006).

### 2.5.4 Types of molecular markers

They include restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites or simple sequence repeats (SSR), single nucleotide polymorphism (SNP) and Diversity Array Technology (DArTs). The RPD, AFLP and SSR markers require direct sequencing of the variable loci (James *et al*., 2008). SSRs show more polymorphism than the restricted fragment length polymorphism (RFLP) markers making them widely adopted in marker-assisted selection (MAS) (Haile *et al*., 2012).

#### 2.5.4.1 Simple sequence repeats (SSR) markers

Most stem rust resistance genes are race specific and are easily tagged with linked microsatellite (SSR) assays. SSR markers are based on 1 to 6 nucleotide core elements which are tandemly repeated. A different “allele” occurs at a micro satellite locus as a result of changes in the number of times the core element is repeated, altering the length of the repeated region. These PCR based SSR markers are highly polymorphic, co-dominant and chromosome or locus specific enabling differentiation of homozygotes and heterozygotes in the early generations (Babiker *et al*., 2009;

Narasimhamoorthy *et al*., 2006; Khan *et al*., 2005). This enhances the breeding process while increasing the selection efficiency (Babiker *et al*., 2009). They are also easily visualized or handled, are stable and evenly distributed throughout the genome. They are also abundant in the pericentromeric DNA and rare in the Euchromatin (Song *et al*., 2005; Peng *et al*., 2000). The allotetraploid wheat genome is large (1C = >16 billion base pairs) and has high percentage of repetitive DNA (Song *et al*., 2005). Locus specific simple sequence repeat (SSR) markers use repeated DNA sequence variation as tools of improving selection efficiency even in very early generation (Hoisington *et al*., 2002). They have been used to search for chromosomal regions associated with adult plant resistance (Vanegas *et al*., 2007). They are also important in the profiling of an entire recombinant inbred line population (Maccaferri *et al*., 2008). Resistance genes introgressed from the wild relatives of wheat are also easily tagged (Narasimhamoorthy *et al*., 2006).

#### 2.5.4.2 Single nucleotide polymorphism

Single nucleotide polymorphism (SNP) consists of a single base difference within a given segment of DNA between two individuals. SNP's contribute about 90% of the genetic variation in living organisms and also provide high density markers. These markers are high throughput, abundant and uniformly distributed throughout the genome (Gupta *et al*., 2008). SNP output is also of binary type enabling easy data interpretation. Moreover, there is no need for electrophoresis and the SNP locus is a qualitative character; the allele is defined by the identity of a particular base in the sequence (Koebner and Summers, 2003).

#### 2.5.4.3 Diversity Arrays Technology

Diversity Arrays Technology (DArT) is a sequence independent micro array-based DNA marker technique (Gupta *et al*., 2008). It is hybridization-based producing

medium-density genome wide discovery and genotyping of any plant species (Akbari *et al*., 2006). This molecular technique scores for the presence or absence of DNA fragments and while quickly and concurrently typing numerous SNP's and insertion/deletion polymorphisms in a single assay from many arbitrary genomic loci (Jing *et al*., 2009). The markers are biallelic and dominant or co-dominant. The technique is high-throughput, economical and is highly polymorphic (James *et al*., 2008). They are obtained by replicating arbitrary DNA fragments of genomic representations (Neumann *et al*., 2011).

DArTs are used to survey genetic diversity of germplasm, parental lines and cultivars and is most preferred for polyploids and does not require prior DNA sequence information. An array for hexaploid wheat has been developed. They also assay a large number of markers and give a high resolution in genetic diversity studies. They are also used to build linkage maps which are usually highly collinear across different populations. The DArT technology is also used to identify QTLs and for association mapping using statistical machine-learning (SML) algorithms (Neumann *et al*., 2011). Novel alleles from exotic germplasm are easily introgressed into adapted backgrounds. This method has been used in bulk segregant analysis to measure allele frequencies in DNA pools. The method is also used to identify and estimate the distance of DArT markers from target locus. Also the markers are used for whole genome selection of complex traits and idiotype based breeding strategies (www.triticarte.com). They are also deployed in marker assisted selection in background and fore ground selection.

DArT assays DNA polymorphism by reproducing a set of DNA fragments from a sample of genomic DNA. In this, genomic DNA is digested with *PstI* and T*aqI*

(wheat) or PstI and BstNI (barley). Adapters are then ligated to the PstI ends followed by the amplification of the adapter-ligated fragments. From this, unknown genotype is decoded through hybridization to a microarray which is built for each species and which contains fragments from a set of genotypes which cover the gene pool of the species. After amplification, part of the polylinker region of the cloning vector is co-amplified. A DNA fragment complementary to this polylinker region is co-hybridized to the array to measure amount of DNA spotted on the array. With this, a marker is polymorphic if hybridization intensity falls into distinct clusters whether absent or present for a certain genotype. To genotype, the algorithm gives an estimate of marker quality of different stringencies for different applications (Wenzl et al., 2006). The use of DArT is associated with high fidelity of restriction enzymes as opposed to primer annealing which leads to methylation filtration due to use of *PstI* and this has enriched genomic regions with low methylation.

#### 2.5.4.4 RAD markers

These are microarray based markers used in the genome wide scanning of variations in plant genomes. The steps involved in the use of the RAD markers have been listed by Gupta *et al* (2008) as follows: (i) the digestion of genomic DNA with a specific restriction enzyme; (ii) ligation of biotinylated linkers to the digested DNA; (iii) random shearing of ligated DNA into fragments smaller, leaving small fragments with restriction sites attached to the biotinylated linkers; (iv) immobilization of these fragments on streptavidin-coated beads; and (v) release of DNA tags from the beads by digestion at the original restriction sites. RAD markers have been developed in many organisms as opposed to the other types of markers.

# CHAPTER THREE

## 3.0 To identify sources of resistance to stem rust in bread wheat

### 3.1 Introduction

The Ug99 race of *Puccinia graminis* f. sp. *tritici* has currently evolved into more virulent forms; TTTSK (Ug99+*Sr36*); TTKST (Ug99+*Sr24*) (Xu *et al*., 2009), TTKSF, TTKSP, PTKSK and PTKST (Singh *et al*., 2011). In addition, along the spore path of the pathogen, high yield losses ranging from 70 to 100% have been reported (Wanyera *et al*., 2004). This has been attributed to the poor agricultural practices; wheat monoculture, use of narrow genetically based wheat and the previous deployment of vertical stem rust resistance in commonly grown wheat cultivars (Wanyera, 2008; Beteselassie *et al*., 2007; Mackenzie**,** 2007).

The Ug99 race has also demonstrated virulence to currently deployed stem rust resistance genes with only 16% of hard spring wheat, 48% hard red winter wheat and 28% soft winter wheat showing resistance. About 25% of the world's wheat crop is vulnerable to the Ug99 races while 90% of the wheat is grown in the Ug99 spore path (Ayliffe *et al*., 2008). The fungus could cause total crop losses due to blockage of vascular tissues in wheat and the entire shriveling of whole wheat plants (Vidal, 2009; Singh *et al*., 2008; Mackenzie, 2007). With the current economic constraints, ineffective crop husbandry practices, inflation and high population growth; use of chemicals is economically and environmentally unviable. Thus, host resistance breeding remains the only feasible option which adds no superfluous cost to the resource constrained small scale wheat farmers.

Previous efforts in host breeding have been hampered by the breakdown of major resistance genes; *Sr31*, *Sr24* and *Sr36* which are race specific. These race specific

genes are only resistant to some stem rust races but susceptible to others. More so, most wheat farmers in East Africa grow wheat throughout the year providing a green bridge for the rust spores (Saari and Prescott, 1985). The current climatic changes have also increased disease inoculums and the emergence of new virulences (Semenov and Halford, 2009). Through mutation (point mutations, short duplication events and indels), continuous breakdown of the *Puccinia graminis* f. sp. *tritici* into more virulent forms has led to the boom and bust cycles (Qamar, 2006). Another key factor is sexual and para-sexual recombinations (Singh *et al*., 2008; Burdon, 1993) and migration of the virulent forms into new regions (Qamar, 2006). Through strong selection pressure, the host cannot recognize the pathogen effector molecule because of the selection of sexual progeny, which do not contain the recognized effector genes due to segregation (Ayliffe *et al*., 2008). The increase in the new mutants has also risen from the widespread use of wheat varieties containing the *Sr31* gene. Thus, this existence is plausible given the gene for gene relationship in the *Puccinia graminis*-wheat pathosystem (Jin *et al*., 2007). The rust fungi appear highly adaptable and their quick evolution leaves many currently grown resistant cultivars vulnerable especially where infection occurs early in the crop growth (Lagudah, 2008). The available resistance to the Ug99 races has limitations for example the *Sr39* (obtained from wild wheat relatives) is associated with linkage drag (Yu *et al*., 2010). The race non specific genes which show resistance to all known pathotypes (Tabassum, 2011) for example; the *Sr2* gene remain very important genes with respect to combating the threat posed by the Ug99 (TTKSK). The race non specific genes appear susceptible at seedling stage but exhibits moderately to highly resistant responses at adult plant stages. This is because it is a slow rusting or race non specific gene which when combined with 4 to 5 genes of minor or additive effect; a near immunity could be

achieved (Njau *et al*., 2009). This type of resistance is also based on minor genes crucial to broaden the wheat genetic base slowing down any possible rust pathogen evolution into more virulent forms (Ali *et al*., 2008). This strategy also deploys resistance loci that do not create extreme selection pressure (McDonald and Linde, 2002).

As such the major emphasis in global wheat breeding today is on the use of *Sr2* which is a durable (race non specific locus) source of resistance to the stem rust derived from the cultivars Yaroslav Emmer and Thatcher (Ayliffe *et al*., 2008). The *Sr2* gene complex has also formed the basis of durable resistance in wheat breeding programs. This gene is expressed on plants as small pustules with necrotic and chlorotic portions suggesting some form of restricted fungal sporulation and colonization of plant tissue (McNeil *et al*., 2008). It is also recessively inherited and is associated with ear head (spike) and stem melanism or blackening of adult plants and this has been used as a morphological marker for the *Sr2* gene. Of equal importance is partial resistance in which plants develop susceptible lesion types but with reduced infection frequency, latent period and rate of spore production (McNeil *et al*., 2008). These resistance genes may be manifested as adult-plant resistance, APR (Navabi *et al*., 2004).

In combating the high wheat yield declines, extensive screening of local and international wheat germplasm for more sources of resistance genes remains a priority. There is need to counter the infection and spread of stem rust (Ug99 and its variants) given the favourable climatic conditions in East African region and the high number of susceptible varieties currently in use. An urgent intervention in identifying good sources of resistance to stem rust is thus necessary. Therefore, this study aimed a) to identify wheat genotypes showing resistance to stem rust, b) to assess selected

wheat germplasm for partial resistance and to test whether the different epidemiological parameters used were adequate in evaluating these lines for partial resistance.

## 3.2 Materials and methods

### 3.2.1 Host material

Twenty five wheat lines were selected from the international wheat screening nursery based at Kenya Agricultural Research Institute (KARI), Njoro (KARI, Njoro) in 2008 and designated as 'Kenya selections 2008 (KSL) (Table 3.1).' The KSL lines were elite lines in terms of resistance to stem rust, good plant height and earliness trait. These lines selected for the study had selections from various nurseries representing six different countries and institutions including CIMMYT and ICARDA nurseries. These lines exhibited different levels of resistance to TTKST (Ug99+Sr24) and as they were derived from different breeding programs, the rationale was assumed that the genes deployed could be different and as the objective was to characterize the materials to identify new sources of resistance which would help us understand if the genes for resistance in these materials were similar or new genes for resistance. Lines selected from CIMMYT, Mexico carry multiple genes for resistance and the materials selected were related as some of the lines had multiple selections from the same cross however their gene combinations could be different. Diverse selections enable one to identify and deploy wider range of both race specific and race nonspecific genes in combinations for breeding varieties with durable resistance. In this study also seven highly susceptible wheat lines were included in the field experiment as checks.

### 3.2.2 Experimental site

KARI, Njoro lies at 0 20'S; $35^0$ 56' E, and 2185 meters above sea level (Ooro *et al*., 2009). The minimum and maximum temperatures of Njoro are 9.7 and $23.5^0$C

respectively while mean annual rainfall is 900mm. These conditions favour infection and spread of *Puccinia graminis.* This site is used for large scale wheat screening for resistance to stem rust and was established by the KARI, Njoro in collaboration with the International Maize and Wheat Improvement Centre (CIMMYT) and the Global Rust Initiative (Singh *et al*., 2009; Jin *et al*., 2007). This site is a quarantine centre and legislatively allowed to carry out artificial inoculation during field experiments. There are two planting seasons at KARI Njoro namely main season running from June to October and off season running from December to April.

### 3.2.3 Field experiments

The twenty five wheat lines and the checks were grown during the 2009-off season and 2010 main season. For each entry, 50g were grown as two 1m row plots spaced at 30cm in an alpha lattice design in two replicates. The lines were sown as 70 cm long twin rows, 20 cm apart, flat bed. Each plot was provisioned with susceptible spreader. Wheat lines sown perpendicular to the twin rows, with a border of 8 spreader rows surrounding the field for uniform disease pressure and inoculum dissemination within experimental plots (Jin *et al*., 2007).

Field inoculations were carried out using freshly collected spores from trap nurseries carrying variety “Kenya Mwamba” which is known to carry Sr24 gene and therefore the race used for inoculating was TTKST(Ug99+Sr24). Reaction of lines with known stem rust resistance genes indicated that the predominant, if not only, race present in the nursery since 2008 was race TTKST (Ug99 + *Sr24* virulence; (Njau et al. 2010). The urediniospores at concentration of ~6x106 spores / mL were suspended in water and injected into spreader plants at 1 m distance prior to booting (growth stage Z35-Z37; (Zadoks et al. 1974). The spreader plants were then sprayed with urediniospores

suspended in light mineral oil Soltrol 170 (Chevron Phillips Chemical Company, The Woodlands, TX). The Soltrol suspensions allows for uniform inoculum distribution. Such system of having 1m spreader rows on four sides of each nursery along with parallel and perpendicular spreaders inside each nursery ensured uniform disease pressure inside the nursery. When there was no rainfall, the plants were repeatedly irrigated to enhance stem rust infection and spread.

#### 3.2.3.1 Data collection and analysis

Stem rust severity scoring began when the spreader rows attained 50% susceptible responses in the two seasons and this was based on the modified Cobbs' scale (Peterson *et al*., 1948). The infection responses (plant response to stem rust infection in the field among the adult plants) were also noted and these were based on pustule size and any associated necrotic and/or chlorotic lesions as follows; TR= trace responses, R = resistant, MR = moderately resistant, RMR = resistant to moderately resistant, MRMS (M) = moderately resistant to moderately susceptible, MSS= moderately susceptible to susceptible, MS = moderately susceptible, and S = susceptible (Roelfs *et al*., 1992).

Other agronomic data collected included days to heading, yellow rust disease severity (based on modified Cobb's scale), pseudo black chaff (as an indicator of the presence of *Sr2* adult plant rust resistance gene), percentage stem lodging, plant height and thousand kernel weight (TKW). The days to 50% flowering were recorded when 50% of spikes completely emerged from the boot as an indicator of maturity. Plant height was measured as the length in centimetres from the soil level to the tip of the spikes of randomly selected four plants per plot. The data on thousand kernel weight was

determined by weighing (in grams) one thousand seeds from each plot. Stem lodging was taken as a percentage of lodged stems per plot.

### 3.2.3.2 Identifying wheat genotypes showing resistance to stem rust

Data were subjected to analysis of variance (ANOVA) to determine the significance of the differences among the wheat lines (genotypes) for the different agronomic traits at $p<0.05$. The wheat genotypes were considered as fixed whereas seasons (otherwise considered in this experiment as environments) were considered as random effects. The least significant difference (P=0.05) test was used to compare genotypic means. A Pearson correlation coefficient was done to establish the relationship between the stem rust severity scores and the agronomic traits measured.

### 3.2.3.3 Assessment of the bread wheat germplasm for partial resistance

The final rust severity (FRS), Area Under Disease progress Curve (AUDPC), coefficient of infection (CI), correlation coefficient between the FRS and AUDPC were used as criterion to identify any possible source of partial resistance to stem rust. The AUDPC is a good indicator of adult plant resistance under field evaluations (Denbel *et al*., 2013). The final rust severity (FRS) puts into consideration all the resistance factors during the disease epidemic (Safavi, 2012). The stem rust disease severity scores taken at different times were used to calculate the area under disease progress curve (AUPDC) of each line following Wilcoxson *et al*., (1975) method.

$$AUDPC = \sum_{i=1}^{n-1} 0.5\left(x_{i+1} + x_i\right)\left(t_{i+1} - t_i\right)$$

**Equation 3.1 Formula used to derive the AUDPC** Values: Where; $X_i$ is the cumulative disease severity; $t_i$ is the time (days after planting) and n is total number of observations

The most susceptible check CCACUKE was used as a reference to obtain the relative AUDPC and relative FRS values due to its complete susceptibility to stem rust. The

coefficient of infection (CI) was obtained by multiplying the final disease severity of each season by the numerical notation for the host response; 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 for infection response of trace responses (TR), resistant (R), moderately resistant (MR), moderately resistant-moderately susceptible (M), moderately susceptible (MS) and susceptible (S), respectively (Ali *et al*, 2009). The coefficient of infection of each entry per each season was then averaged to give the average coefficient of infection, ACI (Afzal, *et al*., 2009). A Pearson correlation coefficient was then used to determine the relationship between the different disease epidemiological parameters namely FRS, the AUDPC, rAUDPC and rFDS.

### 3.2.4 Greenhouse tests

The twenty five lines and the checks described in 3.2.3 (Table 3.1) were grown in pots and placed in the growth chamber in the greenhouse. Standard stem rust differentials carrying individual stem rust resistance genes were also grown to ascertain the race used based on the avirulence/virulence formula (Tsilo *et al*., 2010). When the plants had fully expanded primary leaves, they were inoculated with stem rust spores collected from trap nurseries containing *Sr24* gene. The rationale of using inoculum collected from plants with *Sr24* genes lied in the fact that the predominant race that was prevalent in Kenya since 2006 has been TTKST (Ug99+*Sr24*) due to the susceptibility of the gene *Sr24* in variety "Kenya Mwamba". This gene was also used in high frequency in several breeding programs as it was found to be resistant to the original Ug99 (TTKSK) and screening against the *Sr24* virulent race would enable eliminating materials that were present in the breeding materials owing to the new race. Since Kenya Mwamba was readily available which was susceptible to TTKST therefore this variety was used as a susceptible spreader to increase the frequency of the race in the screening nursery.

Freshly suspended urediniospores from spreaders used to multiply the race TTKST (ug99+*Sr24)* were suspended in light mineral oil Soltrol 170 (Chevron Phillips Chemical Company, The Woodlands, TX) and dispensed by misting spore suspension using pressure pump on to the seedlings at two leaf stage. The pots containing the seedlings were rotated in a clock wise fashion to ensure all seedlings get uniform distribution of spores on leaf surface followed by misting and incubation at 16ºC for 24 hours under polythene hoods to facilitate germination of urediniospores. The seedlings were then moved to a temperature and irrigation-controlled greenhouse rooms for disease scoring after 13-14 days. Disease scoring was made based on 0-4 infection type (IT) scale based on Stakman *et al.,* (1962) and this connoted the expression of host-pathogen interaction (Babiker *et al*., 2009; Beteselassie *et al*., 2007).

## 3.3 Results

### 3.3.1 General performance of the wheat genotypes across the years

There was high disease pressure during the two seasons. The spreader rows used during the experiments had completely susceptible responses with 100% disease severities. They were characterized by total crop death and shriveled and /or no seeds in the two seasons. There was variation in the stem rust severities in the field ranging from 1 to 90% (Table 3.1). The field reactions were diverse ranging from trace resistance (TR) among four entries to susceptible responses among the checks (Figure 3.1). Trace responses were displayed as very small chlorotic flecks coupled with no visible pustules on the wheat stems or stalks. Fourteen of the twenty five wheat lines tested displayed RMR to MR responses and disease severities of up to 30%. These lines were characterized by small to medium sized pustules with chlorosis and rough

texture on the stems. The reactions of the remaining entries were categorized as M (MR-MS) and MS.

Considering other agronomic traits, the line with the highest yellow rust resistance was the susceptible check, CACUKE. The line KSL-19 which displayed trace responses to stem rust, also displayed high resistance to yellow rust. Further on KSL-5 and KSL-19 displayed good earliness and plant height traits. In the field, KSL-5 was characterized by some plants appearing as double dwarf in the years of testing. Thousand kernel weights was a parameter used to assess the effect of stem rust on the grain quality and quantity. KSL-20 showed the highest thousand kernel weight while CACUKE showed the least thousand kernel weights given its high susceptibility to stem rust and the shriveled nature of its grains in the field (Table 3.1). A trait of great importance noted among seven of the wheat lines was the pseudo black chaff trait suggesting the presence of the *Sr2* gene in their background (Table 3.1; Figure 3.2) and was evidently absent on the susceptible check CACUKE (Figure 3.3)

**Figure 3.1 Plants displaying moderately susceptible to susceptible infections**

Figure 3.2 Pseudo black chaff trait on the heads and stems of wheat

**Figure 3.3 Plants showing infected stems which lack the pseudo black chaff trait**

### 3.3.2 Greenhouse test for seedling resistance

The wheat lines and checks were grown in the greenhouse alongside the standard stem rust differentials and the spreader lines. These controls showed compatible host pathogen interaction. The inoculum used was collected from *Sr24* gene containing

trap nurseries and complete susceptibility was noted among these spreader lines implying the inoculation was successful (Table 3.1). Resistant infection types were displayed by all the lines except the susceptible checks which displayed infection types (IT) of 33+.

### 3.3.3 Evaluation of the genotypes for partial resistance

Twenty one wheat lines had compatible host-pathogen responses and showed varied final rust severities and responses. These lines had ACI values ranging from 2.63 to 19.50 compared to the seven checks used in this experiment which had ACI values of 85-90 (Table 3.2). The relative AUDPC of these 21 lines ranged from 8.41% to 24.09% with reference to the checks which had rAUDPC ranging from 91.44% to 100%. For the relative final rust severities (rFRS), these lines showed variation ranging from 12.8 % to 43.1% compared to the checks with rFRS ranging from 94.44% to 100%. The rust infection responses of these wheat lines varied from resistant to moderately resistant (RMR) to moderately susceptible (MS). Some wheat lines with moderately resistant to moderately susceptible (M) responses had statistically low stem rust severity while others with resistant responses had high rust severities in this experiment.

**Table 3.1 Performance of wheat lines tested for resistance to stem rust and other traits at the adult plant stages**

| Entry | Parentage | Origin | Maturity (days) | Plant height (cm) | Stem rust | | Yellow rust | | TKW (g) | Stem Lodging (0-100) | PBC | Infection Types | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Severity (0-100) | FR | Severity (0-100) | FR | | | | 2010 | 2012 |
| KSL-1 | COHUEL/SBE-0050(ARGETINA-MIRANDA) | Argentina | 86.5 | 66.31 | 1 | TR | 16.5 | MS | 20.5 | 70 | + | 0 | 0 |
| KSL-2 | CWANA 1st SR RESIS. ON - ETH - OS71 | Syria | 91.5 | 89.59 | 8.75 | RMR | 7.5 | MR | 19.8 | 0 | + | 1 | 1 |
| KSL-3 | MON'S'/ALD'S'//TOWPE'S' | CWANA | 92.5 | 88.31 | 8.75 | RMR | 8.75 | MR | 22.8 | 0 | + | 0; | ; |
| KSL-4 | HRZ05.0078 | Australia | 86.25 | 78.94 | 8.75 | RMR | 7.5 | M | 27.3 | 0 | - | ;1- | 0 |
| KSL-5 | THELIN#2/ TUKURU CGSS02Y00118S-099M-099Y-099M-16Y-OB | Mexico | 71.75 | 72 | 16.25 | MR | 8.75 | MR | 28.6 | 0 | - | 0; | ; |
| KSL-6 | IGW3207 | Australia | 77.75 | 81.12 | 45 | MS | 11.25 | MR | 27.6 | 0 | - | 0;1p2 | ;1 |
| KSL-7 | SERI.1B*2/3/KAUZ*2/BOW//KAUZ/4/PBW343*2/TUKURU/5/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 87.5 | 67.06 | 1 | TR | 18.75 | MR | 25.7 | 65 | - | 0; | ; |
| KSL-8 | WHEAR/VIVITSI//WHEAR | Mexico | 81.25 | 81.69 | 32.5 | M | 18.75 | M | 27.5 | 0 | - | 1- | 0 |
| KSL-9 | WHEAR/SOKOLL | Mexico | 73.25 | 83.56 | 23.75 | MR | 10 | M | 26.7 | 0 | + | ;1- | 2 |
| KSL-10 | WHEAR/JARU//WHEAR | Mexico | 80.25 | 79.5 | 23.75 | M | 30 | S | 30.9 | 0 | - | 1- | ;1 |
| KSL-11 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 81.75 | 75.25 | 28.75 | MR | 32.5 | MSS | 25 | 0 | - | 1- | 0 |
| KSL-12 | PBW343*2/KUKUNA//PBW343*2/KUKUNA/3/PBW343 | Mexico | 86.5 | 78.38 | 25 | M | 21.25 | M | 25.7 | 0 | - | 1- | 0 |

**Table 3.1 Performance of wheat lines tested for resistance to stem rust and other traits at the adult plant stages**

| Entry | Parentage | Origin | Maturity (days) | Plant height (cm) | Stem rust | | Yellow rust | | TKW (g) | Stem Lodging (0-100) | PBC | Infection Types | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Severity (0-100) | FR | Severity (0-100) | FR | | | | 2010 | 2012 |
| KSL-13 | SUPER SERI#1 | Mexico | 83.25 | 86.88 | 23.75 | MR | 22.5 | M | 28.8 | 0 | + | 1+ | ;1 |
| KSL-14 | WHEAR/VIVITSI//WHEAR | Mexico | 82.25 | 86 | 18.75 | RMR | 36.25 | MSS | 23.6 | 0 | - | ;+ | 0 |
| KSL-15 | WHEAR/KUKUNA//WHEAR | Mexico | 82.5 | 88.12 | 18.75 | RMR | 37.5 | MSS | 33.8 | 0 | - | 1= | ; |
| KSL-16 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 74.5 | 81.47 | 35 | M | 21.25 | M | 23.9 | 30 | + | 0; | 0 |
| KSL-17 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 83.25 | 85.75 | 25 | RMR | 18.75 | M | 28.7 | 0 | - | ;1- | ;1 |
| KSL-18 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 83 | 81.06 | 32.5 | M | 15 | M | 24.6 | 0 | - | 1 | ;1 |
| KSL-19 | SUNCO//TNMU/TUI | Mexico | 72 | 74 | 1 | TR | 7.5 | M | 28.4 | 35 | - | 0; | 1 |
| KSL-20 | CHEN/AEGILOPS SQUARROSA (TAUS)//BCN/3/VEE#7/BOW/4/PASTOR/5/VERDIN CMSS02M00361S-030M-15Y-0M-040Y-6ZTB-0Y-03B-0Y | Mexico | 85 | 76.94 | 17.5 | RMR | 26.25 | MS | 36.5 | 0 | + | 0; | 0 |
| KSL-21 | R07 F4-21258 | Uruguay | 86.5 | 69.5 | 1 | TR | 23.75 | MR | 32 | 0 | - | 0; | 0 |
| KSL-22 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 83.25 | 81.09 | 23.75 | M | 17.5 | M | 28.9 | 0 | - | ;1- | ;1 |
| KSL-23 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 83.5 | 82.38 | 31.25 | MR | 28.75 | MSS | 24.5 | 0 | - | 1- | ; |

**Table 3.1 Performance of wheat lines tested for resistance to stem rust and other traits at the adult plant stages**

| Entry | Parentage | Origin | Maturity (days) | Plant height (cm) | Stem rust | | Yellow rust | | TKW (g) | Stem Lodging (0-100) | PBC | Infection Types | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Severity (0-100) | FR | Severity (0-100) | FR | | | | 2010 | 2012 |
| KSL-24 | CHEN/AEGILOPS SQUARROSA (TAUS)//BCN/3/VEE#7/BOW/4/PASTOR/5/VERDIN CMSS02M00361S-030M-16Y-0M-040Y-16ZTB-0Y-03B-0Y | Mexico | 79.5 | 79.56 | 16.25 | MR | 17.5 | MS | 28.7 | 0 | - | ;+ | 0 |
| KSL-25 | (yield trial 2007) | Mexico | 89 | 85.38 | 23.75 | MR | 11.25 | MR | 30.7 | 0 | - | 1+ | ;22+ |
| CACUKE | CANADIAN/CUNNINGHAM//KENNEDY | | 75 | 80 | 90 | S | 5 | MR | 15 | 0 | - | 33+ | 33+ |
| | Least significant differences | | 6.267 | 11.003 | 10.814 | | 15.139 | | 3.02 | | | | |

Severity based on Modified Cobb's scale (0-100%); FR= field responses based on Roelfs *et al*., (1992); TR= trace responses, R = resistant, MR = moderately resistant, RMR = resistant to moderately resistant, MRMS (M) = moderately resistant to moderately susceptible, MSS= moderately susceptible to susceptible, MS = moderately susceptible and S = susceptible; TKW= thousand kernel weight; PBC= Pseudo black chaff where + implies presence while a minus (-) implies absence: Infection types based on Stakman *et al*., (1962) scale where 0 (immune), ; (fleck), 1 (small uredinia with necrosis), 2 (small uredinia with chlorosis), and 3 (small uredinia without chlorosis or necrosis)

### 3.3.4 Correlation analysis among the agronomic traits and the disease epidemiological parameters used to assess for partial resistance

The correlation analysis showed that stem rust severity and plant height were negatively associated with the earliness trait but positively correlated with thousand kernel weight (Table 3.3). The plant height trait also showed a highly and statistically significant positive correlation with the thousand kernel weight. However, stem rust showed a negative correlation with the yellow rust disease trait. All the epidemiological parameters used to assess the genotypes for partial resistance showed highly significant correlations at $p<0.001$ (Table 3.4). The final rust severity was strongly and positively correlated with AUDPC, $R^2=0.70$; ACI, $R^2= 0.62$ and rAUDPC, $R^2= 0.87$

**Table 3.2 Means of the different epidemiological parameters used to assess the wheat genotypes for partial resistance**

| Entry | Parentage | Origin | ACI | AUDPC | rAUDPC | rFRS |
|---|---|---|---|---|---|---|
| KSL-1 | COHUEL/SBE-0050(ARGETINA-MIRANDA) | Argentina | 0.1 | 21.6 | 1.02 | 1.11 |
| KSL-2 | CWANA 1st SR RESIS. ON - ETH - OS71 | Syria | 4.5 | 217 | 10.11 | 16.7 |
| KSL-3 | MON'S'/ALD'S'//TOWPE'S' | CWANA | 4.5 | 204.4 | 8.89 | 13.9 |
| KSL-4 | HRZ05.0078 | Australia | 2.6 | 215.2 | 11.61 | 19.4 |
| KSL-5 | THELIN#2/ TUKURU CGSS02Y00118S-099M-099Y-099M-16Y-OB | Mexico | 8.3 | 220.2 | 9.04 | 12.8 |
| KSL-6 | IGW3207 | Australia | 20 | 582 | 24.09 | 43.1 |
| KSL-7 | SERI.1B*2/3/KAUZ*2/BOW//KAUZ/4/PBW343*2/TUKURU/5/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 0.1 | 35.5 | 2.01 | 1.11 |
| KSL-8 | WHEAR/VIVITSI//WHEAR | Mexico | 16 | 395.6 | 18.3 | 30.6 |
| KSL-9 | WHEAR/SOKOLL | Mexico | 8.3 | 304.4 | 14.19 | 27.8 |
| KSL-10 | WHEAR/JARU//WHEAR | Mexico | 11 | 413.1 | 20.18 | 26.4 |
| KSL-11 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 9.6 | 345.9 | 15.59 | 27.8 |
| KSL-12 | PBW343*2/KUKUNA//PBW343*2/KUKUNA/3/PBW343 | Mexico | 10 | 293.4 | 13.57 | 33.3 |

**Table 3.2 Means of the different epidemiological parameters used to assess the wheat genotypes for partial resistance**

| Entry | Parentage | Origin | ACI | AUDPC | rAUDPC | rFRS |
|---|---|---|---|---|---|---|
| KSL-13 | SUPER SERI#1 | Mexico | 9.4 | 261.9 | 12.18 | 26.4 |
| KSL-14 | WHEAR/VIVITSI//WHEAR | Mexico | 6.1 | 235 | 11.76 | 20.8 |
| KSL-15 | WHEAR/KUKUNA//WHEAR | Mexico | 6.6 | 240 | 11.73 | 26.4 |
| KSL-16 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 18 | 377.1 | 16.46 | 29.2 |
| KSL-17 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 9.5 | 258.1 | 12.84 | 26.4 |
| KSL-18 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 15 | 423.4 | 19.91 | 36.1 |
| KSL-19 | SUNCO//TNMU/TUI | Mexico | 0.1 | 20.9 | 1.02 | 1.11 |
| KSL-20 | CHEN/AEGILOPS SQUARROSA (TAUS)//BCN/3/VEE#7/BOW/4/PASTOR/5/VERDIN CMSS02M00361S-030M-15Y-0M-040Y-6ZTB-0Y-03B-0Y | Mexico | 5.8 | 170.1 | 8.41 | 19.4 |
| KSL-21 | R07 F4-21258 | Uruguay | 0.1 | 40.2 | 2.58 | 6.11 |
| KSL-22 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 10 | 385.9 | 18.38 | 26.4 |
| KSL-23 | WHEAR/VIVITSI/3/C80.1/3*BATAVIA//2*WBLL1 | Mexico | 15 | 377.1 | 17.83 | 29.2 |
| KSL-24 | CHEN/AEGILOPS SQUARROSA (TAUS)//BCN/3/VEE#7/BOW/4/PASTOR/5/VERDIN CMSS02M00361S-030M-16Y-0M-040Y-16ZTB-0Y-03B-0Y | Mexico | 5.8 | 234.4 | 10.36 | 18.1 |
| KSL-25 | (yield trial 2007) | Mexico | 8 | 417.6 | 18.26 | 26.4 |
| CHECK1 | THELIN/3/BABAX/LR42//BABAX/4/BABAX/LR42//BABAX | | 85 | 1855 | 91.44 | 94.4 |
| CHECK2 | THELIN/3/BABAX/LR42//BABAX/4/BABAX/LR42//BABAX | | 90 | 1850 | 93.58 | 100 |
| CHECK3 | THELIN/3/2*BABAX/LR42//BABAX | | 90 | 1880 | 94.69 | 100 |
| CHECK4 | THELIN/3/2*BABAX/LR42//BABAX | | 90 | 1880 | 94.69 | 100 |
| CHECK5 | THELIN/3/2*BABAX/LR42//BABAX | | 90 | 1880 | 94.69 | 100 |
| CHECK6 | THELIN/3/2*BABAX/LR42//BABAX | | 90 | 1947.5 | 96.61 | 100 |
| CACUKE | CANADIAN/CUNNINGHAM//KENNEDY | | 90 | 2007.5 | 100 | 100 |
| | Least significant differences (l.s.d) | | 6.4 | 176.4 | 10.1 | 20 |

ACI= Average coefficient of infection; AUDPC = area under disease progress; rAUDPC= relative area under disease progress curve; rFRS = relative final rust severity scores

**Table 3.3 Correlations coefficients among the different traits at KARI Njoro**

| | Days to 50% flowering | Plant height | Stem rust | Thousand kernel weight | Yellow rust |
|---|---|---|---|---|---|
| Days to 50% flowering | - | | | | |
| Plant height | -0.4812** | - | | | |
| Stem rust | -0.0226 | 0.2308 | - | | |
| Thousand kernel weight | 0.6717** | 0.6418** | -0.1732 | - | |
| Yellow rust | 0.1052 | 0.0854 | -0.0864 | 0.3114 | - |

**Table 3.4 Correlation coefficients between the partial resistance epidemiological parameters at KARI Njoro**

| | Average Coefficient of Infection | Area Under Disease Progress Curve | Relative Area Under Disease Progress Curve | Relative Final Rust Severity |
|---|---|---|---|---|
| Average Coefficient Of Infection | - | | | |
| Area Under Disease Progress Curve | 0.91*** | - | | |
| Relative Final Rust Severity | 0.89*** | 0.93*** | 0.95*** | - |

*** and ** = significance at $p<0.01$ and $p< 0.05$ respectively

### 3.4 Discussion

KARI Njoro site is a hot spot for stem rust disease as shown by the weather data (Appendix 1) and has Ug99 races currently threatening global wheat production (Singh *et al*., 2011). There was heavy disease pressure during the seasons of testing as indicated by the checks which had 90% susceptibility. However, some lines still showed promising stem rust resistance in the years 2008, 2009 and 2010.

Trace reactions were observed for four of these genotypes and showed no compatible host pathogen interaction. Trace reactions associated with hypersensitivity occur when fungal infections signal a defense mechanism leading to cell collapse further restricting disease spread (Afzal, *et al*., 2009; Singh *et al*., 2006; Rubiales and Nicks,

2000). Resistance often breaks down due to the "arms race" between the fungus and the host plant (Qamar *et al*., 2007). This arises when virulent stem rust races increase in frequency hence strong selection pressure is wielded upon the pathogen population leading to emergence of new dominant races which end up overcoming the available race specific resistance as is the case with the devastating Ug99 races (Wanyera *et al*., 2006). A suitable breeding strategy which may involve use of gene combinations or gene pyramiding could enhance the exploitation of any of these wheat lines which contain race specific genes. Race specific resistance genes could also be combined with genes of minor effects (additive genes). The trace response could also be attributed to the presence of many minor genes of small additive effects which provide near immunity (Afzal *et al*., 2009). However, among the four lines, entry KSL-21 which showed no stem lodging in the two seasons was the most promising.

These lines supposedly have some level of partial resistance and these affected the manner of disease infection and spread at the various wheat growth stages. Despite the compatible host pathogen interaction arising from rust infection initiation and sporulation, the rust development was slowed down and restricted. The available resistance in these materials could have also played a role by influencing the time of disease attack and the resultant small size of pustules. The available resistance genes supposedly overcame the stem rust virulence in the field and led to statistically low disease severities despite presence of visible and compatible interaction between host plant and the pathogen.

All these twenty five wheat lines were initially selected as promising genotypes in 2008 during the main season wheat screening nursery at KARI, Njoro. Furthermore, in two more years of screening (2009 to 2010), they have shown appreciable levels of

stem rust resistance. Moreover, even the wheat lines with M and MS responses were observed to possess statistically low disease severities attributable to a combined effect of all the resistance factors during disease progression (Ali *et al*., 2009; Ali *et al*., 2008). These lines could be good sources of partial or slow rusting resistance to stem rust conditioned by additive gene action if further studies are carried out (Kaur and Bariana, 2010). The reduced selection pressure on the pathogen population could have led to the reduced disease severity among these lines (Khan and Saini, 2009). Use of these lines could highly delay evolution of new rust pathotypes because multiple point mutations will barely occur in normal circumstances (Tsilo *et al*., 2010; Ali *et al*., 2008). Moreover, presence of the pseudo black chaff suggested the presence of the *Sr2* gene in their background which is thought to contribute to reduced receptivity of stem rust infection among the adult plants (Singh *et al*., 2011).

From this study, partially responsive race specific and hypersensitive genes could be controlling the stem rust resistance. It is also possible that these wheat lines have complete resistance genes but due to the multiple pathotypes found at KARI, Njoro (Singh *et al*., 2011); thus there is co-segregation for both the avirulent and virulent types of the pathogen genotypes based on the gene for gene concept (Rubiales and Nicks, 2000). On the other hand, the use of the identified resistance in combinations will increase durability of resistance in commercial wheat varieties. However, further improvement of these lines could lead to the accumulation of more minor genes through intercrossing them with elite Kenyan wheat varieties.

Short wheat lines with earliness trait have shown high resistance to stem rust in this study. These findings corroborate with a report by Singh *et al.*, (2008) that semi dwarf varieties are associated with reduced stem rust inoculum accumulation leading to low

disease epidemics. The study revealed that lines with high yellow rust infection had low stem rust severity probably due to the reduction in photosynthetic area crucial for stem rust infection and spread. Stem rust seems to have a great effect on grain quality leading to shriveling of wheat grains as displayed by the low thousand kernel weight values of the check CACUKE. Work by Ali *et al.*, (2008) also showed that the susceptible check used in their study had the least TKW and the least grain yield. Thus, stem rust poses a serious threat to global wheat production because a clean crop could easily be reduced to a tangle of shriveled seeds leading to total crop failure and even 100% yield losses (Mackenzie, 2007; Wanyera *et al*., 2004).

In this study, a high and strong positive correlation was noted among all the epidemiological parameters; AUDPC, FRS, rAUDPC and rFRS; that were used to assess partial resistance at $p < 0.001$. Thus, these parameters were reliable estimators of partial or slow rusting resistance to stem rust. These epidemiological parameters give a dependable rate of disease increase and are related with components of partial resistance like low receptivity, longer latent period and smaller pustules (McNeil *et al*., 2008). Similar findings have been reported in previous studies (Safavi, 2012; Safavi and Afshari, 2012; Ali *et al*., 2009; Qamar *et al*., 2007; Parlevliet, 1993).

Green house studies were conducted to evaluate the specificity/effectiveness of seedling resistance gene/genes against specific races of the pathogen without any confounding effects/interaction of environment as the parameters were kept constant under study. However the lines that conformed to minor gene adult plant resistance (APR) exhibited significant Genotype X Environment interaction.

### 3.5 Conclusion and Recommendations

The sources of stem rust resistance which combined good agronomic traits are elite and should be integrated in wheat breeding programs given the avirulence/virulence nature of the Ug99 race. This will combat the Ug99 threat due to its ability to adapt and evolve into more virulent pathotypes (Synman *et al*., 2004). Some of the key recommendations include:

1. The further improvement of these lines will expose the nature of resistance making it easier for breeders and pathologists to exploit this genetic variability.
2. The lines could also be used to broaden the genetic diversity of elite Kenyan wheat germplasm through back crosses and bulk population breeding (Bartos *et al*., 2002). The back cross strategy could increase the possibility of maintaining and reselecting desirable genes of the recurrent parent. This will also enable the simultaneous transfer of multiple genes or characters especially of pleiotrophic genes. Ultimately, many plants with good agronomic traits and stem rust resistance are selected thus increasing the possibility to identify transgressive segregants due to larger population sizes.
3. Further screening of these lines for slow rusting components like latent period, receptivity and uredinium size through more greenhouse tests coupled with inheritance studies and marker assisted selection will be crucial to establish the identity of the genes conditioning resistance among these lines.

# CHAPTER FOUR

## 4.0 To determine the genetics of the stem rust resistance genes in bread wheat

### 4.1 Introduction

The general livelihood in sub Saharan Africa (SSA) has worsened due to food price hikes, global inflation, drought, civil strife and malnutrition (Singh *et al*., 2011). On the other hand, crop diseases such as cereal rusts have remained a big impediment to the realization of high yields. In the recent past, heavy yield losses have been reported especially in East Africa where wheat is grown throughout the year. The use of susceptible wheat varieties has tremendously increased the frequency and distribution of the *Puccinia graminis* f. sp. *tritici* especially when grown over vast areas (Babiker *et al*., 2009). The race specific stem rust resistance deployed in currently grown wheat varieties has broken down probably through single step mutations leading to the boom and bust cycles (Khan and Saini, 2009). Resistance to stem rust includes seedling and adult plant resistance (Kaur *et al*., 2009; Messmer *et al*., 2000). Seedling resistance is usually expressed throughout the plant growth stages, is characterized by hypersensitive responses (Navabi *et al*., 2004), is race specific and follows the gene for gene concept (Flor, 1955). Race specific resistance is easily identified under simple genetic control making it highly heritable. However, due to the short life cycle and large population sizes of plant pathogens, rapid selection of rare but virulent alleles occur leading to very high disease epidemics (Prakash and Heather, 1988). The use of single resistance genes has been considered a threat to wheat production due to erosion of the few resistance genes against the Ug99 races due to the arms race between the pathogens and the host. Thus, breeders need to continuously replace cultivars and continually introgress new resistance genes in the cultivars under production (Crute and Pink, 1996).

Due to these limitations of race specific genes, the identification and combination of several stem rust resistance genes could offer durable resistance to stem rust. With durable resistance, a pathogenic race which overcomes an allele of minor effect does not have a selective advantage since the host has more resistance alleles (Polanda *et al*., 2011). Adult plant resistance or race non specific resistance is effective in adult plants; with seedling stages being susceptible (Imtiaz *et al*., 2011). A compatible interaction between the plant and pathogen is displayed by having partially resistant adult plants. It is also characterized by genes with additive effects and non hypersensitive responses (Singh *et al*., 2009; Navabi *et al*., 2004; Prakash and Heather, 1988). Race non specific resistance is also associated with durability where widely grown cultivars under high disease pressure for a long time remain resistant. However, major gene resistance and minor gene resistance are mutual. This complicates efforts aimed at selection for minor or race non specific gene resistance, the estimation and studying of the any genetic variation during inheritance studies. Moreover, the recessive nature of some resistance genes and confounding effects of genes in the wheat germplasm background aggravate the problem (Babiker *et al*., 2009). This calls for proper understanding of the genetics of disease resistance and use of appropriate crosses in order to identify any new resistance sources, broaden the genetic diversity and also help in marker assisted selection (Imtiaz *et al*., 2011). There is need to establish the mode of inheritance of the stem rust resistance genes in the currently grown wheat germplasm.

Different types of crosses involving resistant by susceptible lines and the subsequent evaluation of these crosses under inoculation has revealed wheat genotypes with both adult and seedling resistance genes (Bai *et al*., 2009; Eversmeyer and Kramer, 2000; Liu and Kolmer, 1998). The investigation of the mode of inheritance has made

broadening of the genetic diversity of available wheat germplasm possible by using genes with small additive effects (Ijaz and Khan, 2009; Parlevliet, 1995). In other inheritance studies, new resistance genes have been identified; the leaf rust resistance gene, Lr50 and SrD51 (Brown-Guedira et al., 2003; Yin et al., 2008). Transgressive segregants noted have been attributed to additive gene action with high narrow sense heritability (Skovmand et al., 1978). Inheritance studies have helped to establish the interrelationship among catalogued stem rust resistance genes; for example resistance genes with a masking effect and others with cumulative effects (Babiker *et al*., 2009; Adawy *et al*., 2008; Bahadur *et al*., 2003; Williams and Miller, 1982). Thus, the knowledge of the number of genes controlling a trait ensures breeding efficiency. To establish the number of genes conditioning resistance to stem rust, a comparison between observed and expected Mendelian ratios is done. However, the determination of number of genes for quantitative traits is difficult due to the fact that quantitative resistance is complex in nature, is heterogeneous and suffers from the inability to reliably transmit resistance in the subsequent progenies (Vanegas *et al*., 2007). However, given the major threat of stem rust (Ug99) facing wheat production, the genetic analysis of elite wheat germplasm remains a priority. Thus, this study set out to determine the nature, number and mode of inheritance of the genes controlling stem rust resistance in $F_2$ derived $F_3$ ($F_{2:3}$)wheat lines.

## 4.2 Materials and methods

### 4.2.1 Host material

Among the twenty five wheat lines tested for resistance to stem rust, five of them namely KSL-2, KSL-3, KSL-5, KSL-12 and KSL-19 displayed good levels of resistance (Table 4.1). These five resistant lines were used as parents in a partial diallel with the susceptible parent CACUKE. The parent CACUKE is known to be susceptible to the Ug99 race showing 100% susceptibility (Singh *et al*., 2011). The

use of a fully susceptible line helps to minimize the confounding effects of race specific hypersensitive resistance factors (Rubiales and Nicks, 2000). Furthermore, CACUKE is also used as a spreader row at KARI Njoro during international wheat screening nurseries.

### 4.2.2 Experimental site

The experiments were carried out at Kenya Agricultural Research Institute (KARI), Njoro, described earlier in Chapter 3 (section 3.2.2).

### 4.2.3 Field experiments

#### 4.2.3.1 Development of the wheat crosses

Five wheat parents namely KSL-2, KSL-3, KSL-5, KSL-12 and KSL-19 were crossed in a partial diallel to the susceptible parent CACUKE to develop $F_1$s (Figure 4.1). Four seeds of each $F_1$ were planted in small pots and placed in a growth chamber of a greenhouse at room temperature at KARI, Njoro. The $F_1$'s were then transplanted in the crossing block, self pollinated and harvested from individual plants for each cross at maturity (Figure 4.2). The parents and 200-250 $F_2$ seeds of each $F_1$ cross were planted at a spacing of 10cm in two row plots each measuring 12.5m long and advanced to $F_3$ generation through single seed descent under disease free conditions and no selection was done (Vanegas *et al*., 2007; Liu and Kolmer, 1998) (Figure 4.3). For evaluation, 150 $F_{2:3}$ lines were tested for adult plant resistance.

#### 4.2.3.2 Assessment of adult plant stem rust resistance

About 15 to 20 seeds of each parents and 150$F_{2:3}$ lines were grown on two 0.5m rows in unreplicated plots with intra and inter spacing of 20 cm by 70 cm in 2012 main season and 2012 – 2013 off season. After every twenty plots, a susceptible check CACUKE was included. Spreader rows were also grown perpendicular to the plots and in the perimeter of the experimental plots.

Figure 4.1 Development of the $F_1$ crosses in the crossing block

Figure 4.2 Advancing the $F_1$s into $F_2$ generation

Figure 4.3 Advancement of the $F_2$ into $F_3$ in the crossing block

The spreaders were repeatedly inoculated by injecting the pre-emergent leaves with a water suspension of urediniospores collected from *Sr24* gene containing trap nurseries to create an artificial stem rust epidemic in the field (Hickey *et al*., 2012; Singh *et al*., 2009; Khanna *et al*., 2005; Liu and Kolmer, 1998). All normal crop husbandry practices were observed. The maturity of these families was noted based on 50% days to heading and were recorded when 50% of spikes completely emerged from the boot. The pseudo black chaff trait was also observed among the parents and the $F_3$ crosses.

The modified Cobb's scale (Peterson *et al*., 1948) was used to assess the stem rust severity. Disease scoring began when the susceptible check displayed about 50% susceptible responses. The observations in the $F_{2:3}$ families were genetically grouped into homozygous resistant, segregating and homozygous susceptible depending on their field infection responses (Roelf's scale, 1992). Those displaying disease severity equal to or higher than the susceptible parent CACUKE were considered susceptible (Khanna *et al*., 2005).

### 4.2.4 Greenhouse experiments

The parents and 70 $F_3$ families of each cross were planted in pots and placed in a growth chamber in a greenhouse. When plants attained a two leaf stage, they were inoculated with stem rust spores collected from infected stems of *Sr24* gene containing trap nurseries. Freshly suspended urediniospores from spreaders used to multiply the race TTKST were suspended in light mineral oil Soltrol 170 (Chevron Phillips Chemical Company, The Woodlands, TX) and dispensed by misting spore suspension using pressure pump on to the seedlings at two leaf stage. The pots containing the seedlings were rotated in a clock wise fashion to ensure all seedlings get uniform distribution of spores on leaf surface followed by misting and incubation

at 16ºC for 24 hours to facilitate germination of urediniospores. The inoculated plants were incubated under polythene hoods in natural light at 18-20°C for 48 h. The seedlings were then moved to a temperature and irrigation-controlled greenhouse rooms for disease scoring after 13-14 days. Disease scoring was made based on 0-4 infection type scale based on Stakman *et al.*, (1962); where infection types (IT): 0 (immune),; (fleck), 1 (small uredinia with necrosis), 2 (small uredinia with chlorosis), and 3 (small uredinia without chlorosis or necrosis) were considered resistant, and ITs 3+ to 4 (large uredinia without chlorosis or necrosis) were considered susceptible. The Infection types connoted the expression of host-pathogen interaction (Beteselassie *et al*., 2007). The standard stem rust differentials carrying individual stem rust resistance genes were used as checks to ascertain the race used based on the avirulence/virulence formula (Tsilo *et al*., 2010).

### 4.2.5 Data analysis

Chi square test was done to estimate the number of genes conditioning resistance to stem rust among these populations. The chi square method was used to test the goodness of fit of observed segregations to the expected genetic ratios of 1 homozygous resistant, 2 segregating and 1 homozygous susceptible line (Equation 4.1) (Snedecor and Cochran, 1989).

$$\chi^2 = \frac{\sum(\text{Observed} - \text{Expected})^2}{\text{Expected}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 4.1}$$

## 4.3 Results

There was heavy disease pressure in the field during all the growing seasons. When the five parents and their $F_{2:3}$ families were evaluated for adult plant resistance in the field in 2012 and 2013 seasons, a varied response was noted (Table 4.1). Compared

with the check CACUKE, the five parents were considered resistant and had stem rust severity and infection responses ranging from trace responses (TR) to 25MRMS (M) responses during the 2008, 2009, 2010, 2012 and 2013 screening experiments (Table 4.1). The susceptible line, CACUKE had susceptible responses ranging from 60S to 90S (Table 4.1) and was infected with many pustules (Figure 4.4).

**Figure 4.4 Plants showing susceptible infection responses with teliospores on the check CACUKE**

**Table 4.1 Stem rust disease severity of the wheat parents used in the genetic studies**

| Genotype | Pedigree | origin | [a]Field rust severity (%) and [b]host response across different seasons | | | | | | [c]PBC |
|---|---|---|---|---|---|---|---|---|---|
| | | | 2008/2009 | 2009/2010 | 2010 | 2012- Season | 2012/2013 | Mean | |
| KSL-2 | CWANA 1st SR RESIS. ON - ETH - OS71 | Syria | 10MS | 12.5M | 5RMR | 5R | 5M | 7.5 | + |
| KSL-3 | MON'S'/ALD'S'//TOWPE'S' | CWANA | 10S | 12.5M | 5RMR | 5R | 5MR | 7.5 | + |
| KSL-5 | THELIN#2/ TUKURU CGSS02Y00118S-099M-099Y-099M-16Y-OB | Mexico | TR | 17.5M | 15MR | 5R | 1R | 12.5 | _ |
| KSL-12 | PBW343*2/KUKUNA//PBW343*2/KUKUNA/3/PBW343 | Mexico | 5R | 25RMR | 25M | 10MR -15M | 5MR | 21.7 | _ |
| KSL-19 | SUNCO//TNMU/TUI | Mexico | TR | TR | TR | R -20MSS | 1R | 7.3 | _ |
| CACUKE | CANADIAN/CUNNINGHAM//KENNEDY | | | 80-90S | 80-90S | 80-90S | 60-90S | 90 | _ |
| | Standard error of differences | | | | | | | 5.38 | |

[a] Field rust severity was based on modified Cobb's scale where 0: immune and 100: completely susceptible characterized by shriveled or no kernels and also broken stems; [b]Field responses based on Roelfs *et al*., 1992 scale where TR= trace responses were assumed to give a disease severity of 1; RMR: resistant to moderately resistant; MR: moderately resistant; M: moderately resistant to moderately susceptible; S: susceptible; [c]PBC= pseudo black chaff where a plus (+) implies presence and a minus (–) means absence of the pseudo black chaff

The $F_2$:$_3$ families displayed resistant, segregating and susceptible responses and were thus grouped into these three distinct classes. In 2012 main season, the chi square tests revealed that the segregation data conformed to the expected ratio of 1:2:1 for a single gene model among the parents KSL-2, KSL-5 and KSL-12 at $p < 0.01$ significance level (Table 4.2). The chi square tests for the parents, KSL-3 and KSL-19 conformed to the 12:3:1 genetic ratio. In the 2012/2013 season, the segregation data of the parent KSL-2 conformed to the 1:2:1 genetic ratio whereas that of the other four parents suggested involvement of two genes interacting in different modes (Table 4.2). This experiment also showed segregation with respect to maturity and also presence of the pseudo black chaff (PBC) (Table 4.1; Figure 4.5). However, the PBC was not expressed among the parents KSL-5, KSL-12, KSL-19 and the check CACUKE.

**Figure 4.5 PBC on the wheat head and the stalks**

**Table 4.2 Segregating data for adult plant resistance to stem rust in $F_2$ derived F3 families from crosses involving the five wheat genotypes during the 2012 off season and 2012 -2013 main season at KARI Njoro, Kenya**

| Cross | Parentage | Season | Number of $F_2$ derived $f_3$ families | | | Total | Chi square ($X^2$) | Genetic ratio | Number of genes |
|---|---|---|---|---|---|---|---|---|---|
| | | | Resistant | Segregating | Susceptible | | | | |
| CACUKE/KSL-2 | CACUKE X CWANA 1st SR RESIS. ON - ETH - OS71 | 2012-2013 | 17 | 63 | 28 | 108 | 5.2 | 1:02:01 | 1 gene |
| | | 2012 | 34 | 62 | 18 | 114 | 5.37 | 1:02:01 | 1 gene |
| CACUKE/KSL-3 | CACUKE X MON'S'/ALD'S'//TOWPE' S' | 2012-2013 | 118 | 34 | 2 | 154 | 7.0 | 12:03:01 | 2 genes |
| | | 2012 | 121 | 47 | 12 | 180 | 6.0 | 12:03:01 | 2 genes |
| CACUKE/KSL-5 | CACUKE X THELIN#2/ TUKURU CGSS02Y00118S-099M-099Y-099M-16Y-OB | 2012-2013 | 56 | 75 | 18 | 149 | 7.7 | 7:08:01 | 2 genes |
| | | 2012 | 56 | 73 | 37 | 166 | 6.76 | 1:02:01 | 1 gene |
| CACUKE/KSL-12 | CACUKE X PBW343*2/KUKUNA//P BW343*2/KUKUNA/3/P BW343 | 2012-2013 | 32 | 20 | 25 | 77 | 6.8 | 9:04:03 | 2 genes |
| | | 2012 | 25 | 41 | 19 | 85 | 0.95 | 1:02:01 | 1 gene |
| CACUKE/KSL-19 | CACUKE X SUNCO//TNMU/TUI | 2012-2013 | 80 | 25 | 3 | 108 | 3.2 | 12:03:01 | 2 genes |
| | | 2012 | 79 | 38 | 9 | 126 | 3.36 | 12:03:01 | 2 genes |

[b] $X^2$ at 2df and at probability level p= 0.01 is 9.21

The greenhouse tests among the parents and the $F_{2:3}$ families showed diverse infection types. The parents showed resistant infection types whereas all the $F_{2:3}$ families except the cross between CACUKE and the KSL-2 had infection types ranging from 0 immune (0), fleck (;) to susceptible ones with infection type 4 (Table 4.3; Figure 4.6 and 4.7).

**Table 4.3 The infection types noted among the $F_{2:3}$ families of the five resistant by susceptible crosses**

| Cross | Parentage | Infection types noted in the greenhouse |
|---|---|---|
| KSL-2 | CWANA 1st SR RESIS. ON - ETH - OS71 | 1 |
| KSL-3 | MON'S'/ALD'S'//TOWPE'S' | ; |
| KSL-5 | THELIN#2/ TUKURU CGSS02Y00118S-099M-099Y-099M-16Y-OB | ; |
| KSL-12 | PBW343*2/KUKUNA//PBW343*2/KUKUNA/3/PBW343 | 0 |
| KSL-19 | SUNCO//TNMU/TUI | 1 |
| CACUKE X KSL-2 | CANADIAN/CUNNINGHAM//KENNEDY | 0, ;, 1, 2, 2+, 2X, 3, 3+, 4, 4X |
| CACUKE X KSL-3 | CACUKE X MON'S'/ALD'S'//TOWPE'S' | 0, ;, 1, 1+, 2 |
| CACUKE X KSL-5 | CACUKE X THELIN#2/ TUKURU CGSS02Y00118S-099M-099Y-099M-16Y-OB | 0, ;, 1, 1+, 2, 2+, 3 |
| CACUKE X KSL-12 | CACUKE X PBW343*2/KUKUNA//PBW343*2/KUKUNA/3/PBW343 | 0, ;, 1, 1+, 2, 2+, 3, 3+, |
| CACUKE X KSL-19 | CACUKE X SUNCO//TNMU/TUI | 0, ;, 1, 1+, 2, 2+, 3 |
| CACUKE | CANADIAN/CUNNINGHAM//KENNEDY | 33+ |

**Figure 4.6 Infection types among highly susceptible wheat genotypes**

**Figure 4.7 Chlorotic infection types among the wheat seedlings**

### 4.4 Discussion

The wheat lines involved in this genetic study have shown promising resistance even after four years of field testing at KARI Njoro. These lines were selected for crossing based on their promising resistance during the 2008, 2009 and 2010 growing season at KARI Njoro; a hot spot for stem rust with all known *Puccinia graminis* f. sp. *tritici* races (Singh *et al*., 2011). Despite the fact that heavy stem rust disease pressure characterized all the wheat growing seasons, these five wheat lines have consistently exhibited resistance to stem rust. This implies that these lines are important sources of resistance and could be used to improve the current wheat germplasm as well as broadening their genetic resistance to curb the high global wheat yield losses. Given that even parents with moderately resistant (MR) to moderately susceptible (MS) or M responses have shown promising resistance, the presence of partial resistance seems to be undisputable.

In crop improvement, the nature of population and crosses involved determine the segregation pattern of germplasm (Xian *et al*., 2006). The segregation data for parents KSL-3 and KSL-19 conformed to the genetic ratio 12:3:1 in both seasons implying the presence of complete dominance or dominant epistasis at two gene pairs and had many resistant families. The segregation data for KSL-2 which conformed to the 1:2:1 genetic ratio in both seasons suggested involvement of one dominant gene in conditioning the stem rust resistance. The lack of consistency among KSL-5 and KSL-12 in both seasons could be attributed to the differences in genetic background of the wheat parents and environmental (seasonal) conditions of the research (McNeil *et al*., 2008). The inconsistency could have also risen from the fact that most of the stem rust resistance genes are recessive and have confounding effects in the genetic backgrounds of most

cultivars (Babiker *et al.*, 2009). Thus, the resistance genes in many wheat backgrounds show incomplete penetrance in the presence of the variable and aggressive Ug99 races. The expression of the stem rust resistance has also been found to be very variable under different environmental conditions thus complicating genetic studies even after the phenotypic evaluations (Tsilo *et al*., 2009; Rajender *et al*., 2004; Gold, 1998). Chi square analyses done by several authors have also revealed presence of a single dominant gene (Babiker *et al*., 2009; Yin *et al*., 2008). The genetic ratios 9:4:3, 12:3:1 and 7:8:1 suggested that two different genes with epistatic effects contributed to stem rust resistance among these parents. Epistasis is a form of gene interaction, whereby one gene interferes with the phenotypic expression of another non-allelic gene or genes. Thus, the combined phenotypic effect of two or more genes is either less than (negative epistasis) or greater than (positive epistasis) the sum of effects of individual genes.

Segregation for the expression of the pseudo black chaff was noted in this experiment among the crosses with KSL-2 and KSL-3 during the 2009, 2010, 2012 and 2013 field tests. This supports the fact that it's additive in nature with high heritability (Ginkel and Rajaram, 1993). The pseudo black chaff which develops around stem internodes and the heads is completely linked with the *Sr2* gene; a race non specific stem rust resistance gene (Kaur *et al*., 2009). The race non specific genes are associated with non hypersensitive responses which lead to reduced sporulation and infection frequency. In its presence, there is slow disease progression and less selection pressure is exerted on the pathogen population leading to durability with respect to rust resistance in wheat (Prakash and Heather, 1988).

From this study, it was also noted that there was segregation for stem rust resistance among the resistant by resistant crosses. The genes which are conditioning stem rust resistance among these parents are supposedly different or are non allelic and it's probable that other important stem rust resistance genes could be in the wheat parents' background. However, allelism test should be done to investigate this finding.

### 4.5 Conclusions and Recommendations

The knowledge of the nature and the number of genes revealed among the wheat parents form a core component of the wheat breeding program in ensuring efficient breeding strategy by integrating the wheat parents in breeding for durable resistance to stem rust. Some of the key recommendations include:

1. These parents could be involved in more biparental crosses and advanced into recombinant inbred lines (RILs). These RILs could then be used in QTL mapping to help elucidate the exact gene effects involved and expose the presence of any genes of minor effects. This could then be followed by development of genetic linkage maps with repeat phenotyping to reduce the possibility of recombinants
2. The resistance genes could be isolated and further characterized in order to enhance durability of the available elite wheat germplasm.
3. The diversity revealed in these parents could be exploited effectively in breeding programs if the loci associated with the high resistance could be identified and used in combinations for use in the Ug99 race high risk areas (Khanna *et al*., 2005). This could be achieved by gene pyramiding of major genes and minor gene into elite Kenyan varieties to combat the food and nutritional insecurity. given the imminent threat of Ug99.

# CHAPTER FIVE

## 5.0 To identify molecular markers and the genomic regions harbouring the adult plant stem rust resistance (APR) genes

### 5.1 Introduction

Resistance to stem rust is attributed to major, race-specific resistance/vertical resistance and general resistance, race non-specific resistance, horizontal resistance or adult plant resistance (APR). The vertical or race specific resistance is simply inherited and it provides high protection until the pathogen population evolves to overcome such resistance (Milus *et al*., 2009). It is also based on host pathogen recognition genes (*R*-genes) which are effective at all plant stages and shows hypersensitivity (Maccaferri *et al*., 2008). It is usually expressed at all stages of plant development (overall resistance) following the gene for gene concept and is also associated with the boom and bust cycle (Khlestkina *et al*., 2007). A few race specific stem rust resistance genes are still effective against the Ug99 races including *Sr13, Sr14, Sr22, Sr28, Sr33, Sr35, Sr42* and *Sr45* (Hiebert *et al*., 2010). However, linkage drag associated with some of these resistance genes limits their use in wheat breeding. For example; the *Sr2*2 and *Sr26* pose a yield penalty in wheat production while *Sr25* is associated with undesirable yellow flour (Ejaz *et al*., 2012; Yu *et al*., 2011). The use of single race specific resistance genes could lead to genetic erosion thus reduced genetic diversity. This emphasizes the need for concerted efforts in diversifying the genetic base of available wheat varieties coupled with identifying more resistance sources especially among uncharacterized wheat germplasm. The race non specific resistance leads to increased durability and is associated with multiple additive genes which confer APR thus reducing the selection pressure for pathogen virulence (Milus *et al*., 2009). The use of APR in wheat offers a feasible

sustainable stem rust management strategy. The adult plant resistance (APR) lengthens the time of pathogen colonization while reducing its sporulation. To ensure the efficient utilization of APR in breeding programs, the chromosomal locations of such resistance genes and their biological effects should be identified (Jackson *et al*., 2008). It is also imperative to develop informative or diagnostic markers for use in marker assisted selection (MAS) to facilitate the pyramiding of the race specific genes, use of multilines and partial resistance which could offer sufficient plant stem rust resistance crucial in the Ug99 race high risk areas (Yu *et al.,* 2011; Rosewarne *et al*., 2008).

Many types of molecular markers including restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeat (SSR), single nucleotide polymorphisms (SNPs) and Diversity Array based genotyping technologies (DArTs) have been used for gene tagging and quantitative trait loci (QTL) analysis (Kolmer *et al*., 2011; Crossa *et al*., 2007; Varshney *et al*., 2007; Semagn *et al*., 2006). The Diversity Array based genotyping technologies (DArT) has been used to discover and score genetic polymorphic markers in the whole genome (Akbari *et al*., 2006). Its high multiplexing ability and throughput combined with low cost has made it an edge cutting technology.

The DArT markers are used to identify molecular markers closely linked to genes or QTLs of interest, isolation of genes via map based cloning, genome profiling, genetic map construction, quantitative trait loci (QTL) identification, genetic diversity analysis and identifying of elite varieties, comparative mapping and genome organization studies (Yu *et al*., 2012; Brągoszewska *et al*., 2009; Peleg *et al*., 2008). Quantitative trait loci

(QTL) identification helps to elucidate the inheritance of complex traits which are associated with specific regions of chromosomes called QTL's (Navabi *et al*., 2005). This also helps to establish their action, mode of interaction, number and location of these quantitative traits. Different methods of QTL detection have been identified (Collard *et al*., 2005; Sato *et al*., 2001).

In order to attain a sustainable global wheat production and productivity, the identification of sources of stem rust resistance and the subsequent introgression of this resistance into the adapted wheat germplasm remains critical. This would go hand in hand with the identification of QTL's associated with the stem rust resistance. Thus, this study set out to identify genomic regions involved in adult plant resistance (APR) to stem rust in an $F_6$ PBW343/Juchi recombinant inbred line (RIL) mapping population.

## 5.2 Materials and methods

### 5.2.1 Host germplasm

The mapping population consisted of 107 Recombinant Inbred Lines (RIL) families derived from resistant parent Juchi and susceptible parent PBW343. $F_1$ progeny from the initial cross were advanced into $F_6$ generation through single seed descent. The line "Juchi" (KITE/BOBWHITE//ROMOGA-F-96[3252]) is an advanced spring wheat line developed at CIMMYT, Mexico. This line showed adequate field resistance in the screening nurseries at KARI in 2008, 2009 and 2010 nurseries and seedling tests at CDL, Minnesota confirming that Juchi was susceptible at seedling stage and had no major genes/race specific resistance genes thence the stem rust resistance was conferred by additive minor APR genes.

### 5.2.2 Field experiments

The F6 mapping population was grown during the 2009 main season, 2009/2010 off season, 2010 main season and 2010/2011 off seasons at Kenya Agricultural Research Institute, Njoro (as described in section 3.2.2). The four seasons were treated as different environments in this study because the Ug99 race is a quarantined pathogen and artificial inoculation is not allowed outside of KARI, Njoro screening site. The recombinant inbred lines were grown in two replicates. Every plot comprised two 0.7m rows spaced at 20cm. Spreader rows carrying the *Sr24* and *Sr36* genes were used as secondary sources of inoculum for epidemic development. They were grown perpendicular to the row plots and along the borders of the experiment two weeks before the RILs were planted in order to increase the disease pressure while ensuring uniform dissemination of inoculum. They were later inoculated with urediniospores of the Ug99 spores collected from the trap nurseries with *Sr24* gene carrying lines. Inoculation was done in the evenings when plants were almost booting using a syringe to create an artificial disease epidemic and ensure uniform inoculum dissemination. When there was no rainfall, plants were repeatedly irrigated to enhance stem rust infection and spread (Kaur *et al*., 2009). All other normal crop husbandry practices were observed during the field experiments.

The stem rust disease severity was assessed among the genotypes based on the modified Cobb scale (Peterson *et al*., 1948) combined with recording the stages of plant growth based on Zadok's *et al.,* (1974) scale. The infection responses (plant response to stem rust infection in the field among the adult plants) were noted based on Roelf's *et al*., (1992) scale. The disease assessment continued until the plants attained physiological maturity.

The pseudo black chaff trait was also assessed among the wheat lines for the presence of the *Sr2* gene.

### 5.2.3 Chi square analysis

The stem rust disease severity scores were used to group the genotypes into two distinct classes; non-segregating homozygous resistant and non-segregating homozygous susceptible. The chi square method (Snedecor and Cochran, 1989) was used to test the goodness of fit of observed segregations to the expected genetic ratios of monogenic (1:1) segregation ratio.

### 5.2.4 Genotyping of the mapping population

DNA of the $F_6$ progenies from the Juchi by PBW343 population were extracted from the leaves using the cetyl-trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). DNA of each RIL and the parents comprising 500–1,000 ng of restriction grade DNA was suspended in TE and adjusted to a final concentration of 50–100 ng/lL and sent to Triticarte Pty. Ltd., Canberra, Australia (http://www.triticarte.com.au) for whole genome profiling following the Wenzl *et al.,* (2006) method. This was done to identify a large number of segregating molecular makers. The loci were scored as either present (1) or absent (0). The DArT markers were named starting with the prefix‘XwPt’ and a number corresponding to the particular clone in the genomic representation, where w stands for wheat, P for PstI (primary restriction enzyme) and T for TaqI (secondary restriction enzyme). The markers were named starting with ‘w’ if clones were from wheat, "t" if they were from triticale and ‘r if they were from rye libraries, respectively (Akbari *et al.,* 2006).

### 5.2.5 Trait Analysis

The DArT markers polymorphic in the RIL population were transformed into genotype codes according to the score of the parents which was either resistant (A) or susceptible (B) and loaded onto the Map Disto software version 1.7 (Lorieux, 2007). Mapdisto was used to generate the genetic linkage map for the mapping population and also to establish the significance of the segregation distortion from the expected Mendelian ratio of 1:1 at $P<0.05$ by performing a Chi square test analysis. A maximum distance of 30 centimorgans (cM) and a minimum logarithm of odds (LOD) threshold of 3 were used to partition markers into linkage groups.

The Inclusive composite interval mapping (ICIM) (http://www.isbreeding.net) software version 3.3 was then used to analyze the LOD profiles with the DArT markers as detected by the MapDisto software. The markers were assigned into 25 linkage groups based on their map distances, LOD value and linkages between markers at a significance level of $P = 0.001$ (Alheit *et al.,* 2011). The genetic distances were calculated from the recombination frequencies of all pairs of markers using maximum likelihood estimation procedure by applying the Kosambi mapping function. To determine the LOD threshold value above which a QTL was considered significant, 1,000 permutations were performed ($P = 0.05$) to determine the threshold level for QTL detection. The permutation test identified a LOD score threshold of 2.5 to be significant for QTL detection. The percentage phenotypic variation (%PV) associated with each significant QTL was calculated from the stepwise regression of all the marker information with the phenotypic values which were then adjusted by all markers retained in the regression equation except the two markers flanking the current mapping interval (Semagn *et al*.,

2006). The adjusted phenotypic values were finally used in interval mapping until the testing position moved into a new interval at a LOD score of 2.5 which was the threshold for declaring the presence of QTL. The orientation of the chromosome arms or linkage groups was determined through comparison of the locations of the DArT markers with published high density or consensus wheat maps (Xue *et al.,* 2012; Francki *et al.,* 2009; Crossa *et al.,* 2007; Somers *et al.,* 2004).

## 5.3 Results

### 5.3.1 Phenotypic analysis

The stem rust severities ranged from trace responses to 20% for the resistant parent Juchi while the susceptible parent PBW343 showed severities ranging from 60 to 100% (Figure 5.1). The spreader rows included in the experiment were completely susceptible and were characterized by broken stalks and shriveled or no kernels at all.

### 5.3.2 Genetic analysis of the RIL population

The segregation data among the 107 F6 RILs showed a deviation from the 1:1 Mendelian ratio for all the seasons (Table 5.1). Inheritance studies suggested the involvement of at least 3 to 4 minor APR genes for stem rust resistance. Continuous bell shaped distribution of RIL's in mapping population also suggested quantitative variation due to additive effects (Fig 5.1).

### 5.3.3 Segregation distortion of the markers

From the Mapdisto software, 25 linkage groups and their mapping distances were established. This information was subsequently used in QTL mapping in the ICIM software. The linkage maps spanned 6049.9cM with an average of one marker per 9.3cM and were later assigned to wheat chromosomes using previously published map locations of DArT markers as reference points (Singh *et al*., 2013). The F6 RIL's were genotyped

using DArT markers. Out of the 534 polymorphic marker data obtained, 321 markers were found to be informative for analysis. After genotyping the $F_6$ RILs using 734 DArT markers, one hundred and ninety two DArT markers showed segregation distortion from the expected 1:1 Mendelian ratio at $p<0.05$ (data not shown). Based on 1000 permutations of these datasets, the LOD of the putative QTLs identified ranged from 2.6 to 7.1 (Table 5.2).

**Table 5.1 Segregation for adult-plant resistance to *Puccinia graminis* f. sp. *tritici* in $F_6$ RILs involving Juchi/PBW343 mapping population**

| Mapping Population | Season (Environment) | Resistant | Susceptible | $X^2$ | |
|---|---|---|---|---|---|
| | | | | 3:1 | 15:1 |
| PBW343 × Juchi | 2009 off season (season 1) | 85 | 22 | 1.1 | - |
| | 2009 Main season (season 2) | 93 | 14 | 8.5 | 8.1 |
| | 2010 off (season 3) | 78 | 29 | 0.3 | - |
| | 2010 Main season (season 4) | 90 | 17 | 4.7 | - |

A minus (-) implies significant difference at $p<0.01$

### 5.3.4 QTL analysis for adult plant resistance to stem rust in bread wheat

QTLs were identified through the step wise regression ($R^2$) analysis for the additive mapping using the informative DArT markers. Genetic mapping by ICIM-ADD suggested involvement of regions on chromosomes in stem rust resistance when the threshold significance value for the analysis was set to P value < 0.05. The DArT markers were informative for the ends of the chromosome arms; 1BL, 2BL, 2BS, 3B, 5BL, 6BS and 7BL with LOD scores above 2.5 and %PVE ranging from 8 to 35.9% implying that

they were QTLs of minor effects (Table 5.2 and Figures 5.2-5.5). Three QTLs were identified on different chromosome arms in two seasons of the experiment (Table 5.2).

The QTL on chromosome arm 2BL was flanked by tPt-9767 and wPt-2724 in the first season and the markers wPt-7829 and wPt-2266 in the second season and explained 8.2% and 7.5% of the total phenotypic variation (Figure 5.2). The resistant loci associated with this resistance were derived from the parent Juchi. The QTL detected on chromosome 3B were flanked by the DArT markers wPt-6187 and wPt-5769 in the first season and markers tPt-6872 and wPt-8915 in the third season. These two QTLs explained 8.4 and 8.8% of the total phenotypic variation (Table 5.2). The resistant loci associated with this resistance were derived from the parent Juchi. The third QTL detected across seasons 2 and 3 was identified on the chromosome arm 5BL and was flanked by the markers wPt-0750 and wPt-5896 in the second season and wPt-5896 and wPt-1733 in the third season (Figure 5.3). The QTLs explained 16.4 and 10.6% of the total phenotypic variation. The resistant loci associated with this resistance were derived from the susceptible parent PBW343. Other QTLs detected in one of the seasons of evaluation were mapped on chromosome arms 6BS, 7BL and 2BS.

As determined by the recombination frequencies between parent alleles, the increases in stem rust resistance at these putative QTLs were contributed by the alleles from the resistant parent Juchi except for the QTLs mapped on chromosome 5BL with estimated additive effects of -7.5 and -6.1 for seasons 2 and 3 respectively (Table 5.2). It was also observed that all the markers in this study were mapped to the B genome.

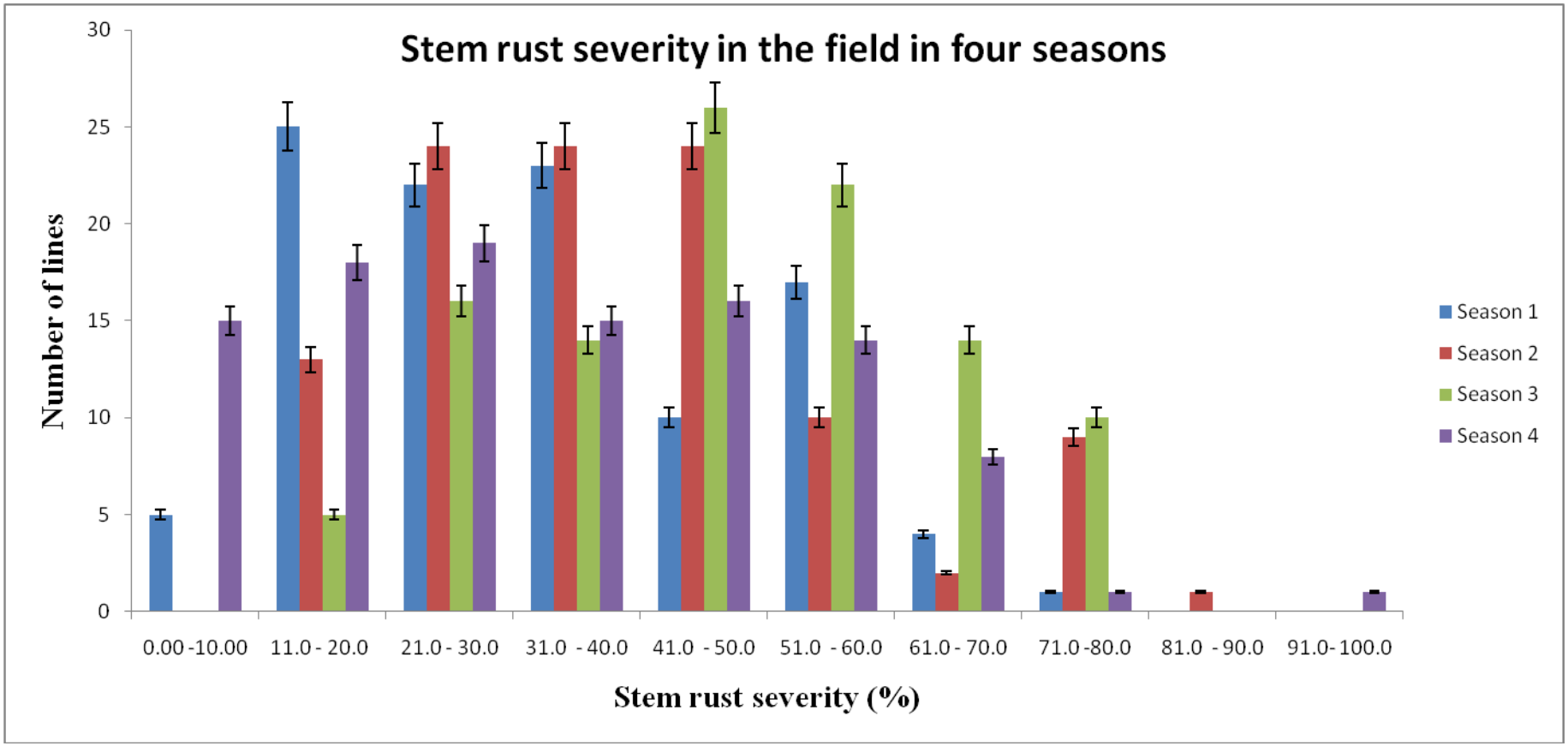


**Figure 5.1 Stem rust disease severity of the PBW343 × Juchi RIL population across four seasons/ environments**

**Table 5. 2 Summary of the QTL explaining stem rust responses variation among the Juchi/PBW343 RIL population across two years of testing**

| Season | Linkage group | Interval size (cM) | Left Marker | Right Marker | LOD | Specific $R^2$ (%PVE) | Estimated additive effects | Chromosome | Cited by | Source | Total $R^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2009/2010 off season | 5 | 142 | tPt-9767 | wPt-2724 | 2.60 | 8.16 | 5.25 | 2BL | Marone *et al*., 2012 | Juchi | |
| 2009/2010 off season | 8 | 3172 | wPt-6187 | wPt-5769 | 2.91 | 8.43 | 5.08 | 3B | Adhikari *et al*., 2012; Francki *et al*., 2009 | Juchi | 35 |
| 2009/2010 off season | 17 | 43 | wPt-6293 | wPt-2964 | 3.04 | 8.79 | 5.09 | 6BS | Marone *et al*., 2012; Vaissayre *et al*., 2012 | Juchi | |
| 2009 Main season | 5 | 152 | wPt-7829 | wPt-2266 | 4.58 | 16.62 | 7.49 | 2BL | Bhavani *et al*., 2011 | Juchi | |
| 2009 Main season | 14 | 472 | wPt-0750 | wPt-5896 | 4.14 | 16.44 | -7.50 | 5BL | Marone *et al*., 2012 | PBW343 | 23 |
| 2010/2011 off season | 4 | 167 | wPt-8326 | wPt-5738 | 7.12 | 35.89 | 10.43 | 2BS | Marone *et al*., 2012 | Juchi | |
| 2010/2011 off season | 8 | 3508 | tPt-6872 | wPt-8915 | 3.06 | 8.65 | 4.98 | 3B | Li *et al*., 2010 | Juchi | 37 |
| 2010/2011 off season | 14 | 473 | wPt-5896 | wPt-1733 | 3.65 | 10.57 | -6.06 | 5BL | Bariana *et al*., 2010; Peleg *et al*., 2008 | PBW343 | |
| 2010 Main season | 2 | 220 | wPt-9028 | wPt-1782 | 4.85 | 21.59 | 9.06 | 1BL | Jing *et al*., 2009; Li *et al*., 2010 | Juchi | |
| 2010 Main season | 20 | 140 | wPt-9493 | wPt-8387 | 5.81 | 21.04 | 8.81 | 7BL | Vaissayre *et al*., 2012; Trebbi *et al*., 2011 | Juchi | 28 |

The reactions were considered as quantitative, and were analysed by QTL Inclusive composite interval mapping (ICIM); LOD: is the logarithm of odds ratio, maximum of QTL peak for the respective QTL; %PVE: Percentage of phenotypic variance explained by QTL as diagnosed with DArT markers; Interval size (cM) support interval of the QTL in cM after starting point of mapping; Estimated Additive Effects: is the estimated additive effect of substituting one allele of Juchi by one allele of PBW343; Negative value indicates the resistance allele is inherited from the parent PBW343; $R^2$: adjusted total phenotypic variance explained by QTL; Source—Parent contributing allele for stem rust resistance

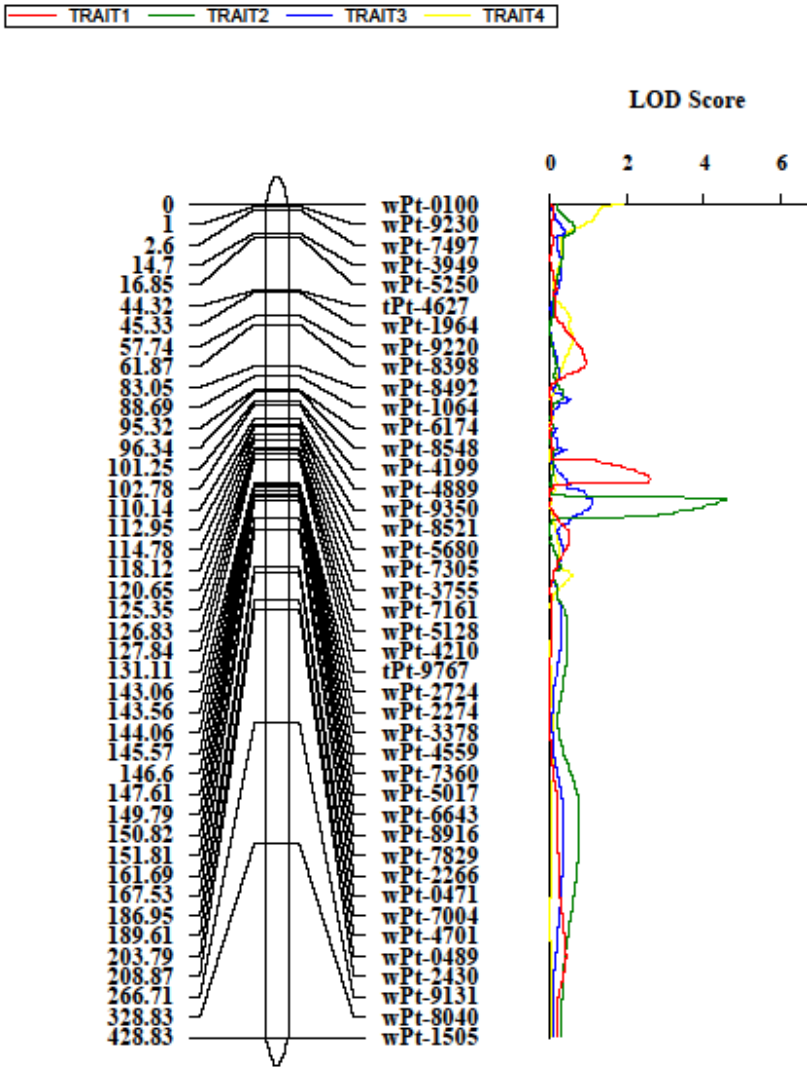


**Figure 5.2 Chromosome 2BL**

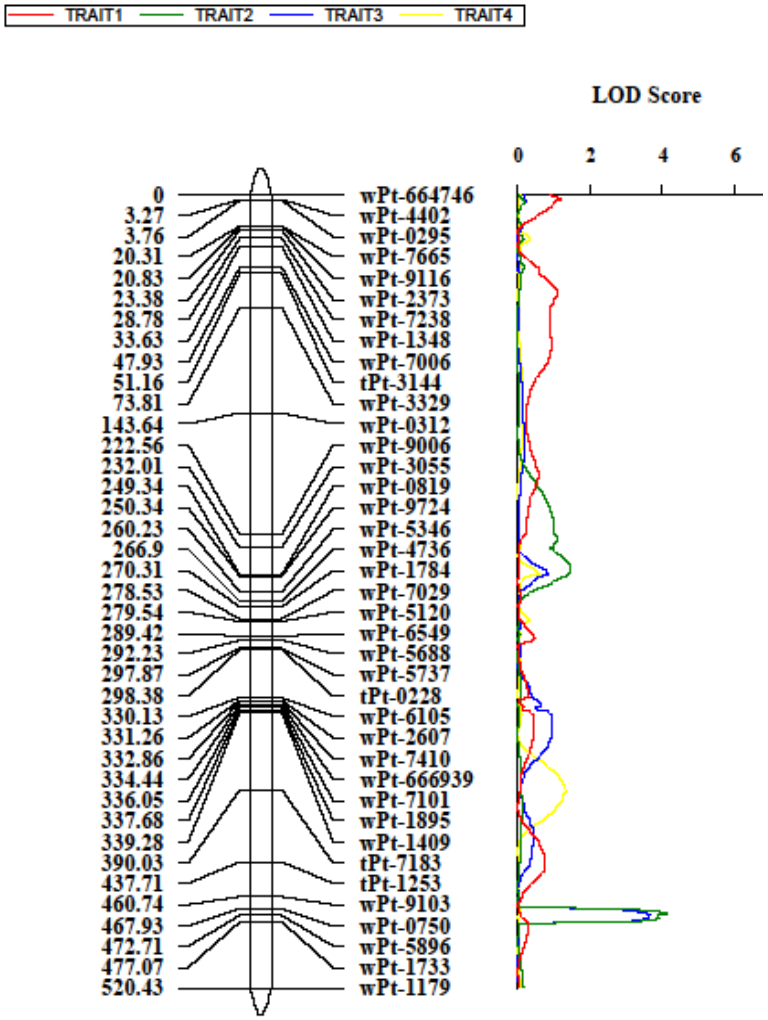


**Figure 5. 3 Chromosome 5BL**

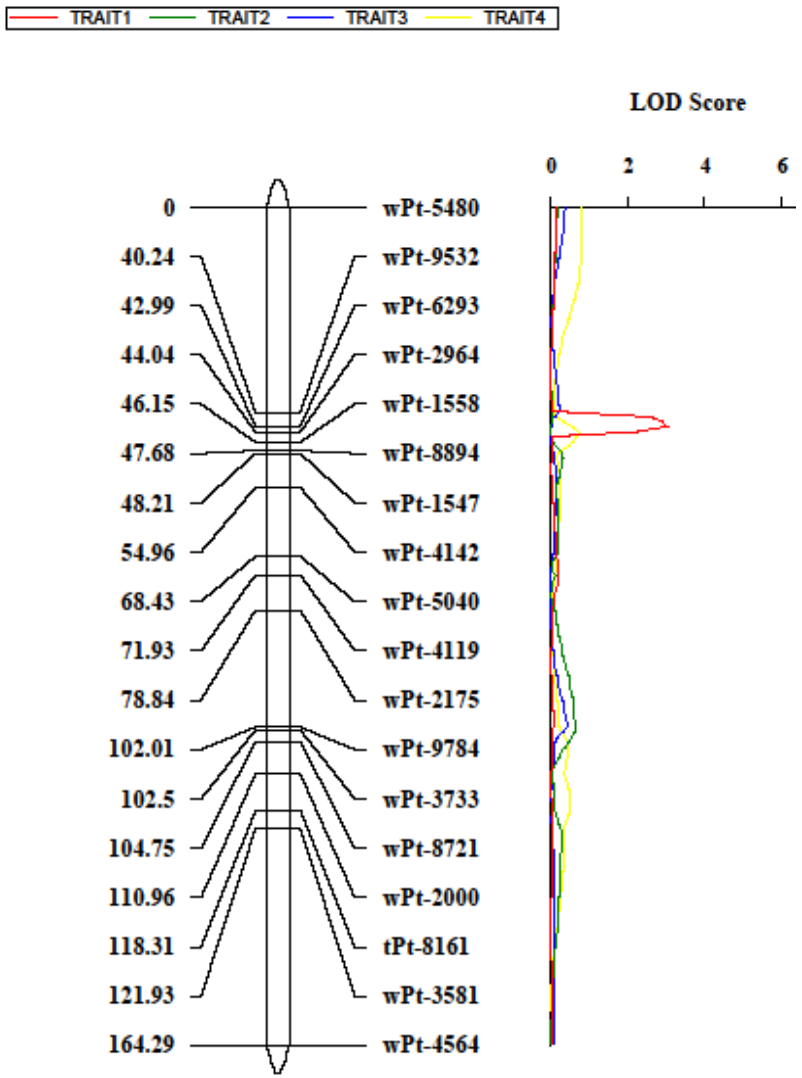


**Figure 5.4 Chromosome 6BS**

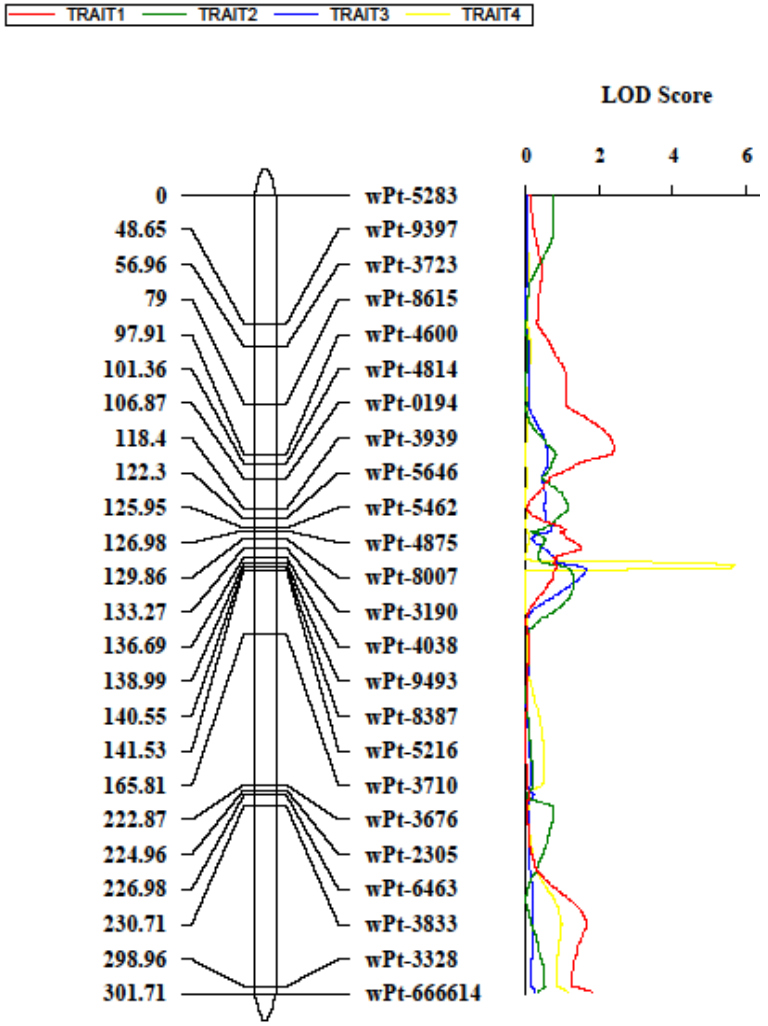


**Figure 5.5 Chromosome 7BL**

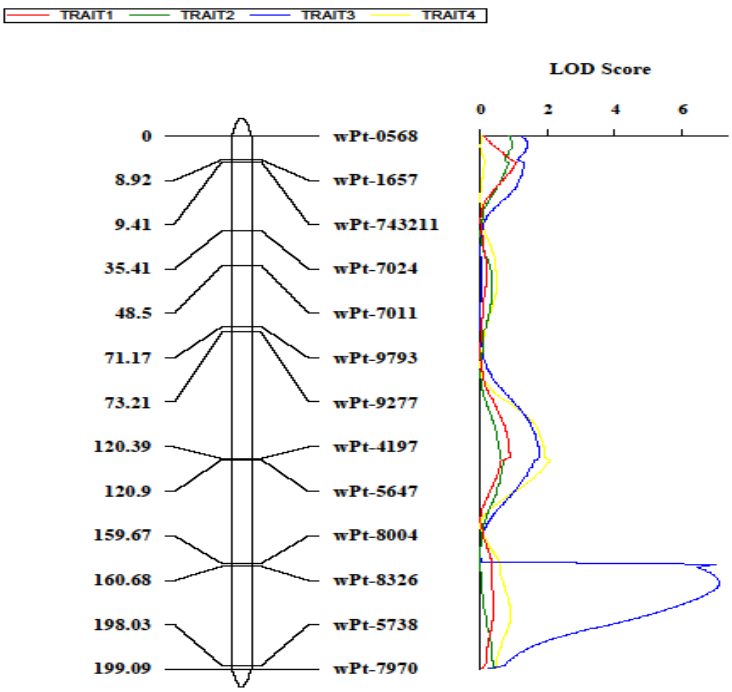


**Figure 5.6 Chromosome2BS**

### 5.4 Discussion

There was a high variation for stem rust resistance among the parents and the RILs with respect to stem rust severity. The frequency distributions of the mean stem rust severity scores in this population across the four seasons (environments) suggested a continuous distribution implying that these parents carry genes with minor or additive effects. Previous studies have reported the involvement of additive gene effects in controlling the inheritance of stem rust resistance and other diseases in wheat (Agenbag *et al*., 2012; Haile *et al*., 2012; Hao *et al*., 2011; Bovill *et al*., 2010). Step wise regression ($R^2$) analysis for additive mapping using all markers explained 52% of phenotypic variance which suggests that the variance was largely due to additive effects of the QTL's. However, the individual effects of minor genes do not confer adequate levels of resistance thus the need for the combination of 4-5 additive minor genes with additive effects leading to near immune phenotype (Singh *et al*, 2005) therefore the individual PVE explained can vary between 5-20%.

The stem rust segregation data suggested the involvement of two to three genes of additive effects corresponding to the 3:1 and 15:1 genetic ratio. This showed a segregation distortion from the expected Mendelian ratio of 1:1 within an $F_6$ segregating population. Segregation distortion was also noted among the DArT markers making them deviate from the Mendelian genetic ratio of 1:1. Previously, distorted markers were usually discarded when carrying out QTL mapping. However, given the current technological advancement for example the use of the Map Disto software which has the capability to adjust the recombination frequencies for deviated markers, distorted markers are included in QTL mapping with a negligible power loss for QTLs with additive effects (Liu *et al*., 2010; Ma *et al*., 2010). The segregation distortion is associated with the presence of segregation distortion loci (SDL). The choice of mapping populations, mode of genetic transmission, non-homologous recombination, gene transfer, transposable elements, reproductive isolation, environmental agents and non-biological factors, such as sample size, marker type and genotyping errors are thought to contribute to segregation distortion (Tang *et al*., 2013; Alheit *et al*., 2011; Xu and Hu, 2009). Gametic and zygotic selection like pollen tube competition, pollen lethal, sterility, chromosome translocation and preferential fertilization lead to segregation distortion (Liu *et al*., 2010). The choice of mapping population for example, recombinant inbred lines are associated with high segregation distortion because they are produced through selfing in several generations (Peleg *et al*., 2008). Through the selfing cycles, competition among the male gametes during fertilization lead to non random fertilization or even abortion of the male gametes or zygotes (Rajender *et al*., 2010) causing the markers to deviate from the Mendelian ratio. This could lead to a negative effect if it's closely linked to important genes. Another factor is the type of markers used. The use of dominant markers for example the

DArTs leads to more biased estimates of recombination frequencies due to the segregation distortion phenomena (Liu *et al*., 2010). The marker loci segregate as a result of the linkage between markers and the segregation distortion loci linking to marker clustering (Xu, 2008). DArT markers have been reported to cluster in several chromosomes in previous studies (Alheit *et al*., 2011). This has been associated with recombination occurring more frequently in the gene-rich regions which are present in clusters and which comprise physically small chromosomal regions accounting for only 5-10% of the wheat genome (Gill *et al*., 1996). Clusters of distorted markers have been reported previously implying that there could be different resistance genes found in those specific QTLs (Haile *et al*., 2012; Francki *et al*., 2009).

Two QTLs were mapped on chromosome 3B. The QTL located on the short arm of chromosome 3B has been associated with the *Sr2* gene which is expressed in the field as pseudo black chaff (PBC) phenotype. The pseudo black chaff appears on the internodes and glumes showing moderately resistant to susceptible responses. Chromosome 3B is also the largest in bread wheat occupying 995 mega-bases with many repetitive elements. This chromosome remains crucial in wheat research and genomics as it harbours 16 genes and QTLs thus aiding in marker development (Horvath *et al*., 2009; Paux *et al*., 2008). The two QTLs mapped on chromosome 3B spanned a total length of 3172cM and 3508cM respectively and were also characterized by the highest marker density (Table 3.2). The chromosome 3BS also carries the slow-rusting gene Yr30 that is also pleiotropic to the *Sr2* gene (Rosewarne *et al*., 2012).

The two QTLs mapped on chromosome 5BL were located 9.14cM apart. However, these two QTLs may or may not necessarily be on same homologues of the chromosome 5B probably

due to the errors involved in assigning QTLs by interval mapping (Boukhatem *et al*., 2002). Consequently, this genomic region could be used to develop wheat cultivars with broad spectrum resistance due to the presence of multiple QTLs mapped on this chromosome (Agenbag *et al*., 2012; Adhikari *et al*., 2011). Epistatic effects were observed for the QTLs mapped on chromosome 5BL with a total phenotypic variation of 13.4%. These epistatic effects were associated with the susceptible allele PBW343. Previous studies have reported the effect of the parent PBW343 in enhancing stem rust resistance (Singh *et al*., 2013). These types of QTLs are detected only if the parents carry different alleles and that the favourable allele may be very specific for one of the parents and cannot be found in other genotypes. Nevertheless, the detected QTLs indicated that an improvement is possible if chromosomal regions with positive effects are combined (Börner *et al*., 2002). In comparison with the additive effects shown on Table 2, the epistatic effects imparted only a small main effect which may have been important as a modifier of other genetic factors given the appearance of chromosome 5BL in the QTL analysis (Singh *et al*., 2013). Previous studies have reported presence of two QTLs on chromosome 5BL (Pumphrey, *et al*., 2012; Singh *et al*., 2013). The gene Sr56 has also been reported to be present on the same chromosome (Bansal *et al*., 2008). On chromosome 5BL, the genes conditioning resistance to Nodorum blotch and also the leaf rust gene Lr18 have been previously reported (Mallard *et al*., 2005).

Some markers identified in this study have been reported in previous mapping studies involving other important traits (Marone *et al*., 2012; Bariana *et al*., 2010). However, some markers were not found to align with the expected region of the genetic map based on previous studies. This could be attributed to either segregation distortion or even of mis-scoring of the marker alleles affecting the overall marker order (Francki *et al*., 2009). The

DArT marker wpt-2266 was located 39.1cM in the chromosome 7A in studies involving mapping for partial resistance to powdery mildew (Lilemo *et al*., 2012). However, this marker was reported on chromosome 2B in studies involving mapping different agronomic traits in bread wheat and has been associated with yield parameters (Neumann *et al*., 2011). Some of these chromosome regions have been associated with other stem rust resistance genes in previous studies; for example; *Sr2* and *Sr12* on 3BS (Yu *et al*., 2011; Kolmer *et al*., 2011); *Sr17* on 7BL (Haile *et al*., 2012; Yu *et al*., 2011); *Sr9g* and *Sr16* on 2BL (Kolmer *et al*., 2011) and Sr19, *Sr2*3, Sr36 and Sr40 on 2BS (Yu *et al*., 2014; Singh *et al*., 2013).

This study also showed that the QTLs were mapped to the B-genome. Other studies have reported the mapping of most QTL in the B genome (Francki *et al*., 2009; Peleg *et al*., 2008). Thus, there is need to diversify the genetic base of the wheat germplasm by developing arrays from *Triticum monoccocum* and *Aegilops tauschii* species to increase the density of DArT markers for the A and D genomes, respectively (Francki *et al*., 2009). This will ensure that dense genetic maps are developed leading to the identification of more molecular markers co-segregating with more resistance genes. This will lead to breeding efficiency with regard to map based cloning, comparative mapping and genetic diversity studies (Peleg *et al*., 2008).

The DArT markers namely wPt-9493, tPt-9767 and tPt-6872 flanking some QTLs have not been identified in published consensus chromosome maps. The prediction of their position was based on either the left or right marker whose position has been determined (Table 5.2). Thus, these novel markers together with the identified QTLs could be used in the co-selection and improvement of agronomically important traits through marker assisted selection (Nagabhushana *et al*., 2006).

In this study, none of the QTLs were consistently detected in all the four seasons (otherwise considered as environments in this study). Variation of phenotypic expression of the RIL population and the expression of QTL only in certain seasons indicated that some of the minor genes were not stable across environments. This may be attributed to host response to the environment, pathogen response to environment, race structure, inoculum level, multiple diseases, as well as interactions of these factors, and systematic error such as varied stem rust severity rating all influence repeatability (Singh *et al*., 2013). Also, the infector/Spreader rows of 1m width along the nursery and inside the nursery ensured that there was heavy buildup of inoculum and the most of the susceptible checks noted disease severities up to 100% which indicated high disease pressure which may or may not occur in natural environment unless the varieties are susceptible, pathogen virulent and environment favoring the establishment of disease in to an epidemic. Thus, this population needs to be evaluated for other agronomic traits and in more environments (Rajender *et al*., 2010). It was also observed that the QTLs identified were mapped on six different chromosomes or homologues implying that the genetic mechanism of resistance to stem rust in this population was multigenic. Other studies have also reported the involvement of many chromosomes or homologues in disease resistance (Haile *et al*., 2012; Li *et al*., 2010).

### 5.5 Conclusion and Recommendations

Both Juchi and PBW343 contributed positive molecular variants. Thus, the resistance in PBW343/Juchi was conditioned by both epistatic and additive genes and this could be exploited through marker assisted selection (MAS), gene pyramiding and other breeding strategies like specific intercrosses, backcrossing and recurrent selection to ensure that QTLs are transferred to elite wheat background (Adhikari *et al*., 2011; Chhuneja *et al*., 2008;

Richardson *et al* 2006). The DArT markers identified in this study will help in the selection of superior wheat genotypes which contain adult plant resistance (APR) (Lillemo *et al*., 2012). Since the APR genes do not confer sufficient levels of resistance on their own, combination with other stem rust resistance genes could enhance their utilization leading to an efficient wheat breeding program (Bariana *et al*., 2010). The information generated from this study adds to the knowledge pool of the breeders, pathologists and molecular genetists leading to a better understanding of the genetic architecture of quantitative resistance to stem rust (Singh *et al*., 2013). Some of the key recommendations include:

1. Development of near isogenic lines with different genetic backgrounds to give a better genetic characterization
2. Positional cloning of genes and QTLs could be done through fine mapping within the gene-rich regions of bread wheat. This could be achieved through saturation of entire genomic regions with additional molecular markers to identify more APR QTLs for better detection, mapping and estimation of gene effects. This will also help to establish any gene/gene combinations × Environment interaction that could be affecting the nature of the resistance in this population and other promising mapping populations.
3. QTLs identified could be used to develop more reliable molecular markers for marker assisted selection
4. Gene pyramiding could also see the transfer of these APR genes and epistatic genes as cassettes through a single transformation step as opposed to the laborious hybridizations and backcrosses. These cassetes could be used to produce multilines with superior alleles which disrupt selection hence slow down the *Puccinia graminis* evolution. Ultimately, the lifespan of lifespan of available stem rust resistance will be prolonged leading to a sustainable host resistance management.

# CHAPTER SIX

## 6.0 General Discussion, Conclusion and Recommendations

In breeding for resistance to stem rust, some of these genotypes showed trace responses with no visible stem rust infections. Some genotypes with moderately resistant to moderately susceptible responses showed low stem rust severities. When these genotypes were assessed for partial resistance, they showed low AUDPC and low coefficient of infection values implying presence of some level of partial resistance. Disease progression was highly retarded among these lines. This type of responses could be attributed to a combined effect of all the resistance factors during disease progression (Ali *et al*., 2008). These lines could be good sources of partial or slow rusting resistance to stem rust conditioned by additive gene action (Kaur and Bariana, 2010). In spite of the resistant infection types at the seedling stage observed in 2010 and 2012, these materials could be containing other stem rust resistance genes in their background responsible for reduced disease severity. Nevertheless, partially responsive race specific and hypersensitive genes coupled with dominant genes could be in control leading to pseudo resistance given the presence of multiple Ug99 races at KARI Njoro. These wheat genotypes are crucial in achieving durable resistance to stem rust given that the most resistant wheat genotypes KSL-2, KSL-3 and KSL-20 also exhibited the pseudo black chaff (PBC) trait the presence of *Sr2* gene in their background.

Genetic studies carried out on five promising lines added to the knowledge pool concerning current *Puccinia graminis* f. sp. *tritici* dynamics. The $F_{2:3}$ derived wheat lines evaluated at KARI Njoro in the 2012 and 2013 cropping seasons showed segregation distortion in that only the parent KSL-2 conformed to the single gene model while the rest of the parents conformed to the two gene model. The parents KSL-2 and KSL-3 and their crosses also

segregated for the presence of the pseudo black chaff. The wheat lines which carry two genes could be backcrossed to the elite Kenyan wheat varieties to enhance wheat production in Kenya. This is because the combined phenotypic effect of two or more genes could enhance the effectiveness of the individual genes with respect to stem rust resistance. Further studies involving the dissection of the QTLs will be instrumental in elucidating the exact gene effects. Also, the diversity revealed in these parents could be exploited effectively in breeding programs if the loci associated with the high resistance is identified and used in combinations for use especially in the Ug99 race high risk areas (Khanna *et al*., 2005). These promising lines could also be used to broaden the genetic diversity of the available Kenyan wheat varieties since by introgression of this resistance into adapted Kenyan varieties through back cross and bulk population breeding.

The mapping of the stem rust resistance among the $F_6$ RILs of the Juchi population revealed new DArT markers which have not been reported in previous studies. The new DArT markers namely wPt-9493, tPt-9767 and tPt-6872 were not assigned to any previously published consensus chromosome maps. Thus, these markers together with the identified QTLs could be used in the co-selection and improvement of agronomically important traits through marker assisted selection (Nagabhushana *et al*., 2006). The study also established that genes with both additive and epistatic effects conditioned resistance to stem rust. The combination of these minor genes with other race specific genes through gene pyramiding will increase durability of stem rust resistance thus slowing down evolution of new races. Through marker assisted selection (MAS), gene pyramiding and crop improvement strategies, QTLs will be transferred to elite wheat background increase wheat production and productivity in Kenya.

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**Appendix 1: Weather conditions at KARI, Njoro from 2010 to 2013**

| Year 2010 | Jan | Feb | March | April | May | June | July | Aug | Sept | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Rainfall (mm)** | 42.9 (9) | 157.0 (14) | 184.1 (21) | 140.4 (15) | 180.8 (15) | 51.8 (11) | 166.1 (18) | 240.0 (22) | 172.2 (21) | 109.9 (21) | 53.1 (12) | 14.3 (4) |
| **Temps (max)** | 23 | 25 | 23 | 23 | 22 | 22 | 21 | 20 | 22 | 22 | 22.6 | 23.6 |
| **Temps (min)** | 8 | 10 | 10 | 10 | 11 | 9 | 8 | 8 | 8 | 10 | 8.4 | 8.1 |

| Year 2011 | Jan | Feb | March | Apri | May | June | July | Aug | Sept | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Rainfall (mm)** | 3.9 (1) | 9. 5(3) | 130.3 (14) | 28.9 (11) | 120.5 (13) | 177.7 (18) | 158.6 (19) | 124.9 (18) | 145.4 (19) | 102.1 (14) | 165.3 (17) | 104.6 (12) |
| **Temps (max)** | 25 | 26 | 26 | 25 | 23 | 21 | 18 | 15 | 22 | 22 | 20 | 16 |
| **Temps (min)** | 8 | 8 | 9 | 9 | 9 | 11 | 11 | 12 | 11 | 11 | 11 | 15 |

| Year 2012 | Jan | Feb | March | April | May | June | July | Aug | Sept | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Rainfall (mm)** | 0 | 13.6 (4) | 11 | 295.0 (26) | 183.7 (22) | 62.1 (13) | 87.3 (18) | 174.7 (14) | 174.9 (22) | 98.3 (18) | 28.0 (6) | 112.7 (14) |
| **Temps (max)** | 23 | 18 | 22 | 24 | 22 | 22 | 20 | 20 | 22 | 23 | 21 | 21 |
| **Temps( min)** | 10 | 16 | 18 | 14 | 12 | 12 | 10 | 10 | 9 | 11 | 14 | 13 |

| Year 2013 | Jan | Feb | March | April | May | June | July | Aug | Sept | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Rainfall (mm)** | 37.8 (6) | 2.5 (1) | 57.5 (9) | 238.3 (21) | 110.9 (14) | 142.7 (16) | 150.1 (17) | 110.6 (13) | 173.3 (20) | 73.9 (13) | 60.6 (17) | 137.5 (11) |
| **Temps (max)** | 23 | 25 | 24 | 20 | 23 | 21 | 21 | 22 | 23 | 21 | 22 | 23 |
| **Temps (min)** | 9 | 13 | 15 | 14.2 | 9 | 11 | 9 | 8 | 9 | 10 | 10 | 10 |

**(In brackets are the number of days recorded when it rained)**