EFFECT OF SALT, DROUGHT AND MECHANICAL WOUNDING STRESS ON GENE EXPRESSION AND HORMONE PROFILES OF *BRACHYPODIUM DISTACHYON*

by

Çağla Görkem Eroğlu

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2019

EFFECT OF SALT, DROUGHT AND MECHANICAL WOUNDING STRESS ON GENE EXPRESSION AND HORMONE PROFILES OF *BRACHYPODIUM DISTACHYON*

APPROVED BY:

Assist. Prof. Bahar Soğutmaz Özdemir .....
(Thesis Supervisor)
(Yeditepe University)

Prof. Dr. Elif Damla Arısan .....
(İstanbul Kültür University)

Assist. Prof. Emrah Nikerel .....
(Yeditepe University)

DATE OF APPROVAL: ..../..../2019

ACKNOWLEDGEMENTS

Firstly, I would like to express my thanks and sincere gratitude to my supervisor Assist. Prof. Dr. Bahar Soğutmaz Özdemir for her guidance, advice, encouragement and continuous support during this study. Her patience, optimism, broad knowledge and expertise helped me throughout my both undergraduate and graduate years. I was very lucky to have a supervisor who is open to new ideas and always motivate me to improve myself.

I would like to thank Assist. Prof. Dr. Emrah Nikerel for his guidance, valuable comments and advice throughout this project. I also would like to thank Prof. Dr. Elif Damla Arısan for her time and suggestions.

I would like to thank Dr. Bihter Güven for her assistance, support and advice which facilitated my laboratory work and helped me to obtain better results.

I would like to thank Ebru Yılmaz for her support in the lab work, especially in HPLC analysis part of the study and her friendship. Her determination gave me the courage to continue without giving up, no matter how frustrating the situations were. I would also like to thank all members of Plant Tissue Culture and Genetics Group for their help and support.

I would like to thank my dear friends Begüm Okutan, Berk Gazioğlu and Cem Derman who always tried to help me in any way possible during my laboratory work and thesis writing process. Their moral support and optimism made even the most stressful times bearable.

Finally, I would like to express my gratitude to my parents, Ayten Eroğlu and Kemal Eroğlu and my beloved sister Doğa Gizem Eroğlu, for their endless love and support.

ABSTRACT

EFFECT OF SALT, DROUGHT AND MECHANICAL WOUNDING STRESS ON GENE EXPRESSION AND HORMONE PROFILES OF *BRACHYPODIUM DISTACHYON*

The rising world population with limited arable lands and the severe effect of climate change require an increase in crop yield. However, abiotic stress factors such as salinity, drought and mechanical wounding also decrease agricultural productivity. The development of crops that can cope with various environmental stress is the key to higher yield. Phytohormones and their cross-talk control many physiological and molecular mechanisms and mediate stress responses through activation of stress-responsive genes. Elucidation of stress-responsive genes and phytohormone signaling are crucial to contribute to a deeper understanding of their roles and relationship in complex response mechanisms of plants under stress. In this study, *Brachypodium distachyon* (Bd21 line), a powerful monocot model plant was used to determine the expression changes in multiple stress-responsive genes by quantitative real-time polymerase chain reaction (qRT-PCR) and quantify the hormonal changes via high-performance liquid chromatography (HPLC) under salinity, drought and time-dependent mechanical wounding stress including local and systemic events. From different gene families, 58 candidate multiple stress-responsive genes were identified by orthology analysis and in accordance with the literature, and eight of these genes were used for gene expression studies. *EXPA2, GF14d, LOX3, P5CS1, PP2CA6* and *WRKY36* genes were up-regulated under drought, salinity and wounding. Under salinity, all genes were up-regulated whereas under drought the genes rather than *ERF1* and *SUT2* were up-regulated. In response to wounding stress, the highest fold-changes were observed at 24^{th} hour at wound sites compared to the gene expression levels at 0^{th} and 6^{th} hours. Besides, wound sites had higher gene expression levels than wound adjacent sites in general. On the other hand, in *B. distachyon*, salicylic acid (SA) levels under drought, and abscisic acid (ABA) levels under salinity and wounding stress showed increasing trends. Indole acetic acid (IAA) levels were significantly reduced at all stress conditions. Characterization and overexpression studies of determined genes can pave the way towards generating multiple stress-tolerant crops.

ÖZET

TUZ, KURAKLIK VE MEKANİK YARALANMA STRESİNİN *BRACHYPODIUM DISTACHYON*' UN GEN EKSPRESYONU VE HORMON PROFİLLERİ ÜZERİNDEKİ ETKİSİ

Artan dünya nüfusu ile birlikte azalan ekilebilir alanlar ve küresel ısınmanın şiddetli etkisi tahıl veriminde artış gerektirmektedir. Diğer taraftan tuzluluk, kuraklık ve mekanik yaralanma gibi abiyotik stres koşulları da tarımsal verimi ciddi bir şekilde düşürmektedir. Çeşitli çevresel streslere dayanıklı tahılların geliştirilmesi daha yüksek verim elde etmenin anahtarıdır. Bitki hormonları ve onların birbirleri ile etkileşimi birçok fizyolojik ve moleküler mekanizmayı ve stres genlerinin aktivasyonu ile stres cevaplarının oluşturulmasını kontrol eder. Strese cevap veren genlerin ve bitki hormon sinyallerinin aydınlatılması, stres altındaki bitkilerin karmaşık cevap mekanizmalarındaki rollerinin ve birbirleriyle olan ilişkilerinin derinlemesine anlaşılmasını sağlayacaktır. Bu çalışmada, güçlü bir monokot bitki modeli olan *Brachypodium distachyon* (Bd21 hattı)'nda birden çok strese cevap veren gen ifadelerinin gerçek zamanlı kantitatif polimeraz zincir reaksiyonu (qRT-PCR) kullanılarak ve hormonal değişimlerin gözlemlenmesi için yüksek performanslı likit kromatografisi (HPLC) analizi yapılarak tuzluluk, kuraklık ve zamana bağlı mekanik yaralama stres cevaplarının (lokal ve sistemik cevaplar dahil) araştırılması amaçlanmıştır. Farklı gen ailelerinden çoklu strese cevap veren 58 aday gen, ortoloji analizi ile literatüre de uyumlu olarak belirlenmiştir. Bunlardan 8 tanesi gen ekspresyon çalışması için kullanılmıştır. *EXPA2, GF14d, LOX3, P5CS1, PP2CA6* ve *WRKY36* genleri tüm stresler altında upregüle olmuştur. Tuzluluk stresi tüm genlerin upregülasyonuna sebep olurken, kuraklıkta *ERF1* ve *SUT2* dışındaki genler upregüle olmuştur. Yaralanma stresi cevabında, gen ekspresyonundaki en yüksek kat değişimi 0. ve 6. saate kıyasla 24. saatte gözlemlenmiştir. Bunun yanı sıra, genel olarak yaralı bölgelerdeki ekspresyon seviyeleri yaralı bölgelere komşu dokulardakine göre daha yüksek bulunmuştur. Kuraklık ve yaralama stresi altında salisilik asit (SA) ve tuzluluk stresi altında absisik asit (ABA) seviyeleri artış trendi göstermiştir. İndol asetik asit (IAA) seviyeleri tüm stresler altında istatistiksel olarak anlamlı oranda azalmıştır. Belirlenen genlerin karakterizasyonu ve gen ifadelerinin arttırılması, çoklu strese toleranslı tahılların üretimine katkı sağlayacaktır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

A. thaliana	*Arabidopsis thaliana*
B. distachyon	*Brachypodium distachyon*
T. aestivum	*Triticum aestivum*
α	Alpha
Å	Angstrom
β	Beta
°C	Degrees celsius
Δ	Delta
μ	Micro
2D	Two dimensional
ABA	Abscisic acid
*AP2/EREBP*s	APETALA2/ethylene-responsive element binding proteins
APX	Ascorbate peroxidase
ASR	Abscisic acid and ripening-induced protein
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
bp	Base pair
BR	Brassinosteroid
bZIP	Basic leucine zipper transcription factor
Ca^{2+}	Calcium ion
$(Ca(NO_3)_2)$	Calcium nitrate
CAT	Catalase
CE	Capillary electrophoresis
cDNA	Complementary DNA
CK	Cytokinin
Cl^-	Chloride ion
CO_3^{-2}	Carbonate
CRISPR	Clustered regularly interspaced short palindromic repeat

Ct	Cycle threshold
D	Drought stress treatment
DC	Drought control
DAP	Differentially accumulated protein
dH_2O	Distilled water
ddH_2O	Double distilled water
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Fe	Iron
Fe-EDTA	Ferric ethylenediaminetetraacetic acid
FISH	Fluorescence in situ hybridization
FLD	Fluorescence detection
ELISA	Enzyme-linked immunosorbent assays
ESI	Electrospray ionization
ET	Ethylene
EtBr	Ethidium bromide
g	Gram
x g	Times gravity
GA	Gibberellin
GC	Gas chromatography
GSH	Reduced glutathione
GR	Glutathione reductase
h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate
HPLC	High performance liquid chromatography
Hsp90	Heat-shock protein 90
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
JA	Jasmonate
JGI	Joint Genome Institute

K	Potassium
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
K_2SO_4	Potassium sulphate
LC	Liquid chromatography
LOD	Limit of detection
M	Molar
MS	Mass spectrometry
MAPK	Mitogen activated protein kinases
MAPKK	Mitogen activated protein kinase kinases
Mg^{+2}	Magnesium ion
mg	Milligram
$MgCl_2$	Magnesium chloride
miRNA	MicroRNA
mL	Milliliter
min	Minute
mM	Millimolar
MPa	Megapascal
*MYB*s	MYB transcription factors
Na	Sodium
Na^+	Sodium ion
NAC	NAC transcription factors
NaCl	Sodium chloride
NCBI	National centre for biotechnology information
ng	Nanogram
nm	Nanometer
NPGS	National plant germplasm system
O^{-2}	Oxide ion
$^1O_2$	Singlet oxygen
OGA	Oligogalacturonide
OH^-	Hydroxide ion
P	Phosphorus

P5CS	Delta 1-pyrroline-5-carboxylate synthase
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
POD	Peroxidase
PP2CA	Type-2C protein phosphatases
PSI	Photosystem I
PSII	Photosystem II
qRT-PCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative trait locus
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
S	Salinity stress treatment
SC	Salinity control
s	Second
SA	Salicylic acid
S-ABA	(+)-cis, trans-Abscisic Acid
sec	Second
SO_4^{-2}	Sulphate ion
SOD	Superoxide dismutase
TAE	Tris-acetate-EDTA
TALEN	Transcription activator-like effector nuclease
U	Unit
UHPLC	Ultra-high performance liquid chromatography
UK	United Kingdom
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
V	Volume
v/v	Volume per volume
µg	Microgram
µL	Microliter
µM	Micromolar

μm	Micrometer
W0	Wounding 0 h
W6	Wounding 6 h
W24	Wounding 24 h
WA0	Wounding adjacent 0 h
WA6	Wounding adjacent 6 h
WA24	Wounding adjacent 24 h
WC	Wounding control
*WRKY*s	WRKY transcription factor family
Zn	Zinc
$ZnSO_4$	Zinc sulphate

1. INTRODUCTION

Model plant systems are required to answer many molecular biology, genetics, biochemistry and physiology questions. For many years, dicot plant *Arabidopsis thaliana* has been widely used for this purpose. However, the crops are different from *Arabidopsis thaliana* in many aspects and some processes are species specific. *Brachypodium distachyon* is a monocot model plant with high agronomic value. It has a close relationship with cereal grains and forage grasses. One of the greatest constrains in crop production is abiotic stress such as salinity, drought, and mechanical wounding. They influence metabolism, growth and development, therefore diminish the yield. Salinity stress causes both osmotic and ionic stress; thereby impair growth and development due to high ion concentrations and toxicity through the excess uptake of ions and their accumulation. Drought negatively affects growth and development, and influences water relations of plants. Mechanical wounding stress causes injuries in the plant tissues, nutrient loss, and leads to further damage by providing entry for the pathogens. To be able to cope with stress, plants induce changes in their metabolism. Plant responses to abiotic stress are highly complex. They involve many stress-inducible genes and both molecular and biochemical mechanisms. Thus, the elucidation of those responses through the discovery of stress-inducible genes and their functions is a significant tool for contributing to the improvement of stress tolerance in crops. Many stress-inducible genes are induced by multiple stress conditions, which suggest that there might be similar mechanisms that control a wide range of stress responses. Since plants experience a combination of stress in their natural environment, it is essential to determine multiple stress-responsive genes to develop crops that can survive under multiple stress conditions.

Phytohormones also play a significant role in the regulation of growth, development, and signaling networks associated with abiotic stress responses. The synergistic and antagonistic activity of phytohormones and their coordination, involving the regulation of hormone synthesis pathways and gene expression patterns, is vital in the adaptation of plants to abiotic stress conditions. Comprehensive studies are required for the explanation of underlying mechanisms of plant defence responses that plays a vital role in gene expression changes and phytohormone signaling pathways under stress conditions.

In this study, in order to determine the expressional changes in selected candidate multiple stress-responsive genes from *ERF*, *EXP*, *SUT*, *P5CS*, *14-3-3* and *LOX* gene families and monitor the changes in phytohormones (ABA, SA and IAA), qRT-PCR and HPLC analysis were conducted, respectively. Both gene expression and phytohormone quantification studies were performed in model plant *Brachypodium distachyon* (Bd21 line) under salinity, drought and time-dependent mechanical wounding stress including local and systemic events by analysing both wounded and unwounded tissues adjacent to wound sites. Since many of the stress-responsive genes have been identified in widely studied species such as *Arabidopsis thaliana*, *Oryza sativa* and *Triticum aestivum*, their predicted orthologs in *Brachypodium distachyon* were determined using nucleotide alignment tool. In accordance with literature, 220 genes were obtained from databases. According to their alignment scores, low scored genes were eliminated and 58 genes were found to be candidate stress responsive genes in *Brachypodium distachyon*. Eight of these genes (*ERF1*, *EXPA2*, *GF14d*, *LOX3*, *P5CS1*, *PP2CA6*, *SUT2* and *WRKY36*) representing different gene families were selected due to their possible multiple abiotic stress responsiveness.

All selected genes were found to be multiple stress-responsive and up-regulated upon salinity and wounding stress. Drought also caused the up-regulation of the genes except for *ERF1*, *LOX3*, and *SUT2*. Moreover, *WRKY36*, *GF14d*, *EXPA2*, *P5CS1*, and *PP2CA6* genes were all up-regulated under all stress conditions. In wounded tissues, gene expression levels were increased as the time passed and the highest fold-changes were observed at 24^{th} hour after wounding at wound sites compared to the gene expression levels at 0^{th} and 6^{th} hours. Wound sites had higher gene expression levels than wound adjacent sites, except *SUT2* gene and this showed the clear difference between local and systemic response. On the other hand, salicylic acid (SA) levels under drought and abscisic acid (ABA) levels under salinity showed increasing trends, however the changes were not significant. Indole acetic acid (IAA) levels were significantly reduced at all stress conditions.

Elucidation of multiple-stress responsive genes and associated signaling molecules will provide a better understanding of the stress response mechanisms in monocot species and thereby pave the way for the crop improvement studies.

2. LITERATURE REVIEW

2.1. PHYLOGENY AND EVOLUTION OF *BRACHYPODIUM DISTACHYON*

Crop production is under threat due to the factors such as misuse of arable lands, urbanization and climate change. In 2050, the population of the world is expected to reach over 9.8 billion according to the United Nations. Thus, global food production needs to be increased by 70 percent until then to be able to meet the drastically increased food and feed demand [1,2]. Focusing on crop research holds the key to obtain higher production rates.

As one of the largest families of flowering plants, the grass family (Poaceae) is composed of 10,000 species and 700 genera including economically important cereals such as wheat, rice, maize, sorghum, barley, rye and oat. Grasses exist in all continents and they show dominance over other plant families [2,3]. They are significant sources for human nutrition and production of sustainable energy [4]. The genus *Brachypodium* belongs to Brachypodieae, a sister group to Triticeae, Aveneae, Poeae and Bromeae tribes. The species *Brachypodium distachyon* belongs to Pooideae subfamily which is one of the 12 subfamilies of Poaceae (Figure 2.1.) [2,5]. Even though it is a native species to Mediterranean and Middle East regions, it has been dispersed all around the world having almost 20 species in temperate regions [6].

Brachypodium genus harbour species with a varying number of chromosomes in somatic cells as 2n= 10, 14, 16, 18, 20, 28 and 30 with basic chromosome numbers of x = 5, 7, 8, 9 and 10. The first karyological analyses of *Brachypodium* showed varying putative ploidies; the three cytotypes were 2n =10, 20 and 30. They thought to be autopolyploid series having the same base chromosome number x= 5 [7]. However, the following cytogenetic studies that used technologies such as fluorescence in situ hybridization (FISH) with total genomic DNA, ribosomal DNA with bacterial artificial chromosomes (BACs) were elicited three somatic chromosome number of 2n=10, 20 and 30 and that showed the existence of three different cytotypes having chromosome numbers of x=10 (*Brachypodium distachyon*) and x=20 (*Brachypodium stacei*) and 2n= 30 (*Brachypodium hybridum*), being their generated allotetraploid [8].

Generally, the grasses possess high synteny within the group that they belong to. Hence, *Brachypodium distachyon* (hereafter *Brachypodium*) also shows high collinearity with cereal grains and forage grasses. It is especially close to rice and wheat. When their sequences are aligned, *Brachypodium* and rice possess whole chromosomes and chromosome arms with high similarity. Besides, when wheat and *Brachypodium* compared, the alignments show abundant rearrangements in sequenced regions and genetic maps. However, in many cases, the smaller the sequenced region compared, the higher the synteny is [9].

Many studies showed that the *Brachypodium* is evolutionarily closer to wheat than the rice is. Rice and wheat underwent divergence 50 to 55 million years ago, whereas *Brachypodium* and wheat diverged from each other 15 million years later. Therefore, the divergence of rice and wheat took place many years before by going through many insertions, deletions, duplications and translocations [9,10].

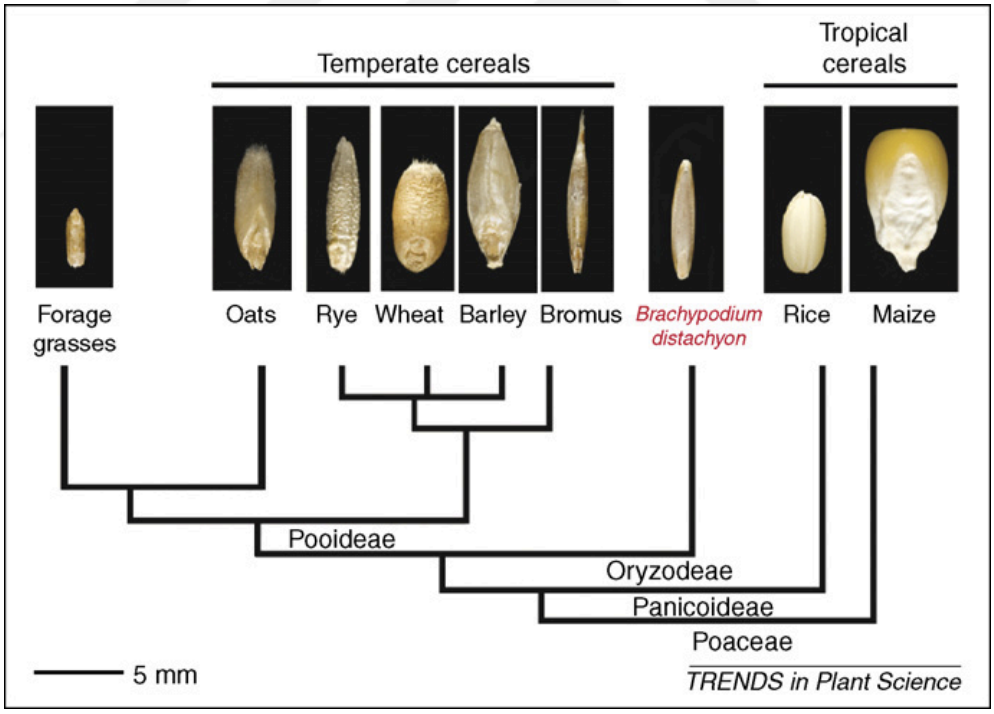

Figure 2.1. The representation of phylogenetic relationships of *Brachypodium* with other small grain cereals [11].

While selections based on obtaining plants with desired attributes such as non-shattering seeds, high seed number and bigger seed size, they also led to unfavorable alterations in genetic diversity due to bottleneck effects. When compared with wild ancestors, many cereal

crops have lost their diversity during domestication. On the other hand, *Brachypodium* was not gone through any domestication process thus protected its genetic diversity [12,13].

2.2. *BRACHYPODIUM DISTACHYON* AS A MODEL ORGANISM

Plant model systems are required to conduct investigations on specific plant families. A model system should have the crucial features such as small stature, quick generation time, high fertility, simple growth requirements, ease of genetic transformation and small genome size (Table 2.1.).

Table 2.1. Comparison of model plant species [4,14,15].

	Brachypodium distachyon	***Arabidopsis thaliana***	***Triticum aestivum***	***Oryza sativa***	***Hordeum vulgare***	***Zea mays***
Genome size	272 Mb	135 Mb	17 Gb	430 Mb	5.3 Gb	2.4 Gb
Chromosome number	10 (2n)	10 (2n)	42 (2n)	24 (2n)	14 (2n)	20 (2n)
Height (cm)	15-20	15-20	50-100	100-120	50-120	120-300
Easy to grow	Yes	Yes	Yes	No	Relatively	Relatively
Generation (weeks)	8-12	8-12	10-20	20-30	16-20	8-20
Pollination	Self	Self	Self	Self	Cross	Self
Transformation	Very efficient	Very easy	Not efficient	Very efficient	Labour intensive	Labour intensive

A. thaliana, a dicotyledonous plant, has been the most widely used plant model by scientists, in a great variety of research areas such as plant biochemistry, genetics physiology and molecular biology. It paved the way for significant contributions to plant science. However, some processes might be specific to families, genera, species or even in some cases, to population [16]. Also, dicots and monocots went through evolutionary separation a long time ago which resulted in important alterations in their genomes [17]. Hence, *Arabidopsis thaliana* has limited adequacy for monocot-specific studies. For the monocot research, cereals such as rice, maize, barley and wheat have been used over time. However, the attributes such as having large and complex genomes or special growth requirements restrict their usage in molecular breeding, genetic and genomic researches. There was an emerging

need for temperate grass model for the elucidation of the questions that arise in the improvement of food and feed crops [18–20].

Brachypodium was introduced as a powerful monocotyledonous model plant species in 2001 for the first time [5]. The small genome of *Brachypodium* holds great agronomic importance since it facilitates genomic studies. The studies for the elucidation of gene functions and the cloning of genes from the crops are significant contributors to the improvement of grain crops. Since it is non-domesticated, conservation of high genetic variability makes *Brachypodium* a strong model for the identification of genes that can be utilized for other crop species with complex genomes [21–23].

2.3. GENOMICS AND RESOURCES OF *BRACHYPODIUM DISTACHYON*

There are broad sets of available tools and resources for *Brachypodium* (Figure 2.2.). *Brachypodium* (Bd21 line) was sequenced using whole-genome shotgun sequencing by the International *Brachypodium* Initiative. High quality ~272 Mb genome sequence of the *Brachypodium* was measured by flow cytometry and verified with cytogenetic analysis, physical maps and BACs. Over 25,000 protein-coding gene loci were estimated. The 5.6 percent of the genes were found to be grass species-specific, while 77-84 percent of the gene families were common in *Brachypodium*, sorghum and rice. The genome structure is quite compact and has a low repeat content. Retrotransposons covers 21.4 percent of the whole genome which is lower than other main crops such as rice, sorghum, maize and wheat [9]. More than 500 miRNAs have been discovered in *Brachypodium* and both species-specific and family specific regulation mechanisms have been shown [24,25].

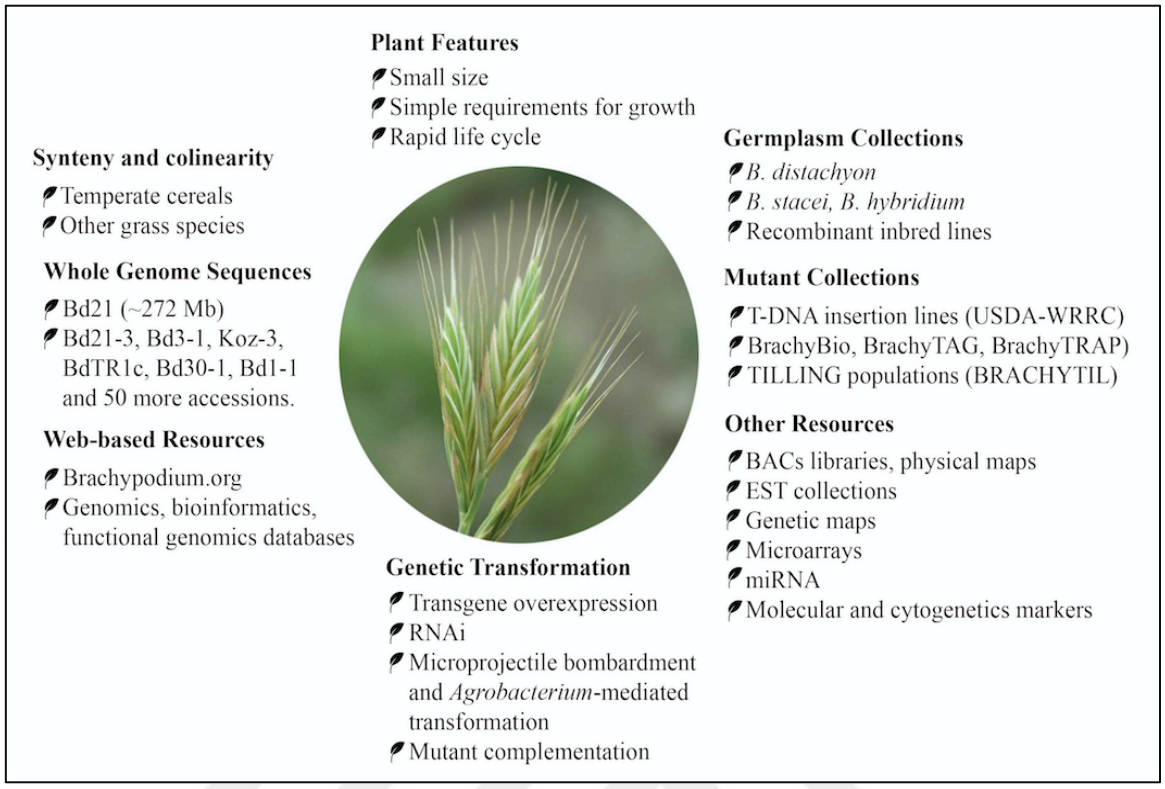

Figure 2.2. Attributes and available tools and resources of *Brachypodium* [18].

Besides the whole genome sequence of Bd21 line, six different lines of *Brachypodium* (Bd1-1, Bd3-1, BdTR12c, Bb21-3, Bd30-1 and Koz-3) were also sequenced. Available genome sequence of different lines made the determination of functional differences and similarities between accessions possible and showed the genetic diversity in *Brachypodium* species annotated [26]. Different germplasm collections were created by the use of nearly 250 wild and inbred accessions and germplasm collections which are mainly native to Turkey [27,28]. They are available in the United States Department of Agriculture's National Plant Germplasm System (USDA- NPGS). Other germplasm collection containing various accessions from different regions is kept at University of Aberystwyth [29].

One of the most significant features for a model plant system is the high-efficiency transformation. Generally, the members of grass family are recalcitrant to genetic transformation. Even though they can be transformed, the efficiency of transformation is remarkably low. However, the transformation of *Brachypodium* is facile and efficient biolistic and *Agrobacterium*-mediated transformation methods are developed and optimized for different genotypes of *Brachypodium*. In 2006, *Agrobacterium*-mediated transformation was reported for the first time [30] and optimized methods were published in the preceding

years for higher efficiency [31–34]. Thousands of *Brachypodium* T-DNA lines were sequenced so far and the collection was made available in Joint Genome Institute (JGI) [35]. Even though, T-DNA mutagenesis is a good genetics tool, it has limitations when it comes to targeting specific sequences in DNA. Therefore, in recent years scientists use targeted gene modification systems; clustered regularly interspaced short palindromic repeat (CRISPR, CRISPR/Cas9) that targets specific DNA sequences and creates double-strand breaks in DNA for mutagenesis by using DNA damage repair as well as transcription activator-like effector nucleases (TALENs) [36,37]. Genetic markers [27], genetic linkage maps and DNA libraries including BACs were constructed from different lines (Bd21 line and Bd3-1) of *Brachypodium* [38,39]. Besides, full-length cDNAs composed of 127, 000 clones, expressed-sequence tag (EST) libraries and mutant collections are available for *Brachypodium* as a great set of tools and resources for the discovery of novel genes and elucidation of their functions [18].

2.4. STRESS FACTORS ON PLANTS

Plants experience stress due to adverse living conditions or different sources that influence their metabolism, growth or development. Throughout their life cycle, plants have to cope with and adapt to those fluctuating unfavorable conditions. Depending on stress source, stress factors could be abiotic or biotic. Abiotic stress includes factors such as salinity, cold, heat, flooding, drought, heavy metals and mechanical wounding while biotic stress is caused by a variety of pathogen infections such as bacteria, fungi, virus, and herbivore attacks [40]. Abiotic stress severely diminishes the yield and causes crop inadequacy worldwide [41]. Salinity and drought are the most common stressors that adversely influence growth, development and productivity of plants [42,43].

Plants possess complex mechanisms to respond and adapt to stress conditions. They alter their physiology, metabolism and gene expression to respond to unfavorable environmental conditions. Stress genes are activated upon stress and the gene products render stress tolerance by the regulation of other genes and metabolic products [44]. Salinity stress has two stages; the first one being the osmotic stress and the second is the ionic stress. Osmotic stress caused by salinity stress hampers the development and growth of plants because if the high concentrations of ions in the environment, which is nearly identical to drought stress

itself. Ionic stress leads to excess uptake of ions, and they accumulate in the plant tissues hence causes ion toxicity problems within the plant. [45,46]. Under drought and salinity conditions, plants close their stomata to diminish the loss of water and to retain their water potential. However, the inhibition of stoma oscillation lowers the rate of photosynthesis due to the limited availability of CO_2 for carbon assimilation. Besides, plant respiration is negatively affected by these changes as well. Hence, plants induce the production of reactive oxygen species (ROS) such as H_2O_2, O^{-2}, $^1O_2$ and OH^- the interaction of ROS with the proteins, lipids, nucleic acid molecules cause oxidative damage in the cells [47,48].

Another frequent stress that the plants encounter, and must be deal with, is wounding. Wounding stress is caused by factors such as rain, wind, hail, snow and herbivore attacks. Wounding not only damages the plant tissues and leads to nutrient loss but also ease the entry of pathogens by creating a passage. Plants have some barriers to prevent damage such as cuticles, tick covers, thorns, waxes, resins and specialized organs. If the plant is wounded, the stress responses have to be activated for the healing process of the tissues. This way plant protects itself from possible further damage caused by infections [49,50].

2.4.1. Drought Stress

The biomass of the most plants, except the ones that have permanent woody stem contains 85-90 percent of water which plays a crucial role in the growth and development of the plant [51]. The long period of water deficiency causes drought stress. Drought is the most common stress that restrains the crop production in many different areas of the world. There are various causes of drought such as low rate of rainfall, soil salinity, extreme temperatures and intense light [52,53]. Drought causes many changes in the, metabolism, morphology, biochemistry and physiology of the plant (Figure 2.3.). The lower rate of cell division, expansion and differentiation restrict the development and growth of the leaves, stems and roots. Imbalances in water and nutrient availability lead to decrease in water use efficiency and crop yield [54,55].

Drought-stressed plants close their stomata for the prevention of water loss. But this process protects plants only to some extent. Since carbon dioxide enters and water exits from the stomata, transpiration and photosynthesis rates fall during stomatal closure [56,57]. Even though the change in stomata oscillations is the main reason for the hindered photosynthesis

and respiration, other alterations such as lower leaf size, higher leaf temperature, early leaf senescence and decrease in relative water content also limit the rate of photosynthesis [54,58]. Under severe and prolonged drought conditions, the metabolism of the plant cannot function properly. Since drought obstructs the Krebs cycle and biosynthesis of ATP, the restrained ATP supply causes the inhibition of many enzymes and pathways especially the ones that play a role in ATP synthesis and fixation of CO_2. The reduced electron transport chain components start to accumulate when the stomata are closed for a long period. The water insufficiency and low CO_2 concentrations under excess light induce the ROS production in thylakoids where the light-harvesting systems; photosystem I (PSI) and photosystem II (PSII), are located. High ROS levels damage the photosynthesis machinery including the key photosynthesis enzymes such as ribulose-1,5-bisphosphate carboxylase (Rubisco). The lipid peroxidation caused by high levels of ROS becomes destructive, and it starts to cause oxidative stress by the production of lipid radicals and lead to oxidative damaging of proteins and nucleic acids [59–61].

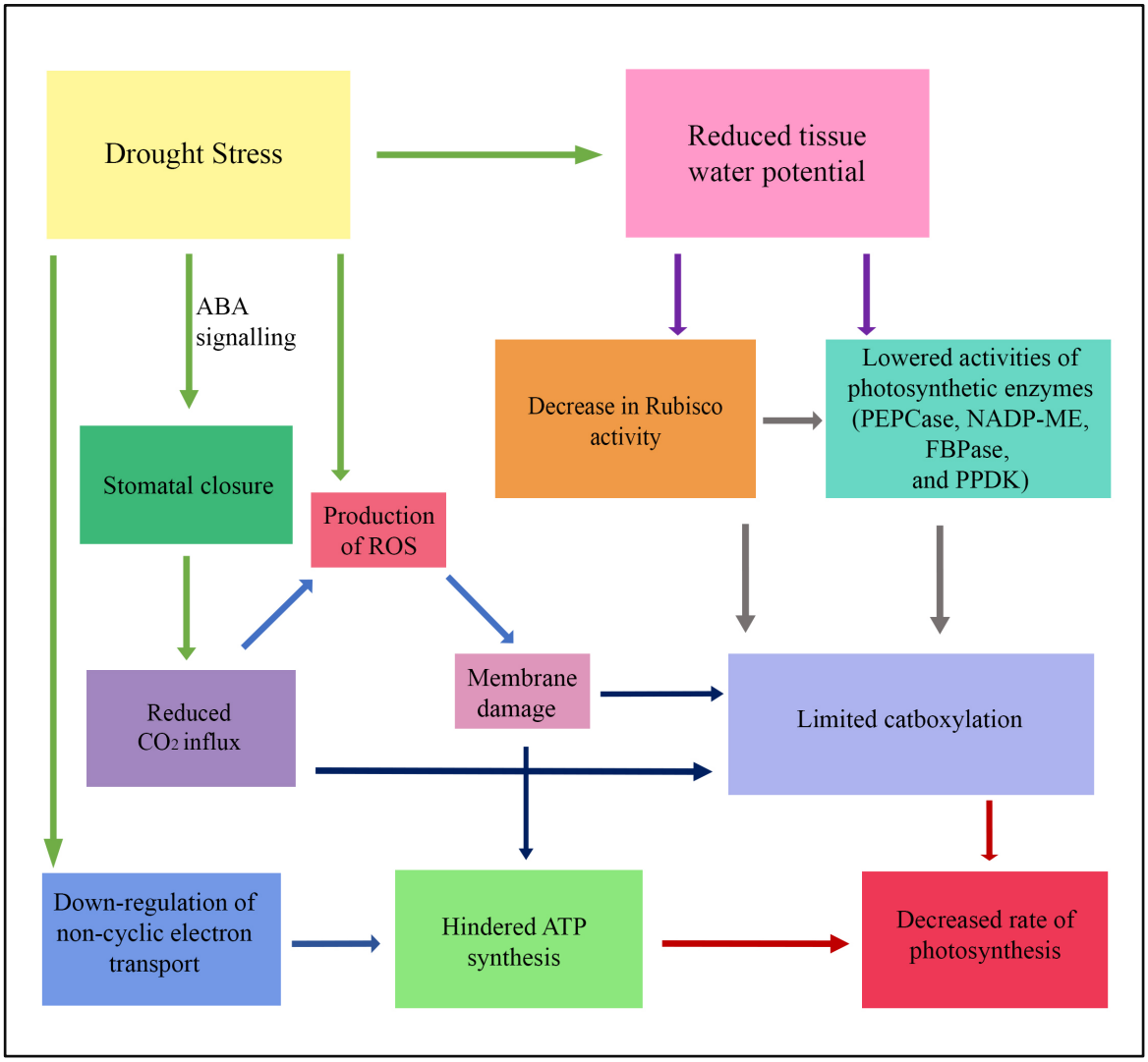

Figure 2.3. Possible mechanisms involved in diminished photosynthesis rate under drought stress [54].

Plants have various strategies for drought resistance to deal with harsh stress conditions. These adaptive attributes of plants can be classified as drought avoidance, escape and tolerance. Through drought escape, plants finish their life cycle before the stress takes place. Therefore, before the detrimental effects of water stress develop, the plant completes its both vegetative and reproductive stages. Plants manage to sustain relatively high tissue water potential under water scarce conditions via stomatal and root system adjustments with drought avoidance. In drought tolerance strategy, plants become capable of maintaining low tissue water potential by sustaining turgor pressure through osmotic adjustment, high elasticity and protoplasmic resistance. [62–64].

Since the ROS in plants causes damage to plant systems, plants produce both enzymatic and non-enzymatic antioxidant components for the detoxification of ROS, as a part of their defence system. Enzymatic antioxidants are ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), peroxidase (POD) and superoxide dismutase (SOD) whereas non-enzymatic antioxidants are ascorbic acid, cysteine and reduced glutathione (GSH), α-tocopherol, phenolics, flavonoids and carotenoids, [42,65].

The osmotic compounds known as compatible solutes and osmoprotectants play a significant role in osmotic adjustments and regulations for drought resistance. The production of amino acids (e.g. aspartic acid, proline and glutamic acid), sugars (e.g. glucose, sucrose, fructose, trehalose and raffinose), the polyols (e.g. sorbitol, pinitol, mannitol, glycerol and inositol) and ammonium compounds (e.g. polyamines and glycine betaine) are induced under water deficit conditions. They play a significant role in the enhancement of plant stress tolerance [66–69].

Under drought, plants change their gene expression patterns to tolerate the stress conditions. While early responses include alterations in transcription factors, signal transduction and translation, late responses include genes associated with the transport of water, osmotic balance, oxidative stress, damage and repair processes [58,70]. The identification, characterization and differential expression profiling of various drought-responsive genes and gene families in *B. distachyon* guide us to understand the molecular mechanism of genes mediated drought stress tolerance. In gene characterization and expression studies of *B. distachyon* abiotic stress responsive gene families; NAC transcription factors (*NAC*s), heat-shock proteins 90 (*Hsp90*s), abscisic acid and ripening-induced proteins (*ASR*s), APETALA2/ethylene-responsive element binding proteins (*AP2/EREBP*s), MYB transcription factors (*MYB*s), WRKY transcription factor family (*WRKY*s), basic leucine zipper transcription factors (*bZIP*s), mitogen activated protein kinases (*MAPK*s), mitogen activated protein kinase kinases (*MAPKK*s), type-2C protein phosphatases (*PP2CA*s) and cystatin genes were found to be induced upon drought [71–79].

Genome-wide transcriptome analysis studies enabled the identification of differentially expressed genes (DEGs) [26,80,81] and the proteomic analyses lead to the discovery of differentially accumulated proteins (DAPs) associated with drought tolerance in *B. distachyon* [82,83]. Metabolic responses associated with drought stress was investigated for the identification of key mechanisms of tolerance [21,84,85]. miRNA screening studies were

conducted for the determination of highly expressed miRNAs under water deficit stress and the ones that are part of drought response and resistance were determined [86–88] and quantitative trait loci (QTLs) related with drought stress tolerance traits were identified in *B. distachyon* [89].

2.4.2. Salinity Stress

Salinity is one of the most challenging environmental factors that hinder the plant growth and productivity severely, especially in arid and semi-arid areas. Accumulation of various soluble salts of Cl^-, CO_3^{-2}, Na^+, SO_4^{-2}, HCO_3^- and Mg^{+2} ions may cause soil salinity [90]. High salt levels in the soil affect approximately 800 million hectares of world's arable lands. Degradation of these lands continues rapidly due to climate change (high temperatures, low precipitation) and wrong irrigation practices (usage of salt-rich or excess amount of water).

The soil falls into the category of saline when its conductivity is equal to 4 dSm^{-1} (which is about 40 mM NaCl with the pressure value of 0.2 MPa) or higher [46]. During their evolution, plants divided into two categories according to their adaptation ability to withstand salt stress as halophytes and glycophytes. Halophytes can grow and complete their life cycle under high salinity conditions. On the other hand, glycophytes cannot survive under high salt stress because unlike halophytes they are not able to tolerate or resist to salinity. Halophytes can tolerate NaCl concentrations above 200 mM through osmotic adjustment and regulation of ions and osmoprotectants. Unfortunately, they constitute only about 1-2 percent of all terrestrial plant species. Crops such as barley and sugar beet are considered highly tolerant whereas wheat, oat, maize and rice are moderately tolerant. Sugarcane, pea and beans are salt-sensitive species [46,91,92].

Salinity stress impairs the metabolic and physiological processes of plants in two ways: 1) Osmotic effect caused by the high salt concentrations in the soil, and 2) Toxic effect through the uptake of high concentrations of salt from the soil, and its accumulation in the tissues of plants (Figure 2.4.). Osmotic stress takes place in the initial phases of salinity stress when the salt concentration is in high levels around the roots and hinders the capacity of plant roots to absorb water. Osmotic effect of salinity stress inhibits the cell division and elongation causing retardation in leaf, stem and shoot growth. Both drought and the initial phase of salinity cause similar effects and reactions within the plant [93]. Deteriorated water relations

and local synthesis of abscisic acid cause stomatal closure. If the stress persists, it leads to decreased rate of photosynthesis, membrane damage and problems in detoxification of ROS [46,94]. Another detrimental effect of salinity is ion toxicity. The excessive amount of Na^+ and Cl^- ions in the soil start to accumulate in tissues of plants to toxic levels. It leads to ion imbalances and deficiency of many important ions required for the plant growth and development. High levels of Na^+ block the uptake of K^+ since it competes with K^+ for the binding sites. High Cl^- levels diminish the water use efficiency of plants and they accumulate in chloroplasts together with Na^+ [94–96]. Salinity impairs the photosynthesis and carbon metabolism of plants through the inhibition of key enzymes. Decreased rate of photosynthesis lead to oxidative stress due to ROS production. High levels of ROS damage proteins, DNA and lipids, hence hinder the cellular functioning of plants.

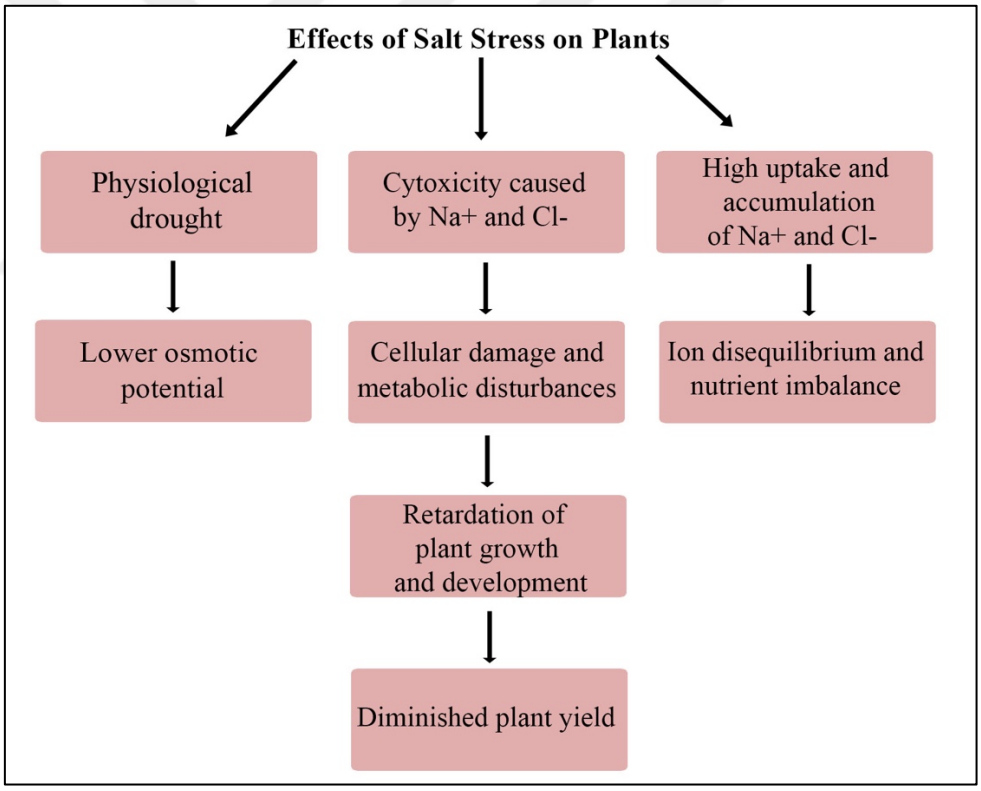

Figure 2.4. Osmotic and ionic effects of salt stress and their consequences [97].

Salt stressed plants activate their tolerance mechanism by 1) removal or the compartmentalization of excess amount of Na^+ into vacuoles to avoid toxicity, 2) accumulation of osmoprotectant molecules for the osmotic adjustment, 3) synthesis of ABA (Abscisic acid) to decrease the water loss and promote the growth under salinity stress and

4) the activation of both enzymatic and non-enzymatic antioxidants for the scavenging of ROS to alleviate both hyperosmotic and hyper ionic stress effects.

The gene expression studies of *B. distachyon* performed to identify genes and gene families responsive to salinity and drought revealed many common genes, considering that they have similar effects and induce similar responses. Members of gene families (e.g. APETALA2/ethylene-responsive element binding proteins (*AP2/EREBPs)*, *Hsp90*, *bZIP*, *MAPK*, *MAPKK*, *NAC* and *MYB*) are found to be both drought and salinity responsive through genome-wide gene expression analyses, generation of transgenic lines and salt tolerance assays [71,72,74,75,77,78]. Overexpression of *BdGF14d* gene (from *14-3-3* gene family) in *Nicotiana tabacum* and *BdPP2AC*s genes (from *PYL* family) in *A. thaliana* exhibited tolerance to salinity [98,99]. Genome-wide differential gene expression via microarray showed up- and down-regulated genes in *B. distachyon* upon various abiotic stress including salinity [80]. By cDNA-AFLP technique, salinity responsive transcripts were identified in *B. distachyon* [93]. For better understanding of salinity stress response and defence mechanisms at both translational and transcriptional levels, many proteins and phosphoproteins were investigated in *B. distachyon*. DEPs (differentially expressed proteins) associated with signal transduction such as *GF14A*, *GF14B* and *14-3-3A*, *ABF2*, *TRAB1* and *SAPK8* involved in ABA signaling were investigated [94].

2.4.3. Wounding Stress

Adverse environmental conditions such as rain, wind, hail, sand, snow, and herbivore and insect attacks cause injuries on plant tissues. Wounding is a continuous threat to plants in nature. It causes nutrient loss and wounded areas facilitate the entry of pathogens and jeopardize the survival of the plant [100]. Early reaction to wounding occurs at the wounding site known as the local response. Later on, the systemic response occurs in the unwounded tissues of the plant via transduction and perception of wounding signals [50].

Local response takes place within few minutes following the wounding event. Since the wounded parts are very susceptible to pathogen attacks, plant regenerates the injured tissues to prevent further damage as a first defence mechanism. Wounding induces ion imbalances, alterations in membrane potential and the production of ROS. As a local response to stress, plants synthesize peptides, oligosaccharides, proteins, oligogalacturonides (OGAs),

phytohormones and phenolic compounds [49,101]. Upon wounding, following the local responses, systemic responses occur in uninjured leaves and organs of the plant. Wound-related signals induce various pathways associated with the metabolism via altering gene expression. Wounding leads to up-regulation of genes that code for vital wounding stress resistance proteins. Mechanical wounding also induces the synthesis of phytohormones such as jasmonate, abscisic acid, and salicylic acid. Accumulation of jasmonic acid in wounded plants induce the expression of many defence genes and regulates wide range of defence mechanisms against stress [49,102]. Hours after wounding, plants up-regulate genes involved in proteinase inhibitors (PIs) and enzymes linked to defence metabolism. The genes encoding PIs are one of the well-characterized genes induced at both wounded and distal sites of the plants. *B. distachyon* proteinase inhibitor gene pin1 (*Bdpin1*) was identified and its coding sequence was characterized through transgenic *Nicotiana tabacum* [103].

2.5. STRESS RESPONSIVE GENE FAMILIES IN PLANTS

The abiotic stress responses of plants are quite complex. They involve biochemical and molecular mechanisms, and up- and down-regulation of various genes. Improvement of crops for abiotic stress tolerance through genetic engineering depends on the elucidation of the functions of these genes and related mechanisms. Plants sense stress by the induction of signaling cascades which activates ion channels, kinase cascades, ROS production and phytohormone synthesis induce defence-related gene expression [104,105]. There are different types of genes that play a role in abiotic stress responses: 1) Genes that code for the production of compounds such as compatible solutes, antioxidants or late embryogenesis abundant proteins (*LEA*s), 2) Genes that play role in signaling cascades, signal transduction pathways kinases or production of transcription factors (*TF*s) and control the transcriptional processes such as mitogen-activated protein kinases (*MAPK*s) or calcium-dependent protein kinases (*CDPK*s), and 3) Genes that are associated with water and ion transport [41,106,107].

For the osmotic adjustment of genes that are involved in osmotic homeostasis are up-regulated under abiotic stress. Accumulation of osmoprotectants such as proline is an important adaptation process under various environmental stress. Proline synthesis induced through the expression of delta 1-pyrroline-5-carboxylate synthases (P5CSs) genes

associated with the enzyme that catalyses the proline biosynthesis under hyperosmotic conditions. The overexpression of genes that are responsible for the synthesis of ROS scavenging enzymes such as GSH and SOD elevates the adverse effects of stress [95,108]. Calcium and ROS work as second messengers in the initial response to abiotic stress conditions. Upon abiotic stress such as drought, salinity and wounding, Ca^{2+} levels rise in the stressed cells of plants. High concentrations of Ca^{2+} within the intracellular space lead to activation of several molecular pathways via calcium interacting proteins such as calmodulin and calcineurin B-like proteins (*CBL*s) and Ca^{2+}-dependent protein kinases (*CDPK*s) [109,110]. The activity of specific proteins that control the expression of abiotic stress-related genes is modulated by the crosstalk between calcium and ROS. *CDPK*s and *MAPK*s cascades are activated in response to abiotic stress and they are involved in signaling important pathways generating defence responses. The transcription factors such as *DREB1A* (Dehydration Response Element Binding) and *EREBP/AP2* were also shown to induce expression of stress tolerance genes [110].

In the majority of gene function studies, *Arabidopsis* was used, so most of the genes reported as stress-responsive are specific to dicots. Unfortunately, the signaling pathways and key genes in the adaptation of stress are poorly understood for monocotyledonous plants that include cereal crops. Even though the fine tuning of molecular responses through gene expressions are staying largely unexplored in *B. distachyon*, the available genome sequence of Bd21 enable the study of several gene families that are involved in stress response of *B. distachyon* such as *AP2/EREBP*, *Hsp90*, *bZIP*, *MAPK*, *MAPKK*, *NAC*, *MYB*, *WRKY*, *14-3-3* and *ASR*.

The *AP2/EREBP* is a superfamily of transcription factors. They play a significant role in growth, development and response to various stress such as drought, salinity, infection, high and low temperatures. Also, they have been reported to be functioning in hormone-related pathways such as ABA, JAs, ethylene and CTK. In the gene expression study of *B. distachyon*, *AP2/EREBP*s were investigated using NGS technologies and microarray and found to be induced in a wide variety of abiotic stress [111].

Hsp90 gene family participates in various abiotic stress-related biological processes and signal transductions such as ABA and endoplasmic reticulum signaling pathways under salinity and drought stress. In a recent study, *Hsp90* gene expression either delayed or

increased upon stress in *B. distachyon*, suggesting that they have important contributions to ABA signaling and processes to protect the plant against stress [72].

*bZIP*s showed significant expression patterns in *B. distachyon* under drought, cold and salinity. Also, the transcription profiles of hormone-treated plants showed that *BdbZIP*s might be regulated by different phytohormones [77].

MAPK cascades that are composed of MAPK kinase kinase (*MAPKKK*s/*MEKK*s), MAPK kinase (*MAPKK*s/*MKK*s) and MAPK protein kinase classes play parts in many developmental and survival processes of plants. MAPK cascade is one of the main signaling pathways involved in abiotic stress. MAP kinases are required in the signal transductions and antioxidant defence responses upon stress. The genomic and bioinformatic analysis of these two gene families and proteins in *B. distachyon* suggested that *MAPK* cascades involved in crucial signaling pathways were required for the survival of the plants under various stress conditions such as cold, salinity, drought and under ABA, H_2O_2 and methyl jasmonate (MeJA) treatments [78].

In another study, *NAC* genes in *B. distachyon* were identified through the expression profiles and phylogenetic analysis. The *in-silico* promoter analysis revealed that NAC genes had stress-related cis-elements in their promoter regions and the transcription levels of *BdNAC*s were investigated upon drought, salinity and phytohormone treatment. The results suggested that *NAC*s in *B. distachyon* might be important in the regulation of complex networks to deal with the unfavorable conditions [71].

MYB transcription family involved in both biotic and abiotic stress responses was studied in different plant species. The studies showed that they contributed to plant development and differentiation, and played a role in the metabolism and defence responses. MYB transcription factors were also identified in *B. distachyon*. The expression profiles of plants treated with hormones and various abiotic stress including drought and salt indicated that most of the *BdMYB* genes might be associated with flower development and abiotic stress responses [75].

WRKY transcription factors are significant regulators of gene expression for plant growth, development and survival under adverse conditions. WRKY proteins can trigger or suppress the transcription of the genes via direct binding to sequences on the promoter. In an expressional and functional study of WRKY genes in *B. distachyon*, *BdWRKY36* genes were

found to be up-regulated upon drought, heat and cold stress. *BdWRKY36* gene was cloned into tobacco plants and its overexpression elevated the drought tolerance [76].

B. distachyon 14-3-3 genes were identified and different expression profiles were observed under environmental stress conditions. The various interaction patterns between *Bd14-3-3s* and transcription factors associated with osmotic stress responses suggested that this gene family had an active role in stress alleviation. Different isoforms of *Bd14-3-3* genes play roles in various abiotic stress responses. *BdGF14e*, *BdGF14f* and *BdGF14g* genes under drought and *BdGF14d* gene under salinity, were up-regulated. Salt stress-induced *BdGF14d* gene was selected and overexpressed in tobacco plants and the results showed that the *BdGF14d* overexpressing plants eliminated the unfavorable conditions with higher ROS scavenging enzyme activity, photosynthesis, water use efficiency and rate of transpiration [98].

In plants, the *PP2C*s regulate ABA-dependent pathways negatively while it positively regulates MAPK cascade pathways, hence plays major roles in stress signal transduction pathways. A recent study confirmed that almost all members of the *PP2C* family in *B. distachyon* exhibited upregulation in response to abiotic stress such as cold, drought, heat and salt. Through genome-wide analysis, gene structure, location and expression patterns were revealed for *PP2C* gene family [112].

The *ASR*s are a family of plant-specific proteins having substantial roles in responses to abiotic stress. *BdASR* genes have been identified via genome-wide analyses in *B. distachyon.* Gene expression studies showed that they were responsive to cold, drought and salt stress. One of the *ASR* genes in *B. distachyon*, *BdASR1* was characterized and overexpressed in tobacco plants. It was proposed that *BdASR1* work as a transcription factor providing drought resistance through the induction of ROS scavenging enzymes and antioxidant compounds [73].

Cystatins are proteins involved in cysteine protease inhibition, growth, development and abiotic stress defence in plants. Recent scientific work performed a thorough analysis of the gene expression and characterization to explain the involvement of cystatin genes in abiotic stress responses. *B. distachyon* cystatin genes (*BdC*s) were up-regulated by cold, salt and hormone application through the regulation of cysteine protease activity. Their involvement

in protein degradation and programmed cell death signaling pathways was triggered by ROS [79].

2.6. PLANT HORMONES IN DEFENCE RESPONSES

Plant hormones (phytohormones) are endogenous molecules that regulate plant growth, development and stress tolerance against a wide range of stress factors. Plants synthesize various hormones including abscisic acid (ABA), auxins, jasmonates (JAs), gibberellins (GAs), cytokinins (CKs), ethylene (ET), salicylic acid (SA), strigolactones and brassinosteroids (BRs). The stress response mechanisms of plants are quite elaborate and require the collaboration of molecular pathways for the activation of responses upon stress. Phytohormones and their cross-talk control many physiological processes, molecular mechanisms and mediate stress responses through their participation in signaling pathways and defence mechanisms [113–115].

ABA is an isoprenoid phytohormone with a weak acid characteristic, synthesized through the plastidial 2-C methyl-D-erythritol-4-phosphate pathway. ABA participates to various processes such as the development of embryos, germination, dormancy and maturation of seeds. ABA also stimulate guard cells for the stomatal closure, and play a role in protein storage and lipid synthesis [116]. ABA levels rapidly increase upon stress and activate associated signaling pathways by changing gene expression levels [117]. ABA plays an essential role in drought and salinity stress response and tolerance. Adverse environmental conditions, especially osmotic stress, boost the ABA biosynthesis. ABA concentrations also show an increase following the wounding stress, primarily at the wound site. It might be related to the dehydration of the plant around the wounded area. However, it is unknown at which step of the mechanical wounding stress defence, the ABA synthesis is activated [118]. When the water is scarce around the roots, ABA enables the signal transductions to minimize the water usage, prevent the water loss and maintain water balance via stomatal closure and reduction in leaf expansion [113,119]. ABA up-regulates the expression of various stress-responsive genes and synthesis of proteins involved in plant protection against the harmful effects of stress such as dehydrins and LEA proteins, osmoprotectants and antioxidant compounds. ABA dramatically increases upon drought, salinity and wounding stress to provide tolerance in various plant species, thereof termed as a stress hormone [116,120].

Auxin hormone plays a pivotal role in the growth and development of the plant. The well-known naturally occurring type of auxin is the indole-3-acetic acid (IAA). IAA is synthesized via tryptophan (Trp)-dependent and Trp-independent biosynthetic pathways. It is involved in various processes such as seed germination, dormancy, cell elongation, organ generation, development of vascular tissues and apical dominance. Auxin modulates plant growth under stress conditions. Synthesized auxins are distributed throughout the plant via a complex cell-to-cell auxin transport system. IAA has been shown to play an essential part in salinity stress adaptation. The growth of roots and shoots of salinity stressed plants increase by the changing auxin accumulation and distribution [121]. The accumulation of IAA in the wound sites activates the synthesis of enzymes that are important in plant defence. By taking part in the transcription and regulation of many genes, known as primary auxin response genes, auxin work as a substantial component in the defence response of plants [122].

Salicylic acid (SA) is a phenolic compound that functions in plant development and growth. Also, they induce the generation of biotic and abiotic stress responses. There are two suggested pathways for the salicylic acid biosynthesis. One is the phenylalanine ammonia lyase pathway and the other is the isochorismate pathway. SA modulates numerous vital physiological processes such as carbon assimilation, nitrogen and proline metabolism, glycine betaine production, antioxidant synthesis and regulation of water balances under adverse conditions. Studies showed that SA improved plant tolerance under salinity and drought through the regulation of the gene expression of defence-related genes [123,124]. SA synthesis pathway is activated upon wounding and initiate defence responses at distal parts to protect unwounded parts of the plant. SA-inducible genes have shown to be induced upon drought to tolerate water deficiency and improve germination under salinity [125]. However, there are controversies regarding the role of SA in salinity stress alleviation. The mechanisms that play a role in abiotic stress defence are not well understood yet. Therefore, further studies are required to elucidate the roles of SA in abiotic stress response and defence pathways [126].

Jasmonates (JAs) are family of oxylipins derived from fatty acids synthesized through the octadecanoid pathway. Mainly, the volatile methyl ester methyl MeJA and free acid from jasmonic acid (JA) are called as jasmonates (JAs). They are vital compounds in the development, reproduction and defence processes of the plant. JAs are involved in the

development of flower and fruit, senescence and production of secondary metabolites as well as direct and indirect defence responses. They mitigate the survival threatening effects of salinity and wounding stress. The endogenous application of JA alleviates the detrimental effects of salinity and drought stress. Plant defence mechanisms induced by wounding are mostly regulated by JAs at both local and systemic tissues. Following wounding, rapid systemic increase in JA levels leads to early transcriptional responses. The perception of JA by its receptor initiates the plant development and defence responses through JAZ (jasmonate ZIM-domain) transcription factors that work as co-receptors and transcriptional repressors at the same time. Many wound-induced adaptive responses are induced by the synthesis of JA. JA modulates systemic wound responses via both cell autonomous and nonautonomous pathways as reported in recent studies. The presence of two different JA dependent and JA independent wound signaling pathways shows that the perception, response and defence mechanisms are highly sophisticated. Thereof, there are still gaps to fill regarding the local and systemic influence of JA in wounded plants for the elucidation of the mechanisms behind the stress responses. There are many wounding studies in dicots such as *Arabidopsis* and tomato plants that provide crucial insights into the significance of JA in plant defence and they are somewhat milestones of wounding research [127–130]. On the other hand, studies on the role of JA in monocot species are not sufficient enough, even though it is quite essential to understand the signaling and synthesis pathways of economically important crops.

Even though for decades, the function of each phytohormone has investigated independently, phytohormones interact with each other in a synergistic or antagonistic way [131]. Each phytohormone interacts with at least one or more hormone and this well-coordinated association orchestrate the biosynthetic pathways and help plants to adapt biotic and abiotic stress [125]. For instance, several genes associated with auxin biosynthesis regulated by ethylene hormone and auxin itself regulates the expression of genes responsible for gibberellin biosynthesis [132,133]. The developmental processes such as root growth are promoted by auxin while cytokinin counteracts this effect. At rapid primary response to drought and salinity stress, stomatal closure occurs via complex signaling pathways regulated by hormonal crosstalk. ABA regulates the expression of genes that induce stomatal closure and interacts with JA for the stimulation of stomatal oscillations [134]. Moreover, many other gene regulations occur under water deficient conditions to minimize and

ameliorate the negative effects of stress such as the genes involved in the interaction of ABA, ethylene, cytokinin, auxin and JA. JA and SA are known to work antagonistically. However, there are also studies that reported the synergistic interactions between them. Together they play a role in the expression of defence-related genes against pathogens [135–137]. Consequently, the defence responses activated against stress depend on the type of crosstalk. In this crosstalk, hormones may antagonistically affect each other in response to stress or they may collaborate by working synergistically to induce defence responses.

2.7. HORMONE PROFILING

Determination of hormone levels in plants is crucial for better understanding of molecular mechanisms and interactions of phytohormones. Since plant hormones may act together synergistically and antagonistically, the ideal way to analyse them is by hormone profiling. Hormone profiling enables to determine the types of hormones, their concentrations, fluctuations and distributions in the tissue at a specific time. However, hormone profiling is quite challenging since phytohormones are found in plants in minute amounts usually around 0.1-50 ng/g fresh weight. Plant extracts contain a wide variety of components that are much higher concentrations and may interfere with the hormone analysis. Hence, the determination of hormones in these complex plant extracts requires effective sample preparation, extraction and purification steps before the final quantification [138,139].

Bioassays, immunoassays, electroanalysis and chromatography techniques have been used for the detection of hormones so far. The bioassay method is one of the oldest methods used in hormone quantification. It is not preferred today due to its limitation in hormone detection. It can detect one hormone at a time and does not let the identification of multiple components. It is also time-consuming and has low accuracy. Immunoassays such as radioimmunoassay and enzyme-linked immunosorbent assays (ELISA) are also used in phytohormone determination studies. However, there are not many available immunoassay kits and the available ones only allow the determination of a specific class of hormones per sample. They are also time and labour-intensive [139–142]. With the advances in analytical techniques, more precise and sensitive qualitative and quantitative analysis is available. Chromatographic methods such as liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE) coupled with many different detectors have been used in

hormone profiling (Table 2.2.). Especially LC coupled with various detectors such as UV-visible, fluorescence detection (FLD) and mass spectrometry (MS) is widely used for hormone analysis since it provides efficient separation and resolution. High-throughput, comprehensive and sensitive techniques are crucial for understanding the role of hormone-regulated signaling networks and their relationship with gene expressions under stress conditions. The primary studies on hormone analysis involve the detection of one or two hormones at a time [143,144]. However, the latter studies quantify many hormones simultaneously in the same plant tissue since the significance of understanding the complex cross-talk and interactions between different hormone signaling pathway is vital. Many phytohormones have acidic characteristics so that the chromatographic detection methods have been reported on the analysis of these class of hormones [145–147]. LC coupled with UV detector was used for the simultaneous determination of phytohormones such as IAA, Indole-3-butyric acid (IBA), ABA, GA, SA, JA and CKs [148–151]. LC coupled with FLD detectors was used as well in several studies on detection of plant hormone such as auxins, ABA, GA, IBA and SA [152–154]. However, the mass spectrometry systems are mostly preferred over other detectors since they provide separation of hormones with high sensitivity and specificity. Many validated protocols using LC-MS systems were reported for hormone profiling in various plant species such as *Arabidopsis*, tomato, banana, lettuce, rosemary, maize and pine [133,142,155–159]. Even though the GC-MS approach is also suitable for the simultaneous analysis of plant hormones, challenging preparation steps in separation, purification and derivatization make it a less preferred system.

Table 2.2. Analysis of plant hormones using HPLC systems.

Plant Species	**Measured Hormones**	**Analysis Instrument**	**Reference**
Lactuca sativa L.	ABA, GAs, CKs, Auxins	HPLC-ESI-MS/MS	[160]
Citrus clementina, *Hordeum vulgare* L., *Carica papaya* L.	ABA, IAA, JA	HPLC–ESI–MS/MS	[147]
Arabidopsis thaliana	ABA, SA, JA	LC/MS/MS	[157]
Arabidopsis thaliana	JAs, SA, Auxins, CKs, ABA, methyl esters, GAs	LC–ESI–MS/MS	[161]
Oryza sativa	ABA, Auxins, CKs, GAs	UPLC-ESI-qMS/MS	[162]
Nicotiana tabacum	JA, SA and related compounds	UPLC–ESI–MS/MS	[163]
Chlorella vulgaris, Duranta repens	IAA, IPA, IBA, NAA	HPLC-FLD	[153]
Nicotiana glauca	Auxins, CKs, ABA	LC/MS/MS	[164]
Arabidopsis thaliana	Auxins, JAs, GAs, CKs, ABA	HPLC–ESI–MS/MS	[142]
Rosmarinus officinalis	IAA, ABA, CTK, GAs, SA, JA	UPLC-ESI-MS/MS	[133]
Natural coconut juice	SA, IAA, ABA, JA	HPLC-UV	[148]
U. fasciata, U. lactuca, U. taeniata, U. linza	Auxins, ABA, IAA, SA	HPLC-UV, HPLC-ESI-MS/MS	[154]
Triticum aestivum L., *Nicotiana tabacum* L., *Arabidopsis thaliana*	IAA, ABA	2D-HPLC	[152]
Solanum lycopersicon	ABA, JAs, SA	UPLC-MS/MS	[165]
Oryza sativa	ABA, IAA, GAs, CKs	UFLC-ESI-MS	[166]
Arabidopsis thaliana	ZA, ABA, JA, SA, BR	UFLC-MS/MS	[158]

2.8. AIM OF THE STUDY

The objectives of this study were to:

i) Identify candidate multiple stress-responsive genes from different gene families (e.g. *ERF*, *EXP*, *SUT*, *P5CS*, *14-3-3* and *LOX*) by orthology analysis and in accordance with literature,
ii) Determine the changes in expression levels of selected 8 multiple stress-responsive genes (*WRKY36*, *GF14d*, *EXPA2*, *P5CS1*, *PP2CA6, ERF1, LOX3* and *SUT2*) by qRT-PCR,
iii) Quantify the changes in phytohormones (IAA, ABA, SA) using HPLC analysis,

under salinity, drought and time-dependent (0^{th}, 6^{th} and 24^{th} hour) mechanical wounding stress including local and systemic events in monocot model plant *B. distachyon* (Bd21 line).

Characterization and overexpression studies of determined genes can pave the way towards generating multiple stress-tolerant crops. Besides, elucidation of multiple-stress responsive genes and associated signaling molecules will provide a better understanding of the stress response mechanisms in monocot species.

3. MATERIALS

3.1. CHEMICALS

Ethanol, absolute (Sigma- Aldrich, USA, cat. no. 34923), 2-Propanol for HPLC, 99.9 percent (Sigma- Aldrich, USA, cat. no.), Dichloromethane (Sigma- Aldrich, cat. no. 270997), Acetic Acid (Sigma- Aldrich, USA, cat. no.), Abscisic Acid (S-ABA) (Duchefa Biochemie, Netherlands, cat. no. A0941), Indole-3-Acetic Acid (Duchefa Biochemie, Netherlands, cat. no. I0901), Jasmonic Acid (Duchefa Biochemie, Netherlands, cat. no. J0936), Methyl Jasmonate (Duchefa Biochemie, Netherlands, cat. no. M0918), Salicylic Acid (Duchefa Biochemie, Netherlands, cat. no. S1367).

3.2. CONSUMABLES

Hard-Shell® 96-well plates (Bio-Rad, USA, cat. no. HSS9601), Microseal® B PCR plate sealing film (Bio-Rad, USA cat. no. MSB1001), Minisart® RC15 Syringe Filter 0.2 µm (Sartorius, Göttingen, Germany, cat. no. 17761-R), 250 µL deactivated glass vial insert with polymer feet (Agilent Technologies California, USA, cat no. 5181-8872), 2 mL screw top autosampler vials, vial caps.

3.3. MOLECULAR BIOLOGY REAGENTS

Water for molecular biology (Sigma- Aldrich, Missouri, USA, cat. no. 95284), DNA Gel Loading Dye (6X) (Thermo Scientific, USA, cat. no. R0611), GeneRuler 1kb DNA ladder (Thermo Scientific, Massachusetts, USA, cat. no. SM0313), 50 bp DNA Ladder (GeneON GmbH, Ludwigshafen, Germany), Primers (Sentromer DNA Teknolojileri, İstanbul, Turkey), dNTP mix (10 mM) (Thermo Scientific, Massachusetts, USA, cat. no. R0192), $MgCl_2$ (25 mM) (Thermo Scientific, Massachusetts, USA, cat. no. R0971), Taq DNA Polymerase PCR Buffer (10X) (Thermo Scientific, Massachusetts, USA, cat. no. 18067017), Taq DNA Polymerase, recombinant (5 U/µL) (Thermo Scientific, Massachusetts, USA, cat. no. EP0402), Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, USA), Prona Agarose

Biomax (Abo, Gdańsk, Poland, cat. no. BS100), Sodium Chloride (Sigma- Aldrich, USA, cat. no. 31434), Ethidium Bromide solution (1percent) (Fisher Scientific, New Hampshire, USA, cat. no. BP1302-10), RedSafe™ Nucleic acid staining solution (20,000x) (iNtRON Biotechnology, Inc., Seongnam, Korea, cat. no. 21141).

3.4. MOLECULAR BIOLOGY KITS

innuPrep RNA Mini Kit (Analytik Jena AG, Jena, Germany, cat. no. 845-KS-2040050), RevertAid First Strand DNA Synthesis Kit (Thermo Scientific, Massachusetts, USA, cat no. K1622), iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA, cat. no. 1755121).

3.5. INSTRUMENTS

-86°C Freezer (Thermo Scientific, Asheville, USA, model number: 88500V), Microwave (Arçelik, İstanbul, Turkey, model number: MD809), Autoclave (Wisd MaXterile™ 60R Daihan Scientific, Seoul, Korea), Ice Flaker (Scotsman AF 80, Ipswich, UK), 4 in 1 soil survey instrument (Microtemp Electrics, Taiwan, model number: AMT-300), Electronic balance (Schimadzu, Kyoto, Korea, model number: TW423L), Vortex Mixer (Velp Scientifica, Milano, Italy), multiSUB Horizontal Electrophoresis System (Cleaver Scientific, Warwickshire, UK), Electrophoresis Power Supply (Cleaver Scientific, Warwickshire, UK, model number: CS-300V), T100™ Thermal Cycler (Bio-Rad, California, USA), NanoDrop 2000 Spectrophotometer (Thermo Scientific, California, USA), PCR cabinet (N-Biotek, Seoul, Korea, model number: NB-603WS), C1000 Touch™ Thermal cycler with CFX96™ Real-Time System (Bio-Rad, California, USA), Milli-Q Water Purification System (Merck Millipore, Massachusetts, USA), Eppendorf Centrifuge 5424 (Hamburg, Germany), UV Transilluminator (Vilber Lourmat, model number: FLX-20M), ChemiDoc XRS+ Imaging System (Bio-Rad, California, USA), Certomat IS Benchtop Shaking Incubator (Sartorius, Göttingen, Germany), Techne Sample Concentrator (Cole-Parmer, Staffordshire, UK), UHPLC-UV system (Shimadzu Nexera XR series consisting of degassing unit (DGU-20A5R), pump, autosampler (SIL-20A XR), column oven (CTO-10AS VP), communications unit (CBM 20A) and UV-Vis detector (SPD-20A/20AV) (Shimadzu,

Kyoto, Japan), ZORBAX Eclipse XDB 80Å C18, 4.6 x 250 mm, 5 µm HPLC column (Agilent Technologies, California, USA, part number: 990967-902).

3.6. PLANT MATERIAL

Brachypodium distachyon seeds (Bd21 line) were provided from the DOE Joint Genome Institute, Walnut Creek, California.

4. METHODS

4.1. GROWTH CONDITIONS OF *BRACHYPODIUM DISTACHYON*

B. distachyon (Bd21 line) seeds were placed on wetted filter papers inside Petri dishes and sealed with parafilm. They were first kept at 4 ºC in the dark for 7 days and then kept at room temperature under the light for 5 days. Germinated seeds were transferred to plug trays filled with peat and soil mixture. Plants were grown in plug trays until the third leaf stage and transferred to plastic pots. Plants were grown inside the greenhouse under controlled conditions (16/8 hours light/ dark photoperiod at 22/25 °C with relative humidity 60-70 percent and a photosynthetic flux of 320 µmol m^{-2} s^{-1} at canopy height provided by the fluorescent lamps in the greenhouse). Plants were treated with 200 mg kg^{-1} N ($Ca(NO_3)_2$), 100 mg kg^{-1} P (KH_2PO_4), 20 mg kg^{-1} S (K_2SO_4), 5 mg kg^{-1} Fe (Fe-EDTA) and 2.5 mg kg^{-1} Zn ($ZnSO_4$) for every 20 days to provide basal fertilization.

4.2. STRESS TREATMENTS: SALINITY, DROUGHT AND MECHANICAL WOUNDING

The experimental design contained 48 pots and each pot was containing 5 individual *B. distachyon* plants (Table 4.1.). When the plants were at their vegetative stage, they were divided into 8 sets each comprising 6 pots. Five sets of plants were stress-treated; the remaining 3 sets were used as control groups by irrigating them with distilled water every day such that each stress treatment had its own control group. One set of plants was subjected to salinity stress (S) by irrigating them once a day with 50 mL of 320 mM NaCl solution for 14 days [167]. Another set of plants was subjected to drought stress by water withheld. Before starting the drought treatment, soil moisture was measured every day using a soil survey instrument. When soil moisture was found to be at normal levels (50-60 percent moisture), the plants were subjected to 12 days of drought stress (D). The control groups were irrigated with distilled water daily (70-80 percent moisture) (Salinity Control: SC, Drought Control: DC, and Wounding Control: WC). Another three sets of plants were subjected to mechanical wounding stress through squeezing each leaf with forceps across the leaf surface with 1 cm intervals. Sample collection was performed at three different time

intervals: 0 h, 6 h and 24 h. (The leaves that were harvested right after wounding (W0), 6 hours after wounding (W6) and finally 24 hours after wounding (W24)). At each harvesting occasion, a different set of plants were used, so each plant was wounded only once, both wounded and unwounded leaves were harvested as the wounded leaves representing local events, and their adjacent unwounded leaves representing the systemic events (WA0, WA6 and WA24).

At each harvesting event of both stress-treated and control plants, immediately after detached, samples were transferred to liquid nitrogen and stored at -86°C in the freezer until the analysis.

Table 4.1. Experimental set-up for stress treatments.

Stress treatments										
SC	**S**	**DC**	**D**	**WC**	**W0**	**W6**	**W24**	**WA0**	**WA6**	**WA24**
6 pots/treatment x 5 plants/pot = 240 plants										
5 plants in each pot were pooled during sample collection										

4.3. DETERMINATION OF STRESS-RESPONSIVE GENES IN *BRACHYPODIUM DISTACHYON* USING BIOINFORMATICS TOOLS

Drought-, salinity- and wounding stress-related 220 gene sequences of *Brachypodium distachyon*, *Triticum aestivum*, *Oryza sativa* and *Arabidopsis thaliana* were downloaded from National Centre for Biotechnology Information (NCBI) and The *Arabidopsis* Information Resource (TAIR) gene databases. Genes from *Triticum aestivum*, *Oryza sativa* and *Arabidopsis thaliana* were aligned with the whole-genome sequence of *Brachypodium distachyon* for orthology analysis using the NCBI Nucleotide BLAST tool. According to alignment scores (query cover, e value, identity percentage), the possibility of responsiveness to multiple stress conditions and availability of transgenic studies of the gene of interest, the candidate stress-responsive genes were selected by eliminating the ones that do not fit the criteria.

4.4. PRIMER DESIGN

Primers for the *ERF1*, *EXPA2*, *SUT2*, *P5CS1*, *GF14d* and *LOX3* genes were designed using NCBI's primer designing tool Primer-BLAST by paying attention to required parameters to obtain optimal primers such as having C or G residue at the 3' end, possessing 40-60 percent GC content and no self-complementarity. The amplified genes and sequences of designed gene-specific primers used in quantitative real-time PCR are given in Table 4.2. As an internal reference gene, Ubiquitin18 (*UBC18*) which encodes an ubiquitin-conjugating enzyme and stably expressed in *B. distachyon* was used [82,168]. The primer sequences for *PP2CA6* and *WRKY36* were obtained from previous research studies [76,99].

Table 4.2. Genes names and gene-specific primers.

Gene names	**Primer sequence (5'3')**	**Product size (bp)**
UBC18	F: GGAGGCACCTCAGGTCATTT	193
	R: ATAGCGGTCATTGTCTTGCG	
ERF1	F: CAAGCACAAACACATCACACG	112
	R: GAGAGCGGGACTACCCAC	
EXPA2	F: TGGGAGGATGCTTGAGCTTG	80
	R: AGAACTGTCCCTTGCCTTCG	
GF14d	F: TGGAAGTATTGACCTTCGCTG	191
	R: TGGAAGTATTGACCTTCGCTG	
LOX3	F: GGAGTCGAGTGAAGGGATGC	162
	R: TCCAGCTTGCTTACTGGTGG	
P5CS1	F: GCATCATCATCAAGGTGGGC	92
	R: CTTCACCTGCTCGCATAGGG	
PP2CA6	F: GCCAGACAGACCTGATGAGATG	103
	R: TGACCTAGAAGTCGCAAGCAC	
SUT2	F: TGCAGCTGTAACTTTAATCGGG	178
	R: TAAAAGGGCACGAGCAGGTC	
WRKY36	F: CGAGCTGGAGTCGGATCCCAT	196
	R: ACCTCCTTTCCCTTGCTCAAATGTG	
F: Forward, R: Reverse		

4.5. RNA ISOLATION

RNA was isolated from leaf tissues using innuPrep RNA Mini Kit 2.0 according to the instructions provided by the manufacturer. 40 mg of frozen leaf sample was ground into powder in liquid nitrogen using mortar and pestle. Powdered tissue was transferred to 1.5 mL Eppendorf tube. 450 µL Lysis Solution RL was added to the tube and shaken. The sample was centrifuged at 21,000 x g for a minute. Spin Filter D was placed into a receiver tube and supernatant was transferred onto a Spin Filter D. Centrifugation was done at 11,000 x g for 2 minutes. Spin filter R was placed into a new receiver tube. The filtrate was mixed with an equal volume of 70 percent ethanol. The sample was transferred to Spin Filter R and centrifuged at 11,000 x g for 2 minutes. Receiver tube was discarded and Spin Filter R was placed into a new Receiver tube. 500 µL Washing Solution HS was added and centrifuged at 11,000 x g for a minute. Receiver tube was discarded and Spin Filter R was placed into a new Receiver tube. 700 µL 80 percent ethanol was added onto Spin Filter R and centrifuged 11,000 x g for a minute. Centrifugation was done at 11,000 x g for 2 min to remove the traces of ethanol. Receiver tube was discarded and Spin Filter R was placed into an elution tube. 50 µL RNase-free water was added, incubated at room temperature for a minute and centrifuged again at 11,000 x g for a minute.

For the determination of concentration and purity of isolated RNA, absorbance measurements of the samples were done using Nanodrop 2000 spectrophotometer. The concentrations were determined as 200-400 ng/µL. The ratio of absorbance at 260 nm and 280 nm (260/280), and 260 nm and 230 nm (260/230) were checked. Both 260/280 and 260/230 were around 2.0. Therefore, they were accepted as pure RNA. Furthermore, each RNA sample was run on 1.5 percent agarose gel in 1X TAE at 110 V to see if the RNA is intact and free from DNA contamination.

4.6. cDNA SYNTHESIS

1 µL of RNA, 1 µL of Oligo (dT) 18 primer and 10 µL nuclease-free water were added into a tube on ice, mixed by pipetting up and down gently, incubated at 65°C for 5 min and chilled on ice for 1 minute. 4 µL of 5X reaction buffer, 1 µL of RiboLock RNase inhibitor (20 U/µL), 2 µL of dNTP mix (10 mM) and 1 µL of RevertAid M-MuLV RT (200 U/µL) were

added into the tube. The reaction mix with 20 µL of total volume was mixed and briefly centrifuged, and then incubated for 60 min at 42°C. The reaction was terminated by incubating at 70°C for 5 min.

4.7. PCR AMPLIFICATION

Conventional gradient PCR was performed before the gene expression analysis to optimize the PCR conditions for the designed primers. PCR reaction was prepared by mixing 2.5 µL of 10X Taq DNA Polymerase PCR Buffer, 0.75 µL of 25 mM $MgCl_2$, 0.5 µL of 10mM dNTP mix, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 0.25 µL of 5 U/µL DNA Taq Polymerase, 100 ng of cDNA and adjusted to a final volume of 25 µL with dH_2O. Thermocycling conditions for the PCR were as follows: initial denaturation at 95°C for 3 min, 34 cycles of 95°C for 30 sec, 53-59°C for 1 min and 72°C for 30 sec, and a final extension at 72°C for 5 min. PCR products were visualized with 1.5 percent agarose gel electrophoresis.

4.8. AGAROSE GEL ELECTROPHORESIS

For agarose gel, 50X TAE buffer was prepared by adding 242 g Tris base and 18.61 g disodium EDTA into 700 mL ddH_2O and stirred until they dissolved. Then, 57.1 ml of glacial acetic acid was added and the volume was completed to 1 L with ddH_2O. 50X TAE was diluted to 1X concentration for use. 1.5 grams of agarose was mixed with 100 mL of 1X TAE in an Erlenmeyer flask and put into a microwave for 2 minutes until boiling point was reached. Then, 1 µL of EtBr was added and poured into a gel tray with a well comb. The tray was set in room temperature for 20 min. The PCR products were mixed with loading dye before loaded to the wells and the gel was ran at 110 V for 40 min. in 1X TAE buffer. Visualization was performed using ChemiDoc imaging system by observing the specific bands for the genes of interest.

4.9. QUANTITATIVE REAL-TIME PCR (qRT-PCR) ANALYSIS

After observation of desired band with the designed primers, qRT-PCR was performed by using the following protocol: 12.5 µL of iTaq SYBR Green Supermix (2x) was mixed with 0.3 µM of forward and reverse primers, nuclease-free water and 100 ng cDNA. qRT-PCR amplifications were done using C1000 Touch™ Thermal cycler with CFX96™ Real-Time System. The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 20 sec, annealing at 54-65°C for 30 sec and extension at 72°C for 30 sec, 65-95°C with 0.5°C increment 5 sec per cycle. Reactions were carried out with both biological and technical replicates. Salinity and drought samples had 3 biological and 3 technical replicates whereas wounding samples had 2 biological and 3 technical replicates. Therefore, Ct values were the mean of 9 values for salinity and drought and 6 for wounding. For the calculation of relative gene expression values, the ΔΔCt method was used. Transcription levels were normalized using the reference gene *UBC18* as an internal control and analysed by Bio-Rad CFX Manager software package.

4.10. PHYTOHORMONE EXTRACTION

Phytohormone extraction was performed via modified protocol [142] 750 mg of leaf tissues from each control and stress treated (salt, drought and mechanical wounding) plants were ground into powder using mortar and pestle. Then, the samples were divided into 15 subsamples each having 50 mg of ground tissue and transferred to 2 mL Eppendorf tubes. 500 µL of extraction solvent (2-propanol/ddH_2O/ HCl (2:1:0.002, v/v/v) were added to each tube. The tubes were placed on a shaker at 100 rpm for 30 min in the cold room at 4°C. 1 mL of dichloromethane was added to each tube and placed again on the shaker for 30 min in the cold room at 4°C. The samples were centrifuged at 13,000 x g for 10 min at 4°C. The lower phases were transferred to Eppendorf tubes and placed into a concentrator. The samples were removed when dried and dissolved in 100 µL of methanol and kept at -20 °C until the analysis.

4.11. QUANTIFICATION OF PHYTOHORMONES (ABA, IAA, SA) VIA HPLC ANALYSIS

Before the analysis, 100 mg/L stock solutions of phytohormones were prepared by dissolving each of them in methanol. Stock solutions were diluted to 50, 20, 10, 5, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.01, 0.005 mg/L, respectively. The lowest concentration that can be detected; the limit of detection (LOD) value for each phytohormone was determined from the chromatograms. The calibration curves of each hormone were constructed by plotting the obtained peak areas versus known concentrations of phytohormones (0.25, 0.5, 1 and 2 mg/L ABA; 0.5, 1, 2 and 4 mg/L IAA; 6, 7, 8 and 9 mg/L SA), which were determined according to their LODs.

Since the plant hormones found in very small amounts in tissues, without the addition of known quantities of phytohormones to the matrix, the concentration in tissues stayed below the detection limit of the HPLC system. Therefore, the extracts were spiked with known concentrations of phytohormones dissolved in methanol to be able to achieve detectable levels. Each sample was prepared by mixing 90 µL of extract with 30 µL of each plant hormone (0.25, 0.5, 1 and 2 mg/L ABA; 0.5, 1, 2 and 4 mg/L IAA; 6, 7, 8 and 9 mg/L SA). The resulting 180 µL aliquots with different concentrations of hormones were transferred to 4 individual 2 ml vials with polymer feet (Table 4.3.). Then, the samples were positioned in a sample tray for the analysis. Chromatographic analysis was performed on a UHPLC system, Shimadzu Nexera XR series consisting of the degassing unit, pump, autosampler, system controller, column oven and S20-UV detector (Shimadzu, Kyoto, Japan). HPLC separations were done by using Agilent ZORBAX Eclipse XDB 80Å C18, 4.6 x 250 mm, 5 µm HPLC column. The column temperature was set to 35 °C. Mobile phases were composed of 0.6 percent acetic acid and methanol in gradient elution mode (Table 4.4.) at a flow rate of 1 mL min^{-1}. The UV detection wavelength was 254 nm and the injection volume was 50 µL. Hormone concentrations were calculated using the equation of the calibration curve of each hormone using the peak areas from HPLC measurements.

Table 4.3. Composition of analysed aliquots.

Sample components	**Vial 1**	**Vial 2**	**Vial 3**	**Vial 4**
90 µL extract	✓	✓	✓	✓
30 µL ABA	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L
30 µL IAA	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L
30 µL SA	6 mg/L	7 mg/L	8 mg/L	9 mg/L

Table 4.4. Gradient elution mode protocol.

Time (minute)	**Mobile phase A (percent) (0.6 percent acetic acid)**	**Mobile phase B (percent) (Methanol)**
0	95	5
13	25	75
15	25	75
20	95	5

4.12. STATISTICAL ANALYSIS

The gene expression changes were calculated as mean ± standard error of the mean (SEM) (n=9 for salinity, drought and their controls; n=6 for mechanical wounding samples and their controls). Statistical analysis performed by multiple t-tests of $\log_2$(fold changes) and volcano plots were constructed with -$\log_{10}$(adjusted p values) against $\log_2$(fold change) where p-value is 0.05 and -log(0.05) =1.301 using GraphPad Prism 8 for MacOS X (Figure 5.25, 5.26, 5.27, 5.28).

The phytohormone concentration results were calculated as mean ± SEM and n=3. Statistical analysis performed by a one-tailed Student's t-test to determine the significant differences relative to controls. When p-value < 0.1, it was concluded that there is a significant difference between compared phytohormone concentrations.

5. RESULTS

5.1. MORPHOLOGY OF THE STRESS-TREATED PLANTS

Abiotic stress causes deficiencies in plants and induces morphological changes that are critical for stress adaptations and survival under unfavorable conditions. The photographs of each plant were taken at the beginning, in the middle and at the end of the stress treatments to monitor those morphological alterations of *B. distachyon* (Bd21 line) plants. A set of plants were subjected to salinity stress by irrigating with 320 mM NaCl solution for 14 days, and their controls were irrigated with distilled water. The growth and development of salt-stressed plants were inhibited by the stress when their morphology compared with their control groups. Salt-stressed plants had lower height, reduced number of leaves and branches than control plants. Also, their leaves were darker and had a greyish surface, due to the accumulation of salts. The images of the plants on the 14th day of salinity stress treatment with 320 mM NaCl solution with its control group are shown in Figure 5.1.

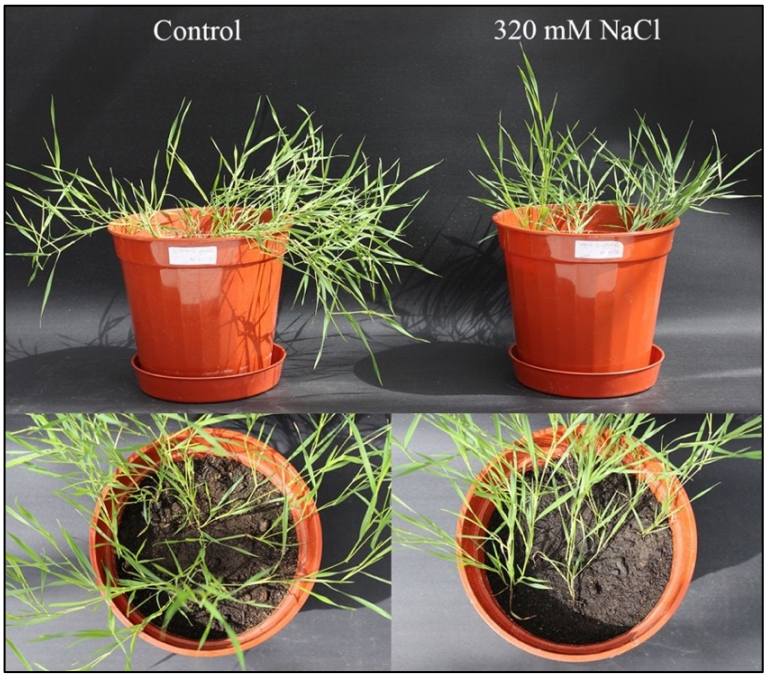

Figure 5.1. *Brachypodium distachyon* (Bd21 line) plant treated with 320 mM NaCl solution every day for 14 days and its control group irrigated with distilled water.

Other sets of plants were subjected to drought stress by water withheld for 12 days, and their controls were irrigated with distilled water. The water withholding negatively affected the growth and development of the plants as well. Drought-stressed plants were smaller in stature and had smaller leaves. Lighter leaf colour, decreased leaf number and branching were observed. Some of the leaves were folded, and all leaf tips were dried and became yellowish in colour. The plants subjected to drought stress through 12 days of water withholding and their control group is shown in Figure 5.2.

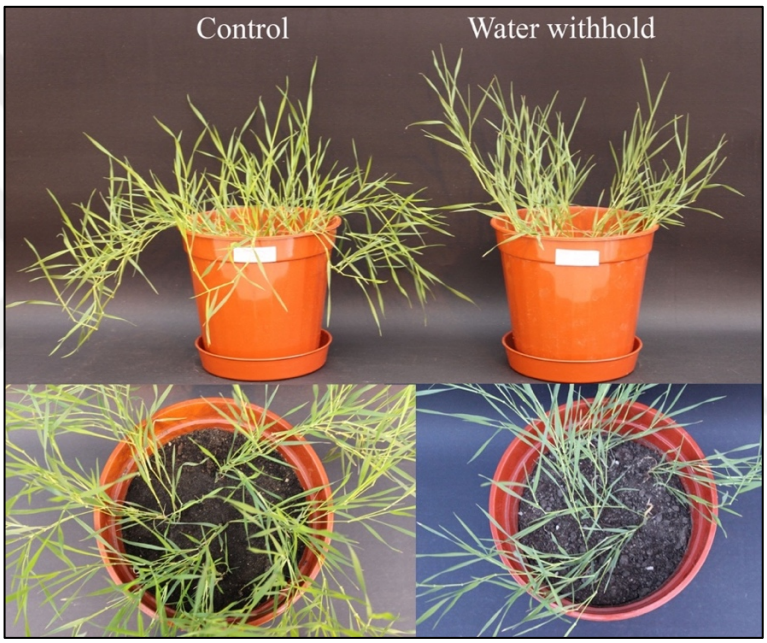

Figure 5.2. *Brachypodium distachyon* (Bd21 line) plant after 12 days of water withholding and its control group irrigated with distilled water.

Another set of plants was subjected to mechanical wounding stress in a time-dependent manner by squeezing each leaf with forceps across the leaf surface. Since mechanical wounding stress cause injuries on the leaf surface, the plants were immediately affected by the damage due to the destruction of the tissues. The wilting was observed in the mechanically wounded plants, and the leaves were shrunk due to the loss of water. The plants that were mechanically wounded by squeezing leaves with forceps are shown in Figure 5.3.

Figure 5.3. *Brachypodium distachyon* (Bd21 line) plants subjected to mechanical wounding stress by squeezing each leaf with forceps across the leaf surface.

5.2. SELECTON OF STRESS-RESPONSIVE GENES

Even though many stress-responsive genes are characterized in the widely used dicotyledonous plant, *Arabidopsis thaliana* and some in monocotyledonous plants such as rice and wheat, there are only a few characterized stress-responsive genes are available for *Brachypodium distachyon*. Therefore, drought, salinity and wounding stress-related 220 genes from *Arabidopsis*, rice and wheat were obtained from databases. Since their orthologs were not available in *Brachypodium distachyon,* each nucleotide sequence was aligned with the *Brachypodium distachyon* genome through NCBI's nucleotide BLAST tool. The search results provided the sequences producing significant alignments. Among all the generated alignments, the corresponding sequences with the best alignment scores (highest query covers, identity percentage and lowest e values) were selected. Since *Arabidopsis thaliana* is a dicot plant, some of the gene sequences resulted in very low alignment scores when aligned with the *Brachypodium distachyon* genome. Thus, the sequences that resulted in low alignment scores were eliminated, and the ones that have higher scores were gathered by also considering the information in the literature (Table 5.1.).

Table 5.1. Candidate stress-responsive genes obtained from databases in accordance with literature showing significant alignments with *Brachypodium distachyon* genome.

Gene/ Gene Family	**Accession number**	**Gene Name**	**Drought**	**Salinity**	**Wounding**	**Query Cover (percent)**	**Ref.**
Calcium dependent kinases (*CDPK*s)	NM_101746	*A. thaliana* Calcium dependent protein kinase 1 (*AtCDPK1*)	✓	✓		67	[169]
	NM_103271	*A. thaliana* Calcium dependent protein kinase 2 (*AtCDPK2*)	✓	✓		67	[169]
	NM_118496	*A. thaliana* Calcium dependent protein kinase 6 (*AtCDPK6*)	✓	✓		51	[170]
MIRO related GTPases (*MIRO*s)	NM_116180	*A. thaliana* MIRO-related GTPase 2 (*AtMIRO 2)*		✓		72	[171]
MAP kinases (*MAPK*s)	NM_118740	*A. thaliana* MAP kinase/ ERK kinase 1 (*AtMK1, AtMEK1*)		✓	✓	54	[172]
	NM_119127	*A. thaliana* MAP kinase 2 (*AtMKK2*)		✓		63	[172]
	NM_129941	*A. thaliana* MAP kinase 6 (*AtMPK6*)		✓		63	[173]
Chitinase-like (*CTL*s)	NM_100466	*A. thaliana* Chitinase-like protein 1 (*AtCTL1*, *AtPOM1*)	✓	✓		46	[174]
Cellulose synthase-interacting (*CSI*s)	NM_127781	*A. thaliana* Cellulose synthase-interactive protein 1 (*AtCSI1*)		✓		76	[175]

Activity of bc1 (*ABC1*) complex	HM773264	*T. aestivum* ABC1 (*TaABC1*)	✓	✓		89	[176]
Acyl-Coa oxidases (*ACX*s)	NM_117778	*A. thaliana* Acyl-coa oxidase 1 (*TaACX1*)			✓	75	[177]
NIN-like proteins (*NLP*s)	NM_118534	*A. thaliana* NIN like protein 7 (*AtNLP7*)	✓			41	[178]
Glucan synthase-like (*GSL*s)	NM_116593.4	*A. thaliana* Glucan synthase- like 5 (*AtGSL5*)			✓	69	[179]
	NM_100436	*A. thaliana* Glucan synthase-like 6 (*AtGSL6*)			✓	88	[179]
	NM_001331632	*A. thaliana* Glucan synthase-like 7 (*AtGSL7*)			✓	86	[180]
CBL-Interacting protein kinases (*CIPK*s)	NM_112631	*A. thaliana* CBL-Interacting protein kinase 1 (*AtCIPK1*)	✓	✓		55	[181]
	NM_179763	*A. thaliana* CBL-Interacting protein kinase 3 (*AtCIPK3*)	✓	✓	✓	56	[182]
	JX243013	*T. aestivum* CBL-Interacting protein kinase 29 (*AtCIPK29*)		✓		99	[183]
Calreticulins (*CRT*s)	HM037186	*T. aestivum* Calreticulin 1 (*TaCRT1*)	✓			94	[184]
	NM_100791	*A. thaliana* Calreticulin 1b (*TaCRT1b, TaCRT2*)	✓			47	[185]
	NM_001198007	*A. thaliana* Calreticulin 3 (*AtCRT3*)		✓		69	[185]

Expansins (*EXP*s)	AY543528	*T. aestivum* Expansin A2 (*TaEXPA2*)	✓	✓		91	[186]
	AY260547	*T. aestivum* Expansin B 23 (*TaEXPB23*)	✓			98	[187]
NAC domain containing proteins (*NAC*s)	AY625683	*T. aestivum* NAC domain containing protein 2 (*TaNAC2*)	✓			87	[188]
	AY625682	*T. aestivum* NAC domain containing protein 69-1 (*TaNAC69-1*)	✓			73	[189]
MYB transcription factors (*MYB*s)	HQ236494	*T. aestivum* MYB3R transcription factor 1 (*TaMYB3R1*)	✓	✓		95	[190]
	JF951913	*Triticum carthlicum* MYB30-R2R3 transcription factor (*TaMYB30*)	✓			100	[191]
	KU674897	*T. aestivum* MYB31 transcription factor (*TaMYB31*)	✓			100	[192]
	JN584645	*T. aestivum* MYB33 transcription factor (*TaMYB33*)	✓	✓		76	[193]
Ethylene Response Factors (*ERF*s)	EF570122	*T. aestivum* Ethylene responsive transcription factor 3 (*TaERF3*)	✓	✓		90	[194]
	AY781352	*T. aestivum* Ethylene response factor 1 (*TaERF1*, *TaEREB1*)	✓	✓		97	[195]

Alkaline Ceramidases (*ACER*s)	NM_118359	*A. thaliana* Alkaline ceramidase 1 (*TaACER*, *TaCES1*)		✓		47	[196]
Ornithine aminotransferase (*Delta-OAT*)	NM_123987	*A. thaliana* Ornithine aminotransferase (*AtDelta-OAT*)		✓		52	[197]
Endonuclease/exonuclease/ phosphatase	NM_100443	*A. thaliana* Inositol polyphosphate 5 phosphatase 13 (*At5PTASE13*)			✓	47	[198]
Gamma-aminobutyric acid transporter	NM_001331761	GABA transporter 1 (*AtGAT1*)			✓	59	[199]
Protein phosphatases (*PP2C*s)	KJ850318	*B. distachyon* Protein phosphatase 2CA 6 (*BdPP2CA6*)	✓			100	[99]
Major facilitator superfamily (*MF*s)	NM_106178	*A. thaliana* Early Responsive to Dehydration-Like 6 (*AtERDL6*)			✓	58	[200]
Sucrose Transporters (*SUC*s *or SUT*s)	NM_126341	*A. thaliana* Sucrose transporter 2 (*AtSUT2*, *AtSUC3*)	✓	✓	✓	52	[201,202]
	NM_100870	*A. thaliana* Sucrose transporter 4 (*AtSUC4*, *AtSUT4*)	✓	✓		45	[201]
Sugar Transporters (*STP*s)	AY052692	*A. thaliana* Sugar transporter 13 (*AtSTP13*)		✓		56	[203]
	X66857	*A. thaliana* Sugar transporter 4 (*AtSTP4*)		✓	✓	59	[204]

Fatty acid desaturases (*FAD*s)	NM_111953	*A. thaliana* Fatty acid desaturase 7 (*AtFAD7*)			✓	49	[100,205]
Lipoxygenases (*LOX*s)	NM_101603	*A. thaliana* Lipoxygenase 3 (*AtLOX3*)		✓	✓	64	[206,207]
	NM_105911	*A. thaliana* Lipoxygenase 4 (*AtLOX4*)			✓	67	[207]
Calmodulins (*CAM*s)	NM_123137	*A. thaliana* Calmodulin 1 (*AtCAM1*)		✓	✓	50	[208,209]
14-3-3	KU933265	*B. distachyon* 14-3-3 Protein G Box Factor 14 d (*BdGF14-E BdGF14d,*)		✓		100	[98]
	KU933266	14-3-3 Protein G Box Factor 14 e (BdGF14-H, *BdGF14e*)	✓			100	[98]
	KU933261	14-3-3 Protein G Box Factor 14 f (*BdGF14-D*, *BdGF14f*)	✓			100	[98]
	KU933263	14-3-3 Protein G Box Factor 14 g (*BdGF14g*)	✓			100	[98]
Serine/Threonine Protein Kinases (*STK*s)	NM_111706	*Arabidopsis thaliana* Serine/Threonine Protein Kinase 19 (*AtPK19*, *AtS6K2*)		✓	✓	43	[210]
Pyrroline-5-carboxylate synthase (*P5CS*)	NM_001202786	*Arabidopsis thaliana* Delta 1-Pyrroline-5-Carboxylate Synthase 1 (*AtP5CS1*)	✓	✓		80	[211,212]
Sodium hydrogen exchangers (*NHX*s)	NM_111375.4	*Arabidopsis thaliana* Sodium hydrogen excharger 2 (*AtNHX2*)		✓		49	[213]

	NM_104315.5	*Arabidopsis thaliana* Sodium hydrogen excharger 5 (*AtNHX5*)		✓			[213]
WRKY transcription factors (*WRKY*s)	EU665425	*T. aestivum* WRKY transcription factor 2 (*TaWRKY2*)	✓	✓		91	[214,215]
	EU665424.1	*T. aestivum* WRKY transcription factor 1 (*TaWRKY1*)		✓		99	[216]
	EU665430	*T. aestivum* WRKY transcription factor 19 (*TaWRKY19*)	✓	✓		100	[214]
	NM_129404	*T. aestivum* WRKY transcription factor 33 (*TaWRKY33*)		✓		100	[216]
		B. distachyon WRKY transcription factor 36 (*BdWRKY36*)		✓		100	[76]

Each gene aligned with *Brachypodium distachyon* belonged to a member of an important gene family associated with multiple abiotic stress. They have been reported to be responsive to at least one or more abiotic stress of interest; drought, salinity and mechanical wounding in various plant species. Some of the selected genes from *Arabidopsis* did not have very high alignment scores. Nevertheless, they were used for further study since the vast majority of wounding stress-responsive genes was identified in dicots rather than monocots. Mechanical wounding stress was investigated time-dependently, including both local and systemic responses, which have not been studied in *Brachypodium*. Eight candidate multiple stress-responsive genes (*ERF1*, *EXPA2*, *GF14d*, *LOX3*, *P5CS1*, *PP2CA6*, *SUT2* and *WRKY36*) were selected according to their responsiveness to multiple stressors. *ERF1* has been studied in wheat under salinity and drought stress and found to be up-regulated under multiple

abiotic stress conditions [195]. However, it has not been studied in *Brachypodium* plants, and the salinity, drought and time-dependent mechanical wounding systemic and local responses were unknown in *Brachypodium*. Therefore, the sequence of *Triticum aestivum ERF1* (*ERF1/EREB1*) was aligned with the *Brachypodium distachyon* genome and among the obtained blast hits, a highly similar sequence with a 97 percent query cover, 1527 bp long predicted *Brachypodium distachyon* ethylene-responsive transcription factor 1 (*ERF1*) sequence with the accession number of XM_003564109 was chosen. Since Expansin A2 (*EXPA2*) gene has not been studied and defined in *Brachypodium distachyon* in any stress conditions but found to confer salinity stress in *Triticum aestivum*, Triticum *aestivum* Expansin A2 (*EXPA2*) nucleotide sequence was aligned with *Brachypodium distachyon* genome and resulted in blast hits on sequences. The top hit was the 933 bp long predicted *Brachypodium distachyon* Expansin A23 (*EXPA23*) with a 68 percent query cover under the accession number XM_003571269. In a previous study, the *GF14d* gene was identified and found to be salinity stress-responsive in *Brachypodium distachyon* [98]. Thus, *Brachypodium distachyon GF14d* gene sequence was available under accession number KU933266. Lipoxygenase 3 (*LOX3*) gene has not been studied in *Brachypodium distachyon*, therefore the gene sequence was not available. However, it has been studied in *Arabidopsis thaliana* and found to be salinity and wounding responsive [206,207]. Hence *LOX3* gene from *Arabidopsis thaliana* was aligned with the *Brachypodium distachyon* genome and with a query cover of 64 percent, 3278 bp long predicted *Brachypodium distachyon* probable lipoxygenase 6 (*LOX6*), which has accession number of XM_003561945, was selected. Delta 1-pyrroline-5-carboxylate synthase 1 (*P5CS1*) gene has not been studied in *Brachypodium distachyon*, so the gene sequence was not available. However, it has been studied in *Arabidopsis thaliana* and found to be salinity and drought-responsive [211,212]. *Arabidopsis thaliana P5CS1* was aligned with the *Brachypodium distachyon* genome, and the alignment resulted in 2785 bp long predicted *Brachypodium distachyon* delta-1-pyrroline-5-carboxylate synthase 1 with the accession number of XM_003568279 and a query cover of 80 percent. After performing BLAST analysis on each of the selected genes, using the corresponding sequences in *Brachypodium distachyon*, gene-specific primers were designed using NCBI's Primer-BLAST tool except for *PP2CA6* and WRKY36. The sequences of primer pairs for protein phosphatase 2C 6 (*PP2CA6*) was obtained from a previous study on *Brachypodium distachyon.* In this study, *PP2CA6* gene was found to be salinity stress-responsive [99]. The nucleotide sequence of *PP2CA6* was available under

accession number KJ850318. The nucleotide sequence of WRKY transcription factor 36 (*WRKY36*) was not publicly available, but in a previous study, the expression levels of the *WRKY36* gene was investigated and *WRKY36* provided drought stress tolerance in transgenic tobacco [76]. So, the primer sequences were obtained from that study.

ABC1, *CIPK3* and *NAC69* genes were also selected for the gene expression study. However, after PCR optimization, they were not studied further due to producing PCR products with low intensity. All selected genes were given in Table 5.2.

Table 5.2. Selected genes for gene expression study in *Brachypodium distachyon*.

Gene/ Gene Family	**Accession number**	**Gene Name**	**Drought**	**Salinity**	**Wounding**	**Query Cover (percent)**	**Ref.**
Ethylene response factors (*ERF*s)	AY781352	*T. aestivum* Ethylene response factor 1 (*TaERF1*)	✓	✓		97	[195]
Expansins (*EXP*s)	AY543528	*T. aestivum* Expansin A2 (*TaEXPA2*)		✓		68	[186]
14-3-3	KU933266	*B. distachyon* Protein G Box Factor 14-3-3 d (*BdGF14d*, *BdGF14-E*)	✓			100	[98]
Lipoxygenases (*LOX*s)	NM_101603	*A. thaliana* Lipoxygenase 3 (*AtLOX3*)		✓	✓	64	[206,207]
Delta-Pyrroline-5-Carboxylate Synthase (*P5CS*)	NM_001202786	*A. thaliana* Delta-Pyrroline-5-Carboxylate Synthase 1 (*AtP5CS1*)	✓	✓		80	[211,212]
Protein phosphatases 2C (PP2Cs)	KJ850318	*B. distachyon* Protein phosphatase 2C 6 (*BdPP2CA6*)		✓		100	[99]
Sucrose Transporters (SUCs or SUTs)	NM_126341	*A. thaliana* Sucrose transporter 2 (*AtSUT2*, *AtSUC3*)	✓	✓	✓	53	[201,202]
WRKY transcription factors (WRKYs)		*B. distachyon* WRKY transcription factor 36 (*BdWRKY36*)	✓			100	[76]
Activity of bc1 (*ABC1*) complex	HM773264	*T. aestivum* ABC1 (*TaABC1*)	✓	✓		89	[176]
CBL-Interacting protein kinases (*CIPK*s)	NM_179763	*A. thaliana* CBL-Interacting protein kinase 3 (*AtCIPK3*)	✓	✓	✓	56	[182]

5.3. OPTIMIZATION OF PCR CONDITIONS

Gene-specific forward and reverse primers were designed via NCBI's primer designing tool (Table 4.2.). Gradient PCR was performed using the primers to amplify the genes of interest and determine the annealing temperature which was ideal for each pair of primer to bind to cDNA template by running PCR products on 1.5 percent agarose gel. The individual bands were checked to see if they resulted in the expected PCR product size. For *ERF1* gene-specific primers, the expected PCR product was 112 bp and it was observed at an annealing temperature of 60.2 °C (Figure 5.4).

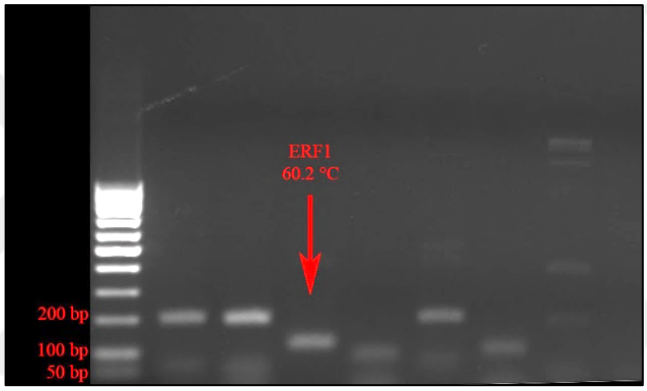

Figure 5.4. Agarose gel (1.5 percent) image of the PCR product with *ERF1* primers. The band shown with red arrow is the expected 112 bp PCR product. Ladder: GeneON 50 bp.

For the *SUT2* gene-specific primers, the optimal annealing temperature was determined as 59.3 °C to obtain a band in expected product size of 178 bp. The expected PCR products of *WRKY36* and *EXPA2* primer pairs were 196 and 80 bp, respectively and they were both observed at an annealing temperature of 65 °C. *GF14d* primer pair was expected to result in a 191 bp PCR product and it was observed at an annealing temperature of 63 °C (Figure 5.5.).

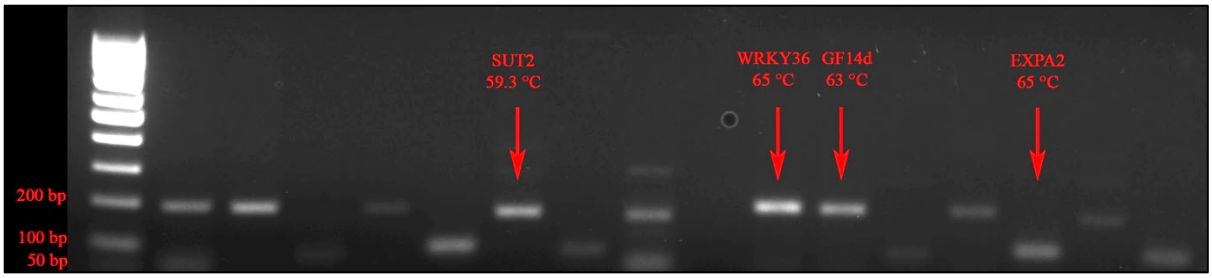

Figure 5.5. Agarose gel (1.5 percent) image of the PCR products with *SUT2* (178 bp), *WRKY36* (196 bp), *GF14d* (191 bp), *EXPA2* (80 bp) primer pairs. Red arrow on top of each band shows the PCR product below in expected size. Ladder: GeneON 50 bp.

With *PP2CA6* gene primer pair, expected 103 bp PCR product was amplified at 62 °C annealing temperature (Figure 5.6.).

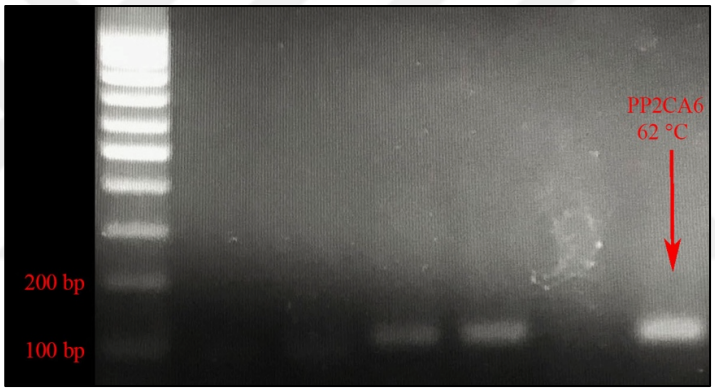

Figure 5.6. Agarose gel (1.5 percent) image of the PCR product with *PP2CA6* (103 bp). The red arrow on top of the band shows the PCR product below in expected size. Ladder: GeneON 50 bp.

The expected PCR product for *P5CS1* gene primer pair was 92 bp and it was observed at annealing temperature of 59.3 °C (Figure 5.7.).

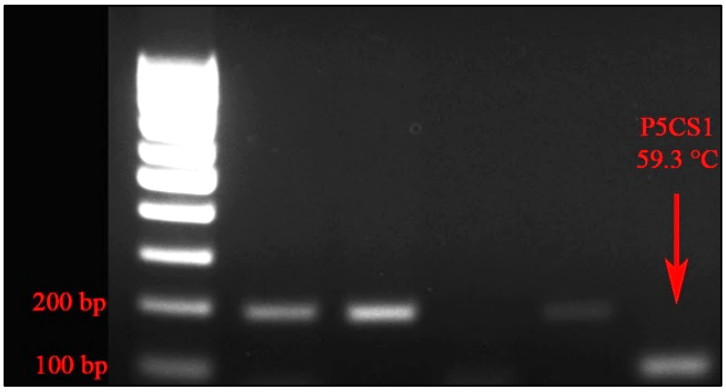

Figure 5.7. Agarose gel (1.5 percent) image of the PCR products with *P5CS1* (92 bp). The red arrow on top of the band shows the PCR product below in expected size. Ladder: GeneON 50 bp.

For *LOX3* gene-specific primers, the expected PCR product was 162 bp and it was observed at 54 °C annealing temperature (Figure 5.8.)

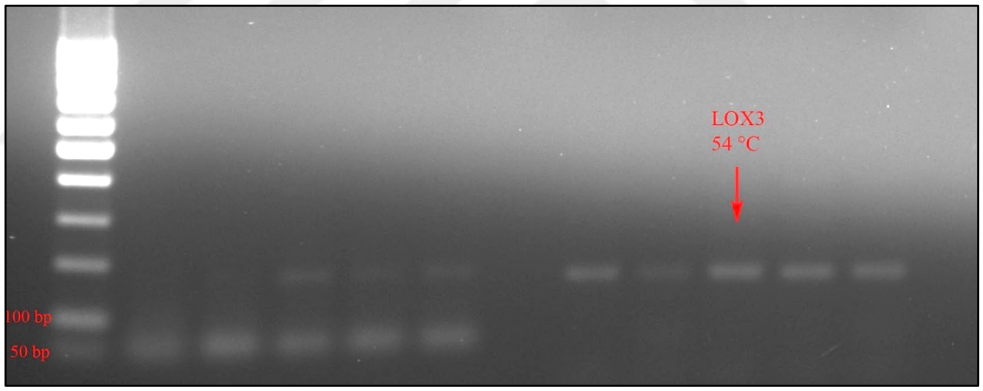

Figure 5.8. Agarose gel (1.5 percent) image of the PCR products with *LOX3* (162 bp). The red arrow on top of the band shows the PCR product below in expected size. Ladder: GeneON 50 bp.

5.4. GENE EXPRESSION CHANGES UNDER ABIOTIC STRESS: SALINITY, DROUGHT AND MECHANICAL WOUNDING

The effect of drought, salinity and mechanical wounding stress on the expression levels of *ERF1*, *EXPA2*, *GF14d*, *LOX3*, *P5CS1*, *PP2CA6*, *SUT2* and *WRKY36* in *Brachypodium distachyon* (Bd21 line) were analysed via qRT-PCR in leaf tissues of salinity stressed plants through the 320 mM NaCl solution application for 14 days (S); drought stressed plants by

water withheld for 12 days (D); wounded plants subjected to mechanical wounding stress through squeezing leaves across the surface with forceps, which collected at 0th (W0), at 6th (W6) and at 24th hour (W24) representing local events; unwounded tissues adjacent to wounded leaves representing the systemic events collected at 0th (WA0), at 6th (WA6) and at 24th hour (WA24); their control groups: salinity control (SC), drought control (DC) and wounding control (WC) plants. The expression levels were normalized to reference gene *UBC18* and the relative expression levels were normalized to control plants. Since *UBC18* was stably expressed in *B. distachyon* plants and previous studies that compared *UBC18* with other housekeeping genes validated *UBC18* as the most suitable gene to use as a reference, especially in the analysis of stress-responsive genes [82,168], The relative expression level data were calculated from the mean and standard deviation of nine (3 biological x 3 technical replicates) Ct values for salinity and drought, and six (2 biological x 3 technical replicates) for mechanical wounding stress. The relative expression levels of genes of interest were represented as fold-changes (Figure 5.9-5.24).

5.4.1. ERF1

The expression levels of *ERF1* gene, which is one of the ethylene response factors induced by ethylene signaling and ethylene response pathway, was investigated under drought and salinity. Salinity significantly increased *ERF1* expression 4.27-fold (Figure 5.9. and 5.25.). However, drought stress only very slightly decreased 0.98-fold (Figure 5.9. and 5.26.).

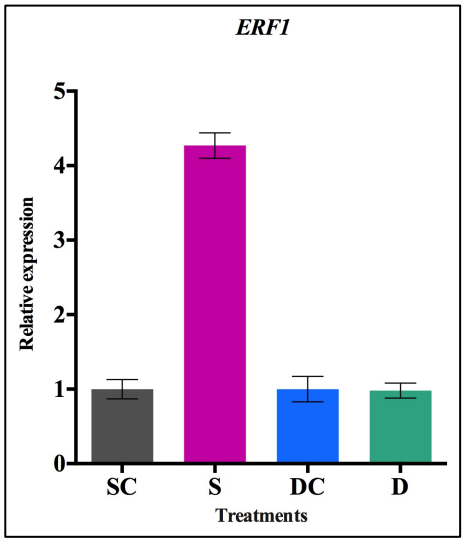

Figure 5.9. Expression levels of *ERF1* under salinity and drought stress.

The expression levels of *ERF1* were also analysed in both wounded and unwounded adjacent leaves, representing local and systemic responses. Wounding stress decreased *ERF1* expression 0.41-fold at 0^{th} h significantly, increased 2.06-fold at 6^{th} h, and increased 6.97-fold at 24^{th} h significantly in the wounded tissues (Figure 5.10. and 5.27.). In the unwounded tissues adjacent to wound sites, gene expression levels were increased 2.12-fold at 0^{th} h, 1.76-fold at 6^{th} h, and significantly increased 4.41-fold (Figure 5.10. and 5.28.).

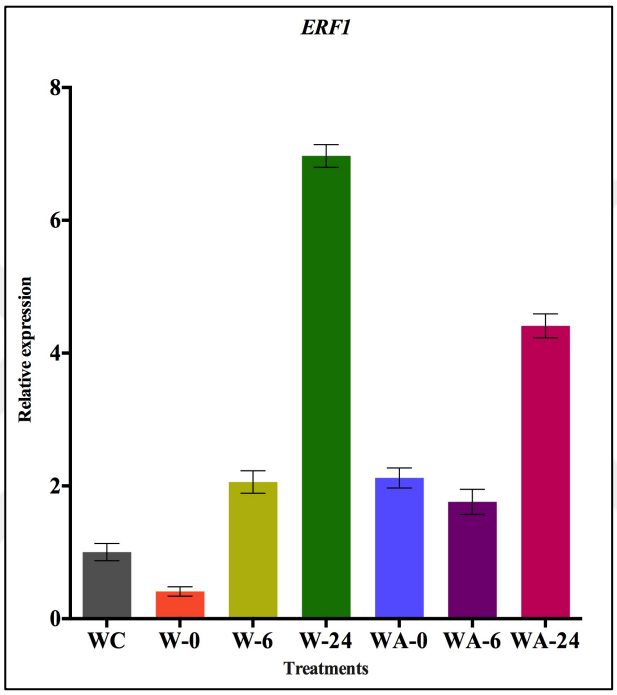

Figure 5.10. Expression levels of *ERF1* under time-dependent (0 h, 6 h and 24 h) wounding stress including local and systemic responses.

5.4.2. EXPA2

The expression levels of *EXPA2,* a gene that encodes expansins (a class of cell wall proteins), were investigated under salinity and drought stress. Expression of *EXPA2* was significantly improved 4.55-fold under salinity (Figure 5.11. and 5.25.) and 1.68-fold under drought stress (Figure 5.11. and 5.26.).

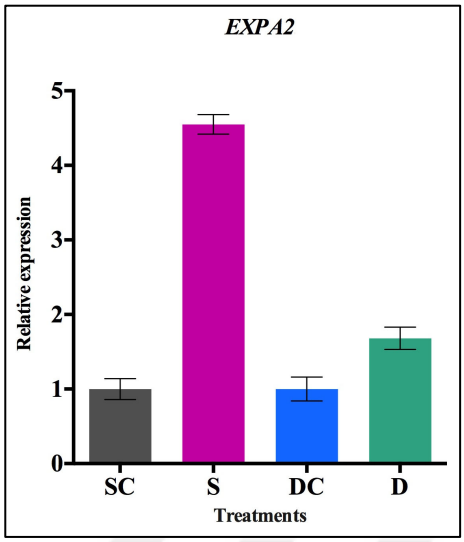

Figure 5.11. Expression levels of *EXPA2* under salinity and drought stress.

Mechanical wounding stress treatment enhanced the expression levels of *EXPA2* 1.77-fold at 0th h in wounded tissues. The expression levels of *EXPA2* was significantly up-regulated 3.32-fold at 6th h and 33.36-fold at 24th h (Figure 5.12. and 5.27.). In the unwounded tissues adjacent to wound sites, the gene expression levels were increased 1.17-fold at 0th h and 2.91-fold at 6th h. Wounding caused a significant 9.82-fold increase in systemic leaf tissues at 24th h (Figure 5.12. and 5.28.).

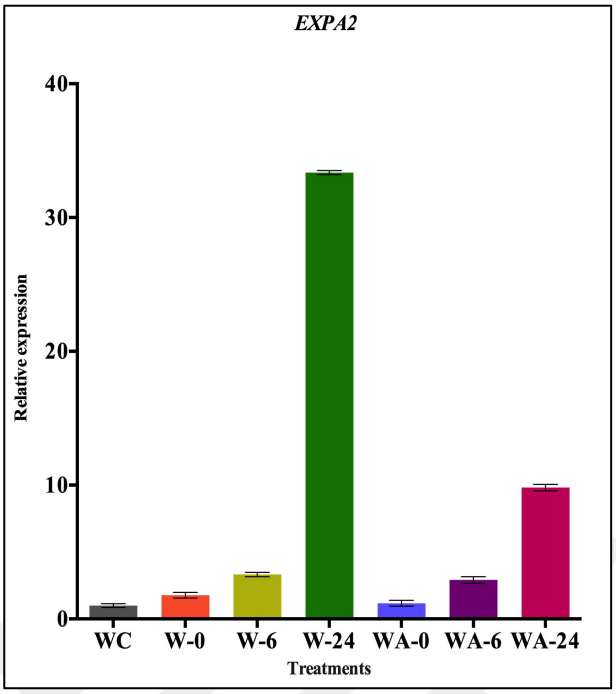

Figure 5.12. Expression levels of *EXPA2* under time-dependent (0 h, 6 h and 24 h) wounding stress including local and systemic responses.

5.4.3. GF14d

GF14d gene is a member of the *14-3-3* gene family, which is composed of genes that code for proteins binding to phosphorylated peptides for the regulation of various metabolic processes. The expression levels of *GF14d* was significantly increased 2.83-fold under salinity stress (Figure 5.13. and 5.25.). Under drought stress conditions, *GF14d* expression levels were increased 1.77-fold (Figure 5.13. and 5.26.).

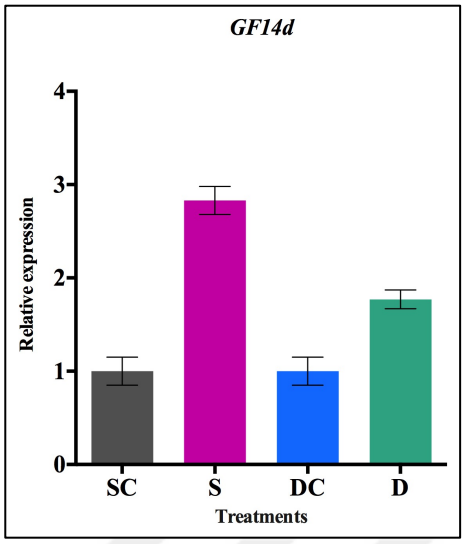

Figure 5.13. Expression levels of *GF14d* under salinity and drought stress.

The effects of mechanical wounding stress on the expression levels of *GF14d* in *Brachypodium* were investigated in wounded tissues at 0th h, and a slight 0.95-fold decrease was observed. Then, *GF14d* expression levels were increased 1.19-fold at 6th h and significantly increased 3.17-fold at 24th h in the wounded tissues (Figure 5.14. and 5.27.). In the unwounded tissues adjacent to wound sites, gene expression levels were decreased 0.67-fold at 0th h, 0.70-fold at 6th h, and increased 1.67-fold at 24th h (Figure 5.14. and 5.28.).

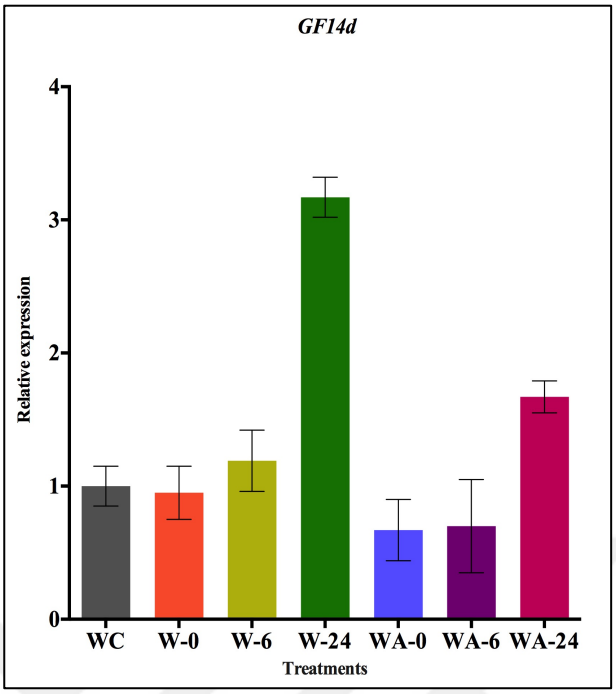

Figure 5.14. Expression levels of *GF14d* under time-dependent (0 h, 6 h and 24 h) wounding stress including local and systemic responses.

5.4.4. LOX3

LOX3 belongs to a lipoxygenase gene family, which codes for enzymes that participate in the synthesis of fatty acid metabolites via their lipid-oxidizing activity. The expression levels of the *LOX3* gene was significantly increased 3.63-fold under salinity stress (Figure 5.15. and 5.25.) and only slightly increased 1.09-fold under drought stress conditions (Figure 5.15. and 5.26.).

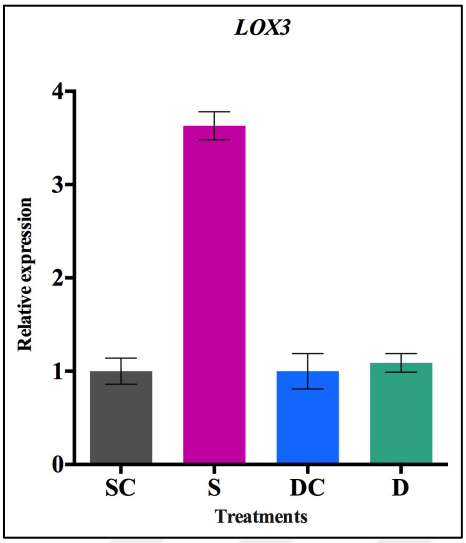

Figure 5.15. Expression levels of *LOX3* under salinity and drought stress.

On the other hand, expression levels *LOX3* gene in the mechanically wounded leaf tissues of *Brachypodium distachyon* was decreased 0.81-fold at 0th h, significantly increased 5.22-fold at 6th h and 11.65-fold at 24th h (Figure 5.16. and 5.27.). In the unwounded tissues adjacent to wound sites, *LOX3* gene expression levels were significantly increased 4.46-fold at 0th h, 3.43-fold at 6th h and 5.18-fold at 24th h (Figure 5.16. and 5.28.).

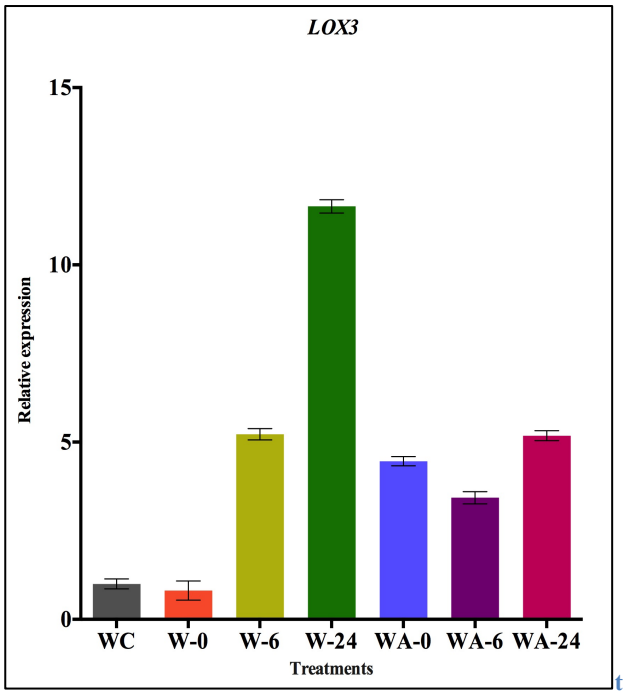

Figure 5.16. Expression levels of *LOX3* under time-dependent (0 h, 6 h and 24 h) wounding stress including local and systemic responses.

5.4.5. P5CS1

P5CS1 gene codes for the important enzyme, delta1-pyrroline-5-carboxylate synthase, which has a role in proline biosynthesis. *P5CS1* gene expression levels were significantly increased 10.44-fold under salinity and 5.95-fold under drought stress (Figure 5.17., 5.25. and 5.26.).

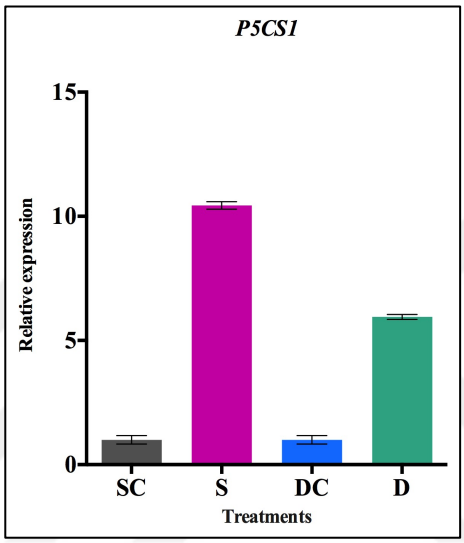

Figure 5.17. Expression levels of *P5CS1* under salinity and drought stress.

The expression levels of *P5CS1* gene were decreased 0.32-fold in mechanically wounded tissues at 0^{th} h but, increased 1.42-fold at 6^{th} h and significantly increased 3.24-fold at 24^{th} h (Figure 5.18. and 5.27.). In the unwounded tissues adjacent to wound sites, gene expression levels were decreased 0.36-fold and 0.78-fold at 0^{th} and 6^{th} h, respectively. However, a 1.68-fold increase was observed at 24^{th} h (Figure 5.18. and 5.28).

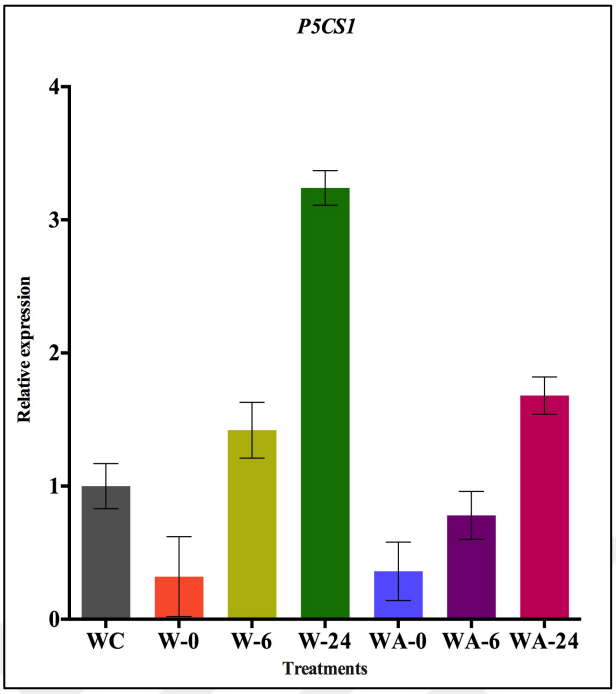

Figure 5.18. Expression levels of *P5CS1* under time-dependent (0 h, 6 h and 24 h) wounding stress including local and systemic responses.

5.4.6. PP2CA6

PP2CA6 is one of the genes, which encodes the type 2C phosphatase (a protein phosphatase that dephosphorylates Serine/Threonine residues). The expression levels of the *PP2CA6* were significantly increased 3.13-fold under salinity (Figure 5.19. and 5.25.), and a 1.85-fold increase was observed under drought stress (Figure 5.19. and 5.26.).

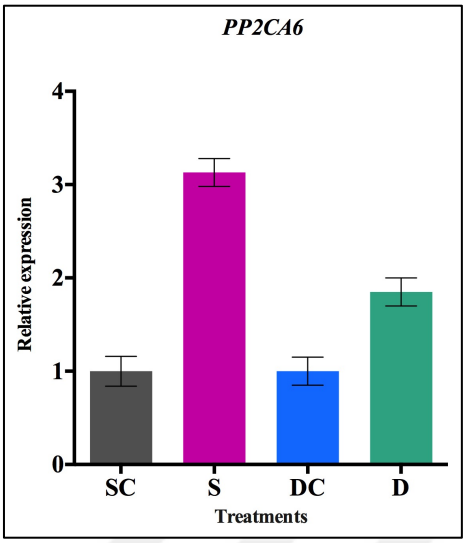

Figure 5.19. Expression levels of *PP2CA6* under salinity and drought stress.

Brachypodium distachyon (Bd21 line) plants were subjected to time-dependent wounding stress, and the expression levels of *PP2CA6* were investigated in both local and systemic tissues. *PP2CA6* expression levels were significantly decreased 0.86-fold in mechanically wounded tissues at 0^{th} h and increased 2.05-fold at 6^{th} h. Then, a significant 6.55-fold increase was observed at 24^{th} h (Figure 5.20. and 5.27.). In the unwounded tissues adjacent to wound sites, *PP2CA6* gene expression levels were decreased 0.60-fold at 0^{th} h and slightly increased 1.07-fold at 6^{th} h. However, it was increased 3.70-fold at 24^{th} h (Figure 5.20. and 5.28.).

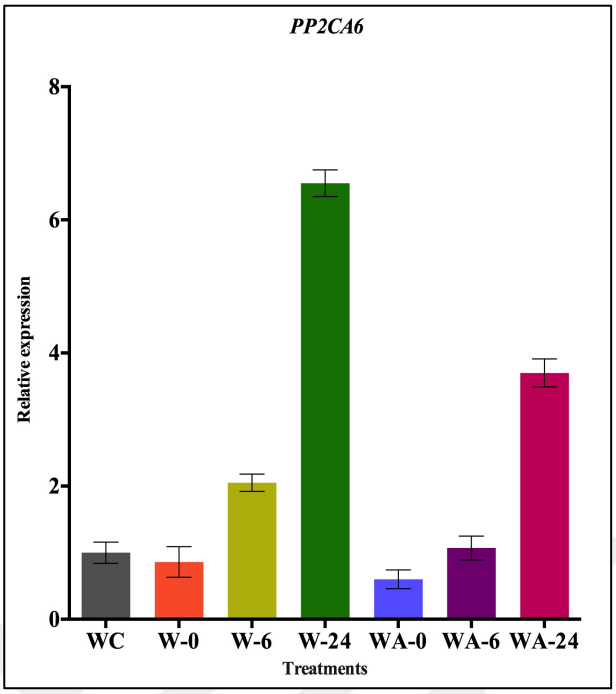

Figure 5.20. Expression levels of *PP2CA6* under time-dependent (0 h, 6 h and 24 h) wounding stress including local and systemic responses.

5.4.7. SUT2

SUT2 is a gene that encodes for sucrose transporters, which are responsible for the allocation of sucrose in plants. The expression levels of the *SUT2* were found to be significantly increased with 1.85-fold under salinity (Figure 5.21. and 5.25.). However, no *SUT2* expression was observed under drought stress conditions (Figure 5.21.).

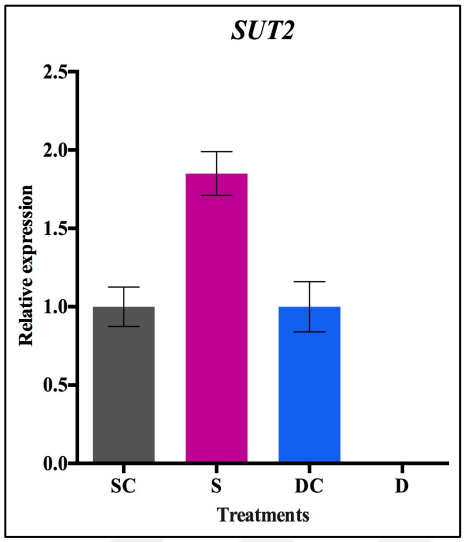

Figure 5.21. Expression levels of *SUT2* under salinity and drought stress treatments.

The effect of time-dependent mechanical wounding stress in *SUT2* gene expression levels in both local and systemic tissues of *Brachypodium distachyon* was also investigated. A significant 0.29-fold decrease in transcript levels was observed in mechanically wounded tissues at 0^{th} h, whereas transcript levels were slightly increased 1.12-fold at 6^{th} h. However, no *SUT2* expression was observed at 24^{th} h (Figure 5.22. and 5.27.). In the unwounded tissues adjacent to wound sites, gene expression levels were increased 2.29-fold at 0^{th} h, 1.89-fold at 6^{th} h, and decreased 0.78-fold at 24^{th} h (Figure 5.22. and 5.28).

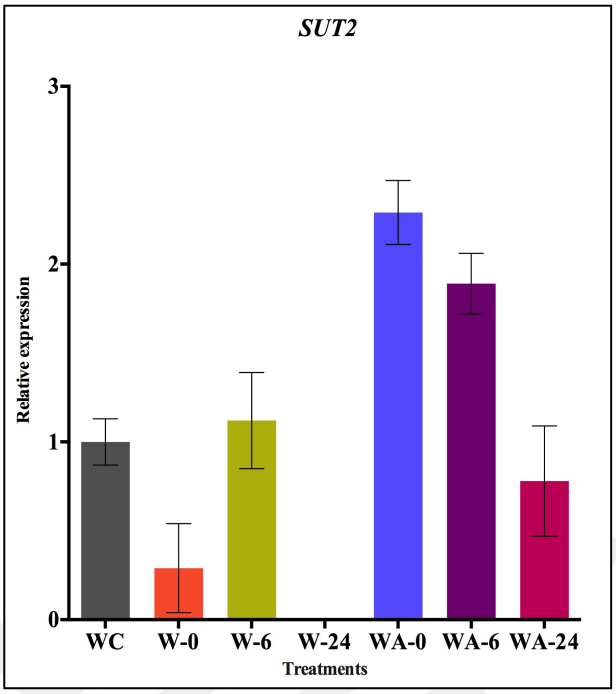

Figure 5.22. Expression levels of *SUT2* under time-dependent (0 h, 6 h and 24 h) wounding stress including local and systemic responses.

5.4.8. WRKY36

The expression levels of the *WRKY36* gene, which encodes for *WRKY* transcription factors, were investigated under salinity and drought stress in *Brachypodium distachyon*. The transcript levels were found to be significantly increased 2.27-fold under salinity (Figure 5.23. and 5.25.), and a 1.50-fold increase was observed under drought stress conditions (Figure 5.23. and 5.26.).

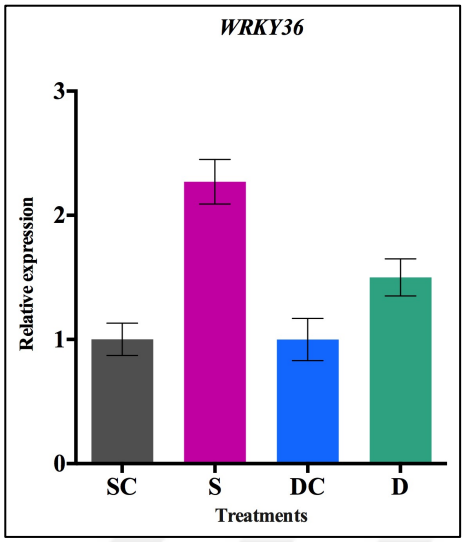

Figure 5.23. Expression levels of *WRKY36* under salinity and drought stress.

The expression levels of *WRKY36* were increased 1.75-fold in mechanically wounded tissues at 0th h, decreased 0.88-fold at 6th h, and a significant 20.21-fold increase was observed at 24th h (Figure 5.24. and 5.27.). In the unwounded tissues adjacent to wound sites, gene expression levels were decreased 0.49-fold at 0th h, increased 1.60-fold at 6th h, and then significantly increased 10.45-fold at 24th h (Figure 5.24. and 5.28.).

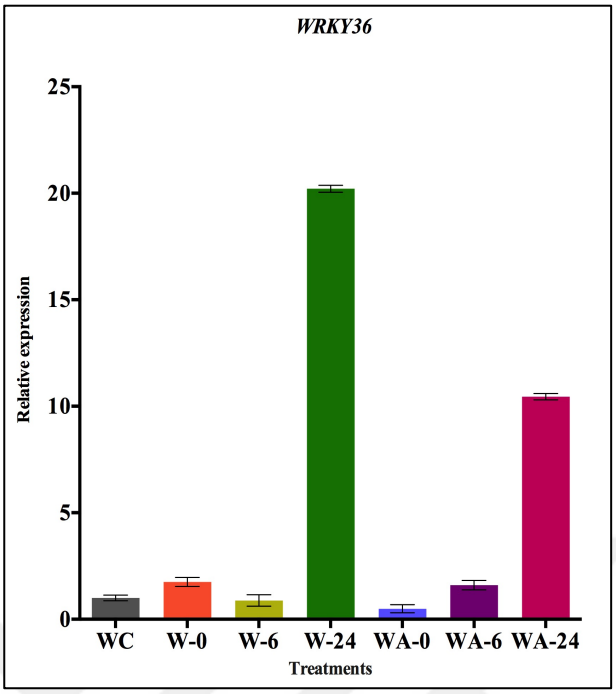

Figure 5.24. Expression levels of *WRKY36* under time-dependent (0 h, 6 h and 24 h) wounding stress including local and systemic responses.

To determine the significant changes in the gene expression levels, multiple t-tests were performed and volcano plots were constructed for each stress; Figure 5.25. for salinity stress, Figure 5.26. for drought stress, Figure 5.27. for wounding local response and Figure 5.28. for wounding systemic response).

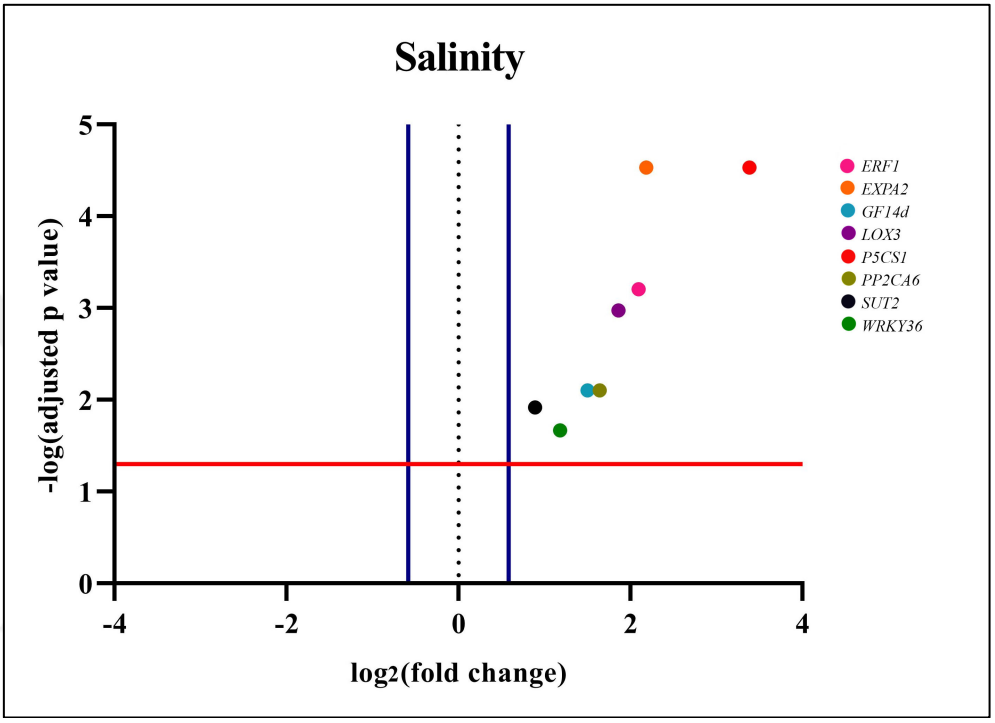

Figure 5.25. Volcano plot from multiple t-tests of gene expression under salinity stress. Each dot represents one gene, and the red line represents the -log(0.05) = 1.301. The values below the red line are non-significant and the ones above the red line are statistically significant. While the dots fall into positive x-axis are up-regulated genes, the dots fall into negative x-axis are down-regulated genes. The dark blue horizontal lines represent the threshold of a 1.5-fold change.

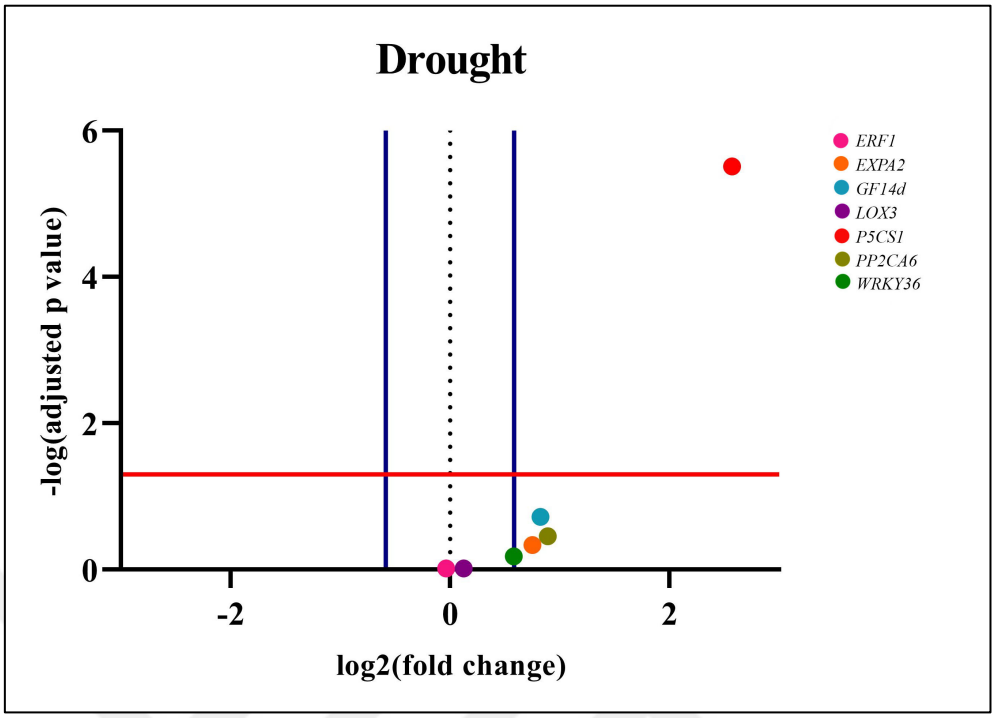

Figure 5.26. Volcano plot from multiple t-tests of gene expression under drought stress. Each dot represents one gene, and the red line represents the -log(0.05) = 1.301. The values below the red line are non-significant and the ones above the red line are statistically significant. While the dots fall into positive x-axis are up-regulated genes, the dots fall into negative x-axis are down-regulated genes. The dark blue horizontal lines represent the threshold of a 1.5-fold change.

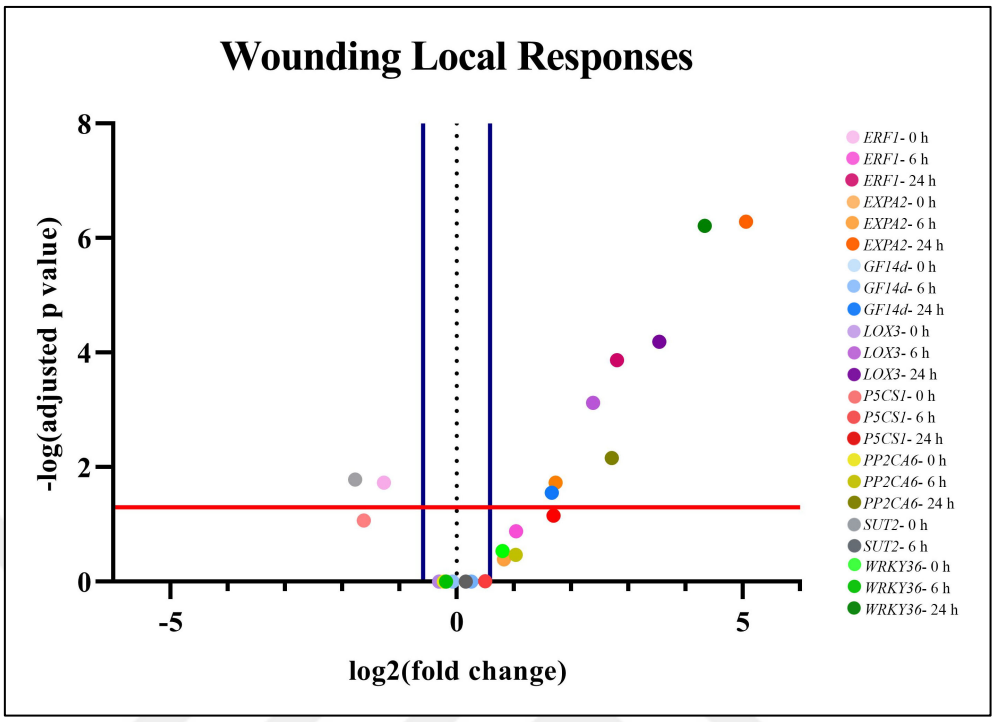

Figure 5.27. Volcano plot from multiple t-tests of gene expression under wounding stress at local tissues. Each dot represents one gene, and the red line represents the -log(0.05) = 1.301. The values below the red line are non-significant and the ones above the red line are statistically significant. While the dots fall into positive x-axis are up-regulated genes, the dots fall into negative x-axis are down-regulated genes. The dark blue horizontal lines represent the threshold of a 1.5-fold change.

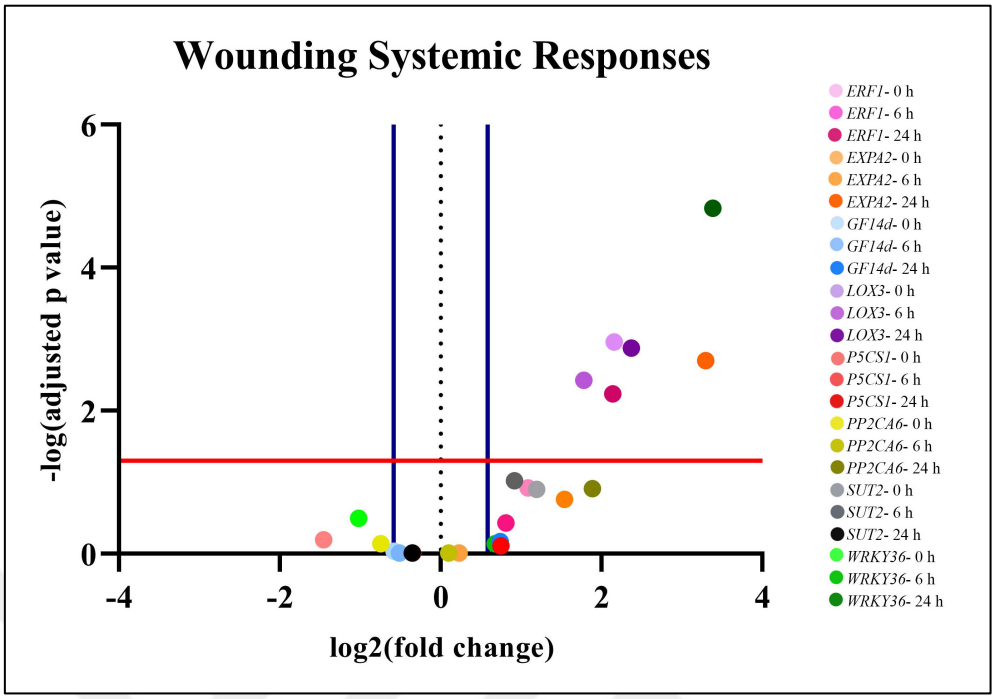

Figure 5. 28. Volcano plot from multiple t-tests of gene expression under wounding stress at systemic tissues. Each dot represents one gene, and the red line represents the -log(0.05) = 1.301. The values below the red line are non-significant and the ones above the red line are statistically significant. While the dots fall into positive x-axis are up-regulated genes, the dots fall into negative x-axis are down-regulated genes. The dark blue horizontal lines represent the threshold of a 1.5-fold change.

5.5. PHYTOHORMONE CHANGES UNDER ABIOTIC STRESS CONDITIONS

Since phytohormones and their cross-talk are pivotal in the molecular mechanisms that regulate environmental stress responses, in the present study the fluctuations of phytohormones were examined under salinity, drought and mechanical wounding stresses. The alterations of phytohormone levels under wounding stress were inspected in both unwounded and wounded leaf tissues at different time points: 0 h, 6 h and 24 h to determine systemic and local phytohormone responses.

The LODs are the lowest phytohormone concentration that can be detected, and their retention times were determined from the chromatograms of standard samples containing phytohormones dissolved in methanol (Table 5.3.).

Table 5.3. LODs of phytohormones.

Phytohormone	Retention time (minute)	LOD (mg/L)
ABA	13.7	0.01
IAA	12.4	0.05
SA	12.8	1

Spiking was performed to be able to measure concentrations below the detection limit. Standard curve of each phytohormone was plotted with peak area vs. known concentrations of phytohormones. From the slope and intercept, the trendline equation was generated. Phytohormone levels were estimated using the trendline equation and the obtained peak area (x). The phytohormone levels of plants subjected to salinity, drought and mechanical wounding stresses were estimated through conducting spiking experiments. HPLC chromatograms of phytohormone extracts from each treatment were given in the Appendix A.

5.5.1. IAA

IAA belongs to auxin family of phytohormones. They participate in phytohormone signaling pathways and regulate responses for plant defence. In order to observe changes in IAA levels under salinity, drought and mechanical wounding stresses, standard curve of IAA was plotted using the peak area obtained from HPLC analysis vs. known concentrations (0.5, 1, 2 and 4 mg/L) of IAA (Figure 5.29).

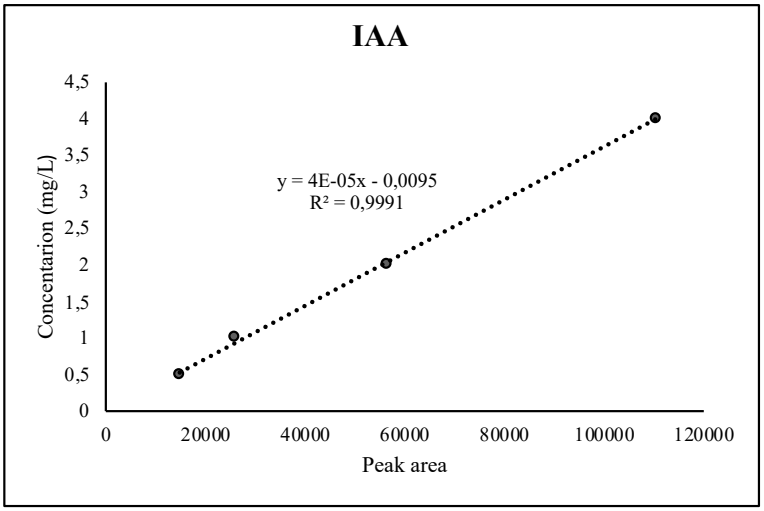

Figure 5.29. The standard curve of IAA.

HPLC analysis was carried out for the stressed and control samples spiked with IAA. There was a reducing trend in IAA levels under salinity stress conditions, yet the decrease was not significant. Nonetheless, under drought conditions, there was a significant decrease in the IAA levels (Figure 5.30).

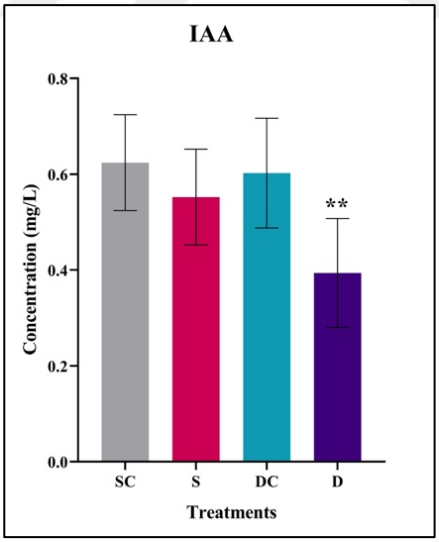

Figure 5.30. IAA levels under salinity and drought stress. Significant at '*' $p<0.1$, '**' $p<0.05$, '***' $p<0.01$.

In addition to drought, IAA hormone levels were significantly declined instantly after mechanical wounding stress ($p<0.05$). At 0 h time point, the IAA levels were halved in comparison to controls. Six hours after wounding, despite being still lower than control plants, IAA levels were increased when compared to 0^{th} h. After 24 h, the IAA levels were

nearly the same with the samples collected at 6th h (Figure 5.31). In the wound adjacent tissues, IAA levels were significantly decreased at 6 ($p<0.1$) and 24 h ($p<0.1$) after wounding, even though IAA levels were low at 0th h as well, the change was not significant.

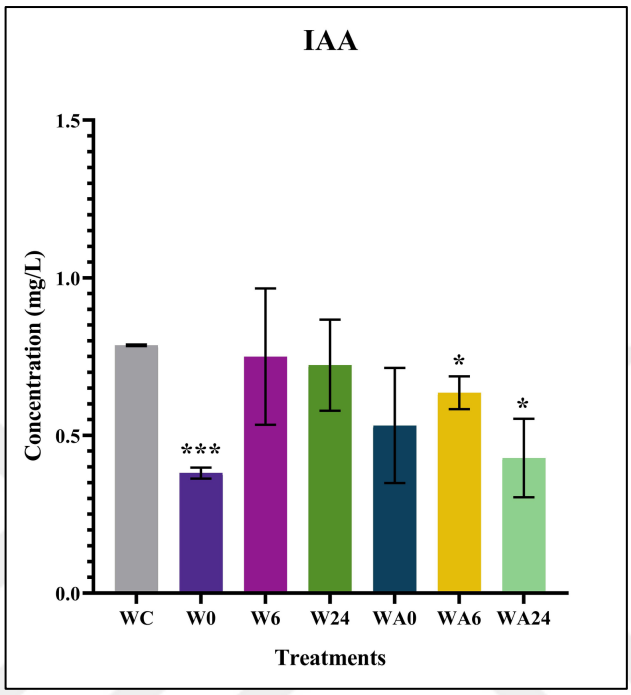

Figure 5.31. IAA levels under mechanical wounding stress in wounded and wound adjacent tissues. Significant at '*' $p<0.1$, '**' $p<0.05$, '***' $p<0.01$.

5.5.2. ABA

ABA is essential for stress response and tolerance, even so it is also referred as stress hormone. So, the ABA levels were monitored under salinity, drought and mechanical wounding stress. A standard curve was plotted for ABA using the peak area obtained from HPLC analysis vs. known concentrations (0.25, 0.5 and 2 mg/L) of ABA (Figure 5.32).

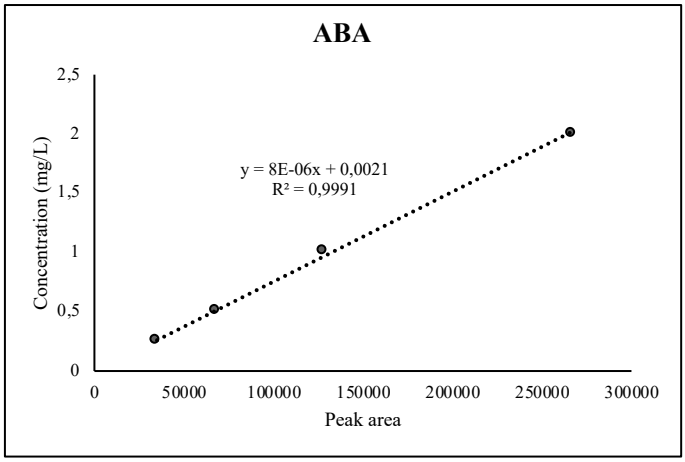

Figure 5.32. Standard curve of ABA.

Marginally significant increase was observed in ABA levels under salinity stress, but drought stress did not change the ABA concentrations (Figure 5.33).

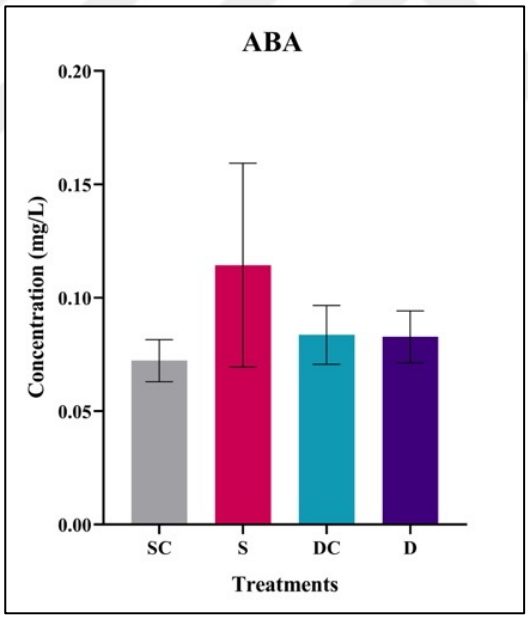

Figure 5.33. ABA levels under salinity and drought stress. Significant at '*' $p<0.1$, '**' $p<0.05$, '***' $p<0.01$.

Under mechanical wounding stress, ABA concentrations were significantly increased at 0^{th} h ($p<0.05$), and marginally increased at 6^{th} and 24^{th} h in wounded tissues. In the wound adjacent tissues, ABA concentrations were higher at 6 and 24 h after wounding than 0 h but none of the fluctuations were significant (Figure 5.34).

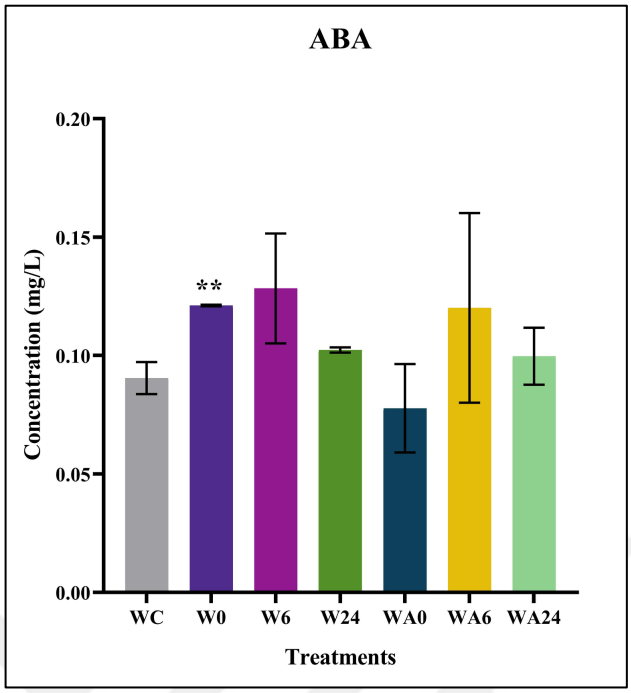

Figure 5.34. ABA levels under mechanical wounding stress in wounded tissues and in wound adjacent tissues. Significant at '*' p<0.1, '**' p<0.05, '***' p<0.01.

5.5.3. SA

Signaling molecule SA that is involved in biotic and abiotic stress tolerance were monitored under various stress treatments: salinity, drought and mechanical wounding. Standard curve for SA was plotted using the peak area obtained from HPLC analysis vs. known concentrations (6, 7, 8 and 9 mg/L) of SA (Figure 5.35).

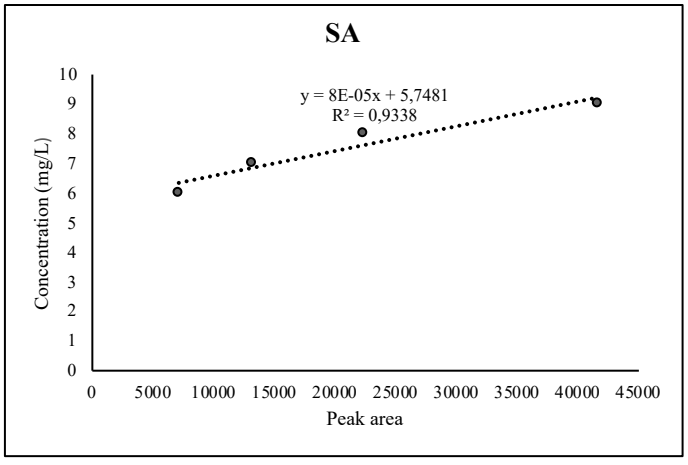

Figure 5.35. Standard curve of SA.

SA levels showed a marginally significant increase upon drought and no significant change was observed under salinity stress. (Figure 5.36).

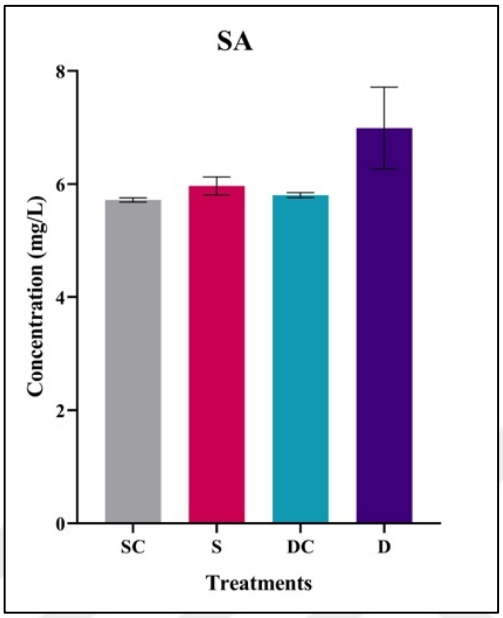

Figure 5.36. SA levels under salinity and drought stress. Significant at '*' $p<0.1$, '**' $p<0.05$, '***' $p<0.01$.

Under mechanical wounding stress, no significant change was observed in SA levels in wounded tissues. There was only a marginally significant increase in SA levels 24 h after wounding in wound adjacent tissues (Figure 5.37).

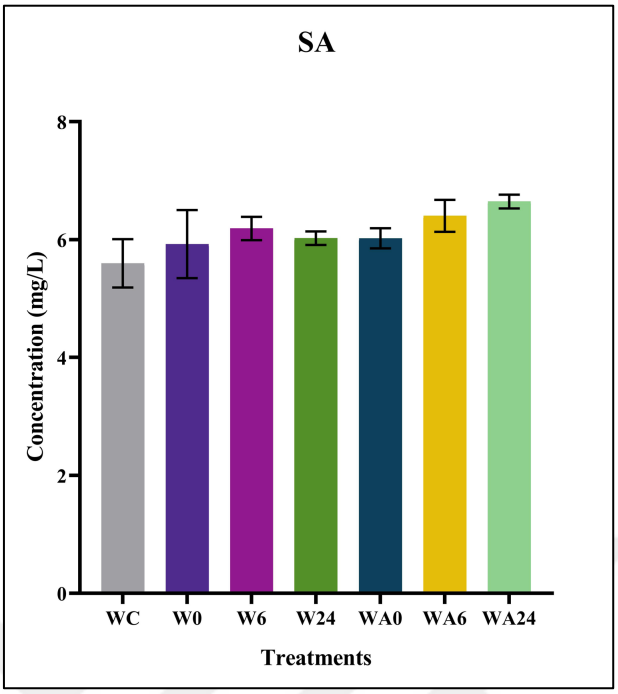

Figure 5.37. SA levels under mechanical wounding stress in wounded tissues and in wound adjacent tissues. Significant at '*' $p<0.1$, '**' $p<0.05$, '***' $p<0.01$.

5.6. THE CHANGES IN PYTOHORMONE LEVELS AND EXPRESSIONS OF STRESS-RESPONSIVE GENES: A SUMMARY

The selected genes for gene expression study were responsive to one or more abiotic stress conditions according to previous studies. The results of our gene expression study showed that those candidate stress-responsive genes were also responsive to the same stress types in *Brachypodium distachyon* (*Bd*) plants except *SUT2* and *ERF1* under drought. Also, *ERF1*, *EXPA2*, *P5CS1*, *PP2CA6*, *WRKY36* were found to be wounding stress-, *EXPA2*, *LOX3*, *PP2CA6* were drought stress-, and *GF14d* and *WRKY36* were salinity stress-responsive in *Brachypodium distachyon* (Table 5.4.).

Table 5.4. Stress-responsiveness of selected genes in this research study compared with previous studies.

	Stress-responsiveness					
	According to previous studies			**According to this study in *Bd***		
Genes	**Drought**	**Salinity**	**Wounding**	**Drought**	**Salinity**	**Wounding**
T. aestivum Ethylene response factor 1 (*TaERF1*)	✓	✓			✓	✓
T. aestivum Expansin A2 (*TaEXPA2*)		✓		✓	✓	✓
B. distachyon Protein G Box Factor 14-3-3 d (*BdGF14d*, *BdGF14-E*)	✓			✓	✓	
A. thaliana Lipoxygenase 3 (*AtLOX3*)		✓	✓	✓	✓	✓
A. thaliana Delta-Pyrroline-5-Carboxylate Synthase 1 (*AtP5CS1*)	✓	✓		✓	✓	✓
B. distachyon Protein phosphatase 2C 6 (*BdPP2CA6*)		✓		✓	✓	✓
A. thaliana Sucrose transporter 2 (*AtSUT2*, *AtSUC3*)	✓	✓	✓		✓	✓
B. distachyon WRKY transcription factor 36 (*BdWRKY36*)	✓			✓	✓	✓

✓: The genes respond to the stress condition above, according to previous studies and this study

✓: The genes respond to stress condition above, according to this study, and have not shown in previous studies.

All the changes observed in transcript levels and phytohormone concentrations of *Brachypodium distachyon* leaves under drought, salinity and time-dependent wounding stress in this study were summarized in Table 5.5.

Table 5.5. Overall gene up- and down-regulations of selected genes, and changes in phytohormone concentrations under each stress treatment in this study.

	Treatments	S	D	W0	W6	W24	WA0	WA6	WA24
Genes	*B. distachyon* Ethylene response factor (*BdERF1*)	↑		↓	↑	↑	↑	↑	↑
	B. distachyon Expansin A2 (*BdEXPA2*)	↑	↑	↑	↑	↑	↑	↑	↑
	B. distachyon Protein G Box Factor 14-3-3 d (*BdGF14d*, *BdGF14-E*)	↑	↑	↓	↑	↑	↓	↓	↑
	B. distachyon Lipoxygenase 3 (*BdLOX3*)	↑	↑	↓	↑	↑	↑	↑	↑
	B. distachyon Delta-Pyrroline-5-Carboxylate Synthase 1 (*BdP5CS1*)	↑	↑	↓	↑	↑	↓	↓	↑
	B. distachyon Protein phosphatase 2C 6 (*BdPP2CA6*)	↑	↑	↓	↑	↑	↓	↑	↑
	B. distachyon Sucrose transporter 2 (*BdSUT2*, *BdSUC3*)	↑		↓	↑		↑	↑	↓
	B. distachyon WRKY transcription factor 36 (*BdWRKY36*)	↑	↑	↑	↓	↑	↓	↑	↑
Phytohormones	IAA		↓	↓				↓	↓
	ABA	↑		↑	↑	↑			
	SA							↑	↑

↑: gene up-regulation / increase in phytohormone concentration

↓: gene down-regulation / decrease in phytohormone concentration

6. DISCUSSION

Due to alterations in the climate and human activities that have an impact on soil quality and plant health, abiotic stress factors threaten food security worldwide [217]. Crop plants are constantly exposed to abiotic stress conditions and suffer from the detrimental effects that lead to low yield and productivity, and threaten plant survival. Plant responses to abiotic stress factors are quite elaborate, comprises many genes, and molecular and biochemical pathways. Investigation of abiotic stress-inducible genes and associated signaling molecules are essential not only to understand stress tolerance mechanisms but also to provide stress-tolerant plants through genetic improvement. Plants possess common genetic mechanisms, and many of the stress-inducible genes are responsive to different stress factors. For instance, many of the drought stress-inducible genes are responsive to salinity, as well [55,218]. Phytohormones are involved in signal transduction pathways associated with abiotic stress responses. Their crosstalk generates synergistic and antagonistic interactions and can quickly alter gene expression levels. Therefore, understanding the phytohormone-mediated regulation of stress responses is important for enhanced stress tolerance [106].

In this study, *B. distachyon*, an important model plant for crop research with an available whole genome sequence was used in gene expression and plant hormone analysis under salinity, drought and time-dependent mechanical wounding stress including local and systemic responses with the aim of determining multiple abiotic stress-responsive genes and the changes in the phytohormones which are the key mediators of abiotic stress responses. Salinity, drought and wounding stress-related genes were obtained from gene databases. Since most of the ortholog gene sequences in *B. distachyon* were not accessible, the obtained sequences from other species such as *O. sativa*, *A. thaliana*, and *T. aestivum* were aligned with *B. distachyon* genome to find orthologous genes. According to alignment scores and possible responsiveness to multiple stress conditions *ERF1*, *EXPA2*, *GF14d*, *LOX3*, *P5CS1*, *PP2CA6*, *SUT2* and *WRKY36* genes were selected for the determination of gene expression patterns under salinity, drought and mechanical wounding stress using qRT-PCR. Changes in IAA, ABA and SA levels were monitored under salinity, drought and wounding stress were determined using HPLC-UV analysis.

ERF1 gene was selected from the ERF transcription factor gene family for gene expression study. *ERF*s play significant roles in various abiotic and biotic stress responses [195]. Since the ethylene hormone is known as one of the vital stress response regulator in plants, ERF transcription factors are induced by stress and take part in signaling pathways through binding GCC-box [195]. Many ERF genes were identified in various plant species such as *O. sativa*, *A. thaliana* and *T. aestivum*, and found to be differentially regulated under abiotic and biotic stress conditions [195,219,220]. The overexpression of various *ERF* genes enhanced abiotic stresses such as salt, drought, heat and cold stress, and also, biotic stresses via providing resistance to various pathogens in *A. thaliana* plants [221]. In an overexpression study, the *T. aestivum ERF1* gene in *A. thaliana* was enhanced under drought, salinity and cold stress [195]. In rice plants, wounding induced the expression of *ERF1*, and *ERF1* expression was also increased slightly upon salinity [220]. Even though previous studies conducted in different plant species showed that the *ERF1* gene was drought-responsive, in this study, gene expression levels of *ERF1* in *B. distachyon* plants were not changed under drought stress conditions. Since *B. distachyon* plants did not undergo domestication, it possesses maximal genetic variability [222]. They have the potential to withstand stresses better than the domesticated cereals. On the other hand, in alignment with previous studies conducted on different plant species [220,224], *ERF1* expression levels were significantly increased 4.27-fold under salinity stress. Following time-dependent mechanical wounding stress, the *ERF1* gene expression levels were significantly decreased 0.41-fold at 0^{th} h. However, a 2.06-fold increase at 6^{th} h and a significant 6.97-fold at 24^{th} h were observed in *ERF1* expression after wounding in wounded tissues. Rather than rapid transcriptomics response, wounding elevated the *ERF1* gene expression levels in the later stages of stress. Also, the increase in the expression levels was observed in a time-dependent manner, while *ERF1* gene expression levels were significantly increased 2.06-fold at 6^{th} h, it was more than tripled at 24^{th} h with a significant 6.97-fold increase. *ERF*s are in an interaction with ABA, JA and ethylene. *ERF1* was reported as a component in the JA signaling pathway in *A. thaliana*, which is a phytohormone rapidly induced upon wounding [223,224]. So, *ERF1* gene products work as key regulators in the defence responses depending on ethylene and jasmonate synthesis. *ERF1* is also a regulator of ABA biosynthesis, but ABA can work as a negative regulator of *ERF1* gene expression [224]. The systemic responses of wounding were different from local responses, in the unwounded tissues adjacent to wound sites, *ERF1* gene expression levels were increased

2.12-fold at 0th h. However, *ERF1* expression was lower with a 1.76-fold increase at 6th h, and a significant 4.41-fold increase was observed in *ERF1* expression at 24th h. Since direct tissue injury causes a rupture in membranes and severe cell damage, local responses are expected to be stronger than systemic ones. Also, wounding causes excessive water loss, therefore, the later *ERF1* gene expression increase might be due to water scarcity in tissues caused by wounding. Overexpression of *ERF1* was also reported to improve expression levels of *P5CS1* and induce proline synthesis, hence provide enhanced abiotic stress tolerance in *A. thaliana* [223].

EXPA2 gene is a member of expansin genes that codes for cell wall loosening proteins, expansins. They provide cell expansion in a non-enzymatic and pH-dependent manner. Since cell walls are barriers that have to interact with environmental stress, they are affected by stress conditions. Expansin genes are often expressed under abiotic stress conditions such as drought, salinity, heat and oxidative stress [225]. Various studies showed that overexpression of expansin genes improved stress tolerance. *Triticum aestivum EXPB23* overexpression improved oxidative and water stress tolerance [226,227], and *TaEXPA2* overexpression improved salt tolerance in tobacco plants [186]. In our study, *Brachypodium distachyon EXPA2* gene expression levels were significantly increased 4.55-fold under salinity stress. Under drought stress conditions, 1.68-fold increase was observed. Mechanical wounding stress also increased the expression levels of *EXPA2* in wounded tissues with a 1.77-fold increase at 0th h and then they were significantly induced 6 h and 24 h after the wounding event with a strong local response of 3.32-fold and 33.36-fold, respectively. Systemic responses were not as strong as local ones, however in the unwounded tissues adjacent to wound sites, gene expression levels of *EXPA2* were increased 1.17-fold at 0th h and 2.91-fold at 6th h. *EXPA2* was significantly up-regulated 9.82-fold at 24th h showing the importance of *EXPA2* gene both at local and systemic wounding response. Although some expansins were studied in different plant species and reported to be involved in abiotic stress response and adaptation mechanisms through mediating cell growth and expansion, the associated mechanisms are still unknown [187]. Under salinity and drought stress conditions, plants undergo dehydration and ion accumulation processes. Therefore, changes in *EXPA2* expression levels could be associated with improved cell wall flexibility provided by expansins. Also, the high expression levels of *EXPA2* under wounding stress might be

explained by the damages in the cell walls of *Brachypodium distachyon* plants and enhanced water loss hours after the injury.

GF14d gene is a member of the *14-3-3* gene family that encodes 14-3-3 proteins. The 14-3-3 family proteins are scaffolding proteins that play vital roles in the regulation of various processes in plants through binding phosphorylated peptides [98,228]. Their interaction with various proteins leads to their participation in many signaling pathways and biosynthesis of important compounds such as phytohormones [229]. 14-3-3 family proteins act as regulators of ABA, IAA and many other phytohormones. They work as a transducer of IAA signaling and key molecules in gene transcription regulated by ABA [229]. Previous studies reported the involvement of *14-3-3* genes in abiotic stress responses by gene expression analysis and generating overexpression mainly in transgenic *Arabidopsis thaliana* plants. While some of the *Arabidopsis thaliana* 14-3-3 family members/ general response factors (*GRF*s) such as *GRF9* improved drought tolerance, other members such as *GFR6* and *GFR8* improved salinity tolerance [230]. Similarly, in a previous study, *Brachypodium distachyon 14-3-3* gene family members were investigated under salinity and drought conditions, and different expression patterns were observed in different isoforms. While PEG-mediated drought application improved *GF14e*, salinity treatment improved *GF14d* expressions [98]. In accordance with the previous study, in our study, the expression levels of *GF14d* was significantly increased 2.83-fold under salinity. However, the findings of our study also indicated that drought stress also increased the *GF14d* expression levels 1.77-fold under drought. This difference might be due to the different methods used to cause stress in plants, and the other parameters such as duration, frequency, and severity of stress, and as wells as sample collection time, which refers to the developmental stage of the plant. While in our study, drought stress treatment conducted by water withhold at the vegetative stage of *Brachypodium distachyon* plant leaf tissues, the previous study used 2-week old seedlings and 3-month old plants, and drought stress was induced by PEG. On the other hand, the wounding responses of *14-3-3* gene family members were unknown. In our study, we investigated the effect of the time-dependent wounding stress as well, including local and systemic responses on the *Brachypodium distachyon GF14d* gene. In the wounded tissues, *GF14d* gene was slightly decreased 0.88-fold at 0^{th} h. However, as time passes, the *GF14d* expression levels were increased 1.19-fold at 6^{th} h and significantly increased 3.17-fold at 24^{th} h. The systemic responses in the unwounded tissues adjacent to wound sites showed that

the gene expression levels of *GF14d* were decreased 0.67-fold at 0th h, 0.70-fold at 6th h, and increased 1.67-fold at 24th h.

LOX gene family members encode the enzymes that catalyse the oxidation reaction of poly-saturated fatty acids into unsaturated fatty acid hydroperoxides [231]. The lipoxygenase pathway is crucial for senescence and defence responses of plants since it regulates lipid peroxidation and produces key products in plant defence such as JA. *LOXs* are up-regulated in wounded tissues and produce a JA dependent response and accumulate ABA. ABA also improves the LOX activity in the case of a mechanical wounding by inducing JA synthesis and lipid peroxide oxidation to cope with stress [232]. Here we selected the *LOX3* gene, which has been reported to be induced under salinity and biotic stress conditions in *Arabidopsis thaliana* [206,207]. In our study, the expression levels of *LOX3* gene was significantly increased 3.63-fold under salinity stress, but only slightly increased 1.09-fold under drought stress conditions. In the local tissues that were directly wounded, *LOX3* expression levels were low, and it was decreased to 0.81-fold at 0th h. So, *LOX3* expression was not affected by the wounding immediately. However, hours after the wounding, the expression levels were rapidly increased. A significant 5.22-fold increase was observed at 6th h, and the expression levels were continued to increase at 24th h with a significant 11.65-fold rise. In the wound adjacent sites, which represent systemic events, interestingly, gene expression levels were significantly increased with 4.46-fold at 0th h, even though no increase was observed at local tissues at 0th h. However, the expression pattern was similar for 6 h and 24 h after wounding. *LOX3* expression levels significantly increased 3.43-fold and 5.18-fold at 6th h and 24th h, respectively.

P5CS1 gene codes for delta1-pyrroline-5-carboxylate synthase, which is a proline biosynthetic enzyme. Since the synthesis and accumulation of proline is part of the adaptive mechanisms of the plants to withstand adverse environmental conditions, *P5CS1* gene overexpression studies have been conducted in *Arabidopsis thaliana* and many other species [233,234]. Studies showed salinity and drought stress-induced expression of *P5CS1*. Besides, *P5CS* mutants showed that *P5CS1* is a key player in proline accumulation under osmotic stress conditions in *Arabidopsis thaliana* [211,212,234]. However, the *P5CS1* gene has not been studied in monocotyledonous plant *Brachypodium distachyon*. To our knowledge, also the mechanical wounding stress response of *P5CS1* gene has not been studied before. The expression levels of *P5CS1* were determined under salinity and drought

conditions. *P5CS1* gene expression levels were significantly increased with 10.44-fold under salinity and 5.95-fold under drought stress. In wounded tissues, the immediate local response of *P5CS1* was down-regulation. The *P5CS1* levels were decreased 0.32-fold at 0^{th} h. But the expression levels were started to increase 1.42-fold at 6^{th} h. The significant 3.24-fold up-regulation of *P5CS1* was observed at 24^{th} h. Concordant with local responses, in unwounded tissues, the systemic responses of *P5CS1* were down-regulation with a 0.36-fold decrease at 0^{th} h and 0.78-fold decrease at 6^{th} h. After 24 h, *P5CS1* gene expression levels were up-regulated with a 1.68-fold increase. This shows that *Brachypodium* plants induced the proline accumulation through the up-regulation of *P5CS1* gene to cope with drought and salinity stress. However, conversely, in the case of mechanical wounding, the *P5CS1* gene expression was down-regulated shortly after wounding and start to increase at the following hours to protect plant from further damage.

PP2CA6 gene is a member of the *PP2C* subfamily that encodes a type 2C phosphatase [99]. They dephosphorylate the serine/threonine residues and inhibit the ABA signal transductions in *Arabidopsis thaliana* [235]. Many studies showed that inhibition of *PP2C* activities positively regulates the signaling pathways associated with abiotic stress responses [99]. However, in contrast with the dicot studies conducted on *PP2C*s, recent studies showed that *PP2C*s positively regulates the abiotic stress responses in monocot species. In *Oryza sativa*, *PP108* gene, a *PP2C* subfamily member, was identified and found to be positively regulated under salinity and drought conditions [236]. In a recent study, *Brachypodium distachyon PP2C* subfamily members were identified as well. Their expression patterns were analysed under salinity and PEG-mediated drought treatments. While *BdPP2CA6* was found to be significantly up-regulated under salinity, *BdPP2CA8* was up-regulated under drought stress. Overexpression of *BdPP2CA6* improved salinity tolerance of *Arabidopsis thaliana* plants [99]. Here, we also investigated *BdPP2CA6* gene expression levels under salinity and drought conditions. But, in addition to those abiotic stresses, we also observed the effect of mechanical wounding on *BdPP2CA6* expression levels. Salinity and drought stress treatment up-regulated the *BdPP2CA6* levels. *BdPP2CA6* expression significantly increased 3.13-fold under salinity and 1.85-fold under drought conditions. In both local and systemic tissues, *BdPP2CA6* expression levels were investigated in a time-dependent manner. *PP2CA6* expression levels were decreased 0.86-fold in mechanically wounded tissues at 0^{th} h. On the other hand, 6 h after wounding a 2.05-fold increase was observed in wounded tissues. Also,

a significant 6.55-fold increase was observed at 24th h. Similar to local tissues, systemic gene responses were low expression levels at 0 h, and higher at the following hours. In wound adjacent tissues, the relative expression levels *PP2CA6* were 0.60-, 1.07- and 3.70-fold at 0th, 6th and 24th h, respectively.

SUT2 or *SUC3* gene encodes sucrose transporters, which play a key role in the export of sucrose from leaves of plants. The allocation and partitioning of sucrose from leaves mostly depend on the photosynthesis and starch metabolism, but also biotic and abiotic stress factors influence the sugar allocation [237]. Exposure to drought and salinity stress has been reported to lead to the up-regulation of *SUT*s. In *Arabidopsis thaliana SUC2, SUC3* and *SUC4* were reported to be induced under salt and drought stress treatments, especially at early stages of the treatments. The expression levels of *SUC3* were not as high as the *SUC2* or *SUC4*. However, *SUC3* interacts with *SUC2* and *SUC4*, and its disruption inhibits the abiotic stress associated sucrose signaling [201]. Wounding stress also enhances the *SUC3* expression levels in *Arabidopsis thaliana*, and the increase was already observed at 3 h after wounding, and it was even detectable at the first hour [202]. The abiotic responses of *SUT*s are unknown in *Brachypodium distachyon* plants. Hence, in our study, *Brachypodium distachyon SUC3* levels were investigated under salinity and drought and mechanical wounding stresses. Salinity stress improved *SUC3* expression levels with a significant 1.85 fold increase, which is very similar to the study conducted on *Arabidopsis thaliana*, and it was reported that the *SUC3* expression levels were increased just above 1.5-fold under salinity stress [201]. However, under drought stress, no *SUC3* expression was observed. Mechanical wounding stress treatment in *Brachypodium distachyon* decreased the *SUC3* levels promptly after wounding in wounded tissues. At 0th h, the *SUC3* expression levels were decreased by 0.29-fold. However, at 6th h, *SUC3* levels were slightly increased 1.12-fold and when 24 h passed after the wounding event, no *SUC3* expression was observed. On the other hand, *SUC3* levels improved by a 2.29-fold increase in wound adjacent tissues at 0th h. *SUC3* expression levels were increased 1.89-fold at 6th h and decreased 0.78-fold at 24th h. These results exhibited that *SUC3* plays a role in salinity and systemic wounding stress responses of *Brachypodium distachyon*. Similar to previous studies, *SUC*s responded to stress conditions in the early stages of the stress and participated in the stress adaptation immediately. Besides, the systemic wounding responses of the *SUC3* gene was stronger than the local ones. It can be suggested that through sucrose signaling induced by local wounding,

unwounded tissues induce sucrose transport quickly through sensing changes to supply required carbohydrates to the cells.

*WRKY*s are transcription factors that have WRKYGQK amino acid sequence at the N-terminus and the C-terminus carrying a zinc-finger motif [238]. Many studies showed the importance of WRKY transcription factors in various molecular processes such as growth, development, and stress response in plants. Even though many *WRKY* genes of *Arabidopsis thaliana* and *Oryza sativa* studied for abiotic stress response, there are only a few characterized *WRKY* genes available for important monocots such as *Triticum aestivum*. In a previous study, a *Brachypodium distachyon WRKY36* gene was characterized. Drought stress-responsiveness was investigated, and *WRKY36* expression levels were found to be enhanced by drought stress but decreased by salinity stress [76]. In our study, *WRKY36* was up-regulated under both salinity and drought. While salinity stress significantly up-regulated the *WRKY36* gene expression levels 2.27-fold, drought stress up-regulated 1.50-fold. *BdWRKY36* expression levels were monitored under mechanical wounding stress for the first time. The expression levels of *WRKY36* were increased 1.75-fold in mechanically wounded tissues at 0^{th} h and slightly decreased by 0.88-fold at 6^{th} h. Mechanical wounding significantly up-regulated the *WRKY36* expression 20.21-fold. In the wound adjacent sites, gene expression levels were decreased 0.49-fold at 0^{th} h, increased 1.60-fold at 6^{th} h, and significantly up-regulated 10.45-fold at 24^{th} h. These results suggested that *WRKY36* not only enhanced drought tolerance in *Brachypodium distachyon* plants but also improved the salinity and mechanical wounding tolerance.

The adaptation processes of plants to stressful conditions involve phytohormones that are the central signaling molecules. The alterations in the environment induce phytohormone synthesis, and they mediate various critical tolerance mechanisms via their cross-talk. The knowledge of the cross-talk and action of phytohormones are mostly supported by the studies carried out on *Arabidopsis thaliana* [131]. Since some processes are not the same in both dicots and monocots, it is necessary to enlighten the phytohormonal stress responses in monocot species. To our knowledge, this is the first research study that monitors phytohormone levels of *Brachypodium distachyon* under abiotic stress. Phytohormone fluctuations of the salinity, drought and mechanical wounding stressed *Brachypodium distachyon* plants were detected using HPLC. Since phytohormones act both synergistically and antagonistically, changes in IAA, ABA and SA were determined. ABA and IAA are

known to work antagonistically in different physiological adjustments upon environmental stress stimuli. For instance, while ABA mediates the stomatal closure, IAA regulates its opening [150]. Hence, quantification of both ABA and IAA would be a relevant tool to detect their cross-talk in the presence of environmental stressors. Drought and salinity stress caused reductions in IAA levels in various plant species such as tomato, rice and soybean [239,240]. Conformably, the present results we obtained in *Brachypodium distachyon* showed a similar pattern. Drought stress significantly reduced the IAA concentrations by 50 percent, and salinity stress led to a marginally significant decrease in IAA concentrations. The decline was also observed in IAA levels promptly right after wounding as a part of the local response. ET and JA are known to be induced under wounding stress conditions. Therefore, the decrease in the IAA levels might be explained through the cross-talk of the hormones. ET and JA hormones may antagonistically affect IAA and suppress its synthesis [241]. In the unwounded tissues, IAA concentrations were reduced at all time points as well. Even though, the lowest concentration was at 0^{th} h, significant changes were observed at 6 and 24 h after wounding as a part of the systemic responses. Both local and systemic responses showed similar trends upon wounding. However, the local IAA responses were quicker and more intense than the systemic ones. That might be due to time passed during the transduction and perception of signals from local parts to systemic parts for the generation of responses [50].

ABA controls many changes in the physiology of the plant and induces stress-responsive genes under stress conditions [158]. ABA concentrations were significantly increased at 0^{th} h and marginally increased at 6 and 24 h after wounding in the wounded tissues. The ABA levels were lower at 24^{th} h than it was at 0 and 6 h after wounding, which might be due to the induced changes for the stress adaptation in the physiology and gene expression levels of stressed plants. Since stress responses were generated as time passes, ABA concentrations were declined to normal levels. ABA concentrations were also marginally increased under salinity stress conditions. ABA plays a critical role in the integration of stress responses under abiotic stress conditions such as drought and salinity that prevent plants from taking up enough water and cause water-deficiency. ABA concentrations increase under abiotic stress, and high ABA concentrations regulate the water status of the plant through the closure of stomata, reduced growth, and induction of stress-responsive genes which code for vital proteins and enzymes for stress tolerance [116].

Although SA levels are known to rise due to environmental stressors, no significant increase was observed in SA levels under any stress. SA is a critical signaling molecule that works in the generation of response abiotic and to biotic stressors, especially herbivores and pathogens. SA is a significant player in wound and pathogen stress-related signaling pathways [138]. However, the present results showed that none of the stress parameters caused a significant upsurge in SA concentrations. Only a marginally significant increase was observed in unwounded tissues of wounded plants at 6^{th} and 24^{th} h. In this study, we measured SA concentrations lower than expected, even though spiking method was used to minimize matrix effect, we suspect that the SA in the sample interacted with other molecules and negatively affected the accuracy of SA measurements. Thus, the measured concentrations were not significantly different from each other, even though a significant increase was expected under abiotic stress conditions, especially wounding. Phytohormones are found in minute amounts in plant tissues, and even the very low concentrations are sufficient for the generation of stress responses. However, the substances that interfere with phytohormone analysis were found in much more higher concentrations in plants, and that makes the analysis challenging. To be able to detect trace amounts of plant hormones with a broad range of features in highly complex matrices, very sensitive and selected detectors were required together with highly efficient extraction techniques.

7. CONCLUSION AND FUTURE PROSPECTS

- Salinity stress significantly up-regulated the expression levels of all selected genes (*ERF1*, *EXPA2*, *GF14d*, *LOX3*, *P5CS1*, *PP2CA6*, *SUT2* and *WRKY36*).
- Drought stress up-regulated the expression levels of all selected genes except for *ERF1* and *SUT2*.
- All the selected genes were significantly up-regulated under mechanical wounding stress at 24th h, except *SUT2*.
- *P5CS1* gene was significantly up-regulated under salinity, drought and mechanical wounding.
- The genes that were up-regulated under salinity, drought and mechanical wounding have a high potential to be used in plant improvement studies for their multiple-stress responsiveness.
- The mechanical wounding stress increased gene expression levels as time passes. The highest fold-changes in gene expression levels were observed 24 h after wounding stress events in both local and systemic tissues when compared to 0th and 6th h.
- *LOX3* gene was highly responsive to mechanical wounding stress at all time points in both local and systemic tissues, except for 0th h in local tissues.
- Phytohormone levels were affected by the stressors. IAA levels were significantly decreased under drought and wounding stress. IAA concentrations were significantly lower in the wounded tissues at 0th h. However, they were increased at 6th h and 24th h after wounding.
- ABA concentrations were significantly increased right after wounding and stayed at high levels at the 6th h. However, they declined to normal levels, 24 h after wounding. Those changes suggest that ABA and IAA rapidly responded to wounding stress, induced signaling pathways, and expression of genes for stress tolerance. Once it is achieved, they returned to normal levels.
- In the unwounded tissues, IAA levels were significantly increased 6 and 24 h after wounding, which showed that local phytohormone responses were much faster and intense than the systemic ones. However, wounding stress still induced the hormone

signaling pathways. Through the perception of signals, systemic responses were constituted as well after a time in wound adjacent tissues.

- Salinity, drought and, time-dependent mechanical wounding stress (both local and systemic) on gene expression levels were investigated in *Brachypodium distachyon* plants for the first time.
- This is the first study that quantifies phytohormones and monitors their concentration changes under abiotic stress conditions in monocot model species *Brachypodium distachyon*.
- The research outputs of this study can be used as a basis for crop improvement studies for abiotic stress tolerance through understanding the changes in expression levels, and direct or indirect involvement of phytohormones to stress defence and adaptations.
- Characterization and overexpression studies of determined genes can pave the way towards generating multiple stress-tolerant crops.

REFERENCES

1. Food and Agriculture Organization of the United Nations (FAO). How to feed the world in 2050. *Insights from an Expert Meet FAO*. 2009;2050(1):1–35.

2. Kellogg EA. The grasses: a case study in macroevolution. *Annual Review of Ecology and Systematics*. 2000;31(1):217–38.

3. Gaut BS. Evolutionary dynamics of grass genomes. *New Phytologist*. 2002;154(1):15–28.

4. Bevan MW, Garvin DF, Vogel JP. *Brachypodium distachyon* genomics for sustainable food and fuel production. *Current Opinion Biotechnology*. 2010;21(2):211–7.

5. Draper J, Mur LAJ, Jenkins G, Ghosh-Biswas GC, Bablak P, Hasterok R, et al. *Brachypodium distachyon* a new model system for functional genomics in grasses. *Plant Physiology*. 2001;127(4):1539–55.

6. Vogel JP. *Genetics and genomics of Brachypodium*. Switzerland: Springer; 2016.

7. Robertson IH. Chromosome numbers in *Brachypodium* Beauv. (Gramineae). *Genetica*. 1981;56(1):55–60.

8. Hasterok R, Draper J. Laying the cytotaxonomic foundations of a new model grass, *Brachypodium distachyon* (L.) Beauv. *Chromosome Research*. 2004; 12(4):397–403.

9. The International *Brachypodium* Initiative. Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature*. 2010;463(7282):763–768.

10. Bossolini E, Wicker T, Knobel PA, Keller B. Comparison of orthologous loci from small grass genomes *Brachypodium* and rice: implications for wheat genomics and grass genome annotation. *The Plant Journal*. 2007;49(4):704–17.

11. Opanowicz M, Vain P, Draper J, Parker D, Doonan JH. *Brachypodium distachyon*: making hay with a wild grass. *Trends in Plant Science*. 2008;13(4):172–7.

12. Buckler ES, Thornsberry JM, Kresovich S. Molecular diversity, structure and domestication of grasses. *Genetics Research*. 2001;77(3):213–8.

13. Girin T, David LC, Chardin C, Sibout R, Krapp A, Ferrario-Méry S, et al. *Brachypodium* : a promising hub between model species and cereals. *Journal of Experimental Botany*. 2014;65(19):5683–6.

14. Brkljacic J, Grotewold E, Scholl R, Mockler T, Garvin DF, Vain P, et al. *Brachypodium* as a model for the grasses: today and the future. *Plant Physiology*. 2011;157(1):3–13.

15. Bevan MW, Garvin DF, Vogel JP. *Brachypodium distachyon* genomics for sustainable food and fuel production. *Current Opninon in Biotechnology*. 2010;21(2):211-7.

16. Kellogg EA. *Brachypodium distachyon* as a genetic model system. *Annual Review of Genetics*. 2015;49(1):1–20.

17. Hayama R. The molecular basis of diversity in the photoperiodic flowering responses of *Arabidopsis* and rice. *Plant Physiology*. 2004;135(2):677–84.

18. Vain P. *Brachypodium* as a model system for grass research. *Journal of Cereal Science*. 2011;54(1):1–7.

19. Ozdemir BS, Hernandez P, Filiz E, Budak H. *Brachypodium* genomics. *International Journal of Plant Genomics*. 2008;(2008): 536-543.

20. Brutnell TP, Bennetzen JL, Vogel JP. *Brachypodium distachyon* and *Setaria viridis* : model genetic systems for the grasses. *Annual Review Plant Biology*. 2015;66(1):465–85.

21. Fisher LH, Han J, Corke FM, Akinyemi A, Didion T, Nielsen KK, et al. Linking dynamic phenotyping with metabolite analysis to study natural variation in drought responses of *Brachypodium distachyon*. *Frontiers in Plant Science*. 2016; 29 (7):1751-66

22. Luo N, Liu J, Yu X, Jiang Y. Natural variation of drought response in *Brachypodium distachyon*. *Physiologia Plantarum*. 2011;141(1):19–29.

23. Chochois V, Vogel JP, Rebetzke GJ, Watt M. Variation in adult plant phenotypes and partitioning among seed and stem-borne roots across *Brachypodium distachyon* accessions to exploit in breeding cereals for well-watered and drought environments. *Plant Physiology*. 2015;168(3):953–67.

24. Nordberg H, Cantor M, Dusheyko S, Hua S, Poliakov A, Shabalov I, et al. The genome portal of the department of energy joint genome institute: 2014 updates. *Nucleic Acids Research*. 2013;42(D1):D26–31.

25. Wei B, Cai T, Zhang R, Li A, Huo N, Li S, et al. Novel microRNAs uncovered by deep sequencing of small RNA transcriptomes in bread wheat (*Triticum aestivum* L.) and *Brachypodium distachyon* (L.) Beauv. *Functional & Integrative Genomics*. 2009;9(4):499–511.

26. Gordon SP, Priest H, Des Marais DL, Schackwitz W, Figueroa M, Martin J, et al. Genome diversity in *Brachypodium distachyon*: deep sequencing of highly diverse inbred lines. *Plant Journal*. 2014;79(3):361–74.

27. Filiz E, Ozdemir BS, Budak F, Vogel JP, Tuna M, Budak H. Molecular, morphological, and cytological analysis of diverse *Brachypodium distachyon* inbred lines. *Genome*. 2009;52(10):876–90.

28. Vogel JP, Tuna M, Budak H, Huo N, Gu YQ, Steinwand MA. Development of SSR markers and analysis of diversity in Turkish populations of *Brachypodium distachyon*. *BMC Plant Biology*. 2009;9(1):88-97.

29. Zwierzykowski Z, Surma M, Kachlicki PJenkins G, Hasterok R DJ. Building the molecular cytogenetic infrastructure of a new model grass. *Application of Novel Cytogenetic and Molecular Techniques in Genetics and Breeding of the Grasses.* 2003:77–84.

30. Vogel JP, Garvin DF, Leong OM, Hayden DM. *Agrobacterium*-mediated transformation and inbred line development in the model grass *Brachypodium*

distachyon. *Plant Cell Tissue Organ Culture*. 2006;84(2):199–211.

31. Vain P, Worland B, Thole V, McKenzie N, Alves SC, Opanowicz M, et al. *Agrobacterium*-mediated transformation of the temperate grass *Brachypodium distachyon* (genotype Bd21) for T-DNA insertional mutagenesis. *Plant Biotechnology Journal*. 2008;6(3):236–45.

32. Vogel J, Hill T. High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3. *Plant Cell Reports*. 2008;27(3):471–8.

33. Păcurar DI, Thordal-Christensen H, Nielsen KK, Lenk I. A high-throughput *Agrobacterium*-mediated transformation system for the grass model species *Brachypodium distachyon* L. *Transgenic Research*. 2008;17(5):965–75.

34. Sogutmaz Ozdemir B, Budak H. Application of tissue culture and transformation techniques in model species *Brachypodium distachyon*. *Brachypodium Genomics: Methods and Protocols*. New York: Humana Press; 2018: 289–310.

35. Bragg JN, Wu J, Gordon SP, Guttman ME, Thilmony R, Lazo GR, et al. Generation and characterization of the western regional research center *Brachypodium* T-DNA insertional mutant collection. *PLoS One*. 2012;7(9):41-91.

36. Shan Q, Wang Y, Chen K, Liang Z, Li J, Zhang Y, et al. Rapid and efficient gene modification in rice and *Brachypodium* using TALENs. *Molecular Plant*. 2013;6(4):1365–8.

37. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012;337(6096):816–21.

38. Farrar K, Donnison IS. Construction and screening of BAC libraries made from *Brachypodium* genomic DNA. *Nature Protocols*. 2007;2(7):1661–74.

39. Garvin DF, McKenzie N, Vogel JP, Mockler TC, Blankenheim ZJ, Wright J, et al. An SSR-based genetic linkage map of the model grass *Brachypodium distachyon*. *Genome*. 2010;53(1):1–13.

40. Zhu JK. Abiotic stress signaling and responses in plants. *Cell*. 2016;167(2):313–24.

41. Bray EA, Baileyserres J, Weretilnyk E. Responses to abiotic stresses. *American Society of Plant Physiology*. 2000;45(54):1158–1203.

42. Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell and Environment*. 2010;33(4):453–67.

43. Zhu J. Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology*. 2002;53(1):247–273.

44. Kavar T, Maras M, Kidrič M, Šuštar-Vozlič J, Meglič V. Identification of genes involved in the response of leaves of *Phaseolus vulgaris* to drought stress. *Molecular Breeding*. 2008;21(2):159–72.

45. Munns R. Comparative physiology of salt and water stress. *Plant Cell and Environment*. 2002;25(2):239–50.

46. Munns R, Tester M. Mechanisms of salinity tolerance. *Annual Review of Plant Biology*. 2008;59(1):651–81.

47. Foyer CH, Fletcher JM. Plant antioxidants: colour me healthy. *Biologist*. 2001;48(3):115–20.

48. Asada K. Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology*. 2006;141(2):391-396.

49. León J, Rojo E, Sánchez-Serrano JJ. Wound signalling in plants. *Journal of Experimental Botany*. 2001;52(354):1–9.

50. Savatin D V, Gramegna G, Modesti V, Cervone F. Wounding in the plant tissue: the defense of a dangerous passage. *Frontiers in Plant Science*. 2014;5(1):470-487.

51. Rehem BC., Bertolde FZ, de Almeida AA. Regulation of gene expression in response to abiotic stress in plants. *Cell Metabolism-Cell Homeostasis and Stress Response*. 2012:82-99.

52. Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*. 2003;218(1):1–14.

53. Hayes M, Svoboda M, Wall N, Widhalm M. The lincoln declaration on drought indices: universal meteorological drought index recommended. *Bulletin of the American Meteorological Society*. 2010;92(4):485–8.

54. Farooq M, Wahid A, Kobayashi N, Fujita D, Basra Smama. Plant drought stress : effects, mechanisms and management. *Agronomy for Sustainable Development*. 2009;29(1):185–212.

55. Seki M, Kamei A, Satou M, Sakurai T, Fujita M, Oono Y, et al. Transcriptome analysis in abiotic stress conditions in higher plants. *Topics in Current Genetics*. 2003;4(1):271–308.

56. Flexas J, Bota J, Loreto F, Cornic G, Sharkey TD. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. *Plant Biology*. 2004;6(3):269–79.

57. Le A. Stomatal responses to drought stress and air humidity. *Abiotic stress in plants-mechanisns and adaptations.* 2011:23-43.

58. Bhargava S, Sawant K. Drought stress adaptation: metabolic adjustment and regulation of gene expression. *Plant Breeding*. 2013;132(1):21–32.

59. Ludlow MM, Muchow RC. A critical evaluation of traits for improving crop yields in water-limited environments. *Advances in agronomy* 1990:107-153.

60. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*. 2004;55(1):373–99.

61. Cruz de Carvalho MH. Drought stress and reactive oxygen species: production, scavenging and signaling. *Plant Signaling and Behavior*. 2008;3(3):156–65.

62. Basu S, Ramegowda V, Kumar A, Pereira A. Plant adaptation to drought stress.

Research. 2016;5(0):1554.

63. Levitt J. Responses of plants to environmental stresses. *Water, radiation, salt, and other stresses.* 1980: 607-626.

64. Morgan JM. Osmoregulation and water stress in higher plants. *Annual Review of Plant Physiology*. 1984;35(1):299–319.

65. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*. 2010;48(12):909–30.

66. Martinelli T, Whittaker A, Bochicchio A, Vazzana C, Suzuki A, Masclaux-Daubresse C. Amino acid pattern and glutamate metabolism during dehydration stress in the 'resurrection' plant *Sporobolus stapfianus*: a comparison between desiccation-sensitive and desiccation-tolerant leaves. *Journal of Experimental Botany*. 2007;58(11):3037–46.

67. Gupta AK, Kaur N. Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *Journal of Biosciences*. 2005;30(5):761–76.

68. Singh M, Kumar J, Singh S, Singh DV, Prasad S. Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. *Reviews in Environmental Science and Bio/Technology*. 2015;14(3):407-426.

69. Smirnoff N. Plant resistance to environmental stress. *Current Opinions in Biotechnology*. 1998;9(2):214–9.

70. Kaur G, Asthir B. Molecular responses to drought stress in plants. *Biologia Plantarum*. 2017;61(2):201–9.

71. You J, Zhang L, Song B, Qi X, Chan Z. Systematic analysis and identification of stress-responsive genes of the NAC gene family in *Brachypodium distachyon*. *PLoS One*. 2015;10(3):1–20.

72. Zhang M, Shen Z, Meng G, Lu Y, Wang Y. Genome-wide analysis of the *Brachypodium distachyon* (L.) P. Beauv. Hsp90 gene family reveals molecular

evolution and expression profiling under drought and salt stresses. *PLoS One*. 2017;12(12):1–18.

73. Wang L, Hu W, Feng J, Yang X, Huang Q, Xiao J, et al. Identification of the ASR gene family from *Brachypodium distachyon* and functional characterization of BdASR1 in response to drought stress. *Plant Cell Reports*. 2016;35(6):1221–34.

74. Cui L, Feng K, Wang M, Wang M, Deng P, Song W, et al. Genome-wide identification, phylogeny and expression analysis of AP2/ERF transcription factors family in *Brachypodium distachyon*. *BMC Genomics*. 2016;17(1):1–19.

75. Chen S, Niu X, Guan Y, Li H. Genome-wide analysis and expression profiles of the MYB genes in *Brachypodium distachyon. Plant Cell Physiology*. 2017;58(10):1777–1788.

76. Sun J, Hu W, Zhou R, Wang L, Wang X, Wang Q, et al. The *Brachypodium distachyon* BdWRKY36 gene confers tolerance to drought stress in transgenic tobacco plants. *Plant Cell Reports*. 2015;34(1):23–35.

77. Liu X, Chu Z. Genome-wide evolutionary characterization and analysis of bZIP transcription factors and their expression profiles in response to multiple abiotic stresses in *Brachypodium distachyon*. *BMC Genomics*. 2015;16(1):227-244.

78. Chen L, Hu W, Tan S, Wang M, Ma Z, Zhou S, et al. Genome-wide identification and analysis of MAPK and MAPKK gene families in *Brachypodium distachyon*. *PLoS One*. 2012;7(10):46744.

79. Subburaj S, Zhu D, Li X, Hu Y, Yan Y. Molecular characterization and expression profiling of *Brachypodium distachyon* L. cystatin genes reveal high evolutionary conservation and functional divergence in response to abiotic stress. *Frontiers in Plant Science*. 2017;8:743-761.

80. Priest HD, Fox SE, Rowley ER, Murray JR, Michael TP, Mockler TC. Analysis of global gene expression in *Brachypodium distachyon* reveals extensive network plasticity in response to abiotic stress. *PLoS One*. 2014;9(1):e87499.

81. Kakei Y, Mochida K, Sakurai T, Yoshida T, Shinozaki K, Shimada Y. Transcriptome

analysis of hormone-induced gene expression in *Brachypodium distachyon*. *Scientific Reports*. 2015;5(1):1–14.

82. Tatli O, Sogutmaz Ozdemir B, Dinler Doganay G. Time-dependent leaf proteome alterations of *Brachypodium distachyon* in response to drought stress. *Plant Molecular Biology*. 2017;94(6):609–623.

83. Bian Y, Deng X, Yan X, Zhou J, Yuan L, Yan Y. Integrated proteomic analysis of *Brachypodium distachyon* roots and leaves reveals a synergistic network in the response to drought stress and recovery. *Scientific Reports*. 2017;7:1–15.

84. Onda Y, Hashimoto K, Yoshida T, Sakurai T, Sawada Y, Hirai MY, et al. Determination of growth stages and metabolic profiles in *Brachypodium distachyon* for comparison of developmental context with Triticeae crops. *Proceedings of the Royal Society B: Biological Sciences*. 2015;282(1811):20150964.

85. Shi H, Jiang C, Ye T, Tan DX, Reiter RJ, Zhang H, et al. Comparative physiological, metabolomic, and transcriptomic analyses reveal mechanisms of improved abiotic stress resistance in bermudagrass [*Cynodon dactylon* (L). Pers.] by exogenous melatonin. *Journal of Experimental Botany*. 2015;66(3):681–694.

86. Bertolini E, Verelst W, Horner DS, Gianfranceschi L, Piccolo V, Inzé D, et al. Addressing the role of microRNAs in reprogramming leaf growth during drought stress in *Brachypodium distachyon. Molecular Plant*. 2013;6(2):423–443.

87. Jeong D-H, Schmidt SA, Rymarquis LA, Park S, Ganssmann M, German MA, et al. Parallel analysis of RNA ends enhances global investigation of microRNAs and target RNAs of *Brachypodium distachyon*. *Genome Biology*. 2013;14(12):145–145.

88. Franke KR, Schmidt SA, Park S, Jeong D-H, Accerbi M, Green PJ. Analysis of *Brachypodium* miRNA targets: evidence for diverse control during stress and conservation in bioenergy crops. *BMC Genomics*. 2018;19(1):547-565.

89. Jiang Y, Wang X, Yu X, Zhao X, Luo N, Pei Z, et al. Quantitative trait loci associated with drought tolerance in *Brachypodium distachyon*. *Frontiers in Plant Science*. 2017;(8):811-822.

90. Eynard A, Lal R, Wiebe K. Crop response in salt-affected soils. *Journal of Sustainable Agriculture*. 2005;27(1):5–50.

91. Flowers TJ, Colmer TD. Salinity tolerance in halophytes. *New Phytologist*. 2008;179(4):945–963.

92. Brouwer C, Goffeau A, Heibloem M. Irrigation water management: training manual No. 1 - Introduction to irrigation. *Food and Agriculture Organization of the United Nations,* Rome, Italy. 1985; 102-103.

93. Bartels D, Sunkar R. Drought and salt tolerance in plants. *Criticals Reviews in Plant Sciences*. 2005;24(1):23–58.

94. Gupta B, Huang B. Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *International Journal of Genomics*. 2014;(2014):1–18.

95. Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ. Plant cellular and molecular responses to high salinity. *Annual Review of Plant Biology*. 2000;51(1):463–499.

96. James RA, Blake C, Byrt CS, Munns R. Major genes for Na+ exclusion, Nax1 and Nax2 (wheat HKT1;4 and HKT1;5), decrease Na+ accumulation in bread wheat leaves under saline and waterlogged conditions. *Journal of Experimental Botany*. 2011;62(8):2939–2947.

97. Evelin H, Kapoor R, Giri B. Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. *Annals of Botany*. 2009;104(7):1263–1280.

98. He Y, Zhang Y, Chen L, Wu C, Luo Q, Zhang F, et al. A member of the 14-3-3 gene family in *Brachypodium distachyon*, BdGF14d, confers salt tolerance in transgenic tobacco plants. *Frontiers in Plant Science*. 2017;13(8):340-353.

99. Zhang F, Wei Q, Shi J, Jin X, He Y, Zhang Y, et al. *Brachypodium distachyon* BdPP2CA6 interacts with BdPYLs and BdSnRK2 and positively regulates salt tolerance in transgenic *Arabidopsis. Frontiers in Plant Science*. 2017;28(8):264-279

100. Reymond P, Weber H, Damond M, Farmer EE . Differential gene expression in

response to mechanical wounding and insect feeding in *Arabidopsis*. *The Plant Cell*. 2000;12(5):707–720.

101. Maffei ME, Mithöfer A, Boland W. Before gene expression: early events in plant-insect interaction. *Trends in Plant Science*. 2007;12(7):310–316.

102. Rojo E, Solano R, Sánchez-Serrano JJ. Interactions between signaling compounds involved in plant defense. *Journal of Plant Growth Regulation*. 2003;22(1):82–98.

103. Mur LAJ, Xu R, Casson SA, Stoddart WM, Routledge APM, Draper J. Characterization of a proteinase inhibitor from *Brachypodium distachyon* suggests the conservation of defence signalling pathways between dicotyledonous plants and grasses. *Molecular Plant Pathology*. 2004;5(4):267–280.

104. Botella MA, Rosado A, Bressan RA, Hasegawa PM. Plant adaptive responses to salinity stress. *Plant Abiotic Stress*. 2005:37–70.

105. Lata C, Yadav A, Pras M. Role of plant transcription factors in abiotic stress tolerance. *Abiotic Stress Response Plants, INTECH Open Access Publishers.* 2011;(10):269-299.

106. Blumwald E. Sodium transport and salt tolerance in plants. *Current Opinion in Cell Biology*. 2000;12(4):431–434.

107. Ciarmiello L, Woodrow P, Fuggi A, Pontecorvo G, Carillo P. Plant genes for abiotic stress. *Abiotic Stress in Plants-Mechanisms and Adaptations*. 2011: 283-308.

108. Delauney AJ, Verma DPS. Proline biosynthesis and osmoregulation in plants. *The Plant Journal*. 1993;4(2):215–223.

109. Takahashi F, Mizoguchi T, Yoshida R, Ichimura K, Shinozaki K. Calmodulin-dependent activation of MAP kinase for ROS homeostasis in *Arabidopsis*. *Molecular Cell*. 2011;41(6):649–660.

110. Fraire-Velázquez S, Rodriguez Guerra R, Sánchez-Calderón L. Abiotic and biotic stress response crosstalk in plants. *Abiotic Stress Response in Plants-Physiological, Biochemical and Genetic Perspectives.* 2011;(29):346-350.

111. Cui L, Feng K, Wang M, Wang M, Deng P, Song W, et al. Genome-wide identification, phylogeny and expression analysis of AP2/ERF transcription factors family in *Brachypodium distachyon*. *BMC Genomics*. 2016;17(1):636-643.

112. Cao J, Jiang M, Li P, Chu Z. Genome-wide identification and evolutionary analyses of the PP2C gene family with their expression profiling in response to multiple stresses in *Brachypodium distachyon*. *BMC Genomics*. 2016;17(1):1–17.

113. Nambara E, Marion-Poll A. Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology*. 2005;56:165–185.

114. Bari R, Jones JDG. Role of plant hormones in plant defence responses. *Plant Molecular Biology*. 2009;69(4):473–488.

115. Nguyen D, Rieu I, Mariani C, van Dam NM. How plants handle multiple stresses: hormonal interactions underlying responses to abiotic stress and insect herbivory. *Plant Molecular Biology*. 2016;91(6):727–740.

116. Wani SH, Kumar V, Shriram V, Sah SK. Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *The Crop Journal*. 2016;4(3):162–176.

117. Nemhauser JL, Hong F, Chory J. Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell*. 2006;126(3):467–475.

118. Birkenmeier GF, Ryan CA. Wound signaling in tomato plants: evidence that ABA is not a primary signal for defense gene activation. *Plant Physiology*. 1998;117(2):687-693.

119. Wilkinson S, Kudoyarova GR, Veselov DS, Arkhipova TN, Davies WJ, Zhang J, et al. Plant hormone interactions: innovative targets for crop breeding and management. *Journal Experimental Botany*. 2012;63(9):3499–509.

120. Sreenivasulu N, Harshavardhan VT, Govind G, Seiler C, Kohli A. Contrapuntal role of ABA: does it mediate stress tolerance or plant growth retardation under long-term drought stress?.*Gene*. 2012;506(2):265–73.

121. Petersson S V, Johansson AI, Kowalczyk M, Makoveychuk A, Wang JY, Moritz T, et al. An auxin gradient and maximum in the *Arabidopsis*; root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. *The Plant Cell*. 2009;21(6):1659-1668.

122. Tanaka Y, Uritani I. Effect of auxin and other hormones on the metabolic response to wounding in sweet potato roots. *Plant and Cell Physiology*. 1979;20(8):1557–1564.

123. Fayez KA, Bazaid SA. Improving drought and salinity tolerance in barley by application of salicylic acid and potassium nitrate. *Journal of Saudi Society of Agricultural Sciences*. 2014;13(1):45–55.

124. Khan MIR, Fatma M, Per TS, Anjum NA, Khan NA. Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. *Frontiers in Plant Science*. 2015;(6):462-473.

125. Verma V, Ravindran P, Kumar PP. Plant hormone-mediated regulation of stress responses. *BMC Plant Biology*. 2016;16(1):86-96.

126. Ma X, Zheng J, Zhang X, Hu Q, Qian R. Salicylic acid alleviates the adverse effects of salt stress on *Dianthus superbus* (Caryophyllaceae) by activating photosynthesis, protecting morphological structure, and enhancing the antioxidant system. *Frontiers Plant Science*. 2017;8:600-613.

127. Farmer EE, Ryan CA. Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proceedings of the National Academy of Sciences*. 1990;87(19):7713–7716.

128. Howe GA, Lightner J, Browse J, Ryan CA. An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. *Plant Cell*. 1996;8(11):2067–77.

129. Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, et al. The JAZ family of repressors is the missing link in jasmonate signalling. *Nature*. 2007;448(7154):666–71.

130. Vijayan P, Shockey J, Lévesque CA, Cook RJ, Browse J. A role for jasmonate in

pathogen defense of *Arabidopsis*. *Proceedings of the National Academy of Sciences.* 1998;95(12):7209–7214.

131. Peleg Z, Blumwald E. Hormone balance and abiotic stress tolerance in crop plants. *Current Opinion in Plant Biology*. 2011;14(3):290–295.

132. Frigerio M, Alabadí D, Pérez-Gómez J, García-Cárcel L, Phillips AL, Hedden P, et al. Transcriptional regulation of gibberellin metabolism genes by auxin signaling in *Arabidopsis*. *Plant Physiology*. 2006;142(2):553-563.

133. Müller M, Munné-Bosch S, Pan X, Welti R, Wang X, Dun E, et al. Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Plant Methods*. 2011;7(1):37-49.

134. Hossain MA, Munemasa S, Uraji M, Nakamura Y, Mori IC, Murata Y. Involvement of endogenous abscisic acid in methyl jasmonate-induced stomatal closure in *Arabidopsis*. *Plant Physiology*. 2011;156(1):430–438.

135. Kohli A, Sreenivasulu N, Lakshmanan P, Kumar PP. The phytohormone crosstalk paradigm takes center stage in understanding how plants respond to abiotic stresses. *Plant Cell Reports*. 2013;32(7):945–957.

136. Schweiger R, Heise A-M, Persicke M, Muller C. Interactions between the jasmonic and salicylic acid pathway modulate the plant metabolome and affect herbivores of different feeding types. *Plant, Cell & Environment*. 2014;37(7):1574–1585.

137. Van der Does D, Leon-Reyes A, Koornneef A, Van Verk MC, Rodenburg N, Pauwels L, Goossens A, Körbes AP, Memelink J, Ritsema T, Van Wees SC. Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *The Plant Cell*. 2013;25(2):744–761.

138. Tarkowská D, Novák O, Floková K, Tarkowski P, Turečková V, Grúz J, et al. Quo vadis plant hormone analysis?. *Planta*. 2014;240(1):55–76.

139. Du F, Ruan G, Liu H. Analytical methods for tracing plant hormones. *Analytical and Bioanalytical Chemistry*. 2012;403(1):55–74.

140. Hedden P. Modern methods for the quantitative analysis of plant hormones. *Annual Review of Plant Biologyl*. 1993;44(1):107–129.

141. Weiler EW. Immunoassay of plant growth regulators. *Annual Review Plant Physiology*. 1984;35(1):85–95.

142. Pan X, Welti R, Wang X. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. *Nature Protocols.* 2010;5(6):986–92.

143. Imbault N, Moritz T, Nilsson O, Chen H-J, Bollmark M, Sandberg G. Separation and identification of cytokinins using combined capillary liquid chromatography/mass spectrometry. *Biological Mass Spectrometry*. 1993;22(3):201–10.

144. Jensen E, Ernstsen A, Sandberg G. Analysis of picogram quantities of indole-3-acetic acid by gas chromatography with fused silica column and flameless nitrogen selective detector. *Plant Growth Regulation.* 1986;4(1):55–63.

145. Muller A, Duchting P, Weiler EW. A multiplex GC-MS/MS technique for the sensitive and quantitative single-run analysis of acidic phytohormones and related compounds, and its application to *Arabidopsis thaliana*. *Planta*. 2002;216(1):44–56.

146. Engelberth J, Schmelz EA, Alborn HT, Cardoza YJ, Huang J, Tumlinson JH. Simultaneous quantification of jasmonic acid and salicylic acid in plants by vapor-phase extraction and gas chromatography-chemical ionization-mass spectrometry. *Analytical Biochemistry*. 2003;312(2):242–50.

147. Durgbanshi A, Arbona V, Pozo O, Miersch O, Sancho J V, Gómez-Cadenas A. Simultaneous determination of multiple phytohormones in plant extracts by liquid chromatography−electrospray tandem mass spectrometry.
Journal of *Agricultural and Food Chemistry*. 2005;53(22):8437–42.

148. Wu Y, Hu B. Simultaneous determination of several phytohormones in natural coconut juice by hollow fiber-based liquid-liquid-liquid microextraction-high performance liquid chromatography. *Journal of Chromatography A*. 2009;1216(45):7657–63.

149. Zhang Y, Li Y, Hu Y, Li G, Chen Y. Preparation of magnetic indole-3-acetic acid imprinted polymer beads with 4-vinylpyridine and beta-cyclodextrin as binary monomer via microwave heating initiated polymerization and their application to trace analysis of auxins in plant tissues. *Journal of Chromatography A*. 2010;1217(47):7337–44.

150. Nakurte I, Keisa A, Rostoks N. Development and validation of a reversed-phase liquid chromatography method for the simultaneous determination of indole-3-acetic acid, indole-3-pyruvic acid, and abscisic acid in Barley (*Hordeum vulgare* L.). *Journal of Analytical Methods in Chemistry*. 2012;1088(1-2):49-56.

151. Wurst M, Přikryl Z, Vokoun J. High-performance liquid chromatography of plant hormones: II. Determination of plant hormones of the indole type. *Journal of Chromatography A*. 1984;286:(23): 237–45.

152. Dobrev PI, Havlíček L, Vágner M, Malbeck J, Kamínek M. Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography. *Journal of Chromatography A*. 2005;1075(1–2):159–66.

153. Lu Q, Chen L, Lu M, Chen G, Zhang L. Extraction and analysis of auxins in plants using dispersive liquid-liquid microextraction followed by high-performance liquid chromatography with fluorescence detection. *Journal* of *Agricultural and Food Chemistry*. 2010;58(5):2763–70.

154. Gupta V, Kumar M, Brahmbhatt H, Reddy CRK, Seth A, Jha B. Simultaneous determination of different endogenetic plant growth regulators in common green seaweeds using dispersive liquid-liquid microextraction method. *Plant Physiology and Biochemistry*. 2011;49(11):1259–63.

155. Almeida Trapp M, De Souza GD, Rodrigues-Filho E, Boland W, Mithöfer A. Validated method for phytohormone quantification in plants. *Frontiers in Plant Science*. 2014;5(8):1–11.

156. Chiwocha SDS, Abrams SR, Ambrose SJ, Cutler AJ, Loewen M, Ross ARS, et al. A method for profiling classes of plant hormones and their metabolites using liquid

chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. *Plant Journal*. 2003;35(3):405–17.

157. Forcat S, Bennett MH, Mansfield JW, Grant MR. A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in plants following biotic and abiotic stress. *Plant Methods*. 2008;4(1):1–8.

158. Kasote DM, Ghosh R, Chung JY, Kim J, Bae I, Bae H. Multiple reaction monitoring mode based liquid chromatography-mass spectrometry method for simultaneous quantification of brassinolide and other plant hormones involved in abiotic stresses. *International Journal* of *Analytical Chemistry*. 2016;(2016): 150-58.

159. Mahouachi J, López-Climent MF, Gómez-Cadenas A. Hormonal and hydroxycinnamic acids profiles in banana leaves in response to various periods of water stress. *Science World Journal*. 2014;(2014):1-9

160. Chiwocha SDS, Abrams SR, Ambrose SJ, Cutler AJ, Loewen M, Ross ARS, et al. A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. *Plant Journal*. 2003;35(3):405–17.

161. Pan X, Welti R, Wang X. Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography-electrospray tandem mass spectrometry. *Phytochemistry*. 2008;69(8):1773–81.

162. Kojima M, Kamada-Nobusada T, Komatsu H, Takei K, Kuroha T, Mizutani M, et al. Highly sensitive and high-throughput analysis of plant hormones using ms-probe modification and liquid chromatographytandem mass spectrometry: an application for hormone profiling in *Oryza sativa*. *Plant and Cell Physiology*. 2009;50(7):1201–14.

163. Matsuura H, Aoi A, Satou C, Nakaya M, Masuta C, Nabeta K. Simultaneous UPLC MS/MS analysis of endogenous jasmonic acid, salicylic acid, and their related compounds. *Plant Growth Regulation*. 2009;57(3):293–301.

164. Giannarelli S, Muscatello B, Bogani P, Spiriti MM, Buiatti M, Fuoco R. Comparative determination of some phytohormones in wild-type and genetically modified plants by gas chromatography-mass spectrometry and high-performance liquid chromatography-tandem mass spectrometry. *Analytical Biochemistry*. 2010;398(1):60–8.

165. Balcke GU, Handrick V, Bergau N, Fichtner M, Henning A, Stellmach H, et al. An UPLC-MS/MS method for highly sensitive high-throughput analysis of phytohormones in plant tissues. *Plant Methods*. 2012;8:(47):100-111.

166. Liu H, Li X, Xiao J, Wang S. A convenient method for simultaneous quantification of multiple phytohormones and metabolites: application in study of rice-bacterium interaction. *Plant Methods*. 2012;8(2):1127-1139

167. Eroglu CG. Physiological and genetic studies on *Brachypodium distachyon* and *Triticum aestivum* under salinity stress. İstanbul. Yeditepe University; 2016.

168. Hong S-Y, Seo PJ, Yang M-S, Xiang F, Park C-M. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. *BMC Plant Biology*. 2008;8:(112):321-332

169. Urao T, Katagiri T, Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N, Shinozaki K. Two genes that encode Ca2+-dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana*. *Molecular and General Genetics*. 1994;244(4):331–40.

170. Xu J, Tian YS, Peng RH, Xiong AS, Zhu B, Jin XF, et al. AtCPK6, a functionally redundant and positive regulator involved in salt/drought stress tolerance in *Arabidopsis*. *Planta*. 2010;231(6):1251–60.

171. Jayasekaran K, Kim KN, Vivekanandan M, Shin JS, Ok SH. Novel calcium-binding GTPase (AtCBG) involved in ABA-mediated salt stress signaling in *Arabidopsis*. *Plant Cell Reports*. 2006;25(11):1255–62.

172. Qiu J-L, Zhou L, Yun B-W, Nielsen HB, Fiil BK, Petersen K, et al. *Arabidopsis* Mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping

functions in defense signaling sediated by MEKK1, MPK4, and MKS1. *Plant Physiology*. 2008;148(1):212–22.

173. Ichimura K, Mizoguchi T, Yoshida R, Yuasa T, Shinozaki K. Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. *Plant Journal*. 2000;24(5):655–65.

174. Kwon YR, Kim SH, Jung MS, Kim MS, Oh JE, Ju HW, et al. *Arabidopsis* hot2 encodes an endochitinase-like protein that is essential for tolerance to heat, salt and drought stresses. *Plant Journal*. 2007;49(2):184–93.

175. Zhang S-S, Sun L, Dong X, Lu S-J, Tian W, Liu J-X. Cellulose synthesis genes CESA6 and CSI1 are important for salt stress tolerance in *Arabidopsis*. *Journal of Integrative Plant Biology*. 2016;58(7):623–6.

176. Wang C, Jing R, Mao X, Chang X, Li A. TaABC1, a member of the activity of bc1complex protein kinase family from common wheat, confers enhanced tolerance to abiotic stresses in *Arabidopsis*. *Journal of Experimental Botany*. 2011;62(3):1299–311.

177. Schilmiller AL, Koo AJK, Howe GA. Functional Diversification of Acyl-Coenzyme A Oxidases in Jasmonic Acid Biosynthesis and Action. *Plant Physiology*. 2006;143(2):812–24.

178. Castaings L, Camargo A, Pocholle D, Gaudon V, Texier Y, Boutet-Mercey S, et al. The nodule inception-like protein 7 modulates nitrate sensing and metabolism in *Arabidopsis*. *Plant Journal*. 2009;57(3):426–35.

179. Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-lefert P. An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. *The Plant Cell.* 2003;15(11):2503–13.

180. Barratt DHP, Kolling K, Graf A, Pike M, Calder G, Findlay K, et al. Callose synthase GSL7 is necessary for normal phloem transport and inflorescence growth in *Arabidopsis*. *Plant Physiology*. 2011;155(1):328–41.

181. Cheong YH, Kim K-N, Pandey GK, Gupta R, Grant JJ, Luan S. CBL1, a calcium

sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. *The Plant Cell*. 2003;15(8):1833–1845.

182. Kim K-N, Cheong YH, Grant JJ, Pandey GK, Luan S. CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. *The Plant Cell*. 2003;15(2):411–23.

183. Deng X, Hu W, Wei S, Zhou S, Zhang F, Han J, et al. TaCIPK29, a CBL-interacting protein kinase gene from wheat, confers salt stress tolerance in transgenic tobacco. *PLoS One*. 2013;8(7).

184. Xiang Y, Hai Lu Y, Song M, Wang Y, Xu W, Wu L, et al. Overexpression of a *Triticum aestivum* calreticulin gene (TaCRT1) improves salinity tolerance in tobacco. *PLoS One*. 2015;10(10):1–20.

185. Kim JH, Nguyen NH, Nguyen NT, Hong S-W, Lee H. Loss of all three calreticulins, CRT1, CRT2 and CRT3, causes enhanced sensitivity to water stress in *Arabidopsis*. *Plant Cell Reports*. 2013;32(12):1843–53.

186. Chen Y, Han Y, Kong X, Kang H, Ren Y, Wang W. Ectopic expression of wheat expansin gene TaEXPA2 improved the salt tolerance of transgenic tobacco by regulating Na(+) /K(+) and antioxidant competence. *Physiologia Plantarum*. 2017;159(2):161–77.

187. Li F, Xing S, Guo Q, Zhao M, Zhang J, Gao Q, et al. Drought tolerance through over-expression of the expansin gene TaEXPB23 in transgenic tobacco. *Journal of Plant Physiology*. 2011;168(9):960–6.

188. Mao X, Zhang H, Qian X, Li A, Zhao G, Jing R. TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in *Arabidopsis*. *Journal of Experimental Botany*. 2012;63(8):2933–46.

189. Xue GP, Way HM, Richardson T, Drenth J, Joyce PA, McIntyre CL. Overexpression of TaNAC69 leads to enhanced transcript levels of stress up-regulated genes and dehydration tolerance in bread wheat. *Molecular Plant*. 2011;4(4):697–712.

190. Cai H, Tian S, Liu C, Dong H. Identification of a MYB3R gene involved in drought,

salt and cold stress in wheat (*Triticum aestivum* L.). *Gene*. 2011;485(2):146–52.

191. Zhang L, Zhao G, Xia C, Jia J, Liu X, Kong X. A wheat R2R3-MYB gene, TaMYB30-B, improves drought stress tolerance in transgenic *Arabidopsis*. *Journal of Experimental Botany*. 2012;63(16):5873–85.

192. Zhao Y, Cheng X, Liu X, Wu H, Bi H, Xu H. The wheat MYB transcription factor TaMYB31 is involved in drought stress responses in *Arabidopsis*. *Frontiers in plant science*. 2018;9(9):1–12.

193. Qin Y, Wang M, Tian Y, He W, Han L, Xia G. Over-expression of TaMYB33 encoding a novel wheat MYB transcription factor increases salt and drought tolerance in *Arabidopsis*. *Molecular Biology Reports*. 2012;39(6):7183–92.

194. Rong W, Qi L, Wang A, Ye X, Du L, Liang H, et al. The ERF transcription factor TaERF3 promotes tolerance to salt and drought stresses in wheat. *Plant Biotechnology Journal*. 2014;12(4):468–79.

195. Xu ZS, Xia LQ, Chen M, Cheng XG, Zhang RY, Li LC, et al. Isolation and molecular characterization of the *Triticum aestivum* L. ethylene-responsive factor 1 (*TaERF1*) that increases multiple stress tolerance. *Plant Molecular Biology.* 2007;65(6):719–32.

196. Wu JX, Li J, Liu Z, Yin J, Chang ZY, Rong C, et al. The *Arabidopsis ceramidase* AtACER functions in disease resistance and salt tolerance. *The Plant Journal*. 2015;81(5):767–80.

197. Roosens NH, Thu TT, Iskandar HM, Jacobs M. Isolation of the ornithine-delta-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. *Plant physiology*. 1998;117(1):263–71.

198. Zhong R, Ye Z-H. Molecular and biochemical characterization of three WD-repeat-domain-containing inositol polyphosphate 5-phosphatases in *Arabidopsis thaliana*. *Plant and Cell Physiology*. 2004;45(11):1720–8.

199. Meyer A, Eskandari S, Grallath S, Rentsch D. AtGAT1, a high affinity transporter for gamma-aminobutyric acid in *Arabidopsis thaliana*. *Journal of Biological Chemistry*. 2006;281(11):7197–204.

200. Poschet G, Hannich B, Raab S, Jungkunz I, Klemens PAW, Krueger S, et al. A novel *Arabidopsis* vacuolar glucose exporter is involved in cellular sugar homeostasis and affects the composition of seed storage compounds. *Plant physiology*. 2011;157(4):1664–76.

201. Gong X, Liu M, Zhang L, Ruan Y, Ding R, Ji Y, et al. *Arabidopsis* AtSUC2 and AtSUC4, encoding sucrose transporters, are required for abiotic stress tolerance in an ABA-dependent pathway. *Physiologia Plantarum*. 2015;153(1):119–36.

202. Meyer S, Lauterbach C, Niedermeier M, Barth I, Sjolund RD, Sauer N. Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. *Plant Physiology*. 2004;134(2):684–93.

203. Lemonnier P, Gaillard C, Veillet F, Verbeke J, Lemoine R, Coutos-Thevenot P, et al. Expression of *Arabidopsis* sugar transport protein STP13 differentially affects glucose transport activity and basal resistance to *Botrytis cinerea*. *Plant Molecular Biology*. 2014;85(4–5):473–84.

204. Truernit E, Schmid J, Epple P, Illig J, Sauer N. The sink-specific and stress-regulated *Arabidopsis* STP4 gene: enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge. *Plant Cell*. 1996;8(12):2169–82.

205. Nishiuchi T, Hamada T, Kodama H, Iba K. Wounding changes the spatial expression pattern of the *Arabidopsis* plastid omega-3 fatty acid desaturase gene (FAD7) through different signal transduction pathways. *Plant Cell*. 1997;9(10):1701–12.

206. Ding H, Lai J, Wu Q, Zhang S, Chen L, Dai Y-S, et al. Jasmonate complements the function of *Arabidopsis* lipoxygenase3 in salinity stress response. *Plant Science.* 2016;244(3):1–7.

207. Ozalvo R, Cabrera J, Escobar C, Christensen SA, Borrego EJ, Kolomiets M V, et al. Two closely related members of *Arabidopsis* 13-lipoxygenases (13-LOXs), LOX3 and LOX4, reveal distinct functions in response to plant-parasitic nematode infection. *Molecular Plant Pathology*. 2014;15(4):319–32.

208. Zhou S, Jia L, Chu H, Wu D, Peng X, Liu X, et al. *Arabidopsis* CaM1 and CaM4 promote nitric oxide production and salt resistance by inhibiting S-nitrosoglutathione reductase via direct binding *PLoS Genetics*. 2016;12(9):625-53.

209. Yuenyong W, Chinpongpanich A, Comai L, Chadchawan S, Buaboocha T. Downstream components of the calmodulin signaling pathway in the rice salt stress response revealed by transcriptome profiling and target identification. *BMC Plant Biology*. 2018;18(1):335.

210. Mizoguchi T, Hayashida N, Yamaguchi-Shinozaki K, Kamada H, Shinozaki K. Two genes that encode ribosomal-protein S6 kinase homologs are induced by cold or salinity stress in *Arabidopsis thaliana*. *Febs Letters*. 1995;358(2):199–204.

211. Fu Y, Ma H, Chen S, Gu T, Gong J. Control of proline accumulation under drought via a novel pathway comprising the histone methylase CAU1 and the transcription factor ANAC055. *Journal of Experimental Botany*. 2017;69(3):579–88.

212. Feng XJ, Li JR, Qi SL, Lin QF, Jin JB, Hua XJ. Light affects salt stress-induced transcriptional memory of P5CS1 in *Arabidopsis*. *Proceedings of the National Academy of Sciences*. 2016;113(51):8335–43.

213. Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, et al. Differential expression and function of *Arabidopsis thaliana* NHX Na+/H+ antiporters in the salt stress response. *The Plant Journal*. 2002;30(5):529–39.

214. Niu CF, Wei W, Zhou QY, Tian AG, Hao YJ, Zhang WK, et al. Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic *Arabidopsis* plants. *Plant, Cell & Environment*. 2012;35(6):1156–70.

215. Gao H, Wang Y, Xu P, Zhang Z. Overexpression of a WRKY transcription factor TaWRKY2 enhances drought stress tolerance in transgenic wheat. *Frontiers in Plant Science*. 2018;9:(997):578-88

216. He G-H, Xu J-Y, Wang Y-X, Liu J-M, Li P-S, Chen M, et al. Drought-responsive WRKY transcription factor genes TaWRKY1 and TaWRKY33 from wheat confer drought and/or heat resistance in *Arabidopsis*. *BMC Plant Biology*. 2016;16(1):116.

217. Huang J, Levine A, Wang Z. Plant abiotic stress. *The Scientific World Journal*. 2013 ;Jan(1);13.

218. Carillo P, Annunziata MG, Pontecorvo G, Fuggi A, Woodrow P. Salinity stress and salt tolerance. *Abiotic Stress Plants - Mechanisms and Adaptations*. 2011;21–38.

219. Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *The Plant Cell*. 2000;12(3):393–404.

220. Cao Y, Song F, Goodman RM, Zheng Z. Molecular characterization of four rice genes encoding ethylene-responsive transcriptional factors and their expressions in response to biotic and abiotic stress. *Journal of Plant Physiology*. 2006 Nov 1;163(11):1167-78.

221. Müller M, Munné-Bosch S. Ethylene response factors: a key regulatory hub in hormone and stress signaling. *Plant Physiology*. 2015;169(1):32-41.

222. Girin T, David LC, Chardin C, Sibout R, Krapp A, Ferrario-Méry S, Daniel-Vedele F. Brachypodium: a promising hub between model species and cereals. *Journal of Experimental Botany*. 2014;65(19):5683-96.

223. Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R. ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *The Plant Cell*. 2003;15(1):165-78.

224. Cheng MC, Liao PM, Kuo WW, Lin TP. The *Arabidopsis* ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. *Plant Physiology*. 2013;162(3):1566-82.

225. Marowa P, Ding A, Kong Y. Expansins: roles in plant growth and potential applications in crop improvement. *Plant Cell Reports*. 2016;35(5):949-65.

226. Han Y, Chen Y, Yin S, Zhang M, Wang W. Over-expression of TaEXPB23, a wheat expansin gene, improves oxidative stress tolerance in transgenic tobacco plants. *Journal of Plant Physiology*. 2015;15(173):62-71.

227. Li AX, Han YY, Wang X, Chen YH, Zhao MR, Zhou SM, Wang W. Root-specific expression of wheat expansin gene TaEXPB23 enhances root growth and water stress tolerance in tobacco. *Environmental and Experimental Botany*. 2015;110:73-84.

228. Denison FC, Paul AL, Zupanska AK, Ferl RJ. 14-3-3 proteins in plant physiology. *Seminars in Cell& Developmental Biology*. 2011;22(7):720-727.

229. Camoni L, Visconti S, Aducci P, Marra M. 14-3-3 proteins in plant hormone signaling: Doing several things at once. *Frontiers in Plant Science*. 2018;13(9):297.

230. Zhou H, Lin H, Chen S, Becker K, Yang Y, Zhao J, Kudla J, Schumaker KS, Guo Y. Inhibition of the Arabidopsis salt overly sensitive pathway by 14-3-3 proteins. *The Plant Cell*. 2014;26(3):1166-82.

231. Song H, Wang P, Li C, Han S, Lopez-Baltazar J, Zhang X, et al. Identification of lipoxygenase (LOX) genes from legumes and their responses in wild type and cultivated peanut upon *Aspergillus flavus* infection. *Scientific Reports*. 2016;6(10):1–9.

232. Babenko LM, Shcherbatiuk MM, Skaterna TD, Kosakivska I V. Lipoxygenases and their metabolites in formation of plant stress tolerance. *Ukrainian Biochemical Journal*. 2017;89(1):5–21.

233. Szabados L, Savoure A. Proline: a multifunctional amino acid. *Trends in Plant Science.* 2010;15(2):89–97.

234. Székely G, Ábrahám E, Cséplő Á, Rigó G, Zsigmond L, Csiszár J, et al. Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. *The Plant Journal.* 2008;53(1):11–28.

235. Lee SC, Luan S. ABA signal transduction at the crossroad of biotic and abiotic stress responses. *Plant, Cell & Environment.* 2012;35(1):53–60.

236. Singh A, Jha SK, Bagri J, Pandey GK. ABA inducible rice protein phosphatase 2C confers ABA insensitivity and abiotic stress tolerance in *Arabidopsis*. *PLoS One*. 2015;10(4):e0125168.

237. Xu Q, Chen S, Yunjuan R, Chen S, Liesche J. Regulation of sucrose transporters and phloem loading in response to environmental cues. *Plant Physiology.* 2018;176(1):930–45.

238. Eulgem T, Rushton PJ, Robatzek S, Somssich IE. The WRKY superfamily of plant transcription factors. *Trends in Plant Science.* 2000;5(5):199–206.

239. Javid MG, Sorooshzadeh A, Moradi F, Sanavy SAMM, Allahdadi I. The role of phytohormones in alleviating salt stress in crop plants. *Australian Journal of Crop* Science. 2011;5(6):726–34.

240. Dong S, Jiang Y, Dong Y, Wang L, Wang W, Ma Z, et al. A study on soybean responses to drought stress and rehydration. *Saudi Journal of Biological Sciences*. 2019;26(8):2006-2017.

241. Onkokesung N, Gális I, von Dahl CC, Matsuoka K, Saluz H-P, Baldwin IT. Jasmonic acid and ethylene modulate local responses to wounding and simulated herbivory in *Nicotiana attenuata* leaves. *Plant Physiology*. 2010;153(2):785–98.

APPENDIX A: HPLC CHROMATOGRAMS OF PHYTOHORMONE EXTRACTS

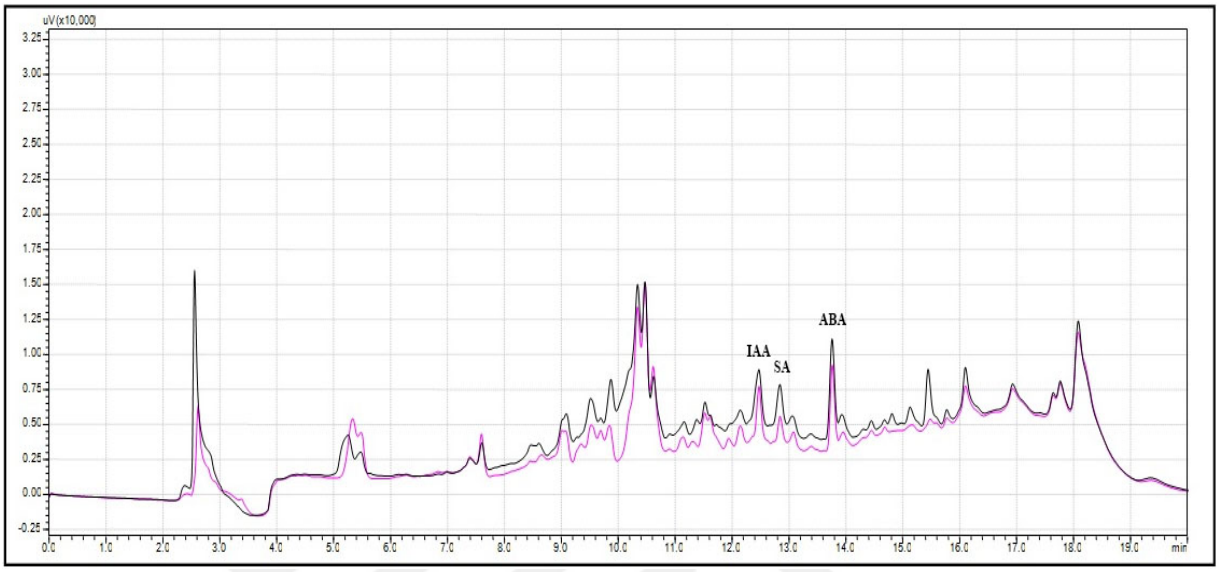

Figure A.1. HPLC chromatogram of phytohormone extracts from salt-stressed (black) and salt stress control (pink) samples at retention time 0-20 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

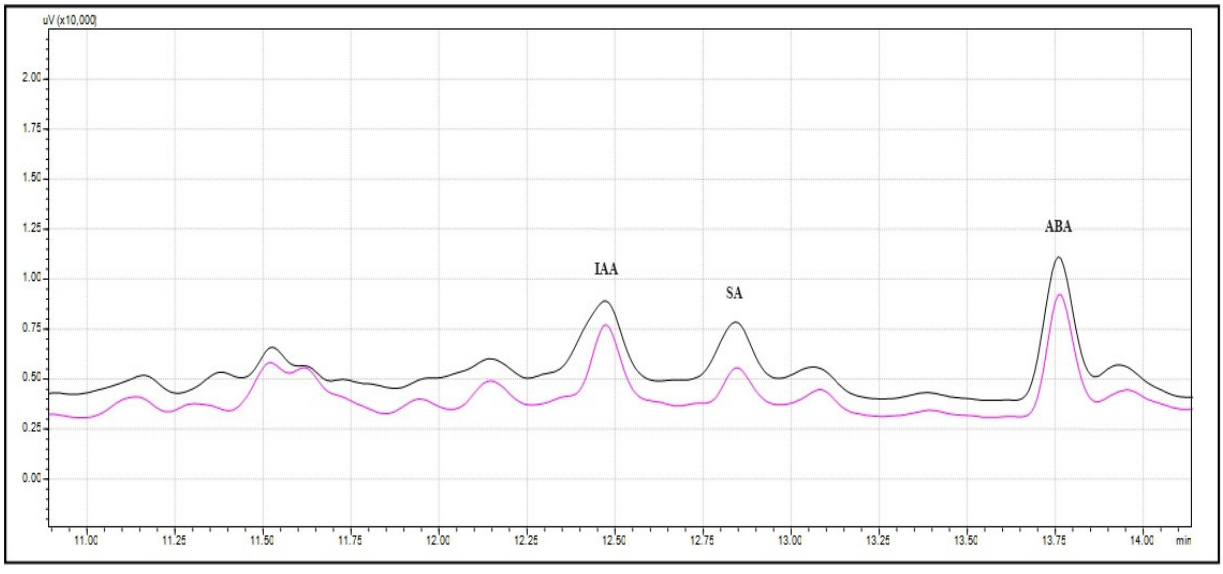

Figure A.2. HPLC chromatogram of phytohormone extracts from salt-stressed (black) and salt stress control (pink) samples at retention time 11-14 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

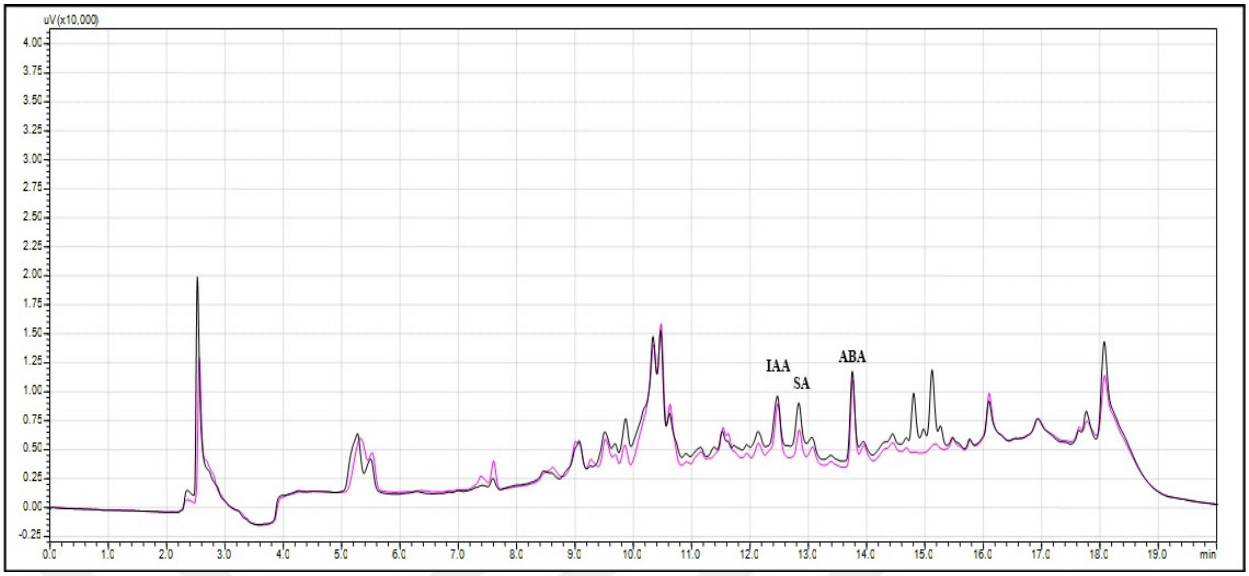

Figure A.3. HPLC chromatogram of phytohormone extracts from drought-stressed (black) and drought stress control (pink) samples at retention time 0-20 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

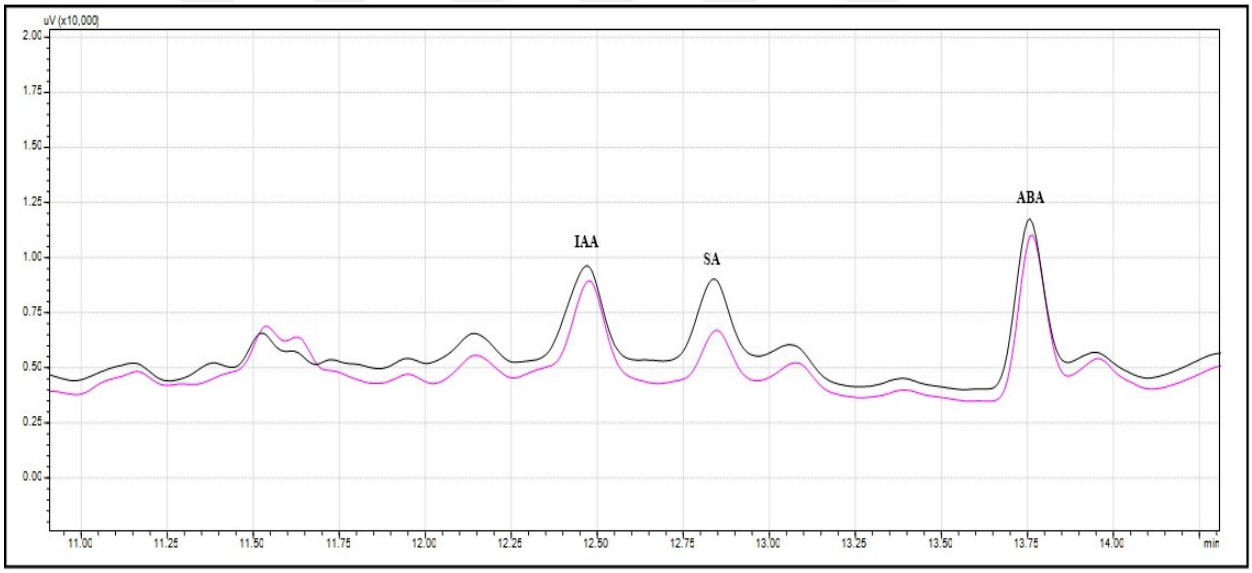

Figure A.4. HPLC chromatogram of phytohormone extracts from drought-stressed (black) and drought stress control (pink) samples at retention time 11-14 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

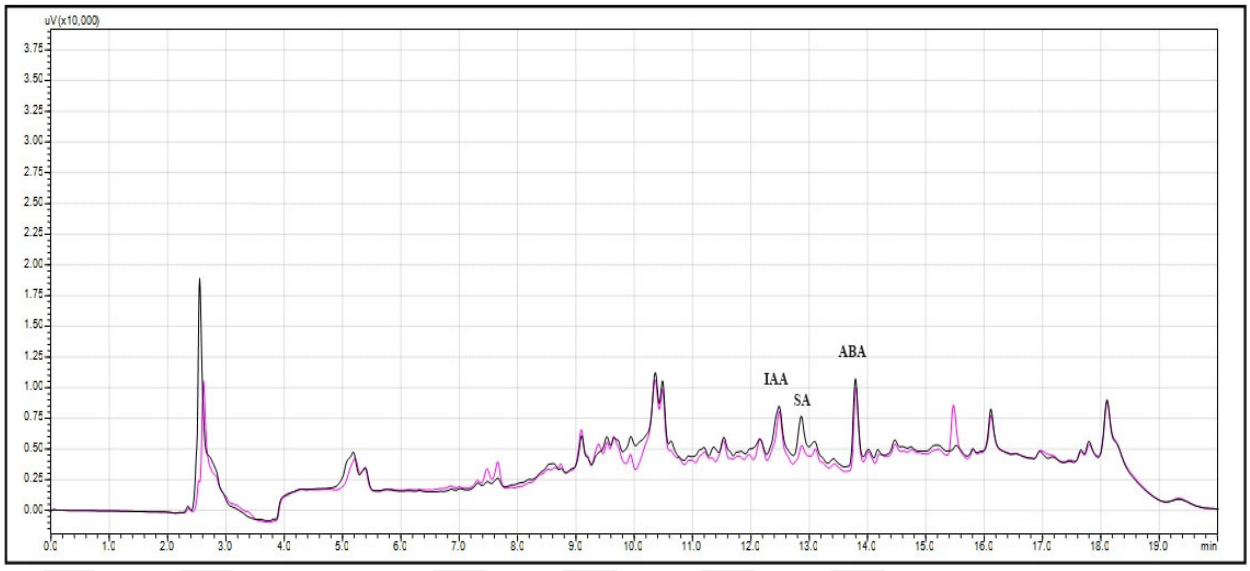

Figure A.5. HPLC chromatogram of phytohormone extracts from mechanically wounded leaves collected at 0th hour (black) and wounding control (pink) samples at retention time 0-20 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

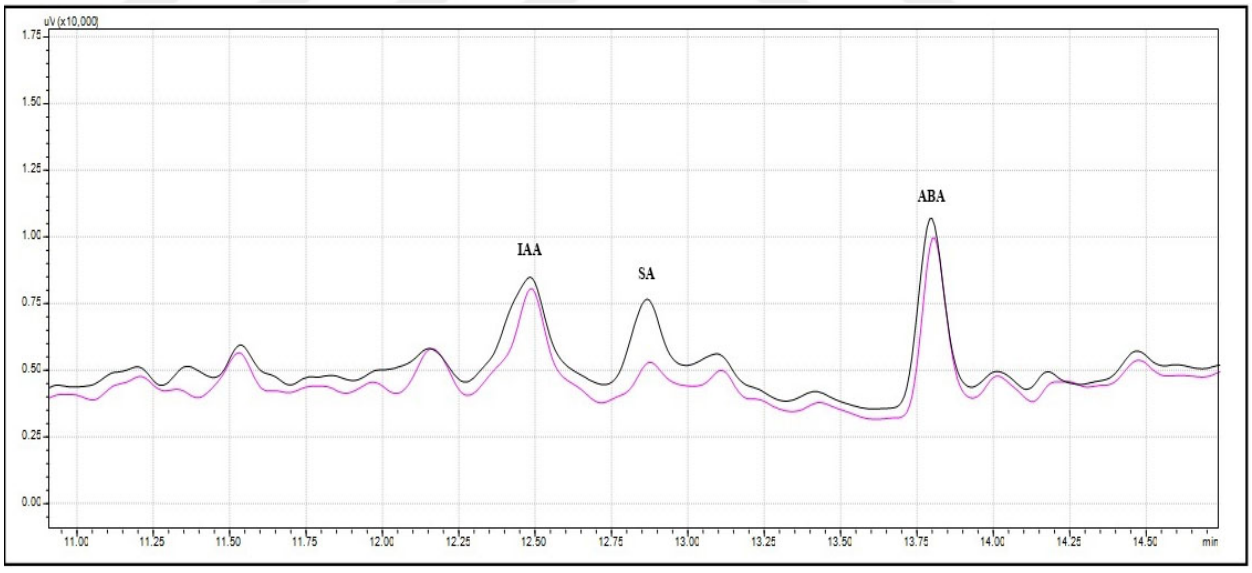

Figure A.6. HPLC chromatogram of phytohormone extracts from mechanically wounded leaves collected at 0th hour (black) and wounding control (pink) samples at retention time 11-14 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

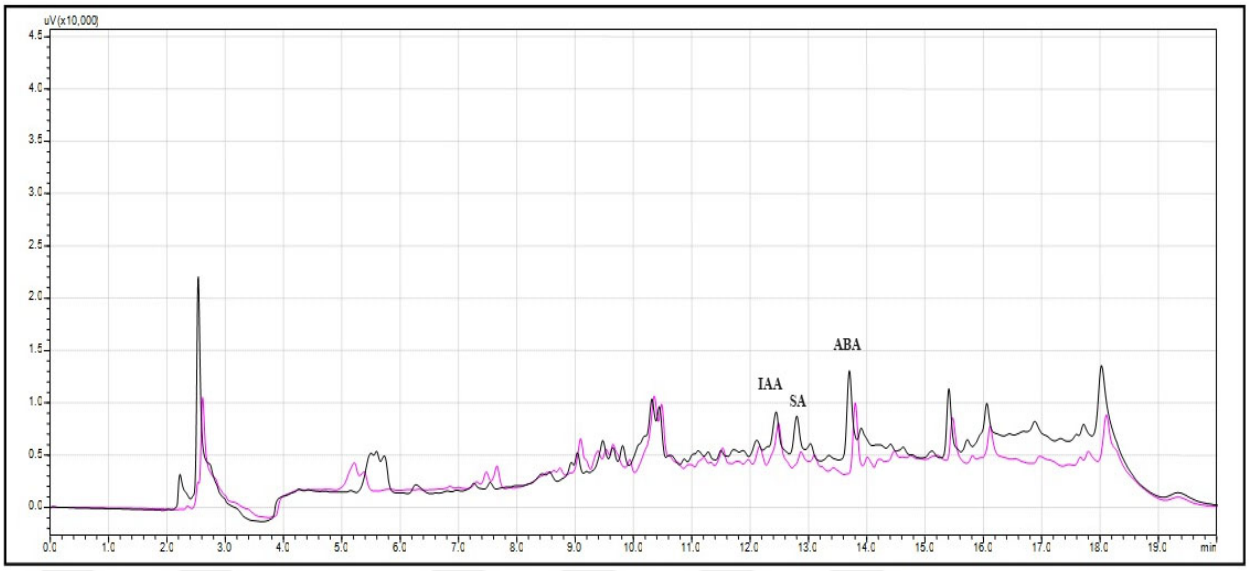

Figure A.7. HPLC chromatogram of phytohormone extracts from mechanically wounded leaves collected at 6th hour (black) and wounding control (pink) samples at retention time 0-20 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

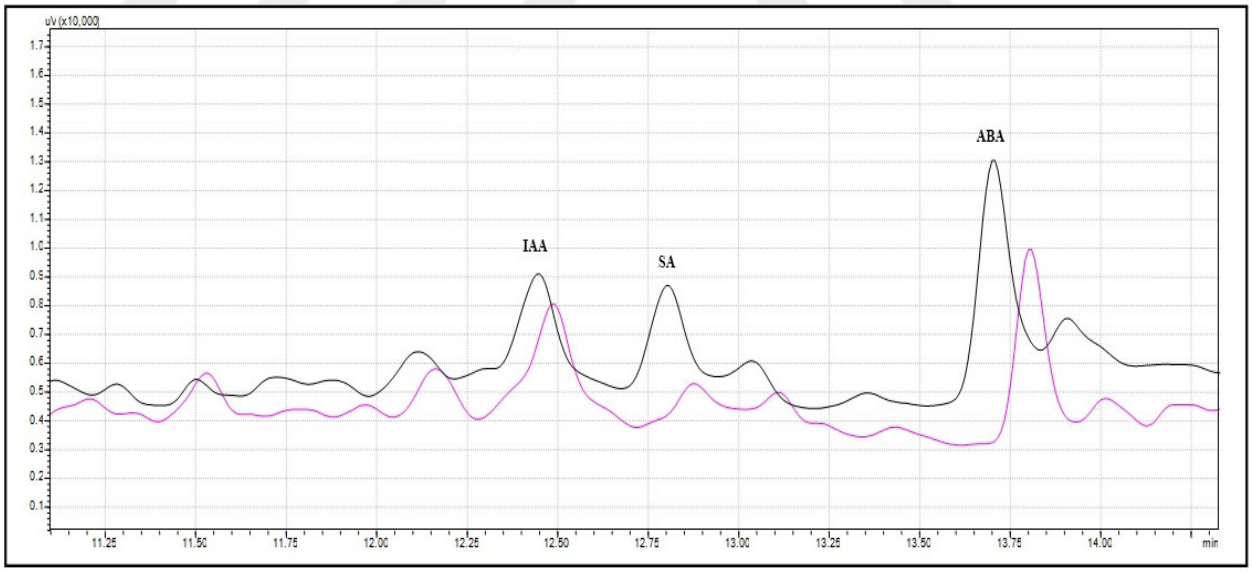

Figure A.8. HPLC chromatogram of phytohormone extracts from mechanically wounded leaves collected at 6th hour (black) and wounding control (pink) samples at retention time 11-14 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

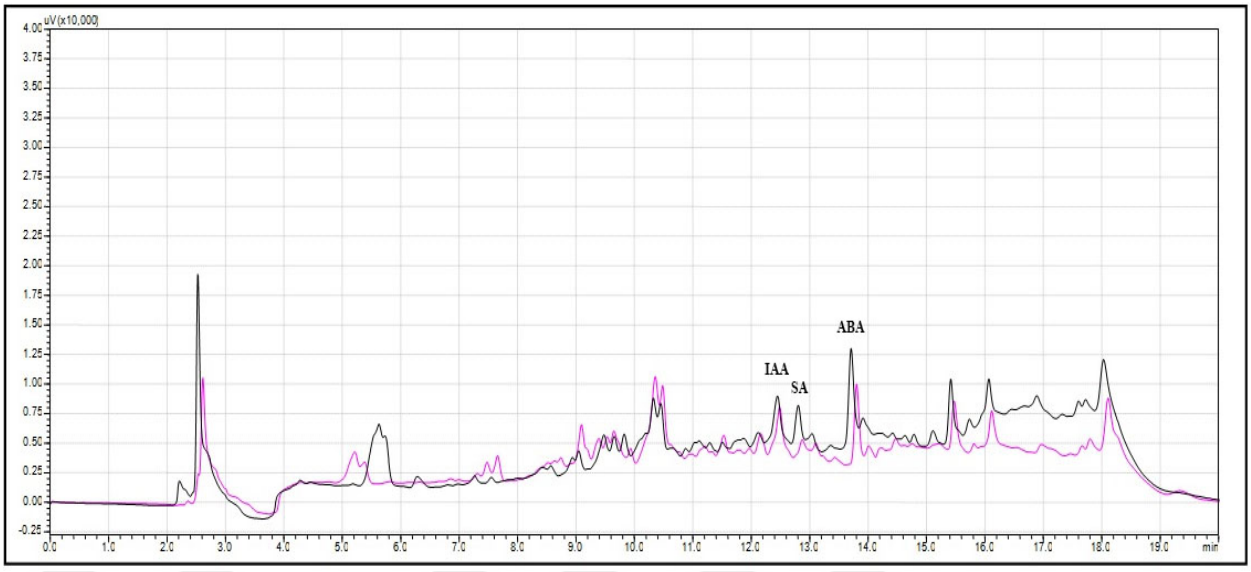

Figure A.9. HPLC chromatogram of phytohormone extracts from mechanically wounded leaves collected at 24th hour (black) and wounding control (pink) samples at retention time 0-20 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

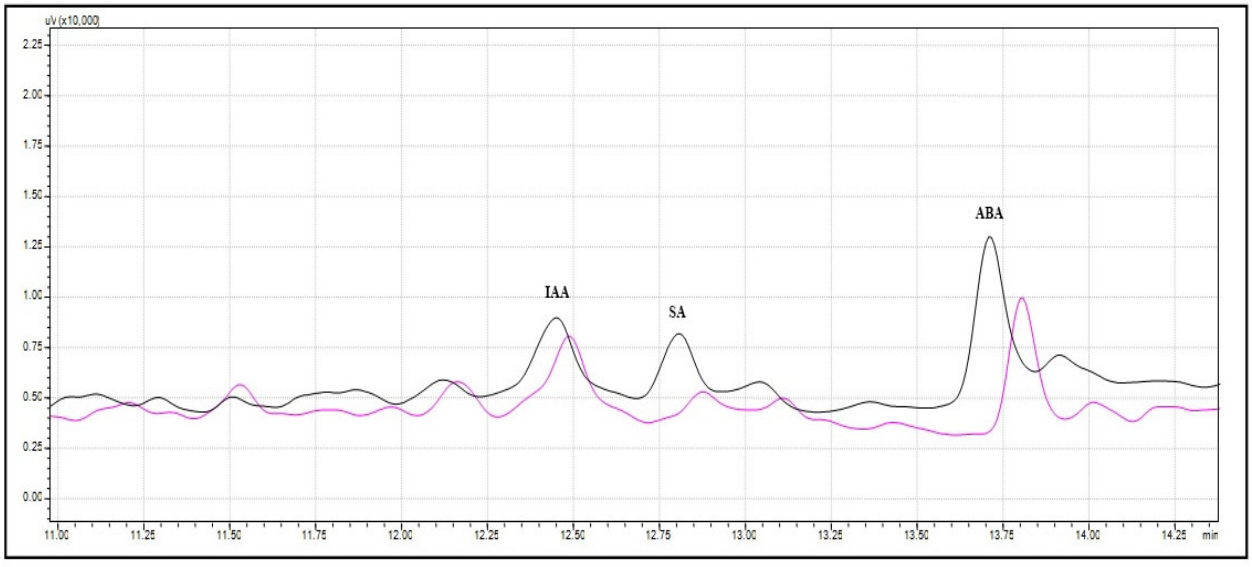

Figure A.10. HPLC chromatogram of phytohormone extracts from mechanically wounded leaves collected at 24th hour (black) and wounding control (pink) samples at retention time 11-14 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

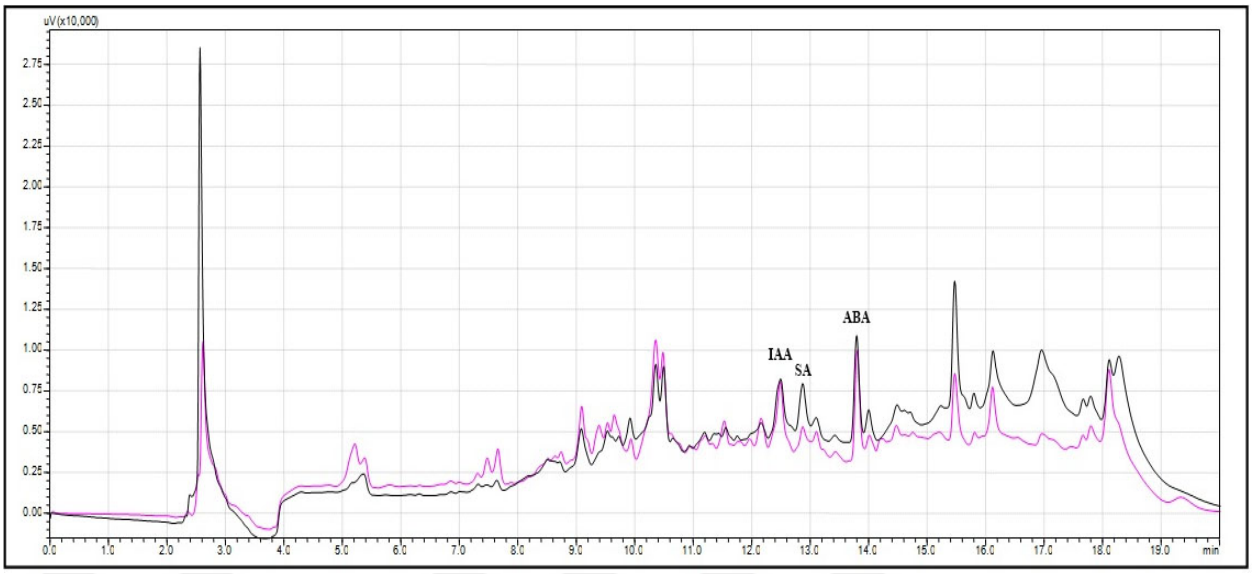

Figure A.11. HPLC chromatogram of phytohormone extracts from unwounded leaves adjacent to wounded leaves collected at 0th hour (black) and wounding control (pink) samples at retention time 0-20 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

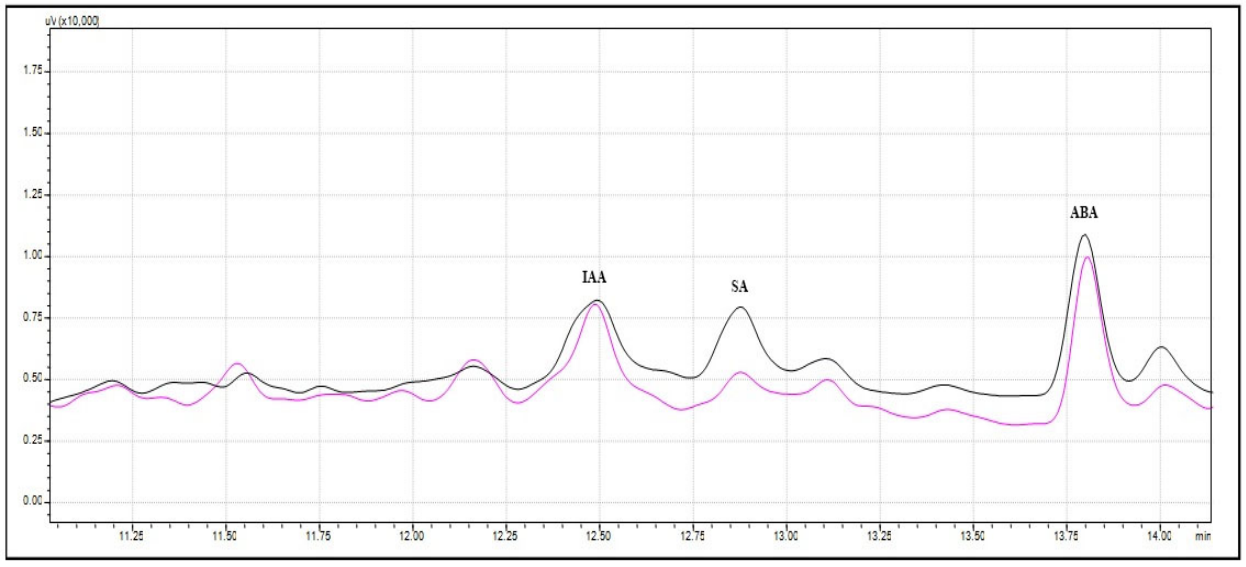

Figure A.12. HPLC chromatogram of phytohormone extracts from unwounded leaves adjacent to wounded leaves collected at 0th hour (black) and wounding control (pink) samples at retention time 11-14 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

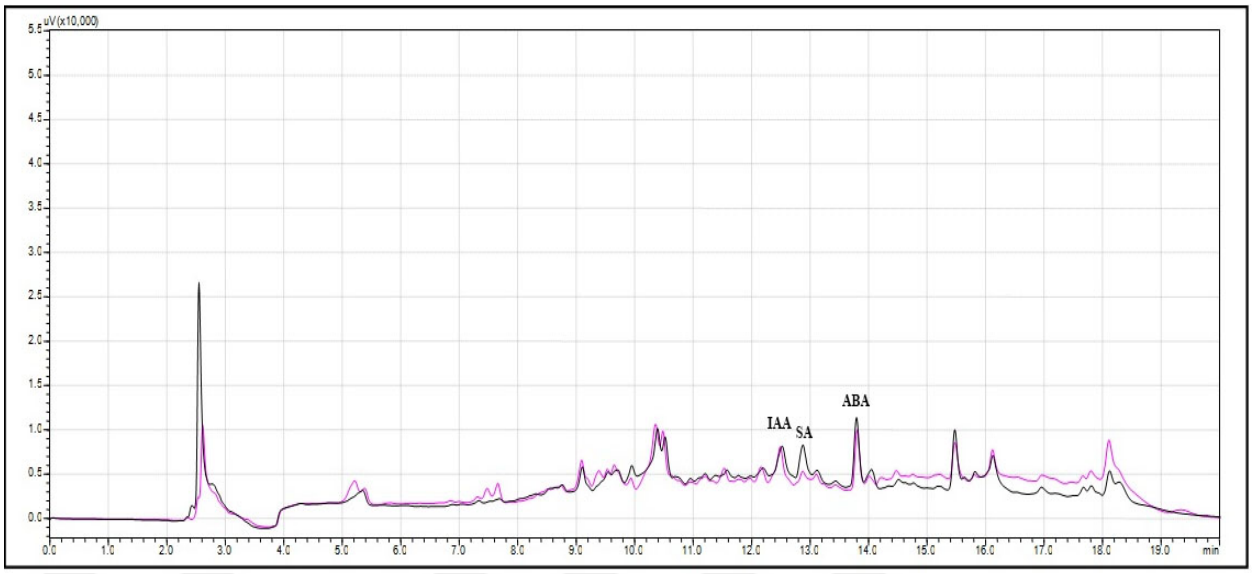

Figure A.13. HPLC chromatogram of phytohormone extracts from unwounded leaves adjacent to wounded leaves collected at 6th hour (black) and wounding control (pink) samples at retention time 0-20 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

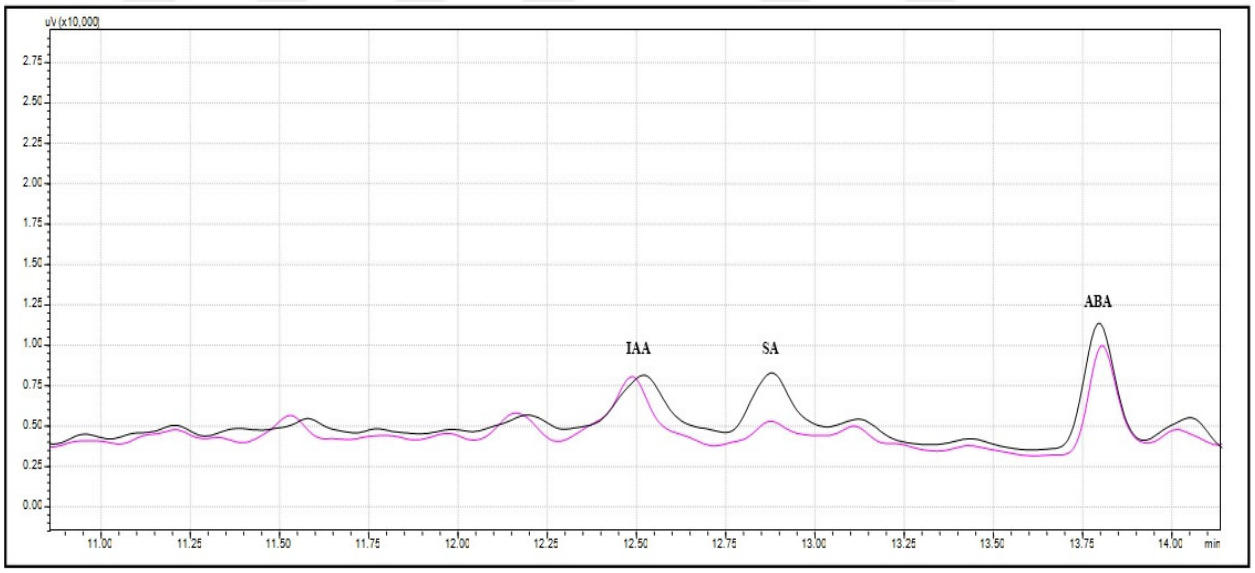

Figure A.14. HPLC chromatogram of phytohormone extracts from unwounded leaves adjacent to wounded leaves collected at 6th hour (black) and wounding control (pink) samples at retention time 11-14 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

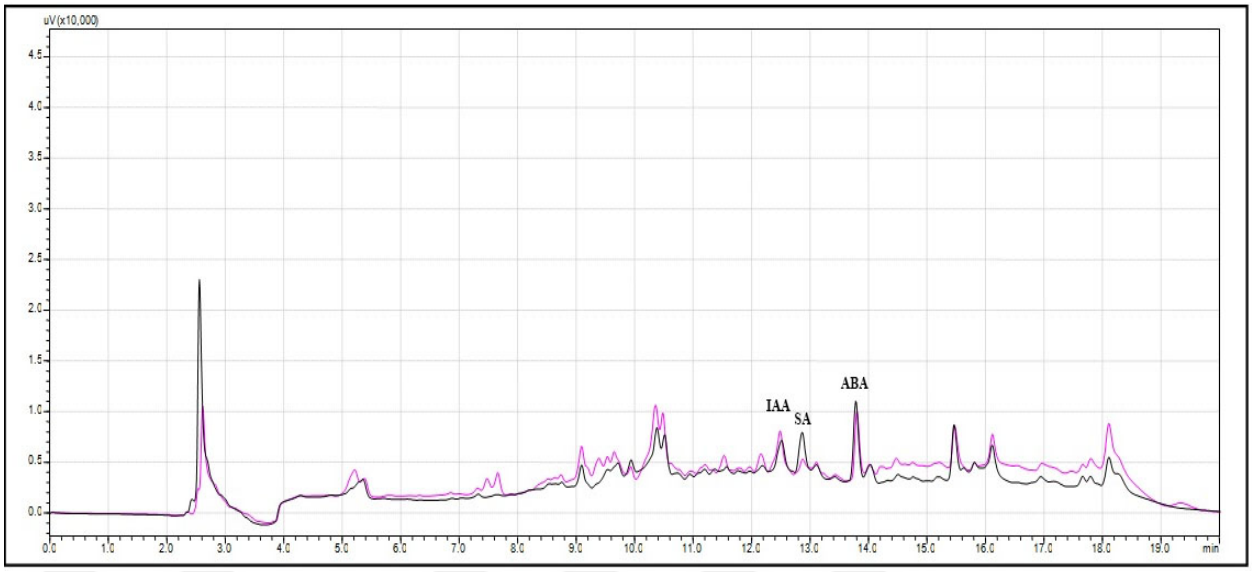

Figure A.15. HPLC chromatogram of phytohormone extracts from unwounded leaves adjacent to wounded leaves collected at 24th hour (black) and wounding control (pink) sample at retention time 0-20 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.

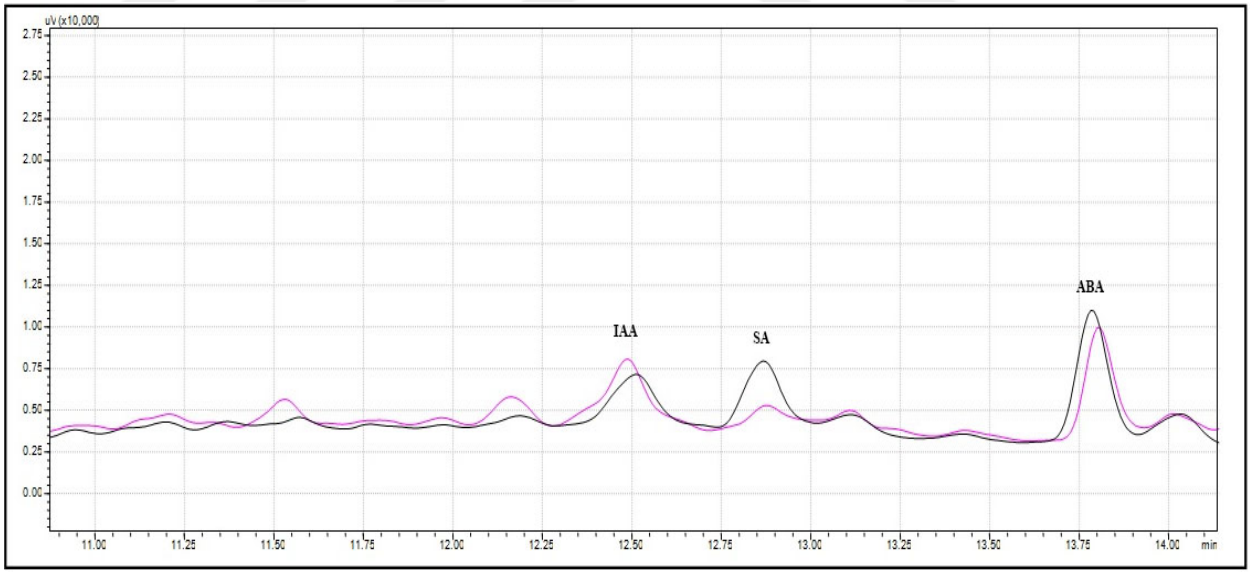

Figure A.16. HPLC chromatogram of phytohormone extracts from unwounded leaves adjacent to wounded leaves collected at 24th hour (black) and wounding control (pink) samples at retention time 11-14 min. Retention times for IAA, SA and ABA were 12.4, 12.8 and 13.7 min., respectively.