DETERMINATION OF THE LEVEL OF EXPRESSION OF OSCIPK15 SALT RESPONSIVE GENE IN SELECTED TANZANIAN RICE LANDRACES

KAMANGA NAOMI OLGA

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT FOR THE REQUIREMENT OF THE DEGREE OF MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

2016

ABSTRACT

This was an experiment to determine the presence and level of expression of OsCIPK15 salt responsive gene in Tanzanian rice breeders' lines. Abiotic stress is one of the factors affecting rice cultivation in Tanzania. The calcineurin B- like protein interacting protein kinases (OsCIPKs) responsive genes have been observed to express in abiotic stress. The calcineurin B- like protein interacting protein kinases-15 (OsCIPK15) salt responsive gene, which is usually a silent gene expresses in saline soils which is abiotic factor affecting yield. In this experiment eighty-four breeders' lines were used for the study. The lines were grown in sterilized sandy soil and grown for two weeks in the greenhouse. After this period the two week old seedlings were uprooted and the roots submerged in saline solution of 200mM concentration for forty-eight hours. Leaf samples were collected exactly twenty-four hours, twenty-nine and forty hours and stored at -80˚C The samples were thereafter analysed using quantitative real-time polymerase chain reaction (QRT-PCR). Of the eighty-four breeders' lines twenty-two gave a quantifiable analysis using the Livak delta analysis. Of the lines CSR 27, TXMS 1-2, TXM 18-1 and TXM 27 were the most tolerant and expressed the gene highly and TXM 13-2-3, GIZA 179, TXMS 14 and TXM 13-2-1 were the least. This experiment proves that Tanzanian breeders' rice have inherent ability to tolerate abiotic stress, such as salinity and the lines studied can be used in breeding programs to develop rice salt tolerant varieties to be cultivated in susceptible areas in order to provide profitable yield for paddy growing rice farmers.

DECLARATION

I, **Olga Naomi Kamanga,** do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and it has neither been submitted nor being concurrently submitted in any other institution.

Olga Naomi Kamanga	Date
(MSc. CandidateMolecular Biology &Biotechnology)	

The declaration is hereby confirmed;

Prof. Paul Gwakisa	Date
(Supervisor)	

Prof. Joseph Ndunguru	Date
(Supervisor)	

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to Mikocheni Agricultural Research Institute (MARI) for the material and technical support during research under the supervision of Prof. Joseph Ndunguru. Dr. Sophie Kashenge for generously donating her rice breeders' line seed material without which this experiment would not have been possible. Mr. Charles Kayuki, Lab Manager for technical support throughout the experiment process.

Secondly I would like to appreciate the academic guidance of the members of staff of the College of Veterinary and Medical Sciences, Sokoine University of Agriculture, in particular Prof. P. Gwakisa, Prof. G. Misinzo – Head, DrKasanga and Dr. H. Ntuntufye. The Intra ACP Programme under the coordination of Prof. D. Kamabarage and Prof. E. Karimuribo for the scholarship that enabled me pursue my studies.

Thirdly my gratitude is extended to the University of Zambia, my employers for providing a conducive environment for me to mature academically. In particular Prof. E. Mwase, Prof. Judith Lungu, Prof Yambayamba, Dr. Martin Simuunza and the late Dr. Cecilia Shinondo I thank you most sincerely for believing in me.

DEDICATION

To the four most important men in my life, JestoryChiheni, dad, friend, critic, confidant who has never doubted my capabilities and my sons Joseph Masuzyo, Paul Panji, Jacob Mapalo you give me purpose and reason to improve myself. Love all you unconditionally.

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LIST OF ABBREVIATIONS AND SYMBOLS

cDNA	complementary deoxyribonucleic acid
CDPK	calcium dependent protein kinase
CGSNL	Committee on Gene Symbolization Nomeclature and Linkage
CIPK	calcineurin B- like protein interacting protein kinase
Ct	Critical value
CTAB	cetylmethylammonium bromide
DNAse	deoxyribonucleic acid enzyme
EDTA	ethylenediaminetetra-acetic acid
gm/gms	gram/grams
MARI	Michocheni Agricultural Research Institute
Min/mins	minutes
ml	milliliter/millilitres
mRNA	messenger ribonucleic acid
^{o}C	degrees Centigrade/ Centrigrades
OsCDPK	Orzya calcium dependent protein kinase
OsCIPK	Orzyacalcineurin B-like protein interacting protein kinase
OsCIPK15	Orzyacalcineurin B-like protein interacting protein kinase 15
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
qPCR	quantitative polymerase chain reaction
RT-qPCR	real time quantitative polymerase chain reaction
RNA	ribonucleic acid
RNAse	ribonucleic acid enzyme
RT	reverse transcriptase

ssddH_2O	sterilized distilled water
Tris-HCL	hydroxyl-methyl –amino methane hydrochloride
USA	United States of America
%	percentage
~	approximately
µl	micro litre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Rice, *Orzya sativa*L*,* belongs to Kingdom Plantae, Division Magnoliophyta, Class; Liliopsidia, Order: Poales of the Genus Orzya. It is an annual, monocot cereal crop grown mainly for its carbohydrate content and is the third most consumed crop after maize and wheat. Rice is considered one of the most staple food in Africa and Asia which is cultivated by most paddy growers as a source of income as well as food (FAO, 2006).According to FAO(2012) rice is the thirdmost important agricultural cash crop in Tanzania. In the recent years, Tanzania has managed to satisfy most of the domestic market and is currently exporting in the region. The domestic market prefers locally grown varieties,which sell at a higher price than imported brands. This was also documented by Ngailo *et al*. (2007) that rice in Tanzanian is grown as an income generating venture by mostpaddy growers.

Paddy rice cultivation has seen low yields,partly due to irregular rainfall patterns leading to drought in some areas, disease manifestation such as blast, rice yellow mottle and weed infestation. Local rice varieties are mainly long maturing requiring a lot of water and highly susceptible to abiotic stresses.Abiotic stress is defined as inanimate components associated with climate, edaphic and physiological factors limiting plant growth (Dobermann *et al.,* 1999). The study byNgailo *et al.*(2007) also noted the need for high yielding seed varieties as well as seed that can tolerate abiotic stress such as drought. Human activity has also had an impact on increasing salt stress in the case of paddy rice cultivation in Tanzania especially as practiced by the small-scale producers. The need to understand the genetic makeup and ability of the Tanzanian rice to be able to adapt to

environmental stress in particular drought and salt tolerance can lead to the breeding strategies of seed having desired traits suited to the local conditions.

Plants can either be referred to as halophytes (salt tolerant) and glycophytes (non-salt tolerant).The degree to which the halophytes can withstand to salinity in the soil and be profitable is dependent on the varying genetic capacity of salt tolerance by the plant(Bhaska, 2015). Rice seedlings are most susceptible to saline stress as it is a glycophytic plant. Salinity with a conductivity as low as 5 – 6 dsm^{-1}can induce retardation in shoot and root elongation as well as dry matter accumulation in susceptible lines,because of physiological changes inhibiting plant growth by osmotic disruptions leading to ionic toxicity(Ali *et al*., 2006). Because of the genetic variability, the degree of physiological damage due to salt will also differ. The stages of stress will start with reduced water absorption capacity by roots followed by high accumulation of salt in the leaves which hyperosmotic stress. This stress results in membrane disruptions, nutrient imbalance, and inability of the plant to reduce reactive oxygen species (ROS). Hyperionic stress then follows which includes accumulation of Na^+ and Cl^- ions leading to ionic imbalance resulting in physiological disorder(Jenks, 2007).

Studies conducted in other parts of the world have indicated that there are responsive genes involved in the ability of plants to withstand abiotic and biotic stress which can be expressed in times of need (Chen *et al*., 2011). The genes are mainly regulated by calcium ions at cellular level. The expression of these calcium sensors can act as signal transducers of the stress signals. The signal transducers include the calcium dependent protein kinases (CDPKs), calcieurin B-like proteins (CBLs) and calmodulin-binding protein. It has been shown that expression of the*Orzya sativa* Ca^{2+}- Dependent Protein Kinase -7 gene (OsCDPK7) in rice was increased during cold, drought and salt stress (Saijo*et al*.,2000).

The calcieurin B-like protein–interacting kinases (CIPKs) genes are known to transcribe proteins in response to abiotic stress (Xiang *et al.,*2007). The understanding of the level of expression pattern of OsCIPK15 during salt stress and the physiological effect on the Tanzania rice lines may assist the breeders to select and breed rice tolerant varieties to be grown in affected areas.Salt stress is one of the abiotic factors, which has contributed to reduced yields in the paddy cultivation of rice in Tanzania.

1.2.2Justification

The rice-breeding program in Tanzania is trying to identify the rice lines that can be used for development breeding for salt tolerant varieties.To understand the process of plant response to stress, there was a need to determine the presence of these genes in Tanzanian breeding rice lines and their expression levels under stress condition. A molecular technique such as the use of QRT-PCR in the determination in local rice lines is a novel and can complement the trials in the fields. In the current situation of global climate change and the repeated practice by paddy farmers has led to the increase in the salinity levels of the growing fields The local varieties offer better starting material as they have adapted to the prevailingclimatic conditions. Furthermore; as observed by Duvick (2005), the use of local varieties has three main benefits. Firstly a narrow genetic base which results in a consistent breeds even though the process may be tedious and slow; secondly use of un-adapted exotic parents give rise to high performing genotypes that are unstable and easily destroyed crosses; and lastly germplasm selection is mainly based on phenotypes. It must be noted that the most popular breeding technique in rice is the backcross method which emphases on the phenotypic traits(Ali *et al*.,2006).

Understanding the genetics and ability to withstand a particular abiotic stress using molecular techniques such as quantitative polymerase chain reaction (QPCR)

undercontrolled conditions in greenhouse can give a picture of the ability of locally grown rice plants to adjust to salt stress. Identification of novel genes, determination of their patterns and understanding of their functions in stress adaption provide the basis of engineering to stress tolerance (Rabbani*et al.,* 2003). Plant breeders need to specifically target the salt tolerant landraces that can be planted in salt affected areas in order to maximize yields. This study was aimed towards determiningOsCIPK 15 salt responsivegene presence andconsistent expressionunder controlled condition in selected Tanzania breeders rice lines using the RT-qPCR.

1.3 Research Objectives

1.3.1 Overall objective

To determine the presence and expression profiles of salt tolerant responsive OsCIPK 15 gene in selected Tanzanian rice breeders' lines.

1.3.2Specific objectives

i. Screen for OsCIPK15 salt responsive gene in selected Tanzanian rice lines
ii. Determine the expression levels of OsCIPK15 genein salt stressed rice lines

1.3.3Hypothesis

i. The OsCIPK15 salt responsiv4e gene is present in all Tanzanian rice varieties
ii. Statistically there is no significant difference in expression of the gene in the selected Tanzanian rice breeders' lines

CHAPTER TWO

2.0LITERATURE REVIEW

2.1Background

Rice, *Orzya sativa*, is a major food crop second only to maize in Tanzania. Rice is not only an important carbohydrate source but it is grown for commercial purposes with Tanzania having the potential of exporting the commodity in the neighboring countries since it has the largest rice cultivated area in the region. Most of the local varieties originated from the Arab regions but have adapted to the local conditions and the palate of the local taste. Local varieties commonly grown in Tanzania include Supa, Behenge, Kula na bwana and Kalata. Theses varieties are usually long maturing and their low tolerance to saline conditions in prolonged cultivated areas has resulted in the reduction in yields. In Tanzania, rice is grown mainly in the Central Corridor, which includes Tabora, Shiyanga,Mbeyaand Morogoro regions amounting to 48% of the rice-cultivated area. Other regions of rice production include Manyanga, Singinda and Dodoma in approximately 230000 households(McLean *et al.,* 2013). The cultivated rice area is approximately 681000 hectares, representing 18% of total cultivated land. Rain fed rice growing is 71% and the rest is grown under irrigated traditional practice. Paddy rice growing, which is 48% in the Central Corridor have been affected mostly by the increasing salinity in the soil.The highest rice paddy areas includeMorogoro 19.7%, Shinyanga 18.5% and Tabora 10.2%. Yields are low approximately one to one and half tons per hectares (as opposed to the expected yields of two to four tons per hectare (Rural Livelihood Development Company, 2009).

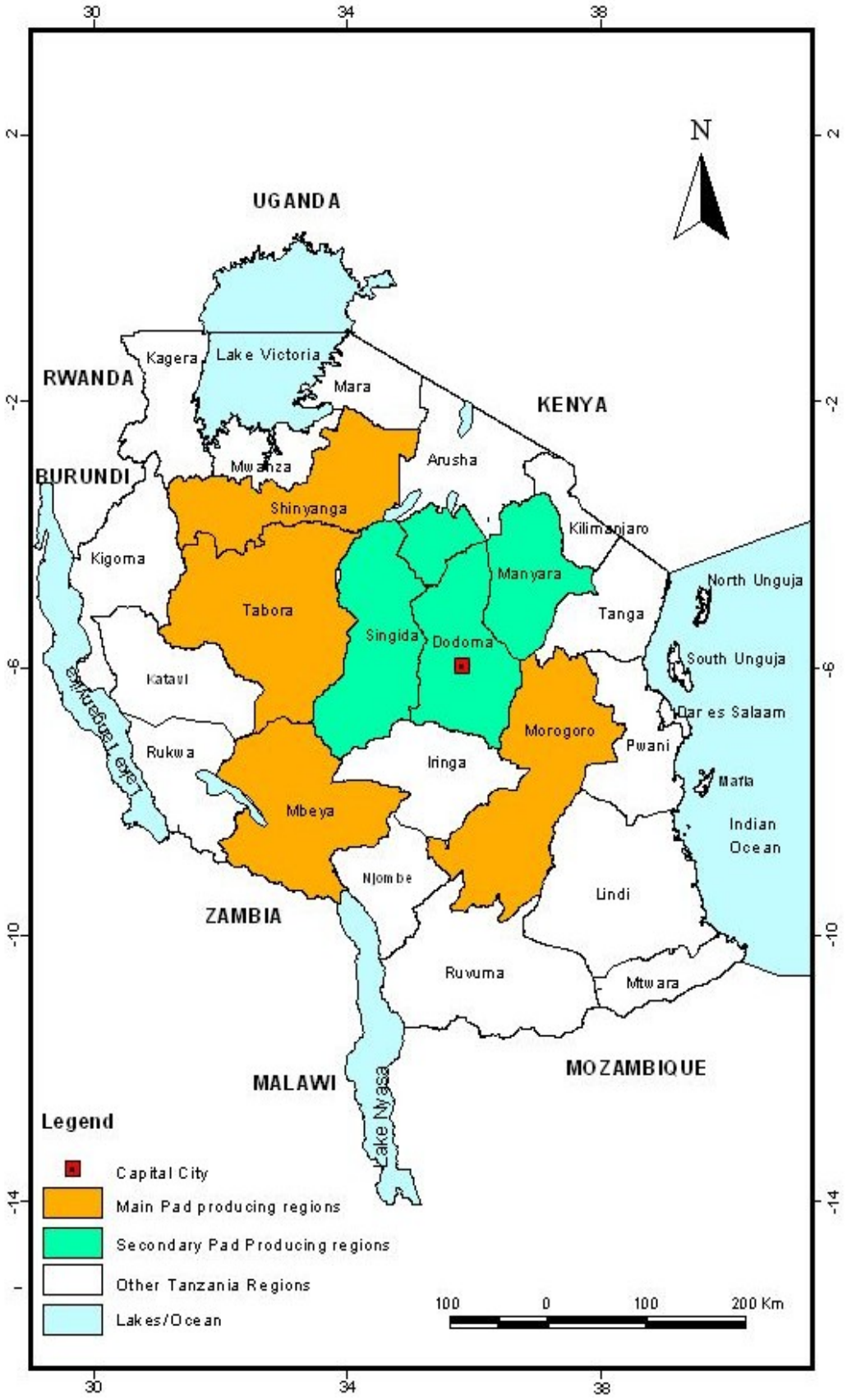

Figure 2.1: A sketch showing map of Tanzania Rice Growing Regions

Source: GIS SUA, Morogoro

2.2Prevailing Situation

In recent years, there has been a dramatic climate change with most areas having shortened rainfall patterns and in some cases drought conditions. There has been a shift therefore by plant breeders to find strategies of not only improving or increasing crop yield but also crops that can perform well under saline conditions requiring minimal water. Limited water sources as well as the salinity in most Tanzanian farming regions has also brought about decreased yields in rice (Mc Lean *et al*., 2013). Abiotic stress has been identified as a contributing factor to low yields. Yields are are as low as one to one and half tons per hectare (1-1.5 tons/ hectare) as opposed to two to four tons per hectare (2 – 4tons/hectare)(Rural Livelihood Development Company, 2009). Important abiotic stresses are light, water, availability, extreme temperature, salinity and cold (Nicot*et al.,* 2005). This experiment was concentrating on the salinity aspect.

2.3Molecular ApproachtoUnderstanding of Abiotic Stress

The molecular biologist is trying to find solutions in the understanding of the genetic makeup of the plants to try to help the breeder improve crop yield. It has been observed that the plant has inherit genetic makeup for physiological properties, which will express only under stress conditions such as drought, salt and cold. Genes involved in salinity tolerance are associated with regulation of transcription, transduction of signals, ionic balance as well as metabolic homoeostasis (Das *et al.*, 2015). Understandingthe mechanisms of expression will help to assist the plant breeder either increase the regulation responses to stress or try the reproduction of the classical metabolic or physiological processes (Cattivelli*et al*., 2008). Glycophytic plants such as rice can be affected by salinity leading to increased reactive oxygen species (ROS) which in turn result in membrane damage, oxidation of proteins and DNA fragmentation (Mishra *et al.*, 2011, Benitez *et al*., 2013). Salinity refers to the adverse effects salt in the soil has

onplants which can be it sodic (alkaline) salts or saline salts(Horie*et al*.,2012). Das *et al.*(2015) observed that salinity induces abscisic acid formation resulting instomatal closure, whose effects are photosynthesis reduction and photo-inhibition. The resulting physiological damage includes growth inhibition, senescence and if prolonged salinity may result in the death of the plant. The inhibition of cell growth is caused by abscisic acid synthesis.

2.4Effects of Salinity on Plant growth

Salinity stress is the result of two major ionic and osmotic stress. Ionic stress is the result of increased sodium ion (Na^+) and deficient potassium ion (K^+) leading to sodium ion toxicity via the root and the effects is seen in the leaf. This Na^+ toxicity causes alteration in leaf senescence disturbances of photosynthetic and protein synthesis pathways as well as enzymatic activity. Dehydration in addition to water uptake restriction,disturbances in leaf development are the effects of osmotic stress. If the signal transduction process between the osmotic and ionic is contained stress is reversible. Ionic homeostasis can be achieved by the removal, separation or reabsorption of Na^+ions.Ionic- , solute and organic compound accumulation leads to osmotic balance. Prolonged saline stress may lead to plant cell death(Horie *et al.*, 2012).

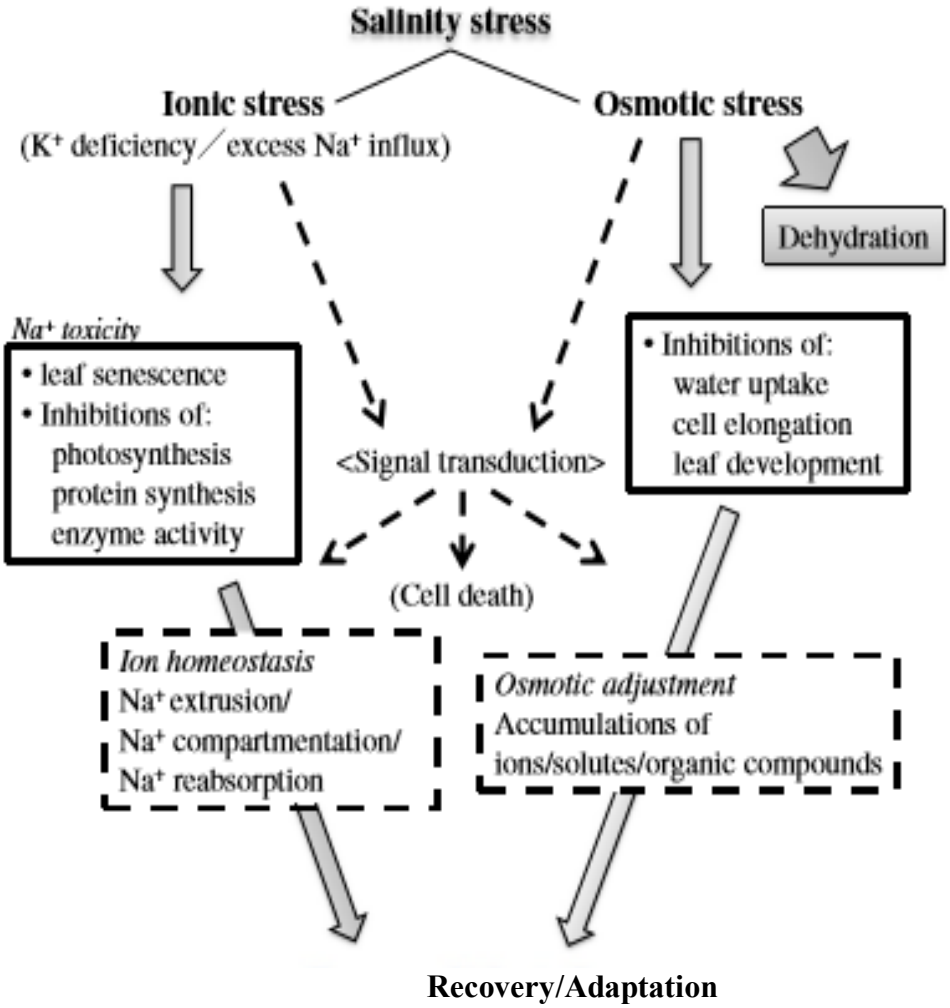

Figure 2.2:A schematic summary of the stresses that plants suffer under high salinity growth condition and the corresponding responses that plants use in order to survive these determental effects

Source: Horie *et al*. (2012)

Studies have shown that the particular stage at which the plant undergoes a particular abiotic stress will determine whether the crop will be able to give a good yield or not, the most critical stage for rice is the seedling stage (Ali *et al.*, 2006). Inability of a plant to tolerate salt stress can affectphysiological factors such as flowering time, plant height, ear type and osmotic adjustments since it is a quantitative trait. Osmotic adjustments are affected by salinity and water deficit a feature, which is currently prevailing in Tanzania hence the need for this experimentto study their effects on the local rice landraces.

Tolerance to salt by the plant is determined by the genes whose expression should be limiting the salt uptake, transportation throughout the plant, adjustment of the ionic and osmotic equilibrium in the root and shoot cells and regulation of leaf development and senescence ie. programmed cell aging (Munns, 2005).

There are mainly two types of salinity as described by Munns (2005).These are natural or primary salinity and secondary or human induced salinity. In natural/ primary salinity salt accumulates through the natural process in the soil or ground water over long periods of time due to weathering. Secondary or induced salinity is mainly due to human activity such as prolonged paddy cultivation practices.

2.5 Genes Involved in Abiotic Stress Mitigation in Rice

For rice plants (*Ozya sativa*) approximately thirty(30) putative OsCIPK (plant calcinerium B-like (CBL)- interacting protein kinases genes have been identified some of whose function has not been well established (Xiang *et al.*,2007). The Ca^{2+}controls the expression of these genes- dependent protein kinase genes(OsCDPK). The OsCDPK 7 isfound in the root and acts as a positive switch for most OsCPIK responsive genes of abiotic stresses. The other responsive genes OsCIPK03, OsCIPK12 and OsCIPK15 have been observed to be useful in stress tolerance improvement due to their expression during cold, drought and salt stress respectively. This study aimed at determination of the levels of the salt responsive gene OsCIPK15 expression in local Tanzania varieties under induced salt stress condition. Other genes involved in cold, drought and salinity as well as wounds and blast disease infection are the NAC transcription containing genes, which were first seen in Arapdopsis species, in rice, the gene was identified as OsNAC 6 (Nakashima *et al.*, 2007). Here it must be noted that resistance to salt or any other abiotic stress is not the function of one gene but is a polygenic feature(Fountain *et al.*, 2010). Salt

tolerance for instance is a quantitative trait, which can be affected by genes controlling flowering time, plant height, ear type of grain and the ability of the plant to adjust to osmotic changes in the soil. It can therefore be expected that the gene for the expression of salt tolerance to be in close proximity with other stress responsive genes such as cold and drought. As explained by Benitez *et al*.(2013) gene function is the involvement of multiple loci and varying loci in the genome which work in coordination to mitigate stress including salinity. This means that the physiological and morphological adjustment of the plant is the product of many gene linkages in the plant genome working in synchrony to adjust in stressful times. As observed by Zeng and Shannon (1998) the effects of salinity are seen in decreased panicle length, reduced spikelet number and reduced grain yields these are the results of delayed panicle emergence and flowering but the study observed that the seed stage was not affected.

Calcium dependent protein kinase (OsCDPK7) gene was identified as one of the calcium dependent protein kinases, which is involved in enhanced expression of stress responsive genes in rice. It has been shown in studies that stress responsive calcineurin B-like protein-interacting protein kinases (CIPKs) initiate the signaling cascade in stress response in the roots by forming complexes with calcium dependent protein kinases(Chen *et al*., 2011). This initial signal activates transcriptional responses to abiotic stress including cold, drought and stress In particularOsCIPK15 genes have been involved in the response against salt stress and were first identified in Arabidopsis species(Chen et al., 2011). The calcineurin B-like protein- interacting protein kinases (CIPKs) contain Ser/Thr protein kinase domain activated through the interaction of Ca^{+2} binding. Upon activation there is a downward transduction by phosphorylation signals (Xiang *etal*., 2007).

Saijo*et al*.(2000) observed that over-expression of a single Ca^{2+}- dependent protein kinase activated the ability of rice plants to endure salt stress due the expression of the

OsCDPK7. The OsCDK7 wasidentified as the positive regulator of abiotic stress responsive genes of the OsCIPKfamily, which includesOsCIPK 15.

Table 1: Classification of OsCD7 and OsCIPK15 Genes in rice

Gene Symbol(S)	CGSNL Gene Name	Gene Name Symbol	Chromosome Number	Trait Class
OsCDPK 7	Calcium-dependent Protein Kinase 7	calcium dependent protein kinase	3	Biochemical character Vegetative organ-root Resistance to disease-Stress tolerance
OsCIPK15	calcineurin B-like protein-interacting protein kinase 15	CBL-interacting protein kinase 15	11	Biochemical character Vegetative organ-leaf Stress tolerance Resistance to disease

Source: Chen *et al.* (2011)

Table 2: Genomic information of OsCIPK 15

Gene Bank Accession Number	Intron number	Chromosome	cDNA length(bp)	Number of Amino acid	Predicted molecular weight (kD)
AB264037	0	11	1305	434	49.5

It must be noted that the exact mechanism in which the plant adjusts to the changes in the environment cannot be fully explained physiologically; the answers may be observed by studying the genomics of the crops at molecular, cellular as well as developmental responses (Tuberosa*et al.,* 2006).

2.6 Gene Expression Analysis

The OsCIPK15 expression protein products were assessed in the analysis of the leaf samples using RT- qPCR (Chen *et al*., 2011). In order to quantify the level of gene

expression the reverse transcription polymerase chain reaction may be used(Livak *et al.,* 2001). Earlier analyses were northern blot procedures which were time consuming and required larger amounts of RNA products (Nicot *et al*., 2005).

According toBrandt (2005) gene expression analyses can be categorized into two groups, which are analysis without amplification and analysis that require amplification prior to analysis. In the former category sequences from the gene of interest are used to transform the plant of interest resulting in transgenics which have vast variety of the gene expressed at uncontrolled and of un-quantified proportions. Techniques used here include fixation, and tissue embedding and the metabolic pathways of the target organism are undisturbed. Whereas in procedures of amplification prior to analysis mostly involve the PCR step, quantification of the target gene is achieved by comparison to a reference gene, which is usually a house keeping gene. In order for the quantification to be made the extracted mRNA has to be reverse transcribed to cDNA. The cDNA of the gene of interest is then comparatively quantified to the cDNA of the reference gene. A reference gene is a house keeping gene whose expression remains constant between the cells of the various tissue in the plant (Jain *et al.,* 2006). Examples of housekeeping genes include β-actin, glyceraldehyde-3-phosphate hydrogenase (GAPDH), ubiquitin (UBQ) and elongation factor -1-alpha (ELF-1-α) (Li *et al.,* 2015).Normalization of the cDNA is also of paramount importance if the quantification is to be of meaning (Wuytswinkel *et al*., 2009). The normalization process takes into account sample variations in total mRNA extracted. The target mRNA quantity is therefore a ratio of target mRNA: total mRNA. This means that the quality, accuracy, quantity of the initial mRNA determines the resultant quality and quantification of the target mRNA readings.

In this study the quantification of the geneOsCIPK 15 was determined by the real time quantification reverse transcriptase polymerase chain reaction (RT-qPCR). The reference

gene used wasEukaryotic elongation factor 1 – alpha AK061464. According to Li *et al*., (2015) a reference gene should be a gene with constant level of expression and not affected by experimental elements in this case salt stress.The procedure included the extraction of total ribonucleic acid (RNA) from fresh leaves converted to complementary deoxyribonucleic acid (cDNA) as per standard procedure in Appendix 2. The RNA quantityincrease/decrease is evidence of the gene activity during the stress period.The OsCIPK 15 gene expression analysis was done using real time quantitative polymerase chain reaction (RT-qPCR), which can be referred to as a closed system.Another example of closed system analysis are microarrays. Microarrays are engraved grid of known DNA segment fragment used for testing and mapping unknown DNA, antibodies or proteins. Other tools of gene expression studies include complementary deoxyribonucleic acid amplified length polymorphisms (cDNA–ALPs), serial analysis of gene expression (SAGE) and massive parallel signature sequencing (MPSS) which are open systems (Benitez *et al*.,2013;Li *et al*.,2015). In this study the QPCRwas chosen due to the fact that the amount of RNA extracted can be amplified even if it was produced in minute quantities as observed by a functional genomics study by Perez-torres*et al*.(2009)as well as considering the time frame in which the study was to be conducted.

2.7Real Time - Quantitative Polymerase Chain Reaction (RT-qPCR)

The RT - QPCR procedure was selected due to the high sensitivity and the ability to detect minute genomic product in real rapid time given the time period the experiment was to be conducted(Nicot *et al*., 2005). RT qPCR is currently the most sensitive tool requiring low quantity of genomic material, apart from that the real time QPCR has the advantage of detecting multiple genes simultaneously. It can be used for disease diagnosis, tissue specific gene expression analysis and plant studies. Since the genes responding to abiotic stress are multigenic, quantitative and complex traits controlled by one quantitative

traitloci (QTL) makes RT – qPCR a valuableprocedure, which can detect the minute subtle changes. The QTL in this study was the OsCDPK7 gene which controls at least thirty(30) putative CIPK genes including the cold (OsCIPK03), drought (OsCIPK12) and salt (OsCIPK15) responsive genes (Xiang *et al*., 2007). The rice was planted and later the two week old seedlings were exposed to saline solution at a concentration of 200mM per litre Gregorio *et al*.(1997) observed that the seedling stage was the most susceptible to salinity causing alteration inmorphological and homeostatic state of the plant.The concentration of 200mM of NaCl. This was determined as by the study done by Gregorio *et al.*(1997). According to Munns (2002), most plants are affected by reduction in biomass by 150 dS/m EC which is at a concentration of 150mM. Of the three most important carbohydrates wheat, maize followed by rice the level of salinity tolerance are in that order. The salt concentration used of200mM, which is approximately 19.6 dS/m EC. Soils are classified as saline when the EC is ≥ 4 dSmwhich is equivalent to 40μM NaCl (Munns, 2005). Das *et al*. (2015) documents that at a salt concentration of 30mM yields rice grain starts to reduce and at an EC of 3.0dsm^{-1} rice seedlings can tolerate and recover, but this is also determined by the amount of water received, soil pH, sunlight, and temperatures prevailing at the time.

Table 3: Salt Concentration to Electrical conductivity Conversion

Solution	EC DS/M
10 mM	1.0
100 mM	9.8
500 mM	42.2

Source: The Impact Salinity Stress, Dr. Rana Munns CSRIO Division Canberra ACT, Austria

CHAPTERTHREE

3.0MATERIALS AND METHODS

3.1Greenhouse Experiment

Sand was heat sterilized by charcoal oven for an hour and allowed to cool. One kilogram of heat sterilized sand was transferred into 1.2 kg capacity plastic pots into which four to six seeds of the breeder' rice line (Appendix 1) were planted. Water (200ml) wasadded daily to each pot for two weeks. Four to six rice seeds were planted in a pot, duplicate for each rice line all in the same room and at the same time with equal volumes of water every day.

3.2 Stress Inducement and Sample Collection

The two-week-old seedlings(saline treated) were uprooted and immersed into saline solution of 100ml volume in 150ml capacity plastic containers. The saline solution was a stock of 200mM concentrationof NaCl, which was a one-time preparation in bulk. Leaf samples were collected exactly after 24 hrs, 29 hours and 48 hours stored at minus eighty (-80°C) degrees for further processing.The first leaf samples taken at exactly 24hrs after submersion in saline were labeled Treatment 0, those taken after 29 hours were labeled, Treatment 2 and the last batch Treatment 3 were collected at 48hrs from the start of sampling. The other duplicate seedlings (non-saline) were not subjected to any saline treatment and sampled only once.

3.3 Extraction of Rice Leaf Total RNA Using the Modified CTAB Protocol

Leaf samples were freeze-dried in liquid nitrogen immediately handground with pestle and mortalthe mRNA was extracted as per the modified cetylmethylammonium bromide (CTAB) protocol as explained in Appendix 23.4 Determination of Extracted Rice Leaf mRNAintegrity.

Quality of mRNA was verified by gel electrophoresis using 1.5% (w/v) agarose gel and 2% ethidium bromide was added for visualization. Sample volume for loading was 5µl and visualization of gels was achieved by using BioDoc- It Imaging Systems Bench top M20V UV trans-illuminator,(California, USA). The quantity and quality was determined using the spectrophotometer (Appendix 3).

3.5Cleaning of Rice leaf mRNA and cDNASynthesis

The mRNA cleaningwas conducted to remove DNA contamination before the cDNA synthesis. The cycler used was the Applied BiosystemsGeneAmp2720 Thermocylers andreagents used in mRNA cleaning and cDNA synthesis were acquired from New England BioLabs.

3.5.1 mRNA Cleaning

Total RNA of 35µl volume was mixed with 4µl 10xDNAse 1X buffer and 1µlDNAse enzyme in a 50µl volume eppendorf tubeand incubated at 37ºC in thermocycler. A voulume of 4µlDNAse deactivator was added, mixed well and vortexed for two minutes. Centrifugation followed at 1000 rpm for 5 min to settle the deactivation agent. Supernatant (clean mRNA) was gently transferred to fresh, sterile eppendorf tube avoiding the pellet at the bottom.

3.5.2cDNA Synthesis

The reverse transcription (RT) was performed in a 25µl volume containing 1.0 µl oligo (dTs), 2.0 µldNTPs, 10.5 µl sterile doubledistilled H_2O and 2.0 µl RNA template at 65ºC incubation for 10 min and stored at 4ºC in the thermocycler. The secondmaster mix contained RT termination stage addition of 2.0 µlsterile double distilled H_2O, 10x RT buffer,RTNxGEN reverse transcriptase and RIBBLOCK 0.5 µlRNAse InhibitorIncubation

was at 42ºC for 50 min. followed by 85ºC The temperature was 85ºC was for enzyme deactivation. The cDNA products were stored at 4ºC in the fridgefor further downward analyses.

3.6Determination of cDNAQuality Using Agarose gel Electrophoresis

Quality of cDNA was verified by gel electrophoresis using 2% (w/v) agarose gel and ethidium bromide was used as a staining agent. Sample of 5µl was loaded and visualization was achieved by using BioDoc- It Imaging Systems Bench top M20V UV trans-illuminator(California USA).

3.7Normalization of cDNA

The concentration and purity of cDNA was checked using Thermo Scientific Nano Drop 2000 UV-Vis Spectrophotometer, (Massachusetts USA)at 260/280nm. Readings are tabulated in Appendix 4. The cDNA was normalized at 100ng/µl to a 50µl volume of sterile distilled water. The normalized cDNA was stored at 4°C for further use.

3.8Determination of QuantitativePolymerase Chain Reaction (QPCR)Conditions andAnalysis

The conditions used for QPCR were modified from the protocol of Chen *et al.*, (2011). Denaturing at 94ºC followed by 40 cycles of 94ºC for 30 sec, 42ºC for 1 min and lastly 72ºC.The SYBR green/ROX qPCR kit (Thermo Scientific,) was as dyes in QPCR.PCR products were stored at -20°C. The QPCR machine used was a model from Agilent Technologies Stratagene System Mx3000 version 4.10. The reaction mixes were as presented in Table 4. The housekeeping gene was Elongation factor 1-α.The primers used are shown in Table 5.

Table 4: Preparation of QPCR mix for the expression profile

Component	Volume for one reaction(µl)
SYBR Green	10
ROX	0.2
F- primer (10 µl)	1.0
R –primer (10 µl)	1.0
ddssH_2O	5.98
plus cDNA template	2.0

The primers used were as follows:-

Table 5: Primer Sequences for detection of OsCIPK15 and Elongation Factor 1-α

Gene	Sequence 5' – 3'
OsCIPK 15	Forward primer GTTACCACTTCCTATCATATCATC
	Reverse primer GTTACCACTTCCTATCATATCATC
Elongation Factor 1-α	Forward primer TTTCACTCTTGGTGTGAAGCAGAT
AK061464	Reverse primer GACTTCCTTCACGATTTCATCGTAA

Primer sequence of gene of interestOsCIPK 15 primerswere all ordered fromInqaba Biotechnical Industries (Pty) Limited, (South Africa).

3.9 Determination of Relative Quantity

Livak and Schmittgen, Method (2^(-ΔΔCT) method.Formula

CT = (CT_1OsCIPKK15–CT*I*ELFA)Time *x* (CT,OsCIPK15 -CT,ELFA)$Time_0$

(Li *et al*., 2015). Assumption of efficiency is 100%. The charts depicting the changes in expression were generated from Ct values using Excel 2007.

Statistical Analysis

The analysis of variance (ANOVA) wasdone using IBM SPSS version 22 software (Appendix 4) The PosHoc analysis was the Duncan test using the same software.

CHAPTER FOUR

4.0RESULTS

4.1 Determination of Presence of Gene Activity Using Conventional PCR

The two-week seedlings were divided into two groups the first group (saline exposed) was subjected to saline solution while the other (non-saline) was not. In both conditions, mRNA was extracted using modified CTAB protocol shown in Appendix 2. The mRNA was converted to cDNA. In the non-saline samples, upon conductingconventional PCR to determine presence of the gene, there was no band present in the gel (Fig. 4.1). M denotes the marker, numbers 1 – 13 are the saline treated samples and the other 1 – 6 are the non saline sample But in the treated samples the bands were present numbered 1 – 20 (Fig.4.2).

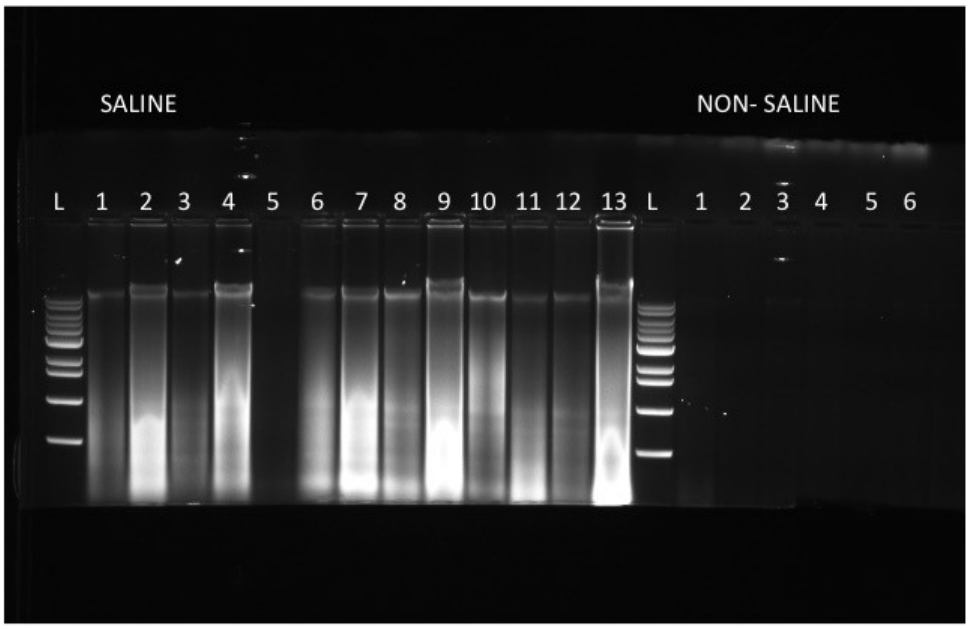

Figure 4.1: Gel electrophoresis picture confirming presence of mRNA in samples

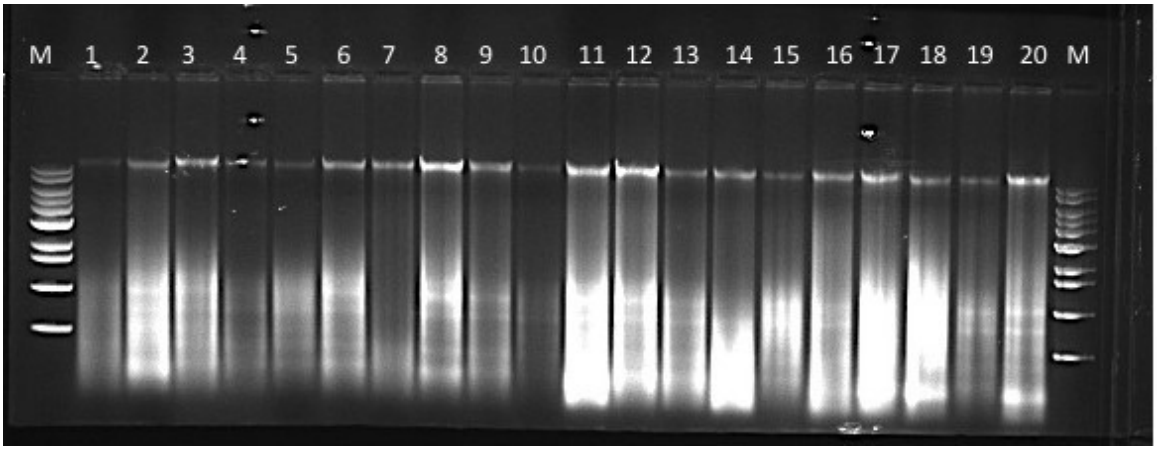

Figure 4.2: **Gel pictures of selected samples of saline exposed rice leaf samples mRNA**

4.2Quality and Quantity of Synthesized cDNA

The quality of the cDNA was investigated using gel electrophoresis The quality was poor in sample number 1, 12 and 13 and 2 but the rest others 2 – 14 the quality was good.The sequence used cDNA synthesis was forward primer GTTACCACTTCCTATCATATCATC and the reverse primer was GTTACCACTTATCATATCATC

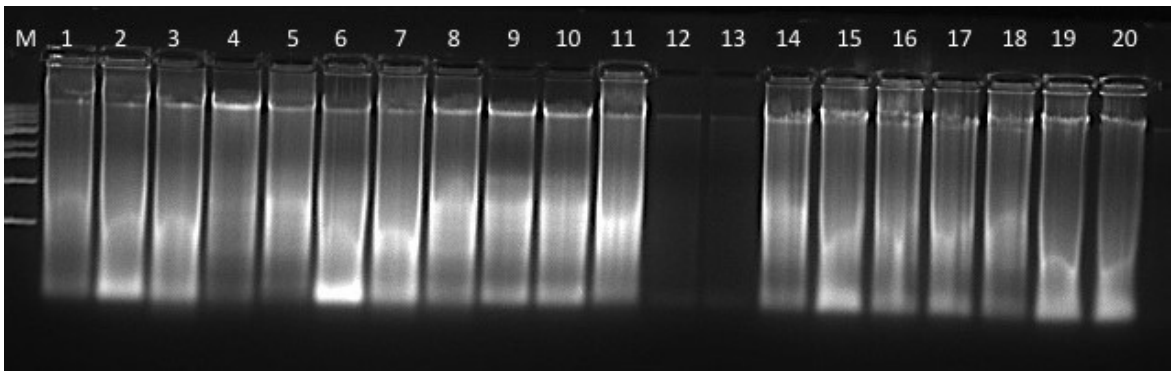

Figure 4.3: Gel electrophoresis of selected cDNA products

The quantity of cDNA wasexamined by spectrophotometry A280/A260 as in the Appendix 3.

4.3QPCR Analysis of cDNA

The suitable QPCR conditions were investigated by convectional PCR using Prime Techne Gradient thermocycler. The PCR products were examined by gel electrophoresis and a suitable temperature for QPCR selected. The PCR product of expected sizes were obtained as shown in Fig. 4.4

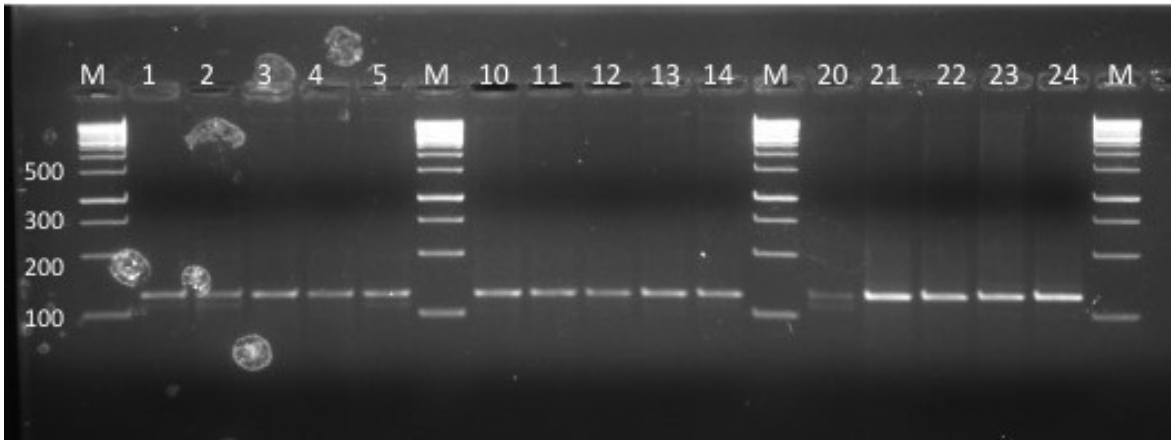

Figure 4.4: **Determination of QCR annealing temperature**

The QPCR reaction conditions were as follows: denaturation was at 94°C for 2 min followed by 40 cycles of 94°C for 30 secs, 42°C for 1min and lastly 72°C for 1 min, modified from Chen *et al*. (2011). The Ct values

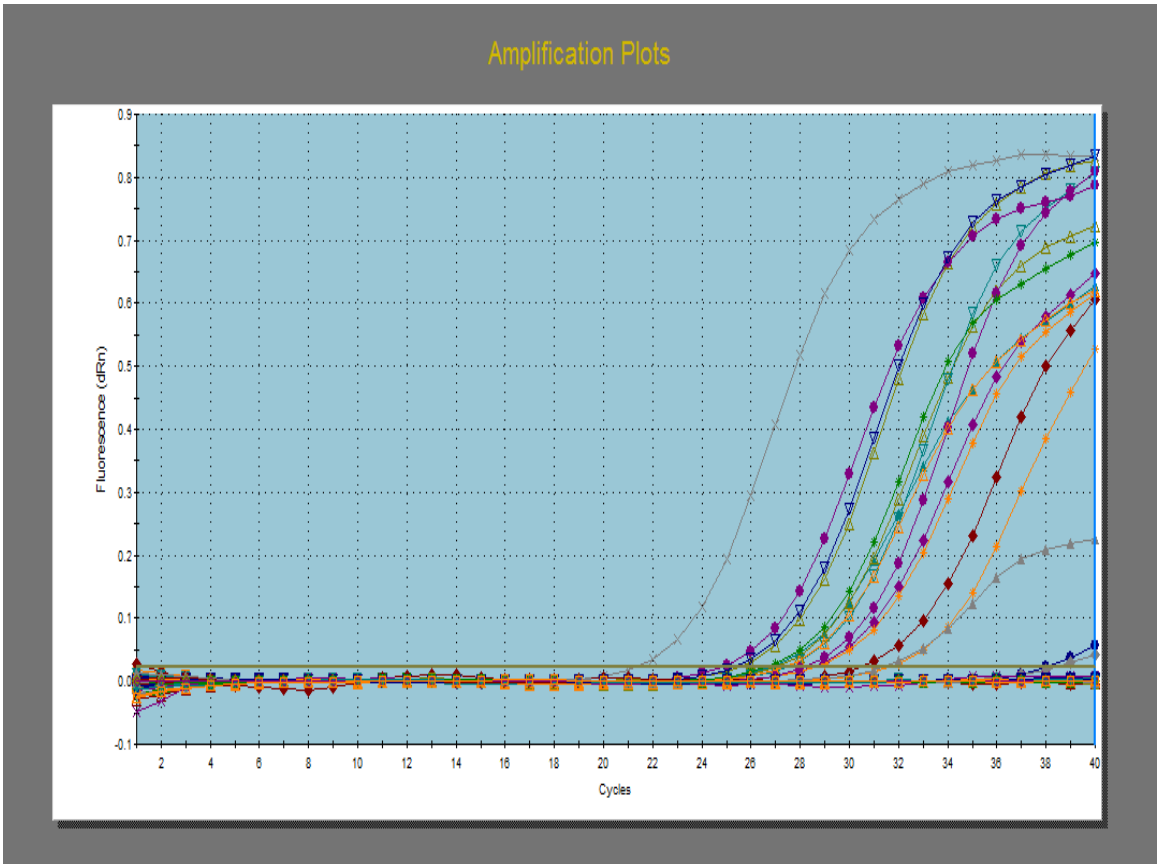

Figure 4.5: **A picture of the amplification plots observed after a QPCR run observed on Agilent Technologies Stratagene System Mx3000**

The Fig. 4.5 depicts the real time quantification polymerase chain reaction picture after completion of running an analysis. The point at which the graphs start t peak is the point of critical point at which the critical (C_t) value is. In Fig. 4.5 the first C_t value is at 21 cycles the last C_t value was generated after 38 cycles.

The initial numbers of rice lines were eight-four (84) out of which after the first analyses 36 lines were able to give quality readings. The first C_t values were obtained at Treatment 0 (after 24hrs) were the control C_t. The figure below is the expression after 5hrs (Fig. 4.6)

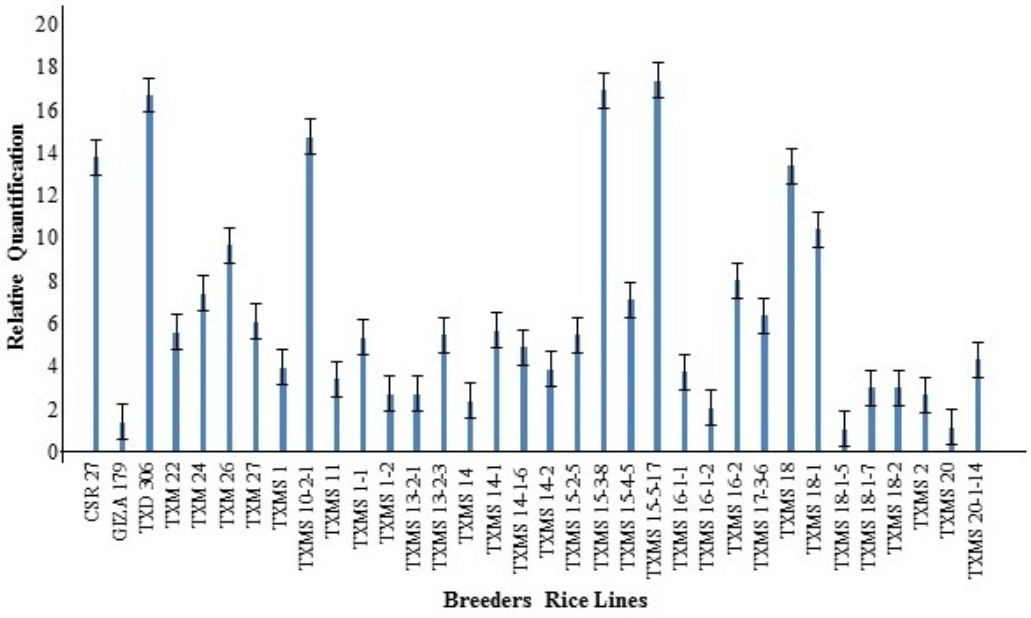

Figure 4.6: OsCIPK 15 Gene Expression Profile in selected Rice Breeders lines after 29 hrs

The second figure(Fig. 4.6) denotes the situation after fourteen hours.Only thirty-four lines out of the eighty-four lines showed expression patterns detectable using QPCR at this time. Here the lines with the highest expression of the gene OsCIPK15 were TXD 306, TXMS 10-2-1, TXMS 15-3-8TXMS 15-5-7 while TXMS 18-1-5 was the least expressed.

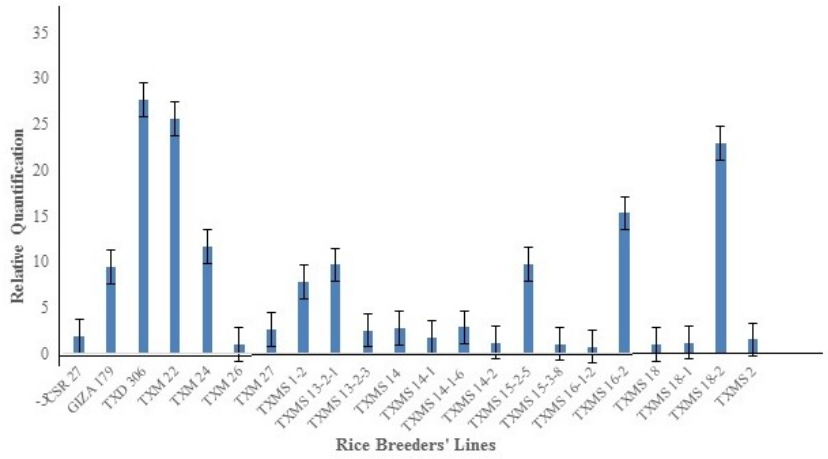

Figure 4.7: OsCIPK 15 Gene expression profiles in twenty-two lines Tanzanian Rice Breeders Lines after 48hrs

This graph (Fig. 4.7) denotes the overall relative quantification pattern after 48hrs of saline stress. Onlytwenty-two (22) lines were able to tolerate the stress up to this stage.Here the lines TXD 306, TXM 22, TXM 18-2 and TXMS 16-2 expressed highest whilst TXM 26, TXM 14-2, TXMS 15 -3-5, TXMS 15-3-8 TXMS 18 were the lines with the lowest expression.

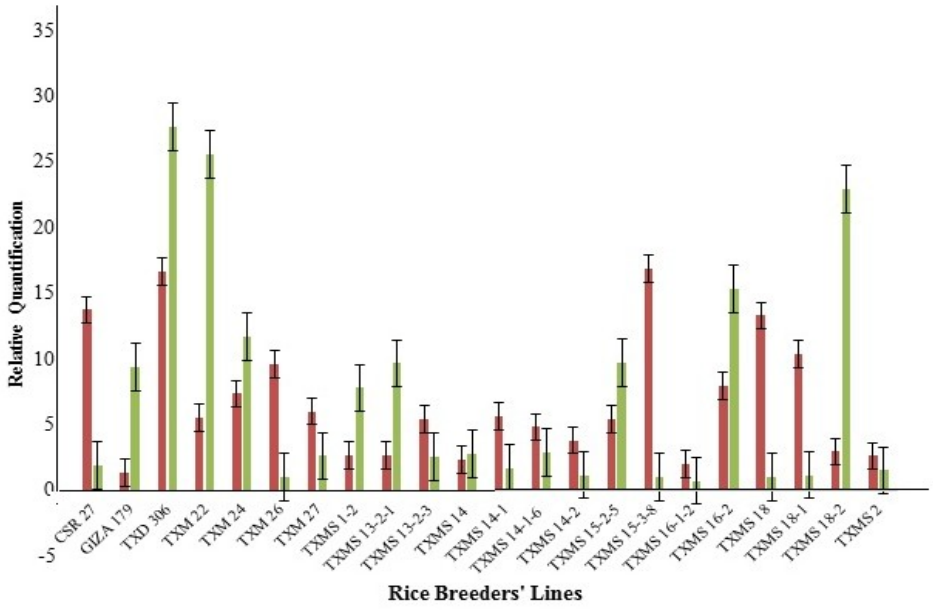

Figure 4.8: Overall OsCIPK 15 Gene expression profiles in Tanzanian rice breeders lines profiles of the twenty-two lines after forty-eight hours of saline exposure

Fig. 4.8 denoted the expression profile of the rice lines after 48 hrs of saline exposure. Here notably 22lines have shown the capacity to with stand saline solution exposure. TXD 306, TXM 22, TXM 18-2 and TXMSW 16-1-2 were the best lines in terms of increased expression over time. On the otherhand CSR 27, TXM 26, TXMS 15-2-5, TXM 18 and TXM 18-1 expression decreased as time elapsed.

4.5 Statistical Analysis Results- ANOVA

When determining effect of the time on the C_t values, at alpha 0.05, therewere significant differences in expression between subjects over time(Appendix 4). Using the Duncan Posthoc analysis, the table below gives an indication of the significant differences between tolerant lines of the experiment(Table 6).

Table 6: Duncan PostHoc Analysis Table

LINES	Mean	Std. Error of Mean
CSR 27	34.62a	±1.58
TXMS 1-2	33.49ab	±0.42
TXM 22	28.38abc	±1.38
TXM 27	29.38abc	±2.92
TXMS 18	29.01abc	±1.17
TXMS 18-1	30.13abc	±1.51
TXMS 18-2	28.94abc	±1.11
TXD 306	27.91cdefghi	±2.46
TXM 14-1	28.72cdefghi	±1.36
TXM 24	25.29defghi	±1.11
TXM 26	24.57cdefghi	±0.93
TXMS 14-1-6	27.65cdefghi	±0.68
TXMS 14-2	26.21cdefghi	±0.79
TXMS 15-2-5	28.13cdefghi	±0.76
TXMS 15-3-8	28.30cdefghi	±1.45
TXMS 16-1-2	27.08cdefghi	±1.00
TXMS 16-2	26.71cdefghi	±0.34
TXMS 2	26.87cdefghi	±0.78
TXMS 13-2-3	24.37fghi	±1.54
GIZA 179	23.75ghi	±0.79
TXMS 13-2-1	22.28i	±0.47

Mean in the column with same letters do not differ significantly from each other at $p<0.05$. Lines arranged in order of expression levels from highest to lowest. The highest expression was CSR 27 followed by TXM 1-2, TXM 22, TXM 27, TXM 18, TXM 18-1 and TXM 18 -2 collectively. The lowest expression was in TXMS 13-2-1.

CHAPTER FIVE

5.0DISCUSSION

The determination of the presence of the gene products in the treated samples, which was the mRNA is an indication of the expression of the salt responsive gene OsCIPK15 as observed in Fig. 4.1and 4.2. The genomic DNA translates to total mRNA, but to confirm that the gene is activated when the plant is subjected to saline conditions, conventional PCR was conducted to amplify the target mRNA. The absence of mRNA bands(Fig. 4.1) in the non saline samples indicates that the OsCIPK15 gene is a silent gene which is responsive when saline content increases and only transcribes the mRNA as seen in presence of bands in the gel pictures of the saline group (Fig. 4.2). This is also proof that the OsCIPK15salt responsive gene is present in the local Tanzanian rice genome. The time taken however for the gene to express cannot be determined exactly but twenty-four hours after subjecting the seedlings, the gene productie mRNA was detectable. It also confirms that the root is involved in the stress effect of the plant since the seedlings were submerged in the saline solution and by transpiration; the saline was transported from root to the leaves. The OsCDPK7 occurs mainly in the root and is the positive regulator of OsCIPK15(Chen *etal*., 2011).

However, the intensities of the bands varied in different saline exposure lines due to the varying degrees of levelsexpression in the various line Fig. 4.2.The differences were also observed in readings after conducting the QPCR and the resulting differences in Ct readings. The lines were eight-four (84) in the beginning of the experiment but after the five (5) hours of saline exposure thirty-four (34) lines had detectable expressionusing the Livak analysis (Fig. 4.6). Here CSR 27, TXD 306, TXMS 10-2-1, TXMS 15-5-7, TXMS 20-2 expressed the highest whilst GIZA 179, TXMS 16-1-2, TXMS 18-1-5 and TXMS 20

had lower quantities After further time lapse of twenty-four hours there were only twenty-two lines tolerant Fig.e 4.7. This means that the twelve lines; which were able to express in the first five (5) hours could not tolerate the saline concentration of 200mM for longer. These were TXMS 1, TXMS 10-2-1, TXMS 11, TXMS 1-1, TXMS 15-4-5, TXMS 15-5-7, TXMS 15-5-7, TXMS 16-1-1, TXMS 17- 3-6, TXMS 18 -1 5, TXMS 18-1-5, TXMS 20 and TXMS 20 – 1-14. Of the lines, which expressed but in reduced levels in subsequent recordings were CSR 27, TXM 26, TXM 27, TXM 15-2-5, TXM 18 and TXMS 18-1 Expression in GIZA 179, TXD 306, TXM 22, TXM 24, TXMS 1-2, TXMS 13-2-1, TXMS 18-2 increased in the third readings. The lines TXMS 14, TXMS 14-1 and TXM 2 did not change.

In order to determine if the lines were statistically different, an ANOVA (Appendix 5) was run and the results showed a significant difference at $p<0.05$. A further posthoc analysis using Duncan Test (Table 5) revealed that statistically TXD 306, TXM 26, TXM 14-1-6, TXMS 14-2, TXMS 15-2-5, TXMS 15-3-8, TXMS 16-1-2, TXMS 16-2 and TXMS 2 were similar in terms of expression profiles. The lines CSR 27, TXMS 1-2, TXMS 18 and TXMS18-1 were the best expressed lines for the salt tolerant responsive gene OsCIPK15.

According to (Munns, 2005.) saline concentration of 150mM can cause a fifty percent (50%) reduction in biomass and affect the plants physiological balance. The concentration used in this experiment was 200mM. In this experiment it must be noted that even if the seedlings didn't withstand the salt concentration exposed to this is an indication that they are not tolerant to salinity stress but the concentration used here was higher than what prevails in the field. According to Das *et al*. (2015) rice yields can be affected by saline concentration of as low as 30mM, since rice is glycophytic plant and a conductivity of as little as 3.0 dsm^{-1} can cause damage but seedlings can recuperate when conditions improve

such as water increase, sunlight, temperature and pH of soil. A study by Gregorio *et al*. (1997) also observed that rice plants are most sensitive at seedling sage but at flowering the effects are not seen but also stated that in order to fully understand the effects of salinity on rice various stages needed to be studied.

Other factors affecting salinity effects on plants are constant such as water and the amount of sunlight,which were controlled in this experiment as the seedlings had been grown in greenhouse. It must be emphased that the effects of salinity are a combination of soil pH,the water supply the plant receives, air humidity and other related factorswhich affect the transpiration process. The OsCIPK15 salt responsive gene is located in the close proximity of other responsive genes to abiotic stress. For instance the other genes in close proximity are OsCIPK03 and OsCIPK12, which respond to cold and drought, respectively are all controlled by OsCDK7. Since the response to abiotic stress is a complex interplay of gene interaction the overstressing of one gene in this case OsCIPK15 expression may be causing an effect on the seedlings physiological homoeostasis.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, the OsCIPK15 salt responsive gene is a silent gene at conducive environment during cultivation and is part of the rice genome including varieties cultivated in Tanzania. The real time quantitative PCR was a valuable tool in quantifying the minute changes in the gene product of the OsCIPK15 salt responsive gene. It can be expressed during stress and varieties which showed consistent expression maybe used in breeding programs to infer tolerance to Tanzanian rice varieties. However, this was an experiment, in the field rice plants can be affected by other factors, which may suppress or increase the tolerance to salinity of the crop.

6.2 Recommendations

In better understanding the abiotic responsive genes of rice, there is need to conduct a QuantitaiveMultiflexGene expression assay analysis of the response simultaneously of allOsCIPK responsive genes in order to determine how the expression profiles will be. This is important since gene expression is a combination of the interaction of gene and environmental conditions at the time. The study interplay of drought and salinity genes, which are the main abiotic, stress factors affecting Tanzanian rice cultivation currently the two genes together would be helpful.

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APPENDICES

Appendix 1: Rice Breeder's Lines

LAB NO	DESIGNATION	ID NO
1	CSR 27	101
2	FANAYE	165
3	GIZA 179	166
4	GIZA TAC	170
5	TXD 306	100
6	TXM 22	90
7	TXM 22	91
8	TXM 24	93
9	TXM 24	92
10	TXM 26	96
11	TXM 26	95
12	TXM 27	98
13	TXM 27	94
14	TXMS 21	88
15	TXMS 1	72
16	TXMS 10-2-1	104
17	TXMS 11	79
18	TXMS 1-1	153
19	TXMS 11-1-1	136
20	TXMS 11-1-2	127
21	TXMS 1-1-14	156
22	TXMS 1-1-14	159
23	TXMS 11-2-1	42
24	TXMS 1-1-4	56
25	TXMS 1-1-4	128
26	TXMS 1-2	113
27	TXMS 1-2-1	61
28	TXMS 1-2-9	134
29	TXMS 13-1	144
30	TXMS 13-1-1	108
31	TXMS 13-2-1	105
32	TXMS 13-2-3	137

33	TXMS 14	80
34	TXMS 14-1	157
35	TXMS 14-1	130
36	TXMS 14-1-1	62
37	TXMS 14-1-2	55
38	TXMS 14-1-6	110
39	TXMS 14-2	139
40	TXMS 14-2	140
41	TXMS 14-2-4	116
42	TXMS 14-3	158
43	TXMS 15	81
44	TXMS 15-1	150
45	TXMS 15-1-5	115
46	TXMS 15-2	148
47	TXMS 15-2-5	1
48	TXMS 15-3-8	2
49	TXMS 15-4-19	4
50	TXMS 15-4-2	3
51	TXMS 15-4-4	33
52	TXMS 15-4-5	34
53	TXMS 15-5-17	5
54	TXMS 16	82
55	TXMS 16-1	160
56	TXMS 16-1-1	46
57	TXMS 16-1-2	114
58	TXMS 16-2	141
59	TXMS 16-2	155
60	TXMS 16-2	124
61	TXMS 16-2-1	43
62	TXMS 16-2-3	63
63	TXMS 17	83
64	TXMS 17	84
65	TXMS 17-1	143
66	TXMS 17-1	123
67	TXMS 17-1-6	48
68	TXMS 17-2-4	53
69	TXMS 17-3-6	69

70	TXMS 17-3-7	70
71	TXMS 18	85
72	TXMS 18-1	164
73	TXMS 18-1-1	107
74	TXMS 18-1-5	52
75	TXMS 18-1-7	129
76	TXMS 18-2	146
77	TXMS 18-2	119
78	TXMS 2	73
79	TXMS 20	86
80	TXMS 20-1-14	6
81	TXMS 20-1-20	51
82	TXMS 20-1-6	50
83	TXMS 20-2	125
84	TXMS 20-2	154

Appendix 2: RNA Extraction Procedure

Before starting sterilization of working surfaces, pipettes, centrifuges and sterilize with ethanol treated with DEPC H_2O. RNAase free tips were used.

(CTAB)EXTRACTION BUFFER PROTOCOL

2% CTAB, 100mM TrisHCl, 20mM EDTA, 2M NaCl. Immediately before extraction, 1% mercapto-ethanol was added

Reagent	Stock solution	Volume (500ml) extraction
PVP		2% 10gms
Sodium sulphite		1% 5gms
CTAB	1M (1000mM)	500x100/1000 50ml
Tris EDTA	0.5M(500mM)	500x20/500 20ml
NaCl		58.55ml
Sterile H_2O make up		500ml

1. Approximately 200gms of leaf was freeze dried using liquid nitrogen and transferred to 1.5ml sterile Eppendorf tubes.
2. 700µl of CTAB buffer containing 2% mecarpto-ethanol warmed at 65°C for 10 mins and votexed.
3. Incubation was done in a Struat heating block at 65°C for 30min, mixing by inversion every 10 mins. The tubes were then left to cool for 5 mins.
4. A volume of 700µl of chloroform: isopropanol (24:1) was added and mixed by shaking briefly for 10 mins
5. The tubes were centrifudged in a Hettich Centrifuge MIKRO 220R at 13000rpm for 10mins
6. Aqueous phase was transferred to clean 1.5ml sterile Eppendorf tubes(~490ml)

7. Cold isopropanol of 700µl was dispensed into each Eppendorf tube and tubes shaken gently.
8. Incubation followed for not less than 30min in a -20°C freezer
9. The tubes were again spun for 10 min and the isopropanol was decanted
10. Washing was done by adding 70% ethanol and tapping, followed by spinning at 13000rpm for 10min. Decanting of ethanol followed later the pellet was left todryfor not less than 40mins
11. Re-suspension of the pellet was done by adding 50µl of RNAse free water.
12. Storage of extracted mRNA was -80°C

Appendix 3:Nanodrop Readings of cDNA

Treatment 1

Sample ID	Nucleic Acid Conc.	Unit	260/280	Sample Type
1	1229.80	ng/µl	1.89	DNA
2	-75.10	ng/µl	1.84	DNA
3	2411.10	ng/µl	1.88	DNA
4	2299.00	ng/µl	1.9	DNA
5	2064.00	ng/µl	1.81	DNA
6	2028.80	ng/µl	1.9	DNA
7	1719.60	ng/µl	1.87	DNA
8	1781.80	ng/µl	1.89	DNA
9	1652.90	ng/µl	1.8	DNA
10	1960.40	ng/µl	1.9	DNA
11	1894.40	ng/µl	1.89	DNA
12	1841.80	ng/µl	1.91	DNA
13	1688.70	ng/µl	1.81	DNA
14	1862.70	ng/µl	1.9	DNA
15	1962.50	ng/µl	1.88	DNA
16	1846.10	ng/µl	1.91	DNA
17	1788.60	ng/µl	1.8	DNA
18	1874.60	ng/µl	1.91	DNA
19	1973.90	ng/µl	1.8	DNA
20	1915.10	ng/µl	1.91	DNA
21	2316.00	ng/µl	1.96	DNA
22	1879.90	ng/µl	1.91	DNA
23	1738.40	ng/µl	1.91	DNA
24	1587.40	ng/µl	1.91	DNA
25	1791.00	ng/µl	1.91	DNA
26	1937.30	ng/µl	1.89	DNA
27	1994.90	ng/µl	1.9	DNA
28	1842.20	ng/µl	1.85	DNA
29	1867.60	ng/µl	1.91	DNA
30	1731.60	ng/µl	1.92	DNA

31	1934.40	ng/µl	1.9	DNA
32	1964.00	ng/µl	1.91	DNA
33	225.60	ng/µl	1.91	DNA
34	1851.50	ng/µl	1.89	DNA
35	2013.50	ng/µl	1.92	DNA
36	1935.50	ng/µl	1.96	DNA
37	1813.90	ng/µl	1.97	DNA
38	2039.30	ng/µl	1.92	DNA
39	2062.80	ng/µl	1.91	DNA
40	2042.30	ng/µl	1.88	DNA
41	1968.40	ng/µl	1.96	DNA
42	1846.00	ng/µl	1.92	DNA
43	2014.00	ng/µl	1.96	DNA
44	2357.40	ng/µl	1.94	DNA
45	1840.50	ng/µl	1.89	DNA
46	1898.80	ng/µl	1.87	DNA
47	1951.20	ng/µl	1.9	DNA
48	2025.20	ng/µl	1.92	DNA
49	1923.30	ng/µl	1.86	DNA
50	2048.90	ng/µl	1.93	DNA
51	1922.10	ng/µl	1.91	DNA
52	1794.00	ng/µl	1.91	DNA
53	3249.80	ng/µl	1.87	DNA
54	2908.60	ng/µl	1.87	DNA
55	2663.20	ng/µl	1.91	DNA
56	1698.70	ng/µl	1.89	DNA
57	2146.30	ng/µl	1.79	DNA
58	1572.00	ng/µl	1.88	DNA
59	1511.50	ng/µl	1.92	DNA
60	1410.40	ng/µl	1.92	DNA
61	1668.90	ng/µl	1.71	DNA
62	178.90	ng/µl	1.85	DNA
63	1850.60	ng/µl	1.86	DNA
64	1790.50	ng/µl	1.93	DNA

65	1523.20	ng/µl	1.7	DNA
66	1941.80	ng/µl	1.87	DNA
67	2116.70	ng/µl	1.96	DNA
68	1875.20	ng/µl	1.9	DNA
69	2064.90	ng/µl	1.85	DNA
70	1779.90	ng/µl	1.97	DNA
71	1894.30	ng/µl	1.96	DNA
72	1857.40	ng/µl	1.97	DNA
73	1806.70	ng/µl	1.96	DNA
74	386.20	ng/µl	1.93	DNA
75	1986.90	ng/µl	1.87	DNA
76	2042.40	ng/µl	1.97	DNA
77	2030.20	ng/µl	1.89	DNA
78	1829.00	ng/µl	1.97	DNA
79	1900.90	ng/µl	1.98	DNA
80	4759.80	ng/µl	1.96	DNA
81	3046.60	ng/µl	1.96	DNA
82	3285.10	ng/µl	1.96	DNA
83	3011.20	ng/µl	1.96	DNA
84	2983.70	ng/µl	1.86	DNA

Treatment 2

Sample ID	**Nucleic Acid Conc.**	**Unit**	**260/280**	**Sample Type**
1	3986.8	ng/µl	1.86	DNA
2	3887	ng/µl	1.96	DNA
3	2505.9	ng/µl	1.93	DNA
4	4262.4	ng/µl	1.98	DNA
5	2464.6	ng/µl	1.93	DNA
6	1091.8	ng/µl	1.86	DNA
7	2439.5	ng/µl	1.92	DNA

8	2363.1	ng/µl	1.8	DNA
9	1299.4	ng/µl	1.84	DNA
10	2615.9	ng/µl	1.88	DNA
11	1349.3	ng/µl	1.87	DNA
12	1545.3	ng/µl	1.96	DNA
13	1708.6	ng/µl	1.93	DNA
14	1545.5	ng/µl	1.9	DNA
15	1910.2	ng/µl	1.94	DNA
16	1759.3	ng/µl	1.93	DNA
17	1450.7	ng/µl	1.93	DNA
18	2017.7	ng/µl	1.92	DNA
19	1995.4	ng/µl	1.96	DNA
20	2030.9	ng/µl	1.94	DNA
21	1548.8	ng/µl	1.97	DNA
22	-523.1	ng/µl	2.25	DNA
23	3422	ng/µl	1.86	DNA
22	3798	ng/µl	1.89	DNA
24	1473.3	ng/µl	1.9	DNA
25	1214.8	ng/µl	1.93	DNA
26	853.3	ng/µl	1.91	DNA
27	2241.7	ng/µl	1.96	DNA
28	2678.9	ng/µl	1.92	DNA
29	1642.3	ng/µl	1.88	DNA
30	1908.2	ng/µl	1.97	DNA
31	2071.6	ng/µl	1.97	DNA

32	2145.4 ng/µl	1.97	DNA
33	1975.4 ng/µl	1.94	DNA
34	1553 ng/µl	1.97	DNA
35	2003.4 ng/µl	1.96	DNA
36	1837.3 ng/µl	1.92	DNA
37	1672 ng/µl	1.96	DNA
38	1245.8 ng/µl	1.89	DNA
39	149.6 ng/µl	1.98	DNA
40	808.2 ng/µl	1.94	DNA
41	1107 ng/µl	1.94	DNA
42	1793.1 ng/µl	1.84	DNA
43	1070.9 ng/µl	1.99	DNA
44	864.9 ng/µl	1.97	DNA
45	324 ng/µl	1.88	DNA
46	300.1 ng/µl	1.88	DNA
47	-302.8 ng/µl	2.49	DNA
48	322.7 ng/µl	1.84	DNA
47	1358.1 ng/µl	1.81	DNA
49	1492.6 ng/µl	1.96	DNA
50	1351.7 ng/µl	1.96	DNA
51	1221 ng/µl	1.99	DNA
52	-253.2 ng/µl	1.79	DNA
53	1138.5 ng/µl	1.99	DNA
52	175 ng/µl	1.94	DNA
54	1807 ng/µl	1.95	DNA

55	125.2	ng/µl	1.85	DNA
56	1067.1	ng/µl	1.91	DNA
57	1063.5	ng/µl	1.94	DNA
58	-314.6	ng/µl	1.97	DNA
59	1231.6	ng/µl	1.96	DNA
58	1070.1	ng/µl	1.96	DNA
60	1603.5	ng/µl	1.91	DNA
61	1034	ng/µl	1.91	DNA
62	-190.4	ng/µl	1.94	DNA
63	1376.1	ng/µl	1.96	DNA
62	722.5	ng/µl	1.93	DNA
64	1601.1	ng/µl	1.8	DNA
65	866.4	ng/µl	1.87	DNA
66	1464.5	ng/µl	1.97	DNA
67	1575.5	ng/µl	1.97	DNA
68	1258	ng/µl	1.98	DNA
69	1286.1	ng/µl	1.82	DNA
70	1464.6	ng/µl	1.89	DNA
71	2192.7	ng/µl	1.85	DNA
72	1513.2	ng/µl	1.87	DNA
73	571.3	ng/µl	1.97	DNA
74	1000.1	ng/µl	1.88	DNA
75	1819.5	ng/µl	1.87	DNA
76	1144.4	ng/µl	1.87	DNA
77	2259.1	ng/µl	1.94	DNA

78	1287.3	ng/µl	1.98	DNA
79	1136.7	ng/µl	1.89	DNA
80	882.2	ng/µl	1.86	DNA
81	672.2	ng/µl	1.86	DNA
82	-474.1	ng/µl	2.07	DNA
83	1976.1	ng/µl	1.87	DNA
82	836.2	ng/µl	1.82	DNA
84	995.1	ng/µl	1.76	DNA

Treatment 3

Sample ID	**Nucleic Acid Conc.**	**Unit**	**260/280**	**260/230**	**Sample Type**
1	4080.1	ng/µl	1.86	1.91	DNA
2	536.2	ng/µl	1.49	1.66	DNA
3	4021.7	ng/µl	1.89	2.12	DNA
4	2882.8	ng/µl	1.87	2.06	DNA
5	3666.6	ng/µl	1.88	2.02	DNA
6	2824.7	ng/µl	1.87	2.11	DNA
7	3603	ng/µl	1.9	2.21	DNA
8	3796.3	ng/µl	1.87	2.15	DNA
9	3669.7	ng/µl	1.89	2.14	DNA
10	4059.9	ng/µl	1.87	2.04	DNA
11	4877.5	ng/µl	1.86	2.03	DNA
12	2869.1	ng/µl	1.79	2.18	DNA
13	2856.1	ng/µl	1.86	2.08	DNA
14	3750.7	ng/µl	1.89	2.04	DNA

15	3394	ng/µl	1.88	2.1	DNA
16	3552.5	ng/µl	1.89	2.22	DNA
17	2176.8	ng/µl	1.53	1.69	DNA
18	3098.5	ng/µl	1.88	2.12	DNA
19	4234.9	ng/µl	1.88	2.11	DNA
20	2949.6	ng/µl	1.86	2.06	DNA
21	3388.7	ng/µl	1.88	2.16	DNA
22	3600	ng/µl	1.87	2.09	DNA
23	3642.5	ng/µl	1.88	2.17	DNA
24	2983.5	ng/µl	1.83	2	DNA
25	2914.8	ng/µl	1.85	2.04	DNA
26	6332.9	ng/µl	1.82	2.05	DNA
27	5834.5	ng/µl	1.9	2.37	DNA
28	81.2	ng/µl	0.86	2.76	DNA
29	3859.8	ng/µl	1.87	2.11	DNA
28	5076.4	ng/µl	1.86	2.11	DNA
30	3332.4	ng/µl	1.88	2.13	DNA
31	3284.5	ng/µl	1.89	2.25	DNA
32	3763.8	ng/µl	1.87	2.09	DNA
33	3889.9	ng/µl	1.88	2.11	DNA
34	3481.9	ng/µl	1.87	1.99	DNA
35	3419.1	ng/µl	1.89	2.13	DNA
36	4000.9	ng/µl	1.78	1.74	DNA
37	3629.1	ng/µl	1.87	1.94	DNA
38	3461.6	ng/µl	1.89	2.14	DNA

39	3261	ng/µl	1.88	2.06	DNA
40	2516.9	ng/µl	1.88	2.05	DNA
41	2638.9	ng/µl	1.88	2.08	DNA
42	2853.7	ng/µl	1.84	1.85	DNA
43	3636.9	ng/µl	1.88	2.11	DNA
44	5039.7	ng/µl	1.46	0.95	DNA
45	2742	ng/µl	1.87	2.15	DNA
46	2802	ng/µl	1.85	2.05	DNA
47	2994.4	ng/µl	1.85	2.08	DNA
48	2230.1	ng/µl	1.89	2.08	DNA
49	2281.3	ng/µl	1.87	1.98	DNA
50	1595.2	ng/µl	1.87	1.91	DNA
51	2674.3	ng/µl	1.83	1.93	DNA
52	2594.5	ng/µl	1.89	2.19	DNA
53	2685.5	ng/µl	1.89	2.31	DNA
54	4188.3	ng/µl	1.88	2.13	DNA
55	2661.9	ng/µl	1.86	2.16	DNA
56	2695	ng/µl	1.86	2.13	DNA
57	3329.4	ng/µl	1.83	2.01	DNA
58	2594.4	ng/µl	1.89	2.11	DNA
59	1787	ng/µl	1.9	2.13	DNA
60	2339.8	ng/µl	1.89	2.21	DNA
61	2624.1	ng/µl	1.89	2.2	DNA
62	5188.6	ng/µl	1.77	2.03	DNA
63	3577.4	ng/µl	1.88	2.16	DNA

64	2547.4	ng/µl	1.87	2.16	DNA
65	3554.2	ng/µl	1.88	2.01	DNA
66	2563.2	ng/µl	1.86	2	DNA
67	2508.5	ng/µl	1.88	2.06	DNA
68	2488.7	ng/µl	1.89	2.16	DNA
69	2380.6	ng/µl	1.91	2.17	DNA
70	2411.1	ng/µl	1.89	2.19	DNA
71	2064.2	ng/µl	1.53	1.68	DNA
72	3486.2	ng/µl	1.87	1.99	DNA
73	2656.3	ng/µl	1.88	2.12	DNA
74	2429.2	ng/µl	1.82	1.92	DNA
75	1750.5	ng/µl	1.91	2.17	DNA
76	2014.9	ng/µl	1.91	2.35	DNA
77	2345.4	ng/µl	1.9	2.15	DNA
78	2554.2	ng/µl	1.88	2.06	DNA
79	2446.8	ng/µl	1.94	2.15	DNA
80	3395.2	ng/µl	2	2.31	DNA
81	2489.6	ng/µl	1.85	2	DNA
82	2403.6	ng/µl	1.88	2.11	DNA
83	5193.7	ng/µl	1.87	2.08	DNA
84	2400.4	ng/µl	1.84	1.97	DNA

Appendix 4:Statistical Analysis - ANOVA

Tests of between – subjects effects

Dependent variable;Ct

Source	Type III sum of squares	df	Mean Square	F	Sig.
Corrected Model	1164.08[a]	21	55.43	5.49	0.00
Intercept	99696.34	1	99696.34	9874.20	0.00
LINES	1164.08	21	55.43	5.40	0.00
Error	1110.63	110	10.10		
Total	101971.05	132			
Corrected Total	2274.71	131			

a R Squared =0.512 (Adjusted R squared =0.419)

Duncan

TIME	N	Subset	
		1	2
14	44	26.5077	
5	44	26.5843	
0	44		29.3548
Sig.		.893	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 7.127.

a. Uses Harmonic Mean Sample Size = 44.000.

b. Alpha = 0.05.