BROWN ALGAL GENES IMPART SALINITY AND HIGH TEMPERATURE STRESS TOLERANCE IN *ARABIDOPSIS THALIANA*

by

Pramod Kumar Rathor

Submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

at

Dalhousie University

Halifax, Nova Scotia

May 2021

With love, I dedicate this thesis to my lovely daughter Chitrita Rathore (Avni) and my wife Renu Rathore

Table of Contents

List of Tables

List of Figures

Abstract

Plants are constantly exposed to unfavorable environmental conditions like salinity, temperature, and water stress, that negatively affects growth and development. The ability of plants to survive these environmental stresses depends on the activation of molecular networks that trigger changes in gene expression and the production of metabolites, mitigating the physiological effects induced by stress. Substantial research has been carried out to develop transgenic plants with improved tolerance to abiotic stresses. Brown alga *Ectocarpus* sp. belongs to Phaeophyceae, a class of macroalgae in the phylum Heterokonta. *Ectocarpus* sp. is a dominant seaweed in the temperate regions around the world, abundant mostly in the intertidal zones that are known for harsh environmental conditions. The aim of this thesis was to determine if genes that were found to be upregulated in *Ectocarpus* sp., when subjected to multiple abiotic stresses, can also impart salinity and high temperature stress tolerance in the model plant *Arabidopsis thaliana.* Mannitol is one the widely studied sugar alcohol, that plays a role in abiotic stress tolerance in some plants and algae. Expression of mannitol biosynthesis genes of *Ectocarpus* sp. strain Ec32 in transgenic *A. thaliana* generated 42.3 – 52.7 nmol g^{-1} fresh weight of mannitol. Mannitol producing transgenic lines exhibited improved tolerance to salinity and high temperature stress. Transcriptomics analysis revealed significant differences in the expression of numerous genes, in both optimum and salinity stress conditions suggesting reprograming of gene expression in the overexpression lines. Besides mannitol biosynthetic genes *EsM1PDH1* and *EsM1Pase2*, two other genes *Esi0379_0027* (that codes HSP70) and *Esi0017_0056* that were upregulated under abiotic stress conditions in *Ectocarpus* sp. Ec 32 were also overexpressed in *A. thaliana*. Bioinformatics analyses indicated that the proteins encoded by *Esi0017_0056* and *Esi0379_0027* are monomeric and soluble. In *A. thaliana* both the genes, under constitutive and stress inducible promoters, improved tolerance to salinity and high temperature stresses. Analysis of the expression of several key abiotic stress-related genes revealed that genes involved in ABA-induced abiotic stress tolerance, K^+ homeostasis, Na^+ sequestration and chaperon activities were significantly up-regulated in the transgenic lines. Transient expression of Esi0017_0056 in *Nicotiana benthamiana* revealed localization of the protein in cytosol. This is the first time *Ectocarpus* sp. genes have been successfully expressed in *A. thaliana* and suggest the possible use of macro-algal genes in improving abiotic stress tolerance in crop plants.

List of Abbreviations Used

FAO	Food and Agriculture Organization
UV	Ultraviolet
Mbp	Millions of Base Pairs
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ATP	Adenosine Tri Phosphate
ROS	Reactive Oxygen Species
M6PR	Mannose 6 Phosphate Reductase
CaMV35S	Cauliflower Mosaic Virus 35S
PMI	Phosphomannose Isomerase
M1PP	Mannitol-1-Phosphate Phosphatase
MtlD	Mannitol Dehydrogenase
M1PDH1	Mannitol-1-Phosphate Dehydrogenase
M2DH	Mannitol-2-Dehydrogenase
HK	Hexokinase
ABA	Abscisic Acid
NSCC	Non-Selective Cation Channels
KORC	Potassium Outward Rectifying Channels
MRKC	Mechanosensitive Receptor Kinase Cyclase
cGMP	Cyclic Guanosine Monophosphate
DINSCC	Depolarization Induced Non-Selective Cation Channels
CNGC	Cyclic Nucleotide Gated Channels
TPC1	Two Pore Channel 1

CDPK	Calcium Dependent Protein Kinase
CBL	Calcineurin B Like
CIPK26	Calcium Induced Protein Kinase
RbohF	Respiratory Burst Oxidase Homolog F
SOS	Salt Overly Sensitive
PP2C	2C Protein Phosphatase
ABI	ABA-Insensitive
SnRK	Sucrose Non-fermenting Related Kinase
RD	Response to Desiccation
HSP	Heat Shock Protein
FLC	Flowering Locus C
GA	Gibberellic Acid
CDF	Cycling D Factor
E. coli	*Escherichia coli*
LB	Luria Broth
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription PCR
RT-qPCR	Real Time Quantitative PCR
W/V	Weight / Volume
ANOVA	Analysis of Variance
LC-MS	Liquid Chromatography Mass Spectrometry
DEPC	Diethyl Pyro Carbonate
GUS	Glucuronidase

GFP	Green Fluorescent Protein
ABRE	ABA Responsive Elements
DRE	Dehydration Responsive Elements
IPCC	International Panel for Climate Change
mM	Millimolar
μM	Micromolar

Acknowledgements

First, I would like to thank my parents and brothers who were very supportive during my studies. I would like to extend my heartfelt sincere thanks to my supervisor Dr. Balakrishnan Prithiviraj for his unconditional love, support, encouragement and guidance throughout the thesis and during all the times I spent with him since my arrival at Dalhousie in Fall, 2011. Sir, I learnt lot of things from you and will continue to do so. You are like my father taking care of me during all the times. I don't have words to say and thank you is very small. Thank you very much for nurturing me, taking care of me and my family during our difficult times. You and Kalyani, both showered lots of love, blessings and support for me to accomplish this, it would never been possible without your support, love and blessings. Thanks very much for all your love, blessings and graciousness.

I would like to extend my heartfelt gratitude to Dr. David Clarke and all his team (Professor and Head, Division of Neurosurgery, Dalhousie University. Chief, Neurosurgery, Nova Scotia Health Authority). Thank you very much Dr. Clarke for your support, flexibility in considering my thesis timeline, positive thoughts, and confidence you provided. Thank you very much for expediting my appointments and fitting me in your very tight schedule to avoid delays in thesis defense. I very much appreciate flexibility you showed and ensuring that my thesis work is completed before you schedule me as your patient. Thank you very much for scheduling me early in your tight schedule and at my convenience. My life was in your hand, and you did such an amazing work operating on me. You provided me better, happy and healthy life. I can never imagine it. I am blessed to be here, and I knew that I was in the best hands in the World. I will owe all achievements to you till my life. You will be always in my heart and mind till my life. I am in paucity to express my gratitude to Dr. Kristein Ikeda, Dr. Mark Sadler, Dr. Antonina Omisade, Karen Legg, Susan Rahey and all the people in division of neurosurgery at QEII Halifax. You all are amazing and thank you very much for help, moral support, positive thoughts, confidence and expediting the process to make me fit and healthy. Thank you very much for all your efforts and because of this now I am living happy and healthy life.

I would like to extend my heartfelt gratitude to my committee members, Dr. Philippe Potin (Station Biologique de Roscoff, France), Dr. Sophia Stone (Department of Biology) and

Dr Svetlana Yurgel (Department of Plant, Food and Environmental Sciences) for their priceless time, support, encouragement and constructive feedbacks throughout thesis.

I would like to express my heartfelt gratitude to Dr. Tudor Borza for the wonderful experiences I had with him. Tudor, you have been my mentor throughout thesis. I learnt lots of new things from you and still many more to learn. You always motivated me to do new things and always advised to do better work and science. You always stood with me whenever I needed to provide the support throughout the work. I appreciate all your efforts, precious time, encouragement, and your valuable experiences you shared. I will never forget the contribution you made and the unforgettable memories I had with you. Thank you very much for taking care of my things during my difficult times and thank you very much for all the support and confidence you provided when I needed it most.

I would like to extend my heartfelt thanks to Dr. Thierry Tonon, Sylvie Rousvoal, Agnes Groisillier, Simon Dittami, Emeline Creis, Celine Conon and Agnes Morin (Station Biologique de Roscoff, France) for their support during work and my stay in Roscoff.

I would like to extend my heartfelt gratitude to Margie Tate for her unconditional support during my thesis. Thank you very much Margie for providing me opportunity to work with you as a teaching assistant in labs for so many years. Many thanks for helping me personally and professionally. You always encouraged me to do hard work and reminded that health is first and then work. Thank you very much for taking care of me during my difficult times. Many thanks for noticing me if I looked tired and reminding that I need to slow down. Thank you very much for pushing me to go and see doctors when you saw me first time feeling unwell. I appreciate you asking my friends in lab if you didn't saw me walking in corridor or in lab even for a single day and asking me next day that if everything is going well. This means lot to me and shows how caring person you are. I will always remember you wherever I will be in future. I would like to extend my heartfelt gratitude to Anne, Paul, Daryl, Fred, Craig, Margaret and Gisele for their unconditional support when I needed it.

I would like to extend my heartfelt thanks to all my fellow friends in lab and those who were there during thesis work. My special thanks to Jai Singh Patel, Vinod Selvaraj and

Aravind who were always there with me during my difficult times. You kept your work a side whenever I needed your help. Thank you very much and I appreciate your helps.

Last, but not least, I would like to express my hearted gratitude my lovely daughter Chitrita Rathore (Avni) and my wife Renu Rathore for their constant support, love and understandings to ensure first I can complete my work before we could plan any outdoor activities or family visits.

Thank you very much everyone for your support, help and providing me courage to accomplish this.

CHAPTER 1

Introduction

Plants are sessile organisms constantly subjected to a changing environment, which is causing stressful or unfavorable conditions for their growth and development. Adverse environmental conditions include various abiotic stresses such as suboptimal climatic and/or edaphic conditions which affect cellular homeostasis and, ultimately, plant growth (Mickelbart et al., 2015). In land plants, these stressors include surplus or deficit of water, ion deficiency and toxicity, ultra-violet (UV) and extreme temperatures (Mickelbart et al., 2015). Among the various abiotic stresses, soil salinity and temperature extremes are the major factors affecting the geographical distribution of plants in nature, thereby limiting agricultural productivity and risk to global food insecurity. The anticipated global demand for food is increasing at a pace that it has become a major concern (FAO, 2008). Concurrent with the growing demand for food, environmental stresses impose adverse conditions that affects productivity of agricultural crops. Irrigated land accounts for ~30% of the food production; to meet the anticipated global food demand, the production on this land should increase to 50% (FAO, 2008). Climate change is happening at a faster rate than predicted, which exacerbates the adverse effects of abiotic stresses. It has been suggested that in the Mediterranean and semi-arid regions of the world, the aridity will increase in the near future and, therefore, the agricultural production will decrease (Parry et al., 2007; Fedoroff et al., 2010).

Understanding the physiological mechanisms of plant responses to water stress is important to develop strategies that reduces the impact of water deficit conditions on crop performance. Water deficit condition is becoming more severe as a greater part of the terrestrial area is being desertified. In the current situation of global water scarcity, salinity and drought are widespread and are expected to affect more than 50% of the arable land area by 2050 (FAO, 2008). Utilization of intensive irrigation practices to expand the land area under cultivation in arid and semi-arid regions will further predispose land to secondary soil salinization. Improving plant tolerance to salinity has been a field of interest for plant breeding and molecular genetics in the last few decades, albeit with very little

success (Zhu, 2016). Although several genes controlling abiotic stress tolerance have been discovered and introgressed into a number of crop plants but have not been deployed in the field and success has been limited. These limitations stem from the fact that tolerance to abiotic stresses is a multigenic trait. Clearly, it is extremely difficult to generate plants harboring all these gene variants, which is the only approach to achieve improved tolerance against a broad range of stresses (Zhu, 2002).

Developing plants with improved tolerance to abiotic stresses is of great interest for plant biologists because plant stress tolerance is extremely critical for agricultural productivity and sustainability (Zhu, 2016). Plants exposed to stresses during the early developmental stages might exhibit slower growth, due to a reduction in cell division and expansion, but this may not have a strong overall impact, that is, to reduce the yield to significant levels. However, the stress imposed during the flowering stage could have more drastic effects and significantly diminish productivity (Mansouri-Far et al., 2010). Moreover, an increase in variability of weather patterns, due to climate change, is causes significant crop losses and threatens global food security. Several climate models predict a significant increase in floods, drought and higher temperatures (Bita and Gerats, 2013; Hirabayashi et al., 2013; Pryor et al., 2013). Integrated climate models, along with crop production models, have projected a severe decline in grain yield for three major crops, i.e., wheat, corn and rice, with serious consequences for world food production in this century (Iizumi et al., 2013; Field, 2014; Rosenzweig et al., 2014). Even though there has been a progressive increase in the production of major crops since 1960s, the risk of the impact of climate change on crop production have also risen in recent years suggesting the need for development and adoption of climate resilient crops, to ensure global food security (Lobell et al., 2014).

Seaweeds, which include the macroscopic, multicellular algae commonly inhabiting the coastal regions of the world, form an integral component of marine ecosystems (Khan et al., 2009). There are ~ 9000 species of macroalgae, which belong to three phyla: Chlorophyta, Phaeophyta and Rhodophyta, or green, brown and red algae, respectively. Phaeophyta comprise approximately 2000 species, are the second most abundant algae (Khan et al., 2009), and most commonly used in agriculture (Blunden and Gordon, 1986).

Phaeophytes are included in the Kingdom Chromalveolata, Division Heterokontophyta, Class Phaeophyceae, and have evolved complex multicellularity (Cock et al., 2010). The group belongs to an important evolutionary lineage, Stramenopiles, which diverged more than a billion year ago from the Archaeplastida, the plastid of Phaeophytes originating through secondary endosymbiosis (Cock et al., 2011). These macro-algae are mostly dominant in intertidal and subtidal zones, which is an environment with higher levels of abiotic stress conditions, such as high salinity, temperature, irradiation and mechanical forces caused by turning tides (Dittami et al., 2009). Brown algae are sessile organisms which during their long evolutionary history, have evolved a number of features that allowed them to survive in these harsh environmental conditions. Brown algal biology differs from that of most terrestrial plants in a number of aspects, including their ability to synthesize C18 and C20 oxylipins (Ritter et al., 2008), and sulfated polysaccharides in their cell wall (Kloareg and Quatrano, 1988; Nyvall et al., 2003; Bartsch et al., 2008). In most green plants the photosynthetically fixed carbon is stored in the form of starch, saccharose and fructans. However, brown algae store it by converting it into laminarin and mannitol (Bonin et al., 2015). Mannitol can make up to 25% of dry biomass in brown algae (Reed et al., 1985). The complete genome sequencing of the filamentous brown algae *Ectocarpus* sp. contributed to the better understanding of brown algal biology and of the strategies utilized by these organisms to cope with abiotic stresses (Cock et al., 2010). Knowledge gained from deciphering brown algal biology can potentially be applied to land plants. Information obtained from the genome sequencing are currently being used in combination with other genetic and biochemical tools to further elucidate of brown algal biology at the molecular level. For example, mannitol biosynthesis pathway in the brown algae *Ectocarpus* sp. was delineated and characterized (Rousvoal et al., 2011; Groisillier et al., 2013). Furthermore, the osmoprotectant role of mannitol in *Ectocarpus* for tolerance to salinity has become apparent (Dittami et al., 2011).

1.1 Model Organisms

1.1.1 *Ectocarpus* sp.

Ectocarpus sp. is a cosmopolitan, small filamentous multicellular brown alga which has relatively small genome size (200 Mb), in contrast to *Fucus serratus* (1095 Mb) and *Laminaria digitate* (640 Mb) (Le Gall et al., 1993; Peters et al., 2004). It was selected for genome sequencing due to its small genome size, high fertility, short life cycle (2-3 month), ease of cultivation in laboratory conditions and for its close phylogenetic relationship to the economically important Laminariales (Draisma et al., 2003). The sequence of *Ectocarpus* sp. was obtained using the strain EC32, which is a meiotic offspring of a field sporophyte collected from San Juan de Marcona Peru in 1988 (Peters et al., 2004). The size of genome for this strain has been estimated to be 214 Mb, which comprised of 18,000 predicted protein coding genes (https://bioinformatics.psb.ugent.be/orcae/) (Cock et al., 2010).

1.1.2 *Arabidopsis thaliana*

It is a small flowering plant of the Brassicaceae family. It is a commonly used model in plant biology due to characteristics such as its small genome size (114.5 Mb/125 Mb total), short life cycle, taking about 45 days to reach seed maturation from germination. The species is prolific in seed production, can be cultivated in restricted spaces, can be easily transformed with *Agrobacterium tumefaciens* and there are a large number of genetic resources and mutant lines available at stock centers (www.arabidopsis.org).

1.2 Purpose of Study

The objective of my thesis was to determine if genes that were found to be upregulated in brown algal model *Ectocarpus* sp., when subjected to multiple abiotic stresses, can be used to improve abiotic stress tolerance in land plants. In the first approach, *Ectocarpus* sp. genes involved in mannitol biosynthesis were introduced into *A. thaliana*. This is the first time that a transgenic plant is generated overexpressing two genes involved in mannitol production, from a brown alga. All previous studies involved either an *E. coli* (*Escherichia coli*) gene or a gene from celery (*Apium graveolens*). Results of this objective are presented

in chapter 3. The second approach was to characterize genes with unknown function or predicted function that have been shown to be consistently up-regulated when *Ectocarpus* sp. is subjected to a variety of abiotic stresses. The results of this objective are presented in chapter 4, 5 and appendix B.

CHAPTER 2

Literature Review

2.1 Mannitol in Abiotic Stress Tolerance

In response to abiotic stress, a number of organisms, including plants, fungi, yeast and algae synthesize and accumulate low molecular weight soluble compounds, commonly referred to as compatible solutes. These compounds are often termed as compatible because they accumulate high concentrations of solutes without interfering with the normal cellular processes (Stoop et al., 1996; Nuccio et al., 1999; Loescher and Everard, 2000; Sakamoto and Murata, 2002; Williamson et al., 2002). The chemical reductions of aldose or ketose sugars, where either the aldehyde or ketone group is reduced to a hydroxy group, produce sugar alcohols or polyols. These are one of the major classes of compatible solutes which accumulate in response to abiotic stresses. Perhaps the most intensively studied plant carbohydrate is sucrose; nonetheless, it has been estimated that ~30% of all photosynthetic assimilate in plants is translocated in the form of polyols (Bieleski, 1982). Polyols are always transported in association with either sucrose or raffinose, even when they are the predominant form of translocated carbohydrate (Williamson et al., 2002). The polyols most commonly reported include six carbon sugar alcohols, such as mannitol, sorbitol and galactitol. Less common polyols reported include the seven carbon sugar alcohols, such as persitol, present in avocado (*Persea americana*), (Liu et al., 1999) and volenitol, present in cowslip (*Primula veris*), (Häfliger et al., 1999). One of the most intensively researched polyols is mannitol, which is chemically related to mannose. Although more than 13 different types of polyols have been isolated and identified in land plants, mannitol is possibly the most widely occurring soluble carbohydrate in living organisms (Patel and Williamson, 2016). Mannitol has been recorded in more than 100 plant species from 70 different families and in several microbes (Lewis and Smith, 1967; Jennings, 1985; Stoop et al., 1996). It is interesting that the biosynthesis of mannitol occurs simultaneously with a sugar, which may be either sucrose, as observed in celery, or raffinose, as in olives (Rumpho et al., 1983; Flora and Madore, 1993). The role of polyols as osmoprotectants has been suggested because of their resemblance with water molecules. The hydroxy (-OH) group of polyols may mimic water structure and thus can maintain an artificial sphere

of hydration surrounding the macromolecules. This prevents the inactivation of metabolic processes under the conditions of osmotic stress (Schobert, 1977; Galinski and Trüper, 1994). Plants that produce and utilize mannitol have a number of advantages over the non-producing plants. In celery, ~50% of the photosynthetically fixed carbon is converted into mannitol and the remaining half of fixed carbon is transformed to sucrose. Both of these photo-assimilates are then translocated to the sinks and used for growth. A higher rate of photosynthesis, similar to that of C4 plants, has been reported in celery, which is a C3 plant. This increase in the photosynthetic activity might be associated with the increased turnover of NADP-NADPH and an additional sink for fixed carbon is provided by mannitol biosynthesis (Patel and Williamson, 2016). The potential advantage of mannitol metabolism in plants might be an efficient utilization of fixed carbon for growth. This might be due to energy production in the first step of mannitol metabolism, where the NADPH is generated, thus contributing to net higher yield of ATP, as compared to the ATP generated from the catabolism of sucrose (Loescher et al., 1995; Pharr et al., 1995). Additionally, plants producing mannitol demonstrate significantly higher levels of abiotic stress tolerance compared to plants lacking mannitol (Tarczynski et al., 1992; Shen et al., 1997; Abebe et al., 2003; Zhifang and Loescher, 2003).

In the plant kingdom, polyols exist both in aliphatic and aromatic forms. The most common aliphatic polyols found in plants include sorbitol, mannitol and glycerol, whereas pinitol and ononitol are aromatic forms of polyols. In most photosynthetic organisms producing polyols, significant amounts of photosynthetically fixed carbon can be converted into polyols, and these make up the significant portion of reactive oxygen species (ROS) scavengers (Bohnert and Jensen, 1996). Mannitol has been reported to play a significant role in hydroxyl radical (OH°) scavenging activity and in stabilizing the macromolecular structures. Shen et al. (1997a) carried out a study to unravel the role of mannitol as a potential ROS scavenger. The authors targeted the expression of the bacterial gene *mannitol-1-phosphate dehydrogenase* to the chloroplast of tobacco by cloning it with a signal sequence which targets the enzyme to plastid. The transgenic tobacco obtained by this process accumulated significantly higher levels of mannitol, as compared to plants overexpressing the same gene in cytosol. Moreover, an improved resistance to oxidative stress induced by the application of methyl viologen, and higher photosynthetic rate due to

improved CO_2 fixation rate, were reported. When the cells of transgenic tobacco containing mannitol in the chloroplasts were observed by introducing a probe for hydroxyl radical dimethyl sulfoxide, they exhibited a significant reduction in the initial production of sulfinic acid, as compared to the wild type plants. These findings suggested that mannitol producing plants have the potential to cope with oxidative stress, thus improving abiotic stress tolerance. In transgenic tobacco, the production and accumulation of mannitol protected the thiol-regulated enzymes, including phosphoribulokinase, ferrodoxin, glutathione and thioredoxin, from the detrimental effects exerted by the hydroxyl radical (Shen et al., 1997b). When *A. thaliana*, which is not a mannitol producing plant, was genetically modified by introducing the *M6PR* (*mannose-6-phosphate reductase*) gene from celery, under the control of the constitutive promoter CaMV35S, all the transformants accumulated mannitol in the range of 0.5 to 6 μmol/g of fresh weight (Zhifang and Loescher, 2003). However, these amounts of mannitol might be significantly smaller than required to have an effect as osmotic regulator. Therefore, the improved tolerance to abiotic stresses, as observed in transgenic *A. thaliana* overexpressing the mannitol biosynthetic genes, might be associated with improved tolerance for secondary stress, such as ROS (Patel and Williamson, 2016).

Despite clear evidence originating from a number of studies on mannitol and its role in protection against osmotic and oxidative stress in biological systems, the understanding of precise mechanisms involved in supporting these effects still remains incomplete. To gain a better understanding of the role of mannitol in oxidative stress, the molecular mechanisms involved in ameliorating this stress must be delineated and well characterized (Patel and Williamson, 2016). Since mannitol is present in high concentrations in some salinity tolerant plants including celery, in many cases it has been assumed to be an osmolyte in transgenic plants as well. However, because the quantified levels of mannitol in transgenic *A. thaliana* plants have been always much lower than the concentration observed in plants that produce mannitol naturally, its function as an osmolyte in transgenic plants is debatable (Patel and Williamson, 2016). The two potential mechanisms proposed to define the role of mannitol as an osmoprotectant without it being an osmolytes are either that it can act as a compatible solute or can act as a ROS scavenger (Patel and Williamson, 2016). The most commonly and widely accepted role of mannitol is that it acts as an antioxidant

(Dat et al., 2000; Parida and Das, 2005; Bernstein et al., 2010). Moreover, in mannitol producing plants, including parsley and snapdragon, mannitol was recorded to be significantly higher in chloroplasts, thus providing additional support to the hypothesis that mannitol acts an antioxidant. Moreover, a higher constant rate for mannitol reaction with OH° has been reported as compared to other solutes (Buxton et al., 1988).

Some research, however, has questioned the role of mannitol as an antioxidant, based on its production and accumulation by plants under abiotic stresses. Smirnoff and Cumbes (1989), demonstrated that a concentration of 35 mM mannitol was required for quenching 60% of OH° generated during *in vitro* experiments. Shen et al. (1997a) performed an experiment on isolated chloroplasts and reported that a concentration of 125 mM mannitol was required to provide complete protection, to an indicator protein, from the detrimental effect exerted by OH°. In addition, a number of earlier studies carried out using transgenic *A. thaliana* obtained by introducing the bacterial or plant mannitol biosynthetic genes showed that mannitol production was rather low, ranging from 0.5 to 6 μmol/g fresh weight; however, these transgenic plants were reported to be tolerant to abiotic stresses (Tarczynski et al., 1992, 1993). These reported concentrations are significantly lower than needed for mannitol to function as an independent osmolyte and to produce the observed phenotype just by acting as an antioxidant under salinity stress. Indeed, 33-125 mM of mannitol was required in *in vitro* studies for ROS scavenging activity (Smirnoff and Cumbes, 1989; Shen et al., 1997a).

When cells generate OH°**,** it reacts instantaneously with any molecule in its close vicinity (Czapski, 1984; Sies, 1997; Davies, 2005). Therefore, if any particular molecule would act as a quencher of OH°**,** it must be present in the proximity of the OH° radical when is produced. To achieve this, the molecule has to have intracellular concentrations high enough to ensure it will be the first molecule to be encountered by the OH°. Czapski (1984), reported that a concentration of mannitol required for 90% protection against cellular OH° would be somewhere from 1.7 to 3.5 molar. If a conclusion has to be drawn from these studies, then it is evident that the amount of mannitol accumulated in transgenic *A. thaliana* is not sufficient to provide protection against abiotic stresses. Therefore, there must be some other mechanism(s) by which mannitol in low concentration imparts abiotic

stress tolerance in transgenic *A. thaliana*. Chan et al. (2011) reported that when transgenic *A. thaliana* were developed by overexpressing the *M6PR* gene from celery, they exhibited improved tolerance to high salinity which was associated with an increased expression of stress inducible genes. Mannitol is known to act as a compatible solute. In osmotolerant organisms, including algae and yeast, the role of compatible solutes was well established during the 1980s (Brown and Simpson, 1972; Brown, 1976; Yancey et al., 1982). When the conditions of low water potential arise, the compatible solutes might act as osmoprotectants to stabilize the cellular proteins by interacting with their hydration shells during low osmotic potential (Yancey, 2005; Yancey and Siebenaller, 2015). Mannitol may be involved in quenching the OH° radicals if present in high concentrations at the specific sites where OH° radicals are generated and/or if they are present in effective concentrations next to specific molecules targeted by OH° radicals (Patel and Williamson, 2016). To explain how mannitol acts as a quencher for OH°, a hypothesis was proposed which states that mannitol, serving as a compatible solute, would cluster in at key primary sites of ROS generation and/or surround the cellular structures or molecules that are highly prone to the toxic effects of free radicals. However, this hypothesis needs further investigations to be widely accepted (Patel and Williamson, 2016).

2.2 Mannitol Biosynthesis in Plants and Algae

The main difference in mannitol biosynthesis in land plants and brown algae is the presence of mannose as an intermediate in both biosynthesis and catabolic reactions in land plants. In biosynthesis pathway fructose-6-phoshate is interconverted to mannose-6-phosphate through phosphomannose isomerase (PMI), which is further oxidized to mannitol-1-phosphate (M1P), through the activity of mannose-6-phosphate reductase (M6PR). Dephosphorylation of M1P by the activity of mannitol-1-phosphate phosphatase (M1PP) produce mannitol. In catabolic pathway mannitol is reduced to mannose and further to mannose-6-phosphate through the activity of mannitol dehydrogenase (MTD) and hexokinase (HK), respectively (Figure 2.1a) (Patel and Williamson, 2016). In brown algae, mannitol metabolism involves two enzymes for biosynthesis and two for recycling. Enzymes for biosynthesis includes mannitol-1-phosphate dehydrogenase (M1PDH1) and

mannitol-1-phosphatase (M1Pase2) while mannitol recycling includes mannitol-2-dehydrogenase (M2DH) and a HK (Figure 2.1b) (Groisillier et al., 2013; Bonin et al., 2015).

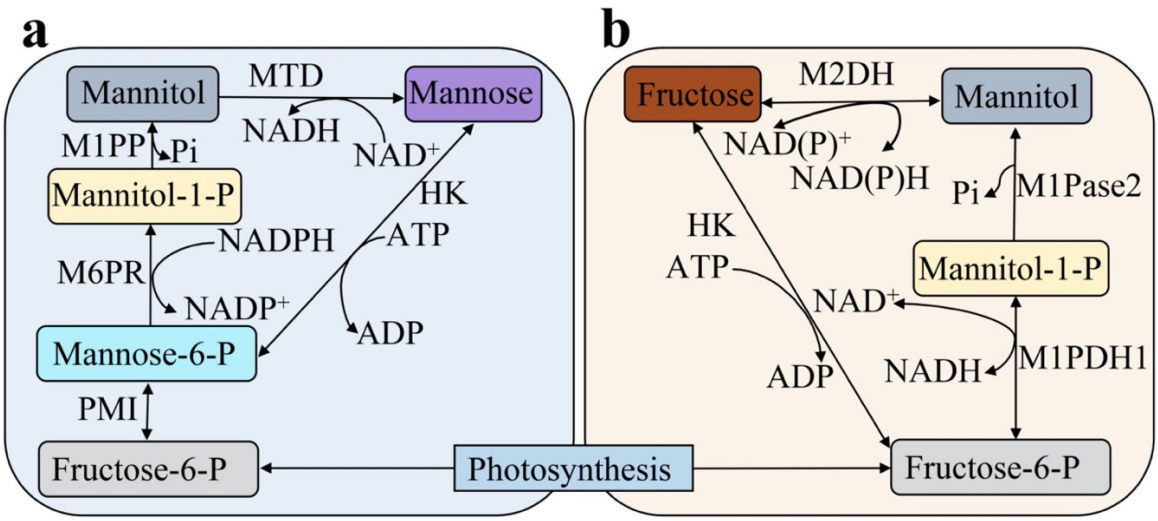


Figure 2.1. Mannitol biosynthesis pathway. **(a)** Land plants. **(b)** Brown algae.

2.3 Mechanisms of High Salinity Stress Tolerance

Response of plants to high salinity include: 1) prevention of further damage caused by salt stress, 2) maintenance or restoration of homeostasis of water potential and ion distribution, and 3) resumption of normal growth (Zhu, 2002). The nature of the damage caused by salt stress on plant tissues is not yet entirely clear. However, cellular membranes, nutrient acquisition, photosynthetic apparatus and enzymes associated with normal growth are known to be susceptible to the toxic effects of Na^+ (Zhu, 2002). Salinity stress inhibits plant growth in different modes and these can be associated with the accumulation of toxic ions in the cells or can be completely independent of ion accumulation (Roy et al., 2014). The different modes of salinity stress can be distinguished experimentally by quantifying the effects instantly or after several days or weeks. Plants exhibit a number of instant responses upon exposure to salinity and these are mainly independent of ion accumulation, whereas the responses observed after several days to weeks are dependent on ion accumulation (Roy et al., 2014). Reduction in shoot growth and in the production of new leaves are the main primary consequences of salt stress in the early phase, also termed the

osmotic phase (Munns and Tester, 2008). The second phase of salinity stress is ion toxicity, due to the accumulation of Na^+ ions in the tissues above the threshold limits, causing premature senescence of the leaves (Munns and Tester, 2008). Plant tolerance to the early phase of salt stress still remains largely unknown, as compared to plant tolerance to ion toxicity, because ion accumulation is a trait that is easy to phenotype, in contrast to osmotic stress, which is relatively complex (Munns and Tester, 2008).

2.3.1 Stress signaling in abiotic stress tolerance

Plants sense alterations which occur in their environments and exhibit dramatic changes in their gene expression, metabolism and physiology to respond to these variations. However, despite breakthroughs in scientific technology, only a few stress sensors have been documented (Zhu, 2016). It is difficult to identify sensor proteins because of the functional redundancy in the genes which encode them (Zhu, 2016); the mutation in one gene does not produce significant changes in the phenotype under stressful conditions. Additionally, in some instances, the sensor may be required for the plant growth and development, therefore the loss of function mutation is lethal. Thus, further investigations to delineate and characterize the roles of sensor proteins in abiotic stress tolerance are precluded (Zhu, 2016). How plants perceive stress signals and translate them to activate the downstream adaptive responses to withstand the adverse climatic conditions are the fundamental biological questions that need to be addressed. In high salinity and drought conditions, it is highly important to distinguish the primary stress signals from secondary signals. During drought stress, the primary stress signal is hyperosmotic, and this is often referred to as osmotic shock. The secondary signals are complex and include oxidative stress due to the accumulation of ROS. The accumulation of ROS damages cellular components, including membrane lipids, proteins and nucleic acids, causing aberrant cell signaling, and leading to metabolic dysfunction (Mittler, 2002). Salinity and drought are hyperosmotic conditions which have unique and overlapping signals because a number of cellular responses in both conditions are triggered by primary signals. Moreover, the hyperosmotic condition triggers the production of abscisic acid (ABA), which in turn induces the adaptive responses at both molecular and physiological levels, as

described in Figure 2.2 (Zhu, 2002). The plant responses to an early phase of salinity stress includes ROS signaling, calcium waves and even electric signals for instant long distance signaling (Maischak et al., 2010; Mittler et al., 2011). The changes noted in plants for tolerance to the osmotic phase might be due to the differences which occurred during the initial perception and those encountered during long distance signaling. The mechanisms of salinity stress tolerance are still unclear and further studies are required to develop a better understanding of osmotic stress tolerance under saline conditions (Roy et al., 2014).

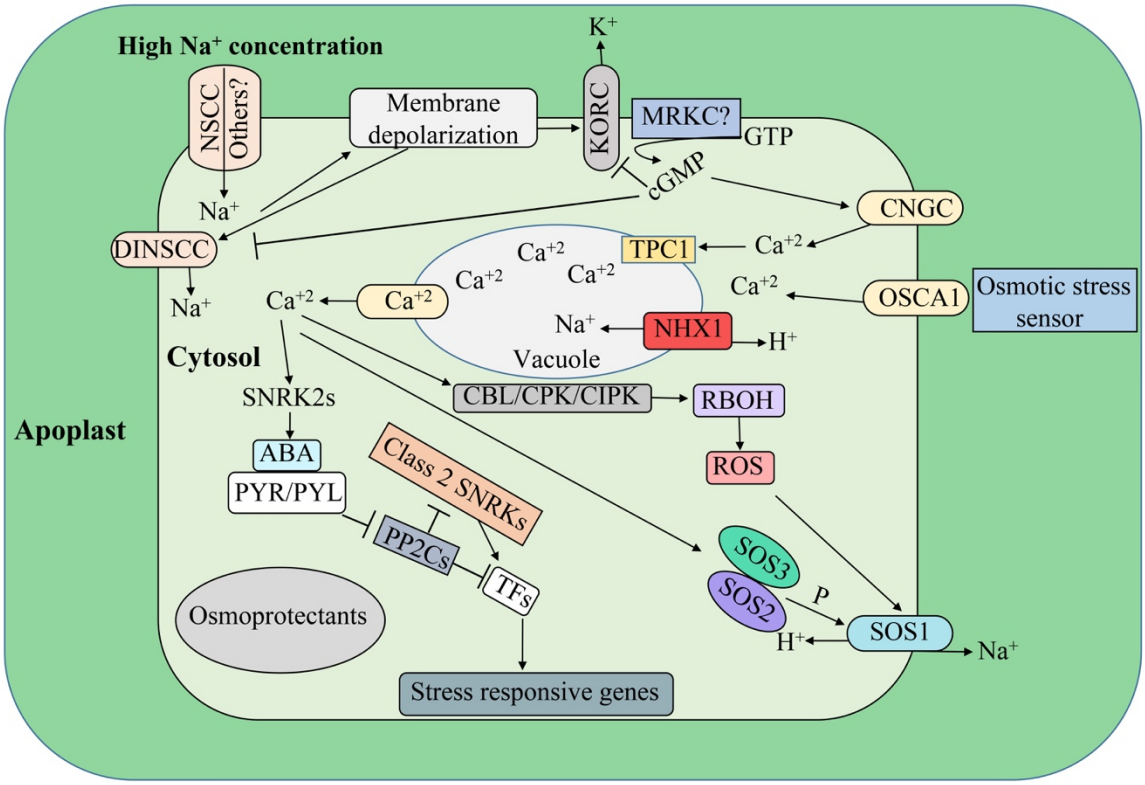


Figure 2.2. Signaling mechanisms in response to salinity, drought stress and ion homeostasis in plants. (Reproduced from Julkowska and Testerink, 2015)

Plant tolerance to high salinity stress involves a number of different mechanisms. When plants are exposed to salinity stress Na^+ concentrations outside the root builds up and this increase in concentration resulting the entry of Na^+ in the roots by the activation of non-selective cation channels (NSCC) (Hua et al., 2003; Tester and Davenport, 2003). A very specific Na^+ transporter has not been yet discovered. In roots, its transport is through the

activity of NSCC, high affinity K^+ transporter, low affinity K^+ transporter and non-selective outward rectifying channels. All of the above-mentioned transporters can mediate the transport of Na^+ and K^+ as both, being monovalent cations, compete with each other. However, many of these transporters have more affinity for K^+ over Na^+ (Blumwald, 2000; Tuteja, 2007). When Na^+ enters inside the cell it causes the depolarization of plasma membrane which induces the activity of K^+ outward rectifying channels (KORC) and therefore reduces the uptake of K^+ via K^+ inward rectifying channels (Shabala and Cuin, 2008). Higher Na^+ concentration increases the osmotic potential which cause the loss of water resulting the cell shrinkage due to loss of turgor pressure. Julkowska and Testerink (2015), mentioned that loss of turgor pressure could be sensed by the activation of mechanosensitive receptor kinase cyclase (MRKC), which can rapidly induce the production and accumulation of cGMP (cyclic guanosine monophosphate). This can reduce the activity of depolarization induced non-selective cation channels (DINSCC) thus reduce the influx of Na^+ in the cytosol (Figure 2.2) (Maathuis and Sanders, 2001). It has been proposed that the accumulation of cGMP can activate the cyclic nucleotide gated channels (CNGC), which in turn can enhance the movement of Ca^{2+} from apoplast to cytosol (Donaldson et al., 2004). Further, increase in concentration of Ca^{2+} in cytosol is also achieved by the activation of vacuolar two pore channels (TPC1). This causes the mobilization of vacuolar calcium into cytosol (Peiter et al., 2005). Calcium signaling transduce the information and turns on the defense cascade including the calcium dependent protein kinases (CDPK), calcineurin B like protein (CBL) and CBL-interacting protein kinases (CIPKs) (Harmon et al., 2000; Boudsocq et al., 2007; Weinl and Kudla, 2009). Activation of these Ca^{2+} sensor proteins transduce and activates different transcription factors involved in the regulation of stress tolerance genes. Increased cytosolic Ca^{2+} concentration induces the activity of calcium binding calmodulin (CBL9) dependent protein kinases. Activation of these kinases induces the plasma membrane localized H^+-ATPase, and this is crucial under salinity stress for repolarization of plasma membrane voltage and to maintain membrane integrity and ion homeostasis (Figure 2.2) (Shabala et al., 2006). Increase in Ca^{2+} concentration in cytosol activates the calcium binding calmodulin (CBL9). This interacts with calcium induced protein kinase 26 (CIPK26). The CBL9-CIPK26 complex targets the respiratory burst oxidase homolog F

(RbohF) which induces the production of ROS (Drerup et al., 2013). To restrict the movement of Na^+ inside the cells, plant activates the SOS (salt overly sensitive) pathway, where the interaction between SOS2-SOS3 phosphorylates the SOS1 localized on plasma membrane maintaining Na^+ homeostasis (Qiu et al., 2002). SOS pathway has been shown to be regulated by ROS signaling (Zhou et al., 2012). ROS was demonstrated to have tight regulation on the mRNA stability of *SOS1* (Chung et al., 2008). SOS2, a key component of SOS pathway has been shown to interact with catalase 2 and catalase 3. This suggests that *SOS2* can be a key player to establish the crosstalk between ROS signaling and salt stress (Verslues et al., 2007). When Na^+ concentrations inside the cells are above the threshold, to avoid metabolic disruptions, Na^+ is transported to vacuoles by the enhanced activity of NHX1, a Na^+/H^+ exchanger localized on tonoplast (Figure 2.2) (Blumwald and Poole, 1985).

2.3.2 ABA signaling in abiotic stress tolerance

In *A. thaliana* 2C protein phosphatases (PP2Cs) clade A type have been demonstrated to act as negative regulators of ABA signaling (Gosti et al., 1999; Merlot et al., 2001; Yoshida et al., 2006; Antoni et al., 2012). These PP2Cs (ABI1, ABI2, HAI1, HAI2) interact with SnRKs (in particular 2.2, 2.3 and 2.6), which are known as key players in ABA signaling, and inhibit them by dephosphorylation (Gómez-Cadenas et al., 1999; Mustilli et al., 2002; Schweighofer et al., 2004; Yoshida et al., 2006; Boudsocq et al., 2007; Fujii et al., 2009). However, during the ABA induction, the ABA receptors pyrabactin resistance like (*PYL*) binds to ABA and suppresses the activity of PP2Cs. This activates the SnRK2s which get autophosphorylated and then phosphorylate the downstream proteins which are involved in abiotic stress responses (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009; Soon et al., 2012). Stressful conditions lead to energy deficiency and this induces the expression of *SnRK1,* which is inhibited by ABI1 and other PP2Cs once the energy is at normal levels (Rodrigues et al., 2013). Even though these PP2Cs are considered as negative regulators they can be considered as important integral components of this signaling pathway because in presence of clade A PP2Cs the binding affinity of ABA to PYL is shown to be increased ~100 fold and therefore these phosphatases can be recognised as the co-receptors (Ma et al., 2009). Moreover, once the energy levels are restored to

normal levels these negative regulators of ABA signaling (Figure 2.2) can reset the signal transduction pathway (Rodrigues et al., 2013).

2.3.3 Sodium homeostasis under salinity stress

Restoring both ionic and water potential homeostasis in stressful environments is the key for improving salt stress tolerance. It is important to prevent the entry of Na^+ into plants to avoid its accumulation in the cytoplasm or organelles, except for the vacuoles. Amtmann and Sanders (1998), proposed that non-selective cation channels (NSCCs) mediate Na^+ influx into the cells, but how these ion channels are working is not yet clearly understood nor all proteins have been identified at the molecular level. One of the important objectives in improving plant salt tolerance should be to discover the specific ion transporters that mediate the Na^+ influx into the cells. By doing this, the ion channels can be blocked, and this has the potential to restrict Na^+ transport into the cells (Zhu, 2016). Additionally, Na^+ efflux should also be considered as an important mechanism for maintaining low Na^+ concentration in the cytoplasm. Plant cells appear to lack the Na^+-ATPases for Na^+ efflux, unlike animal cells or some fungal or algal cells, that possess Na^+-K^+ ATPases and Na^+-ATPases for Na^+ efflux, respectively (Zhu, 2001). *A. thaliana* gene *SOS1* encoding the Na^+-H^+ antiporter in the plasma membrane has been functionally characterized and demonstrated to play an important role in Na^+ efflux and in maintaining the ionic homeostasis in plant cells (Shi et al., 2002). How Na^+ is sensed is not yet clearly understood (Zhu, 2016). Thewes (2014), reported that in the yeast *Saccharomyces cerevisiae,* the calcineurin pathway plays a critical role in Na^+ tolerance and stress signaling. The cytosolic calcium triggered upon exposure to Na^+ stress binds to the EF-hand of calcium binding proteins, which attach to the B subunit of calcineurin and calmodulin. The binding of Ca^{2+} to calmodulin and calcineurin activates the phosphatase catalytic subunit of calcineurin. This in turn dephosphorylates the CRZ1, a zinc finger transcription factor which, upon dephosphorylation, moves to the nucleus and initiates the expression of *ENA1*, a gene coding for the Na^+-ATPase to pump the Na^+ out of the cells, thus maintaining ion homeostasis by reducing the concentration of toxic Na^+ in the cells (Thewes, 2014). Calcineurin proteins are not encoded by the plant genome (Zhu, 2016). Nonetheless, the

term CBL (calcineurin B like) proteins has been widely used by plant biologists to refer to the family of EF-hand Ca^{2+} binding proteins in plants (Yu et al., 2014). To achieve Na^{+} tolerance, plants deploy a Ca^{2+} dependent protein kinase pathway known as the SOS pathway (Figure 2.2). In plants, this is the first well established pathway for salt stress signaling, and the central component of this pathway is the SOS2, which represents an extensive family of protein kinases (Zhu, 2002). The catalytic domain of this protein is similar to the AMP-activated kinase of mammals and sucrose non-fermenting1 (SNF1) of yeast. During protein-protein interaction between SOS2 and SOS3, SOS2 phosphorylates SOS3, and this step of phosphorylation is critical for the activation of kinases (Du et al., 2011). In optimal conditions, without any stress, SOS2 interacts with type 2C protein phosphatase ABI2 and with a protein having the 14-3-3 motif and this keep SOS2 in resting stage or inactive (Ohta et al., 2003; Zhou et al., 2014). The transcript abundance of *SOS1* is recorded to be highest in the xylem, parenchymatous and epidermal cells of the roots. Therefore, the activation of *SOS1* can extrude Na^{+} from plants into the soil. Additionally, the activation of *SOS1* can promote the loading of Na^{+} into the xylem, playing a critical role to facilitate the long distance transport of Na^{+} to leaves through transpirational pull (Shi et al., 2002; Zhu, 2016). To date, despite the intensive research on *SOS1*, only its role in long distance transport is clear. *SOS1* is also expressed in the xylem parenchymatous cells of leaves but its and the role in this tissue is still unclear (Zhu, 2016). However, it has been proposed that the casparian-like structure exists in the xylem parenchymatous cells of the leaves and this prevents the entry of the xylem stream into the apoplastic space of the mesophyll cells. Thus, *SOS1* might be involved in extrusion of Na^{+} from the parenchymatous cells of the xylem to the apoplastic spaces of the mesophyll cells (Zhu, 2016). Another transporter of critical importance in long distance movement of Na^{+} is *HKT1* which encodes a high affinity K^{+} transporter (Mäser et al., 2002). The product of this gene is abundant in parenchymatous cells of the xylem and in other cells of the vascular system throughout the plant (Mäser et al., 2002). The role of the product of *HKT1* in roots is to unload the Na^{+} from xylem, and this provides an aid to prevent a rise in Na^{+} concentration in the transpirational stream. However, in leaves, HKT1 is involved in loading Na^{+} to the phloem, from where it is recirculated back to the roots (Mäser et al., 2002). Plants have evolved the ability to decrease ion toxicity by restricting the movement

of Na^+ into the cells, or by improving their ability to tolerate the ions they have failed to restrict. Plants can tolerate ionic stress by compartmentalization of Na^+ to vacuoles, where it can be stored away from sensitive parts of the cells, including chloroplast and mitochondria, to avoid any interruptions in metabolic processes. Another strategy is to transport Na^+ back to the roots and this process involves a number of transporters localized on the plasma membrane and on the tonoplast of the vacuoles (Figure 2.2) (Flowers and Colmer, 2008; Craig and Møller, 2010).

2.4 Oxidative Stress Tolerance

Plants exposed to high salinity and temperature stress exhibit complex molecular responses, including the biosynthesis and accumulation of compatible osmolytes and activation of pathways leading to stress tolerance (Zhu et al., 1997). The ROS generated by salt stress and temperature stress might be one of the most important causes of damage in plant tissues (Mittler, 2002). Many of the transgenic plants developed for salt tolerance also demonstrated increased ROS scavenging activity. For example, the functional characterization of *A. thaliana* mutant *pst1* provided strong support for the importance of oxidative protection in improving abiotic stress tolerance. The loss of function mutants of *PST1* exhibited more tolerance to salinity, and it was reported that this correlated with an increased potential to tolerate oxidative stress due to scavenging activity (Tsugane et al., 1999).

Plants have been developed for abiotic stress tolerance by genetic engineering the genes for osmolytes, including fructose, trehalose, ononitol, proline, glycine betaine and mannitol. Stress tolerance in these plants might work through the oxidative detoxification strategy because the osmolytes aforementioned have been reported to be highly active in ROS scavenging activities (Shen et al., 1997a). These authors demonstrated that targeted biosynthesis of mannitol in the chloroplast, by fusing the signal sequence in front of the engineered enzyme, resulted in better protection against ROS and improved abiotic stress tolerance. Their study was consistent with the knowledge that the chloroplast is the primary site of ROS generation. It seems that mannitol in transgenic plants acted by enhancing the

mechanism of ROS scavenging because this polyol accumulated at levels that were not high enough to carry out osmotic adjustments (Zhu, 2001).

2.5 Heat Stress Tolerance

Extreme temperatures can alter the fluidity of the cellular membrane and the integral membrane proteins can perceive the associated alterations (Sangwan et al., 2002). High temperature stress causes the denaturation of proteins and the molecular chaperones can bind to these proteins to avoid further damage (Scharf et al., 2012). Heat stress transcription factors (TF) are released from the chaperones following the binding to misfolded proteins and this release of TF activates the heat stress responsive genes. Kumar and Wigge (2010), reported a thermo sensory apparatus known as H2A.Z, which contains a nucleosome found in both plants and yeast. They proposed that this particular nucleosome would wrap the DNA tightly, as compared to H2A containing nucleosome. When exposed to high temperature, this induces the expression of HSPs (heat shock proteins) and other heat stress associated genes by detaching the H2A.Z nucleosome so that the DNA is more accessible to RNA polymerase II (pol II) transcription. However, this proposed hypothesis needs further investigation to be proven.

2.6 Effects of Abiotic Stress on Flowering

The survival of a plant species is largely dependent on flowering and seed production. During the last few decades tremendous progress has been made in research to delineate and characterize the networks and pathways involved in flowering. Large scale reprogramming of genetic and epigenetic factors, and reallocation of biochemical and metabolic resources are required for the successful transition from the vegetative phase to the reproductive phase (Kazan and Lyons, 2015). In *A. thaliana*, the pathways include age, sugar, circadian clock, vernalization, photoperiod, thermo sensory, and gibberellin, and coincides on a few integrator genes which are known to induce flowering (Kazan and Lyons, 2015). The mobile signal florigen, encoded by a key floral integrator gene *flowering locus* (FT), induces the expression of *leafy* (LFY) and *apetala1* (AP1), and the up-

regulation of these genes determines the apical meristem to switch from the vegetative to the reproductive phase.

SPL (SQUAMOSA PROMOTER BINDING PROTEIN LIKE) are a set of transcription factors that are expressed before *LFY* and *AP1* (Cardon et al., 1997; Schmid et al., 2003). These genes play critical role in broad range of developmental processes, such as vegetative phase transition, leaf production, anthocyanin and GA biosynthesis, plant architecture, male fertility and responses to abiotic stresses (Zhang et al., 2007; Yu et al., 2012; Cui et al., 2015; Wang et al., 2015; Chao et al., 2017; Gou et al., 2017; He et al., 2018; Wei et al., 2018). These TFs were first discovered in snapdragon (*Antirrhinum* sp.) and subsequently have been identified in several plant species such as *A. thaliana*, wheat, barley, rice, maize, tomato, cotton, soyabean, poplar and *Brachypodium* (Moreno et al., 1997; Xie et al., 2006; Salinas et al., 2012; Li and Lu, 2014; Zhang et al., 2014; Tripathi et al., 2017; Cai et al., 2018; Tripathi et al., 2018; Tripathi et al., 2020).

A. thaliana genome contains 17 *SPL* genes. Based on their size these are classified in two major categories. SPLs which are greater than 800 amino acid residues (1, 7, 12, 14 and 16) are of class 1 while the remaining SPLs that are shorter than 400 amino acid residues are in class 2 (Cardon et al., 1999; Birkenbihl et al., 2005). Class 2 *SPLs* (2,9,10,11,13 and 15) have been shown to play an important role in shoot development and transition from vegetative to reproductive phase (Xu et al., 2016). Inflorescence of the knockout mutants of *SPL1* and *12* (class 1) genes showed higher susceptibility to heat stress whereas the overexpression plants were tolerant suggesting that both these genes play critical role at reproductive stage (Chao et al., 2017). *SPL3, 4* and *5*, which encode smaller proteins, play critical roles in regulation the flowering time and phase transition (Wang et al., 2009). SPLs proteins contain a conserved SBP domain which harbours 2 zinc binding sites and a C-terminal nuclear localization signal motif (Liu et al., 2017).

Non-coding RNA molecules regulate the expression of several genes by binding to the complementary sequence in the mRNA of their target genes (Chen, 2009; Voinnet, 2009). In *A. thaliana*, 11 *SPL* genes are negatively regulated by miRNA156, which is a pleiotropic regulator of plant developmental processes. Its level decreases with age (Wu and Poethig, 2006; Wang et al., 2009). Except for *SPL8*, all the class 2 *SPLs* have been

shown to be targeted by miRNA156 (Wu and Poethig, 2006; Wang et al., 2009; Wu et al., 2009; Yu et al., 2010). *SPLs* which are targeted by miRNA156 positively regulates the expression of *apetala1* (*AP1*), *fruitful* (*FU*), *suppressor of overexpression of constans* (*SOC1*) and *leafy* (*LFY*), thereby induce the phase transition (Wang et al., 2009; Yamaguchi et al., 2009; Xu et al., 2016). *AP1* and *LFY* are the principal regulators, which controls the flowering at shoot apex (Wang et al., 2009). Knockout mutant of these genes exhibited defective phenotype in flowering where the flowers looked like shoot structure (Lohmann and Weigel, 2002). It has been shown that when *SPL3* and *9* are overexpressed, they show rapid induction of flowering, whereas the reduction in *SPL* activity due to miRNA156 overexpression determines delayed flowering (Cardon et al., 1997; Wu and Poethig, 2006; Gandikota et al., 2007; Schwarz et al., 2008; Wang et al., 2008). In plants, miRNA172 has been shown as essential for the early onset of flowering. *SPL9* and *10* induces the expression of miRNA172 (Wu et al., 2009). *SPL*9 was shown to induce the expression of several genes including *AP1*, *FU*, miRNA172b and *TCL*1 by directly binding to the regulatory sequences of these genes (Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009; Yu et al., 2010).

During long day conditions, the TF *constans* (CO) up-regulates the expression of FT, thus inducing flowering. During long days at ambient temperatures, *flowering locus C* (FLC), a TF that antagonizes the photoperiod and GA (gibberellic acid) pathways by repressing the FT and *suppressor of constans* (SOC1), was found to be down-regulated epigenetically through a vernalization pathway (Andrés and Coupland, 2012; Bluemel et al., 2015; Kazan and Lyons, 2015). Salinity and drought stress during the initiation of flowering cause an early halt of floral development due to reduction in fertility, pollen viability, and opening of flowers and ultimately predisposes the plant to complete sterility (Su et al., 2013). Several studies have demonstrated the key role of floral pathways in modulating stress tolerance (Sherrard and Maherali, 2006; Franks et al., 2007; Bernal et al., 2011; Franks, 2011). For example, in *A. thaliana AtPI4Kγ3*, gene is involved in abiotic stress tolerance and in floral transition by regulating the expression of key genes involved in flowering pathways (Akhter et al., 2016).

The key regulator of the flowering in *A. thaliana* is *gigantea* (GI), the photoperiodic flowering time gene that is known to induce flowering through photoperiod and circadian pathways. During long days, GI is involved in the degradation of the *cycling D factor* (CDF), a transcriptional repressor of the floral integrator gene CO and FT, thus allowing the activation of these genes to promote flowering (Figure 2.3). Additionally, GI can directly bind to the promoter of FT to induce its expression for activation of flowering (Sawa and Kay, 2011).

A. thaliana plants exposed to salinity stress exhibit delayed flowering and a number of different flowering regulators which control this response have been characterized in detail (Kim et al., 2007). Achard et al. (2006) reported that salinity delays flowering in *A. thaliana* through a process dependent on Della proteins. These proteins act as negative regulators of ethylene and gibberellin pathways. Salinity stress down-regulates the expression of CO and FT genes by proteasomal degradation of GI and thereby delays the flowering (Kim et al., 2007; Li et al., 2007). Kim et al. (2013) demonstrated that GI is also a key component of salinity tolerance in plants. The loss of function mutation in GI exhibited improved salinity tolerance. Moreover, GI overexpressing plants were susceptible to salinity, suggesting the role of GI in negative regulation of salt tolerance. Under optimal growth conditions, GI interacts with SOS2, a pivotal and central component of SOS pathway, with protein kinase activity that activates SOS1, a Na^+/H^+ antiporter on plasma membrane to induce salinity tolerance. The interaction between GI and SOS2 prevents the interaction between SOS2 and SOS1, which is required for the induction of salt tolerance response. However, upon exposure to salinity stress, proteasome degrades GI and this enables the interaction between SOS2 and SOS3, and then this forms a protein kinase complex, which directly phosphorylates SOS1 at the plasma membrane to activate the SOS pathways for salinity tolerance (Figure 2.3) (Kim et al., 2013).

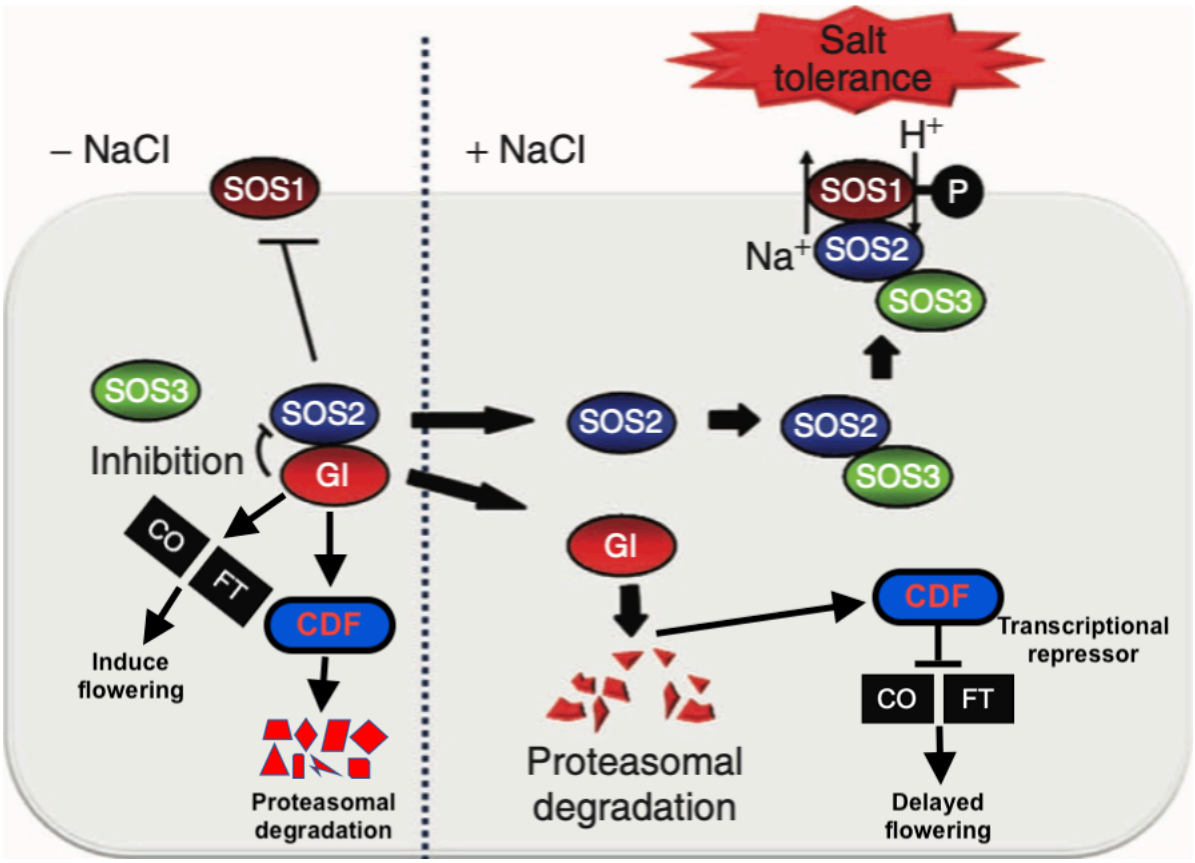


Figure 2.3. Salt stress tolerance and flowering. (Kim et al., 2013, modified)

2.7 Different Approaches to Find Gene Functions

After genome sequencing, it is important to determine the function of genes. Different approaches used to unravel the functions of novel genes include gene mutation, complementation, searching for homologues genes, reporter genes, co-expression and protein-protein interaction analysis.

2.7.1 Searches for homologue genes in other organisms

Breakthrough development in sequencing technologies allowed the sequencing of the genome of numerous organisms. Several thousands of genes have been identified and characterized/or being characterized in different organisms. Information about the gene and protein sequences and their function in organisms are routinely updated on different

databases. Searching the nucleotide or protein sequence of genes on these databases can help to predict the function of gene. However, it becomes difficult to predict the function if the novel protein sequence doesn't have conserved amino acid sequences or specific protein domains in any existing characterized proteins of other organisms.

Changing the genetic material of an organism using various chemicals or techniques represented a major breakthrough in the history of genetics. Several physical and chemical mutagens have been used to knockout the gene function. Ion beam radiations, α-β particles and UV are the major physical mutagens used for mutagenesis. Chemical mutagens used to produce mutants include ethylmethane sulfonate, N-methly-N-nitrousurea, sodium azide and diepoxybutane (reviewed by Viana et al., 2019). *Agrobacterium* mediated transformation led to a major breakthrough in plant biotechnology and genetic engineering. This method has been widely used to generate T-DNA insertion mutants. It inserts the exogenous DNA into the genome which can generate mutations if the inserted sequence can interrupt the coding sequence or the regulatory sequences of the target gene. However, the T-DNA insertions are random in the genome and requires the screening of several clones. Recent advances in technology lead to development of tools for precise sites specific mutagenesis using RNAi (RNA interference) and CRISPR (clustered regularly interspaced short palindromic repeats). Once the large collection of mutants is established, they are screened for the desired trait.

2.7.2 Gain of function mutation

Gain of function mutations can help to predict the function of a gene. These mutations may cause novel phenotypes. These mutations are produced by overexpressing the gene to significantly higher levels than in the normal cells. Overexpression of protein at higher levels may be deleterious and therefore it is important to regulate the expression of gene. The expression is controlled by using the inducible promoters rather than the strong constitutive promoters.

2.7.3 Loss of function mutation

To understand the function of gene it is important to observe the phenotype of organism in absence of the target gene. Once the large collection of mutants is established, they are screened for desired phenotype. However, in some circumstances, this approach might not work because mutation in one gene might not produce significant changes in phenotype or the deletion of target gene may be lethal for the organism, therefore further studies are precluded. In this approach, studying the phenotype from the genotype is much faster and efficient in comparison to understanding the genotype from the observed phenotype.

2.7.4 Reporter genes

The function of a gene can be predicted by investigating and analyzing the expression of protein in different cellular compartments, or in specific tissues of an organisms, at different time intervals. It is mainly examined by measuring the enzymatic activities or by tracking the fluorescence signals produced by reporter genes to see when and where it is produced in the cell, in tissues or in whole organisms. The most common reporter genes used are GFP, YFP, GUS and luciferase. The regulatory sequence of gene can be used to drive the expression of the reporter gene.

2.7.5 Co-expression and protein-protein interactions

Understating and analyzing the expression of genes expressed with target gene can provide clues about the function of the gene. Genes that are up-regulated or down-regulated together with target gene may work together in a cell as they may encode protein which may be the part of a complex network or metabolic pathway.

Co-expression networks are generated by integrating the several expression databases, which connects the multiple genes exhibiting similar expression pattern across the various conditions. The genes are functionally related if they have higher degree of co-expression with other genes that are located on the network. ATTEDII, a co-expression database, provides the relationship of co-regulated genes allowing to predict the gene

function of several plant species including *A. thaliana*, Medicago, field mustard, soybean, rice, poplar, tomato, grapes and maize (https://atted.jp) (Obayashi et al., 2018).

Protein-protein interactions can provide clues about the function of a protein. For interaction among proteins, the noncovalent contacts between the amino acid residues of side chain are critical; these contacts can induce various interactions and associations between the interacting proteins. Based on their persistence, the interactions can be temporary or permanent. If the interaction is temporary then it is a part of signaling pathway and if it is permanent interaction then it will form a protein complex (Zhang, 2009). It has been shown that more than 80% of proteins function in complexes (Berggård et al., 2007). If an unknown function protein interacts with a protein of known function, then it can be used to predict function of protein.

CHAPTER 3

Low Mannitol Concentrations in *Arabidopsis thaliana* Expressing *Ectocarpus* Genes Improve Salt Tolerance

Pramod Rathor [1], Tudor Borza [1], Yanhui Liu [2], Yuan Qin [2], Sophia Stone [3], Junzeng Zhang [4], Joseph P. M. Hui [4], Fabrice Berrue [4], Agnès Groisillier [5], Thierry Tonon [6,7], Svetlana Yurgel [1], Philippe Potin [7] and Balakrishnan Prithiviraj [1,*]

[1] Department of Plant, Food and Environmental Sciences, Dalhousie University, Truro, NS, B2N 5E3 Canada; pramod.rathor@dal.ca (P.R.); tudor.borza@dal.ca (T.B.); syurgel@dal.ca (S.Y.)

[2] Fujian Provincial Key Laboratory of Haixia Applied Plant Systems Biology, State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, Center for Genomics and Biotechnology, College of Life Science, Fujian Agriculture and Forestry University, Fuzhou 350002, China; 2180514010@fafu.edu.cn (Y.L.); yuanqin@fafu.edu.cn (Y.Q.)

[3] Department of Biology, Dalhousie University, Halifax, NS, B3H 4R2 Canada; s.stone@dal.ca

[4] Aquatic and Crop Resource Development Research Centre, National Research Council of Canada, Halifax, NS, B3H 3Z1, Canada; junzeng.zhang@nrc-cnrc.gc.ca (J.Z.); Joseph.Hui@nrc-cnrc.gc.ca (J.P.M.H.); Fabrice.Berrue@nrc-cnrc.gc.ca (F.B.)

[5] Unité Fonctionnalité et Ingénierie des Protéines (UFIP), UMR 6286 CNRS, Université de Nantes, 44322 Nantes, France; agnes.groisillier@univ-nantes.fr

[6] Centre for Novel Agricultural Products, Department of Biology, University of York, Heslington, YO105DD UK; thierry.tonon@york.ac.uk

[7] Sorbonne Université, CNRS, UMR 8227, Integrative Biology of Marine Models (LBI2M), Station Biologique, 29680 Roscoff, France; philippe.potin@sb-roscoff.fr

* Correspondence: bprithiviraj@dal.ca

This chapter is published in Plants: https://doi.org/10.3390/plants9111508

3.1 Abstract

Mannitol is abundant in a wide range of organisms that play an important role in response to biotic and abiotic stresses. Nonetheless, mannitol is not produced by a vast majority of plants, including many important crop plants. Mannitol-producing transgenic plants displayed improved tolerance to salt stresses though mannitol production was rather low, in the μM range, compared to mM range found in plants that innately produce mannitol. Little is known about the molecular mechanisms underlying salt tolerance triggered by low concentrations of mannitol. Reported here is the production of mannitol in Arabidopsis *thaliana*, by expressing two mannitol biosynthesis genes from the brown alga *Ectocarpus* sp. strain Ec32. To date, no brown algal genes have been successfully expressed in land plants. Expression of *mannitol-1-phosphate dehydrogenase* and *mannitol-1-phosphatase genes* was associated with the production of 42.3–52.7 nmol g^{-1} fresh weight of mannitol, which was sufficient to impart salinity and temperature stress tolerance. Transcriptomics revealed significant differences in the expression of numerous genes, in standard and salinity stress conditions, including genes involved in K^+ homeostasis, ROS signaling, plant development, photosynthesis, ABA signaling and secondary metabolism. These results suggest that the improved tolerance to salinity stress observed in transgenic plants producing mannitol in μM range is achieved by the activation of a significant number of genes, many of which are involved in priming and modulating the expression of genes involved in a variety of functions including hormone signaling, osmotic and oxidative stress, and ion homeostasis.

Keywords: mannitol biosynthesis genes; mannitol-1-phosphate dehydrogenase; mannitol-1-phosphatase; *Ectocarpus* sp., *Arabidopsis thaliana*; abiotic stress tolerance; salt stress.

3.2 Introduction

Plants are sessile organisms constantly subjected to abiotic stressors such as soil salinity and temperature extremes, which are considered the major limiting factors to agricultural productivity, posing a risk to global food security. Improving plant tolerance to these abiotic factors is critical for agricultural output and environmental sustainability. In the last

decades, several varieties with improved tolerance to salt stress have been developed; however, the progress has been generally slow because of the multigenic nature of the traits involved in abiotic stress tolerance (Zhu, 2016).

To achieve tolerance to salinity and drought plants produce different osmolytes including sugar alcohols, proline, polyamines, glycine betaine and proteins from the late embryogenesis abundant (LEA) superfamily (Verslues et al., 2006). Mannitol is an ubiquitous sugar alcohol found in a wide range of living organisms including bacteria, fungi, algae, and in over 100 plant species, where it serves as a metabolite, osmolyte and anti-oxidant (Patel and Williamson, 2016; Tonon et al., 2017). Many important crop plants, however, do not produce mannitol (Abebe et al., 2003). In the last 25 years a significant number of mannitol-producing transgenic plants have been generated, using genes of very different origin: *mannitol-1-phosphate dehydrogenase* (*M1PDH*) from *Escherichia coli* and *mannose-6-phosphate reductase* (*M6PR*) from celery (*Apium graveolens*) (Tarczynski et al., 1992, 1993; Abebe et al., 2003; Zhifang and Loescher, 2003; Chiang et al., 2005; Prabhavathi and Rajam, 2007; Sickler et al., 2007; Chan et al., 2011; Patel et al., 2015; Patel and Williamson, 2016). These two different choices for the origin of the genes used to generate transgenic plants reflect the fact that mannitol biosynthesis and catabolism is different in the abovementioned groups of organisms. In *E. coli*, mannitol is interconverted in two steps—phosphorylation and oxidation—to fructose 6-phosphate (F6P), the later step involving M1PDH, the product of *mtlD* gene. In land plants, the interconversion of F6P to mannitol involves mannose as an intermediate in both, the mannitol biosynthetic pathway and the catabolic pathway. In the former pathway, mannose-6-P and mannitol-1-P are produced in reactions catalyzed by phosphomannose isomerase (PMI) and M6PR, respectively. In the last step, mannitol-1-phosphate phosphatase (M1Pase) will generate mannitol. The catabolic pathway proceeds through the formation of mannose and mannose-6-P, in reactions catalyzed by mannitol dehydrogenase, a hexokinase (HX) and PMI. *E. coli mtlD* was introduced into a multitude of plants, in which mannitol is not naturally produced, including important crops such as rice, maize, tomato, eggplant, peanut and tobacco, as well as the model plant *A. thaliana*. The transgenic plants accumulated mannitol and most of them exhibited improved tolerance to salinity stress, in spite of the fact that mannitol concentration in these plants was much lower (generally between 0.1–

10 μmol g^{-1} fresh weight) than in plants that naturally produce this polyol (> 100 μmol g^{-1} fresh weight) (Tarczynski et al., 1992, 1993; Thomas et al., 1995; Abebe et al., 2003; Zhifang and Loescher, 2003; Patel and Williamson, 2016). Celery *M6PR* gene was introduced into *A. thaliana* and mannitol concentrations in the range of 0.5–6 μmol g^{-1} fresh weight resulted in enhanced salt tolerance (Zhifang and Loescher, 2003). Transcriptomics (microarray) analysis of mannitol-producing transgenic *A. thaliana*, carried out under standard and salinity stress conditions, revealed a wide array of pathways being differentially expressed, suggesting that mannitol-enhanced stress tolerance is achieved by global changes in gene expression and not only by increased expression of stress-inducible genes (Chan et al., 2011). Noteworthy, none of the mannitol-producing transgenic plants, generated using *E.coli mtlD* or celery *M6PR*, expressed the phosphatase required to generate mannitol from mannitol-1-phosphate; it has been suggested that a non-specific endogenous phosphatase can perform this essential dephosphorylation step (Tarczynski et al., 1992).

During the evolution of Eukarya, the stramenopiles, the lineage that encompasses brown algae (*Phaeophyceae*) (Coelho et al., 2012), have been evolving for over a billion years independently compared with the most commonly studied multicellular eukaryotes comprising non-photosynthetic opisthokonts (including animals and fungi) and archaeplastida (including red algae and green plants) (Tonon et al., 2017). Large biomass of brown algae is found in the intertidal zone, which is a very harsh environment, with constant fluctuating levels of salinity and temperature. Mannitol is the main photosynthetate in brown algae, which includes seaweeds such as kelp (*Macrocystis* spp. and *Laminaria* spp.), rockweed (*Ascophyllum nodosum*), and *Sargassum.* In these algae, mannitol is the main carbon storage molecule as well as an osmolyte with anti-oxidant potential (Tonon et al., 2017) In late summer, in some kelps, mannitol accounts for up to 25% of dry biomass (Black, 1950). To enable functional studies of brown algae, strain Ec32 of the small filamentous alga *Ectocarpus* sp., formerly included in *Ectocarpus siliculosus* (Montecinos et al., 2017), has been established as a genetic and genomic model (Coelho et al., 2012). In the filamentous alga *Ectocarpus sp*. mannitol content reaches 3.6–6.4% of the dry weight (Gravot et al., 2010). In brown algae, mannitol metabolism involves two enzymes for biosynthesis and two for recycling. Enzymes for biosynthesis includes

M1PDH and M1Pase while mannitol recycling includes mannitol-2-dehydrogenase and a HX. The genome of *Ectocarpus* sp. Ec32 encodes three putative *M1PDH* genes (*EsM1PHD1*, *EsM1PDH2* and *EsM1PDH3*) and two *M1Pases* (*EsM1Pase1* and *EsM1Pase2*). *EsM1PHD1* (EC 1.1.1.17) and *EsM1Pase2* (EC 3.1.3.22) were overproduced in *E. coli* and characterized biochemically (Groisillier et al., 2014; Bonin et al., 2015).

To date, no brown algal genes have been successfully expressed in land plants. Here we report the expression of *EsM1PHD1* and *EsM1Pase2* genes from *Ectocarpus* sp. Ec32 in *A. thaliana*. Transgenic plants showed improved tolerance to salinity and temperature stress, though mannitol concentrations were lower than those reported in transgenic plants expressing *E. coli mtlD* or celery *M6PR* genes. Transcriptomics analysis comparing mannitol-producing plants to wild type plants, grown under standard and salt stress conditions, revealed a significant number of differentially expressed genes involved in hormonal regulation and ABA signaling, defense mechanisms and ROS signaling, plant development, photosynthesis, K^+ homeostasis and secondary metabolism. These findings represent a major contribution to the deciphering of the molecular mechanisms through which low concentrations of mannitol improve plant salt tolerance.

3.3 Materials and Methods

3.3.1 Cloning of *Ectocarpus* sp. *M1PDH1* and *M1Pase2* genes and transformation of *A. thaliana*

Ectocarpus sp. *mannitol-1-phosphatase (M1Pase2)* and codon-optimized *mannitol-1-phosphate dehydrogenase M1PDH1* (Bonin et al., 2015), under separate 35S promoters and OCS terminators, were cloned in pEarleyGate 100 obtained from ABRC (Columbus, OH, USA) using the multisite Gateway Pro Four Fragment recombination technology (Gateway® Technology Multisite, Invitrogen, Mississauga, ON, Canada) (Figure 3.1). The expression plasmid containing both genes was transformed into *Agrobacterium* strain GV310 (pMB90) and then *A. thaliana* (L.) Heynh, ecotype Columbia (Col-0) (Lehle Seeds, Round Rock, TX, USA) was used to generate transgenic plants, using the floral dip method (Clough and Bent, 1998).

Figure 3.1. Construct generated to produce transgenic lines.

3.3.2 Selection of transformants and determination of homozygosity

Positive transformants were selected by seed germination and seedling growth on plates containing half strength Murashige and Skoog medium supplemented with 35 µg/mL ammonium glufosinate (Sigma, Mississauga, ON, Canada). Three independent transgenic lines were selected, and seeds collected from the fifth generation were used for further experiments. Transgene expression was verified by qPCR as described below.

3.3.3 Salinity stress tolerance

For *in vitro* experiments seeds were germinated in half strength MS medium, supplemented with 1% (*w*/*v*) sucrose. Seedlings were maintained at 22 °C, in a 16-h light/8-h dark cycle. After 4 ½ days of growth, uniform seedlings were transferred on plates containing the same medium, in the absence or presence of 100 mM NaCl, and grown in the same conditions as mentioned before. Root length was marked on the day of transfer; after 7 days, leaf chlorosis was recorded, and plates were scanned with a high-resolution scanner (Expression 10000 XL, Epson, Markham, ON, Canada). Root length was measured with Image J software (Research Services Branch, NIH, Bethesda, MD, USA). Fresh weight of seedlings was recorded 11 days after the transfer, then samples were dried. Experiments were repeated three times with 60 seedlings per transgenic line per treatment. Plants grown in these conditions have been referred to as seedlings throughout this paper.

In vivo experiments were carried out using Jiffy peat pellets (Jiffy, Shippagan, NB, Canada). Experiments were started using 15 days old plants. Peat pellets were irrigated once with 40 mL of 200 mM NaCl per plant, giving a final concentration of NaCl in the pellet of 100 mM. After 5 days, 20 mL of water was added to each plant; subsequent watering was done at 4 days interval, for 3 weeks. After 3 weeks plants biomass was recorded. Experiments were repeated three times with 10 plants per transgenic line per treatment. Plants grown in these conditions have been referred to as adult plants throughout

this paper. Data from all experiments were analyzed using ANOVA followed by Tukey's at an $\alpha < 0.05$ in SAS 9.1.2 (2001, SAS Institute, Cary, NC, USA).4.4.

3.3.4 Heat stress tolerance

Seedlings were generated on plates as described earlier. Ten days old seedlings were exposed to 40 °C for 24 h. Seedlings were then grown under standard conditions and biomass was recorded at 8 days after the temperature stress. Experiments were repeated three times with 60 seedlings per transgenic line per treatment.

3.3.5 Electrolyte leakage

Plants were grown on peat pellets as described earlier. Fifteen days old plants were irrigated with 40 mL of 100 mM NaCl per plant and after 24 h the leaves were harvested and placed in scintillation vials containing 20 mL of deionized water. The experiment was repeated two times and each experiment had three biological replicates, each represented by pooled leaves from three plants. Electrolyte leakage was recorded using SympHony SB70C (VWR, Mississauga, ON, Canada) conductivity meter as described by (Cao et al., 2007).

3.3.6 Quantification of mannitol levels in transgenic lines by LC-MS

Leaves from 18-day-old plants, grown on peat pellets, were harvested, frozen in liquid nitrogen, and then lyophilized. Approximately 5 mg of freeze-dried leaves were mixed with 1 mL cold 90% methanol and ribitol (internal standard, Sigma-Aldrich, St. Louis, MO, USA). Samples were dried, cleaned up by suspending in 500 µL of acetonitrile/water (1:1), dried again and then resolubilized in 500 µL methanol for LC-MS analysis.

The LC-MS system used for mannitol quantification included an UltiMate 3000 LC pump (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Exactive™ high resolution mass spectrometer (Thermo Fisher Scientific), equipped with an electrospray ionization

source. Separation was carried out on an Acquity BEH Amide column (2.1 × 100 mm 1.7 µm, Waters, Milford, MA, USA) Peak area based on the accurate mass measurement of mannitol at m/z 181.07176 was used to determine its concentrations in the plant tissue samples. Two extraction replicates and two analytical replicates from three biological samples were analysed for each of the transgenic lines.

3.3.7 Plant samples, RNA isolation, library construction and next generation sequencing

Plants were grown on peat pellet and 15 days old plants were exposed to salt stress as described above. Leaf tissues were collected 24 and 48 h after the exposure to salinity stress and stored in RNA Later solution (Life Technologies, Mississauga, ON, Canada). Leaf samples were ground in liquid nitrogen and RNA isolation was performed using the E.Z.N.A. Plant RNA Kit (Omega Bio-Tek, Shanghai, China). Library construction and sequencing using the Illumina HiSeq2500 technology were done at Beijing Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). A total of 68.03 GB data were generated and the clean data was 66.35 GB. The 24 sequenced libraries were from the wild type and from transgenic line *Es*M3. The experiment had two time points (24 and 48 h after salt stress), in standard conditions (i.e., no salt, samples described as WT and *Es*M) and in presence of salt stress (samples described as WTS and *Es*MS); each time point and condition had three biological replicates.

3.3.8 Next generation sequencing data processing

Sequence trimming (adapters and low-quality reads) was performed by Beijing Novogene Bioinformatics Technology Co. Ltd. Mapping to *A. thaliana* genome was done using TAIR10 (ftp://ftp.arabidopsis.org/home/tair) and TopHat2 software with default parameters. Differential gene expression was performed using Cufflinks, and identification of DEGs was performed using Cuffdiff software with a cutoff p value < 0.05, and a fold change ≥ 2. Functional annotation was done using DAVID Bioinformatics Resources 6.8 website (https://david.ncifcrf.gov). Venn diagrams (Heberle et al., 2015) were produced

from pairwise and multiple comparisons. Heat maps were generated using Heatmapper (Babicki et al., 2016) at http://www.heatmapper.ca; data was analyzed by clustering (average linkage) using the Euclidean distance measurement method. Regulatory networks related to hyperosmotic and salinity stress were generated based on the information available from ATTED-II v. 9.2 (http://atted.jp). Transcriptomics data was submitted to sequence read archive (SRA); submission ID: SUB8462528 and project ID: PRJNA674912.

3.3.9 Real-Time quantitative PCR

Transgenic lines were analyzed by RT-qPCR to assess the expression of *Ectocarpus* sp. *M1PDH1* and *M1Pase2*. Total RNA was extracted using GeneJET plant RNA purification kit (Thermo Scientific, Mississauga, ON, Canada). DNAse treated RNA was converted into cDNA using the RevetAID cDNA Synthesis kit (Thermo Scientific, Mississauga, ON, Canada). The relative transcript levels were determined using the gene specific primers and *ACTIN 2* (AT3G18780) primers (Supplementary Table S1 in Appendix A) as the internal control on the StepOne plus Real-Time PCR system (Applied Biosystems, Mississauga, ON, Canada), using iTaq SYBR Green (Bio-Rad, Mississauga, ON, Canada). Transcript abundance was determined using the comparative C_T method for relative quantification by employing the samples from the time point and the tissue with the lowest gene expression as calibrators.

3.3.10 Infiltration of wild type *A. thaliana* with low concentrations of mannitol

To assess the effect of low concentration of mannitol on *ATPI4KGAMMA3* and *QQS* expression, plants were grown on peat pellets as mentioned earlier. When plants were 20 days old, 3 fully expanded leaves from the 8-10 leaves in the rosette were infiltrated using a syringe with 500 µl of 100 µM D-mannitol (Sigma, ON, Canada). Infiltrated leaves were harvested at 2, 4, 6 and 24 h after infiltration and frozen in liquid nitrogen. Control leaves

were infiltrated using the double distilled water. qPCR analysis was performed as previously described.

3.4 Results

3.4.1 *M1PDH1* and *M1Pase2* expression and mannitol content in *A. thaliana* transgenic lines

The expression of *mannitol-1-phosphate dehydrogenase (M1PDH1)* and *mannitol-1-phosphatase* (*M1Pase2)* in each of the three selected independent double transgenic lines, henceforth named *Es*M1, *Es*M2 and *Es*M3, in which selection was done until the 5th generation, was determined by real-time quantitative polymerase chain reaction (qPCR) (Supplementary Table S1 in Appendix A). Relative gene expression among the transgenic lines varied 3-fold in the case of *M1PDH1* and 2-fold in the case of *M1Pase2*. Transgenic line *Es*M1 showed the overall highest expression while *Es*M3 the lowest (Figure 3.2). Gene expression was relatively high for both *M1PDH1* and *M1Pase2*, the former having a Ct value at 2–4 cycles difference from actin, the reference gene, while the later at 3–4 cycles. The content of mannitol in the transgenic lines, determined by LC-MS, was found to vary between 42.3 and 52.7 nmol g^{-1} fresh weight (Table 3.1).

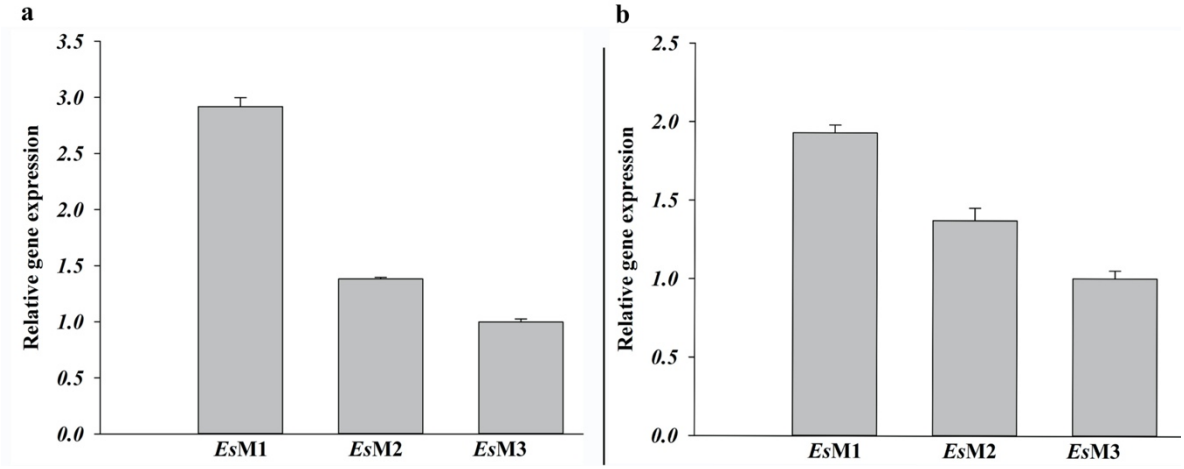


Figure 3.2. Gene expression analysis of *M1PDH1* and *M1Pase2* in transformed *A. thaliana* plants. (a) Relative gene expression of *M1PDH1*, (b) Relative gene expression of *M1Pase2*.

Actin was used as endogenous control and transcript levels were normalized to individual with the lowest expression in the transgenic line *Es*M3. *Es*M1, *Es*M2 and *Es*M3, are the three transgenic lines.

Table 3.1. The amount of mannitol in the three double transgenic *A. thaliana* lines, determined by LC-MS.

Sample	**nmol g^{-1} fresh tissue (mean and SD)**
WT	0
*Es*M1	52.68 ± 21.54
*Es*M2	48.78 ± 0.89
*Es*M3	42.27 ± 3.67

3.4.2 Mannitol producing lines showed enhanced tolerance to salinity and high temperature stress

All T5 transgenic seedlings, exhibited improved tolerance to salinity stress when exposed to 100 mM NaCl (Figure 3.3). Transgenic seedlings subjected to salinity stress showed significantly longer roots, higher number of lateral roots per cm of primary root and reduced leaf chlorosis as compared to the wild type seedlings (Figure 3.3 and Supplementary Table S2). In standard conditions, no significant differences were recorded in the fresh and dry weight of transgenic lines, as compared to the wild type seedlings, while under salinity stress, the transgenic lines accumulated significantly higher biomass (Figure 3.3 and Supplementary Table S3). All transgenic lines grew much better than the wild type on peat pellet when subjected to 100 mM NaCl salt stress. After one week of salinity stress, the transgenic plants displayed less growth inhibition, had significantly higher fresh and dry weight and less chlorosis compared to the wild type plants (Figure 3.4 and Supplementary Table S3).

The effect of salinity stress on membrane intactness was estimated by measuring electrolyte leakage. The 15 days old mannitol-producing transgenic plants, exposed to 100 mM NaCl, showed significantly lower electrolyte leakage compared to wild type plants, suggesting higher membrane stability in transgenic lines (Figure 3.4).

Previous studies have shown that plants tolerant to salinity stress also exhibit tolerance to high temperature stress as both stressors cause *in planta* hyperosmotic conditions, and many of the stress responsive genes are commonly shared (Sakuma et al., 2006). Indeed, when subjected to high temperature stress seedlings from the T5 generation exhibited improved tolerance compared to the wild type seedlings (Figure 3.5). Transgenic seedlings exposed to high temperature stress (40 °C for 24 h) had significantly higher fresh and dry weight as compared to the wild type plants, while no significant differences were noted in plants grown under standard conditions (Figure 3.5 and Supplementary Table S4).

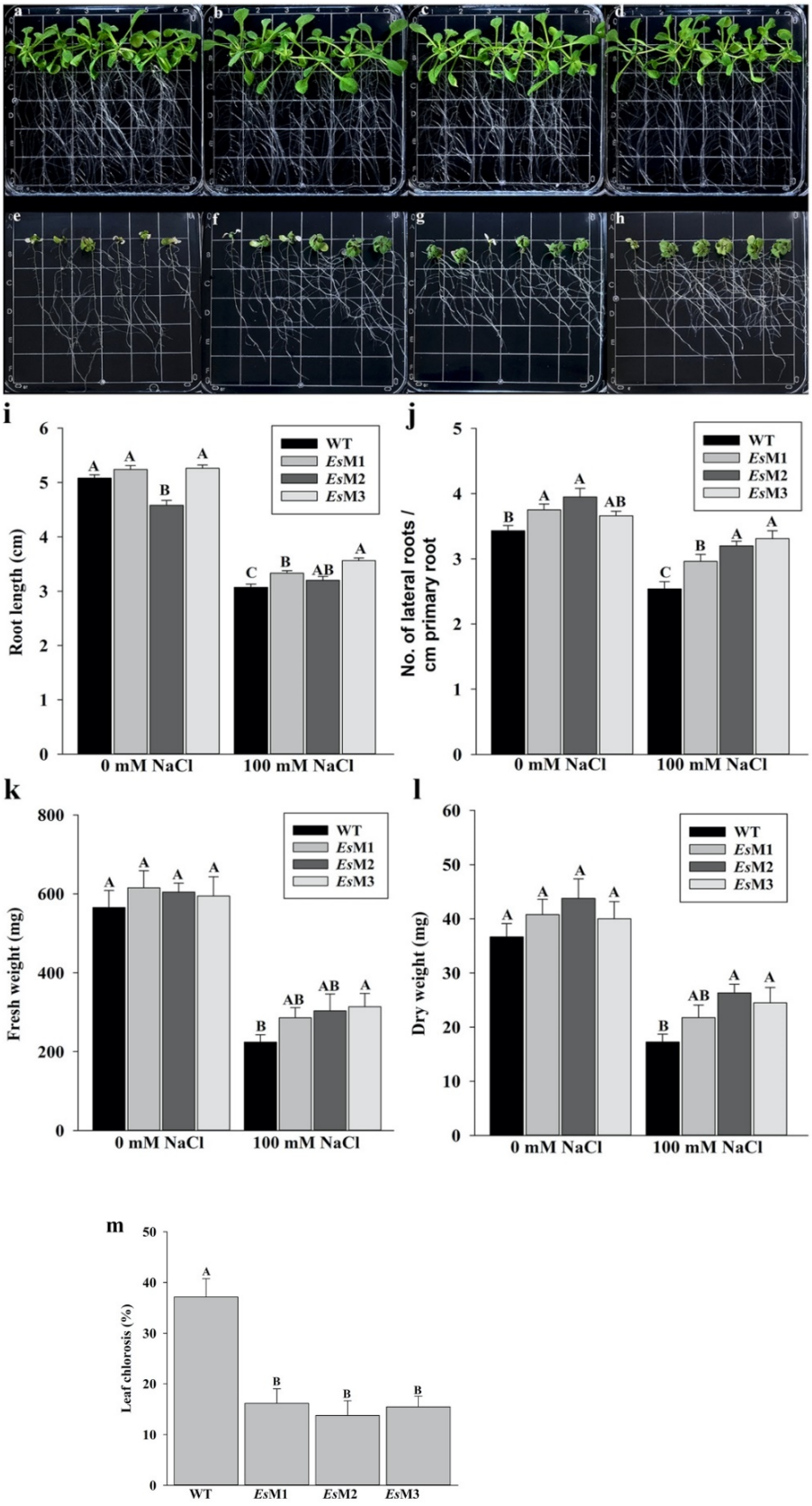
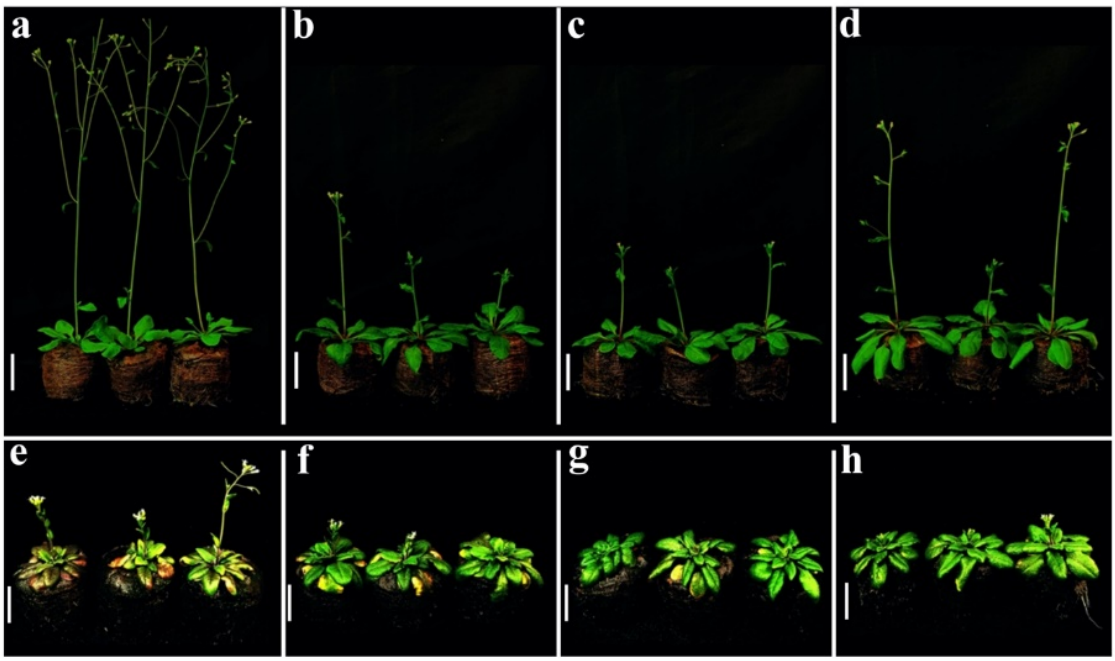

a
b
c
d
e
f
g
h
i
Root length (cm)
WT
EsM1
EsM2
EsM3
0 mM NaCl
100 mM NaCl
j
No. of lateral roots / cm primary root
k
Fresh weight (mg)
l
Dry weight (mg)
m
Leaf chlorosis (%)

Figure 3.3. Seedlings growth, root length, number of lateral roots per cm of primary root, fresh weight (FW), dry weight (DW) and leaf chlorosis of the wild type and 3 independent transgenic lines (*Es*M1, *Es*M2 and *Es*M3), in the absence and presence of 100 mM NaCl. In plates **(a–d)**, seedlings were grown under standard conditions or **(e–h)**, seedlings were grown in the presence of 100 mM NaCl. **(a,e)** WT, **(b,f)** *Es*M1, **(c,g)** *Es*M2 and **(d,h)** *Es*M3. The seedlings were photographed at 11 days after transfer in standard and under salinity stress conditions. Each grid has 13 mm. **(i)** root length, **(j)** number of lateral roots per cm of primary root, **(k)** fresh weight, **(l)** dry weight and **(m)** leaf chlorosis. Columns represents the mean and the bars the standard error; n = 150 for root length, number of lateral roots per cm of primary root and leaf chlorosis and n = 18 for fresh weight and dry weight. Means and SE with the same letter are not significantly different.



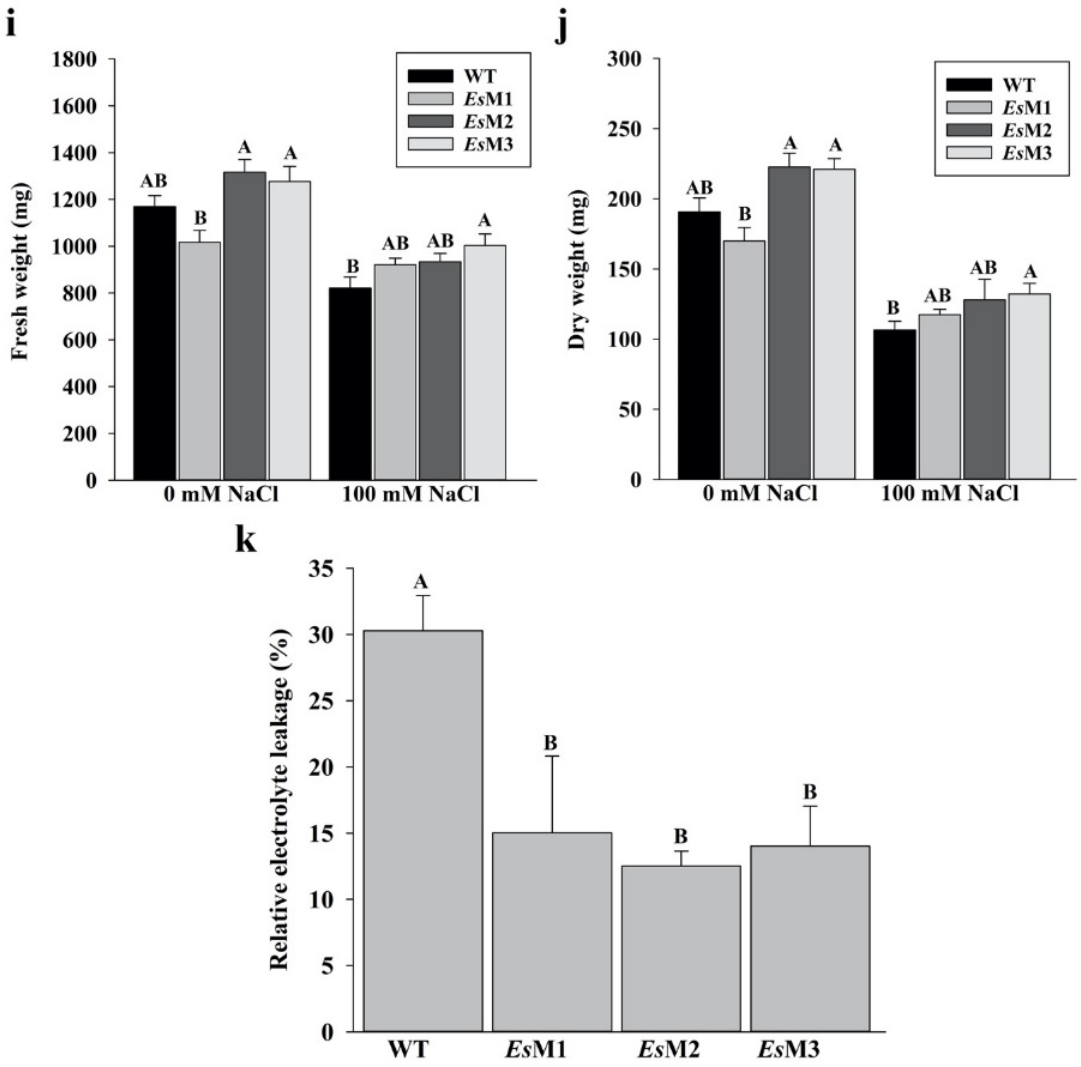


Figure 3.4. Plant growth, fresh weight (FW), dry weight (DW) and electrolyte leakage of the wild type and 3 independent transgenic lines (*Es*M1, *Es*M2 and *Es*M3), grown in the absence and presence of 100 mM NaCl. Plants **(a–d)**, were grown under standard conditions while in **(e–h)**, plants were grown in the presence of 100 mM NaCl. The white bar indicates 3.5 cm. **(a,e)** WT, **(b,f)** *Es*M1, **(c,g)** *Es*M2 and **(d,h)** *Es*M3. The plants were photographed at 10 days after irrigation when they were 25 days old. **(i)** fresh weight, **(j)** dry weight and **(k)** electrolyte leakage. Columns represents the mean and the bars the standard error (n = 30). Means and SE with the same letter are not significantly different.

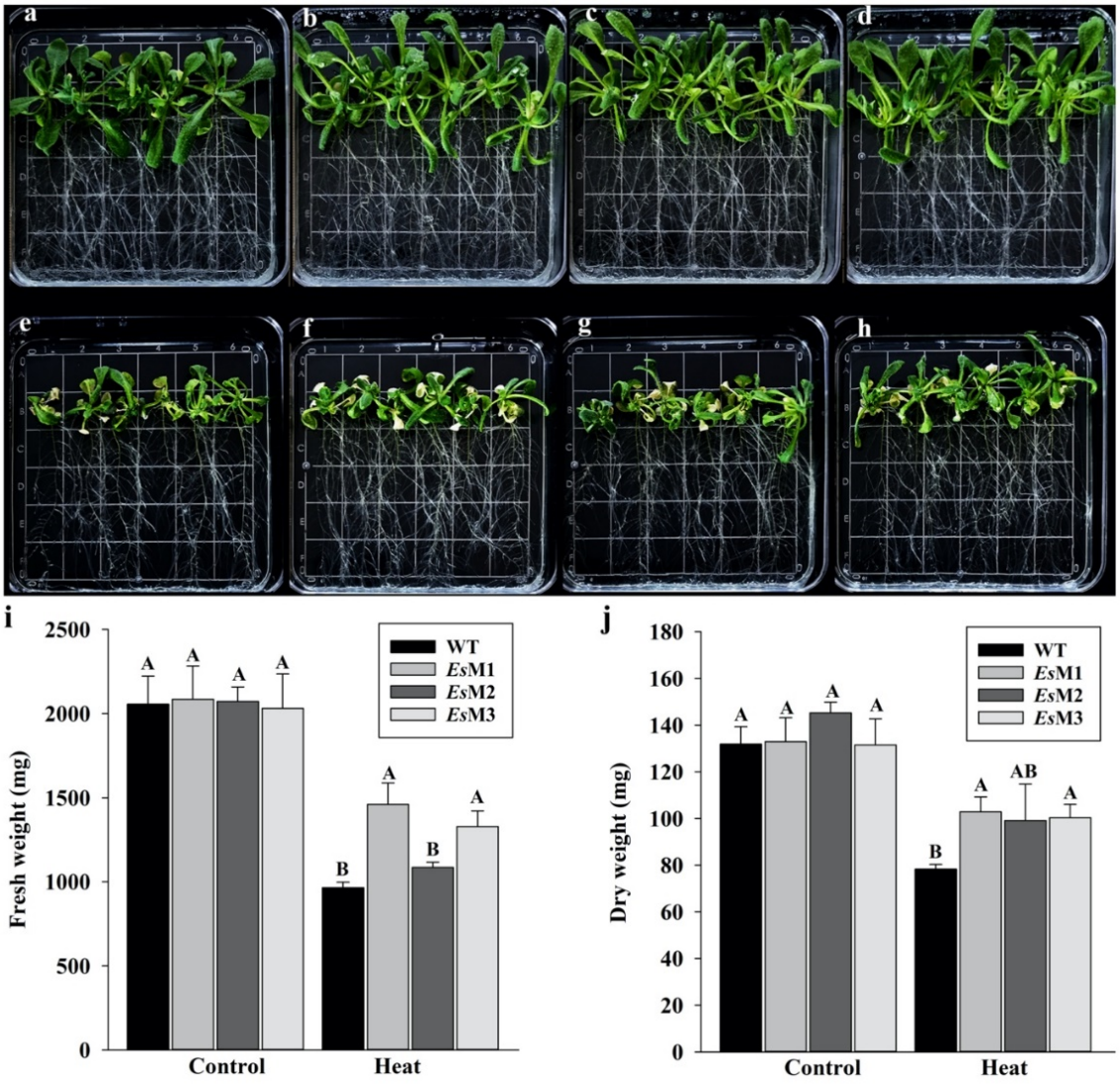


Figure 3.5. Seedlings growth, fresh weight (FW) and dry weight (DW) of the wild type and 3 independent transgenic lines (*Es*M1, *Es*M2 and *Es*M3), in the absence and presence of high temperature stress conditions. In plates **(a–d)**, seedlings were grown under standard conditions; **(e–h)** seedlings subjected to high temperature stress (40 °C for 24 h). **(a,e)** WT, **(b,f)** *Es*M1, **(c,g)** *Es*M2 and **(d,h)** *Es*M3. The 18 days old seedlings were photographed one week after being exposed to high temperature stress. Each grid has 13 mm. **(i)** fresh weight, **(j)** dry weight. Columns represents the mean and the bars the standard error (n = 9). Means and SE with the same letter are not significantly different.

3.4.3 Transcriptomics of a mannitol-producing line and of wild type *A. thaliana* grown in standard conditions and under salt stress

Since we demonstrated that mannitol is produced in transgenic *A. thaliana*, we decided to evaluate to what extent the transcriptome of the transgenic line *Es*M3 is influenced by mannitol, in optimal growth condition and under salt stress. Fifteen days old plants grown on peat pellet were subjected to salinity stress (100 mM NaCl) and leaves were collected 24 and 48 h after treatment. After Illumina sequencing and processing of raw reads, sequences were mapped using TAIR10. A number of 23,387 of *A. thaliana* transcripts were found to be expressed, being present in at least one library from the total number of libraries sequenced. Preliminary global correlation analyses clearly separated the samples (libraries) generated from plants grown in standard conditions from those grown under salt stress (Figure 3.6). Notable differences could also be observed between the 24 h and 48 h time points, after the NaCl treatment. No obvious differences could be observed between the mannitol-producing line and the wild type when plants were grown in standard conditions (Figure 3.6).

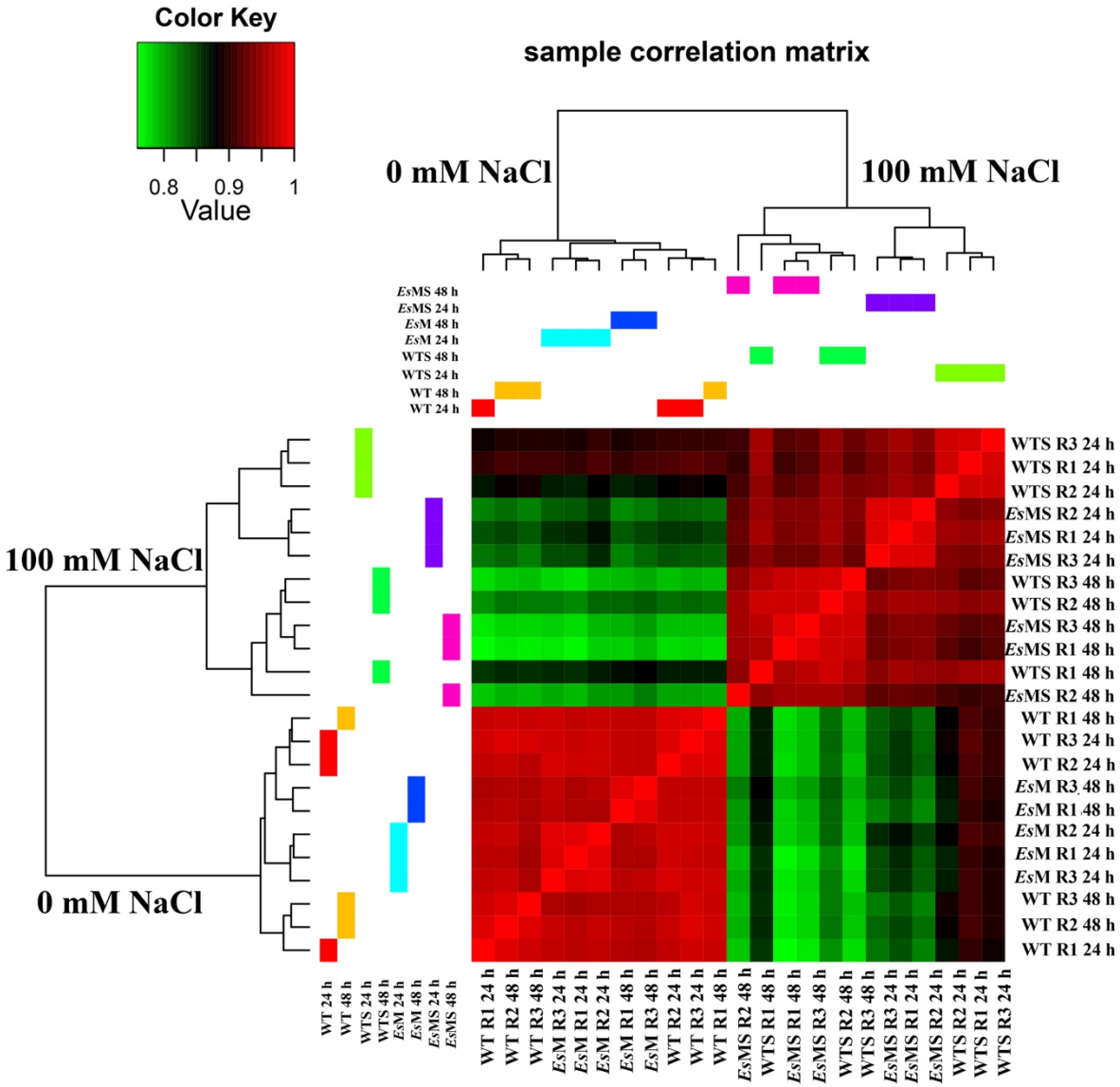


Figure 3.6. Correlation analysis showing separation of libraries in absence and presence of salinity stress at 24 h and 48 h.

Differential gene expression analysis (DGEs) was performed using Cuffdiff software with a cutoff p value < 0.05, and a fold change ≥ 2. DGE between mannitol-producing line (*Es*M) and wild type (WT), under standard conditions, identified 270 genes up-regulated at 24 h and 612 genes at 48 h. A smaller number of genes was found to be down-regulated, i.e., 116 and 223 genes at 24 h and 48 h, respectively (Figure 3.7 and Figure 3.8). These results indicate that mannitol production did not drastically affect overall gene expression in *Es*M *vs*. WT. This view is supported by the fact that in pairwise comparisons WT 24 h

vs. WT 48 h, or in *Es*M 24 h *vs*. *Es*M 48 h, roughly the same number of genes were found to be differentially expressed in standard conditions (Figure 3.8).

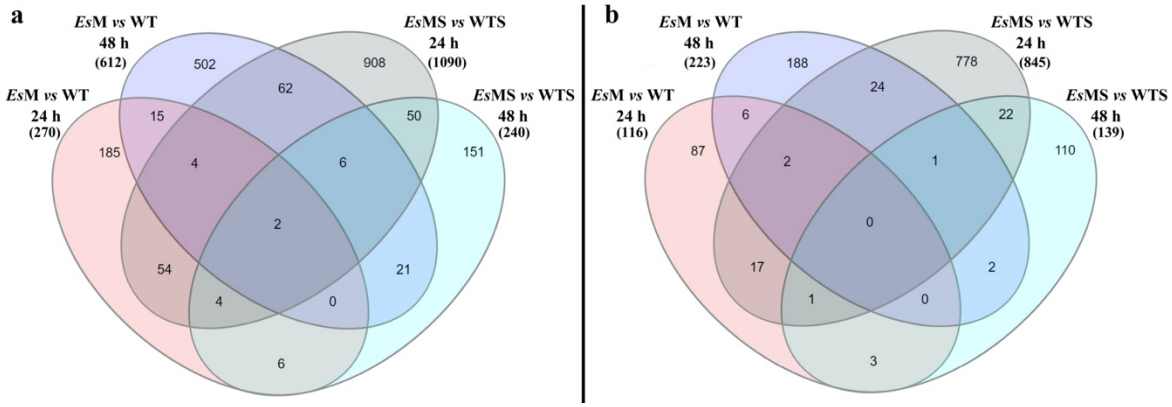


Figure 3.7. Venn diagram showing the differentially expressed genes (> 2-fold up- and down-regulated) in the mannitol-producing transgenic line *vs.* wild type, in standard (*Es*M and WT, respectively) and salinity stress conditions (*Es*MS and WTS, respectively), at 24 and 48 h. **(a)** Genes that were > 2-fold up-regulated, **(b)** Genes that were > 2-fold down-regulated.

DGE between the mannitol-producing line and wild type, under salt stress conditions, henceforth named *Es*MS and WTS, respectively, identified a large number of genes that were substantially increased at 24 h but not at 48 h. A total number of 1090 of genes were identified to be up-regulated at 24 h in *Es*MS *vs.* WTS; at 48 h these differences were much smaller, with only 240 genes displaying up-regulation. A similar DGE pattern was observed for genes identified to be down-regulated, with 845 genes and 139 genes at 24 h and 48 h, respectively (Figure 3.7 and Figure 3.8).

In sharp contrast, the number of genes induced by salinity was quite large, in both the mannitol-producing line and wild type. At 24 h after the salt stress, 1991 genes were up-regulated in WTS *vs.* WT, and 2731 in *Es*MS *vs. Es*M. At 48 h, the number of genes was further increased in WTS *vs.* WT while in *Es*MS *vs. Es*M the number of genes (2585) was almost the same as after 24 h. A rather similar number of genes was found to be down-regulated in both the mannitol-producing line and wild type *A. thaliana* (Figure 3.8).

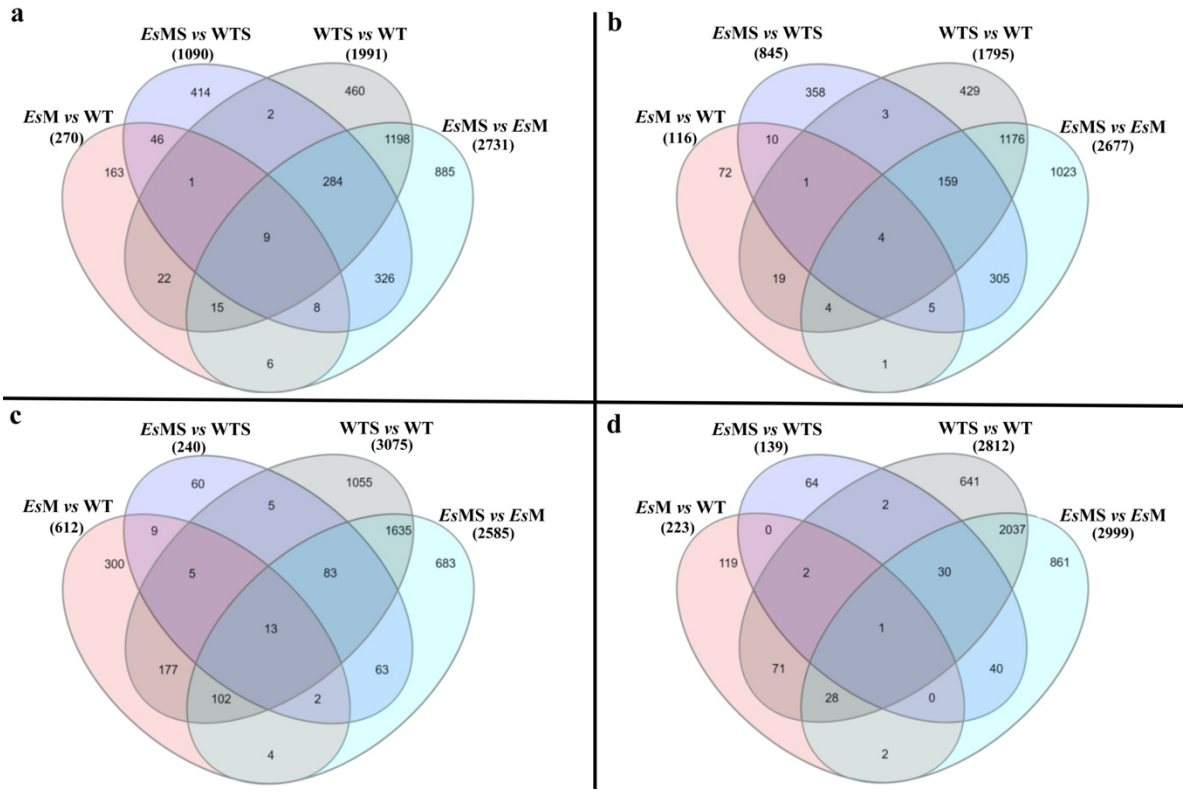


Figure 3.8. Venn diagram showing the differentially expressed genes (> 2-fold up- and down-regulated) in the mannitol-producing transgenic line *vs.* wild type, in standard (*Es*M and WT, respectively) and salinity stress conditions (*Es*MS and WTS, respectively), at 24 h and 48 h. **(a)** Genes that are up-regulated at 24 h, **(b)** Genes that are down-regulated at 24 h, **(c)** Genes that are up-regulated at 48 h, **(d)** Genes that are down-regulated at 48 h.

Functional annotation grouping of DGE, in standard conditions, showed that mannitol enhanced expression of a significant number of genes involved in defense mechanisms, responses to hormones such as auxins, gibberellins, ethylene and abscisic acid, oxidative stress and plant development (Figure 3.9a and Supplementary Dataset S3). Transcript levels of genes involved in categories such as plant development, response to auxin and ethylene, sugar and secondary metabolites and ultraviolet light decreased (Figure 3.9b and Supplementary Dataset S3).

Salt stress up-regulated several genes involved in oxidation-reduction processes, response to abscisic acid and gibberellin, defense mechanisms, responses to water deprivation, salt

stress and temperature and secondary metabolites (Figure 3.9c and Supplementary Dataset S3). Some of these genes involved in ROS homeostasis, ABA signaling, salinity stress, ion transporters, heat stress, LEA proteins, and other functions, that were significantly up-regulated, and acted in concert, are listed in Supplementary Dataset S2. Several genes that were found to be down-regulated belong to categories that contained genes that were found to be up-regulated, for example, response to auxin, gibberellin, cytokinin, oxidation-reduction processes; however, different categories such as photosynthesis, plant development and sugar metabolism have been also identified (Figure 3.9d and Supplementary Dataset S3).

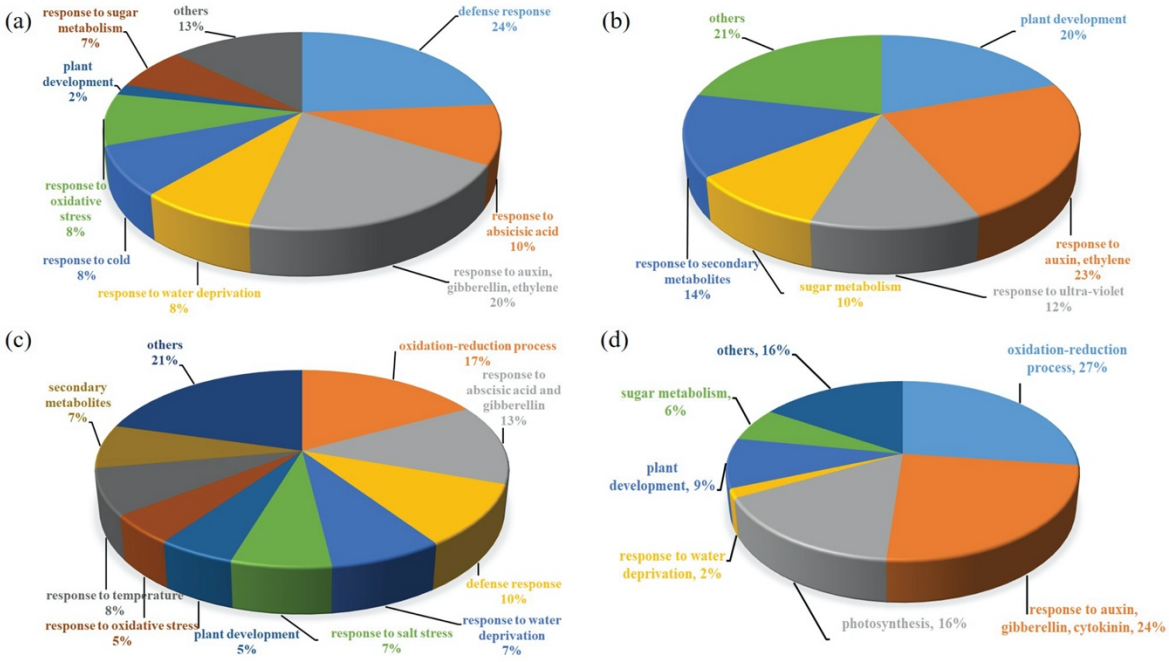


Figure 3.9. Functional categories grouping of genes which showed > 2-fold change in expression, in the mannitol-producing line *vs.* wild type pairwise comparisons, in standard and salinity stress conditions. **(a)** Up-regulated at 24 and 48 h under standard conditions. **(b)** Down-regulated at 24 and 48 h under standard conditions. **(c)** Up-regulated at 24 and 48 h under salinity stress. **(d)** Down-regulated at 24 and 48 h under salinity stress. Percentages for each category represents the number of genes involved in the functions out of total number of genes that were up- and down-regulated > 2-fold at p value < 0.05 for both 24 h and 48 h time points.

To further characterize the effects of mannitol on salinity stress response, transcription factors and genes interconnected within GO:0042538, which comprises all cellular processes related to hyperosmotic salinity stress response, were used to generate a network of co-expressed genes in salinity stress conditions. The relationship among genes was estimated by ATTED-II v. 9.2 (http://atted.jp).

Analysis revealed that genes encoding several transcription factors and their target genes in the network, were significantly up-regulated in *Es*MS in contrast to WTS (Figure 3.10 and Supplementary Dataset S3). For example, one of these transcription factors is *MYB2*, a CaM (calmodulin) binding transcription factor that regulates the expression of stress responsive genes, including that of pyrroline-5-carboxylate synthetase-1 (*P5CS1*), which imparts the salinity stress tolerance by production and accumulation of proline (Yoo et al., 2005). The expression of *MYB2* (AT2G47190) and *P5CS1* (AT4G28330) increased found to be significantly (4.04 and 2.76-fold, respectively) in *Es*MS *vs.* WTS (Figure 3.10 and Supplementary Dataset S3).

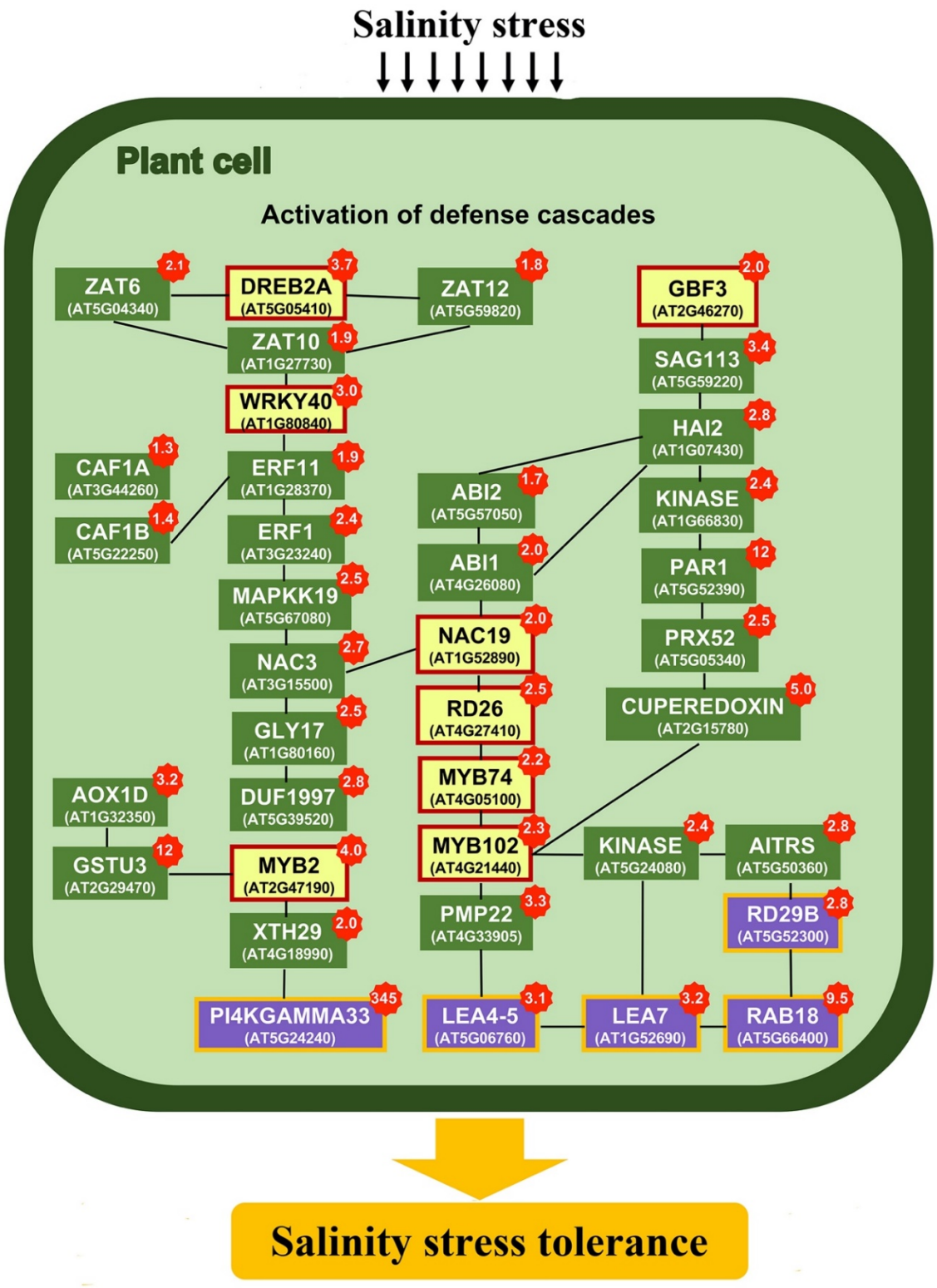


Figure 3.10. Co-expression analysis of genes induced by salinity stress (GO:0042538). Listed in the yellow box are some of the main transcription factors that regulate the

expression of several genes involved in salinity stress tolerance. In green boxes are listed genes involved in the activation of various defense mechanisms. In purple boxes are listed well characterized genes that are up-regulated in salinity and temperature stress in pairwise comparison of *Es*MS *vs.* WTS. Accession number for these genes and fold increase in expression for both time points are also listed in Supplementary Dataset S3. Values highlighted in red are the fold change of genes, in pairwise comparison of *Es*MS *vs.* WTS.

Heat map analysis of the differentially expressed genes, in standard conditions, selected based on pairwise *Es*M *vs.* WT comparison, at 24 h and 48 h, revealed that a large number of genes which were up-regulated in *Es*M (columns 2 and 4) displayed an expression pattern much more similar to that of plants subjected to salinity stress, i.e., to that of *Es*MS (columns 6 and 8) and WTS (columns 5 and 7), as compared to WT (Figures 3.11a,b). This trend was even more noticeable when the expression patterns of *Es*MS and WTS were compared 24 h after the salt stress (columns 5 and 6, respectively) and 48 h (columns 7 and 8, respectively). At 24 h the expression patterns of *Es*MS and WTS were clearly less similar compared to 48 h (Figure 3.11a). This trend was less noticeable for genes that were down regulated in *Es*M, at 24 h and 48 h (Figure 3.11b). However, when comparing the expression pattern of the genes found to be differentially up-regulated or down-regulated, at 24 h and 48 h, in salinity stress, the same trend of delayed changes in gene expression at 24 h in WTS, as compared to *Es*MS, was clearly detected (Figures 3.11c,d).

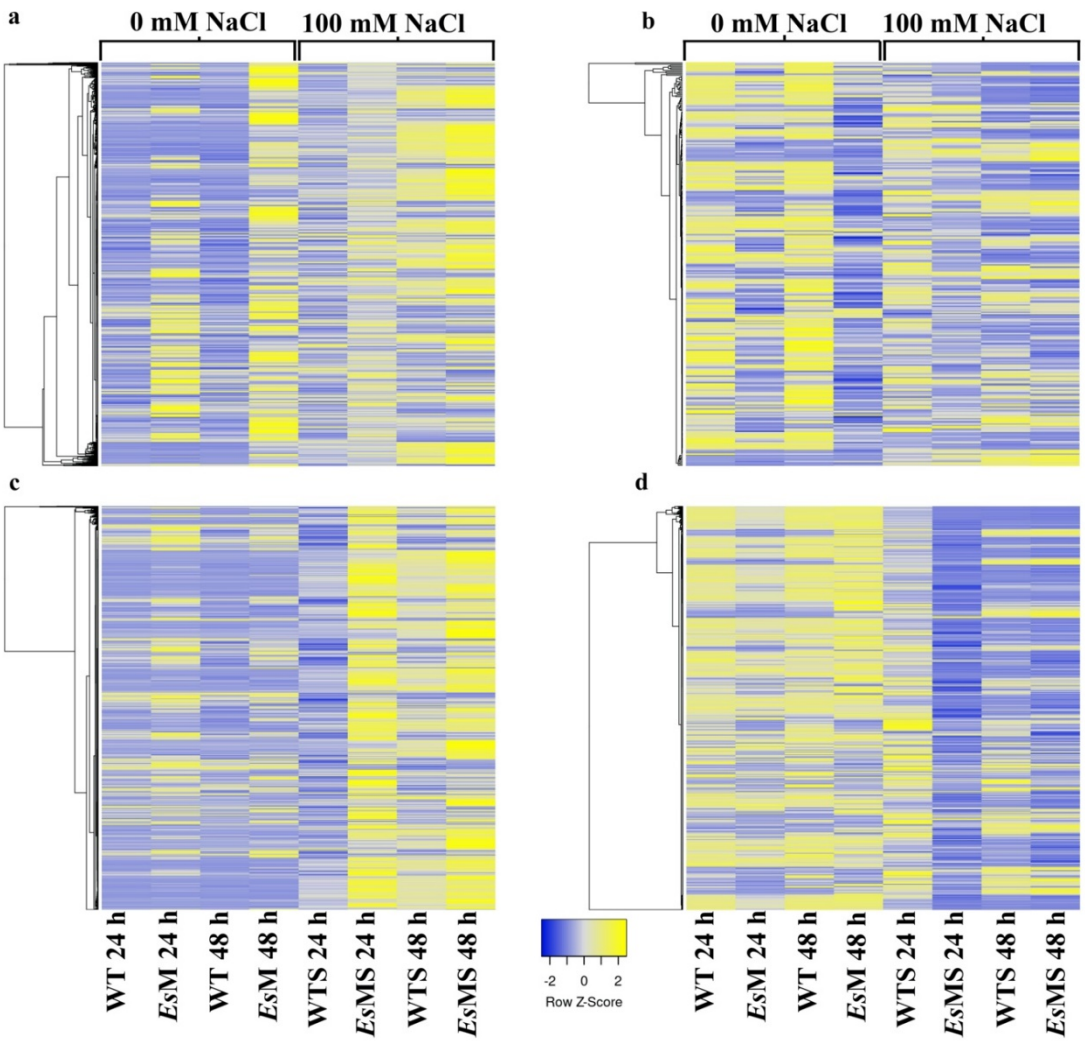


Figure 3.11. Heat map of selected genes in the mannitol-producing line and wild type, in standard (*Es*M and WT) and salinity stress conditions (*Es*MS and WTS). **(a)** Selection based on genes that were up-regulated in *Es*M at 24 and 48 h, under standard conditions (columns 2 and 4). **(b)** Selection based on genes that were down-regulated in *Es*M at 24 and 48 h, under standard conditions (columns 2 and 4). **(c)** Selection based on genes that were up-regulated in *Es*MS at 24 and 48 h, under salinity stress (columns 6 and 8). **(d)** Selection based on genes that were down-regulated in *Es*MS at 24 and 48 h, under salinity stress (columns 6 and 8). Selection of genes was made based on the > 2-fold change in expression in *Es*M *vs.* WT and *Es*MS *vs.* WTS pairwise comparisons.

At 24 h, the expression pattern of WTS (column 5) was found to be totally different from that of *Es*MS (column 6) while at 48 h the expression patterns of *Es*MS and WTS showed

quite a few similarities (WTS, column 7; *Es*MS, column 8) (Figure 3.11c,d). Overall, because the expression patterns in WTS and *Es*MS tend to be more alike at 48 h after salt stress and because the trend is driven by the expression pattern of *Es*MS and not that of WTS these results suggest a faster response to salt stress in the mannitol-producing line as compared to the wild type *A. thaliana*.

Multiple comparisons among time points and conditions identified two genes that showed ≥ 2-fold up-regulation across time points and conditions: *AtPI4KGAMMA3* (AT5G24240), and *QQS* (*Qua-Quine Starch*; AT3G30720). *AtPI4KGAMMA3* was found to be > 100-fold up-regulated while of *QQS* > 3.3-fold up-regulated in standard and salt stress conditions, in all time points; the expression of these genes was not strongly influenced by salinity stress (Supplementary Dataset S1). *ATPI4KGAMMA3* encodes a type II phosphoinositide 4-kinase that is involved in the response to abscisic acid and salt stress as well as in the regulation of flower development (Akhter et al., 2016). In *A. thaliana*, *QQS* was shown to modulate carbon/nitrogen allocation by increasing the protein content and decreasing the total starch content (Li et al., 2015; Qi et al., 2019). Starch degradation is positively corelated with increased abiotic stress tolerance and is associated with the release of oligosaccharides that function as osmolytes, as precursors for other intermediates necessary for energy production or signaling molecules (Rook et al., 2006; Kempa et al., 2008; Thalmann and Santelia, 2017). The expression of several α-amylases and β-amylase, known to involved in starch degradation (Lloyd et al., 2005), was found to be significantly up-regulated in *Es*MS *vs.* WTS.

3.4.4 Leaf infiltration with low concentrations of mannitol augmented the expression of *ATPI4KGAMMA3* and *QQS* in wild type plants

As transcriptomics and RT-qPCR data clearly showed that the expression of *ATPI4KGAMMA3* and *QQS* was significantly higher in the mannitol-producing transgenic plants as compared to the wild type plants, we hypothesized that low amounts of mannitol can trigger the expression of these genes, in wild type *A. thaliana*. the leaves from 20 days old plants were infiltrated with 100 µM mannitol. RT-qPCR analysis showed that both genes were transiently induced, in a time dependent manner, in the leaves of wild plants

infiltrated with 100 µM mannitol. The expression of *ATPI4KGAMMA3* increased at 2 h after infiltration with mannitol (2.1-fold up-regulated), reaching a dramatic peak at 4 h after infiltration (109.4-fold up-regulated), and then quickly reverted, after 6 h, to the pre-infiltration levels. Infiltration with water had little effects on this gene, in all time points (Figure 3.12a). The expression of *QQS* decreased at 2 h after infiltration with mannitol (2.8-fold down-regulated) while after 4 h an opposite trend was observed, i.e., the expression of *QQS* was higher in mannitol-infiltrated plants when compared to control plants (2.1-fold up-regulated). After 6 h, differences between mannitol-infiltrated and control plants were negligible, a situation identical to that observed in the case of *ATPI4KGAMMA3*. In contrast to *ATPI4KGAMMA3,* the expression of *QQS* was found to fluctuate more after water infiltration, the expression being lower at 2 h (1.6-fold down-regulated), higher at 4 h (1.4-fold up-regulated), and again lower at 6 h (1.6-fold down-regulated) as compared to control plants (Figure 3.12b).

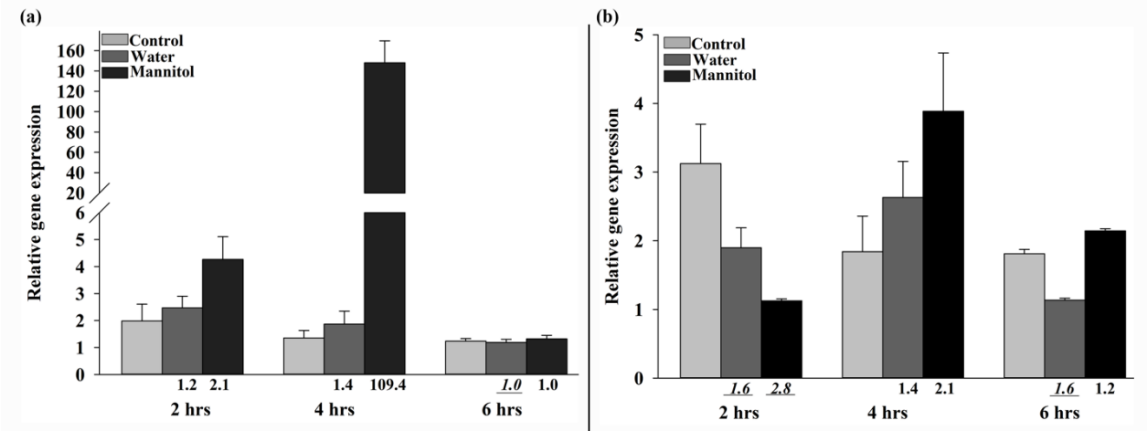


Figure 3.12. Gene expression analysis of *ATPI4KGAMMA3* and *QQS* in wild type plants infiltrated with water or 100 µM of D-mannitol at 2, 4 and 6 h after infiltration with water or mannitol. **(a)** Relative gene expression of *ATPI4KGAMMA3*, **(b)** Relative gene expression of *QQS*. Values under the bars represents fold change; values in bold are up-regulated while values in bold, italicized and underlined, are down-regulated. Data represents mean ± SE from 2 biological replicates at 2 and 6 h, and 6 biological replicates at 4 h.

3.5 Discussion

Studies carried out over the past 25 years, by expressing *E. coli mtlD* or celery *M6PR* in a variety of plants, showed improvement in the tolerance of transgenic plants to salt stress. These results were obtained in spite of the fact that mannitol concentrations in the transgenic plants were significantly lower than in the plants in which this sugar alcohol acts naturally as an osmolyte (Tarczynski et al., 1992, 1993; Thomas et al., 1995; Abebe et al., 2003; Zhifang and Loescher, 2003; Chan et al., 2011; Patel and Williamson, 2016). As a single gene was cloned (*E. coli mtlD* or celery *M6PR*), while the conversion to mannitol requires another dephosphorylation step, all previous studies proposed the presence of a putative endogenous phosphatase that converts M1P to mannitol (Tarczynski et al., 1992, 1993; Thomas et al., 1995; Zhifang and Loescher, 2003). In this study, the genes *EsM1PHD1* and *EsM1Pase2* (Groisillier et al., 2014; Bonin et al., 2015) from the brown algal model *Ectocarpus* sp., were used to produce mannitol in *A. thaliana*. The amount of mannitol was lower than that reported in previous studies (Tarczynski et al., 1992, 1993; Thomas et al., 1995; Abebe et al., 2003; Zhifang and Loescher, 2003; Chan et al., 2011; Patel and Williamson, 2016) suggesting that in *A. thaliana EsM1PHD1* is less efficient compared to *E. coli mtlD* or celery *M6PR.* Nevertheless, the three transgenic lines characterized phenotypically exhibited improved tolerance to salinity and temperature stress. These results, and those reported earlier, suggest that specific networks or pathways are activated by low concentrations of mannitol leading to enhanced abiotic stress tolerance. A microarray study carried out by Chan et al. (2011)*,* at 6 days after the salinity stress (100 mM NaCl), using *A. thaliana* expressing celery *M6PR,* found that improved salinity stress tolerance was likely due to differential expression of a variety of genes involved in processes such as salinity and dehydration stress, oxidoreductive processes, sugar and abscisic acid (ABA) metabolism, and cell wall synthesis.

To our knowledge, no other comprehensive studies, including transcriptomics and/or metabolomics, have been carried out to analyze the response to salinity stress at more than one time point, and early during the stress, in any other mannitol-producing transgenic plant. The complexity of the response at early time points is crucial, as it can reveal if plants are primed for long term survival in osmotic stress and water deficit conditions. In the

current study transcriptomics data was generated at early time points (24 h and 48 h) after exposure to 100 mM salinity stress.

Our study identified hundreds of genes that were up- and down-regulated in the mannitol producing line compared to wild type *A. thaliana* in standard conditions while two to three thousand genes were found to be differentially expressed in salt stress. Heat map analyses suggested that low amounts of mannitol might prime the response of plants to salt stress, as under salt stress conditions the expression pattern of WTS was quite different from that of *Es*MS at 24 h but was much more similar at 48 h. This proposition is also supported by the fact that a much larger number of genes were found to be differentially expressed at 24 h (1090 up- and 845 down-regulated) compared to 48 h (240 up- and 139 down-regulated) in *Es*MS *vs.* WTS. In addition, after 24 h of salinity stress, the total number of differentially expressed genes in mannitol-producing line (*Es*MS *vs. E*sM) was higher (~2700) than in the wild type (WTS *vs.* WT) (< 2000); notably, after 48 h, the number of differentially expressed genes was comparable.

Functional analysis showed that various categories of genes are differentially expressed, and these functions cover a wider range than previously suggested, including hormonal regulation, defense mechanisms, plant development, photosynthesis, and secondary metabolism. These categories comprise a plethora of genes known to be involved in various abiotic stress, including a large number of crucial transcription factors, K^+ homeostasis under salinity conditions, ROS signaling and radical scavengers and ABA signaling. When the results of the current study were compared to the findings of Chan et al. (2011), several notable differences became evident. In our study, under standard conditions, a much lower number of genes were differentially expressed in the mannitol-producing line compared to the wild type. The number of genes found to be > 2-fold up-regulated varied between 270 and 612 and those down-regulated between 116 and 223, at 24 h and 48 h, respectively. In contrast, Chan et al. (2011), reported 1204 genes to be up-regulated and 1068 down-regulated. In salt stress, over 2000 genes were found to be up- or down-regulated, at both time points, while Chan et al. (2011), reported a much lower number of differentially regulated genes (277 up-regulated and 487 down-regulated genes). These contrasting results might be due to differences in the transcriptomics approach, that is, microarrays *vs.*

RNA-seq, time points (6 days *vs.* 24 h and 48 h), and the quantity of mannitol produced (up to 6 µmol g^{-1} fresh weight *vs.* 0.042 µmol g^{-1} fresh weight), when comparing Chan et al. (2011), and the current study, respectively.

3.5.1 Stress responsive genes

Transgenic plants overexpressing *AtNCED3* have higher amount of ABA and were reported to be tolerant to water stress conditions (Qin and Zeevaart, 2002). The expression of *NCED3* (AT3G14440) was significantly higher (3.86-fold) in *Es*MS *vs.* WTS. Similarly, the expression of *RD29A*, *KIN1*, *RD17*, *COR15A* and *ERD10*, that are targets of the salt stress inducible transcription factor *DREB1A* was found to be significantly higher in *Es*MS *vs.* WTS. The expression of the related genes *RD29B* (AT5G52300) and *RAB18* (AT5G66400) was also found to be significantly higher at 24 h (2.81- and 9.51-fold respectively) and at 48 h (1.50- and 2.33-fold, respectively) in *Es*MS *vs.* WTS (Figure 3.10 and Supplementary Dataset S3).

The expression of several heat shock responsive genes was significantly up-regulated in the transgenic line as compared to wild type plant. For instance, *HSP70* (AT3G12580) which has been shown to confer higher tolerance to heat, salinity and drought stress (Wang et al., 2004) is also one of the genes that was found to be up-regulated (2.1-fold) in *Es*MS *vs.* WTS.

Role of LEA proteins in abiotic stress tolerance is well known (Kiyosue et al., 1998; Hundertmark and Hincha, 2008; Jia et al., 2014). *ERD5* and *6* encode a proline dehydrogenase and a sugar transporter, respectively (Kiyosue et al., 1998). The expression of these genes was significantly up-regulated in *Es*M line under salinity stress (Figure 3.10 and Supplementary Dataset S3). Among the different LEA proteins, the early responsive to dehydration (*ERD*) *10* and *14* belongs to the group 2 LEAs, also known as dehydrin (DHN) family proteins. DHNs play an important role in ion sequestration, membrane stabilization and acts as the chaperons (Tunnacliffe and Wise, 2007). The expression of *ERD10* (AT1G20450) and *ERD14* (AT1G76180) was found to be significantly up-regulated (4.50- and 2.16-fold, respectively) in *Es*MS *vs.* WTS.

3.5.2 K^+ homeostasis under salinity stress

Higher concentration of NaCl outside the roots leads to an influx of calcium; its increased cytosolic concentration causes the activation of stress responsive pathways. Lee et al. (2004), showed that ANN1 is a plasma membrane protein whose expression is triggered by hydroxyl radical. The expression of ANN1 (AT1G35720) was significantly up-regulated (3.70-fold) in *Es*MS *vs.* WTS. Accumulation of ANN1 in root has been demonstrated as a negative regulation of Na^+ influx (Guo et al., 2008). Moreover, it reduces the activity of plasma membrane localized guard cell outwardly rectifying K^+ channel (GORK), (Demidchik et al., 2010), therefore playing a role ion homeostasis by preventing the influx of Na^+ in root and efflux of K^+ (Laohavisit et al., 2013).

In plants, Na^+ and K^+/H^+ antiporters play an important role to maintain ion homeostasis. Na^+/H^+ antiporters localized at plasma membrane are involved in Na^+ efflux (Qiu et al., 2003) whereas when localized at tonoplast they are involved in Na^+ compartmentalization into vacuoles (Apse et al., 2003). In *A. thaliana NHX1* (AT5G27150) has been shown to sequester Na^+ into vacuoles (Zhang and Blumwald, 2001), and the expression of this gene was found to be significantly up-regulated (2.15-fold) in *Es*MS *vs.* WTS. *NHX1* was also shown to mediate K^+ transport thus playing a role in K^+ homeostasis (Apse et al., 2003).

The fact that K^+ homeostasis might be slightly different in the *Es*MS when compared to WTS is supported by the fact that the expression of *GORK* (AT5G37500) and *SKOR* (AT3G02850) was significantly higher (3.03- and 2.85-fold, respectively) in *Es*MS *vs.* WTS. In *A. thaliana* these genes encode shaker-like outward-rectifying K^+ channels which play important roles in K^+ hemostasis during the challenging environmental conditions (Pilot et al., 2003). Also, the expression of *KUP6* (AT1G70300) and *KAT1* (AT5G46240), which encode K^+ uptake permeases (Osakabe et al., 2013) was significantly up-regulated (2.68- and 2.21-fold, respectively) in *Es*MS *vs.* WTS.

3.5.3 ROS signaling and radical scavengers

ROS have important roles in signaling; however, to act as a signaling molecule it has to be maintained at steady state levels, otherwise it can cause detrimental effects to the cells (Mittler, 2002). To maintain the steady state levels plant evolved scavenging

mechanisms which involve enzymes such as superoxide dismutases (*SOD*), catalases (*CAT*) and ascorbate peroxidases (*APX*) (Willekens et al., 1997). In response to abiotic stresses the level of ROS can also be reduced by the increased activity of alternative oxidases (*AOXs*). The expression of *AOX1D* (AT1G32350) was found to be significantly higher at 24 and 48 h (3.20- and 5.48-fold, respectively) in *Es*MS as compared to WTS (Figure 3.10 and Supplementary Dataset S3). In plants, ascorbate peroxidases are responsible for the conversion of H_2O_2 into water by oxidizing ascorbate, which is regenerated by mono-dehydroascorbate (*MDHAR*) and de-hydroascorbate reductases (*DHAR*). Increased activity of *MDHARs* has been shown to provide protection against oxidative stress. *DHAR1* (AT1G19570) and *DHAR2* (AT1G75270) were found to be significantly up-regulated (3.09- and 2.07-fold, respectively) while *MDHAR* (AT3G09940) was expressed only in *Es*MS. Moreover, the existence of an ascorbate independent pathway suggests that other antioxidants including glutathione peroxidases (*GPXs*) and glutathione-s-transferases (*GSTs*) can reduce the H_2O_2 and other hydroperoxide radicals without the expense of ascorbate (Chang et al., 2009). In the current study, DGE analysis revealed that some of the genes related to glutathione-s-transferase and peroxidase family were significantly up-regulated in *Es*MS *vs.* WTS. *APX2* (ascorbate peroxidase 2) was shown to improve water use in water deficit conditions (Rossel et al., 2006). The expression of *APX2* (AT3G09640) was found to be 18.5-fold up-regulated in *Es*MS as compared to WTS. The higher expression of these genes and of other genes with similar functions in *Es*MS suggest that mannitol production might be associated with improved ROS detoxification and higher water use efficiency, which can lead to improved tolerance to high salinity and temperature stress. Glyoxalase has been suggested as the direct target of *RD26*, participating in ROS scavenging (Dixon et al., 1998). The expression of *GSTs* family proteins and *RD26* was found to be significantly up-regulated in *Es*MS, indicating another possible mechanism involved in the improved stress tolerance observed in this study.

3.5.4 ABA signaling

In *A. thaliana*, 2C protein phosphatases (PP2Cs) clade A type have been demonstrated to act as negative regulators of ABA signaling (Antoni et al., 2012). These PP2Cs (ABI1, ABI2, HAI1, HAI2) interact with SnRKs (in particular 2.2, 2.3 and 2.6), which are key players in ABA signaling, and inhibit them by dephosphorylation (Fujii et al., 2009). The expression of *ABI1* (AT4G26080), *ABI2* (AT5G57050), *HAI1* (AT5G59220), *HAI2* (AT1G07430) and *HAI3* (AT2G29380) was significantly up-regulated (2.41-, 2.13-, 3.39-, 2.74- and 4.02-fold, respectively) in *Es*MS *vs.* WTS. However, during ABA induction the ABA receptors pyrabactin resistance-like (PYL) bind to ABA, suppressing the activity of PP2Cs. This activates the SnRK2s which get autophosphorylated and then phosphorylate the downstream proteins that are involved in abiotic stress responses (Soon et al., 2012). The expression of *SnRK 2.2* (AT3G50500), *SnRK2.3* (AT5G66880), and *SnRK 2.6* (AT4G33950) was significantly higher (2.14-, 1.40- and 1.68-fold, respectively) in *Es*MS *vs.* WTS. The expression of ABA receptors *PYL2* (AT2G26040), *PYL5* (AT5G05440) and *PYL7* (AT4G01026) was also significantly higher (2.45-, 1.87- and 2.05-fold, respectively) in *Es*MS *vs.* WTS. These results suggest that low amounts of mannitol altered the ABA signaling, and thus, the expression of the downstream stress responsive genes.

3.6 Conclusions

The expression, for the first time, of the two genes involved in mannitol production, from the brown alga *Ectocarpus* sp. in *A. thaliana* resulted in improved tolerance to salinity and temperature stress though mannitol concentrations were not higher than in the plants in which *E. coli mtlD* or celery *M6PR* genes were used to drive mannitol production. Transcriptomics analysis of a mannitol-producing line and of the wild type, grown in standard conditions and under salt stress, revealed a large number of genes that are differentially up- and down-regulated in the mannitol-producing transgenic line. These genes cover a wide range of functions, that were not limited to salt and oxidative stress responses, as previous studies suggested. These findings unravel new facets of the complex

mechanisms through which low concentrations of mannitol improve plant's tolerance to salinity stress.

3.7 Supplementary Materials: Supplementary materials can be found in Appendix A and online at http://www.mdpi.com/2223-7747/9/11/1508/s1

Connection Statement Between Chapter 3 and Chapter 4

Global transcriptomics analysis of brown algal model *Ectocarpus* sp. following the exposure to various abiotic stressors revealed several genes (i.e., *Esi0017_0056, Esi0379_0027, Esi0154_0047, Esi0025_0042, Esi0252_0035, Esi0322_0010, Esi0045_0021, Esi0488_0007, Esi0105_0049, Esi0182_0002, Esi0143_0016, Esi0007_0087, Esi0113_0047, Esi0059_0099, Esi0538_0008, Esi0021_0137, Esi0195_0005, Esi0176_0002, Esi0266_0005, Esi0044_0144, Esi0105_0008*) were up-regulated (Ritter et al., 2014). These genes were overexpressed in *A. thaliana* under 35S promoter to elucidate the potential function of these genes in abiotic stress tolerance (Appendix B). The transgenic lines of all these genes were screened for salinity stress tolerance (Supplementary Table S6 to S14 in Appendix B). Transgenic seedlings of *Esi0017_0056* and of *Esi0379_0027* exhibited significantly improved tolerance to salinity stress as compared to wild type plants (Supplementary Table S7 and S8 in Appendix B). Both these genes were further characterized in detail (Chapter 4 and 5).

CHAPTER 4

A Novel Protein from *Ectocarpus* sp. Improves Salinity and High Temperature Stress Tolerance in *Arabidopsis thaliana*

Pramod Rathor[1], Tudor Borza[1], Sophia Stone[2], Thierry Tonon[3,4], Svetlana Yurgel[1], Philippe Potin[4], Balakrishnan Prithiviraj [1*]

[1]Department of Plant, Food and Environmental Sciences, Dalhousie University, Nova Scotia, Canada

[2]Department of Biology, Dalhousie University, Nova Scotia, Canada

[3]Centre for Novel Agricultural Products, Department of Biology, University of York, UK

[4]Sorbonne Université, CNRS, UMR 8227, Integrative Biology of Marine Models (LBI2M), Station Biologique de Roscoff (SBR), 29680 Roscoff, France.

[1]Correspondence:

*Balakrishnan Prithiviraj

E-mail: bprithiviraj@dal.ca

This chapter is published in International Journal of Molecular Sciences.
https://doi.org/10.3390/ijms22041971

4.1 Abstract

Brown alga *Ectocarpus* sp. belongs to Phaeophyceae, a class of macroalgae that evolved complex multicellularity. *Ectocarpus* sp. is a dominant seaweed in temperate regions, abundant mostly in the intertidal zones, an environment with high levels of abiotic stresses. Previous transcriptomic analysis of *Ectocarpus* sp. revealed several genes consistently induced by various abiotic stresses; one of these genes is *Esi0017_0056*, which encodes a protein with unknown function. Bioinformatics analyses indicated that the protein encoded by *Esi0017_0056* is soluble and monomeric. The protein was successfully expressed in

Escherichia coli, Arabidopsis thaliana and *Nicotiana benthamiana.* In *A. thaliana* the gene was expressed under constitutive and stress inducible promoters which led to improved tolerance to high salinity and temperature stresses. The expression of several key abiotic stress-related genes was studied in transgenic and wild type *A. thaliana* by qPCR. Expression analysis revealed that genes involved in ABA-induced abiotic stress tolerance, K^+ homeostasis, and chaperon activities were significantly up-regulated in the transgenic line. This study is the first report in which an unknown function *Ectocarpus* sp. gene, highly responsive to abiotic stresses, was successfully expressed in *A. thaliana*, leading to improved tolerance to salt and temperature stress.

Keywords: *Arabidopsis thaliana, Ectocarpus* sp., unknown function protein, transgenic plant, salinity, temperature, abiotic stress tolerance

4.2 Introduction

Unfavorable abiotic stress conditions, including high salinity and temperature stress, negatively influence plant performance resulting in significant reduction of agricultural productivity. Due to climate change these stresses are becoming more severe. It has been predicted that in order to feed around 9 billion people agricultural productivity must double in the near future. Completing this demand will be challenging due to the continuous decline in the availability of water for irrigation, changing weather patterns, and the reduction of arable land area.

In the last decades, research carried out using model plants as well as crop plants led to the identification of a large number of genes involved in abiotic stress tolerance; however, much work still remains to be performed to develop climate resilient crops. Brown algae (Phaeophyta) are multicellular organisms in the phylum heterokonts which is highly distantly related from the Archaeplastida and Opisthokonts (Yoon et al., 2004). Brown algae are among the few eukaryotic lineages which have evolved complex multicellularity (Cock et al., 2010). These organisms have been evolving over a billion years, and during evolution, they acquired a number of distinct characteristics that are absent in the other eukaryotic lineages. For instance, phaeophytes, which originated through secondary

endosymbiosis, have complex polysaccharides in their cell wall. Moreover, the secondary endosymbiosis enriched considerably the nuclear genome; as a result, brown algae display a number of distinctive metabolic pathways (Archibald, 2012). Brown algae are constantly subjected to high levels of abiotic stresses arising from the tidal cycles which are associated with temperature extremes, mechanical forces, and irradiation (Dittami et al., 2009). Some of the novel characteristics acquired by these organisms enabled them to survive and flourish in these harsh environmental conditions. The unique features of phaeophytes make this group interesting to explore, to decipher novel pathways and functions that very likely played essential roles in their evolutionary success; clearly, their biology is not well studied compared to that of animals and land plants. *Ectocarpus* sp. is a multicellular brown alga which has relatively small genome size (200 Mb) in contrast to *Fucus serratus* (1095 Mb) and *Laminaria digitata* (640 Mb) (Le Gall et al., 1993; Peters et al., 2004). *Ectocarpus* belongs to Ectocarpales, which is closely related to Laminariales, a group of seaweeds of significant economic importance (Draisma et al., 2003). Ecologically and economically, these seaweeds are of great interest because they are the source of important biomolecules such as fucoidans, laminarin and alginates. A large number of studies reported the benefits of brown algal extracts on plant health and their usage to improve agricultural productivity (Khan et al., 2009; Jithesh et al., 2012; Fan et al., 2014; Battacharyya et al., 2015). The beneficial effects include enhanced seed germination and plant establishment, improved resistance to environmental stresses, improved crop performance and enhanced post-harvest life (Khan et al., 2009; Fan et al., 2014; Battacharyya et al., 2015). However, many aspects of brown algal biology remain largely unknown, including the underlying molecular mechanisms of enhanced tolerance to abiotic stresses. Characterization of genes with novel functions in brown algae will advance knowledge and may lead to discovery of novel unique biomolecules which can contribute to improved, sustainable agricultural production. Complete genome sequencing of the brown algal model *Ectocarpus* sp. constituted an important step in the understanding of phaeophyte biology at the molecular level. Genome analysis followed by searches in protein databases revealed that more than 36% of the proteins were novel, being *Ectocarpus* or phaeophyte specific, with no counterpart in other taxonomic groups. These significant differences suggested that a large number of evolutionary innovations took place in this group, leading to the occurrence of

many novel genes in addition to considerable divergence from homologous sequences (Cock et al., 2012). Therefore, *Ectocarpus* genomic data represent a valuable resource with a great potential to discover novel genes and pathways involved in stress adaptation or specific bioactivities that are absent in other taxonomic groups. However, phaeophyte research is challenging, especially when the aim is to characterize and understand functions of novel genes, coding proteins of unknown function. These limitations are due to the lack of genetic resources such as methods of transformation to generate gain or loss of function mutants, or to determine subcellular localization, thus hampering most molecular studies. Previous transcriptomic analysis of *Ectocarpus* sp*.,* subjected to various abiotic stresses, showed that 76% of the up-regulated genes were encoding proteins of unknown functions, with no significant similarity to any of sequences outside *Ectocarpus* sp. (Ritter et al., 2014). We report here the expression in *A. thaliana* of the unknown function gene *Esi0017_0056* from *Ectocarpus* sp. Its expression in *A. thaliana*, using a constitutive promoter (35S) and an *A. thaliana* stress inducible promoter from the RESPONSIVE TO DESICCATION 29A gene (*RD29A*), led to improved tolerance to salinity and high temperature stress in the model plant. The protein was also successfully expressed in *Escherichia coli* and *Nicotiana benthamiana*.

4.3 Materials and Methods

4.3.1 *Ectocarpus* sp. growth conditions and gene isolation

Ectocarpus sp. (Dilwyn) Lyngbye unialgal strain 32 (accession CCAP 1310/4, isolated in San Juan de Marcona, Peru) was cultured into a 10 L plastic tank filled with filtered and autoclaved natural seawater supplemented with Provasoli nutrient medium at a concentration of 10 mL/L. The tank was maintained at 14 °C with 14 h light/10 h dark cycle, and light intensity of 40 $\mu mol.m^{-2}s^{-1}$. The culture was air bubbled with filtered (0.22 µm filter) compressed air. The algal culture was exposed to salinity stress (1450 mM NaCl) for 6 h. After 6 h, the algal cultures were harvested using filtration, dried and immediately flash frozen into liquid nitrogen. Total RNA was extracted using the method described by (Apt et al., 1995) with slight modifications (Le Bail et al., 2008), treated with Turbo DNAse

(Ambion Austin, USA), and converted into cDNA using a SuperScript IV Reverse Transcriptase (Life Technologies, Saint-Aubin, Essonne, France).

4.3.2 Bioinformatics analysis

Prediction of transmembrane helices or domains in protein was carried out using HMMTOP v. 2.0 (http://www.enzim.hu/hmmtop/; 30 November 2020) and TMHMM v. 2.0 (https://services.healthtech.dtu.dk/; 30 November 2020). Secondary structure homology modelling was performed using PSIPRED (Buchan and Jones, 2019) (http://bioinf.cs.ucl.ac.uk/psipred; 30 November 2020) and JPRED (http://www.compbio.dundee.ac.uk/jpred/; 30 November 2020). To gain more information about the folding and the tertiary structure of Esi0017_0056, protein structure, homology-modelling was carried out using SWISS-MODEL (Waterhouse et al., 2018). The best model (26.71% sequence identity) was built by ProMod v. 3 3.0.0 using the hypothetical protein ybiA from *E. coli* as a template (SMTL ID: 2b3w.1; structure solved by NMR). As the model generated by SWISS-MODEL covered only the DUF1768-domain, another tertiary structure homology-modelling was carried out using DMPfold (Greener et al., 2019). The PDB files generated by ProMod3 v. 3.0.0 and DMPfold were visualized using iCn3D (Wang et al., 2020).

To assess the evolutionary relationship of Esi0017_0056, the amino acid sequence was compared with that of similar protein sequences retrieved from GenBank by running a blastp search. Alignments of selected protein sequences were performed using MUSCLE (Edgar, 2004) implemented in MEGA X (Kumar et al., 2018). Sequences were trimmed to a total of 163 amino acid positions in the final dataset. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.8733)). The analysis involved 71 sequences from different species and that of Esi0017_0056. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Prediction of eukaryotic protein subcellular localization was performed using TargetP 2.0, Signal P, and DeepLoc - 1.0 (https://services.healthtech.dtu.dk; 30 November 2020),

PSORT and iPSORT (http://ipsort.hgc.jp; 30 November 2020), and WoLF PSORT (https://wolfpsort.hgc.jp; 30 November 2020).

4.3.3 Cloning and expression of recombinant protein in *E. coli*

To investigate the expression of Esi0017_0056 protein in *E. coli,* the entry clone was generated using High Fidelity Platinum *Taq* polymerase (Invitrogen, Mississauga, ON, Canada) with attB primers. The attB PCR product was cloned into pDONR221 using the BP Clonase™ II Gateway® (Gateway® Technology with Clonase II, Invitrogen, Mississauga ON, Canada). The entry clone was then introduced into the pDEST17 (N-6xHis) vector using the LR Clonase™ II Gateway®. The expression clone (Figure 4.1a) was transformed into BL21 (DE3) cells. Cells were grown in LB medium containing carbenicillin (50 µg/mL) at 37 °C and 200 rpm on an orbital shaker for 2–3 h to an OD_{600} of 0.4. The cells were then induced using L-arabinose to a final concentration of 0.1 %, and 2% ethanol was added to the medium during induction. The cells were grown overnight at 16 °C with aeration. The cells were centrifuged at 5000× *g* at 4 °C and suspended in 1/10 volume of cold lysis buffer as described by (Iancu et al., 2001). Cells were sonicated on ice using a Qsonica probe sonicator (ThermoFisher Scientific, Mississauga, ON, Canada). Uninduced, induced cells, soluble fraction and pellet were mixed with sample buffer and were used for SDS-PAGE. The gel was stained using Coomassie Brilliant Blue R-250.

4.3.4 Recombinant Esi0017_0056 protein analysis by LC-MS/MS

The excised gel slices were processed for LC-MS/MS as described by (Shevchenko et al., 2006), with minor modifications. The samples were transferred to a 300 µL HPLC vial and were subjected to analysis by LC-MS/MS on a VelosPRO orbitrap mass spectrometer (ThermoFisher Scientific, Mississauga, ON, Canada) equipped with an UltiMate 3000 Nano-LC system (ThermoFisher Scientific, Mississauga, ON, Canada). Chromatographic separation of the digests was performed on PicoFRIT C18 self-packed 75 µm × 60 cm capillary column (New Objective, Woburn, MA, USA) at a flow rate of 300 nl/min. MS and MS/MS data were acquired using a data-dependent acquisition method in which a full scan was obtained at a resolution of 30,000, followed by ten consecutive

MS/MS spectra in both higher-energy collisional dissociation (HCD) and collision-induced dissociation (CID) mode (normalized collision energy 36%). Internal calibration was performed using the ion signal of polysiloxane at *m/z* 445.120025 as a lock mass. Raw MS data were analyzed using Proteome Discoverer 2.2 (ThermoFisher Scientific, Mississauga, ON, Canada). Peak lists were searched against all the available protein databases as well as the cRAP database of common contaminants (Global Proteome Machine Organization). Cysteine carbamidomethylation was set as a fixed modification, while methionine (Met) oxidation, N-terminal Met loss and phosphorylation on serine, threonine and tyrosine were included as variable modifications. A mass accuracy tolerance of 5 ppm was used for precursor ions, while 0.02 Da for HCD fragmentation or 0.6 Da for CID fragmentation was used for product ions. Percolator was used to determine confident peptide identifications using a 0.1% false discovery rate (FDR).

4.3.5 *A. thaliana* seedlings growth conditions

A. thaliana seedlings were produced as described by (Rathor et al., 2020). In brief, Seeds of WT and transgenic *A. thaliana* lines were surface sterilized using 2% (*v/v*) NaOCl and stratified at 4 °C for two days. Seeds were placed on the plates containing half strength Murashige and Skoog (MS) medium (Phytotech, Lenexa, KS, USA), supplemented with 1% (*w/v*) sucrose and solidified with 0.4% (*w/v*) Phytagel (Sigma, Mississauga, ON, Canada). Plates were maintained at 22 °C with 16 h light/8 h dark cycle, with light intensity of 100 $\mu mol.m^{-2}s^{-1}$. Plants grown in these conditions henceforth be referred to as seedlings.

4.3.6 GUS expression analysis in *A. thaliana*

To generate the transformation vector, PMDC140 (C-GUS) (Curtis and Grossniklaus, 2003) was obtained from ABRC (Columbus, OH, USA). Stop codon was removed from the target gene, and entry clone was generated as mentioned earlier. The entry clone was introduced into the PMDC140 as described earlier. The transformation vector (*Esi0017_0056*–GUS) (Figure 4.1b) was transformed into *Agrobacterium* strain GV310 (pMB90) using the freeze and thaw method. The recombinant *Agrobacterium* strain carrying the gene of interest was transformed to flowering *A. thaliana* (L.) Heynh,

ecotype Columbia (Col-0) plants, using the floral dip method, as described by (Clough and Bent, 1998). Positive transformants were selected as described by (Rathor et al., 2020) and allowed to self for 3 generations. Five days old seedlings of F3 generation produced as previously described were used for GUS expression analysis.

4.3.7 Transient expression in tobacco

To generate the transformation vector, pEarleyGate 103 (C-GFP-HIS) (Earley et al., 2006) was obtained from ABRC, and the entry clone used in the previous section was introduced as described earlier. *Agrobacterium* transformed with vector pEarlyGate103 (*Esi0017_0056*-GFP-HIS) (Figure 4.1c) was grown at 28 °C in LB medium containing appropriate antibiotics until it reached an OD_{600} of 0.8. Cells were centrifuged, re-suspended in infiltration medium at a final OD_{600} of 0.1 and infiltrated in tobacco leaves as described by (Sparkes et al., 2006). After two days, several leaves were excised, cut in small pieces and examined for GFP expression using a LSM meta 510 confocal microscope (Carl Zeiss, Mississauga, ON, Canada).

4.3.8 Expression of *Esi0017_0056* in *A. thaliana*

To generate the transformation vectors, pEarleyGate 100 (35S) (Earley et al., 2006) and promoter less Gateway vector pMCS:GW (Michniewicz et al., 2015) were obtained from ABRC. RESPONSIVE TO DESICCATION 29A gene (*RD29A*, a stress inducible gene) promoter sequence, 1000 bp upstream of ATG initiation codon was isolated from wild type *A. thaliana* (Col-0) plants (Yamaguchi-Shinozaki and Shinozaki, 1993). The sequence was isolated using a primer pair with the restriction sites *EcoR1* and *Stu1*. The vector and PCR product were digested and ligated using NEB enzymes (NEB, Mississauga, ON, Canada). The ligated vector was transformed into one shot CcdB survival 2T1R *E. coli* competent cells (Invitrogen, Mississauga, ON, Canada). The entry clone used for recombinant protein in *E. coli* was introduced into pEarleyGate 100 (35S) and pMCS:GW (RD29A) as described above. The transformation vectors 35S:*Esi0017_0056* and RD29A:*Esi0017_0056* (Figure 4.1d and 4.1e, respectively) were transformed into *Agrobacterium* strain GV310 (pMB90), and transgenic plants were generated as described in the GUS expression analysis in *A. thaliana* section.

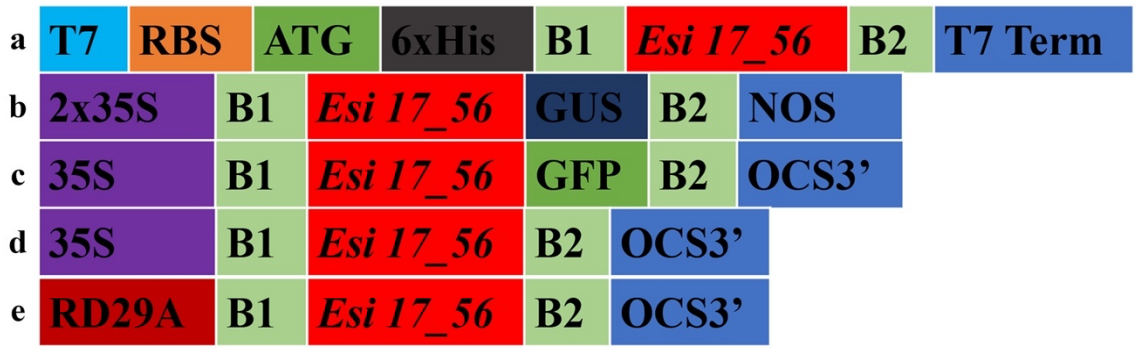


Figure 4.1. Different constructs generated for expression of *Esi0017_0056* in plants and bacteria. **(a)** Expression under N-terminal 6xHis tag fusion. **(b)** C-terminal GUS fusion. **(c)** C-terminal GFP fusion. **(d)** Expression under 35S promoter in *A. thaliana*. **(e)** Expression under *RD29A* promoter in *A. thaliana*.

4.3.9 Selection of transformants and homozygotes

Positive transformants were selected as described by (Rathor et al., 2020). In brief, the seeds collected from transformed plants were grown on plates containing half strength Murashige and Skoog (MS) medium (Sigma, Mississauga, ON, Canada), supplemented with 1% (*w/v*) sucrose and solidified with 0.4% Phytagel (*w/v*) and containing ammonium glufosinate 40 µg/mL. Plates were maintained at 22 °C in a 16-h light/8-h dark cycle. Seeds obtained from the positive lines were allowed to self for 5 generations. Segregation was tested in each generation by growing 100 seeds on half MS plates containing 40 µg/mL of ammonium glufosinate. Expression of the transgene was tested in these plants by RT-qPCR. Two independent lines with the 35S promoter, named as *Es*17-Ox1 and *Es*17-Ox2, and three independent lines with the stress inducible promoter (RD29A), named as *Es*17-A, *Es*17-B and *Es*17-C, were selected for further experiments.

4.3.10 Salinity stress tolerance

Four days old uniform seedlings produced as described earlier were transferred on plates containing half strength MS medium and 100 mM NaCl. Root length was marked on the day of transfer, and on the 7th day, plates were scanned with a high-resolution scanner (Epson Expression 10000 XL, Epson, Markham, ON, Canada). Root length and

the number of lateral roots per cm of primary root were measured using Image J software (Research Services Branch, NIH, Bethesda, MD, USA). Percent leaf chlorosis was visually estimated on 7th day after transfer. Nine-days-old plants were used to determine the fresh and dry weight. The experiment was repeated 3 times with 30 plants in each treatment in all experiments.

To assess the effect of salinity stress, plants were grown on Jiffy peat pellets (Jiffy, Shippagan NB, Canada). After 13 days of growth, uniform plants were selected and used in the experiment. Water-saturated peat pellets were left for 3 days without watering and then were irrigated with 200 mM NaCl (40 mL per plant) to reach a concentration of 100 mM. After five days of growth, plants were watered (20 mL per plant) at three days of interval for three weeks. Plants were photographed after three weeks, and biomass was recorded for individual plant. The experiment was repeated 3 times with 5 replicates in each treatment, in all experiments. The effect of salt stress on membrane intactness was estimated by recording the electrolyte leakage using SympHony SB70C (VWR, Mississauga, ON, Canada) conductivity meter as described by (Cao et al., 2007).

4.3.11 Heat stress tolerance

The experiment was performed according to a method described by (Rathor et al., 2020). In brief, after 9 days of growth on plates containing half strength MS medium, seedlings were exposed to 40 °C for 24 h. The seedlings were then allowed to recover for one week under standard conditions, and biomass was recorded. The experiment was repeated 3 times with 30 plants in each treatment in all experiments.

4.3.12 Real-time quantitative PCR of key stress responsive genes in overexpression line

The expression of 12 stress responsive genes was investigated in 3 independent biological replicates of wild type and overexpression line *Es*17-Ox2 (Supplementary Table S1). Plants were grown and treated as described earlier. Total RNA was extracted from samples using GeneJET plant RNA purification kit (Thermo Scientific, Mississauga, ON, Canada), treated with DNAse and converted into cDNA using the RevetAID cDNA

Synthesis kit (Thermo Scientific, Mississauga, ON, Canada). The relative transcript levels were determined by RT-qPCR, using the gene specific primers and *actin2* as the endogenous control (Supplementary Table S1) on a StepOne Plus Real-Time PCR system (Applied Biosystems, Mississauga, ON, Canada), using iTaq SYBR Green mix (Bio-Rad, Mississauga, ON, Canada). The relative expression was calculated using the delta-delta Ct method, and transcript abundance was normalized to the individual with the lowest expression. The expression of some key genes was also confirmed in *Es*17-Ox1 line.

4.3.13 Statistical analyses

Analysis of Variance (ANOVA) with a confidence level of 95%, followed by Tukey post hoc test with an error rate of 5%, was used to perform multiple mean comparisons. Statistical analyses were performed using Minitab 19.0 (Minitab LLC, State College, PA, USA).

4.4 Results

4.4.1 Protein structure, phylogenetic relationships and intracellular localization of Esi0017_0056

Esi0017_0056 was one of the several *Ectocarpus* sp. proteins (i.e., Esi0379_0027, Esi0154_0047, Esi0025_0042, Esi0488_0007, Esi0059_0099, Esi0322_0010, Esi0252_0035, Esi0045_0021, Esi0105_0049, Esi0182_0002, Esi0143_0016, Esi0007_0087, Esi0113_0047, Esi0538_0008, Esi0021_0137, Esi0195_0005, Esi0176_0002, Esi0266_0005 and Esi0044_0144) that were screened as potential candidates for cloning and expression in *E. coli*, *N. benthamiana* and *A. thaliana*. All these proteins were analyzed first for the presence of transmembrane domains with HMMTOP v. 2.0 (http://www.enzim.hu/hmmtop/; 30 November 2020) and TMHMM v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/; 30 November 2020). The presence of such domains makes protein expression in any system problematic. No transmembrane domains have been predicted for Esi0017_0056.

Homology modelling using PSIPRED provided an overall prediction of the secondary structure of this protein with a MW of 43.1 kDa and an isoelectric point (pI) of 8.74 (Figure 4.2). To gain more information about Esi0017_0056 protein structure, homology-modelling was carried out using the SWISS-MODEL server. The best model (26.71% sequence identity) was built using the hypothetical protein ybiA from *E. coli* as a template. Modelling of the region between residues 132 to 291 suggested that Esi0017_0056 is well-structured with several alpha-helices and short beta-strands (Figure 4.3a and 4.3b). Modelling information and data coming from similarity searches of other proteins harboring domains from the DUF1768 superfamily as well as from the automatic prediction output from PSIPRED, HMMTOP v. 2.0 and TMHMM v. 2.0 indicated that Esi0017_0056 is a soluble, monomeric protein. A full-length tertiary structure, built by DMPfold, revealed a protein with a globular structure, and additional alpha helices (Figure 4.3c).

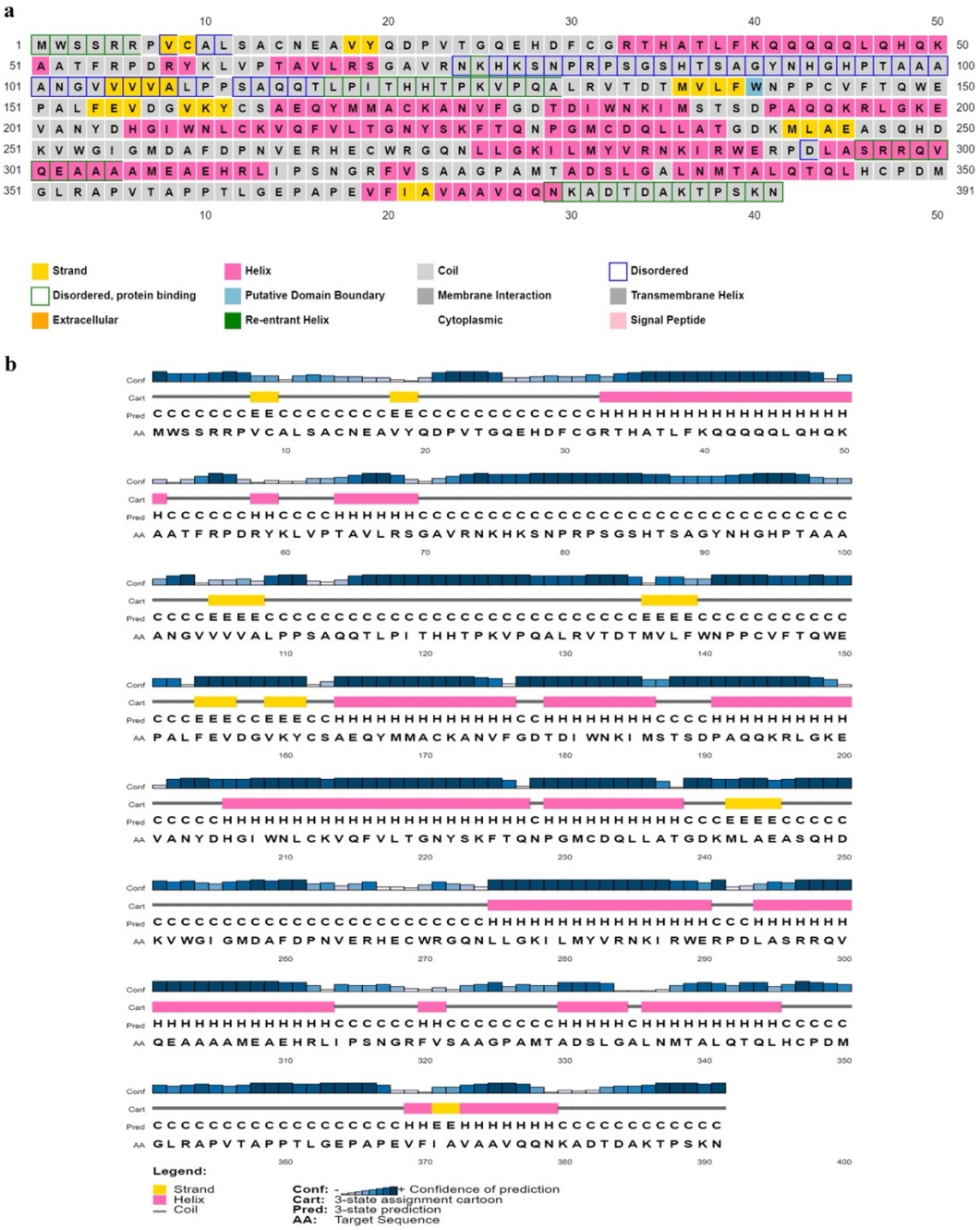


Figure 4.2. PsiPred predictions of the secondary structure (Buchan and Jones, 2019). **(a)** PsiPred sequence plot and **(b)** PsiPred cartoon with confidence of prediction. Models were generated at: http://bioinf.cs.ucl.ac.uk/psipred/.

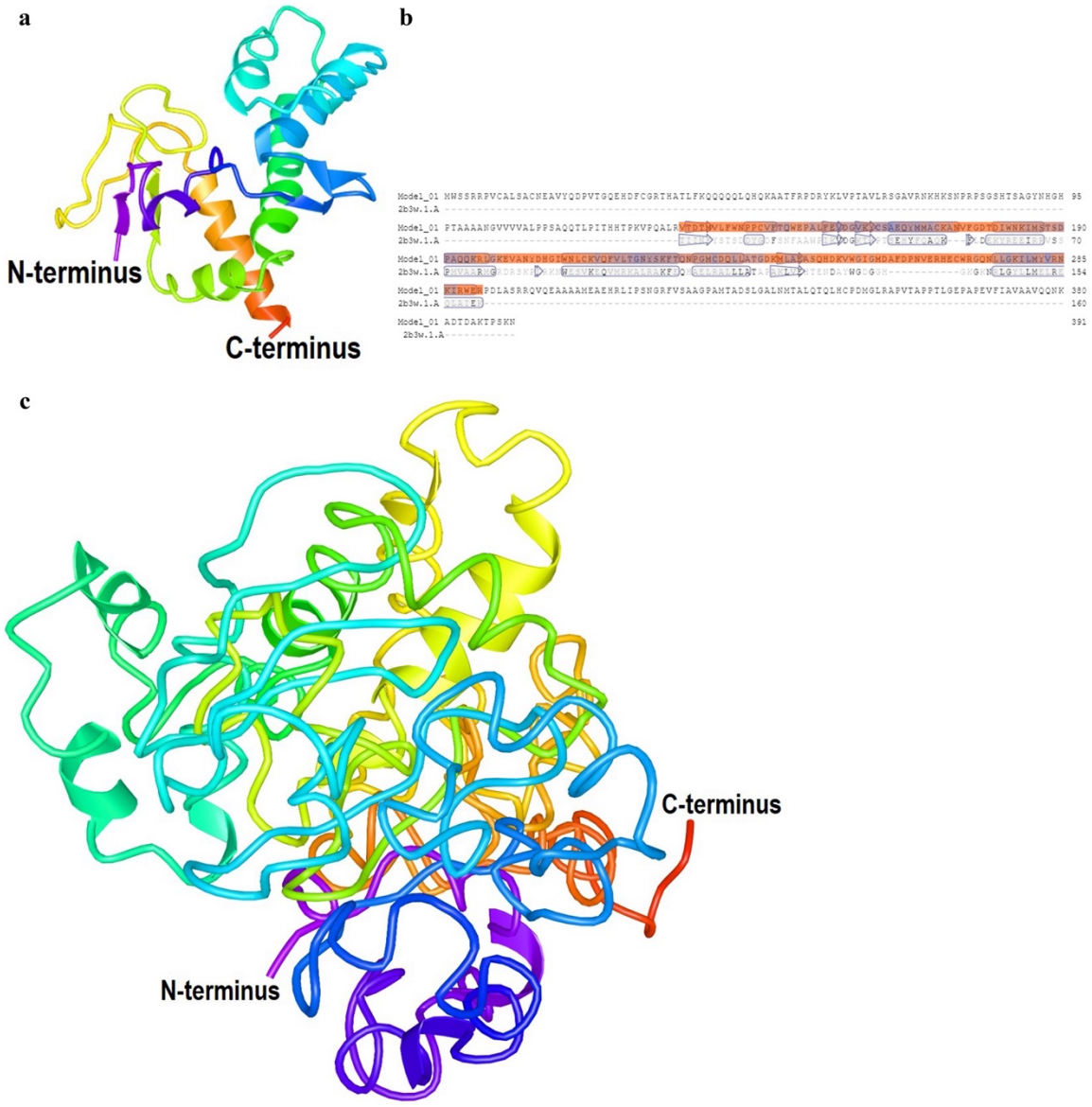


Figure 4.3. Esi0017_0056 tertiary structure modelling. **(a)** Tertiary structure of the DUF1768-domain built with ProMod3 3.0.0 in SWISS-MODEL (Waterhouse et al., 2018) using as template the hypothetical protein ybiA (SMTL ID: 2b3w.1.A); **(b)** The region covering residues 132 – 291 which was used to build the model, based on similarity with ybiA and **(c)** Tertiary structure of the full-length protein built by DMPfold (Greener et al., 2019). The PDB files of the models generated by ProMod3 3.0.0 and DMPfold were visualized using iCn3D (Wang et al., 2020) as ribbon, spectrum colored models, at https://www.ncbi.nlm.nih.gov/structure.

Blastp similarity searches of Esi0017_0056 in GenBank RefSeq non-redundant proteins database revealed that the closest relatives are two other proteins from *Ectocarpus*, which are also labeled as unknown conserved proteins (GenBank accession # CBJ32535 and CBJ27468). The next > 100 organisms harboring protein sequences with significant similarity (E-value: $8x10^{-45}$– $1x10^{-37}$) to these *Ectocarpus* proteins were of prokaryotic origin, including delta proteobacteria, gamma proteobacteria, firmicutes and cyanobacteria. The middle part of the 391 amino acids long Esi0017_0056 protein, that is, residues 132 to 291, was found to have strong similarity with DUF1768, a protein domain of unknown function (pfam08719). DUF1768 contains members such as *E. coli* N-glycosidase YbiA (COG3236), involved in riboflavin biosynthesis, which was initially characterized as a swarming motility protein. More recently, this family was included in the NADAR (NAD and ADP-ribose) superfamily which comprises proteins predicted to be involved in NAD-utilizing pathways, likely to act on ADP-ribose derivatives. Blastp similarity searches of Esi0017_0056 protein in GenBank RefSeq non-redundant proteins database, with restricted searches for eukaryotes, revealed that the closest eukaryotic organisms harboring a similar protein is the apusomonad *Thecamonas trahens* ATCC 50062 (E-value: $1x10^{-38}$). The organisms within this range of similarity were found to be extremely diverse, including the metazoans *Strongylocentrotus purpuratus* and *Dendronephthya gigantea*, the haptophyte *Emiliania huxleyi*, the amoebozoa *Entamoeba invadens* and the fungus *Cercospora berteroae*. The annotation of proteins in these taxa was similar to that found in prokaryotes, that is, DUF1768-domain containing protein, swarming motility protein YbiA-like and riboflavin biosynthesis protein PYRR. All these predictions refer to the same pfam08719.

To test the phylogenetic relationships of Esi0017_0056 to other similar proteins from various prokaryotes and eukaryotes lineages and to assess the likelihood that this gene occurred in *Ectocarpus* sp. through lateral gene transfer, a phylogenetic tree was generated (Figure 4.4). The support for most branches was found to be weak because of the low number of conserved residues present in the DUF1768 superfamily domain. Nevertheless, analysis indicated close relationships between *Ectocarpus* sp. sequences and prokaryotic sequences (proteobacteria), and with eukaryotic apusozoan and haptophytes. Two *Ectocarpus* sp. sequences clustered together with Esi0017_0056. It is therefore likely that

these *Ectocarpus* sp. sequences occurred through lateral transfer from a proteobacteria followed by duplication and divergent evolution. As *Esi0017_0056* gene contains two predicted introns, the possibility of representing a contaminant sequence of prokaryotic origin is excluded (Figure 4.5).

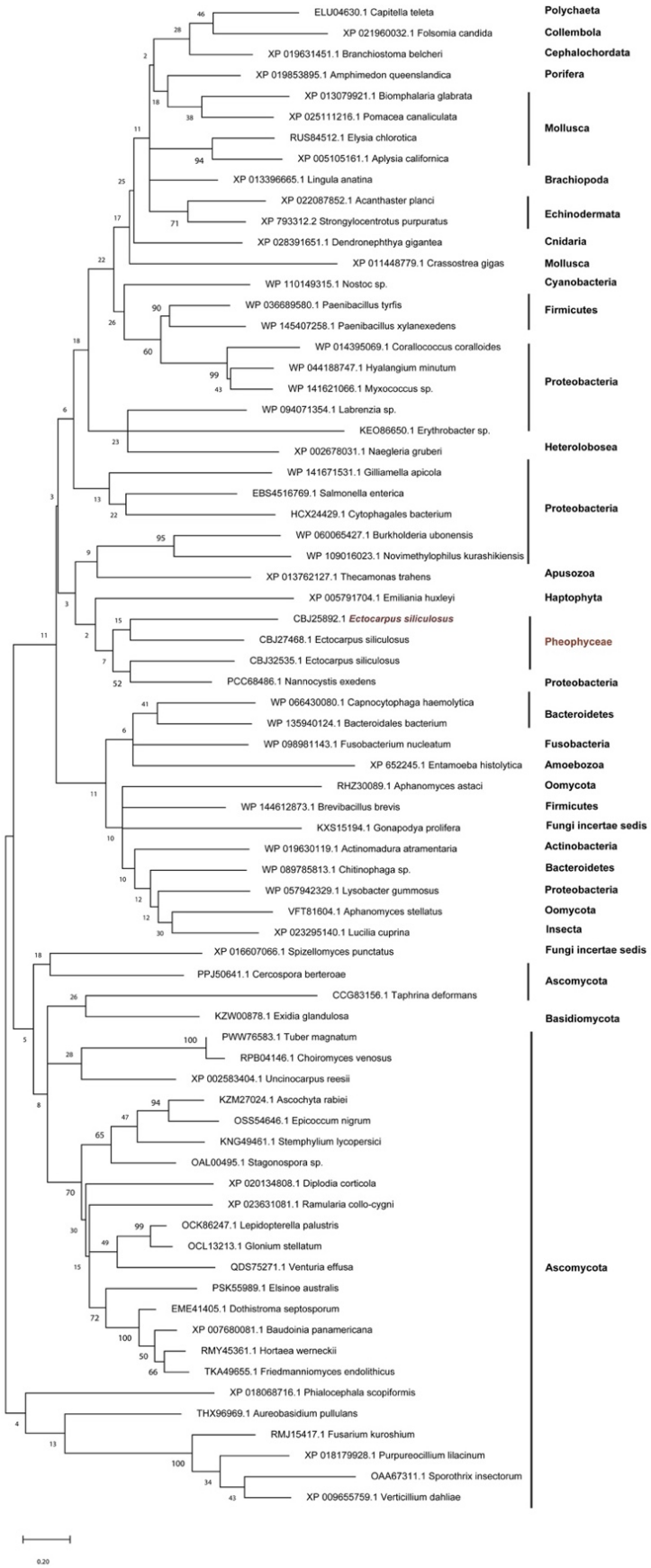

ELU04630.1 Capitella teleta
Polychaeta
XP 021960032.1 Folsomia candida
Collembola
XP 019631451.1 Branchiostoma belcheri
Cephalochordata
XP 019853895.1 Amphimedon queenslandica
Porifera
XP 013079921.1 Biomphalaria glabrata
XP 025111216.1 Pomacea canaliculata
RUS84512.1 Elysia chlorotica
XP 005105161.1 Aplysia californica
Mollusca
XP 013396665.1 Lingula anatina
Brachiopoda
XP 022087852.1 Acanthaster planci
XP 793312.2 Strongylocentrotus purpuratus
Echinodermata
XP 028391651.1 Dendronephthya gigantea
Cnidaria
XP 011448779.1 Crassostrea gigas
Mollusca
WP 110149315.1 Nostoc sp.
Cyanobacteria
WP 036689580.1 Paenibacillus tyrfis
WP 145407258.1 Paenibacillus xylanexedens
Firmicutes
WP 014395069.1 Corallococcus coralloides
WP 044188747.1 Hyalangium minutum
WP 141621066.1 Myxococcus sp.
Proteobacteria
WP 094071354.1 Labrenzia sp.
KEO86650.1 Erythrobacter sp.
Proteobacteria
XP 002678031.1 Naegleria gruberi
Heterolobosea
WP 141671531.1 Gilliamella apicola
EBS4516769.1 Salmonella enterica
HCX24429.1 Cytophagales bacterium
WP 060065427.1 Burkholderia ubonensis
WP 109016023.1 Novimethylophilus kurashikiensis
Proteobacteria
XP 013762127.1 Thecamonas trahens
Apusozoa
XP 005791704.1 Emiliania huxleyi
Haptophyta
CBJ25892.1 Ectocarpus siliculosus
CBJ27468.1 Ectocarpus siliculosus
CBJ32535.1 Ectocarpus siliculosus
Pheophyceae
PCC68486.1 Nannocystis exedens
Proteobacteria
WP 066430080.1 Capnocytophaga haemolytica
WP 135940124.1 Bacteroidales bacterium
Bacteroidetes
WP 098981143.1 Fusobacterium nucleatum
Fusobacteria
XP 652245.1 Entamoeba histolytica
Amoebozoa
RHZ30089.1 Aphanomyces astaci
Oomycota
WP 144612873.1 Brevibacillus brevis
Firmicutes
KXS15194.1 Gonapodya prolifera
Fungi incertae sedis
WP 019630119.1 Actinomadura atramentaria
Actinobacteria
WP 089785813.1 Chitinophaga sp.
Bacteroidetes
WP 057942329.1 Lysobacter gummosus
Proteobacteria
VFT81604.1 Aphanomyces stellatus
Oomycota
XP 023295140.1 Lucilia cuprina
Insecta
XP 016607066.1 Spizellomyces punctatus
Fungi incertae sedis
PPJ50641.1 Cercospora berteroae
CCG83156.1 Taphrina deformans
Ascomycota
KZW00878.1 Exidia glandulosa
Basidiomycota
PWW76583.1 Tuber magnatum
RPB04146.1 Choiromyces venosus
XP 002583404.1 Uncinocarpus reesii
KZM27024.1 Ascochyta rabiei
OSS54646.1 Epicoccum nigrum
KNG49461.1 Stemphylium lycopersici
OAL00495.1 Stagonospora sp.
XP 020134808.1 Diplodia corticola
XP 023631081.1 Ramularia collo-cygni
OCK86247.1 Lepidopterella palustris
OCL13213.1 Glonium stellatum
QDS75271.1 Venturia effusa
PSK55989.1 Elsinoe australis
EME41405.1 Dothistroma septosporum
XP 007680081.1 Baudoinia panamericana
RMY45361.1 Hortaea werneckii
TKA49655.1 Friedmanniomyces endolithicus
Ascomycota
XP 018068716.1 Phialocephala scopiformis
THX96969.1 Aureobasidium pullulans
RMJ15417.1 Fusarium kuroshium
XP 018179928.1 Purpureocillium lilacinum
OAA67311.1 Sporothrix insectorum
XP 009655759.1 Verticillium dahliae
0.20

Figure 4.4. Phylogenetic analysis of Esi0017_0056 protein using the Maximum Likelihood method and JTT matrix-based model. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

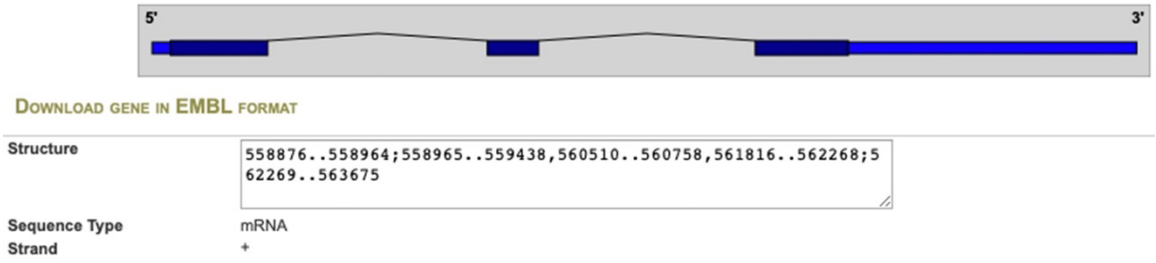


Figure 4.5. Gene structure of *Esi0017_0056* indicating the presence of two introns.

Potential intracellular protein localization was analyzed using several prediction servers. SignalP, iPSORT and TargetP 2.0 ruled out the presence of a signal peptide or of a mitochondrial, chloroplast or thylakoid luminal transfer peptide. WoLF PSORT indicated with low, very similar scores, that the possible location of this protein can be mitochondria, cytosol or chloroplast. PSORT and DeepLoc - 1.0 predicted that the most probable location of this soluble protein is the mitochondria rather than the cytosol, but the support was not strong. It is worth mentioning that DeepLoc - 1.0 predicts eukaryotic protein subcellular localization using deep learning and it can differentiate between 10 different localizations: nucleus, cytoplasm, extracellular, mitochondrion, cell membrane, endoplasmic reticulum, chloroplast, Golgi apparatus, lysosome/vacuole and peroxisome. As Esi0017_0056 was predicted as a soluble protein with no obvious N- or C-terminus extensions, it is likely that in *Ectocarpus,* its translation occurs in the cytosol and in heterologous eukaryotic systems such as *A. thaliana* the process should be similar. However, we cannot formally rule out that its final destination might also depend on its interaction with other macromolecular intracellular structures.

4.4.2 Esi0017_0056 protein is highly expressed in *E. coli*, *A. thaliana* and *N. benthamiana*

Esi0017_0056 was successfully expressed in *E. coli*; however, most of the protein was found in inclusion bodies (Figure 4.6). Sufficient recombinant protein was obtained to carry out LC-MS/MS sequencing, which confirmed the expression of a full length Esi0017_0056. To investigate the expression in plants the C-terminal fusion constructs of *Esi0017_0056* with GUS and GFP tags were introduced in *A. thaliana* and *N. benthamiana*, respectively. In both transgenic plants *Esi0017_0056* was found to be highly expressed (Figure 4.7). GUS activity staining of *A. thaliana* plantlets revealed strong occurrence in roots and many parts of the leaflets (Figure 4.7a–e). Confocal microscopy analysis of *N. benthamiana* leaves section expressing *Esi0017_0056*-GFP revealed strong fluorescence localized throughout the cytoplasm and around nucleus but not in the vacuole (Figure 4.7f).

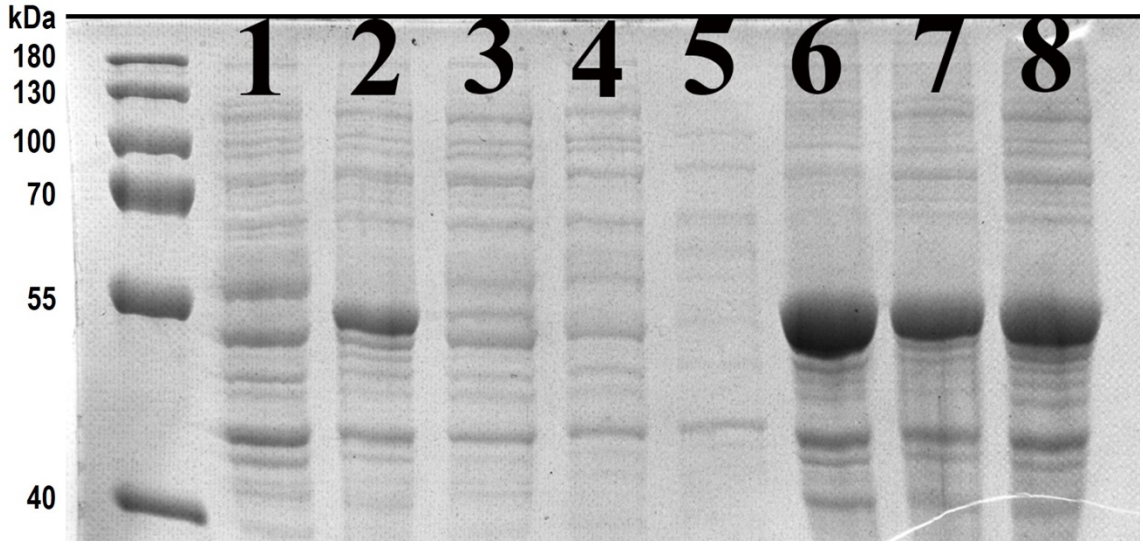


Figure 4.6. Expression of Esi0017_0056 protein with N-6xHis tag in *E. coli* BL21 DE3 cells. Lane 1, uninduced whole cells; lane 2, induced whole cells; lanes 3 to 5, supernatant of cell lysate; and lanes 6 to 8 pellet of cell lysate.

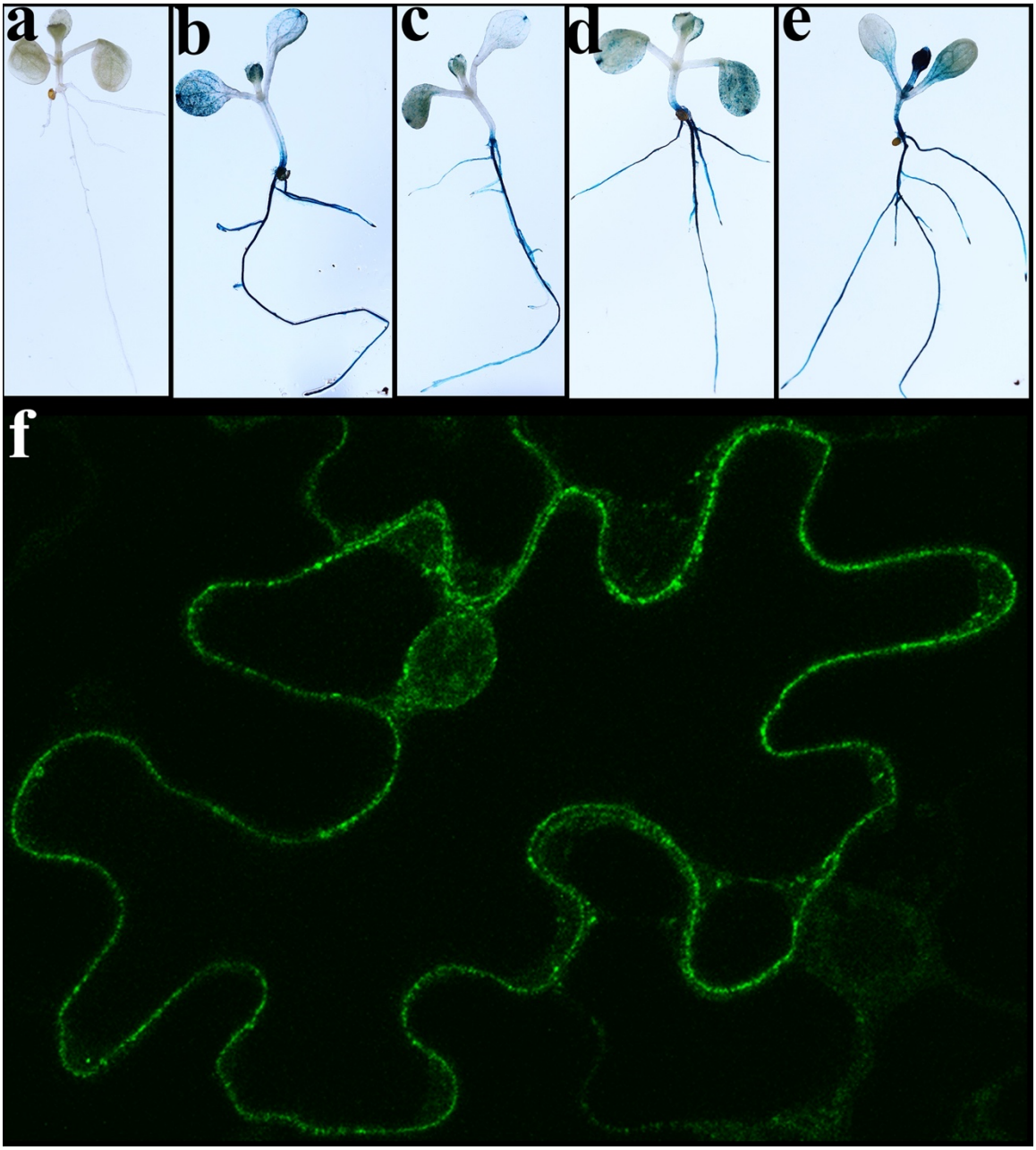


Figure 4.7. Expression of C-terminal fusion proteins Esi0017_0056-GUS and Esi0017_0056-GFP. **(a-e)** GUS expression in *A. thaliana* 5 days old plantlets. **(a)** WT and **(b-e)** independent transgenic lines. **(f)** GFP expression in *N. benthamiana* leaf.

4.4.3 Gene expression of *Esi0017_0056* showed that it is highly expressed in *A. thaliana* under standard and salinity stress conditions

To study the potential role of *Esi0017_0056* in improved tolerance to abiotic stress, the gene was cloned in two different constructs, one in which the expression was driven by the constitutive promoter 35S and the other one by the *A. thaliana* stress inducible promoter RD29A. Expression of *Esi0017_0056* was confirmed in the transgenic lines (Figure 4.8). In normal conditions, the expression in the *Es*17-Ox1 and *Es*17-Ox2 lines, having the 35S promoter, was much higher than that observed in *Es*17-A, *Es*17-B and *Es*17-C lines, having the RD29A promoter (Figure 4.8). It is worth mentioning that the expression of *Es*17-Ox lines, but not of *Es*17-A-C lines, was higher than that of *actin*, the reference gene, which is a highly expressed gene in eukaryotic systems. While Ct values of *actin* varied in the 21-22 cycles range, those of *Es*17-Ox1 and *Es*17-Ox2 were found to be around 19 cycles and 16 cycles, respectively. These results suggest that the transcription of *Esi0017_0056* in *A. thaliana* is efficient and the mRNA is rather stable. Upon exposure to salinity stress, the expression *Esi0017_0056* was found to be strongly up-regulated in the *Es*17-A, *Es*17-B and *Es*17-C lines (19, 9 and 20 times, respectively), while in the *Es*17-Ox1 and *Es*17-Ox2 lines these changes were found to be less pronounced (3 and 7.9 times, respectively) (Figure 4.8). The increase amounts of transcripts in the 35S lines upon exposure to salinity stress might be due to the stability of *Esi0017_0056* mRNA, process observed in other systems as well (Shi et al., 2003).

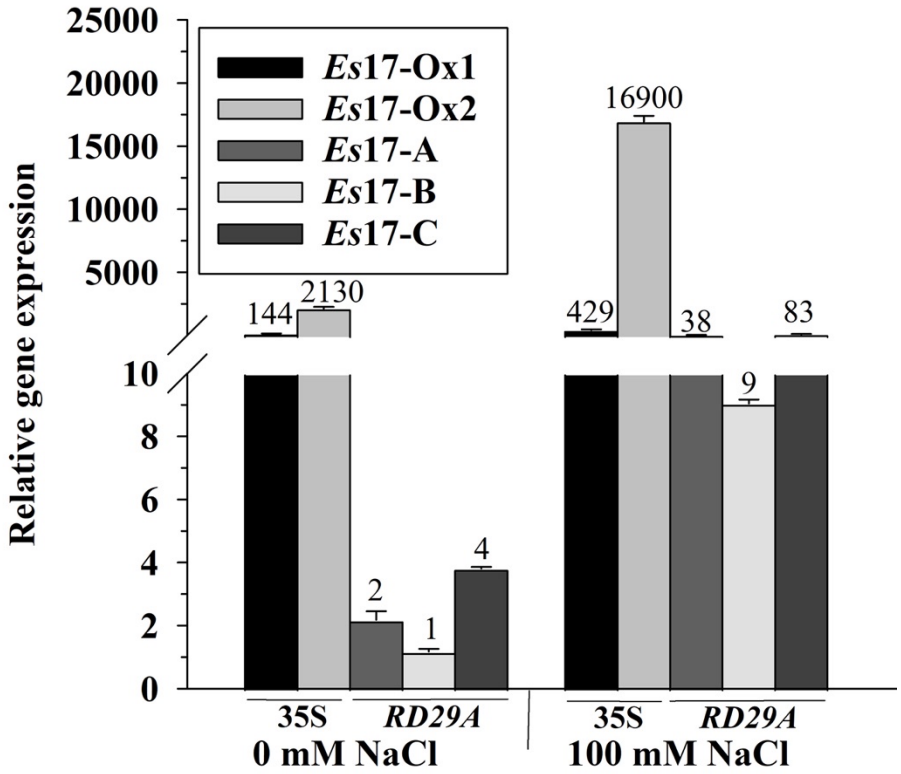


Figure 4.8. Expression of *Esi0017_0056* in two independent *A. thaliana* transgenic lines having the 35S promoter and in three independent transgenic lines having the stress inducible (*RD29A*) promoter under standard and salinity stress conditions. *Actin* was used as the endogenous control and transcript levels were normalized to the individual with the lowest expression. Data represents mean ± SE from 3 biological replicates. Values listed on the bars represent relative expression, fold change ratio *vs* the line with the lowest *Esi0017_0056* expression, that is, line *Es*17-B in absence of NaCl.

4.4.4 Expression of *Esi0017_0056* exhibited better tolerance to salinity stress in *A. thaliana* seedlings and plants

To examine the effects of *Esi0017_0056* expression on salinity stress tolerance of transgenic lines, the seedlings were exposed to 100 mM NaCl. After one week of exposure to 100 mM NaCl, transgenic seedlings had significantly longer roots, higher number of lateral roots per cm of primary root, reduced leaf chlorosis and higher biomass as compared to the wild type seedlings (Figure 4.9).

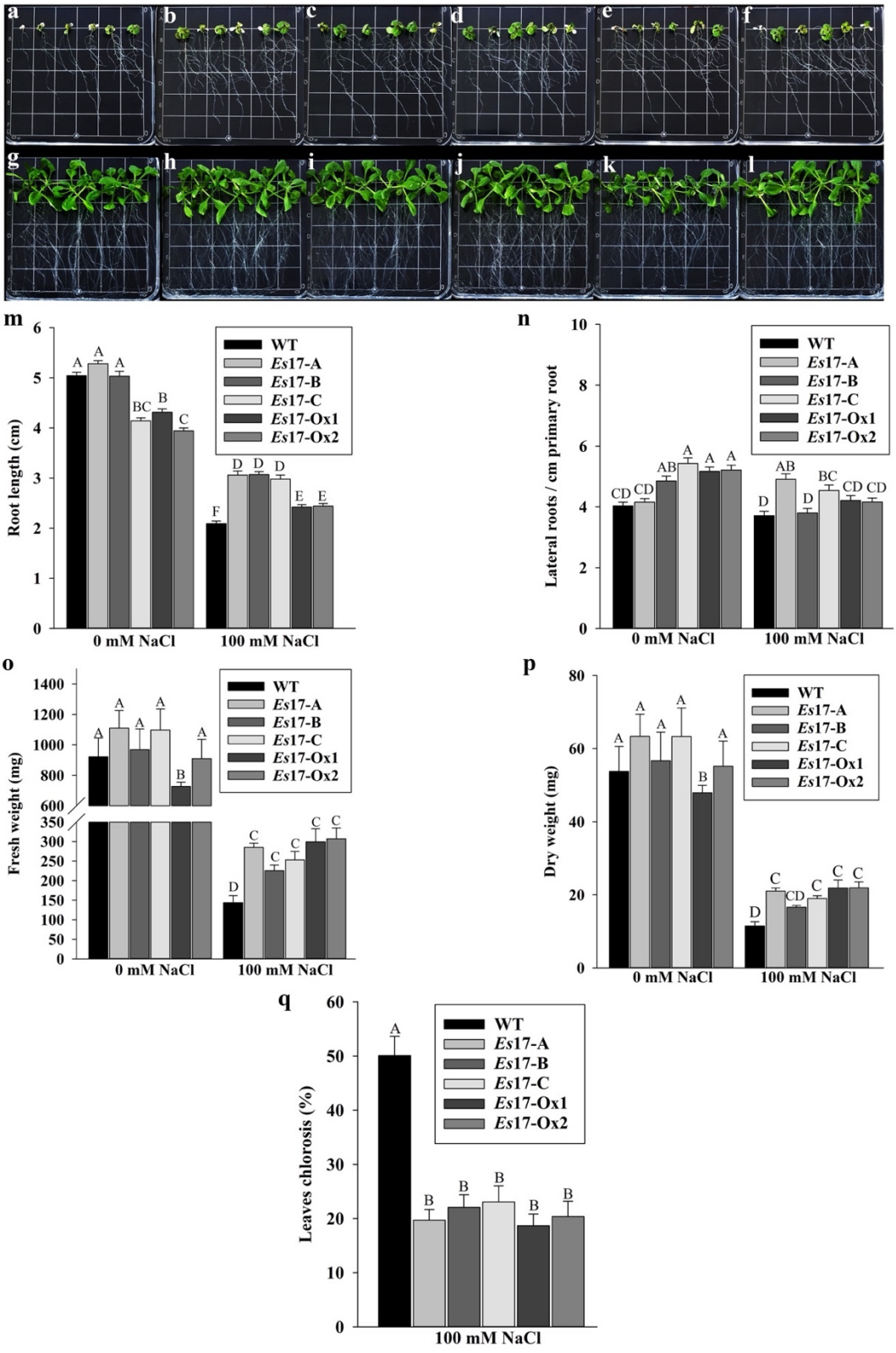


Figure 4.9. Growth of wild type and 2 independent 35S promoter (*Es*17Ox-1-2,) and of 3 independent stress inducible promoter (*Es*17A-C) transgenic *A. thaliana* seedlings,

expressing *Esi0017_0056* in presence and absence of 100 mM NaCl. In plates **(a)** to **(f)**, seedlings were grown under salt stress conditions while in; plates **(g)** to **(l)**, seedlings were grown in standard conditions. **(a, g)** WT, **(b, h)** *Es*17-A, **(c, i)** *Es*17-B, **(d, j)** *Es*17-C, **(e, k)** *Es*17-Ox1, **(f, l)** *Es*17-Ox2, **(m)** root length, **(n)** number of lateral roots per cm of primary root, **(o)** fresh weight, **(p)** dry weight and **(q)** leaf chlorosis. Values represents mean and standard error (n=90) for root length, lateral roots, leaf chlorosis and n=9 for fresh and dry weight. Means and SE followed by the same letter are not significantly different. The plants were photographed at 9 days after transfer in standard and under salinity stress conditions.

In order to verify if the enhanced stress tolerance observed in seedlings can be observed in plants grown in soil, 15-days-old transgenic plants were irrigated with 200 mM NaCl, and concentrations of around 100 mM were maintained in the peat pellet. After one week of exposure to salinity stress, wild type plants exhibited symptoms of growth retardation and leaf chlorosis whereas transgenic plants grew better (Figure 4.10). Analysis of biomass data suggested that transgenic plants had significantly higher fresh weight and dry weight as compared to the wild type plants (Figure 4.10). When the effect of promoters (constitutive and stress-inducible) was contrasted, transgenic lines generated using stress inducible promoter showed slightly reduced fresh weight under standard conditions as compared to the wild type plants, while the lines generated using constitutive promoter showed slightly reduced dry weight. Overall, none of the transgenic plants grew better than the wild type plants under standard conditions, if both fresh weight and dry weight are considered (Figure 4.11).

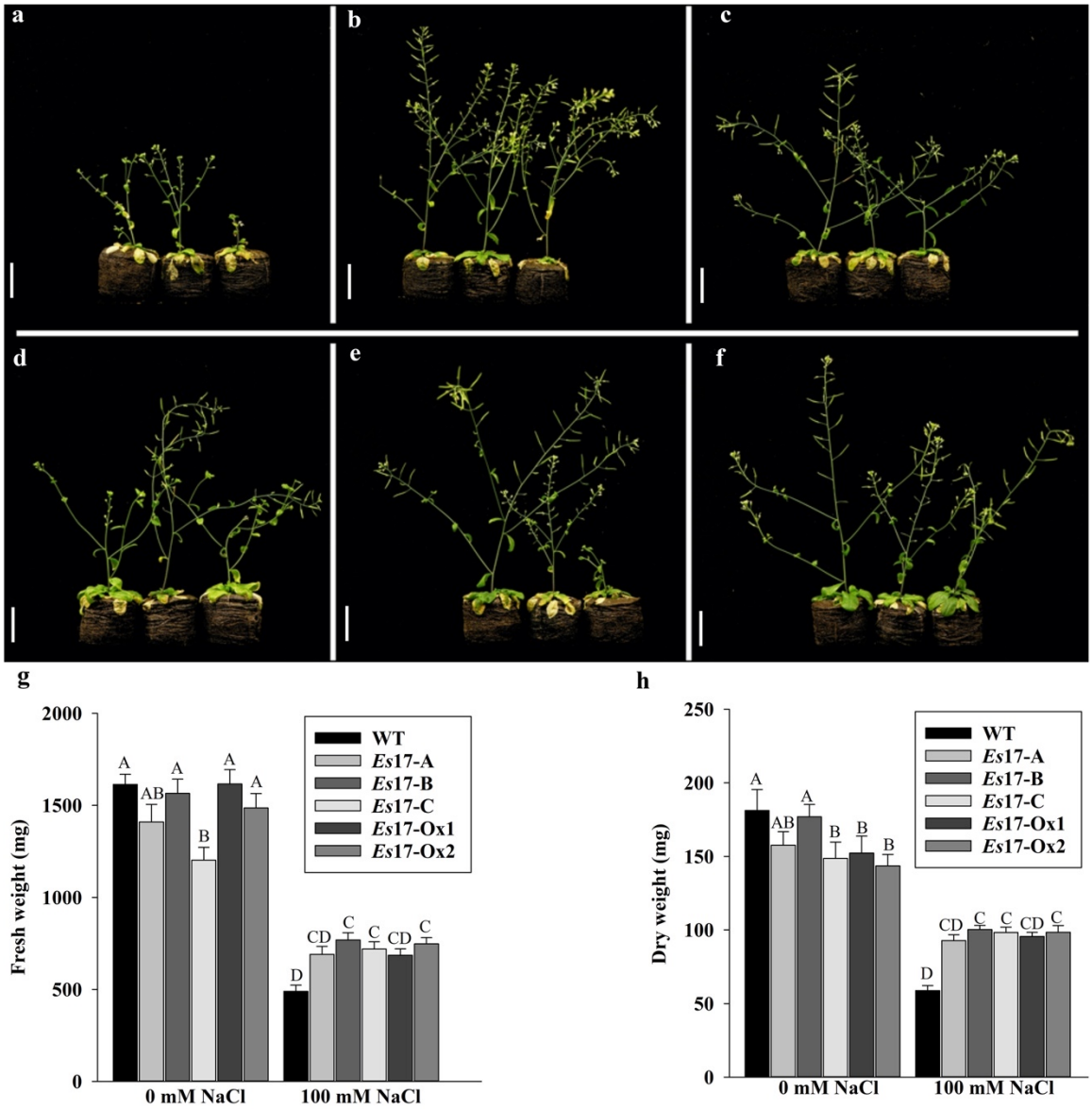


Figure 4.10. Growth of wild type and 2 independent 35S promoter (*Es*17Ox-1-2,) and of 3 independent stress inducible promoter (*Es*17A-C) transgenic *A. thaliana* plants, expressing *Esi0017_0056*, in presence of 100 mM NaCl concentration maintained throughout the experiment. **(a)** WT, **(b)** *Es*17-A, **(c)** *Es*17-B, **(d)** *Es*17-C, **(e)** *Es*17-Ox1, **(f)** *Es17*-Ox2, **(g)** fresh weight and **(h)** dry weight. The plants were photographed at 20 days after irrigation. Values represents mean and standard error (n=15). Means and SE followed by the same letter are not significantly different.

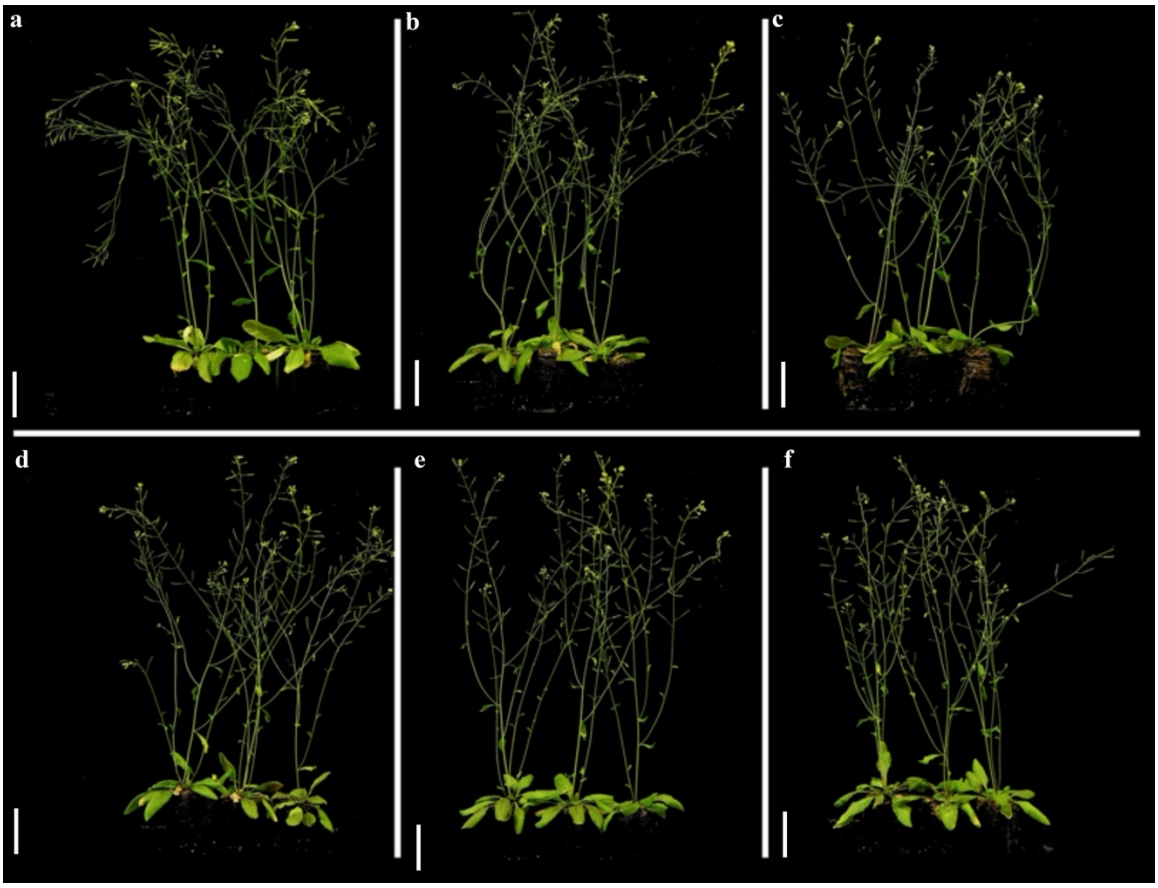


Figure 4.11. Growth of wild type and 2 independent 35S promoter (*Es*17Ox-1-2,) and of 3 independent stress inducible promoter (*Es*17A-C) transgenic *A. thaliana* plants, expressing *Esi0017_0056*, in standard conditions. **(a)** WT, **(b)** *Es*17-A, **(c)** *Es*17-B, **(d)** *Es*17-C, **(e)** *Es*17-Ox1, **(f)** *Es*17-Ox2. The plants were photographed at 20 days after irrigation.

4.4.5 Expression of *Esi0017_0056* exhibited enhanced tolerance to high temperature stress in *A. thaliana* seedlings

To examine the effects of *Esi0017_0056* expression on high temperature stress tolerance of transgenic lines, the seedlings were exposed to 40 °C for 24 h. One week after the high temperature stress, the seedlings of transgenic lines recovered much faster and showed better growth, including significantly higher fresh and dry weight, when compared to the wild type seedlings (Figure 4.12).

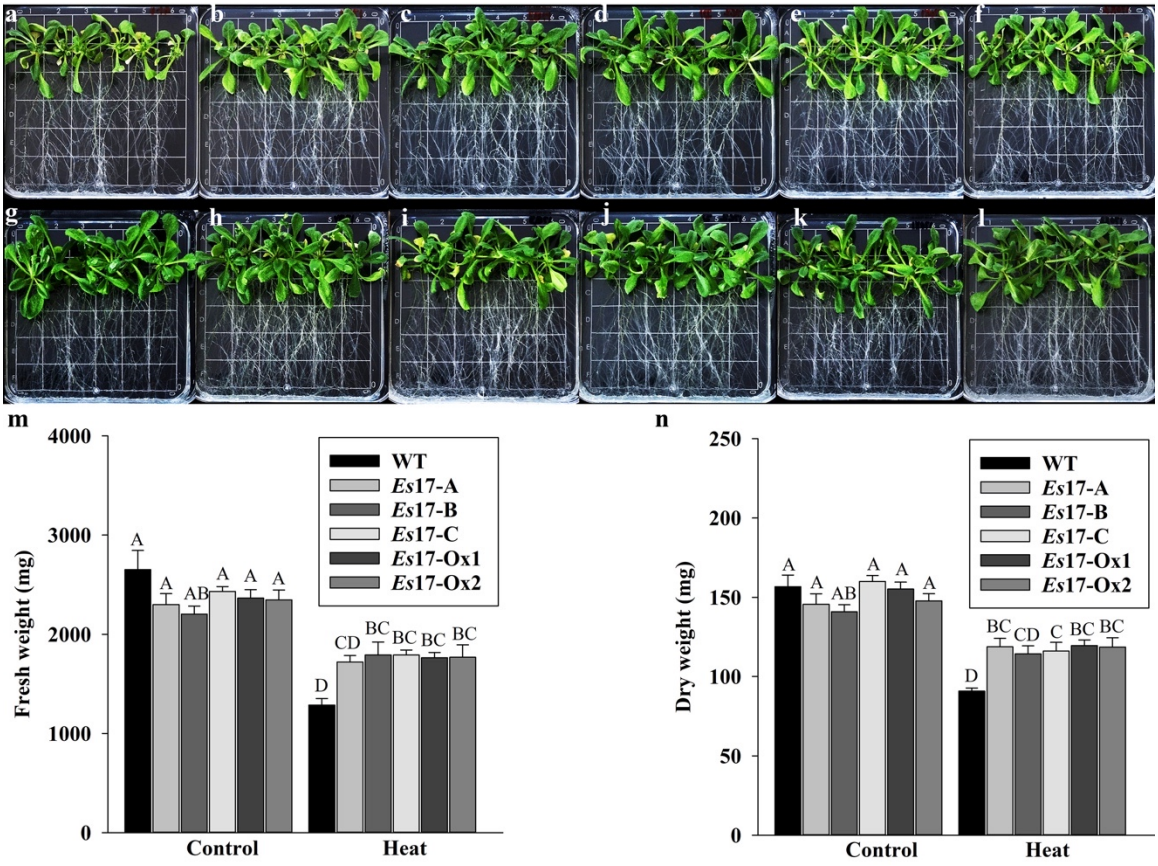


Figure 4.12. Growth of wild type and 2 independent 35S promoter (*Es*17Ox-1-2,) and of 3 independent stress inducible promoter (*Es*17A-C) transgenic *A. thaliana* seedlings under high temperature stress and standard conditions. The seedlings in plates **(a)** to **(f)**, were exposed to high temperature stress while seedlings in plates **(g)** to **(l)**, were grown in standard conditions. **(a, g)** WT, **(b, h)** *Es*17-A, **(c, i)** *Es*17-B, **(d, j)** *Es*17-C, **(e, k)** *Es*17-Ox1, **(f, l)** *Es*17-Ox2, **(m)** Fresh weight and **(n)** dry weight. The 16 days old seedlings were photographed one week after being exposed to high temperature stress. Values represents mean and standard error (n=6). Means and SE followed by the same letter are not significantly different.

4.4.6 *A. thaliana* plants expressing *Esi0017_0056* showed reduced electrolyte leakage

To estimate the effect of salinity stress on membrane stability, electrolyte leakage was measured at 24 and 48 h after the exposure to salinity stress of transgenic plants grown on peat pellets. Both 35S and stress inducible promoter transgenic lines showed significant reduction in leakage of electrolytes compared to the wild type plants, indicating higher membrane stability in transgenic plants (Figure 4.13).

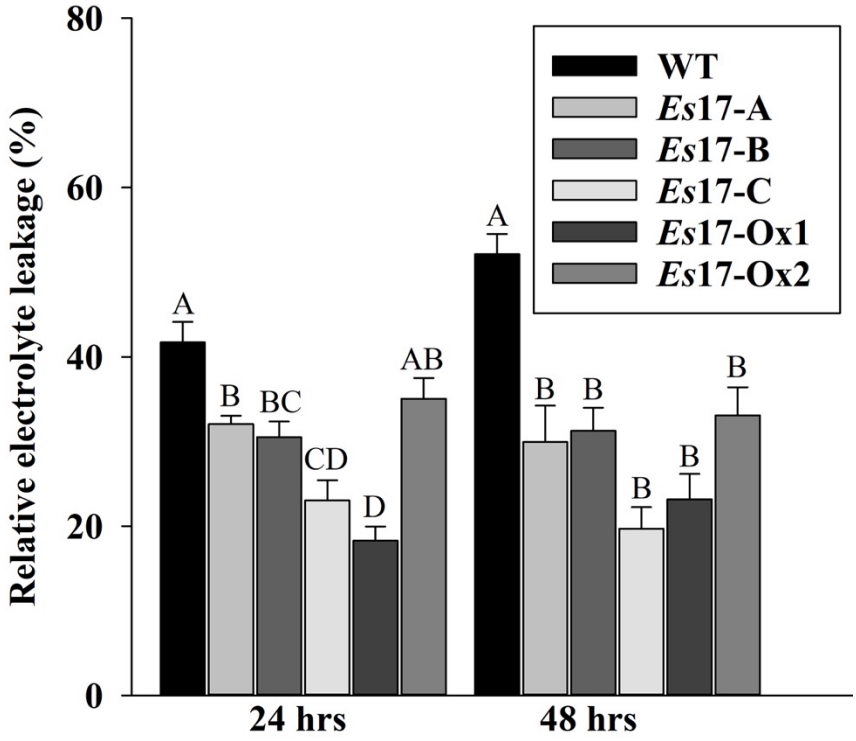


Figure 4.13. Electrolyte leakage of wild type and 2 independent 35S promoter (*Es*17Ox-1-2,) and of 3 independent stress inducible promoter (*Es*17A-C) transgenic *A. thaliana* plants, under 100 mM salinity stress conditions. Values represents mean and standard error (n=15). Means and SE followed by the same letter are not significantly different.

4.4.7 *A. thaliana* plants expressing *Esi0017_0056* exhibited altered expression of stress responsive genes

RT-qPCR analysis was performed to determine whether *Esi0017_0056* has any influence on the expression of several key stress responsive genes of *A. thaliana*. The

expression of 12 genes including *DREB2A* (Dehydration-Responsive Element Binding Protein 2A), *RD29A* (Responsive to Desiccation 29A), *RD29B* (Responsive to Desiccation 29B), *RD26* (Responsive to Desiccation 26), *RD22* (Responsive to Desiccation 22), *RD20* (Responsive to Desiccation 20), *RAB18* (Responsive to Abscisic Acid), *LEA* (Late Embryogenesis Abundant), *LEA14* (Late Embryogenesis Abundant 14), *NHX1* (Sodium/Hydrogen Exchanger), *HSP70* (Heat-Shock Protein 70) and *HSFA1D* (Heat Stress Transcription Factor A-1D)), which were demonstrated to play key roles in salinity and temperature stress tolerance, was analyzed in *Es*17-Ox2. The expression of 3 key genes (*DREB2A*, *RD29A* and *RD29B*) was also confirmed in *Es*17-Ox1. The relative expression of almost all these stress induced marker genes was rather similar in *Es*17-Ox2 and wild type plants in standard conditions, but significantly up-regulated in salinity stress, at both time points (24 and 120 h), in the plants of the transgenic line (Figure 4.14). In normal conditions, the only gene found to be strongly up-regulated in *Es*17-Ox2 *vs.* wild type comparison was that of *HSP70*, coding a ubiquitous *A. thaliana* heat shock protein (Figure 4.14k). Under salinity stress, the expression of the transcription factor *DREB2A* (Dehydration-Responsive Element-Binding protein 2A) was found to be >2 times up-regulated, at both time points, in *Es*17-Ox2 *vs.* wild type comparisons (Figure 4.14a). The expression of RD (Responsive to Desiccation) genes, *RD29B* and *RD26,* was found to be up-regulated >2 fold at both time points (Figure 4.14c,d, respectively), *RD29A* and *RD20* was up-regulated >2 fold only at the first time point (Figure 4.14b,f, respectively) while that of *RD22* was not different in *Es*17-Ox2 and wild type plants. *RAB18* (coding for a protein from the dehydrin family), *LEA* (Late Embryogenesis Abundant) and *LEA14* were also found to be significantly up-regulated in *Es*17-Ox2, at both time points, and this difference in expression was generally >2 fold higher (Figure 4.14g—I, respectively). *NHX1*, an Na^+/H^+ antiporter, and *HSP70* were determined to be significantly up-regulated in *Es*17-Ox2 only at 24 h (Figure 4.14j,k, respectively) while the expression of *HSFA1D*, a member of the Heat Stress Transcription Factor (Hsf) gene family, showed no notable differences at the two time points analyzed (Figure 4.14l).

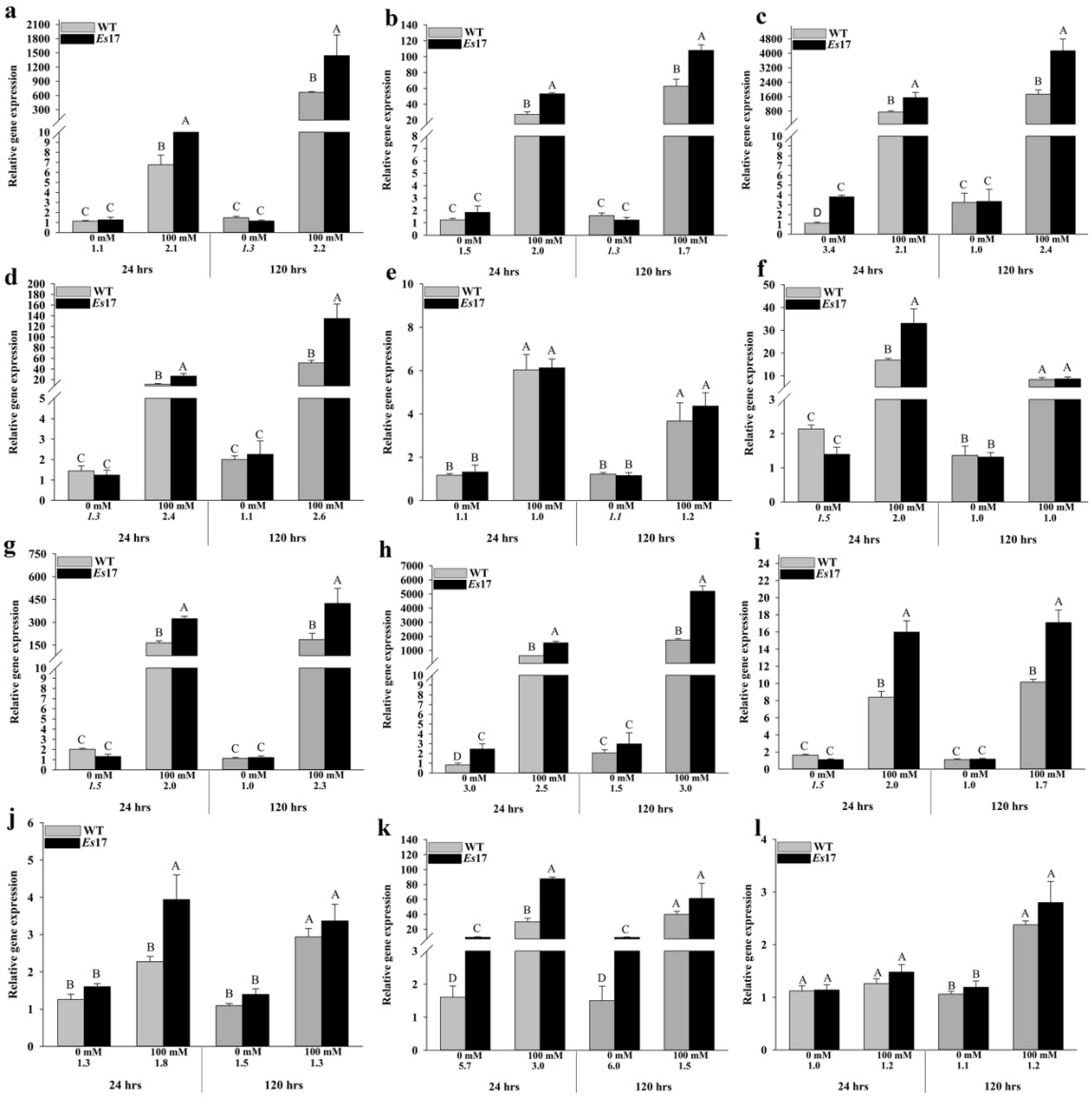


Figure 4.14. Gene expression analysis of stress inducible marker genes in the wild type and transgenic *A. thaliana* plants (*Es*17Ox-2) grown in absence and presence of 100 mM NaCl. Two time points (24 h and 120 h) were studied. *Actin* was used as the endogenous control and transcript levels were normalized to the individual with the lowest expression. Values listed under the bars represent fold difference; default font values represent up-regulation while italicized font values represent down-regulation. Data represents mean ± SE from 3 biological replicates. **(a)** *DREB2A*, **(b)** *RD29A*, **(c)** *RD29B*, **(d)** *RD26*, **(e)** *RD22*, **(f)** *RD20*, **(g)** *RAB 18*, **(h)** *LEA*, **(i)** *LEA14*, **(j)** *NHX1*, **(k)** *HSP70* and **(l)** *HSFA1D*. Means and SE followed by the same letter are not significantly different.

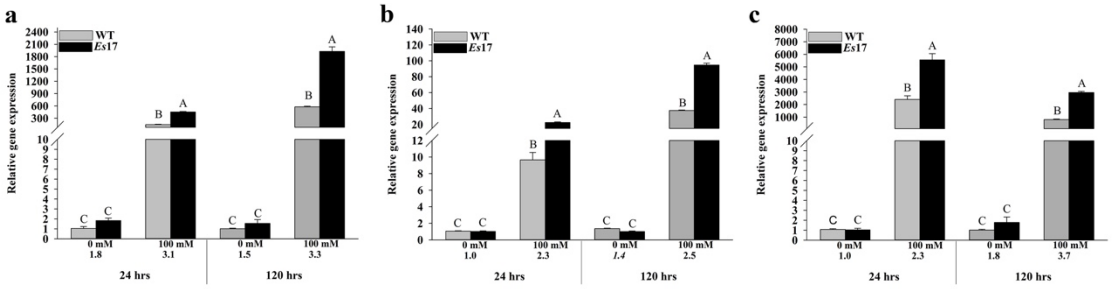


Figure 4.15. Gene expression analysis of stress inducible marker genes in the wild type and transgenic *A. thaliana* plants (*Es*17Ox-1) grown in absence and presence of 100 mM NaCl. Two time points (24 h and 120 h) were studied. *Actin* was used as the endogenous control and transcript levels were normalized to the individual with the lowest expression. Default font values represent up-regulation while italicized font values represent down-regulation. Data represents mean ± SE from 3 biological replicates. **(a)** *DREB2A*, **(b)** *RD29A*, **(c)** *RD29B*. Means and SE followed by the same letter are not significantly different.

4.5 Discussion

Plant response to abiotic stress tolerance involves a complex network of genes and stress signaling pathways. Production of stress signaling molecules is followed by the activation of various molecular mechanisms to protect the plant against stress. In the last decades, tremendous progress has been made to identify and characterize novel gene functions in various photosynthetic organisms, including the plant model *A. thaliana* (Zhu, 2016). From these studies, a large number of genes involved in responses to abiotic stresses have been discovered and used to generate transgenic plants with improved tolerance to these stresses (Zhu, 2016). Several homologs of these genes have been studied and characterized in different crop plants. The aim of this study was to characterize *Esi0017_0056,* an unknown function gene from the brown algal model *Ectocarpus* sp. and to determine if this protein, which in *Ectocarpus* sp. can be induced by different stresses (Ritter et al., 2014), can trigger similar responses in green land plants such as *A. thaliana*. Constitutive expression of heterologous proteins may have negative effects on growth of plants under standard conditions (Shanmugaraj et al., 2020). To test if there was any negative effect of

Esi0017_0056 constitutive expression on growth, transgenic lines under stress inducible promoter were also generated. The results showed that in seedlings grown under standard growth conditions, the 35S constitutive expression lines were associated with reduced root length while decreased biomass was determined in *Es*17-Ox1. In contrast, all the lines that utilized the *RD29A* stress inducible promoter performed similarly to the wild type. This trend was not anymore observed when plants were grown to maturity; no clear-cut differences could be observed between the two promoter lines. In normal conditions, qPCR analyses of mature plants indicated that more *Esi0017_0056* transcripts were produced in 35S lines as compared to RD29A lines; clearly, the difference in transcript abundance was not associated with protein quantity because the phenotype of transgenic plants did not reflect this considerable difference.

Under salinity and temperature stress, both types of transgenic lines performed better than the wild type. Expression of *Esi0017_0056* in *A. thaliana* significantly improved plant tolerance to salinity and high temperature stress in all lines, irrespective of the promoter used.

It is worth mentioning that under salinity conditions, similarly to the normal conditions, no positive correlation could be observed between the *Esi0017_0056* transcript abundance and phenotypic data or between the former condition and electrolyte leakage. We speculate that the elevated level of expression of *Esi0017_0056*, comparable to that of *actin* or better in constitutive expression lines, was already very high for the translational system, which reached a saturation or a plateau level. This process can be observed in many biochemical processes and explains the limited similarities, in certain experimental conditions, observed between transcriptomics and proteomics (Maier et al., 2009; Vogel and Marcotte, 2012; Liu et al., 2016).

Since *Esi0017_0056* has no close relative in green plants, it is extremely difficult to indicate the mechanism through which the product of this gene determines the observed changes in the overall plant growth in normal conditions and how it contributes to the improved tolerance to abiotic stresses. In the current study, we analyzed the expression of several well characterized abiotic stress responsive transcriptional factors and genes regulated by them. In plants, ABA plays a crucial role to improve abiotic stress tolerance (Finkelstein et al., 2002). Several abiotic stress responsive genes require ABA for their

activation and some not; this indicates the existence of both ABA-dependent and ABA-independent stress signal transduction pathways (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). In plants, transcriptional regulation of abiotic stress responsive genes depends on the two major class of cis- acting elements found in promoter region of these genes. These elements are known as ABRE (ABA responsive elements) and DRE (Dehydration Responsive Elements). ABREs are known to participate in ABA dependent and DREs are known to be involved in ABA independent stress signal transduction pathways (Hattori et al., 2002; Agarwal et al., 2006; Nakashima et al., 2009; Fujita et al., 2011). *A. thaliana RD26* gene encodes a NAC transcription factor that has been shown to localize in the nucleus and is induced by drought, salinity and ABA (Fujita et al., 2004). The promoter region of *RD26* has been shown to contain four ABRE, one MYC, two MYB and one DRE recognition sites (Finkelstein et al., 2002; Shinozaki et al., 2003). *RD20* gene functions in ABA dependent stress signaling pathway (Takahashi et al., 2000) and has been shown as a direct target of *RD26* (Fujita et al., 2004). The expression of both these genes, i.e., *RD26* g and *RD20*, was determined to be significantly up-regulated in *Es*17-Ox2 plants as compared to the wild type plants (Figure 4.14d,f, respectively). *A. thaliana DREB2A* gene encodes a transcription factor which regulates the expression of genes induced by salinity and drought stress (Kim et al., 2011). *DREB2A* contains an ERF/AP2 (ethylene responsive element binding factor/APETALA2) DNA binding domain. This domain regulates the expression of downstream genes by interacting with cis-element DRE in their promoter region (Nakashima et al., 2000; Sakuma et al., 2002). *A. thaliana* plants overexpressing constitutively active form of *DREB2A* exhibited up-regulation of *RD29A*, *RD29B* and *LEA14,* suggesting that these genes are the direct target of *DREB2A*. Promoter region of these genes has been shown to carry DRE core motifs (Sakuma et al., 2006). The expression of *DREB2A* (Figure 4.14a) and its abovementioned downstream genes (Figure 4.14b,c,I, respectively) was found to be significantly up-regulated in *Es*17-Ox2 plants in contrast to the wild type plants suggesting that *Esi0017_0056* may possibly modulate, through an unknown mechanism, the expression of *DREB2A.* In *A. thaliana*, *AREB1* gene encodes an ABRE binding protein which is a key transcription factor. *AREB1* is up-regulated by salinity stress and modulates the expression of ABRE dependent stress responsive genes involved in ABA signaling (Fujita et al., 2005; Yoshida et al., 2010).

Overexpression of *AREB1* has been shown to up-regulate the expression of key ABA inducible stress responsive genes belonging to LEA class proteins *At3g17520* (encoding a group 3 LEA class protein), *RD29B/LTI65, RAB18* (Lång and Palva, 1992; Nordin et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1994; Wise, 2003), and ABA regulated *RD20*, which encodes a calcium binding protein (Takahashi et al., 2000). LEA proteins are involved in plant responses to abiotic stress tolerance by stabilizing the cellular membranes, redox homeostasis, nucleic acids and protein structures (Ingram and Bartels, 1996; Veeranagamallaiah et al., 2011; Jia et al., 2014) and have been suggested to be direct targets of *DREB2A* (Sakuma et al., 2006). In this study, the two LEA genes studied (Figure 4.14h,i) as well as *RD29B, RAB18* and *RD20* (Figure 6c,g,f, respectively) were found to be up-regulated in *Es*17-Ox2 plants. Moreover, reduced leakage of electrolytes in all *Esi0017_0056* transgenic lines suggest higher cellular membrane integrity in the transgenic plants compared to that in the wild type plants.

Heat shock proteins (HSPs) act as chaperones playing essential roles in the proper folding and refolding of proteins as well in stabilizing their structure in presence of stress. In *A. thaliana, HSP70* (*At3g12580*) encodes a molecular *HSP70* chaperon which is involved in abiotic stress tolerance by preventing protein aggregation and helping in the refolding of proteins under stressful conditions (Sakuma et al., 2006). The product of this gene is also involved in transport of unstable proteins to lysosomes or proteasomes for their degradation (Wang et al., 2004). Expression of *HSP70* was found to be up-regulated in *Es*17-Ox2 under both normal and salinity stress conditions, at both the time points. HSP70 was reported to be present almost everywhere in the cell, including Golgi apparatus, cell wall, chloroplast, cytoplasm, cytosol, mitochondrion, plasma membrane and vacuolar membrane (Saibil, 2013) (TAIR; https://www.arabidopsis.org; 30 November 2020). As the expression of this HSP70 (*At3g12580)* was found to be increased in the *Es*17Ox-2 line, it is quite possible that this heat shock protein is involved in a specific interaction with Esi0017_0056 which can include assistance for proper folding. Overexpression of *HSP70* has been shown to up-regulate the expression of *DREB2A* and of other target genes of *DREB2A* including *LEA 14, AIL* (group 3 LEA), *RD29A* and of *RD29B* (Sakuma et al., 2006a; Sakuma et al., 2006b), and to improve plant tolerance to high temperature, salinity and drought stress (Wang et al., 2004). In the current study, the expression of all of these genes, i.e., *DREB2A,*

RD29A, RD29B, AIL, LEA14 and *HSP70,* was found to be significantly up-regulated in *Es*17-Ox2 plants as compared to the wild type plants, at both time points (Figure 4.14a–c,h,i,k). At least two main possible scenarios involving HSP70 and Esi0017_0056 can be envisaged: the first one assumes that Esi0017_0056 performs some catalytic activity in *A. thaliana* cells while the second one that *Ectocarpus* protein is inactive in the heterologous expression system. In the first situation the elevated levels of HSP70 are associated with its role as a chaperone, helping Esi0017_0056 to fold properly and, therefore, ensuring that Esi0017_0056 can perform its role, which presumably is an enzymatic activity. As mentioned before, Esi0017_0056 has a DUF1768 domain which based on its similarity with *E. coli* YbiA might exhibit N-glycosidase activity. In *E. coli,* YbiA is involved in riboflavin biosynthesis; however, the family that contains YbiA was included in the NADAR (NAD and ADP-ribose) superfamily which comprises proteins predicted to be involved in NAD-utilizing pathways, possibly using ADP-ribose derivatives as substrates. Nevertheless, based on structure modelling, the N-glycosidase activity is more likely to be present. The N-glycosidase activity, that is, the removal of N-linked oligosaccharides, can occur on a wide range of substrates including glycopeptides, glycoproteins and rRNA; therefore, the effects at cellular level cannot be predicted unless the substrate is identified. Nevertheless, removal of N-linked oligosaccharides is an important step on protein inactivation and subsequent degradation (Suzuki and Fujihira, 2020) which might be associated with increase expression of various heat shock proteins. This scenario explains well the expression pattern of *HSP70 (At3g12580)* and of *DREB2A, RD29A, RD29B, AIL, LEA14*, genes whose expression is up-regulated by HSP70, but what activity Esi0017_0056 performs in the cell remains to be elucidated. The second scenario posits that HSP70 participates in the folding of Esi0017_0056 which is not performing any activity in *A. thaliana* cells in the absence of a suitable substrate in this heterologous system. In this situation, the overall increased abundance of HSP70 is responsible for the observed effects in *A. thaliana* at phenotypic and molecular levels, i.e., increased tolerance to salt and temperature stress and changes in expression of a number of genes including of those aforementioned. Definitely, these propositions involving HSP70 are just two of the many possible scenarios as some direct and indirect interaction with other cytosolic proteins such as RD29A and B, RAB18 and LEA cannot be ruled out. Moreover, most

proteins having the DUF1768 domain are of bacterial origin; therefore, it cannot be ruled out that this molecular pattern is perceived by *A. thaliana* as a non-self, foreign molecule, triggering additional responses, including defense responses, that await characterization. Clearly, future work including an "omics" (transcriptomics or proteomics) approach is needed to understand in depth the effects triggered by the expression of Esi0017_0056 in *A. thaliana.* Additionally, studies of the potential interaction between HSP70 (At3g12580) with Esi0017_0056 and resolving the crystal structure of Esi0017_0056 could contribute to the better understanding of the roles of this protein in *A. thaliana* and *Ectocarpus* sp.

4.6 Conclusions

The expression, for the first time, of the unknown function gene from brown alga *Ectocarpus* sp. in *A. thaliana* resulted in enhanced tolerance to high salinity and high temperature stress. Gene expression analysis revealed that the expression of several key stress markers genes involved in various functions such abscisic acid mediated abiotic stress tolerance, sodium sequestration, chaperon activities and membrane stability was up-regulated in transgenic plants. The protein fused with C-terminal tag was produced in both *A. thaliana* and *Nicotiana benthamiana.* These results suggest that brown algae represent a valuable source of important genes that can be used for generating transgenic land plants with improved tolerance to a wide range of abiotic stresses.

CHAPTER 5

Expression of a Heat Shock Protein 70 from the Brown Alga *Ectocarpus* sp. Imparts Salinity and Temperature Stress Tolerance in *Arabidopsis thaliana*

Pramod Rathor[1], Tudor Borza[1], Sophia Stone[2], Thierry Tonon[3], Svetlana Yurgel[1], Philippe Potin[4], Balakrishnan Prithiviraj [1*]

[1]Marine Bioproducts Research Laboratory, Department of Plant, Food and Environmental Sciences, Dalhousie University, Nova Scotia, Canada

[2]Department of Biology, Dalhousie University, Nova Scotia, Canada

[3]Centre for Novel Agricultural Products, Department of Biology, University of York, UK

[4]Sorbonne Université, CNRS, UMR8227, Integrative Biology of Marine Models (LBI2M), Station Biologique de Roscoff (SBR), 29680 Roscoff, France.

[*]**Corresponding author:**

Balakrishnan Prithiviraj

E-mail: bprithiviraj@dal.ca

This manuscript is in preparation for submission.

5.1 Abstract

Brown alga *Ectocarpus* sp. belongs to Phaeophyceae, a class of macroalgae in the phylum Heterokonts, which is also known as Stramenopiles lineage that made transition from unicellularity to complex multicellularity during long evolutionary history. *Ectocarpus* sp. is a dominant seaweed in the temperate regions around the globe, abundant mostly in the intertidal zones, an environment with harsh environmental conditions resulting from tidal cycles. Analysis of previously generated transcriptomic data of *Ectocarpus* sp. revealed several genes consistently up-regulated by various abiotic stresses; one of these genes was

Esi0379_0027, which encodes a HSP70 protein. Bioinformatics analyses indicated that the HSP70 protein is soluble, monomeric and well conserved in other organisms. The gene was successfully expressed in *A. thaliana* under constitutive and stress inducible promoters. Transgenic plants generated using both promoters exhibited enhanced tolerance to salinity and high temperature stresses. The expression of several key abiotic stress-related genes was analyzed in transgenic and wild type *A. thaliana* by qPCR. Gene expression analysis revealed that genes involved in ABA-induced abiotic stress tolerance, K^+ homeostasis, Na^+ sequestration and chaperon activities were significantly up-regulated in the transgenic plants. This study is the first report in which *HSP70* gene from *Ectocarpus* sp. highly responsive to abiotic stresses, was successfully expressed in *A. thaliana*, leading to improved tolerance to high salinity and temperature stress.

Keywords: *Arabidopsis thaliana, Ectocarpus* sp., heat shock protein, transgenic plant, salinity, temperature, abiotic stress tolerance

5.2 Introduction

Plants being sessile organisms are incapable to avoid stressful or unfavorable growth conditions by changing place. These organisms are constantly exposed to several environmental stresses including salinity, drought, temperature extremes, heavy metal and ultra-violet radiation. These stressors negatively impact plant growth and development resulting in significant loss in productivity. High soil salinity and high temperature stress are the predominant among various abiotic stresses which causes severe crop loss globally. In recent years there has been an increase in global warming is increasing and, causing climate change resulting in changing weather patterns (IPCC, 2014). The effect of global climate change further exasperates the effects of abiotic stresses on agricultural production (Lesk et al., 2016). The crop loss due to these stressors account up to 50% loss in production (Lobell et al., 2011). High salinity inhibits plant growth in multiple ways such as osmotic stress, ion accumulation and secondary stress. The primary stresses, such as salinity, drought and extremes of temperature are often interconnected and lead to secondary

stresses such as oxidative stress. At cellular level, these stresses result in physiological dehydration, which further damages cellular machinery (Wang et al., 2003). Increased soil osmotic potential due to presence of salt reduces the plants ability to uptake water and nutrients (Hasegawa et al., 2000). Accumulation of Na^+ and Cl^- ions in significantly higher concentration than those of outside in the soil helps to maintain turgor pressure but cells must have the strict regulation for these ions in each cellular compartment to avoid inactivation of enzymes thus prevent the metabolic dysfunction (Munns and Gilliham, 2015). Based on their ability to tolerate salt stress plants are classified as halophytes and glycophytes (Flowers et al., 1977); halophytes are highly tolerant to salinity stress. These plants have evolved unique structural features such salt secretory glands in leaves that has the capacity to excrete excess salt which ensures their survival under higher levels of soil salinity. However, most of the crop plants are glycophytic and lacks such structures therefore doesn't exhibit higher level of salinity stress tolerance (Hasegawa et al., 2000; Julkowska and Testerink, 2015). Generally, halophytes have higher concentration of Na^+ and Cl^- ions in leaves than the outside environment this help to maintain higher osmotic potential in the cells and therefore higher turgor pressure. These plants store Na^+ and Cl^- in vacuoles and uses compatible solutes and K^+ ions to equilibrate the osmotic differences in different compartments of cytoplasm (Shabala, 2013). Accumulation of Na^+ and Cl^- ions in some leaves may be deleterious but at whole plant level it might be beneficial as plant can store the excess Na^+ and Cl^- ions in those leaves and later remove these ions via abscission (Munns and Tester, 2008).

High temperature events are becoming more severe and frequent due to the change in the environment caused by climate change. High temperature stress causes denaturation of proteins and changes in membrane fluidity. Plants respond to high temperature stress by increased expression of heat shock proteins that protects protein from misfolding and aggregation of proteins in different cellular compartments (Peng et al., 2004; Echer et al., 2014). Heat shock proteins are classified on the basis of molecular weight. Molecular weight of these proteins ranges from 10 to 200 kDA. Based on molecular weight these proteins are classified into five major categories named as sHSP (small heat shock proteins), HSP60, HSP70, HSP90 and HSP100 (Wang et al., 2004). These proteins are present and conserved in almost all living organisms from prokaryotes to eukaryotes (De

Maio, 1999). Nomenclature of HSPs in bacteria is different, for instance HSP70 in bacteria is called as DnaJ protein. However, the classification is based on the molecular weight (Kotak et al., 2007). Heat shock proteins play an important role in developmental processes and in abiotic stresses such as salinity (Zou et al., 2012), drought (Cho and Choi, 2009) and extremes of temperature (Lopez-Matas et al., 2004). Among several HSPs, HSP70 is a highly conserved protein and has been extensively studied in bacteria, plants and animals. It is the most abundant molecular chaperon which is localized to various cellular compartments including cytosol, nucleus, mitochondria and endoplasmic reticulum protecting proteins by folding of misfolded or unfolded proteins, disassembly of protein complex to translocate in another cellular compartment and in disaggregation of complex aggregates (Saibil, 2013). HSP70 contains two main domains: a substrate binding domain and another ATPase (Kampinga and Craig, 2010; Mayer, 2010; Zuiderweg et al., 2012). The activity of HSP70 depends on the interaction between these two domains and other co-chaperons (Saibil, 2013). Proteins which are not folded correctly in their native state contains several binding sites usually at every 30 to 40 residues for HSP70 (Rüdiger et al., 1997). It is known that HSP70 binds to the 7-residue segment of a polypeptide chain which is hydrophobic in nature (Clerico et al., 2015). Even transient binding of polypeptide segment with HSP70 can prevent the misfolding and aggregation of proteins thus maintains protein in native state (Young, 2010). Once reached to right destination polypeptide regains the native state in free solution by detaching from HSP70. If it fails to refold properly it will lead to rebinding to HSP70. Therefore, the function of HSP70 in proper folding seems to be in stabilization of unfolded proteins until it can refold correctly (Sharma et al., 2010).

Seaweeds are an integral component of marine coastal ecosystem, which includes the macroscopic, multicellular algae commonly inhabiting the coastal regions of the world (Khan et al., 2009). These organisms are very important for marine ecosystem as they make large dense forest in ocean and provides habitat for several organisms. Brown alga are multicellular organisms in the phylum Heterokonts, which is also known as Stramenopiles lineage (Cock et al., 2012). This lineage is distantly related to Archaeplastida (including green plants and red algae) and Opisthokonts (including animal and fungi) (Baldauf, 2003; Brodie et al., 2017). Since each of these lineages have evolved independently it is thought that organisms in each of these lineages have evolved mechanisms necessary for the

development of complex multicellularity. These developmental mechanisms are well studied in land plants and in animals but very little is known in brown alga (Coelho et al., 2012). During the billions year of evolution brown alga have made the transition from unicellularity to complex multicellularity and therefore acquired novel features which are absent in other lineages (Cock et al., 2012; Brodie et al., 2017). Endosymbiotic gene transfer led to the significant incorporation of foreign genes and enriched the nuclear genome. Some of these genes were targeted to specific organelle through N-terminal transit peptide and many of these evolved novel functions in these organisms (Brodie et al., 2017). These novel features make it very interesting to explore and decipher the metabolisms and cellular biology of these organisms. Moreover, genome sequencing demonstrated that more than 36% of genes are of unknown functions and doesn't match to any other existing organisms. This indicates that these are novel genes or got considerably diverged from the homologue genes present in other organisms (Cock et al., 2012). Some of the novel features include metabolisms of halogenated compounds, accumulation of complex polysaccharides and higher tolerance to biotic and abiotic stresses (Charrier et al., 2008). Moreover, in brown algae the photosynthetically fixed carbon is not stored as starch granules instead it is stored as laminarin and mannitol (Craigie, 1974; Davis et al., 2003). In contrast to land plants these organisms produce complex polysaccharides such as alginates and sulfated fucans (Kloareg and Quatrano, 1988). Genome editing tools have been routinely applied to land plants to understand their complex organization. However, their application in other lineages for instance in Stramenopile is very challenging as these are not easily amenable, lack of standardized protocols for genetic transformation, mutation, promoter analysis and subcellular localization. Sequencing of brown algal model *Ectocarpus* sp. provided some insights to brown algal biology however still remains largely unknown. *Ectocarpus* sp. is a cosmopolitan filamentous multicellular brown alga which has relatively small genome size (200 Mb) in contrast to *Fucus serratus* (1095 Mb) and *Laminaria digitate* (640 Mb) (Le Gall et al., 1993; Peters et al., 2004). Previous transcriptomics analysis revealed that several genes were consistently up-regulated in *Ectocarpus* sp. subjected to various abiotic stresses and exhibited elevated levels of stress tolerance. It was reported that 76 % of the up-regulated genes are of unknown functions and doesn't match to any of the sequence outside the *Ectocarpus* sp. available at NCBI or

protein databases (Ritter et al., 2014). One of the gene was *HSP70* (*Esi0379_0027*). In this study we expressed this gene in *A. thaliana*. Expression of this gene exhibited improved tolerance to salinity and high temperature stress. The expression of several stress marker genes was found to be significantly higher in transgenic plants.

5.3 Materials and Methods

5.3.1 *Ectocarpus* sp. growth conditions and gene isolation

The axenic culture of brown algal model *Ectocarpus* sp. (Dilwyn) Lyngbye unialgal strain 32 (accession CCAP 1310/4, origin San Juan de Marcona, Peru) was established in a 10 liters plastic tank filled with filtered, autoclaved natural sea water and supplemented with provasoli nutrient medium at a concentration of 1% (v/v). Culture was maintained in the growth room set at 14 °C with 14-h light/10-h dark, with light intensity of 40 µmol.$m^{-2}s^{-1}$. Compressed air passing through a 0.22 µm filter was used to continuously air bubble the nutrient medium. The algal culture was subjected to high salinity stress at 1450 mM NaCl for 6 h. Following the exposure to high salinity stress culture was collected using filtration, carefully dried on absorbent paper and immediately flash frozen into liquid nitrogen. Total RNA was extracted from ~100 mg of tissue with some changes made to a protocol described by (Apt et al., 1995). The modifications to the protocol were made as described by (Le Bail et al., 2008). RNA concentrations were quantified using Nanodrop 2000 spectrophotometer (Thermo scientific, FRANCE). One microgram of total RNA was treated with Turbo DNAse (Ambion Austin, USA) according to the manufacturer's instruction. The DNAse treated RNA was converted to cDNA, using a SuperScript IV Reverse Transcriptase (Life Technologies, FRANCE), according to the manufacturer's protocol.

5.3.2 Bioinformatics analysis

The protein transmembrane helices or domain were identified using HMMTOP v.2.0 (http://www.enzim.hu/hmmtop/) and TMHMM v.2.0 ((https://services.healthtech.dtu.dk/)

prediction servers. To understand the subcellular localization of protein the amino acid sequences was analyzed using TargetP 2.0, Signal P and DeepLoc-1.0 (https://services.healthtech.dtu.dk/), iPSORT (http://ipsort.hgc.jp), and WoLF PSORT (https://wolfpsort.hgc.jp) prediction servers. Homology modelling for secondary structure was carried out using PSIPRED (Buchan and Jones, 2019) (http://bioinf.cs.ucl.ac.uk/psipred). To obtain information about the tertiary structure and the folding of *Es*HSP70, protein model was generated using SWISS-MODEL (Waterhouse et al., 2018). The best model (77.64 % sequence identity) was built using bovine heat shock cognate 71 kDA as a template (SMTL ID: 4fl9.1; structure solved by X- Ray Diffraction 1.90 Å).

BlastP search was performed and the amino acid sequence of *Es*HSP70 was compared with that of similar protein sequences of different taxa obtained from GenBank. Alignments of some selected protein sequences were performed using MUSCLE (Edgar, 2004) in MEGA X (Kumar et al., 2018). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.8733)) and 500 bootstrap replications were performed. The analysis involved 48 sequences from different species and that of *Es*HSP70. Evolutionary analyses were performed using MEGA X (Kumar et al., 2018).

5.3.3 Expression of *Es*HSP70 in *A. thaliana*

To create the plant transformation vector, pEarleyGate 100 (35S; (Earley et al., 2006)) and a promoter less gateway vector pMCS:GW (Michniewicz et al., 2015) were obtained from ABRC (Columbus, OH, USA). Promoter sequence for a stress inducible gene *RESPONSIVE TO DESSICATION 29A* (*RD29A)* was isolated from the wild type (Col-0) *A. thaliana*. Gateway vector containing stress inducible promoter of *RD29A* gene, was generated as described by (Rathor et al., 2021). The PCR product containing attB sequences was cloned into pDONR221 by the BP recombination reaction using the BP Clonase™ II Gateway®, following the manufacturer's protocol to generate entry clone containing gene of interest (Gateway® Technology with Clonase II, Invitrogen, Ontario,

Canada). The entry clone harboring full gene sequence was then introduced into pEarleyGate 100 (35S) and pMCS:GW (*RD29A* promoter) by LR recombination reaction using the LR Clonase™ II Gateway®, following the manufacturer's protocol to generate the expression clone (Gateway® Technology, Invitrogen, Ontario, Canada). PCR was performed to confirm the sequence of *RD29A* promoter and gene of interest in the expression clone by using the forward primer sequence from the *RD29A* promoter and reverse primer sequence from the gene of interest. The expression clone was then transformed into *Agrobacterium* strain GV310 (pMB90) using the freeze and thaw method and then *A. thaliana* (*Arabidopsis thaliana* (L.) Heynh, ecotype Columbia (Col-0)) flowering plants were transformed using the recombinant *Agrobacterium* strain carrying the gene of interest following a protocol as explained by (Clough and Bent, 1998).

5.3.4 Selection of transformants and homozygotes

Positive transformants were selected as described by (Rathor et al., 2020). Segregation analysis was tested in each generation by placing 100 seeds on solid half MS media amended with 40 µg/ml of ammonium glufosinate and positive 10-15 plants were transferred in soil to collect seeds. Expression of the transgene was analyzed in these lines using qRT-PCR (quantitative real time polymerase chain reaction) as described in later section. For further experiments two independent transgenic lines for 35S promoter named as *Es*HSP-Ox1 and *Es*HSP-Ox2 and three independent lines for stress inducible promoter (*Es*HSP-A, B and C) were selected.

5.3.5 Salinity stress tolerance

Seedlings of wild type and overexpression lines were exposed to 100 mM NaCl. Seedlings were raised as described by (Rathor et al., 2020). After 4 days of growth, uniform seedlings were picked up transferred on plates containing solid half strength Murashige and Skoog (MS) medium (Phytotech, USA) and 100 mM NaCl. Plates were vertically stacked and maintained at 22 °C with 16 h light/8 h dark cycle, with light intensity of 100 $\mu mol.m^{-2}s^{-1}$. Root length was marked on the day of seedling transfer and on 7^{th} day plates were scanned using a high resolution scanner (Epson Expression 10000 XL), (Epson,

Ontario, Canada), and increase in root length was measured using Image J software (Research Services Branch, National Institute of Health, Bethesda, MD). The number of lateral roots per cm of primary root were recorded. Percent leaf chlorosis was recorded visually on 7th day after transfer. Fresh weight of plants was recorded on the 9th day, then samples were dried in a hot air oven at 70 °C for 5 days and dry weight was recorded. The experiment was repeated 3 times with 30 plants in each treatment in all experiments.

To investigate if the phenotypes observed in seedlings were evident in plants, seeds of wild type and of overexpression lines were stratified for 2 days at 4 °C and were sown directly on Jiffy peat pellets (Jiffy, NB, Canada). After 13 days of growth uniform plants were selected and used in this experiment. Peat pellets were completely saturated with water 3 days in advance of the onset of salinity stress to equilibrate amount of water in all peat pellets across the treatments. Then they were left without irrigation for 3 days and then were irrigated with 200 mM NaCl at 40 ml per plant to maintain the concentrations of 100 mM. After 5 days of growth all plants were equally watered at 3 days of interval for 3 weeks. Plants were photographed after 3 weeks. Whole plant was harvested, and biomass was recorded for individual plant. The experiment was repeated 3 times with 5 replicates in each treatment in all experiments. In order to understand the effect of salinity stress on membrane intactness an experiment for electrolyte leakage was performed. Plants were grown and treated as described earlier. Following the exposure to salinity stress rosette leaves were harvested from 3 plants in one biological replicate (5 biological replicates), and carefully placed in scintillation vials containing 20 ml of deionized water. Electrolyte leakage was recorded using SympHony SB70C (VWR, ON, Canada) conductivity meter as described by (Cao et al., 2007).

5.3.6 Heat stress tolerance

Effect of high temperature stress on the growth of overexpression lines was studied following a method described by (Rathor et al., 2020). Briefly, 9 days old seedlings were exposed to 40 °C for 24 h by placing the Petri dishes in growth chamber (Conviron, MB, Canada). The plates were removed and placed under standard growth conditions. After

one week of recovery, plants were photographed, and fresh and dry weight data were recorded as described earlier. The experiment was repeated 3 times with 30 plants in each treatment in all experiments.

5.3.7 Real-time quantitative PCR of key stress responsive genes

In order to understand the expression of abiotic stress associated key marker genes in the overexpression lines *Es*HSP-Ox1 was selected. Plants were grown and treated as described earlier. Leaf tissues were harvested at 24 and 120 h following the exposure to salinity stress and flash frozen in liquid nitrogen. Total RNA was extracted from samples, using GeneJET plant RNA purification kit (Thermoscientific, ON, Canada). RNA concentration was assessed using Nanodrop spectrophotometer (Thermoscientific, ON, Canada), and two micrograms of total RNA was treated using RQ1 RNAse free DNAse by following a protocol as described by manufacturer (Promega, ON, Canada). DNAse treated RNA was converted into cDNA using the RevetAID cDNA Synthesis kit following a protocol described by manufacturer (Thermoscientific, ON, Canada). The cDNA concentration was made to 10 ng/µl, and from that 3 µl was used in qRT-PCR. The relative transcript levels were determined by Real–Time polymerase chain reaction, using the gene specific primers and *actin* (Supplementary Table S1 in Appendix A) as the endogenous control on the StepOne plus Real-Time PCR system (Applied Biosystems, Ontario, Canada), using iTaq SYBR green (Bio-RAD, ON, Canada). The relative expression was calculated using delta-delta ct method and transcripts were normalized to the individual with lowest expression. The expression of few key genes was also confirmed in overexpression line *Es*HSP-Ox2.

5.3.8 Statistical analyses

Multiple mean comparisons were performed using Analysis of Variance (ANOVA) with a confidence level of 95%, followed by Tukey post hoc test with an error rate of 5% in Minitab 19.0 (Minitab LLC, State College, Pennsylvania, US).

5.4 Results

5.4.1 Prediction of transmembrane domain and intracellular localization of *Es*HSP70

*Es*HSP70 (*Esi0379_0027*) was one of the many *Ectocarpus* sp. proteins (i.e. Esi0016_0056, Esi0154_0047, Esi0025_0042, Esi0488_0007, Esi0059_0099, Esi0322_0010, Esi0252_0035, Esi0045_0021, Esi0105_0049, Esi0182_0002, Esi0143_0016, Esi0007_0087, Esi0113_0047, Esi0538_0008, Esi0021_0137, Esi0195_0005, Esi0176_0002, Esi0266_0005 and Esi0044_0144) that were selected and screened for expression in *E. coli* (*Escherichia coli*) and *A. thaliana*. HMMTOP v.2.0 (http://www.enzim.hu/hmmtop/) and TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) prediction servers were used to analyze the presence of transmembrane domains in these proteins as their presence makes protein expression very difficult. For this protein no transmembrane domains have been predicted.

Subcellular localization of protein was analyzed using several prediction servers. SignalP, iPSORT and TargetP 2.0 ruled out the presence of a signal peptide or of a mitochondrial, chloroplast or thylakoid luminal transfer peptide. In plants it is known that endogenous HSP70 is present in different cellular compartments and it is likely that *Es*HSP70 in transgenic plants likely to be same. However, final destination in particular cellular compartment may occur by interaction with other proteins.

Homology modelling using PSIPRED provided an overall prediction of the secondary structure of this protein with a MW of 71.9 kDa and an isoelectric point (pI) of 5.0 (Figure 5.1). To obtain more information about *Es*HSP70 protein structure homology-modelling was performed using the SWISS-MODEL server. The best model (77.64% sequence identity) was built using bovine Heat shock cognate 71 kDA as a template (SMTL ID: 4fl9.1). This suggested that *Es*HSP70 is a well-structured protein (Figure 5.2). Information coming from all these databases indicated that *Es*HSP70 is a soluble, monomeric protein.

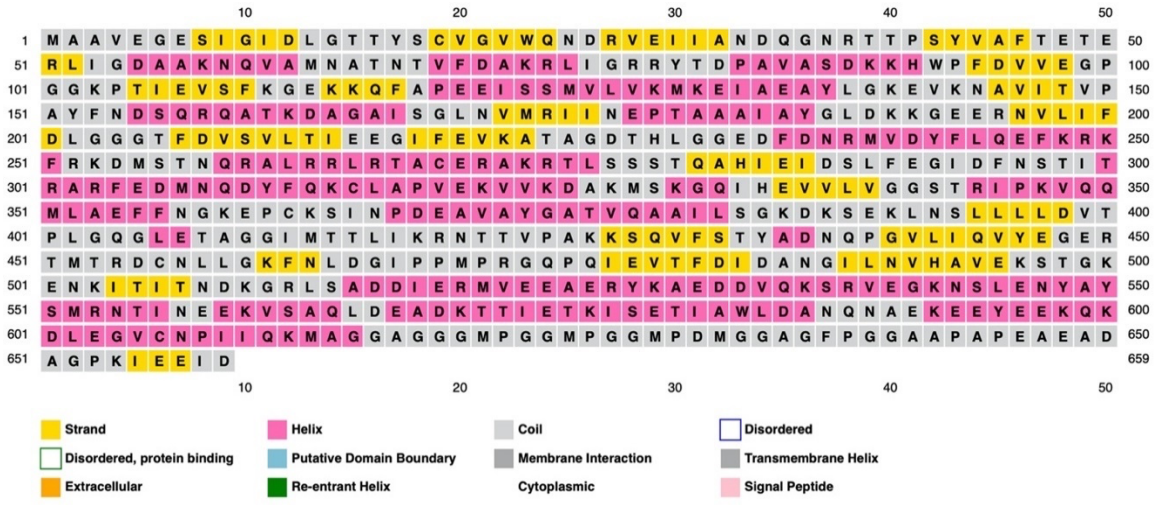
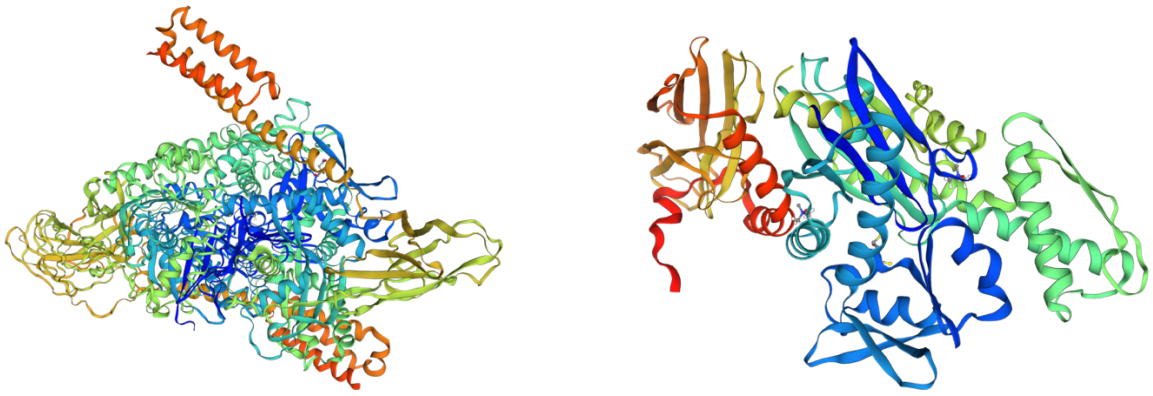


Figure 5.1. PsiPred sequence plot. PsiPred predictions of the secondary structure. Model was generated at: http://bioinf.cs.ucl.ac.uk/psipred/

(a) **(b)**



(c)

Figure 5.2. *Es*HSP70 tertiary structure modelling. **(a)** Tertiary structure of the full length protein generated with ProMod3 3.0.0 in SWISS-MODEL. **(b)** Template (SMTL ID: 4fl9.1). **(c)** Sequence alignment of *Es*HSP70 (Model_02) with bovine Heat shock cognate 71 kDA template.

5.4.2 Phylogenetic analysis indicated that *Es*HSP70 got considerably diverged from land plants

To test the phylogenetic relationships of *Es*HSP70 to other similar proteins from different species a phylogenetic tree was generated (Figure 5.3). The support for most branches was found to be strong. It indicated close relationships between *Ectocarpus* sp. sequences and other two brown alga (*Saccharina japonica* and *Undaria Pinnatifida*). Three *Ectocarpus* sp. sequences clustered together with *Es*HSP70. It is therefore likely that these *Ectocarpus* sp. sequences occurred through duplication and divergent evolution.

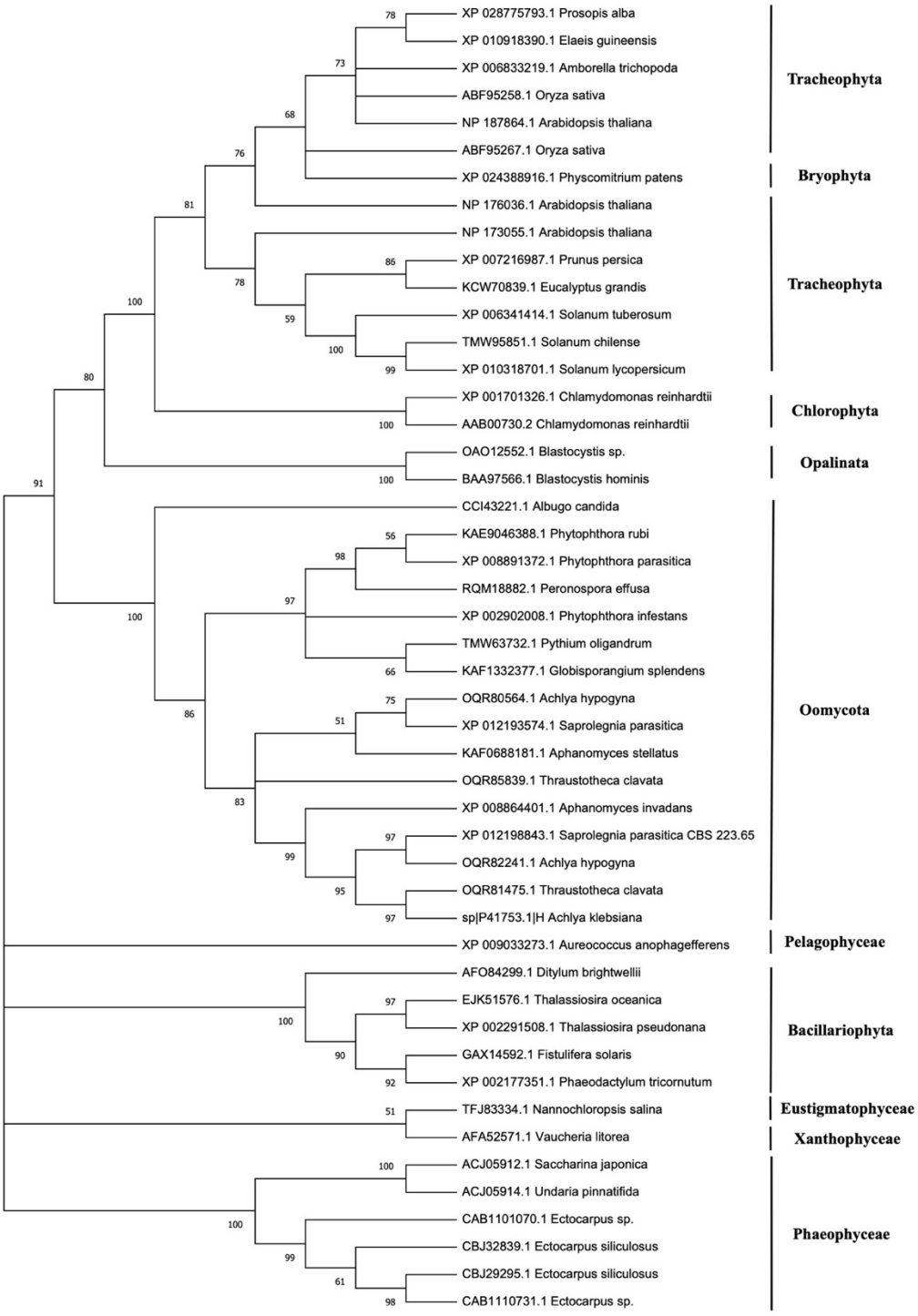


Figure 5.3. Phylogenetic analysis of HSP70 in different species. The numbers of node indicate the percentages of bootstrapping after 500 replications. Tree was generated using Maximum Likelihood method and JTT matrix-based model.

5.4.3 Gene expression analysis exhibited that *Es*HSP70 is abundantly expressed in *A. thaliana* under standard and salt stress conditions

To understand the role of this protein in abiotic stress tolerance it was expressed under 35S and *Responsive to Desiccation 29A* (*RD29A*) stress inducible promoter. Results of qPCR demonstrated that in standard conditions, the expression in the *Es*HSP-Ox1 and *Es*HSP-Ox2 lines was very high as compared to the *Es*HSP-A, *Es*HSP-B and *Es*HSP-C lines (Figure 5.4). In *Es*HSP-Ox lines, having 35S promoter the expression was very close to *actin*, the reference gene. Ct values for *actin* ranged between 21-22 cycle while those of *Es*HSP70 in *Es*HSP-Ox1 and *Es*HSP-Ox2 were 20 and 20.5, respectively. These results suggest the efficient transcription of *Es*HSP70 and higher mRNA stability in *A. thaliana*. When exposed to salinity stress the expression was highly up-regulated in *Es*HSP-A, *Es*HSP-B and *Es*HSP-C lines (57.8, 7.0 and 8.5 times, respectively), whereas in *Es*HSP-Ox1 and *Es*HSP-Ox2 the changes in transcripts level were less (8.0 and 5.8 times, respectively). Further up-regulation of this gene in 35S lines under salinity stress suggest that mRNA is likely more stable under salinity stress (Shi et al., 2003).

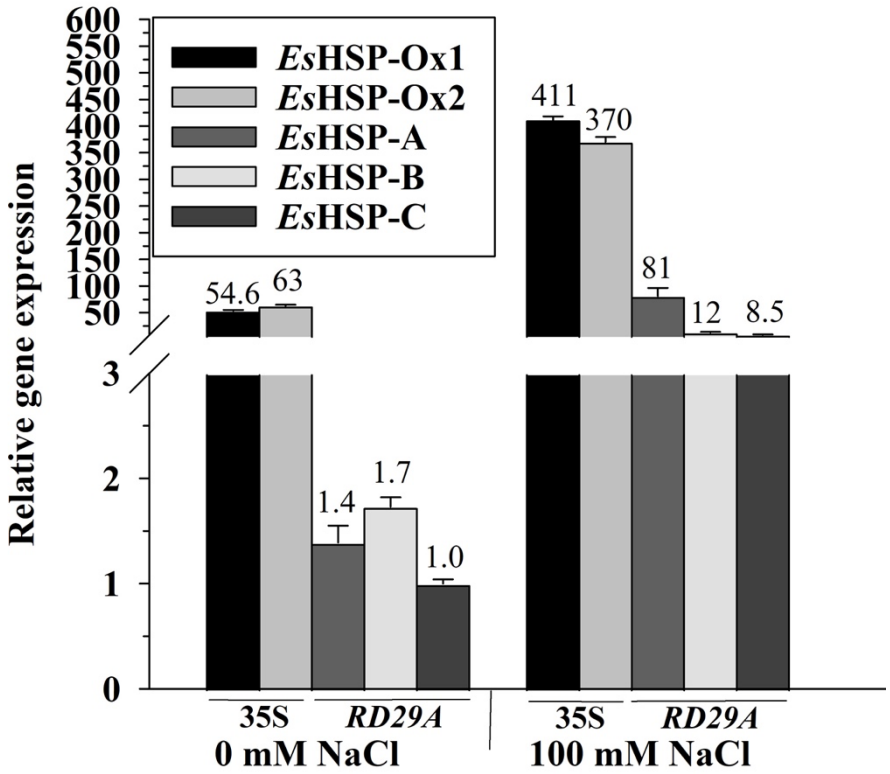


Figure 5.4. Expression of *EsHSP70* in two independent lines having the 35S promoter and in three independent lines having the stress inducible (*RD29A*) promoter under standard and salinity stress conditions. Values listed on the bars represent relative expression, fold change ratio *vs.* the line with the lowest *EsHSP70* expression, that is, line *Es*HSP-C in absence of NaCl.

5.4.4 Expression of *Es*HSP70 demonstrated improved tolerance to high salinity stress

To investigate the salt stress tolerance of overexpression lines 4 days old seedlings were subjected to a treatment of 100 mM NaCl. It was recorded that all the lines exhibited improved tolerance to salinity stress (Figure 5.5). After one week following the exposure to salinity stress it was noticed that seedlings of overexpression lines had significantly longer roots (12-13 and 9-10% increase under standard conditions and 35-36 and 30-40% increase under salinity stress for 35S and stress inducible overexpression lines, respectively), higher number of lateral roots per cm of primary root (16-23 and 7-8% decrease under standard conditions and 15-20 and 17-38% increase under salinity stress for 35S and stress inducible overexpression lines, respectively), reduced leaf chlorosis (50-60 and 50-73% for 35S and stress inducible overexpression lines, respectively), higher fresh weight (10-15 and 10-15% increase under standard conditions and 75-80 and 45-50% increase under salinity stress for 35S and stress inducible overexpression lines, respectively), and higher dry weight (14-15 and 10-15% increase under standard conditions and 70-80 and 40-42% increase under salinity stress for 35S and stress inducible overexpression lines, respectively) in contrast to the wild type plants (Figure 5.5). In order to understand if the similar phenotype can be observed in plants grown in soil, 15 days old plants were exposed to 200 mM NaCl and concentration of 100 mM NaCl was maintained throughout the experiment in peat pellets. After one week it was noticed that wild type plants displayed severe symptoms of growth inhibition in contrast to transgenic plants which continued to grow better (Figure 5.6). Analysis of biomass data revealed that transgenic plants accumulated significantly higher fresh weight (9-10 and 13-14% decrease under standard conditions and 50-60 and 40-50% increase under salinity stress for 35S and

stress inducible overexpression lines, respectively) and dry weight (3 and 5-6% increase under standard conditions and 60-65 and 45-60% increase under salinity stress for 35S and stress inducible overexpression lines, respectively) as compared to the wild type plants (Figure 5.6). When the effect of promoters was compared no significant differences were observed under standard conditions in seedlings and plants of overexpression lines (Figure 5.7).

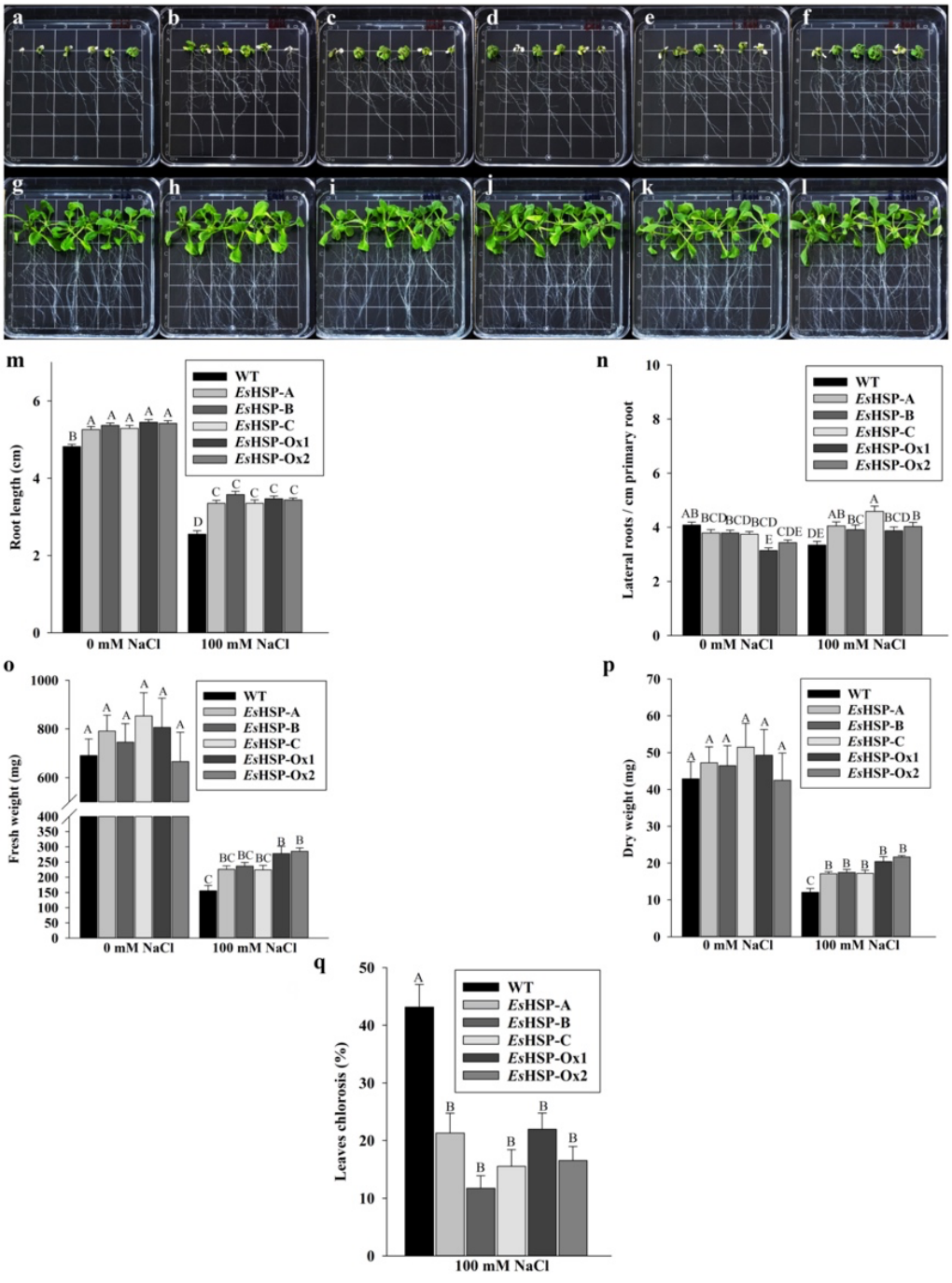


Figure 5.5. Growth of wild type and 2 independent 35S promoter (*Es*HSP-Ox1-2,) and of 3 independent stress inducible promoter (*Es*HSPA-C) transgenic *A. thaliana* seedlings, expressing *Es*HSP70 in presence and absence of 100 mM NaCl. In plates **(a)** to **(f)**, seedlings were grown under salt stress conditions while in; plates **(g)** to **(l)**, seedlings were grown in standard conditions. **(a, g)** WT, **(b, h)** *Es*HSP-A, **(c, i)** *Es*HSP-B, **(d, j)** *Es*HSP-C, **(e, k)** *Es*HSP-Ox1, **(f, l)** *Es*HSP-Ox2, **(m)** root length, **(n)** number of lateral roots per cm of primary root, **(o)** fresh weight, **(p)** dry weight and **(q)** leaf chlorosis. Values represents mean and standard error (n=90) for root length, lateral roots, leaf chlorosis and n=9 for fresh and dry weight. Means and SE followed by the same letter are not significantly different. The seedlings were photographed at 9 days after transfer in standard and under salinity stress conditions.

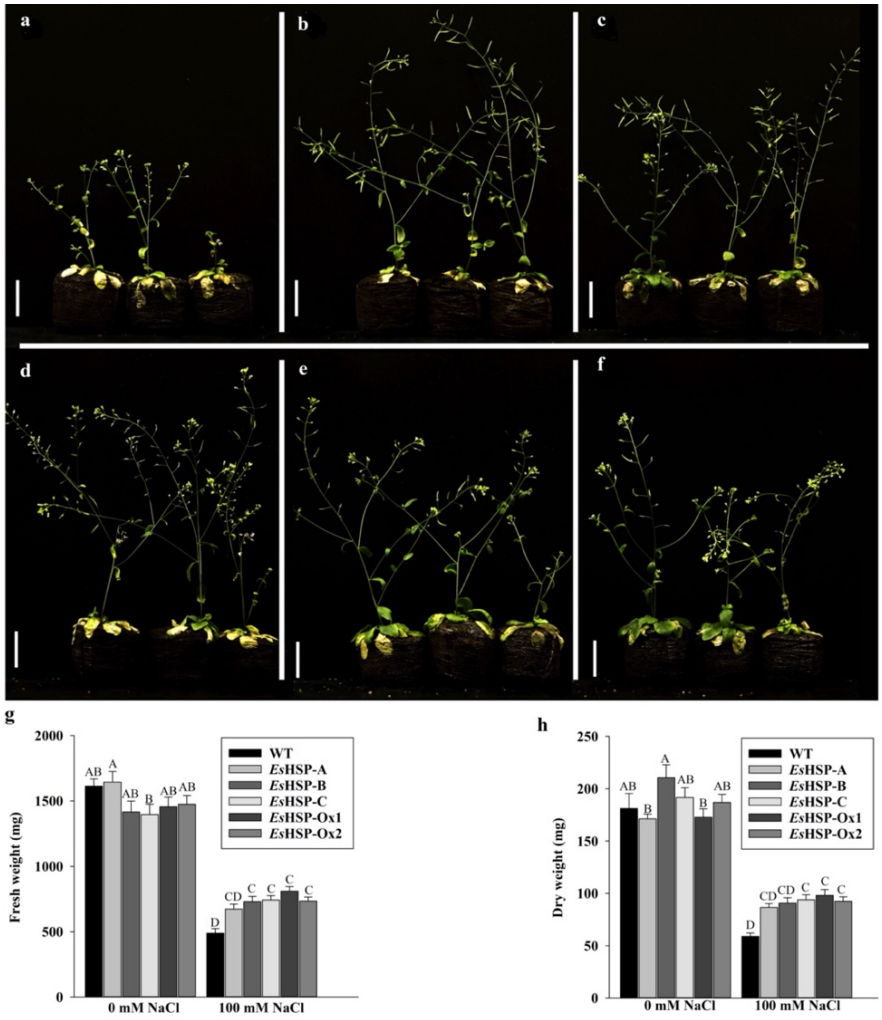


Figure 5.6. Growth of wild type and 2 independent 35S promoter (*Es*HSP-Ox1-2,) and of 3 independent stress inducible promoter (*Es*HSPA-C) transgenic *A. thaliana* plants, expressing *Es*HSP70, in presence of 100 mM NaCl concentration maintained throughout the experiment. **(a)** WT, **(b)** *Es*HSP-A, **(c)** *Es*HSP-B, **(d)** *Es*HSP-C, **(e)** *Es*HSP-Ox1, **(f)** *Es*HSP-Ox2, **(g)** fresh weight and **(h)** dry weight. The plants were photographed at 20 days after irrigation. Values represents mean and standard error (n=15). Means and SE followed by the same letter are not significantly different.

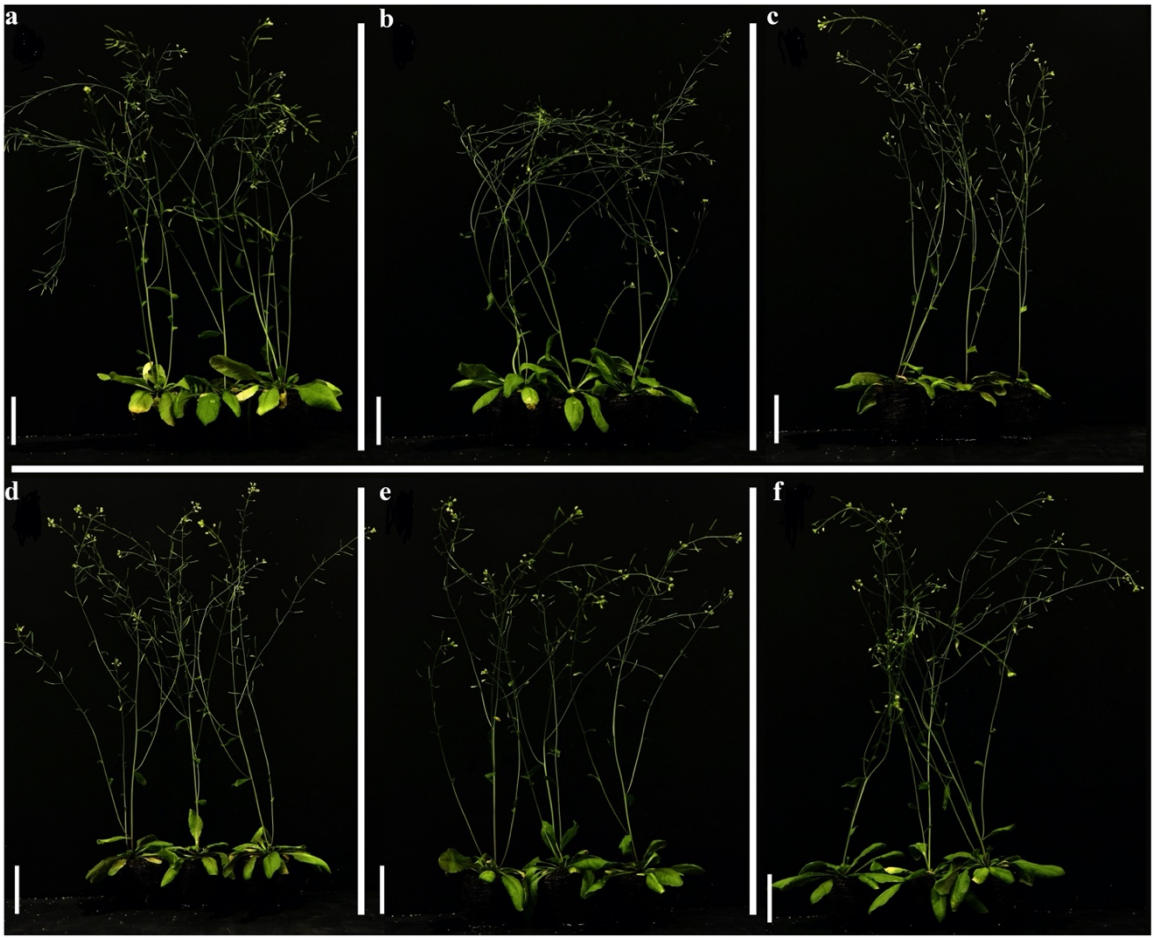


Figure 5.7. Growth of wild type and 2 independent 35S promoter (*Es*HSP-Ox1-2,) and of 3 independent stress inducible promoter (*Es*HSPA-C) transgenic *A. thaliana* plants, expressing *Es*HSP70, in standard conditions. **(a)** WT, **(b)** *Es*HSP-A, **(c)** *Es*HSP-B, **(d)** *Es*HSP-C, **(e)** *Es*HSP-Ox1, **(f)** *Es*HSP-Ox2.

5.4.5 Expression of *Es*HSP70 demonstrated reduced leakage of electrolytes

To understand the effect of salinity stress on membrane stability in overexpression lines 15 days old plants were exposed to 100 mM NaCl. Electrolyte leakage was estimated at 24 and 48 h after challenging with salinity stress. Analysis of electrolyte leakage data showed that both 35S and stress inducible promoter lines had significant reduction of leaked electrolytes at 48 h (50-53 and 52-60% in 35S and stress inducible overexpression lines, respectively) as compared to wild type plants suggesting that membranes were highly intact in overexpression lines (Figure 5.8).

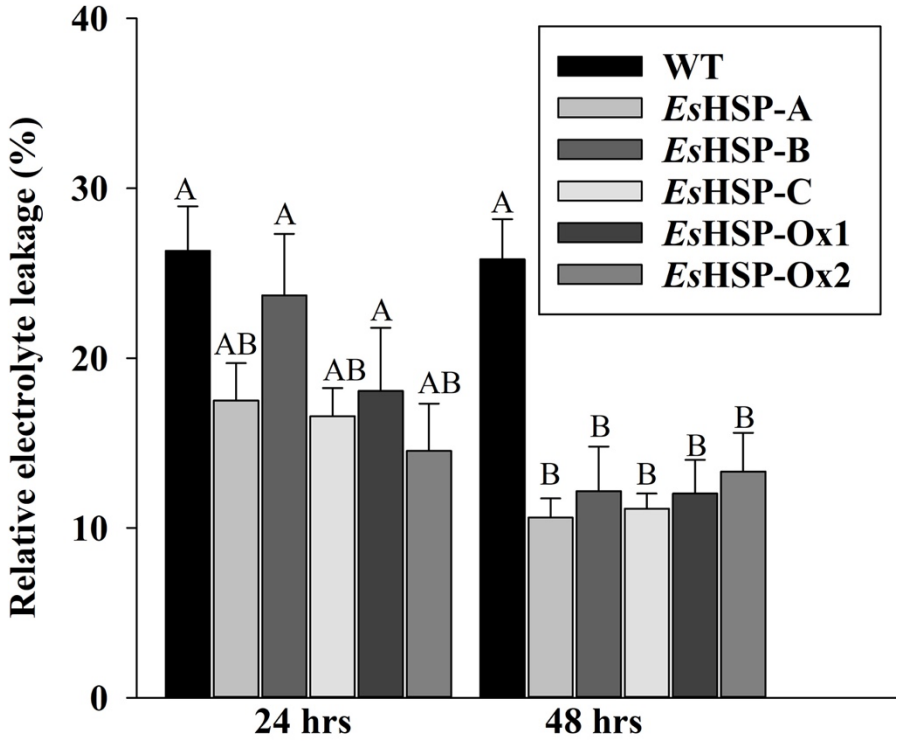


Figure 5.8. Electrolyte leakage of wild type and 2 independent 35S promoter (*Es*HSP-Ox1-2) and of 3 independent stress inducible promoter (*Es*HSPA-C) transgenic *A. thaliana* plants under 100 mM salinity stress conditions. Values represents mean and standard error (n=15). Means and SE followed by the same letter are not significantly different.

5.4.6 Expression of *Es*HSP70 demonstrated enhanced tolerance to high temperature stress

To test the tolerance of overexpression lines to higher temperature stress 9 days old seedlings were exposed to a continuous high temperature stress (40 °C) for 24 h. After one week of recovery under standard conditions it was noticed that transgenic seedlings recovered much faster and grow better as compared to the wild type seedlings (Figure 5.9). Moreover, analysis of biomass data showed that overexpression lines had significantly higher fresh weight (14-16 and 7-9% decrease under standard conditions and 45-50 and 30-40% increase under high temperature stress for 35S and stress inducible overexpression lines, respectively), and dry weight (7-10 and 8-10% decrease under standard conditions and 60-62 and 40-60% increase under high temperature stress for 35S and stress inducible overexpression lines, respectively) as compared to wild type seedlings (Figure 5.9).

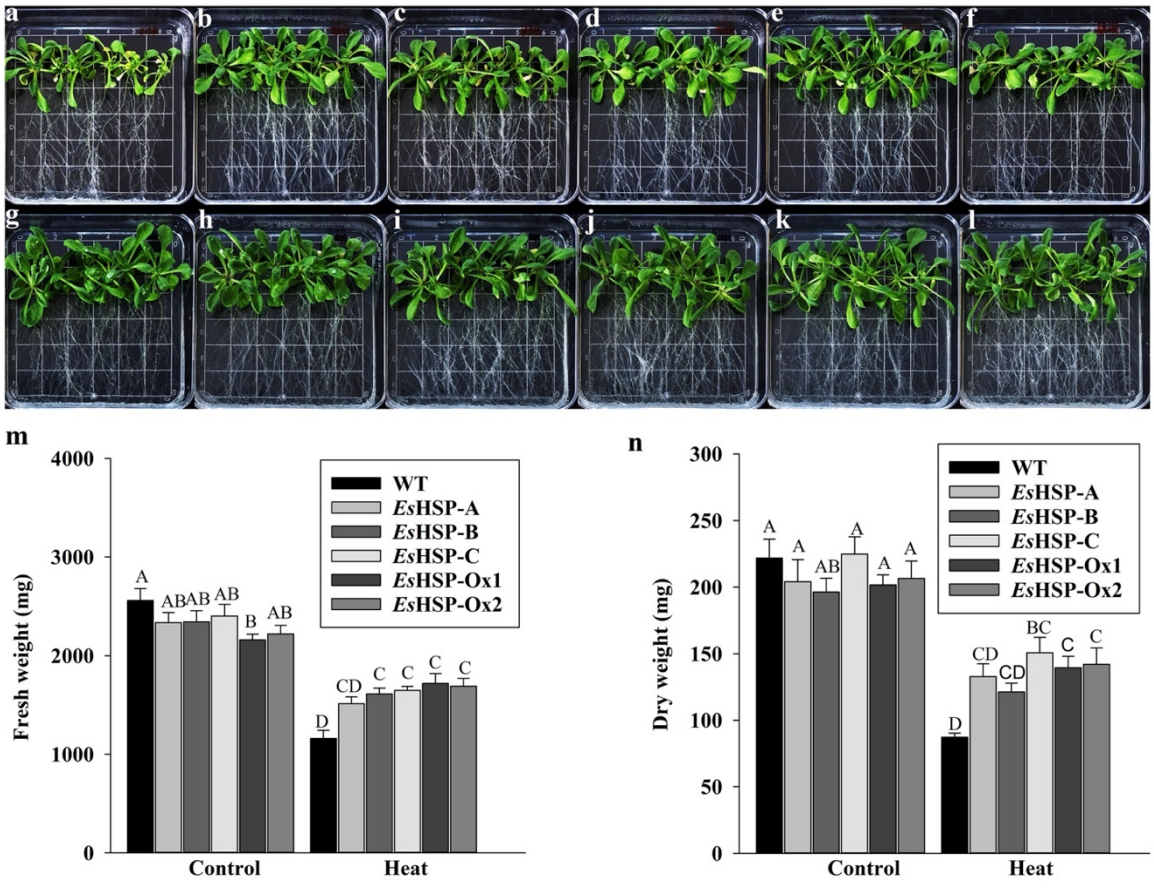


Figure 5.9. Growth of wild type and 2 independent 35S promoter (*Es*HSP-Ox1-2) and of 3 independent stress inducible promoter (*Es*HSPA-C) transgenic *A. thaliana* seedlings

under high temperature stress and standard conditions. Seedlings in plates **(a)** to **(f)**, were exposed to high temperature stress while seedlings in plates **(g)** to **(l)**, were grown in standard conditions. **(a, g)** WT, **(b, h)** *Es*HSP-A, **(c, i)** *Es*HSP-B, **(d, j)** *Es*HSP-C, **(e, k)** *Es*HSP-Ox1, **(f, l)** *Es*HSP-Ox2, **(m)** fresh weight and **(n)** dry weight. The 16 days old plants were photographed one week after being exposed to high temperature stress. Values represents mean and standard error (n=6). Means and SE followed by the same letter are not significantly different.

5.4.7 *A. thaliana* plants expressing *Es*HSP70 exhibited altered expression of stress responsive genes

Gene expression analysis was performed to determine whether *Es*HSP70 affect the expression of key stress responsive genes of *A. thaliana*. The expression of 11 genes (*DREB2A, RD29A, RD29B, RD26, RD22, RD20, RAB18, LEA14, HSP70, SOS1* and *NHX1*), that are known to play key role in salinity and temperature stress tolerance was analyzed in *Es*HSP-Ox1. The relative expression of most of these stress induced marker genes was rather similar in *Es*HSP-Ox1 and wild type plants in standard conditions, but significantly increased under salinity stress, at (24 h and 120 h), in the plants of the overexpression line (Figure 5.10). Under salinity stress, the expression of the transcription factor *DREB2A* (*Dehydration-Responsive Element-Binding protein 2A*) and *HSP70* gene was found to be >2 times up-regulated, at both time points, in *EsHSP*-Ox1 *vs.* wild type comparisons (Figure 5.10a and i, respectively). The expression of RD (Responsive to Desiccation) genes, *RD29A*, *RD29B*, *RD26 and RD20* was found to be up-regulated >2 fold at 24 h and reduced at 120 h (Figure 5.10b c, d, and f, respectively). However, the expression of *RD29A*, and *RD26* was significantly different from wild type at 120 h (Figure 5.10b and d, respectively). Expression of *RD22* was significantly different at 24 h but not at 120 h (Figure 5.10e). The expression of *RAB18* (coding for a protein from the dehydrin family), and *LEA14* (Late Embryogenesis Abundant) were also found to be significantly different in *Es*HSP-Ox1, at both time points, and this difference in expression was 1.9 and 3.0-fold for *RAB18* and 1.6 and 1.9-fold for *LEA14* (Figure 5.10g and h respectively). Expression of *SOS1*, an Na^+/H^+ antiporter, was determined to be significantly up-regulated

in *Es*HSP-Ox1 at both time points and this difference was 1.5 and 2.4-fold, respectively (Figure 5.10j), while the expression of *NHX1*, an Na^+/H^+ antiporter showed significant difference at 24 h and no significant difference was observed at 120 h (Figure 5.10k).

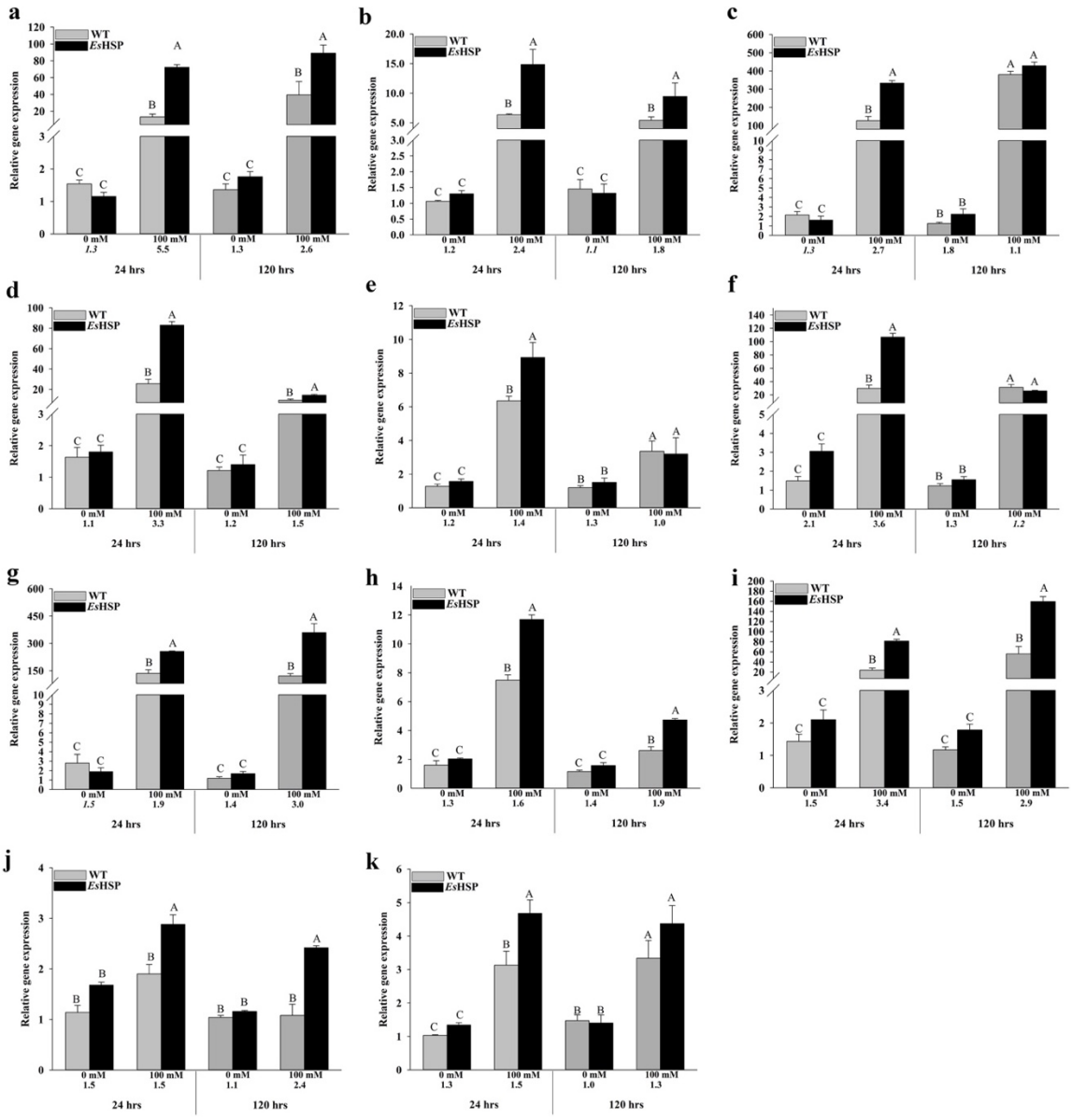


Figure 5.10. Gene expression analysis of stress inducible marker genes in the wild type and transgenic *A. thaliana* plants grown in absence and presence of 100 mM NaCl. Two time points (24 h and 120 h) were studied. *Actin* was used as the endogenous control and transcript levels were normalized to the individual with the lowest expression. Values under the bars represents fold difference. Default font values represent up-regulation while

italicised values represent down-regulation. Data represents mean ± SE from 3 biological replicates. **(a)** *DREB2A*, **(b)** *RD29A*, **(c)** *RD29B*, **(d)** *RD26*, **(e)** *RD22*, **(f)** *RD20*, **(g)** *RAB 18*, **(h)** *LEA14*, **(i)** *HSP70*, **(j)** *SOS1* and **(k)** *NHX1*. Means and SE followed by same letter are not significantly different.

5.5 Discussion

Role of heat shock proteins have been extensively studied in bacteria, plants and animals. Functions of HSP70 protein in response to abiotic stresses has been investigated in several crop plants including rice, soybean, pepper and in model plant *A. thaliana* (Lin et al., 2001; Jung et al., 2013; Sarkar et al., 2013; Zhang et al., 2015; Zhang et al., 2015). However, the physiological role of HSP70 in brown algae have not been documented earlier. Analysis of genome sequencing of brown algal model *Ectocarpus* sp. identified *HSP70* gene in this organism and similarity searches revealed that this protein is well conserved. Multiple sequence alignment indicated that it has no homologs in land plants. This gene was consistently up-regulated in *Ectocarpus* sp. when subjected to various abiotic stresses suggesting that it plays an important role to provide protection against abiotic stresses in this organism (Ritter et al., 2014). To understand the function of this gene we expressed it in *A. thaliana* under 35S and stress inducible promoter *RESPONSIVE TO DESSICATION 29A* (*RD29A*). This is the first report on the overexpression of *Ectocarpus sp. HSP70* improving high salinity and temperature stress tolerance in land plants.

Heat shock proteins also known as chaperons, plays critical role in maintaining the metabolic and structural integrity of cells by ensuring proper folding of misfolded or unfolded proteins and preventing protein aggregation under abiotic stress conditions (Vierling, 1991; Sung and Guy, 2003; Wang et al., 2004). HSPs are shown to be induced by salinity stress in rice (Ngara and Ndimba, 2014), wheat (Sobhanian et al., 2011) and Poplar (Manaa et al., 2011). In past transgenic plants have been generated to improve abiotic stress tolerance by expressing *HSP70* gene from microbes and other land plants. *HSP70* from these two choices of organism had higher sequence similarity to the *HSP70* of other plants. For instance, overexpression of *HSP70* from *Erianthus arundinaceus* has been shown to improve salinity and drought stress tolerance of sugarcane (Augustine et al.,

2015). This increase in tolerance was due to higher membrane stability and up-regulation of abiotic stress responsive genes including *DREB2A, LEA, RD29* and *ERD*. Montero-Barrientos et al. (2010) showed that overexpression of *HSP70* gene (*T34*) from *Trichoderma harzianum* improved salinity and heat stress tolerance in *A*. *thaliana*. Improved tolerance to salinity and heat stress was due to up-regulation of stress responsive genes including Na^+/H^+ exchanger *SOS1* and *APX1* in transgenic plants. HSP70 (T34) had 94.6% amino acid sequence identity with HSP70-2 protein of *Nicotiana tabacum*. Overexpression of *NtHSP70-1* has been shown to improve tolerance to drought stress (Cho and Hong, 2006). Similarly, overexpression of *HSP70* from *Medicago sativa* has been shown to improve drought stress tolerance in *A. thaliana* (Li et al., 2017). Its multiple sequence alignment showed that it had higher sequence similarity with other land plants including wheat, rice, corn and peas. Overexpression of Chrysanthemum *HSP70* has been shown to enhance abiotic stress tolerance in *A. thaliana* (Song et al., 2014). Taken together, all these studies suggest that indeed *HSP70* is an important gene to improve abiotic stress tolerance. However, *HSP70* gene used in these studies had high sequence similarity to the *HSP70* of the plants. In current study we isolated the *HSP70* gene from brown algal model *Ectocarpus*. sp. Phylogenetic analysis indicated that this gene got considerably diverged from *HSP70* in land plants as the close relatives were only two brown algae and other algae from Xanthophyceae family. This gene has no similarity to *HSP70* of the plants suggesting that this is a novel *HSP70*.

Plants face alterations in their growing environment and responds to these variations by adjusting their metabolism and physiology. The most crucial changes include osmotic adjustment by production and accumulation of compatible solutes to maintain turgor pressure, exclusion of Na^+ ions, modulation of root architecture, shoot growth, organization of leaves, leaf senescence, flowering time and biomass accumulation (Munns, 2002; Sun et al., 2008; Park et al., 2013). Phytohormone ABA plays crucial role in plant tolerance to abiotic stresses (Finkelstein et al., 2002). Many stress responsive genes require ABA for their activation and some not; this suggests that both ABA dependent and independent pathways exist in plants (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). Stress responsive genes in plant contain two major classes of cis acting elements referred as ABRE and DRE in the promoter regions. ABRE functions in ABA dependent

signaling pathway and DRE functions in ABA independent pathway (Kim et al., 2011). In current study we analyzed the expression of several well characterized key marker genes associated to abiotic stress tolerance in *A. thaliana* (i.e., *DREB2A, RD29A, RD29B, RD26, RD22, RD20, RAB18, LEA14, HSP70, SOS1* and *NHX1*).

A DRE binding transcription factor *DREB2A* contains ethylene responsive factor elements in promoter region (ERF/APETALA2). It functions as transcriptional activator of downstream stress responsive genes by binding to DRE sequences present in promoter regions (Liu et al., 1998; Nakashima et al., 2000; Sakuma et al., 2002). The expression of *DREB2A* was significantly up-regulated in overexpression line in comparisons to the wild type plants and in general the difference in expression was > 2 fold at both time points (Figure 5.10a). Sakuma et al. (2006) demonstrated that overexpression of constitutively active form of *DREB2A* activated the expression of several downstream genes including *RD29A, RD29B, RD17* and *LEA14*. The expression of these genes i.e., *RD29A, RD29B* and *LEA14* significantly up-regulated in overexpression line in comparison to the wild type plants (Figure 5.10b, c, and h, respectively). Promoter region of these genes contain DRE suggesting that expression of these genes was regulated by *DREB2A* (Sakuma et al., 2006).

RD26 encodes NAC transcription factor functions in ABA dependent pathway as expression of this gene was up-regulated by salinity, drought and ABA (Fujita et al., 2004). *RD20* encodes a Ca^{2+} binding protein functions in ABA dependent signaling pathway. It has been shown as direct target of *RD26* as the expression of this gene was up-regulated in transgenic plants overexpressing *RD26* (Fujita et al., 2004). In this study the expression of *RD26* and its target gene *RD20* was significantly up-regulated in overexpression line at both time points for *RD26* and at 24 h for *RD20* (Figure 5.10d and f, respectively).

Salinity and temperature stress causes disorganization of membrane and therefore results more electrolyte leakage (Tuteja, 2007). Electrolyte leakage is widely used to understand and measure the plant tolerance to abiotic stresses. It represents an important parameter to understand the intactness of cellular membranes in response to abiotic stresses. In this study we observed significant reduction in leakage of electrolytes suggesting higher membrane stability in transgenic lines. Several LEA proteins accumulated in response to abiotic stresses in plants. These proteins protect

macromolecules such as nucleic acid and enzymes by balancing redox and stabilization of cellular membranes (Baker et al., 1988; Ingram and Bartels, 1996; Veeranagamallaiah et al., 2011; Jia et al., 2014). Jia et al. (2014) demonstrated that overexpression of *LEA14* lead to improved tolerance to salinity stress. The expression of this gene significantly up-regulated in transgenic line in contrast to WT (Figure 5.10h).

Overexpression of *HSP70* improved plant tolerance to high salinity, drought and temperature stress in *A. thaliana* (Wang et al., 2004). *HSP70* has been shown to improve plant tolerance to heat stress. For instance, when seeds of *A. thaliana* knockout mutants lacking cPHSC70-1 and 2 were exposed to high temperature during germination plants exhibited defective phenotypes (Su and Li, 2008). Moreover, earlier studies reported that HSP70-15 knockout mutants of *A. thaliana* showed higher susceptibility to high temperature stress whereas overexpression were tolerance to heat (Jungkunz et al., 2011). The expression of *HSP70* was significantly up-regulated in transgenic line in contrast to WT and difference was > 2 fold at both time points (Figure 5.10i).

To improve plant salt tolerance, it is important to prevent the entry of Na^+ into plants to avoid its accumulation in the cytoplasm or organelles, except in the vacuoles. If plant fails to restrict movement of Na^+ from outside environment then it stores these toxic ion into vacuoles by increased activity of transporters (Shabala, 2013), and utilizes K^+ and other compatible solutes to balance the osmotic differences in cytoplasm thus reduces the concentration in cytoplasm to prevent damage to cellular machinery (Shabala, 2013). In *A. thaliana SOS1* encodes Na^+/H^+ antiporter localized on plasma membrane (Shi et al., 2000; Qiu et al., 2002; Quintero et al., 2002; Shi et al., 2002). Based on its expression in different tissues it was suggested that it plays diverse role in Na^+ efflux from roots, reducing concentration of Na^+ ions in cytoplasm (Zhu, 2002). Overexpression of *SOS1* increased tolerance to salt stress (Shi et al., 2003). In *A. thaliana NHX1* encodes Na^+/H^+ antiporter localized on tonoplast membrane of vacuoles. It functions in sequestration of Na^+ ions into vacuoles (Blumwald, 2000). Overexpression of *NHX1* increased plant tolerance to salinity stress in *A. thaliana* (Gaxiola et al., 2001) and in rice (Ohta et al., 2002). The expression of both these genes (i.e., *SOS1* and *NHX1*) was significantly up-regulated at both time point for *SOS1* and at 24 h in case of *NHX1* (Figure 5.10j and k, respectively). Prediction from

DeepLoc-1.0 (https://services.healthtech.dtu.dk/) showed that this protein is localised in cytoplasm. It is highly possible that this protein interacts with other cytosolic proteins including *RD29A, B, HSP70* and *LEA 14*. Together, these results suggest that overexpression of *Es*HSP70 improves salinity stress tolerance by transcriptional activation of *A. thaliana* stress responsive genes.

5.6 Conclusions

The expression, for the first time, of a heat shock protein 70 from brown alga *Ectocarpus* sp. in *A. thaliana* resulted in enhanced tolerance to high salinity and temperature stress. Gene expression analysis showed that the expression of several key stress markers genes involved in several activities including ABA-induced abiotic stress tolerance, K^+ homeostasis, Na^+ sequestration and chaperon activities were significantly up-regulated in the transgenic plants. These results suggest that in addition to land plants and microbe brown algae could be a choice of organism to isolate transgenes to produce transgenic land plants with improved tolerance several abiotic stresses.

CHAPTER 6

Discussion

Agricultural production is negatively impacted by various abiotic stresses such as high salinity, drought, temperature extremes, ultraviolet radiation and heavy metals. It has been predicted that agricultural production will face severe challenges in coming years as global climate change is happening at faster rate than previously predicted and it exacerbates the negative effects of abiotic stresses. Global population is growing at steady pace and is expected to reach ~10 billion by 2050. To feed such a large population, agricultural productivity must double the current production, in spite of the fact that in the last period there is an overall a reduction in the quality and quantity of cultivable land area (Melorose et al., 2015; Mickelbart et al., 2015). Therefore, it is a matter of serious urgency to find solutions to negate the negative effects of environmental stresses on crop production. To acclimatize, and to continue their growth under high salinity stress, plants have evolved various physiological, biochemical and molecular mechanisms. These includes production and accumulation of compatible solutes for osmotic adjustment, prevention of Na^+ ions influx, induction of ROS detoxifying enzymes, activation of stress signaling cascade to transduce the information for activation of stress responsive transcription factors and genes, modulation of root architecture, shoot growth, organization of leaves, leaf senescence, flowering time and biomass accumulation (Munns, 2002; Sun et al., 2008; Park et al., 2013). In the last decades, tireless efforts led to tremendous progress in developing climate resilient crops. This has been largely achieved by traditional breeding but also through transgenic approaches. However, the breeding approach has been very slow to develop traits for improved tolerance to environmental stresses and, in many situations, with little success because of the much longer time required to select plants with desired traits; furthermore, results can be unpredictable due to multigenic complexity of abiotic stress tolerance (Wang et al., 2016). Breakthroughs in genome sequencing technologies, bioinformatics, standardization of cloning protocols and *Agrobacterium* transformation made transgenic approach very popular and successful. The novel strategies to develop transgenic plants for abiotic stress tolerance are mostly dependent on the isolation and overexpression of genes that are involved in signaling and regulatory pathways or of the

genes that encode proteins implicated in stress tolerance or of enzymes involved in synthesis of functional metabolites (Flowers, 2004; Wang et al., 2004). Analysis of genome sequences made it easier to identify gene networks involved in abiotic stress responses and expressing them in crop plants to produce plants with improved tolerance to these stressors. However, choices or organisms considered as source of such genes have been limited. In the past, genes have been identified and expressed largely from bacteria and plant species. Brown algae are well known for their abiotic stress tolerance as these organisms grow and survive in intertidal zones which is an environment with higher levels of abiotic stresses resulting from tidal cycles (Dittami et al., 2009). The extracts of brown algae are well known for improving abiotic stress tolerance in crops. Several studies reported the bio-stimulatory effects of their extracts in plant health and reported improved tolerance to environmental stresses (Khan et al., 2009; Jithesh et al., 2012; Fan et al., 2014; Battacharyya et al., 2015; Kandasamy et al., 2015; Kulshreshtha et al., 2016). In this study we selected brown algal model *Ectocarpus* sp. and isolated genes involved in stress response and expressed them in the model plant *A. thaliana*. Expression of some of these genes improved salinity and high temperature stress tolerance in *A. thaliana* suggesting that this organism could be a valuable resource to isolate genes and express them in land plants to develop climate resilient crops. In the last three decades, several transgenic plants have been developed for abiotic stress tolerance and a number of studies have identified and characterized important plant metabolites such as proline, glycine betaine (GB) and mannitol that provide protection to cellular machinery from the detrimental effects exerted by osmotic shock and osmotic stress created by physiological dehydration (Konstantinova et al., 2002; Capell and Christou, 2004).

Several transgenic crops including rice, maize, tomato, eggplant, peanut and tobacco were generated by introducing the *mtlD* gene from *E. coli*. These plants accumulated mannitol in µM ranges and exhibited improved tolerance to salinity stress. It is known that mannitol in µM ranges cannot act as an osmolyte, therefore there should be some other mechanism(s) by which mannitol improved salinity stress tolerance. (Tarczynski et al., 1992, 1993; Thomas et al., 1995; Abebe et al., 2003; Zhifang and Loescher, 2003; Patel and Williamson, 2016). Chan et al. (2011) developed transgenic *A. thaliana* by introducing *M6PR* gene from Celery. Transgenic plants accumulated mannitol in µM range and

exhibited improved tolerance to salinity stress. They carried out microarray analysis at 6 days after exposure to salinity stress and showed that improved tolerance was associated to global changes in gene expression. In this study, the genes *EsM1PHD1* and *EsM1Pase2* (Groisillier et al., 2013; Bonin et al., 2015) from the brown algal model *Ectocarpus* sp., were used to produce mannitol in *A. thaliana*. The amount of mannitol was lower than that reported in previous studies (Tarczynski et al., 1992, 1993; Thomas et al., 1995; Abebe et al., 2003; Zhifang and Loescher, 2003; Chan et al., 2011; Patel and Williamson, 2016). However, the three independent transgenic lines that were characterized exhibited improved tolerance to salinity stress. We performed RNASeq at early time points (24 h and 48 h) as the early response is crucial for plant survival to stress. Transcriptomics analysis revealed that expression of genes involved in hormonal regulation, defense mechanisms, plant development, photosynthesis, and secondary metabolism, K^+ homeostasis, ROS signaling and radical scavengers and ABA signaling was altered, suggesting that improved tolerance was due to changes in expression of genes involved in several functional categories, not only in stress responses.

The phytohormone ABA plays an important role in abiotic stress tolerance. Several abiotic stress responsive genes require ABA for their activation and some not; this indicates the existence of both ABA-dependent and ABA-independent stress signal transduction pathways (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). In the past, significant efforts have been put to isolate and characterize new genes and tremendous progress have been made to understand and decipher the molecular mechanism(s) of abiotic stress tolerance. Several functional and regulatory genes have been identified and characterized in responses to abiotic stresses (Shinozaki et al., 2003). *A. thaliana RD26* gene encodes a NAC transcription factor and directly targets *RD20*; these genes functions in ABA dependent stress signaling pathway (Takahashi et al., 2000; Fujita et al., 2004). *A. thaliana DREB2A* gene encodes a transcription factor which regulates the expression of genes induced by salinity and drought stress (Kim et al., 2011). *DREB2A* contains an ERF/AP2 (ethylene responsive element binding factor/APETALA2) DNA binding domain. This domain regulates the expression of downstream genes by interacting with cis-element DRE in their promoter region (Nakashima et al., 2000; Sakuma et al., 2002). Sakuma et al. (2006) demonstrated that overexpression of constitutively active form of *DREB2A*

activated the expression of several downstream genes including *RD29A, RD29B, RD17* and *LEA14*. Another important mechanism in plant salinity stress tolerance is reduced accumulation of Na^+ in the cytoplasm or organelles other than vacuoles. If plant fails to restrict the movement of Na^+ from salt contaminated soil, then it stores Na^+ into vacuoles by increased the activity of specific transporters (Shabala, 2013). The next step is to utilize K^+ and other compatible solutes to balance the osmotic differences in the cytoplasm thus reducing the concentration of cations in the cytoplasm to prevent damage of cellular machinery (Shabala, 2013). In *A. thaliana SOS1* encodes a Na^+/H^+ antiporter localized on plasma membrane (Shi et al., 2000; Qiu et al., 2002; Quintero et al., 2002; Shi et al., 2002). Overexpression of *SOS1* increased tolerance to salt stress (Shi et al., 2003). In *A. thaliana*, *NHX1* encodes a Na^+/H^+ antiporter localized on tonoplast membrane of vacuoles. It functions in sequestration of Na^+ ions into vacuoles (Blumwald, 2000). Overexpression of *NHX1* increased plant tolerance to salinity stress in *A. thaliana* (Gaxiola et al., 2001) and in rice (Ohta et al., 2002). The expression of transcription factors including *DREB2A, RD26* and their downstream genes such as, *RD29A, RD29B, RAB 18, RD22, RD20, LEA* class protein, *LEA 14* was found to be up-regulated in transgenic plants in contrast to wild type plants. Moreover, the expression of heat shock protein HSP70 and Na^+ transporters such as *SOS1* and *NHX1* was significantly higher in transgenic lines as compared to the wild type plants. These results suggested that increased tolerance to salinity and high temperature stress of transgenic plants expressing brown algal genes was associated to drastic changes that occurred in the expression of several genes involved in abiotic stress tolerance.

6.1 Future Directions

If the yield reductions caused by abiotic stresses can be minimized, agricultural productivity will thrive. To achieve this, substantially increased and targeted research is required to further identify and implement the most effective ways to confer better protection to plants exposed to abiotic stress conditions. To accelerate the identification and characterization of the most effective mechanisms for stress tolerance, a combination of approaches, including genome wide associations, analyses of gene expression, new gene

discovery, mutation detection and the associated 'omics'-based databases are required. In studies of abiotic stress tolerance, the response of plants to initial osmotic shock should be the priority because initial response can prime the plant to avoid further severe negative effects on growth and development. Tolerance to several abiotic stresses is dependent on complex mechanisms and therefore, the identification and in-depth understanding of those mechanisms, which are involved in stress sensing, and root to shoot communication, will improve the current knowledge and have the great potential to enhance abiotic stress tolerance.

To develop salt tolerant crops, one of the most attractive approaches should be to manipulate the perception, detection, and signaling regulatory networks associated with overall plant salt tolerance. Potentially, there can be significant effects on the downstream processes, including osmotic and tissue tolerance, as well as ion exclusion, if one of the components of the aforementioned regulatory networks can be manipulated. In recent studies it was reported that ROS signaling plays a critical role in regulating the plant responses to abiotic stress tolerance (Mittler et al., 2011; Suzuki et al., 2012). ROS was also found to be involved in controlling the Na^+ accumulation in the shoots by regulating the Na^+ concentration in the vasculature (Jiang et al., 2012). It is important to understand and recognize that when manipulating protein functions, altering gene expression is only one of the possible methods. Additional approaches that can modify plant salt tolerance include post-translational modifications and allelic variations in the protein sequence. These can potentially activate or suppress the protein activity and change its localization within the cell (Mazzucotelli et al., 2008; Weinl and Kudla, 2009; Castro et al., 2012). The best example of such modifications is the SOS pathway, where the post-translational modification can make a plant respond to variable environments more quickly and reversibly (Halfter et al., 2000; Qiu et al., 2002; Luan, 2009; Weinl and Kudla, 2009; Kudla et al., 2010; Huertas et al., 2012; Ji et al., 2013). One of the important objectives of improving plant salt tolerance should be to determine the key ion transporters that mediate Na^+ influx into the cells. By achieving this goal, these ion channels can be blocked, and this will have great potential to restrict Na^+ transport in the cells to improve plant salt tolerance (Zhu, 2016). However, another osmolyte, which can prevent the efflux of water from cells is still needed, because higher Na^+ concentration outside the cells will cause

water to move out of the cells, following the osmotic gradient, which will determine cell plasmolysis.

Regardless of unknowns, engineering mannitol biosynthesis in plants otherwise not able to produce this polyol, using algal genes, is a possible alternate approach because only one or two genes could allow to mitigate abiotic stress tolerance in land plants, in particular for salinity stress. Current development of advanced scientific tools and the availability of genomic sequences for brown algae provide a platform for identifying relevant genes to be expressed in the well-known plant model *A. thaliana*. Since adverse environmental factors are becoming more frequent as a result of global climate change, novel strategies to study abiotic stress tolerance like the one used in this study are of paramount importance.

6.2 Contribution to Science

The research described in this thesis addresses abiotic stress tolerance in plants using brown algal genes and also contributes to the understanding of brown algal biology at the molecular level. Therefore, it provides an alternative organism to consider for the isolation and expression of genes to develop transgenic plants. This research led to the characterization of two mannitol-related genes from *Ectocarpus* sp., of a novel unknown function gene (*Esi0017_0056*), and of a *Ectocarpus* sp. heat shock protein 70, providing insights on the mechanisms related to plant and brown algal abiotic stress tolerance. This study is the first one to report the successful expression and characterization of brown algal genes in land plants.

6.3 This study generated the following manuscripts

6.3.1 Low Mannitol Concentrations in *Arabidopsis thaliana* Expressing *Ectocarpus* Genes Improve Salt Tolerance

Pramod Rathor, Tudor Borza, Yanhui Liu, Yuan Qin, Sophia Stone, Junzeng Zhang, Joseph P. M. Hui, Fabrice Berrue, Agnès Groisillier, Thierry Tonon, Svetlana Yurgel, Phillipe Potin, Balakrishnan Prithiviraj*

This manuscript is published in Plants 9, 1508 (2020).

6.3.2 A Novel Protein from *Ectocarpus* sp. Improves Salinity and High Temperature Stress Tolerance in *Arabidopsis thaliana*

Pramod Rathor, Tudor Borza, Sophia Stone, Thierry Tonon, Svetlana Yurgel, Phillipe Potin, Balakrishnan Prithiviraj*

This manuscript is published in International Journal of Molecular Sciences 22(4), 1971 (2021).

6.3.3 Expression of a Heat Shock Protein 70 from the Brown Alga *Ectocarpus* sp. Imparts Salinity and Temperature Stress Tolerance in *Arabidopsis thaliana*

Pramod Rathor, Tudor Borza, Sophia Stone, Thierry Tonon, Svetlana Yurgel, Phillipe Potin, Balakrishnan Prithiviraj*

This manuscript is in preparation for submission.

References

Abebe T, Guenzi AC, Martin B, Cushman JC (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiol. **131:** 1748-1755

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science **311:** 91-94

Agarwal PK, Agarwal P, Reddy M, Sopory SK (2006) Role of *DREB* transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep. **25:** 1263-1274

Akhter S, Uddin MN, Jeong IS, Kim DW, Liu XM, Bahk JD (2016) Role of Arabidopsis *At PI 4Kγ3*, a type II phosphoinositide 4-kinase, in abiotic stress responses and floral transition. Plant Biotechnol. J. **14:** 215-230

Amtmann A, Sanders D (1998) Mechanisms of Na^+ uptake by plant cells. *In* Adv. Bot. Res., Vol 29. Elsevier, pp 75-112

Andrés F, Coupland G (2012) The genetic basis of flowering responses to seasonal cues. Nat. Rev. Genet. **13:** 627

Antoni R, Gonzalez-Guzman M, Rodriguez L, Rodrigues A, Pizzio GA, Rodriguez PL (2012) Selective inhibition of clade A phosphatases type 2C by PYR/PYL/RCAR abscisic acid receptors. Plant Physiol. **158:** 970-980

Apse MP, Sottosanto JB, Blumwald E (2003) Vacuolar cation/H^+ exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of *AtNHX1*, the Arabidopsis vacuolar Na^+/H^+ antiporter. Plant J. **36:** 229-239

Apt KE, Collier JL, Grossman AR (1995) Evolution of the phycobiliproteins. J. Mol. Biol. **248:** 79-96

Archibald JM (2012) The evolution of algae by secondary and tertiary endosymbiosis. *In* Adv. Bot. Res., Vol 64. Elsevier, pp 87-118

Augustine SM, Narayan JA, Syamaladevi DP, Appunu C, Chakravarthi M, Ravichandran V, Subramonian N (2015) *Erianthus arundinaceus HSP70* (*EaHSP70*) overexpression increases drought and salinity tolerance in sugarcane (*Saccharum* spp. hybrid). Plant Sci. **232:** 23-34

Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, Wishart DS (2016) Heatmapper: web-enabled heat mapping for all. Nucleic Acid Res. **44:** W147-W153

Baker J, Van dennSteele C, Dure L (1988) Sequence and characterization of 6 Lea proteins and their genes from cotton. Plant Mol. Biol. **11:** 277-291

Baldauf S (2003) The deep roots of eukaryotes. Science **300:** 1703-1706

Bartsch I, Wiencke C, Bischof K, Buchholz CM, Buck BH, Eggert A, Feuerpfeil P, Hanelt D, Jacobsen S, Karez R (2008) The genus *Laminaria sensu lato*: recent insights and developments. Eur. J. Phycol. **43:** 1-86

Battacharyya D, Babgohari MZ, Rathor P, Prithiviraj B (2015) Seaweed extracts as biostimulants in horticulture. Sci. Hortic. **196:** 39-48

Berggård T, Linse S, James P (2007) Methods for the detection and analysis of protein–protein interactions. Proteomics **7:** 2833-2842

Bernal M, Estiarte M, Peñuelas J (2011) Drought advances spring growth phenology of the Mediterranean shrub *Erica multiflora*. Plant Biol. **13:** 252-257

Bernstein N, Shoresh M, Xu Y, Huang B (2010) Involvement of the plant antioxidative response in the differential growth sensitivity to salinity of leaves *vs* roots during cell development. Free Radical Biol. Med. **49:** 1161-1171

Bieleski R (1982) Sugar alcohols. *In* Plant Carbohydrates I. Springer, pp 158-192

Birkenbihl RP, Jach G, Saedler H, Huijser P (2005) Functional dissection of the plant-specific SBP-domain: overlap of the DNA-binding and nuclear localization domains. J. Mol. Biol. **352:** 585-596

Bita C, Gerats T (2013) Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front. Plant Sci. **4:** 273

Black WAP (1950) The seasonal variation in weight and chemical composition of the common British *Laminariaceae*. J. Mar. Biol. Assoc. UK **29:** 45-72

Bluemel M, Dally N, Jung C (2015) Flowering time regulation in crops—what did we learn from Arabidopsis? Curr. Opin. Biotechnol. **32:** 121-129

Blumwald E (2000) Sodium transport and salt tolerance in plants. Curr. Opin. Cell Biol. **12:** 431-434

Blumwald E, Poole RJ (1985) Na^+/H^+ antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. Plant Physiol. **78:** 163-167

Blunden G, Gordon SM (1986) Betaines and their sulphonio analogues in marine algae. Progress Phycol. Res. **4:** 39-80

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol. **14:** 89-97

Bonin P, Groisillier A, Raimbault A, Guibert A, Boyen C, Tonon T (2015) Molecular and biochemical characterization of mannitol-1-phosphate dehydrogenase from the model brown alga *Ectocarpus* sp. Phytochem. **117:** 509-520

Boudsocq M, Droillard M-J, Barbier-Brygoo H, Laurière C (2007) Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. Plant Mol. Biol. **63:** 491-503

Brodie J, Chan CX, De Clerck O, Cock JM, Coelho SM, Gachon C, Grossman AR, Mock T, Raven JA, Smith AG (2017) The algal revolution. Trends Plant Sci. **22:** 726-738

Brown A (1976) Microbial water stress. Bacteriol. Rev. **40:** 803

Brown A, Simpson JR (1972) Water relations of sugar-tolerant yeasts: the role of intracellular polyols. Microbiol. **72:** 589-591

Buchan DW, Jones DT (2019) The PSIPRED protein analysis workbench: 20 years on. Nucleic Acid Res. **47:** W402-W407

Buxton GV, Greenstock CL, Helman WP, Ross AB (1988) Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals (·OH/·O− in aqueous solution. J. Phys. Chem. Ref. Data **17:** 513-886

Cai C, Guo W, Zhang B (2018) Genome-wide identification and characterization of *SPL* transcription factor family and their evolution and expression profiling analysis in cotton. Sci. Rep. **8:** 1-13

Cao W-H, Liu J, He X-J, Mu R-L, Zhou H-L, Chen S-Y, Zhang J-S (2007) Modulation of ethylene responses affects plant salt-stress responses. Plant Physiol. **143:** 707-719

Capell T, Christou P (2004) Progress in plant metabolic engineering. Curr. Opin. Biotechnol. **15:** 148-154

Cardon G, Höhmann S, Klein J, Nettesheim K, Saedler H, Huijser P (1999) Molecular characterisation of the Arabidopsis *SBP*-box genes. Gene **237:** 91-104

Cardon GH, Höhmann S, Nettesheim K, Saedler H, Huijser P (1997) Functional analysis of the *Arabidopsis thaliana* SBP-box gene *SPL3*: a novel gene involved in the floral transition. Plant J. **12:** 367-377

Castro PH, Tavares RM, Bejarano ER, Azevedo H (2012) SUMO, a heavyweight player in plant abiotic stress responses. Cell. Mol. Life Sci. **69:** 3269-3283

Chan Z, Grumet R, Loescher W (2011) Global gene expression analysis of transgenic, mannitol-producing, and salt-tolerant *Arabidopsis thaliana* indicates widespread changes in abiotic and biotic stress-related genes. J. Exp. Bot. **62:** 4787-4803

Chang CC, Ślesak I, Jordá L, Sotnikov A, Melzer M, Miszalski Z, Mullineaux PM, Parker JE, Karpińska B, Karpiński S (2009) Arabidopsis chloroplastic *glutathione peroxidases* play a role in cross talk between photooxidative stress and immune responses. Plant Physiol. **150:** 670-683

Change IPOC (2014) IPCC. Climate Change

Chao L-M, Liu Y-Q, Chen D-Y, Xue X-Y, Mao Y-B, Chen X-Y (2017) Arabidopsis transcription factors *SPL1* and *SPL12* confer plant thermotolerance at reproductive stage. Mol. Plant **10:** 735-748

Charrier B, Coelho SM, Le Bail A, Tonon T, Michel G, Potin P, Kloareg B, Boyen C, Peters AF, Cock JM (2008) Development and physiology of the brown alga *Ectocarpus siliculosus*: two centuries of research. New Phytol. **177:** 319-332

Chen X (2009) Small RNAs and their roles in plant development. Annu. Rev. Cell Dev. **25:** 21-44

Chiang Y-J, Stushnoff C, McSay A, ML J, Bohnert H (2005) Overexpression of mannitol-1-phosphate dehydrogenase increases mannitol accumulation and adds protection against chilling injury in *Petunia*. J. Amer. Soc. Hort. Sci. **130:** 605–510

Cho EK, Choi YJ (2009) A nuclear-localized HSP70 confers thermoprotective activity and drought-stress tolerance on plants. Biotechnol. Lett. **31:** 597-606

Cho EK, Hong CB (2006) Over-expression of tobacco *NtHSP70-1* contributes to drought-stress tolerance in plants. Plant Cell Rep. **25:** 349-358

Chomczynski P, Sacchi N (2006) The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. Nat. Protoc. **1:** 581-585

Chung JS, Zhu JK, Bressan RA, Hasegawa PM, Shi H (2008) Reactive oxygen species mediate Na^+ induced *SOS1* mRNA stability in Arabidopsis. Plant J. **53:** 554-565

Clerico EM, Tilitsky JM, Meng W, Gierasch LM (2015) How HSP70 molecular machines interact with their substrates to mediate diverse physiological functions. J. Mol. Biol. **427:** 1575-1588

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735-743

Cock JM, Peters AF, Coelho SM (2011) Brown algae. Curr. Biol. **21:** R573-R575

Cock JM, Sterck L, Ahmed S, Allen AE, Amoutzias G, Anthouard V, Artiguenave F, Arun A, Aury J-M, Badger JH (2012) The *Ectocarpus* genome and brown algal genomics: The *Ectocarpus* genome consortium. *In* Adv. Bot. Res. , Vol 64. Elsevier, pp 141-184

Cock JM, Sterck L, Rouzé P, Scornet D, Allen AE, Amoutzias G, Anthouard V, Artiguenave F, Aury J-M, Badger JH (2010) The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae. Nature **465:** 617

Coelho SM, Scornet D, Rousvoal S, Peters NT, Dartevelle L, Peters AF, Cock JM (2012) *Ectocarpus*: A model organism for the brown algae. Cold Spring Harb. **2012:** pdb.emo065821

Craig PD, Møller IS (2010) Na^+ transport in glycophytic plants: what we know and would like to know. Plant Cell Environ. **33:** 612-626

Craigie J (1974) Storage products. *In* Algal Physiol. Biochem., Vol 10. Blackwell Oxford, pp 206-237

Cui N, Sun X, Sun M, Jia B, Duanmu H, Lv D, Duan X, Zhu Y (2015) Overexpression of *Os*miR156k leads to reduced tolerance to cold stress in rice (*Oryza Sativa*). Mol. Breed. **35:** 1-11

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol. **133:** 462-469

Czapski G (1984) On the use of $OH^{\cdot}$ scavengers in biological systems. Isr. J. Chem. **24:** 29-32

Dat J, Vandenabeele S, Vranová E, Van Montagu M, Inzé D, Van Breusegem F (2000) Dual action of the active oxygen species during plant stress responses. Cell. Mol. Life Sci. **57:** 779-795

Davies MJ (2005) The oxidative environment and protein damage. Biochim. Biophys. Acta Biomembr. Proteins Proteom. **1703:** 93-109

Davis TA, Volesky B, Mucci A (2003) A review of the biochemistry of heavy metal biosorption by brown algae. Water Res. **37:** 4311-4330

De Maio A (1999) Heat shock proteins: facts, thoughts, and dreams. Shock **11:** 1-12

Demidchik V, Cuin TA, Svistunenko D, Smith SJ, Miller AJ, Shabala S, Sokolik A, Yurin V (2010) Arabidopsis root K^+ efflux conductance activated by hydroxyl radicals: single-channel properties, genetic basis and involvement in stress-induced cell death. J. Cell Sci. **123:** 1468-1479

Dittami SM, Aas HT, Paulsen BS, Boyen C, Edvardsen B, Tonon T (2011) Mannitol in six autotrophic Stramenopiles and Micromonas. Plant Signal Behav. **6:** 1237-1239

Dittami SM, Scornet D, Petit J-L, Ségurens B, Da Silva C, Corre E, Dondrup M, Glatting K-H, König R, Sterck L (2009) Global expression analysis of the brown alga *Ectocarpus siliculosus* (Phaeophyceae) reveals large-scale reprogramming of the transcriptome in response to abiotic stress. Genome Biol. **10:** R66

Dixon DP, Cummins I, Cole DJ, Edwards R (1998) Glutathione-mediated detoxification systems in plants. Current Opin. Plant Biol. **1:** 258-266

Donaldson L, Ludidi N, Knight MR, Gehring C, Denby K (2004) Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels. FEBS Lett. **569:** 317-320

Draisma SG, Peters A, Fletcher R (2003) Evolution and taxonomy in the Phaeophyceae: effects of the molecular age on brown algal systematics. Out of the Past**:** 87-102

Drerup MM, Schlücking K, Hashimoto K, Manishankar P, Steinhorst L, Kuchitsu K, Kudla J (2013) The calcineurin B-like calcium sensors CBL1 and CBL9 together with their interacting protein kinase CIPK26 regulate the Arabidopsis NADPH oxidase RBOHF. Mol. Plant **6:** 559-569

Du W, Lin H, Chen S, Wu Y, Zhang J, Fuglsang AT, Palmgren MG, Wu W, Guo Y (2011) Phosphorylation of SOS3-like calcium-binding proteins by their interacting SOS2-like protein kinases is a common regulatory mechanism in Arabidopsis. Plant Physiol. **156:** 2235-2243

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J. **45:** 616-629

Echer F, Oosterhuis D, Loka D, Rosolem C (2014) High night temperatures during the floral bud stage increase the abscission of reproductive structures in cotton. J. Agron. Crop Sci. **200:** 191-198

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acid Res. **32:** 1792-1797

Fan D, Kandasamy S, Hodges DM, Critchley AT, Prithiviraj B (2014) Pre-harvest treatment of spinach with *Ascophyllum nodosum* extract improves post-harvest storage and quality. Sci. Hortic. **170:** 70-74

FAO (2008) Land and plant nutrition management service. ProSoil–Problem Soils Database, FAO (http://www.fao.org)

Fedoroff NV, Battisti DS, Beachy RN, Cooper PJ, Fischhoff DA, Hodges C, Knauf VC, Lobell D, Mazur BJ, Molden D (2010) Radically rethinking agriculture for the 21st century. Science **327:** 833-834

Field CB (2014) Climate change 2014–Impacts, adaptation and vulnerability: Regional aspects. Cambridge University Press

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell **14:** S15-S45

Flora LL, Madore MA (1993) Stachyose and mannitol transport in olive (*Olea europaea* L.). Planta **189:** 484-490

Flowers T (2004) Improving crop salt tolerance. J. Exp. Bot. **55:** 307-319

Flowers T, Troke P, Yeo A (1977) The mechanism of salt tolerance in halophytes. Annu. Rev. Plant Physiol. **28:** 89-121

Flowers TJ, Colmer TD (2008) Salinity tolerance in halophytes. New Phytol.**:** 945-963

Franks SJ (2011) Plasticity and evolution in drought avoidance and escape in the annual plant *Brassica rapa*. New Phytol. **190:** 249-257

Franks SJ, Sim S, Weis AE (2007) Rapid evolution of flowering time by an annual plant in response to a climate fluctuation. Proc. Natl. Acad. Sci. U.S.A. **104:** 1278-1282

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park S-Y, Cutler SR, Sheen J, Rodriguez PL, Zhu J-K (2009) *In vitro* reconstitution of an abscisic acid signalling pathway. Nature **462:** 660

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LSP, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J. **39:** 863-876

Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K (2005) *AREB1* is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. Plant Cell **17:** 3470-3488

Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants. J. Plant Res. **124:** 509-525

Galinski EA, Trüper HG (1994) Microbial behaviour in salt-stressed ecosystems. FEMS Microbiol. Rev. **15:** 95-108

Gandikota M, Birkenbihl RP, Höhmann S, Cardon GH, Saedler H, Huijser P (2007) The miRNA156/157 recognition element in the 3′ UTR of the Arabidopsis SBP box gene *SPL3* prevents early flowering by translational inhibition in seedlings. Plant J. **49:** 683-693

Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought-and salt-tolerant plants result from overexpression of the *AVP1* H^+-pump. Proc. Natl. Acad. Sci. U.S.A. **98:** 11444-11449

Gómez-Cadenas A, Verhey SD, Holappa LD, Shen Q, Ho T-HD, Walker-Simmons M (1999) An abscisic acid-induced protein kinase, PKABA1, mediates abscisic acid-suppressed gene expression in barley aleurone layers. Proc. Natl. Acad. Sci. U.S.A. **96:** 1767-1772

Gosti F, Beaudoin N, Serizet C, Webb AA, Vartanian N, Giraudat J (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. Plant Cell **11:** 1897-1909

Gou J, Fu C, Liu S, Tang C, Debnath S, Flanagan A, Ge Y, Tang Y, Jiang Q, Larson PR (2017) The miR156-SPL4 module predominantly regulates aerial axillary bud formation and controls shoot architecture. New Phytol. **216:** 829-840

Gravot A, Dittami SM, Rousvoal S, Lugan R, Eggert A, Collén J, Boyen C, Bouchereau A, Tonon T (2010) Diurnal oscillations of metabolite abundances and gene analysis provide new insights into central metabolic processes of the brown alga *Ectocarpus siliculosus*. New Phytol. **188:** 98-110

Greener JG, Kandathil SM, Jones DT (2019) Deep learning extends de novo protein modelling coverage of genomes using iteratively predicted structural constraints. Nat. Commun. **10:** 1-13

Groisillier A, Shao Z, Michel G, Goulitquer S, Bonin P, Krahulec S, Nidetzky B, Duan D, Boyen C, Tonon T (2013) Mannitol metabolism in brown algae involves a new phosphatase family. J. Exp. Bot. **65:** 559-570

Guo KM, Babourina O, Christopher DA, Borsics T, Rengel Z (2008) The cyclic nucleotide-gated channel, *AtCNGC10*, influences salt tolerance in Arabidopsis. Physiol. Plant. **134:** 499-507

Häfliger B, Kindhauser E, Keller F (1999) Metabolism of d-Glycero-d-Manno-Heptitol, Volemitol, in Polyanthus. Discovery of a novel ketose reductase1. Plant Physiol. **119:** 191-198

Halfter U, Ishitani M, Zhu J-K (2000) The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc. Natl. Acad. Sci. U.S.A. **97:** 3735-3740

Harmon AC, Gribskov M, Harper JF (2000) CDPKs–a kinase for every Ca^{2+} signal? Trends Plant Sci. **5:** 154-159

Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Biol. **51:** 463-499

Hattori T, Totsuka M, Hobo T, Kagaya Y, Yamamoto-Toyoda A (2002) Experimentally determined sequence requirement of ACGT-containing abscisic acid response element. Plant Cell Physiol. **43:** 136-140

He J, Xu M, Willmann MR, McCormick K, Hu T, Yang L, Starker CG, Voytas DF, Meyers BC, Poethig RS (2018) Threshold-dependent repression of *SPL* gene expression by miR156/miR157 controls vegetative phase change in *Arabidopsis thaliana*. PLoS Genet. **14:** e1007337

Heberle H, Meirelles GV, da Silva FR, Telles GP, Minghim R (2015) InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. BMC Bioinformatics **16:** 169

Hirabayashi Y, Mahendran R, Koirala S, Konoshima L, Yamazaki D, Watanabe S, Kim H, Kanae S (2013) Global flood risk under climate change. Nat. Clim. Change **3:** 816

Höfgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acid Res. **16:** 9877

Hua B-G, Mercier RW, Leng Q, Berkowitz GA (2003) Plants do it differently. A new basis for potassium/sodium selectivity in the pore of an ion channel. Plant Physiol. **132:** 1353-1361

Huertas R, Olias R, Eljakaoui Z, Gálvez FJ, Li J, DE Morales PA, Belver A, Rodríguez-Rosales MP (2012) Overexpression of *SlSOS2* (SlCIPK24) confers salt tolerance to transgenic tomato. Plant Cell Environ. **35:** 1467-1482

Hundertmark M, Hincha DK (2008) LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. BMC Genomics **9:** 118

Iancu CV, Borza T, Choe JY, Fromm HJ, Honzatko RB (2001) Recombinant mouse muscle adenylosuccinate synthetase overexpression, kinetics, and crystal structure. J. Biol. Chem. **276:** 42146-42152

Iizumi T, Sakuma H, Yokozawa M, Luo J-J, Challinor AJ, Brown ME, Sakurai G, Yamagata T (2013) Prediction of seasonal climate-induced variations in global food production. Nat. Clim. Change **3:** 904

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Biol. **47:** 377-403

Jennings D (1985) Polyol metabolism in fungi. *In* Adv. Microb. Physiol., Vol 25. Elsevier, pp 149-193

Ji H, Pardo JM, Batelli G, Van Oosten MJ, Bressan RA, Li X (2013) The Salt Overly Sensitive (SOS) pathway: established and emerging roles. Mol. Plant **6:** 275-286

Jia F, Qi S, Li H, Liu P, Li P, Wu C, Zheng C, Huang J (2014) Overexpression of *Late Embryogenesis Abundant 14* enhances Arabidopsis salt stress tolerance. Biochem. Bioph. Res. Commun. **454:** 505-511

Jiang C, Belfield EJ, Mithani A, Visscher A, Ragoussis J, Mott R, Smith JAC, Harberd NP (2012) ROS-mediated vascular homeostatic control of root-to-shoot soil Na^+ delivery in Arabidopsis. EMBO J. **31:** 4359-4370

Jithesh MN, Wally OS, Manfield I, Critchley AT, Hiltz D, Prithiviraj B (2012) Analysis of seaweed extract-induced transcriptome leads to identification of a negative regulator of salt tolerance in Arabidopsis. HortScience **47:** 704-709

Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. Bioinformatics **8:** 275-282

Julkowska MM, Testerink C (2015) Tuning plant signaling and growth to survive salt. Trends Plant Sci. **20:** 586-594

Jung K-H, Gho H-J, Nguyen MX, Kim S-R, An G (2013) Genome-wide expression analysis of *HSP70* family genes in rice and identification of a cytosolic *HSP70* gene highly induced under heat stress. Funct. Integr. Genomic. **13:** 391-402

Jungkunz I, Link K, Vogel F, Voll LM, Sonnewald S, Sonnewald U (2011) *AtHsp70*-15-deficient Arabidopsis plants are characterized by reduced growth, a constitutive cytosolic protein response and enhanced resistance to TuMV. Plant J. **66:** 983-995

Kampinga HH, Craig EA (2010) The *HSP70* chaperone machinery: J proteins as drivers of functional specificity. Nat. Rev. Mol. Cell Biol. **11:** 579-592

Kandasamy S, Khan W, Kulshreshtha G, Evans F, Critchley AT, Fitton J, Stringer DN, Gardiner V-A, Prithiviraj B (2015) The fucose containing polymer (FCP) rich fraction of *Ascophyllum nodosum* (L.) Le Jol. protects *Caenorhabditis elegans* against *Pseudomonas aeruginosa* by triggering innate immune signaling pathways and suppression of pathogen virulence factors. Algae **30:** 147-161

Kazan K, Lyons R (2015) The link between flowering time and stress tolerance. J. Exp. Bot. **67:** 47-60

Kempa S, Krasensky J, Dal Santo S, Kopka J, Jonak C (2008) A central role of abscisic acid in stress-regulated carbohydrate metabolism. Plos One **3:** e3935

Khan W, Rayirath UP, Subramanian S, Jithesh MN, Rayorath P, Hodges DM, Critchley AT, Craigie JS, Norrie J, Prithiviraj B (2009) Seaweed extracts as biostimulants of plant growth and development. J. Plant Growth Regul. **28:** 386-399

Kim J-S, Mizoi J, Yoshida T, Fujita Y, Nakajima J, Ohori T, Todaka D, Nakashima K, Hirayama T, Shinozaki K (2011) An ABRE promoter sequence is involved in osmotic stress-responsive expression of the *DREB2A* gene, which encodes a transcription factor regulating drought-inducible genes in Arabidopsis. Plant Cell Physiol. **52:** 2136-2146

Kim S-G, Kim S-Y, Park C-M (2007) A membrane-associated NAC transcription factor regulates salt responsive flowering via Flowering Locus T in Arabidopsis. Planta **226:** 647-654

Kim W-Y, Ali Z, Park HJ, Park SJ, Cha J-Y, Perez-Hormaeche J, Quintero FJ, Shin G, Kim MR, Qiang Z (2013) Release of *SOS2* kinase from sequestration with GIGANTEA determines salt tolerance in Arabidopsis. Nat. Commun. **4:** 1352

Kiyosue T, Abe H, Yamaguchi-Shinozaki K, Shinozaki K (1998) *ERD6*, a cDNA clone for an early dehydration-induced gene of Arabidopsis, encodes a putative sugar transporter. BBA Biomembranes **1370:** 187-191

Kloareg B, Quatrano R (1988) Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. Oceanogr. Mar. Biol. **26:** 259-315

Konstantinova T, Parvanova D, Atanassov A, Djilianov D (2002) Freezing tolerant tobacco, transformed to accumulate osmoprotectants. Plant Sci. **163:** 157-164

Kotak S, Larkindale J, Lee U, von Koskull-Döring P, Vierling E, Scharf K-D (2007) Complexity of the heat stress response in plants. Curr. Opin. Plant Biol. **10:** 310-316

Kudla J, Batistič O, Hashimoto K (2010) Calcium signals: the lead currency of plant information processing. Plant Cell **22:** 541-563

Kulshreshtha G, Borza T, Rathgeber B, Stratton GS, Thomas NA, Critchley A, Hafting J, Prithiviraj B (2016) Red seaweeds *Sarcodiotheca gaudichaudii* and *Chondrus crispus* down regulate virulence factors of *Salmonella Enteritidis* and induce immune responses in *Caenorhabditis elegans*. Front. Microbiol. **7:** 421

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. **35:** 1547-1549

Kumar SV, Wigge PA (2010) H2A.Z-containing nucleosomes mediate the thermosensory response in Arabidopsis. Cell **140:** 136-147

Lång V, Palva ET (1992) The expression of a rab-related gene, *rab18*, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. Plant Mol. Biol. **20:** 951-962

Laohavisit A, Richards SL, Shabala L, Chen C, Colaço RD, Swarbreck SM, Shaw E, Dark A, Shabala S, Shang Z (2013) Salinity-induced calcium signaling and root adaptation in Arabidopsis require the calcium regulatory protein annexin1. Plant Physiol. **163:** 253-262

Le Bail A, Dittami SM, De Franco P-O, Rousvoal S, Cock MJ, Tonon T, Charrier B (2008) Normalisation genes for expression analyses in the brown alga model *Ectocarpus siliculosus*. BMC Mol. Biol. **9:** 75

Le Gall Y, Brown S, Marie D, Mejjad M, Kloareg B (1993) Quantification of nuclear DNA and GC content in marine macroalgae by flow cytometry of isolated nuclei. Protoplasma **173:** 123-132

Lee S, Lee EJ, Yang EJ, Lee JE, Park AR, Song WH, Park OK (2004) Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in Arabidopsis. Plant Cell **16:** 1378-1391

Lesk C, Rowhani P, Ramankutty N (2016) Influence of extreme weather disasters on global crop production. Nature **529:** 84-87

Lewis D, Smith D (1967) Sugar alcohols (polyols) in fungi and green plants. I. Distribution, physiology and metabolism. New Phytol. **66:** 143-184

Li C, Lu S (2014) Molecular characterization of the *SPL* gene family in *Populus trichocarpa*. BMC Plant Biol. **14:** 1-15

Li K, Wang Y, Han C, Zhang W, Jia H, Li X (2007) GA signaling and CO/FT regulatory module mediate salt-induced late flowering in *Arabidopsis thaliana*. J. Plant Growth Regul. **53:** 195-206

Li L, Zheng W, Zhu Y, Ye H, Tang B, Arendsee ZW, Jones D, Li R, Ortiz D, Zhao X, Du C, Nettleton D, Scott MP, Salas-Fernandez MG, Yin Y, Wurtele ES (2015) *QQS* orphan gene regulates carbon and nitrogen partitioning across species via NF-YC interactions. Proc. Natl. Acad. Sci. **112:** 14734-14739

Li Z, Long R, Zhang T, Wang Z, Zhang F, Yang Q, Kang J, Sun Y (2017) Molecular cloning and functional analysis of the drought tolerance gene *MsHSP70* from alfalfa (*Medicago sativa* L.). J. Plant Res. **130:** 387-396

Lin B-L, Wang J-S, Liu H-C, Chen R-W, Meyer Y, Barakat A, Delseny M (2001) Genomic analysis of the *HSP70* superfamily in *Arabidopsis thaliana*. Cell Stress Chaperone **6:** 201

Liu N, Tu L, Wang L, Hu H, Xu J, Zhang X (2017) MicroRNA 157-targeted *SPL* genes regulate floral organ size and ovule production in cotton. BMC Plant Biol. **17:** 1-14

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, *DREB1* and *DREB2*, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell **10:** 1391-1406

Liu X, Robinson PW, Madore MA, Witney GW, Arpaia ML (1999) Hass' avocado carbohydrate fluctuations. II. Fruit growth and ripening. J. AM. Soc. Hortic. Sci. **124:** 676-681

Liu Y, Beyer A, Aebersold R (2016) On the dependency of cellular protein levels on mRNA abundance. Cell **165:** 535-550

Lloyd JR, Kossmann J, Ritte G (2005) Leaf starch degradation comes out of the shadows. Trends Plant Sci. **10:** 130-137

Lobell DB, Roberts MJ, Schlenker W, Braun N, Little BB, Rejesus RM, Hammer GL (2014) Greater sensitivity to drought accompanies maize yield increase in the US Midwest. Science **344:** 516-519

Lobell DB, Schlenker W, Costa-Roberts J (2011) Climate trends and global crop production since 1980. Science **333:** 616-620

Loescher WH, Everard JD (2000) Regulation of sugar alcohol biosynthesis. *In* Photosynthesis. Springer, pp 275-299

Loescher WH, Everard JD, Cantini C, Grumet R (1995) Sugar alcohol metabolism in source leaves.

Lohmann JU, Weigel D (2002) Building beauty: the genetic control of floral patterning. Dev. Cell **2:** 135-142

Lopez-Matas M-A, Nunez P, Soto A, Allona I, Casado R, Collada C, Guevara M-A, Aragoncillo C, Gomez L (2004) Protein cryoprotective activity of a cytosolic small heat shock protein that accumulates constitutively in chestnut stems and is up-regulated by low and high temperatures. Plant Physiol. **134:** 1708-1717

Luan S (2009) The CBL–CIPK network in plant calcium signaling. Trends Plant Sci. **14:** 37-42

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science **324:** 1064-1068

Maathuis FJ, Sanders D (2001) Sodium uptake in Arabidopsis roots is regulated by cyclic nucleotides. Plant Physiol. **127:** 1617-1625

Maier T, Güell M, Serrano L (2009) Correlation of mRNA and protein in complex biological samples. FEBS Lett. **583:** 3966-3973

Maischak H, Zimmermann MR, Felle HH, Boland W, Mithöfer A (2010) Alamethicin-induced electrical long distance signaling in plants. Plant Signal Behav. **5:** 988-990

Manaa A, Ben Ahmed H, Valot B, Bouchet J-P, Aschi-Smiti S, Causse M, Faurobert M (2011) Salt and genotype impact on plant physiology and root proteome variations in tomato. J. Exp. Bot. **62:** 2797-2813

Mansouri-Far C, Sanavy SAMM, Saberali SF (2010) Maize yield response to deficit irrigation during low-sensitive growth stages and nitrogen rate under semi-arid climatic conditions. Agric. Water Manag. **97:** 12-22

Mäser P, Eckelman B, Vaidyanathan R, Horie T, Fairbairn DJ, Kubo M, Yamagami M, Yamaguchi K, Nishimura M, Uozumi N (2002) Altered shoot/root Na^+ distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the Na^+ transporter *AtHKT1*. FEBS Lett. **531:** 157-161

Mayer MP (2010) Gymnastics of molecular chaperones. Mol. Cell **39:** 321-331

Mazzucotelli E, Mastrangelo AM, Crosatti C, Guerra D, Stanca AM, Cattivelli L (2008) Abiotic stress response in plants: when post-transcriptional and post-translational regulations control transcription. Plant Sci. **174:** 420-431

Melorose J, Perroy R, Careas S (2015) World population prospects. United Nations **1:** 587-592

Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. Plant J. **25:** 295-303

Michniewicz M, Frick EM, Strader LC (2015) Gateway-compatible tissue-specific vectors for plant transformation. BMC Res. Notes **8:** 63

Mickelbart MV, Hasegawa PM, Bailey-Serres J (2015) Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. Nat. Rev. Genet. **16:** 237

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. **7:** 405-410

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F (2011) ROS signaling: the new wave? Trends Plant Sci. **16:** 300-309

Montecinos AE, Couceiro L, Peters AF, Desrut A, Valero M, Guillemin M-L (2017) Species delimitation and phylogeographic analyses in the *Ectocarpus* subgroup siliculosi (*Ectocarpales*, *Phaeophyceae)*. J. Phycol. **53:** 17-31

Montero-Barrientos M, Hermosa R, Cardoza RE, Gutierrez S, Nicolas C, Monte E (2010) Transgenic expression of the *Trichoderma harzianum* hsp70 gene increases Arabidopsis resistance to heat and other abiotic stresses. J. Plant Physiol. **167:** 659-665

Moreno MA, Harper LC, Krueger RW, Dellaporta SL, Freeling M (1997) *Liguleless1* encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis. Genes Dev. **11:** 616-628

Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ. **25:** 239-250

Munns R, Gilliham M (2015) Salinity tolerance of crops–what is the cost? New Phytol. **208:** 668-673

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. **59:** 651-681

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15:** 473-497

Mustilli A-C, Merlot S, Vavasseur A, Fenzi F, Giraudat J (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell **14:** 3089-3099

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. Plant Physiol. **149:** 88-95

Nakashima K, Shinwari ZK, Sakuma Y, Seki M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2000) Organization and expression of two Arabidopsis *DREB2* genes encoding DRE-binding proteins involved in dehydration-and high-salinity-responsive gene expression. Plant Mol. Biol. **42:** 657-665

Ngara R, Ndimba BK (2014) Understanding the complex nature of salinity and drought-stress response in cereals using proteomics technologies. Proteomics **14:** 611-621

Nordin K, Vahala T, Palva ET (1993) Differential expression of two related, low-temperature-induced genes in *Arabidopsis thaliana* (L.) Heynh. Plant Mol. Biol. **21:** 641-653

Nuccio ML, Rhodes D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr. Opin. Plant Biol. **2:** 128-134

Nyvall P, Corre E, Boisset C, Barbeyron T, Rousvoal S, Scornet D, Kloareg B, Boyen C (2003) Characterization of *mannuronan C-5-epimerase* genes from the brown alga *Laminaria digitata*. Plant Physiol. **133:** 726-735

Obayashi T, Aoki Y, Tadaka S, Kagaya Y, Kinoshita K (2018) ATTED-II in 2018: a plant coexpression database based on investigation of the statistical property of the mutual rank index. Plant Cell Physiol. **59:** e3-e3

Ohta M, Guo Y, Halfter U, Zhu J-K (2003) A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. Proc. Natl. Acad. Sci. U.S.A. **100:** 11771-11776

Ohta M, Hayashi Y, Nakashima A, Hamada A, Tanaka A, Nakamura T, Hayakawa T (2002) Introduction of a Na^+/H^+ antiporter gene from *Atriplex gmelini* confers salt tolerance to rice. FEBS Lett. **532:** 279-282

Osakabe Y, Arinaga N, Umezawa T, Katsura S, Nagamachi K, Tanaka H, Ohiraki H, Yamada K, Seo S-U, Abo M (2013) Osmotic stress responses and plant growth controlled by potassium transporters in Arabidopsis. Plant Cell **25:** 609-624

Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. **60:** 324-349

Park HJ, Lee SS, You YN, Yoon DH, Kim B-G, Ahn JC, Cho HS (2013) A rice immunophilin gene, *OsFKBP16-3,* confers tolerance to environmental stress in Arabidopsis and rice. Int. J. Mol. Sci. **14:** 5899-5919

Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Tsz-fung FC (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science **324:** 1068-1071

Parry M, Parry ML, Canziani O, Palutikof J, Van der Linden P, Hanson C (2007) Climate change 2007-impacts, adaptation and vulnerability: Working group II contribution to the fourth assessment report of the IPCC, Vol 4. Cambridge University Press

Patel T, Krasnyanski S, Allen G, Panthee D, Louws F, Williamson J (2015) Tomato plants overexpressing a celery mannitol dehydrogenase (*mtd*) have decreased susceptibility to *Botrytis cinerea*. Am. J. Plant Sci. **6:** 1116-1125

Patel TK, Williamson JD (2016) Mannitol in plants, fungi, and plant-fungal interactions. Trends Plant Sci. **21:** 486-497

Peiter E, Maathuis FJ, Mills LN, Knight H, Pelloux J, Hetherington AM, Sanders D (2005) The vacuolar Ca^{2+} activated channel *TPC1* regulates germination and stomatal movement. Nature **434:** 404

Peng S, Huang J, Sheehy JE, Laza RC, Visperas RM, Zhong X, Centeno GS, Khush GS, Cassman KG (2004) Rice yields decline with higher night temperature from global warming. Proc. Natl. Acad. Sci. U.S.A. **101:** 9971-9975

Peters AF, Marie D, Scornet D, Kloareg B, Mark Cock J (2004) Proposal of *Ectocarpus siliculosus* (Ectocarpales, Phaeophyceae) as a model organism for brown algal genetics and genomics 1, 2. J. Phycol. **40:** 1079-1088

Pharr D, Stoop J, Williamson J, Feusi MS, Massel M, Conkling M (1995) The dual role of mannitol as osmoprotectant and photoassimilate in celery. HortScience **30:** 1182-1188

Pilot G, Gaymard F, Mouline K, Chérel I, Sentenac H (2003) Regulated expression of Arabidopsis Shaker K^+ channel genes involved in K^+ uptake and distribution in the plant. Plant Mol. Biol. **51:** 773-787

Prabhavathi V, Rajam MV (2007) Mannitol-accumulating transgenic eggplants exhibit enhanced resistance to fungal wilts. Plant Sci. **173:** 50-54

Pryor S, Barthelmie R, Schoof J (2013) High-resolution projections of climate-related risks for the Midwestern USA. Clim. Res. **56:** 61-79

Qi M, Zheng W, Zhao X, Hohenstein JD, Kandel Y, O'Conner S, Wang Y, Du C, Nettleton D, MacIntosh GC, Tylka GL, Wurtele ES, Whitham SA, Li L (2019) *QQS* orphan gene and its interactor NF-YC4 reduce susceptibility to pathogens and pests. Plant Biotechnol. J. **17:** 252-263

Qin X, Zeevaart JA (2002) Overexpression of a *9-cis-epoxycarotenoid dioxygenase* gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. Plant Physiol. **128:** 544-551

Qiu Q-S, Barkla BJ, Vera-Estrella R, Zhu J-K, Schumaker KS (2003) Na^+/H^+ exchange activity in the plasma membrane of Arabidopsis. Plant Physiol. **132:** 1041-1052

Qiu Q-S, Guo Y, Dietrich MA, Schumaker KS, Zhu J-K (2002) Regulation of *SOS1*, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by *SOS2* and *SOS3*. Proc. Natl. Acad. Sci. U.S.A. **99:** 8436-8441

Quintero FJ, Ohta M, Shi H, Zhu J-K, Pardo JM (2002) Reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na^+ homeostasis. Proc. Natl. Acad. Sci. U.S.A. **99:** 9061-9066

Rathor P, Borza T, Liu Y, Qin Y, Stone S, Zhang J, Hui JP, Berrue F, Groisillier A, Tonon T et al. (2020) Low mannitol concentrations in *Arabidopsis thaliana* expressing *Ectocarpus* genes improve salt tolerance. Plants **9:** 1508

Rathor P, Borza T, Stone S, Tonon T, Yurgel S, Potin P, Prithiviraj B (2021) A novel protein from *Ectocarpus* sp. improves salinity and high temperature stress tolerance in *Arabidopsis thaliana*. Int. J. Mol. Sci. **22:** 1971

Reed R, Davison I, Chudek J, Foster R (1985) The osmotic role of mannitol in the Phaeophyta: an appraisal. Phycologia **24:** 35-47

Ritter A, Dittami SM, Goulitquer S, Correa JA, Boyen C, Potin P, Tonon T (2014) Transcriptomic and metabolomic analysis of copper stress acclimation in *Ectocarpus siliculosus* highlights signaling and tolerance mechanisms in brown algae. BMC Plant Biol. **14:** 116

Ritter A, Goulitquer S, Salaün JP, Tonon T, Correa JA, Potin P (2008) Copper stress induces biosynthesis of octadecanoid and eicosanoid oxygenated derivatives in the brown algal kelp *Laminaria digitata*. New Phytol. **180:** 809-821

Rodrigues A, Adamo M, Crozet P, Margalha L, Confraria A, Martinho C, Elias A, Rabissi A, Lumbreras V, González-Guzmán M (2013) ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in Arabidopsis. Plant Cell **25:** 3871-3884

Rook F, Hadingham SA, Li Y, Bevan MW (2006) Sugar and ABA response pathways and the control of gene expression. Plant Cell Environ. **29:** 426-434

Rosenzweig C, Elliott J, Deryng D, Ruane AC, Müller C, Arneth A, Boote KJ, Folberth C, Glotter M, Khabarov N (2014) Assessing agricultural risks of climate change in the 21st century in a global gridded crop model intercomparison. Proc. Natl. Acad. Sci. U.S.A. **111:** 3268-3273

Rossel JB, Walter PB, Hendrickson L, Chow WS, Poole A, Mullineaux PM, Pogson BJ (2006) A mutation affecting *ascorbate peroxidase 2* gene expression reveals a link between responses to high light and drought tolerance. Plant Cell Environ. **29:** 269-281

Rousvoal S, Groisillier A, Dittami SM, Michel G, Boyen C, Tonon T (2011) Mannitol-1-phosphate dehydrogenase activity in *Ectocarpus siliculosus*, a key role for mannitol synthesis in brown algae. Planta **233:** 261-273

Roy SJ, Negrão S, Tester M (2014) Salt resistant crop plants. Curr. Opin. Plant Biol. **26:** 115-124

Rüdiger S, Buchberger A, Bukau B (1997) Interaction of HSP70 chaperones with substrates. Nat. Struct. Biol. **4:** 342-349

Rumpho ME, Edwards GE, Loescher WH (1983) A pathway for photosynthetic carbon flow to mannitol in celery leaves: activity and localization of key enzymes. Plant Physiol. **73:** 869-873

Saibil H (2013) Chaperone machines for protein folding, unfolding and disaggregation. Nat. Rev. Mol. Cell Biol. **14:** 630-642

Sakamoto A, Murata N (2002) The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. Plant Cell Environ. **25:** 163-171

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of Arabidopsis *DREBs*, transcription factors involved in dehydration-and cold-inducible gene expression. Biochem. Biophys. Res. Commun. **290:** 998-1009

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of an Arabidopsis transcription factor, *DREB2A*, involved in drought-responsive gene expression. Plant Cell **18:** 1292-1309

Sakuma Y, Maruyama K, Qin F, Osakabe Y, Shinozaki K, Yamaguchi-Shinozaki K (2006) Dual function of an Arabidopsis transcription factor *DREB2A* in water-stress-responsive and heat-stress-responsive gene expression. Proc. Natl. Acd. Sci. U.S.A **103:** 18822-18827

Salinas M, Xing S, Höhmann S, Berndtgen R, Huijser P (2012) Genomic organization, phylogenetic comparison and differential expression of the SBP-box family of transcription factors in tomato. Planta **235:** 1171-1184

Sangwan V, Örvar BL, Beyerly J, Hirt H, Dhindsa RS (2002) Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. Plant J. **31:** 629-638

Sarkar NK, Kundnani P, Grover A (2013) Functional analysis of HSP70 superfamily proteins of rice (*Oryza sativa*). Cell Stress Chaperone **18:** 427-437

Sawa M, Kay SA (2011) *GIGANTEA* directly activates Flowering Locus T in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U.S.A. **108:** 11698-11703

Scharf K-D, Berberich T, Ebersberger I, Nover L (2012) The plant heat stress transcription factor (*Hsf*) family: structure, function and evolution. Biochim. Biophys. Acta Gene Regul. Mech. **1819:** 104-119

Schmid M, Uhlenhaut NH, Godard F, Demar M, Bressan R, Weigel D, Lohmann JU (2003) Dissection of floral induction pathways using global expression analysis. Development **130:** 6001-6012

Schobert B (1977) Is there an osmotic regulatory mechanism in algae and higher plants? J. Theor. Biol. **68:** 17-26

Schwarz S, Grande AV, Bujdoso N, Saedler H, Huijser P (2008) The microRNA regulated SBP-box genes *SPL9* and *SPL15* control shoot maturation in Arabidopsis. Plant Mol. Biol. **67:** 183-195

Schweighofer A, Hirt H, Meskiene I (2004) Plant PP2C phosphatases: emerging functions in stress signaling. Trends Plant Sci. **9:** 236-243

Shabala S (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann. Bot. **112:** 1209-1221

Shabala S, Cuin TA (2008) Potassium transport and plant salt tolerance. Physiol. Plant. **133:** 651-669

Shabala S, Demidchik V, Shabala L, Cuin TA, Smith SJ, Miller AJ, Davies JM, Newman IA (2006) Extracellular Ca^{2+} ameliorates NaCl induced K^+ loss from Arabidopsis root and leaf cells by controlling plasma membrane K^+ permeable channels. Plant Physiol. **141:** 1653-1665

Shanmugaraj B, I Bulaon CJ, Phoolcharoen W (2020) Plant molecular farming: a viable platform for recombinant biopharmaceutical production. Plants **9:** 842

Sharma SK, De Los Rios P, Christen P, Lustig A, Goloubinoff P (2010) The kinetic parameters and energy cost of the HSP70 chaperone as a polypeptide unfoldase. Nat. Chem. Biol. **6:** 914

Shen B, Jensen RG, Bohnert HJ (1997a) Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. Plant Physiol. **113:** 1177-1183

Shen B, Jensen RG, Bohnert HJ (1997b) Mannitol protects against oxidation by hydroxyl radicals. Plant Physiol. **115:** 527-532

Sherrard ME, Maherali H (2006) The adaptive significance of drought escape in *Avena barbata*, an annual grass. Evolution **60:** 2478-2489

Shevchenko A, Tomas H, Havli J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. **1:** 2856

Shi H, Ishitani M, Kim C, Zhu J-K (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. Proc. Natl. Acad. Sci. U.S.A. **97:** 6896-6901

Shi H, Lee B-h, Wu S-J, Zhu J-K (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nat. Biotechnol. **21:** 81-85

Shi H, Quintero FJ, Pardo JM, Zhu J-K (2002) The putative plasma membrane Na^+/H^+ antiporter *SOS1* controls long-distance Na^+ transport in plants. Plant Cell **14:** 465-477

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant Biol. **6:** 410-417

Sickler CM, Edwards GE, Kiirats O, Gao Z, Loescher W (2007) Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. Funct. Plant Biol. **34:** 382-391

Sies H (1997) Oxidative stress: oxidants and antioxidants. Exp. Physiol. Translation Integration **82:** 291-295

Smirnoff N, Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochem. **28:** 1057-1060

Sobhanian H, Aghaei K, Komatsu S (2011) Changes in the plant proteome resulting from salt stress: toward the creation of salt-tolerant crops? J. Proteom. **74:** 1323-1337

Song A, Zhu X, Chen F, Gao H, Jiang J, Chen S (2014) A chrysanthemum heat shock protein confers tolerance to abiotic stress. Int. J. Mol. Sci. **15:** 5063-5078

Soon F-F, Ng L-M, Zhou XE, West GM, Kovach A, Tan ME, Suino-Powell KM, He Y, Xu Y, Chalmers MJ (2012) Molecular mimicry regulates ABA signaling by SnRK2 kinases and PP2C phosphatases. Science **335:** 85-88

Sparkes IA, Runions J, Kearns A, Hawes C (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nat. Protoc. **1:** 2019

Stoop JM, Williamson JD, Pharr DM (1996) Mannitol metabolism in plants: a method for coping with stress. Trends Plant Sci. **1:** 139-144

Su P-H, Li H-m (2008) Arabidopsis stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. Plant Physiol. **146:** 1231-1241

Su Z, Ma X, Guo H, Sukiran NL, Guo B, Assmann SM, Ma H (2013) Flower development under drought stress: morphological and transcriptomic analyses reveal acute responses and long-term acclimation in Arabidopsis. Plant Cell **25:** 3785-3807

Sun F, Zhang W, Hu H, Li B, Wang Y, Zhao Y, Li K, Liu M, Li X (2008) Salt modulates gravity signaling pathway to regulate growth direction of primary roots in Arabidopsis. Plant Physiol. **146:** 178-188

Sung DY, Guy CL (2003) Physiological and molecular assessment of altered expression of Hsc70-1 in Arabidopsis. Evidence for pleiotropic consequences. Plant Physiol. **132:** 979-987

Suzuki N, Koussevitzky S, Mittler R, Miller G (2012) ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ. **35:** 259-270

Suzuki T, Fujihira H (2020) Folding and quality control of glycoproteins. Reference Module Chem. Mol. Sci. Chem. Eng.

Takahashi S, Katagiri T, Yamaguchi-Shinozaki K, Shinozaki K (2000) An Arabidopsis gene encoding a Ca^{2+} binding protein is induced by abscisic acid during dehydration. Plant Cell Physiol. **41:** 898-903

Tarczynski MC, Jensen RG, Bohnert HJ (1992) Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol. Proc. Natl. Acad. Sci. U.S.A. **89:** 2600-2604

Tarczynski MC, Jensen RG, Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science **259:** 508-510

Tester M, Davenport R (2003) Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot. **91:** 503-527

Thalmann M, Santelia D (2017) Starch as a determinant of plant fitness under abiotic stress. New Phytol. **214:** 943-951

Thewes S (2014) Calcineurin-Crz1 signaling in lower eukaryotes. Eukaryot. Cell **13:** 694-705

Thomas JC, Sepahi M, Arentall B, Bohnert HJ (1995) Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. Plant Cell Environ. **18:** 801-806

Tonon T, Li Y, McQueen-Mason S (2017) Mannitol biosynthesis in algae: more widespread and diverse than previously thought. New Phytol. **213:** 1573-1579

Tripathi RK, Bregitzer P, Singh J (2018) Genome-wide analysis of the SPL/miR156 module and its interaction with the AP2/miR172 unit in barley. Sci. Rep. **8:** 1-13

Tripathi RK, Goel R, Kumari S, Dahuja A (2017) Genomic organization, phylogenetic comparison, and expression profiles of the *SPL* family genes and their regulation in soybean. Dev. Genes Evol. **227:** 101-119

Tripathi RK, Overbeek W, Singh J (2020) Global analysis of SBP gene family in *Brachypodium distachyon* reveals its association with spike development. Sci. Rep. **10:** 1-15

Tsugane K, Kobayashi K, Niwa Y, Ohba Y, Wada K, Kobayashi H (1999) A recessive Arabidopsis mutant that grows photoautotrophically under salt stress shows enhanced active oxygen detoxification. Plant Cell **11:** 1195-1206

Tunnacliffe A, Wise MJ (2007) The continuing conundrum of the LEA proteins. Naturwissenschaften **94:** 791-812

Tuteja N (2007) Mechanisms of high salinity tolerance in plants. *In* Method Enzymol, Vol 428. Elsevier, pp 419-438

Veeranagamallaiah G, Prasanthi J, Reddy KE, Pandurangaiah M, Babu OS, Sudhakar C (2011) Group 1 and 2 LEA protein expression correlates with a decrease in water stress induced protein aggregation in horsegram during germination and seedling growth. J. Plant Physiol. **168:** 671-677

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J. **45:** 523-539

Verslues PE, Batelli G, Grillo S, Agius F, Kim Y-S, Zhu J, Agarwal M, Katiyar-Agarwal S, Zhu J-K (2007) Interaction of SOS2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and H_2O_2 signaling in *Arabidopsis thaliana*. Mol. Cell. Biol. **27:** 7771-7780

Viana VE, Pegoraro C, Busanello C, Costa de Oliveira A (2019) Mutagenesis in rice: the basis for breeding a new super plant. Front. Plant Sci. **10:** 1326

Vierling E (1991) The roles of heat shock proteins in plants. Annual Rev. Plant Biol. **42:** 579-620

Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet. **13:** 227-232

Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell **136:** 669-687

Wang H, Wang H, Shao H, Tang X (2016) Recent advances in utilizing transcription factors to improve plant abiotic stress tolerance by transgenic technology. Front. Plant Sci. **7:** 67

Wang J, Youkharibache P, Zhang D, Lanczycki CJ, Geer RC, Madej T, Phan L, Ward M, Lu S, Marchler GH, Wang Y, Bryant SH, Geer LY, Marchler-Bauer A (2020) iCn3D, a web-based 3D viewer for sharing 1D/2D/3D representations of biomolecular structures. Bioinformatics **36:** 131-135

Wang J-W, Czech B, Weigel D (2009) miR156-regulated *SPL* transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. Cell **138:** 738-749

Wang J-W, Schwab R, Czech B, Mica E, Weigel D (2008) Dual effects of miR156-targeted *SPL* genes and *CYP78A5/KLUH* on plastochron length and organ size in *Arabidopsis thaliana*. Plant Cell **20:** 1231-1243

Wang L, Sun S, Jin J, Fu D, Yang X, Weng X, Xu C, Li X, Xiao J, Zhang Q (2015) Coordinated regulation of vegetative and reproductive branching in rice. Proc. Natl. Acad. Sci. U.S.A. **112:** 15504-15509

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

Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. **9:** 244-252

Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TAP, Rempfer C, Bordoli L (2018) SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acid Res. **46:** W296-W303

Wei H, Zhao Y, Xie Y, Wang H (2018) Exploiting *SPL* genes to improve maize plant architecture tailored for high-density planting. J. Exp. Bot. **69:** 4675-4688

Weinl S, Kudla J (2009) The CBL–CIPK Ca^{2+}-decoding signaling network: function and perspectives. New Phytol. **184:** 517-528

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M, Inzé D, Van Camp W (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants. EMBO J. **16:** 4806-4816

Williamson JD, Jennings DB, Guo W-W, Pharr DM, Ehrenshaft M (2002) Sugar alcohols, salt stress, and fungal resistance: polyols—multifunctional plant protection? J. AM. Soc. Hortic. Sci. **127:** 467-473

Wise MJ (2003) LEAping to conclusions: a computational reanalysis of late embryogenesis abundant proteins and their possible roles. BMC Bioinformatics **4:** 52

Wu G, Park MY, Conway SR, Wang J-W, Weigel D, Poethig RS (2009) The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell **138:** 750-759

Wu G, Poethig RS (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target *SPL3*. Development **133:** 3539-3547

Xie K, Wu C, Xiong L (2006) Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. Plant Physiol. **142:** 280-293

Xu M, Hu T, Zhao J, Park M-Y, Earley KW, Wu G, Yang L, Poethig RS (2016) Developmental functions of miR156-regulated *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* genes in *Arabidopsis thaliana*. PLoS Genet. **12:** e1006263

Yamaguchi A, Wu M-F, Yang L, Wu G, Poethig RS, Wagner D (2009) The microRNA-regulated SBP-Box transcription factor *SPL3* is a direct upstream activator of *LEAFY, FRUITFULL*, and *APETALA1*. Dev. Cell **17:** 268-278

Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of the expression of a desiccation-responsive *RD29A* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol. Gen. Genet. **236:** 331-340

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251-264

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic-and cold-stress-responsive promoters. Trends Plant Sci. **10:** 88-94

Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J. Exp. Biol. **208:** 2819-2830

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science **217:** 1214-1222

Yancey PH, Siebenaller JF (2015) Co-evolution of proteins and solutions: protein adaptation versus cytoprotective micromolecules and their roles in marine organisms. J. Exp. Biol. **218:** 1880-1896

Yoo JH, Park CY, Kim JC, Do Heo W, Cheong MS, Park HC, Kim MC, Moon BC, Choi MS, Kang YH (2005) Direct interaction of a divergent CaM isoform and the transcription factor, *MYB2*, enhances salt tolerance in Arabidopsis. J. Biol. Chem. **280:** 3697-3706

Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol. Biol. Evol. **21:** 809-818

Yoshida R, Umezawa T, Mizoguchi T, Takahashi S, Takahashi F, Shinozaki K (2006) The regulatory domain of SRK2E/OST1/SnRK2. 6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. J. Biol. Chem. **281:** 5310-5318

Yoshida T, Fujita Y, Sayama H, Kidokoro S, Maruyama K, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2010) *AREB1*, *AREB2*, and *ABF3* are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. Plant J. **61:** 672-685

Young JC (2010) Mechanisms of the *HSP70* chaperone system. Biochem. Cell Biol. **88:** 291-300

Yu N, Cai W-J, Wang S, Shan C-M, Wang L-J, Chen X-Y (2010) Temporal control of trichome distribution by microRNA156-targeted *SPL* genes in *Arabidopsis thaliana*. Plant Cell **22:** 2322-2335

Yu Q, An L, Li W (2014) The CBL–CIPK network mediates different signaling pathways in plants. Plant Cell Rep. **33:** 203-214

Yu S, Galvão VC, Zhang Y-C, Horrer D, Zhang T-Q, Hao Y-H, Feng Y-Q, Wang S, Schmid M, Wang J-W (2012) Gibberellin regulates the Arabidopsis floral transition through miR156-targeted *SQUAMOSA PROMOTER BINDING–LIKE* transcription factors. Plant Cell **24:** 3320-3332

Zhang A (2009) Protein interaction networks: computational analysis. Cambridge University Press

Zhang B, Liu X, Zhao G, Mao X, Li A, Jing R (2014) Molecular characterization and expression analysis of *Triticum aestivum* squamosa-promoter binding protein-box genes involved in ear development. J. Integr. Plant Biol. **56:** 571-581

Zhang H-X, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat. Biotechnol. **19:** 765

Zhang J, Liu B, Li J, Zhang L, Wang Y, Zheng H, Lu M, Chen J (2015) *HSF* and *HSP* gene families in Populus: genome-wide identification, organization and correlated expression during development and in stress responses. BMC Genomics **16:** 1-19

Zhang L, Zhao H-K, Dong Q-l, Zhang Y-y, Wang Y-m, Li H-y, Xing G-j, Li Q-y, Dong Y-s (2015) Genome-wide analysis and expression profiling under heat and drought treatments of *HSP70* gene family in soybean (*Glycine max* L.). Front. Plant Sci. **6:** 773

Zhang Y, Schwarz S, Saedler H, Huijser P (2007) *SPL8*, a local regulator in a subset of gibberellin-mediated developmental processes in Arabidopsis. Plant Mol. Biol. **63:** 429-439

Zhifang G, Loescher W (2003) Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer. Plant Cell Environ. **26:** 275-283

Zhou H, Lin H, Chen S, Becker K, Yang Y, Zhao J, Kudla J, Schumaker KS, Guo Y (2014) Inhibition of the Arabidopsis salt overly sensitive pathway by 14-3-3 proteins. Plant Cell **26:** 1166-1182

Zhou H, Zhao J, Yang Y, Chen C, Liu Y, Jin X, Chen L, Li X, Deng XW, Schumaker KS (2012) Ubiquitin-specific protease16 modulates salt tolerance in Arabidopsis by regulating Na^+/H^+ antiport activity and serine hydroxymethyltransferase stability. Plant Cell **24:** 5106-5122

Zhu J-K (2001) Plant salt tolerance. Trends Plant Sci. **6:** 66-71

Zhu J-K (2002) Salt and drought stress signal transduction in plants. Ann. Rev. Plant Biol. **53:** 247-273

Zhu J-K (2016) Abiotic stress signaling and responses in plants. Cell **167:** 313-324

Zhu J-K, Hasegawa PM, Bressan RA, Bohnert HJ (1997) Molecular aspects of osmotic stress in plants. Crit. Rev. Plant Sci. **16:** 253-277

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. **53:** 247-273

Zou J, Liu C, Liu A, Zou D, Chen X (2012) Overexpression of *OsHsp17.0* and *OsHsp23.7* enhances drought and salt tolerance in rice. J. Plant Physiol. **169:** 628-635

Zuiderweg ER, Bertelsen EB, Rousaki A, Mayer MP, Gestwicki JE, Ahmad A (2012) Allostery in the HSP70 chaperone proteins. *In* Mol. Chaperone. Springer, pp 99-153

APPENDIX A

Supplementary Table S1. Primers used to amplify *A. thaliana* key marker genes involved in abiotic stress tolerance and *Ectocarpus* sp. mannitol biosynthesis genes, unknown function gene *Esi0017_0056* and *Esi0379_0027.*

Gene ID	Name	Sequence 5' to 3'
AT3G18780	*Actin2* F	GCACCCTGTTCTTCTTACCG
	Actin2 R	AACCCTCGTAGATTGGCACA
AT5G05410	*DREB2A* F	TTGGCTGAGCGAGTTTGAAC
	DREB2A R	CGGTCCTGATTTAAGCCTGC
AT5G52310	*RD29A* F	TTTGGTGACGAGTCAGGAGC
	RD29A R	CACTACCAAAGCCCATCGGA
AT5G52300	*RD29B* F	CCAGAACTATCTCGTCCCAAAG
	RD29B R	GAAGCTAACTGCTCTGTGTAGG
AT4G27410	*RD26* F	TTGATTGGGCTAGCTTGGCA
	RD26 R	AGTTCTGCTGCCGATTCACA
AT5G25610	*RD22* F	TGGAGAAGGACTTGGTTCGC
	RD22 R	GAACCAGCTTCCACCGAGAA
AT2G33380	*RD20* F	TACACTTCCGAGTTGGGTGC
	RD20 R	AACCGTTAGCGCGTATTTGC
AT5G66400	*RAB18* F	GGGAGGAGGAAGAAGGGAATA
	RAB18 R	CGTAGCCACCAGCATCATATC
AT3G17520	*LEA* F	AGCTAAGGAAGCGGCTAAAC
	LEA R	CCTTAGCTGCACTCGTCATATC
AT1G01470	*LEA14* F	GGCCAAAGTCTCTGTCACCA
	LEA14 R	CTTTCAGAGAACCCGGGTCC
AT5G27150	*NHX1* F	GCCTCGGCCTGACAGTATAC
	NHX1 R	GTACAAAGCCACGACCTCCA
AT3G12580	*HSP70* F	CTTGGGTTTGGAAACTGCCG
	HSP70 R	GTTGTCCTTTGTTCGTGCCC

Gene ID	Name	Sequence 5’ to 3’
AT1G32330	*HSFA1D* F	CGAGCAAGCCAAAGCAATGT
	HSFA1D R	TCCATCTCTGTTCCCTCGGT
Esi0017_0056	*Esi17_56* F	CACGGCATTTGGAACCTGTG
	Esi17_56 R	GGATCAAATGCGTCCATGCC
	M1PDH1 F	GTTCAGGCAGGGATTCCACA
	M1PDH1 R	GCTCTCTGTCCAACAGGCAT
	M1Pase2 F	AGGATAGGAAGGCTGCTGGA
	M1Pase2 R	GCTCAGCGAAGGTCTTGAGT
Esi0379_0027	*EsHSP70* F	AGGACATGAGCACCAACCAG
	EsHSP70 R	CGAACAGGGAGTCGATCTCG

Supplementary Table S2. Root length, number of lateral roots per cm of primary root, and leaf chlorosis of the wild type seedlings and 3 independent transgenic lines (*Es*M1, *Es*M2 and *Es*M3), grown in the presence and absence of 100 mM NaCl. Values represents percentage to wild type plants (n = 150).

	Root length (%)	**Lateral roots (%)**	**Leaf chlorosis (%)**
	0 mM NaCl		
WT	100	100	
*Es*M1	103.15	109.33	
*Es*M2	90.16	115.16	
*Es*M3	103.54	106.71	
	100 mM NaCl		
WT	100	100	100
*Es*M1	108.47	116.54	43.62
*Es*M2	104.23	125.98	37.11
*Es*M3	115.96	130.31	41.67

Supplementary Table S3. Fresh weight and dry weight of the wild type and 3 independent transgenic lines (*Es*M1, *Es*M2 and *Es*M3), grown in the absence and presence of 100 mM NaCl. Values represents percentage to wild type plants (n = 18).

	FW (%)	**DW (%)**	**FW (%)**	**DW (%)**
	Seedlings			
	0 mM NaCl		100 mM NaCl	
WT	100	100	100	100
*Es*M1	108.77	111.21	127.68	126.29
*Es*M2	106.87	119.39	135.49	152.64
*Es*M3	105.08	109.08	140.14	142.08
	Plants			
	0 mM NaCl		100 mM NaCl	
WT	100	100	100	100
*Es*M1	86.97	89.17	112.16	110.24
*Es*M2	112.54	116.81	113.74	120.11
*Es*M3	109.15	115.95	122.16	123.97

Supplementary Table S4. Fresh weight and dry weight of the wild type seedlings and 3 independent transgenic lines (*Es*M1, *Es*M2 and *Es*M3), grown in the absence and presence of high temperature stress conditions. Values represents percentage to wild type plants (n = 9).

	FW (%)	**DW (%)**	**FW (%)**	**DW (%)**
	Control		Heat	
WT	100	100	100	100
*Es*M1	101.37	100.8	151.31	131.43
*Es*M2	100.78	110.15	112.44	126.64
*Es*M3	98.75	99.75	137.69	128.19

Supplementary Dataset S1. List of the genes identified in the transcriptomics data. WT-wild type, *Es*M-mannitol-producing line, WTS-wild type under salinity stress, *Es*MS-mannitol producing line under salinity stress. Tair10 and Araport11 are Arabidopsis information resources database and fragments per kilobase of transcripts per million (FPKM) are expression values. This file is available at Dalspace.

Supplementary Dataset S2. List of selected up-regulated genes in *Es*MS *vs.* WTS grouped in different functional categories. This file is available at Dalspace.

Supplementary Dataset S3. Functional categories grouping of genes which showed > 2-fold change in expression at p value < 0.05, in mannitol-producing line *vs*. wild type pairwise comparisons, in standard and salinity stress conditions. This file is available at Dalspace.

APPENDIX B

Selection of Unknown Function and/or Predicted Function Genes

Global transcriptomics analysis of brown algal model *Ectocarpus* sp. following the exposure to various abiotic stressors revealed numerous genes that were up-regulated (Ritter et al., 2014). Several unknown function and/or predicted function genes which were consistently up-regulated under different abiotic stress conditions were expressed in *A. thaliana* to elucidate the potential function of these genes in abiotic stress tolerance (Supplementary Table S5).

B.1 Materials and Methods

B.1.1 *Ectocarpus* sp. growth conditions and gene isolation

The brown algal model *Ectocarpus* sp. (Dilwyn) Lyngbye unialgal strain 32 (accession CCAP 1310/4, origin San Juan de Marcona, Peru) was cultured into a 10 liters plastic tank filled with filtered and autoclaved natural seawater supplemented with Provasoli nutrient medium at a concentration of 10 ml/L. The tank was placed in a growth chamber set at 14 °C with 14-h light/10-h dark cycle, with light intensity of 40 $\mu mol.m^{-2}s^{-1}$. The culture was air bubbled with filtered (0.22 μm filter) compressed air. The algal culture was exposed to salinity stress (1450 mM NaCl) for 6 h. After 6 h the algal culture was harvested using filtration, carefully dried using paper towels and flash frozen into liquid nitrogen. Approximately 100 mg of fresh tissue was used for total RNA extraction, with some modification to the method described by (Apt et al., 1995). The alteration to the protocol was made as explained by (Le Bail et al., 2008). RNA concentrations were assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, France). One microgram of RNA was treated with Turbo DNAse (Ambion Austin, USA) according to the manufacturer's protocol, to eliminate any possible DNA contaminations. The DNAse treated RNA was converted to cDNA, using a SuperScript IV Reverse Transcriptase (Life

Technologies, France), according to the manufacturer's instructions. The cDNA working concentration was diluted to 10 ng/µl; from that solution 3 µl were used in each RT-PCR reaction. RT-PCR was performed using Takara master mix (Clontech, San Francisco, USA) with full primers on a Bio-Rad thermal cycler (Bio-Rad, France).

Supplementary Table S5. Genes selected for overexpression in *A. thaliana.* Putative function is based on the information obtained from the *Ectocarpus siliculosus* genome database: https://bioinformatics.psb.ugent.be/orcae/overview/EctsiV2

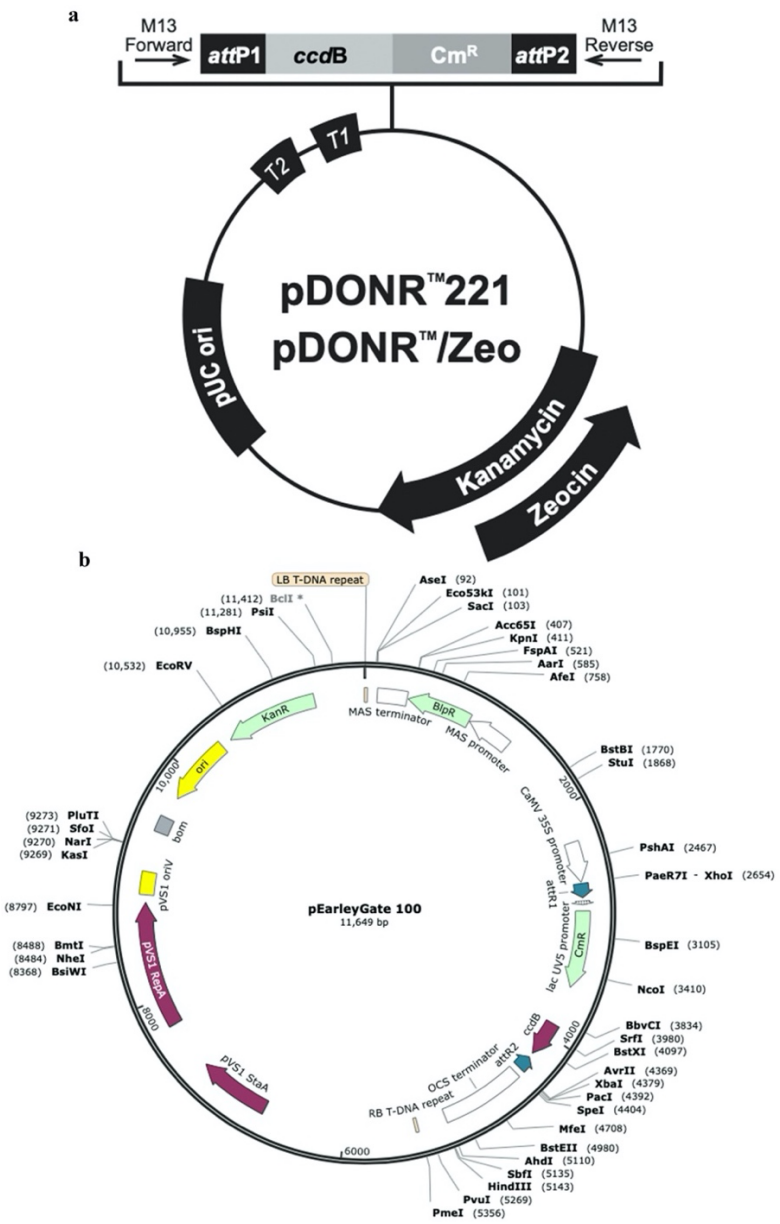
S. No.	Gene	Putative function (last updated May 2021)
1	*Esi0379_0027*	Molecular chaperones HSP70/HSC70-like
2	*Esi0017_0056*	NAD and ADP ribose binding pockets
3	*Esi0154_0047*	Hydroxyacyl Coenzyme A dehydrogenase
4	*Esi0025_0042*	No conserved domains
5	*Esi0252_0035*	No conserved domains
6	*Esi0322_0010*	Lipid metabolism, putative acyltransferase
7	*Esi0045_0021*	No conserved domains
8	*Esi0488_0007*	ubiquitin like
9	*Esi0105_0049*	No conserved domains
10	*Esi0182_0002*	Tegument protein domain
11	*Esi0143_0016*	No conserved domains
12	*Esi0007_0087*	No conserved domains
13	*Esi0113_0047*	SAM-dependent methyltransferase
14	*Esi0059_0099*	DUF4419 domain unknown function

S. No.	Gene	Putative function (last updated May 2021)
15	*Esi0538_0008*	Phox homologous domain
16	*Esi0021_0137*	Putative lipase
17	*Esi0195_0005*	No conserved domains
18	*Esi0176_0002*	Alpha/Beta hydrolase fold 1
19	*Esi0266_0005*	No conserved domains
20	*Esi0044_0144*	Unknown
21	*Esi0105_0008*	Unknown

B.1.2 Cloning and expression in *A. thaliana*

To clone the *Ectocarpus* sp. genes, RT-PCR was performed using a Takara master mix (Clontech, San Francisco, USA) with attB primers using a Bio-Rad thermal cycler (Bio-Rad, France). The attB PCR product was purified using 30% PEG 8000 and 30 mM $MgCl_2$.The entry clone was generated by cloning the attB PCR product into pDONR221 (Supplementary Figure S1a), by the BP recombination reaction using the BP Clonase™ II Gateway®, following the manufacturer's protocol to generate the entry clone containing the target gene (Gateway® Technology with Clonase II, Invitrogen, France). The BP recombination reaction was then transformed into *E. coli DH5α* competent cells by the heat shock method, as described by manufacturer's protocol. The transformed cells were plated on pre-warmed LB plates containing zeocin, 50 µg/ml, and incubated at 37 °C overnight. The next day positive entry clones appeared on the plates and then 3-4 positive entry clones were randomly selected for colony PCR. The positive colonies were inoculated in LB medium containing 50 µg/ml of zeocin and incubated overnight at 37 °C at 200 RPM. Plasmid DNA was isolated from the entry clones by using the plasmid DNA isolation kit (Promega, France). Plasmid DNA concentrations were assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, France). The approximate size of the entry clone was verified by running 2 µl of plasmid DNA on a 0.8% agarose gel in 1X TBE buffer along with GeneRuler 1 Kb DNA ladder (Thermo Scientific, France). To confirm the insert

for target gene, PCR was performed using the Takara master mix (Clontech, San Francisco, USA), with gene specific primers and M13 primers. The PCR product was examined by gel electrophoresis as described earlier. To generate the transformation vector, pEarleyGate 100 (Supplementary Figure S1b) (Earley et al., 2006), was obtained from ABRC (Columbus, OH, USA). The entry clone containing the complete sequence of the target gene and the *attL* sites was then introduced into pEarleyGate 100 containing *attR* sites by LR recombination reaction using the LR Clonase™ II Gateway® kit, following the manufacturer's protocol to generate the expression clone containing the gene of interest, (Gateway® Technology with Clonase II, Invitrogen, France). The LR reaction was transformed into *E. coli DH5α* cells and plated as described earlier. The approximate size of the expression clone was confirmed as previously described. The target gene was confirmed by performing a PCR using gene specific primers as mentioned earlier.



Supplementary Figure S1. Vectors used for cloning. **(a)** pDONR221 used for generating entry clone and **(b)** pEarleyGate100 (35S promoter) used for generating transformation vector.

B.1.3 Generation of transgenic lines expressing brown algal genes

The expression clone containing the target gene was transformed into *Agrobacterium* strain GV310 (pMB90) using the freeze and thaw method (Höfgen and Willmitzer, 1988). The transformed cells were diluted and plated on LB plates containing

50 µg/ml of kanamycin, and 50 µg/ml of gentamycin. Plates were incubated at 28 °C for 3 days. After 3 days, colony PCR was performed to confirm the insert, as mentioned earlier. The recombinant *Agrobacterium* strain carrying the gene of interest was transformed to flowering Col-0 (*Arabidopsis thaliana* (L.) Heynh, ecotype Columbia (Lehle, Round Rock, TX, USA) plants, using the floral dip method, as described by (Clough and Bent, 1998).

B.1.4 Selection of transformants and determination of homozygosity

The seeds collected from the transformed plants were surface sterilized using 2% sodium hypochlorite and stratified at 4 °C for two days. Seeds were placed on plates containing half strength Murashige and Skoog (MS) medium (Sigma, Ontario, Canada) (Murashige and Skoog, 1962), supplemented with 1% (w/v) sucrose and solidified with 0.4% Phytagel (w/v) and containing ammonium glufosinate 40 µg/ml. Plates were maintained at 22 °C in a 16-h light/8-h dark cycle. After one week only positive transformants survived and these were transferred on peat pellets for seed production. Seeds collected from the selected clones were allowed to self for 3 generations to obtain highly stable lines. In each generation segregation was tested by placing 100 seeds on half MS plate containing 40 µg/ml of ammonium glufosinate and positive 10-15 plants were transferred in soil to collect seeds. Expression of the transgene was tested in these plants by qRT-PCR (Quantitative Real Time Polymerase Chain Reaction) as described in later section.

B.1.5 Salinity stress tolerance

Arabidopsis thaliana (L.) Heynh, ecotype Columbia (Col-0) seeds were purchased from Lehle seeds (Round Rock, TX, USA). Seeds of overexpression lines and of wild type (Col-0) were grown as described earlier. After 4 days of growth, uniform seedlings were transferred on plates containing half strength Murashige and Skoog (MS) medium (Sigma, Ontario, Canada) and 100 mM NaCl, supplemented with 1% (w/v) sucrose and solidified with 0.4% (w/v) Phytagel. Plates were vertically stacked and maintained in the aforementioned standard growth conditions. Fresh weight of plants was recorded on the 9th day, then samples were dried in a hot air oven at 70 °C for 5 days and dry weight was

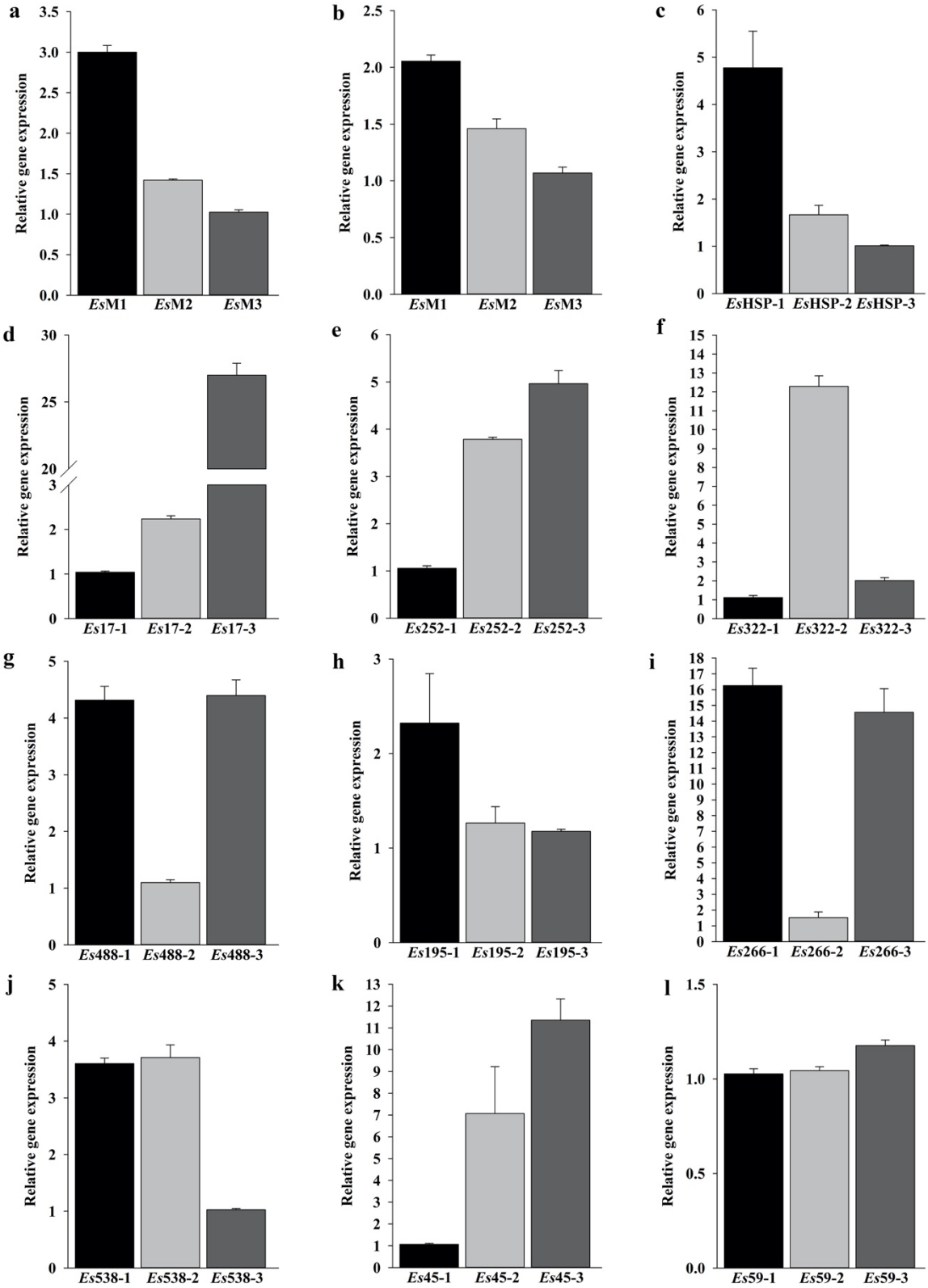
recorded. Data were statistically analyzed, using analysis of variance (ANOVA) followed by Tukey's post hoc test at $\alpha < 0.05$ in Minitab 18 (Minitab LLC, State College, Pennsylvania, US).

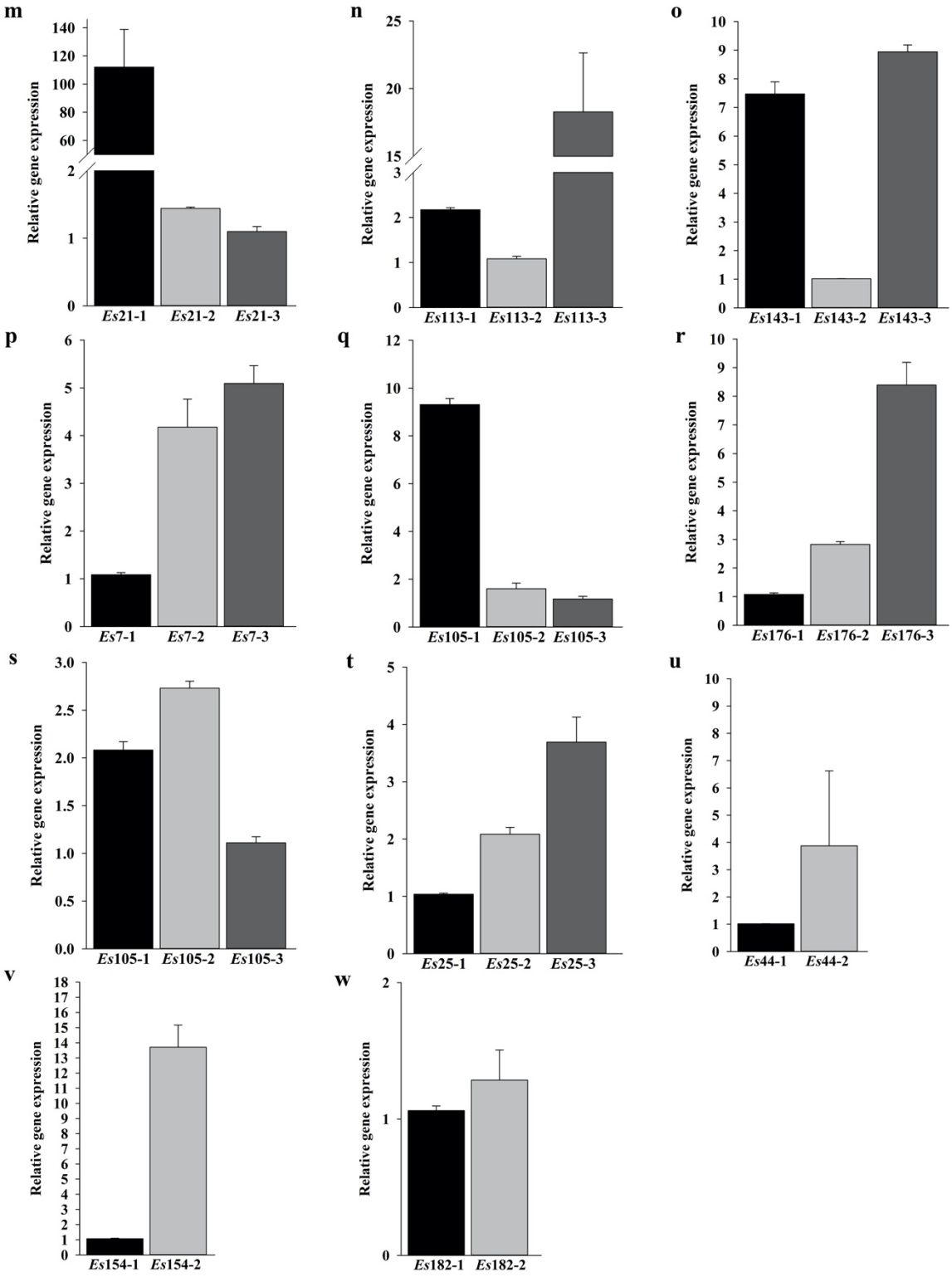
B.1.6 Real-time quantitative PCR of target gene in transgenic lines

To test the expression of target gene in transgenic plants the leaf samples were collected from 3 weeks old plants. Samples were flash frozen in liquid nitrogen and stored at -80 °C until processed. Samples were powdered in liquid nitrogen using a pre-chilled mortar and pestle. Approximately 60-80 mg of plant tissue sample was used for total RNA extraction using the method described by (Chomczynski and Sacchi, 2006) with some minor modifications. The alteration to the protocol was made at the step after adding the 500 µl of isopropanol. The change in the protocol was to incubate the samples at -20 °C for 15 minutes instead of 10 min at room temperature; this modification increased the yield and quality of RNA. The pellet was dissolved in 50 µl of DEPC (Diethylpyrocarbonate) water and gently mixed by using the bench top vortex. RNA concentrations were assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Ontario, Canada). Two micrograms of RNA were treated with 2 units of RQ1 DNAse (Promega, Ontario, Canada), according to the manufacturer's instructions, to eliminate any DNA contamination. The total volume of the reaction was made to 10 µl, using the DEPC water. The DNase-treated RNA was converted to cDNA, using an Applied Biosystems High Capacity cDNA Synthesis Kit (Applied Biosystems Ontario, Canada), according to the manufacturer's protocol. The cDNA working concentration was made to 10 ng/µl, and from that 3µl were used in qRT-PCR. The relative transcript levels were determined by Real–Time PCR using gene specific primers and *ACTIN* as the endogenous control, on the StepOne^{TM} Real-Time PCR system (Applied Biosystems, Ontario, Canada), using iTaq SYBR green mix (Bio-RAD, ON, Canada). Gene specific primer sequences were designed using Primer 3 Plus Software. The transcript levels were normalized to individual with lowest expression.

B.2 Results

Gene expression analysis showed that *Ectocarpus* sp. genes are highly expressed in most transgenic *A. thaliana* lines (Supplementary Figure S2). Preliminary screening analysis revealed that several transgenic lines exhibited improved tolerance to salinity stress when exposed to 100 mM NaCl. Transgenic seedlings had variable phenotypes. Some *Ectocarpus* sp. genes conferred significantly higher tolerance to salinity stress to *A. thaliana* transgenic lines, whereas the presence of some genes was not associated with any significant differences as compared to the wild type seedlings (Supplementary Table S6). The *Ectocarpus* sp. genes that were associated with significantly increased tolerance to salinity stress are listed in Supplementary Tables S7, S8 and S9 while those with limited or no effect on the tolerance to salt stress in Supplementary Tables S10 to S14. Note that some of the genes had obvious negative effects on plant growth in normal conditions, for example, *Esi0143_0016*, *Esi0182_0002* and *Esi195_0005* (Supplementary Table S11, S12 and S13), suggesting that these genes have toxic effects on land plants.


a
Relative gene expression
EsM1
EsM2
EsM3
b
Relative gene expression
EsM1
EsM2
EsM3
c
Relative gene expression
EsHSP-1
EsHSP-2
EsHSP-3
d
Relative gene expression
Es17-1
Es17-2
Es17-3
e
Relative gene expression
Es252-1
Es252-2
Es252-3
f
Relative gene expression
Es322-1
Es322-2
Es322-3
g
Relative gene expression
Es488-1
Es488-2
Es488-3
h
Relative gene expression
Es195-1
Es195-2
Es195-3
i
Relative gene expression
Es266-1
Es266-2
Es266-3
j
Relative gene expression
Es538-1
Es538-2
Es538-3
k
Relative gene expression
Es45-1
Es45-2
Es45-3
l
Relative gene expression
Es59-1
Es59-2
Es59-3


m
Relative gene expression
Es21-1
Es21-2
Es21-3
n
Relative gene expression
Es113-1
Es113-2
Es113-3
o
Relative gene expression
Es143-1
Es143-2
Es143-3
p
Relative gene expression
Es7-1
Es7-2
Es7-3
q
Relative gene expression
Es105-1
Es105-2
Es105-3
r
Relative gene expression
Es176-1
Es176-2
Es176-3
s
Relative gene expression
Es105-1
Es105-2
Es105-3
t
Relative gene expression
Es25-1
Es25-2
Es25-3
u
Relative gene expression
Es44-1
Es44-2
v
Relative gene expression
Es154-1
Es154-2
w
Relative gene expression
Es182-1
Es182-2

Supplementary Figure S2. Gene expression of *Ectocarpus* sp. genes in *A. thaliana* transgenic lines. **(a)** *EsiM1PDH1*, **(b)** *EsiM1Pase2*, **(c)** *Esi0379_0027*, **(d)** *Esi0017_0056*, **(e)** *Esi0252_0035*, **(f)** *Esi0322_0010*, **(g)** *Esi0488_0007*, **(h)** *Esi0195_0005*, **(i)** *Esi0266_0005*, **(j)** *Esi0538_0008*, **(k)** *Esi0045_0021*, **(l)** *Esi0059_0099*, **(m)** *Esi0021_0137*, **(n)** *Esi0113_0047*, **(o)** *Esi0143_0016*, **(p)** *Esi0007_0087*, **(q)** *Esi0105_0049*, **(r)** *Esi0176_0002*, **(s)** *Esi0105_0008*, **(t)** *Esi0025_0042*, **(u)** *Esi0044_0144*, **(v)** *Esi0154_0047* and **(w)** *Esi0182_0002*.

Supplementary Table S6. List of genes screened for salinity tolerance. Phenotype is based on biomass. *** represents >30% increase, ** represents >20% increase and * is no difference.

S. No.	Gene	Putative function	Phenotype
1	*Esi0379_0027*	Molecular chaperones HSP70/HSC70-like	***
2	*Esi0017_0056*	NAD and ADP ribose binding pockets	***
3	*Esi0154_0047*	Hydroxyacyl Coenzyme A dehydrogenase	***
4	*Esi0025_0042*	No conserved domains	***
5	*Esi0252_0035*	No conserved domains	***
6	*Esi0322_0010*	Lipid metabolism, putative acyltransferase	***
7	*Esi0045_0021*	No conserved domains	***
8	*Esi0488_0007*	ubiquitin like	**
9	*Esi0105_0049*	No conserved domains	**
10	*Esi0182_0002*	Tegument protein domain	**
11	*Esi0143_0016*	No conserved domains	*
12	*Esi0007_0087*	No conserved domains	*

S. No.	Gene	Putative function	Phenotype
13	*Esi0113_0047*	SAM-dependent methyltransferase	*
14	*Esi0059_0099*	DUF4419 domain unknown function	*
15	*Esi0538_0008*	Phox homologous domain	*
16	*Esi0021_0137*	Putative lipase	*
17	*Esi0195_0005*	No conserved domains	*
18	*Esi0176_0002*	Alpha/Beta hydrolase fold 1	*
19	*Esi0266_0005*	No conserved domains	*
20	*Esi0044_0144*	Unknown	*
21	*Esi0105_0008*	Unknown	*

Supplementary Table S7. Fresh weight and dry weight of the wild type and of the mannitol-producing (*Es*M) and *HSP70* transgenic seedlings, in the absence and presence of 100 mM NaCl. Values represents mean and standard error. Means and SE followed by the same letter are not significantly different.

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
	0 mM NaCl		100 mM NaCl	
WT	915.50 ± 20.86^{BC}	65.13 ± 1.44^{AB}	131.27 ± 11.87^{D}	13.93 ± 1.51^{C}
*Es*M1	1100.07 ± 24.87^{ABC}	77.90 ± 1.56^{A}	255.50 ± 12.76^{A}	25.27 ± 1.37^{A}
*Es*M2	998.67 ± 16.02^{ABC}	67.33 ± 1.36^{AB}	178.87 ± 1.69^{BC}	16.63 ± 0.32^{BC}
*Es*M3	1009.70 ± 38.08^{ABC}	69.93 ± 2.34^{AB}	237.43 ± 11.72^{A}	21.10 ± 0.79^{AB}
*Es*HSP1	851.87 ± 7.25^{C}	60.70 ± 0.72^{B}	168.27 ± 2.46^{CD}	18.37 ± 0.29^{BC}
*Es*HSP2	1136.47 ± 8.14^{AB}	76.20 ± 0.89^{A}	218.33 ± 8.68^{AB}	20.63 ± 0.99^{B}

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
*Es*HSP3	1235.27 ± 142.16^{A}	79.30 ± 7.25^{A}	257.33 ± 9.59^{A}	25.40 ± 0.53^{A}

Supplementary Table S8. Fresh weight and dry weight of the wild type and of the *Esi0017_0056*, *Esi0154_0047* and *Esi0025_0042* transgenic seedlings, in the absence and presence of 100 mM NaCl. Values represents mean and standard error. Means and SE followed by the same letter are not significantly different.

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
	0 mM NaCl		100 mM NaCl	
WT	1121.67 ± 135.74^{A}	80.30 ± 6.79^{A}	115.40 ± 10.21^{B}	10.67 ± 0.90^{B}
*Es*17-1	1118.47 ± 46.17^{A}	79.33 ± 2.06^{A}	182.50 ± 30.05^{A}	15.70 ± 2.63^{A}
*Es*17-2	1434.17 ± 8.84^{A}	101.67 ± 1.65^{A}	212.53 ± 23.37^{A}	20.53 ± 1.83^{A}
*Es*154-1	1299.13 ± 71.17^{A}	90.87 ± 2.95^{A}	187.13 ± 8.95^{A}	17.77 ± 0.86^{A}
Es 154-2	1237.40 ± 257.54^{A}	79.73 ± 11.17^{A}	135.40 ± 10.47^{AB}	14.33 ± 0.91^{AB}
Es25-1	1147.40 ± 32.50^{A}	88.87 ± 3.28^{A}	199.90 ± 7.97^{A}	20.17 ± 0.94^{A}
Es25-2	1331.20 ± 41.35^{A}	100.80 ± 2.96^{A}	158.47 ± 2.83^{AB}	15.67 ± 0.24^{AB}

Supplementary Table S9. Fresh weight and dry weight of the wild type and of the *Esi0322_0010*, *Esi0488_0007* and *Esi0105_0049* transgenic seedlings, in the absence and presence of 100 mM NaCl. Values represents mean and standard error. Means and SE followed by the same letter are not significantly different.

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
	0 mM NaCl		100 mM NaCl	
WT	1225.77 ± 42.45^{AB}	90.13 ± 1.57^{ABC}	86.07 ± 17.41^{B}	8.47 ± 1.51^{C}

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
*Es*322-1	1321.50 ± 84.10^{AB}	100.37 ± 4.20^{AB}	191.70 ± 8.79^{A}	19.50 ± 0.65^{A}
Es322-2	1395.53 ± 79.09^{AB}	99.53 ± 5.19^{AB}	151.63 ± 4.52^{AB}	14.90 ± 0.12^{AB}
Es488-1	1118.40 ± 51.35^{B}	80.27 ± 3.55^{C}	153.10 ± 7.69^{AB}	14.43 ± 0.50^{AB}
*Es*488-2	1506.60 ± 71.51^{A}	105.33 ± 2.91^{A}	185.27 ± 16.42^{B}	17.33 ± 1.04^{AB}
Es105-1	1368.17 ± 36.11^{AB}	101.90 ± 0.31^{AB}	146.80 ± 22.58^{AB}	14.33 ± 1.70^{AB}
Es105-2	1268.53 ± 109.37^{AB}	86.50 ± 5.95^{BC}	134.97 ± 12.66^{AB}	14.07 ± 1.07^{B}

Supplementary Table S10. Fresh weight and dry weight of the wild type and of the *Esi0113_0047*, *Esi0059_0099* and *Esi0538_0008* transgenic seedlings, in the absence and presence of 100 mM NaCl. Values represents mean and standard error. Means and SE followed by the same letter are not significantly different.

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
	0 mM NaCl		100 mM NaCl	
WT	827.77 ± 51.48^{BC}	54.73 ± 1.97^{AB}	151.20 ± 26.32^{BC}	15.20 ± 1.86^{AB}
Es113-1	1031.23 ± 13.42^{AB}	75.63 ± 2.36^{AB}	179.37 ± 6.66^{ABC}	16.90 ± 1.07^{A}
Es113-2	745.67 ± 54.25^{C}	60.77 ± 3.88^{AB}	83.80 ± 5.18^{D}	8.83 ± 0.38^{C}
Es59-1	833.03 ± 31.22^{BC}	61.33 ± 1.43^{AB}	119.93 ± 11.45^{CD}	11.33 ± 1.18^{BC}
*Es*59-2	1224.80 ± 17.87^{A}	111.73 ± 25.99^{A}	222.73 ± 10.69^{A}	19.77 ± 0.41^{A}
Es538-1	977.37 ± 100.82^{ABC}	50.67 ± 18.37^{B}	157.93 ± 6.29^{BC}	15.63 ± 0.52^{AB}
Es538-2	1216.50 ± 54.74^{A}	81.27 ± 2.30^{AB}	189.33 ± 5.26^{AB}	18.23 ± 0.69^{A}

Supplementary Table S11. Fresh weight and dry weight of the wild type and of the *Esi0143_0016* and *Esi0007_0087* transgenic seedlings, in the absence and presence of 100 mM NaCl. Values represents mean and standard error. Means and SE followed by the same letter are not significantly different.

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
	0 mM NaCl		100 mM NaCl	
WT	1130.37 ± 32.77^{A}	81.93 ± 1.82^{A}	121.97 ± 11.24^{BC}	14.27 ± 1.00^{A}
*Es*143-1	1024.27 ± 72.23^{AB}	76.27 ± 3.62^{AB}	142.77 ± 2.09^{ABC}	18.57 ± 0.32^{A}
*Es*143-2	785.93 ± 55.54^{B}	64.60 ± 3.15^{B}	133.83 ± 11.60^{ABC}	16.73 ± 1.33^{A}
*Es*143-3	1068.10 ± 20.66^{A}	79.53 ± 0.84^{A}	111.47 ± 5.79^{C}	14.53 ± 0.99^{A}
*Es*7-1	1289.90 ± 95.28^{A}	84.20 ± 3.20^{A}	167.20 ± 7.78^{AB}	18.47 ± 0.86^{A}
*Es*7-2	1196.60 ± 42.77^{A}	78.97 ± 0.80^{A}	150.57 ± 21.07^{ABC}	16.77 ± 1.32^{A}
*Es*7-3	1055.67 ± 21.68^{A}	75.33 ± 1.98^{AB}	176.47 ± 2.48^{A}	19.83 ± 6.26^{A}

Supplementary Table S12. Fresh weight and dry weight of the wild type and of the *Esi0182_0002* and *Esi0252_0035* transgenic seedlings, in the absence and presence of 100 mM NaCl. Values represents mean and standard error. Means and SE followed by the same letter are not significantly different.

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
	0 mM NaCl		100 mM NaCl	
WT	1048.57 ± 37.73^{BC}	93.70 ± 14.83^{A}	95.73 ± 10.65^{C}	9.57 ± 1.07^{D}
*Es*182-1	1236.70 ± 68.76^{AB}	92.67 ± 5.62^{A}	222.30 ± 5.27^{A}	23.13 ± 0.64^{A}
*Es*182-2	494.77 ± 44.22^{D}	46.97 ± 4.17^{B}	106.53 ± 5.22^{C}	11.63 ± 0.34^{CD}
*Es*182-3	341.40 ± 37.04^{D}	37.73 ± 3.99^{B}	134.50 ± 1.81^{BC}	13.27 ± 0.41^{BC}

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
Es252-1	1252.50 ± 38.86^{A}	84.40 ± 1.99^{A}	250.57 ± 9.28^{A}	23.87 ± 0.67^{A}
Es252-2	862.03 ± 15.78^{C}	64.53 ± 0.32^{AB}	151.20 ± 13.49^{B}	15.30 ± 1.20^{B}
Es252-3	903.20 ± 19.26^{C}	66.07 ± 0.82^{AB}	123.57 ± 3.43^{BC}	13.73 ± 0.47^{BC}

Supplementary Table S13. Fresh weight and dry weight of the wild type and of the *Esi0195_0005* and *Esi0045_0021* transgenic seedlings, in the absence and presence of 100 mM NaCl. Values represents mean and standard error. Means and SE followed by the same letter are not significantly different.

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
	0 mM NaCl		100 mM NaCl	
WT	712.60 ± 54.20^{BC}	51.70 ± 2.64^{CD}	67.77 ± 4.49^{E}	7.23 ± 0.43^{D}
*Es*195-1	861.07 ± 24.76^{AB}	59.97 ± 1.16^{C}	85.03 ± 2.87^{DE}	9.37 ± 0.20^{CD}
*Es*195-2	529.27 ± 82.31^{BC}	42.90 ± 5.75^{D}	77.27 ± 0.42^{DE}	9.03 ± 0.41^{CD}
*Es*195-3	476.13 ± 37.11^{C}	38.90 ± 2.33^{D}	105.73 ± 8.83^{CD}	10.90 ± 0.79^{BC}
Es45-1	728.80 ± 34.42^{BC}	63.27 ± 3.30^{BC}	116.33 ± 8.58^{BC}	13.00 ± 0.74^{B}
Es45-2	1092.03 ± 53.39^{A}	84.67 ± 0.63^{A}	165.23 ± 8.07^{A}	19.20 ± 0.70^{A}
Es45-3	1204.10 ± 147.24^{A}	77.45 ± 4.65^{AB}	140.10 ± 0.10^{AB}	17.15 ± 0.25^{A}

Supplementary Table S14. Fresh weight and dry weight of the wild type and of the *Esi0021_0137* transgenic seedlings, in the absence and presence of 100 mM NaCl. Values represents mean and standard error. Means and SE followed by the same letter are not significantly different.

	FW (mg)	DW (mg)	FW (mg)	DW (mg)
	0 mM NaCl		100 mM NaCl	
WT	1069.93 ± 72.57^{B}	72.83 ± 4.40^{B}	338.00 ± 40.63^{B}	28.67 ± 2.35^{B}
Es21-1	1427.37 ± 71.72^{A}	89.37 ± 3.49^{A}	347.30 ± 18.68^{B}	32.20 ± 0.72^{AB}
Es21-2	1204.70 ± 60.10^{AB}	74.00 ± 3.21^{AB}	484.87 ± 26.62^{A}	38.70 ± 1.22^{A}