Transcriptional and Post-transcriptional Gene Regulations under Salt-Stress in Wheat

By

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A Thesis Submitted to The Faculty of Science, Agriculture and Engineering, for the degree of

Doctor of Philosophy (Ph.D)

School of Natural and Environmental Sciences

Newcastle Upon Tyne, UK.

October 2023

Abstract

Environmental stressors, including salt stress, reduce significantly cereal crop productivity worldwide, challenging the world's food security. Developing cereal crop varieties that are tolerant to salt stress is required to meet food needs in the future. To attain this goal, understanding the mechanisms underpinning plant responses to salt stress is a pre-requisite. Wheat (*Triticum aestivum*) is widely considered a moderately salt-tolerant species; however, the level of tolerance and the responses that bring about tolerance to salinity vary among different wheat tissues and cultivars. The present study aimed at investigating the main physiological, biochemical and molecular responses to salt stress in different wheat cultivars. Differential responses to salinity were characterised in roots and shoots of three Saudi wheat cultivars including Najran, Mebiah and Qiadh. Results showed that the three wheat cultivars displayed different growth and metabolic and antioxidant responses where Najran wheat exhibited lesser effect of salt on growth and yield and higher accumulation of metabolites and antioxidants in response to the stress. Based on this finding, global profiling of salt-induced changes in the root and shoot transcriptomes as well as salt-induced changes in alternative splicing of pre-mRNA were conducted in Najran wheat. Results revealed that roots respond to a higher extent than shoots to salt stress and that salt-stress induces responses that are organ-specific as well as responses that are common to roots and shoots. Salt stress induced genes that are involved in glutathione metabolism (e.g. GST) and biosynthesis of secondary metabolites such as phenylpropanoids (e.g. PAL) and galactose metabolism (e.g. INV) suggesting that these genes might participate in wheat salt tolerance. In addition, exposing plants to 200 mM NaCl slightly increased the number of AS events by 1.6% and 0.5% in the roots and shoots, respectively, indicating the potential involvement of post-transcriptional regulation in salt-tolerance. Functional enrichment analysis show that a cysteine-type endopeptidase inhibitor was the most significantly enriched term of DSGs shared between the roots and shoots of Najran wheat suggesting that control of proteolysis might participate in the salt-tolerance exhibited in this cultivar.

In the Name of God, the Most Gracious, the Most Merciful

For my parents, Abdullah and Saidah

For my husband, Ali Alahmari

For my son, Basil

To whom I dedicate this thesis.

Norah Alyahya

Acknowledgements

I would like to express my heartfelt gratitude to the following individuals whose unwavering support and encouragement have been instrumental in the completion of this thesis:

My Supervisor, Dr. Tahar Taybi:

Thank you for your invaluable guidance, mentorship, and expertise throughout this research journey. Your dedication to excellence and commitment to fostering my academic growth have been truly inspiring.

My Parents, Abdullah & Saidah:

I am deeply grateful to my parents for their unwavering love, encouragement, continuous prayers and sacrifices that made my educational pursuits possible.

My Husband, Ali:

Special gratitude goes to my love for his unwavering support, patience, and understanding during the ups and downs of this academic effort have been my anchor. Your belief in my abilities and your sacrifices are deeply appreciated.

My Son, Basil:

I want to thank my darling Basil for his boundless love and understanding, even when I couldn't always be present. Your resilience and enthusiasm constantly remind me of the importance of perseverance.

My Brothers and Sisters:

To my dear brothers and sisters, your encouragement and occasional humour provided much-needed breaks during intense research periods. Your belief in my abilities has been a driving force.

This effort would not have been possible without the collective support and belief of these individuals. While their names may appear here, their influence on my life and this work is immeasurable. I am deeply grateful for your presence in my life.

My profound gratitude is extended to my second supervisor Dr. Martin Edwards for his abundant help to access lab appliances and being present when I need him. I am very thankful

to John Casement from the Bioinformatics Unit in Newcastle University for assisting with the analysis of RNA-seq data. I would also like to express my sincere thanks to Dr. Anne Borland and Dr. Vasilios Andriotis from the progression panel for their insightful comments and valuable recommendations. I am thankful to Ministry of Environment, Water & Agriculture in Saudi Arabia for assisting to conduct this research by suppling the wheat seeds. Finally, I acknowledge the financial support provided by the Government of Saudi Arabia and the Ministry of Higher Education. My sincere thanks go to my home university, King Khalid University, for awarding me the scholarship.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

NaCL	Sodium chloride
mM	Millimolar
Na^+	Sodium ions
Cl^-	Chloride ions
Ca^{2+}	Calcium ions
ABA	Abscisic acid
K^+	Potassium ions
NSCCs	Nonselective cation channels
cGMP	Cyclic guanosine monophosphate
Gd^{3+}	Gadolinium cation
La^{3+}	Lanthanum cation
CO_2	Carbon dioxide
ATP	Adenosine triphosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
$O_2^{\bullet-}$	Superoxide
$^1O_2$	Singlet oxygen
OH•	Hydroxyl radical
KCL	Potassium chloride
mtlD	Mannitol-1-phosphate dehydrogenase
P5CS	Pyrroline-5-Carboxylate Synthase
PCD	Programmed cell death
HKT	High-affinity K^+ transporter
SOS	Salt overly sensitive
H^+	Hydrogen ions
CNGCs	Cyclic nucleotide-gated channels
GLRs	Ionotropic glutamate receptors
AVPI	Inorganic H pyrophosphatase family protein
PA	Phosphatidic acid
ANN1	Annexin
MPK6	Mitogen-activated protein kinase 6
NHX	Na^+/H^+ antiporters
CCC	Cation-chloride cotransporter
SKOR	Shaker-type K^+ channel
SIHKT1	Class I HKT1 transporter
MYB	Myeloblastosis family of transcription factors
bZIP	basic region/leucine zipper motif transcription factors
RNA	Ribonucleic acid
AS	Alternative splicing
Pre-mRNA	precursor messenger RNA

SR	Serine/arginine-rich proteins
GRPs	Glycine-rich binding proteins
CBPs	Cap-binding proteins
RL	Root length
SL	Shoot length
RFW	Root fresh weight
SFW	Shoot fresh weight
RDW	Root dry weight
SDW	Shoot dry weight
Na_2CO_3	Sodium carbonate decahydrate
DNA	Deoxyribonucleic acid
bHLH	basic Helix-Loop-Helix transcription factors
AP2/ERF	APETALA2/Ethylene Responsive Factor
WRKY	WRKY transcription factors
NAC	NAC transcription factors
bZIPs	Basic leucine zipper transcription factors
RNA-Seq	RNA sequencing
cDNA	Complementary DNA
QC	Quality control
PE	Paired end reads
KEGG	Kyoto Encyclopedia of Genes and Genomes
DEGs	Differentially expressed genes
GO	Gene ontology
RT-qPCR	Quantitative real-time polymerase chain reaction
FDR	False discovery rate
PCA	Principal component analysis
P5CS	Delta-1-Pyrroline-5-Carboxylate Synthase
GST	Glutathione S-transferase
GSS	Glutathione synthase
GT	Glutathione transferase
APX	Peroxidase
CAT	Catalase
GPX	Glutathione peroxidase
SOD	Superoxide dismutase
POD	Peroxidase
PAL	Phenylalanine ammonia-lyase
CSE	Caffeoyl shikimate esterase
CCR	Cinnamoyl-CoA reductase
CYP84A	Ferulate-5-hydroxylase
INV	Beta-fructofuranosidase
Gb	Gigabyte
SE	Skipping exon
RI	Retained intron
A5	Alternative 5' splice site

A3	Alternative 3' splice site
AD	Alternative donor splice site
AA	Alternative acceptor splice site
TPMs	transcript per million
MX	Mutually exclusive exon
AF	Alternative first exon
AL	Alternative last exon
PSI	Percentage spliced-in
ΔPSI	Delta percentage spliced-in
DSGs	Differentially spliced genes
MF	Molecular function
CC	Cellular component
BP	Biological process
CPI	Cysteine proteinase inhibitor
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
DRFL1a	Drought-responsive factor-like transcription factor
HAT	Histone acetyltransferase
ME	Malic enzyme
DAS	Differentially alternative spliced events
bp	Base pair
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris-HCL	Tris Hydrochloride
SDS	Sodium dodecyl sulphate
rpm	Revolutions per minute
pH	Potential of hydrogen
DTT	Dithiothreitol

Chapter 1 General Introduction

Plant salinity, denoting the elevation of soil salt concentrations, has profound physiological impacts on plants and significant implications for agricultural sustainability. Salinity occurs naturally as a result of geological processes, weathering rocks, and coastal intrusion. Moreover, human activities like excessive irrigation, fertiliser use, inadequate drainage, and poor water management contribute to the accumulation of salt in soil (Munns and Tester, 2008, Shrivastava and Kumar, 2015) which has a substantial impact on agricultural productivity and ecosystem health. This problem is particularly severe in regions with low rainfall and high evaporation rates, such as parts of Australia, the Middle East, North Africa, and some areas of the United States (Qadir et al., 2014). The implications of plant salinity involve economic challenges resulting from decreased agricultural productivity and land deterioration, alongside environmental consequences like limited water availability, decline in biodiversity, and water source pollution (Pitman and Läuchli, 2002). In order to effectively address this problem and improve the situation, it is crucial to possess a comprehensive understanding of the complex relationship between soil salinity, the adaptive responses of plants, and prudent land management practises.

1.1 What is salt stress in plants?

Salt stress is defined as a situation in which an excessive concentration of salts in soil, such as sodium chloride (NaCl) ions, causes reduction in plant growth in general and lower crop yield. Globally, salt is the most toxic substance limiting the vital physiological processes in plants, which in turn affects their growth, development and survival. While salt stress is a considerable threat to all plants, especially those that are sensitive to salts, the harmful effects of salt stress on plants may differ depending on several parameters, such as soil and climate conditions or plant species (Tang et al., 2015). When the electric conductivity in soil media reaches approximately 4 dS/m (i.e. 40 mM NaCl), the soil is termed saline (Munns and Tester, 2008).
High concentrations of salt in the soil cause a water deficit in the plant and osmotic stress as a result of decreased osmotic potential (i.e. increased osmotic pressure) in the soil solution because water is not free or available where it surrounds solute molecules. In other words, soil salinity causes a reduction in soil water potential, limiting water uptake by the plant and therefore causing water stress and nutritional deficiencies (Seleiman et al., 2021). Moreover, salinity causes an ionic imbalance between the two sides of the plasma membrane, affecting

ion transport across the membrane and causing toxicity via enzyme inhibition. Salt stress affects wheat crop productivity through the inhibition of seed germination, a decrease in photosynthesis, the inhibition of enzymes, hormonal imbalance and cell death (Daei et al., 2009, Hasanuzzaman et al., 2017). The reduction in soil water potential and accumulation of Na^+ within the plant can result in hyperosmotic and hyperionic stresses, thereby causing nutritional imbalance (Hajihashemi et al., 2009). It is known that an increase in the salt concentration in the soil makes it difficult for plants to absorb other essential minerals due to the negative effect on the solubility of these minerals. Hajihashemi et al. (2009) have indicated that under conditions of salinity, plants take up ions from soil to different degrees, some ions like Na^+ adversely accumulate in the plant tissues limiting the absorption of necessary ions, such as K^+.

1.2 Plants and salinity

Scientists essentially divide plants into glycophytes and halophytes depending on how much water-soluble salt they can tolerate and still grow or develop (Flowers et al., 1977). Glycophytic plants include most crops that are sensitive or hypersensitive to high salt conditions. Their rate of growth may be reduced or inhibited in the presence of 100–200 mM of NaCl (Munns and Termaat, 1986). Salt-tolerant non-halophytes are a third category between glycophytes and halophytes that can withstand salt concentrations between 200 mM and 300 mM NaCl. Cotton, sugar beets, wheat, and barley are examples of plants whose growth is decreased when exposed to ≥ 200 mM NaCl. Regarding cereals, rye (*Secale cereale*) is considered to be the most salt tolerant, whereas rice (*Oryza sativa* L.) is among the most sensitive cereal crops (Hoang et al., 2016, Gong et al., 2020, Jeong et al., 2022). In terms of fruit trees, avocado and citrus are very susceptible to small amounts of salts.

In contrast to sensitive plants that suffer adverse effects from salt ions, numerous plants have the ability to survive in saline environments due to the adaptive mechanisms they possess. Such plants are called halophytes and can grow in high levels of salinity in excess of 200 mM of NaCl (Kosová et al., 2013). For instance, *Atriplex vesicaria* can complete its life cycle in 58 g/L (1M) of NaCl (Isayenkov, 2012), while *Salicornia bigelovi*, which is considered to be the most saline- tolerant vascular plant, can grow in 70 g/L (1.2M) NaCl (Ayala and O'Leary, 1995). The salt-tolerance features in the majority of halophytic plants are related to their morphological, physiological or anatomical adaptive mechanisms. Since halophytes often grow in extremely salty environments, they have different mechanisms to deal with high levels of toxic ions, such as salt compartmentalization (Hafeez et al., 2021). They frequently accumulate Na^+ and Cl^- ions

to reduce the osmotic potential of cells and therefore draw water in from the soil solution to meet transpirational demands. Most accumulated salts are transferred through the transpirational stream of the xylem to the target leaves. Furthermore, some tolerant plants, such as *Atriplex* (saltbushes), have salt-secreting glands in their stems or leaves enabling them to expel the excess content of salts to their surfaces, where they can be removed by wind or rain and prevent ion toxicity inside the plant (Glenn et al., 1998). Other salt-tolerant plants exclude salts and do not allow them to enter their vascular tissues (Acosta-Motos et al., 2017).

The tolerance level not only differs between glycophytes and halophytes, but also between various species and cultivars. Among the *Triticum* species, *T. aestivum* and *T. turgidum* ssp. *durum* are moderately salt-tolerant wheats, but *T. aestivum* is more tolerant than *Triticum turgidum* ssp. *durum* (Munns and Tester, 2008).

1.3 Effects of salinity on plants

Salinity causes damage to plants which can be recognized by many symptoms. Some of these symptoms can be observed in short-term treatment and others through prolonged exposure. Growth inhibition is the first such type of damage, giving rise to accelerated development, senescence and plant death. Salinization-induced abscisic acid (ABA) works on stomatal closure and results in oxidative stress leading to photosynthesis reduction and ultimately inducing cell death. A salinity experiment reported adverse effects resulting from the salinity of seawater on wheat, including decline in leaf expansion and thereby photosynthetic efficiency (Aldesuquy et al., 2014).

The harmful implications of salt stress are reflected not only in terms of plant phenotype but also the internal and cellular environment of plants, as explained in the following subsections.

1.3.1 Germination

Germination of seeds is a preliminary essential and vital process in the life cycle of all plants because of its importance in determining plant growth and yield. It is regulated by numerous parameters, both internal (such as genotype, hormones, age and dormancy of seeds) and external (such as salinity of soil, light, atmospheric gasses, temperature and moisture) (Wahid et al., 1999, Miransari and Smith, 2014). Several studies have reported on the negative effect of salinity on seed germination, salinity reduces germination and often extends the time needed for seeds to germinate in most plants. These include, for instance, important crops like *T. aestivum* L. (Akbarimoghaddam et al., 2011), *Zea mays* L. (Khodarahmpour et al., 2012),

Helianthus annuus (Mutlu and Bozcuk, 2007) and *Brassica napus* L. (AKRAM and JAMIL, 2007).

Salt stress either inhibits seed germination or leads to a state of dormancy. Afzal et al. (2008) assessed the effect of salinity on germination in two cultivars of wheat (Inqlab-91 and SARC-1) and found that seeds in non-saline conditions germinate quicker than those subjected to saline treatment (125 mM NaCl). Similar results have been found for *T. aestivum* L. cv. Caxton, exposed to five different concentrations of NaCl. Fuller et al. (2012) found the higher the salt concentration, the less the germination rate and the longer the germination time. High amounts of soil salt lead to reduced osmotic potential preventing the ingress of water to seeds. Uptake of salts inhibits the activity of enzymes in charge of metabolic processes required for germination because of ion toxicity. Moreover, the seed hormonal balance can be affected by salt impacting the mobilisation of the seed food reserves (Hasanuzzaman et al., 2013). In general, the effective germination of seeds leads to successful plant growth and higher productivity.

1.3.2 Plant growth

Salinity is one of the most unfavourable stresses to plant growth in most plants. It often affects plant height, the length of the root and shoot systems as well as leaf expansion resulting in decreased fresh and dry weight. These effects result from reduced cell division and cell elongation and the inhibition of photosynthesis. In addition to the negative effects on growth salinity negatively impacts the reproductive fitness of plants. Plants respond to extreme conditions in a systematic way via the deployment of genetic traits allowing them to cope with a number of growth challenges (Majeed et al., 2018). When subjected to salinity, plants respond by reducing their growth because of osmotic stress and water deficiency. The growth response depends on the duration of salt-stress and on the plant sensitivity to salt. When exposed to salinity, initially, plants lose water and therefore their cells shrink. Then, the cells recover their initial hydration level over time, but with a reduced elongation. Persistence of stress over a period of time impacts negatively vegetative and reproductive development via influencing the viability and forming of reproductive organs and changing the time of flowering (Munns, 2002). Plant growth responds to salinization at two stress phases: osmotic-stress phase and ionic-stress phase, as shown in (Figure 1.1). The growth of tolerant and sensitive plants declines during the first phase as a result of osmotic pressure. However, it remains stable in tolerant plants, and the decline increases in sensitive plants during the second phase due to ion toxicity in the leaves.

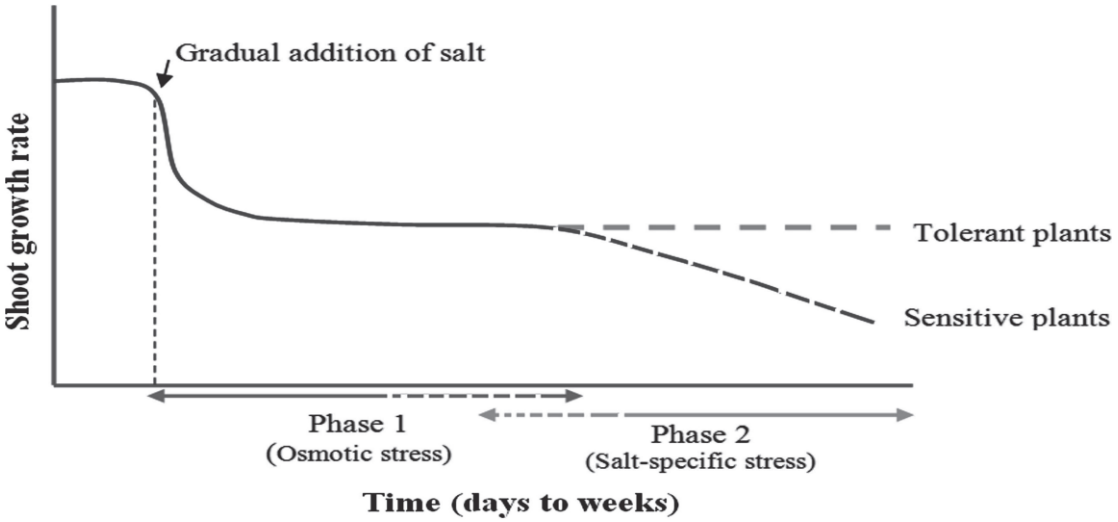


Figure 1.1: The two phases of growth response to salinity in tolerant and sensitive plants: phase 1, caused by osmotic stress is a quicker response, while phase 2 is caused by the ionic stress and is a slower response (Hasanuzzaman et al., 2013).

Salt stress negatively affects plants in all developmental stages and the specific response to salt might differ between growth stages. It has been shown that the responses of two wheat cultivars (S-24 and MH-97) to saline stress differ in the vegetative, booting and reproductive phases through disparity in phenolic content, lipid peroxidation and growth (Ashraf et al., 2010). These different responses are regulated by diverse sets of genes in various growth phases. A study conducted in chickpeas revealed varying gene expression profiles in different genotypes at different vegetative and reproductive phases under salt stress (Garg et al., 2016). Salt stress affects plant growth in conjunction with other abiotic environmental stresses. The expression of an assortment of genes in numerous plant species has been shown to change under different abiotic stresses (Chen et al., 2002, Xiong et al., 2002, Kirch et al., 2005). The expressed genes are associated with various responses, including transcription regulation, protein alteration, metabolism and signalling mechanisms. Deep understanding of how these genes are regulated is required to develop cultivars that are tolerant to salt stress.

1.3.3 **Yield**

The exposure of plants to any amount of salt adversely affects the yield quantity and quality. This is attributed to the injurious effect of salinity on the production of plant leaves and the reduction in photosynthetic rate and then senescence (Wahid et al., 1997). The yields of all susceptible crops and some tolerant plants decrease considerably under salinity conditions. Regarding wheat, it is known that the yield can be dependent on the number of spikes and yield

components, including the number of spikelets, as well as the amount and weight of grain. Asgari et al. (2012) investigated four genotypes of wheat and showed a negative correlation between wheat yield and salinity levels with varying impact on the different components of yield.

In terms of relative yield, a considerable difference has been found between crops in terms of salinity tolerance. For example, sugar beet (a salt-tolerant crop) exhibited 20% decline in dry weight, cotton (a moderately tolerant crop) exhibited a 60% decline and soybean (a sensitive crop) died when treated with 200 mM NaCl (Hasanuzzaman et al., 2013).

1.3.4 **Water uptake and retention**

The hydration of plant cells is necessary for metabolism and all the physiological processes that control plant growth. High concentrations of salt cause a reduction in soil water potential, limiting water uptake by the plant and therefore causing dehydration stress and nutritional deficiencies (Sánchez-Blanco et al., 2004, Chavarria and dos Santos, 2012). In contrast, low concentrations of salt allow plants to extract water from the soil and transport it through the xylem into the leaves due to high soil water potential and osmotic adjustment of the plant. In this case, by accumulating organic and inorganic solutes, plants maintain turgor and cell expansion and thus plant growth is achieved (Munns, 2002, Shahid et al., 2020).

In addition, water uptake in plants is closely related to transpiration which depends on stomatal conductance and stomatal density. In other words, plants lose most of the water absorbed from the soil through transpiration. Consequently, the water potential in leaves is reduced, which means that water flow from the soil depends on the hydraulic gradient in the xylem (Brodribb, 2009). However, once plants are exposed to salinity, they tend to close their stomata to preserve their internal water content (Bañón et al., 2012).

1.3.5 Ion uptake

Not only does soil salinity generate osmotic and dehydration stress, but it also affects the uptake of essential nutrients such as potassium that are important for plant growth through the adverse effects on solubility in soil solution and the competition of Na^+ against the uptake of K^+. This can be observed to a greater extent in sensitive plants than tolerant plants due to the accumulation of Na^+ ions in their cells (Mandhania et al., 2006).

Interestingly, net uptake of ions can be direct, through the apoplastic pathway, or indirect, through the symplastic pathway. In the apoplastic pathway, ions can move between the cells of the root cortex and flow immediately to the xylem, this pathway might be responsible for approximately 50% of Na^+ and Cl^- influx in *Oryza sativa* L. and other plants (PETERSON et

al., 1986, Krishnamurthy et al., 2009, Kronzucker and Britto, 2011, Shi et al., 2013). In contrast, in the symplastic pathway, ions enter the external cells of the root through specific transporters, such as nonselective cation channels (NSCCs), which might be the main channels for Na^+ influx and might be blocked by Ca^{2+} (Leng et al., 2002, Demidchik et al., 2018), or cyclic guanosine monophosphate (cGMP), Gd^{3+} or La^{3+} (Demidchik and Maathuis, 2007). Controlling such channels could make a difference between sensitive and tolerant species. Krishnamurthy et al. (2009) investigated the correlation between Na^+ absorption through roots and the apoplastic barriers in three rice cultivars with different tolerance levels. They found that the tolerant cultivar had the most extensive apoplastic barriers with the lowest Na^+ accumulation.

1.3.6 Photosynthesis and transpiration

Photosynthesis is the most important physiological process in plants, it is necessary for growth and survival. Under salt stress, photosynthesis is limited due to stomatal closure in response to the reduction of guard cells turgor mediated by signal molecules produced by the roots. As a result of stomatal closure, water loss by transpiration is prevented and CO_2 uptake via stomata and leaf mesophyll declines, which in turn limits photosynthetic performance and ultimately causes growth cessation (Flexas et al., 2004, Flexas et al., 2007, Sahu et al., 2023).

Exposure to salinity indirectly disturbs the photosynthetic electron transport chain and de-activates the Rubisco enzyme and therefore affects CO_2 fixation (Meyer and Genty, 1998, Chaves et al., 2009). Under salt stress, the rate of energy production in light-dependent reactions (i.e. ATP and NADPH) is lower than that of energy consumption in the Calvin cycle. Sharkey (1990) noted that some photosynthetic enzymes, such as sucrose-phosphate synthase and nitrate reductase, are inhibited due to the high Na^+ concentration and low CO_2 concentration in the intercellular spaces of plant leaves. In a recent work, Chaves et al. (2009) suggested that alterations in mesophyll conductance result from leaf shrinkage during salt stress, which gives rise to structural changes in the intercellular spaces or membrane permeability. Chlorophyll content which is an important determinant of photosynthetic performance is considered as a biochemical index of salt sensitivity. Stepien and Johnson (2009) and Ashraf and Harris (2013) pointed out that chlorophyll levels under salt conditions rise or do not change in tolerant plants, but reduce in sensitive plants

1.3.7 Plasma membrane damage

Salt stress can effectively increase the production of reactive oxygen species (ROS), which in turn lead to membrane damage. Hydrogen peroxide (H_2O_2) is a toxic ROS, the toxicity of which increases under conditions of salinity and hence adversely affects plant tissues. In addition,

peroxidation of membrane lipids and loss of membrane integrity are among the consequences of saline stress. As a result of lipid peroxidation and H_2O_2 accumulation, the membrane stability index has been shown to decline (Sairam et al., 2002) and electrolyte leakage has been found to increase (Mandhania et al., 2006). It has been shown that these damaging changes in plasma membrane are more obvious in salt-sensitive cultivars than tolerant cultivars of many species: cotton (Gossett et al., 1994), wheat (Mandhania et al., 2006) and rice (Khan and Panda, 2008). Moreover, salt stress also gives rise to membrane rigidity due to a reduction in unsaturated fatty acids, which therefore affects membrane permeability (Neffati and Marzouk, 2008).

1.3.8 Oxidative stress

At the cellular level, it has been shown that NaCl salinity induces oxidative stress in many plants due to its inhibitory action on photosynthesis (Hernández et al., 2001, Mittova et al., 2003, Mittova et al., 2004). Salinity indirectly causes water stress inducing the closure of stomata consequently reducing the uptake of CO_2 by photosynthesis. The lack of CO_2 assimilation under salt stress leads to increased ROS production because of the excessive excitation energy in the chloroplasts (Parida and Das, 2005, Ahmad et al., 2011). Under dehydration stress different ROS molecules are generated including H_2O_2, superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$) and hydroxyl radical (OH•) (Cheeseman, 1988). At high concentrations, ROS molecules are very harmful, however at low concentrations have an important role in mediating various plant responses as signalling molecules. ROS are extremely reactive and their accumulation creates toxicity and cellular damage in the form of DNA mutation, protein degradation or lipid peroxidation (Apel and Hirt, 2004, Ahmad et al., 2010). To mitigate these effects, plants produce a variety of protective enzymes that act as an antioxidative scavenging system. This means that an imbalance between ROS formation and the antioxidant defence system leads to oxidative stress and the consequent molecular and cellular damage (Demiral and Türkan, 2005).

Interestingly, ROS accumulation occurs in different cellular compartments, such as mitochondria and chloroplasts, which are the main sites of ROS production. Moreover, peroxisomes produce ROS (mainly O_2•– and H_2O_2) during their metabolism. Studies have indicated a reduction of H_2O_2 levels in the leaf peroxisomes of salt-tolerant and salt-sensitive *Pisum sativum* (Corpas et al., 1993), as well as in the root peroxisomes of *Lycopersicon esculentum* cultivars (Mittova et al., 2004). This is because of the imbalance between H_2O_2 generation and its detoxification under unfavourable conditions. In the mitochondria of salt-sensitive pea plants, the rate of O_2•– formation is higher than the rate of its scavengers under

70 mM salinity. In this situation, H_2O_2 concentration does not change, a phenomenon attributed to the permeability of mitochondrial membranes to H_2O_2 and the ability of H_2O_2 to accumulate under salt stress (Acosta-Motos et al., 2017). The correlation between the reduction in mitochondrial antioxidative scavenging activity and the increase in protein oxidation and lipid peroxidation has been reported in many works (Hernandez et al., 1993, Gomez et al., 1999).

1.4 Physiological and biochemical adaptation of plants to salt stress

When plants are subjected to high levels of sodium chloride, they suffer from hyperosmotic and hyperionic stresses and therefore nutritional imbalance (Hajihashemi et al., 2009). As a result, plants naturally adapt to these harmful effects through a range of physiological and biochemical responses to survive as best they can. Such responses may include ion sequestration and regulation of ion homeostasis, increased production of osmoprotectants, antioxidants and enzymes like ascorbate peroxidase, catalase etc, these responses are brought about after the induction of signalling pathways (Miransari and Smith, 2019). These responses involve the expression of stress response genes which enhance plant tolerance. A recent review has reported the importance of different biochemical compounds, including osmolytes, polyamines, antioxidants, etc., to alleviate salt stress and enhance growth in rice (Ganie et al., 2019).

1.4.1 Short-term responses

Osmotic stress Under conditions of osmotic stress, such as salinity and drought, plants quickly face a reduction in stomatal conductance and photosynthesis, which affects their growth rate and development, as mentioned in relation to rice (Moradi and Ismail, 2007, Sahu et al., 2023), wheat (Rahnama et al., 2010), barley (Yang et al., 2009a) and maize (Azevedo Neto et al., 2004). Likewise, the application of polyethylene-glycol, mannitol and KCl exerts rather similar effects (Chazen et al., 1995), which suggests that these effects are a result of changes in the water relations between plant and soil. A reduction in growth can also be noticed in the root system (Rodriguez et al., 1997). In this stressed situation, plants display a great range of responses, such as morphological modifications (e.g. enhanced root growth and inhibited shoot growth), ion homeostasis (exclusion, extrusion or compartmentalization) and metabolic modifications (e.g. production of osmolytes and hormones, carbon assimilation etc.). There is no doubt that these responses may be triggered by either primary or secondary osmotic stress signals. The latter could be plant hormones (such as ABA, ethylene, etc.), secondary messengers (such as sugar, phospholipids, etc.), or ROS (Xiong and Zhu, 2002, Rejeb et al., 2014).

An imbalance in water status induces the synthesis of ABA, which acts as an internal signal regulating water status within the plant via guard cells causing stomatal closure and the induction of the expression of ABA-responsive genes (Fahad et al., 2015). ABA is synthesized in plants either in the roots, transported through the transpirational stream in the xylem to the leaves, or within the leaves themselves. ABA might be then loaded into the phloem and circulated back to the roots. It may be recirculated to leaves via the xylem under environmental stresses, such as that caused by excess salinity (Hartung et al., 2002).

Generally, ABA levels correlate with the water potential of soil or leaves (Moons et al., 1995). Zhang et al. (2006) reported that increasing the concentration of this stress hormone, mediates salt-stress responses.

Ionic stress. Soil salinity not only imposes osmotic stress on plants but also ionic stress. It is very well known that sodium chloride is one of the most important inorganic soluble compounds assisting in water absorption by increasing osmotic potential and then maintaining cell turgor. However, an excess accumulation of these ions can cause toxicity in all compartments of the cell (Pardo and Quintero, 2002). Toxicity measurements have been conducted in many studies using a range of methods including X-rays, dyes, microelectrodes and the extraction of entire tissues (Wissing and Smith, 2000, Carden et al., 2003, Chen et al., 2014, Patishtan et al., 2018).

It seems likely that high Na^+ and Cl^- concentrations affect water structure, enzyme activity and nutritional imbalance (Isayenkov and Maathuis, 2019). Early studies found that both halophyte and glycophyte enzymes are susceptible to high salinity, although there are differences between the two categories in terms of tolerance (Flowers, 1972, Greenway and Osmond, 1972). However, a recent study showed that under salt treatment, the enzyme activities of the citric acid cycle increased in the tolerant Grand Brix tomato genotype but decreased in the sensitive Marmande RAF genotype (de la Torre-González et al., 2017). In addition, higher concentrations of Na^+ negatively influence the requirements of many enzymes for K^+ as a cofactor.

Excessive amounts of NaCl entering the plant will transfer via the transpiration stream and eventually injure the plant leaves, particularly the oldest, reducing the plant growth rate. To overcome ion toxicity, salt exclusion is one of the mechanisms used by plants to avoid salts building up within the plant over time. Some halophytic plants compartmentalize high levels of salt in their roots or the vacuoles of leaves, whereas others excrete salt through salt bladders or glands, allowing them to survive for a long time (Munns et al., 2006).

1.4.2 Long-term responses

Osmoregulation. As long as plants are exposed to salinity and osmotic stress, they need to deploy biochemical responses to facilitate absorption of water and retention of water potential within the plant tissues below the level of soil-limiting water efflux and therefore maintain cell turgor and plant growth (Hasegawa et al., 2000). To achieve this, plants osmotically adjust by taking up soil solutes or by synthesizing organic osmolytes, such as soluble sugars, amino acids (e.g. Proline, glutamate, glycine, betaine) and organic acids. Osmolytes are low in molecular weight and are very soluble, assisting plants to survive under various salt-induced stresses. The accumulation of these compatible solutes, even at higher concentrations, is non-toxic to enzymes and metabolic activities, compared to the accumulation of inorganic ions (Sairam et al., 2002).

Not only are these osmoprotectants non-toxic, they also play a critical role in protecting plants from the damage caused by ROS and toxic ions. Liang et al. (2018) have suggested that the synthesis of compatible solutes enhances the osmotic balance at the whole plant and cellular levels, which in turn improves salt tolerance in plants. An early work on transgenic tobacco demonstrated that the accumulation of mannitol had a role in plant response to high salinity (Tarczynski et al., 1993). The role of mannitol in improving plant growth and tolerance to drought and salinity was evidenced by (Abebe et al., 2003). They transferred the mannitol-1-phosphate dehydrogenase (mtlD) gene from tobacco into wheat to produce mannitol as it is not normally synthesized in this plant. They found that an accumulation of mannitol played a great role in osmotic adjustment and the stabilization of macromolecules and scavenging of ROS. These findings are similar to those of other studies modelling plant tobacco, in which it has been suggested that mannitol can minimize oxidative damage and can also be an OH• scavenger (Shen et al., 1997). It has been shown that the levels of compatible solutes differ between species and even among plant organs (Parvaiz and Satyawati, Verbruggen and Hermans, 2008). Studies of other osmoprotectants have also demonstrated that some plants physiologically respond to salt and osmotic stresses with increased production of compatible substances such as Proline. Proline has been found to participate in lowering osmotic potential (Verbruggen and Hermans, 2008), storing carbon and nitrogen (Hare and Cress, 1997), detoxifying ROS (Szabados and Savoure, 2010), protecting the enzyme activities of photosynthesis (Reddy et al., 2015). Also Proline induces adaptive responses by acting as a stress signal (Maggio et al., 2002) under unfavourable conditions. Nxele et al. (2017) investigated Proline content in sorghum plants during salt stress and drought, finding an increases accumulation in response to

these stresses. It has been revealed that during severe conditions, such as salt stress, delta-1-Pyrroline-5-Carboxylate Synthase (P5CS) plays an important role in Proline biosynthesis enhancing osmoregulation in plants (Rai and Penna, 2013). The knockout of the P5CS1 gene leads to a decrease in stress-induced Proline production, demonstrating its contribution to Proline biosynthesis.

***Potassium retention*.** Redundant amounts of Na^+ ions around the root surface disrupt the K^+ nutrition of plants. This can be attributed to the chemical nature of Na^+ and K^+ ions being very similar. Plants take up potassium either through low- or high-affinity transporters. It has been shown that Na^+, as a key competitor of K^+, inhibits the low-affinity K^+ transport system, which has a lower selectivity for potassium ions compared to sodium ions (Schachtman, 2000, Nieves-Cordones et al., 2017). Under salt stress, some plants respond by employing the more selective high-affinity system to maintaining sufficient K^+ nutrition.

It is worth noting that K^+ deficiency results in plant growth inhibition because of its importance in nutrition and maintaining cell turgor pressure, as well as enzyme activity. In case of potassium deficiency, plants become more susceptible to biotic and abiotic stresses (Zörb et al., 2014). Potassium is an essential macronutrient, playing a leading role in plant growth, metabolic processes, cytoplasmic pH and ionic homeostasis, functioning of stomata and stress adaptation (Marschner, 2011). Numerous studies have indicated the importance of K^+ in cell signalling and its relationship with programmed cell death (PCD) under conditions of salinity (Huh et al., 2002, Shabala and Pottosin, 2014, Wu et al., 2018). In the case of salt-sensitive plants, salinity induces K^+ leakage, which worsens with increased Na^+ influx and eventually causes PCD (Demidchik et al., 2014). A recent review has pointed to the role of shaker KOR channels in K^+ loss from the root system (Figure 1.2) (Isayenkov and Maathuis, 2019). K^+ can also lead to modulations in metabolic pathways, which in turn can save energy contributing to the cell defence process, as well as cell repair (Demidchik et al., 2014, Isayenkov and Maathuis, 2019). However, Na^+ ions inhibit several enzyme activities in the cytoplasm and this effect depends on the K^+/Na^+ ratio. In other words, the higher the cytosolic K^+/Na^+ ratio, the lower the damage. Consequently, maintaining this ratio is critical for all plant growth, regardless of whether they are halophytes or glycophytes, as their enzymes are similarly affected by NaCl (Glenn et al., 1999). To maintain this ratio, plants respond using Na^+ exclusion, extrusion or vacuolar compartmentation mechanisms.

The transport systems of K^+ and Na^+ ions determine plants' salt tolerance because of their control over the cytosolic K^+/ Na^+ ratio. One such system is the high-affinity K^+ transporter

(HKT) plasma membrane family, which has the ability to transport K^+ and also Na^+ in some conditions (Rodríguez-Navarro and Rubio, 2006). The key function of HKT1 transporters under salinity stress has been identified in several species such as Arabidopsis, tomato, rice, wheat and sorghum (Nawaz et al., 2019, Jaime-Pérez et al., 2017, Xu et al., 2018, Wang et al., 2014, Golldack et al., 2002). The vital role of HKT1 transporters in K^+ and Na^+ homeostasis in monocots has recently been mentioned (Yao et al., 2010). Moreover, the multiple HKT1 genes in rice, barley and wheat have been characterized as participating in salt stress tolerance (Platten et al., 2006, Munns et al., 2012, Yao et al., 2010). In the Arabidopsis plant, the knockout of the HKT1 gene results in sensitivity to salt, which proves its role in salinity tolerance (Berthomieu et al., 2003, Horie et al., 2005).

Exposure to salinity influences the expression of K^+ transporter genes, they were shown to be down-regulated in rice (Golldack et al., 2003) and up-regulated in *Arabidopsis thaliana* (L.) (Maathuis, 2006). These effects could be at the cell or tissue level. For instance, the mesophyll cells of salt-tolerant wheat and barley have the ability to retain K^+ unlike salt-sensitive cultivars (Wu et al., 2013, Wu et al., 2015). Another example is that K^+ influx in the root apex of barley is higher than that in the mature root zone (Shabala et al., 2006).

Interestingly, exposing plants to high concentrations of NaCl results in a rapid boost in cytosolic Ca^{2+}, which plays a protective role under such environmental conditions. It has been observed that salt induces an increase in Ca^{2+} in the root (Kiegle et al., 2000, Tracy et al., 2008). The positive effects of Ca^{2+} are mediated via a signalling pathway that regulates the expression of Na^+ and K^+ transporters. It has been reported that the SOS3-SOS2 kinase complex is stimulated by Ca^{2+} and as a result the SOS1, Na^+/H^+ exchanger is activated and the sodium taken-up by HKT2 is supressed (Figure 1,2) (Martínez-Atienza et al., 2007, Isayenkov and Maathuis, 2019). In addition, calcium ions directly inhibit sodium uptake by both NSCC channels: cyclic nucleotide-gated channels (CNGCs) and ionotropic glutamate receptors (GLRs) (Figure 1.2) (Demidchik et al., 2002).

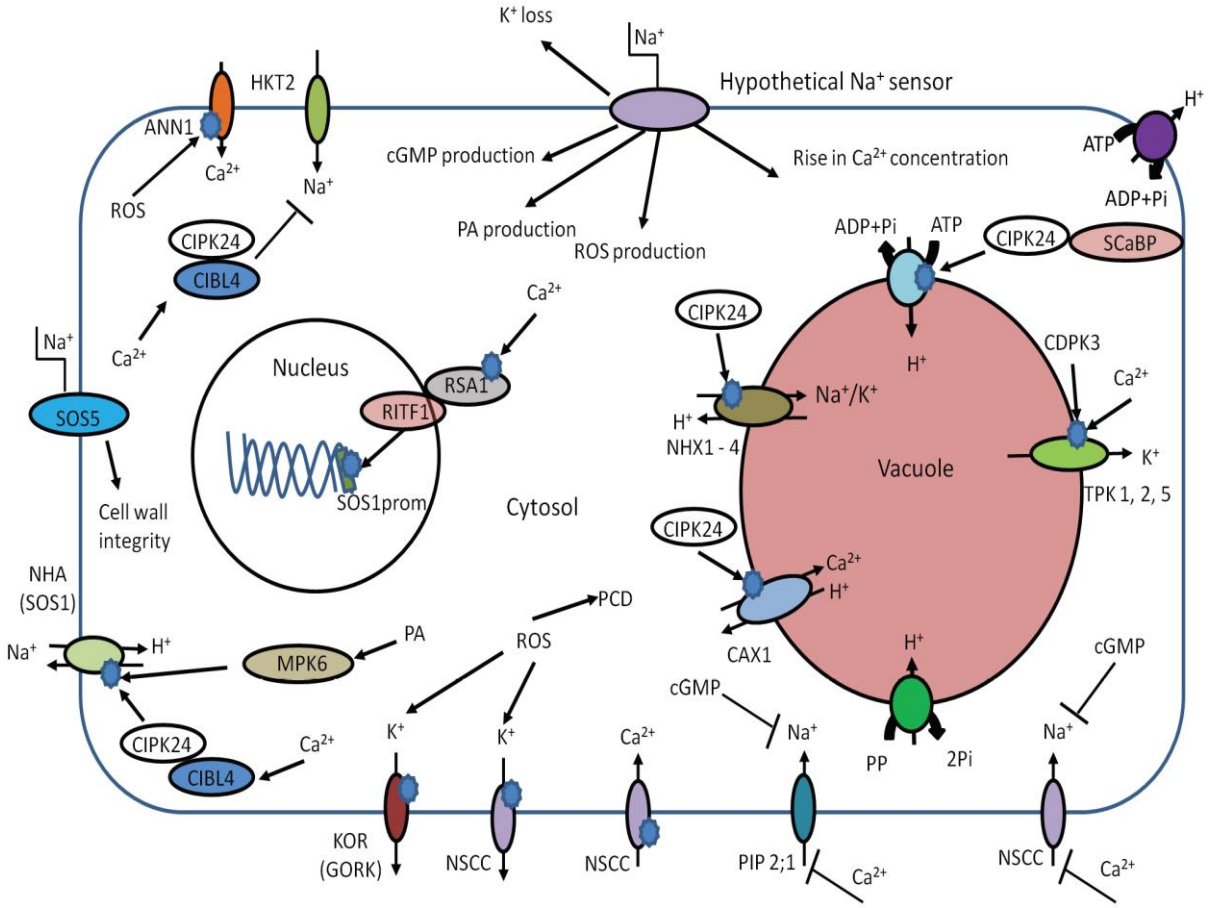


Figure 1.2: Schematic signalling pathway in a plant cell under salt stress. High concentrations of Na^+ induce an increase in cytosolic Ca^{2+} and ROS, cGMP and phosphatidic acid (PA) production within the cell. Ca^{2+} ions stimulate CIBL4-CIPK24 (SOS3-SOS2) complex, which in turn activates SOS1 and supresses Na^+ influx by HKT2. Ca^{2+} ions also inhibit NSCC channels (CNGCs and GLRs) (Isayenkov and Maathuis, 2019).

Antioxidants. As unavoidable consequence of abiotic stresses, including salinity, the accumulation of ROS within the plant cells plays a considerable part in oxidative damage to these cells and ultimately affects plant growth and grain yield in a passive manner (Zhou et al., 2019). To avoid these adverse implications, plants strictly regulate the balance between the generation and elimination of ROS. To put it another way, plants cope by operating an antioxidative defence system that prevents such damage and detoxifies the ROS (Ayvaz et al., 2016). This efficient system consists of reducing enzymatic systems (e.g. glutathione reductase, ascorbate peroxidase, superoxide dismutase, catalase, etc.) and nonenzymatic reductants (metabolites such as glutathione, flavonoids, ascorbic acid, carotenoid, etc.) (Hasanuzzaman et al., 2012). The activity of these components rises under extreme environmental stresses and is more pronounced in tolerant plants than sensitive ones (Hernandez et al., 2000, Sairam et al.,

2000, Zhou et al., 2019), suggesting that the defence system perhaps works more effectively under unfavourable stresses.

Numerous studies have indicated remarkable alterations in the activity levels of scavenging enzymes, such as catalase, guaiacol peroxidase, glutathione reductase and superoxide dismutase in wheat cultivars to mitigate ROS-induced oxidative stress. It is clear, therefore, that the mechanisms of ROS elimination are upregulated in these plants (Huseynova et al., 2014, Kocsy et al., 2002, Mishra et al., 2013, Rao et al., 2013, Srivalli and Khanna-Chopra, 2001). It has also been demonstrated that tolerant genotypes exhibit higher defence activity, leading to lower cellular damage. Responses of wheat may rely on stress extent and its intensity alongside with tissue type (Caverzan et al., 2016).

It has been reported that a higher expression of various antioxidant genes participates in salt tolerance in many plants. For example, *OsECS* (Choe et al., 2013) and *OsMSRA4.1* (Guo et al., 2009) genes in rice, *DHAR*, *OsAPXa* and *OsAPXb* genes in transgenic Arabidopsis (Ushimaru et al., 2006, Lu et al., 2007) and GPX genes in wheat (Zhai et al., 2013). These studies have discovered a positive correlation between this expression and oxidative damage, such that the complex antioxidative system acts as a ROS scavenger and a plant protector.

Signalling molecules. Plants' perception of various environmental factors and their functional responses are among the most crucial properties for survival under stress conditions. When plant cells are exposed to any stress, they need to have the capacity to sense the signals rapidly and respond effectively. This response could be induced by signals deriving from osmotic stress (Chaves et al., 2003) or secondary metabolites, such as plant hormones, second messengers and ROS (Rejeb et al., 2014). It is widely known that phytohormones and metabolites at low concentrations, play a great role in the regulation of plant development. It has been demonstrated that exogenous application of these hormones contributes to alleviating the effects of abiotic stresses such as drought and salinity. For example, the treatment of two *T. aestivum* L. cultivars, Giza 168 and Sohag 3, with gibberellic acid alleviates the harmful effects of salt stress through improving their photosynthetic pigments, growth rate and grain yield (Shaddad et al., 2013). The work of Alasvandyari et al. (2017) indicates that the seed priming of Safflower plants with glycine betaine has a mitigating effect against salinity stress. Glycine betaine is a stress-inducible osmolyte which triggers the antioxidative system, minimizes lipid peroxidation and maintains osmotic regulation and ion homeostasis, eventually protecting the plants from oxidative damage and increasing salt tolerance.

It is known that plants exposed to salt usually send water deficit or hydraulic stress signals from the roots to other organs, causing a reduction in cell turgor. As a result, ABA-independent stomatal closure is triggered to maintain the internal water status and cell turgor (Takahashi and Shinozaki, 2019). Some recent studies have suggested that stomatal closure as a short-term response could be mediated by external Ca^{2+} influx before ABA-dependent stomatal closure is activated as a long-term response (Kudla et al., 2018, Konrad et al., 2018).

In addition, as previously mentioned, ROS at low concentrations participate in salt tolerance as signalling molecules. For instance, H_2O_2, while significant in several biological processes, specifically in metabolism and signal transduction pathways as a freely diffusible secondary messenger (Saxena et al., 2016), has been shown to play a dual role in plant cells, participating in oxidative stress induction and cell death at toxic concentrations (Dat et al., 2003). It is interesting that H_2O_2 might be involved in ABA signalling and stomatal closure under conditions of plant exhaustion. Pei et al. (2000) have reported that ABA triggers H_2O_2 synthesis in guard cells, which in turn activates Ca^{2+} channels in these cells and ultimately leads to stomatal closure.

1.5 Anatomical adaptation of plants to salt stress

In plants, many physiological and anatomical alterations might occur in response to salt stress. Studying these alterations is a good way of knowing the extent to which internal plant tissues are affected. The high amounts of salt in the leaves of dicotyledonous halophytes induce cell succulence due to cell expansion resulting from the accumulation of salts in leaf vacuoles, this is rare in monocotyledonous plants. Salinity adversely affects the anatomical properties of leaves, leading to metabolic disturbances, such as a reduction in photosynthesis. Increasing leaf thickness and succulence values and decreasing intracellular spaces and tissue density are among the common anatomical changes in response to stress (Romero-Aranda et al., 1998). Navarro et al. (2007) evaluated the anatomical alterations in *Arbutus unedo* plants irrigated with different concentrations of NaCl solutions, they correlated the decline in CO_2 assimilation with the reduction in intracellular spaces of mesophyll and stomatal closure. It is also likely that the Cl^- ion is involved in anatomical changes in leaves, causing enlargement in cell size and increasing their succulence (Franco-Navarro et al., 2016). Similar anatomical alterations have recently been observed in many stressed plants: *T. aestivum* L. (Nassar et al., 2020), *Solanum pennellii* (Albaladejo et al., 2017), *Salicornia freitagii* (Akcin et al., 2017), *Acacia karroo* and *Acacia saligna* (Kheloufi and Mansouri, 2019).

Salt stress not only affects leaf thickness and succulence but also leaf ultrastructure, including alterations in the number and size of chloroplasts, changes in starch content, reductions in the number of mitochondria cristae, etc. (Hernandez et al., 1993, Acosta-Motos et al., 2017). Based on anatomical studies of leaves, it is clear that salt-sensitive plants are subjected to more damage than tolerant plants. Hernhdeza et al. (1995) studied the effect of salinity on two pea cultivars with different degrees of tolerance to NaCl. They found a considerable decline in the chlorophyll content and chloroplast integrity of sensitive cultivars compared to tolerant cultivars. In addition, they noted that the starch content decreased only in tolerant cultivars, which might be attributed to the fact that these plants consume starch in several physiological processes, such as osmotic adjustment, which enhances their tolerance to salt stress (Munns and Gilliham, 2015, Sánchez-Blanco et al., 2004). There is evidence that the chloroplast ultrastructure changes and thylakoids being dilated thus the photosynthetic rate is reduced under salt-stress in NaCl-susceptible plants (Navarro et al., 2007).

1.6 Sodium concentration in the cytosol

High Na^+ concentrations in the plant cytosol mainly cause salt toxicity, particularly in the leaves, resulting in damage to plant growth. Numerous physiological and metabolic processes, and molecular functions are affected under pronounced Na^+ elevations (Xue, 2002). It has been claimed in some papers that the threshold level of cytosolic sodium concentration is ~30 mM (Munns and Tester, 2008, Tester and Davenport, 2003), whereas other articles report a range from 50 to 200 mM (Kronzucker and Britto, 2011, Flowers et al., 2015). Halperin and Lynch (2003) undertook an experiment that measured Na^+ accumulation in the salt-exposed root hairs of *Arabidopsis thaliana* and found it to be lower than 65 mM. However, to control the inhibitory effect of high Na^+ concentrations, plants usually maintain ion homeostasis by excluding Na^+ influx, sequestering sodium in vacuoles, or re-exporting excess content of Na^+.

***Na^+ exclusion*.** As high levels of sodium ions are harmful and toxic, the Na^+ exclusion mechanism in protecting plants has been widely studied (Liu et al., 2019b, Chen et al., 2020). It has been determined that Na^+ exclusion in the plant root is one of the crucial determinants of salt tolerance in plants. Net salt accumulation within plants is more likely to be determined by the influx and efflux of Na+. It is well known that NSCC and HKT channels (Rodríguez-Navarro and Rubio, 2006) mediate Na^+ influx, while Na^+/H^+ antiporter (SOS1) mediates efflux (Ali and Yun, 2017). SOS1, SOS2 and SOS3 proteins function to balance the Na^+ ion in plants

under saline environmental constraints by controlling its efflux from cell cytosol to apoplast (Figure 1.2) (Zhu, 2003). It has been reported that SOS1 overexpression results in increased salt tolerance in transgenic tobacco and Arabidopsis plants (Yue et al., 2012, Yang et al., 2009b, Oh et al., 2009) and the expression of SOS1 has been shown to be at its highest level in the root apex. Nevertheless, the knockout genes involved in the SOS pathway cause hyper-salt sensitivity in halophytic and glycophytic plants (Shi et al., 2000, Oh et al., 2009), suggesting that SOS1 mainly participates in sodium exclusion.

In an experiment conducted by Cuin et al. (2011), the wheat genotype Kharchia 65 was the most tolerant to salinity among the genotypes studied as it exhibited the highest Na^+ exclusion and highest SOS1 activity in the root region. The ability of the genotype to control sodium transport to the target leaves and prevent its accumulation in the metabolically active cytosol is a critical trait of NaCl tolerance in plants.

***Na^+ compartmentation*.** The large vacuoles in plant cells store Na^+ away from metabolic activities in the cytoplasm and mitigating against its toxic concentrations in the cytosol. Na^+ sequestration, which is mediated by vacuolar Na^+/H^+ exchangers, is an efficient mechanism for avoiding the damaging effects resulting from high levels of Na^+ in the cytoplasm (Rahnama et al., 2011). In addition, this mechanism contributes to an increase in the intracellular osmotic potential through the accumulation of sodium ions as osmotica, resulting in water uptake into the cells (Blumwald et al., 2000). As a result, the vacuolar volume will expand, further enhancing Na^+ compartmentalization.

Many studies have shown that the NHX family and AVP1 are crucial transporters playing a great role in Na^+ ion homeostasis in several species (Gouiaa et al., 2012, Munns and Tester, 2008). Overexpression of these proteins enhances the depositing of vacuolar Na^+, leading to improved salt tolerance in plants (Zhang and Blumwald, 2001, Chen et al., 2007). Importantly, it has been observed *in vitro* that the enzymes of both halophytic and non-halophytic plants are susceptible to salt stress (de la Torre-González et al., 2017), suggesting the significance of the compartmentalization process in all plants, regardless of their degree of tolerance to salinity.

***Na^+ Excretion*.** Besides the compartmentalization of vacuolar Na^+, high concentrations of Na^+ arriving in the leaves of dicotyledonous halophytes can be disposed of by specialized glands (modified trichomes) or epidermal bladder cells. This secretion mechanism has been shown to mitigate the toxic effects of salt in leaves and thus enhance salt tolerance (Flowers, 2019). Salt

glands are a superior feature that improve salinity tolerance in halophytes, allowing them to store and eliminate excessive amounts of salt from their tissues. Plants using this system are termed “recretohalophytes”, referring to the salt disposal mechanism (secretion) by salt glands (Lüttge, 2019). Some mangrove species, such as *Aegiceras corniculatum,* can restrict salt content by secreting it through salt glands on their leaf surfaces to form salt crystals (Liang et al., 2008).

In contrast, approximately 50% of halophytes deposit sodium ions in external bladder cells that are ten times larger than normal epidermal cells, enabling them to sequester more ions. Within naturally NaCl-adapted plants, salt bladders are very widespread, for example in *Atriplex lentiformis* leaves and *Chenopodium quinoa* stems, storing toxic ions away from metabolically active tissues (Acosta-Motos et al., 2017).

1.7 Na^+ sensing and transport systems in plants

In contrast to animal cells, the specific mechanism of sodium sensing is yet unknown in plants. However, it has been reported that high concentrations of external NaCl could cause an increase in cytosolic Ca^{2+}, ROS, cGMP and phosphatidic acid (PA). Data from different plants suggest that Annexin 1 (ANN1) and SOS5 function as extracellular Na^+ sensors. Under high sodium conditions, ANN1 has been found to mediate ROS-triggered Ca^{2+} uptake through the cell membrane (Laohavisit et al., 2013). In contrast, SOS1 has been proposed to sense intracellular Na^+ (Shabala et al., 2005). SOS2-SOS3 complex is involved in the activation of SOS1 via phosphorylation (Martínez-Atienza et al., 2007). Furthermore, mitogen-activated protein kinase 6 (MPK6), activated by PA, directly phosphorylates the Na^+/H^+ antiporter (Yu et al., 2010). Later studies have revealed that cGMP might be one of the early response components of salinity. cGMP inhibits sodium influx by both NSCC channels, CNGCs and GLRs, which are blocked by external Ca^{2+} (Figure 1.2) (Isayenkov and Maathuis, 2019).

It is noteworthy that Na^+ enters plant roots passively through NSCCs and some HKT family transporters. Net sodium ions move toward the stele to reach the transpiration stream in the xylem. However, not all these ions reach the xylem, some being sequestered in the vacuoles of root cells (i.e. epidermis, cortex and endodermis cells), a process that is mediated by tonoplast Na^+/H^+ antiporters (NHX family). Information afforded by X-ray microanalysis suggests that the vacuolar Na^+ compartmentalization rate in wheat plants is more pronounced in the epidermis than in the endodermis (Munns and Tester, 2008). Besides sodium sequestration, most Na^+ ions taken up in glycophytes are actively pumped back out into the soil solution,

consuming significant energy, through Na^+/H^+ antiporters (SOS1) located in the plasma membrane (Tester and Davenport, 2003). The remaining Na^+ ions is transported or loaded from stelar cells to the xylem via the SOS1 plasma membrane. It has also been suggested that CCC co-transporters and SKOR channels play an efficient role in xylem sodium loading (Figure 1.3) (Wu, 2018).

In contrast, sodium unloading from the xylem is an important way of protecting the shoot from Na^+ toxicity and controlling salinity tolerance. This recirculation of Na^+ is achieved by HKT-type transporters, which probably participate in both Na^+ loading and unloading (Figure 1.3) (Davenport et al., 2007). The expression of *AtHKT1* (in Arabidopsis) and *SIHKT1, 2* genes (in tomato) has been found to be associated with Na^+ retrieval from the xylem (Berthomieu et al., 2003, Jaime-Pérez et al., 2017). Ions loaded to the xylem transfer through the transpirational stream to transpiring leaves, where their ultimate fate is either to be deposited in the leaf vacuoles or secreted out of the leaf.

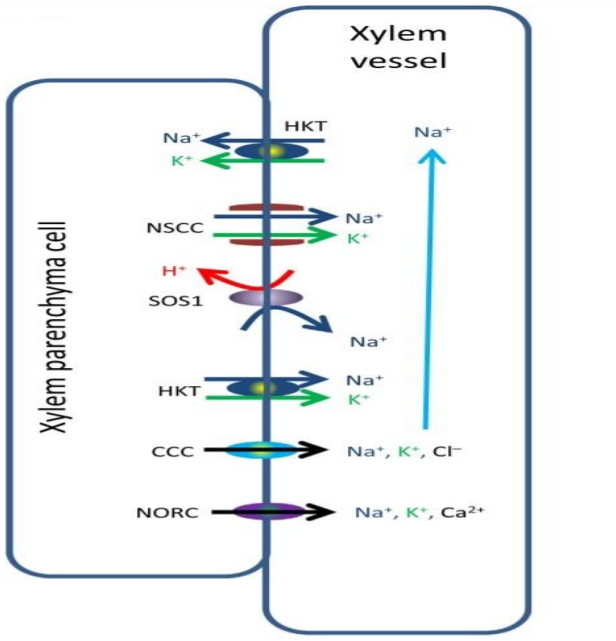


Figure 1.3: Schematic presentation of the loading and unloading of sodium ions showing the different transport systems of sodium in and out of the xylem (Wu, 2018).

1.8 Transcriptional regulation improves salt tolerance in plants

In response to salinity, plants deploy various molecular, physiological, morphological, and anatomical responses against salt-stress (Marin et al., 2006). The complexity of the different alterations induced by salt-stress makes it difficult to understand the molecular basis of plant responses to salinity. This makes it difficult to devise an efficient strategy to produce salt-tolerant crops with high productivity. Although significant discoveries have been made recently, more effort is needed to develop novel strategies that might help mitigate the harmful effects of these stresses. However, the levels of transcriptional changes under abiotic stresses vary between plant tissues, being observed to a greater extent in those sensing stress early rather than those subsequently detecting it. In recent research on drought-treated Arabidopsis plants, root cells exhibited higher levels of transcriptional alterations compared to shoot cells, indicating the involvement of numerous candidate genes that play a regulatory role in plant drought responses (Bashir et al., 2018).

At the genetic level, salt stress induces a variety of responsive genes in plants, classified by Munns (2005) into three sets, including genes encoding NaCl transporters (as mentioned previously), osmotic-induced genes (such as those encoding osmolytes or protective proteins) and growth-enhancing genes (such as those encoding transcription factors, plant hormones and protein kinases or phosphatases). The expression level of these genes has been shown to change in response to different unfavourable environmental cues in many species. For example, ABA is synthesized under dehydration conditions, inducing several genes as an adaptive cellular response to osmotic stress induced by drought or high salinity stresses. ABA accumulation within tissues and its transport has been demonstrated to play a great role in plant stress tolerance as it enables plants to regulate their internal water status (Osakabe et al., 2014). This occurs through the signal cascades of ABA, which lead to the activation of downstream components, such as transcription factors and ion transporters. These play roles in the adaptive responses of plants, including stomatal closure, stress-responsive gene induction or the production of compatible solutes, which eventually give plants the capability to survive under stress conditions (Laloum et al., 2018). Many transcription factors have been identified as positively regulating ABA-dependent gene expression in response to salt stress. For instance, the overexpression of *ZmMYB3R*, a maize MYB transcription factor, in transgenic Arabidopsis increases the growth rate and ABA content under salinity and drought stresses to a greater extent than is the case for wild type plants. In interpreting these findings, Wu et al. (2019) attributed the plant salt and drought tolerance to the upregulation of stress/ABA gene expression

by *ZmMYB3R*. According to Agarwal et al. (2019), bZIP transcription factors play a regulatory role in the expression of genes involved in tolerance to different stresses, such as salinity, cold and drought.

The intricate manner that plants respond to salt stress involves interactions of numerous genes, proteins, different metabolic and signalling pathways. Developing salt tolerant cultivars requires identifying different key genes and metabolic pathways associated with salinity tolerance. High-throughput sequencing methods like RNA sequencing (RNA-Seq) and microarray can help understanding how crops respond to salinity stress by identifying salt responsive genes and their functional and biological pathways. Transcriptomics, a global gene-transcript sequencing approach, can provide insights into stress associated genes with their post-transcriptional alterations. RNA-seq and microarrays are two techniques commonly used to study the gene expression patterns, with RNA-seq being more common due to its accuracy and rapidity (Kumar et al., 2022). These techniques have been used in studies on wheat (Amirbakhtiar et al., 2021, Liu et al., 2019a) and other crops such as rice (Chandran et al., 2019), maize (Qian et al., 2020) and sorghum (Chen et al., 2022) to identify salt candidate genes and related pathways.

1.9 Post-transcriptional regulation of salt responsive genes

Alternative splicing (AS) is one of the essential post-transcriptional mechanisms in eukaryotic genomes, including plants. AS, discovered in the late 1970s, is a process by which exons and introns in pre-mRNAs can be included or excluded from the mature mRNA (Figure 1.4). This process is important for complex organisms and can change under different conditions, such as stress. It is an important process in controlling mRNA polymorphism and then boosting proteome diversity, which derives from the same gene (Kelemen et al., 2013). This process occurs in the nucleus when the spliceosome differentially recognizes splice sites in pre-mRNA, resulting in multiple transcripts with exon skipping, intron retention or inclusion of cryptic sequences of exon. Furthermore, this regulatory mechanism leads to fine tuning of the gene expression and eventually enhances plant functions. It has been estimated that almost 70% of multi-intron genes in plants undergo such regulatory AS, with the predominant event being intron retention (Chaudhary et al., 2019). Retained intronic regions affecting stability, localization and translation of transcripts (Wong et al., 2013) largely produce non-sense mRNAs with premature termination codons that are recognized by specific proteins during the translation process, leading to the degradation of these transcripts through the non-sense-

mediated mRNA decay system (Laloum et al., 2018). The non-degraded transcripts with retained introns have been reported to produce truncated proteins, be sequestered in cell nucleus and then released on demand or act as protein-coding introns called exitrons (Filichkin et al., 2015, Staiger and Simpson, 2015).

Gene expression patterns in plants are fine-tuned by a post-transcriptional mechanism in various developmental stages and based on different environmental cues. Transcriptome data have confirmed that abiotic stresses clearly change AS and thereby control both the ratios and timing of sense (functional) and non-sense (intron-retaining) transcripts (Filichkin et al., 2015). However, the way in which the ratios and timing of splicing in plants to adapt to environmental stresses is yet unclear. A relatively recent study revealed the effect of salt stress on increasing the AS frequency in the root of a wheat cultivar, Arg (Amirbakhtiar et al., 2019), confirming the significant role of this post-transcriptional modulation in response to adverse stresses. Through AS analysis, 1,482 alternatively spliced genes have been identified, generating 4,041 different isoforms involved in various pathways responding to salt stress. Similarly, Fu et al. (2019) identified 40 and 33 alternatively spliced genes in barley roots and shoots, respectively, which enhance salt tolerance through their participation in transcription regulation and the metabolic pathways and functions associated with the concerned genes.

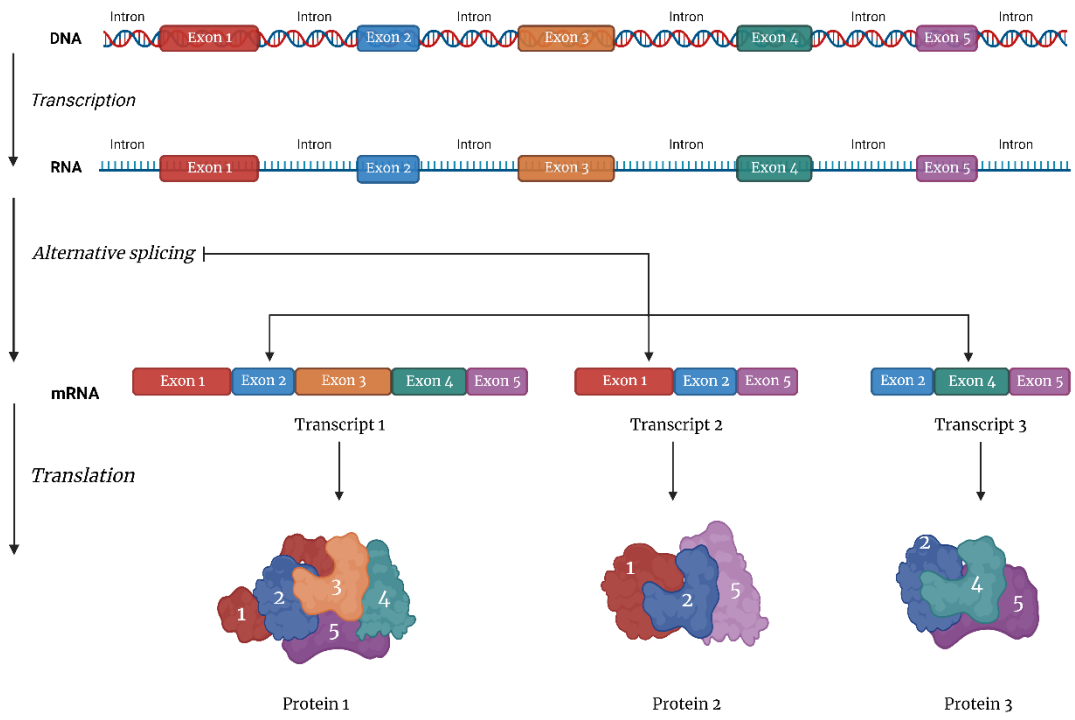


Figure 1.4: Schematic presentation of the Alternative Splicing process showing a range of unique proteins produced from the same pre-mRNA.

Several splicing factors or regulators thought to influence plant stress responses have been explored in a number of studies. In recent years, there has been notable amount of literature published on serine/arginine-rich (SR) proteins, glycine-rich binding proteins (GRPs), cap-binding proteins (CBPs) and different spliceosomal components. An example of the key contributory role of SR proteins to splicing regulation under environmental stresses was shown by (Zhang et al., 2014). They investigated the role of SR34b in tolerance of Arabidopsis to cadmium and found that SR34b mutant plants were more prone to cadmium toxicity due to its accumulation within these plants. This was attributed to the mis-splicing of the IRT1 gene, which encodes the cadmium transporter, emphasizing the importance of SR34b in the fine-tuning of IRT1 expression and therefore cadmium tolerance. As another example, under drought stress, the GRP2 and GRP7 of Arabidopsis positively regulate grain yields in rice, functioning as mediators of drought stress responses (Yang et al., 2014). However, controlling the significant stress-induced genes at the post-transcriptional level is determined by the activation and levels of these splicing factors.

ABA is considered to be an essential coordinator of plant responses under unfavourable stresses that induce plant osmotic stress and then ABA biosynthesis. Splicing factors, activated by the ABA hormone, have been reported in several studies to regulate spliced mRNAs which encode the components of ABA signal transduction. For instance, the ABA-activated splicing regulator RBM25 binds to HAB1 PP2C, controlling its AS and then its function in the ABA signalling pathway (Zhan et al., 2015, Wang et al., 2015). Therefore, the identification of pre-mRNAs involved in ABA signalling and targeted splicing regulators, as well as the determination of upstream components that control the activation of these regulators, might hold promise for understanding how AS regulates salt stress responses particularly during the establishment of wheat seedlings. This will inform strategies for enhancing salt tolerance in wheat and other crops and maintain yield either quantitatively or qualitatively, reducing the salinity threat to the global crop yield security.

1.10 Project hypothesis and aims

There is a relatively large variation in terms of salt-tolerance among wheat cultivars and the basis of this variation is yet to be understood. Indeed, understanding the molecular basis of this variation should lead the way towards the development of new wheat cultivars with higher salt-tolerance.

This project aims at

1) comparing three different Saudi wheat cultivars (**Najran, Mebiah** and **Qiadh**) in terms of their salt-tolerance and characterizing the differential responses in the root and shoot tissues of these cultivars.

2) attempting to determine the key mechanisms behind variation in salt-tolerance in wheat through using different approaches including physiology, biochemistry, transcriptomics, post-transcriptomics, and bioinformatics.

3) investigating the global profiles of transcriptome and post-transcriptome in the roots and shoots of salt-tolerant cultivar (Najran) and unravelling the most significant salt candidate genes and their related functions.

4) targeting the most important differentially spliced gene to salt tolerance expressed in both roots and shoots, and conducting a deeper analysis from transcriptomic to proteomic levels.

Chapter 2 Characterisation of Physiological and Biochemical Mechanisms Underpinning Salt-tolerance in Some Wheat Cultivars

2.1 Introduction

Wheat *(T. aestivum)* is one of the most essential staple food crops since thousands of years with a massive economic importance worldwide (Borlu et al., 2018). Wheat in Saudi Arabia has a major role in baking industry and its production was around 3-4 million tonnes during the beginning of 1990s. However, wheat yield has decreased to 2.63 million tonnes since 1993 due to various limiting environmental factors (Howladar and Dennett, 2014). Soil salinity is one of these factors which are becoming more severe in the brackish water-irrigated lands constituting a global threat for food production. High salinity represents a considerable constraint to crop production limiting the yield and quality of the crop (Munns et al., 2020). Therefore, it is becoming a hard challenge to boost crop output and meet food security under increasing salinity conditions.

High levels of sodium chloride in soil interfere with plant growth imposing various types of stresses, such as osmotic and ionic stresses. Plants have evolved several physiological and biochemical mechanisms as essential responses to adapt to these stresses. Stomatal closure has been reported for being one of the most common responses to osmotic stress. The stomatal closure gives rise to an initial reduction in plant biomass as a consequence of carbon starvation, and over a period of time leads to early senescence followed by plant death because of the ionic toxicity. It has been revealed that accelerated senescence is an adaptive way which stressed plants use to reduce their canopy size and consume carbon and nutrients in their reproductive parts to produce seeds. Although this way is efficient for next generation survival, it leads to a yield decline in annual crops (Sade et al., 2018). In addition, Osmotic adjustment within stressed plant cells has been evidenced as a crucial contributor mechanism in acclimation to salt stress in various plant species; sugar beet (Ghoulam et al., 2002), cotton (Meloni et al., 2001), durum wheat (Borrelli et al., 2018) and bean (Farhangi-Abriz and Torabian, 2017). Under salt stress, plants osmotically adjust to maintain their cellular turgor and the structural integrity of their membranes and protect themselves from the damage caused by ROS and toxic ions. Moreover, antioxidative defence system is another important protective mechanism that is induced under salt stress, which prevents the cellular damage caused by ROS accumulation (Ayvaz et al., 2016).

Wheat has been widely considered to be a moderately salt tolerant plant and its tolerance and responses to salinity stress vary among different tissues and cultivars (Saddiq et al., 2021). Given the fact that salt-tolerance is among the most physiologically complex traits in plants, regulated by a number of mechanisms, it seems that plants operate not all the salt tolerance-key mechanisms to overcome salt stress but a few, depending on different cultivars or species (Wang et al., 2017). Wheat cultivars, which have been developed through selective breeding programmes and genetic selection, demonstrate diverse levels of tolerance to environmental stresses including salt stress. This indicates the presence of underlying genetic diversity within the species. The exploration of variations in salt tolerance among different wheat cultivars has become a crucial objective in modern agriculture (Li et al., 2021), as it offers a strategic avenue and potential solutions to mitigate the impact of salt stress and enhance wheat production in salt-affected areas. Various cultivars of wheat have been documented to exhibit differences in their growth and yield outcomes, leading to different levels of tolerance to salt stress (Ghonaim et al., 2021, Tao et al., 2021). Some wheat cultivars show remarkable resilience, exhibiting minimal decrease in growth and yield upon exposure to salinity conditions. In contrast, other cultivars are more susceptible to the stress and thus suffer significant losses (Al-Ashkar et al., 2020). Zeeshan et al. (2020) previously investigated the variations in physiological and biochemical responses between two wheat cultivars under salt stress. They found that the salt-tolerant wheat cultivar (Suntop) showed lower reductions in growth and photosynthetic efficiency and higher activities of antioxidant enzymes, exhibiting higher tolerance to salinity compared to the salt-sensitive cultivar (Sunmate).

Hundreds of wheat cultivars have been grown in different regions of Saudi Arabia since hundreds of years. Many of these cultivars have evolved adaptations to prevailing local conditions, thus, they represent an invaluable germplasm resource that needs proper characterisation. Few studies have been conducted to evaluate the tolerance responses of different Saudi wheat cultivars to biotic and abiotic stresses. For examples, responses of agronomic performance and yield potentials to water stress (Boutraa et al., 2010, Akhkha et al., 2011, Albokari et al., 2016), growth and physiological responses to heat stress (Boutraa et al., 2015), responses to pathogen attacks (Dawabah et al., 2015), and responses of morphological traits to gamma irradiations (Albokari and Almuwalid, 2015). Very limited studies have attempted to investigate the different responses of typical Saudi wheat to salt stress (Howladar, 2010, Almaghrabi, 2012, Alshaharni, 2022). Therefore, this investigation was conducted to characterize the differential responses to salt-stress in three Saudi wheat cultivars, Najran,

Mebiah and Qiadh) in cultivation in different regions of the Kingdom to potentially reveal the underlying mechanisms for salt tolerance in wheat. The study investigated variation in the physiological and biochemical responses, as well as antioxidant scavenging capacity via phenolics accumulation among the three cultivars. The obtained knowledge constitutes an important addition towards understanding the different salt-tolerance mechanisms in wheat and potentially help to develop wheat cultivars with higher salt-tolerance.

2.2 Materials and Methods

2.2.1 Plant materials and salt stress treatment

The experimental design of this study is shown in Figure 2.1. Seeds of three genotypes of wheat (*T. aestivum.* Cv Najran, Mebiah and Qiadh) were collected from Ministry of Environment, Water & Agriculture, Saudi Arabia. Prior to sowing, seeds were wrapped by aluminium foil to block out light and then incubated at 4^{o}C for 3 days to break seed dormancy and stimulate germination. Six cold-stratified seeds were sown in 2L plastic pot filled with a mixture of John Innes soil compost No. 2, vermiculite 2-5 mm and grit sand in a volume ratio of 2:1:1, respectively. Pots were irrigated with either tap water for control set, 100 mM NaCl solution for yield stage set or 200 mM NaCl solution for seedling stage set and then sealed with cling film to maintain moisture. Pots were placed in a controlled growth cabinet at (20 °C day/15 °C night) and photoperiod (16 h light/8 h dark cycle) with constant 70% humidity. After germination, three randomized seedlings from each pot were retained and watered 3 times a week. One-month old plants were harvested at midday (i.e. 15:00 pm) to conduct growth, biochemical measurements, and RNA extraction. The other plants set was harvested after grain filling to assess the effect of salt stress on crop output.

2.2.2 Growth and yield analysis

Root and shoot of thirty-day old Najran, Mebiah and Qiadh wheat plants were harvested separately, and roots rinsed with tap water. Different growth parameters such as root length (RL), shoot length (SL), root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW) and shoot dry weight (SDW) were recorded. Roots and shoots of each cultivar were grouped into three replicates (each sample has a duplicate of plants) and then frozen in liquid nitrogen and stored at -80 °C after grinding them to be used in various analyses. Dry weight was determined after drying plant tissues in an oven at 80 °C for 7 days. To evaluate the extent to which the yield is affected by salinity, number of spikes, number of seeds per plant and seeds weight were calculated.

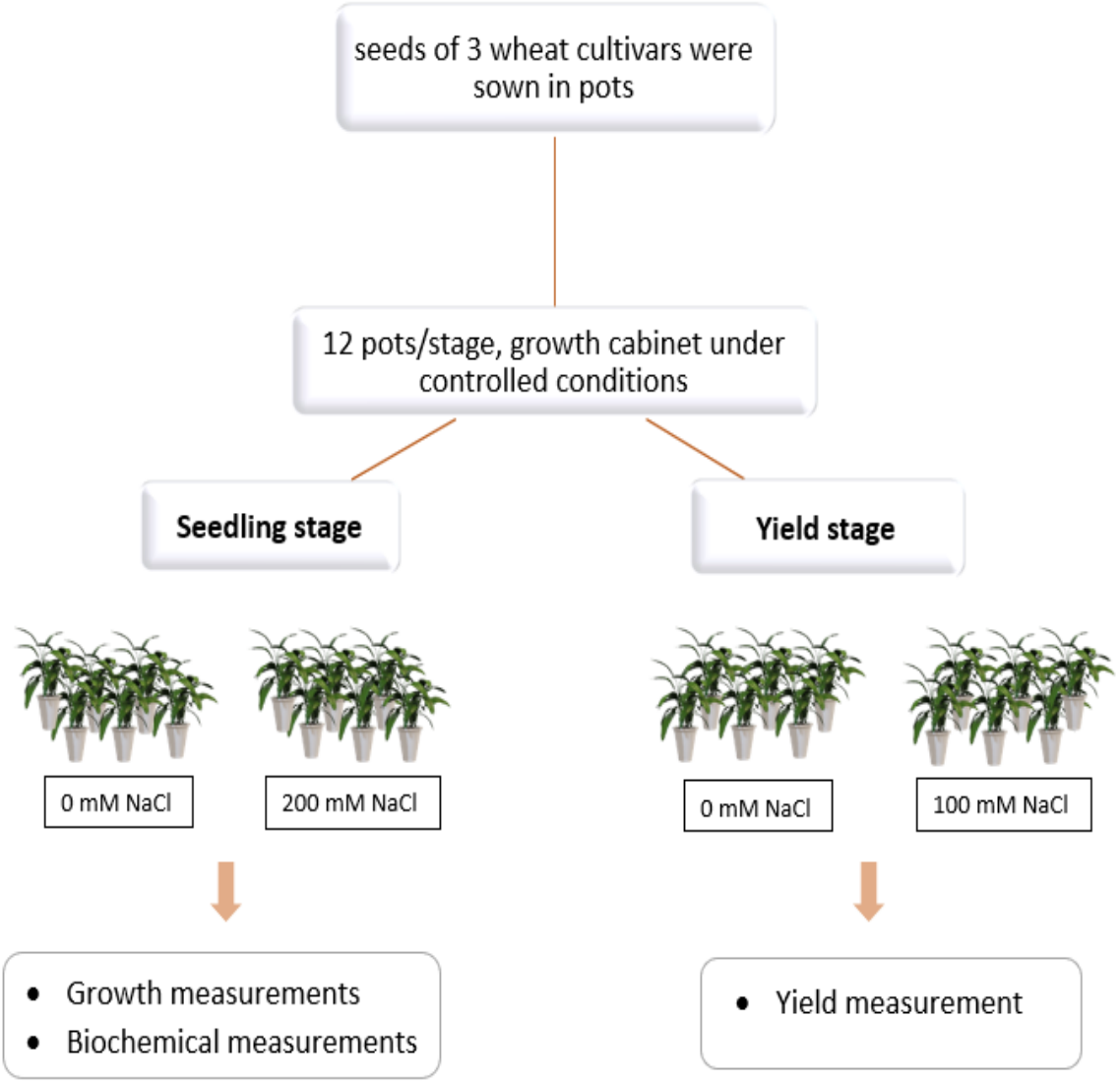


Figure 2.1: Schematic diagram representing the experimental design of salt stress experiment in three wheat cultivars (Najran, Mebiah and Qiadh).

2.2.3 Measurement of Proline content

Total free Proline content in control and salt-stressed plants of the three wheat cultivars was measured using a modified colorimetric method described by (Claussen, 2005). Ground root or shoot samples from each treatment (100 mg each) were transferred to a 2 ml micro centrifuge tube, then homogenized in 1 ml of 3% (w/v) sulphosalicylic acid. The homogenate was clarified by centrifugation at 10,000 g for 3 minutes at room temperature. A volume of 500 µl of each supernatant was mixed with 500 µl of glacial acetic acid and 500 µl of acidic ninhydrin reagent in a 2 ml micro centrifuge tube. To make the nihydrin reagent, 2.5 g ninhydrin was dissolved in 0.1 L of a solution containing 0.06 L glacial acetic acid, 0.03 L diH_2O and 0.01 L 85% orthophosphoric acid. The tubes of reaction mixture were incubated in a heat block at 98 ºC for 1 hour then cooled at room temperature. After cooling, absorbance of the red colour developed in samples was read spectrophotometry at 546 nm. The concentration of Proline in each sample was measured using a standard curve made using commercial pure L-proline and calculated on a dry weight basis (µg Proline mg^{-1} DW).

2.2.4 Measurement of soluble sugars and starch level

Soluble and insoluble carbohydrates were quantified in salt-stressed and unstressed plants from all wheat cultivars using the phenol/sulfuric acid method (Dubois et al., 1956) based on a colorimetric assay. From ground root and shoot samples, 100 mg plant tissue was homogenized in 1 ml of 80% methanol in an Eppendorf tube and then heated at 80 °C for 40 min. The homogenate was centrifuged at 13000 rpm for 10 min at room temperature, then supernatant was transferred to a new tube to be used in soluble sugar assay and the remaining plant tissue was kept for measuring starch level. To extract starch, the remaining tissue was washed several times with acetate buffer to remove any traces of glucose. After that, 1.2 ml acetate buffer and 0.2 ml enzyme cocktail were added, to digest starch molecules into glucose equivalent, and incubated overnight at 45 ºC. For enzyme cocktail, 26 mg (300 units) amyloglucosidase and 9 mg (25 units) amylase (Sigma-Aldrich, UK) were mixed in 20 ml acetate buffer. After incubation, the homogenate was centrifuged at 13000 rpm for 10 min at room temperature. Exactly 0.5 ml of each supernatant prepared for either soluble sugar or starch assays was transferred to a glass tube, then 0.5 ml diH_2O, 0.5 ml 5% phenol and 2.5 ml sulfuric acid were added, respectively and left to cool for 15 min at room temperature. The absorbance of reaction mixtures was read using a spectrophotometer at 483 nm and then plotted against a standard curve created using commercial glucose with different known concentrations.

2.2.5 Measurement of total organic acids

The content of organic acids in the root and shoot tissues from control and salt treated plants which had been frozen at -80 °C was assessed using a basic titration method. Plant tissues were extracted with 1 ml 80% methanol and then heated at 80 °C for 40 min. The extracts were centrifuged at 13000 rpm for 10 min and the supernatants were collected. A 20 µl aliquot of plant extract was transferred to a small vial to this, 970 µl distilled water and 10 µl phenolphthalein (10 $mg.ml^{-1}$) as a pH indicator were added then the total acidity mixture was neutralized with 0.1 N sodium hydroxide, added from a titration burette, until a pink colour was obtained. The volume of sodium hydroxide used was obtained by reading the burette and the titration data was calculated and expressed on a dry weight basis ($\mu mol.mg^{-1}$ DW).

2.2.6 Measurement of phenolics content

Total phenolics in root and shoot plant materials of the three different wheat cultivars were estimated using the Folin-Ciocalteu (F-C) reagent. To 20 µl of plant extracts, prepared in previous experiment and stored at -20 °C, 200 µl of 10% F-C reagent and 800 µl of 0.7 M Na_2CO_3 were added and mixed thoroughly in a 2 ml tube. The mixture tubes were incubated at room temperature for 120 min. After incubation, tubes content was transferred into cuvettes and absorbance readings were taken using a spectrophotometer at 265 nm. The levels of phenolic compounds were determined from a standard curve plotted using gallic acid at 0, 5, 10, 15, 20, 25, 50 and 100 nmol concentrations.

2.3 Results

2.3.1 Plant growth and development

Growth performance of the three *T. aestivum* cultivars under salt-stress and control conditions was evaluated by measuring different parameters including RFW, SFW, RDW, SDW, RL and SL. All these growth parameters were at similar levels in the examined cultivars under unstressed conditions, however significant difference appeared under salt-stress between cultivars (Figure 2.2). Fresh and dry weight under salinity treatment in both roots and shoots were significantly lower than those in control plants. Qiadh has shown the highest reduction in fresh and dry weight, about 8.9-, 9.8-fold in the roots, and 8.1-, 9.8-fold in the shoots, respectively ($P < 0.01$ - $P < 0.001$). However, Najran has shown the lowest salt effect on fresh and dry weight, approximately 8.3-, 9.3-fold in the roots, and 7.8-, 8.7-fold in the shoots, respectively ($P < 0.05$ - $P < 0.001$). In contrast, RL decreased more in Najran (39%, $P < 0.001$) than in Mebiah (23%, $P < 0.01$), whereas SL was more reduced in Mebiah (40%, $P < 0.001$) than in Najran (36%, $P < 0.001$).

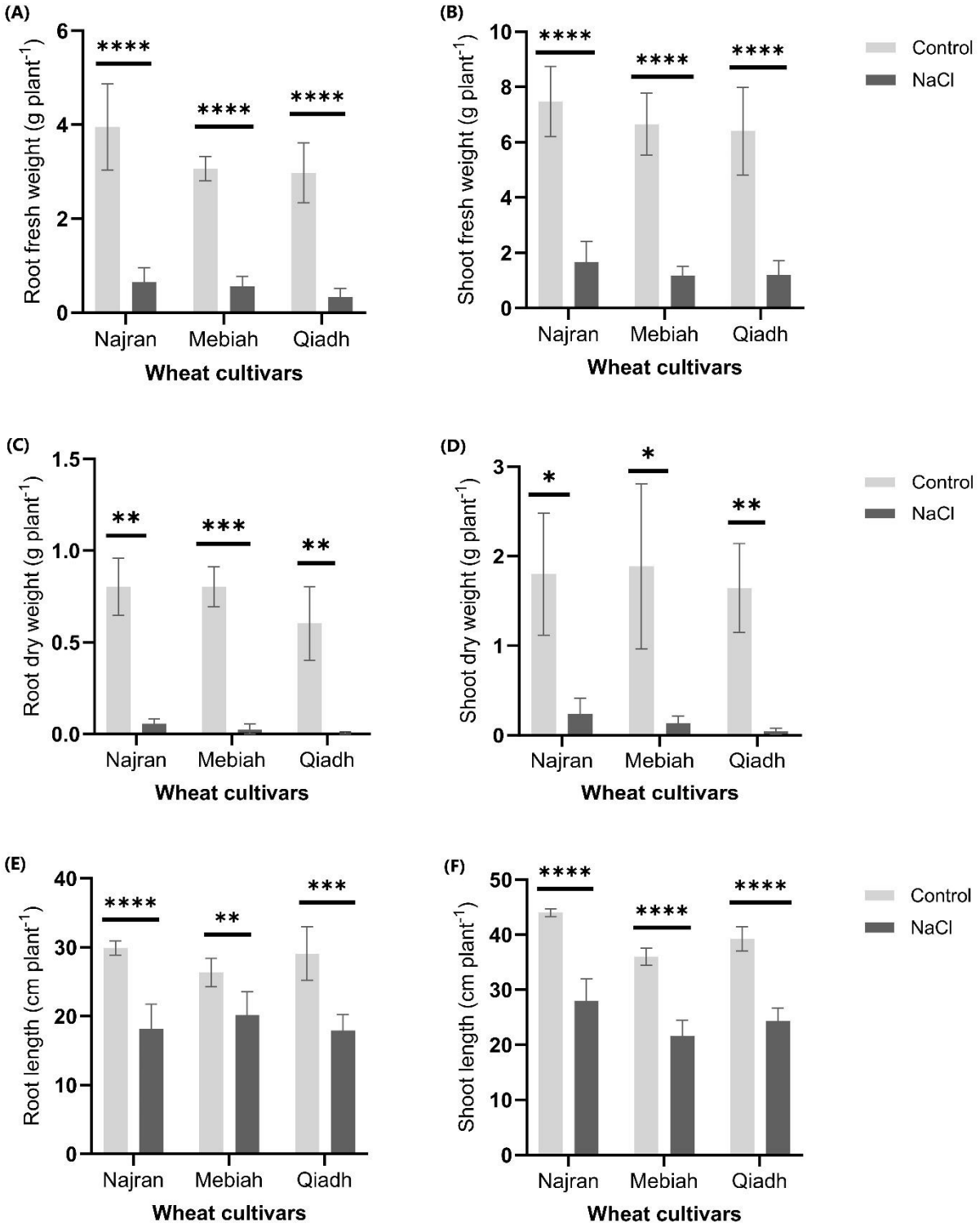


Figure 2.2: Effect of salt-stress on (A&B) root and shoot fresh weight, (C&D) root and shoot dry weight, and (E&F) root and shoot lengths of three wheat *(Triticum aestivum)* cultivars, Najran, Mebiah and Qiadh (n=6 +/- S.E). Salt-treated plants were watered with 200 mM NaCl whereas control plants were watered with 0 mM NaCl (tap water). Asterisks refer to significant differences at confidence levels of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.3.2 Grain yield

As shown in Figure 2.3, there was a significant difference between the three wheat cultivars regarding their spike and seed numbers. Qiadh had the largest number of spikes in control and saline treated plants (3 spikes), and the largest number of seeds in control plant (54 seeds), however it had the smallest number of seeds in NaCl-treated plants (17 seeds). On the other hand, Najran had the lowest number of spikes (1 spike) and seeds (17 seeds) in control plants, while Mebiah had more seeds (43 seeds) and fewer spikes (2 spikes) in salt treated plants. In addition, salt treatment had a positive effect on spike and seed number in Najran and Mebiah, whereas Qiadh displayed a negative salt-effect on both parameters. This result reveals that Qiadh was the most affected cultivar by salt-stress as the number of seeds decreased dramatically ($P < 0.001$) and the number of spikes reduced slightly ($P > 0.05$), while the seeds number increased slightly, and the spikes number increased significantly ($P < 0.01$) in Najran cultivar.

Not only the number of seeds was affected under saline conditions but also the weight of seeds where all wheat cultivars exhibited a significant decline in seed weight ($P < 0.001$) in comparison to control plants. As shown in figure 2.3, Najran cultivar has shown the highest reduction of seed weight.

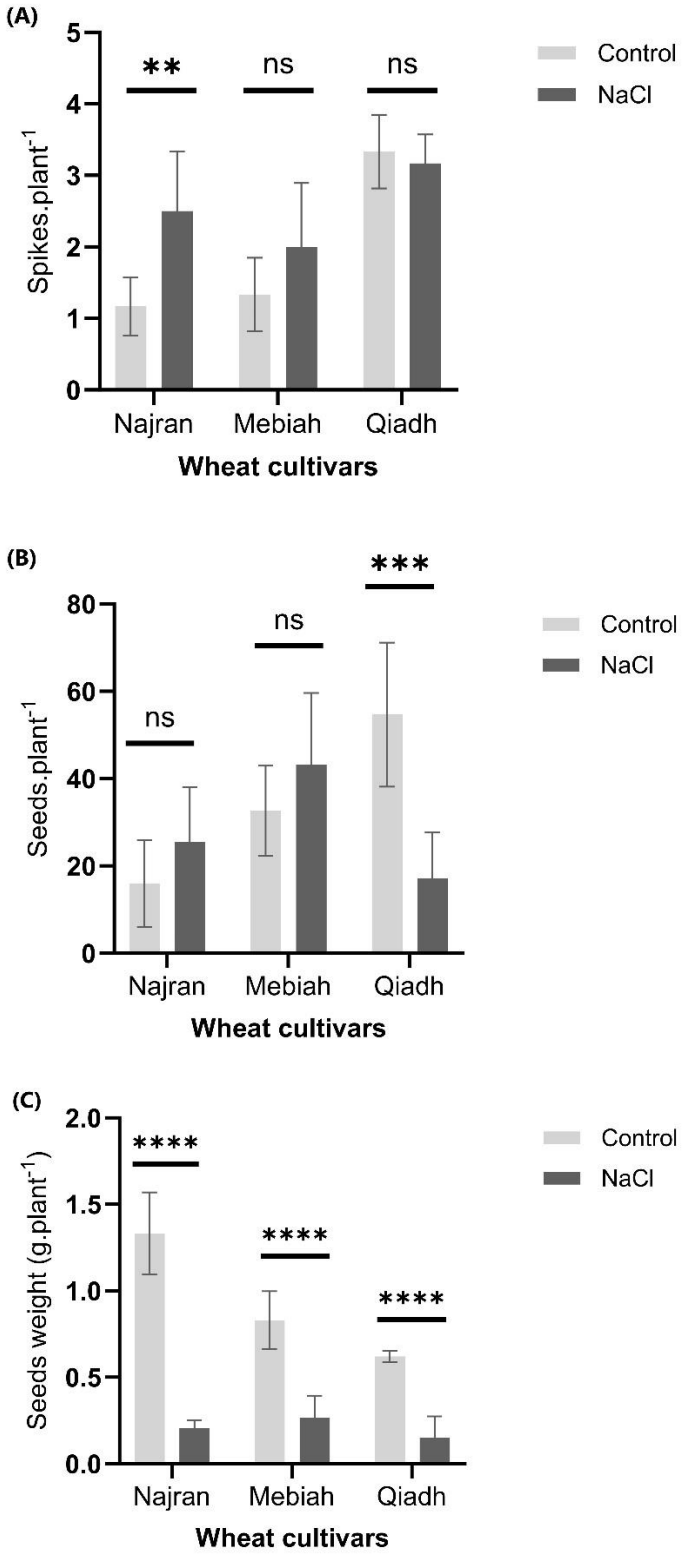


Figure 2.3: Effect of salt-stress on (A) spikes number, (B) seeds number and (C) seeds weight of three wheat *(Triticum aestivum)* cultivars, Najran, Mebiah and Qiadh. Salt-treated plants were watered with 200 mM NaCl whereas control plants were watered with 0 mM NaCl (tap water). Asterisks refer to significant differences at confidence levels of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.3.3 Proline content

Plants subjected to salt treatment displayed an increased accumulation of Proline in roots and shoots compared to control plants. As shown in Figure 2.4, under un-stressed conditions the three wheat cultivars had little Proline content to be measured in their roots and shoots except Qiadh which had a tiny amount of Proline only in its shoot tissues (0.01 µg mg^{-1} DW). Salt-stress induced an important increase in proline content in both roots and shoots of the three wheats (Figure 2.4). However, a significant difference in free Proline content in root and shoot tissues was observed between the three wheat cultivars under salt treatment ($P < 0.01$). In response to salt stress, Mebiah had the highest whereas Qiadh had the lowest Proline content in root, 0.17 and 0.01 µg mg^{-1} DW, respectively. Moreover, Mebiah had the largest content of Proline in its shoots followed by Qiadh and Najran, at 0.86, 0.66 and 0.39 µg mg^{-1} DW of the metabolite respectively.

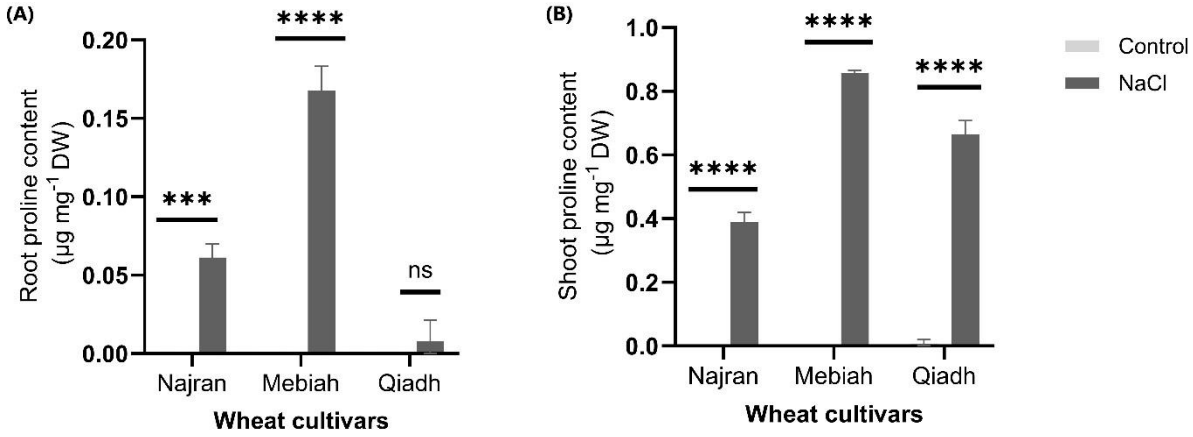


Figure 2.4: Effect of salt-stress on Proline content in (A) root and (B) shoot of three wheat *(Triticum aestivum)* cultivars, Najran Mebiah, and Qiadh (n=3 +/- S.E). Salt-treated plants were watered with 200 mM NaCl whereas control plants were watered with 0 mM NaCl (tap water). Asterisks refer to significant differences at confidence levels of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.3.4 Soluble sugars and starch level

There was no significant difference between levels of soluble sugars in the three wheat cultivars under unstressed conditions (Figure 2.5). However, soluble sugar content raised under salt-stress in roots, by 7.6-fold in Najran, 5.3-fold in Mebiah and 4.5-fold in Qiadh cultivar, as well as in shoots, by 1.9-fold in Najran, 4.6-fold in Mebiah and 4.9-fold in Qiadh cultivar. Soluble sugars content differed significantly among salt-treated plants of the three cultivars.

In contrast, there was a significant variation between wheat cultivars regarding the starch level in root and shoot of control plants (Figure 2.5). Qiadh displayed almost no starch in roots while Najran exhibited 0.06 µg mg^{-1} DW of starch in its roots, while 0.04 and 0.16 µg mg^{-1} DW accumulated in the shoots of the two cultivars respectively. Salt stress led to a significant boost in starch accumulation where Qiadh had the most increase in level of starch in both roots and shoots whereas Mebiah and Najran had the lowest starch levels in their roots and shoots, respectively relatively to the control.

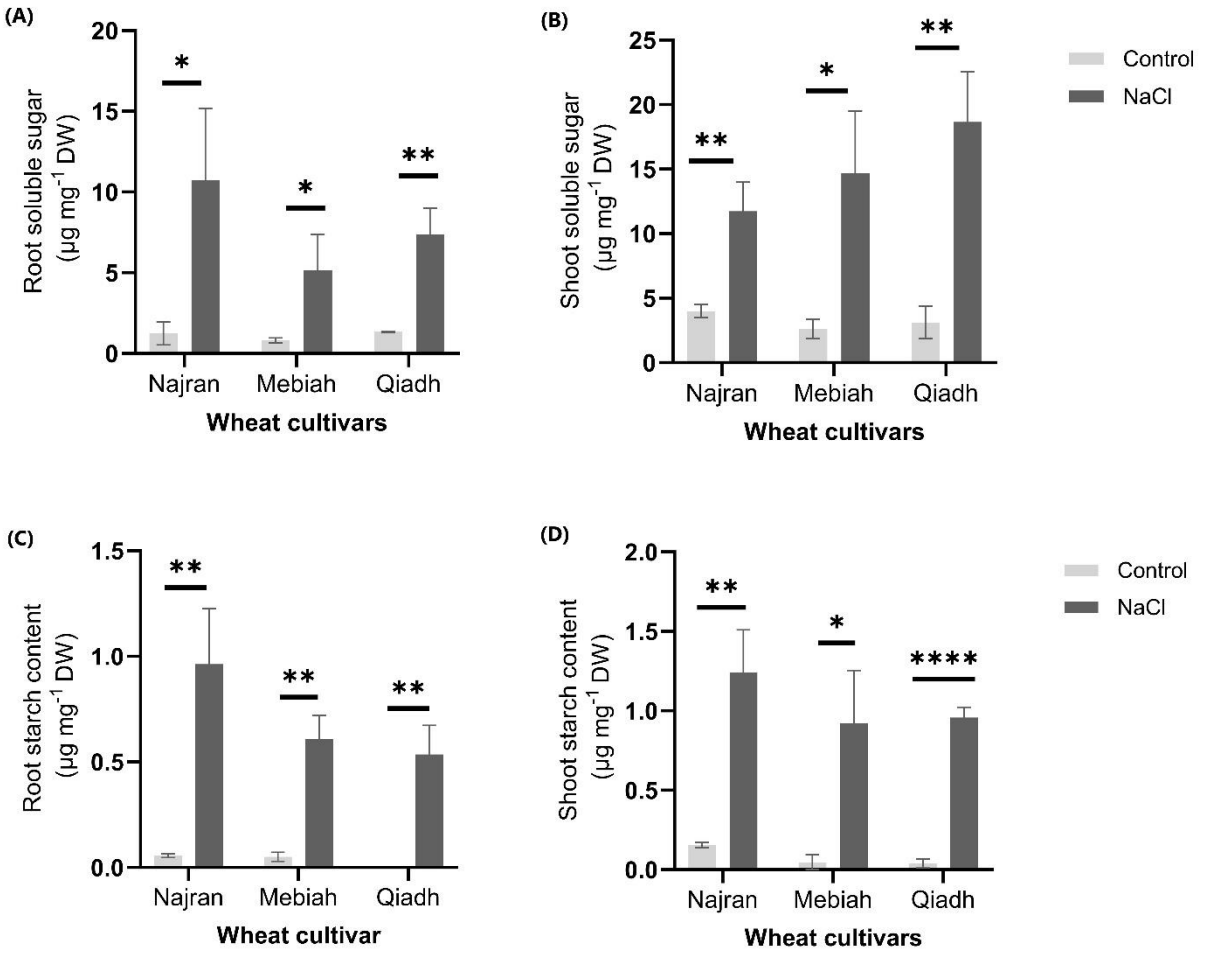


Figure 2.5: Effect of salt-stress on (A&B) levels of soluble sugars and (C&D) starch content in root and shoot of three wheat *(Triticum aestivum)* cultivars, Najran, Mediah and Qiadh (n=3 +/- S.E). Salt-treated plants were watered with 200 mM NaCl whereas control plants were watered with 0 mM NaCl (tap water). Asterisks refer to significant differences at confidence levels of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.3.5 Total organic acids

Levels of total organic acids in the roots and shoots of control plants were significantly different between the three *T. aestivum* cultivars ($P < 0.01$). Salt-stress resulted in a big increase in total organic acids in the three wheat cultivars ($P < 0.05$) (Figure 2.6). In the Najran cultivar which has shown higher salt tolerance, salt-stress increased content of organic acids 6.3 folds in the root whereas in Qiadh which exhibited less stress tolerance, only a 1.7-fold increase was measured under salt-stress. Salt-stress induced the highest increases in total organic acids of 35 folds and 19.3 folds in the shoots of Qiadh and Najran, respectively.

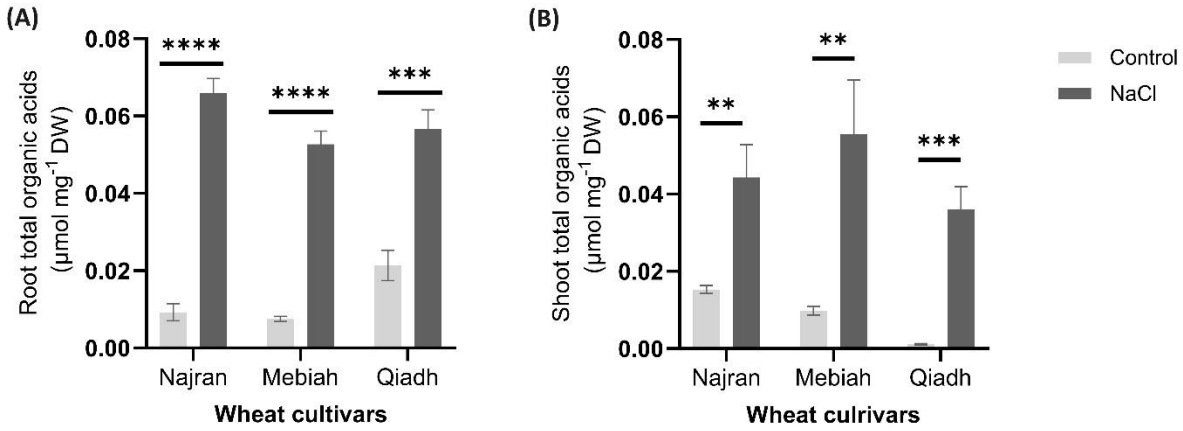


Figure 2.6: Effect of sat-stress on total organic acids in (A) root and (B) shoot of three wheat *(Triticum aestivum)* cultivars, Najran, Mediah and Qiadh (n=3 +/- S.E). Salt-treated plants were watered with 200 mM NaCl whereas control plants were watered with 0 mM NaCl (tap water). Asterisks refer to significant differences at confidence levels of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.3.6 Phenolics content

NaCl treatment significantly enhanced the production of phenolics in root and shoot tissues of Najran, Mebiah and Qiadh wheats. As seen in Figure 2.7, a pronounced increase of phenolics content was observed in the roots and shoots of salt-treated plants of the three cultivars compared to the control. Higher levels of phenolics content of 3.48 and 3.20 nmol.mg^{-1} DW were recorded in Najran whereas Qiadh showed lower values of phenolic compounds of 1.83 and 1.87 nmol.mg^{-1} DW in their roots and shoots, respectively.

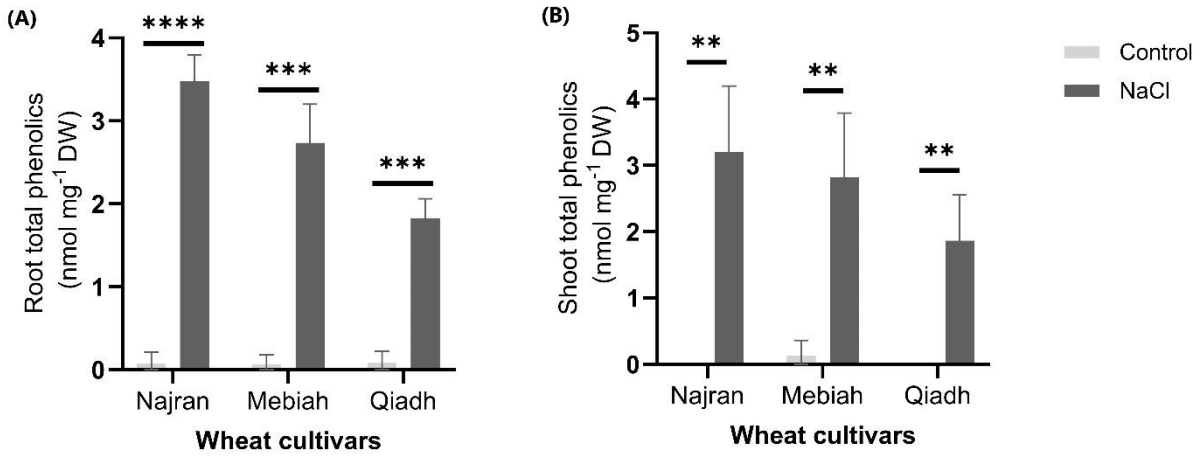


Figure 2.7: Effect of salt-stress on total phenolics content in (A) root and (B) shoot of three wheat *(Triticum aestivum)* cultivars, Najran, Mediah and Qiadh (n=3 +/- S.E). Salt-treated plants were watered with 200 mM NaCl whereas control plants were watered with 0 mM NaCl (tap water). Asterisks refer to significant differences at confidence levels of * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.4 Discussion

2.4.1 Effect of salt stress on plant growth and yield outputs

Salinity has the ability to alter plant growth and development. Increasing NaCl concentrations in the growth medium results in an adverse effect on crop survival and potential yield. In addition, Salinity leads to severe impact on the physiology traits of wheat plants including total biomass of the plant. Indeed, wheat cultivars vary in their sensitivity and their differential responses to NaCl stress (Ashraf et al., 2010). In the current study, a distinctive variation in salt tolerance was observed between three cultivars, Najran was the most tolerant to saline cues while Qiadh was the most susceptible cultivar. Under salt stress, a dramatic decline in mean fresh and dry weights as well as lengths of root and shoot tissues was noticed in all cultivars, but the reduction was significantly lower in the NaCl-tolerant cultivar Najran than in the NaCl-sensitive cultivar Qiadh. This decline in all growth measures might be a consequence of the increased salt concentration around root area which in turn causes a water deficit, nutritional imbalance and osmotic stress in the plant (Chavarria and dos Santos, 2012). Moreover, prolonged exposure of plants to salinity leads to ion toxicity in the leaves and severely affects cell division and cell elongation which results in a reduction in root and shoot lengths (Munns, 2002).

It is well known that salt stress influences negatively the production of plant leaves causing a reduction in photosynthetic capacity which in turn affects the quantity and quality of grain yield. The results obtained in the present study confirm this, where Qiadh cultivar showed the highest reduction in fresh and dry weights of the shoot as well as in yield production compared to the other two cultivars. In contrast, salinity had a positive effect on spike and seed numbers in Najran and Mebiah whereas seed weight had been negatively affected by NaCl. These findings are in line with those of Ghonaim et al. (2021), who found a significant decline in yield outputs of all tested wheat cultivars except Sakha 94 and Sids 13 cultivars, suggesting that while most wheat cultivars are sensitive to salinity some cultivars were salt tolerant, our results suggest that Najran wheat is among the salt-tolerant cultivars.

2.4.2 Simultaneous production of different osmotic adjustment substances is not essentially required for salt tolerance

Prolonged and high salt concentrations impair plants growth due to the resulting hyperionic and hyperosmotic stresses. Plants respond to these stresses by implementing biochemical mechanisms to facilitate water uptake and therefore maintain cell turgor and plant growth (Hasegawa et al., 2000). Osmotic adjustment is one of the crucial biochemical strategies in plant

acclimation to salt stress. Proline, soluble sugars, starch and organic acids are of the main organic osmotica which are synthesized within plants to assist surviving under various salt-induced stresses. It is demonstrated in this study that different *T. aestivum* cultivars might employ various mechanisms to alleviate the harmful effects of saline stress. For example, Mebiah was the highest in free Proline content, whereas Qiadh and Najran were the lowest in Proline concentration of the root and shoot tissues, respectively. These results were interpreted that Mebiah responded to the high level of NaCl by producing Proline which plays on one side a great role in osmoregulation and on the other side protects plants from the damage caused by ROS and toxic ions. Proline has been found to participate in lowering osmotic potential (Verbruggen and Hermans, 2008), storing carbon and nitrogen (Hare and Cress, 1997), detoxifying ROS (Szabados and Savoure, 2010), protecting the enzyme activities of photosynthesis and production of antioxidants (Reddy et al., 2015) and inducing adaptive responses by acting as a stress signal (Maggio et al., 2002) under unfavourable conditions. Another example, Najran exhibited more accumulation of soluble sugars in the roots and shoots while Qiadh revealed less content of these sugars. This accumulation may participate in increasing the photosynthetic activity leading to high plant biomass as these soluble sugars act as building blocks of macromolecules. These findings were accompanied with what we had found in growth analysis where Najran showed the highest fresh and dry weights under salt treatment compared to other cultivars. In contrast, Qiadh displayed the most increased level of starch in both root and shoot in comparison with the two other cultivars. Increasing the level of soluble sugars and decreasing starch level is considered to be a critical trait in salt tolerant cultivars. Similar results were reported by Boriboonkaset et al. (2013), who found a higher accumulation of soluble sugars in salt tolerant rice genotype, Pokkali, suggesting their important role in osmotic adjustment and enhancing the carbon energy reserves in plants. Chen et al. (2008) has pointed out a decline in starch concentration in salt-treated leaves of *Oryza sativa* L. as a result of carbon limitation due to the poor photosynthetic activity under salt-stress, the decline in this case might be a result of the suppression of starch biosynthesis.

Organic acids are ubiquitous metabolites in plants which are accumulated in response to salt stress to act as compatible solutes for osmoregulation and as ROS scavenger as well as plant protectors. The accumulation of total organic acids in the salt stressed wheat cultivars was obvious in the roots, however it decreased in the shoot of Najran whereas Qiadh showed the opposite results. These findings are consistent with a previous study that confirmed the increase of organic acids in root tissues and their depletion in the leaves under saline treatment,

suggesting that these different levels of organic acids might be attributed to organ-specific functions (Zhao et al., 2014b). In the case of salt stress, roots uptake excessive amounts of sodium cations which require anions to balance the charge. Thus, organic acids are more accumulated in the roots to enhance the cation–anion balance. Furthermore, their high level in the roots assist plants to osmotically adjust under salinity conditions.

2.4.3 Salt stress enhances plant antioxidant activities

Salt-stressed plants respond to oxidative stress resulting from the accumulations of ROS by operating an antioxidant defence system that prevents damage caused by ROS and detoxifies ROS molecules. Phenolics are one of the nonenzymatic antioxidants produced to mainly protect plants against various stresses and act as ROS scavengers. It would seem that the activity of the defence system components rises under extreme environmental stresses and is more pronounced in tolerant plants than sensitive ones (Hernandez et al., 2000, Sairam et al., 2000, Zhou et al., 2019), suggesting that the defence system perhaps works more effectively under unfavourable stresses. In the current study, prolonged saline stress has shown significant accumulation of total phenolics in all wheat cultivars and was more pronounced in Najran followed by Mebiah and Qiadh, confirming that wheat cultivars with different sensitivity to NaCl stress exhibit different metabolites alteration.

2.5 Conclusion

In the current study, three Saudi wheat cultivars; **Najran, Mebiah** and **Qiadh** varying in their salt-tolerance have been investigated for the effect of salt stress on the following parameters:

- Growth measurements including fresh weight, dry weight and plant length for both roots and shoots under control (0 mM NaCl) and salt treatment (200 mM NaCl) conditions.
- Yield measurements including number of spikes as well as seeds, and weight of seeds under control (0 mM NaCl) and salt treatment (100 mM NaCl) conditions.
- Biochemical measurements including Proline accumulation, total sugars and starch levels, total organic acids and antioxidants (phenolics) under control (0 mM NaCl) and salt treatment (200 mM NaCl) conditions.

Salt stress caused differential reduction in physiological activities and grain yield depending on wheat cultivars. Moreover, shoot and root tissues from different wheat cultivars with different sensitivity to NaCl stress exhibited different metabolic alterations and antioxidative responses. These salinity effects were less pronounced in the Najran cultivar potentially due to its high osmotic and antioxidant responses therefore it was characterized as the most tolerant cultivar to salt stress. This cultivar was used to do a deep analysis at the transcriptional and post transcriptional levels to reveal the salt responsive genes involved in important pathways for salt tolerance (chapter 3 & 4).

Chapter 3 Comparative Transcriptomic Profiling Reveals Differentially Expressed Genes and Important Related Metabolic Pathways in a Saudi Wheat Cultivar (Najran) under Salinity Stress

3.1 Introduction

High salinity of soil is a threatening constraint for agricultural output worldwide. It has been estimated that salt stress could severely restrict the productivity of about 30% arable land by 2050 (Wang et al., 2018b). Ranked as the third most important crop, wheat (*T. aestivum*) is used as stapple food in many parts of the world. Unfortunately wheat production, is currently challenged by salinity stress that causes up to 40% yield loss, seriously compromising the global food security (Singh et al., 2020).

A considerable number of studies has examined the harmful effects of salt stress on plant life (Borrelli et al., 2018, Hniličková et al., 2019, Tanveer et al., 2020). Accumulation of toxic salts in the cell causes inhibition of enzymes which together with stomatal closure results in reduced photosynthetic efficiency, reduced cell elongation and division and thereby decreased biomass accumulation. Stomatal closure results in the generation of ROS, causing cellular damage in the form of DNA mutations, protein degradation or lipid peroxidation (Apel and Hirt, 2004, Ahmad et al., 2010). Plants respond to oxidative stress, by synthesizing a variety of protective enzymes that act as an antioxidant scavenging systems (Ayvaz et al., 2016). Extensive research has indicated remarkable increases in the activity levels of scavenging enzymes, such as ascorbate peroxidase, guaiacol peroxidase, glutathione reductase, superoxide dismutase and catalase, observed across various wheat cultivars (Esfandiari et al., 2007, Mandhania et al., 2006, Rao et al., 2013). This heightened enzymatic activity serves as a crucial defence mechanism aimed at mitigating the detrimental effects of ROS-induced oxidative stress within the plant system under different environmental stresses (Zhou et al., 2019).

Other acclimation responses include osmotic adjustment, increased biosynthesis of secondary metabolites, which help plants stay hydrated and maintain ion homeostasis under stress conditions (Ashraf et al., 2008). All these responses implicate thousands of genes which may be directly or indirectly involved in plant salt tolerance by regulating ion influx and efflux (Li et al., 2020), production and accumulation of osmotica or compatible solutes (Singh et al., 2018), biosynthesis of signalling and regulatory enzymatic and nonenzymatic elements (Thabet et al., 2021). To understand the complexity of the various plant's responses to salt stress, gene

expression profiling can be used to identify salt-responsive genes. RNA sequencing is a high throughput sequencing technology which has been used in recent years as a robust and accurate approach to explore RNA quantity and sequences in biological samples. This allows access to comprehensive insights in the cell's inner alterations at the transcriptional level (Marguerat and Bähler, 2010, Sicilia et al., 2019). RNA-Seq in *T. aestivum* has received much attention in the past decade to analyse changes in the transcriptome profile of wheat under salt stress, Goyal et al. (2016) have sequenced root transcriptome of wheat (Kharchia Local cultivar) showing that 17,911 unigenes and 310 different metabolic pathways were responsive to salt-stress. Transcriptomic work on the root of a wheat cultivar called Arg, exposed to 150 mM NaCl after three weeks of planting revealed 5128 differentially expressed genes involved in response to salinity stress, comprising 1995 and 3133 up- and down-regulated genes, respectively (Amirbakhtiar et al., 2019). Within this set of genes, there existed genes responsible for sensing and signalling of salt stress, genes coding for transcriptional regulators and genes associated with the process of adapting to salt stress. Luo et al. (2019) have analysed the differences in transcriptome responses of two different varieties of wheat, Zhongmai 175 and Xiaoyan 60 to salt stress. They found that photosynthesis and energy metabolisms and glucosinolate biosynthesis were the most important pathways in the response of Zhongmai 175 to salt-stress, while polyunsaturated fatty acid metabolism and glucosinolate biosynthesis were significantly enriched in Xiaoyan 60 under salt-stress, suggesting the importance of polyunsaturated fatty acids in salt tolerance of wheat through the enhancement of jasmonic acid-related pathways and the photosynthetic system.

Despite the latest interest in transcriptome analysis of wheat under salt stress, many studies have only focused either on roots or shoots. Fewer research has analysed both roots and shoots simultaneously at the transcriptional level. In this study, a global RNA-Seq analysis was performed in the root and shoot of a salt-tolerant wheat cultivar (Najran) under two conditions: control (0 mM NaCl) and salt treatment (200 mM NaCl). The current study revealed differentially expressed genes revealing key biological pathways involved in plant responses to salt stress in the two different plant organs, providing more insights into the molecular mechanisms underlying salt tolerance in wheat.

3.2 Material and methods

3.2.1 Plant growth and salt stress treatment

Seeds of Najran wheat cultivar (accession No. 193) were obtained from Ministry of Environment, Water & Agriculture, Saudi Arabia. Cold-stratified seeds were germinated as three seeds per pot in 2L pots filled with 2:1:1 (v/v/v) mix of John Innes soil compost No. 2, vermiculite, and grit sand, respectively. After germination plants were divided into two groups, each group having a salt-treated batch and water-control batch. The first group consisted of (1) unstressed control plants, watered with tap water and (2) salt-treated plants, watered with 200 mM NaCl solution. These plants were grown for 4 weeks from germination to full vegetative growth. Plants from this group were used to measure growth and extract RNA for sequencing. The second group consisted of (1) unstressed control plants, watered with tap-water and (2) salt-treated plants watered with 100 mM NaCl solution. These plants were grown until flowering and seed production. Spikes were harvested when seeds became dry. All plants were watered every other day and kept under controlled conditions; 16 h light at 20°C, 8 h dark at 15°C and 70% humidity.

3.2.2 RNA extraction and quantification

Total RNA was extracted from twelve root and shoot samples using Plant/Fungi Total RNA Purification Kit, according to the manufacturer's instructions (Cat. 25800; Norgen, Canada) and treated with RNase-Free DNase I Kit (Cat. 25710, Norgen) during RNA purification. RNA purity and quality were assessed using NanoDrop 1000 spectrophotometer whereas its integrity was examined by RNA 6000 Nano Kit for Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). High quality RNA samples with RIN ≥ 6.5 (Figure S1 & 2) were sent to Admera Health LLC (New Jersey, USA) for cDNA library construction and sequencing.

3.2.3 Illumina library construction and transcriptome sequencing

According to Admera Health LLC, the quality of 20 µl RNA samples was assessed by High Sensitivity RNA Tapestation (Agilent Technologies Inc., California, USA) and quantified by Qubit 2.0 RNA HS assay (ThermoFisher, Massachusetts, USA). Paramagnetic beads coupled with oligo d(T)25 were combined with total RNA to isolate poly (A^+) transcripts based on NEBNext® Poly(A) mRNA Magnetic Isolation Module manual (New England BioLabs Inc., Massachusetts, USA). Prior to first strand synthesis, samples were randomly primed (5′ d(N6) 3′ [N=A,C,G,T]) and fragmented based on manufacturer's recommendations. The first strand was synthesized with the Protoscript II Reverse Transcriptase with a longer extension period, approximately 40 minutes at 42 ºC. All remaining steps for library construction were done

according to the NEBNext® Ultra™ II *Non-Directional* RNA Library Prep Kit for Illumina® (New England BioLabs Inc., Massachusetts, USA). Final libraries quantity was assessed by Qubit 2.0 (ThermoFisher, Massachusetts, USA) and quality was assessed by TapeStation D1000 ScreenTape (Agilent Technologies Inc., California, USA). Final library size was about 430bp with an insert size of about 300bp. Illumina® 8-nt dual-indices were used. Equimolar pooling of libraries was performed based on QC values and sequenced on an Illumina® Novaseq S4 (Illumina, California, USA) with a read length configuration of 150 PE for *40*M total reads per sample (*20*M in each direction).

3.2.4 RNA-Seq data analysis

The workflow of RNA-Seq data analysis is presented in (Figure 3.1), starting with the pre-processing of the raw reads and ending with identifying enriched KEGG pathways for the DEGs. All data presented and discussed in this study have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE225565 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE225565).

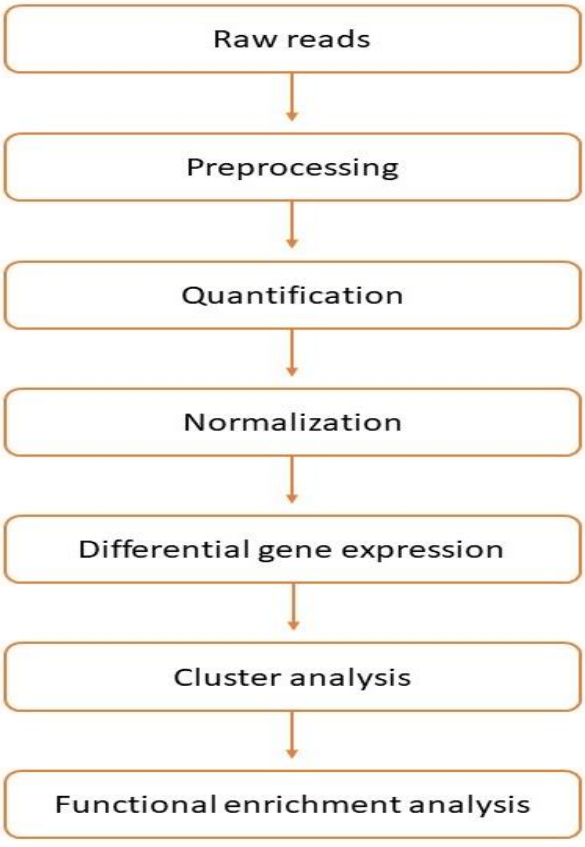


Figure 3.1: RNA-Seq data analysis workflow

3.2.4.1 Quality control and read quantification

Raw sequences from FASTQ files generated by Illumina were assessed with Fast QC (version 11.8, http://www.bioinformatics.babraham.ac.uk/projects/fastqc). A total of 136281942 and 138608660 paired-end reads were obtained from twelve control and salt stressed samples of root and shoot tissues, respectively (Table 3.1). Trimmomatic (version 0.36, http://www.usadellab.org/cms/?page=trimmomatic) was used for trimming out contaminating adaptor sequences and producing filtered reads (Bolger et al., 2014). High-quality reads were quantified against transcripts derived from the Ensembl *T. aestivum* genome (version 51, https://plants.ensembl.org/Triticum_aestivum/Info/Index). Salmon (version 0.12.0, https://combine-lab.github.io/salmon); a tool which performs 'quasi-alignment' was used to quantify expression of transcripts from RNA-Seq data via read abundances. To quantify the levels of gene expression (gene-level counts), the R package 'tximport' (version 1.18.0, http://bioconductor.org/packages/release/bioc/html/tximport.html) was performed.

Table 3.1: Summary of sequencing output, clean reads and mapping to the wheat genome. R indicates root and S indicates shoot.

Sample	Total reads	High quality reads	Mapped reads
Ctrl_R_1	23001104	22838034	19425012
Ctrl_R_2	26022495	25836862	21983090
Ctrl_R_3	21950892	21726586	18437481
Salt_R_1	24008611	23818150	20066908
Salt_R_2	20462131	20265845	17006769
Salt_R_3	20836709	20702466	17395220
Ctrl_S_1	20340235	20188148	14449739
Ctrl_S_2	27874023	27720205	21256010
Ctrl_S_3	26033823	25804393	19259820
Salt_S_1	20727638	20590369	15766118
Salt_S_2	20869668	20721980	16374493
Salt_S_3	22763273	22575139	17764005

3.2.4.2 Differential gene expression analysis

Differentially expressed genes (DEGs) were identified using the R package DESeq2 (version 1.30.1, http://bioconductor.org/packages/devel/bioc/vignettes/DESeq2/inst/doc/DESeq2.html). DESeq2 estimates fold change between experimental conditions (Table 3.2) in the root and

shoot samples using a Negative Binomial GLM (General Linear Model) with a logarithmic link function. According to that, genes which have base 2 logarithmic fold change ≥1 and a cut-off of adjusted P-value (false discovery rate (FDR)) < 0.01 were considered as upregulated genes, whereas genes with a fold-changes ≤ −2 ($Log_2 \leq -1$) have been indicated as downregulated genes.

3.2.4.3 Gene Ontology terms analysis

The gene ontology (GO) terms enrichment analysis was done by searching the PANTHER classification system (http://www.pantherdb.org/panther/ontologies.jsp) using the obtained DEGs against the wheat repository. Testing for enriched GO terms was carried out using the hyper GTest function from the R package GO stats, with a p-value test at cut-off of 0.05.

3.2.4.4 Functional enrichment analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) was searched to identify enriched KEGG pathways for the DEGs. KEGG pathways for *T. aestivum* were obtained using the R package 'KEGGREST' (version 1.34.0, https://bioconductor.org/packages/release/bioc/html/KEGGREST.html). Protein sequences for wheat genes were downloaded in FASTA format using the ID mapping tool from UniProt (https://www.uniprot.org/uploadlists/). KEGG's 'GhostKOALA' search (https://www.kegg.jp/ghostkoala) was used to find KEGG orthology IDs for wheat genes based on the UniProt FASTA download. The KEGG orthology numbers enabled a mapping from wheat genes to KEGG pathways. Enriched KEGG pathways for the lists of significantly DEGs were calculated using a hypergeometric test with the corrected P-value < 0.05.

3.2.5 Validation of RNA-Sequencing results using Quantitative real-time PCR analysis (RT-qPCR)

To validate the gene expression profiles obtained from the analysis of RNA-Seq data, eight genes differentially expressed under salt-stress were randomly chosen and their transcript levels monitored by RT-qPCR using the same RNA samples to RNA-Seq. The genes included four genes upregulated in both roots and shoots, two genes down-regulated in roots and shoots, one gene upregulated in roots and downregulated in shoots and one gene down regulated in roots and up-regulated in shoots (Table S1). Gene-specific primers (Table S2) for RT–qPCR (18–21 bp) were designed using Primer3(v. 0.4.0). cDNA was synthesized from twelve purified RNA samples using Tetro™ cDNA Synthesis Kit (Bioline, UK) according to the manufacturer's procedure using Oligo dT primer. The SensiFAST™ SYBR Hi-ROX Kit (Bioline, UK) was used to perform qPCR in Rotor Gene Q5 & Qiagility Robot instrument (Qiagen, UK) based on

the manual of SYBR Hi- ROX Kit (Bioline, UK) using cDNA as template. Normalization of gene expression level was done using CJ705892 gene as a housekeeping (internal control) gene from wheat (Dudziak et al., 2020). Primer efficiency was determined using serial dilutions of the cDNA for each gene, it ranged from 95% to 103% (Figure S3). Relative transcript levels of the target genes were quantified using the standard $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001).

3.3 Results

3.3.1 Transcriptome profiling and sequencing statistics

In order to investigate the regulatory mechanism of wheat responses to salinity stress at transcriptional level, RNA sequencing was carried out in the root and shoot tissues of Najran cultivar. Three biological replicates for each tissue and treatment were used in Illumina high-throughput sequencing. The number of raw reads obtained varied from 20.34 million to 27.87 million per sample for the 12 samples with a mean of 22.90 million. A total of 272788177 high-quality paired-end reads (99% of the raw reads) were generated from all samples; 135187943 and 137600234 reads from roots and shoots (Table 3.1), respectively. All the clean reads were quantified against transcripts of *T. aestivum* genome IWGSC_V51, resulting in mapped reads ranging from 83.9% to 85% (17006769 to 21983090 reads) in the roots and 71.6% to 79% (14449739 to 16374493 reads) in the shoots (Table 3.1).

3.3.2 Differential gene expression analysis

Transcript abundance of each gene expressed under 0 or 200 mM NaCl conditions and mapped was normalized, then the significance of difference in transcript abundance in the root and shoot tissues was determined based on the thresholds of adjusted P-value (FDR < 0.01). Interestingly, a total of 5829 genes were differentially expressed in the roots under salt treatment, including 38.19% up-regulated genes and 61.81% down-regulated genes (as obviously depicted in the volcano plot Figure 3.2 A). On the other hand, 3495 DEGs were revealed between the control and salt treated shoots, 42.15% of them were up-regulated, while 57.85% were down-regulated (Figure 3.2 B). Only 1205 from 5829 genes expressed in roots and 733 from 3495 genes in shoots had annotated functions (Table 3.2).

Table 3.2: Number of total differentially expressed genes (DEGs) and up/down-regulated genes in the root and shoot tissues of Najran wheat cultivar under 0 mM NaCl (control) and 200 mM NaCl (salt treatment) conditions.

	Total DEGs	Up-regulated	Down-regulated	Up_differences % ratio	Down_differences % ratio
Salt treated Root vs Control Root	5829	2226	3603	38.19	61.81
Salt treated Shoot vs Control Shoot	3495	1473	2022	42.15	57.85

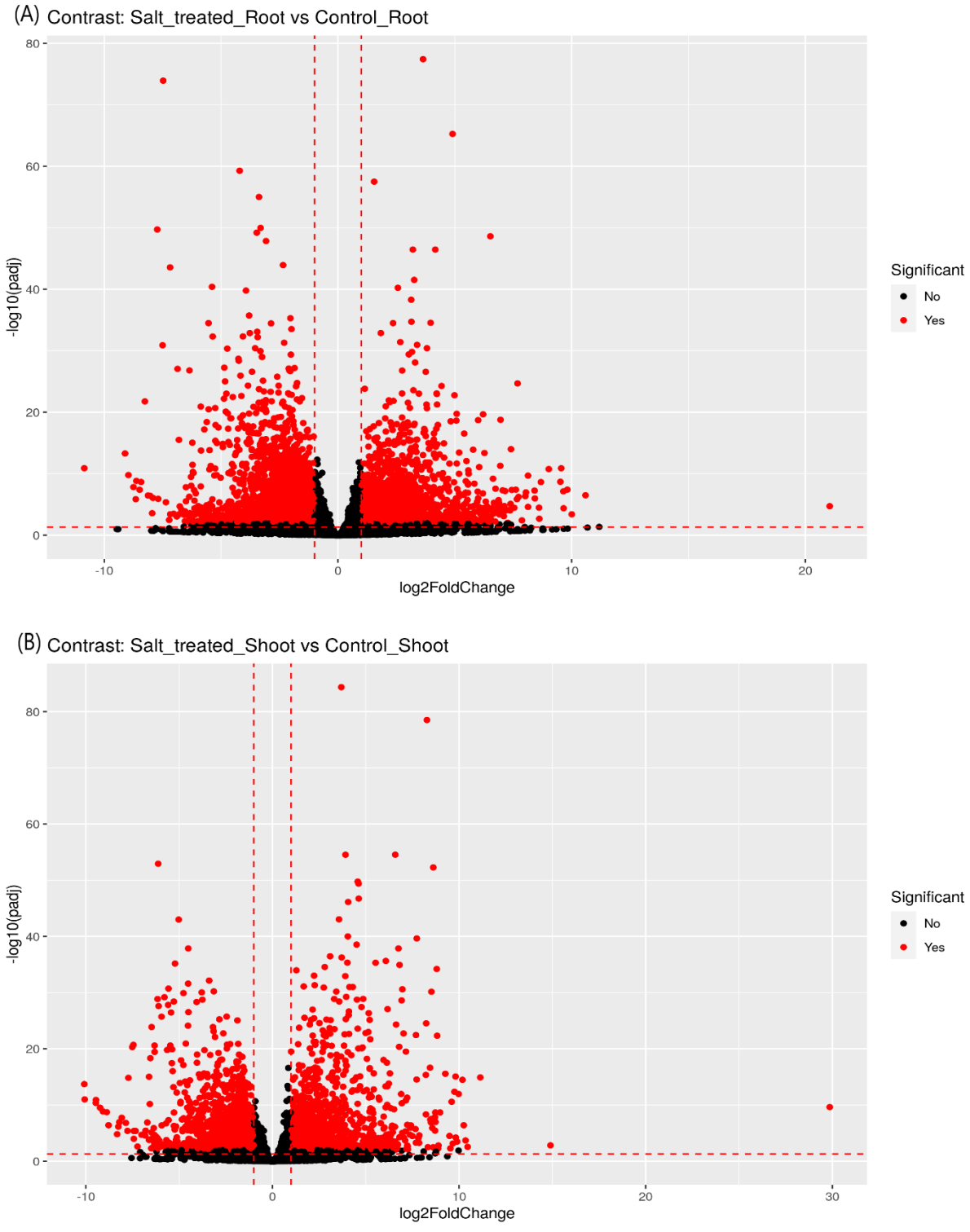


Figure: 3.2: The volcano plot (log_2 FC ≥1 or ≤ -1, p-adj ≤ 0.01) of differentially expressed genes (DEGs) in (A) salt-treated root versus control root and (B) salt-treated shoot versus control shoot. Red symbols towards the right indicate statistically significant-upregulated genes, red symbols towards the left indicate statistically significant-downregulated genes and black symbols indicate non-significant genes.

The number of genes that were identified as DEGs in roots and shoots was compared and the overlap of DEGs in the two organs analysed using a Venn diagram (Figure 3.3). The Venn diagram showed that only 1158 genes were overlapping genes expressed in roots and shoots under salt-stress conditions.

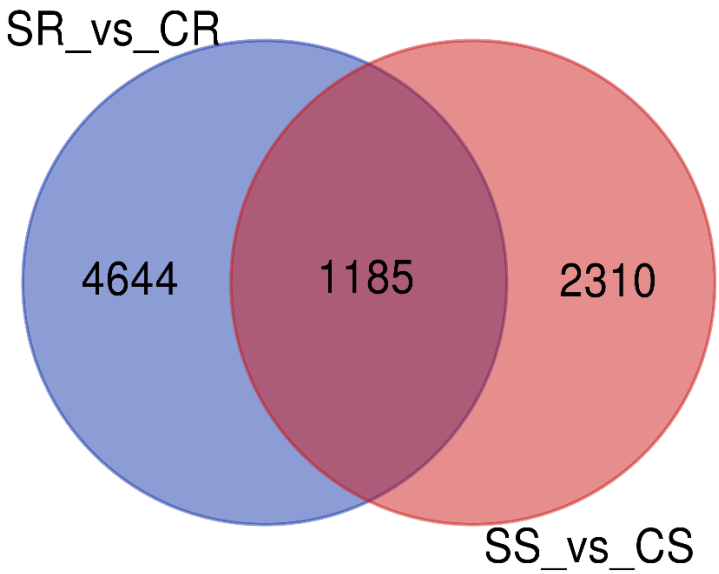


Figure 3.3: Venn diagram of Differentially Expressed Genes (DEGs) in roots and shoots of Najran wheat (*Triticum aestivum*) under salt-stress (200mM NaCl). The numbers in the Venn diagram indicate DEGs in salt treated-roots vs. control-roots (SR_vs_CR,) and salt-treated shoots vs. control-shoots (SS_vs_CS).

3.3.3 Cluster analysis of RNA-sequencing data

To have an overview of the relationship of expression patterns between the control and salt stressed samples as well as between the root and shoot tissues, Principal Component Analysis (PCA) and hierarchical clustering methods were used. PCA, showed that 98% variance was observed between the root and shoot samples (Figure 3.4). Moreover, these two distinct groups formed based on their expression profile; one group comprised control and salt-treated roots and the other group comprised shoots from non-stressed and stressed plants. On the other hand, the PCA plot in Figure 5B demonstrated that the outlier (Salt-treated root) at the middle west

of the plot is almost entirely responsible for the variation seen in the second principal component (1% of the variation in the entire dataset).

The heatmap visualisation of gene expression, combined with clustering method grouped the 12 samples based on similarity of their gene expression pattern and these classifications were consistent with the PCA findings. The colour and intensity of heatmap boxes is used to represent the similarity in gene expression, the darker the blue colour, the more similarity between samples. The heatmap analysis indicated the notable difference between the control and salt treated samples in both roots and shoots and a massive difference between the gene expression profiles of the roots and shoots (Figure 3.5). These variations in gene expression patterns among wheat samples are a result of exposing plants to salt stress as well as differences in plant organs.

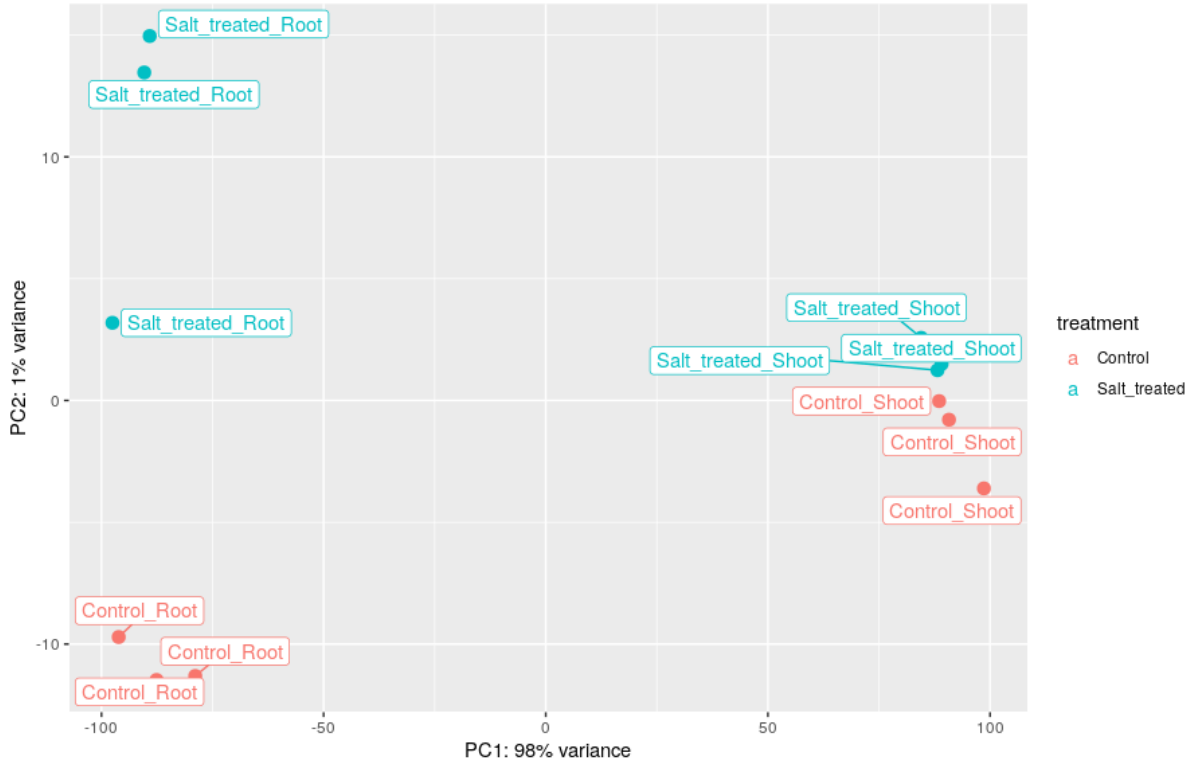


Figure 3.4: Principal component analysis (PCA) of Najran cultivar (*Triticum aestivum*) samples showing the relationship between control and salt stressed roots along with shoots. Orange colour refers to control samples and blue colour to salt treated samples.

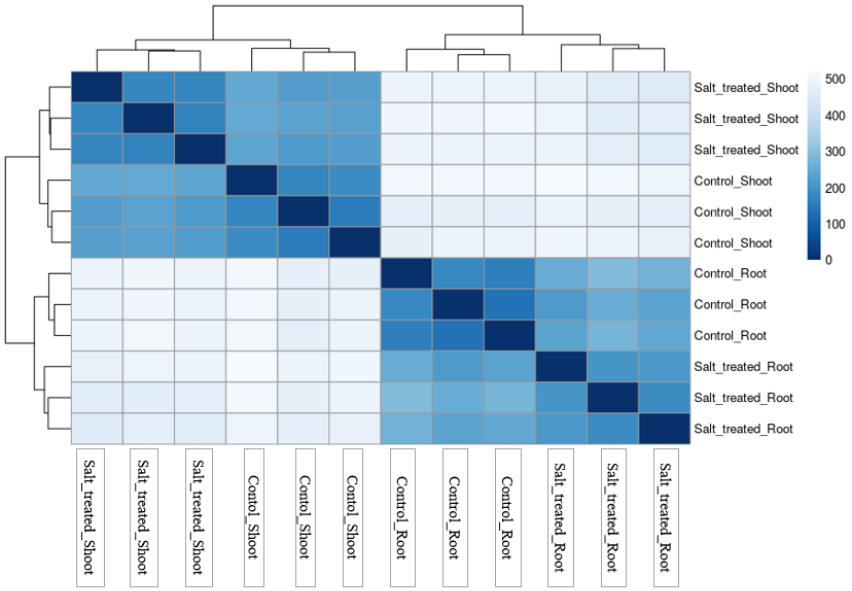


Figure 3.5: Heatmap of 12 Najran cultivar (*Triticum aestivum*) samples indicates the similarity of their gene expression pattern. Darker colour refers to more similarity between experiment samples.

3.3.4 GO terms enrichment of DEGs

To determine the functional meaning of the salt-induced changes in the transcriptome in roots and shoots of Najran Wheat, we performed the GO terms enrichment for the DEGs in the two organs. As shown in the heatmap of Figure 3.6, the DEGs in roots and/or shoots were associated with 76 functional categories in total. The DEGs were associated with 48 “biological process” categories, 19 “molecular function” categories and 9 “cellular component” categories. Exactly 35 categories were present only in roots and 16 were present only in shoots while 25 categories were present in both organs. Among the categories enriched in roots there were processes involved in cell ionic homeostasis, oxidative stress responses including Glutathione synthesis, osmotic stress response, hormonal signalling, carbohydrate transport etc. Among the categories enriched in shoots there were protein folding, photosynthesis, synthesis of secondary metabolites, response to Abscisic acid, hormonal-signalling. Among the categories enriched in both organs we find response to oxidative stress, glutathione synthesis and carbohydrate transport.

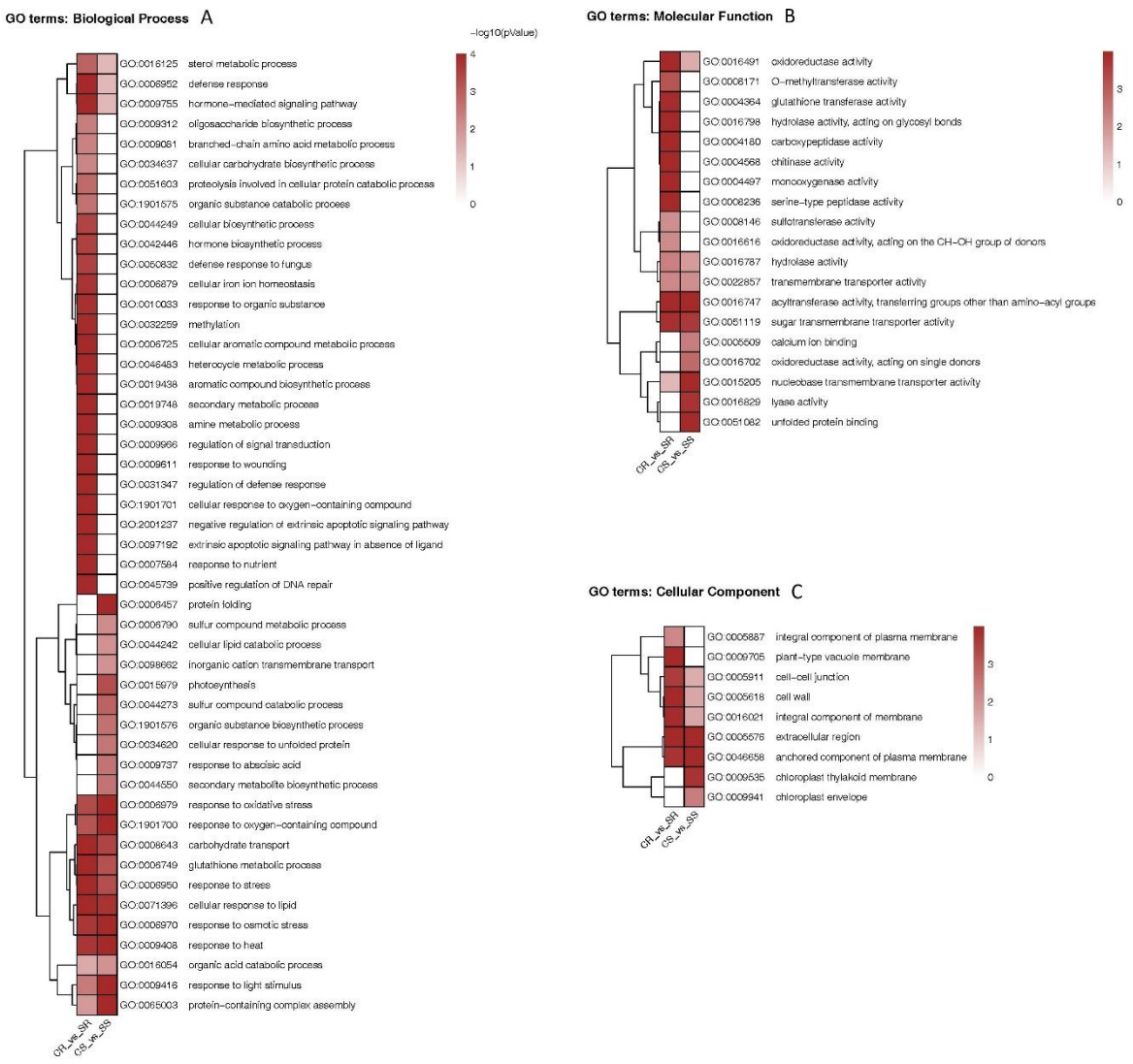


Figure 3.6: Heatmap comparison of Gene Ontology classifications of DEGs in roots and shoots of Najran Wheat plants under salt stress (200 mM NaCl) versus unstressed control. The heatmaps representing GO terms based on **(A)** biological process (45 categories), **(B)** molecular function (19 categories) and **(C)** cellular component (9 categories). The color scale indicates different levels of significance of enriched terms (P-value < 0.05).

3.3.5 KEGG enrichment analysis of DEGs and functional annotations

To identify which biological pathways for the DEGs were potentially enriched in the roots and shoots of Najran cultivar transcriptome, KEGG enrichment analysis was done for all pairwise comparisons. The KEGG analysis revealed that all DEGs in both roots and shoots could be classified into 25 enriched KEGG pathways which located in three main KEGG classes; metabolism, organismal systems; environmental adaptation and Genetic information processing; folding, sorting and degradation (Figure 3.7A, Tables S3, S4). Among them, the number of DEGs was the largest in metabolism category, which included 4044 genes involved in different pathways such as Biosynthesis of secondary metabolites, Amino acid metabolism, Carbohydrate metabolism, Metabolism of cofactors and vitamins and other compounds. Pathways relating to glutathione metabolism, galactose metabolism and thiamine metabolism were significantly upregulated in the roots under salt stress (Figure 3.7B), where glutathione metabolism had the highest number of DEGs (Figure S4). In contrast, the significantly enriched pathways in the salt treated shoot were biosynthesis of secondary metabolites, protein processing in endoplasmic reticulum, starch and sucrose metabolism, phenylpropanoid biosynthesis, galactose metabolism and phenylalanine metabolism pathways (Figure 3.7C). Most of DEGs were related to biosynthesis of secondary metabolites such as genes associated with phenylpropanoids (Figure S5).

Comparing salt-stress to control plants revealed three and six different pathways preferably enriched in salt treated roots vs control roots and salt treated shoots vs control shoots, respectively (Table S4). Interestingly, the only common pathway between salt treated roots vs control roots and salt treated shoots vs control shoots was galactose metabolism (Figure S6), which is a part of carbohydrate metabolism and has been reported to have a role in salt tolerance (Darko et al., 2019).

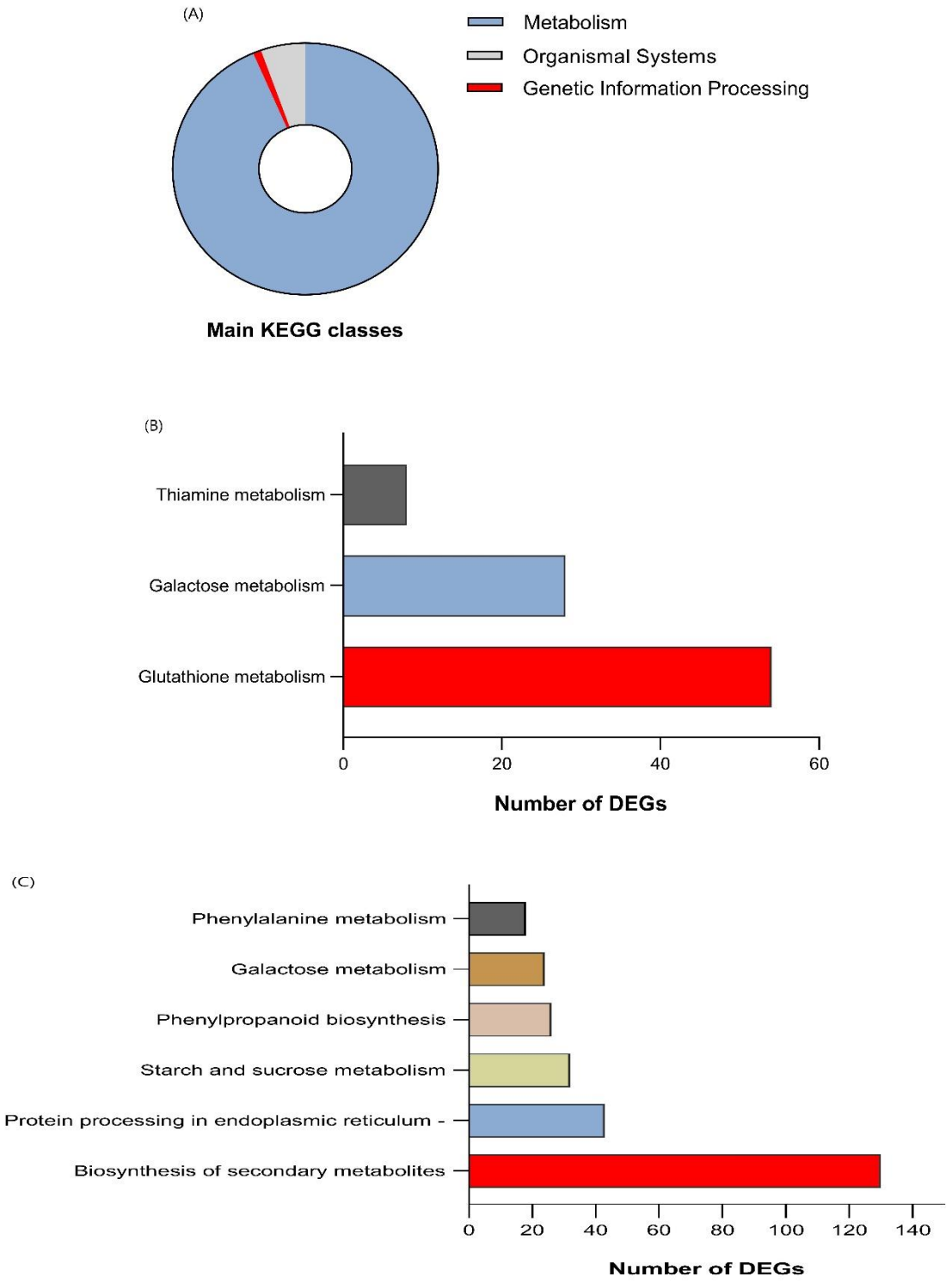


Figure 3.7: **(A)** Categorization of identified Differentially Expressed Genes in both roots and shoots of Najran wheat (*Triticum aestivum)* in three chief KEGG classes. The top significantly enriched pathways of identified DEGs in roots **(B)** and shoots **(C)** in response to salinity stress.

3.3.6 Validation of RNA-Seq results using Quantitative real-time PCR analysis (qRT-PCR).

To validate the change in transcript-levels revealed by RNA-Seq data, transcript levels of eight DEGs in both roots and shoots of Najran wheat were measured by RT-qPCR. The results of this analysis (Figure 3.8) confirmed that change (up or down-regulation depending on gene) in transcript levels was consistent with that obtained by RNA-Seq for the selected genes including heat shock protein 90, dirigent protein, delta-1-pyrroline-5-carboxylate synthase, flavincontaining monooxygenase, glutamate receptor, lipoxygenase, bidirectional sugar transporter SWEET and ABA inducible protein. The log2 fold change in transcript levels for the eight randomly selected genes measured by RT-qPCR and RNA-Seq is shown in Figure 3.8.

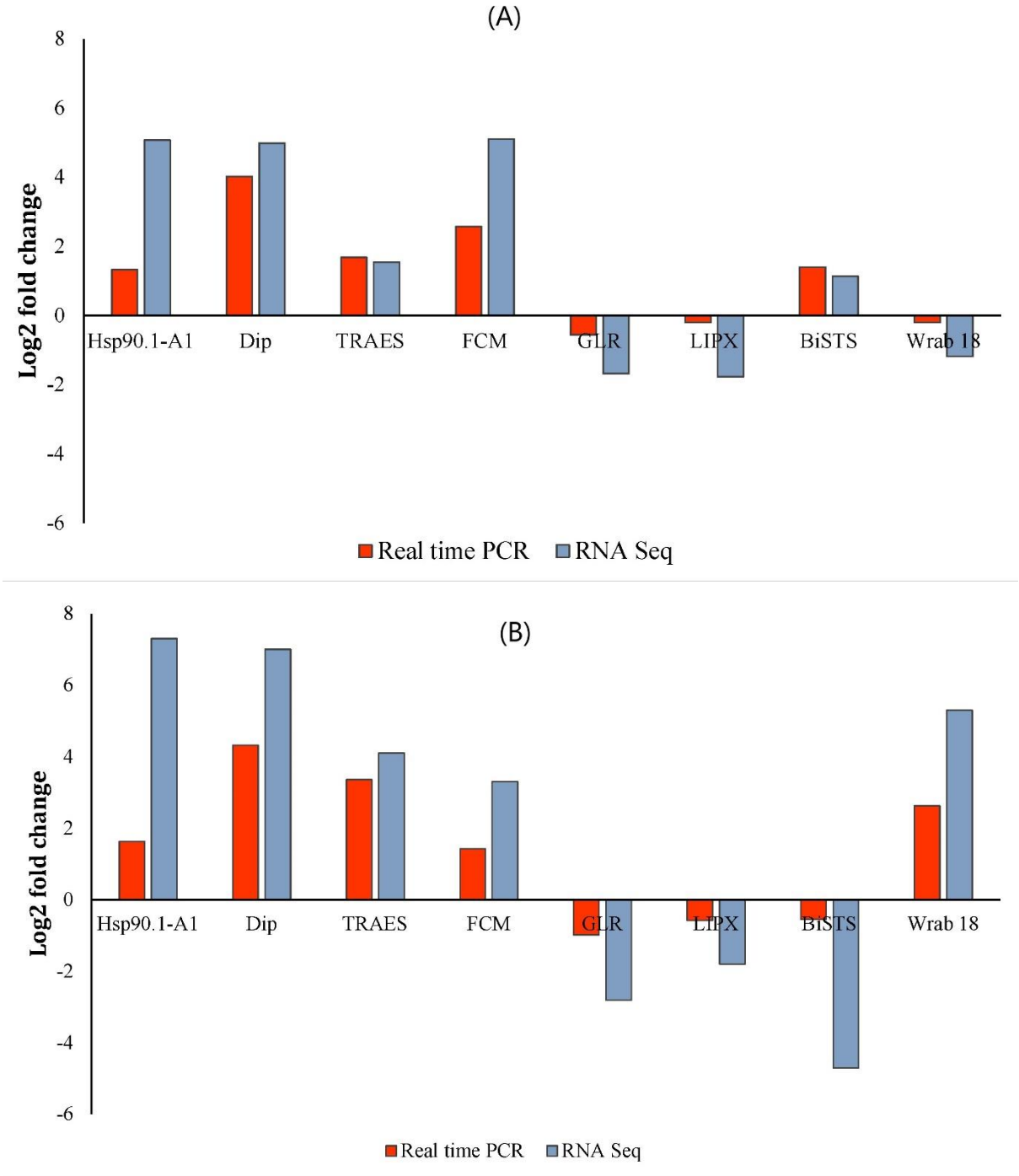


Figure 3.8: RT-qPCR validation of the salt-induced transcript change in Najran wheat (*Triticum aestivum*) subjected to salt- stress (200 mM NaCl). Log2 fold change in transcript levels is shown in root **(A)** and shoot **(B)** for eight genes including four up-regulated genes in roots and shoots (*TaHSP 90*, *TaDip*, Tres CFD, *TaCFM*), two genes down regulated in roots and shoots (*TaGLR* and *TaLIPX*), one gene upregulated in roots and down regulated in shoots (*TaBiSTS*) and one gene down regulated in roots and up-regulated in shoots (*TaWrab18*). Red bars represent qPCR results, whereas blue bars represent the results of RNA sequencing.

3.4 Discussion

Global profiling of the transcriptome of roots and shoots of Najran wheat revealed profound changes in gene expression under salt-stress as shown by the high number of DEGs. The expression of about 6.2% and 3.7% of the estimated 94000 wheat genes changed in roots and shoots respectively under salt-stress. Among the identified DEGs, candidate genes related to key pathways with important roles in adaptation and responses to salt stress were identified. It is worth noting that the levels of transcriptional changes under abiotic stresses vary between plant tissues, this being observed to a greater extent in tissues sensing stress early rather than those subsequently detecting it. Roots of drought-treated Arabidopsis plants, undergo higher levels of transcriptional alterations compared to shoots under water-stress, suggesting that the physiological adjustments induced in roots were deeper to those induced in shoots (Bashir et al., 2018). Goyal et al. (2016) have showed that 17,911 genes were differentially expressed under salt-stress in roots of Kharchia wheat. Similarly, Amirbakhtiar et al. (2019) have shown that 5128 were differentially expressed under salt-stress in roots of Arg wheat. Among these genes, there were genes for sensing and signalling of salt stress such as genes that encode calcium transporters (*Ta.ANN4*, *Ta.ACA7*, *Ta.NCL2* and *Ta.GLR*) and SOS1 (Na^+/H^+ antiporter), genes coding for transcriptional regulators such as transcription factors (MYB, bHLH, AP2/ERF, WRKY, NAC, and bZIPs) as well as genes related to salt stress adaptation, including genes coding for LEA proteins, Aquaporins, P5CS, dehydrins, ABA and K+ transporters (*Ta.ABAC15*, *Ta.HAK25*), catalases, glutathione-S-transferases. Roots as the first organ encountering salinity in the surrounding media often respond by deploying specific responses to maintain water and nutrient uptake while limiting the uptake of toxic ions and potentially extruding them (Rajaei et al., 2009). In contrast, plant shoots detect and respond to salt-stress at a later stage, they might deploy specific responses to keep cells hydrated and protect metabolism from inhibition including photosynthetic reactions to keep growth. Shoots have higher sensitivity to salinity than the roots (Esechie et al., 2002). Functional enrichment of the DEGs in roots and shoots of Najran wheat revealed important biological and molecular functions that may be key to the survival and development of this wheat cultivar under salinity. Genes associated with glutathione metabolism, galactose metabolism and thiamine metabolism were differentially regulated in roots of salt treated Najran wheat compared to the control. The highest number of DEGs was in glutathione metabolism pathway. These DEGS included fifty genes coding for glutathione S-transferase (GST) and one gene coding for glutathione dehydrogenase. Three genes encoded glutathione synthase (GSS) were however downregulated

in response to salinity stress. Exposure of plants to salt stress may increase the production of ROS which might lead to cellular damage via protein denaturation, peroxidation of membrane lipids, DNA mutation, pigment breakdown and carbohydrate oxidation (Noctor and Foyer, 1998; Ahmad et al., 2010). To alleviate ROS-destructive effects, plants have evolved ROS-scavenging systems, including regulatory enzymes e.g. glutathione transferase (GT), ascorbate peroxidase (APX), catalase (CAT), and nonenzymatic elements (metabolites such as phenolics, glutathione, flavonoids, ascorbic acid, carotenoid). The detoxifying role of these enzymes and antioxidants has been reported to enhance salt tolerance in many plants such as GST in Arabidopsis (Qi et al., 2010), glutathione peroxidase (GPX) in rice (Paiva et al., 2019), phenolic acids in cabbage (Linić et al., 2019), APX, CAT, superoxide dismutase (SOD) and peroxidase (POD) in Tobacco (Li C et al., 2020) and flavonoids in *Ginkgo biloba* (Xu et al., 2020). The significantly enriched pathways in shoots of salt-treated plants included biosynthesis of secondary metabolites, protein processing in endoplasmic reticulum, starch and sucrose metabolism, phenylpropanoid biosynthesis, galactose metabolism and phenylalanine metabolism pathways. Most DEGs in shoots of Najran wheat were related to the biosynthesis of secondary metabolites pathway. Secondary metabolites play important roles in plants acclimation to different environmental stresses. In this study, genes associated with phenylpropanoid, galactose metabolism, fatty acid elongation, flavonoid biosynthesis, starch and sucrose metabolism and lignin synthesis pathways were shown to be significantly regulated in shoots under NaCl stress. As an example of the salt-regulated genes in phenylpropanoid biosynthesis there were genes encoding phenylalanine ammonia-lyase (PAL), two isogenes encoding for caffeoyl shikimate esterase (CSE), one isogene encoding for cinnamoyl-CoA reductase (CCR) and one isogene encoding ferulate-5-hydroxylase (CYP84A). All these genes have previously been documented to participate in salt tolerance in (Amirbakhtiar et al., 2021; Kong et al., 2021). In phenylpropanoid biosynthesis, PAL is involved in the first step of synthesizing trans-cinnamate from Lphenylalanine and acts as one of the antioxidative components produced under stress to minimize oxidative damage induced by salt stress (Gholizadeh and Kohnehrouz, 2010). The final products in this metabolic pathway are lignin components, including syringyl lignin, 5-hydroxy-guaiacyl-lignin and guaiacyl lignin. It is widely known that plants synthesize lignin to maintain the structural integrity of cell wall and the rigidity of the stem helping the plants to cope with various environmental stresses (Rao et al., 2017). More recent work highlighted the important regulatory role of NAC transcription factor (*AgNAC1*) in lignin biosynthesis which ultimately enhances salt tolerance in *Arabidopsis*

thaliana plants (Duan et al., 2020). In the present study, one NAC domain containing gene was significantly up-regulated in wheat shoots and five genes were down-regulated in the roots. Another study conducted on *Betula platyphylla* suggested the positive correlation between overexpression of *BpNAC012* and lignin biosynthesis and its crucial role in the tolerance of both salt and osmotic stresses (Hu et al., 2019). Luo et al. (2019) carried out functional enrichment analysis for DEGs induced under salinity stress in new leaf, old leaf, and root tissues of two wheat varieties, Zhongmai 175 and Xiaoyan 60, they found that metabolic pathways including those for phenylpropanoid biosynthesis, biosynthesis of secondary metabolites, benzoxazinoid biosynthesis and starch and sucrose metabolism were significantly enriched in the three organ types. DEGs under salt stress were classified into 5 key categories of KEGG pathways in roots (Amirbakhtiar et al., 2019) and leaves (Amirbakhtiar et al., 2021) of Arg wheat cultivar namely metabolism, genetic information processing, environmental information processing, cellular processes and organismal systems. Metabolism in these studies has been reported to have the greatest enrichment, far larger than the other enriched pathways. Similarly, our findings revealed that most DEGs in the roots and shoots were enriched in three main KEGG classes: metabolism, organismal systems and genetic information processing pathways. Among them, the number of DEGs was largest in metabolism category, including 4044 genes involved in different pathways such as biosynthesis of secondary metabolites, amino acid metabolism, carbohydrate metabolism, metabolism of cofactors and vitamins. Abiotic stresses including salt stress usually result in high accumulation of sugars such as glucose, sucrose and galactose which has a great role in osmoregulation, homeostasis, stabilization of protein structure and carbon storage (Singh et al., 2015; Sami et al., 2016). Darko et al. (2019) investigated the content of different metabolites in wheat seedlings and found that plants exposed to NaCl exhibited higher levels of sucrose and galactose in root and shoot tissues, suggesting the participation of L-galactose in ascorbic acid pathway. Ascorbic acid has been shown to protect plants from stress-induced oxidative damage, acting as an antioxidant, and to enhance the growth and development of plants (Zhang et al., 2015). The results obtained in this study confirm these findings where two genes involved in galactose metabolism, and which encode beta-fructofuranosidase (INV) and raffinose synthase were identified to be regulated in the roots and shoots of Najran wheat under NaCl stress.

3.5 Conclusion

This investigation used RNA sequencing to profile salt-induced changes in the transcriptome of roots and shoots of a salt-tolerant wheat, the Najran Cultivar from Saudi Arabia. Previous studies used the same approach to study changes in the transcriptome of roots or shoots of different wheat plants under salt-stress or water-stress, to our knowledge no previous study analysed these changes simultaneously in roots and shoots of same wheat plants. Our results, show that roots respond to a higher extent than shoots to salt-stress and that salt-stress induces organ specific responses as well as responses that are common to roots and shoots. These results suggest that an efficient attempt to improve tolerance to salt-stress should consider starting with optimizing antioxidants responses including the production of glutathione and phenolics particularly phenylpropanoids as well as galactose metabolism in roots and shoots. A transcriptomic approach such as the one used in this work is vital to map the global changes induced by salt-stress to the cellular functions and metabolic pathways. This needs however to be complemented with detailed expression- and functional analysis of key genes of these functions and pathways to inform an integrated and efficient approach to improve salt-tolerance in wheat and potentially other plant species.

Chapter 4 Transcriptome-wide Characterization of Alternative Splicing Regulation in Roots and Shoots of Wheat under Salt Stress

4.1 Introduction

In recent years, climate change has had a direct impact on agricultural production and quality of yields by increasing the frequency and severity of several environmental stresses (Ahsan et al., 2020). Salt stress is one of these stresses impacting 20% of the world's cultivable land and can contribute to around 50% decrease in crop outputs (Egamberdieva et al., 2019, Ha-Tran et al., 2021). Wheat is the second most globally cultivated crop and is a main source of vegetable proteins and daily calories required for human consumption (Saddiq et al., 2021). Exposure of wheat to salinity leads to many physiological, morphological, and biochemical modifications along with considerable changes in the transcriptome profiles. The hexaploid bread wheat has a very complicated large genome of around 17 Gb, consisting of 94,000- 96,000 genes with different complex roles (Brenchley et al., 2012). Undoubtedly, the landscape of AS in bread wheat and its influence on proteome diversification is more intricate compared to that in other plants. Therefore, comprehending the regulatory mechanisms underlying intricate responses to salt stress holds significance in enhancing the quality and yield potential of wheat.

AS is a critical co-transcriptional and post-transcriptional process that selectively includes or excludes certain exons and introns from the mRNA transcript. In a simplified term, AS enables a single gene to produce several mRNA transcripts, which in turn can result in the generation of various protein isoforms (Tognacca et al., 2023). This process is controlled by a complex interaction between splicing *trans*-acting factors and regulatory *cis*-elements (existed in the intronic and exonic regions of precursor mRNA) that determines which exons and introns are included or omitted in the final mRNA. The resulting alternatively spliced transcripts can be translated into a variety of protein isoforms with different functions, structures or sub-cellular locations, giving rise to proteomic diversity in the plant cells from a relatively limited genome (Kelemen et al., 2013). Skipping exon (SE), retained intron (RI), alternative donor splice site (alternative 5' splice site, A5 or AD) and alternative acceptor splice site (alternative 3' splice site, A3 or AA) are the four fundamental types of AS (Misra et al., 2023), where RI is the most abundant event in plants and SE is the most predominant form of AS in animals (Petrillo, 2023). The AS process occurs in the nucleus when the spliceosome differentially recognizes splice sites in pre-mRNA, failures in exon or intron recognition can lead to either shorter (SE) or longer (RI) mRNA isoforms, respectively. It has been estimated that almost 70% of multi-intron

genes in plants undergo such regulatory mechanism and approximately 50% of the resulted events are RI, followed by A3, A5 and SE (Zhang et al., 2017, Yu et al., 2021, Zhang et al., 2022). Retained intronic regions have been reported to play a vital role in regulating the gene expression timing (Jacob and Smith, 2017, Wong and Schmitz, 2022). Indeed, retained introns can offer specific features to the transcripts which contain them. Many introns possess stop codons, which can result in the production of transcripts containing premature termination codons upon their retention. The occurrence of premature termination codons may cause transcript degradation through the nonsense-mediated mRNA decay pathway (Laloum et al., 2018). However, transcripts with retained introns, which are not degraded through the decay system, might be kept in the nucleus and then released on demand resuming the splicing process in response to internal or external signals, circadian rhythms or developmental stage (Filichkin et al., 2015). On the other hand, some introns, called exitrons, have characteristics that make them more similar to exons and they act as protein-coding introns. Therefore, in addition to the contribution of retained intron transcripts in the transcriptome and proteome diversification, they have a distinct effect on stability, localization and translation of transcripts (Cecchini et al., 2022, Petrillo, 2023).

There is growing evidence that AS is essential for a number of plant developmental processes and stress responses such as the regulation of flowering transition (Wang et al., 2020), defence against pathogen attacks, and responses to environmental stresses (Zhang et al., 2019a, Ling et al., 2017). In addition, AS might have possible functions specific to different plant tissues. Despite the fact that the vast majority of AS isoforms can be expressed in all plant organs, the differentiation and unique functions of each organ explain the variations in some AS isoforms among different organs. For instance, leaves, stems, roots, flowers, and fruits each have distinct cellular compositions, metabolic activities, and environmental interactions. As a result, certain AS isoforms may exhibit differential expression levels or splicing patterns across different organs to meet the specific requirements of each organ (Qulsum et al., 2023). These variations in AS isoform abundance or composition can contribute to organ-specific traits, such as leaf morphology, stem architecture, root architecture, floral development, and fruit ripening. More recent high-throughput study (Hazra et al., 2023) identified thousands of AS events and genes in *Vigna mungo* involved in various regulatory functions, including transcription factors, highlighting their differentially expressions across seed, root and leave tissues. Similarly, Zhu et al. (2018) analysed AS dynamics across eight developing tissues in tea plants and proposed similarities in AS genes between buds and young leaves as well as large variations between

summer mature leaves and winter old leaves. This can be attributed to the extent to which these tissues are molecularly similar or diverse, respectively, implying the different roles of AS during tissue development.

Tissue-specific AS modulation has been intensively investigated recently in several model and non-model plants under different biotic and abiotic stresses (Shen et al., 2014, Filichkin et al., 2018, Zhu et al., 2018, Zhang et al., 2019c, Martín et al., 2021). However, up to now, no study has analysed the global AS variations among different tissues in response to salt stress in wheat plants. This study therefore was designed to 1) investigate the comprehensive profiling of AS in the root and shoot tissues of Najran wheat cultivar treated with 200 mM NaCl, 2) compare the contribution of AS and expression regulation in response to salinity cues 3) characterize tissue-specific AS regulation under salt stress and relevant associated molecular functions.

4.2 Material and methods

4.2.1 Plant material, growth and stress treatment

Plant growth and treatment of salt-tolerant wheat cultivar (Najran) with salt stress (200 mM NaCl) were done as what has been described in the previous (transcriptomic) chapter (3.2.1). Roots and shoots of one month old plants were collected, washed, and then ground immediately in liquid nitrogen and stored at -80 °C for RNA extraction.

4.2.2 RNA extraction, purification and high-throughput sequencing

RNA extraction, quality control, library construction and sequencing from wheat samples were done following the procedure used in the previous chapter (section 3.2.2 & 3.2.3).

4.2.3 Quantification of RNA-Seq data

The obtained paired-end reads were assessed and then filtered from contaminating adaptor sequences by Fast QC (version 11.8, http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and Trimmomatic (version 0.36, http://www.usadellab.org/cms/?page=trimmomatic), respectively. The clean trimmed reads were quantified against wheat reference gene annotation (Ensembl *T. aestivum* genome version 51) and normalized to generate transcript expression (TPMs, transcript per million) files. The abundance of each transcript was estimated for each sample using Salmon (version 0.12.0, https://combine-lab.github.io/salmon).

4.2.4 AS event profiling and PSI calculation

The AS analysis was carried out with SUPPA2 (Trincado et al., 2018). All potential AS events including SE, RI, A5, A3, mutually exclusive exon (MX), alternative first exon (AF) and alternative last exon (AL) (Figure 4.1) were generated from the wheat genome GTF file (*Triticum_aestivum*.IWGSC.51.gtf) using the SUPPA2 command 'generateEvents'. This produces an event file (referred to as an ioe file) which includes the IDs of transcripts that define each event per gene. The SUPPA2 command ‘psiPerEvent’ uses the transcripts in the ioe file and the transcript abundances (TPM values) calculated by Salmon to compute the relative abundance of AS events or what has been known by percentage spliced-in (PSI) values for each event.

4.2.5 Analysis of differential Alternative Splicing events

The SUPPA2 command 'diffSplice' was employed to calculate differential splicing events in the roots or shoots of wheat under salt stress conditions, using PSI values, event files, and transcript expression files. Differential splicing is measured in terms of delta-PSI (ΔPSI), the difference in PSI measured between tested groups (salt treated root vs control root or salt treated

shoot vs control shoot). By comparing the distribution of ΔPSI between groups, SUPPA2 calculates p-values which are adjusted using the Benjamin-Hochberg method. Events with an adjusted p-value less than 0.05 were considered to be significantly differentially spliced between groups.

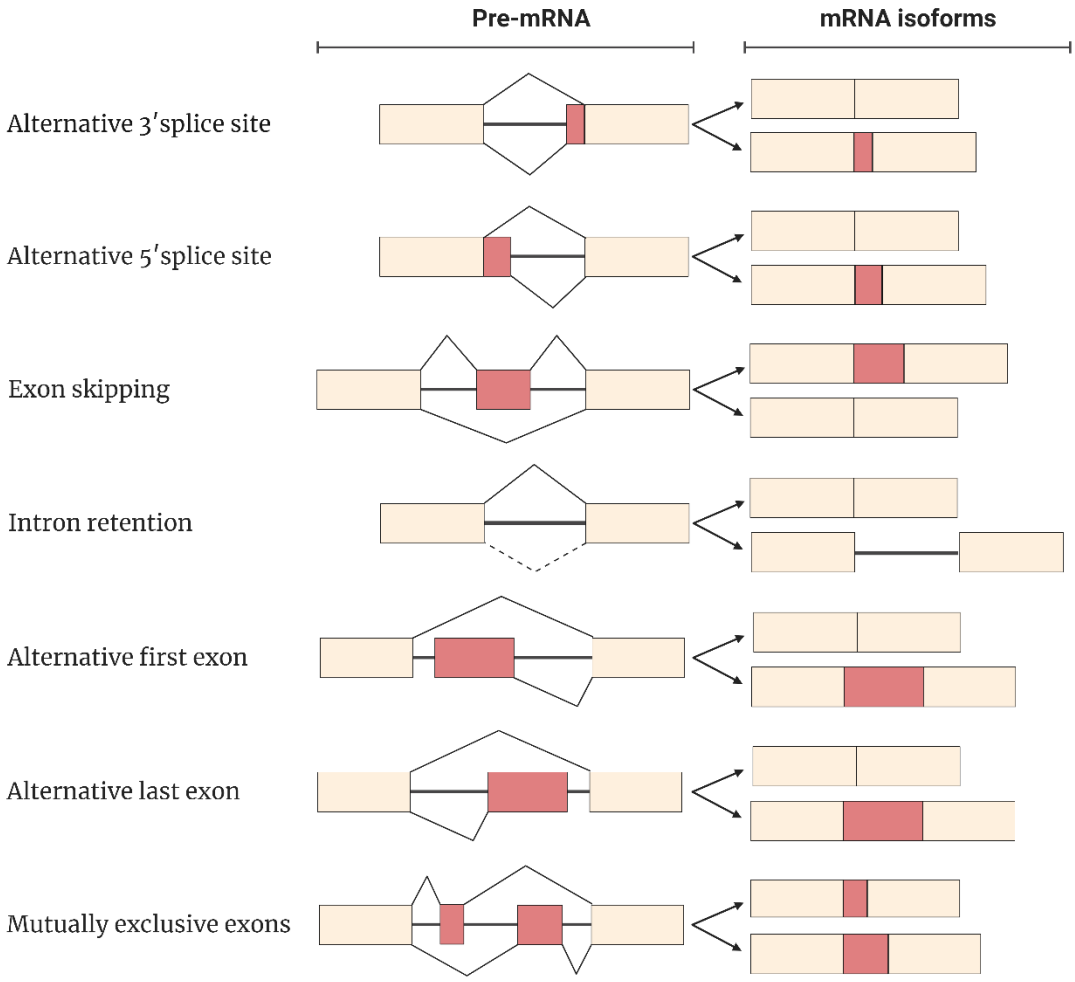


Figure 4.1: Schematic representation of different modes of Alternative Splicing events in Najran wheat cultivar under salt stress. Beige boxes represent constitutively expressed exons while red boxes represent alternatively spliced exons. Introns are illustrated as black lines.

4.2.6 Gene ontology enrichment analysis

GO analysis was carried out to identify enriched GO terms for the differentially spliced genes (DSGs). GO terms were assigned to the DSGs using the PANTHER classification system (http://www.pantherdb.org/panther/ontologies.jsp). PANTHER classifies DSGs by molecular function (MF), cellular component (CC) and biological process (BP). The significantly overrepresented GO terms for the lists of DSGs were calculated using a Fisher exact test with the corrected *P*-value (FDR) < 0.05.

4.2.7 Correlation analysis of salt stress and AS

According to GO analysis, cysteine-type endopeptidase inhibitor activity (GO:0004869) was the most significantly enriched term which was shared between roots and shoots of Najran wheat. Cysteine proteinase inhibitor (CPI) with SE was the only DSG associated with this GO term in our RNA-Seq data under salt stress. To explore the relationship between salt stress and AS, the two differently sized transcripts of CPI gene (SE event) from samples treated with salt at two levels: 1) different salt concentrations (0, 50 and 200 mM NaCl) and 2) different time points of salt treatment (0, 1, 3, 6 and 12 days) were monitored by RT-PCR and visualised using 2% agarose gel. Roots and shoots total RNAs (5 µg) were reverse transcribed with 200 U of Tetro Reverse Transcriptase and Oligo $(dT)_{18}$ primers using Tetro™ cDNA Synthesis Kit (Bioline Reagents Ltd, UK) according to the manufacturer's procedure. PCR was performed using MyTaq™ Mix (Bioline Reagents Ltd, UK) in 50 µl reaction mixtures; 2 µl cDNA, 25 µl My Taq Mix (2x), 1 µl of each CPI-specific primer (10 µM) and 21 µl double distilled water. The thermal profile used of the PCR was as follows: 95°C for 1 min, then 40 PCR cycles at 95°C for 15 s, 63.8°C for 30 s to allow primers annealing to the target cDNA sequence, and 72°C for 1 min. The amplified products were then examined on 2% agarose gel stained with GelRed® Nucleic Acid Stain. Afterwards, densitometric analysis of gel bands was done using ImageJ software (https://imagej.nih.gov/ij/) to measure the approximate quantity of the two splice isoforms in all samples.

4.2.8 Validation of alternatively spliced genes

To validate the AS profiles obtained from RNA-Seq data, four DSGs expressed in both roots and shoots were randomly selected for the RT-PCR test (Table S5). AS patterns of these genes were RI event for genes encoded for drought-responsive factor-like transcription factor (DRFL1a) and histone acetyltransferase (HAT), A5 event for malic enzyme (ME) gene and SE event for CPI gene. Gene-specific primers (Table 4.1) were designed using Primer3 (v. 0.4.0) to amplify both differently sized alternative splicing isoforms (Figure 4.2) and then checked

using automated web site (http://www.bioinformatics.org/sms2/pcr_primer_stats.html). Before running the RT-PCR, cDNA was synthesized, from the same RNA samples used in the RNA-Seq analysis, using Tetro™ cDNA Synthesis Kit (Bioline Reagents Ltd, UK) according to the manufacturer's instructions. MyTaq™ Mix was used to perform RT-PCR in BIO-RAD T100™ Thermal Cycler based on the manual of the kit. Annealing temperature for all tested genes was optimised using a temperature gradient ranged from 50°C to 65°C (Figure S7). The PCR program was run (as described above in 4.2.7) with 55.7°C, 62°C or 63.8°C annealing temperature for HAT, DRFL1a & ME or CPI-specific primers, respectively. After running RT-PCR, 2% agarose gel electrophoresis was utilised to visualise the different spliced isoforms.

Table 4.1: Primer pairs used for the validation of Alternative Splicing in HAT, DRFL1a, ME and CPI genes.

Gene ID	**Primers**	**Primer Sequence（5'-3'）**
TraesCS6A02G107300	HAT- F	TGAGGTTTTGGATCAGTCAGAA
	HAT-R	CTTGCATAGAATGGCTGGAA
TraesCS5D02G200900	DRFL1a-F	TCCAAGAACGGCAAGAAGTC
	DRFL1a-R	GGTACTTGGTGAGGGCGAAG
TraesCS3B02G128000	ME-F	GCCCACAGGGTCTGTATGTC
	ME-R	CCAAGGGCAGTGTACAGAGA
TraesCS1A02G256400	CPI-F	CAAGGCAGTTGTCGAGTTCC
	CPI-R	CTTCACGCCTTCCAGTTCCA

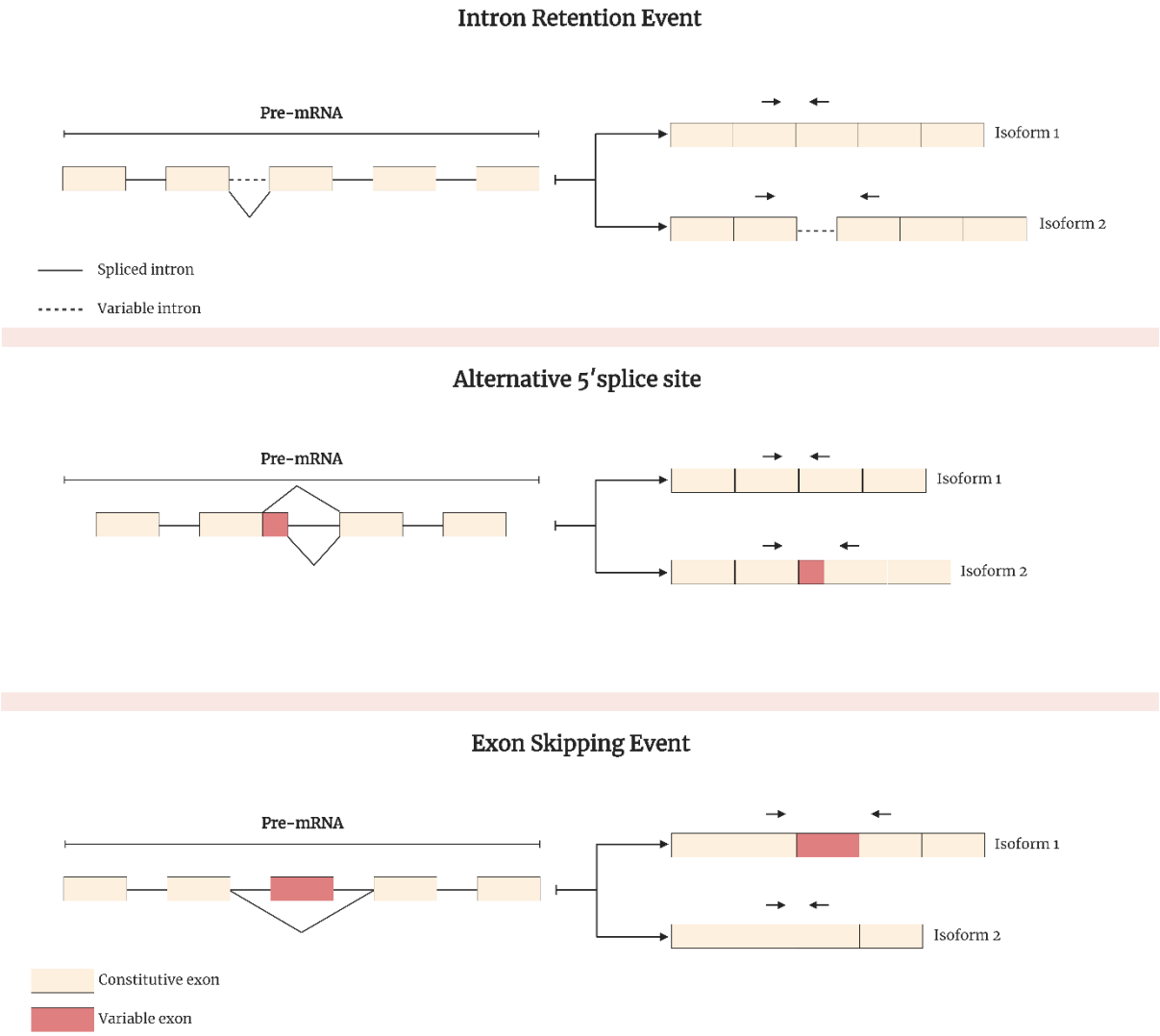


Figure 4.2: Reverse transcription PCR for investigating different splice isoforms. The two arrows indicate primer pairs that flank variable sequence of respective exon or intron and thus amplify both splice isoforms. Beige boxes represent constitutively expressed exons while red boxes represent alternatively spliced exons. Spliced introns are illustrated as black lines while variable introns are depicted in dashed lines.

4.3 Results

4.3.1 Transcriptome-wide identification of AS profiles in wheat

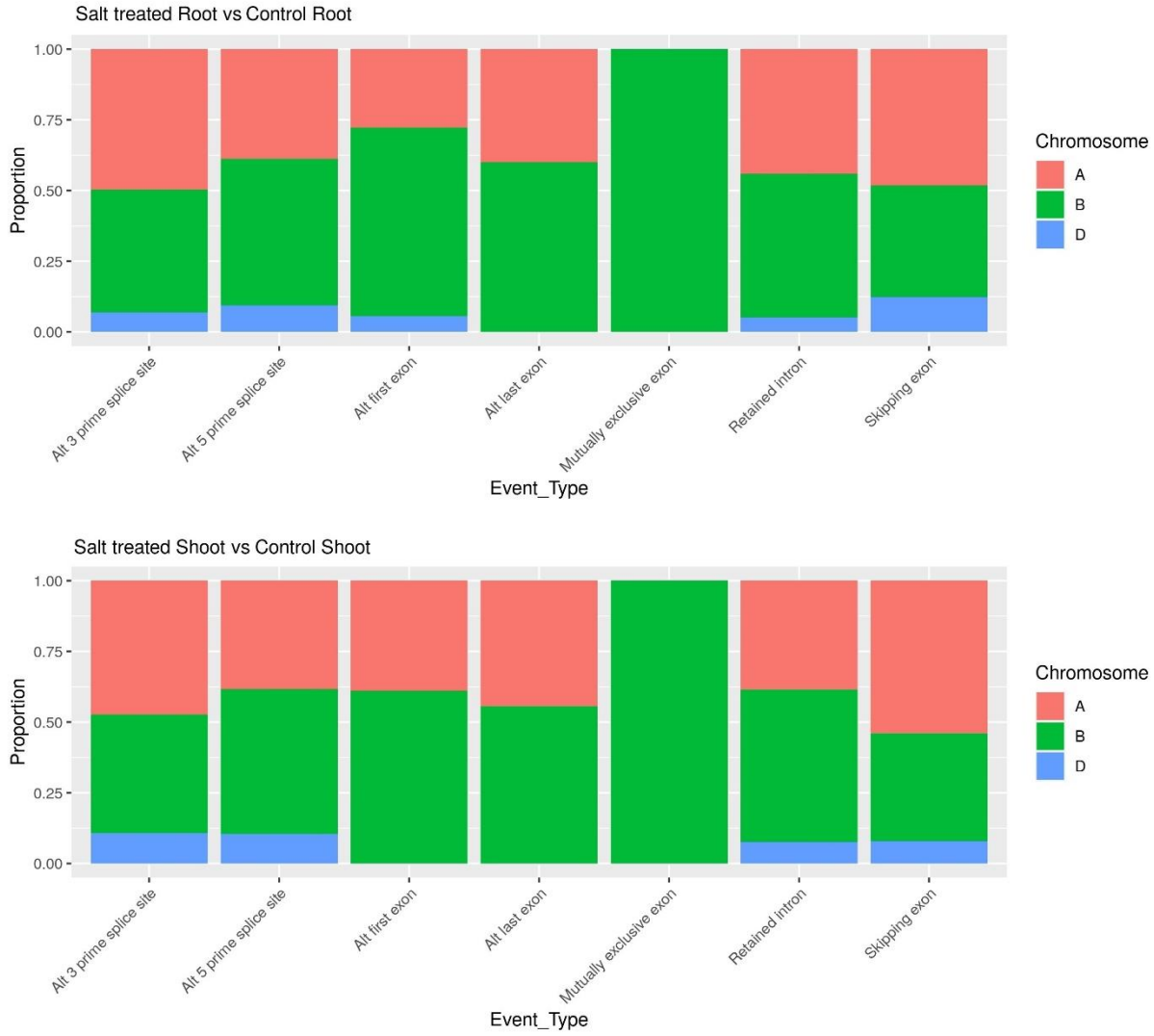
To understand how mRNA splicing was regulated in the roots and shoots of wheat (Najran cultivar) under salt stress, the global profiles of AS landscape was identified using the RNA-Seq data obtained in the previous chapter. A total of 135187943 (99% of all roots raw reads) and 137600234 (99% of shoots raw reads) high quality reads were quantified against transcripts of *T. aestivum* genome IWGSC_V51 and all potential AS events were then recognized by using the SUPPA2 tool. According to that, 39823 AS events corresponding to 32268 AS genes were identified in the roots as well as 39424 AS events corresponding to 31941 genes were found in the shoots under control and salt stress conditions (Table S6). All identified AS genes counted for about 22.5% and 23.1% of expressed genes in the roots and shoots, respectively. Wheat genome consists of three sub-genomes (A, B and D) having 21 pairs of chromosomes. To have an overview of the global AS events in the whole genome, the distribution of different modes of AS events among the three sub-genomes was investigated. Most AS events were found in sub-genome B (197, 181) comparing with sub-genome A (191, 176) and D (34, 35) in the root and shoot samples, respectively (Table 4.2).

The Abundance of AS types among wheat sub-genomes and chromosomes are shown in Figure 4.3, A3 was the most frequent AS event (45.0, 40.3%), followed by SE (20.4, 23.3%), A5 (17.3, 18.8%), RI (13.6, 11.4%) and AF (2.6, 4.0%) in the root and shoot samples. In addition, more DSGs were in sub-genome B (349), whereas 340 and 63 DSGs were found to be located on sub-genomes A and D, respectively (Figure 4.4). Approximately 59.9% of these DSGs had two transcript isoforms, whereas 40.1% exhibited more than two AS variants (an average of 2.84 isoforms per gene).

Table 4.2: Proportion of differential AS events and DSGs in A, B and D sub-genomes of *Triticum aestivum* (Najran cultivar) under salt stress.

	Sub-genome A		**Sub-genome B**		**Sub-genome D**	
	DSGs	**AS events**	**DSGs**	**AS events**	**DSGs**	**AS events**
Salt treated Root vs. Control Root	45.1%	45.2%	46.1%	46.6%	8.4%	8.0%
Salt treated Shoot vs. Control Shoot	45.0%	44.8%	46.4%	46.1%	8.3%	8.9%

(A)



(B)

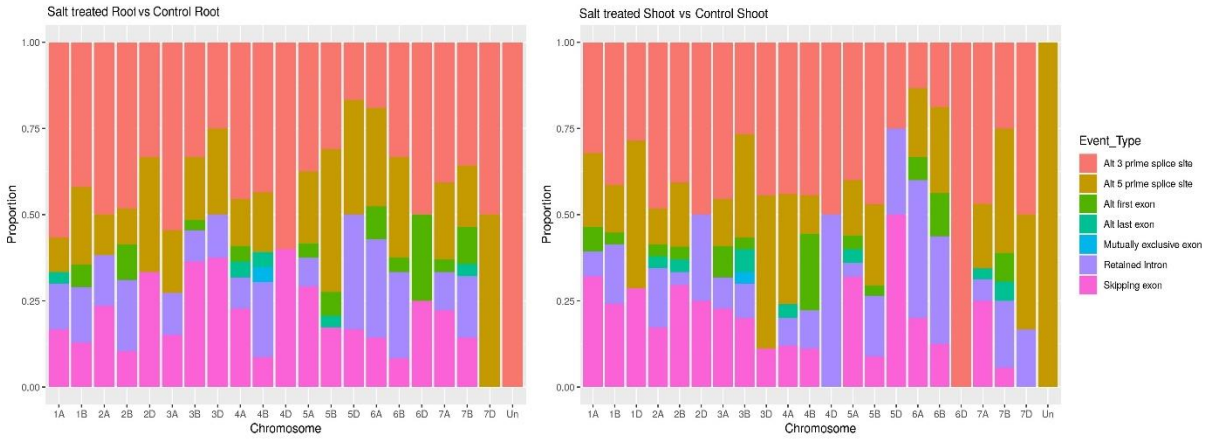


Figure 4.3: Frequency of differential Alternative Splicing modes in (A) wheat sub-genomes A, B and D and (B) different chromosomes in the roots and shoots of Najran cultivar.

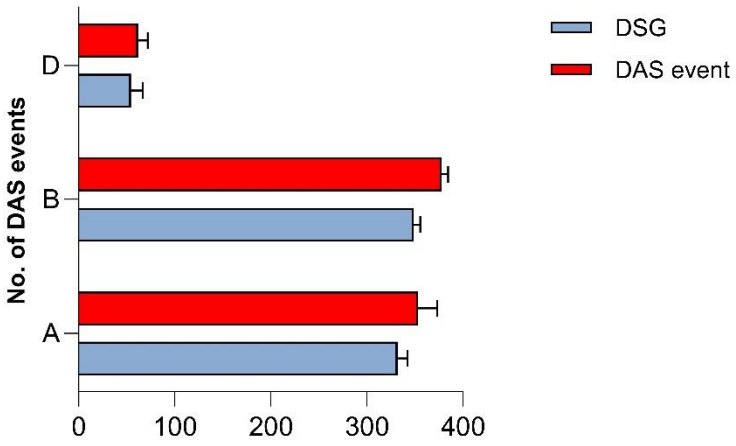


Figure 4.4: The total number of Differential Alternative Splicing events and corresponding differentially alternatively spliced genes in wheat sub-genomes A, B and D (n=3 +/- S.E).

Moreover, the total number of AS events that were identified as stress responsive in each organ was compared and the overlaps between different comparisons in each sub-genome were analysed using a Venn diagram (Figure 4.5). The Venn diagram showed 38, 36 and 3 overlapping AS events between the salt treated roots vs. control roots and salt treated shoots vs. control shoots in sub-genome A, B and D, respectively.

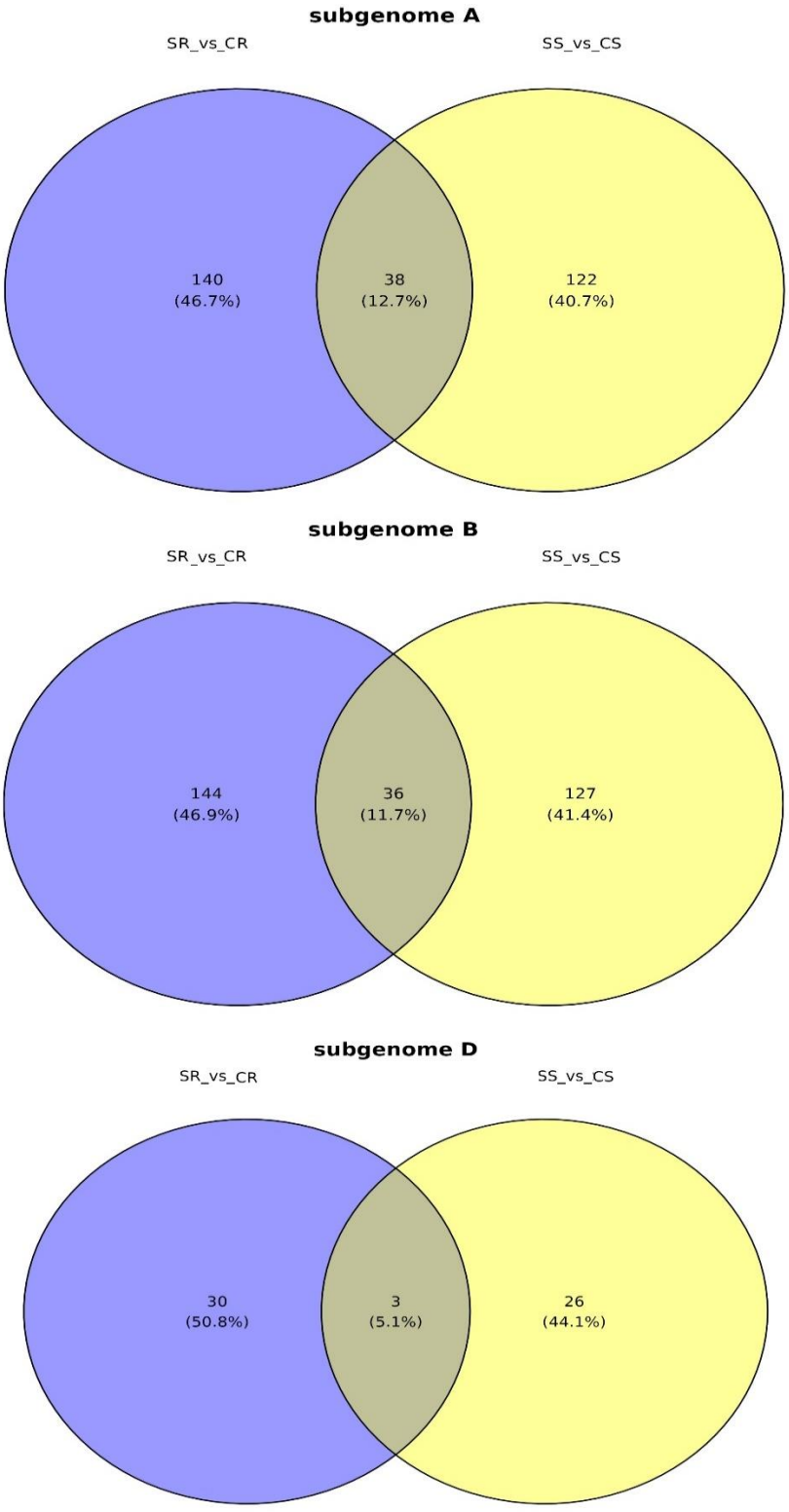


Figure 4.5: Venn diagrams of Alternative Splicing events in Najran wheat cultivar. Venn diagram shows the overlaps of AS events between salt treated roots vs. control roots and salt treated shoots vs. control shoots in sub-genomes A, B and D. SR refers to salt treated roots; CR, control roots; SS, salt treated shoots; CS, control shoots.

4.3.2 Analysis of differentially spliced events

To determine the modulation of AS in the roots and shoots of wheat in response to salt stress, ΔPSI was computed based on the difference of mean PSI between control and salt treated samples. PSI refers to the splicing efficiency of a specific exon into transcript isoforms of a gene, indicating the relative abundance of AS events. Differential splicing was then measured based on the difference of these relative abundances between different conditions. A total of 816 stress responsive differentially alternative spliced (DAS) events were found to be regulated under stress, including 423 and 393 DAS events in salt treated roots vs. control roots and salt treated shoots vs. control shoots (Figure 4.6), corresponding to 392 and 353 genes, respectively. Of these DAS events, 86.3% and 83.2% have already been annotated in the wheat genome, while 13.7% and 16.8% were identified as novel AS events in the roots and shoots, respectively. Although the constant modes of splicing in the roots and shoots, a slight difference on the ratio of AS event types was observed between the root and shoot tissues. Further distribution analysis of DAS event types showed that A3 represented 41.1% and 38.2% of total events in the salt treated roots and shoots, respectively, followed by A5 (20.1% and 22.1%), SE (19.1% and 19.3%), RI (13.9% and 13.2%), AF (4.3% and 4.6%), AL (1.2% and 2.3%) and MX (0.2% and 0.3%) (Table 4.3). The analysis's results revealed that A3 was the most abundant event and MX was the less prevalent form of AS in both tissues.

The boxplots in Figure 4.7 show the range of ΔPSI values for different modes of AS events in the roots and shoots of Najran cultivar. Delta-PSI is the difference in PSI between groups, and is the data used to determine differential splicing. For example, in the salt treated roots vs. control roots comparison (Figure 4.7 B1) SUPPA works out the difference (ΔPSI) between PSI values for (for example A3 event) in the salt treated root samples and PSI values for A3 in the control root samples and uses this to decide which A3 events are significantly different between the treated roots and control roots samples. Comparing the different modes of AS events between the roots and shoots (Figure 4.7 A) revealed that there was not a significant difference in terms of how the range of delta-PSI values differs between these two tissues. However, a clear variation was observed on the distribution of different AS events within each tissue type.

Table 4.3: Summary of the percentage of the different types of Alternative Splicing events in the roots and shoots of Najran wheat cultivar under salt stress.

	Salt treated Roots vs. Control Roots	Salt treated Shoots vs. Control Shoots
A3	174 (41.1%)	150 (38.2%)
A5	85 (20.1%)	87 (22.1%)
AF	18 (4.3%)	18 (4.6%)
AL	5 (1.2%)	9 (2.3%)
MX	1 (0.2%)	1 (0.3%)
RI	59 (13.9%)	52 (13.2%)
SE	81 (19.1%)	76 (19.3%)
Total	423	393

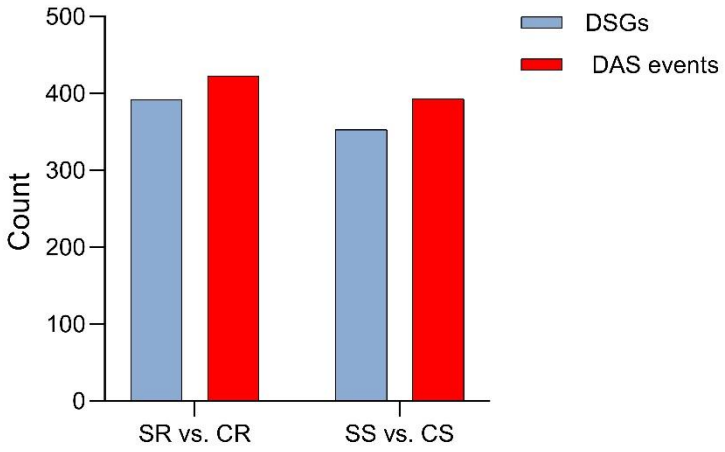


Figure 4.6: Total number of Differential Alternative Splicing events and corresponding differentially alternatively spliced genes in response to salt stress in wheat. SR indicates salt treated root; CR, control root; SS, salt treated shoot; CS, control shoot.

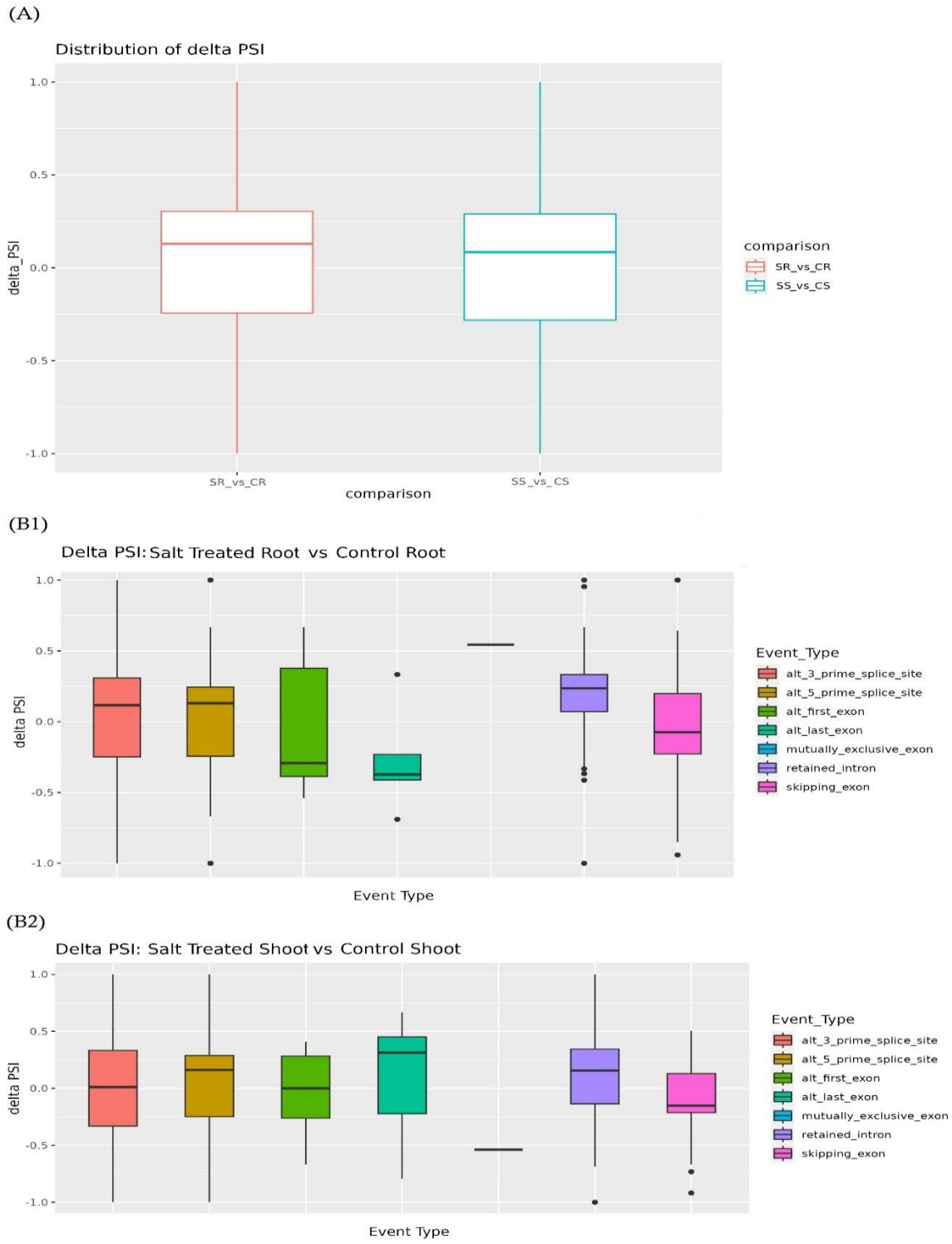


Figure 4.7: Distribution of (A) overall ΔPSI in the roots and shoots (B) ΔPSI of seven modes of AS events within (1) salt treated roots vs. control roots and (2) salt treated shoots vs. control shoots. Delta PSI was calculated based on the difference of mean PSI between different groups (*p*-adj < 0.05). The middle bar is the median, the top of the box is the upper quartile, and the bottom of the box is the lower quartile, the whiskers go from each quartile towards the minimum or maximum value, dots outside the whiskers represent the outliers. CR refers to control root; SR, salt treated root; CS, control shoot; SS, salt treated shoot.

Under salinity stress, the number of AS events in both root and shoot tissues were a bit higher in comparison to control conditions (1.6% in roots and 0.5% in shoots), indicating the potential effect of salt stress on post-transcriptional regulation by inducing AS of pre-mRNA to generate protein isoforms with distinct functions in order to allow the plant to adapt to salt stress (Figure 4.8).

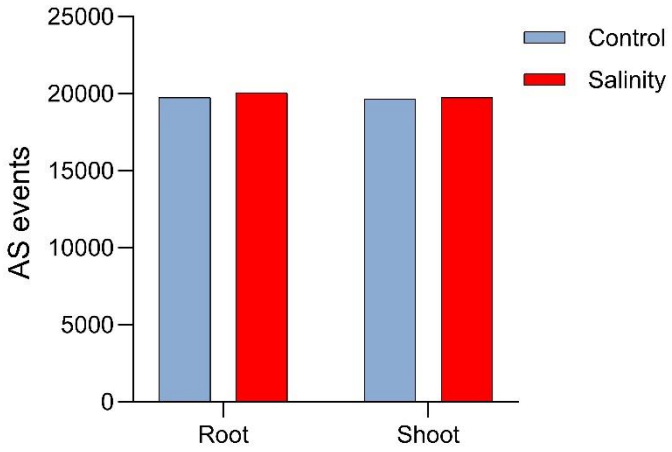


Figure 4.8: Total number of Alternative Splicing events in the roots and shoots of Najran wheat cultivar under control and salt stress conditions.

4.3.3 Comparative analysis of DEGs and DSGs under salt stress

To further elucidate the relationship between transcriptional and post-transcriptional regulations under salt-stress in Najran Wheat, a comparison was done between the genes whose transcript levels were significantly changed and the genes whose mRNAs were significantly spliced in response to salt stress (Figure 4.9). In the previous chapter, the number of genes which were identified as significantly differentially expressed was 5829 and 3495 genes in salt treated roots vs. control roots and salt treated shoots vs. control shoots, respectively. In response to salt stress, only 52 genes whose mRNA levels were changed have been found to exhibit modulation in AS patterns, accounting for 0.8% of all DEGs in the roots. Similarly, the number of overlapping genes between DEGs and DSGs in the shoots was 26 (0.7%) genes. The low ratios of overlapping genes in terms of change in expression and AS under salt-stress indicates a weak relation between the regulation of AS and transcriptional regulation suggesting these two regulations might work independently and potentially in a complementary way under salt stress in this wheat cultivar. Moreover, 340 and 327 genes have been found to be regulated only by AS in responsive to salt stress, accounting for 86.7% and 92.6% of all DSGs in salt treated roots vs. control roots and salt treated shoots vs. control shoots, respectively.

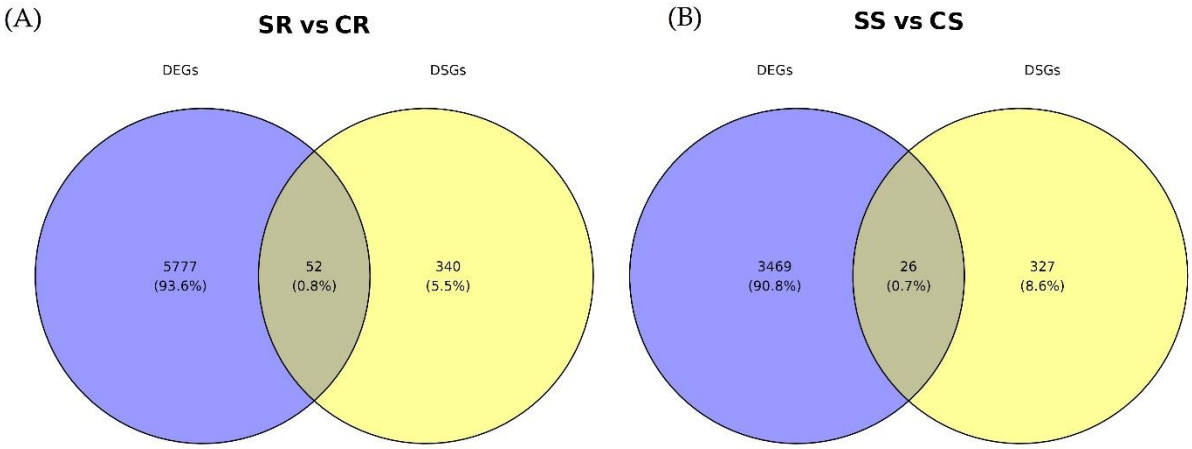


Figure 4.9: Comparisons of Differentially Expressed Genes and Differentially Spliced Genes percentages in response to salt stress in Najran wheat cultivar. The Venn diagrams show the overlaps of genes subjected to transcriptional and post-transcriptional regulation in (A) salt treated root vs. control root and (B) salt treated shoot vs. control shoot.

4.3.4 Functional enrichment analysis of salt responsive DSGs

In the previous transcriptomic study (chapter 3), 4644 and 2310 DEGs were identified as tissue-specific genes in the roots and shoots, respectively. The expression of these genes was found to be strongly associated with the physiological condition of the respective tissue. For example, cell ionic homeostasis, oxidative and osmotic stress responses, hormonal signalling etc were among the most enriched GO terms in roots whereas photosynthesis, response to Abscisic acid and other terms were enriched in shoots. In the present study, 909 root-specific isoforms corresponding to 325 DSGs and 840 shoot-specific isoforms corresponding to 287 DSGs were detected (Figure 4.10 B). Although the small common set of 74 AS events were produced under salt stress in both roots and shoots, most genes have undergone AS in a tissue-specific manner (Figure 4.10 A). The prevalence of unique AS events was found to be higher in root tissues (349 events) compared to shoot tissues (319 events), indicating the potential regulatory role of AS in tissue specialisation.

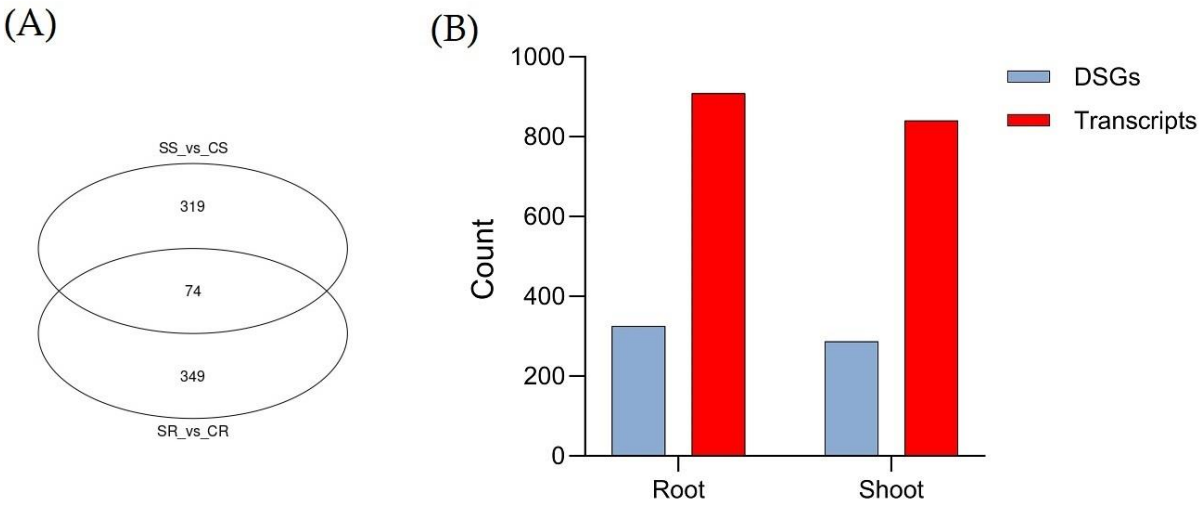


Figure 4.10: Characterization of (A) tissue-specific and common Alternative Splicing events and (B) tissue-specific Differentially Spliced Genes and corresponding transcripts in the roots and shoots of Najran wheat cultivar.

To gain more insight into the functional and biological relevance of DSGs regulated by AS under salt stress, enrichment analysis of GO terms was performed using the PANTHER classification system. PANTHER classifies DSGs of different contrasts by Biological Process (BP) (Figure 4.11 A), Molecular Function (MF) (Figure 4.11 B) and Cellular Component (CC) (Figure 4.11 C). In roots, significant overrepresentation of DSGs was found in 6 BP enriched terms such as microtubule-based process (GO:0007017), purine ribonucleoside monophosphate metabolic process (GO:0009167), actin filament-based movement (GO:0030048) etc, and 3 MF GO terms; cysteine-type endopeptidase inhibitor activity (GO:0004869), oxidoreductase activity, acting on NAD(P)H, heme protein as acceptor (GO:0016653) and cytoskeletal motor activity (GO:0003774).

In shoots, most of the significant DSGs were found to be enriched in 7 BP GO terms including a set of metabolic-related GO terms (e.g. catabolic process (GO:0009056), organic hydroxy compound metabolic process (GO:1901615), monocarboxylic acid metabolic process (GO:0032787) etc), and stimulus-related GO terms (e.g. response to gibberellin; GO:0009739). Besides, one MF GO term (cysteine-type endopeptidase inhibitor activity; GO:0004869) and one CC enriched term (elongator holoenzyme complex; GO:0033588) were significantly overrepresented in DSGs during salt treatment (Figure 4.11 A, B and C).

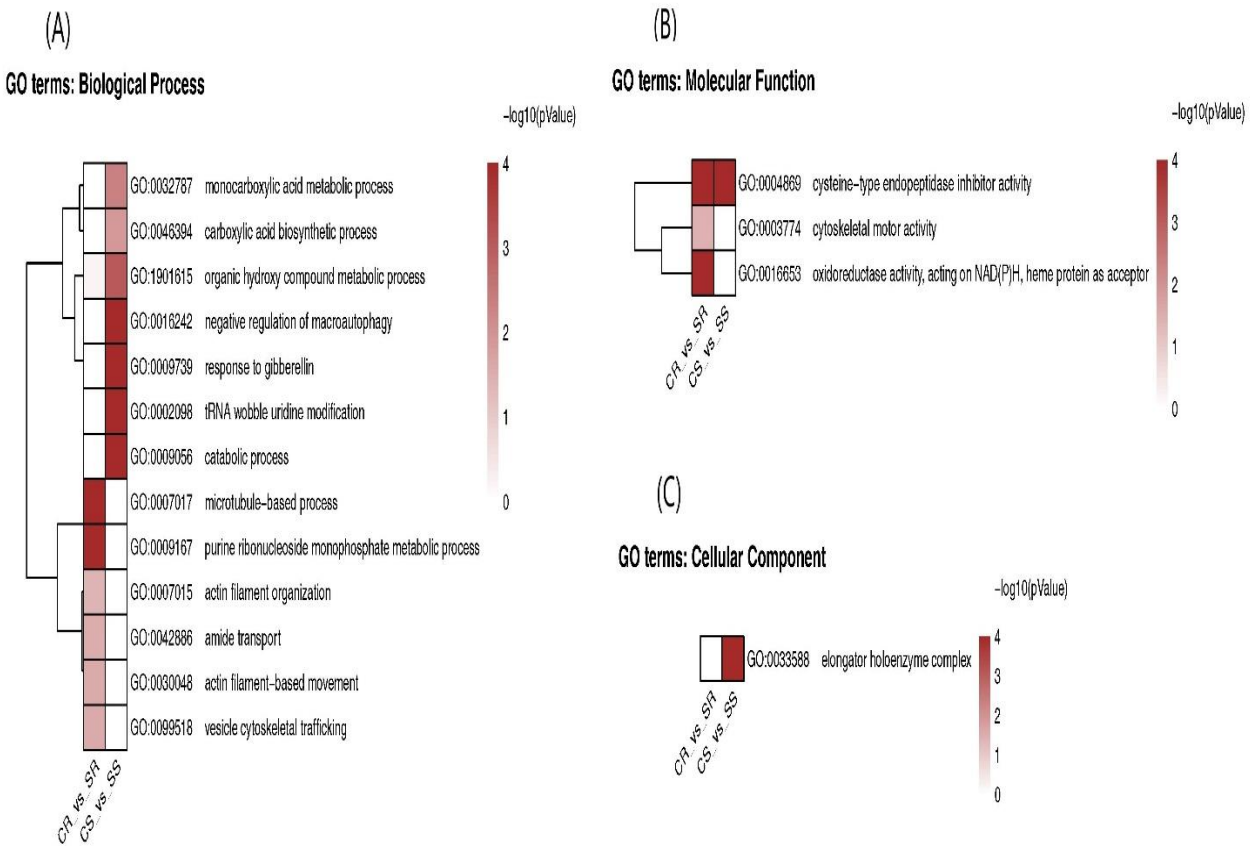


Figure 4.11: Enriched Gene Ontology terms of Differentially Spliced Genes in Najran wheat cultivar. The heatmaps representing GO terms based on (A) biological process, (B) molecular function and (C) cellular component. The colour scale indicates different levels of significance of enriched terms (*P*-value < 0.05).

4.3.5 Correlation analysis of salt stress and AS

To investigate the relation between salt stress and AS, total RNAs were extracted from the roots and shoots of wheat plants treated with different concentrations of salt (0, 50 and 200 mM NaCl) and plants treated with 200 mM NaCl for different times (0, 1, 3, 6 and 12 days). The CPI gene with SE event had two differently sized transcripts; isoform 1 (226 bp) and isoform 2 (134 bp) which were apparent on a 2% agarose gel. The results showed that subjecting wheat roots to 50 mM and 200 mM NaCl increased splicing by 8.79% and 2.13%, respectively. However, salt stress caused a 0.52% splicing decrease in the shoots treated with 50 mM NaCl and a 7.22% splicing increase in the shoots treated with 200 mM NaCl compared to control plants (Figure 4.12 A&B), indicating the non-consistent relationship between AS and level of salt stress.

Similarly, there was a poor correlation between AS and time duration of salt stress where salt treated roots induced AS by 2.82%, 4.45%, 4.35%, 4.46% during 1, 3, 6 and 12 days of salt stress. In addition, the expression levels of alternatively spliced isoform (isoform 2) have shown 1.26%, 1.49% increases in wheat shoots subjected to salt stress for 1, 3 days and 0.31%, 2.67% decline in the shoots treated with salt stress for 6 and 12 days, respectively (Figure 4.13 A&B).

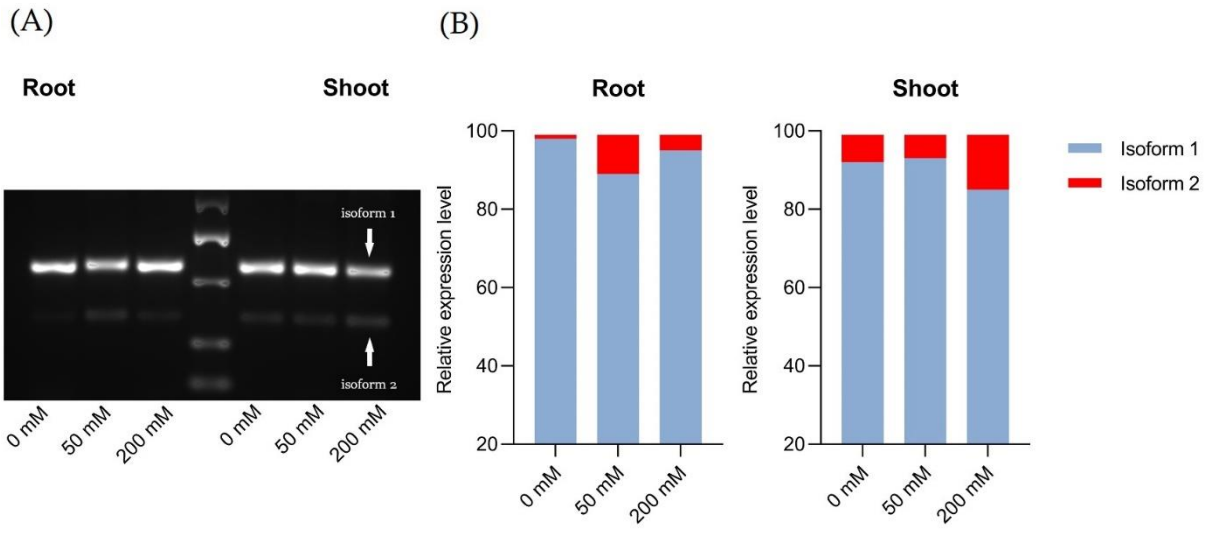


Figure 4.12: Correlation of Alternative Splicing and salt stress in the roots and shoots of Najran wheat cultivar treated with different salt concentrations (0, 50 and 200 mM NaCl). (A) The RT-PCR (left panel) shows the presence of fully spliced transcript (isoform 1, 226 bp) and alternatively spliced transcript (isoform 2, 134 bp) of CPI gene using Agarose gel. (B) The bar graphs (right panel) represent the relative expression levels of isoform 1 and isoform 2 under control and salt stress.

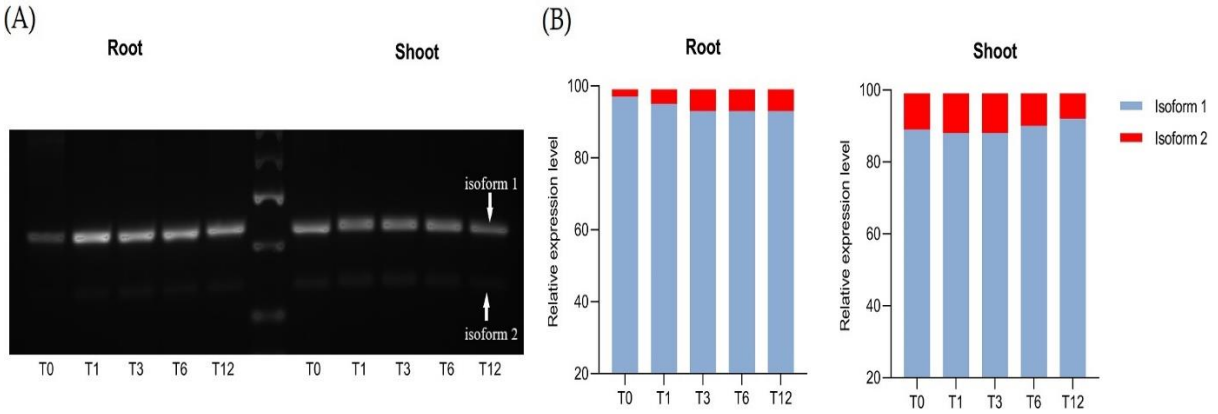
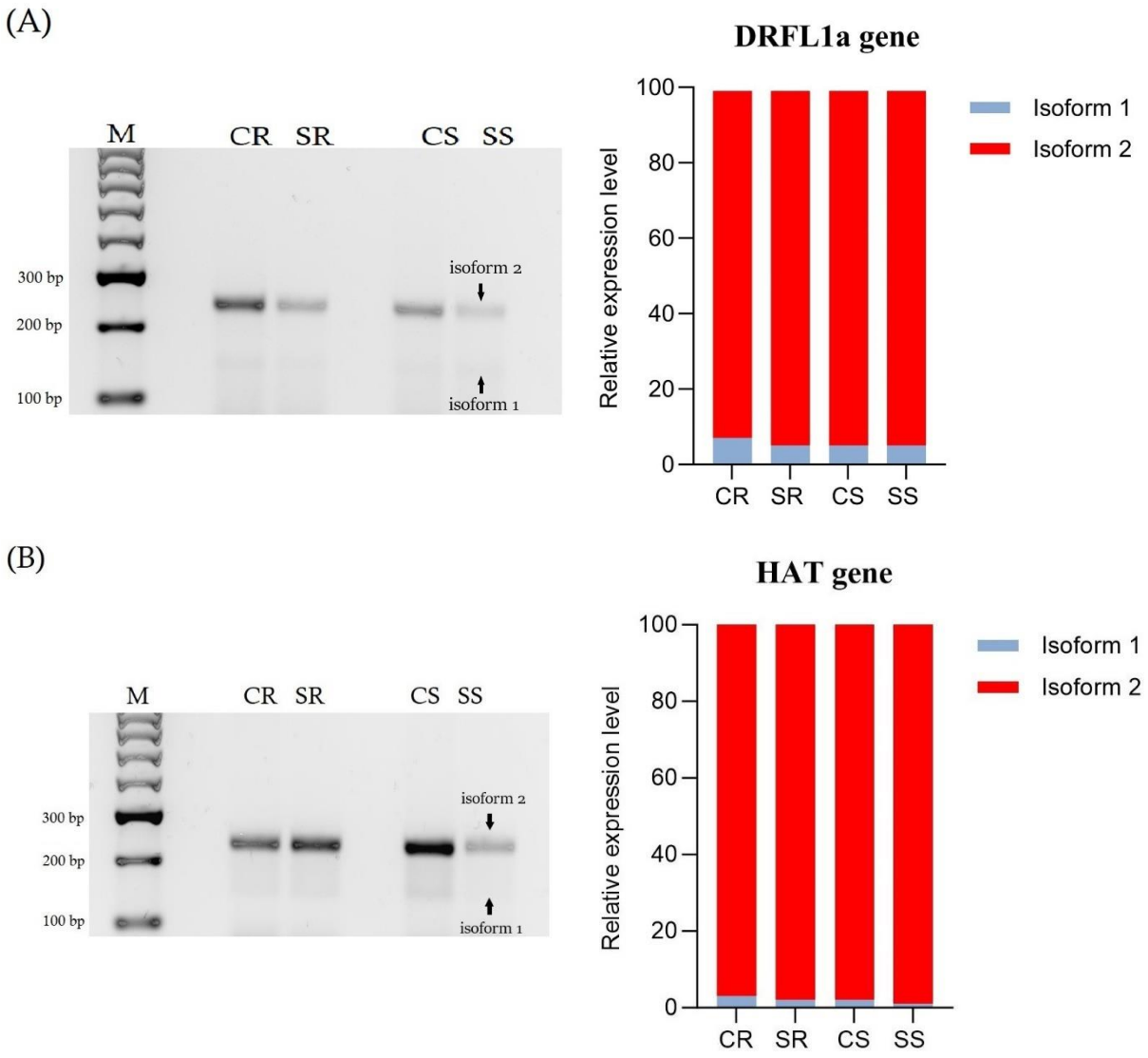


Figure 4.13: Correlation of Alternative Splicing and salt stress in the roots and shoots of Najran wheat cultivar treated with 200 mM NaCl at different points of time (0, 1, 3, 6 and 12 days). (A) The RT-PCR (left panel) shows the presence of fully spliced transcript (isoform 1, 226 bp) and alternatively spliced transcript (isoform 2, 134 bp) of CPI gene using Agarose gel. (B) The bar graphs (right panel) represent the relative expression levels of isoform 1 and isoform 2 under control and salt stress.

4.3.6 Validation of alternative spliced genes

To verify the accuracy and reliability of RNA-Seq analysis, RT-PCR was used to validate the AS transcripts of four randomly selected genes: DRFL1a, HAT, ME, and CPI. Differences in the two alternatively spliced isoforms of each gene can be recognised by size and visualised using 2% agarose gel electrophoresis (Figure 4.14). For example, a RI event occurred in the DRFL1a gene (TraesCS5D02G200900) under control and salt stress conditions in both roots and shoots, where the retained intron amplicon was 252 bp and the fully spliced transcript was 156 bp. As shown in Figure 4.14A, the alternatively spliced transcripts were more abundant than the fully spliced isoforms, which were consistent with RNA-Seq data.



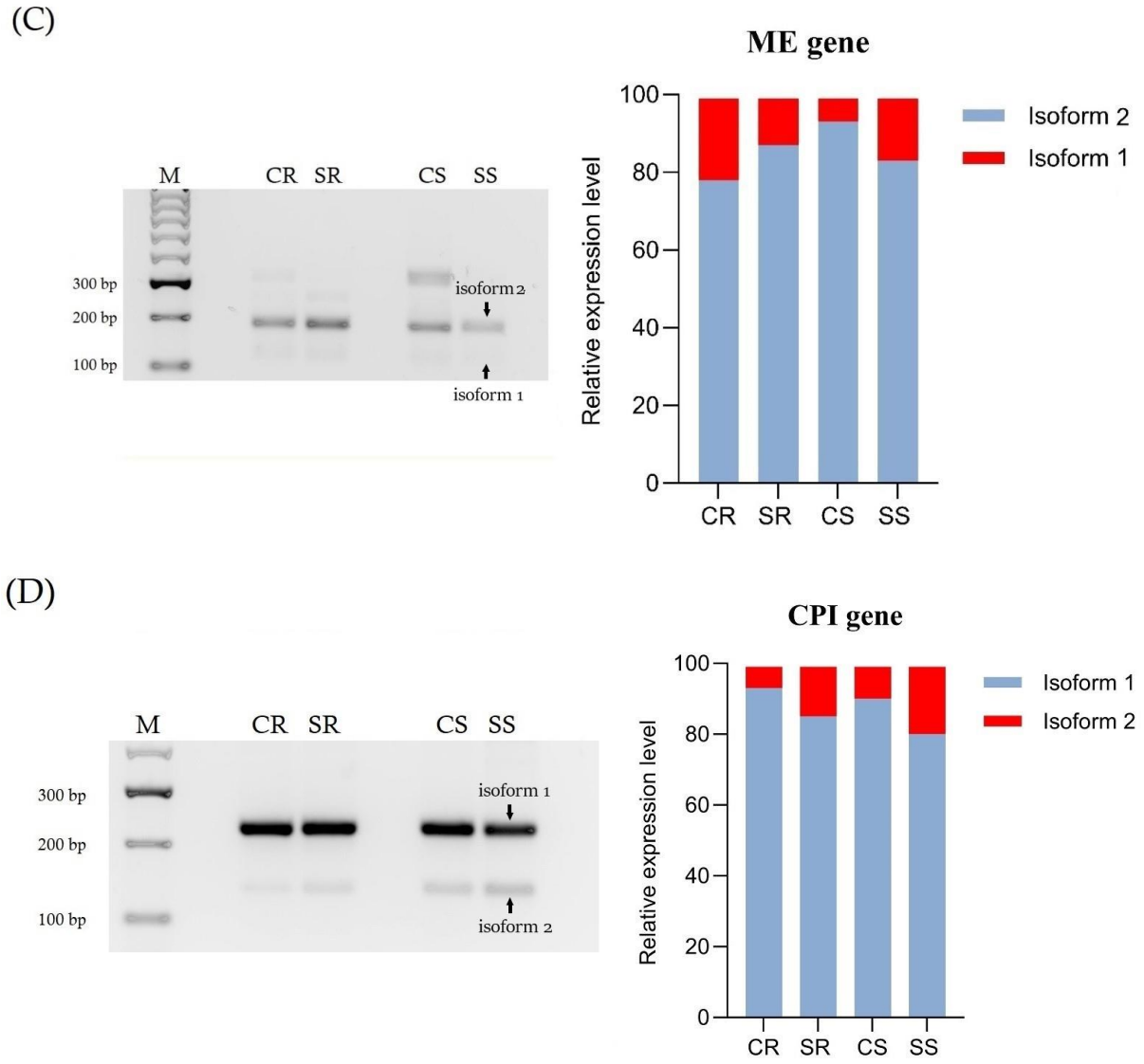


Figure 4.14: RT-PCR validation of the salt-induced Alternative Splicing change in Najran wheat (*Triticum aestivum*) subjected to salt- stress (200 mM NaCl). Relative transcript levels (right panel) are shown in control roots (CR), salt-treated roots (SR), control shoots (CS) and salt-treated shoots (SS) for four randomly selected genes: DRFL1a and HAT (intron retention) (A) and (B), ME (alternative 5' splice site) (C), and CPI (exon skipping) (D). Fully spliced transcript (isoform 1) and alternatively spliced transcript (isoform 2) are visualized on Agarose gel (left panel).

4.4 Discussion

4.4.1 AS landscape and transcriptome adaptation

AS is one of plants molecular responses that enhances the complexity of the transcriptome and serves as a crucial factor in regulating gene expression (Yang et al., 2022). In this study, a total of 39,823 AS events were observed in the roots under both control and salt stress conditions, corresponding to 32,268 AS genes. Similarly in the shoots, 39,424 AS events were detected, corresponding to 31,941 genes. The proportion of identified AS genes accounted for approximately 22.5% and 23.1% of the expressed genes in the roots and shoots, respectively. The occurrence proportion of AS is significantly lower than that observed in *Arabidopsis thaliana* (62.2–66.4%), *Oryza sativa* (~32%), *Zea mays* (45%), and *Sorghum bicolor* (38.5%) (Wei et al., 2017, Wang et al., 2018a, Martín et al., 2021), but slightly greater compared to the percentages reported in *T. aestivum*, cultivar Xiaoyan 6 (18.23%) (Mao et al., 2020). This low ratio of AS shown by the Najran wheat cultivar might be attributed to the small sample size, where we used the roots and shoots from one growth stage that was subjected to one type of stress. The AS ratio was found to be related to many factors, including the size of the sample, the stage of growth, and the range of stresses that plants are exposed to during their lives (Shen et al., 2014). Consistent with findings from Fu et al. (2019), Zhang et al. (2019c), and (Zuo et al., 2023), it was observed that A3 was the most abundant AS event in roots and shoots of the Najran wheat cultivar, whereas MX has the less frequent. However, this finding is different to what was concluded in previous studies, which have suggested that intron retention is the most prevalent AS event in plants (Sun and Xiao, 2015, Wei et al., 2017, Li et al., 2022).

Wheat has three subgenomes (A, B, and D), with the B subgenome exhibiting more AS events than the other genomes. This finding is consistent with that of (Guo et al., 2020), who found most of the spliced events on subgenome B, followed by A and D, suggesting sub-functionalization of homologous genes. Hence, in wheat, AS can contribute to transcriptome reprogramming in response to abiotic challenges, potentially playing a significant molecular role in enabling plants to acclimate to unfavourable environments. An average of 2.84 transcript isoforms per gene were detected in this cultivar, which is somewhat similar to those observed in cotton (2.14) (Zhang et al., 2019b) and Arabidopsis (2.4) (Zhang et al., 2017).

4.4.2 Differential tissue-specific Alternative Spliced genes induced by salt stress

AS plays a significant role in modifying the transcriptome to improve tolerance in plants against environmental stresses. It has been observed that several AS events are regulated at the post-transcriptional level in a tissue-specific manner (Barbazuk et al., 2008, Gu et al., 2020, Martín

et al., 2021). The findings of our investigation indicated that around 82% of these events exhibited differential expression patterns among tissues. For example, 349 DAS events were identified in the roots compared to 319 DAS events in the shoots, suggesting distinct molecular strategies in these two organs to combat the environmental salinity challenge. The greater frequency of AS events in roots compared to shoots can be explained by the differences in specific functions, developmental processes, and environmental adaptations between these two different organs (Hu et al., 2020, Hossain et al., 2022). The higher prevalence of AS observed in roots is collectively driven by the complex interplay between tissue-specific functions, environmental challenges, and regulatory mechanisms (Booth et al., 2022). Consistent with the literature (Gao et al., 2013, Szakonyi and Duque, 2018, Qulsum et al., 2023), this study confirms that each plant's organ has its own unique AS transcript isoforms that are related to specific functions for example organ differentiation. In roots, for example, several salt-induced DSGs were enriched in cytoskeletal-related categories such as microtubule-based processes, actin filament-based movements, and cytoskeletal motor activity. Microtubules and actin filaments are significant constituents of the cytoskeleton and have been observed to perform diverse roles in various essential biological processes, including but not limited to providing structural support, facilitating intracellular transport, enabling cell movement, and ensuring proper DNA segregation (Garner et al., 2004, Fang et al., 2016). On the other hand, in shoots, the DSGs were found to be overrepresented in biological categories related to metabolic and stimulus processes such as catabolic processes, organic hydroxy compound metabolic processes, response to gibberellin, etc. A previous transcriptomic study (Amirbakhtiar et al., 2019), conducted on a salt-tolerant wheat cultivar, Arg, has reported the involvement of salt-responsive DSGs in several biological terms, including metabolic processes related to nucleic acids, response to stimulus, oxidative stress response, metabolic processes related to RNA regulation, and other processes. Although most genes in this study had undergone AS in a tissue-specific manner, a few genes (74 genes) were differentially spliced in both roots and shoots. Interestingly, GO enrichment revealed cysteine-type endopeptidase inhibitor activity as the most significant molecular term enriched in both organs. The shared genes between roots and shoots can serve as a positive control to detect differential AS. To verify this and validate the reliability of AS in RNA-Seq analysis, AS transcripts of DRFL1a, HAT, ME, and CPI genes were checked in the two organs using RT-PCR, confirming the consistency of these findings with RNA-Seq results.

4.4.3 Alternative Splicing as an independent and efficient layer of gene regulation

The results presented in this study highlight the relationship between transcriptional regulation and post-transcriptional regulation in response to salt stress in the Najran wheat cultivar. By comparing the genes that exhibited changes in transcript levels (DEGs) with those showing changes in mRNA splicing patterns (DSGs), this study attempted to understand the coordination and interplay between these two regulatory mechanisms. The results showed that a relatively small proportion of root genes (0.8%) and shoot genes (0.7%) exhibited dual regulation. This indicates that these two modes of gene regulation (transcriptional and post-transcriptional) are largely independent of each other in their responses to salt stress. Several studies have demonstrated that post-transcriptional mechanisms, such as AS, can fine-tune gene expression and contribute to the overall stress response, even though transcriptional alterations are the primary means of gene regulation in response to stress (Qu et al., 2016, Martín et al., 2021, Hazra et al., 2023). Recent analysis of the AS and transcriptome in Arabidopsis and rice (Guo et al., 2023) showed that the overlap between DEGs and DSGs was relatively limited, confirming the independence of the transcriptional and post-transcriptional regulation mechanisms.

In order to gain a comprehensive understanding of the relationship between AS and salt stress, the profiles of AS transcripts for the CPI gene were investigated under different concentrations of salt and under different slat-stress durations. Cysteine protease inhibitors have been extensively documented to have a crucial role in the defence mechanisms of plants under biotic and abiotic stressful conditions (Pernas et al., 2000), as well as in various aspects of plant growth and development, including the germination and maturation of plant seeds, fruit ripening, and PCD (Zhao et al., 2014a, Usman et al., 2021). The CPI gene has been significantly induced here in the roots and shoots of wheat in response to salt stress. However, the increase in the level of spliced transcripts was not consistent at various salt concentrations and over different times of salt exposure. These results are in agreement with those obtained in a previous study by Ding et al. (2014), who examined the AS in some candidate genes related to salt stress response in Arabidopsis plants treated with 0, 50, 150, and 300 mM NaCl. They observed an incompatible increase in AS patterns under stress, proposing that the elevation of AS levels might be attributed to splicing errors or inaccuracies in splicing regulation as a consequence of the stress.

4.5 Conclusion

The process of AS of pre-mRNA is a crucial mechanism in plants that contributes to the enhancement of protein complexity. This mechanism leads to a wide range of transcriptome and proteome expressions in a tissue-specific way. The present study investigated the transcriptome-wide salt-induced changes in AS profiles of root and shoot tissues of the Najran wheat cultivar. In response to salt stress, a higher frequency of AS events was observed in roots compared to shoots, where tissue-specific isoforms were related to specific functions including organ differentiation. Surprisingly, roots and shoots showed the same trend in splicing modes, with a slight difference in the ratio of AS event types. A3 was the most abundant event, and MX was the less prevalent form of AS in both tissues. In addition, the findings obtained in this study revealed the individual transcriptional or post-transcriptional regulation for many genes differentially induced in response to salt stress, suggesting the important role of AS in fine-tuning gene expression. The study offered valuable insights into the AS events that occur in response to salt stress in the roots and shoots of the Najran cultivar. Additionally, it emphasised the potential regulatory roles of AS in modulating the expression of stress-responsive genes in this wheat cultivar. Further functional investigations into AS transcripts of salt-responsive genes will contribute to a better understanding of the underlying mechanisms of gene regulation in wheat under stressful salt conditions.

Chapter 5 Comparative Analysis of Protein Degradation and Proteolytic Activity in Roots and Shoots of a Salt-tolerant Cultivar (Najran) and a Salt-susceptible Cultivar (Qiadh)

5.1 Introduction

Plants typically respond to salt stress by synthesizing compatible solutes and antioxidants, inhibiting photosynthesis, adjusting source/sink allocation, ion homeostasis, acceleration of senescence and synthesizing defence proteins such as thionins, lectins and protease inhibitors (Arif et al., 2020, Jain et al., 2022). Moreover, protein degradation has been identified as a significant mechanism by which plants respond to abiotic stresses (Zhang et al., 2015, Jurkiewicz and Batoko, 2018). Protein degradation in plants is facilitated by a class of enzymes known as proteases. Proteases are enzymes that catalyse the hydrolysis of certain peptide bonds inside proteins. These enzymes are categorised according to the type of the active site residue in the target protein, which includes cysteine, glutamic, threonine, peptidases, aspartic, metallopeptidases and serine proteases (David Troncoso et al., 2022). It has been reported that cellular housekeeping and stress response mechanisms are regulated by proteolytic enzymes, which function by breaking down misfolded and possibly non-functional proteins. This degradation process yields amino acids that can be utilised for the synthesis of new proteins (Rocha et al., 2017). To mitigate the degradation of valuable proteins under saline conditions, significant modifications in several proteases, proteasome components, and protease inhibitors have been observed (Mansour and Hassan, 2022). Proteases, serving as pivotal regulators, govern several processes in accordance with developmental and environmental stimuli in addition to controlling destiny of other proteins (Van Der Hoorn, 2008). Cysteine proteases are a class of proteolytic enzymes which have been documented to be involved in many biological processes such as senescence, PCD, protein mobilisation in seeds and tubers, and fruit ripening (Grudkowska and Zagdańska, 2004, Rocha et al., 2017, Yu et al., 2023). Furthermore, the upregulation of cysteine proteases genes is observed in response to several environmental stressors, which induce metabolic reorganisation, cellular protein component remodelling, degradation of impaired or excess proteins, and remobilization of nutrients (Liu et al., 2021, Zhou et al., 2023).

Proteases, while playing a crucial role in cellular processes, can exhibit detrimental effects when they are overexpressed, resulting in the destruction of several proteins in response to

environmental stimuli. Consequently, tight regulatory mechanisms are in place to control protease activity, both in space and time. The occurrence of cellular damage caused by proteases triggers the induction of protease inhibitors, which serve to regulate and mitigate the undesired proteolysis mediated by proteases (Huang et al., 2007). Plant proteinase inhibitors are categorised based on two main criteria: their reaction mechanism, which includes competitive, uncompetitive, non-competitive, and suicide inhibitors, and the specific type of protease that they inhibit, such as cysteine, serine, metalloproteinases and aspartic (Li et al., 2015). Several protease inhibitors have been identified from many plants like tea plants (Bhardwaj et al., 2021), ashwagandha (Tripathi et al., 2021), wheat (Benbow et al., 2019), tomato (Fan et al., 2019) and rice (Huang et al., 2007). Increased gene expression for protease inhibitors is a common response under different environmental stresses (Zhang et al., 2008, Malefo et al., 2020), which play a crucial role in maintaining physiological homeostasis and serving as an integral component of the plant's innate defence system (Hellinger and Gruber, 2019). Cysteine protease inhibitors (CPIs), commonly referred to as cystatins, are one of plant protease inhibitors which have been investigated in many plants for their protective and regulatory roles within plant tissues (Gaddour et al., 2001, Diop et al., 2004, Alomrani, 2020). CPIs have a crucial role in the regulation of proteolytic activity of cysteine proteases during seed development (Fernandes et al., 1991) and in PCD (Belenghi et al., 2003). In addition, CPIs have been shown to enhance plants' capacity to withstand environmental stresses by inhibiting cysteine protease activity, which is triggered by different abiotic stimuli and contributes to PCD acceleration (Solomon et al., 1999, Zhang et al., 2008, Kidrič et al., 2014, Sun et al., 2014).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the prevailing technology employed for analytical separation of protein mixtures with high resolution. Denaturation of constituent proteins, as the first part of the process, is done by using an anionic detergent that also exhibits binding affinity towards these proteins. As a result, all proteins acquire a negative charge that is directly proportionate to their molecular mass. Subsequently, electrophoresis is conducted using a porous acrylamide gel matrix, which effectively segregates proteins based on their molecular mass, resulting in high-resolution separation (Walker, 2002, Gallagher and Wiley, 2012). SDS-PAGE copolymerized with protein substrate such as Gelatin is the most common method used to investigate the proteolytic activity of proteases and other enzymes. Zymography has been widely used for the analysis of enzyme activity under nonreducing conditions (Kleiner and Stetlerstevenson, 1994, Srivastava et al., 2009, Ioannou and Labrou, 2022).

In the current study, a preliminary attempt was made to understand the proteolytic mechanism plants employ to protect against adverse salt stress. Total protein hydrolysis and proteolytic activity of proteases were examined in the roots and shoots of two wheat cultivars differing in their sensitivity to salt stress (Najran and Qiadh).

5.2 Material and methods

5.2.1 Plant material, growth and stress treatment

Plants of a salt-sensitive, Qiadh and salt-tolerant, Najran wheat cultivars were grown under control (0 mM NaCl) and salt stress (200 mM NaCl) conditions (Figure S8). Growth conditions and plant sampling were done as what has been described in the transcriptomic chapter (section 3.2.1). Ground root and shoot samples stored at -80 °C were used for both RNA and protein extractions.

5.2.2 RNA extraction and relative gene expression analysis

Extraction of RNA from the roots and shoots of wheat cultivars was done using the same RNA purification kit and RNase-Free DNase I Kit used previously in chapter 4 (section 4.2.2, P70). Purified samples with absorbance A260/280 ratios ranged from 2.12 to 2.23 were used in the reverse transcription quantitative PCR (RT-qPCR). Transcript levels of *TaCPI* gene that was induced under salt stress (see section 4.2.7) were quantified in roots and shoots of Qiadh and Najran wheat cultivars using two-step RT-qPCR method. Roots and shoots total RNAs were reverse transcribed into cDNAs by reverse transcription, and the resulting cDNAs were then used as templates for qPCR amplification (The used kits were mentioned in section 3.2.6). According to Dudziak et al. (2020), *CJ705892* gene is the best reference gene showing stable expression level in wheat seedlings under drought conditions. CJ705892 gene was used here as a control gene to normalize the transcript level of *TaCPI* gene. Exactly 5 µg total RNAs with 0.8 µl of each gene-specific primer pairs (10 µM), 10 µl SensiFAST™ SYBR Hi-ROX mix and H_2O were used in 20 µl final volume of reactions. The cycling program performed for qPCR was as follows: 95°C for 2 min to activate the polymerase, then 40 PCR cycles at 95°C for 5 sec, 62°C annealing temperature for 10 sec, and 72°C for 20 sec, final extension at 72°C for 90 sec. After the completion of amplification cycles, melt curve (between 72 ºC and 95 °C) was produced to confirm the generation of a single amplicon by qPCR to indicate specificity of the amplification reaction.

TaCPI-specific primers (Table 5.1) for RT–qPCR (20 bp) were designed using Primer3 (v. 0.4.0). Primer efficiency for PCR amplification was calculated based on the efficiency equation below, using a 1:10 serial dilution of cDNA template synthesized from stressed and unstressed root and shoot samples.

Efficiency (%) = $(10^{(-1/Slope)} -1) *100$

Relative expression of target gene (*TaCPI*) was quantified using standard $2^{-(\Delta\Delta Ct)}$ method (Livak & Schmittgen, 2001).

$$\mathbf{2^{-(\Delta\Delta Ct)} = 2^{-(\Delta Ct\ (treated\ sample) - \Delta Ct\ (control\ sample))}}$$

Where;

ΔCt = Ct (gene of interest) – Ct (reference gene)

Table 5.1: Oligonucleotide primers used in reverse transcription quantitative PCR analysis to monitor the transcript levels of the *TaCPI* gene in Qiadh and Najran wheats.

Gene ID	Annotation	Primer Name	Primer Sequence (5'→3')	Amplicon length (bp)
TraesCS1A02G256400	*TaCPI*	*TaCPI*-F *TaCPI*-R	CTGTCACACGGACATGCTTT TCGTCGAACAACATGCCTTA	183
CJ705892 (Reference gene) (Dudziak, 2020)	*TaCJ70*	*TaCJ70*-F *TaCJ70*-R	GCCTCAGTGGTAGGAGCATT TTCAGCAAATGCGGTGGTTG	116

5.2.3 Protein extraction and SDS-PAGE analysis

Total proteins were extracted from the roots and shoots of Qiadh and Najran wheats using an ice-cold protein extraction buffer with and without a protease inhibitor cocktail (Sigma-Aldrich, UK). The ground frozen samples (100 mg) were homogenised in 250 µl extraction buffer containing in 100 mM Tris-HCl, pH 8.3, 100 mM NaCl, and 10 mM DTT. For samples extracted with protease inhibitors, 2.5 µl of protease inhibitor cocktail was added to the extraction buffer before homogenization. Sample extracts were clarified by centrifugation at 14,000 rpm for 10 min at 4°C. After centrifugation, sterile glycerol followed by SDS were added to the supernatant in final concentrations of 10% and 25% (v/v), respectively. An aliquot of protein samples extracted with or without protease inhibitors was incubated at 37°C for 21 h. All protein extracts (30 µg) were boiled for 5 min after adding Laemmli sample buffer mixed with 2-mercaptoethanol and then immediately loaded in a 12% precast SDS-PAGE gel (20 µl). After running the electrophoresis, gels were fixed in 40% ethanol and 10% acetic acid for 15 min then stained overnight with QC Colloidal Coomassie stain (BIO-RAD, cat# 1610803). Gels were then distained in water for 3 hours changing the water every hour. Protein hydrolysis in

the samples was monitored by inspecting gel images taken using a BIO-RAD Gel-Documentation system.

The Bradford assay was used to determine total protein concentration using bovine serum albumin as a standard (Bradford, 1976).

5.2.4 Gelatin Zymography

Proteases activity in Qiadh and Najran wheat cultivars was checked in 10 % SDS-PAGE gel co-polymerized with 0.1 % (w/v) Gelatin. Root and shoot proteins were extracted under non-denaturing conditions (in absence of SDS) to maintain the biological activity of proteases. Protein extracts (30 µg) were mixed with Laemmli sample buffer (without adding 2-mercaptoethanol to ensure full enzymatic activity of proteases) and incubated at room temperature for 10 min before loading samples on the gel. Following the electrophoresis procedure, gels were gently agitated in 1X Zymogram renaturing buffer for 30 min at room temperature to remove SDS, followed by agitation in 1X Zymogram developing buffer for 30 min. Subsequently, the gels were subjected to overnight incubation in developing buffer at 37°C and then were stained with QC Colloidal Coomassie stain. Proteases activity of Gelatin digestion was visualized as clear bands against a dark background. A relevant control for the inhibitory effect of *TaCPI* on proteolytic activity was considered. Corresponding protein samples that were extracted with the protease inhibitor cocktail were used to confirm the results.

5.3 Results

5.3.1 Gene expression profiles of Cysteine proteinase inhibitor (*TaCPI*)

Observed changes in AS of *Ta*CPI gene (see section 4.3.5) prompted us to investigate their potential relation to changes in gene expression levels of the same gene. In the current study, the transcript levels of *TaCPI* gene were analysed in the roots and shoots of the salt-tolerant wheat cultivar (Najran) and the relatively salt-sensitive wheat cultivar (Qiadh) using real-time RT-qPCR. No significant change in the transcript level of the *TaCPI* gene was seen in the roots and shoots of both cultivars when subjected to salt treatment. It was observed that *TaCPI* exhibited -0.01- and 0.85-fold changes in the roots of Najran and Qiadh wheat cultivars under salt stress, respectively (Figure 5.1 A). Clearly, the transcript level was approximately two times higher in the salt-treated roots in Qiadh than their respective control levels. In addition, the transcript levels of the *TaCPI* gene were shown to be slightly lower in the shoots of tolerant cultivar (-0.74-fold change) and the shoots of the sensitive cultivar (-0.22-fold change) under salt-stress compared to the control (Figure 5.1 B). These findings were further correlated with proteolytic activity using Zymogram gel (Figure 5.3).

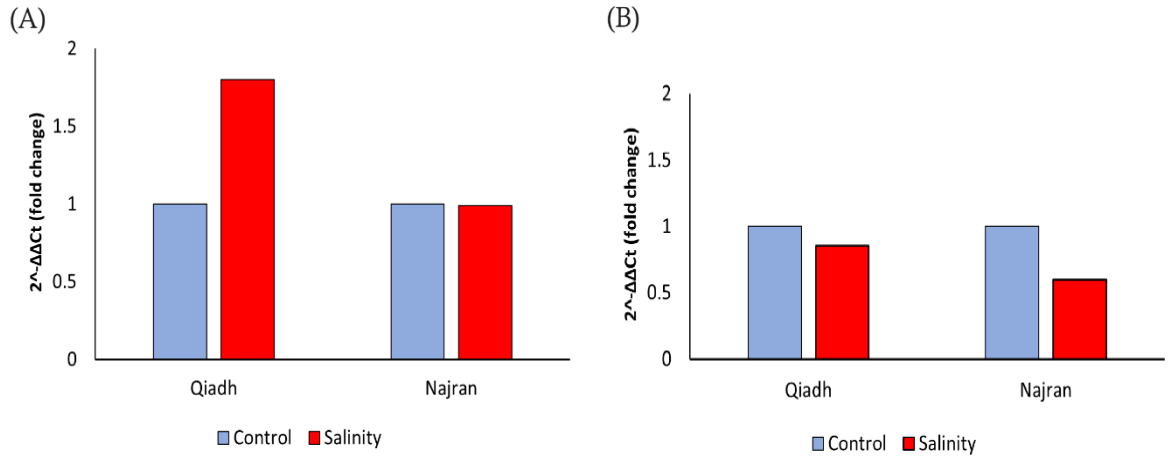


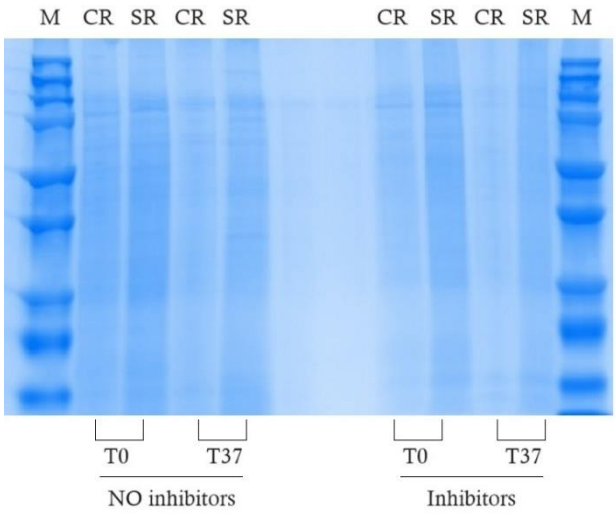
Figure 5.1: Transcript levels of *TaCPI* gene in roots (A) and shoots (B) of two wheat (*Triticum aestivum*) cultivars, a salt-sensitive cultivar (Qiadh) and a salt-tolerant cultivar (Najran) subjected to salt-stress (200 mM NaCl).

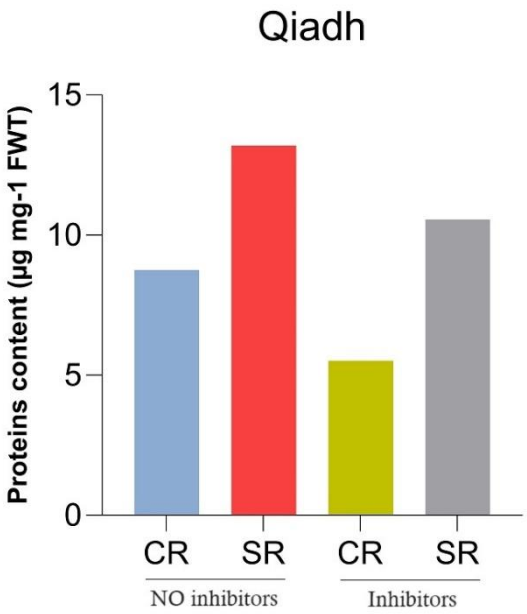
5.3.2 Protein hydrolysis profiles

Hydrolysis of protein extracts in the roots and shoots of wheat cultivars was examined in SDS-PAGE gels. Results in Figure (5.2 A) showed that the hydrolysis of proteins in Qiadh cultivar was less in the salt-treated roots compared to the control and this was obvious after incubating samples at 37°C. Root proteins extracted in the presence of an inhibitor cocktail exhibited more degradation which was less under salt stress. In contrast, the protein profiles observed in Najran cultivar exhibited no visible differences between the control and salt-stressed roots. Following the incubation period, a greater degree of protein hydrolysis was found, particularly in the control samples. Furthermore, it was shown that the protein degradation was more pronounced in samples that were mixed with an inhibitor cocktail in comparison to samples that were free from the inhibitor cocktail during protein extraction (Figure 5.2 A&C).

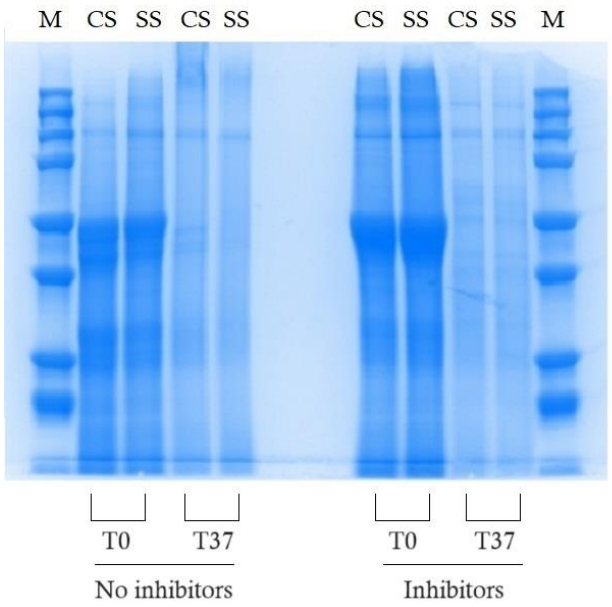
The analysis of protein patterns in the salt-treated shoots of Qiadh and Najran, when compared to the control shoots, revealed no significant discernible degradation in both samples that were extracted with and without the inhibitor cocktail. Shoots extracted with inhibitor cocktail showed stronger protein bands than shoots extracted without inhibitor cocktail in both cultivars. After incubation, all samples exhibited an increase in protein hydrolysis (Figure 5.2 B&D). Taken together, these results suggest that there is more protein degradation observed in the shoots than in the roots in both cultivars.

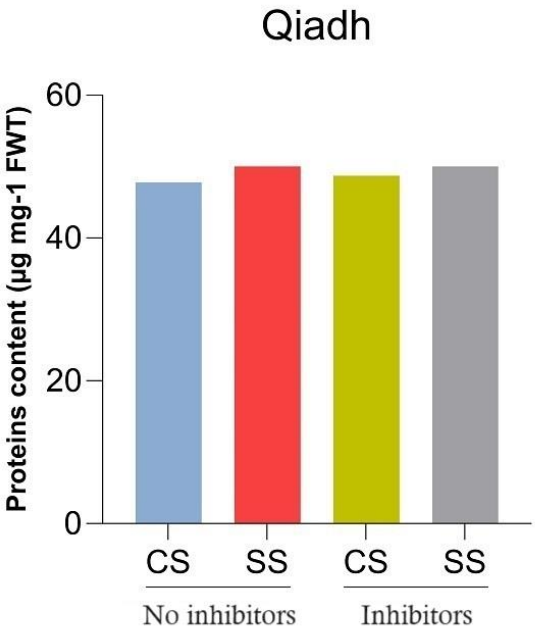
(A)


M CR SR CR SR CR SR CR SR M
T0 T37 T0 T37
NO inhibitors Inhibitors


Qiadh
Proteins content (µg mg-1 FWT)
15
10
5
0
CR SR CR SR
NO inhibitors Inhibitors

(B)


M CS SS CS SS CS SS CS SS M
T0 T37 T0 T37
No inhibitors Inhibitors


Qiadh
Proteins content (µg mg-1 FWT)
60
40
20
0
CS SS CS SS
No inhibitors Inhibitors

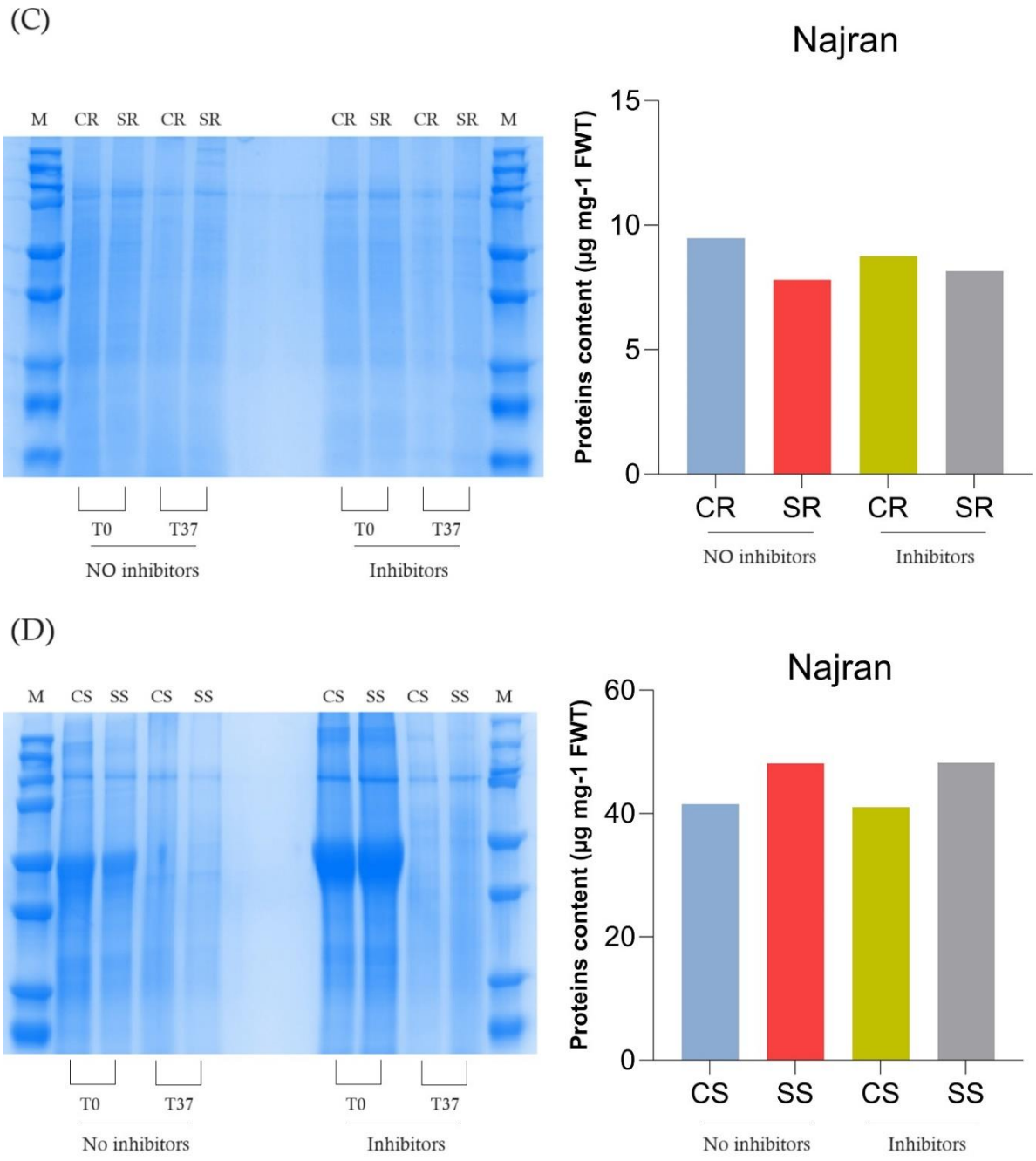


Figure 5.2: SDS-PAGE analysis and protein content in roots (A&C) and shoots (B&D) of two wheat cultivars (*Triticum aestivum*), salt-sensitive cultivar (Qiadh) and salt-tolerant cultivar (Najran) subjected to 200 mM NaCl. M: protein marker, C: control, S: salt treatment, R: root, S: shoot, T0: non-incubated samples, T37: samples incubated at 37°C for 21 hours. No inhibitor: protein samples extracted without inhibitors, Inhibitor, protein samples extracted with inhibitor cocktail.

5.3.3 Proteolytic Activities under salt stress

To detect the proteolytic activity of proteases under salt stress, protein extracts from root and shoot tissues of Qiadh and Najran cultivars were examined. Proteins were firstly separated by SDS-PAGE gel electrophoresis through a matrix containing Gelatin as a substrate, renatured and allowed to degrade the Gelatin. The zymogram analysis depicted in (Figure 5.3) demonstrates a reduced proteolytic activity in the roots of Qiadh cultivar in response to salinity. In contrast, the proteolytic activity present in the salt-treated roots of the Najran cultivar exhibited Gelatin hydrolysis similar to that observed in the control roots. In addition, the analysis of proteolytic profiles in the shoots indicated that there was no observed increase in proteolytic activity in either cultivar when subjected to salt stress.

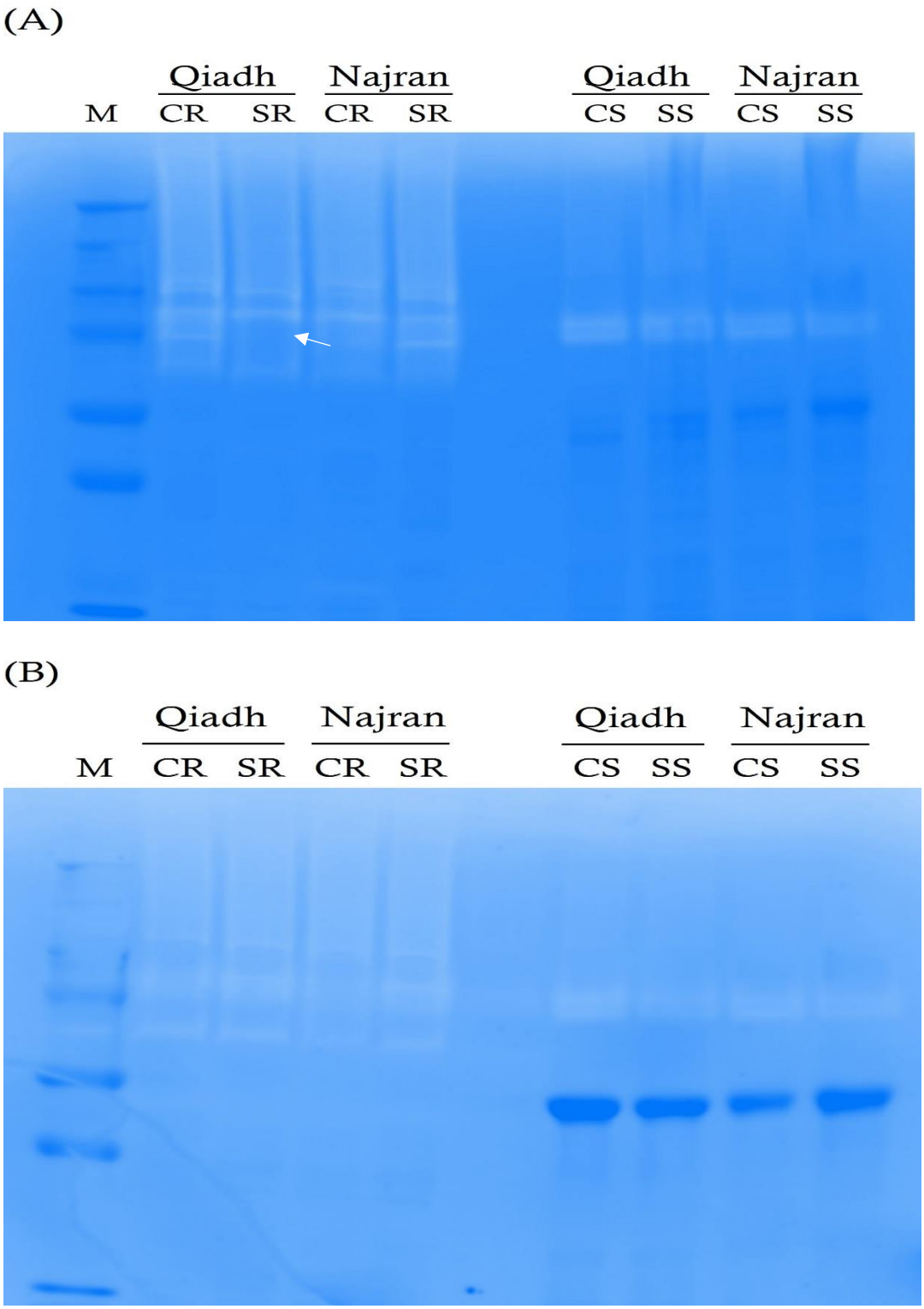


Figure 5.3: (A) Gelatin Zymography in roots and shoots of two wheat cultivars (*Triticum aestivum*), salt-sensitive cultivar (Qiadh) and salt-tolerant cultivar (Najran) subjected to 200 mM NaCl. M: protein marker, C: control, S: salt treatment, R: root, S: shoot. Arrow shows missing protease activity. (B) Control samples that were extracted with protease inhibitor cocktail.

5.4 Discussion

The TaCPI gene was found to be differentially spliced under salt-stress in both roots and shoots of Najran wheat as shown in Figure 4.11. To see if the gene was also regulated at the transcriptional level, the transcript levels of *TaCPI* were examined in salt-tolerant and salt-sensitive wheat cultivars by RT-qPCR. Results showed that there was no significant alteration in the transcript abundance of the gene in the roots and shoots of both cultivars following exposure to salt treatment. The slight increase in transcript levels in the roots of the salt sensitive wheat cultivar, Qiadh (0.85-fold), was not considered as significant because below 1-fold change ($Log_2 <1$). The RT–qPCR findings in this study are consistent with the results obtained from RNA-Seq data of Najran cultivar. It is therefore likely that the regulation of *TaCPI* in wheat under salt stress relies more on post-transcriptional than transcriptional regulation. These results are in line with those of a previous study conducted on wheat under drought conditions, Vaseva et al. (2016) found that the transcript level of cysteine protease inhibitor (WC1) in dehydrated leaves was similar to the control leaves in a drought tolerant cultivar, Katya. They suggest that the relatively stable abundance of cysteine protease inhibitor is due to the lower endogenous proteolytic activity observed for this wheat cultivar during drought stress.

Based on the existing literature the dominant proteases in wheat leaves that respond to drought conditions are of the cysteine type. Additionally, it has been observed that winter wheat cultivars that are sensitive to drought have higher levels of proteolysis in comparison to tolerant cultivars (Simova-Stoilova et al., 2010). However, proteolysis profiles obtained in this investigation showed less protein degradation in roots of the salt susceptible cultivar (Qiadh) compared to the salt tolerant cultivar (Najran) under salt stress, as depicted by the protein content presented in the right panels of Figure 5.2 A&C. In addition, a lower protein degradation was observed in the salt-treated roots in both cultivars in comparison to the control roots. Together these results might suggest that the reduction in protein biosynthesis that takes place in plants experiencing salt stress may be counterbalanced by a decrease in protein degradation, thereby contributing to salt tolerance (Mansour and Hassan, 2022). This explanation is in accordance with the decreased protein content in the stressed roots of salt tolerant cultivar, Najran (Figure 5.2 C). Although no significant protein hydrolysis was revealed in the salt-treated shoots of both cultivars, a slight increase in the proteins content was seen under salt stress. These results seem to be consistent with (Tammam et al., 2008) who investigated protein content in roots, shoots and spikes of a moderately salt-adapted wheat cultivar (Banysoif 1) under different concentrations of salt. They found that protein content in roots decreased with

elevated levels of salinity, whereas a significant increase in protein content was observed in shoots and spikes. The observed rise in protein accumulation in shoots and spikes suggests that they may have a greater ability than roots to mitigate the effects of salt stress through osmotic adjustment and maintaining growth (Abdel Latef, 2010). Moreover, our results obtained in (chapter 2, section 2.3.1) confirm this finding, where both cultivars exhibited more reduction of growth in the stressed roots compared to shoots, which was linked here to the lower content of proteins in root tissues.

Under challenging plant stresses, proteases play a crucial role in controlling the degradation of misfolded or damaged proteins, facilitating the recycling of essential amino acids and energy conservation (Velez et al., 2017). It has been reported that proteolytic activity of proteases increases under environmental stresses including salt stress, drought and low temperature (Hieng et al., 2004, Kidrič et al., 2014, Savić et al., 2019, Mangena, 2020, Yingqi et al., 2022). Zymogram analysis conducted in this study demonstrated a decrease in proteolytic activity in the roots of salt-susceptible wheat cultivar when compared to the tolerant cultivar under salinity. However, there was no apparent significant increase in proteolytic activity in the shoots of both cultivars when subjected to salt treatment. The findings of this study indicate a negative correlation between the transcript levels of *TaCPI* and proteolysis revealed in the Qiadh and Najran cultivars. Nevertheless, there is a lack of conclusive evidence demonstrating a direct correlation between the observed activity and the expression of *TaCPI*. An examination of thiol protease activities in oilseed plants, as documented by Gogna and Bhatla (2020) demonstrated a significant contrast between salt-tolerant and salt-sensitive varieties. The salt-tolerant variety exhibited a remarkably elevated protease activity in comparison to the sensitive variety. Although proteolytic activity appeared to remain unaffected by salt stress in the salinity tolerant cultivar, it has however decreased in the sensitive variety when subjected to salt stress. Therefore, there is a positive relationship between salt tolerance and the extent of proteolytic activity by thiol protease. Similarly, Simova-Stoilova et al. (2010) investigated total proteolytic activity in three wheat cultivars that are various in their sensitivity to water stress. They found a remarkable increase of proteolytic activity in the cultivar that displayed the highest tolerance to drought compared to other cultivars.

5.5 Conclusion

In this chapter, a preliminary investigation of protein hydrolysis and proteolytic activity was conducted in the roots and shoots of salt tolerant and sensitive wheat cultivars. The observed decrease in protein breakdown and proteolytic activity in the treated roots of Qiadh, as compared to Najran, could potentially be attributable to the relative increase in transcript level of *TaCPI* gene in the roots of Qiadh cultivar. The study contributes to our understanding of the significant regulatory role of protease inhibitors in modulating protein metabolism in response to environmental stresses. Further proteomic analysis and Western blot analysis might help identifying the specific proteases induced under salt stress in these cultivars.

Chapter 6 General Discussion

6.1 Introduction

Plants are continuously subjected to a multitude of environmental stressors, which have detrimental impacts on plant's performance and eventually on agricultural yield. Plants therefore respond effectively and promptly to these environmental stimuli in order to maintain survival and eventually growth (Zandalinas and Mittler, 2022). This research project investigated the differential responses of the roots and shoots of three Saudi bread wheat cultivars to salt stress in terms of growth, yield and biochemical responses. Subsequently, the transcriptional and post-transcriptional responses of both tissues of a salt-tolerant wheat cultivar (Najran) were investigated to deeply understand the molecular mechanisms underpinning the responses of wheat to salt stress. In addition, this research compared the proteolysis activity under salt-stress between Najran, a salt-tolerant cultivar, and Qiadh, a salt-susceptible cultivar, to expand our understanding of their potential contribution to salt tolerance mechanisms. The findings provided in this study helped to elucidate the intricate network of wheat responses to salt-stress including at the physiological, biochemical, and molecular (transcriptome, alternative splicing and proteolysis) levels under conditions of salt stress. Findings from this holistic approach of analysis could guide future efforts to enhance salt tolerance in crops, contributing to global food security in the face of changing environmental conditions (Zhao et al., 2020).

6.2 Do different wheat cultivars exhibit similar responses to salt stress under comparable conditions?

Within *T. aestivum* species, different cultivars of wheat respond variably to biotic and abiotic stresses (Baloch et al., 2012, Abid et al., 2016, Garcia de Leon et al., 2020). Understanding these differences is essential for crop improvement, it can inform the efforts aiming at developing salt-tolerant crops including wheat and can help optimize agricultural practices in salt-affected regions such as Saudi Arabia. The variability in salt stress responses among wheat cultivars might be attributed to differences in the genetic background, these differences control salt stress perception and signalling pathways, osmotic adjustment capacity, ion transport and compartmentalization, and the activation of stress-response genes (Munns and Tester, 2008, Gupta and Huang, 2014).

As demonstrated in this study, Najran, Mebiah and Qiadh wheat cultivars responded differently to salt stress, with the former displaying superior adaptation to salinity. Although the response mechanisms might be similar between cultivars, the extent of stress responses was different between the tested wheat cultivars. For example, Najran cultivar adapted to salinity by accumulating higher levels of soluble sugars and total phenolics compared to Mebiah and Qiadh. This suggests that the accumulation of soluble sugars in Najran may contribute to its ability to tolerate salt stress and maintain higher growth rates. The higher biomass observed in Najran could be attributed to its efficient utilization of these soluble sugars for energy production and growth (Boriboonkaset et al., 2013). Additionally, the production of phenolics may be up-regulated via specific genes that might be induced to higher extent in response to environmental stresses, further enhancing the ability of Najran wheat to prevent ROS damage. On the other hand, Mebiah and Qiadh responded to salinity by increasing the levels of proline and starch, respectively as a mean of osmoregulation to assist survival under salt conditions (Hasegawa et al., 2000). This observed variability in the responses to salt stress among wheat cultivars is in line with numerous studies (Aly et al., 2019, Zeeshan et al., 2020, Ghonaim et al., 2021, Tao et al., 2021). It is worth noting that the diversity in salt stress responses among different wheat cultivars offers a promising avenue for enhancing crop productivity. Salt-tolerant cultivars can serve as genetic sources for breeding programs aimed at developing new varieties with enhanced salt tolerance. Identifying and exploiting the genetic determinants responsible for salt tolerance in specific cultivars can facilitate the development of other resilient wheat cultivars that can thrive in saline environments.

6.3 How does salt stress impact molecular responses in Najran wheat cultivar?

Salinity profoundly affected the molecular responses in Najran wheat, triggering changes at multiple levels of gene regulation including transcriptional and post-transcriptional levels. These complex molecular mechanisms enabled Najran wheat to adapt and survive high salt conditions. Firstly, at the transcriptional level, salt stress induced changes in the gene expression profiles of roots and shoots of salt-treated Najran wheat. Approximately 6.2% and 3.7% of the estimated 94,000 wheat genes exhibited altered expression in the roots and shoots, respectively, in response to salt stress. These changes in gene expression were involved in various biological processes, such as glutathione metabolism, biosynthesis of secondary metabolites such as phenylpropanoids, and galactose metabolism. Although some pathways like glutathione metabolism biosynthesis of secondary metabolites are commonly up-regulated in different wheat cultivars in response to salt stress (Goyal et al., 2016, Amirbakhtiar et al., 2019, Li et al.,

2023), the response of pathways like galactose metabolism to stress can be specific to certain wheat cultivars (Ma et al., 2022). It is worth noting that by studying the common responses to a stress factor like salinity, researchers can identify key genes and pathways that play a crucial role in the tolerance to the stress across different wheat cultivars. This knowledge can then be used to develop targeted breeding approaches, using marker-assisted selection or gene editing strategies to enhance salt tolerance in specific cultivars. Additionally, understanding cultivar-specific responses allows breeders to select and cross plants with desirable traits, ultimately leading to the development of new varieties with improved salt-tolerance under different growing conditions and environments.

Secondly, at the post-transcriptional level, salt stress induced AS in Najran wheat, contributing to the complexity of its transcriptome (Yang et al., 2022). Out of the total number of genes that were expressed, a proportion of 32,268 genes (22.5%) in the roots and 31,941 genes (23.1%) in the shoots were identified as being subject to AS. However, salt-stress caused a 1.6% and 0.5% increase in AS events in the roots and shoots, respectively. This suggests that salt stress has the potential to influence post-transcriptional regulation by triggering AS of pre-mRNA, leading to the production of protein isoforms with distinct functions, enabling the plant to regulate different biochemical and physiological processes for adaptation to the challenges posed by salt stress (Zhang et al., 2019c). In this study, A3 was shown to be the most frequent AS event in Najran wheat, whereas MX was found to be the least common. Different wheat cultivars may prioritize different types of AS events in response to environmental stresses. While Najran predominantly showed A3 as the most frequent AS event, other cultivars may exhibit different AS events, such as intron retention as the most prevalent event (Yu et al., 2020). These differences might be attributed to various factors, including the genetic background, stress tolerance levels, environmental conditions, and other factors unique to each cultivar.

6.4 Is post-transcriptional regulation a complementary mechanism to gene expression for salt-tolerance in Najran wheat?

Integrating the findings from both the transcriptional and post-transcriptional regulations under salt-tress revealed a complex interplay. While a proportion of genes exhibited dual regulation under salt stress, the majority of them showed independent responses at transcriptional or post-transcriptional levels. This suggests that AS plays a distinct role in fine-tuning gene expression under salt stress, enabling a more efficient response to changing environmental conditions

(Martín et al., 2021, Hazra et al., 2023). In this study, the *TaCPI* gene was used as an example to demonstrate the most non-dual regulation of gene expression in the Najran wheat cultivar in response to salt stress. The AS of the *TaCPI* gene significantly increased, whereas the transcript level of this gene did not change under salt stress. This suggests the independence between the transcriptional and post-transcriptional regulatory mechanisms in their response to salt stress (Guo et al., 2023).

Salt stress reduced protein degradation in the roots, which was more pronounced in the salt-sensitive wheat cultivar, Qiadh than in the salt-tolerant wheat cultivar, Najran. In addition, the study found that the roots of Qiadh cultivar had lower proteolytic activity compared to the Najran cultivar when exposed to salinity. However, no apparent increase in protein hydrolysis was observed in the shoots of salt treated plants compared to unstressed plants in both cultivars. The observed decrease in protein degradation could be attributed to salt-induced changes in several proteases, proteasome components, and protease inhibitors. These alterations suggest that the decrease in protein degradation serves as a compensatory mechanism for the observed decline in protein biosynthesis in plants experiencing salt stress, thereby contributing to their ability to tolerate saline conditions (Mansour and Hassan, 2022). In addition, the obtained results suggest that the observed decline in protein hydrolysis and proteolytic activity in the treated roots of Qiadh cultivar may be due to a higher expression level of the *TaCPI* gene which plays important role in the regulation of proteolytic activity of cysteine proteases (Fernandes et al., 1991). Further investigation is needed to determine the specific mechanisms by which salinity regulates *TaCPI* gene expression and post-transcriptional modulations in wheat.

6.5 How does the spatio-temporal regulation of genes participate in the responses of Najran wheat to salt stress?

Spatio-temporal regulation of genes is a critical component playing an important role in shaping plant's response to salt stress. This phenomenon allows various plant organs to fine-tune their adaptation mechanisms in response to salt stress. The current comprehensive study uncovered the complicated interplay between various aspects of plant biology in the roots and shoots, providing insights into how these different organs responded to salt stress. Roots of Najran cultivar experienced larger reduction in growth than shoots, which might be associated in this study with the lower content of proteins in the roots and the higher content in the shoots under salt stress. These observations might be attributed to the more enhanced capacity of shoots to alleviate the impacts of salt-stress by means of osmotic adjustment resulting in sustained growth

(Abdel Latef, 2010). Root systems often use ion exclusion strategies to maintain low sodium levels and prevent cellular damage, while shoots effectively adjust osmotic conditions, using osmoprotectants to prevent water loss and protect metabolism from photosynthetic inhibition to maintain growth (Rajaei et al., 2009). Transcriptional responses in Najran wheat mirror this tissue-specific patterns, with a set of genes that were differentially expressed for specialized functions indicating salt stress adaptation. Functional enrichment analysis revealed that the most significant DEGs in the roots were involved in cell ionic homeostasis, oxidative stress responses including Glutathione synthesis, osmotic stress response, hormonal signalling, carbohydrate transport etc. Meanwhile, the DEGs in the shoots were associated with protein folding, photosynthesis, synthesis of secondary metabolites, response to Abscisic acid, and hormonal-signalling. At the post-transcriptional level, AS events were induced by salt stress for some genes, with roots and shoots exhibiting differences in the frequency and extent of AS, being higher in the roots compared to the shoots. AS generates protein isoforms with distinct functions, adding an additional layer of tissue-specific regulation. In roots several salt-induced DSGs were enriched in cytoskeletal-related categories, whereas the DSGs in shoots were found to be overrepresented in biological categories related to metabolic and signalling processes. Although most genes in this study were transcriptionally and post-transcriptionally regulated in a tissue-specific manner, a few genes were differentially expressed or spliced in both roots and shoots. GO enrichment revealed anti-oxidative stress response e.g glutathione synthesis and carbohydrate transport (for DEGs) and cysteine-type endopeptidase inhibitor activity (for DSGs) as the most significant molecular terms enriched in both organs. The observations revealed in this study are consistent with findings in the literature, which emphasise the significance of tissue-specific responses in improving the overall salt tolerance of wheat plants (Nagaraju et al., 2019, Gu et al., 2020, Martín et al., 2021).

6.6 Future perspectives

In conclusion, the results presented in this study enhance our understanding of salt tolerance mechanisms in wheat and provide promising insights for future investigations and crop improvement efforts. Comprehending these mechanisms is critical for developing salt-tolerant wheat cultivars for sustainable agriculture practices in the face of boosting soil salinity.

This work provides a foundation for future research aimed at enhancing salt tolerance in wheat and other crop species. Several avenues for further investigation emerge from this study:

1. Functional Validation: In-depth functional studies are needed to confirm the roles of key genes and AS events identified in salt tolerance including *TaCPI* gene. To assess the function of a specific gene, the gene expression can be manipulated using genetic techniques. For instance, RNA interference (RNAi) or CRISPR/Cas9 gene editing can be performed to knock down or out the gene's expression and observe the plant's response to salt stress. Conversely, the gene can be overexpressed in a salt-sensitive cultivar and evaluate whether it enhances salt tolerance.

2. Proteomic Analysis: Complementing transcriptomics with proteomic analysis can provide a comprehensive view of protein regulation in response to salt stress. This would allow the identification of specific proteins and their function under stress conditions.

3. Genetic Engineering: The insights gained from this study can guide genetic engineering efforts to improve salt tolerance in wheat and other crops. Targeting key genes and pathways identified here such as those involved in Glutathione pathway, phenylpropanoids, galactose metabolism and proteinase inhibitor activity may enhance crop tolerance to salinity.

4. Multi-Omics Integration: Integrating transcriptomics, proteomics, and metabolomics data can provide a holistic understanding of the salt stress response. This multi-omics approach can uncover regulatory networks and metabolic pathways critical for salt tolerance.

Appendix

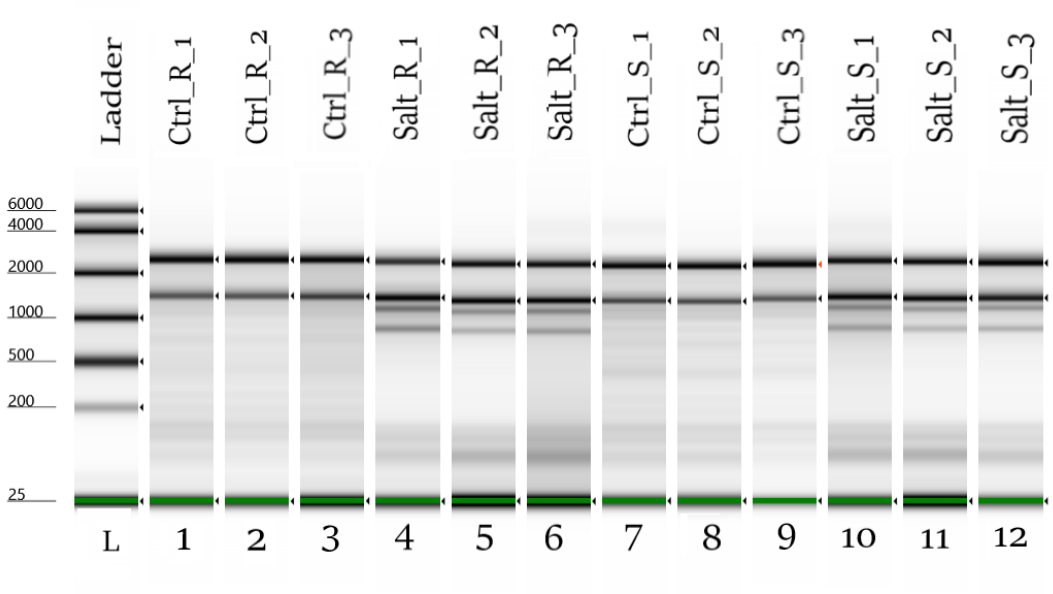


Figure S1: Gel images of Agilent Tapestation system from high quality RNA wheat samples with RNA integrity number (RIN) ranged from 6.5 to 8.1. R indicates roots and S indicates shoots.

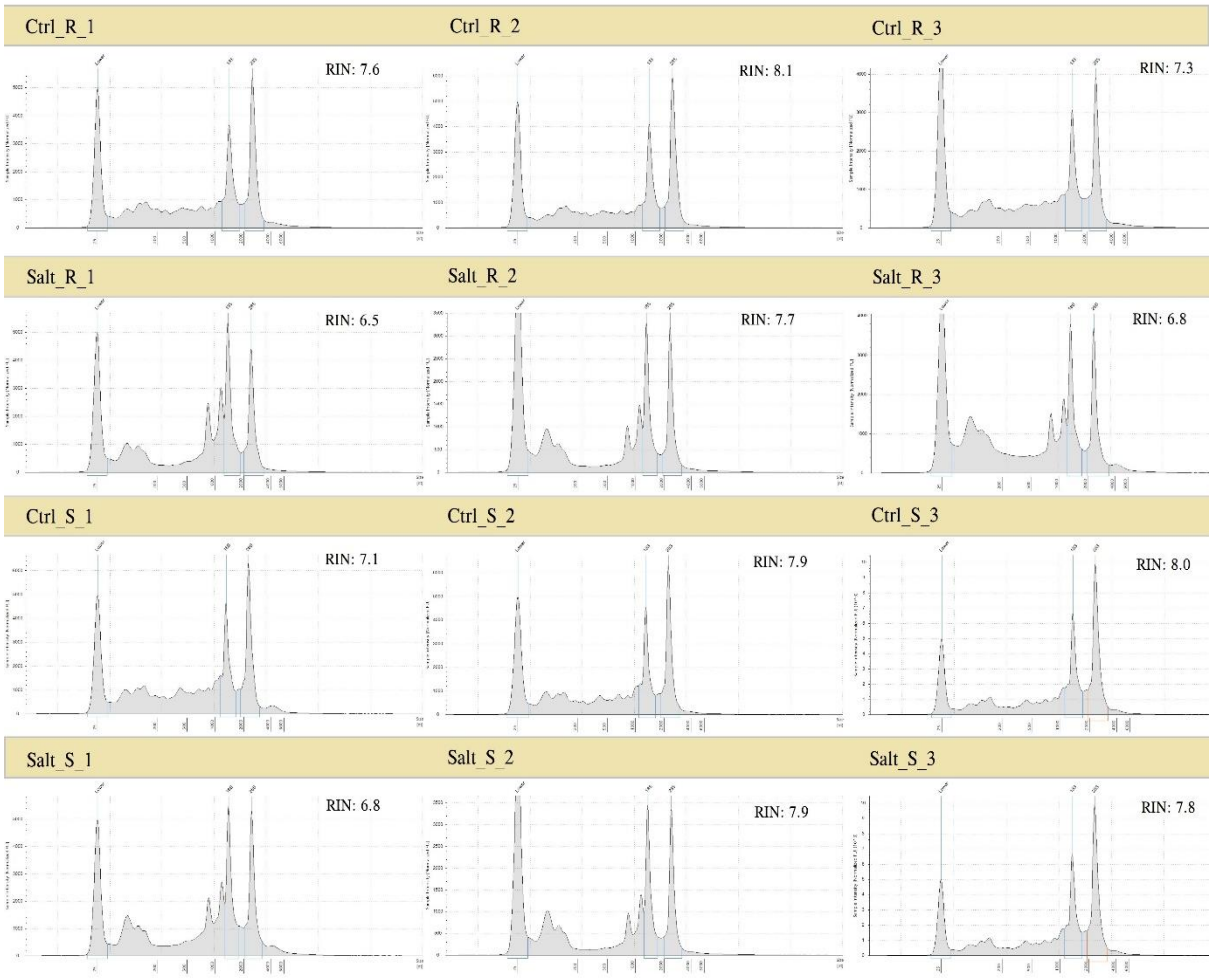
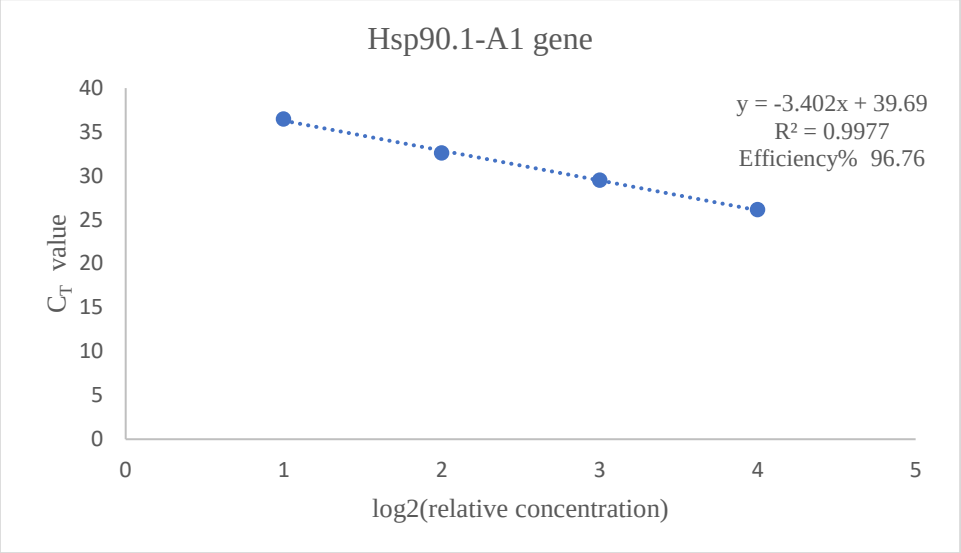
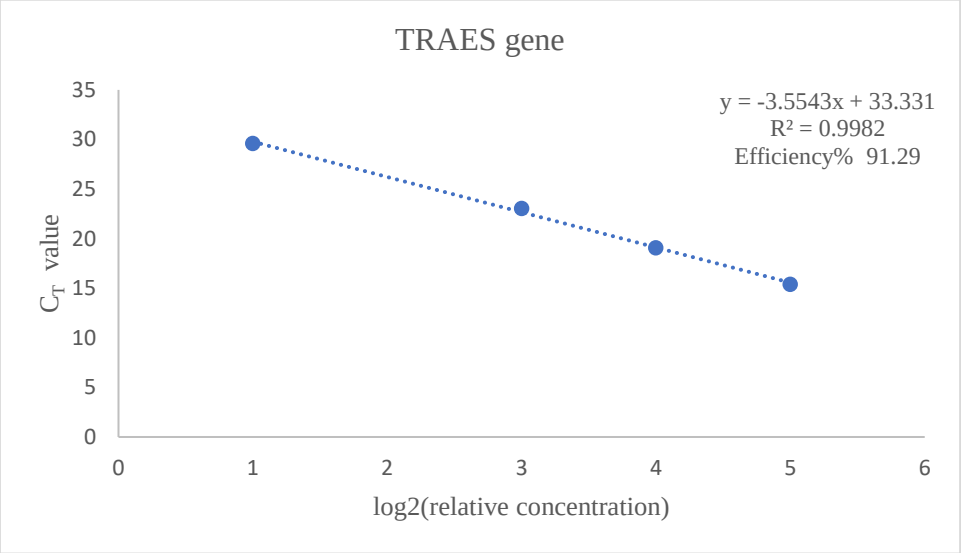
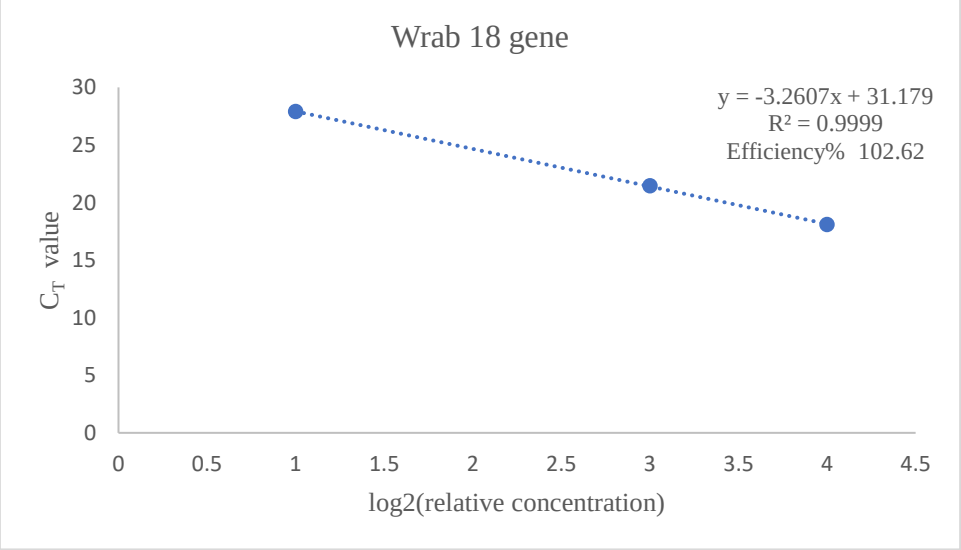
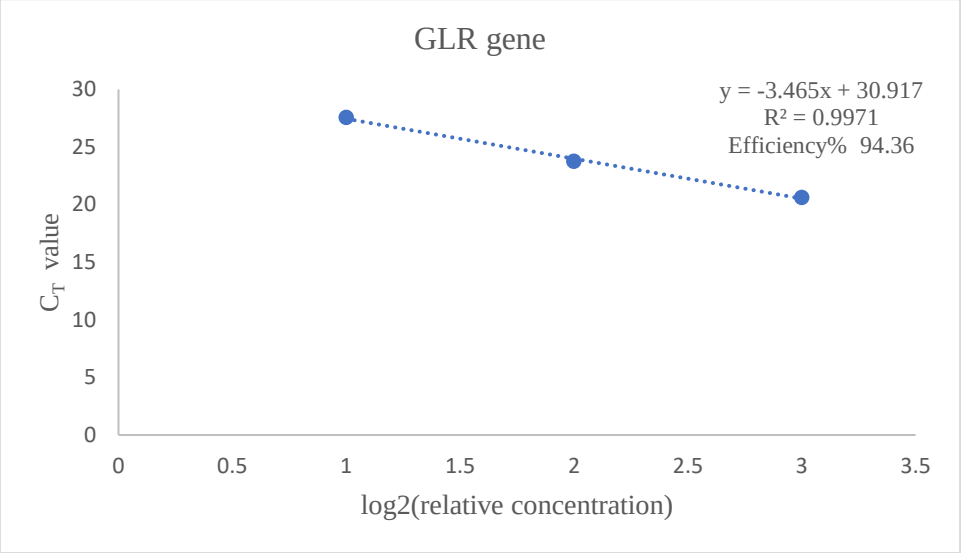


Figure S2: Quality control of 12 RNA samples from the roots and shoots of Najran wheat cultivar using Tapestation High Sensitivity D1000 Assay (Agilent Technologies, CA, USA). R indicates roots and S indicates shoots.


Hsp90.1-A1 gene
y = -3.402x + 39.69
R² = 0.9977
Efficiency% 96.76
C_T value
log2(relative concentration)


TRAES gene
y = -3.5543x + 33.331
R² = 0.9982
Efficiency% 91.29
C_T value
log2(relative concentration)


Wrab 18 gene
y = -3.2607x + 31.179
R² = 0.9999
Efficiency% 102.62
C_T value
log2(relative concentration)



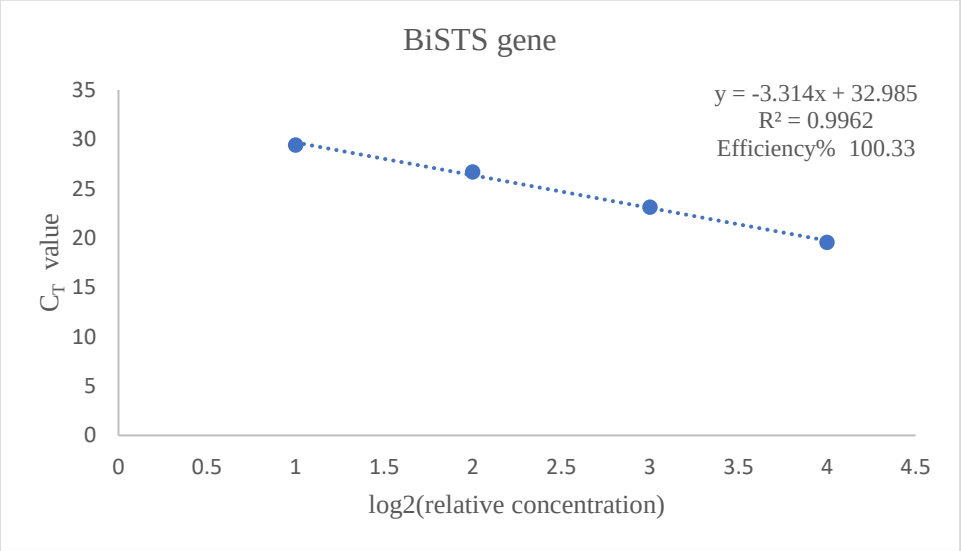


Figure S3: PCR efficiency for some of genes differentially expressed under salt-stress and used in RT-qPCR validation. Primer efficiency was determined using serial dilutions of the cDNA for each gene and calculated based on the efficiency equation (Efficiency (%) = $(10^{(-1/Slope)} -1) *100$).

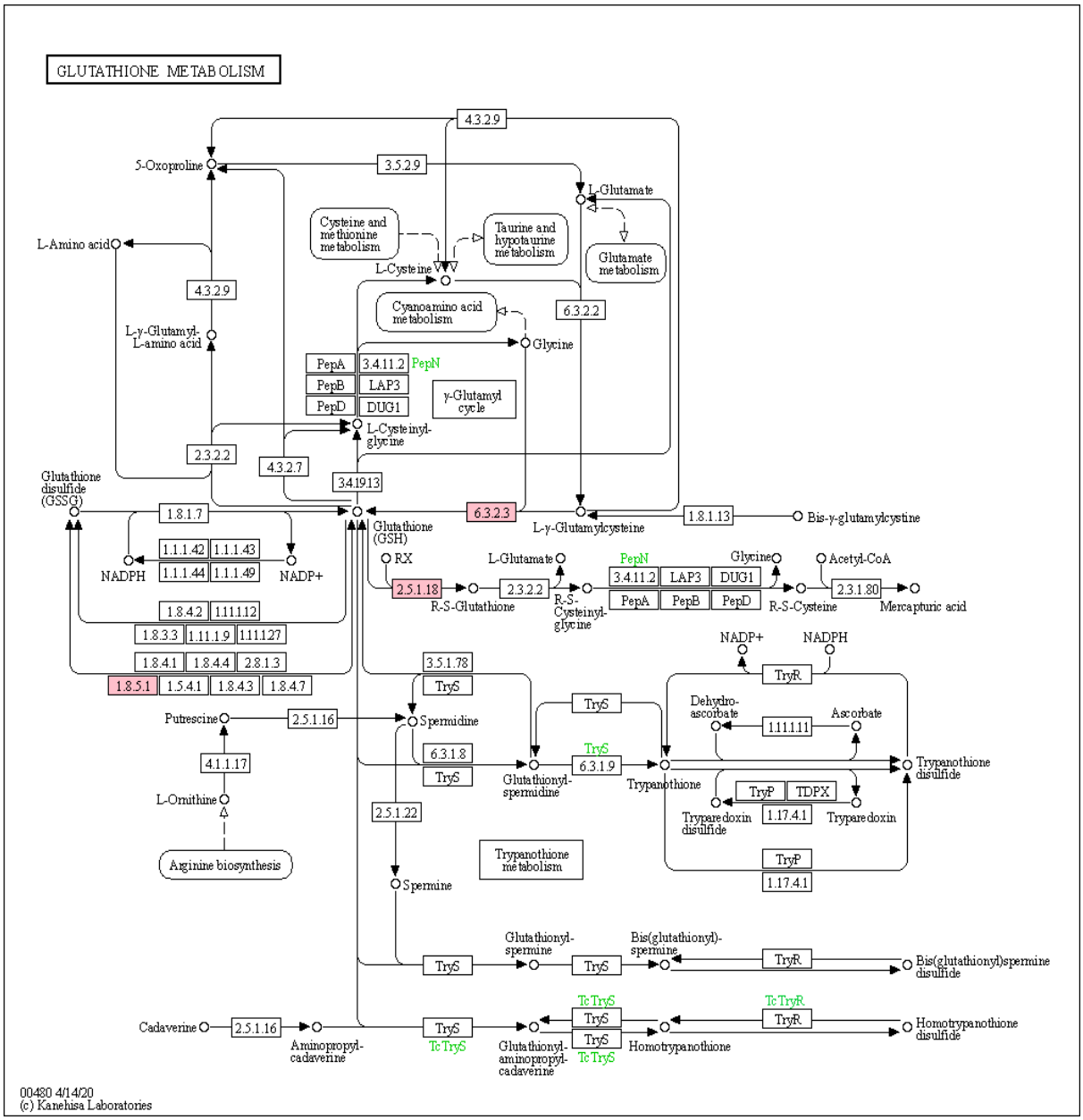


Figure S4: KEGG map of glutathione pathway regulated in the root of wheat (*Triticum aestivum*) under salt stress conditions.

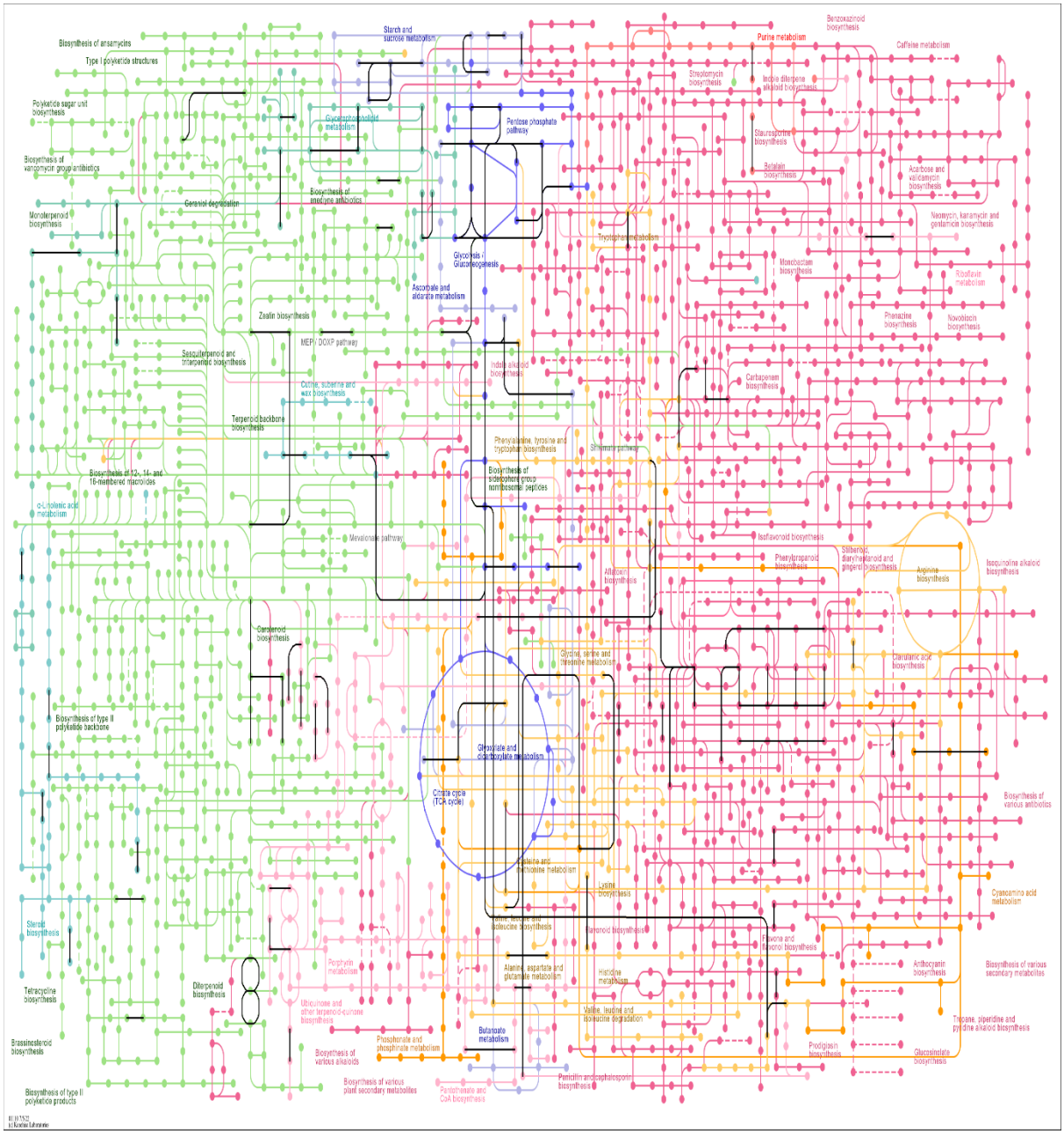


Figure S5: KEGG map of biosynthesis of secondary metabolites pathway regulated in the shoot of wheat (*Triticum aestivum*) under salt stress conditions.

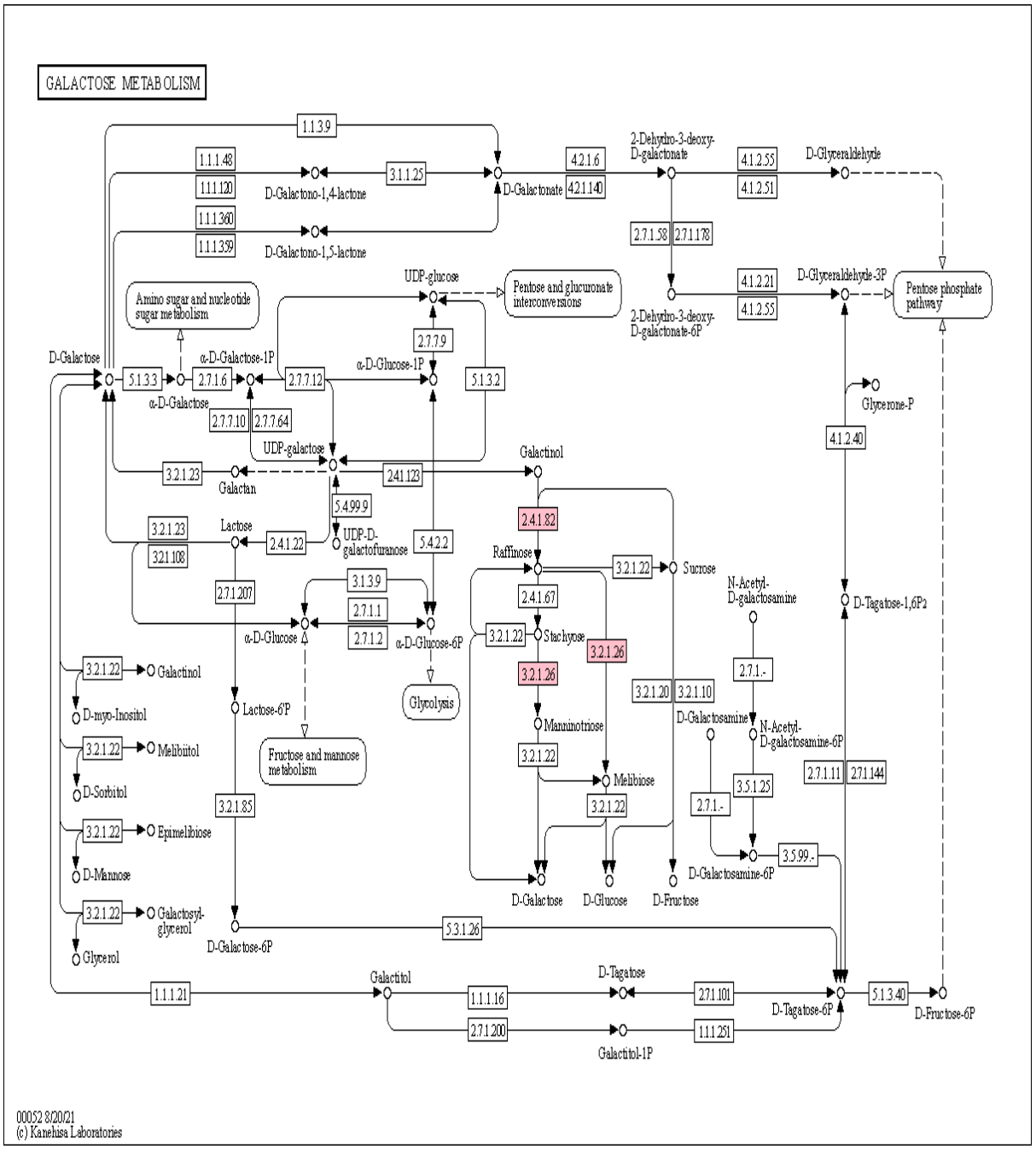


Figure S6: KEGG map of galactose pathway regulated in the root and shoot of wheat (*Triticum aestivum*) under salt stress conditions.

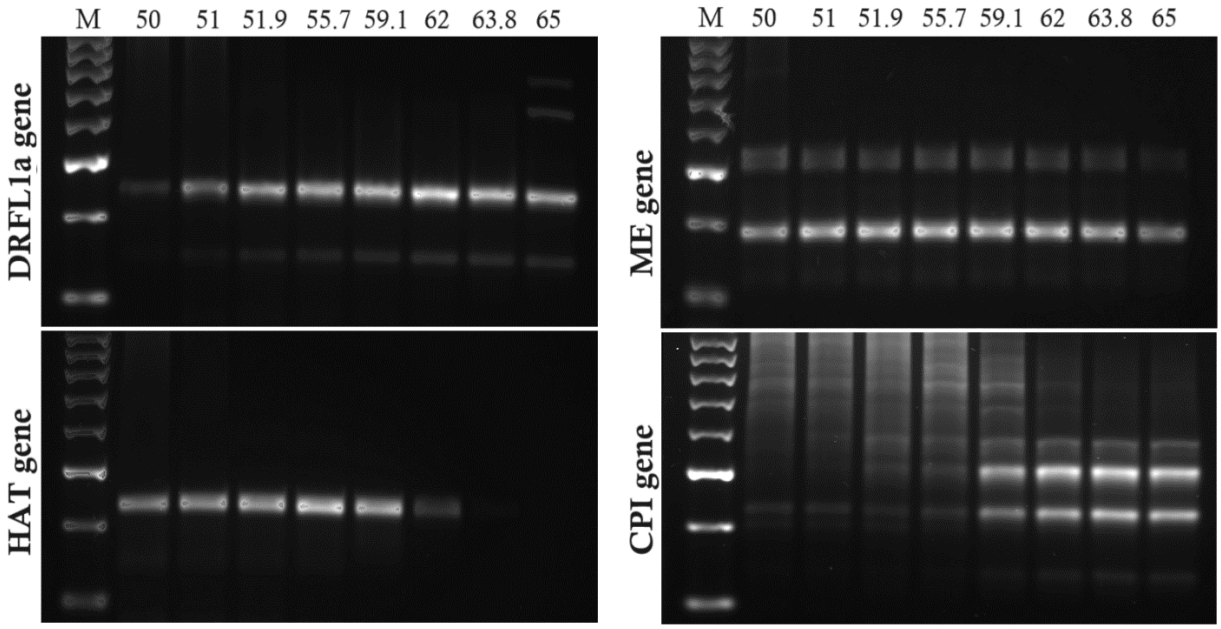


Figure S7: Temperature gradient ranged from 50°C to 65°C for optimization of primers used in validation of alternative spliced genes.

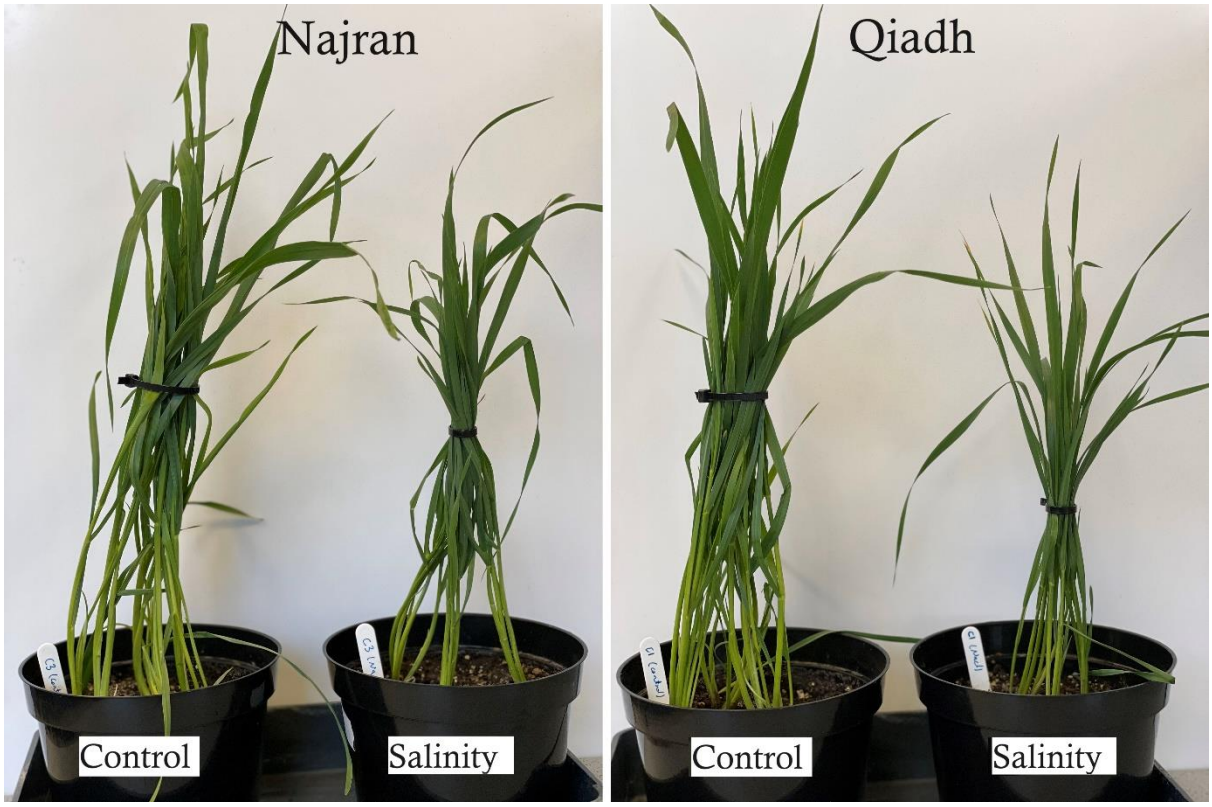


Figure S8: Growth of wheat cultivars (*Triticum aestivum),* Najran Qiadh. Control: plants grown under normal conditions. Salinity: plants grown under 200 mM NaCl.

Table S1: The eight DEGs randomly selected for the RT-qPCR

Gene ID	**Gene name**	**Description**	**Log2 fold change (Root)**	**Log2 fold change (Shoot)**
TraesCS2A02G033700	Hsp90.1-A1	Heat shock protein 90 [Source:UniProtKB/TrEMBL%3BAcc:F4Y589]	5.07376836	7.365718292
TraesCS4A02G431400	*N/A*	Dirigent protein [Source:UniProtKB/TrEMBL%3BAcc:A0A1D5WYI3]	4.987534773	7.024568308
TraesCS3B02G395900	TRAES_3BF065800020CFD_c1	Delta-1-pyrroline-5-carboxylate synthase [Source:UniProtKB/TrEMBL%3BAcc:A0A077RXE4]	1.542868089	4.129638563
TraesCS5A02G348400	*N/A*	Flavin-containing monooxygenase [Source:UniProtKB/TrEMBL%3BAcc:A0A1D5YP16]	5.106458059	3.346230427
TraesCS5B02G217500	*N/A*	Glutamate receptor [Source:UniProtKB/TrEMBL%3BAcc:A0A1D5ZBQ4]	-1.6837149	-2.820227478
TraesCS2B02G333600	*N/A*	Lipoxygenase [Source:UniProtKB/TrEMBL%3BAcc:A0A1D5U5U4]	-1.771863296	-1.892795301
TraesCS6A02G009100	*N/A*	Bidirectional sugar transporter SWEET [Source:UniProtKB/TrEMBL%3BAcc:W5GC93]	1.147319828	-4.77364506
TraesCS1B02G381500	Wrab18	ABA inducible protein [Source:UniProtKB/TrEMBL%3BAcc:Q7XAP5]	-1.189341477	5.395800134

Table S2: Oligonucleotide primers used in RT-qPCR confirmation. Forward primer is marked in yellow and reverse primer is marked in blue.

Gene ID	**Annotation**	**Primer Name**	**Primer Sequence (5'→3')**
TraesCS2A02G033700	*TaHsp90.1-A1*	Hsp90.1-A1-**F**	GCAGTGTCGTGTCTGTCCAT
		Hsp90.1-A1-**R**	TCCCTCCGTTCTCACATCTC
TraesCS4A02G431400	*TaDip*	Dip-**F**	TTCAGTATTCGACCGTGCTG
		Dip-**R**	AGGCATAAACAACGGGACAC
TraesCS3B02G395900	*TaTRAES_3BF065800020CFD*	TRAES-**F**	TCTCTCTGTGCGAGGAATGA
	_c1	TRAES-**R**	AGCATAGATGTACCGATGCAA
TraesCS5A02G348400		FCM-**F**	CAGAAACATTGCCATTCCAG
	TaFCM	FCM-**R**	GGAGGACCGGTAACAAGAGT
TraesCS5B02G217500		GLR-**F**	CGATGACGATGACGATGAAT
	TaGLR	GLR-**R**	GCACCGACTGAGACTTCTGAC
TraesCS2B02G333600		LIPX-**F**	GGGAATTTTTCAGCCTCGAT
	TaLIPX	LIPX-**R**	GATTCGGCCTTCGTAGTGAA
TraesCS6A02G009100		BiSTS-**F**	GCAGTCATGCGTTCATGC
	TaBiSTS	BiSTS-**R**	AAAATAACTGGCTCGAGCATC
TraesCS1B02G381500		Wrab18-**F**	CATGCGTCCAATCTTGCTAA
	TaWrab18	Wrab18-**R**	CATTACAGAACCGGACACGA
CJ705892		CJ70-**F**	GCCTCAGTGGTAGGAGCATT
(Reference gene)	*TaCJ70*	CJ70-**R**	TTCAGCAAATGCGGTGGTTG
(Dudziak, 2020)			

1- TraesCS2A02G033700 (*TaHsp90.1-A1*)

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2- TraesCS4A02G431400 (*TaDip*)

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5- Glutamate receptor (*TaGLR*)

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6- TraesCS2B02G333600 (*TaLIPX*)

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ATCACGGAGGAGCACATCATCGGGAACCTGGACGGCATGTCGGTGCAGCAGGCG
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TGGACCGGATCAACTCGCTGGACGGCCGCAAGGCCTACGGGACGCGCACGCTCTT
CTTCCTGACGGCCGGTGGCACGCTGAAGCCGATCGCGATCGAGCTGTGCCTGCCG
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TGAGAACATCATAATAATGGAAAGTAATTCATAGTAAGACCTCAAAATAATTCAG
TGT

7- TraesCS6A02G009100 (*TaBiSTS*)

AAGCAACAACCTCAAGTAGTAGGCTGGCAGTTCTTCTCTTCCCATTGAACCATCT
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CCTACCTGGCCCCACTGCCGACATTCTACCGGATCTACCGGAGCAAGTCGACGCA
GGGGTTCCAGTCGGTCCCTTACGTGGTGGCGCTCTTCAGCGCGATGTTGTGGATCT
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CTGTGTCATTGAGACCATCTACATCATCATCTACCTCACCTATGCACCAAAGCAA
GCCAAGCTCTTCACGGCGAAGATCCTCCTCCTCCTGAATGTGGGTGTGTTCGGGC
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CCGCCTTGTGGTGCGTACCCGGAGTGTGGAGTTCATGCCCTTTTCCCTCTCCCTCT
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AGAGAAATGCATGCACGTGGGCTTGTGATTTCCACTATGGACGTTCCATTAGATG
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ATGTAATGATCTTATTATAAAAATTGCCATTTTCT

8- TraesCS1B02G381500 (*TaWrab18*)

ATCGAATCAGCAGCTCGTGATATGGGTGAGCGATCGAGCTTCGCGAAGCTACTTT
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TATAAATGCCAACGGCTCCAATCTCCTTTCCAACACACACAAGCAGTCGATCGAT
TCATCCAAGCCAGAGTTGAGCAATACTAGCAGTGAGATTTACAGTGATTTCACTT
CGTGTTTGTTGGTGAGAGAGAAGAGCAGAGAAGACAAGATGGCCTCCAACCAGA
ACCAGGCGAGCTACGCGGCCGGCGAGACCAAGGCCCGCACTGAGGAGAAGACCG
GGCAGATGATGGACAAGGCGGGGCAGGCCACGGAGGCCACCAAGCAGAAGGCC
GGAGAGGCCAAGGACAAGACGGCCCAGACGGCGCAGGCGGCCAAGGACCGCGC
CGCCGAGAGCAAGGACCAGACCGGCAGCTTCCTCGGCGAGAAGACGGAGGCGGC
CAAGCAGAAGACCGCCGAGGCAACCGATGCGGCCAAGCAGAAGGCGTCGGAGA
CGGCCCAGTACGCGCAGGAGAGGTCCTCCGACGCGGCGCAGTACACCAAGGAGT
CCGCCGTGGCCGGCAAGGACAAGACCGGCAGCGTGCTCCAGCAGGCCGGCGAGA
CGGTGGTGAGCGCCGTGGTGGGCGCCAAGGACGCCGTGGCGAACACGCTGGGCA
TGGGCGGCGACAACACCAACACCGCCAAGGACAGCACCACCGAGAAGATCACTA
GGGATCACTAGACGCATACATGCGTCCAATCTTGCTAATTTGCTTCCTTTACTCGT
TTGGTCGTTCGCGGGCCCTTTACATATTTGTATGTTTCCCTCTTTTGTGATTTCCGC
TCATTTAGTGTAAGTTTGCCTTCGATTTGATGTACTACTCGTGTCCGGTTCTGTAA
TGAGTTACTTATAATCCATGTTTTACTTTGGTGTAAATGGATAACGAGGACAGTC
GAAGGTGTCAATAAAGTTCTTTTAGCACG

Table S3: The significantly enriched pathways of identified DEGs in both roots and shoots of *Triticum aestivum* cultivar (Najran) under control and salinity stress conditions.

	Enriched KEGG Pathways	**Main pathway**	**Pathway ID**	**Observed Significant Genes**	**Expected Significant Genes**
Salt treated root vs control root	Glutathione metabolism	Amino acid metabolism	path:taes00480	54	35
	Thiamine metabolism	Metabolism of cofactors and vitamins	path:taes00730	8	2
	Galactose metabolism	Carbohydrate metabolism	path:taes00052	28	14
Salt treated Shoot vs Control Shoot	Phenylalanine metabolism	Amino acid metabolism	path:taes00360	18	8
	Phenylpropanoid biosynthesis	Biosynthesis of other secondary metabolites	path:taes00940	26	12
	Biosynthesis of secondary metabolites		path:taes01110	130	96
	Starch and sucrose metabolism	Carbohydrate metabolism	path:taes00500	32	16
	Galactose metabolism		path:taes00052	24	9
	Protein processing in endoplasmic reticulum -	Genetic Information Processing; Folding, sorting and degradation	path:taes04141	43	20

Table S4: Most commonly identified KEGG pathways in different comparisons.

Enriched KEGG Pathways	**Pathway ID**	**Salt treated Root vs Control Root**	**Salt treated Shoot vs Control Shoot**
Glutathione metabolism	taes00480	✓	
Thiamine metabolism	taes00730	✓	
Galactose metabolism	taes00052	✓	✓
Phenylalanine metabolism	taes00360		✓
Phenylpropanoid biosynthesis	taes00940		✓
Biosynthesis of secondary metabolites	taes01110		✓
Starch and sucrose metabolism	taes00500		✓
Protein processing in endoplasmic reticulum -	taes04141		✓

Table S5: Differentially spliced genes randomly selected for RT-PCR run.

Gene ID	**Description**	**AS event type**	**ΔPSI (Root)**	**ΔPSI (Shoot)**
TraesCS6A02G107300	Histone acetyltransferase (EC 2.3.1.48)	RI	0.2790	0.2336
TraesCS5D02G200900	Drought-responsive factor-like transcription factor DRFL1a	RI	-0.0768	0.1098
TraesCS3B02G128000	Malic enzyme	A5	0.1600	-0.1175
TraesCS1A02G256400	Cysteine proteinase inhibitor	SE	-0.0872	-0.0872

Table S6: Summary of Alternative Splicing events and corresponding genes (given in brackets) in different comparisons of Najran wheat cultivar.

	Salt treated Root vs. Control Root	**Salt treated Shoot vs. Control Shoot**
A3	17662 (13539)	17475 (13419)
A5	8145 (7011)	8108 (6961)
AF	2382 (1600)	2405 (1609)
AL	1120 (943)	1093 (922)
MX	86 (84)	88 (87)
RI	4615 (4069)	4504 (3975)
SE	5813 (5022)	5751 (4968)
Total	39823 (32268)	39424 (31941)

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