Elucidating the Possible Mechanism Underlying Salinity Tolerance in Indigenous Rice Korgut (*Oryza sativa* L.)

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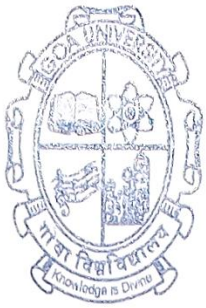

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I hereby declare that the data presented in this Dissertation/ Internship report entitled, **"Elucidating the Possible Mechanism Underlying Salinity Tolerance in Indigenous Rice Korgut (*Oryza sativa* L.)"** is based on the result of investigations carried out by me in the Botany at the School of Biological Sciences and Biotechnology, Goa University under the supervision of **Dr. Siddhi K. Jalmi** and the same has not been submitted elsewhere for the award of any degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations/ experimental or other findings given in the dissertation.

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This is to certify that the dissertation report **"Elucidating the Possible Mechanism Underlying Salinity Tolerance in Indigenous Rice Korgut (*Oryza sativa* L.)"** is a bonafide work carried out by **Ms. Kelkar Mrunal Madhav** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of M.Sc. in the Discipline Botany at the School of Biological Sciences and Biotechnology, Goa University.

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ABSTRACT

Salinity is considered a global threat to agriculture and causes a significant reduction in crop yield. In particular, salinity stress promotes reactive oxygen species (ROS) accumulation and ionic imbalance in cells, leading to oxidative stress and even cell death. Plants perceive the environmental stress with the help of signalling pathway and respond to salinity stress through a series of molecular mechanisms including activation of defence response genes, production of antioxidant enzymes, regulation of hormone pathway, osmolyte production, and activation of signalling, which can be further seen in physiological differences. Plant defence systems modulate osmotic and ionic balance, the overproduction of ROS and maintain growth and development of the plant through the activation of stress responsive-genes. This study discusses the possible regulatory mechanism of salt stress response in Goan indigenous salt tolerant rice variety Korgut, comparing it to salinity sensitive rice variety IR64.

CHAPTER I

INTRODUCTION

1. INTRODUCTION

1.1 Salt tolerant Rice Varieties of Goa

Rice (*Oryza sativa* L.) is the most widely consumed grain for more than half of the world's population. It is forecast that the global production volume in 2022-2023 will be about 503 million metric tons. India stands second in global Rice production, producing over 195 million metric tons and is the largest exporter of rice with 21.5 million metric tons export providing a significant contribution to the Indian economy. India is ranked second with 108.5 million tons of rice consumed in the abovementioned period. Rice is the most important food crop with respect to caloric intake and human nutrition. It tallies up to more than one-fifth of the global human calorie consumption.

In India, rice is grown under widely diverse climatic conditions. Rice crop requires a hot and humid climate. It grows well in regions with high humidity, prolonged sunshine and ample water. The average temperature requirement throughout the crop's life cycle ranges from 21 to 37ºC. It can tolerate a maximum temperature of about 42ºC. Many rice species grow well at pH 6 (Yu, 1991).

Rice is the prime staple food crop of Goa, grown over an area of more than 30 per cent (52,191 ha) of the net cultivated area in the state. Rice cultivation in Goa is carried out in both ***Kharif*** (34,261 ha) and ***Rabi*** (17,930 ha) seasons. During Kharif (rain-fed) season, rice is cultivated in three distinct ecologies: Morod lands or the lateritic uplands, Midlands or Kher lands and Khazan lands, with soil pH varying between 4.5 to 6.5. (Korikanthmath et al., 2010). Rabi or *vaigan* is an irrigated crop.

The present study deals with the Rice varieties, namely Korgut, IR64 and Pokkali. **Korgut** is a local salt-tolerant variety indigenous to the state of Goa and is grown in the *Khazan* lands, having an electrical conductance of 12 dSm^{-1} (Srivastava and Sharma., 2021). While the salinity of the fields varies from season to season, Korgut gives an average yield of 1.5 to 2.0 t/ha (Korikanthmath et al., 2010). Although Korgut has been cultivated, conserved by the locals, and entitled as 'Salt Tolerant' (Manohara et al., 2015), the tolerance mechanism has not been evaluated and remains unidentified. **IR64** is an IRRI-developed semi-dwarf, high-yielding salt-sensitive *indica* variety. (Mackill et al., 2018). Comparative analysis of these genotypes offered a better understanding of salinity tolerance in Korgut.

1.2 Environmental stresses affecting rice productivity

Stress is usually defined as an external factor that negatively influences the plant (Taiz and Zeiger, 2002). Environmental stress is a major area of scientific concern as it hampers plant metabolism, development and crop productivity. Plants in their natural habitat are exposed to adverse biotic and abiotic stresses. Biotic stresses are caused by biological units like diseases, insects, etc. (Verma et al., 2013), causing a global 30% yield loss per hotspot (Savary et al., 2019). The primary weeds in rice fields are grasses such as *Panicum repens*, *Echinochloa colona*. In Goa, rice is infected by several pests. The most common diseases are blast, blight, gall midge, cutworm, brown plant-hopper, leaf roller, or leaf folder. (Bhonsle and Krishnan, 2012).

Abiotic stresses are caused by either physical or chemical factors such as salinity, sunlight, temperature, cold, floods and drought. About 150 million hectares of rice worldwide are currently threatened by high temperatures, and the world's rice yield loss will be 40% in the next century due to the undesirable effects of high temperatures (Jagadish et al., 2015). It is

reported that more than 15 million hectares of rice are threatened by cold weather globally, and rice cultivation is impossible in nearly 7 million hectares of land in the south and Southeast Asia due to cold stress (Pradhan et al., 2019). Despite being a short-day plant and usually grown in the wet season when solar radiation decreases to 40%–60% of its peak, long- term low light or dark caused by cloudy and rainy conditions often reduces rice production by up to 50%. Generally, low light adversely affects photosynthesis and induces premature leaf senescence, resulting in reduced yield and quality (Gad et al., 2021). By 2025, over 15-20 million hectares of irrigated rice have been predicted to suffer from some degree of drought (Bouman et al., 2007). Salt stress affects cereal yield worldwide, takes up to 1.5 million hectares of farmland out of production annually and reduces productivity by approx. 46 million additional hectares (FAO, 2021; 2022).

1.3 Salinity stress affecting rice productivity and yield

Salt stress is a detrimental factor affecting plant growth and productivity, as it obstructs more than 20% of the total irrigated land. Over 800 million hectares of land across the globe is salt-affected (Munns, 2005), which makes up 6% per cent of the world's land area. (Parihar et al., 2014). 2.1% of India's geographical land is salt affected, of which 2.96 million ha are saline (Arora, 2017). Thus, salinity stress seems to be the chief constraint to plant and crop productivity. Soil is classified as saline when it has a high concentration of soluble salts and Electrical conductivity (EC_e) more than or equal to

$4dSm^{-1}$, equivalent to 40 mM NaCl (Munns, 2005). Among cereals, rice is the staple crop most sensitive to salinity stress. Rice is one of the most salt-sensitive cereal crops and highly sensitive in the early seedling. In rice, salt stress induces both toxicity and osmotic stress,

resulting in growth inhibition, developmental changes, metabolic adaptations, and ion sequestration or exclusion (Munns and Tester, 2008; Zelm et al., 2020; Wang et al., 2021).

The detrimental impact of NaCl on plants is due to both the reduction of water availability as sodium accumulates in the soil and the toxic effects of sodium and chlorine ions on plants (Zelm et al., 2020).

In the State of Goa, "*Khazans*", the coastal saline lands are affected by the coastal salinity through the inundation of sea water. Nearly 18,000 ha along the seacoast is affected by the coastal salinity through the ingression of sea water (Manohara et al., 2015). It is interesting how the traditional landraces such as Assgo and Korgut thrive in this ecosystem despite the saline nature of the soil.

1.4 How does salinity affect plant growth?

Salinity affects plant growth in two segments of toxicity (**Fig.1.1**) **Osmotic** and **Ionic** (Gupta et al., 2020). Low osmotic potential is developed in the rooting zone due to dissolved solutes, which lowers the soil water potential. As a result, the "downhill" gradient of water potential between the soil and the leaves cannot be maintained, and the normal water balance of plants is affected. This leads to a reduction in the overall growth rate. This is basically the osmotic or water-deficit effect of salinity. Entry of excessive amounts of salt in the transpiration stream causes injury to cells in the transpiring leaves, which causes a further decline in growth, leaf death, and reduced yields. This is referred to as the salt specific or ion-excess effect of salinity (Parihar et al., 2015).

These effects lead to a two-phase growth response to salinity. The first is the result of **the salt outside the plants**. Leaf growth is affected to a greater extent than root growth. The

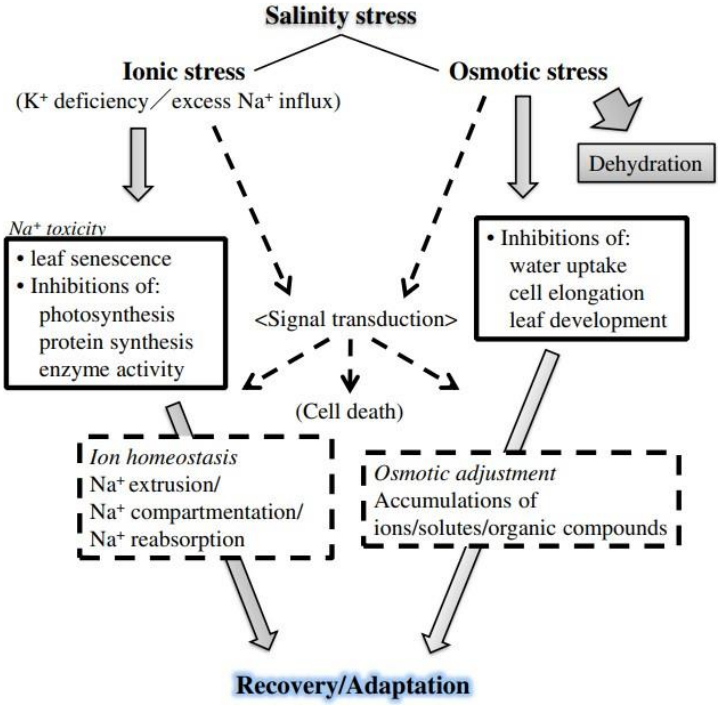


Figure 1.1 A schematic representation of the stresses that plants suffer under high salinity and the corresponding responses that plants use in order to survive these detrimental effects. (Horie et al.,2012).

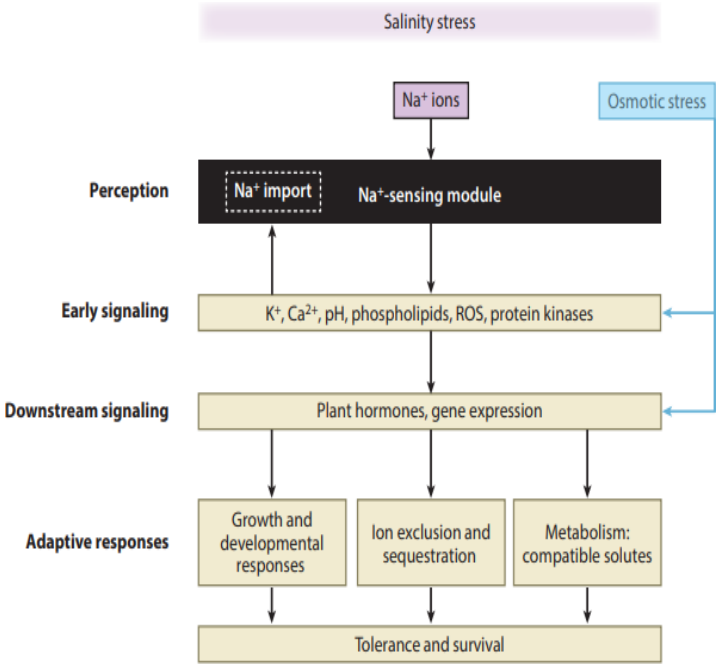


Figure 2.1 Illustrative representation of Perception, early signaling, Downstream signaling, and adaptive responses to salinity exhibited by plants.

second phase results from the **toxic effect of salt inside the plant**. The salt taken up by the plant gets accumulates in older leaves. This accumulation of sodium and chloride ions in the plant tissues disrupts cellular processes and causes damage to the plant. Salt accumulation in the cytoplasm and cell walls inhibits enzyme activity and dehydrates the cell, leading to tissue damage and death.

Moreover, the effects of salt on plant growth and productivity are further exacerbated by the accompanying water stress. As salt accumulates in the soil, it reduces water availability to the plant, leading to drought stress. This, in turn, exacerbates the toxic effects of salt on plant growth and productivity.

1.5 Mechanisms of Salt Tolerance in Plants

Plants have evolved different mechanisms to cope with salt stress that involve exclusion, compartmentalization, osmotic adjustment, antioxidant defense, and hormonal regulation. These mechanisms allow plants to adapt to various environmental conditions and survive in saline soils. Complex signal transduction pathways and response mechanisms are induced by specific functional and regulatory proteins. **Functional proteins** responding to stress include **membrane proteins** like transporters and water channel proteins, **osmolyte biosynthesis enzymes** required to produce proline, betaine, soluble sugars, etc., **detoxification enzymes** such as catalase, superoxide dismutase, ascorbate peroxidase, glutathione S-transferase, etc., and other proteins that help protect macromolecules including LEA protein, osmotin, antifreeze proteins, mRNA binding protein, etc. **Regulatory proteins** respond to stress and include **transcription factors** (bZIP, MYC, MYB, DREB, etc.), **protein kinases** like receptor protein kinase, MAP kinase, CDP kinase, transcription-regulation protein kinase, etc., and **proteinases** like phospholipase C, phosphoesterases, etc.

(Agarwal et al., 2006). Among the regulatory proteins, transcription factors (TFs) play core roles in abiotic stress responses. Specifically, they activate or repress the expression of stress-response genes by recognising and binding to cis-elements in the promoters of their targets. They are the main targets of genetic engineering for enhancing stress tolerance in crop plants (Chai et al., 2020).

CHAPTER II

REVIEW OF LITERATURE

2. Review of Literature

2.1 Perception of salt stress by plants and salinity tolerance mechanism exhibited by functional proteins

Na^+ induces specific downstream responses, but the sodium-sensing mechanism of plants remains to be identified. Sensing could occur either inter- or extracellularly, before or after Na+ import. After the initial perception, early signalling responses are induced, including K^+ transport, Ca^{2+} signalling, H^+ transport, phospholipid modifications, reactive oxygen species (ROS) induction, and protein kinase activity. In turn, these early Na^+-induced signals reduce sodium import. Downstream of the early signalling phase, phytohormone levels change through both biosynthesis and transport, and gene expression levels are altered in a manner dependent on and independent of phytohormones. Finally, the salt-induced signalling cascade results in adaptive responses, such as modulation of growth and development, ion transport and production of compatible solutes to compensate for the osmotic pressure of Na^+. Every step in the signalling chain plays a role in mounting an adequate response to salinity and survival on saline soils. Part of the early and downstream signalling response overlaps with osmotic stress-induced pathways (**Fig. 2.1**).

2.2 Regulation of ionic homeostasis inside plant cell by SOS signal transduction pathway

During salinity stress, the Calcium transporter proteins in the plasma membrane are activated. Thus, Ca^+ ions are transported inside the cytoplasm of the plant cell. Ca^+ concentration rises in the cytoplasm, consequently. These ions activate SOS3 (Calcium regulated Protein Phosphatase), which in turn activates SOS2 (Serine Threonine Kinase), which further activates SOS1. Ca^+ ions act as secondary messengers during the signal

transduction pathway. SOS3 activates K^+ along with SOS2 so K^+ ions can enter the cytoplasm. Activated SOS2 activates SOS1 and inhibits AtHKT1 (The transporter that allows the entry of Na+ inside the cytoplasm). Therefore, the Entry of Na^+ is blocked. The activated SOS1 is responsible for excluding the Na^+ from the cytoplasm to the outside of the cell. Overall, the concentration of Na^+ starts decreasing in the cytoplasm (**Fig. 2.2**).

2.3 Role of HKT1 in salinity stress

Members of HKT form the second step in the movement or transport of Na ions inside the plant, and unlike SOS1, they are involved in the efflux of Na ions from the inner half of the root cells to the xylem and its influx back into these cells from xylem preventing its delivery into the shoots through the transpiration stream. (Munns and Tester 2008). HKT1 family preferentially transport Na^+.

SOS1 forms the final component of PM-H^+ ATPase-mediated efflux of the Na^+ module and is considered a critical biochemical molecule determining the salt tolerance ability of plants (Quintero et al., 2011). SOS1 is thought to primarily mediate the efflux of Na^+ at the root epidermis and thus prevention of transport of the ion from roots to shoots (Shi et al., 2002; Olias et al., 2009).

2.4 PIP (Plasma Membrane Intrinsic Protein) in salt stress

Aquaporins (AQPs) belong to the major intrinsic protein (MIP) family. AQPs play a significant role in water transport channels and are able to direct water transport across membranes between the protoplasm. AQPs in higher plants are divided into five subfamilies-plasma membrane intrinsic proteins (PIPs), tonoplast membrane intrinsic proteins (TIPs), nodulin26-like major intrinsic proteins (NIPs), small and essential intrinsic proteins (SIPs)

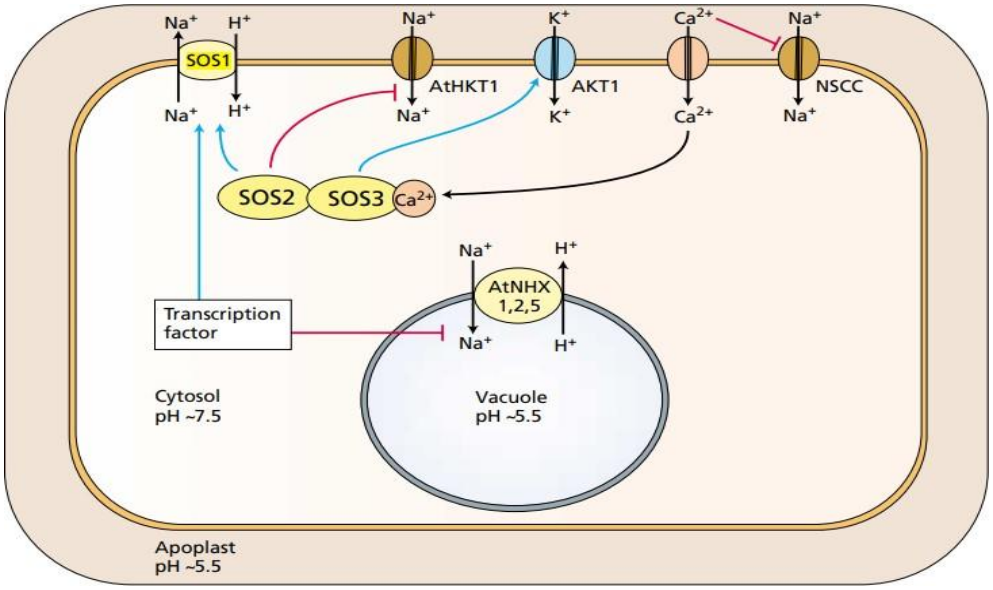


Figure 2.2. A schematic representation of SOS transduction pathway regulating ionic homeostasis inside plant cell (Taiz and Zeiger, 2002).

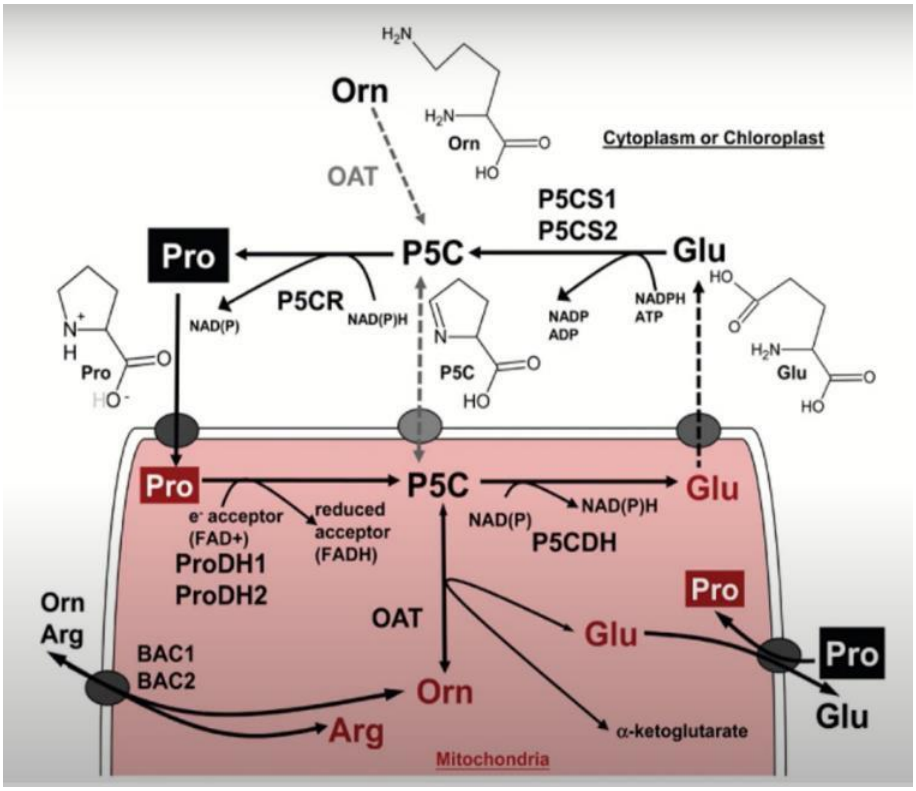


Figure 2.3. A schematic representation of proline biosynthesis pathway (Verslues and Sharma., 2010).

and X intrinsic proteins (XIPs). Based on the N-terminal length of the proteins, PIPs are divided into PIP1s and PIP2s, which have different roles in plants when they respond to various stresses. Plasma membrane intrinsic proteins (PIPs) are plant channel proteins involved in water deficit and salinity tolerance. PIPs play a major role in plant cell water balance and responses to salt stress.

2.5 P5CS1 (Delta 1-Pyrroline-5-Carboxylate Synthase)

Accumulation of proline is a common physiological response in plants exposed to various abiotic stresses (Kaura et al., 2022). P5CS1 appears involved in salt stress responses related to proline accumulation, including protection from reactive oxygen species.

The proline biosynthesis pathway involves compartmentalized metabolism of Proline between three organelles- Cytoplasm, Chloroplast and Mitochondria. Glutamate acts as a precursor for Proline biosynthesis and gets converted into an intermediate compound Pyrroline-5-Carboxylate in the presence of an enzyme Pyrroline 5 Carboxylase. Pyrroline-5-Carboxylate is reduced to Proline in the presence of the enzyme Pyrroline 5 Carboxy Reductase. Once Proline is synthesized in the cytosol, it enters mitochondria, which gets oxidized back to Pyrroline 5 Carboxylate in the presence of enzymes Proline Dehydrogenase 1 and 2. The Pyrroline 5 Carboxylate has converted back to glutamate in the presence of Pyrroline 5 Carboxy Dehydrogenase and is then exported back to cytosol. This cyclic process continues depending on the requirement of the plant (**Fig.2.3**).

2.6 Transcription factors responding to salt stress

2.6.1 OsDREB2A encodes DRE binding proteins

The DREBs (Dehydration Responsive Element Binding Proteins) are cis-acting elements (Liu et al., 1998) and are members of the ERF (Ethylene Responsive Element Binding Factors) family of transcription factors and follow the ABA-independent signal transduction pathway. The DREBs are significant transcription factors that induce abiotic stress-related genes and impart stress tolerance to plants. The two subclasses of DREB, DREB1 and DREB2, are separately involved in cold and dehydration, and high salt stress, respectively (Agarwal et al., 2006) (**Fig. 2.4**).

2.6.2 MYC

Late-embryogenesis-abundant (LEA) proteins induced by osmotic stresses in vegetative tissues impart dehydration tolerance to vegetative tissues of plants. These LEA-type proteins are encoded by different genes, including *RD* (responsive dehydration), *ERD* (early responsive to dehydration), and *RAB* (responsive to ABA) in different plant species. Accumulation levels of these proteins correlate with stress tolerance in various plant species suggesting protective roles under osmotic stress. Promoters of LEA-like genes contain dehydration-responsive elements, ABA-responsive elements, and MYB/MYC recognition elements. ABA-dependent pathways regulate LEA-type genes through MYC/MYB and bZIP-type transcription factors. IP3 and Ca^{2+} mediate ABA-dependent signalling.

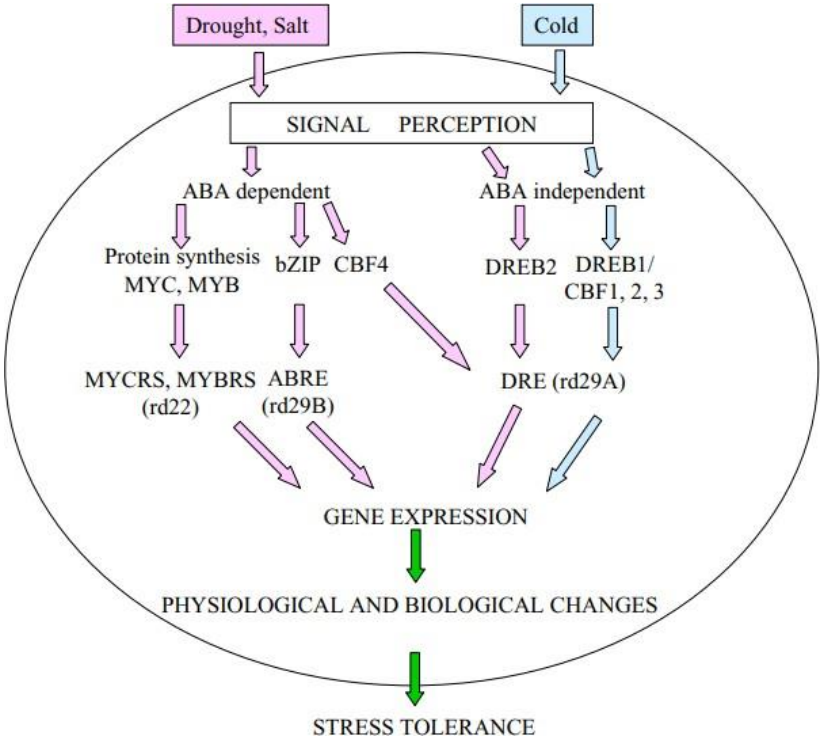


Figure 2.4 Schematic representation of DREB signal transduction pathway (Agarwal et al.,2006).

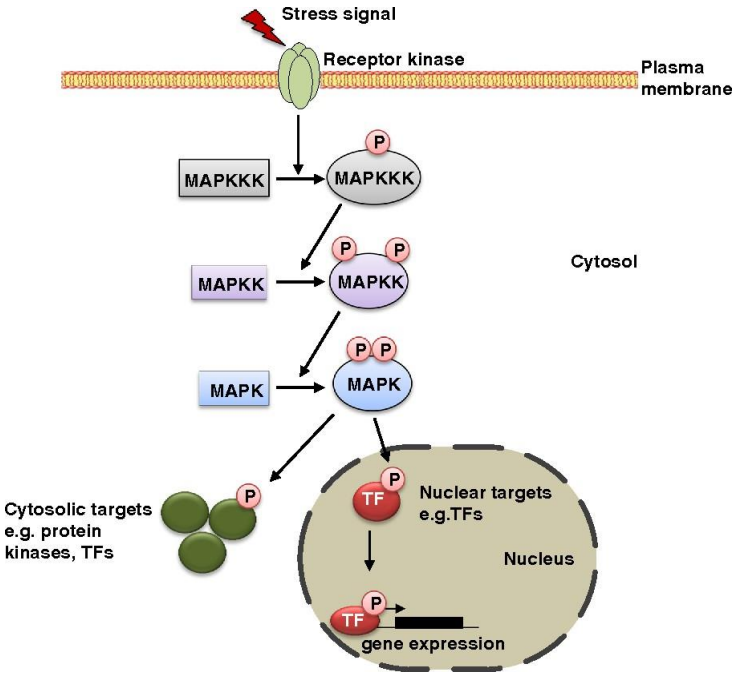


Figure 2.5 Mitogen Activated Protein Kinase (MAPK) signalling (Danquah et al., 2014)

2.7 Protein kinases involved in the salt stress-responsive mechanism

Mitogen-activated protein kinase (MAPK/MPK) cascades play key roles in the signal perception and transduction of salt stress signalling in plants by transducing extracellular stimuli through sequential phosphorylation phosphorylating substrates and modifying their activities. The rice genome contains 17 MAPK, 8 MAPKK, and 75 MAPKKK coding genes. Recently, several MPK cascades mediating the rice salt response have been identified. OsMKK1, whose kinase activity was induced by salinity, positively regulated rice salt tolerance via activation of the downstream substrate OsMAPK4 (also known as OsMPK6) (Wang et al., 2014). OsMAPKKK63 is associated with OsMKK1 to enhance rice resistance to salt stress. Overexpression of constitutively activated OsMPKK6 enhanced rice salt tolerance, and OsMPK3 and OsMPK6 were activated in salt stress response. The kinase activity of OsMPK6 was upregulated by OsMKK4 in response to various stresses (Shen et al., 2010). OsMAPK3 positively affected salt tolerance by attenuating the reactive oxygen species accumulation (Zhang et al., 2018) (**Fig.2.5**).

The present study aims to investigate the genetic basis of salt tolerance in rice by analysing different varieties that showed varying degrees of tolerance to salinity. The study anticipates shedding light on the mechanisms underlying variation in salt tolerance by identifying the specific genes important for salt tolerance.

Munns et al. (2005) discussed various aspects of salinity stress in rice, the genes responsible, and their mechanisms contributing to salinity tolerance. Proteins that control the uptake of Na^+ from the soil and the following transport of Na^+ within the plant are embedded in the lipid bilayer of the membrane, which otherwise is fairly impermeable to ions. Na^+ is thought to be entering the cells through nonselective cation channels, and at high salinity, possibly

through K^+ channels or transporters that are incompletely selective for K^+ (Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Tyerman and Skerrett, 1999; Tester and Davenport, 2003). Active ion transport takes place via antiporters and symporters that transport ions. These are usually highly selective for ions other than Na^+. The rate of transporters is much less than that in channels. Passive ion transport occurs through channels, which are membrane proteins with ion-selective pores. These are usually highly selective for a specific ion. Some non-channel transporters like HKT1 allow passive transport under specific conditions. Cytosolic Na^+ concentrations of the root are probably in the order of 10-30 mM. Leaf Na^+ cytosolic concentrations are considered to be much less than 100 mM. Plants transpire around 50 times more water than they retain in their leaves. About 98% of the salt in the soil solution is excluded and only about 2% is transported in the xylem to the shoots. 94% Na^+ ion exclusion was observed in rice cultivar IR36 when grown in 50mM NaCl.

It is established that short-term cellular changes lasting from minutes to hours are due to rapid induction of the signal perception machinery and associated protein phosphorylation events. In contrast, late response requires the synthesis of new mRNA and protein that result in metabolic readjustments, setting the stage for long-term acclimatisation to stress. Moreover, these late adaptive responses have a genetic basis and are crucial in differentiating between a sensitive and a tolerant cultivar (Munns, 2005). Gene expression analyses in shoot and root tissues show that P5CS, the gene for proline synthesis, is commonly detected and was upregulated 8.5-fold by 100 mM NaCl (Atienza et al., 2004; Ueda et al., 2004). Genetic variation in salt tolerance of the whole plant correlates with the degree to which the plant can limit the rate of Na^+ transport to leaves.

Liu et al. (1998) isolated two cDNA clones that encode DRE binding proteins, DREB1A and DREB2A. The binding of the DREB1A and DREB2A proteins specifically to the DRE sequence and activation of transcription of the β-glucuronidase reporter gene driven by the DRE in Arabidopsis leaf protoplasts was detected. Low temperature-induced expression of the DREB1A gene, whereas expression of the DREB2A gene was induced by dehydration. The expression patterns were analysed using RNA gel blot hybridisation. DREB2A gene expression was induced within 10 min after dehydration and was strongly expressed after 2 hours. Significant DREB2A mRNA accumulation within 10 minutes after 250mM NaCl treatment was seen.

A review by Agarwal et al. (2006) interpreted the role of DREB transcription factors in abiotic and biotic tolerance in plants and summarised that DREBs are essential transcription factors regulating stress-responsive gene expression through DRE/CRT cis-elements and its DNA binding domain, provide tolerance to multiple stresses and display overlapping responses to various stress conditions. The specific biological functions of the DREB proteins are due to the highly conserved domains.

Studies conducted by Ibragimova et al. (2015) showed that the expression level of P5CS1 increases in response to environmental factors such as salinity, drought, and cold. The proteins encoded by P5CS1 are detected in the cytoplasm and chloroplasts of leaf mesophyll tissue. In a study (Kavi Kishor et al., 1995), introducing the P5CS1 cowpea gene into the tobacco genome (*Nicotiana tabacum* L.) showed an increase in the constitutive level of proline by 10-18 times compared to the control. Another study (Chen et al., 2013) showed a 2.5fold increase in proline content in NaCl-treated plants was observed in transformants of

A. thaliana carrying P5CS1 and P5CS2 genes of beans, while P5CS2 showed more proline accumulation than P5CS1 gene.

The authors (Ibragimova et al., 2014) dealt with evaluating the salt tolerance of transgenic tobacco plants bearing the P5CS1 gene of *Arabidopsis thaliana*. The results of this study confirmed the known data on the positive correlation between an increased cellular content of free proline and resistance to high NaCl concentrations, as well as on the participation of P5CS1 in the molecular mechanisms of plant stress resistance.

HKT1;5 is a vital transporter protein that recirculates Na^+ to maintain the homeostasis of $Na^{+/}K^+$ in higher plants. In rice, this transporter is identified as the mediator for Na+ retrieval from the xylem to the xylem parenchyma. The study conducted by Shohan et al. (2019) gene expression of HKT1;5 and its association with salt tolerance across rice genotypes. Pokkali and IR29 were given salt stress of 100 mM and 200 mM. Microarray data, mRNAseq data and real-time expression analysis showed that the HKT1;5 genes are expressed mainly in root than in shoot. Despite of similar increase of 24 fold in relative expression at 24 hours, IR29 could not survive in 100mM stress, while tolerant Pokkali could. This indicated that Na^+ could not recirculate from shoot to root with ease in IR29, unlike Pokkali leading to a higher Na^+/K^+ ratio in high salt stress.

Srivastava and Sharma (2021) evaluated two contrasting rice varieties: Jaya and Korgut were treated with NaCl of 40, 80, 120 and 160 mM to analyse their morpho-physiological and biochemical tolerance mechanisms to salinity. It was found that plants of the Korgut variety exhibited tolerance to NaCl stress. The salt-tolerant nature of Korgut depends on its ability to maintain water and ionic homeostasis under saline conditions, prevent oxidative stress, and neutralise the toxic effects of high concentrations of inorganic ions. The morphological

and physiological adaptations in Korgut were facilitated by a reduction in stomatal size and an increase in the number of trichomes, shoot and root length and the rate of photosynthesis, compared to Jaya, which was found to be more severely affected by NaCl stress. A more significant accumulation of Na^+ and Cl^- ions was observed in Jaya, suggesting that salt toxicity as Na^+ is the primary toxic ion as it competes with the uptake of $K^{+,}$ resulting in K^+ deficiency in the Jaya variety but not in Korgut. Proline estimation revealed 26fold more accumulation in treated leaf samples of Korgut than under control conditions.

According to the studies undertaken by Wang et al. (2019), stress responses triggered by the Jasmonic acid signalling pathway result in the activation of MYC transcription factors, which target defence-related genes. (Koene et al., 2022). The JAZ-MYC module plays a central role in the JA signalling pathway by integrating regulatory transcription factors and related genes in the JA response to the abiotic stress process. It was found that endogenous JA enhanced salt tolerance in tomatoes, mainly through homeostasis maintenance among reactive oxygen species (ROS) (Abouelsaad et al.,2018). However, some studies have reported that exogenous JA treatments reduced salt-induced damage to various plants via increased photosynthetic rates, proline contents, ABA levels (Bandurska et al., 2003), and antioxidant enzyme activity (Walia et al., 2007), or via reductions in Na^+ accumulation rates in shoots (Khan et al., 2012), (Shahzad et al., 2015) observed that exogenous JA could improve Na^+ exclusion in the root by decreasing Na^+ uptake, facilitating surface salt stress tolerance in two maise genotypes. These results show that JA could facilitate salt stress tolerance by enhancing the concentrations of antioxidative compounds and antioxidant enzyme activity.

Calzone et al. (2021) evaluated the transcriptional regulation of genes *NHX1*, *SOS1* and *HKT1* in two Pomegranate cultivars, Wonderful and Parfianka; both cultivars are considered tolerant to moderate levels of salt (100 mM NaCl), and in response to salt stress. At 10 and 24 hours of NaCl treatment, mature leaves (ML) of Wonderful showed a significant decrease in SOS1 levels. In contrast, salt treatment significantly increased the relative expression levels of SOS1 at all time points in YL (Young leaves) of Wonderful. In Parfianka ML, SOS1 gene expression was upregulated at 3 h, downregulated at 24 h and not significantly altered at 10 h after the beginning of the irrigation. In Parfianka YL, a highly variable pattern was also observed with SOS1 downregulation at 3 h, upregulation at 10 h, and no differences at 24 h. In Wonderful roots, HKT1 gene expression was upregulated at 0.5 and 3 h, downregulated at 10 h and again upregulated at 24 h after the beginning of the irrigation. In Parfianka, a highly variable pattern was observed with HKT1 upregulation at 3 h and downregulation from 3 h onwards. In Wonderful roots, a significant decrease of SOS1 levels was observed at 0.5 and 10 h, whereas only a brief upregulation was found in Parfianka roots at 0.5 h. This data confirmed that Wonderful plants preferred redirection of Na^+ to roots and ML, hence maintaining low Na^+ concentration in YL to prevent Na^+ accumulation and K^+ loss. On the other hand, Parfianka plants seemed to be able to avoid Na^+ accumulation, significantly improving high K^+ concentration in ML and maintaining unchanged Na^+ concentration in YL.

Investigative studies by Gupta et al. (2020) dealt with the biochemical and molecular characterisations of salt tolerance components in rice varieties tolerant and sensitive to NaCl and the relevance of Na^+ exclusion in salt tolerance in the varieties Nona Bokra, Pokkali, IR64 and IR29.

Nona Bokra and Pokkali were classified as salt tolerant, whereas IR64 and IR29 were classified as salt sensitive by the authors. The expression of SOS1 was in harmony with the literature that proposes the upregulation of gene expression due to salinity. A decline in its expression in roots of IR64 and IR29 indicated that salt inducibility is necessary for a variety of rice to be salt-tolerant; Poor expression of SOS1 in shoots indicated a higher accumulation of Na^+. The insignificant expression of SOS1 in shoots of the tolerant varieties suggests its function in regulating ion loading into the xylem, which leads to controlled delivery to shoots besides governing the net uptake of ions, which corresponds to the reduced accumulation of Na^+. The HKT gene expression was rather contrasting. The expression of most of the HKT isoforms was downregulated in the salt-sensitive rice varieties IR64 and IR29, suggesting the important role of HKTs in salt tolerance. The authors concluded that as far as rice is concerned, the HKTs response to NaCl could be specific to cultivars and organs as the importance of HKTs in salt tolerance in plants is undisputable. OsCIPK 24 and CBL10 showed highly significant NaCl-induced expression in the roots of Nona Bokra and Pokkali, clearly suggesting efficient functioning of the Na^+ efflux. In the case of IR64 and IR29, insignificant expression or downregulation of OsCIPK24, OsCIPK15 and CBL10 was observed in roots and shoots. Salt induction significantly increased the expression of OsCIPK24 and OsCIPK15, and CBL10 in shoots of tolerant varieties signifying efficient functioning of the $Na^{+/}H^+$ antiporter in shoot tissue. The authors determined that the findings indicated competent functioning of the SOS1/PM-H^+ ATPase Na^+ exclusion module as the primary factor determining salt tolerance in rice.

With the objective of unveiling the major genetic contributors to the complex physiological processes involved in salt stress tolerance, Kumari et al. (2009) tried to establish the

contrasting nature of the two genotypes- IR64 and Pokkali. Salinity stress was induced with 200 mM NaCl treatment in 4 days old seedlings. Visual comparison of salinity-treated seedlings of Pokkali showed better tolerance ability. IR64 seedlings showed almost a 25% reduction in shoot length, whereas shoot length in Pokkali was reduced by 12% only. Estimation of proline content also contributed to establishing the contrasting nature of IR64 and Pokkali, showing 41% and 61% gain in proline content in the two varieties, respectively. The transcripts were classified into 4 groups based on a detailed analysis of the transcriptome data obtained through macroarrays. Genes showing high inducible expression in the early phase of stress constituted Group 1. including kinases, such as *mitogen-activated protein kinase (EF575932), serine-threonine kinase (EF576082), CBL interacting protein kinase (EF576522), and transcription factor Myb (EF576247)*.

Early inducible gene expression was exhibited by almost 66% of genes in IR64, while 50% of these transcripts were induced in Pokkali. 36% of the genes were commonly upregulated in both IR64 and Pokkali. Among these, 7% were commonly down-regulated in both cultivars. A higher level of upregulation was seen in IR64 compared to that in Pokkali. Genes induced during the late phase of salinity stress were placed in Group 2. Only 23% of genes were common between the two genotypes, with many genes involved in general plant metabolism. Upregulation of around 45-46% of the total genes was observed during the late phase of stress in the two cultivars Pokkali displayed an upregulation of most stress-protective genes, such as *thioredoxin* and *cyclophilin*. Group 3 included genes downregulated in the early phase of stress with about 19% genes in IR64 and 32% in Pokkali. The genes found to be downregulated in the late phase of salt stress were placed under Group 4. The majority of these were those involved in photosynthesis and protein synthesis. IR64

contributed to 30% of the genes, while Pokkali demonstrated 40% of genes in this category, of which 12% were common. Another prominent feature of the study was the ability of the transcripts in Pokkali to maintain a steady-state expression level for most of the transcripts even after 3 days of stress, while the transcripts altered only transiently in IR64. This agrees with the findings of Kawasaki et al. (2001), where stressed IR29 resulted in death due to an early decrease in transcript levels, while Pokkali recovered due to a more efficient response of late-induced genes. This study defined a set of known abiotic stress-inducible genes expressed at high levels in Pokkali even in the absence of stress, further proposing that these genes may prove as potential candidates to improve salinity tolerance in crop plants using a transgenic approach. Based on this background, the following objectives were formulated to understand the possible mechanism of salt stress tolerance in the indigenous least-explored salt-tolerant rice variety Korgut.

OBJECTIVE

OBJECTIVES

- To understand the physiology of Korgut in saline conditions.
- To study the biochemical parameters underlying the salt stress tolerance in Korgut
- To decipher the molecular pathways undergoing in Korgut in saline conditions.

CHAPTER III

MATERIALS

&

METHODS

3. Materials and Methods

3.1 Rice Varieties, growth conditions and salinity treatment

The seeds of the rice (*Oryza sativa* L. ssp. *indica*) varieties- Korgut, referred to as salt-tolerant at the seedling stage, and IR64, referred to as salt-sensitive, were obtained from local farmers, and National Institute of Plant Genome Research (NIPGR), New Delhi, respectively. The seeds were washed and soaked overnight in water. The soaked seeds were sown in plastic pots filled with sterile soil in a growth room under controlled conditions at 28ºC and 70% relative humidity. 10 days old Rice seedlings were treated with 300mM NaCl (8.766g in 500mL distilled water). The seedlings not receiving NaCl served as Control. The shoot portion was harvested after 1 hour, 3, and 6 hours of treatment and were frozen in liquid Nitrogen until further use (**Fig. 3.1**).

3.2 Physiological parameters

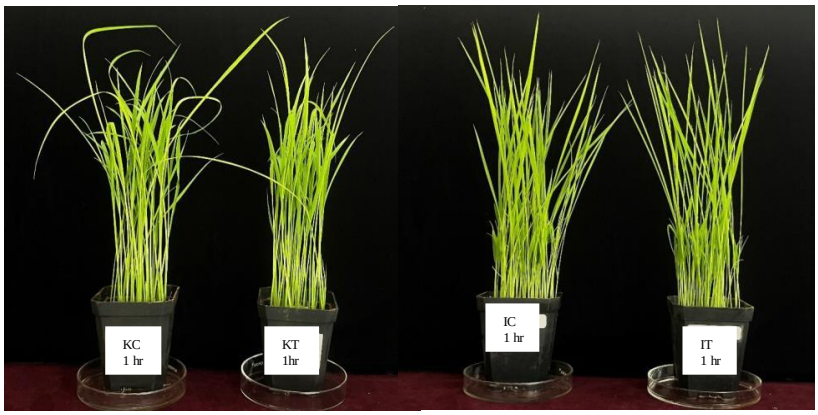
3.2.1 Root and Shoot Length Measurements

In this experiment, the plants were treated with 100-500mM NaCl concentrations to figure out relative effects of salinity on the characters under study. Root and shoot lengths of the plants were measured after 7 days of NaCl treatment to seek the growth differences in response to NaCl stress. A salt-tolerant rice variety, namely Pokkali, served as a positive control. Physiological indices like Salt tolerance index (STI), Shoot length stress index (SLSI) and Root length stress index (RLSI) were calculated using the following formulas-

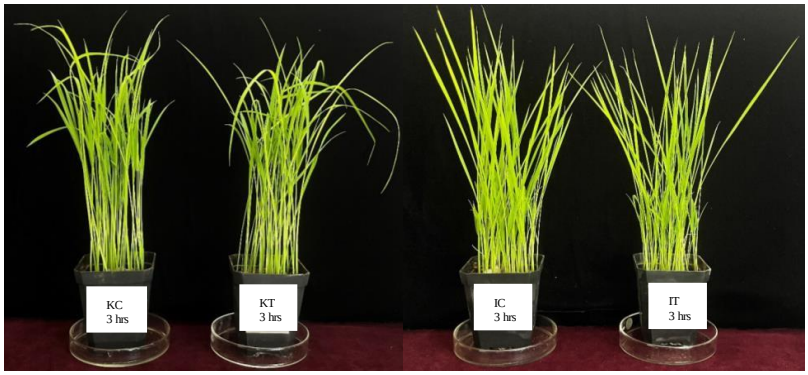
$$\text{STI (shoot)} = \left(\frac{\text{Shoot length of treated plant}}{\text{Shoot length of Control plant}} \times 100\right) - 100$$

$$\text{STI (root)} = \left(\frac{\text{Root length of treated plants}}{\text{Root length of Control plants}} \times 100\right) - 100$$

(A)



(B)



(C)

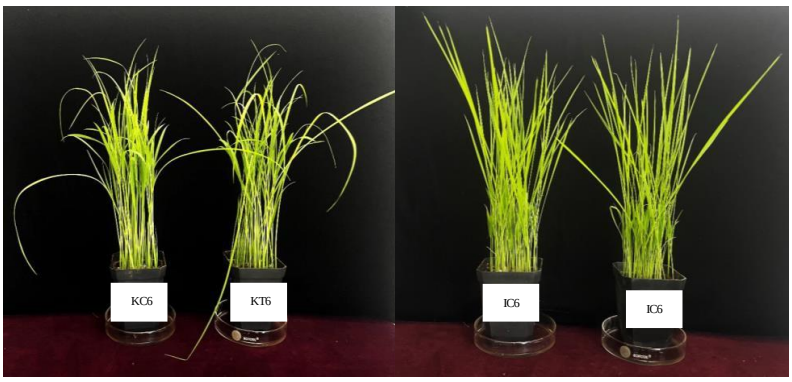


Figure 3.1 Effect of 300mM NaCl treatment in Korgut (Salinity tolerant) and IR64 (Salinity sensitive) rice varieties after **(A)** 1 hour of NaCl treatment, **(B)** 3 hours of treatment, **(C)** 6 hours of treatment

$$\text{SLSI} = \frac{\text{Shoot length of treated plants}}{\text{Shoot length of control plants}} \times 100$$

$$\text{RLSI} = \frac{\text{Root length of treated plants}}{\text{Root length of control plants}} \times 100$$

3.3 Biochemical parameters

3.3.1 Estimation of Water Content

Leaf tissues were weighed to note the fresh weight, were further oven dried at 80ºC for 24hrs, cooled and reweighed to find out the dry weight. Water content and water content percentage were calculated using the following formulae-

$$\text{Water content} = \text{Dry weight} - \text{Fresh weight}$$

$$\text{Water content \%} = \frac{\text{Water content}}{\text{Fresh weight}} \times 100$$

3.3.2 Measurement of Proline Content

The proline content in the leaf tissue was determined using the ninhydrin method given by Bates et al. (1973). 500mg of leaf tissue was homogenised in 5mL 3% sulfosalicylic acid (3g Sulfosalicylic acid+ 100mL distilled water). This was followed by centrifugation at 5000rpm for 5 minutes. 1mL of supernatant was mixed with 1mL acid ninhydrin (1.25g Ninhydrin, 30mM Glacial acetic acid, 20mL 6M Phosphoric acid) and 1 mL glacial acetic acid. The mixture was then incubated at 90ºC for an hour and cooled at room temperature. Chromophore (the developed colour) was extracted in 5mL toluene by vigorous shaking. The mixture was allowed to settle, and absorbance was measured spectrophotometrically at 520 nm against toluene using an ultraviolet (UV) visible spectrophotometer (UV 2450,

Shimadzu). A standard curve with L-proline was used to quantify the concentrations obtained.

3.3.3 Detection of Hydrogen Peroxide by DAB Staining

1. Preparation of DAB staining solution- To prepare a final 1mg mL^{-1} DAB solution, 350 mg DAB was added to 315 mL sterile distilled water. pH was adjusted to 3.0 with 0.2 M HCl and stirred using a magnetic stirrer to dissolve DAB. The tubes were covered with aluminium foil as DAB is light-sensitive.

2. Staining roots with DAB solution- Roots of plants grown under both control and NaCl treated conditions were harvested and were immersed in ~10mL (volume enough to immerse the roots completely) DAB staining solution, and the Falcon tubes were covered with aluminium foil. To ensure that the DAB solution is taken up by the roots, the tubes were placed in a desiccator to carry out vacuum infiltration. A minute hole was perforated on the tube wall to facilitate vacuum formation. After an hour of vacuum filtration, the samples were visualised for DAB staining under an optical microscope, and photographs were taken.

3.4. Gene expression studies

3.4.1 Total RNA isolation

The frozen shoot tissues of control and treated plants were powdered separately in liquid Nitrogen in a mortar and pestle. 1mL Trizol (RNA-Xpress™ reagent) and crushed until it mixed thoroughly. The mixture was left to thaw for 5 minutes, followed by centrifugation at 12,000rpm for 15 minutes at 4ºC. 500 µL chloroform was added to the supernatant, mixed, and allowed to stand at room temperature for 5 minutes. The mixture was then centrifuged at 12000 rpm for 10 minutes at 4ºC. The upper aqueous phase was mixed with 500µL

isopropanol, mixed and was allowed to stand at room temperature for 15 minutes. This was followed by centrifugation at 12000rpm for 15 minutes at 4ºC. The obtained pellet was washed with 500 µL of 5% ethanol prepared in DEPC-treated water, mixed, and centrifuged at 7500rpm for 5 minutes at 4ºC. Washing was repeated twice. The pellet was dried at room temperature and dissolved in 25µL of DEPC-treated water. The obtained RNA was stored at -20ºC (**Table. 3.1**).

3.4.2 Purity and Integrity of RNA

The purity of isolated RNA was checked spectrophotometrically by measuring its absorbance at 260 nm and 280 nm on an ultraviolet (UV) visible spectrophotometer (UV 2450, Shimadzu) (**Fig. 3.2 A**). RNA concentration was calculated using the formula-

$$\text{Concentration} = \text{Absorbance}_{260} \times 40 \times 100$$

The integrity of RNA was confirmed by Agarose Gel Electrophoresis and Bands Visualization.

1. Preparation of gel

50mL10X Tris Borate EDTA (Thermo Scientific) electrophoresis buffer was diluted in 450mL DEPC treated water to prepare 1X buffer. Agarose gel was prepared by dissolving 2.25g agarose in 150 mL 1X TBE buffer followed by boiling at 90ºC and the addition of Ethidium bromide (10mg Ethidium bromide in 10mL DEPC treated water).

(A)

(B)

(C)

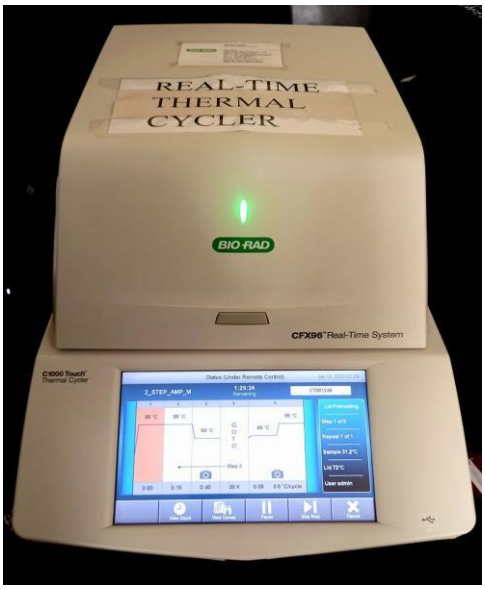


Figure 3.2. Instruments operated to carry out experiments under study **(A)** UV Visible spectrophotometer (UV 2450, Shimadzu), **(B)** Thermocycler (MiniAmp^{TM}Plus Thermal cycler), **(C)** BioRad Real-Time PCR

Table 3.1 List of chemicals used for the study

Type	**Materials**	**Source**
Fine Chemicals	Agarose, Chloroform, DAB, DEPC, EtBr, Ethanol, Glacial, Isopropanol, L- Proline, NaCl, Ninhydrin, Phosphoric acid, Sulfosalicylic acid, SYBR®green master mix TBE electrophoresis buffer, TE buffer, TRIzol	Himedia; Duchefa Biochemie; Thermo Scientific; Bio-Rad
Kits	RevertAid First Strand cDNA synthesis kit	Thermo Scientific
Enzymes	RNase free DNase	Thermo Scientific
	Revert Aid reverse transcriptase	Thermo Scientific
	Taq DNA polymerase	Thermo Scientific
Oligonucleotide/Primers	Gene specific	Eurofins Genomics

2. Preparation and loading of sample

2 µL of the sample was mixed with 4 µL loading dye, and the final volume was made up to 20 µL by the addition of 14 µL of DEPC-treated water. On solidification of the gel, it was placed in the electrophoresis chamber, which was then filled with 1X TBE buffer. The samples were loaded onto respective wells. The gel was run at 100-150 V for around 30 minutes.

3. Visualisation of bands

The gel was placed on a UV transilluminator for the visualisation of RNA bands, and photographs were taken.

3.4.3 cDNA synthesis

cDNA synthesis was performed in a thermocycler (MiniAmp^{TM}Plus Thermal cycler) using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Fig. 3.2 B). RNA equivalent was calculated and mixed with Nuclease free water to make the final volume 8 µL, followed by 1µL DNase and 1µL DNase buffer. The mixtures were incubated at 37ºC for 30 minutes. The reaction was terminated with the addition of 1µL EDTA stop solution. The reaction mix was incubated at 65ºC for 10 minutes. This was followed by adding 1 µL Primer oligo dT, incubating at 65ºC for 5 minutes, And chilling on ice. 8 µL master mix containing 4 µL of 5X reaction buffer, 1µL RiboLock RNase inhibitor, 2µL 10mM dNTP mix and 1µL Revert Aid M-MuLVRT were added to each reaction mix.

3.4.4 Primer Design for Quantitative Reverse Transcriptase PCR Analysis

Primers were designed for *SOS1*, *HKT1*, *PIP2A*, *DREB2A*, *P5CS1*, *OsMYC2*, *MPK3*, *MPK4* and *MPK6* genes based on genomic sequences of *Oryza sativa* based on the sequence available on National Center for Biotechnology Information (NCBI). All primers were designed using Primer3 Plus software and synthesised by Eurofins Genomics (**Table.3.2**).

3.4.5 Semi-Quantitative RT-PCR

Primer stock solutions (100 µM) were prepared in TE buffer (Duchefa Biochemie) containing TRIS, EDTA Disodium.$2H_2O$. The TE buffer solution was prepared by dissolving 15.8mg TE buffer powder in 10 mL of sterile distilled water. Prepared stock solutions were stored at -20ºC for future use. The primers were diluted to 10µM (5µL of 100µM stock solution, 45µL sterile distilled water) to use for sqRTPCR and qRTPCR. The master mix contained 14 µL Nuclease free water, 2 µL 10X DNA polymerase buffer, 0.5 µL 10mM dNTP mix, 1 µL forward primer, 1 µL reverse primer and 0.5 µL DNA polymerase. To prepare the reaction mix, 19 µL of the master mix was added to each PCR tube and 1 µL template cDNA. Actin served as control. In the thermocycler (MiniAmp^{TM}Plus Thermal cycler), the reaction mix was subjected to the initial denaturation stage at 95ºC for 2 minutes; denaturation at 95ºC for 30 seconds, annealing at 51ºC for 30 seconds, the number of cycles for this stage was 30; extension at 72ºC for 30 seconds and final extension stage at 72ºC for 5 minutes (**Fig. 3.2 B**). On completion of the reaction, the tubes were stored at -20º. The integrity of resultant amplicons was verified on agarose gel electrophoresis.

Table 3.2 List of primers for semi q-RTPCR and qRTPCR

Gene	**Forward primer**	**Reverse primer**
OsActin	CGGTGTGATGGTTGGTATGG	GCCTCAGTCAGCAACACAGG
SOS1	CACATGGCAGCACTTTGG	GAGCAAAAGCCTGGCAAC
HKT1	CAGCTTGCCTGCATCTCA	CTCCACCATCCGGACAAG
PIP2A	GCACCTTCGTGCTCGTCT	CCACGCCTTCTCGTTGTT
DREB2A	ATACGATGAGGCGGCAAG	GCCACGATGAAAGGAGGA
P5CS1	CTTGGGCATGCTGATGGT	TTGTGCGCAATAGGTCCA
OsMYC2	AACGACGCCAAGAGCAAC	CTCCAGGTCCGAGTGGTC
MPK3	GCTCCAACCAAGAACTGC	AGTCGCAGATCTTGAGG
MPK4	CGAGGTCTCCTCCAAGTACG	AGCAGCTTGATCTCCCTGAG
MPK6	AGGTCACCGCCAAGTACAAG	AGCAGCTTGATCTCCCTGAG

4.7 Real-Time PCR Analysis

Real-time PCR for the individual genes was carried out using SsoAdvanced Universal SYBR Green Supermix on BioRad instrument, and cDNA prepared from independent biological samples was considered for real-time PCR of each gene *MPK3*, *MPK4*, *MPK6*, *DREB1*, *SOS1*, *MYC*, *PIP*, *P5CS1*, *HKT1*. The expression of genes was represented as fold change in the shoot tissue of the NaCl-stressed seedlings over that in the control seedlings using the $2^{-\Delta\Delta CT}$ method and taking Actin-1 as the reference gene (**Fig. 3.2 C**).

4.8 *In silico* analysis

In silico analysis was done to further elucidate the selected genes' regulation in salt stress. The study of different protein-protein interactions was done using *in silico* tool STRING 9.0 (https://string-db.org/). Promoter analysis was done to find out whether the selected transcription factors play a role in the activation of promoters of SOS1 and PIP2A. Promoter sequences were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/), and promoter analysis was carried out using PlantRegMap (http://plantregmap.gao-lab.org/network.php).

CHAPTER IV

RESULTS

4. RESULTS

Based on preliminary studies performed by (Manohara et al., 2015), Korgut is reported to be a salt-tolerant rice variety. To assess the pathways and possible mechanisms involved in the salt tolerance of Korgut, we first analysed the physiological parameters affected by salinity in Korgut and IR64. IR64 is a salt susceptible cultivar used as a control in our study. Physiological parameters studied were root and shoot length measurements, and biochemical parameters tested were water content, proline content and detection of hydrogen peroxide. Since environmental signals perceived by plants are transmitted by the intricate signalling pathways working inside the cells and the response towards these stresses is regulated by the transcription factors, the gene expression study of signalling pathway genes and transcription factors were performed in Korgut and IR64 under control and salt treated conditions.

4.1. Physiological parameters

4.1.1 Root length and shoot length measurements

The response toward salt stress varies depending on the type of plant species, variety, cultivar, etc. Some salt-tolerant varieties show a decrease in root length as one of the physiological responses to lower sodium salt's effect. Previous studies have observed decreased root and shoot lengths with increased salt concentration. (Momayezi et al., 2009). The reduction of growth results from the inhibition of cell cycle progression and a reduction in root apical meristem size (West et al., 2004). However, the decrease of root length is seen to be more in the case of salt-tolerant varieties due to the tolerance mechanism shown by these varieties to avoid higher concentrations of NaCl. To understand the mechanism of salt stress tolerance in Korgut, we performed a shoot and root length measurement study of NaCl

treated and untreated in Korgut and IR64 seedlings. Root length and shoot length measurements were taken at the seedling stage on the 7^{th} day of NaCl treatment. Physiological indices like the Salt tolerance index (STI), Shoot length stress index (SLSI) and Root length stress index (RLSI) were calculated in both varieties.

For the shoot and root length analysis experiment under salt stress, a salt-tolerant rice variety named Pokkali was used as a positive control. The effect of NaCl on Korgut physiology was studied in comparison to Pokkali and IR64. With this experiment, it was observed that the root and shoot length in all three varieties showed a decrease. However, in Pokkali and Korgut, the decrease was more than IR64. IR64, a semi-dwarf rice variety, showed shorter shoot length and longer root length than Pokkali and Korgut under control conditions without adding external NaCl. However, the treatment with gradually increasing NaCl concentration suggested a drastic decrease in root length of Pokkali and Korgut as compared to IR64, which showed less decrease with an increase in salt concentration (**Fig. 4.1, 4.2**). This suggested that Korgut employs the same mechanism as Pokkali for giving salt tolerance. However, with NaCl treatment, the shoot length was also decreased in all the varieties with more decrease in IR64 (**Fig. 4.3**). These observations suggested that the indigenous rice variety Korgut was less affected by even 400mM NaCl than IR64 and could tolerate the salt stress as salt tolerant rice variety Pokkali. This experiment also ascertained that Korgut was a salt-tolerant rice variety and could even tolerate a concentration of up to 400mM NaCl. The next question was, what biochemical factors are changing in Korgut in saline conditions (**Table. 4.1**).

Figure 4.1. Root and shoot length measurement analysis in Control (left) and 300 mM NaCl treated (Right) in **(A)** Korgut and **(B)** IR64

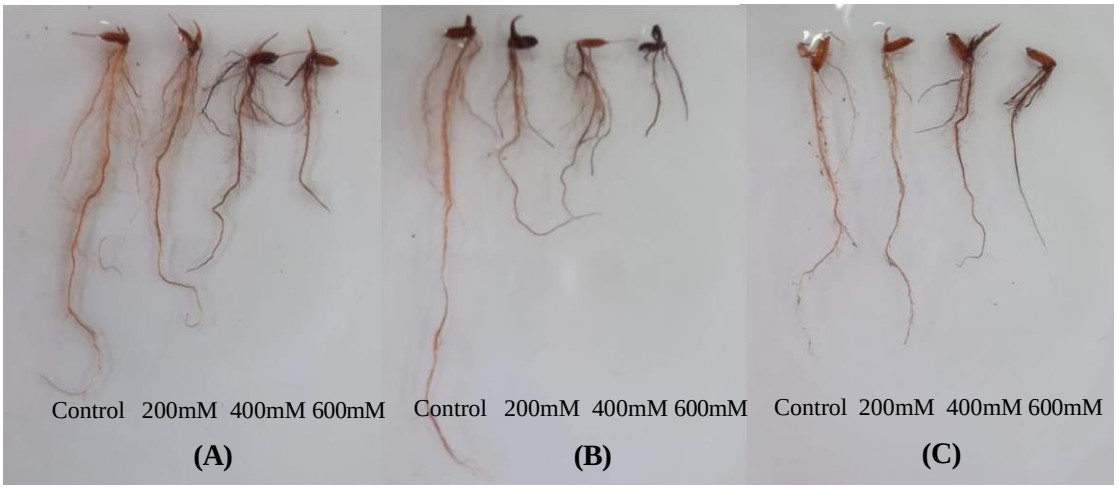


Figure 4.2 Root length decline with increase in NaCl treatments in **(A)** Pokkali, **(B)** Korgut and **(C)** IR64.

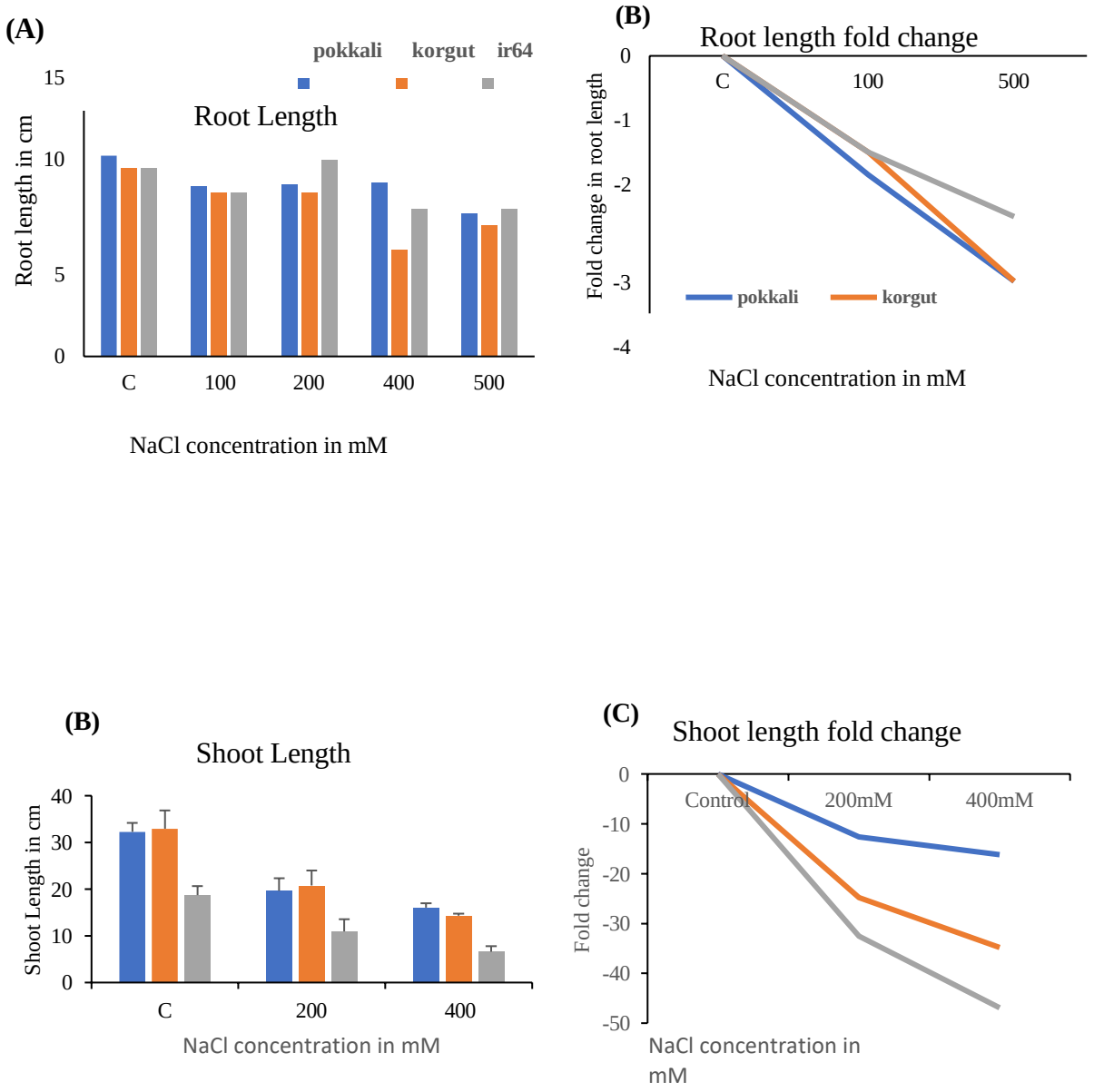


Figure 4.3 Effects of salinity on physiological parameters-Root length and shoot length in Untreated and NaCl treated seedlings of Pokkali (Positive control), Korgut (Salinity tolerant) and IR64 (Salinity sensitive) **(A)** Root length, **(B)** Fold change in root length, **(C)** Shoot length , **(D)** Fold change in shoot length.

Table 4.1 Root and shoot length measurements, **(A)** Average root and shoot length under control and

NaCl (100-500mM) treatment, **(B)** Salt tolerance index in roots and shoots of rice varieties- Pokkali, Korgut and IR64, **(C)** Shoot length stress index and root length stress index

Average root length under control and NaCl treatment						
Rice varieties	**Control**	**100mM**	**200mM**	**300mM**	**400mM**	**500mM**
Pokkali	9.75	8.55	8.2	7.4	7.4	7.5
Korgut	9	7.15	7.65	7.6	6.45	7.25
IR64	10.75	10	9	8.25	7.5	8.5
Average shoot length under control and NaCl treatment						
Rice varieties	**Control**	**100mM**	**200mM**	**300mM**	**400mM**	**500mM**
Pokkali	32.25	18.4	19.6	17.7	16.05	11.35
Korgut	32.9	22.05	20.75	18.55	14.25	10.4
IR64	18.7	20.2	10.95	15.5	6.6	9.9

Salt tolerance index in roots					
Rice varieties	**100mM**	**200mM**	**300mM**	**400mM**	**500mM**
Pokkali	-12.31	-15.90	-24.10	-24.10	-23.08
Korgut	-20.56	-15.00	-15.56	-28.33	-19.44
IR64	-6.98	-16.28	-23.26	-30.23	-20.93
Salt tolerance index in shoots					
Rice varieties	**100mM**	**200mM**	**300mM**	**400mM**	**500mM**
Pokkali	-42.95	-39.22	-45.12	-50.23	-64.81
Korgut	-32.98	-36.93	-43.62	-56.69	-68.39
IR64	8.02	-41.44	-17.11	-64.71	-47.06

Shoot length stress index					
Rice varieties	**100mM**	**200mM**	**300mM**	**400mM**	**500mM**
Pokkali	87.69	84.10	75.90	75.90	76.92
Korgut	79.44	85.00	84.44	71.67	80.56
IR64	93.02	83.72	76.74	69.77	79.07
Root length stress index					
Rice varieties	**100mM**	**200mM**	**300mM**	**400mM**	**500mM**
Pokkali	57.05	60.78	54.88	49.77	35.19
Korgut	67.02	63.07	56.38	43.31	31.61
IR64	108.02	58.56	82.89	35.29	52.94

4.2 Biochemical parameters

4.2.1 Water content

Water content measures plant water status in terms of the physiological consequence of cellular water deficit. High concentration of salts in the soil water causes water to flow from the plant roots back into the soil. This results in dehydration of the plant, causing yield decline or even death of the plant. A relative decrease in water content percentage was seen in the samples treated with 300mM NaCl, in comparison with the control samples, in both varieties. In the salt-tolerant Korgut variety, the WC% decline was relatively higher in the case of the Korgut variety when compared to that in the IR64 variety (**Fig. 4.4C**). This gives us an idea that water transporters like aquaporins and ion channels are working differentially in Korgut compared to the salt susceptible rice variety. Further study of these water channels in Korgut would be interesting to understand the mechanism of salt tolerance in Korgut (**Table. 4.2 A**).

4.2.2 Determination of Proline Content

Proline accumulation to counteract the effects of osmotic stress is a common adaptive mechanism for plant responses to stress conditions. Proline content was determined using the proline estimation method by Bates et al. (1973). The extracted proline reacted with ninhydrin in acidic conditions and formed a chromophore, which was spectroscopically determined at a wavelength of 520 nm using a UV-visible spectrophotometer. Proline concentration was calculated using the L-proline standard and expressed as µmol/g FW.

The proline level was measured in Korgut and IR64 under control and saline conditions, as proline has been shown to play a significant role in ROS scavenging in plants under salinity

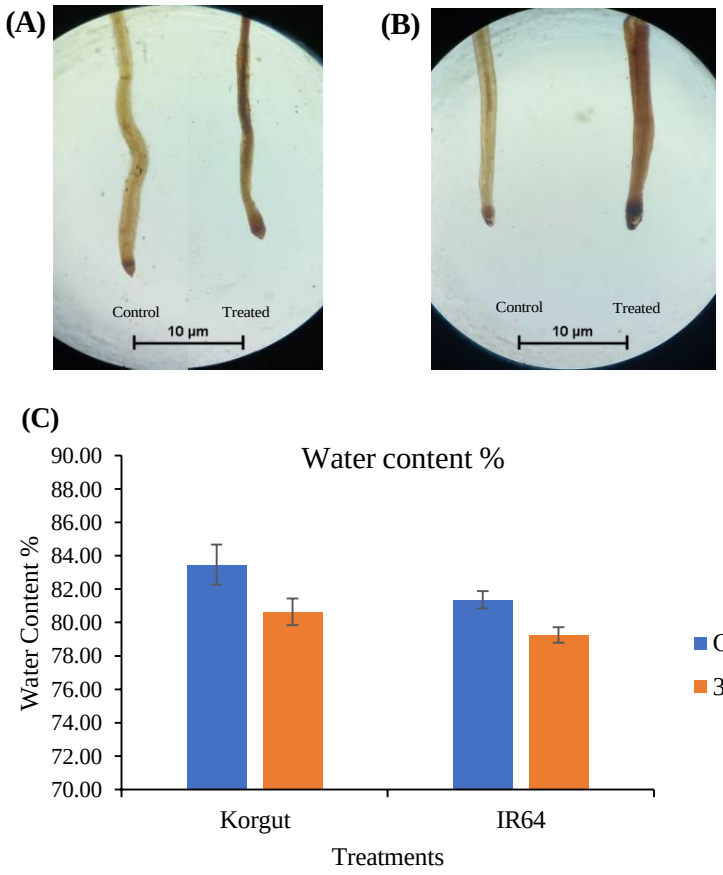


Figure 4.4 Effects of salinity on biochemical parameters studied in leaf tissue of Control and NaCl treated Korgut (Salinity tolerant) and IR64 (Salinity sensitive) varieties , **(A)** Detection of H_2O_2 by DAB staining in Korgut and IR64 **(B)** Water content % and **(C)** Proline content

Table 4.2 (A) Water content percentage in leaves of Korgut and IR64 leaves under control and NaCl (300mM) stress
(B) Proline content in leaves of Korgut and IR64 under control and NaCl (300mM)

Water content of Korgut leaves under control and NaCl stress					
Treatment	**FW(g)**	**DW(g)**	**Water Content(g)**	**WC%**	**Average**
Korgut Control	0.051	0.008	0.043	84.31	83.46
Korgut Control	0.053	0.009	0.044	82.61	
Korgut+300mM NaCl	0.053	0.011	0.042	80.08	80.64
Korgut+300mM NaCl	0.052	0.010	0.042	81.20	
Water content of IR64 leaves under control and NaCl stress					
IR64 Control	0.053	0.010	0.043	80.99	81.36
IR64 Control	0.053	0.010	0.043	81.73	
IR64+300mM NaCl	0.056	0.011	0.044	79.57	79.24
IR64+300mM NaCl	0.057	0.012	0.045	78.91	

Proline content in Korgut under control and salt treatment				
Treatments	**OD $_{520\ nm}$**	**y value**	**µmol/gFW**	**Average**
Control$_1$	0.267	0.8407	6.35	5.10
Control$_2$	0.2115	0.8407	5.03	
Control$_3$	0.165	0.8407	3.93	
300mM NaCl$_1$	0.296	0.8407	7.04	6.74
300mM NaCl$_2$	0.2835	0.8407	6.74	
300mM NaCl$_3$	0.2705	0.8407	6.44	
Proline content in IR64 under control and salt treatment				
Treatments	**OD $_{520\ nm}$**	**y value**	**µmol/gFW**	**Average**
Control$_1$	0.2465	0.8407	5.86	4.24
Control$_2$	0.1805	0.8407	4.29	
Control$_3$	0.1075	0.8407	2.56	
300mM NaCl$_1$	0.248	0.8407	5.90	5.92
300mM NaCl$_2$	0.248	0.8407	5.90	
300mM NaCl$_3$	0.25	0.8407	5.95	

(Khanna-Chopra et al., 2019). The proline content in Korgut was 0.9 times higher than in IR64 under control conditions. Upon salt treatment, this content was further increased by 1.6 times (**Fig. 4.4D**). The higher level of proline in Korgut suggested that salt tolerance in Korgut is possibly regulated with a pathway involving proline synthesis. Also, the higher amount of proline in Korgut than in IR64 suggests that the genes and proteins involved in proline synthesis are expressed more in Korgut than in IR64 rice (**Table. 4.3 B**).

4.2.3 Detection of hydrogen peroxide (H_2O_2) by 3,3-diaminobenzidine (DAB) staining

The level of ROS (Reactive Oxygen Species) determines whether it will be a defensive or destructive molecule, and its level is maintained through coordination between ROS production and turnover (Mittler et al., 2004; Miller et al., 2007). The function of ROS is also governed by its site of production, site of action and duration of action. When environmental stress becomes detrimental to the plant, it activates a genetically controlled process called programmed cell death to eliminate damaged tissues specifically. In this process, plants produce excess ROS, which helps in programmed cell death of damaged tissue and also initiates various signalling in plants. Signal transduction pathways regulate the level of ROS production, thereby protecting the plants from the adverse effects of ROS (Bowler and Fluhr, 2000; Mittler et al., 2004). In situ detection of hydrogen peroxide was carried out in rice seedlings by staining with 3,3′-diaminobenzidine (DAB). The levels of H_2O_2 increased in response to salinity in the roots of both the genotypes, as inferred from the dark brown precipitate formed due to the oxidation of DAB. The precipitate of dark brown colour was more in Korgut than in the roots of IR64, suggesting a higher accumulation of H_2O_2 in the former. In addition, we also observed a few dark brown spots of DAB in the unstressed roots of Korgut but not in IR64 (**Fig.4.4 A and B**). The accumulation of ROS was

observed to be more also in salt tolerant Pokkali cultivar, similar to what was observed in Korgut. The increase in ROS in the roots of Korgut, Pokkali as compared to salt-sensitive IR64 suggests that the increase in ROS might be responsible for initiating signalling pathways, which also suggests the study of antioxidant enzymes and ROS-induced cell death in salt-treated Korgut, Pokkali and IR64.

4.3 Analysis of gene expression

The physiological and biochemical analysis suggested the salinity tolerance of Korgut over the IR64 salt susceptible rice. However, the possible mechanism that Korgut utilises to combat salt stress is still unknown. This can be understood by analysing molecular pathways and genetic regulations of stress-responsive genes. We know that plant perceives any extracellular signal and transmit it with the help of signalling pathways, which ultimately regulate the expression of the stress response or defence response genes and, thereby, the plant gives stress response. In order to understand the stress response, it is worth studying the expression of genes encoding signalling pathway proteins and transcription factors in salt-tolerant variety Korgut and susceptible variety IR64. Hence, we began our analysis of transcriptional gene expression of some important signalling pathway components and transcription factors. One of the important signalling pathways that transmit extracellular signals is Mitogen-Activated Protein Kinase (MAPK) pathway. This pathway consists of MAPKKK, MAPKK and MAPK, which transmit signals by a phosphorelay from the upstream receptor to the nucleus. Among these MAPKs, we chose MPK3, MPK4, and MPK6 for studying their expression in Korgut. Also, these MAPK are known to activate many downstream transcription factors and other proteins; hence we chose some important

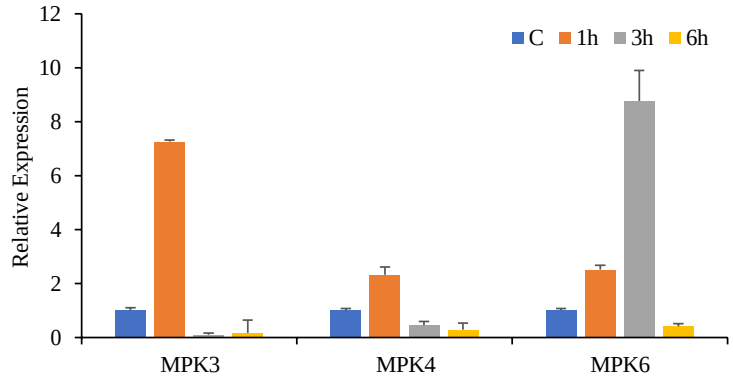
transcription factors like DREB (Dehydration responsive element binding), MYC and some transporters and other genes showing a role in salinity stress tolerance.

The expression study of MAPK genes in IR64 was conducted by Real-Time PCR (RT-PCR) by using the rice seedlings treated with 300mM NaCl for a period of 1 hour, 3 hours, and 6 hours and by using the primers designed for genes of MPK3, MPK4 and MPK6. This expression analysis showed that the expression of MPK3, MPK4 and MPK6 increased in response to salinity stress, suggesting their involvement in transmitting signals related to salinity. In this *MPK3* and *MPK4* genes showed an increase in gene expression within 1 hour of NaCl treatment, while MPK6 showed increased expression after 3 hours of NaCl treatment. This showed that MPK3 and MPK4 are early-responsive genes that respond earlier than MPK6 under salt treatment (**Fig. 4.5 A**).

These MAPKs are known to activate many transcription factors and other proteins, and kinases by phosphorylating them. The activation of transcription factors further leads to the upregulation of many stress response genes, which help plants to respond under stress conditions. Hence, in order to understand the salt tolerance in Korgut rice, gene expression analysis of important transcription factors like DREB and MYC was studied. For this, Korgut and IR64 rice seedlings treated with 300mM NaCl were harvested after 1 hour, 3 hours, and 6 hours of the treatment, wherein without NaCl, treated seedlings were used as control. The total RNA was isolated (**Fig. 4.6 A**), and cDNA was prepared using a reverted cDNA synthesis kit. Real-time PCR was performed using the designed primers for the transcription factors DREB, and MYC. Also, gene expression of other proteins like transporters HKT (High-affinity Potassium Transporters), PIP2A (Plasma Membrane Intrinsic Proteins), and proteins involved in the signal transduction pathway regulating ioni

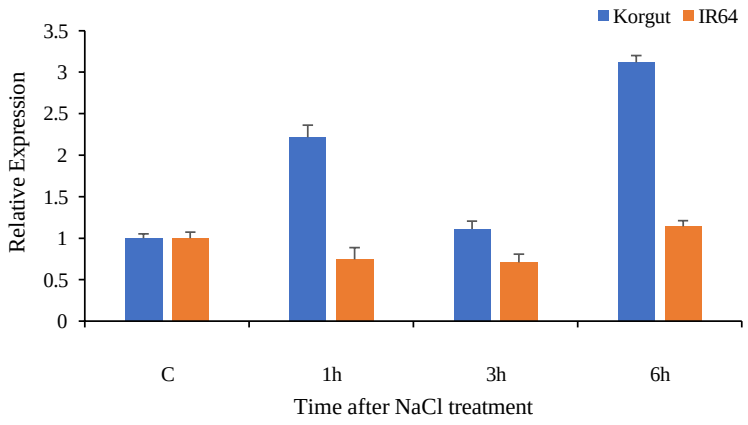
(A)

MAPK gene expression in IR64



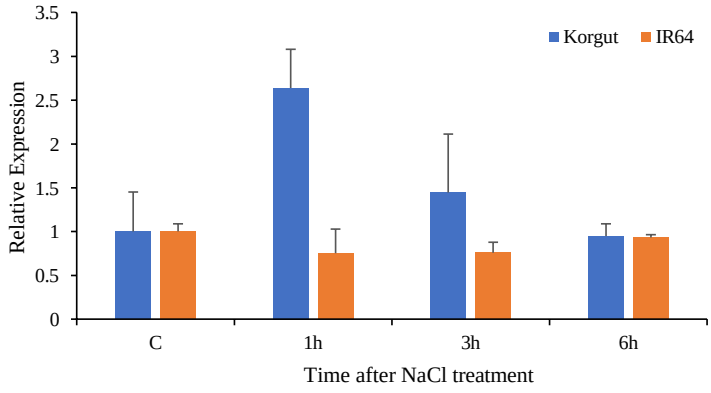
(B)

MYC2 gene expression

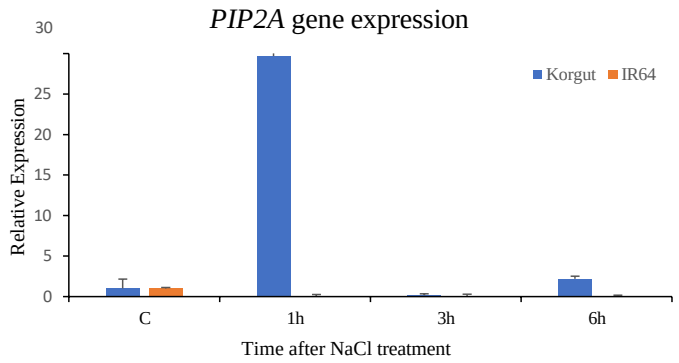


(C)

DREB2A gene expression



(D)



(E)

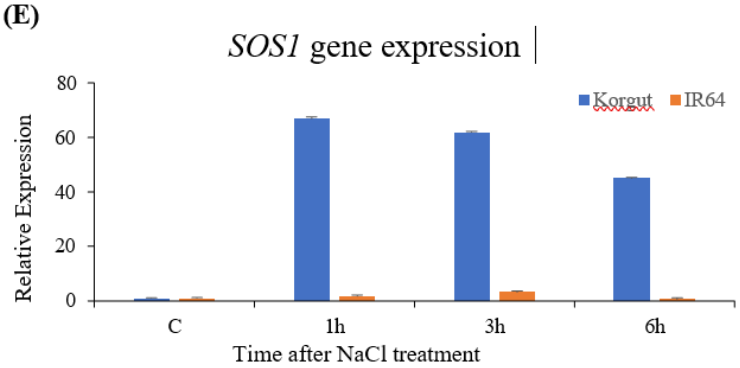
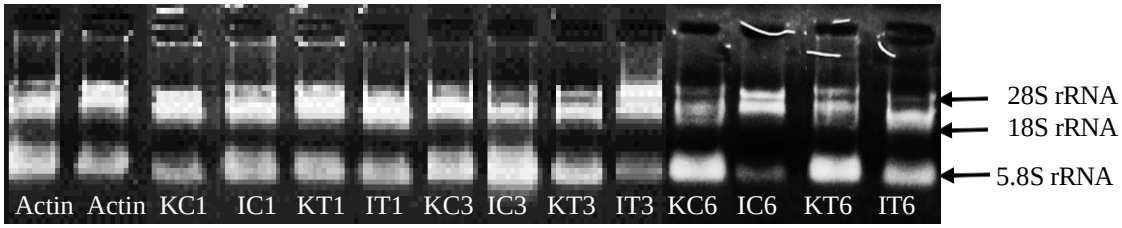


Figure 4.5 Gene expression analysis in leaf tissue of Control and NaCl treated Korgut (Salinity tolerant) and IR64 (Salinity sensitive) Rice varieties for **(A)** MAP Kinases MPK3, MPK4, MPK6; **(B)** Transcription factors MYC and **(C)** DREB2A, **(D)** PIP2A and **(E)** SOS1

(A)



(B)

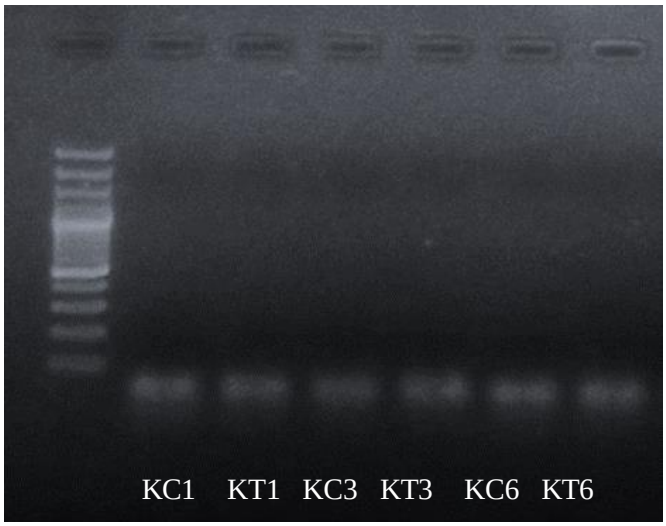


Figure 4.6 (A) Agarose gel electrophoresis showing integrity of isolated RNA bands, **(B)** Agarose gel electrophoresis showing integrity of amplicons obtained in RTPCR

homeostasis inside plant cells like SOS1 (Salt overly sensitive) was studied. According to the gene expression analysis, transcription factors DREB and MYC were upregulated under NaCl treatment in Korgut compared to IR64 NaCl-treated seedlings. The increase in DREB and MYC gene expression was observed within 1hour of NaCl treatment. Also, the expression of Plasma Membrane Intrinsic Protein PIP2A and SOS1 was drastically increased within 1 hour of NaCl treatment in Korgut seedlings. The upregulation of transcription factors DREB2A and MYC2 gene expression suggested their possible involvement in salt stress tolerance in Korgut. This can be done by activating many downstream defence response genes by these two transcription factors in Korgut in saline conditions (**Fig. 4.5 B and C**). Also, the upregulation of the PIP2A transporter and SOS1 suggested their role in giving salt stress tolerance (**Fig 4.5 D and E**). SOS1 is responsible for excluding the Na^+ from the cytoplasm to the outside of the cell, while Plasma membrane intrinsic proteins (PIPs) plant channel proteins that play a major role in plant cell water balance and responses to salt stress.

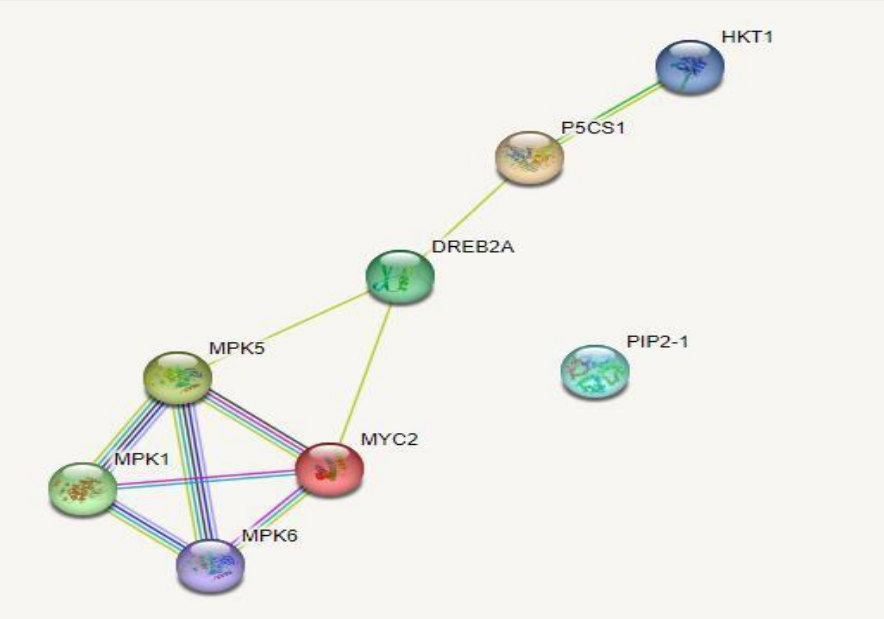
4. *In-silico* analysis

This gene expression analysis also gave us an idea about the inter-regulation and cross-talk among these selected genes. To assess whether the MAPK signalling pathway components have any role in activating the transcription factors DREB2A and MYC2, we performed *in silico* analysis. In this, to check the regulation of transcription factors by MAPKs, we accessed their interaction by using *in silico* tool STRING 9.0. In this, it was found that MYC2 interacted with all the three MAPKs, i.e. MPK3, MPK4 and MPK6, while DREB2A interacted with MPK3. Interestingly the interaction of DREB with MYC2 and P5CS1 was also observed. DREB2A and MYC2 interaction suggests their regulation on each other and

DREB interaction with P5CS1, an important enzyme in proline biosynthesis, suggests its involvement in proline biosynthesis, which correlates with more proline production in Korgut under salt treatment (**Fig 4.7 A and B**; **Table.4.3**).

Further, it was worth studying whether the DREB2A and MYC2 transcription factors activate SOS1 and PIP2A. Hence, to explore this possibility, we performed promoter analysis of SOS1 and PIP2A for binding sites of different transcription factors. Interestingly, it was observed that the SOS1 promoter showed a binding site for DREB2A along with many other transcription factors belonging to the family of Dof, C2H2, GATA, ERF, etc. Also, DREB2A showed a binding site in the promoter of the PIP2A gene. These observations suggested that DREB2A might be possibly involved in upregulating the transcript level of PIP2A and SOS1 genes. This also gives an idea about the possible mechanism that Korgut employs in salt tolerance. This study provides a basic idea for further exploration of the mechanism of salt tolerance in the indigenous unexplored rice variety Korgut (**Fig 4.8 A and B**).

(A)



(B)

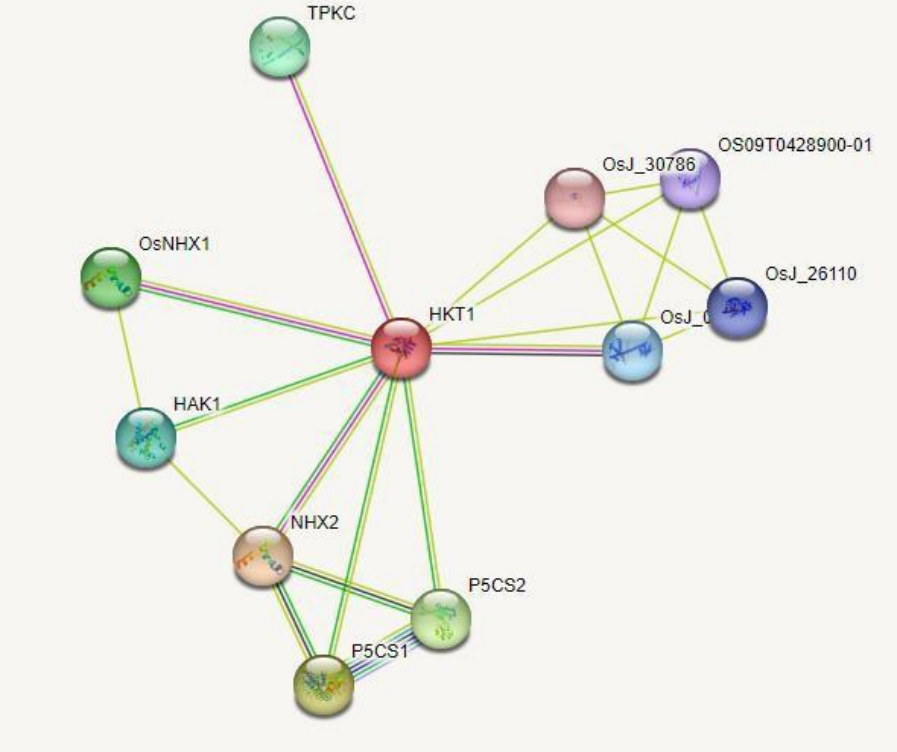
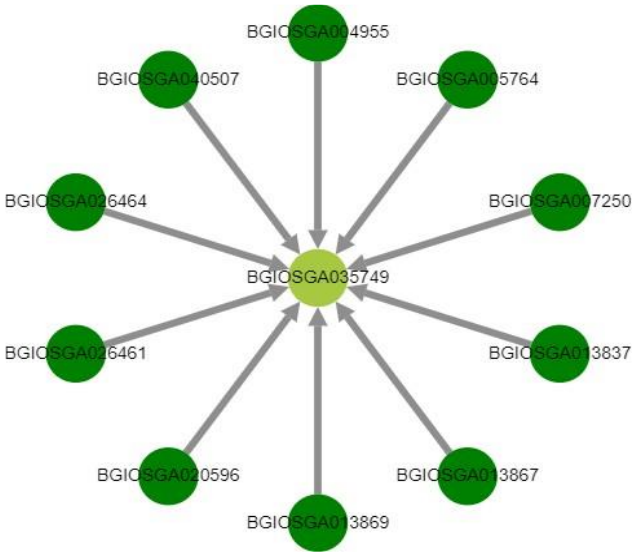


Figure 4.7 Protein-protein interaction network of **(A)** MPK3, MPK4, MPK6, DREB2A, P5CS1, HKT1 and **(B)** Interacting proteins of HKT1 and P5CS1, and others important proteins synthesised in response to salinity stress

(A)



(B)

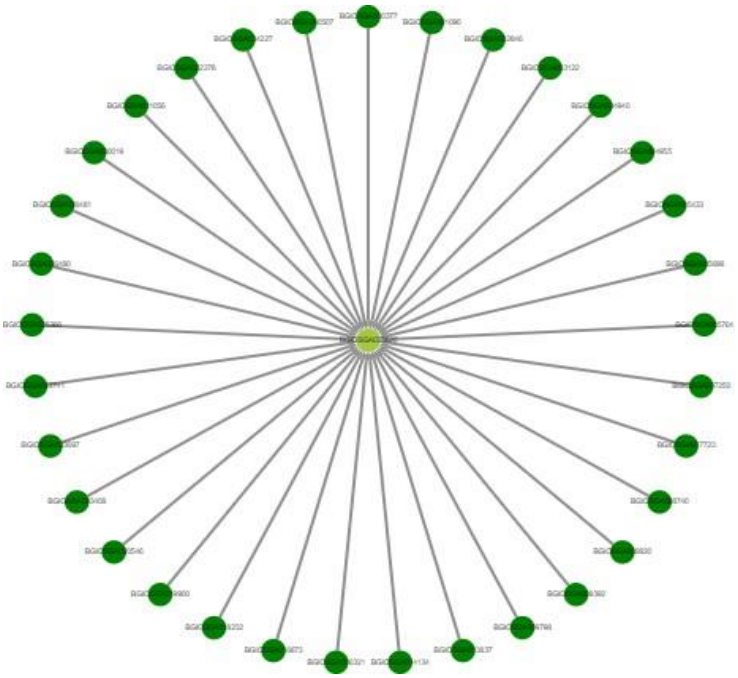


Figure 4.8 Promoter analysis illustrating transcription factors binding to **(A)** *SOS1* and **(B)** *PIP2A*

Table 4.3 Transcription factors binding to promoter of **(A)** SOS1, **(B)** PIP2A

(A)

TF	Target	TF Family
BGIOSGA004955-PA	SOS1	Dof
BGIOSGA005764-PA	SOS1	Dof
BGIOSGA007250-PA	SOS1	C2H2
BGIOSGA013837-PA	SOS1	Dof
BGIOSGA013867-PA	SOS1	GATA
BGIOSGA013869-PA	SOS1	GATA
BGIOSGA020596-PA	SOS1	GATA
BGIOSGA026461-PA	SOS1	ERF
BGIOSGA026464-PA	SOS1	ERF
BGIOSGA040507-PA	SOS1	Dof
BGIOSGA002846-PA	SOS1	ERF

(B)

TF	Target	TF Family
BGIOSGA008976-PA	PIP2A	HD-ZIP
BGIOSGA011366-PA	PIP2A	HD-ZIP
BGIOSGA016852-PA	PIP2A	HD-ZIP
BGIOSGA026014-PA	PIP2A	HD-ZIP
BGIOSGA028019-PA	PIP2A	MYB_related
BGIOSGA000377-PA	PIP2A	MIKC MADS
BGIOSGA001090-PA	PIP2A	ERF
BGIOSGA002846-PA	PIP2A	ERF
BGIOSGA003122-PA	PIP2A	C2H2
BGIOSGA004940-PA	PIP2A	MIKC_MADS
BGIOSGA004955-PA	PIP2A	LBD
BGIOSGA005433-PA	PIP2A	ERF
BGIOSGA005698-PA	PIP2A	Dof
BGIOSGA005764-PA	PIP2A	Dof
BGIOSGA007250-PA	PIP2A	C2H2
BGIOSGA007723-PA	PIP2A	C3H
BGIOSGA008740-PA	PIP2A	ERF
BGIOSGA008820-PA	PIP2A	MIKC_MADS
BGIOSGA009392-PA	PIP2A	ERF
BGIOSGA009798-PA	PIP2A	MIKC_MADS
BGIOSGA013837-PA	PIP2A	Dof
BGIOSGA014134-PA	PIP2A	ERF
BGIOSGA032378-PA	PIP2A	BBR-BPC
BGIOSGA026481-PA	PIP2A	NAC
BGIOSGA026480-PA	PIP2A	C2H2
BGIOSGA023408-PA	PIP2A	SBP

CHAPTER V

DISCUSSION

5. DISCUSSION

Like other abiotic stresses, salinity stress is a significant threat to agriculture. Salinity is believed to have a detrimental effect on the growth and yield of rice, mainly due to the deposition of excess salts (Shereen et al. 2005). Flowers et al. (1985) and Munns et al. (1995) have shown that the reduction in the growth and yield of crops under salinity is mainly due to loss of water potential, cell turgor pressure, closure of stomata, followed by a reduction in photosynthesis.

Numerous plant genes have been reported to respond to abiotic stresses, and proteins encoded by these genes are expected to augment the tolerance level of plants to these stresses. The analyses of these stress-responsive genes and their corresponding proteins shed light on the possibility of developing stress-resistant plants. The development of salinity-tolerant high-yielding rice genotypes will be possible with a prior understanding of the physiological, biochemical, and molecular events in rice seedlings when exposed to salinity. The availability of contrasting genotypes of rice viz. Korgut being tolerant and IR64 being sensitive to salinity offers a brilliant model to dissect these details in cellular response enabling the former to tolerate high salinity.

In the present study, we attempted to go deeper and decipher the machinery that enables the seedlings of Korgut to flourish in saline soil. Reduction of growth in both the genotypes with an increase in the duration of salinity did not seem astonishing since the extent of reduction was significantly greater in IR64 than in Korgut.

Plants have evolved strategies to dynamically regulate their spatial growth and reshape their architecture in response to environmental changes. Roots need to adapt to maintain growth and regulate nutrient and water uptake, as these are the frontline organs that encounter salt in the soil. Salt stress is known to reduce root mass and modify the

distribution of various root system architecture (RSA) components, differentially affecting the growth rate of the primary root and lateral roots and inhibiting lateral root formation. Inhibition of both primary and lateral roots was observed in salt-stressed *Arabidopsis thaliana* seedlings after 4-8 days of treatment (Duan et al., 2013, Julkowska et al., 2014). The reduction of growth results from the inhibition of cell cycle progression and a reduction in root apical meristem size (West et al., 2004). Different RSA strategies are found to be partially associated with abscisic acid sensitivity and different Na^+ /K^+ ratios in shoots of seedlings grown under mild salt stress. Plant growth is known to be inhibited by salt stress that challenges the plant's osmotic and ionic stress-counteracting capacities. Plant responses to salt stress involve the activation of ion pumps to either limit Na^+ influx into the plant (Salt Overly Sensitive1) or to compartmentalise the excess of Na^+ into tissues (High affinity K^+ Transporter1) or vacuoles (vacuolar Na^+ /H^+ exchanger1; Munns and Tester, 2008). Shoots of the plant overexpressing SOS1 show reduced accumulation of Na^+ because of its function in regulating the loading of the ion into the xylem leading to limited delivery to shoots besides controlling the net uptake of the ion (Martínez-Atienza et al., 2007, Mahi et al., 2019). Considering the reported role of SOS1 in the controlled delivery of Na^+ to the shoots, the noteworthy NaCl-induced expression of SOS1 in the shoots of Korgut seen in the present study can be correlated with the trivial decrease in root and shoot length of Korgut in comparison with IR64(**Fig. 4.3**)

The growth and development of aboveground tissues are limited by salt stress, although the molecular mechanisms are not as profoundly studied as those for roots. Thus far, it is not clear how the shoot apical meristem and shoot architecture are regulated by salt (Zelm et al., 2020). The present study showed a decrease in IR64 shoot length compared to Pokkali and Korgut (**Fig. 4.3C and D**), which can be linked with lower Na^+ accumulation

in Pokkali and Korgut than salt-sensitive IR64, as discussed in root length analysis. Whereas the root length of Korgut decreased to a larger extent than that of salt-tolerant Pokkali when compared to IR 64 root length under salt treatment, which suggests that Korgut is employing the same mechanism as that of Pokkali to avoid the excess of NaCl present on the soil.

The degree of growth reduction under stress is thought to have a direct correlation with the imbalance in the biochemical composition of the plants (Anjum et al., 2011; Hasanuzzaman et al., 2013). The early shock response of salinity causes oxidative stress, leading to excess ROS generation (Mittler, 2002; Neill et al., 2002). Accumulation of ROS further leads to membrane damage; hence, to battle this, plants accumulate several ROS-scavenging enzymes such as SOD, APX, GSH, and CAT (Foyer, 2018). SOD reduces the accumulation of H_2O_2; However, H_2O_2 also acts as a secondary messenger that activates adaptive signalling and defence pathway at the physiological and molecular levels (Orabi et al., 2015). As inferred from the slightly darker DAB staining in Korgut roots, a higher accumulation of H_2O_2 is seen in the tolerant variety compared to IR64.

The observations of Fatima et al. (2020) exhibited a higher basal level of H_2O_2 in Pokkali seedlings, as inferred from the higher DAB staining in the control root, showing an effective adaptive mechanism against salinity. The level of H_2O_2 was higher in Pokkali than in IR64, even under stress. In the current study, Korgut roots showed slightly darker staining in control and the NaCl-treated roots. This observation demonstrates the ability of Korgut to maintain the level of H_2O_2 production, under both control and stress conditions, to protect against membrane oxidation under salinity.

The water content percentage of both the genotypes decreased in the shoot tissue of control and NaCl-treated seedlings. However, the degree of the drop was much greater

in IR64 than in Korgut (**Fig.4.4C**), indicating a higher sensitivity of IR64 variety towards salinity.

During stress, solute such as proline (a nontoxic organic molecule) accumulation helps maintain the turgor pressure and provides cellular osmolarity (Matysik et al., 2002; Wani et al., 2013). An increase in the duration of salinity encourages proline to adjust the osmotic potential of plant cells and protect the proteins and cellular structures against denaturation (Fariduddin et al., 2013). Proline accumulation to counteract the effects of osmotic stress is a common adaptive mechanism for plant responses to stress conditions. Proline content increased under stressed conditions in transgenic plants and WT; however, it was significantly higher in transgenic plants than in WT. Consequently, the transcription levels of two proline biosynthesis genes, *OsP5CS1* and *OsP5CS2*, were detected in transgenic plants and WT. The expression levels showed non-significant differences between the transgenic plants and WT under normal growth conditions. However, it was significantly higher in transgenic plants than WT under drought-stress conditions. These results indicated that the overexpression of *OsMYB48-1* could increase proline production by regulating proline biosynthesis genes in rice, especially under drought-stress conditions (Xiong et al., 2014).

Fatima et al. (2020) observed that the proline accumulation was higher in the salinity-tolerant genotype, Pokkali, compared to the sensitive cultivar, IR64, in both root and shoot samples. Similar results were obtained in the present study, as proline content was higher in the shoot tissue of Korgut, the tolerant variety, than in IR64.

A total of nine genes were studied for their gene expression; we were able to report upregulation of *SOS1*, *DREB2A*, *MYC2* and *PIP2A* in Korgut. The extent of expression was higher in *DREB2A*, *PIP2A* and *SOS1* in the early hours of NaCl treatment,

suggesting their early responsive nature. While *MYC2* showed higher expression after 6 hours of treatment, signifying its late-responsive nature. *MPK3* and *MPK4* showed greater expression in the early hours of treatment, whereas expression of MPK6 was found to be significant after 6 hours of treatment in IR64. No proper results were obtained in the case of MPK genes in Korgut, thought to be due to cultivar differences resulting from the difference in binding sites. This branches out a pioneer area for future research in the field.

In silico promoter analysis of *SOS1*, they revealed binding sites for many TFs belonging to families Dof, C2H2, GATA, ERF, etc. The presence of these binding sites for TFs in the promoter region of the genes under study indicates their possible involvement in the salt stress mechanism of rice.

Dof (DNA-binding with one finger) is a group of plant-specific transcription factors that play a role in the transcriptional regulatory networks acting on the vascular system's development and functioning. The Dof TFs are hypothesised to act at the cross-talk of various developmental pathways directly or indirectly linked to vascular development and/or functioning (Le and Bellinni, 2013; **Silva et al., 2017**). Limited root and shoot growth in the present study, in IR64, could be due to the insignificant expression of *SOS1*.

C2H2 is one of the most prominent transcription factor families comprising Zinc finger proteins. These are known for their finger-like structure and ability to bind Zn^{+} and are involved in plant growth, development, and stress signal transduction. *OsZFP213* enhanced the salt tolerance in rice seedlings. Overexpression of *OsZFP213* enhanced ROS scavenging ability and decreased H_2O_2 and O_2^- accumulation in hypersensitive transgenic plants (Zhang et al., 2018). This supports our results showing lightly DAB-stained IR64 roots.

GATA represents a highly conserved family of transcription factors. A member of this family, *OsGATA8*, localised within the Saltol QTL in rice, has been stated to be induced by salinity, drought, and ABA. It increases tolerance to abiotic stresses in both Arabidopsis and rice. Overexpression of OsGATA8 showed an improved phenotype under salinity stress in terms of higher biomass and an increase in RWC, electrolyte leakage, proline content, and K+/Na+ ratio, all indicating a higher tolerance to salinity stress. (Nutan et al.,2020). This adds to the possible reasons for the salt tolerance of Korgut.

APETALA2/Ethylene Response Factor (AP2/ERF) includes subfamilies ethylene-response factor (ERF) and dehydration-responsive element (DRE)-binding; Plays crucial roles in transcriptional regulation and defence response of plant growth and development processes. Studies have revealed the roles of AP2/ERF TFs in improving the resistance of rice to various stresses, pests and diseases. The ERF subfamily proteins are associated with abiotic stress in plants and are affected by drought, low temperature and high salinity. Important functions of ERF proteins have been demonstrated in the transcriptional regulation of various biological processes related to growth and development and various responses to environmental stimuli. *TaERF3* was reported to positively regulate salinity tolerance in wheat (Rong et al., 2014). *SlERF5* plays an essential role in the adaptation to abiotic stress in tomatoes. The transgenic plants overexpressing the *SlERF5* gene showed increased salt and drought stress resistance. (Pan et al., 2012). AP2/ERF family genes, such as *OsERF48* and *ERF3*, are involved in root formation and development (Xie et al., 2022). Enhanced expression of SOS1 and its interaction with ERF transcription factors might be the reason for a slight decline in root length of salt-tolerant Korgut, compared to a moderately higher decrease in IR64 in the present study.

Members of the DREB/CBF subfamily of the AP2/ERF TFs have been documented for a decade for their roles in stress tolerance via ABA-dependent and -independent pathways and for their regulation of a stress-response with more than a hundred target genes. Considering the observations of Zhang et al. (2013), where the overexpression of *OsDREB2A* in Soybeans enhanced salt tolerance by accumulating osmolytes, such as soluble sugars and free proline, Higher proline content in Korgut can also be linked to the interaction of DREB2A with SOS1.

HD-ZIP (Homeodomain-leucine Zipper) belongs to the homeobox superfamily. HD-ZIP genes respond to various abiotic and biotic environmental stimuli by regulating the defence response of plants. HD-ZIP genes regulate plant architecture, organogenesis, and reproductive processes. Most HD-ZIP genes in transgenic research showed distinct effects against drought and salinity.

Oshox22 is strongly induced by salt stress. *Oshox22* affects ABA biosynthesis and regulates drought and salt responses through ABA-mediated signal transduction pathways. It acts as a negative regulator in stress responses. (Zhang et al., 2012).

MYB-type transcription factors (TFs) play crucial roles in plant growth and development and respond to environmental stresses. MYB functions have been extensively investigated in different plant species, as reviewed by Fang et al. (2015). MYB TFs are active factors in abiotic stress signalling; MYBs have been found to regulate downstream genes in response to abiotic stresses and potentially act at both the transcriptional and post-transcriptional levels.

In a study about MYB TF functions in drought stress tolerance in rice, the authors reported that a novel gene, *OsMYB48-1*, was induced by ABA, H_2O_2, dehydration, and PEG was slightly expressed under salt and cold stress treatment.

In maise, *ZmMYB30* was induced by four stress treatments, and transgenic Arabidopsis expressing *ZmMYB30* enhanced salt tolerance and increased expression levels of several stress-related genes. MYB-related type TF *OsMYB48-1* overexpression increased LEA protein and proline content while the reduced rate of water loss in transgenic plants to confer drought tolerance (Xiong et al., 2014), suggesting its possible role in maintaining higher water content in Korgut.

The NAC (NAM, CUC, and ATAF) TF family is one of the most prominent plant-specific TF families. The abiotic stress response gene *SbSNAC1* is reportedly induced by drought, salinity, and ABA, with *SbSNAC1* overexpression leading to enhanced drought stress tolerance in transgenic Arabidopsis plants. In wheat, Zhang et al. (2016) reported that the *TaNAC47* overexpression led to several physiological and biochemical changes, including the soluble sugars and proline content, due to the activation of downstream genes such as *AtRD29A*, *AtRD29B*, and *AtP5CS1*. Zhou et al. (2018) reported that transgenic rice plants overexpressing the *SNAC3* gene had lower levels of malondialdehyde, hydrogen peroxide (H_2O_2), and relative electrolyte leakage compared to the wild-type control under heat stress treatment. The authors proposed that *SNAC3* may confer tolerance to these stresses by modulating ROS homeostasis. *EcNAC67* overexpression in rice helped maintain a high water content and sustainable grain yield under drought conditions. This adds to the possible reasons for the higher water content and darkly DAB stained roots of Korgut.

MADS-box genes are well recognised for their functions in floral induction and development. Overexpression of *OsMADS57* in rice increases the rate of seed germination and root elongation in response to salt stress conditions (Wu et al., 2021). *OsMADS25* regulates nitrogen transporter genes, demonstrating that this TF is a key regulator of primary and lateral root formation in rice (Yu et al., 2015). *OsMADS47* is

expressed at high levels in seedling leaves, whereas the transcription of *OsMADS55* is highest in mature leaves. As a result of these contrasting expression patterns, *OsMADS55* and *OsMADS47* are the primary negative regulators of brassinosteroid responses in leaves (Lee et al., 2008).

The LBD protein family represents a new class of DNA-binding transcription factors recognising the cis-element GCGGCG. *PvLBD12* enhanced the salt tolerance by increasing proline accumulation, improving K^+ accumulation, and decreasing reactive oxygen species levels in switchgrass (Wang et al., 2021).

Basic Pentacysteine1 (BPC1) regulates the homeotic *Arabidopsis thaliana* gene SEEDSTICK (STK), which controls ovule identity. BPC1 can induce conformational changes by cooperative binding to purine-rich elements present in the STK regulatory sequence. Analysis of STK expression in the *bpc1* mutant showed upregulation of STK. The regulation of *BPC1* gene expression in plants provides the basis for further studies to understand the mechanisms that control ovule identity in Arabidopsis. BBR (Barley B Recombinant) is nuclear targeted and is a characterised nuclear localisation signal (NLS) sequence. In co-transfection experiments, *BBR* activates (GA/TC)8-containing promoters, and its overexpression in tobacco leads to a pronounced leaf shape modification. In Arabidopsis (GA/TC), 8 repeats occur, particularly within 1500 bp upstream of gene start codons in some homeodomain genes of different classes.

A combination of biochemical and genetic evidence in the model plants *Brachypodium distachyon* and *Arabidopsis thaliana* supports a role for Coumarate 3-hydroxylase (C3H) Coumarate 3-hydroxylase (C3H). Barros et al. (2019) identified C3H as the only non-membrane-bound hydroxylase in the lignin pathway. The cell wall lignin deposition in endodermal and ectodermal cells changes under salt stress (Byrt et al., 2018). These

molecular and physiological changes enhance plant adaptation to salt stress by preventing water loss and altering ion (i.e., Na+, Cl-, and K+) transporting pathways.

SQUAMOSA promoter binding protein (SBP)-box proteins are plant-specific transcription factors (TFs) and play crucial roles in a variety of physiological processes, including plant growth and development, signal transduction, and stress response (Teng et al., 2022). In pineapple, SBP family genes were proven to respond to abiotic stresses such as cold, heat, salt and drought (Ali et al., 2017).

Taking together the roles of all the abovementioned genes, the fact that salinity tolerance is an inter-regulated and multigenic response was verified in Korgut.

CONCLUSION

6. CONCLUSION

The present study revealed that although all the components that determine salt tolerance in plants independently or cumulatively are present or expressed in salt-tolerant and sensitive rice varieties, these do not ensure their salt tolerance unless regulated correctly. A clear understanding of salt-tolerant components regulation is key to finding valuable targets for improving plant salt tolerance through genetic engineering. Selected MAPKs were found to be activating TFs and interacting with DREB, MYC2, and P5CS1. The binding site for DREB2A was found in the promoter region of *SOS1* and *PIP2A*. Clarifying possible mechanisms employed by Korgut in salt tolerance. Protein-protein interactions and promoter analysis of genes under study revealed multiple genes at work to regulate salinity tolerance. The study also helped classify the genes under study as early and late responsive with respect to salinity tolerance in Korgut. Correlation between physiological, biochemical and molecular mechanisms inducing salinity tolerance was deciphered. This study provided the basic idea for further exploration of the mechanism of salt tolerance in the indigenous unexplored rice variety Korgut.

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