SCREENING FOR STEM RUST RESISTANCE IN MUTANT BARLEY (*Hordeum vulgare* L.) LINES

BY

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Reg. NO:AGR/PGB/05/10

A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PLANT BREEDING AND BIOTECHNOLOGY

UNIVERSITY OF ELDORET, KENYA.

JULY, 2013

DECLARATION

DECLARATION BY THE CANDIDATE

This thesis is my original work and has not been presented in this or any other university for the award of any degree.

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DEDICATION

This thesis is dedicated to my beloved father Obed Obare Machogu and my mother Margret Bwari Obare for bringing me up with encouragement to learn and siblings Ian, Ira and Ida.

ABSTRACT

Stem rust is a devastating disease in barley that is caused by a fungi (*Puccinia graminis* f.sp *tritici*, Eriks. and E. Henn).The disease has been controlled for quite some time due to the presence of cultivars carrying the resistant gene *Rpg1* .This was so until the emergence of the race Ug99 from Uganda in the year 1998. This race did break all the resistant genes that were there hence the need to get new sources of resistance. In the current study mutation breeding was used to create variation for stem rust resistance (Ug99) and screen the barley lines for resistance. Seeds of barley (Nguzo variety- M_O) were sent to Vienna in Austria for irradiation at the International Atomic Energy Agency at a dosage of 250 gray. The M_1 seeds were multiplied at the University of Eldoret farm. A thousand plants were selected from the M_1 population and two ears harvested and divided into two corresponding groups. The seeds from these ears were designated as M_2. One group was planted at University of Eldoret experimental field while the corresponding groups of a thousand ears were planted at KARI Njoro. Each ear formed a line. A susceptible line of wheat was planted as a spreader and inoculated with stem rust Ug99 in both sites. After the disease pressure was high selection was done basing on severity and infection type. A total of 183 lines were selected from the two sites which showed acceptable level of resistance. These lines were again replanted in University of Eldoret as M_3 in a RCBD design with three replicates to determine the effect of mutation on agronomic traits, and the parent as a control. it was found that mutation had a significant effect on a number of tillers, height, weight of 1,000 seeds, spike length and days to 50% heading ($p<0.001$).The following mutant lines showed superiority in terms of the agronomic traits studied. Molecular screening of the lines was done using SSR markers and 12 mutant barley lines were found to be probably carrying the resistant genes for Ug99 race of stem rust. In terms of correlation, the number of tillers of the mutant barley lines had a positive correlation with spike length at $P<0.05$,Spike length had a positive correlation with weight of 1000 grains of the mutant barley lines at $P<0.001$.The height of the mutant barley lines correlated positively with the number of tillers at $P<0.01$ and with days to 50% heading. These lines could be positively selected for further stabilizing and evaluation of other agronomic traits.

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LIST OF ABREVIATION

FAO Food and Agriculture organization

IAEA International Atomic Energy Agency.

SSR Simple Sequence Repeats.

DNA Deoxyribonucleic acid.

RH Relative humidity.

R*pg* Stem rust resistant gene in barley.

SED Standard error of difference.

KARI Kenya agricultural research institute.

NCPB National cereals and poduce board.

KML Kenya Maltings limited.

EABL East Africa breweries limited.

GOK Government of Kenya.

Sr stem rust resistant gene in wheat.

EMS Ethylmethane sulfonate.

ACKNOWLEDGEMENT

I express my sincere gratitude to University of Eldoret for giving me the opportunity to pursue the Master of Science degree in Plant breeding and Biotechnology. In addition, I appreciate the contribution of the entire staff of the School of Agriculture and biotechnology, and Department of Biotechnology who supported and helped me to achieve my academic goals.

Special thanks go to my supervisors Prof Miriam Kinyua and Dr Oliver Kiplagat for their ever presence, encouragement, guidance and assistance. Moi University-East Africa Breweries limited project for funding my studies.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Barley (*Hordeum vulgare* L.) is one of the founder crops of Old World agriculture. Genetic markers point to the origin of barley being the Fertile Crescent especially the Israel-Jordan area in the southern part of the Fertile Crescent where there is the highest diversity of the species (Badret *et al*., 2000). This is the area that has the highest probability of being the geographical area within which wild barley was domesticated about 8000 B.C. (Zohary and Hopf, 1993).The Agriculture Sector, which is the backbone of Kenya's economy and its growth is dependent on increasing barley production amongst other crops and livestock (GOK, 2001). The importance of barley as a major crop in Kenya cannot be overlooked because it is the fourth most important cereal in Kenya and the world after maize(*Zea mays*), wheat(*Triticum aesetivum)* and rice (*Oryza Sativum*) (FAO, 2004). Some of the major barley producing areas in Kenya include; Timau, Moiben, Nakuru, on the wetter escarpment of Samburu District near Maralal Town, Molo, and Mau Narok (KML, 2007). Three cultivars of barley Sabini, Nguzo and Bima are recommended for production in Kenya while Karne, another local barley variety, is no longer used because it is prone to fungal attacks (KML, 2007). The total land area that is deemed suitable for barley production in Kenya is 85,000 hectares. Only about 20,000 hectares of this total land area suitable for barley production is under barley production, thus 65,000 hectares has not been utilized (EABL, 2005). Barley production in Kenya was estimated to be 75,000 MT in 2007, (NCPB, 2007). Rust fungi a major problem world-wide is one of

the contributions to the sub-optimal yields and breeding for resistance is the principal method of protection (Hovmøller and Justesen, 2007).

Stem rust of barley and wheat, caused by (*Puccinia graminis* f.sp *tritici*, Eriks. and E. Henn.) is one of the most devastating barley diseases worldwide. A new race with virulence to a number of wheat stem rust genes, *sr31, sr24* and *sr36* in addition to barley stem rust genes, that were used to confer resistance to stem rust of wheat and barley was discovered in Uganda in 1999 and named stem rust race TTKS or "Ug99" in 2001 (CIMMYT, 2005). In 2006, stem rust monitoring in Kenya confirmed that isolates of Ug99 were already in the country and causing heavy yield losses (Jin *et al*., 2007). Tests also indicate that the race TTKS is virulent on Midwestern barley cultivars carrying the durable stem rust resistance gene *Rpg1* which is one of the important genes that confer resistance to stem rust of barley. The search for more stem rust resistance genes has stagnated due to unavailability of new genes that are effective against virulent races of stem rust in barley.

1.2 Statement of the Problem

Stem rust, caused by *Puccinia graminis* f.sp *tritici,* is one of the most destructive diseases of barley and wheat.The disease was then effectively controlled through the use of host plant resistance. It is however the nature of the pathogen to evolve virulent pathotypes that overcomes host resistance genes by sexual recombination and mutation. In 1999, a new race of stem rust appeared in Uganda (designated as race TTKS or Ug99) and has since spread to Kenya (Pretorious *et al.*, 2000). It is responsible for an estimated yield loss of over 70% of wheat annually. The emergence of Ug99 implies that the package of resistance genes bred into many barley and almost all wheat varieties are no longer effective. The high epidemics lead to yield

losses given that the conventional control strategies (fungicides) are effective, are environmentally unsafe, costly and a temporary solution because of their short residue period coupled by the pathogen polycyclic life cycle. Almost all barley genotypes are susceptible to stem rust race Ug99 and there is need to search for resistance in other genotypes and also through mutation of existing barley genotypes to create variation for stem rust resistance. Induced mutation in hexaploid wheat by treating seeds with ethylmethane sulfonate (EMS) produced mutant lines with resistance to 13 races of stem rust out of 15 races (Williams *et al.*,1992) Little work has been done to evaluate barley mutants for resistance to virulent stem rust race TTKS or Ug99.

1.3 Objectives

1.3.1 Broad objectives

To contribute towards management of stem rust and increase barley yield by identifying new sources of resistance to stem rust among mutated barley germplasm.

1.3.2 Specific objectives

1. To evaluate for ug99 resistance among mutant lines at M_2 and M_3 in the field at adult plant stage.

2. To evaluate for ug99 resistance among mutant lines at M_3 in the greenhouse at seedling stage.

3. To determine effects of mutation on agronomic traits on mutant lines at M_3.

4. To screen mutant lines at M_3 using Simple Sequence Repeats markers.

1.4 Hypotheses

1. H_o There is no resistance to ug99 among mutant lines at M_2 and M_3 in the field at adult plant stage.

2. H_o There is no resistance to ug99 among mutant lines at M_2 in the greenhouse at seedling level.

3. H_o Mutation has no effects on the agronomic traits in mutant barley lines.

4. H_o SSR markers cannot be used to screen the mutant barley lines at M_3.

1.5 Justification

Barley (*Hordeum vulgare* L) is used for livestock feed, for human food and as hay crop in some areas. Its importance as a crop for both human food and animal feed production is known because it is one of the principal raw materials used to process malt, a vital ingredient for beer brewing. The industry is one of the highest corporate taxpayers with annual turnover of Kshs. 28.9 billion and employs more than 1600 people across the region (EABL, 2005). Barley improvement for both yield and quality involves selection for many traits among them disease resistance. The ability of a crop to resist infection by a given disease means that it will translate into minimal yield losses and therefore will lead to improved yields(Agrios,2005). Fungal diseases especially stem rust can reduce profitability of barley production by reducing both the quantity and quality of barley yield, both of which result in lower returns to the grower (Chelkowski *et al.,* 2003; Williams, 2003). The major control method for stem rust in barley is the use of scheduled fungicidal sprays. This method of stem rust control though effective, is expensive and leads to destruction of environmental quality for habitation by both man and other beneficial life forms and therefore not recommended for long-term use in rust management. The search for and deployment of resistant cultivars therefore continues to be just important as its selection for quantity and quality attributes in malting barley improvement (Emebiri, 2005). No Barley varieties has resistance to the new virulent stem rust race Ug99 and other emerging virulent races and there is need to screen for new sources of resistance including developing mutant populations of barley and screening them for the Ug99 resistance(Cimmyt 2005). Barley being one of the crops affected by stem rust need to be screened for resistance to the stem rust physiological races including Ug99, so that breeding programs can be effectively sustained in barley.

CHAPTER TWO

LITERATURE REVIEW

2.1 Botany and Genetics of Barley

Barley (*Hordeum vulgare* L.), is one of the most important temperate cereals grown worldwide. Barley belongs to the family Poacea also known as Gramineae and genus *Hordeum* and has been a subject of various genetic studies (Abeledo *et. al*., 2003). Barley is a self-fertilizing monocotyledonous plant and a true diploid (2n = 2x = 14) (Caldwell *et al*., 2004). Barley originated from the fertile crescent in the near Middle East where the largest diversity for the species is (Pourkheirandish and Komatsuda, 2007). It is the fourth most produced cereal worldwide (after maize, wheat and rice). Barley is used as animal feed, human food in breakfast cereal and in beer making for malting (Belcredi *et al*., 2009). Although almost half of the area where barley is grown is a high-risk area and crop failures are common and resources are limited, it has remained a predominant grain crop and a source of livelihood to many and this makes breeding for increased yield imperative (Shakhatreh *et al*., 2001; Pswarayi *et al*., 2008). Barley growth is influenced by the genetic makeup and the environment hence a single plant will produce three to six stems which are 10 cm to more than 150 cm in length. Single leaves consist of a sheath, ligule, auricle and blade but each leaf node of the stem and are borne alternatively on opposite sides. Each spikelet has one flower consisting of two glumes and floret. Three spikelets are attached at each node of the rachis. In two – rowed barley, only the central floret is fertile, while in six-rowed barleys, all three florets are fertile. The number of kernels that develop per head varies from 25 to 60 in six – rowed types and from 15 to 30 in two-rowed types (Mathre, 1982).

Barley has dominant genes such as *Rpg1* that control stem rust epidemic in Northern Great Plains since 1942. This gene was mapped to the short arm of barley chromosome 1 (7H) from cultivar Morex (Steffenson *et al*., 1992). Moreover the knowledge on allele sequencing confirmed that *Rpg1* have 14 axons in 4,466 bp of genomic sequence encoding an 837 amino acid protein. However barley cultivars indicated no amino acid sequence polymorphism (Brueggeman *et al*., 1992).

2.2 Stem Rust Disease: Distribution and Taxonomy

The stem rust fungus has for several years coevolved with barley and wheat throughout the world, (Walker, 1957; Agrios, 2005). In Kenya barley and wheat is produced in various parts of the country; often in different seasons; hence it can be said that growth of barley in Kenya is throughout the year. The disease is one of the most important diseases affecting barley and wheat in Kenya. In wheat the disease contributes to approximate 70% reduction in yield.

Puccinia graminis is a rust fungus in the division *Basidiomycota*, class *Basidiomycetes*, order *Uredinales*, and family *Pucciniaceae* which contains 17 genera and about 4121 species, of which the majority are in the genus *Puccinia* (Kirk *et al*., 2001). A subdivision of *P. graminis* into subspecies, varieties and *formae speciales* has been suggested although based on spore size and host range. Crossing studies and DNA sequence comparisons support the separation into two subspecies. The host range of *P. graminis* is very broad compared with that of most *Puccinia spp*; it includes at least 365 species of cereals and grasses in 54 genera (Anikster, 1984). Wheat stem rust, *P. graminis f. sp. tritici* , was shown to infect 74 species in 34 genera in artificial inoculations of seedlings, but only 28 of those species belonging to eight genera were known to be natural hosts of the fungus. Other *formae speciales* of *P. graminis* have narrower host ranges than *P. graminis f. sp. tritici* (Kurt 2005).

2.3 Etiology of stem rust disease

The causal organism of stem rust is *Puccinia graminis f.sp.tritici*, a fungus belonging to the family of the *Basidiomycetes* that are ranked among the true fungi. They are also called perfect fungi because they possess the sexual stage in their life cycle. Physiological races, these are biotypes of a pathogen that vary in their ability to infect different varieties of the same host species. Walker (1957) reported 200 distinct races known, while Agrios (2005) reported several hundreds of the pathogen with new races appearing every year. Of importance is the recent appearance of a pathotype ug99 which is virulent to all previously resistant varieties of wheat and barley. In fungi, production of new physiological race is achieved through various mechanisms including parasexual recombination, mutation, sexual reproduction, and heterokaryosis.

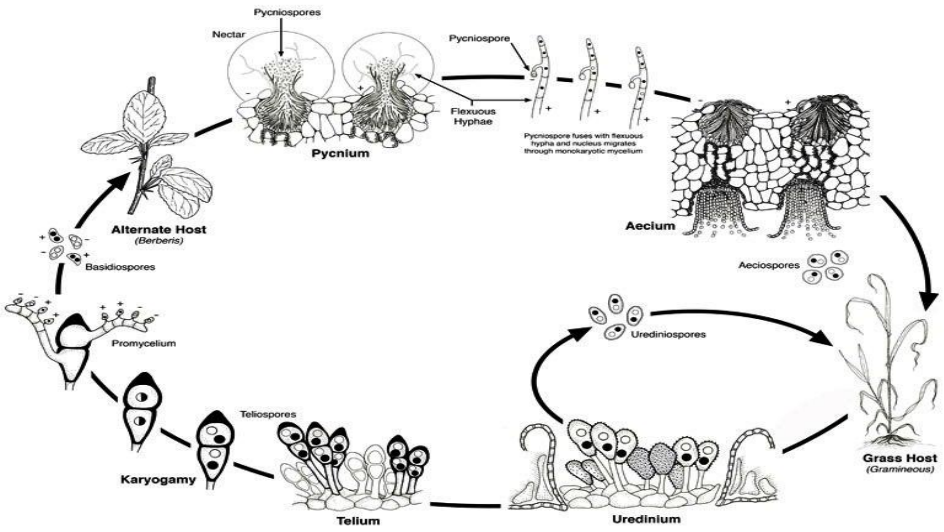

Plate 2 *Puccinia graminis* fsp *tritici* life cycle (Roelfs *et al*., 1992)

The fungus *P. graminis f.sp. tritici* undergoes several cycles (macrocyclic) in various alternate hosts (Figure 1). The alternate hosts affected besides barley include species of Barberry and Mahonia (Agrios, 2005; Wiese, 1977). The fungus produces aecia and spermagonia on Barberry while it produces telia and uredia on barley (Agrios, 2005). Other hosts affected include wheat, wild barley, oats, and rye. Several grasses including rhye grass are also affected and may serve as source of primary inoculum (Wiese, 1977). The fungus undergoes full/ complete life when the alternate host; Barberry is present, (Nyvall, 1979). In the absence of this alternate host, the fungus undergoes asexual life cycle; Angus, (DeMillano, 1983). As aresult of the wide variations in cropping seasons in Kenya (and other parts of East Africa) rust inoculum is present throughout the year (Harder *et al*, 1972).

2.4 Epidemiology of stem rust

Stem rust needs green plant tissue to be able to infect and is favored by hot (25 to 30ºC) days, mild (15 to 20ºC) nights and frequent dews. The urediniospores, which are the asexual infective spores for cereal and grass hosts, are spread over long distances by wind and deposited by rain (USDA, 2008). They can also be moved as contaminants on human clothing (Singh *et al.*, 2006). The level of disease injury/ epidemic in a given area and season is a function of the crop and the environment. The crop affects the level of epidemic through its susceptibility or resistance to the pathogen which determines the amount of inoculum. The environment affects disease development through levels of moisture, temperatures and prevailing winds. As a result of the diverse cropping seasons and hence a constant level of inoculum. In Kenya the pathogen is available throughout hence it causes infection, (Harder *et al*, 1972).

2.5 Stem rust Disease symptoms

Infections in cereals or grasses occur mainly on stems and leaf sheaths, but occasionally they may be found on leaf blades and glumes.(Agrios,2005) The first macroscopic symptom is usually a small chlorotic fleck, which appears a few days after infection. About 8–10 days after infection, a pustule several millimetres long and a few millimeters wide is formed by rupture of the host epidermis from pressure of a mass of brick-red urediniospores produced in the infection. These uredinial pustules are generally linear or diamond shaped and may enlarge up to 10 mm long. The powdery masses of urediniospores appear similar to rust spots on a weathered iron surface. With age, the infection ceases production of brick-red urediniospores and produces a layer of black teliospores in their place, causing the stems of heavily infected plants to appear blackened late in the season (Kurt, 2005).

2.6 Crop losses due to stem rust of barley

Stem rust typically causes losses of 10–50 %, but this can increase to up to 90 per cent when it occurs in early spring (Ciara *et al*., 2006). In 1994 in Ethiopia wheat stem rust reduced the yield of Enkoy a wheat variety which was widely grown in the highlands, by 60 to 100% while in Kenya yield losses up to 80% were recorded (KARI, 2005; UNDP, 1994). Stem rust is primarily a warm weather disease, but it can cause great damage to susceptible barley crops over broad geographical regions. A crop that appears healthy 3 weeks before harvest can be devastated by explosive buildup of stem rust if sufficient inoculum arrives from a heavily infected crop in some distant region. Severe infection of stems interrupts nutrient flow to the developing heads, resulting in shriveled grain. In addition, stems weakened by rust infection are prone to lodging and further loss of grain (Roelfs *et al*., 1992). In extreme cases, infected plants may die. Infected plants may also show increased water loss because they

transpire more due to the loss of water through ruptured tissues. Photosynthetic area is also reduced leading to reduced photosynthesis. Seed filling is affected through induction of early maturity; affecting seed size, quality and number (Agrios, 2005).

2.7 Disease Management

2.7.1 Barberry(*Berberis Vulgaris*) eradication

Being an important alternative host eradication of Barberry has had significant positive effects on the control of stem rust epidemics. First, it removes a significant, early source of inoculum. A single barberry plant can produce as many as 64 billion aeciospores. Second, it reduces the genetic variation in the fungal population by eliminating the sexual cycle, leaving only asexual urediniospores to maintain the fungus. (APS. 1977).

2.7.2 Chemical control

In some areas where disease pressure is high, fungicides are applied to barley to control rust diseases. Fungicides that inhibit the synthesis of sterols [i.e., sterol biosynthesis inhibitors (SBIs) or demethylation inhibitors (DMIs)] are particularly effective, but the cost of application is generally prohibitive for routine use in most barley-growing areas. In the developing world, treatment with fungicide when epidemics occur would not be feasible for many farmers and countries given the constraints to mounting well-coordinated mobilization campaigns (Marasas *et al*., 2004).

2.7.3 Potential approaches to management

The infection process starts when spores land on barley surface. The spore absorbs moisture and produces a germ tube which must find a way of breeching the cuticle and epidermal cells into the mesophyll layer. The germtube can only infect barley only through the stomata (Agrios, 2005). Scientists have studied how germinating urediniospores locate stomata on leaf surfaces. Although several factors are involved, the germ tube is able to detect the guard cells by their physical dimensions relative to the epidermal cells. Once a stoma is found, an aspersorium is produced and infection begins. In the future, it may be possible to breed barley that has altered stomata thus limiting recognition by the fungal germtube. This would render the barley resistant to urediniospore infection and therefore disease will not develop.

2.7.4. Genetic resistance/ Tolerance and SSR markers.

Genetic resistance is the most commonly used and the most effective means to control stem rust (Agrios 2005). The currently used resistance genes should not be expected to remain effective as new races of the fungus begin to appear. Even without the presence of alternate hosts, the fungus is capable of overcoming resistance genes, primarily through mutation. For this reason, plant pathologists monitor the race populations each year and advice breeders about which resistance genes will best protect the barley crop in various areas. Barley breeders use a combination of vertical resistance genes against specific races of *P. graminis* and horizontal resistance genes that slow the development of the epidemic by offering some resistance to all pathogen races.

In plant-pathogen interactions, there are two broad types of genetically determined resistance to infection in host plants: quantitative (representing the combined effect of many minor genes), and qualitative resistance, which is controlled by major genes (single genes with large effects). Beginning with Harold Flor's elegant work in the 1940s and 1950s, it has been repeatedly shown that in systems characterized by qualitative host resistance, the associated pathogens have corresponding major genes that determine virulence (the ability to infect a given host genotype). As a consequence, such systems are typically referred to as 'gene-for-gene' interactions (Flor 1956). The central assumption is that each resistance (R) gene in the host interacts specifically with a corresponding avirulence (Av) gene in the parasite, with resistance being dominant to susceptibility and avirulence dominant to virulence (Flor 1956 and 1971). For resistance to occur, both genes for resistance in the host, as well as the corresponding Av genes in the pathogen, must be present. Plant race-specific disease resistance genes function by recognizing the avirulence gene product of a pathogen and, presumably, by activating signaling cascades leading to resistance (Hammond *et al*., 1997, Hulbert *et al*., 2001). In barley, 7 stem rust resistance genes have been described (*Rpg1–5, RpgBH, and RphU*) (Sun and Steffenson 2005), but only 3 have been mapped on the barley genome. The *rpg4* locus confers resistance to the *P. graminis f. sp. tritici* pathotype, Pgt-QCC and the *Rpg5* locus confers resistance to the rye stem rust pathogen *Puccinia graminis f. sp. secalis*, isolate 92-MN-90. *Rpg5* is unique among plant disease resistance genes in that it encodes the NBS-LRR and the protein kinase domains all in one gene. The barley stem rust resistance gene R*pg*1 confers resistance to many pathotypes of the stem rust fungus *Puccinia graminis f. sp. tritici*. (Rostoks *et al*., 2004). Unfortunately race TTKS is not only virulent for wheat cultivars with Sr31, but also barley cultivars with the widely used gene *Rpg1*,

(Steffenson *et al*., 2007). Resistance has been identified in cultivated, Swiss landrace, and wild barleyQ2861 possesses a high level of resistance to race TTKS This resistance is conferred by a single gene that co segregates with QCC and rye stem rust resistance in bin 13 of chromosome 7(5H) (Steffenson *et al*., 2007).

Simple sequence repeat (SSR) markers are repeats of short nucleotide sequences, usually equal to or less than six bases in length, that vary in number (Rafalski *et al.*, 1996). SSR markers have become quite useful in various aspects of molecular genetic studies in the past decade, including assessment of genetic diversity (Amsellem *et al*., 2001; Ashley *et al*., 2003), fingerprinting (Rongwen *et al*., 1995), ecological-genetic studies (Li *et al.,* 2000), marker-assisted selection (Fazio *et al*., 2003), and genetic linkage mapping (Akkaya *et al.,* 1995). They are desirable because they are often co dominant, highly reproducible, frequent in most eukaryotes, and reveal high allelic diversity (Mohan *et al*., 1997). SSR markers are amplified using the PCR, thus allowing for the rapid generation of data from a relatively small amount of plant tissue.

2.8 Screening for stem rust resistance

Improved grain yield, grain quality and resistances to various biotic and abiotic stresses are the most important breeding objectives in most crop improvement programs (Singh *et al*., 1998) The agronomic performance of crop varieties and advanced lines could be evaluated by multi-environment trial, genotype environment interaction analysis, yield with its component analysis, agronomic trait analysis, correlation analysis, additive main effects and multiplicative interaction (Ozgen, 1993; Truberg, 2002; Wamatu and Thomas, 2002; Voltas *et al*., 2005).

Effective genetic control of rust disease requires a coordinated effort, including race monitoring, collection and characterization of sources of resistance and resistance

breeding (McIntosh *et al*., 1995). This ensures continued genetic diversity within cultivar arrays to buffer the extent of loss when relevant pathogenic changes occur. In wheat seedlings are often used to screen sources of resistance and to study the genetics of resistance (Lui and Harder, 1996). This method is also applicable for barley. Adult plant resistance assessment and the timely detection of stem rust races with virulence are important to producers aiming at maximum yields (McIntosh *et al*., 1995). The mode of inheritance of resistance to physiological races of stem rust appears to involve one or two major genes (Singh, 2004). Most of which are rapidly being broken down, putting multiple race-specific genes in a single genotype minimizes the probability that a single mutation in the pathogen can overcome all the resistance genes (Huang *et al*., 1997; McIntosh and Brown, 1997; Mundt, 1991). Breeding for multigene resistance to Ug99 can take at least five years (Anon, 2008). It involves crossing disease-resistant lines with barley varieties adapted to local conditions in the world's barley-growing countries. Seed multiplication then follows to produce sufficient planting material for areas at risk from Ug99 (Anon, 2008). A substantial effort on the identification and characterization of rust resistance genes occurred in the second half of the last century (McIntosh *et al*., 2003).The solid plant cell wall itself presents a passive barrier to pathogen entry. However, attempted entry by fungal pathogens usually induces in the attacked cells a set of responses that suggest there are active defense mechanisms also at play (Heath 1997; Zeyen *et al*., 2002; Koh *et al*. 2005).Pathologists and breeders must continually be vigilant for virulence changes in the stem rust population. A case in point is TTKS (i.e. isolate Ug99), a new race of *P. g. f. sp. tritici*, which was first discovered in Uganda in 1999 (Pretorius *et al*., 2000) and has now spread in east Africa (Wanyera *et al*. 2006). Race TTKS is virulent on many of the wheat and barley cultivars grown throughout the

world (Pretorius *et al*. 2000; Jin and Singh 2006; Singh *et al*. 2006; Steffenson and Jin 2006). *Hordeum vulgare* sp. spontaneum is the progenitor of cultivated barley (von Bothmer *et al*., 2003). Wild barley grows over a remarkably wide range of ecological habitats, not surprisingly; it exhibits a tremendous level of genetic diversity for both abiotic and biotic stresses (von Bothmer *et al*., 2003). The long co-evolution of this subspecies with various pathogens in its natural range makes it one of the most important sources of disease resistance genes for cultivated barley (Fetch *et al*. 2003). In particular, wild barley is considered a rich source of resistance genes to leaf rust, stem rust, and stripe rust (Moseman *et al*., 1990; Fetch *et al*., 2003). There is a great concern about the lack of durability of disease resistance (Johnson, 1992). Several strategies can be adopted to prolong the durability of the stem rust resistance genes, such as gene pyramiding, diversification and application of cultivar mixtures (McDonald and Linde, 2002). Another possibility would be to introduce resistance types that are intrinsically durable like partial resistance, which is characterized by a slow epidemic build-up despite a high infection type (IT) (non hypersensitive type of resistance) (Parlevliet and Van Ommeren, 1975).

2.9 Mutation as a source of host plant resistance

Application of nuclear in food and agriculture has contributed in enhancing production of seed and vegetative propagated crops (Jain,2005) More than 2300 mutant varieties have officially been released in many counties (Arabi,2004). Mutation may occur in nature or can be induced by human using mutagenic agents that are either chemical or physical mutagens. Inducing mutation using chemical or physical mutagenic agents is ideal for augmenting natural variation in germplasm and as an alternative to hybridization and recombination in plant breeding. Mutation provides new starting material for the production of new cultivars (Adamu *et al.*,

2007) and on the other hand they offer excellent tools for identifying new genes for studying nature of genes and their biochemical pathways (Wenzel, 1985). A trait arising as a result of a mutation has to be phenotypically expressed to be selectable; all other mutations are only of scientific interest. Induced mutations has great potentials, it is a complimentary approach in genetic improvements of crops (Mahandjiev *et al.,* 2001). The main strategy in mutation-based breeding is to upgrade the well adapted plant varieties by improving a few desirable major traits such as yield component, tolerance to biotic and abiotic stress and for consumer preferences (Ahloowalia *et al* 2004). In Kenya, mutation breeding has not been widely adopted as a method of breeding, however, few crops have been developed through mutation such as cowpea(Pathak *et al.,*1996) and wheat (Kinyua *et al*.,2000;2008) Resistant genes can be induced through mutagenesis to create variability (George,2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 SITE DESCRIPTION

The study was conducted at the University of Eldoret, (0 30′ 0″ North, 35^0 15′ 0″ East.), 10 Km from Eldoret town, in Uasin Gishu county of Kenya. It is located at an altitude of 2180 m above sea level; it consists primarily of an agro-ecological zone Lower Highland 3 (LH3). The site is among the major wheat growing regions in Kenya. The University of Eldoret receives a unimodal rainfall which begins in March. The average annual rainfall range is between 900 mm and 1100 mm and mean annual temperature of 16.6^0C. The soils are shallow, ferralsol, well drained, non humic cambisols with low nutrient availability and moisture storage (Jaetzol and Schmidt, 1983).

The study was also conducted at Kenya Agricultural Research institute, Njoro in Nakuru county, (0°20'S 35°56'E), located in the lower highlands (LH3), at an altitude of 2166 m above sea level. The temperature ranged between 18-28^0C during the period of study, while the average annual rainfall is about 1,000 mm. The soils are deep, well drained, fertile *Vitric Mollic Andosols* (Jaetzold and Schmidt, 1983)

3.2 Irradiation

Six hundred grams of M_0 seeds (non mutated seeds) of the barley variety Nguzo, obtained from East African Maltings in Molo, Kenya were sent to International Atomic Energy Agency in Vienna and subjected to gamma radiation at an irradiation dose of ld_{50} at 250 gy (gray) to obtain M_1(mutated seed that gives rise to the first generation of mutants).

3.3 Seed Multiplication and Selection

The land to be planted was disc ploughed and harrowed to fine tilth suitable for barley planting. The irradiated M_1 seeds were planted at the University of Eldoret for seed multiplication. The mutated seed were drilled on a plot measuring 125 m by 40 m. Drills were 5 cm apart and all the agronomic practices like insect, disease and weed control were done up to harvest time to ensure good crop establishment. At harvest, a thousand plants were selected and two ears from each of these plants harvested. The chimaeras and deformed plants were not selected. The harvested ears were put in individual envelopes and labeled with corresponding numbers. One group was planted at the University of Eldoret experimental field while the corresponding group of a thousand ears was planted at KARI Njoro. The seeds from the ears were designated as M_2. Each ear formed a line.

3.4 Preliminary Evaluation of Barley Mutants for Stem Rust Race Ug99 Resistance

3.4.1 Planting and field management

The M_2 seed from each ear was sown in 1m rows for the 1000 ears on either sites. Sowing was done at an equivalent seeding rate of 125 Kg/ Ha and it was done by hand. To facilitate inoculum build-up and uniform dissemination within the nursery, a continuous row of stem rust spreader (variety Chozi and ks Mwamba) were planted perpendicular to all lines. At planting time, Di-ammonium phosphate fertilizer was applied at an equivalent rate of 125kg/ha. Weeds growth was restricted by applying both pre - and post - emergence herbicides. Immediately after sowing, Stomp® 500 EC (Pendimethalin) a broad spectrum, pre-emergent herbicide was applied at an equivalent rate of 2.5 l/ha. At tillering stage (Zadok's Growth stage 20-29) (Zadok

et. al., 1979) the plots were sprayed with Buctril MC (bromoxynil + MCPA) at an equivalent rate of 1.5 l/ha to control broad-leaved weeds. The trial was top dressed with Calcium Ammonium Nitrate (CAN) at stem elongation stage (Zadok's GS 30).

3.4.2 Inoculum collection, preparation and inoculation of test material

The inoculum was collected (a mixture of susceptible varieties.) from the diseased trap nursery in the field. The diseased plants were cut into pieces and suspended in distilled water. The inoculum was filtered and drained in a dispenser and sprayed on the test material while another batch was injected into the stem of the spreaders.

3.4.3. Preliminary data collection and selection

Rust development was closely monitored on the test plants and response to rust infection at the adult plant stage was termed "infection response". According to the size of the pustules and associated necrosis or chlorosis infection responses were classified into four categories; R = resistant, MR = moderately resistant, MS = moderately susceptible and S = susceptible (Roelfs *et al*., 1992). Stem rust severity was assessed using the modified Cobb scale (Peterson *et al*., 1948). The mutant lines were scored for response to infection and stem rust severity between heading and plant maturity. The lines that did show acceptable level of resistance were selected. They were harvested to be advanced to M_3. Seventy four lines were selected from Njoro whereas at the University of Eldoret, one hundred and nine lines were selected. Each of these lines were harvested and kept in a separate bag to avoid mechanical mixture. The rest of the materials were bulked together.

3.5 Field screening for stem rust race Ug99 resistance in the field

3.5.1 Genotypes

One hundred and eighty three lines selected from the preliminary evaluation of barley mutants for stem rust resistance at Kari Njoro and at the University of Eldoret field were used as the genotypes for further evaluation at M_3. A susceptible control Nguzo (non mutant-parent) was also included.

3.5.2 Experimental Procedure

A field experiment was established on land previously under wheat and was disc ploughed and harrowed to fine tilth suitable for barley planting. Each line was sown in double rows measuring 0.2 × 0.75m forming an experimental unit. The experimental units were separated by 0.3 m and 0.5 m wide alleyways within and between the blocks, respectively. Sowing was done at an appropriate seeding rate. To facilitate inoculum build-up and uniform dissemination within the nursery, a continuous row of stem rust spreader was planted perpendicular to all lines. The lines were randomly assigned within a block. The experiment was laid out in a Randomized Complete Block Design (RCBD) replicated three times. The experiment was managed as described above in section 3.4.1 for agronomic management and inoculation done as described for preliminary evaluation (section 3.4.2).

3.5.3 Data Collection

Plant response to rust infection at the adult plant stage was done following "infection response". This is an evaluation based on the size of the pustules and the associated necrosis or chlorosis. The infection response score was classified into four categories; R = resistant, MR = moderately resistant, MS = moderately susceptible and S = susceptible as described by Roelfs *et al.*, (1992). Stem rust severity was assessed

following the modified Cobb scale (Peterson *et al*., 1948). Lines were evaluated for response to infection and stem rust severity between heading and plant maturity. At physiological maturity 5 plants /plot were selected randomly and used to measure plant height (from plant base to the tip of spike excluding awns), spike length, number of tillers and days to 50% heading. Spike length was measured from the base of the ear to the tip of the spike (excluding the awns) based on an evaluation of all the spikes from the five plants. A sample of 1000 seeds per line was taken and weighed to give 1000-seed weight at harvest maturity.

3.5.4 Data Analysis

The data was analyzed using GenStat software 12th Edition and means separated using Duncan Multiple Range test. Correlation was done by SAS Pearson correlation Coefficients.

The following Statistical model was used;

$\mathbf{X_{ijk}=\mu+t_i+\beta_j+e_{ijk}}$

Where,

$\mathbf{X_{ijk}}$= observation
$\boldsymbol{\mu}$=overall mean
$\mathbf{t_i}$=treatment effect(mutant lines)
$\boldsymbol{\beta_j}$=block effect.
$\mathbf{e_{ijk}}$=experimental error

3.6 Seedling screening of M3 plants for resistance to stem rust in the greenhouse

Greenhouse experiment was conducted at the University of Eldoret greenhouse. It was put in a Completely Randomized Design (CRD) with three replicates and repeated three times. The selected 183 lines were evaluated for seedling resistance to Ug99 plus their parent as a check. They were planted in Plastic cups measuring 4 cm

diameter by 6 cm height filled with about 200g of a mixture of soil and sand in a ratio of 3:1. The plastic cups were placed in a greenhouse that was maintained at 23^0C and 60% relative humidity (RH). In each pot, 3-4 seeds were planted at approximately 2 cm deep and the cups were then placed in large non-draining trays measuring 60cm × 240cm and watered to field capacity. The seedlings were inoculated with urediniospores after 14 days using a hand sprayer when the first leaf was fully developed and placed in dark moist chamber maintained at 100% relative humidity, temperature at 13^0C for 18 hours. Thereafter, the seedlings were then transferred to a growth chamber that was maintained at about 22^0C day and 20 - 21^0C night temperature. After the disease had developed, scoring was done according to Stakman *et al* 1962.

Table 1 Major Infection type classes for stem rust

Infection type	Host Response	Symptoms
0	Immune	No visible uredia
;	Very resistant	Hypersensitive flecks
1	Resistant	Small uredia with necrosis
2	Resistant to moderately resistant	Small to medium sized uredia with green islands and surrounded by necrosis or chlorosis
3	Moderately resistant/ moderately susceptible	Medium sized uredia with or without chlorosis
4	Susceptible	Large uredia without chlorosis
X	Resistant	Heterogeneous, similarly distributed over the leaves

3.6.1 Data collection

Seedlings were assessed for disease infection type 14 days after inoculation using 0-4 infection types (IT) scale (Stakman *et al* 1962) when uredinia on the susceptible line appeared fully developed (Roelfs *et. al*., 1994; McIntosh *et.al*., 1995). ITs "0", "1" and "2" were considered low ITs where as "3" to "4" were considered high ITs.

3.7 Molecular screening using SSR markers

The hundred and eighty four lines selected were screened using four SSR markers of the *Rpg4* and *Rpg5* genes to know their identities and to confirm the presence of any known Ug99 resistance genes. DNA was isolated from seedling of the genotypes that showed resistance to stem rust race Ug99. Two, 10 cm leaf segments were harvested from the primary leaves of each seedling at four leaf stages (Zadok *et al*., 1974). Two hundred and fifty milliliter bottles with air-tight screws containing 50g blue silica gel were used to store the leaf samples for DNA isolation. A sample of 5g leaf tissues from each seedling were cut into small pieces and sealed in the bottles for about 24 hours. When the gel turned white in color, the tissues were transferred to bottles containing fresh silica gel to ensure thorough drying before DNA isolation. DNA isolation was done according to the method of Dellaporta and Woods, (1983) (See Appendix IV). DNA was quantified by a spectrophotometer. DNA quality was checked by 1% agarose gel electrophoresis, against lambda DNA of known quantity. Two microliters of DNA from each sample was mixed with 3 µl of 6X DNA loading buffer and loaded in wells including 3µl of each lambda DNA of known quantity. The gels were run at 100 volts for 40 minutes. The bands were visualized under a UV

transilluminator. The products were captured on a camera and transferred to a computer. DNA was diluted to a working stock of 50 ng/µl for PCR reactions.

3.8 PCR amplification.

The PCR reactions were performed in a thermo cycler (eppendorf /AB2720) in a total of 10-ul reaction volume. The PCR Mix stock contained 10x buffer,10mM dNTP, 50mM $Mgcl_2$,10mM forward pimer,10mM reverse primer, 5 U Taq,ddH_20 and 50ng/ul DNA. The volume per sample was as follows 0.4mM dNTP, 0.6mM $Mgcl_2$, 0.8mM reverse and forward primer, 4.9 ddH_20 and 1.4ng/ul of DNA. The PCR cycles consisted of initial denaturation of 95^0C for 3minutes, denaturation of 98^0C for 30 seconds. Extension was at 72^0C for 30 seconds and final extension was for 10 min at 72^0C .The DNA fragments were separated 0n 2% agarose gel run 1 hour at 100 volts using 1x TBE buffer. The DNA fragments in gel was visualized by staining in 0.5/mg ethidium bromide for 30 seconds and rinsed in distilled water for 20 minutes, visualized and photographed on ultraviolet (UV) transilluminator. Scoring was done on either presence or absence of the band.

Chapter four

Results

4.1 Effects of Mutation on agronomic traits

There was a notable variation among the mutant lines for the agronomic traits evaluated including number of tillers, height, spike length, days to 50% heading and weight of the 1000 seeds.

4.1.1 Effect of mutation on number of tillers on mutant barley lines

Mutation had a significant effect at $P<0.001$ on a number of tillers on the various mutant barley lines (Table 2). Mutant line 23 had the highest mean of 14 tillers and line 126 had the lowest mean of 6 tillers and the parent (non mutant) line 184 had a mean of 9.

4.1.2 Effect of mutation on height of mutant barley lines.

Mutation had a significant effect at $P<0.001$ on height of the various mutant barley lines (Table 2). Line 156 had the highest mean of 110cm and line 154 had the lowest mean of 75cm and the parent (non mutant) line 184 had a mean of 99.33cm.

4.1.3 Effect of mutation on spike length of mutant barley

Mutation had a significant effect on spike length of the various mutant barley lines at $P<.001$. Line 156 had the highest mean of 11.5cm and line 116 had the lowest mean of 8.5cm and the parent (non mutant) line 184 had a mean of 9.233cm (Table 2).

4.1.4 Effect of mutation on weight of 1000 seeds per mutant barley line

Mutation had a significant effect on weight of 1000 seeds per mutant line of the various mutant barley lines at P<.001. Line 173 had the highest mean of 52.5g and line 163 had the lowest mean of 33.4g and the parent (non mutant) line 184 had a mean of 44.33g.

4.1.5 Effect of mutation days to 50% heading of mutant barley lines

Mutation had a significant effect on days to 50% heading of mutant barley lines at P<.001. Line 90 took 58 days t 50% heading while line 5 took 80 days to 50% heading. The Non mutant parent line took 68 days to 50% heading.

Table 2-Summary table of means of Agronomic traits

Mutant lines	Tillers	Height (cm)	Spike length(cm)	Days to 50% heading	Weight of 1000seeds(g
1	11ab	93.33a-k	9.667a-h	77a-d	45.7a-i
2	10a-d	97a-j	9.667a-h	79.33ab	43a-i
5	9a-d	93.33b-k	10.33a-e	80.33a	35.43jkl
7	12ab	92.33b-i	9.5a-h	77.33a-c	50.87a-c
8	11a-d	96.33a-k	10.43a-e	77a-d	42.8a-l
9	12ab	95.33a-k	9a-h	75.67a-g	44.03a-l
21	11a-d	89b-m	8.9a-j	72b-q	35.6i-l
23	14a	96.33a-k	11ab	72b-p	44.5a-l
26	8.a-d	97a-j	8.9a-j	70c-w	35.8h-l
27	11a-d	105.33ab	9.7a-h	66.67l-x	42.8a-l
34	9a-d	101a-h	9.5a-h	63v-A	48.9a-f
36	8b-d	97.33a-j	9a-j	64s-A	44.97a-l
41	11a-d	98a-j	8.667b-j	71c-t	37.87d-l
44	12a-c	92.33b-l	7.667e-j	66.33m-x	47.6a-j
49	10a-d	92.33b-l	9.5a-h	65.33o-y	48.13a-i
54	11a-d	99a-j	7.933d-j	69f-x	51.93a-b
55	11a-d	96.67a-k	9.533a-h	66n-x	40.3a-l
58	8a-d	96.67a-k	8.167c-j	68.67g-x	46.27a-k
59	11a-d	94.33a-k	9.5a-h	66.67l-x	45.77a-l
62	8b-d	97a-j	9.2a-j	62x-A	43.07a-l
69	9a-d	96a-k	8d-j	66n-x	43.3a-l
76	8a-d	99a-j	9.467a-h	67.33j-x	47.13a-j
78	9.33a-d	95.67a-k	9.267a-i	67k-x	45.2a-l
90	8b-d	95.67a-k	8.133c-j	58Y-a	38.57c-l
95	9a-d	94a-k	10.9a-c	68.33g-x	40.97a-l
124	6d	89.7b-m	9a-j	70.67c-u	40.13a-l
126	6d	92.33b-l	9.5a-h	71.67c-r	39.2c-l
130	8b-d	89.3b-m	7.1h-j	65p-A	36.67f-l
156	9a-d	110a	11.5a	75a-i	42.17a-l
161	9a-d	104a-c	10.167a-g	71.67c-r	42.23a-l
163	10a-d	89b-m	9.5a-h	75.67a-g	33.4l
165	9a-d	97.67a-j	9.5a-h	66.67l-x	50.4a-d
173	8a-d	87.7c-m	8.6b-j	72b-q	52.5a
184	9a-d	99.33a-i	9.233a-i	67.67i-x	44.33a-l
Grandmean	9	94.344	9.0189	69.3	43.518
SED	1.2317	2.922	0.6021	3.543	2.748
CV%	16.1	5.2	8.2	4.6	7.7

4.2 Correlation Coefficient between the agronomic traits

Height was positively and significantly correlated to the number of tillers, and to spike length (Table 3). Spike length had a positive correlation with number of tillers at $p<0.05$, and weight of 1000 grains of mutant barley lines at $p<0.001$and negatively correlated with height and days to 50% heading of mutant barley lines at $p<0.001$.

Weight of 1000 grains of the mutant barley lines had a positive correlation with spike length at $p<0.001$,weight of 1000 grains per mutant line had a negative correlation with days to 50% heading of the mutant barley lines at $p<0.001$.Plant height of the mutant barley lines positively correlated with tillers at $p<0.01$, height of the mutant barley lines also had a positive correlation with days to 50% heading at $p<0.001$.Plant height of the mutant barley lines had a negative correlation with spike length at $p<0.001$.Days to 50% heading of the mutant lines had a positive correlation with height at $p<0.001$ and a negative correlation with spike length and weight of 1000 grains per mutant barley lines at $p<0.001$.

Table 3 Correlation coefficient between the agronomic traits.

	Tillers	height	spike length	weight	days 50%
Tillers	1	0.11**	0.08*	0.02	0.03
Height		1	-0.26***	-0.02	0.16***
Spike length			1	0.26***	-0.41***
weight				1	-0.35***
Days 50%					1

*=significant at p<0.05, **=significant at p<0.01, ***=significant at p<0.001

4.3 Screening of the mutant barley lines using SSR molecular markers.

Four SSR molecular markers were used in the screening for stem rust (Ug99) resistance in the mutant barley and the parent used as a check. The following results were observed.12 mutant barley lines showed polymorphism, as shown below.

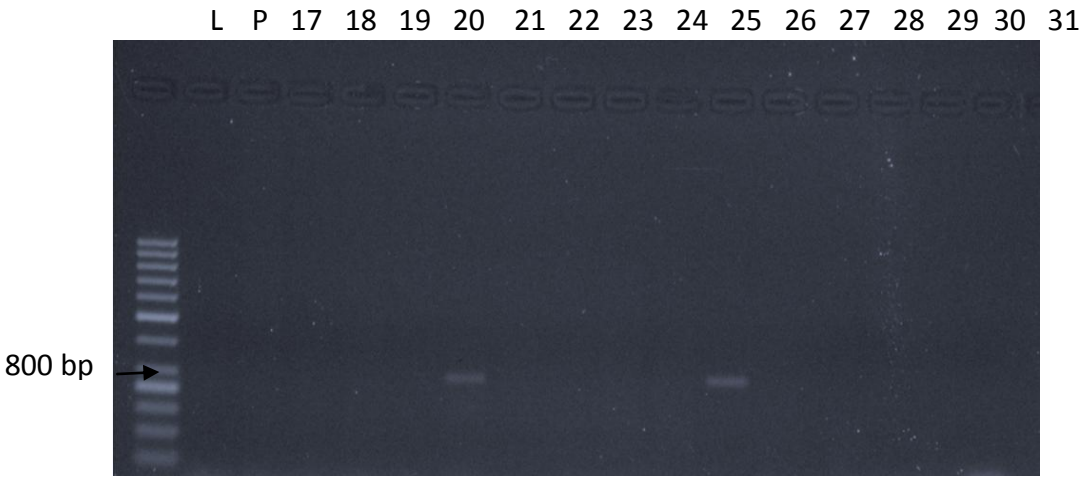

Plate 2-gel picture for primer r4 amplified at 800bp (source, author 2012)

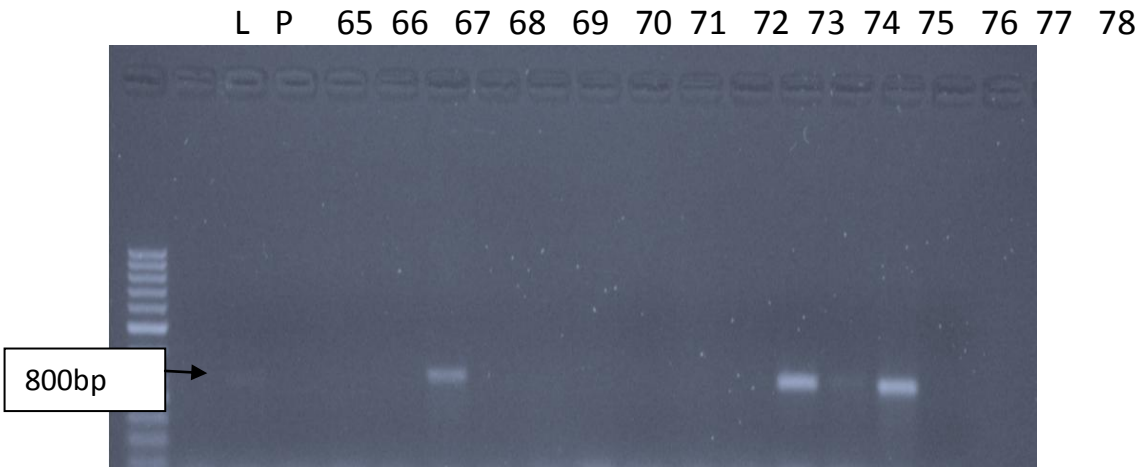

Plate 3-gel picture for primer r4 amplified at 800bp (source, author 2012)

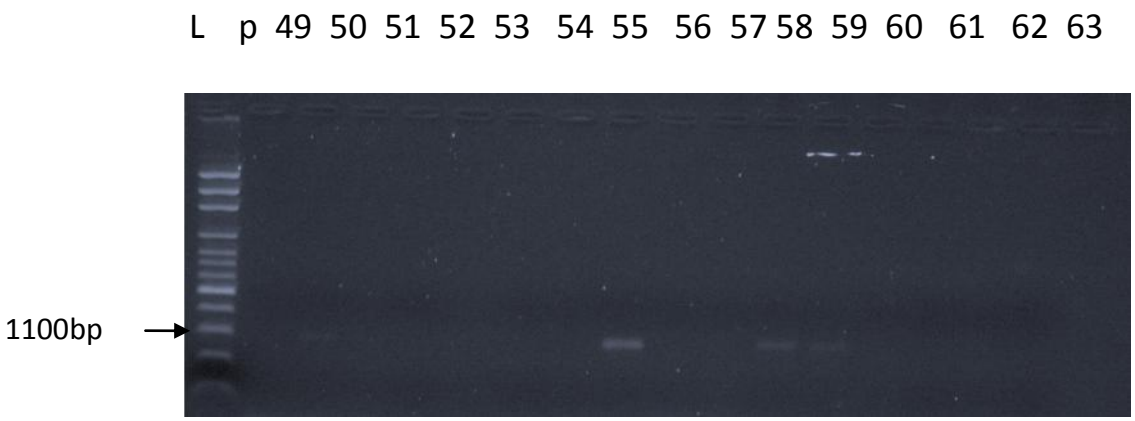

Plate 4-gel picture for primer A12 amplified at 1100bp (source, author 2012)

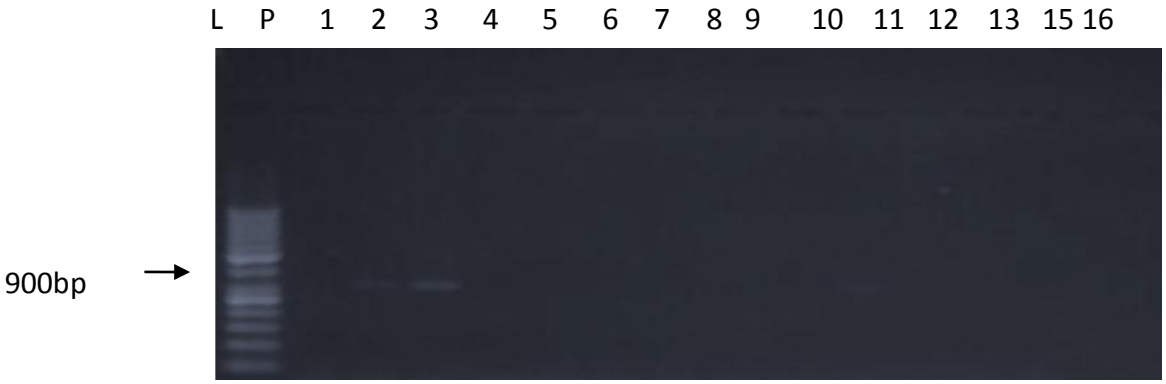

Plate 5-gel picture for primer RB amplified at 900bp (source, author 2012

NB-The arrow points the Presence of a band (Polymorphism).

The infection type at seedling level ranged from low (1 and 2) infection type and high infection type (3 and 4).Mutant lines 1,2,21,26,34,36 and 41 had low infection types while lines 5,7,44 and 124 had high infection types (Table 4).The the infection response in adult plants ranged from Moderate Resistance (MR), Moderate Susceptible (MS) and Susceptible(S).The severity of the disease also ranged from 5% to 40% (Table 4).Polymorphism of the various molecular markers was also observed

(Table 4) where + denoted the presence of the of the band of the molecular marker and – denoted the absence of the band of the marker.

Table 4-Summary of seedling resistance infection type and adult plant resistance of the mutant barley lines and Polymorphism of the primers used.

Mutant lines	Seedling resistance infection type(IT)	APR (Severity % and infection type)	Polymorphism of Primer RB	Polymorphism of Primer A12	Polymorphism of Primer r4
1	1	5MR	+	-	-
2	2	20MR	+	-	-
5	4	30S	-	-	-
7	4	20MS	-	-	-
8	3	MS	-	-	-
9	2	15MR	+	-	-
21	1	20MR	-	-	+
23	2	20MS	-	-	-
26	2	10MR	-	-	+
27	3	40MS	-	-	-
34	2	35MS	-	-	-
36	2	15MS	-	-	-
41	2	20MS	-	-	-
44	3	10MS	-	-	-
49	2	10MR	-	-	-
54	2	20MS	-	-	-
55	2	25MR	-	+	-
58	2	20MR	-	+	-
59	1	25MR	-	+	-
62	1	5MR	-	-	-
69	1	10MR	-	-	-
76	1	5MR	-	-	-
78	2	20MR	-	-	-
90	3	45MS	-	-	-
95	1	5MR	-	-	-
124	3	20MS	-	-	-
126	2	30MS	-	-	-
130	3	25MS	-	-	-
156	3	20MS	-	-	-
161	3	15MS	-	-	-
163	2	35MS	-	-	-
165	2	20MR	-	-	-
173	2	30MS	-	-	-
184	3	40S	-	-	-

KEY: S-Susceptible, MS-Moderate Susceptible, MR-Moderate Resistant, R-Resistant.
+ - Presence of polymorphism.
- -Absence of polymorphism

CHAPTER FIVE

DISCUSSION

5.1 Mutation on agronomic traits.

Mutant lines showed wide variation in terms of number of tillers, some lines showed superiority over other mutant lines and the control which was the parent material (non mutant). Similar results were previously observed by Mucci (1962) in wheat. (Millado *et* al 1972) also reported the variation in terms of number of tillers observed in mutant lines upon usage of the various doses of gamma radiation. The variation in terms of number of tillers varied from 14 tillers which was the highest to 6 tillers which was the least. Mutant line number 23 was the one that had the highest number of tillers and mutant line number 126 had lowest number of tillers. The lines with most number of tillers could be positively selected for high yield; this is after evaluation on their potentiality in terms of yield. Mutation produced variability among the mutant barley lines in terms of height. This ranged from tall plants to short plant compared to the check, the parent material (non mutant).The short plants could be positively selected and be used in breeding for against lodging in crossing programmes. Mucci (1962) observed similar variation in terms of plant height in irradiated wheat population. (Millado *et* al 1972) also reported the variation in terms of number of tillers observed in mutant lines upon usage of the various doses of gamma radiation. in the present study mutant line 156 had the highest mean of 110cm and line 154 had the lowest mean of 75cm. Mutation had significant effect on spike length of the mutant barley lines as observed from the above results. The effects were in comparison to the check

i.e the non mutant material (parent).This results are in agreement with those of Morad *et al*. (2011) they concluded that gamma radiation induced greater variability and improvements in different traits such as spike length and yield per spike. In the present study, mutant line 156 had the longest spike length of 11.5cm and mutant line 116 had the shortest spike length of 8.5cm. Mutation had significant effect on the weight of 1000 seeds per mutant barley line as observed from the results, and this was in comparison with the check (non mutant parent material) the lines with higher weight as compared to the non mutant material could be positively selected and advanced. These results are in agreement with those of Morad *et al*. (2011) who found that gamma radiation produced greater variability on yield related parameters in wheat. Rahimi *et al.* (2011) also reported similar results on effects of gamma radiations on 1000 grain weight and grain yield of wheat crop. The variation in terms of weight varied from 52.5g to 33.4g, this were shown by line number 173 and 163 respectively. Mutation had significant effect on 50%heading as observed from the results. some lines showed earliness in heading compared to the parent lines while others showed delayed heading compared to the non mutant parent lines, similar results were reported by shah *et al* (1987). Jamil and Khan (2002) reported that radiation intensity delays heading and increases maturity period. In the present study variation in terms of days to 50% heading was from 58 days to 80 days. This were shown by mutant line number 90 and 5 respectively

5.2 Correlation coefficient among traits

Spike length showed positive correlation with weight of 1000 grains per mutant barley lines, this to mean that the longer the spike length the more the weight of the 1000grains per mutant barley line. This results are in agreement with those of shahid

et al ,(2002),saleem *et al*.,(2006) and khokar *et al* (2010).This could mean that the lines with longer spike length could be positively selected and advanced.

There was a negative correlation between days to 50% heading and weight of 1000 grain weight per mutant line, this a results are in agreement with those of Bilgin *et al.*,(2011) who found a negative correlation between days to 50 % heading and weight of 1000 grain in durum wheat. Days to 50% heading had a positive and significant correlation with height of mutant barley lines, this meant that the more the days, the more height of a plant increases.

5.3 Molecular screening of mutant barley lines using SSR markers for stem resistance (Ug99)

In the present study the markers associated with this (*Rpg4* and *Rpg5*) genes were used to screen the presence or absence of this genes in mutant barley lines. Simple sequence repeats (SSR) markers are simple PCR based co-dominant markers which are extremely polymorphic and highly informative due to the number and frequency of alleles, they are the most utilized ones among the molecular markers (Hillel *et al*., 1990) in many grass species (Budak *et al.*, 2004; Budak *et al*., 2003; Budak *et al*., 2005) SSR markers can be used for selection if they are found to be completely associated or even responsible for the targeted trait (Sorrells and Wilson1997). Out of the 184 lines screened with the SSR markers 12 lines(1,2,9,21,26,49,55,58,59,69,76,78) were found to have shown presence of the bands hence concluding probably the presence of this genes. The check line which was the parent (non mutant) didn't show any band, this meant that it didn't have the genes which were conferring resistance to Ug99.These markers were for the *Rpg4* and *Rpg5* genes. *Rpg4* and *Rpg5* genes confer resistance to Ug99 (Brueggeman *et al* 2009). In barley effective control of stem rust has been achieved through the

deployment of cultivars with the durable resistance gene *Rpg1* and is effective against many races of *P. graminis* f. sp. *tritici*;(Steffenson, 1992) in 1999 and 2001 a new race Ug99 or TTKS with virulence to stem rust resistant cultivars, was detected in Uganda and Kenya respectively (Wanyera *et al*., 2006; Pretorius *et al.*, 2000). The lines which have shown the presence of the markers linked of *Rpg4* and *Rpg5* genes can be validated and used in Marker assisted selection for Ug99. This *Rpg4* and *Rpg5* genes are in the large group of R genes expressed by plants which confers resistance to diseases. Plants can activate a very effective defense response, comprised of genetically programmed suicide of infected cells, as well as tissue reinforcement and antibiotic production at the site of infection (Hammond and Jonnes, 1996). Plant cells autonomously maintain a constant vigilance against pathogens by expressing large arrays of 'R genes' (R, resistance) (Dangl and jones, 2001). R genes encode putative receptors that respond to the products of 'Avr genes' (Avr, avirulence) expressed by the pathogen during infection. R genes have been used in conventional resistance breeding programs for decades (pink, 2002).

5.4 Mutation and disease resistance.

Mutations are used to study the nature and function of genes which are the building blocks and basis of plant growth and development, thereby producing raw materials for genetic improvement of economic crops (Adamu *et al*., 2007) Mutation has been used to produce many cultivars with improved economic value and study of genetics and plant developmental phenomena (Van, Den-Bulk *et al*., 1990; Bertagne- Sagnard *et al*., 1996). In the present study gamma rays were used to induce variation in barley lines especially for the gene governing stem rust (Ug99) resistance. The induced variations that were brought about by the gamma rays had some molecular basis i.e. change in the base sequence of the DNA molecule coding for the protein. The change

in the base sequence can be through base substitution, base addition or deletions. This could explain the induced variations in terms of Ug99 resistance in the barley lines. There some lines which had low infection type in their seedling screening and were showing resistance to Ug99 in the field at adult plant stage, such lines didn't show any polymorphism during molecular screening. In such a situation, maybe the gene was there but the marker didn't amplify.

CHAPTER SIX

6.1 Conclusions and recommendation

6.1.1 Conclusions

Mutation by irradiation was successfully applied and generated the much needed variability that inferred resistance to the mutant barley lines at M_2 and M_3 at the seedling level and adult plant level. The following lines did show resistance both at the seedling level and adult plant level (1,2,9,21,26,49,55,58,59,69,76 and 78).

Mutation had an effect on the agronomic traits in the mutant barley lines. The following lines did show superiority in terms of the agronomic traits studied (7,23,27,34,44,54,90,95,130,156,161,165 and 173).

6.1.2 Recommendations

The study recommends the following.

- Continual screening of these lines as this race may mutate further((1,2,9,21,26,49,55,58,59,69,76 and 78).
- Stabilization of these mutant lines through double haploid techniques and backcrossing to reduce the effects of mutation.
- Other agronomic traits should be evaluated on the resistant lines to identify superior lines for release for commercial purposes.

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APPENDICES

Appendix I table of seedling resistance infection type and Adult plant resistance severity in%and infection type

MUTANT LINE	Seedling resistance infection type (IT)	APR(Severity% and Infection type)
1	1	5MR
2	2	20MR
3	3	50MS
4	3	40MS
5	4	30S
6	3	40MS
7	4	20MS
8	3	50MS
9	2	15MR
10	2	30MS
11	2	20MR
12	4	60MS
13	2	10MS
14	2	20MS
15	3	60S
16	2	30MS
17	2	20MS
18	3	70S
19	2	50MS
20	2	25MS
21	1	30MR
22	2	25MS
23	2	20MS
24	3	60S
25	3	20MS
26	2	10MR
27	3	40MS
28	4	70S
29	2	20MS
30	3	10MS
31	3	35MS
32	3	65S
33	2	20MS
34	2	35MS
35	3	55S
36	2	15MS
37	3	25MS
38	3	10MS
39	3	60S

40	4	70S
41	2	20MS
42	3	20MS
43	3	15MS
44	3	10MS
45	3	45S
46	2	5MR
47	1	10MS
48	3	5MS
49	2	15MS
50	3	25MS
51	3	65S
52	2	10MS
53	1	5MR
54	2	20MS
55	2	45MS
56	2	15MS
57	1	10MS
58	2	20MR
59	1	25MR
60	3	45S
61	2	10MS
62	1	5MR
63	1	10MR
64	3	35MS
65	4	65S
66	2	20MS
67	3	15MS
68	2	25MS
69	1	10MR
70	3	55S
71	2	15MS
72	4	60S
73	2	20MR
74	2	20MS
75	2	25MS
76	1	5MR
77	4	70MS
78	2	20MR
79	2	5MS
80	2	20MS
81	3	35MS
82	1	10MR
83	3	5MS
84	3	35MS
85	4	60S
86	1	5MR
87	2	10MS

88	3	10MS
89	2	25MS
90	3	45MS
91	3	30MS
92	3	25MS
93	2	10MS
94	2	10MS
95	1	5MR
96	4	60S
97	3	50MS
98	2	45MS
99	3	20MS
100	2	45MS
101	2	40MS
102	3	25MS
103	3	20MS
104	3	20MS
105	2	45MS
106	3	30MS
107	2	10MS
108	3	40S
109	2	20MS
110	3	45MS
111	3	25MS
112	2	20MS
113	1	10MS
114	2	35MS
115	3	20MS
116	3	25MS
117	3	50MS
118	3	20MS
119	2	15MS
120	1	10MR
121	3	20MS
122	2	35MS
123	2	20MS
124	3	20MS
125	3	45MS
126	2	30MS
127	3	35MS
128	3	40S
129	2	15MS
130	3	25MS
131	2	45MS
132	3	20MS
133	2	35MS
134	2	25MS
135	3	50S

136	4	60S
137	2	35MS
138	2	45MS
139	3	35MS
140	2	50MS
141	4	60S
142	2	25MR
143	3	50S
144	2	25MS
145	2	30MS
146	2	40MS
147	3	25MS
148	2	25MS
149	3	50S
150	3	30S
151	3	30S
152	4	60MS
153	2	25MS
154	2	20MS
155	3	20MS
156	3	55MS
157	2	20MS
158	2	15MS
159	3	30MS
160	2	20MS
161	3	15MS
162	2	30MS
163	2	35MS
164	2	45MS
165	2	20MR
166	4	25MS
167	1	30MS
168	2	40MS
169	3	45S
170	4	20S
171	4	25S
172	3	20MS
173	2	30MS
174	4	50MS
175	3	20MS
176	2	25MS
177	4	30S
178	3	35MS
179	2	40MS
180	2	25MS
181	4	30MS
182	3	40MS
183	3	25MS

184	3	40S

Appendix II table of means.

Mu-lines	Tillers	Height	Spike length	Days to 50% heading	Weight 1000 seeds
1	11.333a-d	93.33b-k	9.667a-h	77a-d	45.7a-l
2	10a-d	97a-j	9.667a-h	79.33a-b	43a-l
3	10.333a-d	99a-j	9.333a-h	71 c-t	38.23c-l
4	10a-d	95.33a-k	9.833a-h	71c-t	42.67a-l
5	9.333a-d	93.33b-k	10.333a-e	80.33a	35.43j-l
6	11.667a-d	96a-k	9.167a-i	76.33a-f	39.9a-l
7	12.667a-b	92.33b-l	9.5a-h	77.33a-c	50.87a-c
8	11.333a-d	96.33a-k	10.433a-e	77a-d	42.8a-l
9	12.667a-b	95.33a-k	9.5a-h	75.67a-g	44.03a-l
10	9.333a-d	96.33a-k	9.5a-h	72.67b-o	49.37a-e
11	11.333a-d	93.67a-k	9.667a-h	71.67c-r	45.53a-l
12	10.333a-d	95.33a-k	9a-j	69.67d-w	40.33a-l
13	11.667a-d	102.33a-f	10a-g	74a-l	39.97a-l
14	11.333a-d	91.33b-m	9.333a-h	72b-q	43a-l
15	10.333a-d	96.67a-k	10.233a-g	69.67d-w	38.63c-l
16	7.667b-d	87e-m	9.5a-h	68h-x	43.10a-l
17	10.667a-d	89.33b-m	9.167a-i	69.33e-x	43.43a-l
18	10.333a-d	93b-k	10a-g	72.67b-o	41.43a-l
19	11.333a-d	93b-k	9.667a-h	72.67b-o	44.8a-l
20	10a-d	90.67b-m	10.1a-g	72.67b-o	41.1a-l
21	11.667a-d	89b-m	8.9a-j	72b-q	35.6i-l
22	10.333a-d	94.33a-k	8.433b-j	72.33b-p	41.43a-l
23	14a	96.33a-k	11a-b	72b-q	44.5a-l
24	9.333a-d	97.33a-j	9.767a-h	75a-i	41.97a-l
25	11.667a-d	94a-k	9.5a-h	68.67g-x	39.07c-l
26	8.333a-d	97a-j	8.9a-j	70c-w	35.8h-l
27	11a-d	105.33a-b	9.7a-h	66.67l-x	42.8a-l
28	10.333a-d	102.33a-f	9.333a-h	67k-x	42.13a-l
29	11.333a-d	100.33a-i	9.667a-h	68h-x	44.63a-l
30	10a-d	95.67a-k	8.933a-j	66.67l-x	50a-e
31	10.333a-d	85.33h-m	9.7a-h	65.67o-x	38.43c-l
32	11.333a-d	96.33a-k	9.167a-i	75.67a-g	49.8a-e
33	10.333a-d	95.33a-k	8.933a-j	72.33b-p	45.1a-l
34	9.333a-d	101a-h	9.5a-h	63v-A	48.9a-f
35	9a-d	94.33a-k	9.167a-i	67k-x	34.3k-l
36	8b-d	97.33a-j	9a-j	64s-A	44.97a-l
37	8.333a-d	99.67a-i	9.167a-i	70.67c-u	44.37a-l
38	9.333a-d	93.67a-k	9.5a-h	66.67l-x	45.97a-l
39	10.333a-d	94.33a-k	8.333-j	70c-w	43.43a-l
40	10.333a-d	98a-j	9.7a-h	67.33j-x	42.1a-l
41	11.667a-d	98a-j	8.667b-j	71c-t	37.87d-l
42	12a-c	98a-j	9a-j	65p-A	40.3a-l
43	9.667a-d	98a-j	8.567b-j	67.67i-x	48.3a-h

44	12a-c	92.33b-l	7.667e-j	66.33m-x	47.6a-j
45	9a-d	92b-l	8.667b-j	64.67q-A	48a-j
46	9a-d	96a-k	9.667a-h	65p-A	43.4a-l
47	9.667a-d	102a-g	7.667e-j	64s-A	44.77a-l
48	10.667a-d	92.67b-k	9.3a-h	67.33j-x	43.2a-l
49	10.667a-d	92.33b-l	9.5a-h	65.33o-y	48.13a-i
50	10a-d	94.67a-k	9.567a-h	64.67q-A	44.3a-l
51	9a-d	99.67a-i	9.567a-h	67k-w	41.87a-l
52	10.33a-d	102.33a-f	9.733a-h	66n-x	45.63a-l
53	8b-d	102a-g	8.667b-j	65.67o-x	46.47a-k
54	11a-d	99a-j	7.933d-j	69f-x	51.93a-b
55	11.333a-d	96.67a-k	9.533a-h	66n-x	40.3a-l
56	8.667a-d	94.67a-k	9.667a-h	70.67c-u	44a-l
57	9.333a-d	100.33a-i	10.033a-g	64stuvwxyzA	46.37a-k
58	8.667a-d	96.67a-k	8.167c-j	68.67g-x	46.27a-k
59	11a-d	94.33a-k	9.5a-h	66.67l-x	45.77a-l
60	9.333a-d	97.33a-j	8.667b-j	66n-x	45.73a-l
61	9a-d	92b-l	8.6b-j	66.67l-x	44.57a-l
62	8.333a-d	97a-j	9.2a-i	62x-A	43.07a-l
63	8.667a-d	96.67a-k	8.533b-j	64s-A	44.67a-l
64	7.667b-d	94.67a-k	9a-j	63.67t-z	46.67a-k
65	9.667a-d	95a-k	8.367b-j	66n-x	48.5a-g
66	9.333ad	88c-m	8d-j	63.33u-z	40.23a-l
67	9a-d	95a-k	9.2a-i	69f-x	41.1a-l
68	9.333a-d	98a-j	9.767a-h	66n-x	46.9a-k
69	9a-d	96a-k	8d-j	66n-x	43.3a-l
70	10a-d	91.67b-l	9.3a-h	70c-w	47.87a-j
71	11a-d	97.33a-j	7.967d-j	70.33c-v	44.27a-l
72	10a-d	104a-c	10.3a-f	64.33r-A	44.33a-l
73	10a-d	93.33b-k	9.233a-i	70c-w	59.93a-b
74	10a-d	98a-j	8.533b-j	71.33c-s	38.63c-l
75	9.667a-d	98.33a-j	9.133a-i	65p-A	46.57a-k
76	8b-d	99a-j	9.467a-h	67.33j-x	47.13a-j
77	10a-d	96.33a-k	7.667e-j	65.33o-z	48.13a-i
78	9.333a-d	95.67a-k	9.267a-i	67k-x	45.2a-l
79	10a-d	92b-l	8.633b-j	66.67l-x	49.5a-e
80	8.667a-d	89.33b-m	8.5b-j	67k-x	47.2a-j
81	9.667a-d	95.67a-k	8.9a-j	64.67q-A	43.10a-l
82	9.667a-d	93.67a-k	7.767e-j	66.33m-x	50.40a-d
83	9a-d	92.33b-l	8.833a-j	65p-A	40.97a-l
84	8b-d	91.33b-m	8d-j	63.33u-A	49.53a-e
85	8.667a-d	97.33a-j	8.767a-j	64.33r-A	43.63a-l
86	7.333b-d	93.67a-k	8.767a-j	66.67l-x	43.93a-l
87	11a-d	97a-j	9.667a-h	71.67c-r	39.13c-l
88	10.333a-d	97.33a-j	7.833e-j	68.33g-x	42.9a-l
89	10a-d	102.33a-f	8.433b-j	65.67o-x	44.93a-l
90	8b-d	95.67a-k	8.133c-j	58Y-a	38.57c-l
91	7.333b-d	99a-j	8.567b-j	65.67o-x	49.97a-e
92	8b-d	96a-k	9.367a-h	68h-x	43.83a-l
93	10.333abcd	91.33b-m	8.933a-j	68h-x	44.77a-l

94	8b-d	82.67j-m	9.067a-j	68.67g-x	45.13a-l
95	9a-d	94a-k	10.9a-c	68.33g-x	40.97a-l
96	7.667b-d	89b-m	7.533f-j	67.67i-x	46.4a-k
97	9a-d	96.67a-k	10.833a-c	70.33c-v	39.47b-l
98	8b-d	95.67a-k	8.833a-j	73.33a-n	39.53a-l
99	9.667a-d	102.33a-f	9.233a-i	69.33e-x	45.7a-l
100	8b-d	101.67a-g	8.933a-j	69.67d-w	46.77a-k
101	10.667a-d	86.33f-m	9.167a-i	64s-A	40.7a-l
102	9.667a-d	87.33d-m	9.2a-i	67k-x	47.87a-j
103	10.667a-d	87e-m	9.333a-h	69f-x	44.13a-l
104	8b-d	91.67b-l	8.833a-j	73.33a-n	43.32a-l
105	8b-d	91.67b-l	8.333b-j	69f-x	45.63a-l
106	7.667b-d	97a-j	9.133a-i	65p-A	44.63a-l
107	9a-d	90.33b-m	8.833a-j	72.67b-o	45a-l
108	8b-d	90.67b-m	9.167a-i	73.33a-n	47.13a-j
109	8b-d	94.33a-k	9.333a-h	77a-d	43.79a-l
110	8.333a-d	95.33a-k	10a-g	69f-x	44.97a-l
111	8b-d	94.67a-k	8.5b-j	67.67i-x	44.87a-l
112	8.333a-d	99.33a-i	9.067a-j	68h-x	47.83a-j
113	9.667a-d	93b-k	8.833a-j	70c-w	44.2a-l
114	6.333c-d	84i-m	7.5g-j	75.33a-h	40.03a-l
115	8.667a-d	101.67a-h	7.833e-j	71c-t	43.5a-l
116	8.3a-d	91.67b-l	8.5b-j	64s-A	49.67a-e
117	7b-d	92.67b-k	7.167h-j	69f-x	44.3a-l
118	7.333b-d	85.67g-m	7.767e-j	70.67c-u	34.27k –l
119	7.333b-d	80.33k-m	7.5g-j	65.33o-z	38.77c-l
120	8b-d	87.33d-m	8.667b-j	72.33b-p	41.53a-l
121	8.667a-d	96.33a-k	8.333b-j	71.33c-s	38.57c-l
122	8b-d	84.33i-m	6.5i-j	68.33g-x	43.83a-l
123	8b-d	84i-m	7.167h-j	73.33a-n	43.4a-l
124	6.667c-d	89.67b-m	9a-j	70.67c-u	40.13a-l
125	9.333a-d	94a-k	8.667b-j	72bc-q	44.13a-l
126	6d	92.33b-l	9.5a-h	71.67c-r	39.2c-l
127	10.333a-d	94.33a-k	8.167c-j	70.67c-u	41.5a-l
128	8.333a-d	76l-m	8.5b-j	71.33c-s	40.13a-l
129	8.667a-d	89.33b-m	8.167c-j	62x-A	43.53a-l
130	8b-d	89.33b-m	7.1h-j	65p-A	36.67f-l
131	8.333a-d	93b-k	8.267b-j	71.33c-s	45.13a-l
132	7b-d	99.33a-i	9a-j	69f-x	43.2a-l
133	9.333a-d	92b-l	9.667a-h	74a-l	38.2c-l
134	8b-d	87.33d-m	8d-j	68.33g-w	41a-l
135	9.667a-d	93b-k	8d-j	73.33a-n	37.7e-l
136	8.333a-d	95a-k	9a-j	72bc-q	39.9a-l
137	7.667b-d	97a-j	9.667a-h	71.67c-r	46.63a-k
138	9.333a-d	97.33a-j	8.833a-j	71c-t	46.67a-k
139	9a-d	96.33a-k	8.667b-j	68.67g-x	43.2a-l
140	7.667b-d	99.33a-i	9.833a-h	74.33a-k	45.20a-l
141	9a-d	96a-k	8.2c-j	71.67c-r	44.37a-l
142	7.333b-d	91.33b-m	9.067a-j	71c-t	40.3a-l
143	11.333a-d	97a-j	9.067a-j	67.67i-x	43.87a-l

144	10a-d	91.67b-l	8.467b-j	71c-t	42.3a-l
145	8b-d	94a-k	8.667b-j	72.67b-o	41.4a-l
146	9.333a-d	99.33a-i	9.5a-h	75.33a-h	41.13a-l
147	10.333a-d	96a-k	9.233a-i	75.33a-h	39.53b-l
148	9a-d	87.33d-m	8.2c-j	72.67b-o	44.5a-l
149	11.333a-d	96.33a-k	10a-g	75a-i	48.3a-g
150	9a-d	90.67b-m	9.433a-h	73.67a-m	41.9a-l
151	9ab-d	97.67a-j	9.267a-i	69.67d-w	43.4a-l
152	10.667a-d	84i-m	8.967a-j	66n-x	47a –j
153	7.333b-d	95a-k	9.167a-i	62.67w-A	43.87a-l
154	12a-c	75m	6.333j	64.67r-A	46.03a-l
155	7.667b-d	100a-h	10.267a-g	68h-x	42.73a-l
156	9.667a-d	110a	11.5a	75a-i	42.17a-l
157	7.667b-d	95a-k	9.167a-i	68.67g-w	43.17a-l
158	10a-d	97.67a-j	8.667b-j	64.33r-A	43a-l
159	9a-d	76l-m	9.167a-i	69f-x	38.2c-l
160	10.667a-d	85.67g-m	8.333b-j	66.67l-x	45.97a-l
161	9.333a-d	104a-c	10.167a-g	71.67c-r	42.23a-l
162	8.667a-d	93.33b-k	9.5a-h	64s-A	39.23c-l
163	10a-d	89b-m	9.5a-h	75.67a-g	33.4l
164	10.667a-d	103.67a-d	10.310a-f	75.33a-h	36.4f-l
165	9a-d	97.67a-j	9.5a-h	66.67l-x	50.40a-d
166	10a-d	103.33a-e	10.667a-d	73.33a-n	43.57a-l
167	8.333a-d	88c-m	9.167a-i	69f-x	45.37a-l
168	8.667a-d	89.33b-m	9.5a-h	72b-q	41.8a-l
169	9.333a-d	98.33a-j	9.333a-h	70.67c-u	45.37a-l
170	8.667a-d	93.67a-k	8d-j	71.33c-s	43.5a-l
171	8.667a-d	97a-j	9.267a-i	73.33a-n	45.27a-l
172	9.667a-d	90b-m	9a-j	74.67a-i	42.37a-l
173	8.333a-d	87.67c-m	8.6b-j	72b-q	52.5a
174	7.667b-d	94.33a-k	10.833a-c	72.67b-o	38.4c-l
175	8.667a-d	91.96b-l	9.5a-h	72.33b-p	45.4a-l
176	8b-d	89.46b-m	8.133c-j	68h-x	43.03a-l
177	11.667a-d	99a-j	9.167a-i	66.33m-x	44.28a-l
178	8b-d	98.33a-j	9a-j	69.67d-w	46.5a-k
179	10.667a-d	95.67a-k	8.7b-j	75.67a-g	40.83a-l
180	7.333b-d	94.67a-k	8.5b-j	76.33a-f	36.17g-l
181	8b-d	97.33a-j	8.667b-j	76.67a-e	46.4a-k
182	9.667a-d	88c-m	9.667a-h	80.33a	43.77a-l
183	9.667a-d	92.67b-k	9.1a-j	72.67b-o	41.27a-l
184	9.333a-d	99.33a-i	9.233a-i	67.67i-x	44.33a-l

APPENDIX III-Correlation table.

The SAS System 10:55 Sunday, January 20, 2002 1

The CORR Procedure

5 Variables: tillers hgt spike wgt days

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
tillers	549	9.36066	1.90686	5139	5.00000	16.00000
hgt	549	94.16576	7.40831	51697	7.00000	114.00000
spike	549	9.15209	2.37244	5025	5.50000	51.60000
wgt	549	43.64372	5.01154	23960	23.90000	73.00000
days	549	69.15483	6.70954	37966	9.00000	84.00000

Pearson Correlation Coefficients, N = 549
Prob > |r| under H0: Rho=0

	tillers	hgt	spike	wgt	days
tillers	1.00000	0.11060	0.07680	0.02325	0.03485
		0.0095	0.0722	0.5868	0.4151
hgt	0.11060	1.00000	-0.25920	-0.01917	0.16197
	0.0095		<.0001	0.6541	0.0001
spike	0.07680	-0.25920	1.00000	0.25845	-0.40632
	0.0722	<.0001		<.0001	<.0001
wgt	0.02325	-0.01917	0.25845	1.00000	-0.34696
	0.5868	0.6541	<.0001		<.0001
days	0.03485	0.16197	-0.40632	-0.34696	1.00000
	0.4151	0.0001	<.0001	<.0001	

APPENDIX IV: Dellaporta DNA extraction protocol

A. Reagent Preparation

Extraction (Dellaporta) buffer

To make 500mls of working buffer from stocks solutions use

- 100mls of 1M Tris-HCL, (pH 8.0)
- 100mls of 0.5M EDTA, (pH 8.0)
- 100mls of 5M NaCl

Bring the final volume to 500mls and autoclave at 121°C at 15psi/15minutes.

- Add 700µl β-mercaptoethanol just before use while in fume hood into the autoclaved buffer ***(This is because mercaptoethanol degrade with heat).***

1M Tris-HCl (pH 8.0)

- Dissolve 121.14g Tris (hydroxymethyl) aminomethane/Tris base (MW=121.14) in 450ml distilled water (ddH_2O)
- Adjust pH to the desired value by adding concentrated solution of HCl till 8.0
- Adjust volume to 500mls with ddH_2O
- Sterilize by autoclaving and store at room temperature

0.5M EDTA (MW 372.24) pH 8.0

For 250mls

- Dissolve 46.53g into 200ml of ddH_2O + 5 pellets of NaOH and stir to dissolve
- Make pH to 8.0 with NaOH solution which will be close
- Add ddH_2O to 250mls
- Autoclave and store at room temperature

5M NaCl (MW 58.44)

For 250mls

- Dissolve 11.7g of NaCl into 200ml using dH_2O
- Adjust volume to 250ml with dH_2O
- Autoclave at 121°C, 15psi/15 min and store at room temperature

Sodium Dodecyl Sulfate (SDS), 20%

To make 100ml working stock

- Dissolve 20g Sodium Dodecyl Sulfate crystals (SDS) in 90ml sterile dH_2O
- Heat to 68°C to solute the crystals
- Adjust pH to 7.2
- Make volume to 100mls with sterile dH_2O
- Store at room temperature

5M Potassium acetate

To make 250mls of working stock of 5M Potassium acetate

- Dissolve 122.67g Potassium acetate (KOAc, MW=98.14) in 200ml of dH_2O
- Adjust volume to 250 litres with dH_2O and autoclave

Isopropanol (Propan-2-nol, keep at -20°C in the freezer to be chilled before beginning extraction)

70% Ethanol

To make 200ml working solution

- 99.9% Absolute ethanol (alcohol) 140mls
- Distilled water 60mls

Mix thoroughly and label the bottle properly

Gel Electophoresis

50 X TAE Stock solutions is used as running buffer and for preparing gel

For 1 litre stock solution

- 242g Tris Base (121.1g FW)
- 57.1 mls of Glacial acetic acid
- 100mls of 0.5 EDTA (pH 8.0)
- Mix Tris with stirrer to dissolve in 600mls of dH_2O
- Store at room temperature

(final 1x concentration is used for running gel in electrophoresis machine. So dilute 50X using the basic formula of C1V1=C2V2)

B. Procedure

- 700µls of Dellaporta extraction buffer freshly added with β-mercaptoethanol was put in motor containing 0.3g of leaf sample and ground to a fine extract. The fine extract was transferred to clean eppendorf tubes.
- 42µl of 20% SDS was added to the eppendorf tubes containing samples and mixing done by turning the tubes up down. Incubation followed at 65°C for 10 minutes in a water bath. **(Mixing was done for maximum effects every after 5 minutes between 10 minutes).**
- The tubes were removed from the water bath and 160µls of 5M Potassium acetate added, thorough mixing was done and the tubes transferred on ice or in freezer for 10 minutes.
- The tubes were remove from the ice and condensational water wiped so as not to damage the rotor. The samples were centrifuged at 13, 000 rpm for 10 minutes.

- 600µls of upper phase of clear supernatant was withdrawn carefully and transferred into pre-labeled new tubes. This step was repeated once to get extremely clear DNA.
- 600µls of pre-chilled isopropanol was added into each tube with supernatant and mixing done slowly by swirling the tubes while viewing to see if cobwebs-like structures formed. This shows that DNA has precipitated.
- Centrifugation of the precipitated DNA followed for 10 minutes at 13, 000 rpm to pellet the DNA.
- After centrifugation the isopropanol was decanted and white pellets could be observed at the bottom of the tubes. The supernatant was then carefully poured out leaving behind DNA pellet.
- Pellets were washed by adding in 500µls of 70% ethanol, and centrifuged at full speed 13, 000 rpm for 5 minutes.
- Decanting of 70% ethanol is then done and briefly air-drying for 15-20 minutes.
- The DNA was resuspended in 100µls of 1XTE buffer.
- 2µls of RNAse A enzyme was added and incubation in a water bath set at 37°C for 1 hour and storing followed at -20°C.

APPENDIX V SEQUENCES OF PRIMERS

Primers	**Sequence 5′–3′**
RB_SSR1-F1	CACATCCACCCATGGTTGTTGAGAG
RB_ SSR1-R1	CTTCACTGGTACCAGTTCGACCGAG
r4- F22	CGGATATAGGGAGAAGGGTTTGATG
r4- R22	GTCGTCCATCTTGTACACCACGAAC
QK-F6	GGTGGATCGAAGAGAATGGAACTGC
R5-R7.3	GCAACCTTCATTCTGACAGACCATG
A12-F13	CGCCCGACGAAAGAGAACGACAATG
A12-R13	GGGCCACCGACACTGTAGCACTC

APPENDIX VI ANOVA TABLES

Variate: spike_lngth

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
rep stratum	2		10.5024	5.2512	9.66	
rep.*Units* stratum						
trt	183		359.8706	1.9665	3.62	<.001
Residual	365	(1)	198.4723	0.5438		
Total	550	(1)	567.3837			

Standard errors of differences of means

Table	trt
rep.	3
d.f.	365
s.e.d.	0.6021

Stratum standard errors and coefficients of variation

Variate: spike_lngth

Stratum	d.f.	s.e.	cv%
rep	2	0.1689	1.9
rep.*Units*	365	0.7374	8.2

Variate: tillers

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	133.808	66.904	29.40	
rep.*Units* stratum					
trt	183	1026.592	5.610	2.47	<.001
Residual	366	832.859	2.276		
Total	551	1993.259			

Standard errors of differences of means

Table	trt
rep.	3
d.f.	366
s.e.d.	1.2317

Stratum standard errors and coefficients of variation

Variate: tillers

Stratum	d.f.	s.e.	cv%
rep	2	0.6030	6.4
rep.*Units*	366	1.5085	16.1

Variate: wgt

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
rep stratum	2		217.19	108.60	9.59	
rep.*Units* stratum						
trt	183		7299.97	39.89	3.52	<.001
Residual	365	(1)	4133.29	11.32		
Total	550	(1)	11650.18			

Standard errors of differences of means

Table	trt
rep.	3
d.f.	365
s.e.d.	2.748

Stratum standard errors and coefficients of variation

Variate: wgt

Stratum	d.f.	s.e.	cv%
rep	2	0.768	1.8
rep.*Units*	365	3.365	7.7

Variate: days_50%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	601.16	300.58	23.46	
rep.*Units* stratum					
trt	183	8635.97	47.19	3.68	<.001
Residual	366	4688.84	12.81		
Total	551	13925.97			

Stratum standard errors and coefficients of variation

Variate: days_50%

Stratum	d.f.	s.e.	cv%
rep	2	1.278	1.8
rep.*Units*	366	3.579	5.2

Standard errors of differences of means

Table	trt
rep.	3
d.f.	366
s.e.d.	2.922

Variate: hgt

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
rep stratum	2		312.86	156.43	8.31	
rep.*Units* stratum						
trt	183		15380.63	84.05	4.46	<.001
Residual	364	(2)	6854.23	18.83		
Total	549	(2)	22530.05			

Standard errors of differences of means

Table	trt
rep.	3
d.f.	364
s.e.d.	3.543

Stratum standard errors and coefficients of variation

Variate: hgt

Stratum	d.f.	s.e.	cv%
rep	2	0.922	1.0
rep.*Units*	364	4.339	4.6