***Arabidopsis thaliana* Glycine Rich Ribosome Binding Protein 3**

***(AtGR-RBP3)* is a Negative Regulator of Salinity Stress**

By

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I dedicate this thesis to my parents and my supervisor Dr. Balakrishnan Prithiviraj who have always been supportive to me. Their love and blessings were always motivation for me to set higher targets and work to achieve them.

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Abstract

Salinity is one of the major abiotic stresses that affect crop production. Mechanism(s) of plant tolerance to salinity is complex and is mediated by multiple biochemical pathways. Our earlier studies have shown that *Ascophyllum nodosum* extracts (ANE) impart salinity tolerance in plants. To understand the molecular mechanism of salinity tolerance induced by ANE, the whole genome transcriptome of *Arabidopsis thaliana* was examined through microarray analysis. Analysis of the ANE-induced transcriptome under salinity stress led to the identification of a number of positive and negative regulators of salinity tolerance in Arabidopsis. I systematically screened Arabidopsis single gene knockout mutants corresponding to genes that were significantly down regulated in ANE-mediated salinity tolerance. In this study it was observed that plants carrying a mutation in the gene *Arabidopsis thaliana Glycine Rich-Ribosome Binding Protein 3* (*AtGR-RBP3*) exhibited a marked salt tolerance compared to wild type Arabidopsis Col-0. *AtGR-RBP3* mutants, *gr-rbp3-1* and *gr-rbp3-2,* accumulated significantly less sodium (36 and 37.76 µg mg^{-1} of dry weight respectively) in their leaf tissue as compared wild type plants (57.73 µg mg^{-1}) when grown under high salt conditions. Also, the increase in sodium:potassium (Na$^+$/K$^+$) ratio which was observed in wild type (7.0) was not observed in *gr-rbp3-1* and *gr-rbp3-2* (3.12 and 3.54 respectively). In this research an increase in the expression of stress responsive genes; *RD29A, RD22* and *SOS1* was evident in *gr-rbp3-1* and *gr-rbp3-2.* Subcellular localization study revealed that ATGR-RBP3 was localized in the endoplasmic reticulum. Taken together, these results indicate that *AtGR-RBP3* is a negative regulator of salinity stress in Arabidopsis.

List of Abbreviations and Symbols Used

ANE	*Ascophyllum nodosum* extract
AtGR-RBP3	*Arabidopsis thaliana glycine rich ribosome binding protein3*
ABRC	Arabidopsis biological resource center
RD	Response to dehydration
SOS	Salt overlay sensitive
AtCLH	*Arabidopsis thaliana chlorophyllase*
Hrs	Hours
%	Percentage
~	Approximately
$T\ ha^{-1}$	Tonnes per hectare
FAO	Food and Agricultural Organization
Mha	Million hectares
H_2O_2	Hydrogen peroxide
CO_2	Carbon dioxide
NaCl	Sodium chloride
LiCl	Lithium Chloride
$MgCl_2$	Magnesium chloride
IP_3	Inositol trisphosphate
PIP_2	Phosphatidyl inositol bisphosphate
DAG	Diacylglycerol
PLC	Phospholipase C
DEPC	Diethylpyrocarbonate

PEP	Phosphoenolpyruvate
MEP	Methylerythritol phosphate
Tm	Tunicamycin
DTT	Dithiothreitol
PEG	Polyethylene glycol
TBE	Tris borate EDTA buffer
EDTA	Ethylenediaminetetraacetic acid
MS	Murashige and Skoog
LB	Luria broath
SO_4^{2-}	Sulfate ion
HCO_3^-	Bicarbonate ion
CO_3^{2-}	Carbonate ion
Cl^-	Chloride ion
Ca^{2+}	Calcium ion
Mg^{2+}	Magnesium ion
Na^+	Sodium ion
K^+	Potassium ion
H^+	Proton
Na^+/K^+	Sodium potassium ratio
O_2^-	Superoxide
$\mu g\ mg^{-1}$	Microgram per milligram
$\mu g\ ml^{-1}$	Microgram per milliliter
$\mu mol\ m^{-2}s^{-1}$	Micromole per meter square per second

dsm^{-1}	Decisiemen per meter
µl	Microliter
ng/µl	Nanogram per microliter
ml	Milliliter
mM	Millimolar
µM	Micromolar
mg	Milligrams
g	grams
cm	Centimeter
v/v	Volume/volume
w/v	Weight/volume
Mbp	Mega base pairs
mv	millivolts
˚C	Degree centigrade
OD	Optical density
EC	Electrical conductivity
SAR	Sodium adsorption ratio
SAS	Statistical analysis system
RPM	Revolution per minute
Col-0	Wild type Arabidopsis (Colombia)
ROS	Reactive oxygen species
PSII	Photosystem II
SE	Standard error

AAS	Atomic absorption spectroscopy
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
T-DNA	Transfer DNA
LB1.3	Left border primer
A	Adenine
T	Thymine
G	Guanine
C	Cytosine
P	Pyrimidine base
HKT	*Histidine kinase transporter*
SOS3	*Calcium sensor*
SOS2	*Serine/Threonine protein kinase*
SOS1	*Na^+/H^+ antiporter*
NHX1	*Vacuolar Na^+/H^+ exchanger*
CAX1	*H^+/Ca^+ antiporter*
cDNA	Complementary DNA
qPCR	Quantitative polymerase chain reaction
ABA	Abscisic acid
ABRE	ABA responsive elements
bZIP	Basic leucine zipper
DRE	Dehydration responsive element
MYC	Myelocytomatosis

MT	Mitochondria
GB	Golgi bodies
CHL	Chloroplast
PX	Peroxisomes
ER	Endoplasmic reticulum
PT	Plastids
CFP	Color fluorescent protein
YFP	Yellow fluorescent protein
OE	Overexpression
RRM	RNA recognition motif
2-D PAGE	2-dimensional polyacrylamide gel electrophoresis
WAK1	*Wall associated kinase 1*
PME1	*Pectin methyl esterase*

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Chapter 1 INTRODUCTION

Plants are sessile organisms subject to a range of abiotic stresses, such as salinity, drought, and temperature extremes (Ahmad et al., 2012). Abiotic stresses are a major constraint to agricultural production; on a global scale, it is estimated that abiotic stresses account for more than a 50% of the losses in production (Mayra et al., 2005). The global population is expected to be more than 9 billion in 2050 and to feed this growing population, world agricultural productivity needs to grow to 5 t ha^{-1} (tonnes per hectare) from the current 3 t ha^{-1} (Jajoo, 2013). Concurrently, environmental stresses are creating adverse conditions for crop survival and significantly depleting areas under cultivation. According to the FAO Land and Nutrient Management Service (2008), approximately 900 Mha of arable land has been affected by high salinity and it is expected that if current trends perpetuate, it will result in the loss of up to 50% of the arable land throughout the world by the turn of this century (Wang et al., 2003).

The effect of soil salinity on plant health depends on the concentration of salt in the soil. From the past research, it is clear that no element threatens agriculture more than sodium (Zhu, 2007). Sodium concentrations beyond threshold levels are extremely toxic to plants and prolonged exposure to high sodium concentration leads to death (Munns and Tester, 2008). Additionally, sodium ions restrict the uptake of potassium because both are monovalent cations and are transported by the same protein transporter. This significantly

reduces the potassium content, leading to a decrease in the K^+/Na^+ (potassium:sodium) ratio in the cytoplasm. An increase in Na^+ (sodium) and a decrease in K^+ (potassium) inhibit enzymatic reactions and result in a loss of turgor pressure (Zhu, 2007). Ion imbalance and resultant hyperosmotic stress leads to oxidative damage predominantly due to the accumulation of reactive oxygen species (ROS), such as O_2^- and H_2O_2 (Mittler, 2002). High concentrations of ROS damage both cytoplasmic and organelle membranes as well as affecting the secondary and tertiary structures of macromolecules such as proteins and nucleic acids leading to aberrant cell signaling (Arora et al., 2002).

Earlier research in our laboratory has shown that ANE treatment imparts salinity tolerance in plants (Jithesh et al., 2012). To get a better understanding of the molecular mechanism(s) of ANE-induced salinity tolerance, the complete genome transcriptome of *Arabidopsis thaliana* was examined using a cDNA microarray (Mundya narayanan and Prithiviraj, unpublished data). This study led to the identification of a number of positive and negative regulators of salinity stress (Jithesh et al., 2012). T-DNA insertion mutants in the genes that were significantly down-regulated under ANE-mediated salinity tolerance were screened in this project. The hypothesis for this study was that the genes that were down-regulated during ANE-mediated salinity tolerance are negative regulators of salinity tolerance in Arabidopsis. The objective of this study was to characterize an *A. thaliana* T-DNA insertion mutant in one gene that exhibited a significant tolerance to salinity stress.

Chapter 2 LITERATURE REVIEW

2.1 *Arabidopsis thaliana* (L.) Heynh

Arabidopsis thaliana is a small flowering plant of the Brassicaceae family. It is a commonly used model in plant biology due to the following characteristics:

- Its genome size (114.5 Mb/125 Mb total) is small (www.arabidopsis.org).

- It has a short life cycle, taking about 45 days to reach seed maturation from germination.

- It is a prolific seed producer and can be cultivated very easily in restricted spaces.

- It provides an efficient method of transformation with the help of *Agrobacterium tumefaciens* (www.arabidopsis.org).

- There are a large number of genetic resources and mutant lines available at stock centers.

2.2 Soil Salinity

Soil salinity occurs through natural, as well as human activities. Natural salinity arises over time due to the accumulation of salts in soil or ground water. This is mostly due to the weathering of parent rocks containing soluble salts. Other factors that contribute to salinity are the intrusion of sea water inland and the deposition of sea salt carried by rain and wind from sea to land (Rasool et al., 2013). Human activity also contributes to soil salinity largely due to inappropriate agricultural practices, such as improper irrigation, over use of fertilizers, replacement of native plants with exotic species, and the application of poor

quality water for irrigation (Rasool et al., 2013). According to Richard (1954), salt affected soils are classified into three types; saline soils, sodic soils, and saline-sodic soils, based on electrical conductivity (EC) and sodium adsorption ratio (SAR). Most of the salt affected soils in the world are either saline or sodic. Saline soils have an EC over 4 dSm^{-1} (deci Siemens per meter) and a SAR less than 13. Sodic soils have a SAR over 13 and an EC less than 4 dSm^{-1} (Richard, 1954). In sodic and saline soils, sodium is the dominant exchangeable cation. Currently, approximately 50% of salt affected soils are sodic soils where ~15% of the contribution to cation exchange capacity comes from sodium (Rozema and Flowers, 2008; Brady and Weil, 2010). Saline and sodic soils have a poor structure and can prevent the movement of water into plants and through the soil (Richard, 1954). Soil salinity could also be associated with higher concentrations of SO_4^2 (sulfate), HCO_3^- (bicarbonate), CO_3^{2-} (carbonate) or Cl^- (chloride) salts of Ca^{2+} (calcium), K^+ (potassium), Mg^+ (magnesium) and Na^+ (sodium) but in general, sodium chloride (NaCl) is the major cause of soil salinity (Flowers et al., 1977, 1986; Rengasamy, 2002; Epstein and Bloom, 2005; Munns and Tester, 2008; Rozema and Flowers, 2008; Brady and Weil, 2010; Yadav et al., 2011). Among the above mentioned salts in soil, sodium chloride is the one that has been the most intensively researched (Kronzucker et al., 2013).

2.3 Soil Salinity as a Global Issue

The global population is expected to be more than 9 billion in 2050 (FAO, 2008). To feed this population, world agricultural productivity must increase by 70% (FAO, 2008). Concurrently, environmental stresses that limit crop productivity are

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worsening due to climate change. Further, the land area under cultivation is decreasing due to urbanization and the limited availability of good quality water for irrigation. According to the FAO's Land and Nutrient Management Service (2008), approximately 900 Mha of arable land are affected by high soil salinity (Table 1) and it is estimated that if current trend continues, it will result in a loss of up to 50% of the arable land area by the turn of this century (Wang et al., 2003). Globally, irrigated land available for cultivation is ~311 Mha and out of this, ~30 Mha are affected by high soil salinity and, ~1 Mha more is salinized every year (FAO, 2008). This is a major global concern as 40% of the agricultural production comes from irrigated land.

Table 1. Geographic distribution of land area affected by soil salinity

Region	Total Area (Mha)	Saline soils (Mha)	Saline soils (%)	Sodic soils (Mha)	Sodic soils (%)
Africa	1,899	39	2.0	34	1.8
Asia, Pacific and Australia	3,107	195	6.3	249	8.0
Europe	2,011	7	0.3	73	3.6
Latin America	2,039	61	3.0	51	2.5
Near East	1,802	92	5.1	14	0.8
North America	1,924	5	0.2	15	0.8
Total	12,781	397	3.1	434	3.4

(FAO Land and Nutrient Management Service, 2008)

2.4 Farming Practices Contributing to Soil Salinity

Intensive farming practices involve the clearing of deep rooted native vegetation and replacing them with shallow rooted crop plants, contributing to soil salinity. When irrigated, shallow rooted crops absorbed less water than the natural vegetation, which results in increased ground water and a rise in the water table. This, in turn, can bring salts from the lower layer of the soil to the surface layer upper (Sadava et al., 2009). Environmental calamities also contribute to salinity. The tsunami disaster in India, which happened on December 26th, 2004, caused

a large area of land to be affected by salinity due to sea water intrusion inland, and high sea waves caused soil erosion and deposition of soluble salts (Sadava et al., 2009).

2.5 Effects of Soil Salinity on Plants

A number of studies have documented the negative effect of sodium on cellular ionic homeostasis (Bazihizina et al., 2012; Cheeseman, 2013). Sodium exerts its toxic effects on critical processes, such as photosynthesis, transpiration, reactive oxygen species (ROS), and ultimately affects plant growth and productivity as discussed later in this section. Previous studies indicated that a high concentration of sodium in the growth medium disrupts the integrity of the cell membrane. This is followed by more ion specific effects. Flowers et al. (1991), observed that older leaves were affected first as they were no longer able to dilute the excess sodium because they are not expanding. They noted that plants also sent the sodium to older leaves to limit its toxic effect on newly developing meristems. The main effect of salt stress on the plants is hyperosmolality, which creates a water deficit and ionic disequilibrium, adversely affecting physiological and metabolic functions, and ultimately leading to cell death (Hasegawa et al., 2000a; Rivero et al., 2007; Flowers and Colmer, 2008; Munns and Tester, 2008; Aleman et al., 2011). Plants exposed to a high salinity stress, quickly transmitted a water deficit symptoms from the roots to other parts of the plant, leading to a reduction in turgor pressure and impeding cell expansion (Matthews et al., 1984; Munns et al., 1995; Munns and Tester, 2008). High salinity created low water potential in plants, leading to the depolarization of guard cells and a decrease in

stomatal aperture and conductance (Schroeder et al., 2001; Yoo et al., 2009; Kim et al., 2010). The reduction in stomatal conductance led to a reduction in photosynthesis and carbon assimilation, which ultimately decreased yield (Smith and Stitt, 2007; Salekdeh et al., 2009; Yoo et al., 2009; Tardieu et al., 2011). In addition, the reduction in stomatal conductance lowered the transpiration rate which exerted a negative effect on nutrient uptake and evaporative cooling of leaves, resulting in an increase in temperature and, increased stress in the plant (Wilkinson and Davies, 2002; Christmann et al., 2007; Yoo et al., 2009; Kim et al., 2010). Moreover, water deficit and ion toxicity had negative effects on aerobic metabolism, which resulted in the accumulation of ROS above physiological concentration (Mittler, 2002; Zhu, 2002; Apel and Hirt, 2004).

Shabala et al. (2006), determined that when plants were under high salinity stress; K^+ concentration in the cytoplasm was reduced. The mechanisms discussed below may explain the decrease in K^+ concentrations when plants are exposed to high Na^+ concentrations (Britto et al., 2010; Coskun et al., 2013). It is possible that under high salinity, Na^+ induce a depolarization of the plasma membrane which stimulated the efflux of K^+ through the outward-rectifying channels. Higher concentrations of Na^+ resulted in the disintegration of the membranes because of osmotic stress and ionic displacement (especially Ca^{+2}), which further resulted in the release of cytoplasmic contents, including water and K^+ (Nassery, 1975; Lynch and Lauchi, 1984; Cramer et al., 1985; Britto et al., 2010; Coskun et al., 2013).

2.6 Sodium as a Micronutrient and Toxicant

Some terrestrial plants, especially C_4 plants, use Na^+ ions for cellular processes and benefit from the presence of Na^+ in the growth medium as long as the concentration remains below threshold levels (Kronzucker et al., 2013). In earlier studies, it was shown that Na^+ played an important role in the conversion of pyruvate to phosphoenolpyruvate (PEP) in the mesophyll cells (Johnston et al., 1988). They determined that Na^+ deficiency in *Amaranthus tricolor* led to an increase in the C_3 metabolites alanine and pyruvate, and a decrease in C_4 metabolites malate, aspartate and PEP. Interestingly, in C_3 plants such as tomato, the concentration of these metabolites was not affected by Na^+. Grof et al. (1989), and Johnstone et al. (1989), showed that Na^+ deficiency in the C_4 plants *Atriplex tricolor* and *Kochia childsii*, altered the activity of photosystem II (PSII) in the chloroplasts of mesophyll cells, but did not affect PSII activity in the chloroplasts of bundle sheath cells. Ohta et al. (1989), observed that Na^+ increased nitrate uptake and nitrate assimilation in the roots of *Amaranthus tricolor* and this may contribute to the beneficial effects observed.

Most C_4 plants benefit from low levels of Na^+ concentrations, but exceptions include corn and sorghum (Ohta et al., 1989; Ohnishi et al., 1990; Subbarao et al., 2003). The possible explanations for this observation are discussed in section 2.6.1.

2.6.1 Sodium Mediated Pyruvate Transport in Chloroplast of C_4 Plants

In C_4 plants, the import of pyruvate to the chloroplasts is crucial for the MEP (Methyl-Erythritol- Phosphate) pathway, where Na^+- coupling is established. This facilitates pyruvate uptake into chloroplasts and was first demonstrated in *Panicum miliaceum,* where uptake of pyruvate was directly proportional to the external concentration of Na^+ supplied (Ohnishi et al., 1987). Black Jr (1973), observed that pyruvate plays a central role in CO_2 fixation in C_4 plant species. It is one of the precursors for several biochemical pathways, such as fatty acid biosynthesis and isoprenoid metabolism. It was also shown to be the end product of glycolysis and positioned as the important key intermediate for primary and secondary metabolism. However, at the functional level, this Na^+ coupled pyruvate transport pathway was not observed in corn (Ohnishi et al., 1987). Furumoto et al. (2011), identified and characterized the *BASS2* gene, which was localized to chloroplasts in *Flaveria* and *Cleome* (a C_4 plant). This gene is involved in the Na^+ pyruvate co-transport mechanism and is widespread in various C_4 species. However, it was absent from corn at the protein level, supporting the previous observations made by Ohnishi and Kanai (1987). This provides a partial explanation for why only some C_4 plant species depend on the Na^+ as a nutrient (Ohinishi et al., 1990; Aoki et al., 1992; Weber and Caemmerer, 2010; Langdale, 2011). Although C_4 plants benefit from low levels of Na^+, how Na^+ enters the plant is unknown. The possible answer to this question is discussed in section 2.7, under molecular mechanisms.

2.7 Mechanisms of Salinity Tolerance in Plants

Mechanisms of plant abiotic stress tolerance are complex and are mediated by multiple biochemical pathways. The most common mechanism for salt tolerance is the accumulation of osmolytes or osmoprotectants, such as glycinebetaines, proline and sugars (particularly sucrose), in proportion to the change in external osmolarity (de Oliveira et al., 2013). The accumulation of these osmolytes results in a negative water potential, compared to the external environment, thereby increasing water uptake (Rhodes et al., 2002). Abscisic acid (ABA) is an important phytohormone which plays a central role in a plant's response to abiotic stresses. ABA regulates plant water content through guard cells in stoma, and by upregulating genes, which encode enzymes, and proteins that act to confer tolerance to cellular dehydration (Zhang et al., 2006). In earlier studies, it was shown that ABA functions as a long-path dehydration signal in perceiving soil dryness (Davies and Zhang, 1991). During soil dryness, ABA is produced in dehydrated roots and transported to xylem, where changes in pH regulate stomatal opening and leaf growth in shoots (Zhang et al., 1987; Zhang and Davies, 1989; Hartung et al., 2002).

2.7.1 Signal Transduction Pathway in Response to Cellular Dehydration

Exogenous applications of ABA induce a number of genes that respond to salt, drought and cold (Shinozaki and Yamaguchi-Shinozaki, 1996; Bray et al., 1997). This suggests that ABA biosynthesis is triggered by dehydration, which in turn induce the genes. Earlier studies have shown that the several genes, which are induced in response to dehydration, do not respond to exogenous ABA. These

studies demonstrated the existence of ABA-dependent and ABA-independent signal transduction pathway between the perception of initial signal of water stress and the expression of specific genes (Shinozaki and Yamaguchi-Shinozaki, 1996; Bray et al., 1997). Previous studies carried out using ABA-deficient (*aba*) or ABA-insensitive (*abi*) mutants of Arabidopsis have demonstrated that some of stress induced genes do not require an accumulation of endogenous ABA under dehydration conditions, suggesting that not only ABA-dependent but ABA-independent pathway is also involved in response to dehydration. Gene expression analysis of ABA induced genes exhibited that various genes require protein biosynthesis for their induction by ABA. This suggests that stress-induced ABA regulates the expression of a number of genes in two different routes, depending on whether or not new protein synthesis is necessary (Giraudat et al., 1994; Shinozaki, 1997; Bray, 2002). In the route where new protein biosynthesis is not required for the expression of water stress inducible genes, the candidate genes harbor ABA Responsive Elements (ABREs) in their promoter region. The ABREs functions as a cis acting DNA element involved in ABA regulated gene expression (Giraudat et al., 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996). The other route in ABA-dependent pathway is followed where new protein biosynthesis is required for the expression of dehydration stress responsive genes. The expression of an Arabidopsis dehydration stress inducible gene *RD22*, is mediated by ABA and it requires protein biosynthesis for its activation by ABA-dependent pathway (Shinozaki and Yamaguchi-Shinozaki, 1996). In the

promoter region of *RD22* a 67 bp region along with several conserved motifs of DNA binding proteins, such as *MYC/MYB* (myelocytomatosis/myeloblastosis) is necessary for the ABA-responsive expression but does not carry ABREs (Iwasaki et al., 1995). Several genes induced in response to dehydration stress do not respond to exogenous ABA. *RD29A*, a commonly used marker gene for dehydration stress, is independent of ABA (Schinozaki, et al., 2000). Under abiotic stress conditions, a 9 base pair conserved sequence (TACCGACAT), called the dehydration responsive element (DRE), is required as a cis-acting element for the expression of *RD29A*.
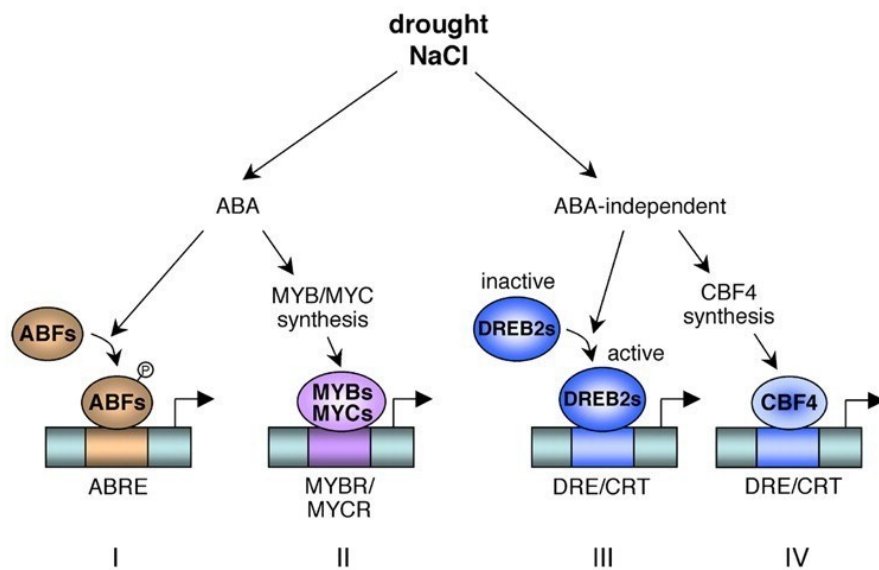
(Giraudat et al., 1994; Schinozaki, 1997; Uno et al., 2000)

Figure 1. Signal transduction pathway between the perception of water stress and gene expression in response to cellular dehydration. There are two signal transduction pathway based on the response of stress inducible genes to ABA. Genes, which require an accumulation of endogenous ABA for their expression in response to dehydration, follow ABA-dependent pathway. Genes, which do not

require an accumulation of endogenous ABA for their expression in response to dehydration, follow ABA-independent signal transduction pathway. In ABA-independent pathway in one route DRE sequence is involved in regulating the gene expression in salt, drought and cold but another route is controlled by salt and drought but not cold.

2.7.2 *SOS* (salt overlay sensitive) Pathway in Response to Salinity Stress

One of the most cited mechanisms for plant salt tolerance is sodium exclusion from the plant cell by the different antiporter channels and transporters located across the plasma membrane (Figure 2A). When plants are subjected to high salinity, sodium alters the ionic homeostasis in the cytoplasm by reducing K^+ and increasing Na^+, altering the H^+ electrochemical gradient (Czempinski et al., 1999). The well characterized H^+ electrochemical gradient has been shown to lower Na^+ concentration in cytosol, relative to the apoplasts or vacuoles (Niu et al., 1995; Blumwald et al., 2000). To date, there is no well characterized system, which mediates Na+ uptake in plants, but some evidence exists (in grasses) that the *HKT* (histidine kinase transporter) gene family (particularly *HKT2*) plays an important role in sodium transport (Sunarpi et al., 2005; Moller et al., 2009). It was documented that the gene *HKT1* is involved in intra-plant sodium transport from roots to shoots when potassium is the limiting factor (Hauser and Horie, 2010, 2011; Horie et al., 2001; Laurie et al., 2002; Munns and Tester, 2008). Interestingly, *HKT2* has not been identified in most plant species, including *A. thaliana* which serve as the current plant model system for genetics and molecular biology. *A. thaliana* harbours only one gene (*HKT1*) from the *HKT*

gene family, as compared to the grasses which have both *HKT1* and *HKT2*, suggesting that *HKT1* might be the common feature in dicotyledonous plants (Sunarpi et al., 2005; Munns and Tester, 2008). Previous physiological data suggested that Na^+ competes with K^+ as both are monovalent cations and are transported by the same proteins (Amtmann et al., 1998). Once Na^+ overtakes K^+, it is transported to vacuoles for compartmentalization by the increased activity of the Na^+/H^+ antiporters localized on tonoplast (Shi et al., 2002).

Plant tolerance to salt stress is the result of multiple stress responsive genes. In plants, Na^+/K^+ homeostasis involves the *SOS* (salt overlay sensitive) pathway (Figure 2B), and under high salinity stress conditions, this pathway is activated to maintain ionic homeostasis in the cytoplasm. This pathway leads to changes in the activities of transporters for Na^+, K^+ and H^+ ions. It is important for the plants to have high affinity for K^+ to maintain Na^+ homeostasis under high salinity stress (Mahajan et al., 2008). When plants are exposed to high salinity, they absorb more salt and this increases cytosolic Na^+ levels, which disrupts cellular processes. When there is high Na^+ in cytoplasm, it induces calcium signaling which activates the *SOS* pathway. The Ca^{+2} signal first activates phospholipase C (*PLC*), which hydrolyzes the phosphatidylinositol bisphosphate (PIP_2) to inositol triphosphate (IP_3), and diacylglycerol (DAG), resulting in an increased cytosolic Ca^{+2} concentration. The changes in Ca^{+2} concentration are perceived by *SOS3* (calcium sensor, encodes for calcineurin B-like protein) and it interacts with *SOS2* (serine/ threonine protein kinase). Both are then directed to the plasma membrane by a peptide called myristoidal fatty acid chain as a

complex where they directly phosphorylate the *SOS1*, a Na⁺/H⁺ antiporter, which efflux the sodium from the cytoplasm. This *SOS3-SOS2* complex reduces the activity of *HKT1*, a low affinity Na⁺ transporter which blocks Na⁺ entry into the cytoplasm and concurrently increases the activity of *NHX1,* leading to the sequestration of Na⁺ into vacuoles where they are stored away from sensitive parts of the cells (Zhu, 2002; Tuteja, 2007; Mahajan et al., 2008). Other calcium binding proteins, including calnexin and calmodulin, also sense the increased cytosolic calcium concentration and, activate *NHX1* (vacuolar Na⁺/H⁺ exchanger to sequester Na⁺ in vacuoles), and target the activity of *SOS2* to maintain the Ca^{+2} homeostasis, as depicted in Figure 2B (Zhu, 2002; Tuteja, 2007; Mahajan et al., 2008). The mechanism of dealing with cytosolic sodium is to store it in the vacuoles, away from cytoplasmic enzymes, and this mechanism is achieved by the activity of sodium/proton antiporters on the tonoplast (Zhu, 2007).

(A)

(B)
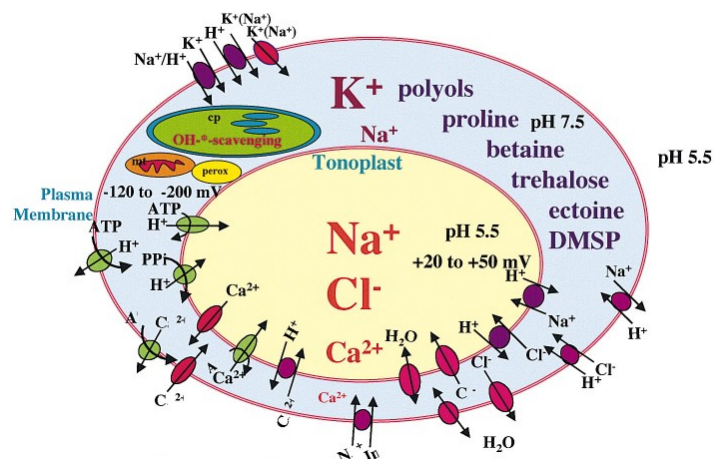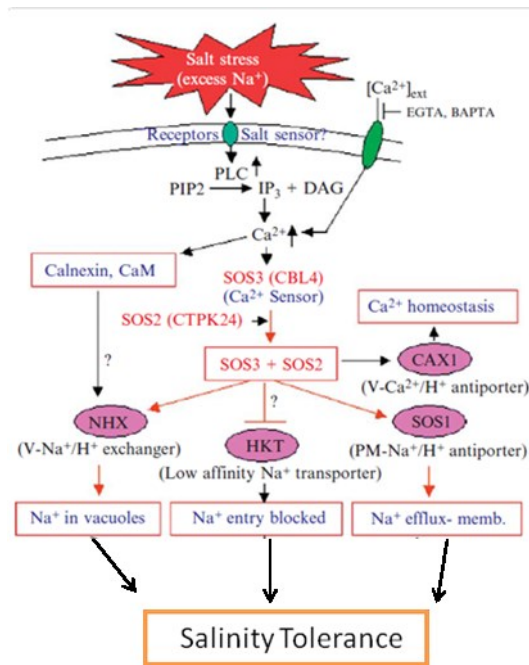
(Zhu, 2002; Tuteja, 2007; Mahajan, 2008)

Figure 2. Various ion pumps and *SOS* pathway maintains ion homeostasis in salinity stress. **(A)** In salinity stress the signals are perceived by receptor present on plasma membrane. The signal activates the various ion pumps located on plasma membrane and vacuolar membrane. The pumps/channels involved in maintaining ion homeostasis are KIRC (potassium inward rectifying channel), HKT (histidine kinase transporter), NSCC (non-selective cation channels) and KORC (potassium outward rectifying channel). The NSCC is a voltage independent channel, which act as a gate for the entry of sodium in plant cells. Plasma membrane hyperpolarization leads to activation of KIRC, which mediates

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the influx of potassium over sodium. However, depolarization of plasma membrane opens the KORC resulting efflux of potassium and influx of sodium. **(B)** The salinity stress signal activates the *SOS* pathway for maintaining ion homeostasis. High salinity induces calcium signaling, which activates phospholipase C (PLC) leading to increase calcium concentration in cytoplasm. The increase in calcium concentration is perceived by *SOS3* (calcium sensor). *SOS3* interacts with *SOS2* and this complex is targeted to plasma membrane, where *SOS3*-*SOS2* directly phosphorylates *SOS1* (Na^+/H^+ antiporter). *SOS1* retrieves sodium from xylem and reduce uptake of sodium in plant. This complex increases the activity of *NHX1* (Na^+/H^+ exchanger) at tonoplast resulting in sequestration of excess sodium in vacuoles. The *CAX1* (H^+/Ca^{2+} antiporter) is a target for *SOS2* activity, which is involved in Calcium homeostasis.

2.8 Seaweed and Salinity

Earlier studies showed that the application of *Ascophyllum nodosum* extracts (ANE) enhanced tolerance against abiotic stresses, such as drought, salinity and temperature extremes (Nabati et al., 1994; Rayirath et al., 2009; Spann and Little, 2011), but the molecular mechanism and the chemistry of the bioactive compounds that elicit the stress tolerance remain largely unknown. To understand the molecular mechanism of salinity tolerance induced by ANE, the entire transcriptome of *A. thaliana* was examined by a cDNA microarray analysis. The cDNA microarray analysis of ANE-mediated salinity tolerance led to identification of several positive and negative regulators of salinity stress (Mundya narayanan and Prithiviraj unpublished data).

2.9 Glycine Rich Ribosome Binding Protein3 (GR-RBP3)

Arabidopsis thaliana genome encodes 196 RRM (RNA Recognition Motifs) containing proteins, and from these eight were classified as *GR-RBPs* (Lorvic and Barta, 2002). Proteins containing RRM at N-terminal, and glycine rich region at C-terminal have been described as *GR-RBPs* (Gomez et al., 1988). The characteristic structural features of *GR-RBP3* are 309 amino acid residues, making it the largest *GR-RBP* of *GR-RBPs* gene family members of Arabidopsis (www.arabidopsis.org). *GR-RBP3* contains numerous glycine residues at the C-terminal end if compared to other *GR-RBPs* in the family. *GR-RBP3* is involved in regulation of flower development and in response to cold (www.arabisopsis.org).

Chapter 3 MATERIALS AND METHODS

3.1 Screening of Arabidopsis Mutants for Salinity Tolerance

Arabidopsis thaliana (L.) Heynh, ecotype Columbia (Col-0) seeds were purchased from Lehle seeds (Round Rock, TX, USA) and T-DNA insertion mutant lines for the genes that were downregulated during ANE mediated salinity tolerance were obtained from Salk institute La Jolla, CA 92037, USA. A comprehensive screen of eleven mutant lines in four different genes (Table 2), was carried out to repeat the preliminary screen completed by Jitesh et al., (2012). Seeds were surface sterilized with NaOCl (sodium hypochlorite) 2% (v/v), and stratified at 4 °C for 3 days. Seeds were placed on the plates containing half strength Murashige and Skoog (Murashige and Skoog, 1962) medium (Sigma, St. Louis, MO), supplemented with 1% (w/v) sucrose and solidified with 0.8% (w/v) phytagel. Plates were vertically stacked in the growth chamber set at 22 °C with 16-h light/8-h dark cycle, with light intensity of 100µmol.m^{-2}s^{-1}. After 4 days growth, plants were transferred on plates containing half strength Murashige and Skoog (MS) medium (Sigma, St. Louis, MO) and 0 to 150mM NaCl, supplemented with 1% (w/v) sucrose and solidified with 0.8% (w/v) phytagel. Plates were vertically stacked in the growth chamber and maintained at 22 °C with 16-h light/8-h dark cycle, with light intensity of 100µmol.m^{-2}s^{-1}. Root length was marked on Days 1, 3, 5, 7, 9 after transfer and for each plant, the number of lateral roots per cm of primary root were recorded as described by Dubrovsky and Forde, 2012. The bleached plants were recorded visually on Days 3, 5, 7, 9 after transfer. Plates were scanned with a high resolution scanner (Epson

Expression 10000 XL), (Epson, Ontario, Canada), and root length was measured with Image J software (Research Services Branch, National Institute of Health, Bethesda, MD). Fresh weight of plants was recorded on the 9th day, then they were dried in a hot air oven at 70 °C for 48 hrs and dry weight was recorded. Data for root length, number of lateral roots, percentage plants bleached, fresh weight and dry weight were compared with the wild type (Col-0) plants.

Table 2. Genes which were down-regulated during *ANE-* mediated salinity tolerance and corresponding mutant lines screened in this study.

Gene name	T-DNA line	Insertion	Accession
GR-RBP3	**Salk_078242C**	**Intron**	*AT5G61030*
GR-RBP3	**Salk_007455C**	**Promoter**	*AT5G61030*
GR-RBP3	**Salk_078146**	**Intron**	*AT5G61030*
WAK1	**Salk_107175**	**Exon**	*AT1G21250*
CSLE1	**Salk_011984**	**Exon**	*AT1G55850*
CSLE1	**Salk_008597C**	**Exon**	*AT4G24000*
GR-RBP3	**Salk_078245**	**Exon**	*AT5G61030*
GR-RBP3	**Salk_078241**	**Exon**	*AT5G61030*
GR-RBP3	**Salk_007592**	**Exon**	*AT5G61030*
GR-RBP3	**Salk_098675**	**Exon**	*AT5G61030*
PMEI	**Salk_007858C**	**Promoter**	*AT1G62760*

Data on root length, number of lateral roots and percentage plants bleached were analyzed, using repeated measure PROC mixed procedure at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001). Data for biomass accumulation were compared with the wild type (Col-0) and were statistically analyzed, using analysis of variance Tukey's at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001).

3.2 Quantitative Reverse Transcription - Polymerase Chain Reaction Analysis

To test whether T-DNA insertion mutants were knockout or knockdown mutants, the expression of *GR-RBP3* was quantified, using qRT-PCR. Leaf samples were collected from wild type and mutant plants. Samples were flash frozen in liquid nitrogen and stored at -80 °C until processed. The samples were powdered in liquid nitrogen using a pre-chilled mortar and pestle. Approximately 60-80 mg of sample was used for total RNA extraction, with some modification to the method described by Chomczynski and Sacchi, (1987). The alteration to the protocol was made at the step after adding the 500µl of Isopropanol. The protocol was to incubate the samples at room temperature for 10 minutes and instead of this, the samples were incubated at -20 °C for 15 minutes, which increased the yield and quality of RNA. The pellet was dissolved in 50µl of DEPC (Diethylpyrocarbonate) water and gently mixed by using the bench top vortex. RNA concentrations were assessed using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Ontario, Canada). Two micrograms of RNA was treated with 2 units of RQ1 DNAse (Promega, Ontario, Canada), according to the manufacturer's instructions, to eliminate any DNA contamination. The total volume of the

reaction was made to 10µl, using the DEPC water. The DNAse-treated RNA was converted to cDNA, using an Applied Biosystems High Capacity cDNA Synthesis Kit (Applied Biosystems Ontario, Canada), according to the manufacturer's protocol. The incubation program was 25 °C for 10 minutes, 37 °C for 2 hours and 85 °C for 5 minutes. The cDNA working concentration was made to 100ng/µl, and from that; 3µl was used in RT-PCR. RT-PCR was performed using the EconoTaq® PLUS GREEN Master Mix (Lucigen, Ontario, Canada) with gene specific primers (Table 3) and *ACTIN* as the endogenous control (Table 3) on MJ Mini™ Personal Thermal Cycler (Bio-Rad, Ontario, Canada). The PCR program was set to 94 °C for 2min., followed by 30 cycles of 94 °C for 30sec., 55 °C for 30sec., 72 °C for 30sec., and a final extension of 72 °C for 10 minutes. The PCR product was examined using gel electrophoresis on a 2% (w/v) agarose gel in 1X TBE buffer at 80V for 40-50 minutes. The gel was photographed in GelDoc™ XR + with image lab software (Bio-Rad, Ontario, Canada). The relative transcript levels were determined by Real–Time polymerase chain reaction, using the gene specific primers and *ACTIN* as the endogenous control, on the StepOne™ Real-Time PCR system (Applied Biosystems, Ontario, Canada), using SYBR green (Applied Biosystems). Gene specific primer sequences were designed using Primer 3 Plus Software. The primers flanked an intron-spanning region.

Table 3. List of genes selected and primers set used in this research.

Gene	Primer	Primer Sequence 5' to 3'
RD29A	Forward	GAATGGTGCGACTAAGATGTTTAGGAA
	Reverse	GTACAGATTCAGTGGGTTTGGTGTAAT
RD22	Forward	AGGGCTGTTTCCACTGAGG
	Reverse	CACCACAGATTTATCGTCAGACA
SOS1	Forward	CTGCTTGCTACATTTCTGCTG
	Reverse	TGCTTCCTCTCCTTCCTTTTC
CLH1	Forward	GAGTGTAAGGCGACGAAAGC
	Reverse	AGAAACGCAACCACAATTCC
GR-RBP3	Forward	ACCTCTTCAGAAGCGGCATC
	Reverse	TAGCCACCACCTCCAAAACC
Actin	Forward	TGAGCAAAGAAATCACAGCACT
	Reverse	CCTGGACCTGCCTCATCATAC
attb	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGGCTTTTTTGAGTAAATTCGGGA
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAAGCTCTTT TGGCAACATCT
M13	Forward	GTAAAACGACGGCCAG
	Reverse	CAGGAAACAGCTATGAC
gr-rbp3-1	Forward	CTGCGTAGTGAGTGGCTTAGC
	Reverse	GAAACCTGAGCATTGAGTTGC
gr-rbp3-2	Forward	TTCACCGTATTTGGTGAAAGC
	Reverse	ATTTTCAGCAAATCAAATGGG
LB1.3		ATTTTGCCGATTTCGGAAC

3.3 Confirmation of T-DNA Insertion

To confirm T-DNA insertion sites in the mutant lines, total genomic DNA was isolated following a method described by Dellaporta et al. (1983). The DNA concentrations were assessed using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Ontario, Canada). The working concentration was made to 100ng/µl by dilution with DEPC water, and from this 2µl was used in PCR. PCR was performed using the EconoTaq® PLUS Master Mix (Lucigen, Ontario, Canada) with the left and right primers in Col-0, and LB1.3 and right primers (Table 3) in mutant lines on MJ Mini™ Personal Thermal Cycler (BIO-RAD,

Ontario, Canada). The PCR program was set to 94 °C for 2min., followed by 30 cycles of 94 °C for 45sec., 58 °C for 30sec., 72 °C for 1min., and a final extension of 72 °C for 10 minutes. The PCR product was examined using gel electrophoresis on a 1% (w/v) agarose gel as described in section 3.2. To confirm T-DNA insertion sites, PCR product was purified using the Qiagen PCR purification kit (Qiagen, Ontario, Canada) and sequenced, as described by Alonso et al. (2003). The sequences were analyzed with the BLAST (Basic Local Alignment Search Tool; Alonso et al. 2003), at NCBI (National Center for Biotechnology Information). Left primer, right primer and LB1.3 (Table 3) were obtained from the SIGnAL website (Salk T-DNA).

3.4 Screening Mutant Lines for Ions Specificity and Osmotic Stress

T- DNA insertion mutant lines Salk_078242C (*gr-rbp3-1*) and Salk_007455C (*gr-rbp3-2*), which exhibited enhanced NaCl tolerance, were screened with 10mM LiCl (Lithium Chloride), as described earlier in section 3.1 to determine whether the mutation in *GR-RBP3* was specific to sodium or whether it tolerated other ionic stresses. To study the effect of mutation in *GR-RBP3* on osmotic stress, *gr-rbp3-1* and *gr-rbp3-2* plants were exposed to with 250mM mannitol to investigate their tolerance to osmotic stress, as described earlier.

3.5 Phenotypic Characterization of Salt Tolerant Mutants

To study the growth characteristics of mutant plants, the biomass accumulation was estimated under optimal growth conditions, as well as under salinity stress. Seeds of wild type and mutant lines were stratified at 4 °C for 3 days. Seeds were planted in Jiffy peat pellets (Jiffy, NB, Canada) and maintained in a growth chamber as mentioned earlier. After two weeks of growth, uniform plants were selected for the experiment. They were treated by placing individual plants in saucers and irrigating with 40 ml of 200mM NaCl solution. There were four replications per treatment and ten plants in each replicate. The fresh weight was recorded on Days 1, 3, 5, 7 and 9 after treatment. Dry weight was recorded after drying the samples in a hot air oven at 70 °C for 48 hours. Data were compared to wild type (Col-0) plants and statistically analyzed using repeated measure PROC mixed procedure at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001).

3.6 Analysis of Sodium and Potassium Content in Plants

The sodium and potassium concentrations were analyzed using high temperature dry oxidation of organic matter, as described by Kalra (2010). Plants were grown as mentioned in section 3.5. Two weeks old plants were selected and used in the experiment. The plants were treated with 200mM NaCl (40 ml/plant). Destructive sampling of treated plants was carried out on Days 1, 5, and 7. There were three replications for each treatment and 20 plants per replicate. After collection, samples were dried in a hot air oven at 70 °C for 72 hours. The samples were then made into a fine powder and 200 mg of powder was used to analyze concentration of sodium and potassium. All the glassware

was acid washed and rinsed 5-6 times with millipore water. Samples were placed in porcelain crucibles and pre-ashed to avoid smoke in the muffle furnace, where they were then placed for further ashing. The temperature of the furnace was gradually increased to 500 °C and samples were ashed for 5 hours. Ash was dissolved in 100 ml 1.0 N HCL and filtered through Whatman filter paper. Elemental analysis was carried out using Atomic Absorption Spectroscopy (AA240FS, Fast Sequential Atomic Absorption Spectrometer, California, US). Analyte concentration was recorded in µg ml^{-1} and it was calculated by using the following formulae:

$$Na^+ = (\text{Sodium content (µg ml}^{-1}) / \text{dry weight}) \times 100$$

$$K^+ = (\text{Potassium content (µg ml}^{-1}) / \text{dry weight}) \times 100$$

After calculations, data were compared to the wild type plants and analyzed; using repeated measure PROC mixed procedure at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001).

3.7 Molecular Analysis of Effects of Mutation in *GR-RBP3* on Salt Stress Responsive Genes

To study the influence of *GR-RBP3* on the transcripts of stress responsive genes, the candidate genes *RD29A, RD22* and *SOS1* were selected as these have been well characterized as being salt stress responsive (Shi et al., 2002a; Jakab et al., 2005). After two weeks, uniform plants were selected and used in the experiment. Each plant was placed in a saucer and treated with 40 ml of 200mM NaCl solution. Samples were collected at 0, 24 and 48 hours after

treatment and immediately flash frozen in liquid nitrogen. To study the differential gene expression, plants were grown hydroponically in half strength Murashige and Skoog (MS) medium (Sigma, St. Louis, MO), supplemented with 1% (w/v) sucrose in jars. Jars were placed in a growth chamber set at 22 °C, with a 16-h light/8-h dark cycle and a light intensity of 100µmol.m^{-2}s^{1}. After 10 days, plants were transferred in the jars containing half strength Murashige and Skoog (MS) medium (Sigma, St. Louis, MO), supplemented with 1% (w/v) sucrose and containing 100mM NaCl. Samples of roots and shoots were collected separately at 24 and 48 hours after treatment and flash frozen in liquid nitrogen. Total RNA was extracted from samples ground in liquid nitrogen, following a method described by Chomczynski and Sacchi (1987), as discussed earlier in section 3.2. Two micrograms of RNA was treated with RQ1 DNAse and converted into cDNA as described in section 3.2. The cDNA working concentration was made to 100ng/µl, and from that 3µl was used in qRT-PCR. The relative transcript levels were determined by Real–Time polymerase chain reaction, using the above mentioned stress responsive genes along with gene specific primers (Table 3) and *ACTIN* as the endogenous control on the StepOne^{Tm} Real-Time PCR system (Applied Biosystems, Ontario, Canada), using SYBR green (Applied Biosystems).

The data were compared to the wild type plants and statistically analyzed, using analysis of variance Tukey's at α < 0.05 in SAS 9.1.2 (SAS, 2001). Gene specific primer sequences were designed using the Primer 3 Plus Software (Table 3). The primers flanked an intron – spanning region.

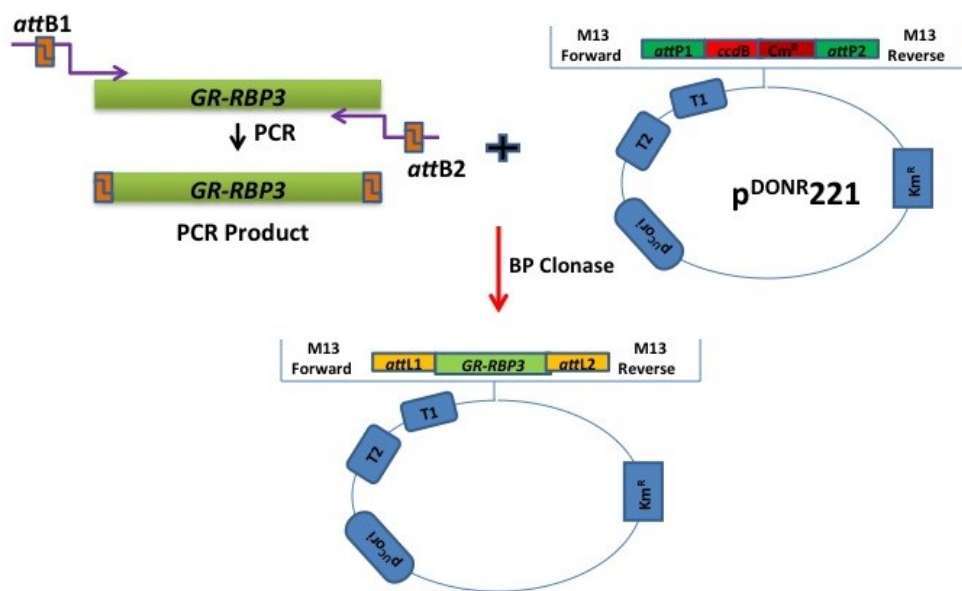## 3.8 Subcellular Localization and Generation of Transgenic Lines

The clone (U21056 in pUNI51), which contained the complete gene of interest was obtained from ABRC (Columbus, OH, USA) and streaked on LB plates containing 50μg/ml kanamycin. The plates were incubated at 37 °C overnight. Next day, one colony was picked from the plate with a sterile loop and transferred into 5 ml LB liquid medium containing 50μg/ml kanamycin, and incubated at 37 °C overnight at 200 RPM (revolution per minute). Plasmid DNA was isolated from the overnight *E. coli* liquid culture, using a plasmid isolation kit (Invitrogen, Ontario, Canada). Plasmid DNA concentrations were assessed using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Ontario, Canada). To clone the complete gene of interest, PCR was performed using the Takara master mix (Clontech, San Francisco, USA) with attB primers (Table 3) and plasmid DNA (Fig. 3A) on MJ Mini™ Personal Thermal Cycler (Bio-Rad, Ontario, Canada). The PCR program was set to 94 °C for 2min., followed by 30 cycles of 94 °C for 90sec., 55 °C for 90sec., 72 °C for 80 seconds and a final extension of 72 °C for 10 minutes. The PCR product was examined using gel electrophoresis as described in section 5.3. The attb PCR product was restriction digested with *Dpn1* (Invitrogen, Ontario, Canada) and purified by using 30% PEG 8000/30mM MgCl$_2$. The attB PCR product, which has attB sites, was cloned into pDONR221 (Fig. 3A), which has attP sites, by the BP recombination reaction using the BP Clonase™ II Gateway®, following the manufacturer's instructions to create the entry clone containing the gene of interest (Fig.3A) (Gateway® Technology with Clonase II, Invitrogen, ON, Canada). The BP recombination reaction was then

transformed into *E.coli DH5α* competent cells by the heat shock method, as described by manufacturer's protocol. The transformed cells were diluted to 1/10 and 1/00, and 100µl of each was plated on pre-warmed LB plates containing kanamycin, 50µg/ml and incubated at 37 °C overnight. The positive entry clones appeared on the plates the next day. Then, 10 positive entry clones were randomly inoculated in LB medium containing 50µg/ml kanamycin and incubated overnight at 37 °C at 200 RPM. Plasmid DNA was isolated from the entry clones by using the plasmid DNA isolation kit (Invitrogen, Ontario, Canada). Plasmid DNA concentrations were assessed using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Ontario, Canada). The approximate size of the entry clone was verified by running 2µl of plasmid DNA on a 0.8% agarose gel in 1X TBE buffer along with GeneRuler 1Kb DNA ladder (Thermo Scientific, Ontario, Canada), at 80V for 90 minutes. To confirm the insert for gene of interest (*GR-RBP3*), PCR was performed using the EconoTaq® PLUS GREEN Master Mix (Lucigen, Ontario, Canada), with gene specific primers (Table 3) on MJ Mini™ Personal Thermal Cycler (BIO-RAD, Ontario, Canada). The PCR program was set to 94 °C for 2min., followed by 30 cycles of 94 °C for 1min., 55 °C for 30sec., 72 °C for 30sec., and a final extension of 72 °C for 10 minutes. The PCR product was examined using gel electrophoresis as described earlier in section 3.2. To confirm the correct orientation, the entry clone plasmids were sequenced using the M13 left and right primers (Table 3) and sequences were analyzed by BLAST at NCBI. To generate the transformation vector, pEarleyGate 104 (N-YFP; Earley, 2005) was obtained from ABRC (Columbus, OH, USA) and

first linearized by restriction digestion with *pVU1*, which cuts the kanamycin gene (Fig. 3B). The positive entry clone containing *attL* sites and complete sequence of the gene of interest was then cloned into pEarleyGate 104 (N-YFP), containing *attR* sites, by LR recombination reaction using the LR Clonase^{TM} II Gateway$^®$, following the manufacturer's instruction to create the expression clone containing the gene of interest (Fig. 3B), (Gateway$^®$ Technology with Clonase II, Invitrogen, ON, Canada). The LR reaction was transformed into *E.coli DH5α* cells, and the transformed cells diluted and plated, as discussed earlier in this section. The approximate size of the expression clone was confirmed as previously described. The insert for the gene of interest was confirmed by performing a PCR using gene specific primers as mentioned earlier in this section. The expression clone containing the gene of interest was transformed into *Agrobacterium* strain GV310 (pMB90) using the freeze and thaw method as described by Hofgen and Willmitzer (1988). The transformed cells were plated on LB plates containing 50µg/ml of kanamycin, and 50µg/ml of gentamycin. The plates were incubated at 28 ˚C for 3 days. After 3 days, colony PCR was performed to confirm the insert, as mentioned earlier in this section. The recombinant *Agrobacterium* strain carrying the gene of interest was transformed to flowering Col-0 plants, using the floral dip method, as described by Clough and Steven (1998). Seeds were collected from the floral dipped transgenic plants, planted in the Jiffy peat pellets (Jiffy, NB, Canada), and placed in a growth chamber set at 22 °C with a 16-h light/8-h dark cycle, and a light intensity of 100µmol.m^{-2}s^{-1}. After 10 days of growth, the plants were sprayed with 250µM ammonium glufosinate (Sigma,

Ontario, Canada) as the expression clone harbored the *Basta* resistant gene and ammonium glufosinate is the active ingredient of *Basta*. After one week, plants started to turn yellow and then they were again sprayed with ammonium glufosinate, as mentioned above. After this, only positive plants survived, and gene expression was confirmed as described in section 3.2 by excising one leaf from each positive plant.

(A)

(B)
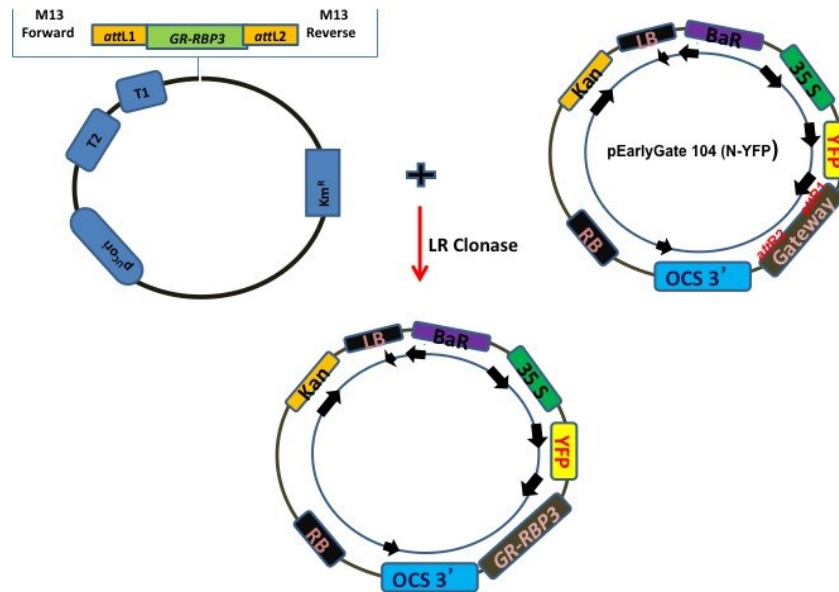
Figure 3 Gateway Technology system to clone *GR-RBP3* in pEarleyGate 104 (N-YFP), to study the subcellular localization and overexpression in the wild type plants. The clone containing the complete insert sequence was purchased from ABRC (OH, USA). Plasmid isolated from cDNA clone was used to generate the attB PCR product using the attB primers and then, the PCR product was fused into pDONR[221] using BP Clonase. The pDONR[221] harboring *GR-RBP3* was then fused into pEarleyGate 104 (N-YFP) using LR Clonase to generate the expression clone carrying *GR-RBP3* sequence. **(A)** Generation of *GR-RBP3* entry clone using the BP recombination reaction. **(B)** Generation of *GR-RBP3* expression clone using the LR recombination reaction.

3.9 Transient expression of *GR-RBP3* in *Nicotiana benthamiana*

To study subcellular localization of *GR-RBP3*, the organ specific markers for mitochondria (MT), Golgi bodies (GB), chloroplasts (Chl), endoplasmic reticulum (ER), plastids (PT) and peroxisomes (PX) were obtained from ABRC (Columbus, OH, USA; Nelson, 2007). The markers were streaked on LB plates containing 50µg/ml kanamycin and, plates incubated at 37 °C overnight. Next day, one colony was selected from each plate by sterile loop, transferred in to 5 ml LB liquid medium containing 50µg/ml kanamycin, and incubated at 37°C for overnight at 200 RPM (revolution per minute). Plasmid DNA was isolated from the overnight *E. coli* liquid culture by using the plasmid isolation kit (Invitrogen, Ontario, Canada). Plasmid DNA concentration was assessed using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Ontario, Canada). Plasmid DNA for each marker was transformed to *Agrobacterium* strain GV310 (pMB90), and positive clones were selected as described in the previous section. To study the co-localization tobacco plants were grown in the pots filled with promix and maintained in controlled conditions as mentioned in the earlier section. When plants were one month old, and leaves were large enough to infiltrate then, they were used in experiment. Transformed vector pEarlyGate 104 (N-YFP) carrying the gene of interest in *Agrobacterium* and organ specific markers in *Agrobacterium* were grown in liquid LB medium containing 50µg/ml of kanamycin, and 50µg/ml of gentamycin at 28°C at 200 RPM until it reached an OD_{600} of 0.8 as described by Sparkes et al. (2006). The cells were collected by centrifugation, and rinsed with sterile water and recentrifuged. The cells were re-

suspended in infiltration medium prepared as described by Sparkes et al. (2006). The final 0.1 (OD_{600}) of expression clone and 0.1(OD_{600}) of above mentioned organ specific markers was mixed well and co-infiltrated in cleaned tobacco leaves as described by Sparkes et al., (2006). For positive and negative controls organ specific markers and expression clone were infiltrated in separate leaves. After 24 and 48 hrs the leaves were cut in to pieces and examined for co-localization using a confocal microscope (Carl Zeiss, Ontario, Canada), at the CMDI (center for molecular digital imaging) Dalhousie University, Halifax.
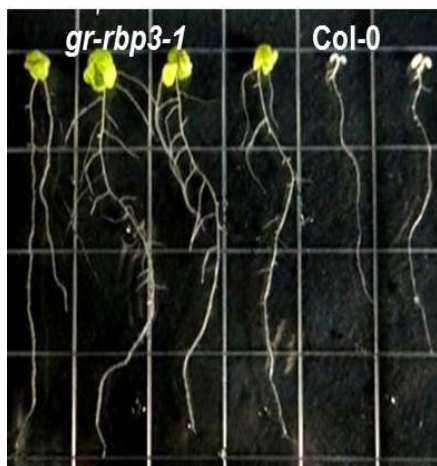
Chapter 4 RESULTS

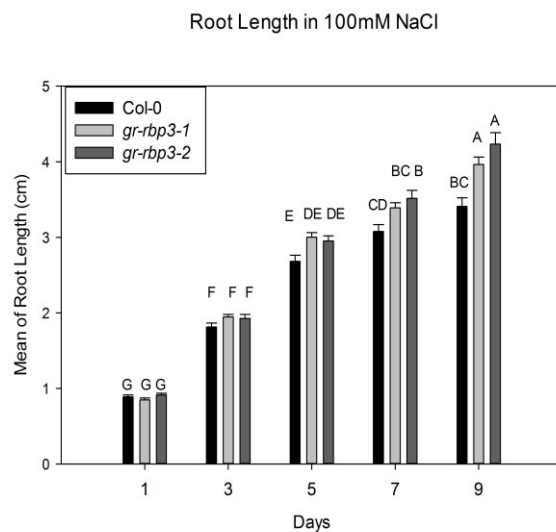**4.1 Identification of Mutants Exhibiting Altered Phenotype for Salt Tolerance**

Candidate genes were selected from the previous study of seaweed extract induced transcriptome of *A. thaliana* under salinity stress (Mundya narayanan and Prithiviraj, unpublished data). T-DNA insertion lines corresponding to genes downregulated by ANE under salt stress were obtained from Salk institute La Jolla, CA 92037, USA. These mutant lines were screened for salt tolerance at different concentrations of NaCl, ranging from 0 to 150mM. Salk_007858C (*PME1*), Salk_107175 (*WAK1*) and Salk_078242C (*GR-RBP3*) exhibited enhanced salt tolerance as compared to the wild type plants. In normal growth conditions no difference was recorded in mutant lines as compared to Col-0. Salk_078242C carrying a T-DNA insertion in *GR-RBP3* (encoding for Glycine Rich-Ribosome Binding Protein3*;* At5G61030), (Alonso et al., 2003), exhibited stronger phenotype in 100mM NaCl, as compared to the wild type and other mutant lines (Figure 4A). This line was named as *gr-rbp3-1*, and selected for further characterization. In the salt tolerance screen, *gr-rbp3-1* plants had significantly longer roots (4.0 cm ± 0.10) and more lateral roots per cm of primary root (1.83 ± 0.025), as compared to the wild type plants (3.4 cm ± 0.11 and 1.31 ± 0.034, respectively), (Figure 4B and Figure 4C). It was also observed that there was significantly reduced plant bleaching in *gr-rbp3-1* (59%), as compared to the wild type (77%) plants (Figure 4D). Furthermore, *gr-rbp3-1* plants showed significantly increased fresh weight (63 mg ± 2.0) and dry weight (7.12 mg ±

0.20), as compared to the wild type (42 mg ± 2.0 and 4.25 mg ± 0.23, respectively) plants (Figure 4E and 4F). To confirm that the altered salt tolerance was caused by a mutation in the *GR-RBP3* gene and not by an unlinked mutation, a second mutant line Salk_007455C (*gr-rbp3-2*) also carrying a mutation in *GR-RBP3,* was examined under salt stress. It was noted that *gr-rbp3-2* plants also exhibited marked salt tolerance (Figure 4G). To further investigate whether the salt tolerance of *gr-rbp3-1* and *gr-rbp3-2* plants was evident in plants grown in soil, 2–week old plants grown on peat pellets were irrigated with 40 ml of 200mM NaCl. After one week, wild type plants exhibited severe symptoms of chlorosis and growth inhibition, whereas *gr-rbp3-1* and *gr-rbp3-2* plants showed reduced chlorosis (Figure 4H).
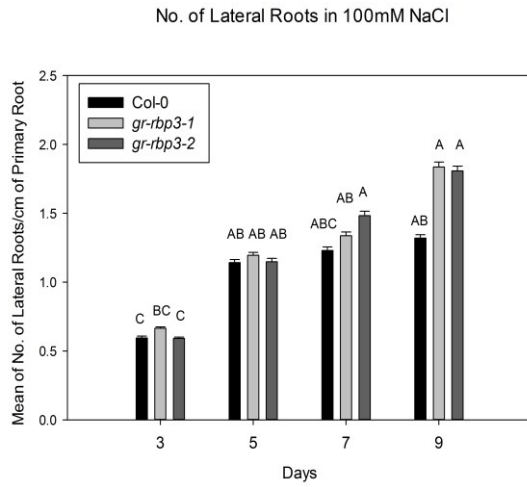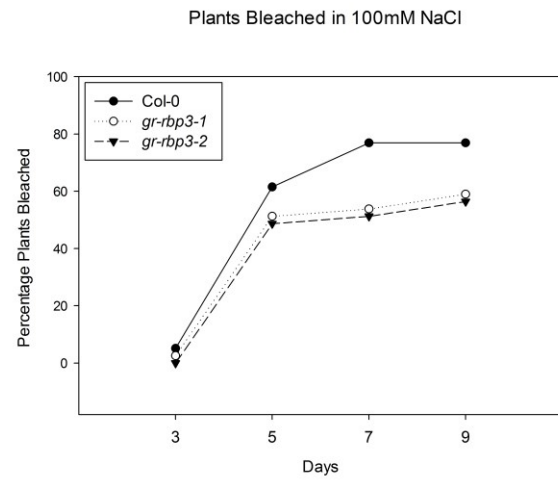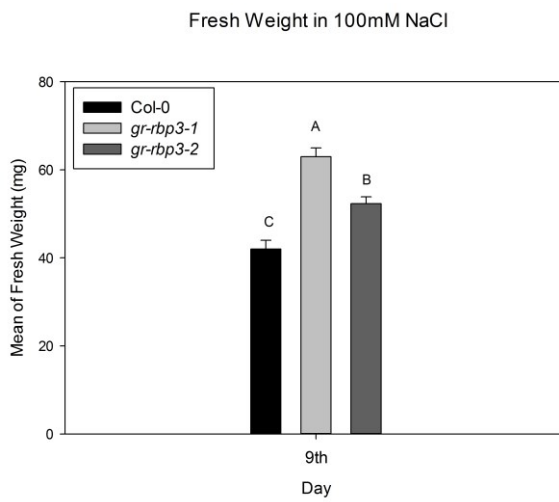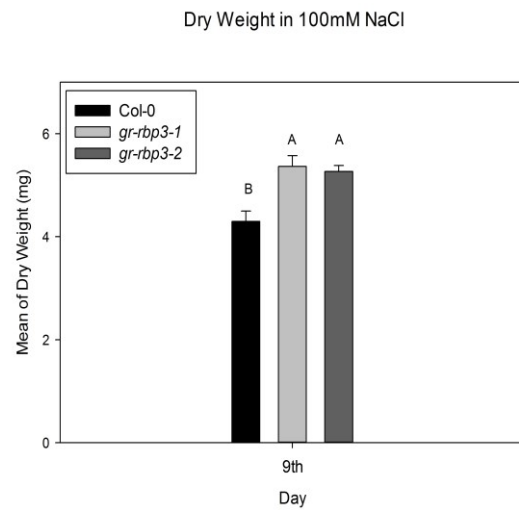
(A)

(B)

(C)

No. of Lateral Roots in 100mM NaCl

(D)

Plants Bleached in 100mM NaCl

(E)

Fresh Weight in 100mM NaCl

(F)

Dry Weight in 100mM NaCl

(G)
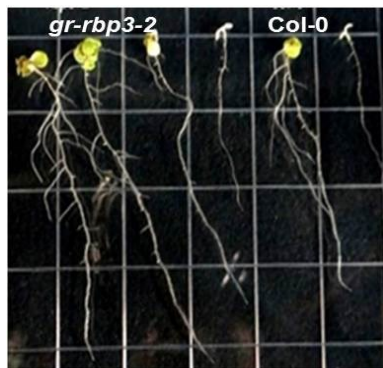
(H)
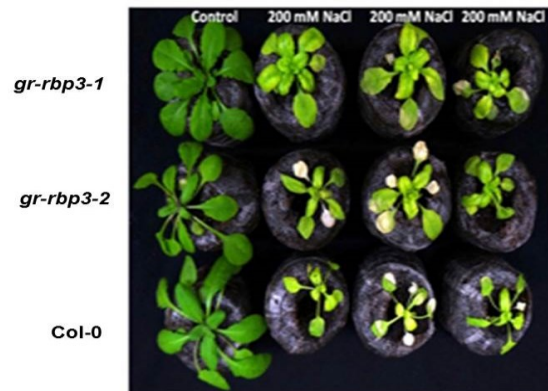
Figure 4 Mutation in *AtGR-RBP3* showed marked tolerance to sodium chloride in the mutant lines Salk_078242C (*gr-rbp3-1*) and Salk_007455C (*gr-rbp3-2*), as compared to the wild type (Col-0) plants. Four days old seedlings grown on half strength MS medium supplemented with 1% (w/v) sucrose and solidified with 0.8% (w/v) phytagel) were transferred to half strength MS medium supplied with 100mM NaCl. **(A)** Salk_078242C (*gr-rbp3-1*) plants were photographed on Day 9 after transfer on 100mM NaCl amended medium. **(B)** Mutation in *AtGR-RBP3* exhibited increased root length in 100mM NaCl amended medium. **(C)** More lateral roots per cm of primary root. **(D)** Reduced plant bleaching (increased plant survival) **(E)** Increased Fresh weight. **(F)** Increased Dry weight. **(G)** Salk_007455C (*gr-rbp3-2*) plants were photographed on Day 9 after transfer. **(H)** Mutation in *AtGR-RBP3* increased plant survival in mutant lines Salk_078242C (*gr-rbp3-1*) and Salk_007455C (*gr-rbp3-2*), as compared to the wild type (Col-0) when grown in soil. Two-week old plants grown on Jiffy peat pellets were irrigated with 40 ml of 200mM NaCl. Plants were photographed on the 7th day after irrigation. Data were analyzed using repeated measure PROC mixed procedure at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001). Data for biomass were statistically analyzed using analysis of variance Tukey's at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001).

Values are the mean ± SE (n≥ 45) from three independent experiments. Means sharing the different letters are significantly different from each other.

4.2 Molecular Characterization of *gr-rbp3-1* and *gr-rbp3-2*

Genotyping through PCR using genomic DNA confirmed that *gr-rbp3-1* carried a T-DNA insertion in the first intron (Figure 5A), whereas *gr-rbp3-2* carried a T-DNA insertion in the promoter region of the *GR-RBP3* gene (Figure 5B) Earlier studies demonstrated that mutation in 5' untranslated region immediate upstream of starting codon can disrupt the gene function (Bird et al., 2007; Kim et al., 2013). qRT-PCR analysis of At5G61030 *(GR-RBP3)* expression revealed that *gr-rbp3-1* and *gr-rbp3-2* are knockdown lines (Figure 5D and 5E).

(C)

WT LP+ RP   *rbp3*-1,2 LB1.3 + RP

1500 bp →

500 bp →

(D)

Col-0   *gr-rbp3-1*   *gr-rbp3-2*

AtGR-RBP3

ACTIN

(E)

At5G61030 Expression

- Col-0
- *gr-rbp3-1*
- *gr-rbp3-2*

Relative Gene Expression
(*GR-RBP3/ACTIN*)

Control
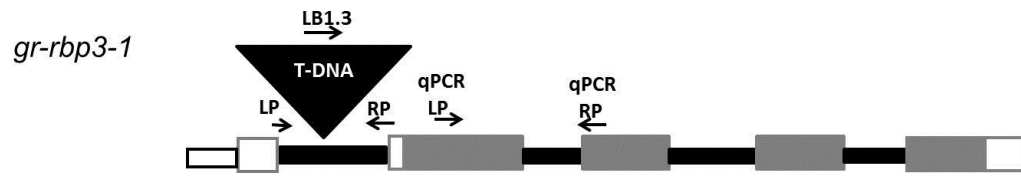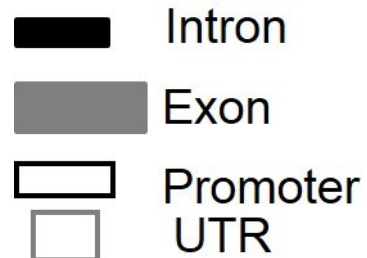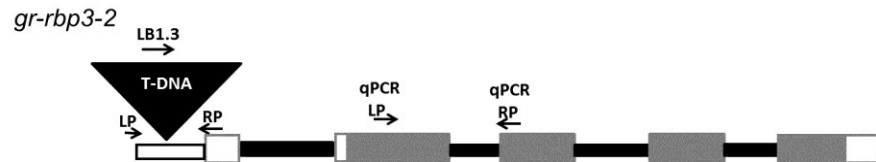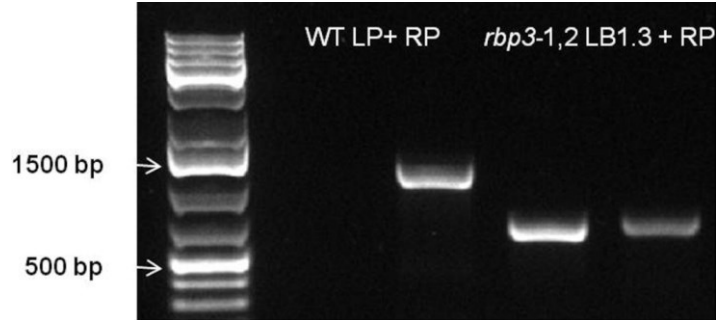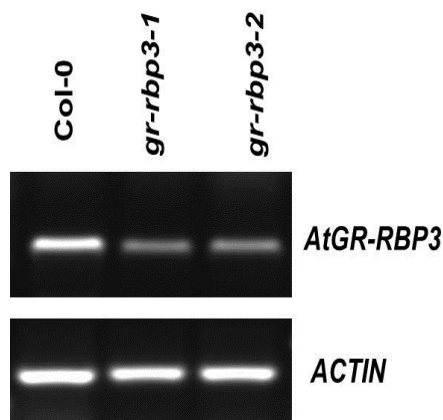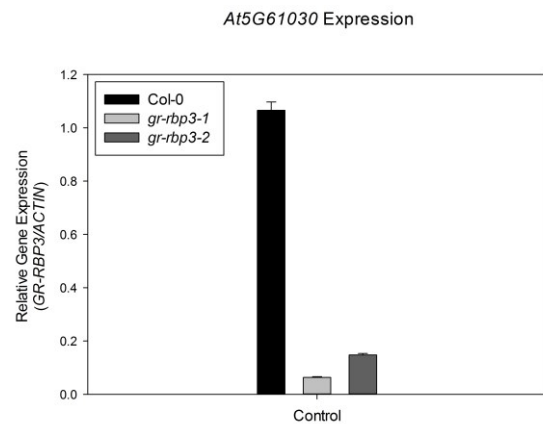
Figure 5 T-DNA insertion sites were confirmed by PCR. Total genomic DNA was isolated, following the method described by Dellaporta Plant DNA isolation protocol. PCR was performed with left and right primers in Col-0 and, LB1.3 and right primers in mutants. PCR product was sequenced as described by Alonso et al., (2003). Rectangular boxes represent exons and thick black line represents introns. To confirm if mutant lines were knockout or knockdown, total RNA was isolated following the method described by Chomczynski (1987), and

converted to cDNA. RT-PCR was performed using a target gene-specific primer and *ACTIN* as endogenous control. **(A)** Physical map of the *At5G61030* locus and T-DNA insertion site in Salk_078242C (*gr-rbp3-1*). **(B)** Physical map of the *At5G61030* locus and T-DNA insertion site in Salk_007455C (*gr-rbp3-2*). **(C)** Genotyping by PCR exhibited expected product size for Salk_078242C (*gr-rbp3-1*) and Salk_007455C (*gr-rbp3-2*), (Salk T-DNA). **(D and E)** qRT-PCR using *AtGR-RBP3* primers showed Salk_078242C (*gr-rbp3-1)* and Salk_007455C (*gr-rbp3-2*) are reduced expression lines. *ACTIN* was used as endogenous control and transcripts levels were normalized to Col-0 control.

4.3 *GR-RBP3* Mutants Showed Altered Phenotype on Lithium Chloride (LiCl) and Mannitol Amended Medium

To determine if the tolerance induced by mutation in *GR-RBP3* is specific to Na^+ or is capable of tolerating other ionic and osmotic stresses, mutant plants were subjected to a treatment with 10mM LiCl and 250mM mannitol. It was noted that *GR-RBP3* mutant plants exhibited enhanced tolerance to both LiCl (Figure 6A) and mannitol (Figure 7A). Mutant plants exposed to both LiCl and Mannitol showed significantly longer roots (Figure 6B and Figure 7B, respectively), more lateral roots per cm of primary root (Figure 6C and 7C, respectively), reduced plant bleaching (Figure 6D), increased fresh weight (Figure 6E and 7D, respectively), and dry weight (Figure 6F and 7E, respectively).

(A)

(B)

Root Length in 10mM LiCl

(C)

No. of Lateral Roots in 10mM LiCl

(D)

Plants Bleached in 10mM LiCl

(E)

Fresh Weight in 10mM LiCl

(F)

Dry Weight in 10mM LiCl
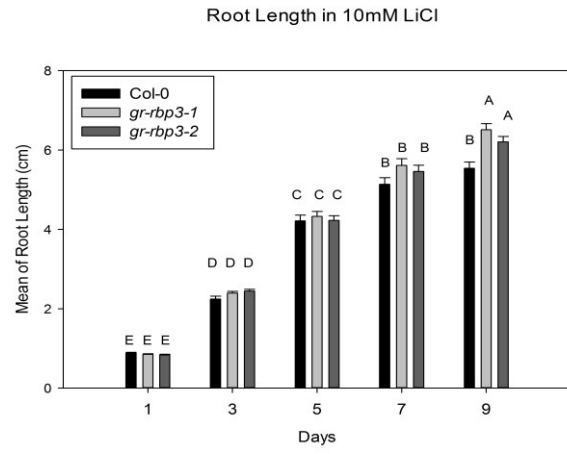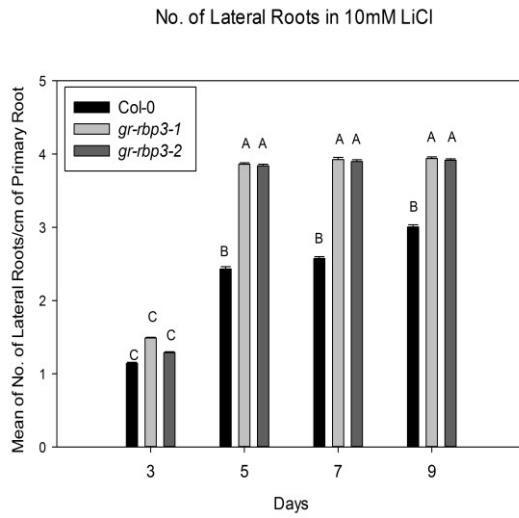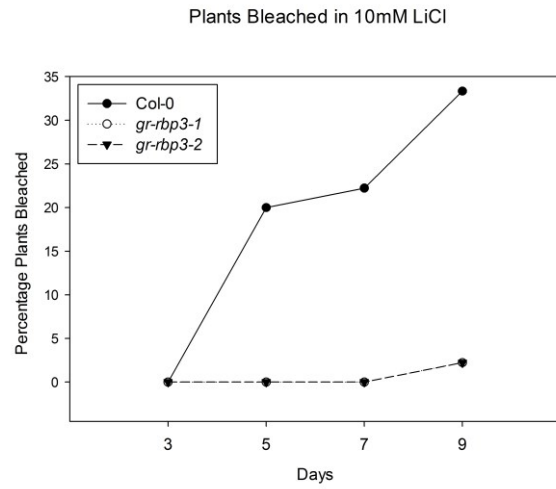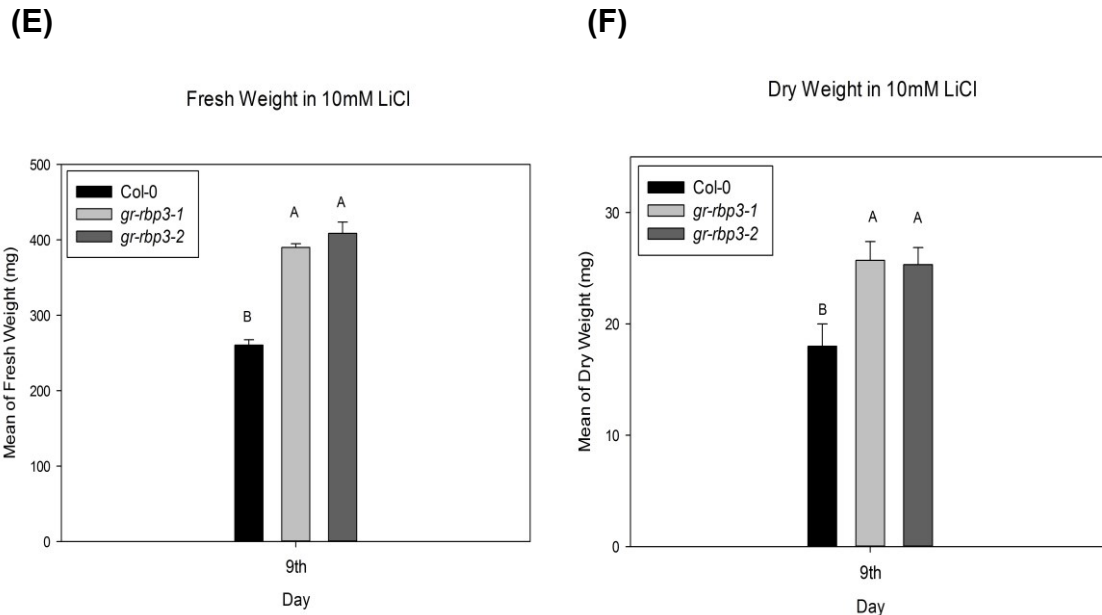
Figure 6 Mutation in *AtGR-RBP3* showed marked tolerance to lithium chloride in mutant lines Salk_078242C (*gr-rbp3-1*) and Salk_007455C (*gr-rbp3-2*). 4 days old seedlings grown on half strength MS medium supplemented with 1% (w/v) sucrose and solidified with 0.8% (w/v) phytagel) were transferred to half strength MS medium supplied with 10mM LiCl. **(A)** Salk_078242C (*gr-rbp3-1*) plants were photographed on 9th day after transfer. Mutation in *AtGR-RBP3* exhibited **(B)** Increased root length. **(C)** More lateral roots per cm of primary root. **(D)** Reduced plant bleaching (increased plant survival). **(E)** Increased fresh weight. **(F)** Increased Dry weight. Data were analyzed using repeated measure PROC mixed procedure at α < 0.05 in SAS 9.1.2 (SAS, 2001). Data for biomass were statistically analyzed using analysis of variance Tukey's at α < 0.05 in SAS 9.1.2 (SAS, 2001).

Values are the mean ± SE (n≥ 45) from three independent experiments.
Means sharing the different letters are significantly different from each other.

(A)

(B)

Root Length in 250mM Mannitol

(C)

No. of Lateral Roots in 250mM Mannitol

(D)

Fresh Weight in 250mM Mannitol

(E)

Dry Weight in 250mM Mannitol

Figure 7 Mutation in *AtGR-RBP3* demonstrated enhanced tolerance to mannitol in mutant lines Salk_078242C (*gr-rbp3-1*) and Salk_007455C (*gr-rbp3-2*). 4 days old seedlings grown on half strength MS medium supplemented with 1% (w/v) sucrose and solidified with 0.8% (w/v) phytagel) were transferred to half strength MS medium supplied with 250mM mannitol. **(A)** Salk_078242C (*gr-rbp3-1*) plants were photographed on 9th day after transfer. Mutation in *AtGR-RBP3* exhibited **(B)** Increased root length. **(C)** More lateral roots per cm of primary root. **(D)** Increased fresh weight. **(E)** Increased dry weight. Data were analyzed using repeated measure PROC mixed procedure at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001). Data for biomass were statistically analyzed using analysis of variance Tukey's at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001).

Values are the mean $\pm$ SE (n$\geq$ 45) from three independent experiments. Means sharing the different letters are significantly different from each other.

4.4 Phenotypic Characterization of Salt Tolerant Mutants

To study the growth characteristics of the mutant plants, the biomass accumulation was studied under optimal growth and salinity stress conditions. Two weeks old plants, grown on Jiffy peat pellets, were irrigated with 200mM NaCl at 40 ml per plant. Samples were collected by on Days 1, 3, 5, 7 and 9 after irrigation and fresh weight and dry weight was recorded as discussed in material and methods. No difference was recorded in mutant lines as compared to Col-0 in normal growth conditions. It was observed that *gr-rbp3-1* and *gr-rbp3-2* accumulated significantly higher biomass (329 mg ± 19.16 and 297 mg ± 15.14 of fresh weight; 66 mg ± 3.5 and 55 mg ± 2.61 of dry weigh, respectively) as compared to the wild type plants (251 mg ± 10.2 of fresh weight and 43 mg ± 0.45 of dry weight), (Figure 8A and 8B). Data were compared to the wild type (Col-0) and statistically analyzed using repeated measure PROC mixed procedure at α < 0.05 in SAS 9.1.2 (SAS, 2001).

(A)   **(B)**
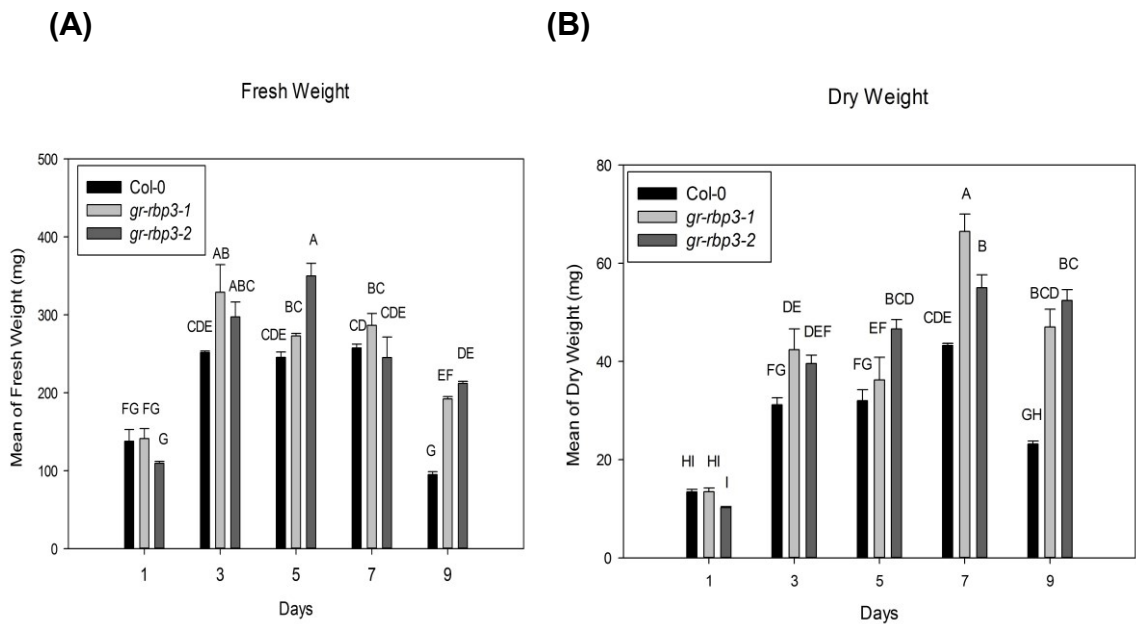
Figure 8 Mutation in *AtGR-RBP3* exhibited higher biomass in mutant lines Salk_078242C (*gr-rbp3-1*) and Salk_007455C (*gr-rbp3-2*) when grown in soil. Two weeks old plants grown on Jiffy peat pellets were irrigated with 40 ml of 200mM NaCl. **(A)** Mutation in *AtGR-RBP3* exhibited enhanced fresh weight. **(B)** Mutation in *AtGR-RBP3* exhibited enhanced dry weight. Data were analyzed using repeated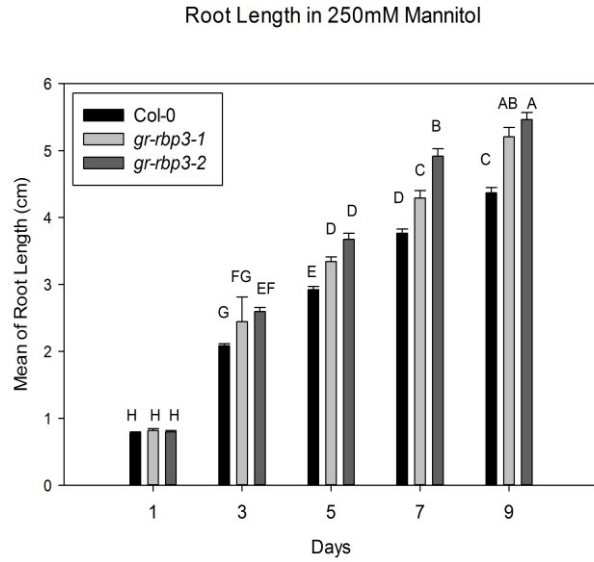 measure PROC mixed procedure at α < 0.05 in SAS 9.1.2 (SAS, 2001). Values are the mean ± SE (n≥ 15) from three independent experiments. Means sharing the different letters are significantly different from each other.
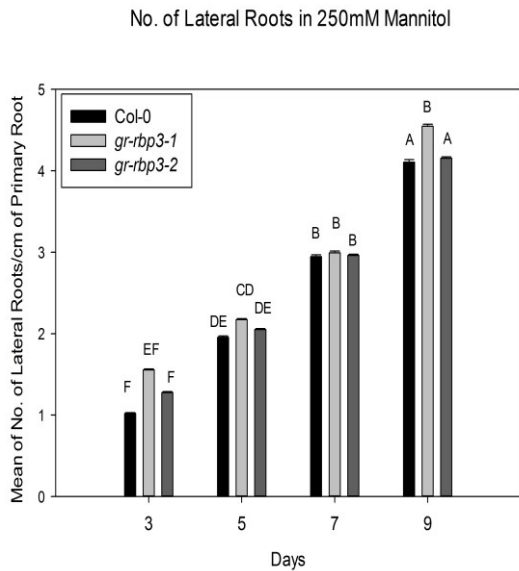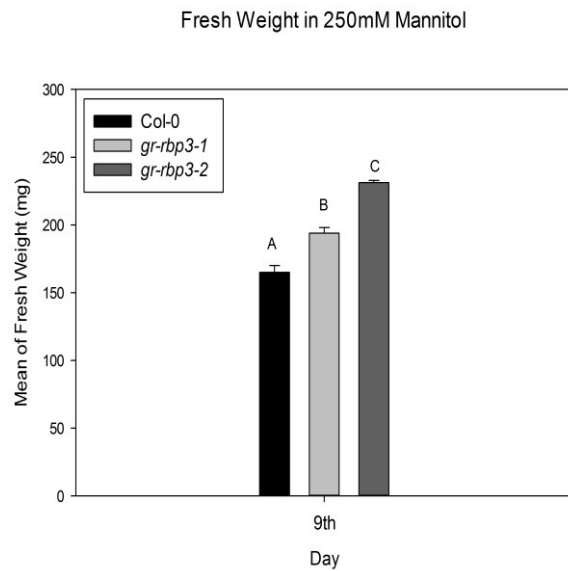
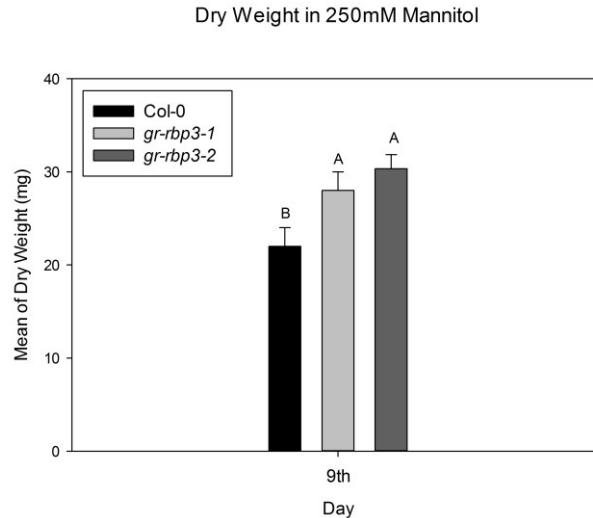## 4.5 Mutation in *GR-RBP3* Reduced Na^+ and Na^+/K^+ Ratio

To investigate, whether the mutations in *GR-RBP3* had reduced Na^+ accumulation and Na^+/K^+ ratio, the Na^+ and K^+ concentration in plants was analysed using AAS (Atomic Absorption Spectroscopy). Two weeks old plants grown on Jiffy peat pellets were irrigated with 40 ml of 200mM NaCl. No significant difference in Na^+ and K^+ concentration was recorded in samples collected on Day 1 following the exposure to 200mM NaCl in both wild type and mutant plants. However, Na^+ significantly increased in wild type plants (57.73 µg mg^{-1} of dry weight ± 2.87) as compared to *gr-rbp3-1* (36 µg mg^{-1} of dry weight ± 2.0) and *gr-rbp3-2* (37.76 µg mg^{-1} of dry weight ± 3.5) plants on day 5 (Figure 9A). Furthermore, higher K^+ concentrations were recorded in *gr-rbp3-1* (11.84 µg mg^{-1} of dry weight ± 0.2) and *gr-rbp3-2* (10.59 µg mg^{-1} of dry weight ± 0.5) as compared to the wild type plants on day 5 (9.13 µg mg^{-1} of dry weight ± 0.68), (Figure 9B). It was also noted that the Na^+/K^+ ratios were significantly reduced in

gr-rbp3-1 (3.12 ± 0.16) and *gr-rbp3-2* (3.54 ± 0.4) as compared to the wild type

(7.0 ± 0.62) plants (Figure 9C).

(A)

(B)

(C)
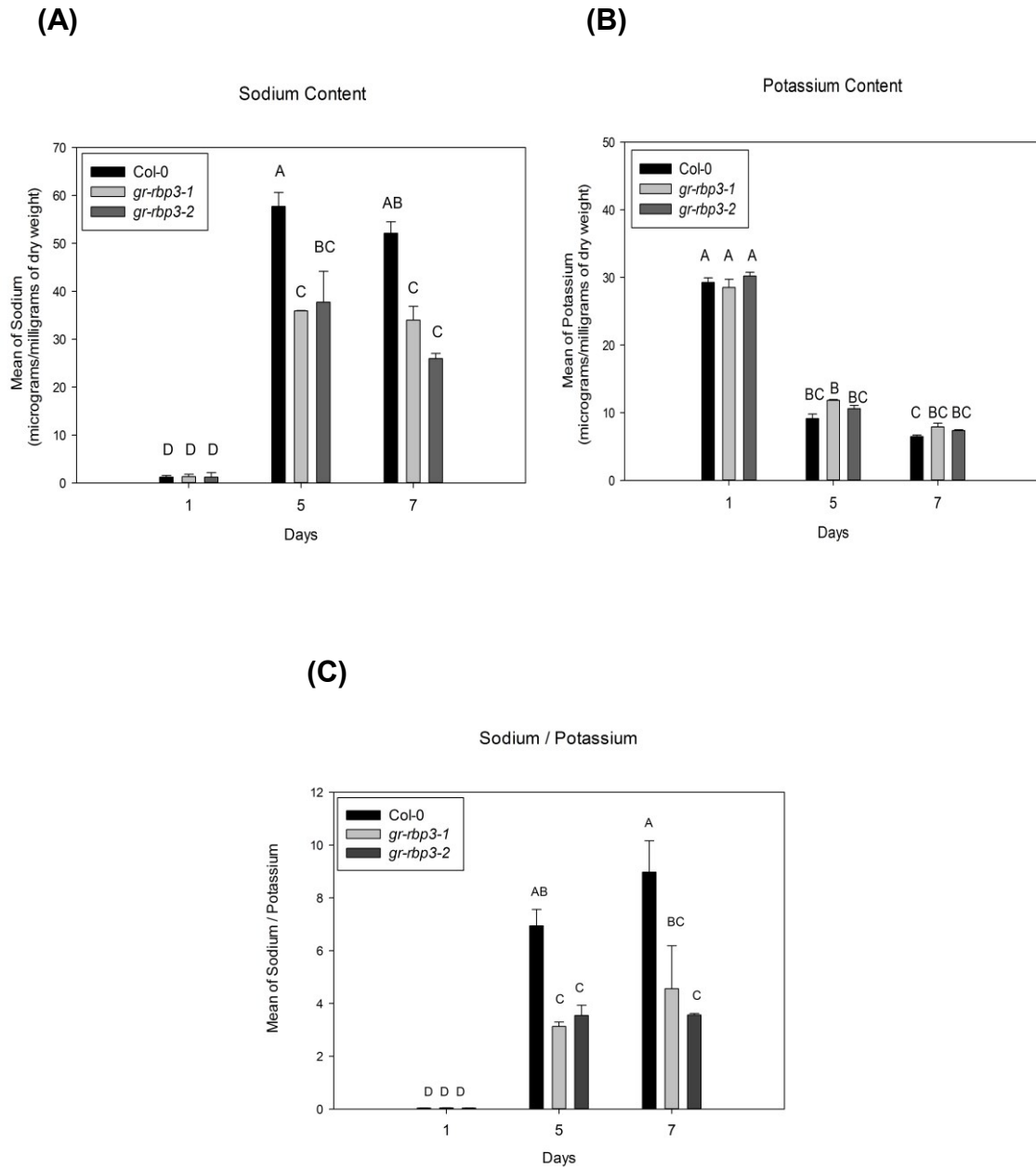
Figure 9 Mutation in *AtGR-RBP3* exhibited reduced sodium accumulation and reduced sodium/potassium ratio in mutant lines Salk_078242C (*gr-rbp3-1*) and Salk_007455C (*gr-rbp3-2*) when grown in soil. **(A)** Mutation in *AtGR-RBP3* exhibited reduced sodium accumulation. **(B)** Increased potassium content. **(C)**

Reduced sodium/potassium ratio. Data were analyzed using repeated measure PROC mixed procedure at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001). Values are the mean ± SE (n≥ 3) from two independent experiments. Means sharing the different letters are significantly different from each other.

4.6 Reverse Transcription – Polymerase Chain Reaction Revealed Altered Salt Stress Signaling in *gr-rbp3-1* and *gr-rbp3-2*

To examine the possible association between known salt tolerance pathways and *AtGR-RBP3*, known candidate genes that are responsive to salt stress were selected. *RD29A, RD22,* and *SOS1* were selected as these are well characterized salt stress responsive genes (Shi et al., 2002a; Jakab et al., 2005). The transcripts abundance of *RD22* and *SOS1* were significantly higher in both *gr-rbp3-1* (~4 and ~3 fold from normal, respectively), and *gr-rbp3-2* (~4.3 and ~2 fold from normal, respectively), as compared to the wild type plants (~2.7 and ~1.2 fold from normal, respectively) at 48 hrs after the exposure to 200mM NaCl (Figure 10A and 10B). Whereas, the transcript levels of *RD29A* were significantly higher in *gr-rbp3-1* (~10.5 fold from normal) but no significant difference was observed in *gr-rbp3-2* as compared to the wild type plants at 48 hrs after treatment (Figure 10C). It was also observed that the transcripts abundance of *Chlorophyllase1 (AtCLH1)* were significantly higher in wild type plants (~2.5 fold from normal) as compared to mutant plants (Figure 10D). Furthermore, it was also confirmed that *GR-RBP3* transcript levels increased following the 200mM NaCl treatment in the wild type plants (~3 fold from normal), (Figure 10E). The differential expression of *GR-RBP3* in the shoot and root of wild type and mutant

plants were studied. Interestingly, *GR-RBP3* was highly expressed in shoots (~5.5 fold from normal) as compared to roots (~2.0 fold from normal) in wild type plants (Figure 10F and Figure 10G respectively).

(A)

RD22 Expression

(B)

AtSOS1 Expression

(C)

RD29A Expression

(D)

AtCLH1 Expression

(E)

AtGR-RBP3 Expression

(F)

AtGR-RBP3 Expression in Shoots

(G)

AtGR-RBP3 Expression in Roots
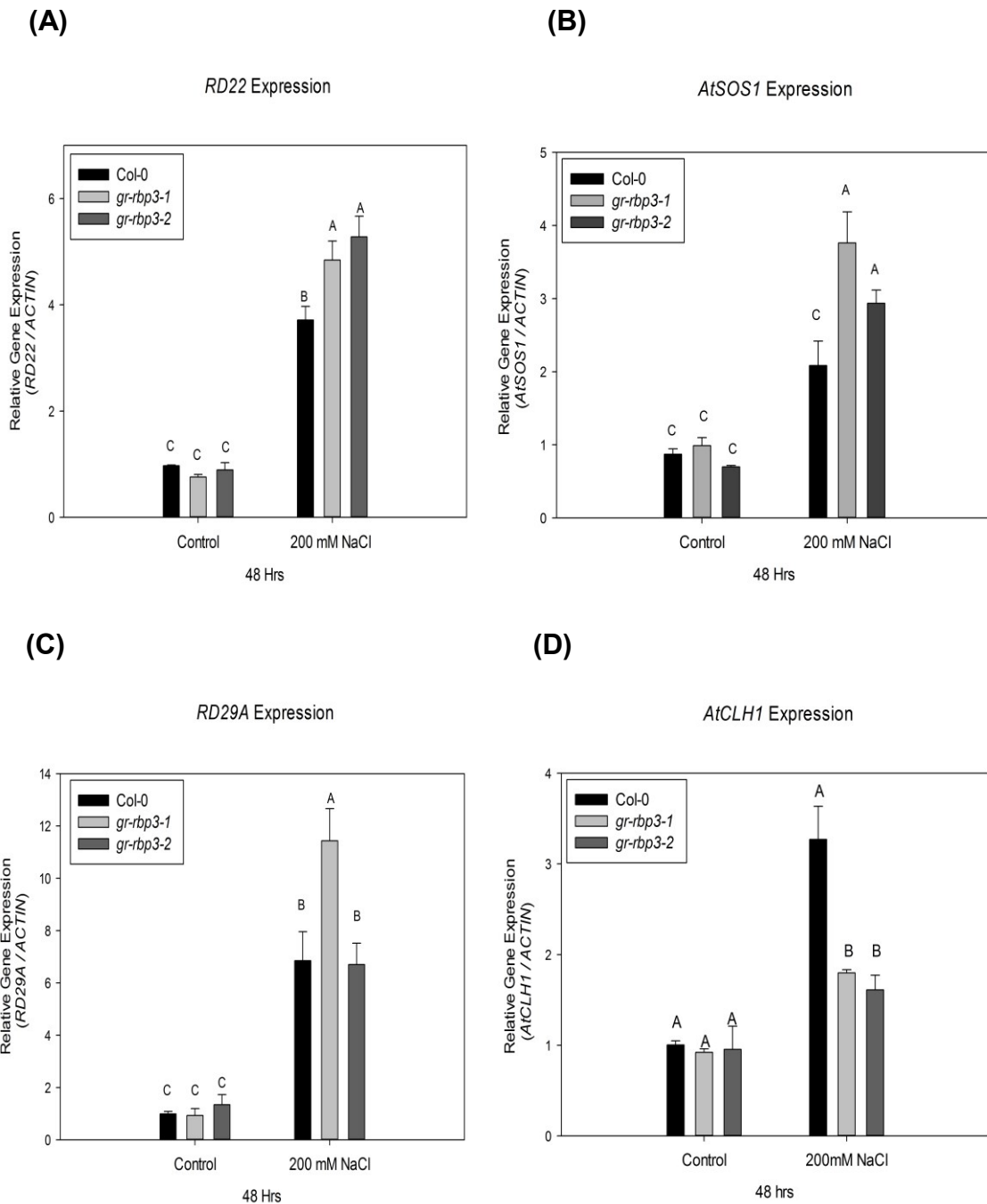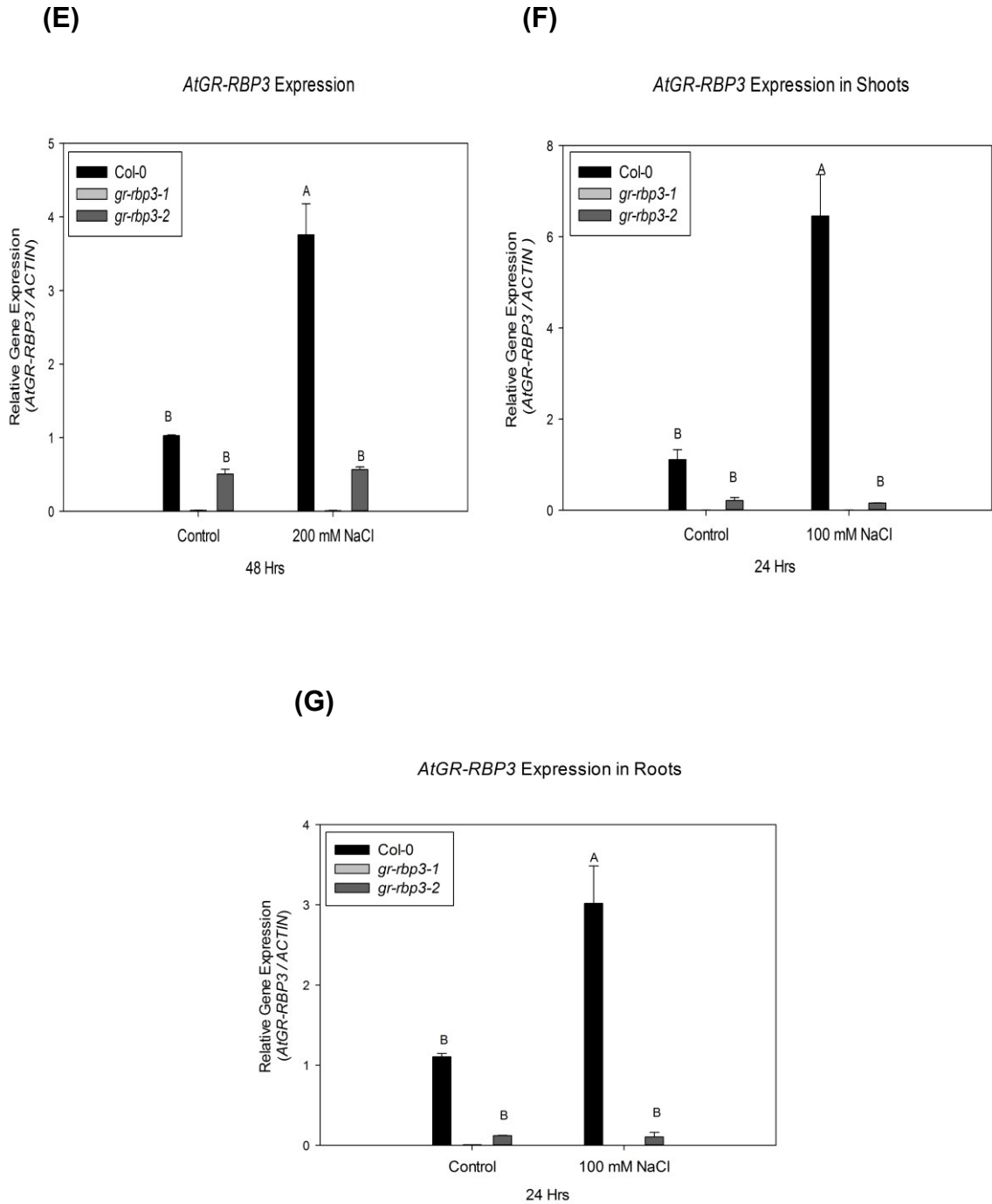
Figure 10 Gene expression studies of different stress responsive genes in wild type and mutant plants following the exposure to sodium chloride. Two-week old plants grown on Jiffy peat pellets were irrigated with 40 ml of 200mM NaCl.

To study differential expression of *AtGR-RBP3* in shoots and roots plants were grown hydroponically. Total RNA was isolated and converted to cDNA. qRT-PCR was performed to study the expression level of different stress responsive genes **(A)** *RD22* expression. **(B)** *AtSOS1* expression. **(C)** *RD29A* expression. **(D)** *AtCLH1* expression. **(E)** *AtGR-RBP3* expression. **(F)** *AtGR-RBP3* expression in shoots. **(G)** *AtGR-RBP3* expression in roots. *ACTIN* was used as endogenous control and transcripts levels were normalized to Col-0 control. Data were statistically analyzed using analysis of variance Tukey's at $\alpha < 0.05$ in SAS 9.1.2 (SAS, 2001). Data represents the mean ± SE (n≥ 5) from three independent biological replicates. Means sharing different letters are significantly different.

4.7 Subcellular Localization of GR-RBP3

To study the localization of GR-RBP3 in the plant cells, the gene was inserted into the plant transformation vector (pEarleyGate104-N-YFP, Earley, 2005) using Gateway Technology (Gateway® Technology with Clonase II, Invitrogen, ON, Canada), and the plasmid was incorporated into *Agrobacterium*. The subcellular localization of YFP tagged GR-RBP3 was examined in tobacco leaves through confocal microscopy as described by Sparkes et al., (2006). The subcellular localization study revealed that YFP-GR-RBP3 was localized in the endoplasmic reticulum (Figure 11). Nelson et al. (2007) reported the use of this endoplasmic reticulum marker for subcellular localization studies.
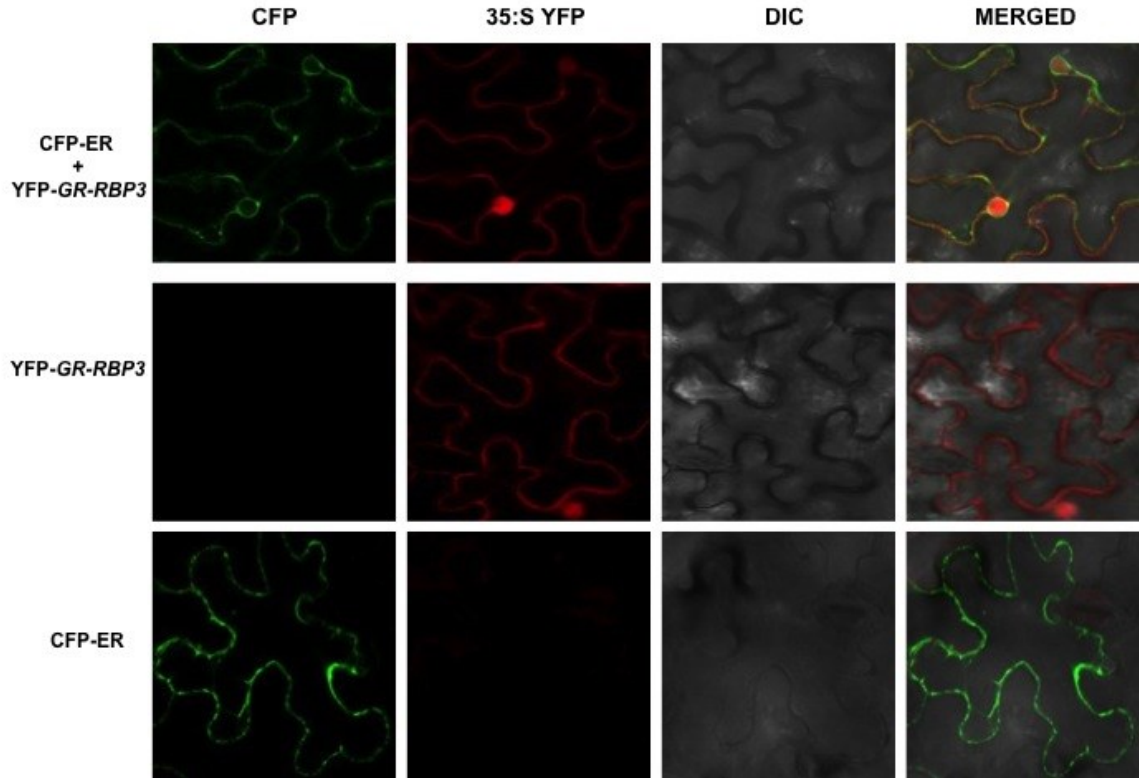
Figure 11 Subcellular localization of GR-RBP3 revealed that it is localized to endoplasmic reticulum. The full length sequence of *AtGR-RBP3* was cloned in pEarleyGate 104 (N-YFP) using the Gateway Technology. The construct pEarleyGate 104 (N-YFP) harboring gene of interest along with endoplasmic reticulum (Nelson et al., 2007) was infiltrated in tobacco leaves and examined through confocal microscopy.

4.8 Overexpression of *GR-RBP3*

GR-RBP3 was overexpressed in the wild type plants (Clough and Steven, 1998) to further study the phenotype of overexpression lines under salinity stress. In this study, two overexpression lines (OE1, OE2) were identified by qRT-PCR. The over-expression lines OE1 and OE2 had higher transcript abundance of *GR-*

RBP3 (~50 fold and ~80 fold, respectively) as compared to the wild type (~1 fold) under optimal conditions (Figure 12).
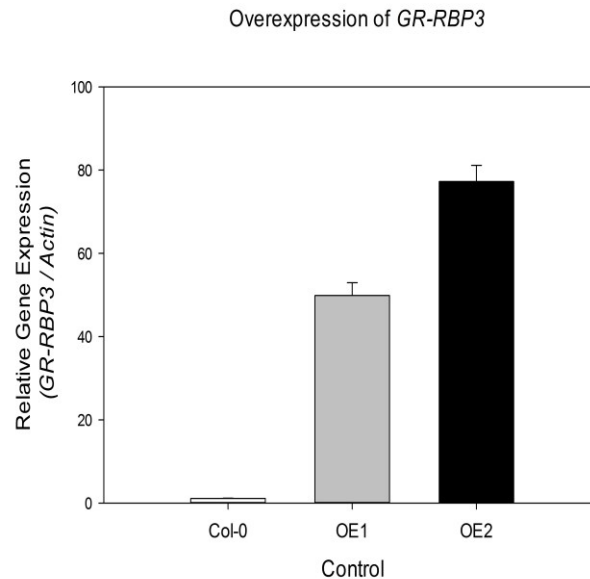
Figure 12 *GR-RBP3* was overexpressed in wild type plants by floral dip method. The pEarleyGate 104 (N-YFP) construct carrying gene of interest was transformed into *Agrobacterium* by freeze and thaw method. The wild type flowering plants were then, floral dipped in 0.1 (OD_{600}) of *Agrobacterium* harboring gene of interest. Seeds collected from transformed plants were planted on Jiffy Peat Pellets. Plants were sprayed with 200µM ammonium glufosinate (active ingredient of Basta). The positive transgenic plants were further confirmed by qRT-PCR.

Chapter 5 DISCUSSION AND CONCLUSION

Soil salinity is detrimental to plant growth leading to a significant loss in agricultural production (Rodríguez et al., 2005). Under conditions of salinity stress, the reduced expression of *GR-RBP3* in ANE treated plants as compared to wild type plants suggest the potential role of *GR-RBP3* in saline environments.

5.1 Mutants Exhibited Altered Phenotype for Salt Tolerance

The loss of function mutants in *GR-RBP3* (At5G61030), *gr-rbp3-1* and *gr-rbp3-2* exhibited marked tolerance to 100mM NaCl in comparison to wild type plants (Figure 4A and 4G). Furthermore, in this research it was noted that when mutant plants were subjected to 250mM mannitol and 10mM LiCl, they had significantly longer root length, more lateral roots per cm of primary root and increased biomass accumulation (Figure 6 and Figure 7) demonstrating the plant tolerance to salt and osmotic stress. Wild type plants were susceptible to NaCl, LiCl and mannitol in comparison to mutants, and this is possibly associated with turgor pressure. The turgor pressure of normal root cells ranges from 0.4– 0.6MPa and the osmotic pressure of 100mM NaCl is 0.5MPa. If the turgor pressure of cells is only 0.4 (mostly in older root cells), then the exposure of cells to 100mM NaCl or 200 mM mannitol will plasmolyse the cell because 200 mM mannitol also has the osmotic pressure of 0.5MPa at 100mM NaCl (Pritchard et al., 1991). In earlier studies when plants were subjected to osmotic agents, such as KCl, or mannitol at an osmotic pressure similar to NaCl, they exerted the same effect as NaCl (Yeo et al., 1991). A rapid and transient reduction in root growth was observed immediately after exposure to NaCl, KCl and mannitol. This suggests that the

reduction in leaf and root growth was entirely associated with a sudden change in plant water status rather than an ion-specific effect (Frensch and Hsiao, 1994, &1995; Rodriguez et al., 1997). As mentioned earlier, that 200mM mannitol induced the same osmotic pressure as 100mM NaCl, and in this study it was observed that mutant plants exhibited enhanced tolerance to 100mM NaCl, 250mM mannitol and 10mM LiCl. This demonstrated that mutation in *GR-RBP3* was not specific to sodium.

Salinity tolerance is mostly assessed on the basis of biomass production (Greenway and Munns, 1980). In this study, it was observed that *gr-rbp3-1* and *gr-rbp3-2* had significantly higher fresh and dry weight (Figure 8A and 8B, respectively) demonstrating tolerance to high salinity.

5.2 *GR-RBPs* in Abiotic Stress Tolerance

Lorković and Barta (2002) reported that the *Arabidopsis thaliana* genome encodes196 RRM (RNA Recognition Motifs) containing proteins and out of these, eight were classified as *GR-RBPs*. The characteristic structural features of *GR-RBP3* are 309 amino acid residues, making it the largest *GR-RBP* of the *GR-RBPs* gene family members of *A. thaliana* (http://www.arabidopsis.org/). *AtGR-RBP3* contains numerous glycine residues at the C– terminal end if compared to other *GR-RBPs* in the family. In recent years, it was reported that *GR-RBP2*, *GR-RBP4* and *GR-RBP7* play roles in response to abiotic stresses, and it was shown that *GR-RBP4* also played a role in biogenesis, RNA metabolism of actively proliferation organs, and negative regulation under salinity stress (Kwak et al., 2005; Kim et al., 2007; Kim et al., 2008).

In eukaryotes, gene regulation has been shown to play a central role for adaptation to different environmental stresses (Jiang et al., 2013). Post-transcriptional gene regulation was reported to involve a number of co-transcriptional processes such as pre-mRNA splicing, capping, polyadenylation, mRNA transport, stability and translation (Jiang et al., 2013). Over the last decade, research has focused on the RBPs and it was discovered that N-terminal of RBPs had one or more RNA Recognition Motifs (RRMs), while the C–terminal had different kinds of auxiliary motifs such as glycine-rich, arginine-rich, SR-repeats and RD-repeats (Kenan et al., 1991; Burd and Dreyfuss, 1994; Alba and Pages, 1998). Proteins containing RRM at N–terminal, and glycine rich regions at C–terminal have been described as *GR-RBPs* (Gomez et al., 1988). Since the first gene encoding a GR-RBP protein was identified in maize (Gomez et al., 1988), other cDNAs encoding homologous proteins have been identified in *Arabidopsis thaliana* (vanNocker and Vierstra, 1993; Carpenter et al., 1994). However, these proteins were not characterized in detail and it was hypothesized that these might be involved in responses to different environmental stresses as their mRNA levels were upregulated concomitant to the exposure to abiotic stresses (Sachetto-Martins et al., 2000).

5.3 Mutation in *GR-RBP3* Altered Salt Stress Signaling in Mutants

High salinity, drought, and cold share the common features of creating a water deficit in plants. Kreps et al. (2002) reported form microarray analysis that ~30% of genes were common under osmotic, salt, and cold stress. Under water deficit conditions, a complex of responses was elicited with the perception of stress, leading to activation of the signal transduction pathway (Bray, 1993). The plant's molecular responses to a water deficit condition raised a number of interesting questions, such as how does a physical phenomenon, such as loss of water from cells lead to the activation of stress response genes. The answer to this question still remains unclear. It is thought that a water deficit decreases the turgor pressure at the cellular levels. This decrease in turgor pressure leads to a change in osmotic potential across the plasma membrane. The change in osmotic potential across the plasma membrane triggered the activation of stress responsive genes (Wurgler-Murphy and Saito, 1977; Shinozaki and Yamaguchi-Shinozaki, 1997). In plant cell cytosolic Ca^{2+} plays an important role for turgor regulation (Cote, 1995; Niu et al., 1995). When plants were exposed to salinity stress, endogenous ABA levels were increased (Ingram and Bartels, 1996; Bray, 1997). As a result of this *RD22*, an ABA mediated stress response gene was induced (Shinozaki and Yamaguchi-Shinozaki, 1996). In a study carried out by Yamaguchi-Shinozaki and Shinozaki (1994), a number of genes were induced in ABA deficit mutants in response to cold, salt and drought. This exhibited that in these genes ABA did not required for the induction in stress response. Among these genes, *RD29A* has been extensively studied, and it was discovered that a

9-bp sequence TACCGACAT, called *DRE/CRT*, was required for the induction of this gene under salinity stress. In further studies it was identified that promoter domain in *RD29A* harbours the ABRE (ABA Responsive Elements), which functions in ABA-dependent expression, making activation of *RD29A* partial ABA–dependent and ABA-independent (Thomshow, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). Under salinity stress, ABA is synthesized *via* the carotenoid biosynthetic pathway. Where 9'-cis-neoxanthin is cleaved to xanthoxin and the xanthoxin is oxidized to ABA-aldehyde. This is further converted to ABA by ABA-aldehyde oxidase (Parry, 1993). In salinity stress, the first step of ABA synthesis pathway is regulated. The transcription/translation is required for the biosynthesis of enzymes and proteins to elevate the ABA levels (Parry, 1993). In this study qRT-PCR analysis of *RD22*, *SOS1* and *RD29A* (Figure 10A, 10B and 10C, respectively), revealed a significant increase in mRNA levels following the exposure to salinity stress. In most of the studies related to salinity stress, *RD29A* and *RD22* were used as marker genes and have been shown to have a protective function in salinity stress (Shinozaki and Yamaguchi-Shinozaki, 2000).

5.4 Mutation in *GR-RBP3* Reduced Na^+ and Na^+/K^+ Ratio

In plants, Na^+ exclusion has been cited as an important mechanism for salinity tolerance (Munns and Tester, 2008). The identification of the *SOS3* gene led to the discovery of Ca^{2+} dependent pathway of ion homeostasis, and high salinity tolerance. Zhu (2001), showed that the *SOS3* was required for the activation of *SOS2*, which regulated the transcripts of *SOS1*. The double knockout mutant exhibited that the *SOS1, SOS2 and SOS3* functions in a linear pathway (Zhu,

2001). *SOS3* encodes for the calcium sensor, which is a calcineurin B like subunit protein. This increased the possibility that this gene was involved in controlling K^+/Na^+ transport through Ca^{2+} dependent pathway. Under salinity stress cytosolic Ca^{2+} concentration was elicited by phosphoinositide signaling (Blat et al., 1990). Calcineurin was a protein phosphatase, which played an important role in ion homeostasis and salt tolerance in yeast (Mendoza, 1994), and plants (Liu and Zhu, 1998; Pardo, 1998). Halfer et al. (2000), reported that *SOS3* was involved in regulating *SOS2*, which encodes for serine/threonine protein kinase containing a kinase domain on N-terminal which is sufficient for interaction with *SOS3* at the C-terminal end. Plants carrying a mutation in *SOS1* were highly susceptible to salt and had higher amounts of salt in tissues compared to wild type plants. The *SOS1* was down-regulated in *SOS2* and *SOS3* mutant plants in response to NaCl suggesting that *SOS1* was controlled by *SOS2-SOS3*. This was further evident from *SOS* gene interactions. The interaction in *SOS2-SOS3* was essential for the phosphorylation and consequent activation of *SOS1*, which encodes for a plasma membrane Na^+/H^+ antiporter (Liu and Zhu, 1997; Shi, 2000; Qiu, 2002). In this study, it was observed that mutants accumulated less sodium (Figure 9A), and had a low Na^+/K^+ (Figure 9C), ratio, and high concentration of potassium (Figure 9B) suggesting Na^+ exclusion from mutant plants. To better understand this, the *SOS1* mRNA levels were probed in wild type and mutant plants under salinity stress using qRT-PCR. A significant increase in *SOS1* mRNA level (Figure 10B) was noted in mutant plants at 48 hours after treatment. *SOS1* contributes to ion homeostasis and high

salinity tolerance by retrieving Na$^+$ from xylem and regulating the activity of *NHX1* for vacuole compartmentalization on tonoplast (Shi et al., 2003; Qiu et al., 2004).

5.5 Mutation in *GR-RBP3* Reduced Chlorophyll Degradation

In phenotype screening, this study found that wild type plants exhibited a significant increase in chlorosis under salt stress, (Figure 4D). This is mostly associated with a loss of chlorophyll. The qRT-PCR analysis showed that *AtCLH1* (*Chlorophyllase1*), which is the first enzyme in the chlorophyll degradation pathway, catalyzing the dissociation of phytol residues and the porphyrin ring of the chlorophyll molecule (Benedetti et al., 1998; Tsuchiya et al., 1999; Benedetti and Arruda, 2002), was upregulated (Figure 10D) in wild type plants as compared to the mutant plants, demonstrating less chlorophyll degradation in mutants.

5.6 *GR-RBP3* is Differentially Expressed in Shoots and Roots

To get better insight into the mechanism of salinity tolerance induced by mutation in *GR-RBP3*, the transcripts of *GR-RBP3* in the shoot and the roots were studied. The qRT-PCR results of differential gene expression revealed that in wild type plants *GR-RBP3* transcripts were significantly upregulated in shoots (~6 fold from normal), as compared to the roots (~2 fold from normal), at 24 hours after salt treatment (Figure 10F and 10G). The plants might have evolved the differential expression of *GR-RBP3* (i.e. less expression in roots and higher expression in shoots, under the salinity stress) because in salt stressed plants have the higher priority to maintain the normal roots growth as compared to the shoots growth for

maintaining the water status in plant (Hsiao and Xu, 2000; Munns and Sharp, 1993). When plant has increased root growth over shoot growth (higher root:shoot), plant can maintain normal water status by reducing the transpiration and increasing the uptake of water, which is resulting from increased root:shoot in stressed conditions (Munns and Sharp, 1993). In plants roots are often seen as the most vulnerable part as they are directly exposed to high salt concentration but surprisingly, they are very robust. Roots often have a lower Na^+ and Cl^- concentration compared to external solutions (Munns, 2002). It is often demonstrated that leaf growth was lower under high salinity compared to root growth.

5.7 GR-RBP3 Protein is localized to Endoplasmic Reticulum

In this study, subcellular localization carried out using confocal microscopy revealed that *GR-RBP3* was localized to endoplasmic reticulum (Figure 11). According to Lin et al. (2011), proteins were modified and subjected to proper folding in endoplasmic reticulum. Earlier Shubert et al. (2000), demonstrated that the misfolded proteins remain in endoplasmic reticulum until they get properly folded or degraded by the endoplasmic reticulum associated degradation (ERAD) system. Lee et al. (2001), noted that misfolded proteins were accumulated to greater extent in ER following the exposure to high salinity stress, resulted in an increased expression of unfolded protein response (UPR), which increased the expression of ER chaperon and ERAD system to facilitate the degradation of misfolded proteins (Kamauchi et al., 2005; Martinez et al., 2003; Wang et al., 2010). Furthermore, they noted that after the application of ER stress inducers,

tunicamycin (Tm), and dithiothreitol (DTT), the expression of a number of stress related genes was upregulated, suggesting the involvement of ER stress in plants response to salinity stress.

5.8. Future Directions

In this research, it was hypothesized that the negative effect exerted by *GR-RBP3* in salinity stress might result from, it directly binding to the target genes in Arabidopsis and, negatively regulating the genes, which are responsible for normal seedling growth under salinity stress. In further studies, discovery of RNA targets and the investigation of protein patterns in Arabidopsis, using 2-D PAGE and probed peptide mapping (Kwak et al., 2005), could be a way to isolate targeted genes, and their translated products influenced by *GR-RBP3*. This may give a better insight to its functional role in the survival of plants under salinity stress. Also, in future research, an examination of the protein structure of *GR-RBP3* in endoplasmic reticulum following exposure to salinity stress might lead to a better understanding of the localization of *GR-RBP3* in endoplasmic reticulum.

In conclusion, this study provided novel information concerning the role of *GR-RBP3* in salinity tolerance, suggesting that such approaches can be used to study the molecular mechanisms and improve salinity tolerance in plants.

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