UNDERSTANDING THE COMMON BEAN (*PHASEOLUS VULGARIS* L.) TRANSCRIPTOME IN RESPONSE TO SALINITY STRESS

by

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A THESIS

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DEDICATION

I would like to take the time to dedicate my thesis work to my parents Gwendolyn and Lynford Brown who have sacrificed a lot to support me in college. My parents were never rich but tried their very best to give me the education that they never had, so that I can be a better individual. They would do extra work on the side to make ends meet and for that I will forever be grateful. I am who I am now because of the training I received at home. My mom taught me to work hard for what I want in life and my dad taught me to be persistent. I would also like to dedicate my thesis to my little sister who is just behind me completing her undergraduate degree. I have always tried to live a life that is an example for her to follow to be a great young lady.

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Understanding the Common bean (*Phaseolus vulgaris* L.) Transcriptome in Response to Salinity Stress

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ABSTRACT

Salinity is a grave threat to agriculture as it affects crop growth globally; it is known as one of the major stressors that hinders common bean production. Common bean (*Phaseolus vulgaris* L.*)* is the main source of human dietary vegetarian protein that constitutes approximately half of the consumed grain legumes globally. Additionally, common bean is of great economic weight as it provides income for millions of small farmers; however, the crop is known to be sensitive to salinity. In this study, common bean plants were salt stressed in a hydroponic system at 0 mM, 50 mM and 150 mM of NaCl for ten days (early stress) and five weeks (prolonged-flowering stage) in an effort to identify salt responsive genes. Exposure of plants to salt for several hours to a few days induces osmotic stress genes; lengthier salt stress treatments from a few days up to several weeks will ultimately induce salt specific genes that are involved in the plant's adaptation to, and development under stress. Therefore, we isolated root RNA from ten days and five weeks salt stressed bean tissues and sequenced their transcriptomes. RNA-Seq analysis identified more differentially expressed genes (DEGs) in roots at five weeks compared to ten days salt treatment of 0 mM, 50 mM and 150 mM of NaCl. Some early and delayed salt responsive genes were identified which includes: Salt Tolerance Homolog 2 (*STH2),* Sodium Hydrogen Exchanger *(NHX1),* Chloride Chanel A *(CLC-A),* Late Embryogenesis

Abundant *(LEA)* & Proline Transporter 1 *(PROT1)*.The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed the Mitogen-Activated Protein Kinase (*MAPK*) signaling pathway which contained ten DEGs including protein kinase (*CTR1*), *calmodulin 1* and *WRKY33.* Transcription factors (TFs) that are known to be involved in flower development and flowering time such as *WRKY, MADS-box* and *bZIP* were observed to be up-regulated both at ten days and five weeks of salt stress when compared to the control (no salt treatment). The up-regulated genes identified were functionally classified using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system for Gene Ontology (GO) Slim (general) categories. Additionally, PANTHER overrepresentation test was done to identify granular (specific) terms that were enriched for both upregulated and down regulated genes. This first report on common bean under prolonged salt stress of five weeks has identified potential candidate genes that are perhaps needed for acclimation to salt stress and hence will serve as a genetic resource for plant salt tolerance.

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LIST OF ABBREVIATIONS

Bp	Base pair
CR	Control Root (0 mM of NaCl)
DEGs	Differentially Expressed Genes
FC	Fold Change
MS	Moderate Stress (50 mM of NaCl)
PCR	Polymerase Chain Reaction
qRTPCR	Real-time quantitative reverse transcriptase polymerase chain reaction
HS	High Stress (150 mM of NaCl)
TFs	Transcription Factors

CHAPTER I: INTRODUCTION

Soil salinity is a global problem which poses a major threat to agriculture worldwide as it decreases the crop yield in the areas that are affected (Kumar, 2013). A soil is recognized to be saline if the electrical conductivity (EC) of the saturation extract in the root zones is over four deciSiemens per metre (4 dS/m) which is roughly 40 mM NaCl (Shrivastava & Kumar 2015). The unfavorable result of surplus minerals such as Na^+ and/or Cl^- on plant is referred to as salt stress (Munns, 2005). Salt stress is one of the most consequential restraining factors for plant development and production (Zhu, 2001). Numerous physiological conditions and deleterious effects on plants are caused by the major ions Na^+ and Cl^-. However, Na^+ is the main ion as it intrudes on the uptake of potassium (K^+) ion and interrupts stomatal regulation that eventually causes water loss; simultaneously chlorophyll production is disturbed by the Cl^- ion and therefore causes chlorotic toxicity (Tavakkoli et al., 2011). Plants have adapted primarily two types of tolerance mechanisms to cope under salt stress by restricting the entry of salt through the roots and by regulating its concentration and distribution. Breeding crops with enhanced yield performance under salinity stress can be achieved through a better understanding of these tolerance mechanisms (Hanin et al., 2016)

1.1 Types, Causes and Classes of Salinity

Natural or primary salinity

Primary salinity is caused from the build-up of salts over lengthy time periods by natural courses in groundwater or soil. The two main natural processes are: a) Breaking down of parent rocks comprising of soluble salts; soluble salts consist of diverse types primarily chlorides of sodium, calcium, and magnesium, and to a minor range, sulfates and carbonates, all of which can

be released by the breaking down of rocks. b) Oceanic salts deposited inland by wind and rainfall are made up primarily of sodium chloride (Parihar et al., 2015)

Secondary or human-induced salinity

Secondary salinization is caused by human actions that alter the hydrologic equilibrium of the soil between water applied (irrigation or rainfall) and water utilized by crops (Manchanda & Garg, 2008). The two common causes are: a. Clearing of land and using annual crops to replace perennial vegetation b. The use of salt-rich irrigation water for irrigation systems or having inadequate drainage (Parihar et al., 2015). As per the Food and Agriculture Organization (FAO) Land and Plant Nutrition Management Service, salinity affects over 6% of the world's land. Currently there are 230 million hectares of irrigated land globally of which salt affects 45 million hectares (19.5%); of the 1,500 million hectares worldwide that are considered dry land agriculture, 32 million are salt-affected to variable levels (2.1%) (Parihar et al., 2015).

Classes of Salinity

Salinity Class	EC range (dS/m)	Description
Non-saline	0-2	A wide array of plants is present with no effect on vegetation
Low salinity	2-4	Normally, a reduction in number and vigor is displayed by salt sensitive plants. There is no evidence of salt stain/ crystals on bare ground
Moderate salinity	4-8	The vegetation community is slowly being dominated by salt tolerant species and salt sensitive plants are noticeably affected by the soil salinity levels. There is the likelihood of visible salt stain/crystals on bare soil
High Salinity	8-16	Vegetation is dominated by a few high salt tolerant plant species that remain unaffected by salt. There is the occurrence of bare saline areas having salt stains or crystals
Severe Salinity	16-32	Two or three salt tolerant species may dominate the area with the survival of highly salt tolerant plant species
Extreme salinity	>32	Widespread bare saline areas with evidence of salt stains and or crystals. Dying/death of trees.

(Rasool et al., 2013 & Victoria State Government, 2018)

1.2 The Effects of Salinity on Plants

Two rationales are recognized for the inhibition of plant growth in saline soils: a. The existence of salt in the soil solution decreases the potential of the plant to take up water hence results in reductions in the growth rate; this is known as the osmotic or water-deficit outcome of salinity. b. If extreme quantities of salt penetrate the plant in the transpiration stream, there will be damage to cells in the transpiring leaves and this may result in more growth reductions. This is referred to as the salt-specific, or ion-excess effect of salinity (Greenway & Munns, 1980). The two effects cause a rise in a two-stage growth response to salinity (Munns 1993 & Munns, 2005). These stages are: Stage 1: The effect of salt outside the plant causes the first phase of growth response. The salt in the soil solution causes a decrease in leaf growth (Munns, 1993) and root growth (Läuchli & Grattan, 2007). The cellular (metabolic) activities involved are familiar to

drought-affected plants. Stage 2: This stage of growth response is caused from the toxic consequence of salt inside the plant. Salt taken up by the plants accumulates in the older leaves first; over a long period of time, continuous transport into transpiring leaves will ultimately results in very high levels of Na^+ and Cl^- and the leaves will die. The cause of damage is possibly the salt load being greater than the ability of the cells to separate salts in the vacuole. Salts would then accumulate quickly in the cytoplasm and prevent enzyme activity; on the other hand, they might accumulate in the cell walls and cause the cell to be dehydrated. The extreme salt concentration ultimately heightens the osmotic potential of the soil that inhibits plant water uptake. The effects caused by salinity are three-fold: a. it decreases water potential b. causes ion variation and c. disruptions in ion homeostasis and toxicity. This changed water status results in primary growth reduction and restriction of plant productivity. The undesirable effect is detected at the whole-plant level as death of plants or reduction in yield. Main processes that are affected by salt stress include germination, growth, photosynthetic pigments and photosynthesis, water relation, nutrient imbalance, oxidative stress, and yield (Parihar et al., 2015).

1.3 Salt Tolerance Mechanisms

Plants utilize several mechanisms to cope with salt stress; the most common includes a reduction of the quantity of salt gathered by the roots and its apportioning at the tissue and cellular levels to prevent accumulation of toxic concentrations in the cytosol of efficient leaves. Ion homeostasis, osmotic homeostasis, redox equilibrium, and growth regulation are among the distinct mechanisms that plants employ to deal with salt stress. These mechanisms are attained through adapting physiological and biochemical modifications enabled by the expression of several salt-responsive genes. These involve activation of functional protein-coding genes, comprising of osmoregulatory genes, antioxidant proteins, transporters/antiporters, transcription

factors (TFs), and signal-associated protein kinases (Ali et al., 2012). These strategies and mechanisms provide diverse levels of tolerance to different plants. Plants that are considered stress-tolerant are able to show effective mechanisms of stress sensing, signal transduction, and programs of gene expression, or feasibly disparate metabolic pathways (Bartels & Sunkar, 2005). A slow and gradual acclimation might need to be employed by sensitive plants to attain suitable expression of genes that are responsible for this case (Zhu, 2001).

1.4 Salt Stress on Common Bean (*Phaseolus vulgaris* L.)

Globally, salinity is one of the main severe abiotic stresses that affects the production of legumes (Bayuelo-Jiménez et al., 2002; Wang et al., 2003). Salinity adversely affects the symbiosis between legumes and rhizobia through osmotic and/or ionic effects which results in an inhibition of several physiological and biochemical processes as well as limitations on host plant growth, and proliferation of rhizobia and nodulation (Farissi et al., 2011; Farissi et al., 2014; Latrach et al., 2014). The ability of legumes to tolerate salinity stress relies on modifications of many physiological and molecular processes which includes: a. sequestration of sodium ion (Na^+), b. buildup of osmoprotectants, c. hormone biosynthesis and d. production of antioxidative stress responses (Faghire et al., 2013; Farissi et al., 2014; Latrach et al., 2014). Common bean (*Phaseolus vulgaris* L.) is the most significant grain legume grown globally for direct human consumption (Chen et al., 2016); it is the most essential source of vegetarian protein in the developing world (Castro-Guerrero et al., 2016). Legumes such as common bean are also essential crops in agriculture as these plants initiate root nodules via symbiotic associations with nitrogen fixing bacteria (Broughton et al., 2003). However, common bean is recognized to be sensitive to numerous environmental factors such as salinity which is noted as one of the major factors restricting plant growth and productivity (Ghoulam et al., 2002). The productivity of

common bean is known to be reduced up to 20% at 1 dS/m salinity level which is approximately ten mM NaCl (Chinnusamy et al., 2005). Plant acclimation to salinity is an intricate process, which involves extreme alterations than just reduced growth. At the cellular level it entails adjustment of gene expression, particularly those that encode transporter proteins, temporary increases in Abscisic Acid (ABA) concentration, buildup of compatible solutes and protective proteins, raised levels of antioxidant and repression of energy-consuming pathways (Chaves et al., 2009).

While studies have been carried out to investigate common bean's acclimation to salinity at the cellular level using high-throughput approaches (Büyük et al., 2016; Hernández-Lucero et al., 2014; Hiz et al., 2014; Kavas et al., 2016; Verdoy et al., 2004); these studies have been limited to 'onset' of salt stress treatment (hours to a few days). Studies attempting to understand the effect of prolonged salt stress (up to the flowering stage) and its effect on gene expression have not been carried out. The flower is the reproductive organ of the plant and so the flowering stage is fundamental to plant breeding. Therefore, the goal of the present study was to examine transcriptomic variations in common bean cv. "Sierra", exposed to 0 mM (0 dS/m), 50 mM (5 dS/m) and 150 mM (15 dS/m) NaCl (sodium chloride) for ten days (onset stress) and up to five weeks (prolonged stress) at which the plants were flowering. According to Gao et al (2018), in hostile environments, plants have evolved vital defensive responses which allow for survival and reproduction; these responses can be triggered within minutes and up to several weeks after contact of high salt conditions. Roots are the first-line of recognition and detriment for dehydration stresses which includes salinity and drought. In several cases it is the root's sensitivity to the stress that limits the productivity of the whole plant (Atkin et al., 1973). We aimed to identify differentially expressed genes (DEGs), transcription factors (TFs) and

molecular interaction pathways in the roots of common bean under salt stress using the Illumina high-throughput RNA-sequencing platform. To our knowledge this will be the first transcriptomic salt study on common bean at the flowering stage. Hence, the data generated in this study should identify key players for plants' adaptation to salt stress and ultimately the molecular mechanisms that are involved in salt stress tolerance.

1.5 Objectives

- Identify Differentially Expressed Genes (DEGs) in the roots of common bean cv. 'Sierra' at ten days and five weeks after salt treatment using RNA-sequencing
- Compare differences in expression of genes in 0 mM (0 dS/m), 50 mM (5 dS/m) and 150 mM (15 dS/m) salt treated roots of common bean cv. Sierra using RNA-sequencing

1.6 Hypothesis

Null hypothesis (1)

There will be no difference in the number of differentially expressed salt genes in the roots of common bean at ten days (onset) and five weeks (prolong-flowering) of stress.

Alternative hypothesis (1)

There will be more differentially expressed genes in the roots of common bean at five weeks (prolonged-flowering) compared to ten days (onset) under salt stress.

Null hypothesis (2)

Salt responsive genes of 50 mM (5 ds/m) and 150 mM (15 ds/m) NaCl will display the same levels of expression

Alternative hypothesis (2)

Salt responsive genes of 50 mM (5 ds/m) and 150 mM (15 ds/m) NaCl will display varying levels of expression

CHAPTER II: LITERATURE REVIEW

The common bean (*Phaseolus vulgaris* L.) is a primary grain legume important for direct human consumption; it is also known for its rich source of protein, vitamins, minerals, and fiber, particularly for economically fragile populations in Africa and Latin America (Broughton et al., 2003). The fiber content of common bean helps in alleviating blood sugar and cholesterol, aiding with reducing obesity and diabetes (Chandalia et al., 2000). Along with its nutritional significance, beans have great economic value as it produces income for millions of small farmers (Hernández-Lucero et al., 2014). As a result of its nutritional and economical importance, common bean is necessary for worldwide food security in many developed and developing countries all over the world, for instance eastern Africa (Buruchara et al., 2011) and Latin America (Petry et al., 2015). Annually, there is a global production of over 12 million metric tons of beans with the majority, of 5.5 million metric tons being produced in Latin America (Petry et al., 2015). The crop value of common bean in the US only is over $1 Billion dollars (Bailey, 2014). Common bean is recognized to be susceptible to numerous environmental factors including salinity; salinity is recorded as one of the main factors restricting common beans' growth and productivity (Ghoulam et al., 2002). Salinity is known to have unfavorable effects on germination, plant vigor and crop yield (Rana Munns & Tester, 2008).

2.1 Salt Stress Sensors

Salt stress is sensed by plants through ionic (Na^+) and osmotic stress signals. An excess of Na^+ is known to be sensed in two ways: i) on the exterior of the plasma membrane by a transmembrane protein or ii) on the inside of the cell by membrane proteins or by enzymes that are Na^+ sensitive (Zhu, 2003). The plasma membrane Na^+/H^+ antiporter *SOS1* (Salt Overly Sensitive 1) is known for its role as an antiporter which contains 10-12 transmembrane domain and a lengthy cytoplasmic tail which may suggest that it is also acting as a Na^+ sensor (Zhu, 2003). Membrane depolarization may occur because of Na^+ entry through nonspecific ion channels under salinity that eventually activates Ca^{2+} channels (Sanders et al., 1999) which generates Ca^{2+} oscillations, and signals salt stress (Chinnusamy et al., 2005). Salinity-induced hyperosmotic stress results in turgor loss which decreases the cell volume leading to retraction of the plasma membrane from the cell wall; possibly this is sensed by stretch-activated channels and transmembrane protein kinases which include two component histidine kinases and wall-associated kinases (Kreps et al., 2002; Urao et al., 1999). Exposure to salt resulted in an upregulation of the biosynthesis of the plant stress hormone ABA (Jia et al., 2002; Xiong & Zhu, 2003) and cause a buildup of reactive oxygen species (ROS) (Hernández et al., 2001; Smirnoff, 1993). Ionic and osmotic homeostasis is also regulated by ABA and ROS in addition to stress damage control and repair processes (Chinnusamy et al., 2005). To maintain preferable K^+ Na^+ ratios in the cytosol plants utilized some common strategies such as the regulation of K^+ uptake and/or the avoidance of Na^+ entry, outflow of Na^+ from the cell and exploitation of Na^+ for osmotic alteration. Osmotic homeostasis is achieved through Na+ compartmentation into the vacuole or by the biosynthesis and buildup of compatible solutes (Chinnusamy et al., 2005).

ROS detoxification systems and stress proteins that belong to the LEA protein family are some contributory factors in the inhibition of salt stress damage (Zhu, 2002).

2.2 Effects of Salt Stress on Plant Growth

Studies have been carried out to investigate the physiological and ionic toxicity effects of salt on plants. Ndakidemi and Makoi (2009) observed leaf injury and changes in leaf color under salt stress; they also reported a reduction in plant height and dry matter yield of common bean under elevated NaCl concentrations. In a previous study by Guan et al (2011) the seablite's (*Suaeda salsa*) plant height, number of branches, length of branches, and diameter of shoot were notably affected by salt stress because of an increase level of Na^+ and Cl^-. Dolatabadian et al (2011) detected that salinity stress remarkably reduce shoot and root weight, total biomass, plant height and leaf number of soybean plant. According to Khan et al (2000) salinity stress causes a rise in the levels of Na^+ and Cl^- in salt bush (*Atriplex griffithii)* root, stem and leaves with the highest ion built-up found in leaves, stem, and root respectively which proposes a supportive relationship between Na^+ and Cl^- concentration. Additionally, there was a reduction in the Ca^{2+} content in the shoots and leaves of salt bush (*A. griffithii)* plants grown under extreme salinity but it was observed to be stable in roots. Specifically, in leaves, there was a decrease in K^+ with increased levels of salinity. Mg^{2+} concentration was not really affected in stems and roots but a reduction in leaf was more prominent (Khan et al., 2000). Tavakkoli et al (2011) used four barley genotypes to investigate the effect of ion toxicity of Na^+ and Cl^- on growth in saline soils under different salinity treatments. Increase levels of Na^+, Cl^-, and NaCl individually decrease the growth of barley; however, the greatest decrease in growth and photosynthesis were under NaCl stress. The study also revealed that the eliminations of Na^+ and Cl^- among barley genotypes are two disparate mechanisms and different genotypes depict distinct combinations of the two

mechanisms. Postnikova et al (2013) carried out salt ion measurements in their study in which there was less accumulation of sodium in the salt-tolerant genotype of alfalfa under salinity stress than the salt susceptible genotype. Also, the salt tolerant genotype had a better K^+/Na^+ ratio in roots in relation to the salt sensitive genotype. Likewise, Karan & Subudhi (2012) found that the K^+/Na^+ ratio was higher in tolerant Arabidopsis genotypes in relation to susceptible genotypes under both salt and control stress.

Scientific studies have demonstrated that salinity stress has a negative impact on germination. Kaveh et al (2011) discovered a notable undesirable association between salinity and germination rate and percentage which caused a delay in germination and a decreased germination percentage in tomato (*Solanum lycopersicum)*. Bybordi (2010) stated that there was a significant reduction of germination percentage in *Brassica napus* at 150 and 200 mM NaCl. Germination rate was also reduced with increased salinity levels. In comparison with controls, germination percentage and speed of germination were lessened by 38% and 33% respectively by 200 mM NaCl. Khodarahmpour et al (2012) observed notable decrease in germination rate (32%), length of radicle (80%), and plumule (78%), seedling length (78%), and seed vigor (95%) in *Z. mays* seeds exposed to 240 mM NaCl. Photosynthetic rates are also affected under salt stress when elevated concentrations of Na^+ and/or Cl^- accumulate in the chloroplasts (Parihar et al., 2015). When exposed to increasing concentrations of NaCl treatments, *Vigna radiate* displayed a linear decrease in the levels of total chlorophyll (Saha et al., 2010). Khan et al (2013) revealed that an increase in NaCl levels has resulted in a significant decrease in total leaf chlorophyll in cucumber plants. Additionally, a reduction in net photosynthetic rate, stomatal conductance, performance of PSII and photosynthetic efficiency has resulted in decreased growth in citrus plant under salinity stress (Lopez-Climent et al., 2008).

2.3 Salt Stress Gene Expression

Salinity stress ultimately results in gene expression changes that involves a broad range of mechanisms that are utilized by plants to upregulate and downregulate the production of definite gene products (protein) (Gupta and Huang, 2014). Comprehensive knowledge about gene expression at the mRNA level is provided by transcriptomic analysis which is extensively used to identify candidate genes playing a role in stress responses. Transcriptomic and genomic approaches have been used to identify and characterize numerous salt-responsive transcription factors and genes which are upregulated or downregulated in response to salinity stress (Gupta & Huang, 2014). Salt responsive genes are primarily classified into four functional categories namely: i) ion transport or homeostasis (e.g., Salt Overly Sensitive (*SOS)* genes, Sodium Hydrogen Exchanger (*AtNHX1)*, and H^+ -*ATPase*), ii) senescence-associated genes (*SAG*), iii) molecular chaperones (Heat Shock Proteins -*HSP)* genes) and iv) dehydration related transcription factors (Dehydration responsive element-binding protein -*DREB*). Amid the stress responsive genes, the *SOS* genes family is known to play a very interesting role in ion homeostasis, hence conferring salt tolerance (Gupta & Huang, 2014; Kawasaki et al., 2001; J. Liu et al., 2000). Transcription factors (TFs) are known as the most significant regulators controlling gene expressions. *bZIP, WRKY, AP2, NAC, C2H2* zinc finger genes and *DREB* families are among the significant transcription factors that include many stress-responsive members (Gupta and Huang, 2014). When exposed to long-term salinity Johnson et al (2002) detected the expression of *bZIP* genes to be upregulated in a salt-sensitive wheat cultivar but was observed to be decreased in the salt tolerant variety. Overexpression of a *NAC* transcription factor is known to confer salt tolerance in rice and wheat plants (Nakashima et al., 2007). Song et al., (2011) reported an upregulation of transcription factors *QsNAC5* and *ZFP179* under salinity

stress; these TFs possibly control the synthesis and accumulation of proline, sugar and LEA proteins which are presumed to play a fundamental part in stress tolerance (Song et al., 2011). *AtWRKY8* is seen to be upregulated under salt stress in *Arabidopsis*; this TF directly attaches with the promoter of RD29A which suggests it to be one of the target genes of *AtWRKY8* (Hu et al., 2013). In some plant species, a few ROS scavenging and osmotic-regulating genes are upregulated by salinity. For instance, an upregulation of glutathione-S-transferase and ascorbate peroxidase was observed in rice plants under constant exposure to salinity for around 24 hours; also, an increase in the length of exposure to salt cause an upregulation of metallothionein and water channel proteins (Kawasaki et al., 2001). Ten genes that are associated with osmotic regulation were identified to be upregulated in the halophyte plant species *Spartina alterniflora* when exposed to salt stress (Baisakh et al., 2006). Mukherjee et al (2006) identified *OSBZ8* which is a *bZIP* class of *ABRE* binding transcription factor and is seen to be highly expressed in salt tolerant rice cultivars than in salt sensitive ones. A rice (*Oryza sativa)* transcription factor named *SALT-RESPONSIVE ERF1 (SERF1)* was identified by Schmidt et al (2013) in which it displayed a root-specific induction under salt and H_2O_2 treatment. They also verified that plants lacking *SERF1* are more sensitive to salt stress in comparison with the wild type and salinity tolerance is improved by constitutive overexpression of *SERF1*. Salt stress also cause a growth of numerous proteins with protective functions which includes chaperones from *HSP90* family in tomato roots (Manaa et al., 2011) and in *A. thaliana, HSP 70* family, *Hsc70* (heat-shock cognate) proteins were found (Pang et al., 2010). Large quantities of some small *HSPs* (mitochondrial small *HSP*, chloroplast *HSP*, 17.8 kDa class I small *HSP*, *HSP20*) was found to be expressed in salt treated tomato hypocotyls (Chen et al., 2009).

2.4 Transcriptomic Studies of Salt Stress

Differentially expressed genes (DEGs) and pathways involved in salt stress were identified by a comprehensive analysis of the transcriptomes of common bean leaf and root tissues (Hiz et al., 2014). There were more down than up-regulated genes in both leaf and root tissues collected at five days after stress. Secondary metabolite metabolism and membrane transport activity are GO terms that were enriched by up-regulated genes of leaf tissues while the macromolecular energy metabolism related terms were enriched by the down-regulated genes. Hiz et al (2014) also identified transcription factors that were salt responsive with ten most abundant TF families namely: *AP2_EREBP, bHLH, PHD, HB, (R1) R2R3_Myb, WRKY_Zn, NAC, bZIP, C3H-TypeI*, and *Myb*_related. Previously, work has been done to detect genes that are induced during saline stress in a tolerant bean cultivar (Pinto Villa) using suppression subtractive hybridization (Hernández-Lucero et al., 2014). Salinity stress of 0 mM and 200 mM NaCl was applied to 30-day-old Pinto Villa plants and RNA was extracted after at days 2 and 5 from leaves, stems and roots to identify genes in early and late responses to saline stress. Heat shock proteins including *PvDnaJ3* and *PvHSP90*, late embryogenesis abundant proteins such as *PvLEA-18* and dehydrin PvERD10, transcription factor *PvATHB-7* and a protein of unidentified role that comprises a short glycine-rich domain (*PvGRDP1*) are all unigenes that were chosen to examine for differential expression by real-time quantitative PCR (qRT-PCR). The genes were found differentially expressed in a tissue and time specific manner in the bean plant under salt stress. *PvLEA18* was remarkably expressed in stems by salt stress at day 2 and at day 5 it was also induced in root tissues of salt treated plants. *PvERD 10* was highly expressed at day 5 in stems but decreased levels in leaves for both days. The highest expression of *PvHSP90* gene was detected two days after treatment in salt stress roots (Hernández-Lucero et al., 2014).

In a recent article by Goyal et al (2016), there was a study of differential root transcriptome analysis to identify salt stress responsive genes in *Triticum aestivum* cv. Kharchia Local. In response to salt stress, zinc finger, *bHLH*, *bZIP* transcription factors were up-regulated (Goyal et al., 2016). Current research on salinity responsive *HSP70s* in common bean has revealed that there was more *PvHSP70* genes expression in salt treated leaf tissue in comparison to root tissues. Two common bean cultivars, Zulbiye (sensitive), and Yakutiye (resistant) to drought stress were used to evaluate the salt tolerance capacity of both varieties under acute salt stress conditions. 250 mM and 400 mM NaCl was applied for nine days after which leaves and root tissues were harvested for qRT-PCR which then revealed that the transcript absorptions of upregulated PvHSP70 genes were highly expressed in leaves of Zubiye than those of Yakutiye (Buyuk et al., 2016).

Kumari et al (2009) demonstrated a transcriptomic study by using a salt-sensitive rice line IR64 and a salt-tolerant rice Pokkali; a set of salinity responsive genes were identified including *GST*, *LEA*, *V-ATPase*, *OSAP1* zinc finger protein and transcription factor *HB1B*. Higher expressions of these were displayed by Pokkali than IR64 and probably play a part in higher salinity tolerance in Pokkali. Bioinformatics analysis specified that critical transcription factor (TF) families associated with stress responses and growth regulation (*MYB, bHLH, WRKY*) were differentially expressed in Bermuda grass root tips under salinity (Hu et al., 2015). Postnikova et al (2013) used two alfalfa genotypes namely AZ-88NDC (salt susceptible) and AZ-GERM SALT -11 (salt tolerant at the germination stage) to identify a wide-ranging spectrum of genes that are affected by salt stress. RNA- seq was carried out to identify the total size and composition of the alfalfa root transcriptome. Bioinformatics analysis demonstrated that the expression of 1,165 genes together with 86 transcription factors was greatly changed under salt

stress. Findings of the study indicated that there were many common differentially expressed genes as well as transcription factors in both genotypes. Transcription factor genes encoding *AP2/ERF, MYB*, *NAC*, and *WRKY* displayed a significant enrichment of expression in root apexes under salt stress in contrast to whole roots in *M. truncatula* (Postnikova et al., 2013). Another study used *Medicago truncatula* genotypes (having contrasting responses to salt stress) to carry out a comparative transcriptomic analysis of salt adaptation in roots. The salt adapted genotype TN1.11 displayed increased root growth and differential built-up of sodium ions compared to the other genotype (A17) under saline stress. Transcriptomic analysis showed specific gene clusters that are favorably controlled by salt in the root apices of TN1.11. Several genes that encode for transcription factors (TFs) were differentially regulated as well between the genotypes under salt stress; there was an overexpression of the *bHLH-type* TF in the roots of the A17 genotype (Zahaf et al., 2012).

2.5 Genetic Engineering for Salt Tolerance

Manipulation of ion homeostasis, osmoprotectant accumulation, *LEA-type* proteins, and ROS scavenging capacity are transgenic approaches that have confirmed the competences of engineering salt-tolerant crops. Even though multiple genes are considered to play a part in abiotic stress tolerance, it was revealed that notable increases in salt tolerance can be attained by single gene manipulations such as *SOS1* (Shi et al., 2003) and *NHX1* (Apse et al., 1999; Zhang et al., 2001; Zhang and Blumwald, 2001) overexpressing transgenic plants. These transgenic plants have the capability to grow and produce flowers at salt levels of up to 200 mM NaCl (20 dS/m) which is considered lethal for wild-type plants. Most crop plants are susceptible to this concentration of salinity. Additionally, these transgenic plants did not show any visible growth abnormalities or alterations in the quality of the consumable product which is comparable to the

results with *NHX1* overexpressing transgenic tomato and *Brassica* plants. Therefore, genetic engineering for ion homeostasis by use of tissue specific overexpression of *SOS1*, *NHX1*, and their positive controller which is the active form of *SOS2*, will aid in substantial enhancement in salt tolerance (Chinnusamy et al., 2005). It is known that certain developmental phases of plants are more susceptible to salt stress than others. For instance, in rice, seedling growth, seedling survival, and fertility are all unfavorably affected by salinity concentrations (EC_e) higher than 1.9, 3.4 and 4.5 dS m^{-1}, respectively (Zeng & Shannon, 2000).Therefore, it is important to comprehend the tissue and developmental specificity of salt-stress tolerance. Genetic and transgenic analyses have proven the case that manipulation of upstream transcription factors or signaling genes can result in the expression of multiple target tolerance effector genes, and thereby greatly enhance abiotic stress tolerance (Chinnusamy et al., 2005).

CHAPTER III: MATERIALS AND METHODS

3.1 Plant Growth Conditions

The seeds of common bean cv. "Sierra" were supplied by Dr. Venu (Kal) Kalavacharla (Delaware State University, Department of Agriculture and Natural Resources, Dover DE). (As a note, the germination, hydroponic growth, salt treatment, and collection of tissues were carried out by Adrianne Brown, a former graduate student in the Molecular Genetics & EpiGenomics Laboratory at DSU). Further processing of collected tissue material for the molecular genetics and genomics was carried out for this MS thesis research. The seeds were surface sterilized with sodium hypochlorite for 5 min and rinsed three times with distilled water. Seeds were slightly scarified for faster germination and then germinated in rock wool cubes. After 14 days, germinated seeds were then placed in hydroponic Hoagland nutrient solution (Hoagland & Arnon, 1950); the solution was changed every 3 days. Plants were left to acclimatize in the nutrient solution for 7 days before salt stress was applied. Subsequently, plants were subjected to salt stress of 0 mM (control-no stress), 50 mM (moderate salinity), and 150 mM (high salinity) of NaCl for 10 days and five weeks; steady step adjustment of NaCl was carried out to reduce the possibility of plasmolysis because of osmotic shock during salt treatment (Shavrukov Y. , 2013). Therefore, for the 150 mM salt treatment, 50 mM of NaCl was given daily until it reached 150 mM on day 3. A pH meter was used to test the pH of the solution which should be 6-6.5 (sodium hydroxide, NaOH was used to increase pH when it was low and acetic acid, CH_3COOH was used to lower the pH when it was high). The experiment was conducted in the growth chamber at Delaware State University with temperatures of 25 ± 3 °C and relative humidity 50%–60%, light

250 µmol of photons $m^{-2} \cdot s^{-1}$ on a 16/8-h day/night cycle. Randomized block design was utilized for the experiment with three biological replicates and three technical replicates for each treatment and each stress time points.

3.2 Sample Collection and RNA Isolation

The root tissues from salt treated and control plants were collected at ten days and five weeks after salt treatment. Samples were frozen in liquid nitrogen and stored at -80°C prior to RNA extractions. Total RNA was isolated from control, moderate and high salt stressed root samples collected at ten days and five weeks after stress using NucleoSpin® RNA Plant (Macherey-Nagel Inc. Bethlehem, PA, USA) according to the manufacturer's protocol. Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used to assess reagent contamination (A260/A230 nm ratios) and protein contamination (A260/A280) of all RNA samples; only those samples with OD260/280 >1.8 were used for sequencing and downstream validation. The RNA purity/quality was determined by agarose gel electrophoresis (1.5%) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) based on 28S/18S rRNA band intensity and RNA integrity number respectively. The concentrations of all RNA samples were measured by Qubit® fluorometer (Invitrogen, Catalog # Q32857).

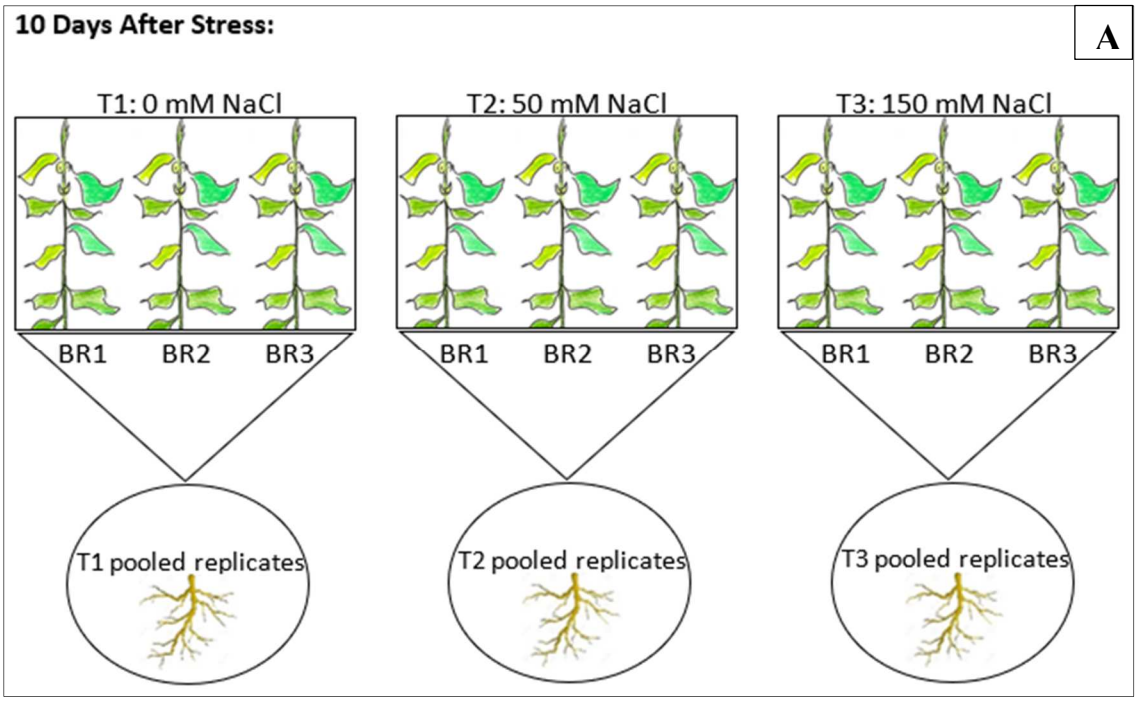
3.3 Library Construction and Sequencing

Total RNA (700 ng) was used to build sequencing libraries with the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA) as per the guidelines of the Low Sample (LS) protocol. The steps involved in the library construction included: the purification and fragmentation of mRNA from total RNA, first and second strand cDNA synthesis, end repair, adapter ligation, PCR amplification, library validation, normalization and pooling. Eighteen

libraries were constructed from the experiment which included three treatments, three biological replicates for each treatment and two collection time points **(Table 1).** The Illumina HiSeq 2500 platform was used to sequence the eighteen libraries, which generated paired-end 101 nucleotide reads. Libraries were sequenced at the University of Delaware Sequencing and Genotyping Center at the Delaware Biotechnology Institute (DBI-Newark, DE, USA).

3.4 Transcriptome Analysis

CLC Genomics workbench v10 (Qiagen, København, Denmark) was used to analyze the reads that were sequenced at DBI. Sequenced reads were downloaded and imported; then the RNA-seq mapping tool was used to process reads (at this point the reads for three biological replicates for each treatment as well as each time point were pooled together to be processed- so biological replicates 1, 2 & 3 for 0 mM collected at 10 days were pooled together and the same was carried out for 50 mM and 150 mM for 10 days as well as 5 weeks) (**Fig. 1**). Reads were mapped to the common bean genome v2 (https://phytozome.jgi.doe.gov/pz/portal.html) annotated with genes and transcripts. Expression values were calculated using Transcripts per Kilobase Million (TPM). Mapped read samples of control (0 mM), moderate (50 mM) and high (150 mM) NaCl collected at ten days and five weeks of salt stress were compared to one another by setting up an experiment to determine differential gene expression across the root transcriptome.



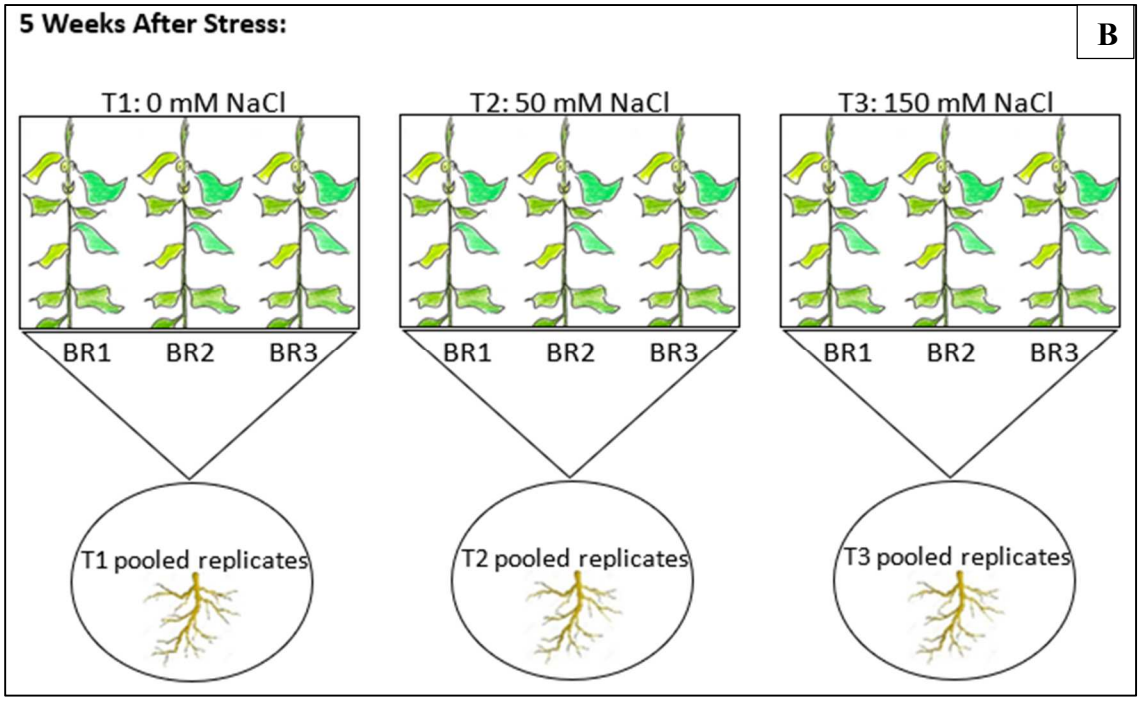


Figure 1. Pooling of biological replicates for reads processing: A. 10 days salt stressed plants. B. 5 weeks salt stressed plants. Biological replicates (1, 2 & 3) for each treatment were pooled as one for processing and mapping to the genome. T1-Treatment 1 (0 mM), T2- Treatment 2 (50 mM), T3- Treatment 3 (150 mM). BR1- Biological replicate 1, BR2- Biological replicate 2 and BR3- Biological replicate 3.

3.5 Functional Classification, Gene enrichment and Pathway Analysis

PANTHER classification was used to perform functional classification on the differentially up-regulated genes; this involved PANTHER GO-Slim categories of biological processes, molecular function and cellular components (Mi et al., 2016). Furthermore, Gene Ontology (GO) term enrichment was carried out on the up- and down-regulated genes using PANTHER overrepresentation test with Bonferroni correction for multiple testing of $P<0.05$ (Consortium et al., 2000; Mi et al., 2016). Pathway analysis was performed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa et al., 2015).

3.6 Validation of RNA-Seq data by Real Time Quantitative RT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) validation was carried out to quantitatively measure the amplification of cDNAs, as an indicator of gene expression by using ABI 7500 real-time PCR (Applied Biosystems, Foster City, CA). We selected five salt responsive genes that were differentially expressed based on RNA-Seq analysis for corroboration. The details of genes and respective primers can be found in **Table 10**. The primers for the selected genes were designed by using PrimerQuest Tool (Integrated DNA Technology, Coralville, IA, USA). The RNAs that were derived from control, moderate, and high salt treatments collected at ten days and five weeks after stress were reverse transcribed to complementary DNA (cDNA) using ProtoScript II (NEB, Ipswich, MA, USA) according to the manufacturer's instruction. qRT-PCR was performed in 25µl reactions that contained 10ng of cDNA (2µL), 12.5µL of Power SYBR Master mix, 0.5µL each of forward and reverse primer and 9.5 µL of nuclease free water. Actin11 was used as an endogenous internal control for each qRT-PCR run. PCR conditions for qRT-PCR were as follows: 95°C for ten min, followed by 40 cycles of 95°C for 15 s and 55°C for one min. In our study, we used three biological replicates,

three treatments (0 mM- control, 50 mM- moderate & 150 mM- high salt stress), two collection time points after treatment (ten days and five weeks). There were three technical replicates quantified for each sample. Fold change differences due to treatment for each sample in each experiment were calculated using the 2-ΔΔCT method (Schmittgen & Livak, 2008).

CHAPTER IV: RESULTS AND DISCUSSION

Unfavorable environmental stresses such as elevated salinity have caused plants to develop a range of complex mechanisms at various levels that are needed for survival (Yamaguchi-Shinozaki & Shinozaki, 2006). An intricate signaling network is efficiently and timely instigated upon the sensing of external stress signals which leads to a shift in the expression of a huge set of stress-responsive genes (Golldack et al., 2014; Nakashima et al., 2014; Shinozaki & Yamaguchi-Shinozaki, 2007; Zhu, 2002). This shift in the expression of stress-responsive genes at the whole genome level regularly stimulates several cellular, physiological, biochemical, and metabolic processes such as stomatal closure and suppression of cell growth and photosynthesis (Hu & Xiong, 2014; Shinozaki & Yamaguchi-Shinozaki, 2007). Under salt stress plants sense the signals primarily in the roots; hence immediate sensing and response in roots is crucial for survival. Two key phases are used to describe a plant's response to salinity at the basic level: 1. Initially, there is the shoot ion-independent response that occurs within minutes to days and is perceived to be associated with Na^+ sensing and signaling (Gilroy et al., 2014; Roy et al., 2014). In this primary phase, the impact of salinity on water relations can be significant, triggering stomatal closure and the hindrance of leaf expansion (Munns & Termaat, 1986). In the secondary phase, the ion-dependent response to salinity progresses over a lengthier period (from days to weeks) and includes the accumulation of ions to toxic concentrations in the shoot, mainly in older leaves which results in pre-mature senescence of leaves and eventually decreased yield or even death of the plant (Munns &Tester, 2008). Three associated plant activities are required to achieve salt tolerance: prevention or reduction of damage, re-establishment of homeostasis in the new, stressful conditions and resumed plant

growth even at a slow rate after the adjustments at the cellular and whole-plant levels (Amudha & Balasubramani, 2011; Zhu, 2001).

4.1 Physiological Effects of Salt Stress

The hydroponics system setup contained three blocks which represents biological replicate 1, 2 & 3 **(Fig. 2 A)**. According to Pandey & Penna (2017), plants employ several strategies for adjustment for their survival which include a change in root and shoot length. A decrease in shoot growth is a noticeable sign of salinity stress which ultimately can alter the distribution of biomass between roots and shoots (Negrão et al., 2017). Läuchli & Grattan (2007) mentioned that root and shoot growth is repressed under salinity and that added Ca partially mitigates the growth inhibition. In our study it was evident that salinity stress does affect root growth, as the higher the intensity of the salt stress (from 0 mM-150 mM NaCl) the less root growth **(Fig. 2).** This study was in agreement with others that identified reduced root growth in common bean and soybean under salinity stress (Gama et al., 2007 and Wei et al., 2009). The ionic phase of salt stress leads to chlorosis and necrosis of plant leaves which is as a result of Na^+ buildup that impedes on several physiological processes in plants (Munns, 2002). Wahid et al (2004) observed enhancement of chlorotic and necrotic effects on leaves as the ionic content increases. Necrotic and chlorotic effect was evident in our study at five weeks (ionic phase) after salt treatment **(Fig. 3 A & B).**

A. 

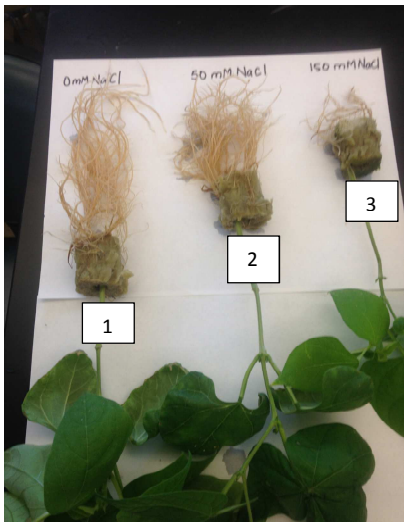
B. 

Figure 2. Experiment setup and root growth under salinity stress: A. Hydroponic system set up of experiment; three biological replicates. **B.** Root length under different concentrations of salt- 1. 0 mM NaCl (0 dS/m) 2. 50 mM NaCl (5 dS/m) 3. 150 mM NaCl (15 dS/m)

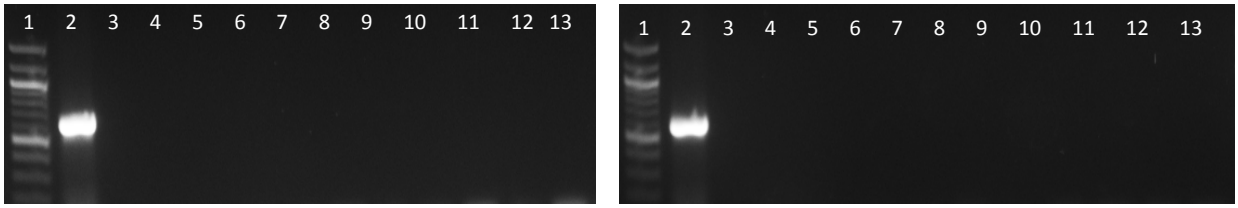
A. 

B. 

Figure 3. Necrotic and chlorotic effect: A. Necrotic effect of salinity (death of older leaves). B. Chlorotic effect of salinity (yellowing of leaves)

4.2 Tests for DNA contamination in reverse transcriptase PCR

To confirm that the RNAs used in these experiments were free of DNA contamination, we amplified genomic DNA (gDNA) and cDNA with SK14, a common bean molecular marker that will only amplify gDNA as it is likely from the intronic region of the gene (Kalavacharla et al., 2011). Primers that were derived from the SK14 sequence were used to amplify a 600 bp product from genomic DNA and cDNA. SK14 amplified in gDNA, but not cDNA **(Fig. 4).**



A. **B.**

Figure 4. DNA contamination testing: SK14 used to test for DNA contamination in RNA samples **(A) Ten days salt treated samples**; Lane 1- 100bp ladder, Lane 2-gDNA of SK14, Lane 3- Negative control-1 (no reverse transcriptase was added to cDNA synthesis), Lane 4- Negative control-2 (H_2O), Lane 5- 0 mM Rep 1, Lane 6- 0 mM Rep 2, Lane 7- 0 mM Rep 3, Lane 8- 50 mM Rep 1, Lane 9- 50 mM Rep 2, Lane 10- 50 mM Rep 3, Lane 11- 150 mM Rep 1, Lane 12- 150 mM Rep 2, Lane 13-150 mM Rep 3. **(B) Five weeks salt treated samples**; Lane 1- 100bp ladder, Lane 2-gDNA of SK14, Lane 3- Negative control-1 (no reverse transcriptase was added to cDNA synthesis), Lane 4- Negative control-2 (H_2O), Lane 5- 0 mM Rep 1, Lane 6- 0 mM Rep 2, Lane 7- 0 mM Rep 3, Lane 8- 50 mM Rep 1, Lane 9- 50 mM Rep 2, Lane 10- 50 mM Rep 3, Lane 11- 150 mM Rep 1, Lane 12- 150 mM Rep 2, Lane 13-150 mM Rep 3.

4.3 Library Preparation

Libraries were prepared using the roots of control and salt treated bean plants (0 mM, 50 mM and 150 mM of NaCl) collected ten days and five weeks after stress. This resulted in a ~260pb product which was used for sequencing (**Fig. 5**)

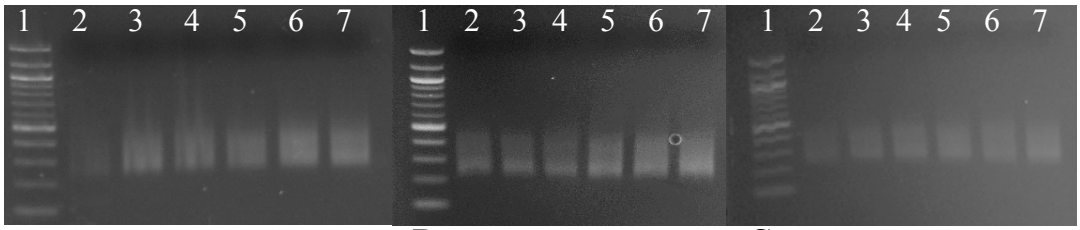


Figure 5. Gel images of libraries: Eighteen (18) libraries were constructed from the roots of salt treated and control plants. **A. Biological replicates 1**; Lane 1-100bp ladder, Lane 2- ten days 0 mM NaCl root, Lane 3- ten days 50 mM NaCl root, Lane 4- ten days150 mM NaCl root, Lane 5- five weeks 0 mM NaCl root, Lane 6- five weeks 50 mM NaCl root, Lane 7- five weeks 150 mM NaCl root. **B. Biological replicates** 2; Lane 1-100bp ladder, Lane 2- ten days 0 mM NaCl root, Lane 3- ten days 50 mM NaCl root, Lane 4- ten days 150 mM NaCl root, Lane 5- five weeks 0 mM NaCl root, Lane 6- five weeks 50 mM NaCl root, Lane 7- five weeks 150 mM NaCl root. **C. Biological replicates 3**; Lane 1-100bp ladder, Lane 2- ten days 0 mM NaCl root, Lane 3- ten days 50 mM NaCl root, Lane 4- ten days150 mM NaCl root, Lane 5- five weeks 0 mM NaCl root, Lane 6- five weeks 50 mM NaCl root, Lane 7- five weeks 150 mM NaCl root.

4.4 Data Collection and Pre-processing

Eighteen (18) libraries were generated from the root samples collected from three biological replicates, three treatment conditions and two collection time points after treatment. RNA-Seq of these 18 libraries resulted in ~ 912 million reads (Table 1). Biological replicates 2 & 3 for high stressed root samples (150 mM NaCl) collected at five weeks after treatment had low mapping percentage **(Table 1);** according to CLC Genomics workbench v10 (Qiagen, København, Denmark) there could possibly be a problem with the sample quality, which is potentially due to the stress that the plants are undergoing. When isolating RNA from these salt stressed roots (150 mM NaCl) their appearance was different from the others; the roots were dry as a result of the prolonged salt stress. However, the RNA quality of these samples were good as they had a RNA Integrity Number (RIN) of $\geq$7.5. In the subsequent analysis, we still worked with the low mapped reads, as at least one of the biological replicate in this high salinity stress show high mapping percentages. Further research needs to be conducted and repetitions of experiments are needed to identify genes involved at high salinity stress.

Table 1. Summary statistics of RNA-Seq (Illumina/HiSeq 2500): Total number of reads collected, mapped and mapping percentage of three biological replicates of control and salt treated common bean. 0 mM (Control), 50 mM (Moderate salt stress) and 150 mM (high salt stress). Eighteen libraries generated.

Treatments & time points	Replicate	# of reads collected	# of reads mapped to the reference genome	% of mapped reads
10 days 0 mM	Replicate 1	45,177,178	35,380,345	78.32
10 days 0 mM	Replicate 2	46,955,698	40,525,227	86.31
10 days 0 mM	Replicate 3	52,818,744	43,480,845	86.11
10 days 50 mM	Replicate 1	50,648,134	43,432,425	85.75
10 days 50 mM	Replicate 2	50,370,210	44,215,468	87.78
10 days 50 mM	Replicate 3	57,518,888	46,199,443	80.32
10 days 150 mM	Replicate 1	54,968,046	46,199,731	84.04
10 days 150 mM	Replicate 2	45,974,504	36,602,855	79.62
10 days 150 mM	Replicate 3	45,521,614	39,342,802	86.43
5 weeks 0 mM	Replicate 1	64,127,856	55,048,050	85.85
5 weeks 0 mM	Replicate 2	61,544,460	52,814,108	85.81
5 weeks 0 mM	Replicate 3	58,396,700	50,031,646	85.67
5 weeks 50 mM	Replicate 1	56,490,560	49,554,500	87.72
5 weeks 50 mM	Replicate 2	7,770,926	6,916,906	89.01
5 weeks 50 mM	Replicate 3	46,213,748	39,974,962	86.5
5 weeks 150 mM	Replicate 1	53,755,242	41,602,742	77.39
5 weeks 150 mM	Replicate 2	57,804,592	94,758,977	8.23
5 weeks 150 mM	Replicate 3	56,392,232	1,776,355	3.15

4.5 Differentially Expressed Genes

Differentially Expressed Genes (DEGs) were identified based on fold change (FC) due to treatment with upregulated genes having a FC of ≥2 and downregulated genes of FC ≤ -2. There were more DEGs in roots at five weeks in comparison to ten days under moderate (MS-50 mM) and high (HS-150 mM) salt stress **(Fig. 6 & 5 A, B)**. Additionally, there were also more DEGs in the high salt stress treatment compared to moderate salt treatment at ten days and five weeks **(Fig. 6)**, suggesting that the five weeks (prolonged) salt stress as well as high salt treatment undergo more intricate transcript regulation when compared to ten days (onset) salt stress and moderate salt treatment respectively. Interestingly, there were more down-regulated genes than up-regulated genes under high salt stress at ten days and five weeks and moderate stress at ten days; however, the downregulation was significant at five weeks under high salt treatment **(Fig 6).** These results imply that salt stress, particularly prolonged high salt stress tends to deter gene expression. Furthermore, there were more up-regulated genes at five weeks (prolonged stress) compared to ten days (onset of stress) under both moderate and high salt stress **(Fig. 6 & 5 A, B).;** similarly there were more up-regulated TFs at five weeks than ten days under both moderate and high salt stress **(Fig. 9).** Shavrukov (2012) mentioned that in response to osmotic stress under salt conditions, there is an initiation of fairly smooth fluctuations in gene expression but in response to the ionic phase there is more distinct change in expression of major numbers of genes. When considering up-regulated genes, our results could indicate that prolonged salt stress, which is the ionic phase of salt stress causes more genes to be expressed than the onset of stress.

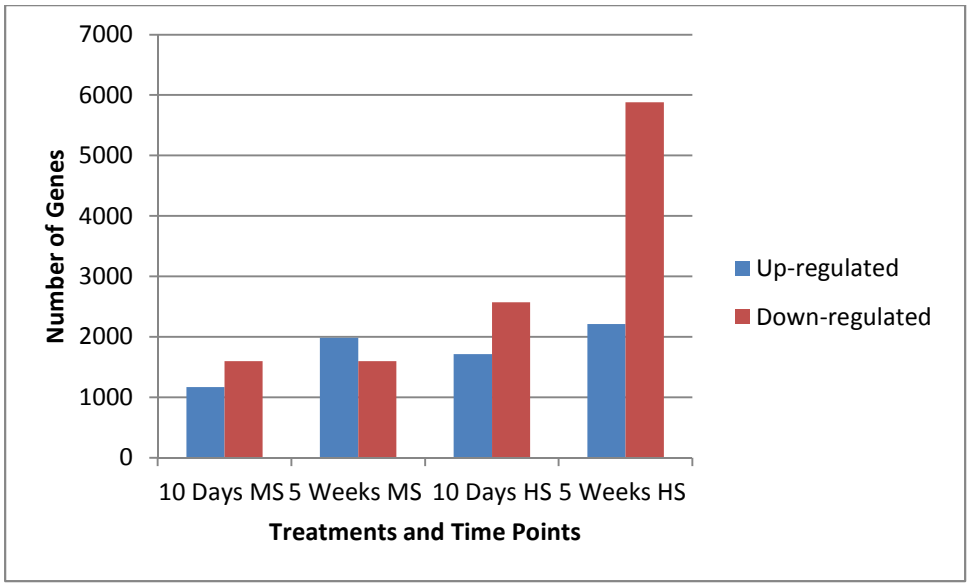
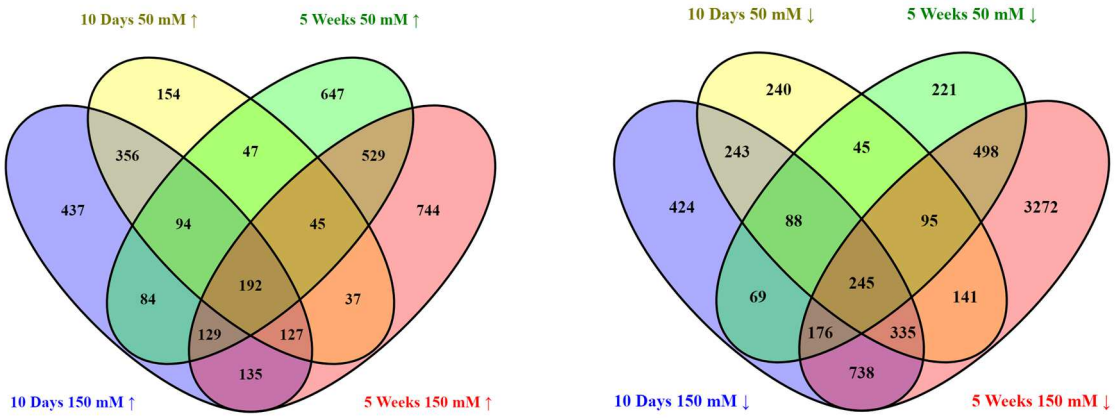


Figure 6. Differentially Expressed Genes (DEGs): Total number of genes that were upregulated and downregulated at ten days and five weeks of moderate (MS-50 mM) and high salt stress (HS-150 mM).



A. **B.**

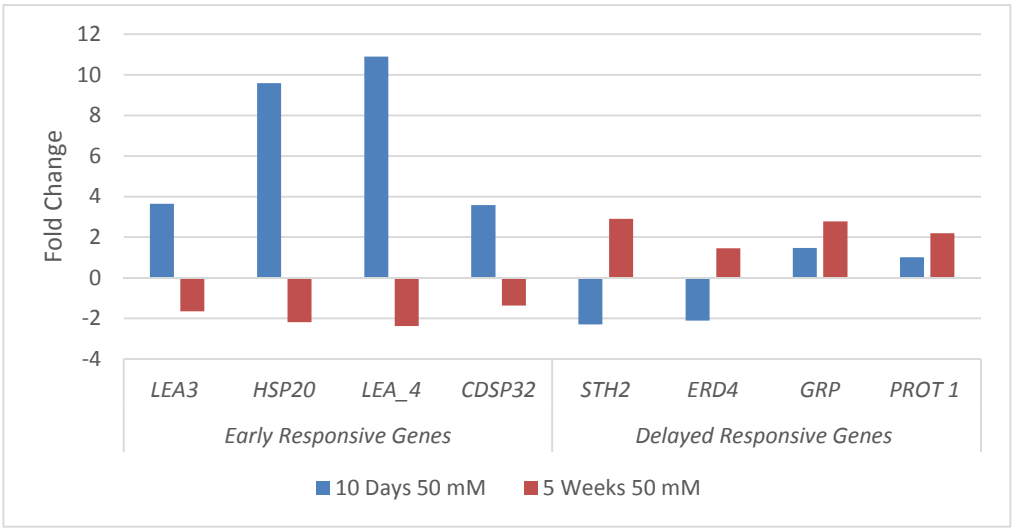
Figure 7. Four-way venn diagrams showing the number of common and specific differential gene expression of 50 mM and 150 mM of salt stress in root tissues of common bean: A- Differentially up-regulated (↑) genes of 50 mM and 150 mM salt stress at ten days and five weeks of salt treatment. **B-** Differentially down-regulated (↓) genes of 50 mM and 150 mM salt stress at ten days and five weeks of salt treatment.

4.6 Early and Delayed Salt/Drought Genes

Increased levels of salt in the soil is a possibility for the initiation of drought stress; likewise, the co-occurrence of salinity and drought is very common (Czolpinska and Rurek, 2018). Manchanda and Garg (2008) reported that a water deficit condition is induced by salt stress as a result of the salt concentration in the rhizosphere. Global transcriptional changes are employed by the plants in response to stress which can be temporary or continual over time with early and delayed responsive gene expression variations (Caldana et al., 2011). Kaur & Gupta (2005) reported that stress responsive genes have been categorized as either early or delayed response genes based on their expression at the molecular level. Early response genes are known to be activated rapidly and most times temporarily after stress detection. There is a slower activation of delayed response genes and their expression is frequently maintained throughout stress conditions (Kaur and Gupta, 2005). Based on these known facts, some early and delayed salt/drought responsive genes were identified in our study based on their fold change values at ten days vs five weeks of 50 mM and 150 mM salt stress. Some early response genes that were identified include: Heat Shock Protein 20 (*HSP20*), Late Embryogenesis Abundant (*LEA3 & LEA_4*), Sodium Hydrogen Exchanger 1 (*NHX1*), *Na+ symporter* and Chloroplastic Drought-Induced Stress Protein of 32kD (*CDSP32*) **(Fig. 8 A & B).** These genes were grouped as early responsive genes as they were upregulated at ten days of salt stress but down-regulated at five weeks. Among the salt/drought responsive genes that were identified, the following were classified as delayed response genes: Glycine-Rich Protein (*GRP*), Proline Transporter 1 (*PROT1*), Salt Tolerance Homolog 2 (*STH2*), Early Responsive to Dehydration 4 (*ERD4*), *DnaJ*/Heat Shock Protein 40 (*HSP40*) and Chloride Channel A (*CLC-A*) **(Fig. 8 A & B).** These genes were classified as delayed responsive because they were either upregulated at ten days and

maintained their expression up to five weeks or they were downregulated at ten days but up-regulated at five weeks.

A.



B.

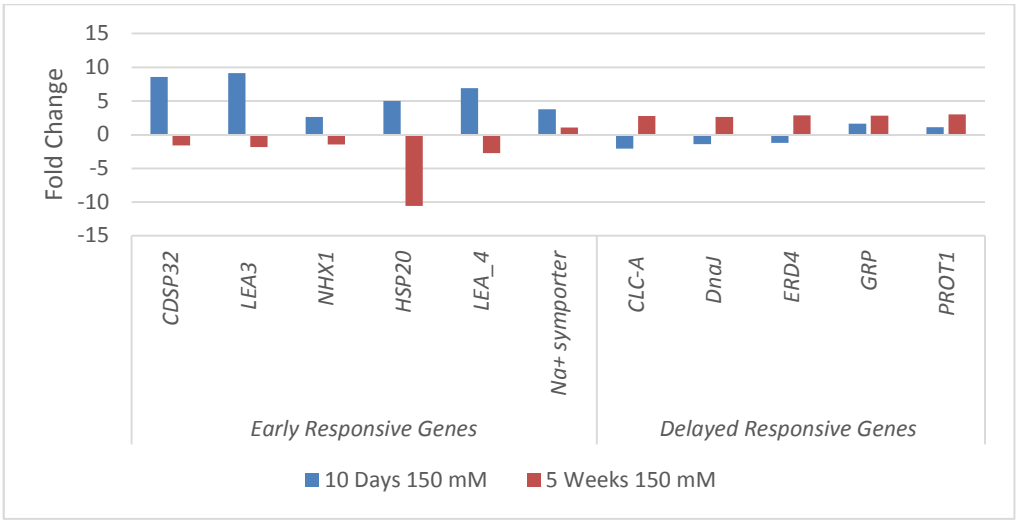


Figure 8. Salt/drought response genes: Early and delayed salt/drought responsive genes of 50 mM salt stress **(A)** and 150 mM salt stress **(B).** These genes were classified as early or delayed based on their fold change expression values.

Table 2. Salt related genes: These genes identified in our study were neither classified as early nor delayed based on their fold changes (FC).

Gene ID	**Description**	**10 Days 50 mM NaCl (FC)**	**5 Weeks 50 mM NaCl (FC)**
Phvul.008G136700.1	Sodium Bile acid symporter family	-1.28612	-2.82508
Phvul.010G150800.1	Heat shock protein 70 (*Hsp 70*) family protein	1.495019	-6.90019
Gene ID	**Description**	**10 Days 150 mM NaCl (FC)**	**5 Weeks 150 mM NaCl (FC)**
Phvul.001G221700.1	Low temperature and salt responsive protein family (*RC12B*)	-1.14093	-10.2875
Phvul.002G185150.2	sodium proton exchanger, putative (*NHX7*) (*SOS1*)	1.480136	-179.614

In response to dehydration and other various stresses the early responsive to dehydration (*ERD*) genes can possibly be induced (Ayyappan et al., 2015; Liu et al., 2009). According to Rai et al (2016) Early Responsive to Dehydration (*ERD*) genes are a group of plant genes that play roles in plant stress tolerance and development. A maize Early Responsive to Dehydration gene, (*ZmERD4*) was identified by Liu et al (2009) which provided improved salt tolerance in *Arabidopsis.* Also, *ERD 4* was identified to confer salt tolerance and enhanced plant growth in *Arabidopsis* (Rai et al., 2016). We noted that the *ERD4* gene was a delayed responsive gene under 50 mM & 150 mM salt stressed plants that was only up-regulated at five weeks **(Fig. 8A & 8B)** indicating that this gene aids the plant in growth and adaptation under prolonged salt stress. *LEA* proteins belong to a group of hydrophilic and thermostable proteins that are initiated by dehydration from salt, drought, cold and heat stresses. Previous biochemical, biophysical and bioinformatics research have indicated the likely roles of *LEA* proteins in osmotic stresses which includes functioning as: (i) antioxidants (ii) membrane and protein stabilizers and (iii) "space filler" to avoid cellular collapse (Tunnacliffe and Wise, 2007). The *LEA* genes identified in our study were classified as early responsive genes that were only up-regulated at ten days under

both 50 mM and 150 mM of salt stress and significantly down-regulated at five weeks **(Fig. 8 A & B).** According to Broin et al (2000) Chloroplastic Drought-Induced Stress Protein of 32kD (*CDSP32)* can sustain chloroplastic structures against oxidative damage upon drought stress. In the present study *CDSP32* was significantly upregulated at ten days of 50 mM and 150 mM salt stress but was down-regulated at five weeks **(Fig. 8 A & B).** An earlier study by Eymery & Rey (1999) discovered that *CDSP32* was significantly synthesized in *Solanum tuberosum* plants under water deficit condition.

Synthesis of Heat Shock Proteins is common in all cells in response to several types of environmental stresses. It is recognized that under stress conditions such as heat and NaCl, Heat Shock Proteins are induced (Zhichang et al., 2010). *HSP* play a critical role in plant defense against stress by regenerating standard protein conformation and hence cellular homeostasis (W. Wang et al., 2004). In Arabidopsis and some other plant species, salinity stress was identified to trigger the synthesis of *HSPs* (Swindell, Huebner, & Weber 2007); for instance it was reported by Zhichang et al (2010) that the overexpression of *Arabidopsis DnaJ* (*HSP40*) contributed to NaCl-stress tolerance. In our study *DnaJ* was a unique delayed responsive gene of high salt stress that was only upregulated at five weeks **(Fig. 8 B)**, while *HSP20* was considered an early response gene **(Fig. 8 A & 8 B)**. According to Sachetto-Martins et al (2000) plant Glycine Rich Protein (*GRP*) genes display developmentally controlled expression patterns and these patterns are also adjusted by abiotic factors. Additionally, *GRP* gene expression is regulated in the main developmental stages, for instance pollen and embryo development (Czolpinska and Rurek, 2018). This *GRP* gene in our study was expressed both at ten days and five weeks of 50 mM and 150 mM salt stress; however, its expression was significant at five weeks **(Fig. 8 A & B)** which indicates that this gene may play a role in plant development under salt stress. Proline

homeostasis is vital for actively dividing cells as it aids to uphold continual growth during long-term stress (Kavi Kishor & Sreenivasulu, 2014). Upon stressful conditions proline transporters represent a significant factor for proline homeostasis (Kishor & Sreenivasulu, 2014). Continual growth was evident in our study as most of the plants maintained growth up to five weeks of 50 mM and 150 mM salt stress indicating the role that the Proline Transporter (*PROT1*) may have played as it was upregulated both at ten days and five weeks of 50 mM and 150 mM of salt stress treatment **(Fig. 8 A & B)**. Proline buildup is a regular response to salt stress in numerous plants (H. Wang et al. 2015). Likewise, Kishor et al (2015) suggested that proline possibly play apart throughout flower formation and its successive development.

Sodium Hydrogen Exchanger (*NHX*) is a gene that is identified to be involved in the sequestration of Na in vacuoles which possibly apportion surplus Na in root and leaf vacuoles. A recent study showed that a lack in expression of *NHX2* resulted in non-sequestration of Na into the vacuoles in the roots and that upregulation of *NHX2* was significantly greater in salt treated roots compared to control of G01 genotype of alfalfa (Sandhu et al., 2017). *NHX1* was seen to be playing a role only under high salt stress (150 mM) compared to moderate (50 mM) **(Fig. 8A & 8B)** where it was only upregulated at ten days and down-regulated at five weeks. This result may suggest that there was more Na accumulation under 150 mM (high) salt stress compared to 50 mM (moderate). The Chloride Channels (*CLCs*) protein family is known to primarily moderate Cl^-transport (Zifarelli and Pusch 2010). Nguyen et al (2015) reported that plants utilize transporters such as members of the *CLC* family to clear the cytoplasm of Cl^- to offset chloride toxicity. In Arabidopsis *AtCCC* and *AtCLCc* encode for chloride channels or transporters which have been recognized to be vital for Cl^- homeostasis under high salinity (Colmenero-Flores et al., 2007; Jossier et al., 2010). In a recent study done by Wei et al (2016)

the NaCl-treated plants used the anion transporter *CLCs* to alter and decrease Cl^- buildup in the cell cytoplasm in a response to Cl^- toxicity under salt stress. Additionally, salt tolerance was conferred by the overexpression of *ZmCLC-d* in Arabidopsis by a decrease in the accumulation of Cl^- in the transgenic plants when compared to wild type (Wang et al., 2015). In our study the chloride channel A transporter was only observed to be upregulated at five weeks of 150 mM salt stress. This was a unique delayed gene of high (150 mM) salt stress (**Fig. 8 B**), suggesting that the high salt stressed plants encountered chloride toxicity during prolonged stress compared to onset of stress in which this transporter gene was downregulated at that time.

Other salt genes that were identified in our study includes: Sodium Proton Exchanger (*NHX7, SOS1*), Sodium Bile Acid Symporter family (*BASS*), heat shock protein 70 (*HSP70*), and low temperature and salt responsive protein family (*RC12B*) (**Table 2**). The Na^+/H^+ antiporters (*NHXs*) are secondary ion transporters that are involved in the exchange of H^+ and transport of Na^+ or K^+ across membrane, they have key roles in plant development and stress responses (Tian et al., 2017). Upon detrimental accumulation of Na^+ , *NHX* (*NHX7/SOS1*) actively release Na^+ out of the cell and the Vac-class *NHX* facilitate the apportioning of Na^+ into vacuole (Apse and Blumwald, 2007; Pardo et al., 2006; Shi et al., 2002). *SOS1* was shown to decrease Na^+ accumulation and enhance salt tolerance of the mutant cells when expressed in a yeast mutant deficient in endogenous Na^+ transporters (Shi et al., 2003). Also, *SOS1* overexpression in Arabidopsis revealed enhanced level of salinity tolerance (Yang et al., 2009). Elevated levels of Na+ possibly will encumber the activity of the plastidic bile acid/sodium symporter family protein 2 (*BASS2*) (Müller et al., 2014). *HSP 70* gene family has functional roles in guarding plant cells or tissues from environmental stresses through degradation of misfolded and condensed proteins as molecular chaperones (Guo et al., 2015).

According to Medina et al (2001) *RC12B* is involved in upholding membrane function and/or stability under water stress situations that is triggered by environmental conditions that decrease water availability.

4.7 Transcription Factors

Plants can activate various molecular, cellular, and physiological modifications in order to respond and acclimatize to salt stress. Numerous salt-related genes are induced throughout these responses and adaptations (Sakamoto et al., 2004) such as transcription factors (TFs) that play crucial roles in salt stress responses by transcriptional control of the downstream genes that are accountable for salt tolerance (Sun et al., 2010). Stress-responsive transcription factors largely connect with promoter elements of stress genes which ultimately results in expression of numerous functional genes (Ramegowda et al., 2012).

Data mining was carried out to highlight the TFs in our study; then a Venn diagram software- Venny 2.1 (Oliveros, 2015) was used to identify temporal differences in expression of the up-regulated (FC= ≥2) TFs at ten days and five weeks under moderate and high salt stress. A total of 42 TFs were recognized to be significantly upregulated under moderate stress (MS) ten days and five weeks **(Fig. 9, Table 4)**. There were more significantly upregulated TFs in MS at five weeks (32) compared to ten days (15) **(Fig. 9)**. While a total of 47 TFs were recognized to be significantly upregulated under high salt stress (HS) ten days and five weeks of salt stress **(Fig. 9, Table 5)**. There were more significantly upregulated TFs in HS at five weeks (31) compared to ten days (26) **(Fig.7)**. Amid the many transcription factors that are involved in abiotic stress responses, it is important to identify 'primary or master' regulators which will reveal the hierarchy of molecular mechanisms in the regulatory network (Bhattacharjee and Jain, 2013). In our study 'master' regulators were identified based on prolonged expressions of the

transcription factor from ten days to five weeks of stress. Five 'master' transcription factor families were found to be upregulated both at ten days and five weeks of moderate salt stress (MS) which includes TFIIE, zinc finger, MADS-box and GATA transcription factor **(Fig. 9, Table 4).** Ten 'master' TFs were found to be upregulated both at ten days and five weeks of high salt stress (HS) including WRKY type TFs, TFIIE, MADS-box, bZIP, DPB, PHD, NAC, GRAS, HSF **(Fig. 9, Table 5)**.

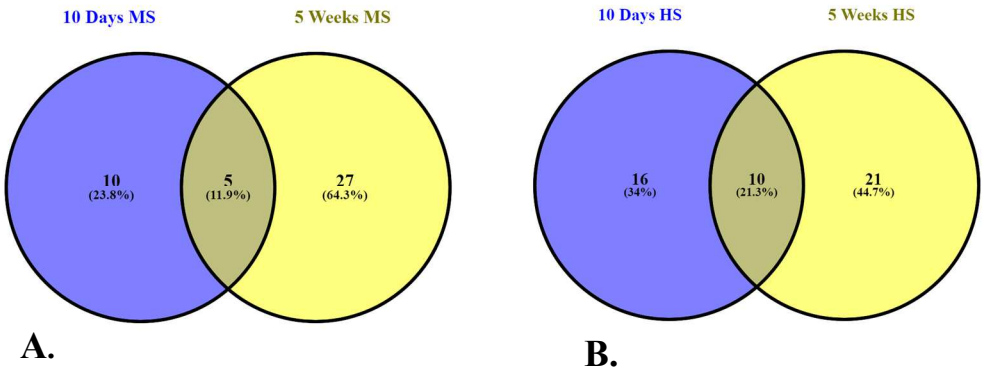


Figure 9. Differentially expressed transcription factors (TFs) that were identified based on fold change ≥ 2: The number of transcription factors that were upregulated at a certain time point whether at ten days or five weeks of **A.** Moderate stress (MS-50 mM) and **B.** High salt stress (HS-150 mM) and those that are upregulated both at ten days and at five weeks.

Table 3. 'Master' transcription factors (TFs): Transcription factors that were upregulated both at ten days and five weeks under moderate (MS) and high (HS) salt stress based on fold change (FC).

Gene ID	**Description**	**10 days MS FC**	**5 Weeks MS FC**
Phvul.009G200700.1	Transcription factor TFIIE, alpha subunit	18.09596	7.163745
Phvul.006G193200.3	protein binding / transcription factor/ zinc ion binding protein	14.50848	2.031565
Phvul.003G182800.1	Agamous-like mads-box protein agl3-related	4.651172	2.235964
Phvul.007G065100.1	K-box region and MADS-box transcription factor family protein	2.196151	2.260276
Phvul.008G157600.1	GATA transcription factor	2.034888	8.695414
Gene ID	**Description**	**10 days HS FC**	**5 weeks HS FC**
Phvul.007G209000.1	WRKY family transcription factor	2.000472	4.907752
Phvul.009G200700.1	Transcription factor TFIIE, alpha subunit	12.5247	3.235535
Phvul.006G202300.2	K-box region and MADS-box transcription factor family protein	8.189224	7.704029
Phvul.007G075400.1	WRKY family transcription factor	3.519552	2.026265
Phvul.001G140100.2	bZIP transcription factor family protein bZIP65,	3.514929	2.191233
Phvul.002G039800.2	Transcription factor DP	3.415826	3.424013
Phvul.008G260100.1	PHD finger transcription factor,	3.372034	4.280016
Phvul.010G120850.1	NAC transcription factor-like 9 NTL9	3.179346	4.280016
Phvul.007G278200.1	heat shock transcription factor A6B	2.635821	2.775679
Phvul.003G291500.1	GRAS family transcription factor	2.141893	2.867777

The ‘master’ regulators that were identified in our study may suggest an involvement of these TFs in plant adaptation for survival under salt stress, as these TFs maintained their expression throughout from ten days to five weeks of stress. Three of these TFs (WRKY, MADS-box and bZIP) are known to be involved in flower development and flowering time (Ali et al., 2016; Cai et al., 2014; Li et al., 2016; Michaels et al., 2003). According to Budak et al (2013) the WRKY transcription factor is a part of a very huge family of transcription factors that is possibly involved in drought/salt stress response. WRKY-type transcription factors are essential for regulating a surplus of downstream stress-related genes, causing biochemical and physiological variations needed for plant adaptation (Ouyang et al., 2007; Phukan, Jeena, and Shukla, 2016). In our study *WRKY* transcription factors maintained their expression from ten days to five weeks under high salt stress **(Table 3)**. Numerous reports have demonstrated the regulating role of WRKY TFs in signaling pathways and variation of diverse molecular and physiological processes such as flowering time and plant height (Cai et al., 2014). Similarly, Li et al (2016) recognized the significance of WRKY proteins in flowering time. Previously, Gupta et al (2011) reported that a few MADS transcription factors played essential roles as regulators in responding to abiotic stresses. Mads-box transcription factors (TFs) are essential in floral organ specification as well as some other features of plant growth and development (G. Saha et al., 2015). Michaels et al (2003) also observed MADS-box genes to be key players in regulating flowering time. Ali et al (2016) reported that Basic Leucine Zipper Protein (bZIP) belongs to a huge transcriptional factor family that plays a role in several developmental and stress responses such as flower development, maturation of seeds, biotic and abiotic stress signaling (Ali et al., 2016).

NAC TFs reveal significant roles during plant development and responses to abiotic stress (Nakashima et al., 2009). A transcriptional profiling analysis done be Jiang & Deyholos, (2006) discovered at least 33 *Arabidopsis* NAC genes that were accountable to abiotic stresses which includes elevated salinity. According to Wei et al (2009) PHD finger proteins are needed for either transcriptional activation or transcriptional repression; under salt stress PHD TF was found to be one of the most abundant TF families (Hiz et al., 2014). Zinc finger proteins are specific class of proteins that play a part in plant responses to abiotic stresses (Martin et al., 2012). Constitutive expression of a Zinc Finger Protein gene *(ZFP3)* displayed improved salt and osmotic stress tolerance in plants (Zhang et al., 2016). When exposed to salt stress ZFP179 gene which is a salt-responsive zinc finger protein displayed increased salt tolerance in rice (Sun et al., 2010). Guo et al (2015) states that heat shock factors (*HSFs*) play essential roles in defense and plant developmental processes. In response to a number of abiotic stresses, plant *HSFs* play a critical role in controlling the expression of stress-responsive genes, for instance Heat Shock Proteins (*HSPs*) (Guo et al., 2016). Recently, Wen et al (2017) discovered that the Arabidopsis HSFA1 facilitated the initiation of *HSP* genes upon salt, osmotic and oxidative stresses and tolerance was conferred throughout plant growth and development. Furthermore, Liu et al (2011) discovered that *HSFA1s* are involved in tolerance and response to salt, osmotic, and oxidative stresses.

Table 4. Differentially expressed transcription factors: Differences in expression of upregulated TFs of ten days and five weeks under moderate salt stress (MS-50 mM NaCl). **Fold change (FC)**

Gene ID	Description	10 days MS (FC)	5 weeks MS (FC)
Phvul.009G200700.1	Transcription factor TFIIE, alpha subunit	18.09596	7.163745
Phvul.006G193200.3	Zinc finger, RING-type;Transcription factor jumonji/aspartyl beta-hydroxylase B160	14.50848	2.031565
Phvul.003G182800.1	K-box region and MADS-box transcription factor family protein AGL2,SEP1	4.651172	2.235964
Phvul.007G065100.1	K-box region and MADS-box transcription factor family protein	2.196151	2.260276
Phvul.008G157600.1	GATA transcription factor 9 GATA9	2.034888	8.695414
Phvul.008G212100.1	KNOX/ELK homeobox transcription factor BUM,BUM1,SHL,STM,WAM,WAM1	4.709311	-1.72505
Phvul.003G124000.1	WRKY family transcription factor AtWRKY42,WRKY42	2.158065	1.396458
Phvul.001G131000.1	heat shock transcription factor B3 AT-HSFB3,HSFB3	3.693577	-1.54598
Phvul.011G035700.2	Basic-leucine zipper (bZIP) transcription factor family protein bZIP23	12.88926	1.691095
Phvul.008G260100.1	PHD finger transcription factor, putative	2.616284	∞
Phvul.003G135400.1	heat shock transcription factor A2 ATHSFA2,HSFA2	2.543609	-1.72037
Phvul.008G270400.2	transcription factor IIIA TFIIIA	2.27928	-1.07322
Phvul.009G142900.2	Basic-leucine zipper (bZIP) transcription factor family protein	2.180237	∞
Phvul.002G061300.1	PLATZ transcription factor family protein	2.093027	-1.25652
Phvul.009G165100.2	Squamosa promoter-binding protein-like (SBP domain) transcription factor family protein	2.011444	-27.8883
Phvul.005G097200.6	TCP family transcription factor 4 MEE35,TCP4	1	49.56386
Phvul.010G134100.4	ATPases;nucleotide binding;ATP binding;nucleoside-triphosphatases;transcription factor binding	-2.67555	7.536026
Phvul.007G025500.2	bZIP transcription factor family protein PAN	1	6.260698
Phvul.006G169600.1	K-box region and MADS-box transcription factor family protein AG	∞	5.217249
Phvul.011G100300.2	Plant-specific transcription factor YABBY family protein YAB2	1	5.217249
Phvul.002G287604.1	GATA type zinc finger transcription factor family protein	-1.14666	4.347707
Phvul.009G165100.3	Squamosa promoter-binding protein-like (SBP domain) transcription factor family protein	-2.03033	3.975046

Phvul.002G297100.1	WRKY family transcription factor ATWRKY53,WRKY53	-1.16957	3.916148
Phvul.002G069700.1	Plant-specific transcription factor YABBY family protein INO	-3.43999	3.478166
Phvul.008G048100.1	WRKY family transcription factor	-1.55619	3.203574
Phvul.011G136115.4	TCP family transcription factor 4 MEE35,TCP4	-2.29333	2.782533
Phvul.011G116800.2	Basic-leucine zipper (bZIP) transcription factor family protein ATBZIP61,BZIP61	-1.28937	2.634658
Phvul.004G037700.1	plastid transcription factor 1 PTF1,TCP13,TFPD	1	2.562859
Phvul.003G291500.1	GRAS family transcription factor family protein GAI,RGA2	1.855229	2.540956
Phvul.002G039800.4	Transcription factor DP ATDPB,DPB	1.630438	2.434716
Phvul.008G190600.1	K-box region and MADS-box transcription factor family protein AGL5,SHP2	∞	2.318777
Phvul.002G251900.1	Transcription factor jumonji (jmj) family protein / zinc finger (C5HC2 type) family protein	1.090118	2.271455
Phvul.008G169000.2	bZIP transcription factor family protein	-1.24717	2.266717
Phvul.003G236300.1	GRAS family transcription factor	-1.24123	2.252903
Phvul.007G223401.1	heat shock transcription factor A3 AT-HSFA3,HSFA3	1.370866	2.231357
Phvul.001G226500.1	GATA transcription factor 16 GATA16	1.966488	2.230563
Phvul.007G174800.1	heat shock transcription factor A6B AT-HSFA6B,HSFA6B	-1.48886	2.207489
Phvul.009G013900.1	K-box region and MADS-box transcription factor family protein AP3,ATAP3	-1.34608	2.086899
Phvul.006G171700.2	heat shock transcription factor A4A AT-HSFA4A,HSF A4A	-2.80296	2.07568
Phvul.004G163300.1	heat shock transcription factor C1 AT-HSFC1,HSFC1	1.36867	2.059402
Phvul.009G213800.1	MADS-box transcription factor family protein	-1.94051	3.043395
Phvul.005G153900.1	Basic-leucine zipper (bZIP) transcription factor family protein	-1.70775	2.549944

Table 5. Differentially expressed transcription factors: Differences in expression of upregulated TFs of ten days and five weeks under high salt stress (HS-150 mM NaCl). **Fold Change (FC)**

Gene ID	Description	10 days HS FC	5 weeks HS FC
Phvul.007G209000.1	WRKY family transcription factor	2.000472	4.907752
Phvul.009G200700.1	Transcription factor TFIIE, alpha subunit	12.5247	3.235535
Phvul.006G202300.2	K-box region and MADS-box transcription factor family protein	8.189224	7.704029
Phvul.007G075400.1	WRKY family transcription factor	3.519552	2.026265
Phvul.001G140100.2	bZIP transcription factor family protein	3.514929	2.191233
Phvul.002G039800.2	Transcription factor DP	3.415826	3.424013
Phvul.008G260100.1	PHD finger transcription factor	3.372034	4.280016
Phvul.010G120850.1	NAC transcription factor-like 9	3.179346	4.280016
Phvul.007G278200.1	heat shock transcription factor A6B	2.635821	2.775679
Phvul.003G291500.1	GRAS family transcription factor family protein	2.141893	2.867777
Phvul.006G171700.1	heat shock transcription factor A4A	2.025467	1.487
Phvul.002G112200.3	K-box region and MADS-box transcription factor family protein	18.305325	∞
Phvul.011G035700.2	Basic-leucine zipper (bZIP) transcription factor family protein	15.970212	1.832943
Phvul.001G131000.1	heat shock transcription factor	9.3948102	1.057708
Phvul.001G037000.2	heat shock transcription factor A2 HSFA2	5.0799467	1.194423
Phvul.004G037700.3	Trancription factor TCP13	4.9242395	1.017034
Phvul.002G227900.2	transcription factor-related	4.6131687	1.21051
Phvul.002G113100.3	Transcription factor IIIC, subunit 5	3.6002163	1.576848
Phvul.009G165100.5	Squamosa promoter-binding protein-like (SBP domain) transcription factor family protein	3.5326066	∞
Phvul.007G065100.1	K-box region and MADS-box transcription factor family protein	2.6160511	1.371699
Phvul.008G157600.1	GATA transcription factor 9	2.5691685	∞
Phvul.002G061300.1	PLATZ transcription factor family protein	2.5049392	1.091041
Phvul.006G147800.1	WRKY family transcription factor, WRKY6	2.3039034	1.019909
Phvul.010G129400.1	GRAS family transcription factor	2.0748266	1.914587

Phvul.002G228400.1	heat shock transcription factor B2A, HSFB2A	2.0354328	-1.20327
Phvul.003G252200.1	GRAS family transcription factor	2.0097059	-2.61056
Phvul.009G142900.2	Basic-leucine zipper (bZIP) transcription factor family	∞	116.4164
Phvul.006G193200.1	Zinc finger, RING-type; Transcription factor jumonji/aspartyl beta-hydroxylase B160	-1.50725	26.04325
Phvul.009G213800.1	MADS-box transcription factor family protein	-1.42718	4.451217
Phvul.006G169600.1	K-box region and MADS-box transcription factor family protein	∞	99.29637
Phvul.010G134100.2	ATPases;nucleotide binding; ATP binding; nucleoside-triphosphatases;transcription factor binding	-6.227696	28.37039
Phvul.007G154900.6	myb family transcription factor	-2.933335	9.107874
Phvul.001G226500.1	GATA transcription factor 16, GATA 16	1.6057303	6.32698
Phvul.002G287604.1	GATA type zinc finger transcription factor family	∞	5.136019
Phvul.006G146500.1	Transcription factor jumonji (jmjC) domain-containing protein	1.2387062	4.357835
Phvul.002G297100.1	WRKY family transcription factor, WRKY53	-1.324298	4.13572
Phvul.005G025300.1	transcription factor IIIA, TFIIIA	∞	3.54486
Phvul.008G048100.2	WRKY family transcription factor	1.5563232	3.424013
Phvul.003G081800.1	KNOX/ELK homeobox transcription factor	-2.395268	3.424013
Phvul.003G166100.1	plant-specific transcription factor YABBY family protein	∞	3.424013
Phvul.001G189400.1	Basic-leucine zipper (bZIP) transcription factor family protein	1.7578521	2.793826
Phvul.003G222900.1	myb-like transcription factor family protein	1.1629096	2.758253
Phvul.009G003800.1	GATA transcription factor 7	-1.181867	2.475178
Phvul.007G196300.1	Zinc finger, RING-type;Transcription factor jumonji/aspartyl beta-hydroxylase	-1.046325	2.279657
Phvul.002G024600.2	nucleic acid binding;sequence-specific DNA binding transcription factors;zinc ion binding	-2.996142	2.249408
Phvul.011G063100.1	GRAS family transcription factor	1.3466238	2.043652
Phvul.002G157500.1	transcription factor jumonji (jmjC) domain-containing protein	1.2248203	2.040781

4.8 Pathway Analysis

Pathway analysis was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG) KEGG Pathway Database (Kanehisa et al., 2015) on the up-regulated genes (FC= ≥2) of 50 mM and 150 mM of NaCl collected at ten days and five weeks after stress. The four most enriched pathways that were identified in our salt treated samples were; i) metabolic pathways ii) biosynthesis of secondary metabolites iii) plant hormone signal transduction & iv) phenylpropanoid biosynthesis. More genes were involved in these pathways under 150 mM of salt stress at ten days and five weeks compared to 50 mM **(Table 6)**; one possible explanation for this is 150 mM (high) salt stress when compared to 50 mM (moderate) stress produced more upregulated genes at both time points **(Fig. 6)** and hence more of these genes in the pathways under 150 mM of NaCl. Yang et al (2017) found that metabolic pathways and plant hormone signal transduction were enriched under salt stress. Furthermore, Chen et al (2016) discovered biosynthesis of secondary metabolites, phenylpropanoid biosynthesis and metabolic pathways to be enriched under salt stress.

According to Im et al (2012) when plants are exposed to salt stress, they mainly activate three signaling pathways: calcium-dependent protein kinase (*CDPK*) pathway, salt overly sensitive (*SOS*) pathway, and the mitogen-activated protein kinase (*MAPK*) pathway. These three pathways were also mentioned by Che-Othman et al (2017) when highlighting adaptive mechanisms and signaling during salt exposure. Based on KEGG pathway analysis, the *MAPK* pathway was identified in our salt stressed plants **(Fig 8)**. *MAPK* signaling pathway is crucial in salt stress sensing and signaling (Conde et al., 2011). Martin et al (2012) reported that activation of the *MAPK* pathway is one of the survival strategies that are employed by the plant in response to increased salinity to cope with the osmotic stress. *MAP* kinases in plants are characterized by

multigene families and are a part of effective transmission of distinct stimuli as well as the control of the antioxidant defense system in reacting to stress signaling (Sinha et al., 2011). MAPKs are induced in the presence of drought and other environmental stressors. The *MAPK* cascade comprise of three functionally intertwined protein kinases: *MAPKKK*, *MAPKK*, and *MAPK* (Agrawal et al., 2003). *MAPK* phosphorylation system acts as a connection between upstream receptors and down-stream signaling components that initiates cellular response (**Fig. 10**) (Kaur & Gupta , 2005). Our study has identified ten (10) differentially expressed genes in the *MAPK* pathway namely: i. Leucine-rich receptor ii. *EIN3* iii. *EIN3* binding F-box protein iv. Calmodulin 5 v. Ethylene receptor vi. Abscisic acid receptor vii. Pathogenesis-related protein viii. Protein kinase (*CTR1*) ix. Ethylene response factor 1 x.WRKY33 **(Table 7)**.

Table 6. The four most enriched pathways identified in our study: KEGG pathway analysis was done on upregulated genes (Fold change = ≥2) of 50 mM & 150 mM of NaCl. These four pathways top the list of each treatment as enriched pathways.

Pathways	**Pathway ID**	**10 days 50 mM # of genes**	**5 weeks 50 mM # of genes**	**10 days 150 mM # of genes**	**5 weeks 150 mM # of genes**
Metabolic pathways	ko01100	527	578	684	668
Biosynthesis of secondary metabolites	ko01110	404	393	528	457
Plant hormone signal transduction	ko04075	141	204	238	234
Phenylpropanoid biosynthesis	ko00940	179	146	188	148

Table 7. Identification of MAPK pathway genes: Differentially expressed *MAPK* pathway genes of 50 mM & 150 mM NaCl stress at ten days and five weeks of salt treatment. The values represent fold change of each gene. The comparison was carried out done across rows separately for each gene. Green indicates highest expression of the gene, red indicates lowest expression of the gene and a yellow/orange color indicates values that are intermediate.

Gene Name	**Description**	**10 days 50mM**	**5 weeks 50mM**	**10 days 150mM**	**5 weeks 150mM**
Phvul.002G196200.1	Leucine-rich receptor	-3.86681	1.005663	-3.05842	1.350274
Phvul.002G253900.1	*EIN3*	4.360473	-5.45015	19.26876	-25.4672
Phvul.003G165500.1	*EIN3* -binding F-box protein	1.090118	-2.30006	1.242328	-3.18239
Phvul.004G076400.1	calmodulin 1	1.453491	-1.4114	3.670241	72.03109
Phvul.006G106400.1	ethylene receptor	201.8899	-1.3417	2.408595	-2.04438
Phvul.006G175700.1	abscisic acid receptor *PYR/PYL* family	-2.7171	-1.19531	-4.19024	-1.92756
Phvul.006G196900.1	pathogenesis-related protein 1	2.328293	-1.26279	3.284169	1.817019
Phvul.007G245500.2	Protein kinase *CTR1*	2.984502	22.01313	2.601283	12.795
Phvul.007G273000.1	ethylene response factor 1	2.068905	-1.43946	3.361784	1.039514
Phvul.010G062500.1	*WRKY 33*	-1.81032	2.444545	-1.10107	2.30317

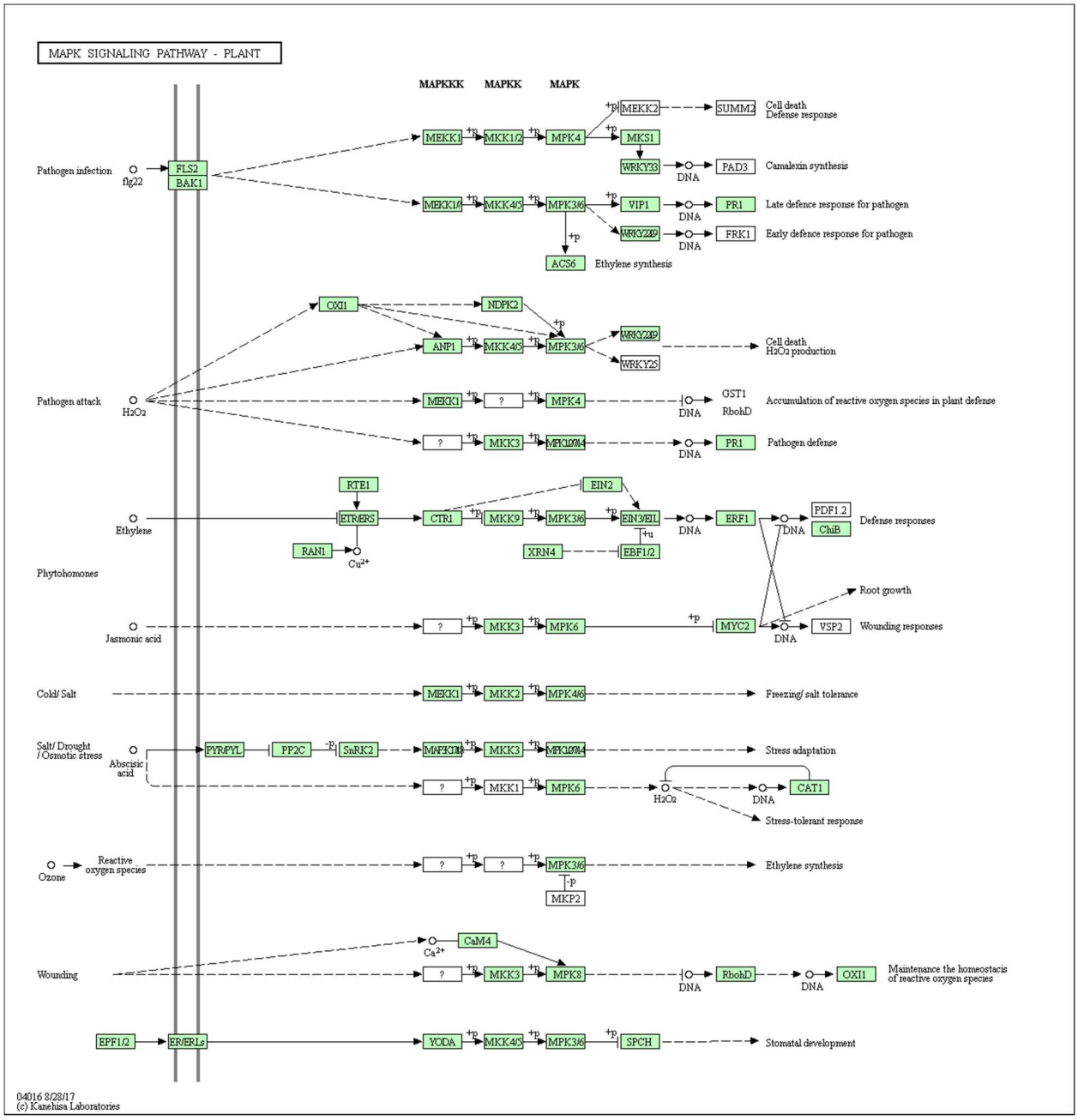


Figure 10. Putative KEGG pathway (ID:04016): MAPK pathway identified in our salt treated common bean plants. The boxes highlighted in green are hyperlinked to GENE entries on the database.

Transcription factors (TFs) play significant roles in stress signaling and compose an essential part of signaling networks. *WRKY* proteins are among the major TFs that take part in crucial roles of regulating transcriptional reprogramming related to stress responses (Muthamilarasan et al., 2015). Comprehensive studies on signaling mechanisms and transcriptional regulation has revealed the connection of the *WRKY* proteins to mitogen-activated protein kinases (*MAPKs*) (Rushton et al., 2010). *WRKY 33* was observed in our *MAPK* pathway **(Table 7)** and was only up-regulated at five weeks for both 50 mM (moderate) and 150 mM (high) salt stress while down-regulated at 10 days. These findings support the idea that *WRKY 33* is a delayed response transcription factor. Elevated salt and dehydration treatments have induced *ZmWRKY33* and overexpression of the *ZmWRKY33* in Arabidopsis triggered stress induced genes and enhanced salt stress tolerance (Li et al., 2013). Jiang and Deyholos (2009) also found that overexpression of *AtWRKY33* gene in *Arabidopsis* conferred salt tolerance. Pathogenesis-related (*PR*) proteins that are involved in signaling pathways were identified under salt stress by Jain et al (2006) where high levels of *PR10* proteins were found in saline-tolerant peanut. Salt tolerance of transgenic *Arabidopsis* plants was enhanced by heterogeneous overexpression of pathogenesis related proteins *(SMPR1)* taken from *S. matsudana* (Han et al., 2017).

The Ethylene Response Factor (*ERF*) proteins have important functions in the transcriptional regulation of plant growth and development, as well as plant responses to various environmental elements (Nakano et al., 2006; Zhuang et al., 2011). Achard et al (2006) demonstrated that Ethylene Insensitive3 (*EIN3*) promotes salt tolerance as they found that the ein3-1 mutants (lacking *EIN3*) showed reduced salt tolerance at high salt concentration. *EIN3* was only upregulated at ten days under 50 mM and 150 mM salt stress while downregulated at five weeks under both stresses **(Table 7)**. The Ethylene Response Factor (*ERF*) proteins play key

roles in transcriptional control of plant growth and development, as well as responses of plants to several environmental conditions (Nakano et al., 2006; Zhuang et al., 2011). Current studies have identified a few *ERFs* that are connected to dehydration-responsive elements and act as a primary hub in directing plants' responses to abiotic stresses (Müller &Munné-Bosch, 2015). Previously Rong et al (2014) documented that constitutive expression of the wheat *ERF* genes, *TaERF3* in wheat augments tolerance to salt and drought stress (Rong et al., 2014). In addition, Wang et al., (2017) reported that NaCl treatment induced the expression of *ERF96* in the wild type *Arabidopsis thaliana* (cv. Col-0) seedlings and overexpression of *ERF96* in transgenic plants displayed more tolerance to salt stress. Ethylene receptors possibly function in response to stress through mediating salt-responsive gene expressions (Cao et al., 2008). An ethylene receptor gene *(NTHK1*) was recognized in controlling salt stress responses by impacting ion accumulation and associated gene expressions (Chen et al., 2016).

Protein kinases of different types and families are main unifiers of plant stress signaling. These kinases regulate ionic and osmotic homeostasis by linking cellular metabolic signaling to stress adaptive physiological processes (Golldack et al., 2014). Salinity stress triggers numerous protein kinases that are closely involved in salt adaptation signaling cascades (Kulik et al., 2011). Constitutive triple response 1 (*CTR1*), a protein kinase is known to adversely control ethylene responses in *Arabidopsis* (Testerink et al., 2007). There were significantly higher expressions of protein kinase (*CTR1*) in our study at five weeks compared to ten days under 50 mM and 150 mM of salt stress **(Table 7)**; this was in accordance with Huai et al., (2008) who identified enhanced expression of protein kinases under salt stress in maize plants. Similarly, Shiozaki et al (2005) found a *CTR1*-like protein kinase gene to be induced by salt stress. There was no significant expression of Leucine-Rich Repeats (*LRR*) in our study (down regulated at ten days

and little expression at five weeks under 50 mM and 150 mM salt stress) **(Table 7)**. Leucine-rich repeat receptor-like kinases (*LRR-RLKs*) were seen to have significant roles in plant growth and development and in stress responses (Wang et al., 2017). de Lorenzo et al (2009) identified a barrel clover (*Medicago truncatula)* leucine-rich repeat *RLK* gene, *(Srlk)* which was shown to enhance plant roots salt stress tolerance by gathering less sodium ions and lessening the level of expression of numerous salt-responsive genes.

Calmodulin-like (*CML*) proteins are essential Ca^{2+} sensors that play notable roles in controlling plant stress tolerance (Munir et al., 2016) and are also identified as a defense responsive gene (Ayyappan et al., 2015). Calmodulin was significantly upregulated at ten days and five weeks of 150 mM salt stress compared to 50 mM salt stress in our study **(Table 7)**; this finding coincides with Pandey et al (2002) who observed that there was an upregulation of calmodulin, Ca2+/CaM-dependent protein kinase (*PsCCaMK)* in pea (*Pisum sativa*) roots under increased salinity stress. Additionally, expression of *OsMSR2* which is a novel calmodulin-like protein gene was recognized to confer improved tolerance to elevated salt and drought in *Arabidopsis thaliana* (Xu et al., 2011). In response to biotic and abiotic stresses, two ABA signal pathway models were proposed, from stress signaling to stomatal closure, controlling pyrabactin resistance (*PYR*)/*PYR*-like (*PYL*) (Lim et al., 2015). According to Dorosh et al (2013) protein pyrabactin resistance 1 (*PYR1*) is a member of *PYR1*-like (*PYL*) proteins that control plant development and responses to drought and salinity conditions. The *PYR* gene in our study was observed to be down-regulated at ten days and five weeks of 50 mM and 150 mM of salt stress **(Table 7).**

4.9 Functional classification and Gene Ontology (GO) term enrichment

Functional classification was carried out using the PANTHER classification system (Mi et al., 2016) to perform PANTHER GO-Slim categories (biological process (BP), molecular function (MF) & cellular component (CC) of salt treated plants collected at ten days and five weeks after stress **(Fig. 11 & 12)**. More upregulated genes were involved in each GO-Slim category at five weeks compared to ten days of stress for both moderate and high salt treated plants **(Fig. 11 & 12)**. This could be as a result of a larger number of expressed genes during prolonged stress compared to onset of stress (Fig. 6), resulting in more genes in each category at five weeks. Gene Ontology (GO) term enrichment was carried out on the up (FC= $\geq$2) and downregulated (FC=$\leq$ -2) genes using PANTHER gene list analysis tools (Consortium et al., 2000; Mi et al., 2016) ; this result shows granular (specific) terms that are overrepresented in each GO category **(Table 8 & 9)**. Identical protein binding (Molecular Function) and plasma membrane (Cellular Component) were both overrepresented at five weeks of 50 mM and 150 mM of salt stress for upregulated genes **(Table 8)**. In the downregulated gene analysis, response to wounding (Biological Process) was enriched in 50 mM NaCl at ten days and five weeks while plant-type secondary cell wall biogenesis and microtubule-based movement (BP) were enriched in 150 mM NaCl at ten days and five weeks **(Table 9)**. Oxidoreductase (MF) was enriched in 50 mM NaCl at ten days and five weeks while microtubule binding (MF) were enriched in 150 mM NaCl at ten days and five weeks **(Table 9)**. In the cellular component category plasma membrane was enriched for 50 mM NaCl both at ten days and five weeks while Kinesin complex was enriched at ten days and five weeks of 150 mM NaCl **(Table 9)**.

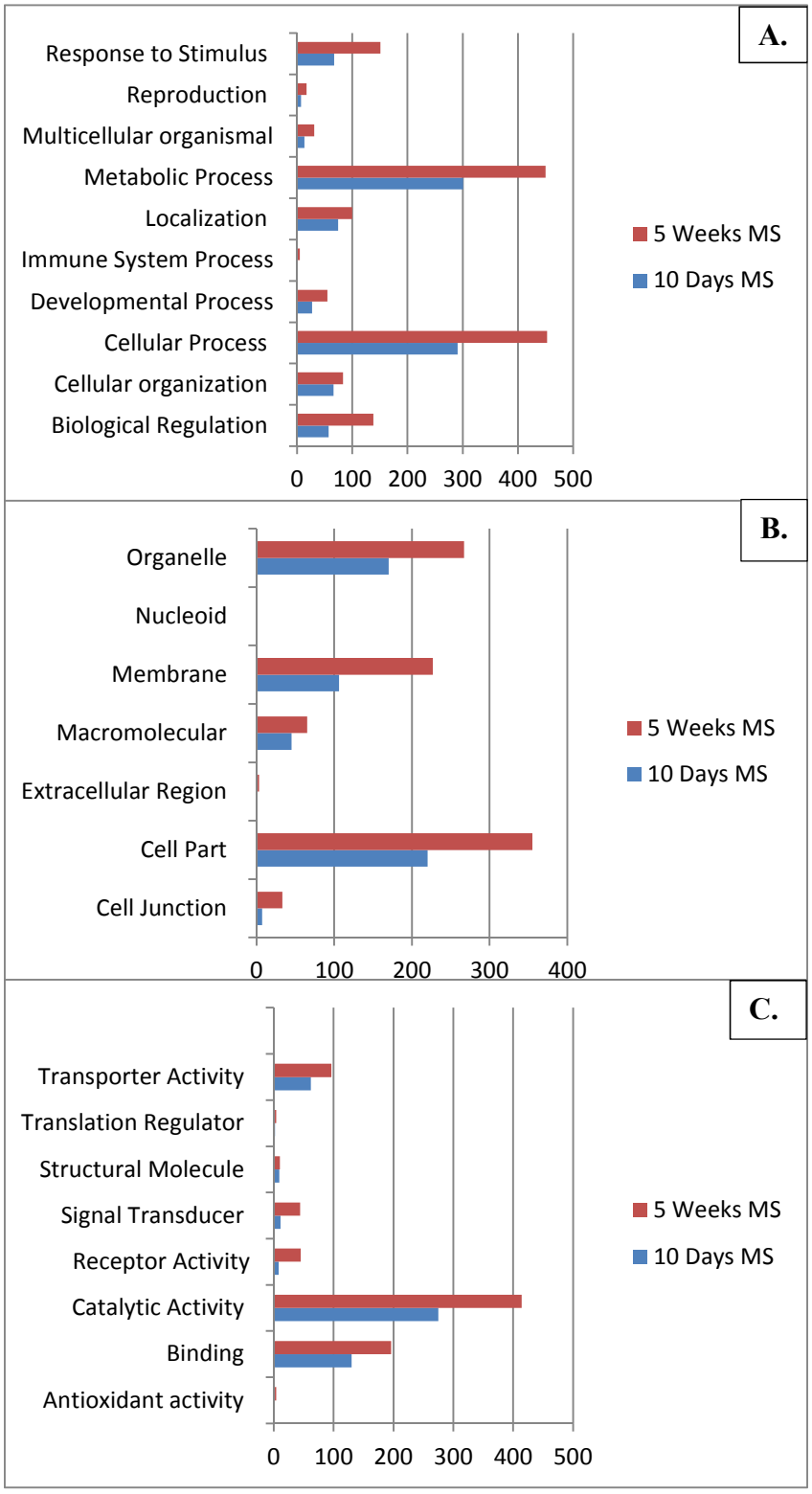


Figure 11. The distribution of GO-Slim categories: GO-Slim categories between the three main GO domains under ten days and five weeks moderate salt stress (MS-50 mM) in common bean roots. The genes were categorized based on the GO categories of (A) biological processes (B) cellular components and (C) molecular functions. The bar represents the total number of upregulated (≥2) genes involved in each GO term

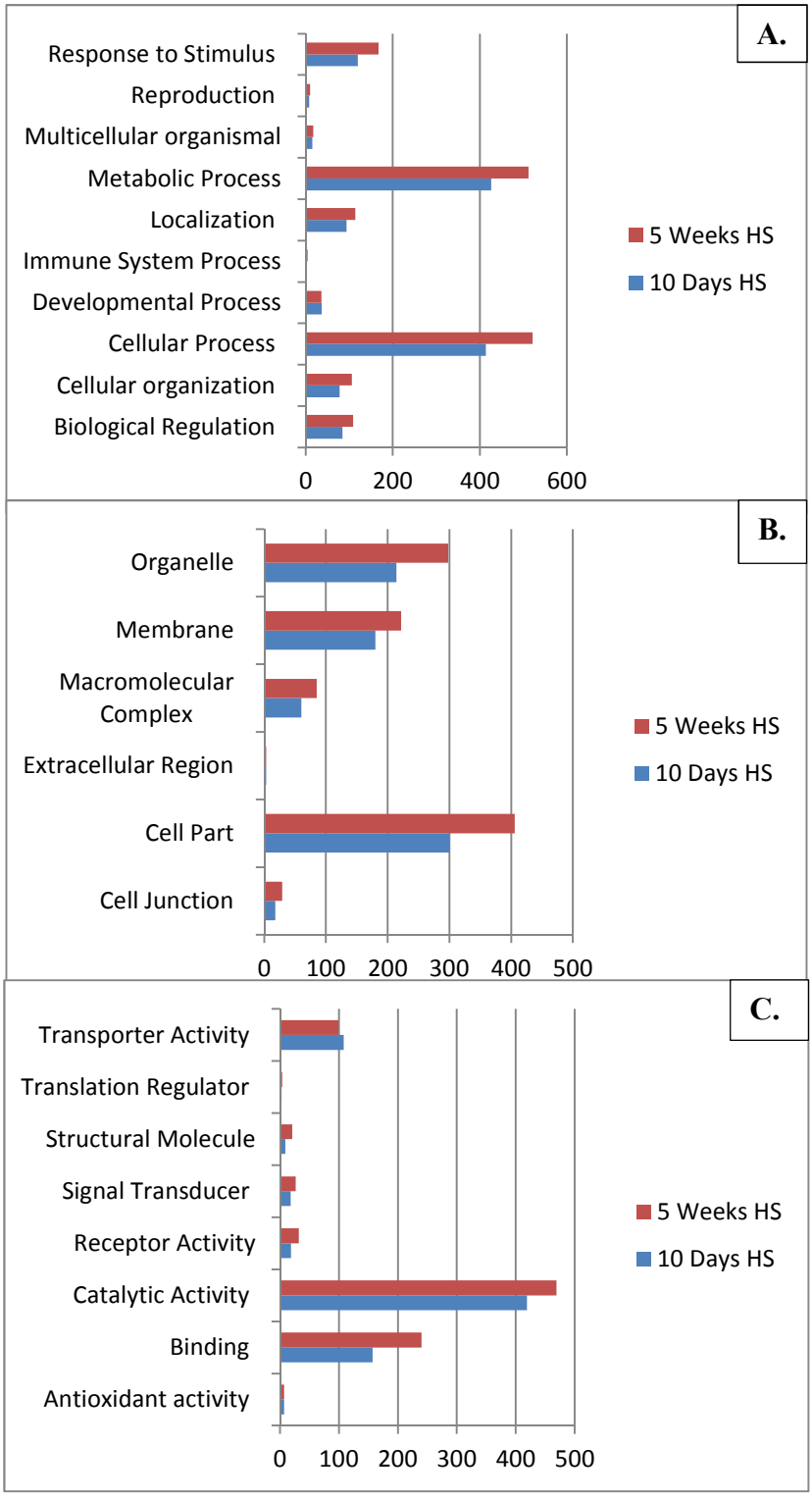


Figure 12. The distribution of GO-Slim categories: GO-Slim categories between the three main GO domains under ten days and five weeks high salt stress (HS-50 mM) in common bean roots. The genes were categorized based on the GO categories of (A) biological processes (B) cellular components and (c) molecular functions. The bar represents the total number of upregulated (≥2) genes involved in each GO term

Table 8. PANTHER overrepresentation test: Enrichment of specific GO terms of upregulated genes at ten days and five weeks of 50 mM and 150 mM salt stress. The table displays the top 5 overrepresented terms in each GO category.

Treatments	Biological Process	Molecular Function	Cellular Component
10 days 50 mM	Respond to wounding	Protein homodimerization activity	Integral component of plasma membrane
	Defense response, incompatible interaction	Oxidoreductase activity	
	Response to water deprivation	Cofactor binding	
	Secondary metabolic process	Transmembrane transporter activity	
	Metabolic process	Drug binding	
5 weeks 50 mM	Plant-type hypersensitive response	UDP-glucosyltransferase activity	Plant-type vacuole
	Transmembrane receptor protein tyrosine kinase signaling pathway	Transmembrane receptor protein kinase activity	Plasma membrane
	Cellular response to jasmonic acid stimulus	Oxidoreductase activity	Integral component of membrane
	Phloem or xylem histogenesis	Oxygen binding	
	Phenylpropanoid biosynthetic process	Identical protein binding	
10 days 150 mM	Response to hydrogen peroxide	Dioxygenase activity	Integral component of plasma membrane
	Amino acid transport	UDP-glucosyltransferase activity	Cell Wall
	Response to karrikin	ATPase activity	
	Response to chitin	Anion transmembrane transporter activity	
	Phenylpropanoid biosynthetic process	Coenzyme binding	
5 weeks 150 mM	Protein-chromophore linkage	ADP binding	NAD(P)H dehydrogenase complex
	Response to chitin	Calcium ion binding	Tubulin complex
	Response to light intensity	Identical protein binding	Photosystem I
	Cell death	Molecular transducer activity	Chloroplast thylakoid membrane
	Protein auto phosphorylation	Protein dimerization activity	Plasma membrane

Table 9. PANTHER overrepresentation test: Enrichment of specific GO terms of downregulated genes at ten days and five weeks of 50 mM and 150 mM salt stress. The table displays the top 5 overrepresented terms in each GO category.

Treatments	Biological Process	Molecular Function	Cellular Component
10 days 50 mM	Regulation of jasmonic acid mediated signaling	Protein serine/threonine kinase activity	Plasma membrane
	Response to wounding	Oxidoreductase	
	Cellular response to acid chemical	Protein binding	
	Response to abscisic acid	Ion binding	
	Protein phosphorylation		
5 weeks 50 mM	Divalent inorganic anion homeostasis	Oxidoreductase activity, acting on peroxide as acceptor	Cell Wall
	Oxylipin biosynthetic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Plasma Membrane
	Response to wounding	Cofactor binding	
	Hormone biosynthetic process	Inorganic molecular entity transmembrane transporter activity	
	Response to water deprivation	Ion transmembrane transporter activity	
10 days 150 mM	Microtubule-based movement	Microtubule motor activity	Kinesin complex
	Xylan metabolic process	Microtubule binding	Photosystem
	Plant-type secondary cell wall biogenesis	Oxidoreductase activity	Microtubule
	Cellular process involved in reproduction	Transmembrane transporter activity	Plant-type cell wall
	Male gamate generation	DNA-binding transcription factor activity	Apoplast
5 weeks 150 mM	DNA replication initiation	ATP-dependent microtubule motor activity	Condensed Chromosome Kinetochore
	Xylan biosynthesis process	Microtubule binding	Condensed nuclear chromosome
	Plant-type secondary cell wall biogenesis	Copper ion binding	Mitochondrial respiratory chain complex I
	Meiotic chromosome segregation	Heme binding	Kinesin Complex
	Microtubule-based movement	Structural constituent of ribosome	Cytosolic large ribosomal subunit

4.10 Real-time RT-PCR validation of RNA-Seq analysis

To validate the differential expression identified by the Illumina RNA-Seq data, quantitative real-time PCR (qRT-PCR) was carried out on the same RNA pools that were used for the next-generation sequencing. Five genes that were differentially expressed based on RNA-Seq were randomly selected for real-time RT-PCR analysis **(Fig. 13).**

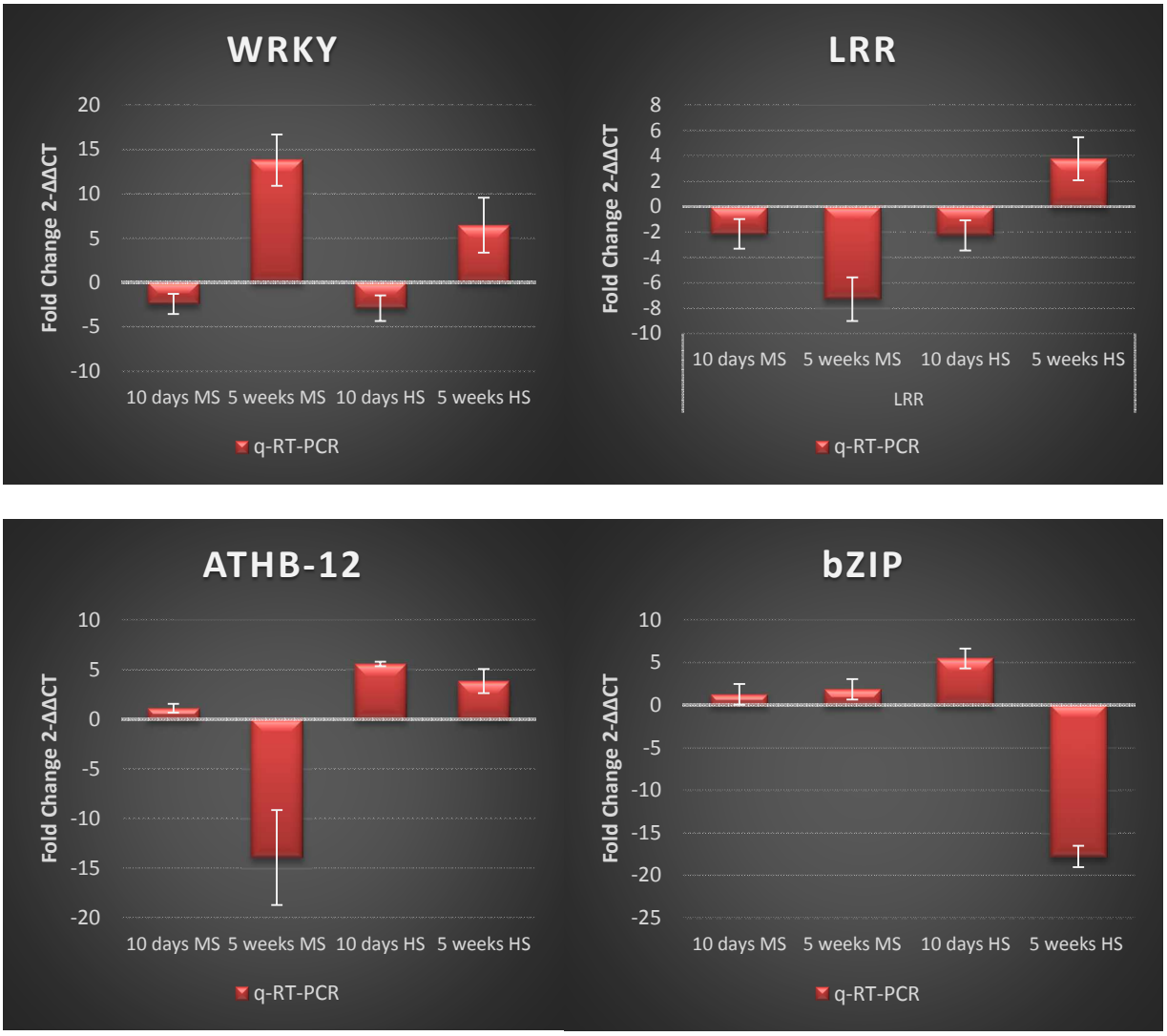
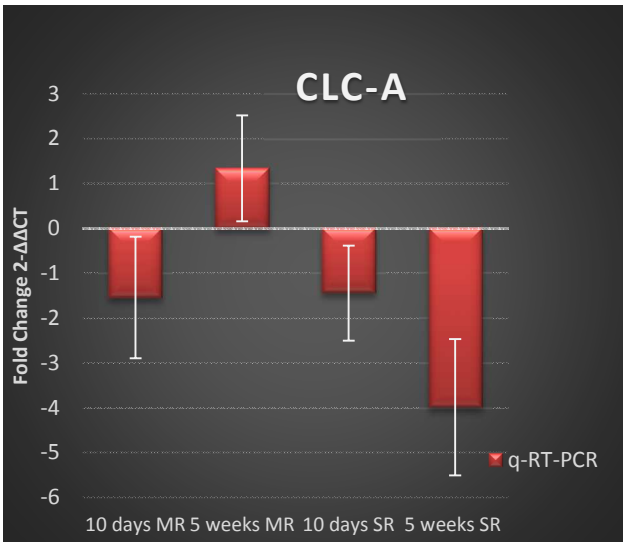
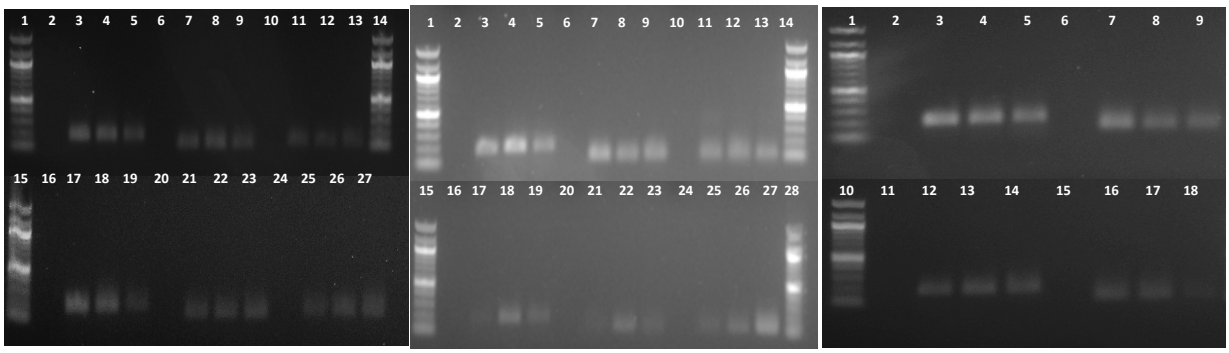


Figure 13. Validation of RNA-seq data: Five Differentially Expressed Genes (DEGs) that were obtained from the RNA-seq data were chosen for validation by quantitative real-time PCR (qRT-PCR) analysis. The trends of expression patterns for both ten days and five weeks of moderate (MS) and high (HS) salt stress were found to be similar to those in the RNA-Seq analysis. The x-axis shows treatments at different time points and y-axis shows fold change value of 2-ΔΔCT.



Continue-Figure 13. Validation of RNA-seq data: Five differentially expressed genes (DEGs) that were obtained from the RNA-seq data were chosen for validation by quantitative real-time PCR (qRT-PCR) analysis. The trends of expression patterns for both ten days and five weeks of moderate (MS) and high (HS) salt stress were found to be similar to those in the RNA-Seq analysis. The x-axis shows treatments at different time points and y-axis shows fold change value of 2-ΔΔCT.



A **B** **C**

Figure 14. Quantitative Real-time PCR (qRT-PCR) gel pictures: Five genes were used to corroborate the RNA-seq data using RT-qPCR. The genes included LRR, CLC-A, WRKY, bZIP & ATHB-12.**CR-(Control root 0 mM), MS- (Moderate stress 50 mM), HS (High stress 150 mM) A- (Lanes 1-14 ten days salt stress)** Lane 1-100bp ladder, lane 2-actin negative, lane 3-actin CR, lane 4-actin MS, lane 5-actin HS, lane 6- CLC-A negative, lane 7-CLC-A CR, lane 8-CLC-A MS, lane 9-CLC-A HS, lane 10-LRR negative, lane 11-LRR CR, lane 12-LRR MS, lane 13-LRR HS, lane 14-100bp ladder, **(Lanes 15-27 five weeks salt stress)** Lane 15-100bp ladder, lane 16-actin negative, lane 17-actin CR, lane 18-actin MS, lane 19-actin HS, lane 20-CLC-A negative, lane 21-CLC-A CR, lane 22-CLC-A MS, lane 23-CLC-A HS, lane 24-LRR negative, lane 25-LRR CR, lane 26-LRR MS, lane 27-LRR HS. **B-(Lanes 1-14 ten days salt stress)** Lane 1-100bp ladder, lane 2-actin negative, lane 3-actin CR, lane 4-actin MS, lane 5-actin HS, lane 6- WRKY negative, lane 7- WRKY CR, lane 8- WRKY MS, lane 9- WRKY HS, lane 10-ATHB-12 negative, lane 11- ATHB-12 CR, lane 12- ATHB-12 MS, lane 13-ATHB-12 HS, lane 14-100bp ladder, **(Lanes 15-28 five weeks salt stress)** Lane 15-100bp ladder, lane 16-actin negative, lane 17-actin CR, lane 18-actin MS, lane 19-actin HS, lane 20-WRKY negative, lane 21-WRKY CR, lane 22-WRKY MS, lane 23-WRKY HS, lane 24-ATHB-12 negative, lane 25-ATHB-12 CR, lane 26-ATHB-12 MS, lane 27-ATHB-12 HS,

C- (Lanes 1-9 ten days salt stress) Lane 1-100bp ladder, Lane 2- actin 11 negative, Lane 3-actin 11 CR, Lane 4-actin 11 MS, Lane 5-actin 11 HS, Lane 6-bZIP negative, Lane 7-bZIP CR, Lane 8-bZIP MS, Lane 9-bZIP HS, **(Lanes 10-18 five weeks salt stress)** Lane 10-100bp ladder**,** Lane 11-actin 11 negative, Lane 12- actin11 CR, Lane 13-actin11 MS, Lane 14-actin11 HS, Lane 15-bZIP negative, Lane 16-actin11 CR, Lane 17- actin11 MS, Lane 18- actin 11 HS

Table 10. Primer Sequences: Oligonucleotides designed for qRT-PCR analysis

Locus	Functional Annotation	Primer Sequences
Phvul.002G196200	Leucine-rich receptor-like protein kinase family protein (LRR)	5'-TGGTGCATCTCAACCTTTC-3' 3'-GCATGGCGGTAGGAATTTA-5'
Phvul.001G094700	chloride channel A (CLC-A)	5'-TGTCCACCTGGCTTCTAT-3' 3'-GAGGGACAAGGGTTGATATTC-5'
Phvul.011G035700	Basic-leucine zipper (bZIP)	5'-TGGGTCCTTTCCGTATCA-3' 3'-CCGCAATACACCCTATCATC-5'
Phvul.006G116000	homeobox-leucine zipper protein ATHB-12	5'-GAGAGTGAAGCGGGAAATG-3' 3'-GCTGTCACATTCCGAGATAC -5'
Phvul.002G297100	WRKY family transcription factor	5'-GCACAGGCATCACAAGAA-3' 3'-CAGATGAAGTCGAAGGGAAAG-5'
Phvul.008G011000	Actin11 (Reference gene)	5'-TGCATACGTTGGTGATGAGG-3' 3'-AGCCTTGGGGTTAAGAGGAG5'

CHAPTER V: CONCLUSIONS AND FUTURE RECOMMENDATIONS

As far as we know, this is the first transcriptomic analysis of common bean under prolonged salt stress (five weeks) which extended to the flowering stage. The flower is the reproductive organ of the plant, and therefore, it is crucial to understand the acclimation mechanism that is employed by the plant to cope under salt stress on a molecular scale. Comprehensively, this study has identified more differentially expressed genes under prolonged salt stress (five weeks) when compared to onset of stress (10 days) supporting the idea that more intricate transcript regulation takes place under continuous salt stress. Some early (*LEA4, HSP20, CDSP32, NHX1*) and delayed (*CLC-A, STH2, GRP, PROT1*) salt responsive genes were recognized based on the time of their expression. 'Master' transcription factors (*MADS-box, WRKY, TFIIE, PHD, TF DP, & bZIP*) that maintained their expression from the onset of stress through prolonged stress were also identified which may suggest that these TFs play a part in salt acclimation and tolerance. Additionally, we identified in our data, the MAPK pathway which is crucial in salt sensing and signaling; several genes found in the pathway were observed to be differentially expressed such as *WRKY33, CTR1, EIN3*, Calmodulin 1 & ethylene receptor. The potential candidate genes and pathway identified in our study provide a basis for further understanding of salt acclimation and tolerance in common bean.

Further studies on the common bean response to salt stress would be advantageous for breeding of salt tolerant cultivars. A physiological and molecular comparison of more than one common bean genotype exposed to varying levels of salt stress would be valuable to breeders and ultimately farmers. Tolerant cultivars can grow and produce higher yield under stress in comparison to sensitive cultivars. Hence, differential expression in genes will be recognized among genotypes that will eventually aid in the selection of genes needed to produce salt

tolerance. As a next step for future studies, an osmolality measure can be carried out on the NaCl solutions used for the experiment. Primarily, this will provide the concentration of the ions in the solution, and an understanding of the osmotic pressure for different salt concentrations that ultimately affects the availability of water to the plant.. Studies have identified some genes to be time specific as well as tissue specific; hence, a transcriptomic study on the common bean root, stem, leaf and flower would identify tissue specific responses of genes that may play a role in plants' survival under stress. Growing the plants in salty environments or the practice of utilizing actual saline soil could replace the hydroponic system; this is a way to mimic the natural environment. This will display a true representation of plants response to salinity-physiologically and genetically. Globally, hydroponic systems have become a popular method for crop production; and so, a comparison of the use of hydroponic system and saline soil would be of interest as the water used in the system may possibly be rich in salt. Additionally, Chromatin Immunoprecipitation Sequencing (ChIP Seq) can be carried out along with RNA sequencing to identify genome-wide DNA binding sites for the salt responsive transcription factors and genes. This will allow for the recognition of gene regulatory networks that play a role in plant development under stress. The genes and transcription factors identified in our study that aid in the plants' acclimation to salt stress could be further examined for biological functions through studies and breeding. These findings will also be available via online databases and since common bean is a legume, these results could be applicable to other leguminous plants such as soybean, chickpea and alfalfa.

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