GENETIC CHARACTERIZATION OF HEAT TOLERANT (HT) UPLAND   MUTANT RICE (*Oryza sativa* L.) LINES SELECTED FROM RICE GENOTYPES

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EXTENDED ABSTRACT

Rice (*Oryza sativa* L.) is the one of the most important cereal crop and staple food of over half the world's population that provides 45-60% of the dietary calories. The global climate changes including increased heat affect negatively rice production and other crops resulting into increased food insecurity. The analysis of Induced gamma rays mutations from upland rice mutant lines was done to discover mutations in heat tolerant genes (HSPs genes). Out of 64 putative heat HT mutant upland rice lines characterized for mutations in HSPs genes, 34 lines discovered to have mutations in that gene by using TILLING (Targeting Induced Local Lesions IN Genomes) technique with SNP Markers; gene specific primers. The results of nucleotides sequenced of mutant rice lines DNA, indicated that most mutations discovered were base pair substitution and InDels 50% and 41% in OS_HSP90_1 and 23% and 35% in OS_HSP17.9 respectively. Mutant rice lines identified to have HSPs genes were evaluated for growth performance, yield and yield components in order to select the promising HT mutant rice lines which can produce economic yield under heat and drought stress conditions. The 8 Mutant rice lines produced economical yield under heat stress condition selected for further breeding as donor materials for heat tolerant. This study aimed to determine the genetic factors associated with heat tolerance in mutant upland rice lines for variety development by Marker Assisted Selection (MAS). Understanding genetic mechanisms for heat stress tolerance in mutant rice lines will help further breeding and selection of suitable rice breeding materials for heat tolerance in order to improve rice productivity hence to reduce food insecurity.

DECLARATION

I Neema Yona, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and has neither been submitted nor being concurrently submitted in any other Institution.

………………………..   ………………………………

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DEDICATION

This work is dedicated to the Family of Yona Yohana Mwiru (My Late Father) and my lovely Mother Elizabeth S. Kipandwa for they laid a strong base to my life, gave me the golden gift of education and molded me to what I am today. May the Almighty God bless them abundantly.

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LIST OF ABBREVIATIONS AND SYMBOLS

CEL I	Celery nuclease
DNA	Deoxyribonucleic acid
dNTP's	Deoxyribonucleotide tri-phosphate
EDTA	Ethylenediaminetetraacetic acetate
EtBr	Ethidium bromide
g	Gram
Gy	Gamma rays
ha	Hectares
ha^{-1}	Per hectare
HCL	Hydrochloric acid
HS	Heat susceptible
HSPs	Heat Shock Proteins
HT	Heat tolerant
IAEA	International Atomic Energy Agency
InDels	Insertions/Deletions
IPCC	Intergovernmental Panel on Climate Change
kDa	Kilo Dalton
KR	Kihogo Red
MAS	Markers Assisted Selection
PARSESNP	Project Aligned Related Sequences and evaluate SNP
PBGL	Plant Breeding and Genetics Laboratory
PCR	Chain Polymerase reaction

rpm	Rotations per minute
SDS	Sodium dodecylsulphate
sHSPs	Small Heat Shock Proteins
SNPs	Single Nucleotide Polymorphisms
TBE	Tris-Borite EDTA
TE	Tris-EDTA
TILLING	Targeting Induced Local Lesions IN Genomes
TOSCI	Tanzania Official Seed Certification Institute
ton	Tonnage
UV	ultra violate
µl	Microliter
^{o}C	Degree Celsius
%	Percentage
bp	Base pair
et al	And others
w/v	Weight by volume

CHAPTER ONE

1.0   INTRODUCTION

Rice (*Oryza sativa* L.) is the one of the most important cereal crops and a staple food of over half the world's population that provides 45-60% of the dietary calories (IRRI, 2006). It is a cash and food crop among the most cultivated crops in the world ranking second in production after maize in Tanzania (MAFC, 2009; Mghase *et al.*, 2010) and worldwide after wheat (Moukoumbi, 2011). Rice is a rapidly growing food source in most of the African Countries as a result of high population growth, urbanization and change in dietary pattern in rural areas (Luzi-Kihupi *et al.,* 2009). The average of rice productivity in Tanzania is estimated to be 1.6 ton/ha which is low compared with 4 ton/ha potential yield (FAO, 2011). The low rice productivity in Tanzania is attributed to various factors such as abiotic (drought, heat stress, soil infertility, salinity) and biotic factors (diseases and pests).

Among the abiotic factors, heat stress is a serious constrain in many rice production regions worldwide (Hall, 2001). Global mean temperature increased by 0.5°C in the 20th Century and is further increasing by 1.5 to 5.8°C in the 21st Century (IPCC, 2007). The yields of the major food crops are expected to decline in many areas in the future due to increased global warming trends and climate change (Lobell *et al*., 2011). The global climate changes affect negatively rice production and other crops. This results in increased food insecurity and low income generation in Tanzania (Jones *et al*., 1999)**.** McSweeney *et al*. (2010) reported that the mean annual temperature in Tanzania is projected to increase by 1.0 to 3°C by the 2060s, and 1.5 to 4.3°C by the 2090s. This increase in temperature has exposed most of the world's

food crops including rice to the heat stress during some stages of their life cycle. Wopereis *et al*, (2008) reported that high temperature above 27-35°C at panicle development, heading and flowering stages cause a high percentage of spikelet sterility. In the tropical and subtropical countries in general, rice crop yields may fall by 10 to 20% by 2050 because of high temperature and drought, but there are places where yield losses may be more severe (CGIAR, 2012).

In order to improve rice productivity, most advanced plant breeding technologies intended for developing improved rice varieties must be employed. Among the strategies, Mutation breeding by Induced mutations is best suited for development of such varieties (Till *et al*., 2007a). Induced mutations by gamma rays mutagenic agent were used to develop the variant mutants of different agronomic traits of interest including heat tolerance from existing local cultivars (Till *et al*., 2006)

Putative HT upland rice lines have been developed from four rice genotypes; Kihogo Red, CG14, WAB 50-56 and WAB 50-104 (Masanche, 2014). They were classified as putative HT upland rice lines that require characterization for their genetic mechanisms that are involving in heat stress tolerance. Understanding genetic mechanisms of mutant rice lines adaptation to heat stress would facilitate the development of heat-tolerant rice varieties for improving rice productivity in warm climatic regions. Although researches have been done in understanding physical mechanism of heat stress tolerance (Shah, 2011), further studies should focus on understanding genetic mechanisms of heat stress tolerance at both vegetative and reproductive stage of rice growth. This study aims to understand the genetic

mechanisms of the heat stress tolerance in Gamma Irradiated mutant upland rice. This will help further breeding and selection of suitable breeding rice lines for heat stress tolerance using Markers Assisted selection (MAS) techniques. Genetic characterization of mutant rice lines for heat tolerant by using TILLING (Targeting Induced Local Lesions IN Genomes is proposed as the best and easiest strategy for rice crop improvement (Warner and Erwin, 2005).

1.1   Objectives

1.1.1   Overall objective

To determine the genetic factors associated with heat tolerance in mutant upland rice lines for variety development by Marker Assisted Selection (MAS).

1.1.2   Specific Objectives

i.   To identify induced and natural nucleotide variation associated with heat shock proteins (HSPs) genes in Gamma rays irradiated mutant upland rice lines.

ii.   To evaluate the agronomic traits of HT mutant rice (*Oryza sativa* L.) lines for growth, yield and yield components under the field conditions.

CHAPTER TWO

2.0   LITERATURE REVIEW

2.1   Heat Stress

2.1.1   Heat stress on rice production

Rice (*Oryza sativa* L.) is widely crop grown throughout the tropical, subtropical and temperate zones of all continents; Temperature, solar radiation and rainfall are important climatic components which affect growth, yield and yield components (Fageria *et al.*, 1997; Wopereis *et al.*, 2008). High temperature is a major environmental stress that limits plant growth, metabolism, and productivity worldwide (Hasanuzzaman, 2013; IPCC, 2007). The optimum temperature for the normal rice growth and development ranges from 27°C to 35°C (Shah *et al*., 2011). High temperatures exceeding optimum level affects almost all growth stages of rice and thus decreases rice grain quality and yield (Nakagawa *et al.,* 2003; Matsui *et al.,* 1997a). Increasing in temperatures 1-2°C than the optimum level during reproductive stage result in shorter grain filling periods and negatively affect yield and other yield components of cereal crops including rice (Zhang *et al.,* 2013).

High temperature during vegetative stage, may damage leaf gas exchange properties of the rice plant including reducing carbon dioxide gas (CO_2) assimilation rates (Shah *et al.,* 2011). High temperatures may affect the whole growth period of rice, especially the reproductive stage; high temperatures during this stage can cause serious damage to the traits related to the rice grain yield. Extreme high temperatures during anthesis, affects anther dehiscence, pollination and pollen germination, leading to spikelet sterility. High temperature during booting stage can lead to

abnormal pollen development and floret deformity, hence resulting in abnormal fertilization (Jiang-lin *et al.,* 2011). Heat stress during the flowering period of heat sensitive rice varieties causes decrease in pollen production or increase pollen sterility, thus, and later on leading to a serious low yield due to low pollen fertilization (Matsui *et al.,* 2001).

Among the major consequences of heat stress in plant cells is the excess accumulation of reactive oxygen species (ROS) which includes hydrogen peroxide, superoxide, singlet oxygen, and hydroxyl radical (Hasanuzzaman *et al.,* 2013). Overproduction of these oxygen species brings about oxidative damage to most important plant cellular components that are lipids, membrane, proteins, pigments, enzymes and nucleic acids (Sade *et al.,* 2011). However, oxidative damage of these cellular components which are found in chloroplast and mitochondria may lead to plant death.

The plant protection system may involve genetic heat resistance mechanism that is associated with synthesis and accumulation of specific proteins is known as heat shock proteins (HSPs) reported by John, 2001. The rapid accumulation of HSPs in the sensitive organs can play an important role in the protection of the metabolic apparatus of the cell, thereby acting as a key factor for plants adaptation to, and survival under heat stress (Wahid *et al.,* 2007). The products of stress-inducible genes (HSPs) can function in both sides during the initial stress response and in establishing plant stress tolerance (Shinozaki and Yamaguchi, 2006). Hasanuzzaman *et al.* (2013) reported that heat stress causes alterations in expression of genes

involved in direct protection of plant cellular organelles from high temperature stress include genes responsible for the expression of osmo-protectants, detoxifying enzymes, transporters, and regulatory proteins. Singh, (2002) reported that heat tolerance in plants refers to the ability of the plant to grow and produce economic yield when subjected to higher temperature condition.

2.1.2   Mechanism of heat stress to induce sterility

The mechanism of high temperature to induce sterility in rice involves decreasing ability of the pollen grains to swell, which results in poor anther dehiscence (Matsui *et al.,* 2000). This swelling of pollen grains is the driving force for anther dehiscence (Matsui *et al.,* 1999a). Sheehy *et al,* (2005) found that the different levels of temperature increments altered the responses of rice genotypes in terms of spikelet fertility. Matsui *et al,* (2001) found that variation of temperatures of 3°C cause rice sterility of about 50% between the most tolerant and susceptible cultivars. Other possible reasons for decreasing spikelet fertility at high temperature are altered hormonal balance in the floret and changes in the activities of starch and sugar biosynthesis enzymes (Shah *et al*., 2011). The greater the temperature increments, the higher the proportions of spikelet sterility in rice crop (Shah *et al*., 2011).

2.2   Genetics of Heat Tolerance in Rice

Genetic analysis of heat stress tolerance will help the plant breeders to produce rice varieties that will adapt to the future climates (Singh, 2002). Heat Shock Proteins (HSPs) are the type of plant protein which is synthesized by HSP genes during high temperature stress. High temperature generally induces the expression of HSPs and,

suppression at least in part, whereby the normal cellular proteins synthesis occurs (Shah *et al.,* 2011). Waters *et al*, (1996) reported that there are five major classes of HSPs due to its structure. These HSPs are found also in different molecular weights and synthesized by eukaryotes including HSP100, HSP90, HSP70, HSP60 and smallHSPs (sHSPs) (~17-30 kDa). High molecular weight HSPs (HSP100, HSP90, HSP70, HSP60) are characterized to have high sequence homology but small HSPs shows great difference in plants among eukaryotes (Efeoğlu, 2009). However, even closely related HSP gene families, show difference in terms of their specific function. The specific role of high molecular weight HSPs like HSP90 in plant is to manage protein folding but also plays a key role in protein degradation, protein trafficking and signal transduction networks (Chen *et al*., 2006). Waters, (1996) reported that sHSPs facilitate reactivation of denatured enzymes in preventing heat-induced aggregation or reverse inactivation of protein substrates. Waters *et al*., (1996) report was revealed that HSPs functioning as molecular Chaperones; molecular chaperones are proteins that bind to partially folded or denatured substrate proteins and thereby prevent irreversible aggregation or promote correct folding of proteins.

Chang *et al.,* 2007 reported that, response of HSPs allows rice plant to become tolerant to high temperature conditions. HSPs are not only induced in response to short-term stress but their production is also essential for heat acclimation (Timperio *et al*., 2008; Shah *et al.,* 2011). Accumulation of HSPs in plant during high temperature leads to increased thermo-tolerance. The rapid accumulation of HSPs in the sensitive organs indicated that can play an important role in protection of the

metabolic apparatus of the cell, thereby acting as a key factor for plants adaptation to, and survival under heat stress condition (Shah *et al.,* 2011).

2.3   Breeding for Heat Tolerant rice

2.3.1   Conventional breeding

Plant breeding is defined as identifying and selecting desirable traits in plants and combining those traits into single plant. It is well known that all traits of the plants are controlled by genes located on chromosomes, hence conventional plant breeding can be considered as the manipulation of the combination of chromosomes (Singh, 2002). Heat tolerance in plants is the ability of the plant(s) to grow and produce economic yield when subjected to higher temperature condition. In rice breeding program, the heat tolerant materials for breeding can be selected from breeding germplasm (Singh, 2002). The process of selection continues until the desired phenotype dominates the population however this can take several years.

Shah *et al*, (2011) reported that length of the anther and size of its basal pore are some of the morphological traits that can be easily identified and can be used in breeding as a screening tool for selection of rice germplasms for high-temperature tolerant. Selection for heat tolerant rice genotypes can be done for those rice breeding materials which tolerate temperatures higher than 32°C (Shah *et al*., 2011). The selected rice genotypes survived under high temperature and retain other desirable traits including high yield and disease resistance. Such materials are either released directly as a new variety or used as genetic donors for breeding. Conventional breeding is time consuming also depending on environmental

conditions. Breeding new variety may take more than eight years compared with breeding using Molecular marker technology like Marker Assisted Selection which takes few months to identify desirable trait of interest (Greenpeace International, 2009).

2.3.2 Molecular Breeding and Marker-Assisted Selection

Molecular plant breeding can be defined as use of genetic techniques performed at DNA molecular level to improve agronomic characters of interest in plants including gene manipulation, molecular marker-assisted selection, and genomic selection (John, 2004). The use of molecular techniques for plant genetic analysis has increased the knowledge and understanding of the plants genomes that led to understand the plant behavior. These molecular techniques, in particular uses of the molecular markers have been used to examine DNA sequence variations among species and, create new sources of genetic diversity by introducing new trait of interest from related species.

2.3.3 TILLING technique for mutation screening

TILLING (Targeting Induced Local Lesions In Genomes) technique is a reverse genetic technique that uses mismatch-specific endonuclease (CEL I) to detect induced DNA polymorphisms in rice (Gilchrist and Haughn, 2005). This technique is a technology developed for detecting unknown point mutations in mutated populations based on CEL I cleavage of mismatches base pairs (Wang *et al*., 2010; Till *et al*., 2006; Colbert *et al.,* 2001). It is also used to detect SNPs and InDels in genes of interest created from a mismatch in a mutated population. However, it examines the effect of various mutations in a genes and their link to a particular

phenotype (Barkley and Wang, 2013). Although originally TILLING was developed for use with Arabidopsis only, but now the technique has been applied to a wide range of plants, including rice (*Oryza sativa* L.) as reported by Till *et al*, (2004a). McCallum *et al.,* (2000) narrated that uses of TILLING for the detection of mutations in rice genomes, the method can be used to screen genes for specific mutations using a PCR assay with specific primers in presence of CEL I (Colbert *et al.,* 2001; Comai and Henikoff, 2006). The technique allows the identification of single-base pair (bp) allelic variation of target gene in a high-throughput manner (Ozhan and Yildiz, 2010). Mutation identified in DNA with single base pair changes and small InDel (<50bp) detected through enzymatic mismatch cleavage using a CEL I (Till *et al*., 2006). The advantage of TILLING technique over other reverse genetic techniques is that, it can be applied to any plant species, regardless of its genome size or ploidy level (Till *et al*., 2006). The technique has been confirmed to be successful to detect DNA polymorphisms (SNPs) including variations in nucleotides number in genomes (Till *et al*., 2006).

2.4   Mutation breeding

2.4.1   Induction Mutation in rice crop

Plant breeding requires genetic variation of useful traits for rice crop improvement (Singh, 2002). Induced mutations by chemical or physical mutagenic agents have been used by many plant breeders worldwide to generate genetic variations and produce some desirable traits of rice varieties (Akbar & Manzoor, 2003; Khin, 2006). These induced mutations can facilitate an association between a genotype and a particular phenotype of the plant (Barkley and Wang, 2013).

2.4.2 Effects of Gamma rays

Gamma rays is an energetic form of electromagnetic radiations, are known to be the most popular mutagens for their simple application, good penetration, reproducibility, high mutation frequency and less disposal problems (Shagufta, 2013). Physical mutagen agents like gamma rays, offered great possibilities for increasing genetic variability of quantitative traits such as heat stress tolerance and other agronomic traits of interest. The understanding of the effects of gamma rays radiation on plants is a broad and complex field. Gamma irradiation was found to influence plant growth and development by inducing biochemical, cytological, genetically, physiological and morphogenetic changes in cells and tissue depending on plant species and the dose of irradiation (Shah, 2011). It is one of the important physical mutational agents used to improve the growth and productivity of many plants. The effects of gamma rays includes changes in the plant cellular structure and metabolism such as dilation of thylakoid membranes, alteration in photosynthesis, accumulation of phenolic compounds and modulation of the anti-oxidative system as reported by (Kim *et al.,* 2004; Wi *et al.*, 2005).

The effect of Gamma rays in plant genomes result in point mutations. Sato *et al,* (2006) reported that half of the point mutations induced by this mutagen agent were deletions, which can cause a frame-shift; affects the function of the gene (Jing and Pauline, 2012) if located in exons. The study of Rashid *et al,* (2011) reported that detection rate of knockout mutations generated by gamma ray radiation is much higher than in a TILLING experiment in which chemical mutagens were applied. Induction Mutation techniques can be used to increase the genetic diversity which enables plant breeders to make the selection of the genotype of the desired traits.

Gamma radiations were used by rice breeders to increase genetic variations in plants, and to render them more productive and resistant/tolerant to various stresses including heat stress. Induction mutations especially gamma rays irradiation in rice crop has become a proven way of creating genetic variations of important agronomic traits for several years (Luzi-Kihupi *et al*., 2009). Mutation breeding techniques can be used to increase rice genetic diversity enabling the breeders to make selection for the genotype of desired agronomic trait of interest like heat stress tolerance. The mutant rice lines were developed using Induced Gamma rays irradiation for the aim of enhancing the agronomic trait (s) of the rice plant including tolerance to heat and drought stress. The TILLING technique with SNP markers was used to detect the Gamma rays induced mutations in heat tolerant genes (HSPs genes) from putative HT mutant rice lines.

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CHAPTER THREE

3.0   Identification of induced and natural mutation associated with heat tolerance in Gamma rays irradiated mutant upland rice lines

3.1   Abstract

TILLING-reverse genetic method has been used for characterizing of putative HT mutant upland rice lines from gamma rays induced mutation and target mutation in genes associated with heat stress tolerance. Out of 64 putative rice lines screened for heat tolerance, 34 mutant rice lines were identified to have mutations in heat tolerant genes (HSPs genes) using TILLING techniques with gene specific primers. Nucleotide Sequencing was done to identify nucleotides variations; single nucleotide polymorphism (SNP) and/or InDel in HT upland rice mutant lines. The Sequence Analysis done using PARSESNP on Gamma Irradiated mutants DNA which were amplified with OS_HSP 90_1 showed that 50% were base substitution, 23.08% were deletion, 19.23% were insertion and 7.69% were inversion while from the DNA samples amplified with OS_HSP17.9 the results indicated that 41.38% were base substitution, 34.48% were deletion and 24.14% were insertion. Gamma radiation dosage of 150Gy and 200Gy were investigated to increase genetic variations in rice lines, and make them more productive and resistant/tolerant to various environmental stresses such as heat stress. Cluster analysis was used to group the individual mutant lines due to their similarities and differences. Individual mutant rice lines were grouped into two main groups due to genetic similarities from both primers; OS_HSP 90_1 and OS_HSP 17.9.

Key words: *Oryza sativa* L.*,* Induction mutation, heat tolerance, gamma irradiation, TILLING, Heat Shock proteins, InDels).

3.2   Introduction

Rice (*Oryza sativa* L.) is one of the most important staple food crop in the world and principle model crop for other monocotyledon species (Lee I *et al.,* 2011). Although rice has been used as a model plant crop for many years, its growth and productivity to the environment stresses is still a problem (Shah *et al.,* 2011). Rice productivity is affected negatively by environmental stresses including heat and drought stress due to climate change effects (Zang *et al.,* 2013). High temperature is a major environmental stress that limits plant growth, metabolism and productivity of plants worldwide (IPCC, 2007). High temperatures exceeding optimum level (27-35°C) affects almost all growth stages of rice and thus decreases rice grain quality and yield (Nakagawa *et al.,* 2003; Matsui *et al.,* 1997a). The plants try to struggle for survival under such stress condition but may tolerate to some extent by physical changes that occurs within the plant body and frequently by creating signals for changing their metabolism as reported by John, (2001). The plant protection system may involve heat resistance mechanism that is associated with synthesis and accumulation of specific proteins known as heat shock proteins (HSPs) reported by John, 2001. Many studies of plant stress tolerance have been performed**.** However, the genetic mechanisms making rice plant cells survive from heat stress are very complicated and are controlled by many genes; hence further information is needed. In this research the TILLING technique was used to discover mutations in heat tolerant genes (HSPs genes) from gamma rays irradiated HT mutant upland rice lines focusing on determining the genetic factors associated with heat tolerance in mutant upland rice lines for variety development by Marker Assisted Selection (MAS).

This technique also is used to detect Single Nucleotide Polymorphisms (SNPs) and Insertions/Deletions (InDels) in genes of interest created from a mismatch in a mutated population (Till *et al*., 2006). It also examines the effect of various mutations in a genes and their link to a particular phenotype (Barkley and Wang, 2013). The technique allows the identification of single-base pair (bp) variation of target gene in a high-throughput manner (Ozhan and Yildiz, 2010). Although originally TILLING was developed for use with Arabidopsis only, now the technique has been applied to a wide range of plants, including rice (Till *et al*., 2007). McCallum *et al.,* (2000) reported its use for detection of mutations in rice genomes, TILLING method can be used to screen genes for specific mutations using a PCR assay then enzymatic digestion of PCR products by CEL I (Till *et al*., 2007). The advantages of using TILLING technique over other reverse genetic techniques is that, it can be applied to any plant species, regardless of its genome size or ploidy level (Till *et al*., 2006). The aim of this work was to identify induced and natural nucleotide variation associated with heat shock proteins (HSPs) genes in Gamma rays irradiated mutant upland rice lines.

3.3   Material and Methods

3.3.1   Plant Materials

The parent rice materials were treated by gamma rays as mutagenic agent at different dosage rate (0Gy, 100Gy, 150Gy, 200Gy and 250Gy). The breeding rice lines; Kihogo Red (KR), CG14 and WAB 56_50 with desired trait of interest (heat stress tolerance) were selected from 150Gy dosage while WAB 56_104 lines were selected from 200Gy dosage. The Mutant upland rice lines were developed from four rice genotypes (parent materials); Kihogo red, CG 14, WAB 56_50 and WAB 56_104.

Rice materials were raised to M_3 generation then exposed at 45°C in higher heat regulated chamber modulated for 6 hours in consecutive 6 days and recovered after 10 days in tropical screen house. The total of sixty four (64) HT mutant upland rice lines including 6 Kihogo Red, 12 WAB 56_104, 35 WAB 56_50 *(Oryza sativa),* 11 CG_14 lines *(Oryza glaberrima)* and 4 parents/controls (heat susceptible) were used in this study. The materials used in this study were obtained from the Rice Mutation Breeding Project for Heat Tolerance Climate Proof Project at the Department of Crop Science and Production of Sokoine University of Agriculture. The list of 64 putative HT Mutant upland rice lines which were developed from four rice genotypes used in this research are indicated in the table 1 whereby gamma rays (Gy) dosage (LD50) for each rice line were shown in Table 1

Table 1: List of 64 Putative HT Mutant upland rice lines used in the study

S/No.	Rice line Name	Gy dosage
1	KR 3_1	150Gy
2	KR 10_1	150Gy
3	KR 27_1	150Gy
4	KR 38_1	150Gy
5	KR 38_5	150Gy
6	KR 38_6	150Gy
7	CG 14_5_2	150Gy
8	CG 14_13_1	150Gy
9	CG 14_16_1	150Gy
10	CG 14_20_1	150Gy
11	CG 14_21_1	150Gy
12	CG 14_54_1	150Gy
13	CG 14_59_1	150Gy
14	CG 14_61_3	150Gy
15	CG 14_62_1	150Gy
16	CG 14_63_1	150Gy
17	CG 14_63_2	150Gy
18	CG 14_64_1	150Gy
19	WAB 56_104_18_1	200Gy
20	WAB 56_104_36_1	200Gy
21	WAB 56_104_43_1	200Gy
22	WAB 56_104_76_1	200Gy
23	WAB 56_104_76_2	200Gy
24	WAB 56_104_141_1	200Gy
25	WAB 56_104_141_2	200Gy
26	WAB 56_104_141_3	200Gy
27	WAB 56_104_141_4	200Gy
28	WAB 56_104_150_1	200Gy
29	WAB 56_104_150_2	200Gy
30	WAB 56_50_ 48_1	150Gy
31	WAB 56_50_48_2	150Gy
32	WAB 56_50_48_3	150Gy
33	WAB 56_50_51_1	150Gy
34	WAB 56_50_56_1	150Gy
35	WAB 56_50_56_2	150Gy
36	WAB 56_50_56_3	150Gy
37	WAB 56_50_74_1	150Gy
38	WAB 56_50_82_1	150Gy
39	WAB 56_50_85_2	150Gy
40	WAB 56_50_85_3	150Gy
41	WAB 56_50_97_2	150Gy
42	WAB 56_50_97_3	150Gy
43	WAB 56_50_97_4	150Gy
44	WAB 56_50_98_1	150Gy
45	WAB 56_50_98_3	150Gy
46	WAB 56_50_123_1	150Gy
47	WAB 56_50_123_2	150Gy
48	WAB 56_50_123_3	150Gy
49	WAB 56_50_127_1	150Gy
50	WAB 56_50_127_3	150Gy
51	WAB 56_50_127_4	150Gy
52	WAB 56_50_127_5	150Gy
53	WAB 56_50_127_6	150Gy
54	WAB 56_50_127_7	150Gy
55	WAB 56_50_127_8	150Gy
56	WAB 56_50_127_9	150Gy
57	WAB 56_50_135_1	150Gy
58	WAB 56_50_135_2	150Gy
59	WAB 56_50_141_1	150Gy
60	WAB 56_50_141_2	150Gy
61	WAB 56_50_141_3	150Gy
62	WAB 56_50_152_1	150Gy
63	WAB 56_50_152_2	150Gy
64	WAB 56_50_152_3	150Gy

3.3.2 Seedlings establishment

Ten (10) seeds were sown in petri dish with moist blotter paper inside from each HT mutant upland rice line to enhance seed germination. Petri dishes with seeds were kept inside the box; dark condition to facilitate germination. Water was applied to ensure germination. Healthy rice seedlings were transferred after 14 days to the hydroponic condition. Hydroponic rice solution used was prepared according to Yoshida *et al.* (1976) with adaptation made by PBGL (2013). The rice solution for hydroponics comprised of five macro elements and one composition of eight micro elements (the list of five macro elements and one composition of 8 micro elements are indicated in table 2). The amount of fertilizers (by chemical name) for making five litres for stock solution was prepared as shown in Table 2.

Table 2: **Components of stock solution for rice hydroponic solution**

Stock No.	Fertilizer Chemical formula	Fertilizer Element	Amount (g or ml) for 5 litres
1	NH_4NO_3	N	457
2	NaH_2POH_2O	P	201.5
3	K_2SO_4	K	357
4	$CaCl_2$	Ca	443
5	$MgSO_47H_2O$	Mg	1 620
6A	$MnC_{12}4H_2O$	Mn	7.5
B	$(NH_4)6Mo7O_24H_2O$	Mo	0.37
C	H_3BO_3	Bo	4.67
D	$ZnSO_47H_2O$	Zn	0.175
E	$CuSO_45H_2O$	Cu	0.155
F	$FeCl_36H_2O$	Fe	38.5
G	$C_6H_8O7H_2O$	C	59.5
H	$1M\ H_2SO_4$	S	250ml

The working hydroponic solution was prepared as described by Yoshida *et al.,* 1976 with modifications made by PBGL, (2013). The amount and concentration of

Hydroponics working solution to make 120 litre drum was prepared (Table 3). The pH of working solution was adjusted to 5.0 by using 1N Sodium hydroxide (NaOH) and 1N Hydrochloric acid (HCL) with continuous stirring by water pump to ensure the solution is homogenized. Hydroponics solution distributed to 20L recovery tank (planting box with 40×30×17cm outside measurement) which was covered with platforms with holes on top (2.2 cm diameter). Four healthy seedlings from each mutant rice line were fixed in the hole by mattress wool and then level of hydroponic solution was controlled by adding fresh solution in case evaporation took place.

Table 3: **Stock solutions; main elements and amounts required to make the working hydroponic solution**

S/No.	Macro Element	Amount of stock solutions needed to make 1tank(20L)	Amount of stock solutions needed to make1 drum (120L)	Conc. Of elements in working solution (mg/l)
1	N	25	150	40
2	P	25	150	10
3	K	25	150	40
4	Ca	25	150	40
5	Mg	25	150	40

Source; (PBGL, 2013)

Key; N; Nitrogen, P; Phosphorus, K; Potassium Ca; Calcium, Mg; Magnesium

3.3.3 DNA extraction

Genomic DNA was extracted from young leaves of HT mutant rice seedlings by using Low Cost-DNA extraction protocol (PBGL, 2013). About 2 leaves were collected from mutant rice seedlings grown in hydroponic in Tropical Glasshouse at PBGL Seibersdorf, Vienna. The leaf samples were placed between thin tissue paper then kept into the tray containing silica gel orange for drying (for maximum of 3

days). After 3 days leaves were already dried and silica gel orange turned to white colour. The dried leaves samples were removed from silica gel tray and placed into 1.5ml eppendorf tubes with 3 metal tungsten beads. Then 1.5 ml eppendorf tubes with dried leaves and 3 metal tungsten beads inside were closed and taped on the vortex machine and vortex for 30 minutes at high speed until leave samples became fine powder. 800 μl of lysis buffer and 4 μl RNAse were added to each tube then mixed well by vortex at high speed for 2 minutes. Sample mixture was incubated for 10 minutes at room temperature, followed by addition of 200 μl (3M) Sodium Acetate and mixing done by inverting tubes and then incubated on ice for 5 minutes. After 5 minutes incubated samples were held for centrifugation at 13 200 rpm for 5 minutes at room temperature. The supernatant/liquid phase transferred into the tubes containing 700 μl silica binding solution (SBS). Silica powder was completely suspended by vortex and inversion of tubes for 20 sec. followed by incubation which was done at room temperature for 15 min and then centrifugation done at 13 200 rpm for 3 min. Then liquid (supernatant) transferred into appropriate labeled SBS containing tubes followed by completely suspend the silica powder by vortexing and inversion of tubes (20 sec). The mixture incubated for 15 min at room temperature (on a shaker at 400 rpm and/or invert tubes from time to time) followed centrifugation in 13 200 rpm for 3 minutes (to pellet the silica). The supernatant removed with a pipette and discarded in this step DNA bounding to the silica). 500 μl of freshly prepared wash buffer (70% Ethanol) were added to each tube to suspend the silica powder by vortexing and inverting tubes (approx. for 20 sec) followed with centrifugation at 13 200 rpm for 3 minutes to pellet the silica. Supernatant removed and the pellet kept. This step repeated twice to insure DNA pellet bound to silica.

Pellet centrifuged for 30 seconds and any residual ethanol removed with pipette and then pellets dried by keeping the open eppendorf tubes in fume hood for 30 minutes. 200 μL TE buffer (10 mM Tris HCl, 0.1 Mm EDTA) added to each tube to elute the DNA while silica powder was completely suspended by vortex and inversion of tubes (approx. 20 sec). Then incubation done at room temperature for 5 minutes followed with centrifugation at 13 200 rpm for 5 minutes to pellet the silica. The supernatant containing DNA collected and placed into 1.5 ml new eppendorf tubes labeled accordingly. Finally DNA was stored temporarily at 4°C for further processes.

3.3.4 Genomic DNA Quantification

The DNA quantification done on 1% (w/v) Agarose gel; 3g agarose was dissolved into 300 ml of 1×TBE Buffer (0.04m Tris-Borite, 0.001m EDTA, and pH 8.0). The mixture was heated on microwave at 750°C for 3 minutes in order to dissolve the mixture completely, mixture allowed to cool on magnetic plate to 50°C then 6 μl EtBr (gel staining dye) was added, after that gel was poured into prepared gel casting try which fitted with specific comb to create wells. Of the extracted DNA, 5μl sample, 1 μl l6x gel loading dye (Bromophenol Blue) and seven DNA standards Ladder with different concentrations were loaded in 1% (w/v) agarose gel. Gel image for amplified bands were visualized using UV trans-illuminator.

3.3.5 Gene specific primers (HSPs gene) design and Validation

Total of six Heat shock proteins primers (Table 4) were designed to detect single nucleotide polymorphism (SNP) and InDels in HSPs genes of HT mutant upland rice

lines. Gene specific primers based on SNP were designed to screen the putative heat tolerant mutant rice lines. It were designed using Primer3 program (Steve and Helen, 2000) and NCBI database (http://www.ncbi.nlm.nih.gov) and, then sequences were subjected to the BLAST computer software (http://www.ncbi.nlm.nih.gov/BLAST). The primers (HSPs primers) were designed to amplify about 1 500 bp or less of targeted HSPs genomic DNA fragment. Primers were validated and tested using pooled mutant DNA samples and, were showing positive results; amplified the rice Mutant DNA whereby from each primer, two DNA samples and one negative control (water) were loaded. The list of six OS_HSPs primers and its sequences were used to amplify the Genomic DNA from putative HT mutant rice lines were indicated in the Table 4.

Table 4: List of 6 gene specific primers (HSP genes) for heat stress tolerance

S/No	PRIMER NAME	FORWARD PRIMER	REVERSE PRIMER	TM (°C)	Gene bank Seq. Ref.
1	Os_hsp17.9	GGCCCCATTTTACTATGGCCCACAT	TTAGGTTTTCGGCAACGAGCATTGG	52	AY034057.1
2	Os_sp17	CAGCTTCATCAGGCAGCTCAACACC	CACGAACTATGAATCCGCGCAAATG	56	AB050097.1
3	Os_hsp70	TGCAGCCTGTGATGGCAAAAGTACC	GGACAGCAGCACCATAAGCAACAGC	52	X67711.2
4	Os_hsp90_1	TGGCGACACATCACTACAGCTCGTC	ATGAAGCCAGGGCAGAAGGACATC	56	AC091774.7
5	Os_hsp90_2	ACTGGCCCTTTTTCTCCTCCATTGC	GCATGAATTGCTGCCTTTGGTTGAA	52	AC091774.7
6	Os_hsp90_3	CAGCTGGCACTTCCACATCAACCTC	TTTTGGGCGAAGGTGACACTGCTAA	48	AC091774.7

3.3.6 PCR Analysis

The screening for mutations by using PCR based on TILLING procedures began with amplification of target DNA fragments (Till, 2006) with pooled DNA (2 folds; mutants and parent DNA) using gene specific primers (Colbert *et al.,* 2001) designed for heat tolerant genes. The genomic DNA pooled prior to PCR reaction (Henikoff & Comai, 2003). The PCR conducted in a reaction volume of 20μl containing 5.0 μl of 0.1 ng/µl DNA template and 15 µl of PCR mix. PCR mix per single reaction were mixture of 8.0 µl PCR water, 10 µl of 10xEx Taq Buffer, 8 µl of 2.5 mM dNTPs mix, 1 µl of each 10 μM primer (forward and reverse) and 0.25 µl TaKaRa HS Taq (*Taq* DNA polymerase). The pooled DNA were arranged into 96 well Microtiter plates then incubated to the PCR machine for the process of melting and annealing (PBGL, 2013).

The PCR thermocycler program consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of amplification with DNA templates denaturation at 94°C for 30 minutes, primers annealing done at 55°C for 1 minute and primer extension at 72°C for 2 minutes followed by a final elongation at 72°C for 10 min. and a 4 °C hold until electrophoresis and visualization on gels (Colbert *et al.,* 2001). The amplified product were separated by electrophoresis on agarose 1.5% (w/v) gel and stained with Ethidium bromide and analyzed under UV light.

3.3.7 Enzymatic mismatch cleavage-CEL I digestion

Crude Celery Juice Extract (CJE) with CEL I nuclease was used to digest mismatched base pairs or heteroduplexes after PCR amplification, which represent

induced single nucleotide polymorphisms (SNPs). Enzyme master mix was prepared with the following composition (for single reaction); 16.25 µl PCR water, 3.0 µl 10xCEL I Buffer and 0.75 µl CEL I nuclease. The mixture of 10µL of PCR product from each sample and 20µl of enzyme master mix mixed together vortexes and then mixture was incubated at 45°C for 15 minutes in Thermal cycler (EXOSAP program). After PCR, the plate was placed on ice and enzymes reaction was stopped by adding 5µl of 0.25M EDTA per sample then samples kept into -20°C for further procedures (PBGL, 2013).

3.3.8   PCR products (DNA) purification for Sequencing

HT mutant rice DNA samples that showed polymorphic bands after electrophoresis were indicating mutation regions on target gene were eventually used for sequencing and confirmation of mutations. The selected DNA samples for sequencing were purified by using enzymatic digestion of free (single stranded) primers with nuclease 1 and removal phosphates from dNTPs with Shrimp Alkaline Phosphatase. Procedure for DNA purification done by adding 10ul of PCR product, 5µl of Exonuclease I and 1 µl of Shrimp Alkaline Phosphatase (Fermentase) into a new PCR tube, then mixture mixed by pipetting up and down several times. The mixture was incubated to thermo cycler at 37°C for 15 minutes (PBGL, 2013).

3.3.9   DNA Sequencing, Alignment and BLAST

DNA sequencing was done on mutant rice DNA samples scored with polymorphism bands and good quality DNA, whereby DNA sequences variations determined from mutant rice genomes after alignment. The PARSESNP computer programme (Taylor

and Greene, 2003) was used for sequencing mutant rice DNA samples which expressed polymorphic bands. The DNA sequences for alignment and Cluster analysis were trimmed by using BioEdit software and then aligned by using MEGA6 Computer software (Tamura *et al.,* 2013). DNA sequences were used for Cluster analysis to identify different groups of HT mutant rice lines caused by InDels and single nucleotide polymorphisms in HT mutant rice.

The nucleotide sequences of targeted DNA fragments (HSPs gene) were subjected to the BLAST (Basic Local Alignment Search Tool) Computer software (www.ncbi.blast) to determine genes associated with heat tolerance in rice lines. Phylogenetic trees were constructed by Unweighted Pair-Group Method of Arithmetic mean (UPGMA) (Michener and Sokal, 1957) using MEGA6 software to create groups for the HT mutant rice lines with similar nucleotide sequences in gene of interest due to SNPs and InDels caused by gamma rays Induced mutation. Nucleotide sequences used were from mutant rice DNA samples were amplified with OS_HSP90_1 and OS_HSP17.9 respectively and, were indicated to have DNA band polymorphisms.

3.4   Data collection and Analysis

The DNA bands visualized after electrophoresis were scored from the PCR products with polymorphic bands that indicating CEL I nuclease cut the mismatched base pairs in the targeted genes. The DNA band polymorphisms were expected that were the SNPs or InDels due to Gamma rays-Induced mutation in gene of interest were selected for sequencing. DNA sequence analyzed using PARSESNP computer

programme (Taylor and Greene, 2003) and alignment done with reference gene sequence and its parent DNA sequences data scored. The phylogenetic tree for mutant rice sequences were constructed to show their similarities and differences.

3.5 RESULTS

3.5.1 Genomic DNA quantification

The Genomic DNA used for amplification had concentration which ranked between 15-40 ng/μl (Plate 1). The quality of Genomic DNA was determined using 1% (w/v) agarose gel whereby bands length taken in comparison with seven different concentrations of DNA Standards Ladder (S1-S7) as indicated in plate 1.The final DNA concentrations for PCR amplification from each sample were standardized to 0.1ng/μl.
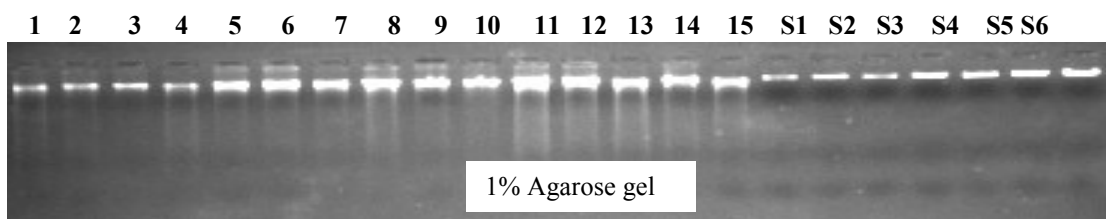
Plate 1: Agarose gel 1% (w/v) image for 15 HT mutant Genomic DNA samples

3.5.2 Primer Validation

Six (6) HSPs gene specific primers were designed for heat tolerant have been tested and amplified rice DNA samples with approximately 900bps as indicated with an arrow in Plate 2. After validation, DNA separated in 1% (w/v) agarose gel (Plate 2) after electrophoresis and then DNA bands were observed under UV light machine
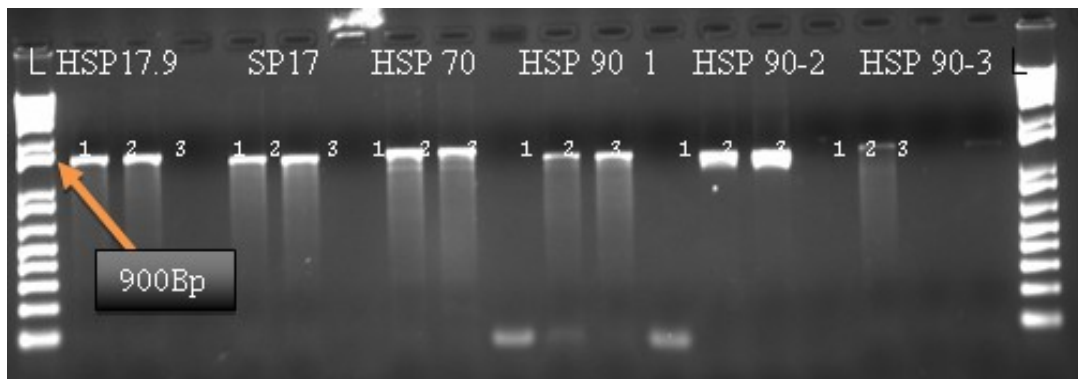
Plate 2: **Agarose 1% (w/v) gel image showing six heat shock protein (HSPs) primers tested in three samples (Lane 1&2 DNA samples and Lane 3; water)**

3.5.3 DNA Amplification and Mutation discovery in HSP genes

Mutation discovery in mutant rice lines by using TILLING technique was started with amplification of targeted HSPs DNA fragment of approximately 900bps using gene-specific primers. The designed 6 gene-specific primers (Table 2) were used to amplify the DNA fragments for heat stress tolerant gene (HSPs) in putative HT mutant upland rice lines. Out of 6 primers, 2 primers including OS_HSP90_1 and OS_HSP17.9 were amplified mutant DNA samples and showed band polymorphisms (Plate 3, 4 & Table 5). The primers were amplified genomic HSPs fragments and showed DNA band polymorphisms were preferred over others.

Out of 68 DNA samples amplified, 20 DNA samples from both Primers were selected for sequencing (Plate 3 & 4) also their 4 control (non-irradiated rice genotypes) were included. Polymorphic bands indicated mutant candidates with mutations in HSP genes. The Individual mutant rice lines showed band polymorphisms in its targeted DNA fragments were considered as the mutants with

point mutations in HSPs gene in their genomes. Samples with band polymorphisms are pointed with orange arrows in agarose gel images as indicated in Plate 3 & 4, also data for polymorphic bands scored are summarized in Table 5.
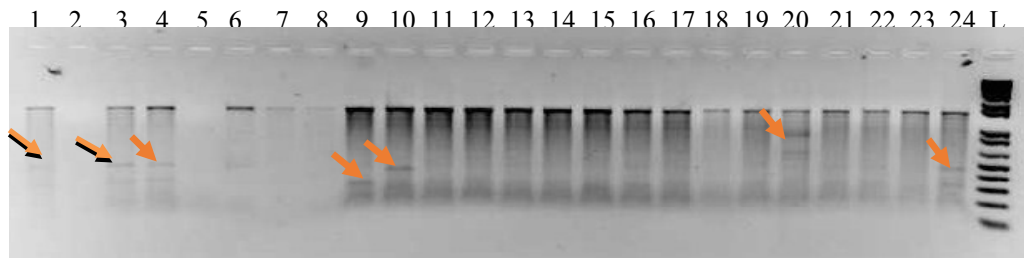
(a)
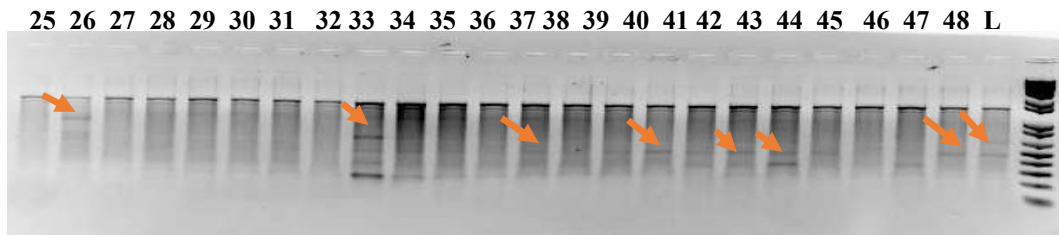
(b)
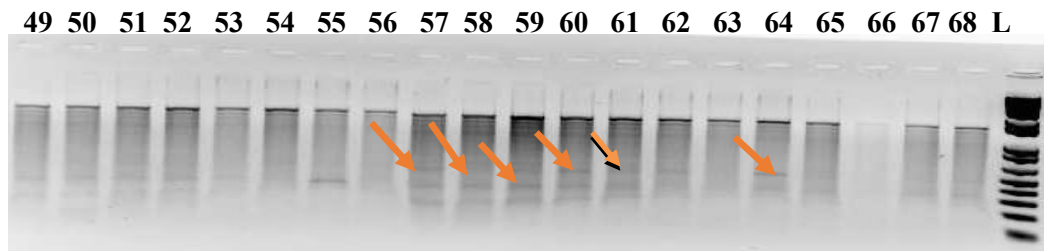
(c)

Plate 3(a, b & c): Agarose gel 1.5% (w/v) image for 68 PCR products after enzyme digestion from rice DNA samples amplified with Primer OS_HSP90_1
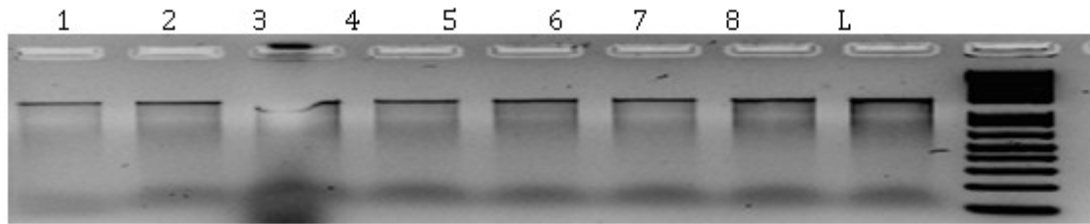
(a)
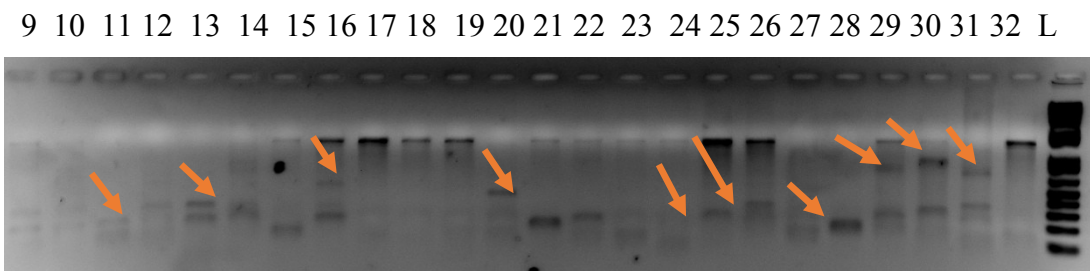
(b)
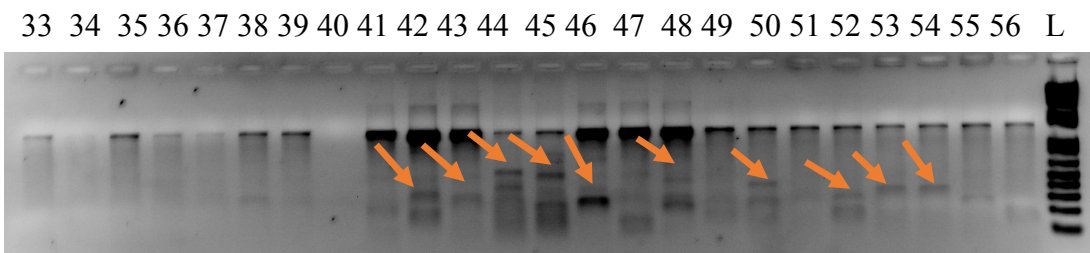
(c)
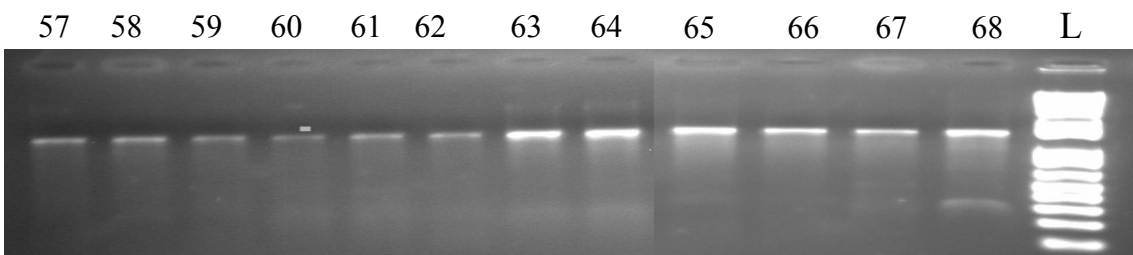
(d)

Plate 4(a, b, c & d): **Agarose gel 1.5% image for 68 PCR products after Enzyme digestion for DNA samples amplified with Primer OS_HSP17.**9.

Table 5: **Summary of mutant rice lines with band polymorphisms scored after DNA amplification with OS_HSP90_1 and OS_HSP17.9 primers**

Sample ID	Rice line name	OS_HSP90_1	OS_HSP17.9
1	KR 3_1	+	+
2	KR 10_1	+	+
3	KR 27_1	++	+
4	KR 38_1	++	+
5	KR 38_5	+	+
6	KR 38_6	+	+
7	CG 14_5_2	+	+
8	CG 14_13_1	+	+
9	CG 14_16_1	++	+
10	CG 14_20_1	++	+
11	CG 14_21_1	+	+
12	CG 14_54_1	+	+
13	CG 54_59_1	+	++
14	CG 14_61_3	++	+
15	CG14_62_1	+	+
16	CG 14_63_1	+	++
17	CG 14_63_2	+	++
18	CG 14_64_1	+	+
19	WAB 56_104_18_1	+	+
20	WAB 56_104_36_1	++	+
21	WAB 56_104_43_1	+	+
22	WAB 56_104_76_1	+	+
23	WAB 56_104_76_2	+	+
24	WAB 56_104_141_1	++	+
25	WAB 56_104_141_2	+	++
26	WAB 56_104_141_3	++	++
27	WAB 56_104_141_4	+	+
28	WAB 56_104_150_1	+	+
29	WAB 56_104_150_2	+	++
30	WAB 56_50_48_1	+	+
31	WAB 56_50_48_2	+	+
32	WAB 56_50_48_3	+	+
33	WAB 56_50_51_1	++	+
34	WAB 56_50_56_1	++	+
35	WAB 56_50_56_2	+	++
36	WAB 56_50_56_3	+	++
37	WAB 56_50_74_1	++	+
38	WAB 56_50_82_1	+	++
39	WAB 56_50_85_2	+	++
40	WAB 56_50_85_3	++	+
41	WAB 56_50_97_2	++	++
42	WAB 56_50_97_3	++	++
43	WAB 56_50_97_4	++	++
44	WAB 56_50_98_1	+	++
45	WAB 56_50_98_3	+	++
46	WAB 56_50_123_1	+	++
47	WAB 56_50_123_2	+	++
48	WAB 56_50_123_3	++	++
49	WAB 56_50_127_1	+	+
50	WAB 56_50_127_3	+	++
51	WAB 56_50_127_4	+	+
52	WAB 56_50_127_5	+	++
53	WAB 56_50_127_6	+	+
54	WAB 56_50_127_7	+	+
55	WAB 56_50_127_8	+	+
56	WAB 56_50_127_9	+	+
57	WAB 56_50_135_1	++	+
58	WAB 56_50_135_2	+	+
59	WAB 56_50_141_1	++	+
60	WAB 56_50_141_2	++	+
61	WAB 56_50_141_3	+	+
62	WAB 56_50_152_1	+	+
63	WAB 56_50_152_2	+	+
64	WAB 56_50_152_3	++	+

Key: ++ Samples with polymorphic bands and + samples with (monomorphic) single band

3.5.4 Purified PCR products for sequencing

DNA samples amplified with gene specific primers; OS_HSP90_1 and OS_HSP17.9 respectively showed polymorphic bands after enzyme cut and electrophoresis (Plate 3 & 4), its PCR products were purified with enzymatic digection of free primer and, thereafter DNA fragements were separated in agarose gel 1.5% (w/v). The PCR products with good quality and high DNA concentration (≥40ng/µl) were determined in 1.5% agarose gel (Plate 5 and 6) by comparing its bands with Low DNA Mass[TM] Ladder (4µl/ng) bands, also Quibit 2.0 Fluorometer was used to check the quantitiy of PCR products from purified mutant DNA samples. The DNA samples from mutant rice lines and its parent with good quality sequencing were presented on Plate 5 and 6.
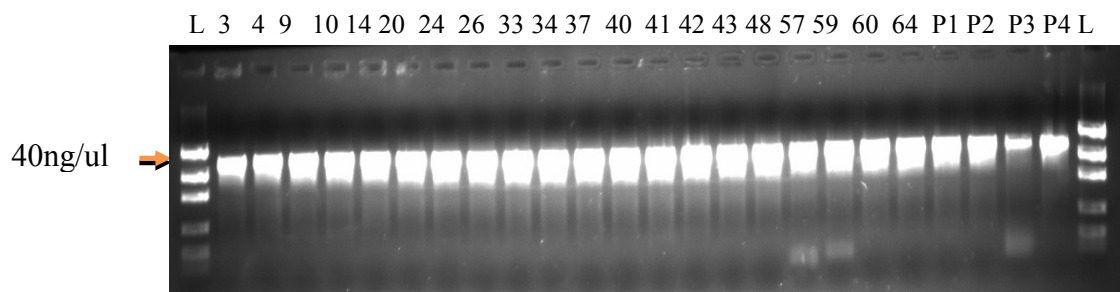
Plate 5: **Agarose gel image for 20 PCR products of mutant rice amplified with OS_HSP90_1 and showed polymorphic bands were sequenced**
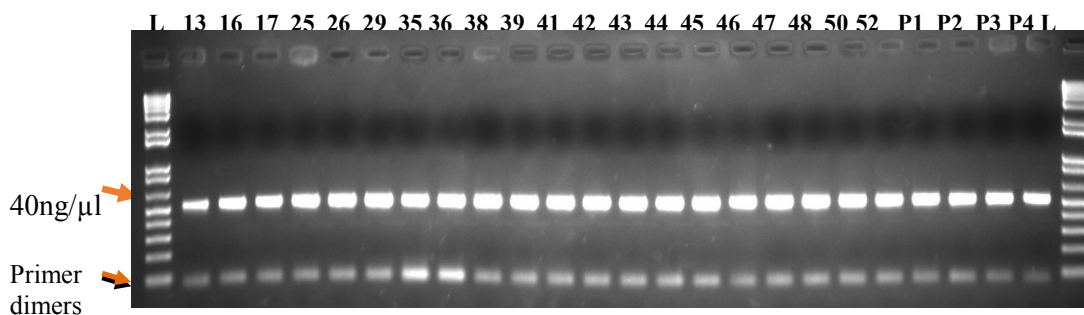
Plate 6: Agarose gel image for 20 PCR products of mutant rice amplified with OS_HSP 17.9 and showed polymorphic bands were sequenced

3.5.5 PCR products (DNA) sequencing and Alignments

Nucleotides were sequenced from purified and good quality (≥40ng/µl) PCR products of HT mutant rice lines which have been identified to have mutations in genes of interest (HSPs genes) through amplifications with gene specific primers. Sequencing were done from mutant rice lines DNA sequence with gene sequences respectively in both directions (forward and reverse) to minimize the false SNP due to sequencing artifacts (Garg *et al.,* 2012). The DNA sequencing was done to determine nucleotides variations in mutant rice genomes occurred due to Induced Gamma mutation and may be due to natural mutation (Table 6, appendix 1 & 2). The target SNPs were selected following a SNP discovery effort that provides information about the frequency of polymorphism in mutant's genomes. The mutants and control sequence reads were aligned to the respective reference gene sequence; sequences of genes which were used to amplify the targeted DNA fragment (Appendix 1 & 2). The nucleotides variations were detected by nucleotide alignment procedures with reference gene sequence and hence the nucleotide matrix (Appendix 1 & 2) was obtained for both primers. Number of nucleotides changed in gene of interest from both directions were indicated in Table 6. The results revealed that most of SNPs detected per targeted DNA fragment in each rice line were ranged between 0-6 bp and 2-19 bps in DNA samples amplified with OS_HSP90_1 and OS_HSP17.9 primer respectively (Appendix 1 & 2). The highest number of nucleotide variations (SNP and InDel) was observed in samples amplified with OS_HSP17.9 primer, therefore OS_HSP17.9 primer confirmed to be more polymorphic marker for heat tolerant compared to OS_HSP90_1.

Table 6: Number of nucleotides changed in mutant rice detected following the sequencing and alignment with reference gene sequences

S/No.	Rice Line name	Forward (F)/ Reverse (R)	OS_HSP90_1 Number of nucleotides changed	OS_HSP17.9 Number of nucleotides changed
1	CG 14 Control	F	1	18
2	CG 14 Control	R	4	13
3	CG 14_16_1	F	3	6
4	CG 14_16_1	R	2	16
5	CG 14_20_1	F	6	19
6	CG 14_20_1	R	2	16
7	CG 14_61_3	F	4	19
8	CG 14_61_3	R	6	13
9	KR Control	F	1	9
10	KR Control	R	1	13
11	KR 27_1	F	3	19
12	KR 27_1	R	3	8
13	KR 38_1	F	2	19
14	KR 38_1	R	3	16
15	WAB 56_104 Control	F	4	19
16	WAB 56_104 Control	R	4	13
17	WAB 56_104_141_1	F	4	19
18	WAB 56_104_141_1	R	3	8
19	WAB 56_104_141_3	F	3	19
20	WAB 56_104_141_3	R	3	13
21	WAB 56_104_36_1	F	2	14
22	WAB 56_104_36_1	R	2	13
23	WAB 56_50_123_2	F	3	19
24	WAB 56_50_123_2	R	1	19
25	WAB 56_50_127_9	F	4	11
26	WAB 56_50_127_9	R	2	6
27	WAB 56_50_135_2	F	1	18
28	WAB 56_50_135_2	R	2	16
29	WAB 56_50_141_1	F	0	19
30	WAB 56_50_152_2	F	0	18
31	WAB 56_50_152_2	R	2	8
32	WAB 56_50_51_1	F	6	13
33	WAB 56_50_51_1	R	3	13
34	WAB 56_50_56_1	F	2	19
35	WAB 56_50_56_1	R	3	18
36	WAB 56_50_74_1	F	2	18
37	WAB 56_50_85_3	F	1	15
38	WAB 56_50_85_3	R	3	5
39	WAB 56_50_97_2	F	2	18
40	WAB 56_50_97_2	R	4	19
41	WAB 56_50_97_3	F	4	18
42	WAB 56_50_97_3	R	3	19
43	WAB 56_50_97_4	F	3	13
44	WAB 56_50_97_4	R	5	18
45	WAB 56_50 Control	F	0	13
46	WAB 56_50 Control	R	3	6

3.5.6 Point mutations discovery from Gamma Irradiated rice lines

3.5.6.1 Mutant DNA sequence aligned with reference gene sequence

Mutations discovery after Sequences analysis which was done by PARSESNP computer software (Taylor and Greene, 2003) and aligned with reference gene sequences, pointed that four types of point mutations occurred in the mutant rice genomes. These point mutations were base pair substitutions, insertions, deletions and inversion (Table 7, 8, Appendix 1 and 2). Sequence Analysis of DNA from Gamma Irradiated rice lines which was amplified with OS_HSP 90_1 and aligned with reference gene sequence showed that 50% were base substitution, 23.08% deletion and, 19.23% insertion (42.31% InDel) and inversion were 7.69% (Table 7). From the DNA samples amplified with OS_HSP 17.9, results indicating that 41.38% were base substitution, 34.48% deletion and 24.14% insertion (Table 7). Among four type of mutation discovered, the highest mutation proportion of 59% were InDels in samples amplified with OS_HSP17.9 and (50%) were base substitution in samples amplified with OS_HSP 90_1. Generally highest point mutations (SNP and InDel) were from samples amplified by OS_HSP 90_1. The summary of four types of point mutations and its proposition (percentage) discovered as a results of induced mutation by gamma irradiation were indicated in Table 7 below.

Table 7: **Summary of types, number and proportion of point mutations discovered after aligning all mutant rice DNA sequences with reference gene sequence**

DNAs amplified with OS_HSP 90_1			DNAs amplified with OS_HSP 17. 9	
Type of point mutation	Number of mutants	Proportion (%)	Number of mutants	Proportion (%)
Substitution	13	50%	12	41%
Deletion	6	23%	10	35%
Insertion	5	19%	7	24%
Inversion	2	8%	0	0%
Total	**26**	**100%**	**29**	**100**

3.5.6.2 Mutant DNA sequence aligned with parent's sequence

The alignment of the DNA sequence from mutant rice lines DNA amplified OS_HSP90 and OS_HSP 17.9 respectively were also done with the sequences of their parents respectively; for stance DNA sequences of mutant CG 14 lines were aligned with sequence of CG 14 non-irradiated/parent. The results revealed that nucleotide substitution point mutation were observed in high proportion than deletion and inversion point mutation discovered in both genes. From the investigation done on mutant DNA amplified with OS_HSP90 and OS_HSP17.9 respectively and sequence were aligned with their parents respectively in each mutant rice line were indicated that base pair substitution occurred in high proportion among rice lines (Table 8).

The DNA from 4 mutant rice lines genotypes amplified with OS_HSP17.9, sequenced and aligned with respectively parent sequences, the results showed that the highest point mutation proportional discovered was substitution followed by insertion and deletion from all lines (Table 8). Nucleotides substitution is a type

of point mutation in which a single nucleotide is exchanged with a different nucleotide that may result in alteration of sequence of amino acid during translation and may render the newly synthesized protein ineffective.

Table 8 (a&b): Summary of types of point mutations discovered from all gamma irradiated rice DNA amplified with OS_HSP90_1 and OS_HSP17. 9 respectively

(a)

Types of point mutation	CG 14 LINES		WAB 56_104 LINES		WAB 56_50 LINES		KR LINES	
	No. of variants	Proportion (%)	No. of variants	Proportion (%)	No. of variants	Proportion (%)	No. of variants	Proportion (%)
Substitution	9	90	2	100	4	50	5	62.5
Insertion	0	0	0	0	3	37.5	1	12.5
Deletion	1	10	0	0	1	12.5	2	25
Total	10	100	2	100	8	100	8	100

(b)

Types of point mutation	CG 14 LINES		WAB 56_104 LINES		WAB 56_50 LINES		KR LINES	
	No. of variants	Proportion (%)	No. of variants	Proportion (%)	No. of variants	Proportion (%)	No. of variants	Proportion (%)
Substitution	10	83.33	11	73.33	12	57.14	0	0.00
Insertion	2	16.67	3	20.00	7	33.33	0	0.00
Deletion	0	0	1	6.67	2	9.52	0	0.00
Total	12	100	15	100	21	100.00	0	0.00

3.5.7   Cluster Analysis

3.5.7.1 Cluster Analysis for samples amplified by OS_HSP90_1

A phylogenetic tree was constructed to examine similarities and differences among heat tolerant mutant rice lines. The clustering based on DNA sequences of HT mutant rice lines amplified with OS_HSP90_1. The results revealed that two main groups of mutant rice lines were formed (Fig. 2). The first group (Cluster I) comprised of 18 rice and the second group (Cluster II) comprised of two mutant rice lines (WAB 56_50_141_1 and WAB 56_50_97_3). From main group one, four subgroups were formed; first subgroup comprised of 15 mutant rice lines and, second, third and fourth subgroups comprised of single HT mutant rice lines; WAB 56_50_127_9, CG14 _16_1 and WAB 56_50_51_1 respectively, more details were indicated in Figure 1 & 2. Induced mutation caused base pair substitution, insertions and deletions in HSPs gene of HT mutant rice lines (Fig.1); these effects of mutation in gene of interest created genetic differences among mutant rice lines as shown in figure 2. WAB 56_50_141_1 and WAB 56_50_97_3 were grouped together because they showed similar base pair substitution and some insertions in gene of interest (Fig. 1 and 2). KR 27_1 and WAB 56_50_127_9 indicated the deletion of nucleotides (**CC**) in the same position in gene of interest (Fig. 1).

*Indicates the same nucleotides sequences in OS_HSP90_1 gene in some positions, A, G, T and C are nucleotides

Figure 1: **Base pair substitution, deletion and insertions among 20 HT mutant rice lines DNA amplified with OS_HSP90_1**

Figure 2: A dendogram diagram showing relatedness of 20 mutant rice lines DNA amplified with OS_HSP90_1

3.5.7.2 Cluster Analysis for samples amplified with OS_HSP 17.9

The genetic similarities and differences from HT mutant rice lines which were selected following expression of band polymorphisms were discovered after alignment of HT mutant rice DNA sequences. DNA sequences of HT mutant upland rice lines showed some base pair substitutions, deletions and insertions which resulted DNA sequences to differ from one another (Fig. 3). WAB 56_50_104_150_2 had a nucleotide substitution of G/A and some deletions (Fig. 3) and was grouped alone (Fig. 4). Only WAB 56_50_85_2 had insertion of T nucleotide and was grouped differently from members of group one (Fig. 3 & 4). Individual mutant rice lines with similar DNA sequences were grouped into the same group (Fig. 3 & 4). The clusters based on Mutant rice DNA sequences of samples that were amplified with OS_HSP17.9 showed that mutant rice lines were clustered into two main groups; group I comprised of 19 mutant rice lines and group II comprised of one mutant rice line (Fig. 4). It was found that group I had two sub-groups; first subgroup included 18 mutant rice lines and second sub group had 1 (WAB 56_50_85_2) HT mutant rice line and, group II had no sub-groups (Fig. 4). Genetic similarities among mutant rice lines ranged from 0.0 to 0.6 coefficients (Fig. 4).

Key; A, G, T and C are nucleotides in fragment of OS_HSP17.9 gene sequence

Figure 3: Base pair substitution, deletion and insertions among 20 HT mutant rice lines in DNA amplified with OS_HSP17.9
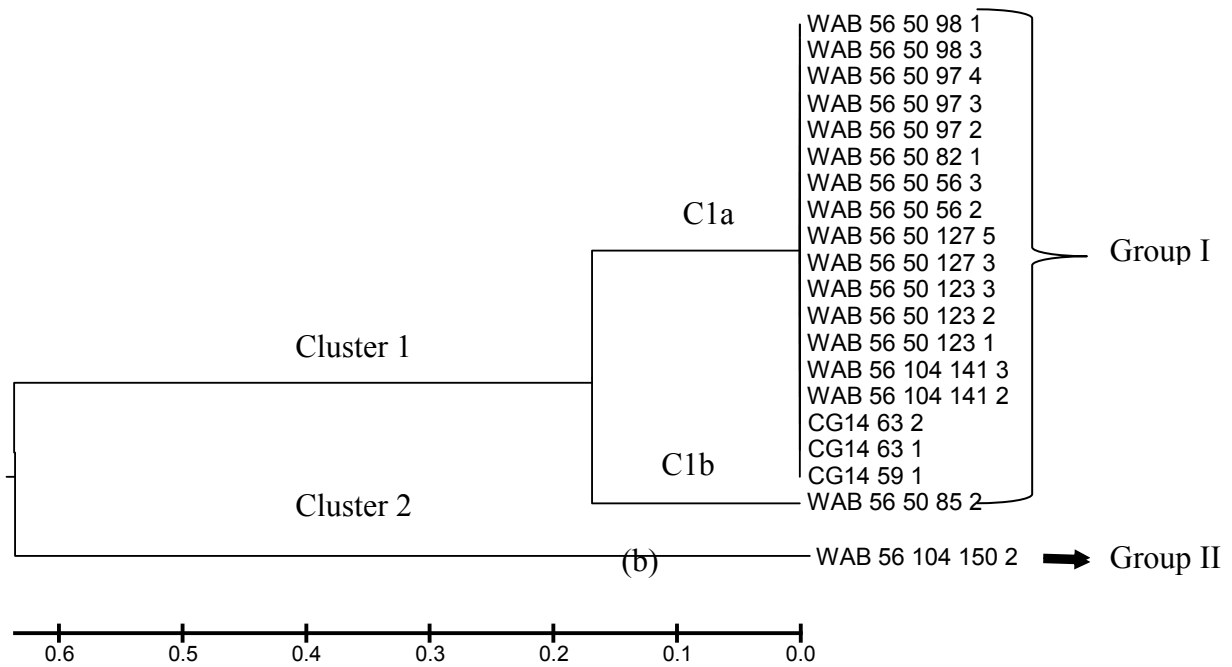
Figure 4: **Phylogenetic tree showing relatedness of 20 mutant rice lines, DNA were amplified with OS_HSP17.9**

3.5.8 Estimates of Evolutionary divergence among HT mutant rice lines

The genetic distance similarities among HT mutant rice lines were estimated by using MEGA6 program whereby pair wise distance (Tamura *et al.,* 2013) of DNA sequences from DNA samples amplified with OS_HSP90_1 and OS_HSP17.9 respectively (Table 9a & 9b) were estimated. The evolutionary difference estimate between sequences of HT mutant rice lines was done to quantify the effect of deletion, insertion and base pair substitution due to induction mutation changes among HT mutant rice lines. The different nucleotide variation rate was estimated among the sequences of HT mutant rice lines; however there were no equal distribution of mutations in HSPs genes (OS_HSP90_1 and OS_HSP17.9) in mutant rice genomes. The HT mutant rice lines with zero (0) genetic distance coefficient seems to have the same genetic make-up. The minimum and maximum genetic distance among mutant rice samples amplified with OS_HSP90_1 and OS_HSP17.9 were 0.0 and 2.53, and 0.0 and 1.34 respectively (Table 9a & b). In samples amplified with OS_HSP90_1 and OS_HSP17.9, WAB_56_50_141_1 and WAB_56_104_150_2 respectively were observed to be have high genetic distance compared to others

Table 9 (a & b): Estimates of Evolutionary Divergence between Sequences from HT mutant rice DNA samples amplified with OS_HSP90_1 and OS_HSP17.9

(a)

S/N	Rice lines	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	CG14_20_1																				
2	CG14_61_3	0.03																			
3	CG14_16_1	0.03	0.00																		
4	KR_27_1	0.03	0.00	0.00																	
5	KR_38_1	0.03	0.00	0.00	0.00																
6	WAB_56_104_141_1	0.03	0.00	0.00	0.00	0.00															
7	WAB_56_104_141_3	0.03	0.00	0.00	0.00	0.00	0.00														
8	WAB_56_104_36_1	0.03	0.00	0.00	0.00	0.00	0.00	0.00													
9	WAB_56_104_123_2	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00												
10	WAB_56_50_127_9	0.04	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01											
11	WAB_56_50_135_2	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01										
12	WAB_56_50_141_1	2.53	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.44	2.30									
13	WAB_56_50_152_1	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	2.30								
14	WAB_56_50_51_1	0.06	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	2.44	0.04							
15	WAB_56_50_56_1	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	2.30	0.00	0.04						
16	WAB_56_50_74_1	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	2.30	0.00	0.04	0.00					
17	WAB_56_50_85_3	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	2.30	0.00	0.04	0.00	0.00				
18	WAB_56_50_97_2	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	2.30	0.00	0.04	0.00	0.00	0.00			
19	WAB_56_50_97_3	1.73	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.64	1.58	1.64	0.68	1.64	1.66	1.64	1.64	1.64	1.64		
20	WAB_56_50_97_4	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	2.30	0.00	0.04	0.00	0.00	0.00	0.00	1.64	

(b)

Sample ID	Rice lines	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	CG14_59_1																				
2	CG14_63_1	0.00																			
3	CG14_63_2	0.00	0.00																		
4	WAB_56_104_141_2	0.00	0.00	0.00																	
5	WAB_56_104_141_3	0.00	0.00	0.00	0.00																
6	WAB_56_104_150_2	1.27	1.27	1.27	1.27	1.27															
7	WAB_56_50_123_1	0.00	0.00	0.00	0.00	0.00	1.27														
8	WAB_56_50_123_2	0.00	0.00	0.00	0.00	0.00	1.27	0.00													
9	WAB_56_50_123_3	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00												
10	WAB_56_50_127_3	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00											
11	WAB_56_50_127_5	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00										
12	WAB_56_50_56_2	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00									
13	WAB_56_50_56_3	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00								
14	WAB_56_50_82_1	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00							
15	WAB_56_50_85_2	0.05	0.05	0.05	0.05	0.05	1.34	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05						
16	WAB_56_50_97_2	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05					
17	WAB_56_50_97_3	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00				
18	WAB_56_50_97_4	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00			
19	WAB_56_50_98_1	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00		
20	WAB_56_50_98_3	0.00	0.00	0.00	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	

3.6   DISCUSSION

3.6.1   Heat shock proteins (HSPs) gene

Heat Shock Proteins (HSPs) are the type of plant proteins which are synthesized by HSPs genes during high temperature stress and help the plant to survive under heat stress condition. Waters *et al,* (1996) reported that HSPs gene protect plant cells from the detrimental effects of high temperature, hence accumulation of HSPs leads to increase in thermo-tolerance. The mechanisms of HSPs genes to protect the plant from heat stress is due to its ability of protein folding, assembly, translocation and degradation in many cellular processes also stabilizing proteins and membranes, and assist in protein refolding under stressed conditions including high temperatures (Garg *et al.,* 2012).

The DNA samples amplified with HSPs primers including OS_HSP17.9 and OS_HSP90_1 and, expressed band polymorphisms indicated the mutant rice lines with HSPs genes in their genomes (Table 5) which influence thermo tolerance in mutant rice lines. The individuals identified to have DNA band polymorphisms with comparison to its parents after electrophoresis were indicating differences in DNA fragments after CEL I cleave the mismatched base pairs as consequence of Induced-gamma Irradiation and natural mutation in HSPs gene. The cleavage of mismatched base pairs resulted in polymorphic bands in mutant rice DNA which indicated that SNPs and/or InDel was caused by Gamma rays mutations (Till *et al.,*2006) in upland rice lines genomes (Plate 3 &4). SNPs are the most abundant DNA polymorphisms in genome which may play vital roles in induction of phenotypic variations in plants. However, the positions of SNPs or InDels on plant genome sequence may affect the

gene expression and function especially if nonsense mutation occurred in exon regions or frame-shift mutations. Nonsense mutation is type of mutation whereby premature stop codons were introduced into the part of the gene that encodes a protein (Hirakawa *et al.*, 2013). Frame-shift mutation is a kind of mutation caused by insertion or deletion of nucleotides; thus causing changing in the reading frame of DNA sequence (Jing and Pauline, 2012). Frame-shift result in nucleotides sequence reading to change, consequently is causing a much larger effect on protein structure as well as gene function.

3.6.2 Nucleotide sequence and alignment

Sequencing analysis showed that most of mutations which occurred in mutant rice genomes was due to induced mutations by gamma rays and was point mutations. The results showed that the highest frequency of point mutation observed was base pair substitution compared to other point mutations (deletion, insertions and inversions) discovered from both primers as demonstrated in Table 7 & 8. The detected number of nucleotides changed in mutant rice DNA amplified with OS_HSP17.9 were high compared to the nucleotides changed from the DNA samples amplified with OS_HSP90_1. This may confirm that OS_HSP17.9 was more polymorphic marker for heat stress tolerance compared to the OS_HSP90_1 (Table 1). The nucleotides variations observed in mutant rice lines were due to induced and natural mutations because it is difficult to differentiate these two types of mutations. The occurrence of the nucleotides changes in the same position in different genomes from the different mutant rice lines were expected may be due to natural mutations (Appendix 1 & 2) which may have no significant effects in physiological processes of the plant. In

some HT mutant rice lines including WAB 56_50_141_1, WAB 56_50_152_2 and WAB 56_50 control, were observed to have no nucleotides variations (0 bp) occurred, this means that there was no effect of induced or natural mutations in amplified DNA fragments compared with reference gene sequence. The nucleotide sequence differences were observed among mutant rice lines after comparing with reference genes sequence (Table 6, Appendix 1 & 2). The nucleotide variations were detected after alignment of HT mutant rice line sequence done with reference genes; OS_HSP90_1 & OS_HSP17. 9 respectively (Table 9) and parents sequences (Table 8). The nucleotide sequencing and alignment confirmed the effect of Gamma rays mutations and or natural mutations in HSP genes from mutant rice genomes.

3.6.3   Mutation discovery from Mutant rice lines

Induced mutations can cause changes in nucleotide arrangements in plant genome; can have no effect in plant genomes but it may alter the product of a gene, or can prevent the gene to function properly or completely. Gamma rays irradiation caused point mutations; change in one nucleotide or a few nucleotides in rice genomes. Most findings reported that Gamma ray Irradiation causes nucleotide polymorphisms and InDel (Till *et al.,* 2006). Induced mutations caused by gamma rays, chemical mutagenesis or any mutagenesis agent have been examined to alter base pair arrangements of plant DNA (Till *et al*., 2006).

The effects of Gamma rays mutation were discovered in mutant rice lines using TILLING procedures with SNP Markers and then followed by nucleotide sequencing. The Gamma rays induced mutations in DNA from mutant rice were

detected after incubation of amplified DNA fragments (PCR products) with CEL I nuclease (Till *at al.,* 2006) for digesting the mismatched base pairs in genes of interest. The bands more than one in the same lane which visualized in agarose 1.5% gel after electrophoresis were representing cleavage products at the site of mutations in gene of interest (PBGL, 2013) these were marked by arrows in Plate 3 & 4. The induced mutations cause changes in base pair variation in plant genome as result of base pair insertion, deletion, substitution, inversion or Single Nucleotide Polymorphism (SNP) thus inducing gene functions to change (Comai and Henikoff, 2006). Change in gene functions has direct useful effect to the plant physiology and morphology. The mutation analysis and sequencing of DNA from Putative HT mutant rice lines showed that, nucleotides variations were a result of induced and natural mutations demonstrated by altered nucleotides arrangements after nucleotides alignments done with reference HSP gene sequences; sequences of genes which were used in amplification of respective DNA. It were expected that the mutant rice candidates with nucleotides variations in HSPs genes were due to mutations which impact increase tolerance to heat stress and will sustain and produce economic yield under high temperature condition.

3.6.4 Genetic diversity of HT Mutant rice lines

Molecular characterization of mutant rice lines by using sequencing approach and alignment was used to identify similarities and differences in genetic information as expressed in nucleic acids (DNA) among the HT mutant rice lines. The phylogenetic trees were created for HT mutant rice DNA sequences of samples were amplified with OS_HSP17.9 and OS_HSP90_1 respectively. The HT mutant rice lines were

displayed in two cluster ranging from 0.0 to 0.6 and 0.0 to 0.8 coefficient of similarity from samples amplified with OS_HSP17.9 and OS_HSP90_1 respectively (Fig. 1 & 2). HT mutant rice lines were grouped into two main groups and few subgroups formed in both primers as shown in Figure 1 & 2. The groups formed of the HT mutant rice lines confirmed that mutants with similar DNA sequences were grouped together.

It was expected that mutant rice lines would have the homologous DNA sequences in amplified genes of interest (HSP genes), but it was found to have different DNA sequences. These variations observed in DNA sequences were due to deletions, insertion and SNP of nucleotides as results of Induced mutations by gamma rays. Induced mutation creates genetic variation (Novak and Brunner, 1992) thus different groups of HT rice mutant lines were formed due to variations in DNA sequences in genes of interest (HSPs genes).

Deletion or insertion of nucleotide (s) in a DNA sequence affects the encoding of amino acids which coding for the proteins (Jing and Pauline, 2012). Deletion of base pair led to a frame-shift mutation causing all of the codons occurring after the deletion to be read incorrectly during translation, and hence results in non-functional protein (Jing and Pauline, 2012). The SNP, insertion and deletion of amino acid in DNA sequences led to the loss of chaperone activities in mutant rice genome (Garg et al., 2012) and hence influencing the mutant rice lines to be heat tolerant and more productive.

The evidence from genetic distance among HT mutant rice lines sequences from DNA samples amplified with OS_HSP17.9 suggested that all rice mutants lines were closely related in DNA sequences except few Mutants including WAB 56_50_104_150_2 and WAB 56_50_85_2 which were different in 1.27 and 1.34 coefficient distance respectively. These two mutants differed greatly from others due to base pair substitutions and insertion occurred in gene of interest due to induction mutation by gamma rays. WAB 56_50_141_1 and WAB 56_50_97_3 showed to have highest genetic distance of 2.53 and 1.73 coefficients respectively among HT mutant rice lines in DNA samples amplified with OS_HSP90_1.

The genetic distance coefficients of the mutant upland rice lines could range from 0 to 1, however some mutant lines including WAB 56_50_141_1, WAB 56_50_97_3 and WAB 56_50_104_150_2 showed greater genetic distance coefficients than 1, this was because pairs were overlapped in more dimensions (Bayardo *et al.,* 2007). The results from both primers indicated that mutant with many similar DNA sequences in genes of interest have small genetic distances; thus showing that they are closely related and were not affected by induced mutation and vice versa is true for those large genetic distances (Nei, 1972).

3.7   Conclusion

Induced mutation is a powerful tool for crop improvement and it has been applied to develop new mutant rice lines with heat stress tolerant and other agronomic traits of interest. It is a simple and fast plant breeding technique of creating genetic variants among species or variety of the rice crop. Gamma rays irradiations were used to

increase genetic variations in rice, and to cause them to be more productive and resistant/tolerant to various stresses including heat stress. HT mutant rice lines with point mutations in heat shock proteins genes (heat - stress tolerant genes) were identified. The 4 types of point mutations were discovered from HT mutant upland rice lines studied. The changes in DNA sequences of HT mutant rice lines due to Induction mutation seems to influence mutant rice lines to be more tolerant in heat stress and productivity compared non irradiated rice materials studied.

The point mutations induced by gamma rays were observed to be associated with HSPs genes on the mutant rice lines and were responsible for the heat stress tolerance. TILLING technique with SNP markers should be the best and easy tool to discover point mutations caused by Gamma rays irradiation in genes of interest (HSPs genes) from mutated populations. This technique also allowed the identification of single base pair (bp)/allelic variation of target gene from Induced mutation populations. InDel and base pair substitutions on HT mutant rice genome affected amino acids reading as well as gene expression and function.

The nucleotide variations in DNA sequences of HT mutant rice lines as results of Induced and natural mutations were confirmed by DNA sequencing and alignment of HSPs genes fragments. The DNA sequence analysis approach allowed estimating the effects of mutations with very small effects in genes of interest. The genetic distances results revealed that, mutant rice lines with many similar DNA sequences in genes of interest have small genetic distances. The nucleotides variations observed

in HT mutant rice lines against non-irradiated rice genotype were expected to change the gene functions of agronomic traits of the interest.

 The current study recommends TILLING technique with SNP markers (gene specific primers) to be used to discover Induced and natural mutations in gene of interest. Mutation breeding is a type of breeding method of inducing genetic diversity thus can be used as a means of introducing genetic variations in an elite germplasm without the need to acquire variation from exotic cultivars, consequently, avoiding the introduction of undesirable agronomic traits. The DNA sequences from HT mutant rice lines were used in creating phylogenetic tree to group the mutant rice individuals due to their similarities and differences.

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Appendix 1: Nucleotides Alignment Matrix for HT mutant rice and control DNA amplified by Primer OS_HSP_90_1

Bp. position Covered	299	299.1	345	781	789	812	836	850	856	912	953	953.1	954	955	960.1	1,279	1,284	1,286	1,287	1,296	1,296.1	1,296.2	1,319	1,335	1,342	1,393	1,401	Nucleotide changed rice line⁻¹
Ref. gene sequence	T	:	T	A	A	G	G	A	A	C	G	:	A	G	:	C	C	A	A	A	:	:	A	G	G	T	T	
CG 14 control		T		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1
CG 14 control	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T	T				A	A				X	X	4
CG 14_16_1		T	G			A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	3
CG 14_16_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				:					T	X	X	2
CG 14_20_1		T	G			A	A	:	G	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	6
CG 14_20_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				:					T	X	X	2
CG 14_61_3		T	G			A	A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	4
CG 14_61_3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T	C			A	A			T	:	X	6
KR Control		T		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1
KR Control	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X					A					X	X	1
KR 27_1	:					A				T	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	3
KR 27_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T					A	A				X	X	3
KR 38_1		T				A		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2
KR 38_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T	T				A						X	3
WAB 56_104						A				T		A	:		X	X	X	X	X	X	X	X	X	X	X	X	X	4
WAB 56_104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				A	A	C	C		X	X	4
WAB 56_104_141_1						A				T	:		A				X	X	X	X	X	X	X	X	X	X	X	4
WAB 56_104_141_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T					A	A				X	X	3
WAB 56_104_141_3						A				T		A			X	X	X	X	X	X	X	X	X	X	X	X	X	3
WAB 56_104_141_3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T				A	A				X	X	3
WAB 56_104_36_1						A				T	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	# Variants
WAB 56_104_36_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X					A	A				X	X	2
WAB 56_50_123_2		T		N		A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	3
WAB 56_50_123_2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X					A					X	X	1
WAB 56_50_127_9						A				T	:	A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	4
WAB 56_50_127_9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X					A	A				X	X	2
WAB 56_50_135_2		T				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1
WAB 56_50_135_2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T					A					X	X	2
WAB 56_50_141_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0
WAB 56_50_152_2		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0
WAB 56_50_152_2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X					A	A					X	2
WAB 56_50_51_1						A				T	:	A		A	G	X	X	X	X	X	X	X	X	X	X	X	X	6
WAB 56_50_51_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T					A	A				X	X	3
WAB 56_50_56_1						A				T	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2
WAB 56_50_56_1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T					A	A				X	X	3
WAB 56_50_74_1						A				T		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2
WAB 56_50_85_3						A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1
WAB 56_50_85_3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T					A	A					X	3
WAB 56_50_97_2						A				T	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2
WAB 56_50_97_2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T	T				A	A				X	X	4
WAB 56_50_97_3						A				T	:	A		X	X	X	X	X	X	X	X	X	X	X	X	X	X	4
WAB 56_50_97_3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T					A	A				X	X	3
WAB 56_50_97_4		T			C	A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	3
WAB 56_50_97_4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T	T				A	A					G	5
WAB 56_56 control						X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0
WAB 56_56 control	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	T					A	A					X	3
# Variants	**1**	**9**	**3**	**1**	**1**	**18**	**2**	**1**	**1**	**11**	**4**	**6**	**1**	**1**	**1**	**8**	**9**	**1**	**1**	**2**	**20**	**16**	**1**	**1**	**3**	**1**	**1**	

Key; X representing nucleotides which are the same with nucleotides from reference gene sequence, : (colons) is insertion/Deletion, letters including A, G, T and C show nucleotides varied from reference gene due to induced and natural mutation

Appendix 2: Nucleotides Alignment Matrix for HT mutant rice lines and its parent DNA amplified by Primer OS_HSP17. 9 (Hypothetical gene) and analysed by PARSESNP

Bp Position coverage	133	136.1	136.2	136.3	137.1	138	144	145	146	149	151	172	184.1	245	394	402	427	565	566	640	642	643	644	645	646.1	647	648	649	651	652	655	657.1
Ref. gene sequence	T	:	:	:	:	G	G	T	G	A	G	A	:	G	C	G	T	G	G	A	C	A	G	T	:	T	C	C	T	G	C	:
CG 14 control	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	:	A	A	A	C	A	X
CG 14 control	X	X	X	X	X	X	:	:	A	:	:	C	T	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
CG 14_63_1	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
CG 14_63_1	X	C	C	A	T	:	:	:	A	:	:	C	T	A	T	A	:	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CG 14_63_2	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	:	:	A	A	C	A	A
CG 14_63_2	X	C	C	A	T	:	:	:	A	:	:	C	T	A	T	A	:	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CG 54_59_1	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	A	A	A	A	C	A	A
CG 54_59_1	X	X	X	X	X	X	:	:	A	:	:	C	T	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
KR Control	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	X	X	X	X	X	X	X	X	X	X
KR Control	X	X	X	X	X	X	:	:	A	:	:	C	T	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
WAB 56_104 co	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	:	:	A	A	C	A	G
WAB 56_104	X	X	X	X	X	X	X	X	X	X		C	T	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
WAB56_104_141_2	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	:	:	A	A	C	A	A
WAB56_104_141_2	X	C	C	A	T	:	:	:	A	:	:	C	T	A	T	A	:	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
WAB56_104_141_3	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	:	:	A	A	C	A	A
WAB56_104_141_3	X	X	X	X	X	X	:	:	A	:	:	C	T	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
WAB 56_50 control	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	A	A	A	A	C	A	A
WAB 56_50 control	X	X	X	X	X	X	X	X	X	X	X	C	T	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
WAB 56_50_123_1	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	:	:	A	A	C	A	A
WAB 56_50_123_1	X	X	X	X	X	X	:	:	A	:	:	C	T	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
WAB 56_50_123_3	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	:	:	X	X	X	X	X
WAB 56_50_123_3	X	X	X	X	X	X	:	:	A	:	:	C	T	A	T	A	:	:	:	X	X	X	X	X	X	X	X	X	X	X	X	X
WAB 56_50_127_3	X	X	X	X	X	X	X	X	X	X	X	X	X	A	T	A	:	:	:	G	T	G	C	C	A	:	:	A	A	C	A	C

```
WAB 56_50_127_3   G  C  C  A  T  :  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_127_5   X  X  X  X  X  X  X  X  X  X  X  X  A  T  A  :  :  :  G  T  G  C  C  X  X  X  X  X  X  X  X
WAB 56_50_127_5   X  X  X  X  X  X  X  X  X  X     C  T  A  T  A  :  X  X  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_56_2    X  X  X  X  X  X  X  X  X  X  X  X  X  X  T  A  :  :  :  G  T  G  C  C  A  :  :  A  A  C  A  G
WAB 56_50_56_2    X  C  C  A  T  :  :  :  A  :  :  C  T  A  T  A  :  X  X  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_56_3    X  X  X  X  X  X  X  X  X  X  X  X  A  T  A  :  :  :  G  T  G  C  C  A  :  :  A  A  C  A  A
WAB 56_50_56_3    X  C  C  A  T  T  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_82_1    X  X  X  X  X  X  X  X  X  X  X  X  A  T  A  :  :  :  G  T  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_82_1    X  X  X  X  X  X  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_85_2    X  X  X  X  X  X  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_97_2    X  X  X  X  X  X  X  X  X  X  X  X  A  T  A  :  :  :  G  T  G  C  C  A  :  :  A  A  C  A  A
WAB 56_50_97_2    X  C  C  A  T  :  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_97_3    X  X  X  X  X  X  X  X  X  X  X  X  A  T  A  :  :  :  G  T  G  C  C  A  :  :  A  A  C  A  X
WAB 56_50_97_3    X  X  X  X  T  T  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_97_4    X  X  X  X  X  X  X  X  X  X  X  X  X  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_97_4    X  C  C  A  T  :  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_98_1    X  X  X  X  X  X  X  X  X  X  X  X  A  T  A  :  :  :  G  T  G  C  C  A  :  :  A  A  C  A  A
WAB 56_50_98_1    X  C  C  A  T  :  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
WAB 56_50_98_3    X  X  X  X  X  X  X  X  X  X  X  X  A  T  A  :  :  :  G  T  G  C  C  A  :  A  A  A  C  A  A
WAB 56_50_98_3    X  X  X  X  X  X  :  :  A  :  :  C  T  A  T  A  :  :  :  X  X  X  X  X  X  X  X  X  X  X  X
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Key; X representing nucleotides which are the same with nucleotides from reference gene sequence, : (colons) is insertion/Deletion, letters including A, G, T and C show nucleotides varied from reference gene due to induced and natural mutation

CHAPTER FOUR

4.0 **Agronomic evaluation of HT mutant upland rice (*Oryza sativa* L) lines for growth performance, yield and yield components under field conditions**

4.1 **Abstract**

Field experiment was conducted in 2014/2015 during hot and dry season at the crop Museum of the Department of Crop Science and Production, Sokoine University of Agriculture Morogoro, located at Latitude 6° 50' 55" S and longitude 37° 39' 22" E at 300m above sea level (masl). The study aimed to evaluate the growth parameters, yield and yield components of Gamma irradiated mutant upland rice lines developed from Kihogo red, WAB 56-50, WAB 56-104 and CG-14 rice genotypes. Mutant rice lines from these genotypes were identified and selected based on polymorphic expression of heat shock protein genes (HSPs) for heat tolerance. The minimum and maximum temperature and rainfall during conducting experiment were $20^{o}C$ and $35^{o}C$, 32.7 mm and 155.5 mm respectively. Data collected and analyzed for evaluation were days to early and 50% flowering, days to physical maturity, plant height, number of tillers, number of panicles, spikelet, filled grains, unfilled grains and 1000 grains weight. Significant differences ($P \leq 0.05$) among the treatments were observed on grain yield, spikelet sterility and other variables. These variables can be used as criteria for selecting heat and drought tolerant rice lines. Eight heat and drought tolerant mutant rice lines with high yields ≥ 3.5 ton ha^{-1} and low spikelet sterility were selected to be used as donor materials in rice breeding programs.

Key words; *Oryza sativa* L, heat tolerance, mutation, growth, yield and yield components

4.2   Introduction

Rice is a staple food for more than 50% of the world's population, mostly in developing countries, and is the most important cereal crop worldwide (Fageria *et al.,* 2011). High temperature as a result of climate change may affect the plant growth, development and yield of some crops including rice. Climate changes due to Global warming have negative significant effects on rice production. Zhang *et al,* (2013) stated that extreme climate changes associated with high temperature will occur more frequently with longer duration in many rice producing regions worldwide. Increase in global temperature is bound to affect the growth and development of rice crop (Manneh *et al.,* 2007). Heat stress causes alterations in plant growth, development and physiological processes (Hasanuzzaman *et al.,* 2013). High temperatures affect almost all growth stages of rice crop; high temperatures during vegetative, flowering, booting and grain filling stages of rice adversely affect the plant growth and development, grain quality and yield in many rice production regions worldwide (IPCC, 2007).

As part of the strategies for adaptation and or mitigation of climate change that might impart crop production, a concerted effort for development of climate proof crop varieties is one of the approach to stabilize crop production in face of climate change. HT mutant rice lines were developed through Induction mutation using gamma rays at Seibersdorf Laboratories, Vienna, Austria. The parents of these lines were subjected to the extreme heat stress and subsequent survivals were genetically characterized for expression of heat shock proteins to identify point mutations on the different positions of HSP17.9 and HSP90 genes (GeneBank accession No.

AY034057.1 and AC091774.7 respectively) as indicated in Table 4. Rice being one of the most demanded food-crop in the tropics and temperate regions, and therefore development of heat and drought stress tolerance rice was the major focus.

Tolerance to heat and drought stress in plants including rice is a complex phenomenon and controlled by multiple genes imparting number of physiological and biochemical changes in plant cells (John, 2001). John (2001) reported that plants may continue to struggle for survival under such stress conditions but may tolerate to some extent by physical changes occurring within the plant body and frequently by creating signals for changing metabolism but in adverse condition plant may die. The protection system may involve heat and drought resistance mechanisms associated with synthesis and accumulation of specific proteins known as heat shock proteins (HSPs) as reported by John, (2001). The response of HSPs allows rice plant to become tolerant to stresses including heat and drought (Chang et al., 2007). Several molecular plant breeding based approaches have been proposed to identify the genes influencing agronomic traits of interest for improving the rice productivity. The use of these approaches for evaluation of yield and yield components, continue to be important to measure the success of a rice genotypes in heat and drought stressed environments (Garg et al., 2012). The HT mutant upland rice lines have been identified to have heat tolerant genes (heat shock proteins genes) using TILLING technique whereby local lesions or point mutations were identified among the HT mutants on the HSPs genes. The main objective of the current study was to evaluate the growth, yield and yield components of the HT mutant rice lines that expressed various point mutations on the HSPs genes.

4.3   Material and Methods

4.3.1   Experimental Site, Climate, Design and Plant Materials

Field experiment was conducted during 2014/2015 a few weeks before short rainfall season "vuli" which normally starts on December and ending on February before long rainfall season "Masika" which starts just after short rain season. The experiment was conducted at the rice experimental plots of the Department of Crop Science and Production of Sokoine University of Agriculture, Morogoro located at Latitude 6° 50' 55" S and longitude 37° 39' 22" E. Daily mean maximum and minimum temperatures and rainfall were recorded for duration of three months. The minimum and maximum mean temperature were 20°C and 35.5°C respectively while the minimum and maximum rainfall were 32.7mm and 155.5mm respectively in December 2014, January, February and March, 2015. A total rainfall and average minimum and maximum temperature for 4 months are indicated in Fig.1
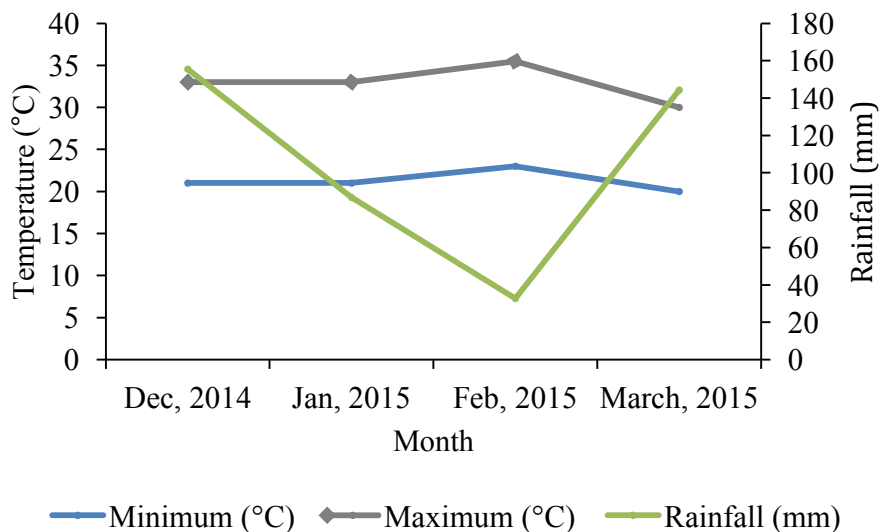
Figure 1: Climate trend for four months in SUA, Morogoro 2014/2015

The experiment was laid out in randomized complete block design (RCBD) with three replications to evaluate the performance of mutant heat tolerant upland rice lines under natural uncontrolled heat and water deficit stress field conditions. Single row planting with fifteen hills with spacing of 20x20cm, for each mutant rice line was treated as a plot (0.2mx3m). A total number of thirty four advanced HT mutant (M_5) upland rice lines and their 4 parents were used in this experiment (Table 1). The mutant rice lines indicated in table one below were derived from four rice genotypes including Kihogo Red (*O. sativa*), CG 14 (*O. glaberrima*), WAB 56-50 (*O. sativa*) and WAB 56-104 (*O. sativa*).

Table 1: The list of HT mutant upland rice lines with HSPs genes were used

Sample ID	Mutant rice line
1	KR 27_1
2	KR 38_1
3	CG 14_16_1
4	CG 14_20_1
5	CG 14_58_1
6	CG 14_61_3
7	CG 14_63_1
8	CG 14_63_2
9	WAB 56_104_36_1
10	WAB 56_104_141_1
11	WAB 56_104_141_2
12	WAB 56_104_141_3
13	WAB 56_104_150_2
14	WAB 56_50_51_1
15	WAB 56_50_56_1
16	WAB 56_50_56_2
17	WAB 56_50_74_1
18	WAB 56_50_82_1
19	WAB 56_50_85_2
20	WAB 56_50_85_3
21	WAB 56_50_97_2
22	WAB 56_50_97_3
23	WAB 56_50_97_4
24	WAB 56_50_98_1
25	WAB 56_50_98_3
26	WAB 56_50_123_1
27	WAB 56_50_123_2
28	WAB 56_50_123_3
29	WAB 56_50_127_3
30	WAB 56_50_127_5
31	WAB 56_50_135_1
32	WAB 56_50_141_1
33	WAB 56_50_141_2
34	WAB 56_50_152_3
35	KR Control
36	CG 14 Control
37	WAB 56_104 Control
38	WAB 56_56 Control

4.3.2 Sowing and experiment management

Seeds of HT mutant upland rice lines were sown in the field on 20/12/2014 dry and hot season before short rainfall season "Vuli". Four seeds from each rice line were sown directly in soil 3cm deep hill and water applied to enhance germination. After 3 weeks, thinning and gap filling were done to remain with single seedling per hill.

Fertilizers were broadcasted at the recommended rate of 120, 60 and 60 kg N (Urea), P_2O_5 (TSP) and K_2O (Muriate of potash) ha^{-1} respectively, with half dose of nitrogen and full dose of phosphorous and potassium worked in to the soil during seedbed preparation using NPK 15: 15: 15: and second dose of 80 kg N ha^{-1} Urea (46% N) was applied prior to tillering. Weeds were controlled manually with hand hoe to ensure plots were free from weeds. Birds were controlled by scaring though they attacked the crop in some plots during milking growth stage. No Disease outbreak was observed.

4.4 Data collected

Data for rice growth performance, yield and yield components evaluation were recorded on; number of reproductive tillers per plant, days to first and 50% flowering, days to 85% physical maturity, plant height (cm), panicle number per plant, panicle length (cm), number of spikelet per panicle, number of filled and unfilled grains per panicle, spikelet sterility percentage (sterility %), weight of 1000 grains and grain yield per plot. The crop was harvested manually from the net plot at physiological maturity using sickles when 90% of the panicles had turned brown. Seed were extracted from spikelet by hands and followed by cleaning through winnowing. The procedures undertaken for collecting data of each variable are narrated as follows;

4.4.1 Early flowering and Days to 50% flowering

The days at which the first flowers appeared and the day when at least half of the rice plants exerted a fully opened panicle per plot was recorded as first and 50% days of flowering respectively.

4.4.2 Number of tillers per plant

Number of tillers per plant for each treatment was counted at the maximum tillering stage, whereby number of tillers per row were counted and recorded. From the total number of tillers obtained in each plot, its average was calculated by dividing the total number of tillers by the number of plants in a plot to get number of tillers plant^{-1}.

4.4.3 Number of panicles and spikelet

The number of panicles was recorded at maturity stage in each treatment. The number of panicles per plant was counted by taking five plant samples from each treatment. Also number of spikelet was obtained by counting spikelet per panicle in each treatment using the same five plant samples.

4.4.4 Plant height

Plant height in centimeter (cm) from five plants per treatment was measured during maturity growth stage of rice using a ruler from the collar of the plant to the longest leaf and averages were recorded.

4.4.5 Panicle length

From this variable length was taken by measuring five panicles randomly in a plot by using a ruler before harvesting, and then an average was recorded as panicle length of the plant in centimeter (cm).

4.4.6 Days to physical maturity

This was counted from the day of seeding until more than 85% of rice grain turned from green to brown.

4.4.7 Number of filled, unfilled and damaged grains per panicle

Number of filled, unfilled and damaged seeds by birds per panicle per plant was counted from the whole plot after harvest. Dried filled, unfilled and damaged grains by birds separated manually and then, its numbers determined using automated Seed counter (SEEDBURO™ 801 COUNT-A-PAK®).

4.4.8 Spikelet Sterility and bird loss percentage

The spikelet sterility percentage was obtained through a relationship between the number of unfilled grains and total number of grains per panicle per plant also grain loss caused by birds obtained by dividing number of damaged grains with total number of grains per panicle per plant times hundred. The spikelet sterility and bird loss percentage (%) was calculated (as adopted by Masanche, 2014) by using the following formula below;

$$\% \text{ sterility} = \frac{\text{Number of Unfilled grains/plant}}{\text{Total number of grains/plant}} \text{X}100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$\% \text{ Bird loss} = \frac{\text{Number of damaged grains/plant}}{\text{Total number of grains/plant}} \text{X}100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

The weights of 1000 grains per plot were determined from sun dried, threshed, cleaned and filled grains. 1000 filled grains were counted using Seed Counter and then, its weight determined using sensitive Digital weighing balance OHAUS Pioneer™.

4.4.10 Grain yield (t /ha)

Grain yield per plot was recorded by weighing the filled grain per plant per plot and then was converted to grain yields in tonnage per hectare (t /ha).

4.5   Data Analysis

Data analysis was done using GenStat software version 15. The treatment means were separated by Duncan's Multiple Range Test (DMRT) at 5% probability level.

The basic assumption in ANOVA is that each observation (Yij) is constituted of the mean, treatment effects, block effect and random error. The statistical model used was;

Yij = U + Ti + Bj + Eij

Where;

U = Overall mean of the experiment

Ti = the effect of i^{th} treatments among the treatments

Bi = Block effects of the blocks j

Eij = Random error effects to each observations in the i^{th} treatments and j^{th} block

4.6   RESULTS

4.6.1   The growth performance, yield and yield components evaluation of HT mutant rice lines from all 4 rice genotypes

The variables considered for HT mutant rice lines evaluation were days to early flowering, days to 50% flowering, plant height, number of tillers per plant, panicle number per plant, spikelet per panicle, filled grains per panicle, unfilled grains per panicle, 1 000 grains weight and grain yield (t/ha). The variables data were scored at

different growth stages of mutant rice during hot dry field conditions (Fig. 2). Mutant rice lines were exposed to the high temperature in different growth stages; vegetative (early and late), reproductive (flowering) and maturity stages (Fig. 2) were the critical stages in this study. The high temperature above optimum level (27-35^{o}C) affects all growth and development stages of the heat susceptible rice genotypes and, thus decreases their grain quality and yield (Shah *et al.*, 2011). According to IPCC, (2007) report, it is expected that the abnormal temperature increase will affect rice production worldwide. Zhang *et al.,* (2013) also reported that an increase of temperatures 1-2°C than the optimum level during reproductive stage results in shortening grain filling periods and negatively affecting yield and other yield components of unimproved cereal crops including rice.

| Early vegetative stage | Late vegetative stage |
| Flowering stage | Maturity stage |

Figure 2: **Different growth stages of mutant rice lines during agronomic evaluation at dry hot field condition.**

4.6.1.1   Days to early and 50% flowering, physical maturity, plant height and panicle length

There were significant differences at P≤0.05 level of significance in days to early flowering and plant height (cm), but there was no significant difference (P≤0.05) in days to 50% flowering, days to 85% physical maturity and panicle length (cm) among the treatments (Table 2). Both CG 14_16_1 and WAB 56_50_56_2 were

observed to have short duration of 74 days from seeding to first flowering among treatments while KR 38_1 took long period of 108 days to show anthers. The short duration of 81 days to 50% flowering was observed in WAB 56_50_98_1 but the longest duration of 125 days were observed in KR 38_1 among the treatments. Both WAB 56_50_97_4 and WAB 56_50_127_5 took 98 days after seeding (DAS) to reached physical maturity among the treatments but KR 38_1 took long period of 149 DAS to attain 85% physical maturity. The highest mean value for plant height was 131cm, which was observed from KR 38_1 but the lowest mean value for the plant height was observed in CG 14_63_2 among the treatments. The highest panicle length of 36cm among the treatments was recorded in WAB 56_50_51_1 while shorter panicle length of 20 cm was recorded from several mutant rice lines including CG 14_63_1 (Table 2).
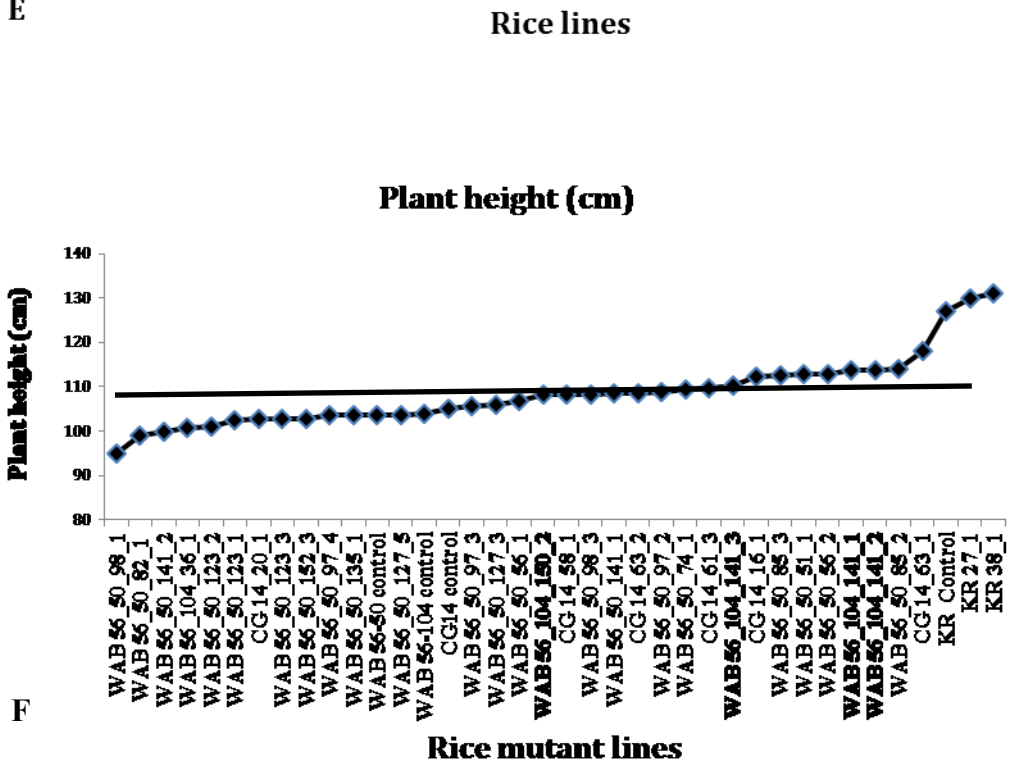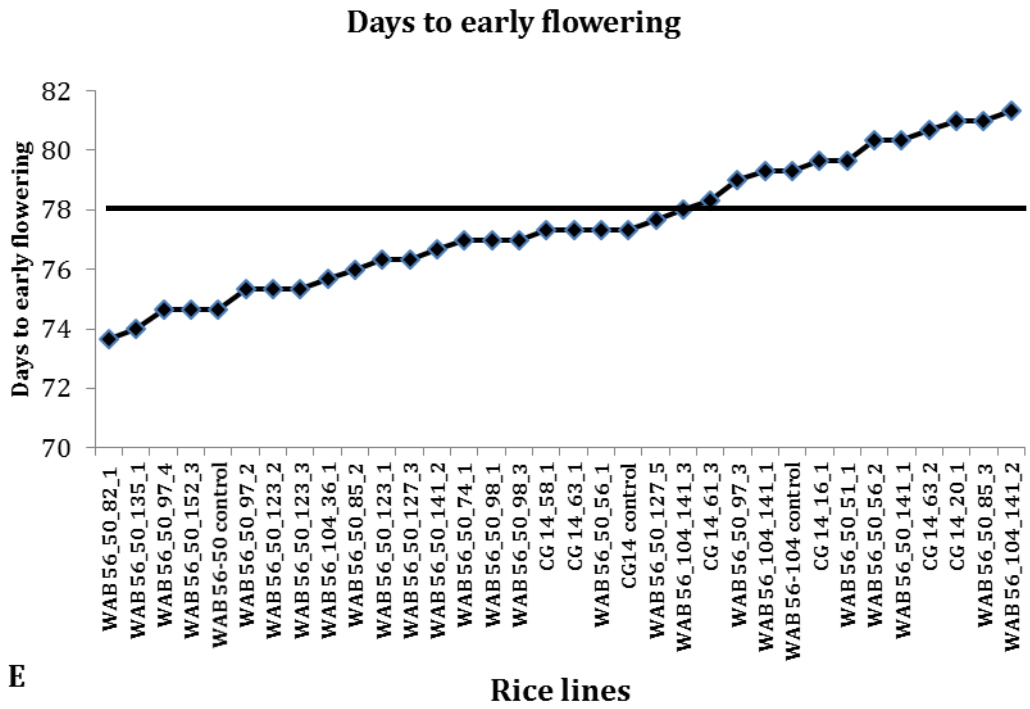
Days to early flowering

E

Plant height (cm)

F

Key; Horizontal lines represent the cut off points which are the overall means of the
mutant lines for the respective variables

**Figure 3: (E & F) Number of days to early flowering and plant height (cm) for
all mutant rice lines evaluated**

4.6.1.2   Number of spikelet, tillers number, panicle number, filled grains and unfilled grains per plant

There were highly significant differences at P≤0.05 level of significance in number of spikelet per panicle, number of tillers per plant, panicle number per plant, filled and unfilled grains per panicle per plant among all treatments (Table 2).

The highest mean number of spikelet (13) per panicle was observed in CG 14_16_1 and the minimum mean number of 9 spikelet per panicle were observed in both WAB 56_104 Control and CG 14_20_1. Number of tillers per plant was observed to be higher (33) in WAB 56_104_150_2 and lowest mean number of 6 tillers was observed from CG 14_16_1. The highest panicle number of 27 was observed in both WAB 56_104 Control and WAB 56_104_150_2 but the lowest mean number of panicles per plant was counted in several lines including CG 14_63_1 (Table 1). The highest average number of filled grains (759) per plant was observed in WAB 56_50_51_1 among treatments while few filled grains (122 grains) per plant counted in WAB 56_104 Control (Table 5). Treatments with the lowest unfilled grains among all treatments were WAB 56_50_127_5, having 74 unfilled grains (Fig. 4).

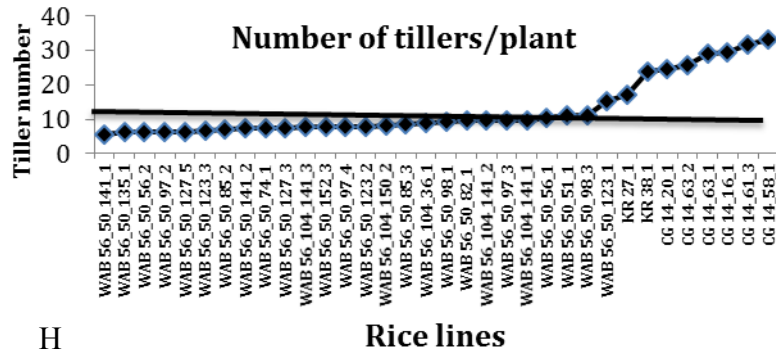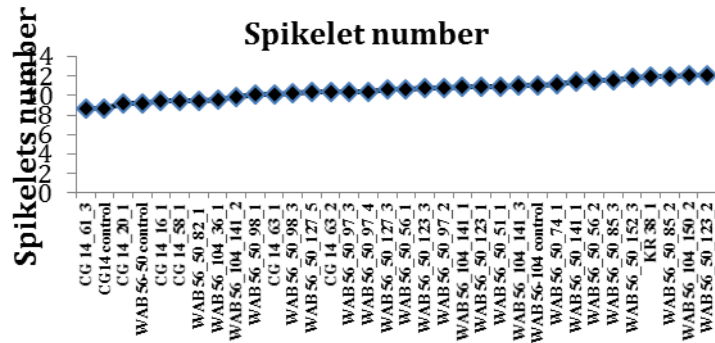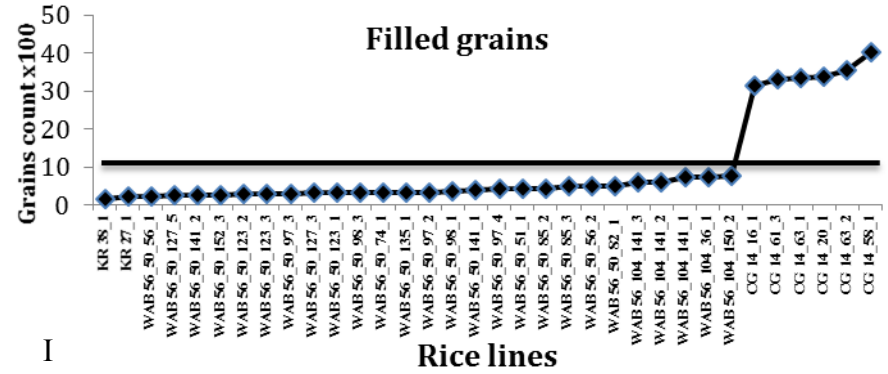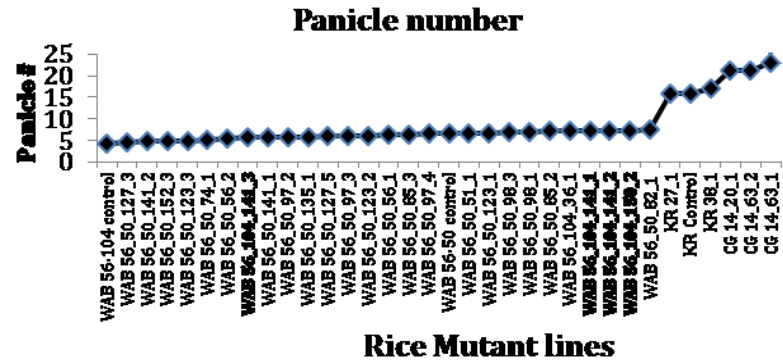**Figure 4: (H, I, J & K) Number of spikelet, number of tillers, number of panicles, filled grains of mutant rice lines**

Key; Horizontal lines represent the cut off points which are the overall means of the mutant lines for the respective variable

4.6.1.3 Grain yield, 1 000 grains weight, bird loss and percentage spikelet

        sterility

In the upland rice mutant lines evaluated for the growth, yield and yield components under the hot field conditions the results showed that there were significant differences among the treatments in grain yield, bird loss and spikelet sterility percentage but there was no significant differences in 1 000 grains weight (g) at $P \leq 0.05$ level of significant (Table 2).

The highest mean of grains' yield (t ha^{-1}) was observed on CG 14_63_1 with 6.8 t ha^{-1} while 2 t ha^{-1} was observed on both WAB 56_50_123_3 and WAB 56_50_127_5 mutant rice lines as lowest grain yield among the treatments evaluated. The highest weight of 1000 grains of 33g among treatments was observed on WAB 56_104_36_1 but the lowest weight of 1000 grains was 25g on KR 27_1. WAB 56_50_141_1 indicated to have lowest sterility percentage of 9% but the highest sterility of 28% counted in WAB 56_50_98_1 among the assessed mutant rice lines. Bird loss in percentage was observed to be high (52%) on WAB 56_50_123_2 while the lowest bird loss of 2% counted in both CG 14_63_1 and CG 14 control (Fig. 5).
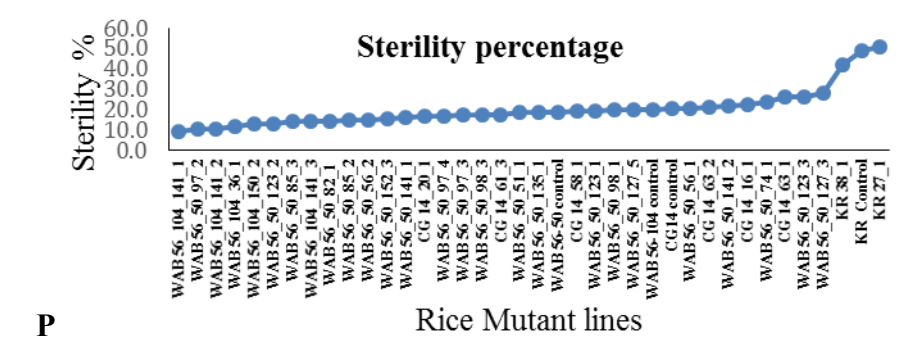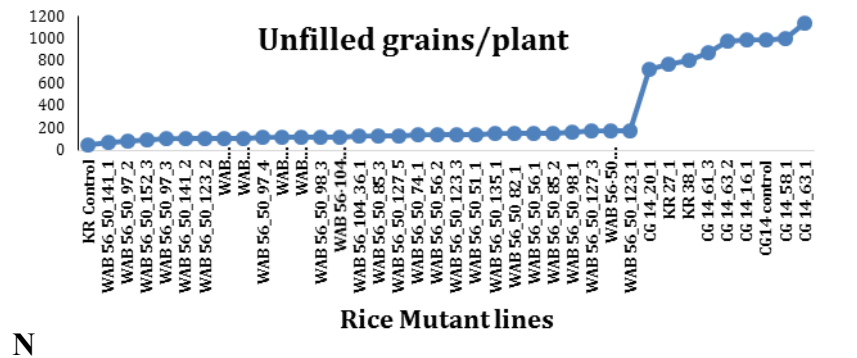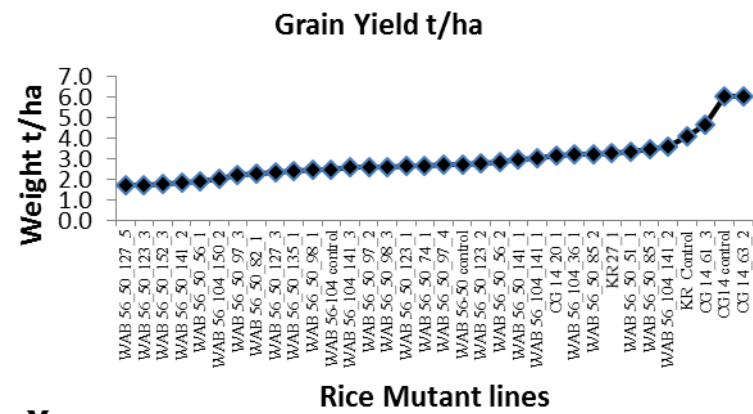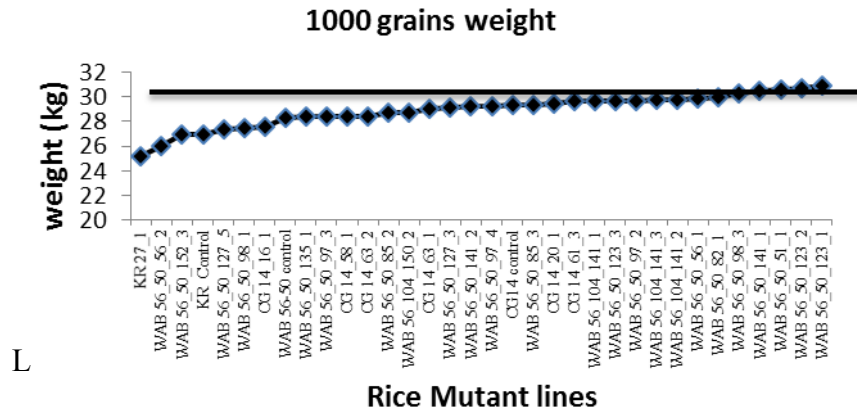
Figure 5: (L, M, N & P) 1000 grains weight (g), grain yield (t/ha), Spikelet sterility and bird loss of rice mutant lines

Key: Horizontal lines represent the cut off points which are the overall means of the mutant lines for the respective variables.

Table 2: **A summary mean of Analysis of variance of all rice mutant lines evaluated for growth performance, yield and yield components**

| Rice line	DEF	D50%F	DPM	PH (cm)	PL(cm)	SN	TN	PN	NFG	NUG	%SS	1000GW	GY
KR 27-1	95d	104bc	147c	130g	23abcd	13g	17abc	16b	14a	48a	50de	25abc	3abc
KR 38-1	108de	125cd	149c	131g	21abc	12efg	24cd	17b	10a	46a	43d	31bcd	6.5de
CG 14_16_1	74a	85a	108abc	104abcde	21abc	13g	6a	6a	343a	146a	21abc	28abc	6.6e
CG 14_20_1	77abc	82a	104abc	105abcde	23abcd	9ab	28cd	24bc	3791bc	989cd	26bc	29abcd	3abc
CG 14_58_1	81cd	89ab	109abc	113cdef	21abc	12efg	9a	6a	507a	128a	18abc	28abc	6.5e
CG 14_61_3	79abcd	87ab	110abc	114ef	35bcd	11abcdefg	10ab	7a	744a	109a	14abc	30abcd	5cd
CG 14_63_1	77abc	88ab	100ab	100ab	20ab	12efg	7a	5a	256a	101a	22abc	29abcd	6.8e
CG 14_63_2	77abc	82a	107abc	95a	22abcd	10abcdef	9ab	7a	355a	164a	19abc	28abc	6de
WAB 56_104_36_1	81cd	89ab	109abc	114ef	20ab	10abcde	10ab	7a	615a	107a	14abc	33d	3abc
WAB 56_104_141_1	81bcd	97b	108abc	109bcdef	25abcd	10abcdef	26c	21b	3540bc	973cd	13ab	30abcd	3abc
WAB 56_104_141_2	78abcd	85a	106abc	110bcdef	21abc	11abcdefg	8a	6a	610a	116a	14abc	30abcd	4bc
WAB 56_104_141_3	75abc	83a	107abc	109bcdef	24abcd	11abcdefg	6a	6a	351a	78a	18abc	30abcd	3ab
WAB 56_104_150_2	77abc	84a	107abc	108bcdef	24abcd	9abcd	33d	27c	4029c	995cd	11a	29abc	2ab
WAB 56_50_51_1	76abc	80a	106abc	101abc	36d	10abcd	9ab	7a	759a	123a	14abc	31bcd	3abc
WAB 56_50_56_1	80abcd	89ab	108abc	113def	20ab	11abcdefg	11ab	7a	435a	144a	15abc	30abcd	2ab
WAB 56_50_56_2	74a	82a	111bc	99ab	21ab	9abcd	10ab	7a	514a	149a	17abc	26a	3ab
WAB 56_50_74_1	77abc	85a	110abc	118f	25abcd	10abcdef	29cd	23bc	3351bc	1130d	13ab	31cd	3ab
WAB 56_50_82_1	77abc	83a	111bc	107bcdef	21ab	11abcdefg	11ab	6a	252a	150a	16abc	30abcd	3ab
WAB 56_50_85_2	79abcd	85a	105abc	106abcde	23abcd	10abcdef	10ab	6a	314a	101a	19abc	29abc	3abc
WAB 56_50_85_3	76abc	86ab	111bc	114ef	22abc	12efg	7a	7a	448a	154a	17abc	29abcd	3abc
WAB 56_50_97_2	76abc	84a	113c	106abcde	23abcd	11abcdef	7a	5a	325a	172a	20abc	30abcd	3ab
WAB 56_50_97_3	77abc	84a	110abc	110bcdef	21ab	11acdefg	7a	5a	341a	134a	17abc	28abc	2ab
WAB 56_50_97_4	75ab	87ab	98a	103abcde	21ab	12defg	8a	5a	263a	91a	23abc	29abcd	3ab

WAB 56_50_98_1	75ab	81a	111bc	104abcde	21ab	9abc	8a	7a	346a	174a	28c	27abc	2ab
WAB 56_50_98_3	77abc	91ab	108abc	108bcdef	22abcd	10abcdef	11ab	7a	335a	117a	19abc	30bcd	3ab
WAB 56_50_123_1	75abc	84a	106abc	103abcde	22abcd	11abcdefg	7a	5a	307a	140a	20abc	31bcd	3ab
WAB 56_50_123_2	75ab	79a	104abc	104abcde	22abc	10abcdef	8a	7a	431a	111.5a	19abc	31bcd	3ab
WAB 56_50_123_3	84d	86ab	111bc	108bcdef	20ab	12efg	8a	7a	789a	112a	15abc	30abcd	2a
WAB 56_50_127_3	78abc	82a	108abc	104abcde	22abcd	10abcdef	6a	6a	254a	130a	21abc	29abcd	2ab
WAB 56_50_127_5	80bcd	85a	98a	108bcdef	21ab	11cdefg	6a	6a	410a	74a	22abc	27abc	2a
WAB 56_50_135_1	75abc	81a	109abc	101abcd	21ab	12efg	8a	6a	288a	107a	20abc	28abc	2ab
WAB 56_50_141_1	80abcd	85a	105abc	112cdef	25abcd	9abc	30cd	25bc	3160b	985cd	9a	31bcd	3abc
WAB 56_50_141_2	79abcd	85a	109abc	104abcde	20a	11abcdefg	6a	4a	389a	121a	26bc	29abcd	2ab
WAB 56_50_152_3	81cd	86ab	108abc	103abcde	36cd	9abc	25c	21b	3390bc	718b	11a	27ab	2ab
KR control	109e	122b	148d	127g	23abcd	13cdefg	21a	16ab	240a	752bc	48d	27ab	3.7abc
CG14 Control	80bcd	91ab	106abc	113cdef	21abc	12cdefg	6a	6a	510a	135a	17abc	29abcd	6de
WAB 56-104 Control	78abcd	84a	107abc	110bcdef	23abcd	9a	32d	27c	3305bc	873bc	12ab	31cd	2ab
WAB 56-50 Control	76abc	83a	106abc	103abcde	22abc	11abcdefg	15b	7a	332a	178a	19abc	28abc	3ab
Grand mean	**78**	**85**	**107**	**107**	**23**	**11**	**12**	**10**	**1040**	**292**	**18**	**29**	**3**
S.E	**3.11**	**6.04**	**6.13**	**6**	**7.3**	**1.2**	**3.3**	**2.7**	**396**	**96.5**	**7.12**	**1.95**	**0.92**
CV	**4.0**	**7.1**	**5.7**	**5.6**	**31.6**	**11.5**	**26.7**	**27.3**	**38.1**	**33**	**40.3**	**6.6**	**28.4**
F value	**0.017***	**0.372**	**0.54**	**0.002***	**0.531**	**0.002***	**<.001*****	**<.001*****	**<.001*****	**<.001*****	**0.043***	**0.117**	**<.001*****

*, **, *** Significant difference at P≤ 0.05, P≤ 0.01, P≤ 0.001 respectively,

Figures followed by the same letter (s) in columns are not significantly different at P≤ 0.05 according to DMRT

DEF=Days to early flowering, D50%F=Days to 50% flowering, DPM=Days to physical maturity, PH=Plant height (cm), PL=Panicle length (cm), SN=Spikelet number, TN=Tiller number, PN=Panicle number, NFG= Number of filled grains, NUG=Number of unfilled grains, %SS=Percentage Spikelet Sterility, 1000GW= 1000 grains weight.

4.6.2 **The growth performance, yield and yield components evaluated from**

   HT mutant rice lines within the genotype

The agronomic performance evaluation of HT mutant rice lines from the same genotype was done by comparing Analysis of variance average means of mutant upland rice lines against non-irradiated genotype (control) shown in Fig. 6, 7, 8 & 9.

4.6.2.1 **The growth performance, yield and yield components evaluated for**

   the HT mutant rice lines from WAB 56_50 rice genotype

The means from ANOVA for days to early flowering, days to 50% flowering, plant height (cm), panicle number/plant, spikelet/panicle, filled grains and unfilled grains/panicle, 1 000 grains weight (g) and grain yield (t/ha), bird loss percentage were observed to be higher in mutant rice lines compared to non-irradiated rice but spikelet sterility and days to physical maturity were low in mutant rice lines compared to its control but also number of tillers/plant observed to be the same (Fig. 6a and 6b).

	Seed #/panicle	Days to maturity	Plant height (cm)	50% flowering	Days to early flowering
■ WAB 56_50 Mutant (μ)	119	107	106	85	77
▫ WAB 56_50 Non-mutant (μ)	127	111	104	81	75

WAB 56_50 Mutants and non-mutants

(a)

	Bird loss (%)	1000 grains wt(g)	Panicle length (cm)	Panicle Sterility	Spikelets #/panicle	Tillers #	Panicle #	G.Yield ton/ha
■ WAB 56_50 Mutant (μ)	32	29	22	18	12	8	6	3
▫ WAB 56_50 Non mutant (μ)	31	28	21	20	9	8	7	3

WAB 56_50 Mutants and non-mutant

(b)

Figure 6: (a & b) Growth, yield and yield components means differences from Mutant rice lines against non-irradiated of WAB 56_50 rice genotype

4.6.2.2 **The growth performance, yield and yield components evaluation for mutant rice lines of WAB 56_104 rice genotype**

From the variables analyzed for agronomic performance evaluation of HT mutant rice lines from WAB 56_104 genotype; its means from ANOVA was compared against non-irradiated genotype (Appendix 2). The means for days to early flowering, days to physical maturity, plant height (cm), panicle number/plant, filled and unfilled grains/panicle, 1000 grains weight and grain yield t/ha were counted to be higher in mutant rice lines compared to non-irradiated genotype, but spikelet sterility percentage and days to physical maturity were high in non-mutant rice genotype compared to HT mutant rice lines but also number of days to 50% flowering and spikelet/panicle were observed to be the same in HT mutant lines and non-irradiated rice genotype (Fig.7a & 7b).

	Unfilled seed/panicle	Plant height (cm)	85% Maturity	Filled seed/panicle	Days to 50% flowering	Days to early flowering
WAB56_104 mutant (µ)	113	109	108	101	85	80
WAB56_104 non mutant (µ)	121	104	109	89	85	79

WAB 56-50 Mutants and Non-mutants rice

(a)

	1000 grains wt. (g)	Sterility %	Panicle length	Spikelets /panicle	Tillers #	Yield (t/ha)	
WAB56_104 mutant (µ)	30	28	27	11	9	3	
WAB56_104 non mutant (µ)	31	40	20	11	6	2	
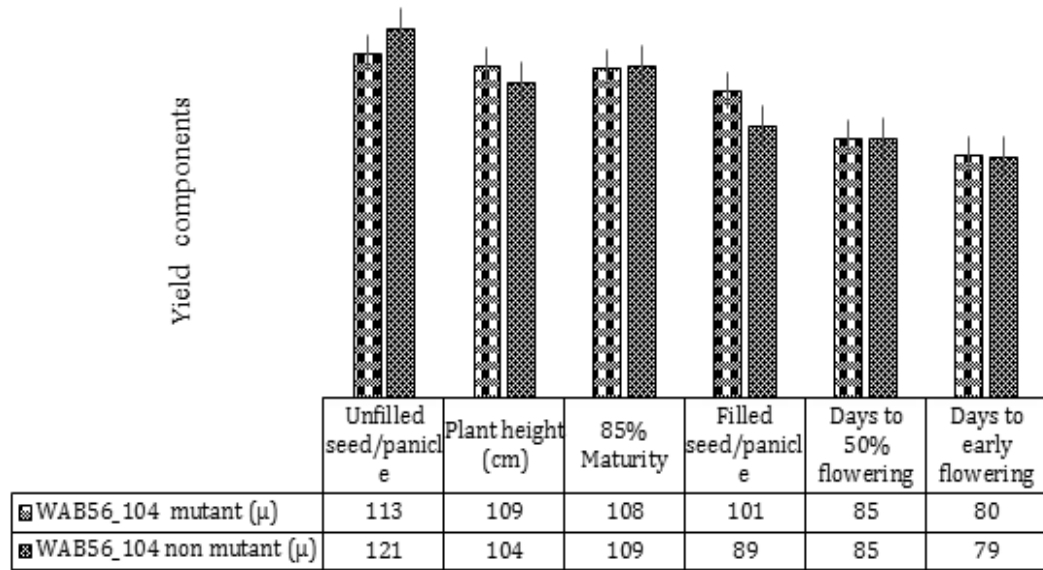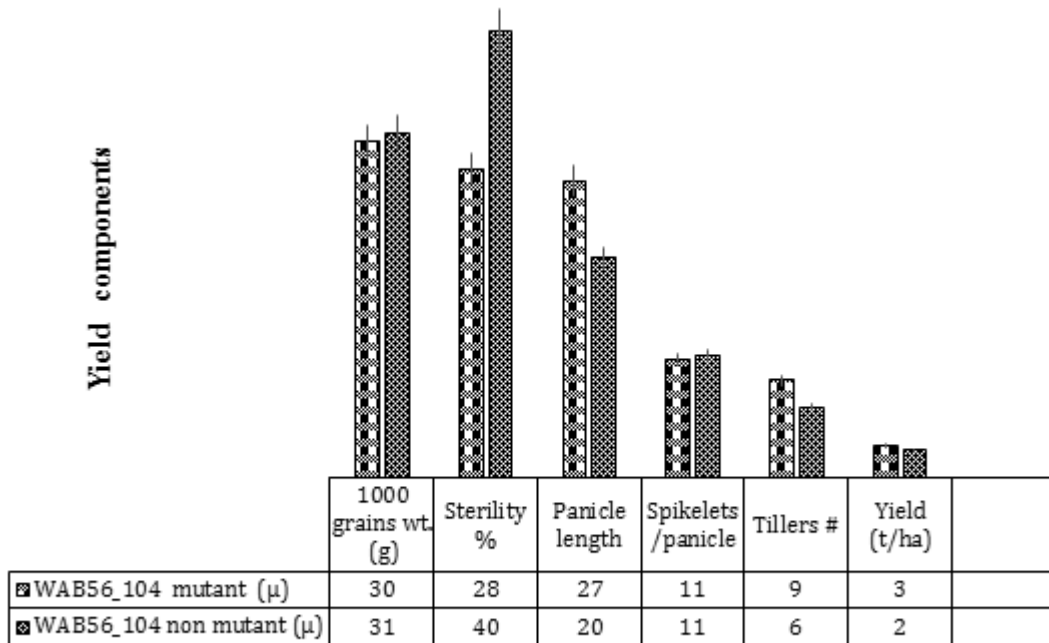
WAB 56_104 Mutant and Non-

(b)

Figure 7: (a & b) Yield and yield components of mutant rice lines against non-mutant (its parent) from WAB 56_104 genotype

4.6.2.3   The growth performance, yield and yield components evaluated from HT mutant Rice lines of CG 14 rice genotype

HT mutant CG 14 rice lines were evaluated for agronomic performance; growth, yield and yield components, and its means from ANOVA were compared against non-irradiated rice genotype (Appendix 3). The means for days to early flowering, days to 50% flowering, days to physical maturity, plant height (cm), spikelet/panicle, filled grains, Bird loss (%) and grain yield t/ha were observed to be higher compared to non-mutant but unfilled grains/panicle and spikelet sterility percentage were low in mutant rice lines compared to its control but panicle number/plant and 1000-grains weight (g) were observed to be the same in both rice categories as indicated in Fig.8(a & b).

Yield components

	Filled grains/panicle	Plant height (cm)	Days to 85% maturity	Days to 50% flowering	Days to early flowering
CG 14 Mutants (μ)	159	110	107	86	79
CG 14 Non mutant (μ)	148	105	104	82	77

CG 14 Mutants and Non mutants rice

(a)

Yield components

	Unfilled grains	1000 grains wt. (g)	Tiller number	Panicle number	Panicle length	Sterility %	Spikelets	Bird loss %	Yield t/ha
CG 14 Mutants	43	31	29	26	26	11	10	7	6
CG 14 Non mutant	41	29	26	24	23	13	9	2	5
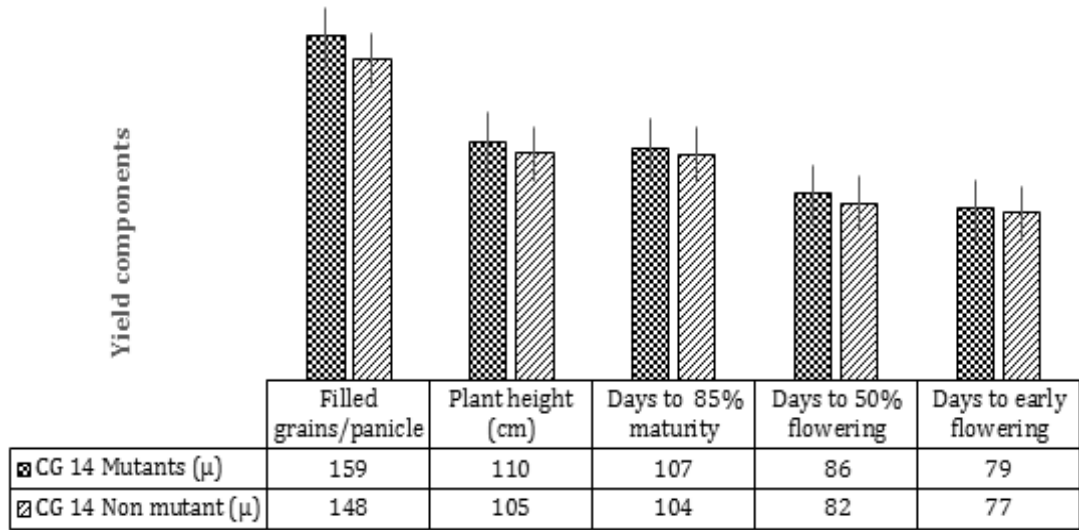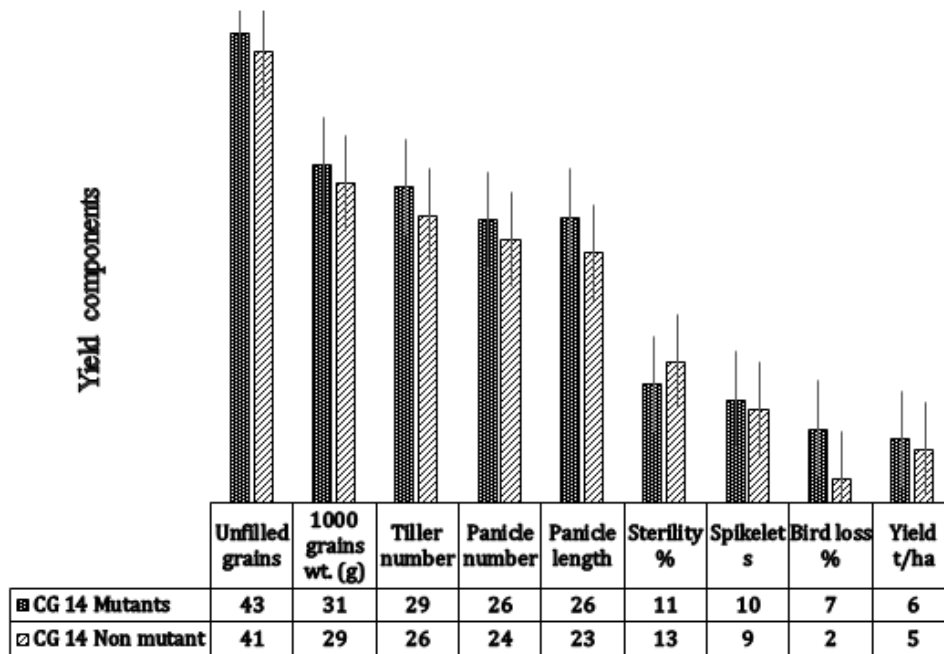
CG 14 Mutants and Non-Mutants rice

(b)

Figure 8: (a&b) The growth, yield and yield components of CG 14 mutant rice against non-mutant rice CG 14 genotype.
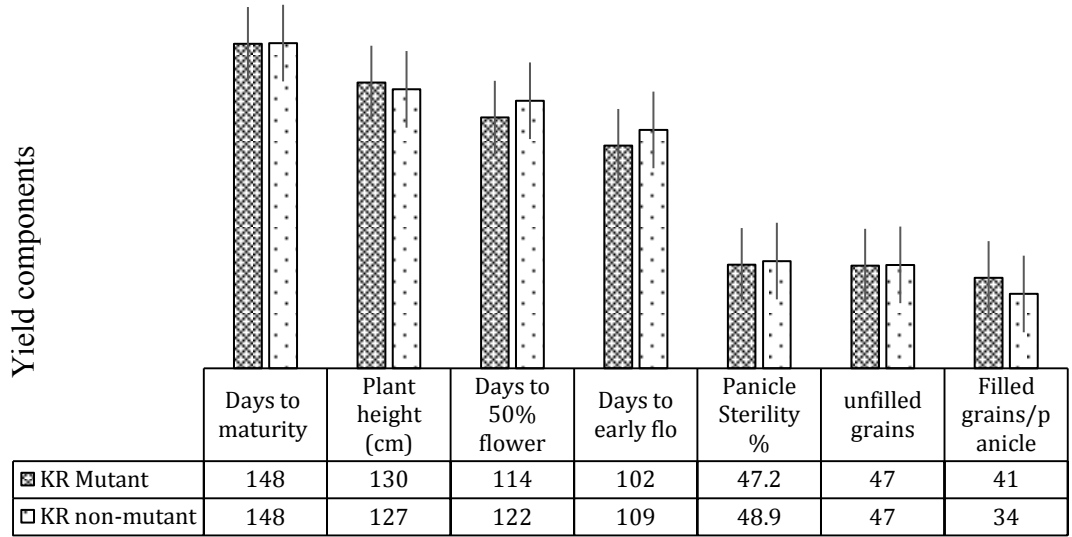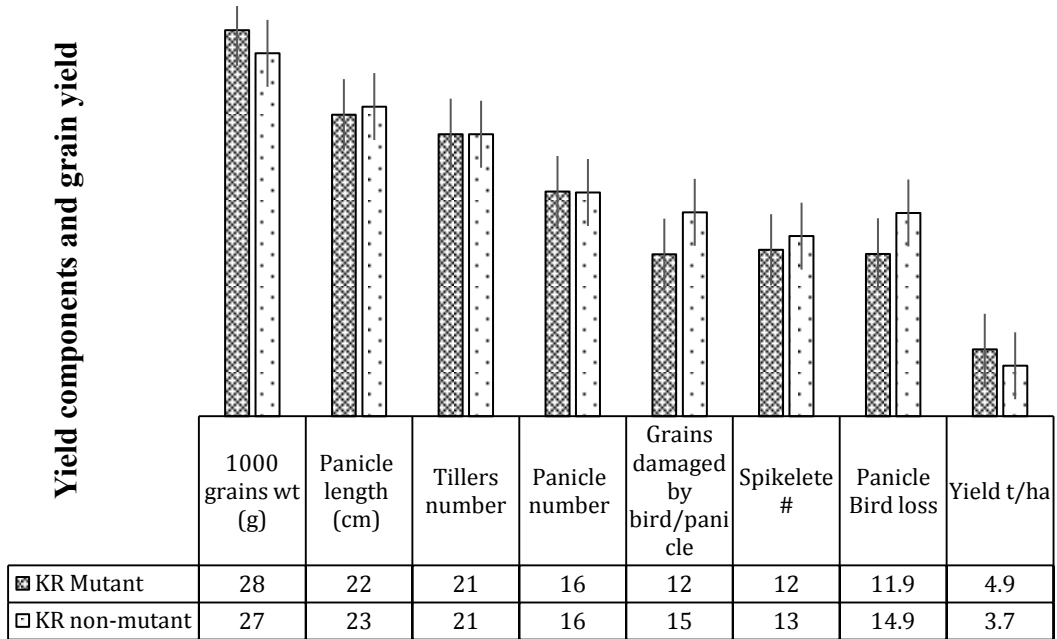
4.6.2.4 **The growth performance, yield and yield components evaluated from**

HT mutant rice lines of Kihogo (KR) rice genotype

The evaluation of the agronomic performance of the HT KR mutant rice lines for the

all analyzed variables was done by comparison of HT mutant rice lines means from

ANOVA against the means of non-irradiated genotype (Appendix 4). The average

mean for plant height (cm), filled grains/panicle, 1000-grains weight (g) and grain

yield t/ha were high in HT mutant rice lines compared to non-irradiated genotype

while days to early flowering, days to 50% flowering, panicle length (cm),

spikelet/panicle, unfilled grains/panicle, bird loss and spikelet sterility percentage

were observed to be low in mutant rice lines compared to its control but also number

of tillers/plant, panicle number/plant and days to physical maturity were found to be

the same between mutant and non-mutant (Fig. 9 (a & b) and Appendix 5).

4.7   DISCUSSION

4.7.1   The effects of induced mutations on growth performance of the HT mutant rice lines

The evaluation of the effects of induced mutation in growth performance of the HT mutant rice lines was done by assessing the plant height, panicle length, days to early flowering, days to 50% flowering and days to 85% physical maturity. Days to early flowering and plant height revealed that there were significant differences in 5% probability level but there were no significant differences in panicle length, days to 50% flowering and days to 85% physical maturity. The induced mutation may affect positively or negatively the growth characteristics of the plant. According to the current study results, it indicates that Induced mutations enhance the plant growth in respects to plant height. Highest plant height was observed in all mutant rice lines evaluated compared to non-irradiated rice genotypes (parents - heat susceptible rice genotypes). The higher the plant can maximize the utilization of the resources especially sun light and hence, allowing the production of economical grain yield (Sangoi and Salvador, 1997). This study also agrees with results reported by Kabir and Sarkar, (2008) that, plant height differs significantly among studied HT mutant upland rice lines may be due to differences in genetic make-up.

The Mutant rice lines flowered early compared to the non-irradiated rice genotypes which may confirm that mutations is associated with shortening the growth period of the rice. The shorter the period for flowering will results mutant rice lines to flower early before the high temperature duration to attain (Shah *et al.,* 2011) in future flowering and fertilization processes will be not affected by high temperature (Matsui

et al., 2001). This is because high temperature during reproductive stage affects the flowering, fertilization, grain filling and hence, resulting into low pollen production, fertilization, increasing spikelet sterility and hence lower the grain yield of rice crop (Matsui *et al.,* 2001). Jiang-lin *et al,* (2011) confirmed that the plant growth parameters of heat susceptible rice genotypes can be negatively affected by high temperature in all growth stages compared to HT rice, however using HT rice will help to improve rice productivity.

4.7.2 The effects of induced mutations on number of tillers, panicle number, number of spikelet, filled grains and grain yield in mutant rice lines

The results of the current study revealed that there were significant differences at $P \leq 0.05$ level of significance in number of spikelets per panicle, number of tillers per plant, panicle number per plant, filled and unfilled grains per panicle among the treatments (Table 6). The results of the HT Irradiated gamma rays mutant rice lines indicated that, this kind of mutation associated with increase in number of tillers, number of panicles, number spikelet and number of filled grains per plant in rice crop.

This findings agrees with the findings of Cheema and Atta, (2003) which narrated that, the effects of the Induced mutations tend to influence increase in yield and yield components of the rice. Increase of reproductive tillers per plant results in the increase of panicle number per plant and spikelet per panicle which together result into increase of number of grains per panicle so high grain yield. The findings by De Datta, (1975) confirmed that tillering is the major factor that determines the yield in

rice crop. Surek and Beser, (2003) reported that there is positive association between grain yield/plant and yield components: total spikelet/panicle, filled grains/panicle and 1000 grains weight. Increase of these yield components influenced increase in grain yield of the mutant rice lines as well as productivity under the heat and drought stressed environment. Surek and Beser, (2003) suggested that individual yield component may contribute valuable information in rice breeding for yield. In general, an increase of the yield components was observed in all the HT mutant rice lines compared to its control (heat susceptible rice genotypes) which led to increase in grain yield of mutant rice lines.

4.7.3   The effects of induced mutations on grains yield, 1000 grains weight and percentage spikelet sterility in mutant upland rice lines

The results showed that there were significant differences among all rice lines in grain yield and spikelet sterility percentage but there was no significant differences among treatments in 1000 grains weight at $P \leq 0.05$ level of significant (Table 2). Surek and Beser, (2003) found that, the effects of mutations enhance increase of number of tillers per plant, number of spikelet per panicle, number of panicles per plant and filled grains and these factors are associated directly with increase in grain yield of rice. The grain yield was observed to be high in all HT mutant rice lines compared to non-irradiated rice genotypes, this indicated that heat tolerant mutant rice lines were able to tolerate the heat stress conditions and this increase its production capacity.

The percentage of spikelet sterility in mutant rice lines were observed to be very low in all evaluated HT mutant rice lines (Table 1) compared to its controls (heat susceptible genotype). The low sterility in mutant rice lines compared to non-mutant rice was due to its ability (heat and drought tolerance) to sustain under heat and drought stressed and to produce high number of filled grains than unfilled grains. The effects of Induced mutations by gamma rays were observed to help the rice plants to sustain under heat and drought stress condition and produce the economical grain yield. However, Porter and Semenov, (2005); Mahmood *et al,* (2010) reported that, both grain number and grain yield in many temperate cereal crops including rice appeared to be impacted by heat stress, with a decline in grain yield directly proportional with increasing temperatures during flowering and grain filling stage in heat susceptible genotypes. The high temperature above optimum level (35^{o}C) raised during panicle development, heading and flowering stages cause a high percentage of spikelet sterility in heat susceptible rice genotypes (Shah *et al*., 2011; Wopereis *et al.*, 2008; Sheehy *et al.,* 2005). High temperature during booting stage and flowering period lead to abnormal pollen development (Jiang-lin *et al.,* 2011) and increase pollen sterility (Matsui *et al.,* 2001) respectively, thus leading to a serious panicle infertility and decrease in grain yield in heat susceptible rice varieties. The results indicated that there was no significant differences in 1000 grains weight at 5% probability level among the mutant lines of different genotypes studied though there was significant differences within the lines of the same genotype such as WAB 56_104 (Appendix 2) and KR (Appendix 4). Generally this result indicates that the size of rice grains were not affected by the high temperature stress among all mutant rice lines.

4.8   Conclusion

The results of the current experiment indicate that Induced mutations by gamma rays created genetic variations among mutant rice lines studied. These variations were observed in growth performance, yield and yield components of the HT mutant rice lines. The induced mutation has a great impact in changing the genetic make of the rice plant as well as its physiological functions being affected and thus resulted into more productive and tolerance to environment stresses including heat and drought. This was observed from high grain yield and other variables produced from the mutant rice lines compared to non-mutant rice genotypes (its parents) in experiment conducted during hot and dry conditions.

There were significant differences at 5% probability level in number of tillers, number of panicles, number of spikelet per plant, sterility percentage and grain yield among the mutant rice lines. These variables can be used as criteria for selecting mutant rice lines which produce economic yield under heat and drought stress conditions. Eight mutant rice lines (KR 38_1, CG 14_63_1, CG 14_58_1, CG 14_16_1, CG 14_63_2, CG 14_61_3, WAB 56_104_141_2 and WAB 56_50_85_3) with high grain yield ≥ 3.5 t ha^{-1} and low spikelet sterility were selected. These mutant rice lines can be used as donor materials in Breeding Programs or can be released direct as a new high yielding, heat and drought tolerant mutant rice varieties. Seeds from these lines were processed and are reserved for increase and rescreening for stringent heat and drought stress under controlled environment chamber. This study recommend use MAS plant breeding so as genes associated with heat stress-tolerance can be easily identified and then incorporated to the adopted varieties hence improved varieties can be produced within short time as well as productivity be enhanced.

4.9 References

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CHAPTER FIVE

5.0   CONCLUSIONS AND RECOMMENDATIONS

5.1   Conclusions

Markers Assisted selection (MAS) approaches including TILLING with SNP markers should be applied for the purpose of improving crop productivity as well as increasing food security in order to combat the climate changes. The TILLING technique knowledge in mutation discovery of gene of interest assist plant breeders to enhance the crop breeding programs. DNA sequencing done from Mutant rice lines helped to get information to which nucleotide varied in heat tolerant genes due to Gamma rays-induced and natural mutations. Nucleotide variations in mutant rice lines resulted changes in genes functioning as well as agronomic traits of interest hence influenced mutant rice lines to be more productivity and tolerance to environmental stresses. The current study confirmed that there is association between the mutation effects and physiological processes (heat-stress tolerance) of the rice plant.

The clustering of the mutant rice lines based on DNA sequences helped to group together the mutant rice lines of the similar genetic makeup (DNA sequences). The point mutations discovered were found to be associated with HSPs genes including OS_HSP_17.9 and OS_HSP_90_1 in mutant rice lines. Local lesion (point mutation) discovered using TILLING techniques were revealed that has influence in the growth performance, yield and yield components of the mutant rice lines. The effects of mutations to hinder the gene function (s) it depends on its position in that gene;

mutation at coding regions or exon affect the gene expression but if occurred in non-coding regions or introns do not affect the gene expression. The high grain yields (6.8 t/ha) were observed from the HT mutant rice line (CG 14_16_1) which expressed point mutations (pair base substitutions) in Heat Shock Proteins gene.

5.2   Recommendation

TILLING technique is a simple reverse genetic tool which is used to discover mutations in gene of trait of interest using SNP markers (gene specific primers). This technique will be helpful in developing countries where there were no sophisticated molecular laboratory equipment for mutant discovery. DNA sequencing helps the Breeders to understand the nucleotides changed in gene of trait of interest due to mutation in relation to physiological changes of the intended crop. The sequencing of the targeted amplified DNA segments with specific gene primer(s) gives us information where mutations occurred in gene of interest but whole genome sequencing can give us the information where mutation occurred in the whole genomes of the mutant rice.

This findings recommend further study to be conducted on selected heat tolerant rice lines to sequence the whole genome to discover more mutations. Molecular markers for heat tolerance of rice to be developed also heritability study for HSPs genes to be conducted. This findings also recommend the study to be conducted on introgression of the HSPs genes to elite rice varieties preferred by the farmers in Tanzania hence to improve rice productivity. Refined HT mutant rice lines to the multi-locations to be released as HT mutant rice varieties and to be used by farmers.

APPENDICES

Appendix 1: Means summary of WAB 56_50 Mutant rice lines for yield and yield components

Rice line	Days to early flowering	Days to 50% flowering	Days to maturity	Plant height (cm)	Tillers number plant^{-1}	Panicle length (cm)	Panicle plant^{-1}	number Spikelet panicle^{-1}	Grains panicle^{-1}	1000 Grain (g)	Spikelet sterility	Grains Yield t/ha
WAB 56_50_51_1	80abcde	89abc	108abc	113cd	11ab	20ab	7abcd	11abcd	122abcd	31bc	18ab	3.3abc
WAB 56_50_56_1	77abcde	83abc	111bc	107abcd	11ab	21ab	6abcd	11abc	109abcd	30bc	21ab	1.9ab
WAB 56_50_56_2	80bcde	91bc	106abc	13cd	6a	21abc	6abcd	12abcd	127abcd	25a	15ab	3.3abc
WAB 56_50_74_1	77abcde	84abc	110abc	110bcd	7a	21ab	5abc	11abcd	112abcd	31bc	23ab	2.7abc
WAB 56_50_82_1	74a	82abc	111abc	99ab	10ab	21ab	7d	9a	147d	30bc	14ab	3.9c
WAB 56_50_85_2	76abcde	86abc	111abc	114d	7a	21abc	7cd	12bcd	138abcd	29abc	14ab	3.2abc
WAB 56_50_85_3	80ce	89abc	109abc	113cd	9a	21abc	6abcd	12abcd	140bcd	29abc	14ab	3.7bc
WAB 56_50_97_2	75abcde	83abc	107abc	109bcd	6a	24c	6abcd	11abcd	131abcd	30bc	11a	2.6abc
WAB 56_50_97_3	79abcde	85abc	105abc	106abcd	10ab	23bc	6abcd	10abc	105abcd	28abc	17ab	2.2abc
WAB 56_50_97_4	75abc	79a	104abc	104abcd	8a	22abc	7abcd	10abc	111abcd	28abc	18ab	3.1abc
WAB 56_50_98_1	77abcde	82abc	107abc	95a	9ab	22abc	7bcd	10ab	122abcd	27abc	19ab	2.4abc
WAB 56_50_98_3	77abcde	91c	108abc	108bcd	11ab	22abc	7abcd	10abc	96abc	30bc	17ab	2.5abc
WAB 56_50_123_1	76abcde	83abc	106abc	103abcd	15b	22abc	7abcd	11abcd	144cd	31bc	19ab	2.6abc
WAB 56_50_123_2	75abcde	81ab	109abc	101abc	8a	21abc	6abcd	12bcd	136abcd	31bc	13ab	2.2abc
WAB 56_50_123_3	75abcde	84abc	106abc	103abcd	7a	22abc	5abc	11abcd	118abcd	30bc	26ab	2.2abc
WAB 56_50_127_3	76abcde	84abc	113c	106abcd	7a	23bc	5a	11abc	111abcd	29bc	28b	2.4abc
WAB 56_50_127_5	78abcde	82abc	108abc	104abcd	6a	22abc	6abcd	10abc	110abcd	27abc	20ab	1.8a
WAB 56_50_135_1	74ab	85abc	108abc	104abcd	6a	21abc	6abcd	13d	136abcd	28abc	19ab	2.4abc
WAB 56_50_141_1	80bcde	85abc	98a	108bcd	6a	21abc	6abcd	11abcd	90a	31bc	16ab	3.1abc
WAB 56_50_141_2	77abcde	88abc	100ab	100ab	7a	20a	5ab	12cd	104abcd	29bc	22ab	1.8ab
WAB 56_50_152_3	75abc	87abc	98a	103abcd	8a	21abc	5ab	12bcd	94ab	27ab	15ab	1.8a
WAB 56_50 Control	75abcd	81abc	111abc	104abcd	8a	21ab	7abcd	9a	127abcd	28abc	20ab	2.5abc
Grand mean	76.79	84.76	106.92	106	8	22	6	11	119	29.06	18.2	2.62
S.E	3.173	5.296	6.647	6.3	3.2	1.2	1.2	1.2	24.2	2.138	7.85	0.961
CV	4.1	6.2	6.2	5.9	38.3	5.6	19.6	11	20.3	7.4	43.1	36.6
P0.05	0.196	0.345	0.343	0.037	0.178	0.142	0.145	11	0.168	0.106	0.632	0.245

Figures followed by the same letter (s) in columns are not significantly different at P≤ 0.05 according to DMRT

Appendix 2: Means summary of WAB 56_104 Mutant rice lines for yield and yield components

Rice line	Days to early flowering	Plant height (cm)	1000 grains wt. (g)	Grain Bird loss
WAB 56_104_36_1	76a	104ab	31b	14a
WAB 56_104_141_1	79ab	114b	30ab	18ab
WAB 56_104_141_2	81ab	101a	33c	12a
WAB 56_104_141_3	78a	110ab	30ab	25ab
WAB 56_104_150_2	84b	114b	30ab	24ab
WAB56_104 Control	79ab	108ab	29a	32b
Grand mean	**80**	**108**	**30**	**20.8**
S.E	**2.91**	**5.70**	**1.01**	**7.79**
CV	**3.70**	**5.30**	**3.30**	**37.40**
P0.05	**0.07**	**0.100**	**0.008**	**0.079**

Figures followed by the same letter (s) in columns are not significantly different at $P \leq 0.05$ according to DMRT

Appendix 3: Means summary of CG 14 Mutant rice lines for growth performance, yield and yield components

Rice line	Plant height (cm)	Tiller number plant[-1]	Panicle number plant[-1]	Sterility %	Bird loss %	Yield t ha[-1]
CG 14_16_1	112ab	30ab	25ab	10.5a	7.4ab	6.6bc
CG 14_20_1	103a	25a	21a	10.0a	7.2ab	3.2a
CG 14_58_1	108a	33b	27b	9.1a	6.0ab	6.5bc
CG 14_61_3	110ab	32b	27b	24.4b	14.3b	4.7ab
CG 14_63_1	118b	29ab	23ab	2.6a	1.9a	6.8c
CG 14_63_2	109ab	26a	21a	6.6a	4.6a	6.0bc
CG 14 Control	105a	28ab	24ab	3.0a	2.4a	4.0bc
Grand mean	**109**	**29**	**24**	**9.5**	**6.2**	**5.9**
S.E	**5.0**	**2.8**	**2.7**	**7.04**	**4.43**	**1.37**
CV	**4.6**	**9.8**	**11.4**	**74.3**	**70.9**	**23.4**
P0.05	**0.051**	**0.034**	**0.114**	**0.039**	**0.072**	**0.022**

*Figures followed by the same letter (s) in columns are not significantly different at $P \leq 0.05$ according to DMRT

Appendix 4: Means summary of KR Mutant rice lines for growth performance, yield and yield components

Rice line	Days 50% flowering	Grains/panicle	1000 grains weight (g)	Spikelet sterility %	Grain yield t/ha
KR 27-1	104a	33a	25a	50.7b	3.4a
KR 38-1	124.7b	49b	31b	43.6a	6.5b
KR control	122b	34a	27a	48.9a	3.7a
Gr mean	116.9	34	27.73	47.7	4.53
S.E	7.21	10.2	1.667	4.86	1.321
CV	6.2	26.2	6	10.2	29.2
P0.05	0.046	0.205	0.014	0.284	0.087

*Figures followed by the same letter (s) in columns are not significantly different at P≤ 0.05 according to DMRT