Differential Regulation of Salt Tolerance Mechanisms in *Arabidopsis thaliana* and *Thellungiella halophila (salsuginea)*

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Declaration page

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ABSTRACT

High salt concentrations in soil are the leading cause of salt stress restraining crop production in different parts of the globe. It is anticipated that stresses from abiotic factors including salinity will result in over 50% decrease in average yield of major crops under current agricultural practices by 2050. Therefore, extensive work has been conducted during the last 20 years to understand the basic mechanisms for stress-tolerance to develop plants that can survive under extreme environmental conditions including salinity. The key mechanisms for salt-tolerance are now well known and they involve osmoregulation via increased production of compatible solutes (e.g. proline, glycine betaine), sequestration of salts in the vacuole, exclusion of salts by the roots and extrusion of salts from the roots and/or leaves as well as alleviation of the negative effects of salt-stress. It is becoming clear that these mechanisms are expressed in most plants, with differential and spatiotemporal regulation of the expression of these mechanisms being the key to the salt-tolerance trait. It is, however, not clear as to what is behind the differential expression of these mechanisms and the research already conducted in this field lacks detail in terms of the responses to salt-stress.

This project aimed at exploring in depth the differences in salt-responses shown by two close relatives, *Arabidopsis thaliana* (salt-sensitive) and *Thellungiella halophila* (salt-tolerant). It also aimed at understanding the regulatory processes behind the observed differential responses by exploring the regulation of genes playing key roles under salt-stress in the two plant species. Detailed analysis of the kinetics of responses to salt-stress were conducted in the two plant species including physiological responses (growth, photosynthesis), metabolic responses (production of osmoregulators, accumulation of sugars, uptake of salts), gene responses (*P5CS1* and *SOS1*) and role of regulatory components in *A. thaliana* null mutants (signalling elements and transcription factors). *T. halophila* showed faster and stronger responses to salt-treatment in the regulation of the accumulation of key compatible metabolites such as sucrose, fructose, inositol and proline compared to *A. thaliana*. The difference in proline accumulation between the two species was mirrored by *P5CS1* transcript abundance. Along with *P5CS1* gene the *SUS3*, *UGP2, FBA1* and *PPC1*genes showed higher transcript levels under saline conditions in *T. halophila*. Analysis of the *P5CS1* gene suggests the possibility of the presence of two isogenes in *T. halophila* as suggested by the promoter regions as well as the numbers of introns. Moreover differential splicing of the *P5CS1* transcripts under salt-treatment occurred between *T. halophila* and *A.*

thaliana. Finally targeted screening for potential key signalling elements (protein kinases*: NPK15, CPK11* and *ORG1*) and transcription factors (*Rp2.4f*) using *A. thaliana* null-mutants for these genes suggested these components had an indirect role in the regulation of the responses to salt-treatment, probably via the regulation of the metabolic background of the plant. The results suggest that along with differential gene regulation between glycophytes and halophytes, salt tolerance also depends upon the level of metabolic plasticity of the plant to mount rapidly appropriate responses to salt stress and the capacity of the plant to modulate the response.

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List of Abbreviations

ABA	Abscisic acid
ABREs	ABA response elements
AP1	Adapter primer 1
AP2	Adapter primer 2
ATP	Adenosine triphosphate
ARMS	Arabidopsis thaliana relative model system
Ca^{2+}	Calcium
Cl^-	Chloride
DHAP	Dihydroxyacetone phosphate
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
DMSP	Dimethylsulphopropionate
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FBA	Fructose-bisphosphate aldolase
FBP	Fructose 1,6-bisphosphatase
FW	Fresh weight
GA	Gibberellin
GC/MS	Gas chromatography/mass spectrometry
G3P	D-glyceraldehyde-3-phosphate
Glc-1-P	Glucose-1-phosphate
HKT	High affinity K^+ transporter
K^+	Potassium
KCl	Potassium chloride
KOH	Potassium hydroxide
HPLC	High-performance liquid chromatography

LC/MS	Liquid chromatography/mass spectrometry
MDH	Malic dehydrogenase
mM	Millimolar
mRNA	Messenger RNA
$MgSO_4$	Magnesium sulphate
$MgCl_2$	Magnesium chloride
nM	Nanomolar
Na^+	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAD	β-Nicotinamide adenine dinucleotide (sodium salt)
NCBI	National Center for Biotechnological Information
OD	Optical density
P5CS	Pyrroline-5-carboxylate synthase
P5CS1	Δ^1- Pyrroline-5-carboxylate synthase 1 gene
P5CS2	Δ^1- Pyrroline-5-carboxylate synthase 2 gene
PCR	Polymerase chain reaction
PDH	Proline dehydrogenase
PEG	Polyethylene glycol
PEPC	Phosphoenolpyruvate carboxylase
PLACE	Plant Cis-acting Regulatory DNA Elements
PSII	Photosystem II
Q-RT-PCR	Quantitative reverse transcriptase polymerase chain reaction
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
RE	Response element
SOS	Salt overly sensitive
SOS1	Salt overly sensitive 1 gene
SOS2	Salt overly sensitive 2 gene
SOS3	Salt overly sensitive 3 gene
SUSY	Sucrose Synthase

TBE	Tris/Borate/EDTA
UBQ10	Ubiquitin 10
UDP-Glc	UDP-Glucose
UGP	UDP-Glucose pyrophosphorylase
WC	Water content

Chapter 1

Introducing Salt stress

1.1 Introduction

According to United Nations population estimates, the World Population stood at 7.2 billion people in 2012 and is projected to exceed 9 billion by 2050 (United Nations, 2012). The projected population increase implies that food production has to increase with same level to feed increasing number of mouths. Strategic studies show that 57% more food production is required by 2050 from the same land area available now due to low possibility of cultivated-land extension (Wild, 2003). A major threat to food production is abiotic stress conditions, e.g. drought and salinity that are prevailing and increasing over vast land-area across the Globe (Wang et al., 2003). Increasing saline conditions in the major agricultural regions will make about 50% of the arable land saline by 2050; thereby adversely affecting the production of major crops making it impractical to fulfill the needed food production (Mahajan and Tuteja, 2005). To meet these challenges, it is required to select/develop crops with increased salt tolerance from relatively new genetic sources and to use biotechnological tools to improve existing crops in terms of their salt-tolerance capacities. For this end, it is vital to have a thorough understanding of the different adaptation mechanisms that have evolved in many plants to cope with salinity and to have a good knowledge of the regulation of these mechanisms. Unfortunately most studies to understand the physiological and molecular responses to salt-stress mechanisms have been conducted in salt-sensitive plants like *A. thaliana* and studies in salt-tolerant plants are needed to get a better insight into these mechanisms. Adaptation to saline conditions is found in species from different lineages as a result of convergent evolution; similar changes have taken place in phylogenetically unrelated species resulting in the salt tolerance trait. This suggests that the genetic changes that occurred in the salt-sensitive ancestors of salt tolerant species are relatively simple. Thus, our aim is the comparison of salt-tolerant plants with salt-sensitive relatives that can lead to a better and more comprehensive understanding of the mechanisms underpinning salt-tolerance. In addition, this will provide the means and tools for genetically engineering salt-tolerance in various salt-sensitive crop plant species by incorporating these mechanisms or their regulation into salt sensitive crop plants. This chapter focuses on introducing the problem of salinity as an abiotic stress on plants, the responses exhibited by the plants, and will give the hypothesis upon which the research described in the thesis was based as well as the scope and the structure of the research conducted.

1.2 Effects of salinity on plants

Presence of excessive amounts of salts in soil or water or both causes salinity (salt-stress). Salt stress is most commonly caused by high amounts of Na^+ and Cl^-, and results in a threefold effect on plants. Salinity decreases the water potential of the soil reducing water uptake by the roots, disrupts the ion homeostasis of the cell causing ionic imbalance between the different cellular compartments, and finally inhibits many enzymes causing toxicity. The distorted water status of the plant leads to limited initial growth and altered development weakening plant productivity. The suppression of plant growth is directly related to both the osmotic and ionic stress caused by salinity (Greenway and Munns, 1980; Hayashi and Murata, 1998). Growth suppression takes place in all plants but the extent of suppression varies due to different salt tolerance levels and lethal salt concentrations vary among different plant species. Inhibition of cell division and expansion and acceleration of plant cell death result from the processes following the change in the water status of the cell (Hasegawa et al., 2000). Important plant processes like photosynthesis, protein synthesis and lipid and energy metabolism are all affected by salinity stress at varying levels, directly or indirectly leading to growth reduction. These effects are discussed under separate headings below.

1.2.1 Effect on growth

Salinity immediately reduces the rate of leaf surface expansion and with increasing salt-concentration may lead to its cessation (Wang and Nil, 2000). Also there is a clear stunting of plants as a result of salt stress (Takemuraa et al., 2000). The mangrove *Rhizophora mucronata* shows optimal growth at 50% seawater salt concentration but with increase in salinity its growth rate declines drastically (Aziz and Khan, 2001). In some plants fresh and dry weights increase with salinity up to 200 mM NaCl but with further increase in salinity they show the opposite (Aziz and Khan, 2001). Salt stress greatly affects the fresh and dry weights of leaves, stems and roots (Chartzoulakis and Klapaki, 2000). According to experimental studies performed on two mangrove species, *Bruguiera parviflora,* a salt non-secretor, and *Aegiceras corniculatum,* a salt secretor, there is a significant difference in the lethal concentrations of NaCl for the two species. *B. parviflora* can grow optimally at 100 mM NaCl, but dies at 500 mM NaCl, whereas *A. corniculatum* can tolerate up to 250 mM NaCl and 300 mM NaCl is a lethal dose (Parida et al., 2004). Increasing salinity is usually correlated with identifiable reductions in shoot weight, plant height, and root length, number of leaves per plant and root surface area with varying degrees of amplitude depending on the plant species.

1.2.2 Effect on water relations

With increase in salinity, plants' water and osmotic potential decrease but the turgor pressure can increase (Romero-Aranda and Soria, 2001). According to Aziz and Khan (2001), in *Rhizophora mucronata* there is a decrease in leaf osmotic and water potential but an increase in xylem tension with increase in salinity. In the case of jute plants short term exposure to salinity decreases relative water content, leaf water potential, water uptake, water retention, transpiration rate and water use efficiency (Chaudhuri and Choudhuri, 1997). The mode of salt application and the water potential of the rooting medium influence the decline of the leaf water and osmotic potential. In the case of prolonged or progressive NaCl stress, a greater decline is noticed in osmotic potential when compared to total water potential that leads to the maintenance of turgor pressure, in plants (Rajasekaran et al., 2001)

1.2.3 Effect on leaf and cell anatomy

In plant species like bean and cotton, increase in salinity increases epidermal thickness, mesophyll thickness and spongy cell diameter in leaves (Longstreth and Nobel, 1979). In contrast, *Bruguiera parviflora*, a mangrove species, experiences reduction in both epidermal and mesophyll thickness, as well as a decrease in intercellular spaces when treated with high levels of NaCl (Parida et al., 2004). According to Delfine et al. (1998), salinity greatly reduces the intercellular spaces in leaves of plants. Leaves of sweet potato under salt stress face major adverse effects like vacuolation development and partial swelling of the endoplasmic reticulum, mitochondrial swelling and decrease in mitochondrial cristae, tonoplast fragmentation and vesiculation and cytoplasm degradation (Mitsuya et al., 2000). In another study rounding of cells, reduction in chloroplast number and reduction in intercellular spaces were reported (Bruns and Hecht-Buchholz, 1990). In addition reduction in stomatal density and leaf surface area were linked to salinity in plants like tomato (Romero-Aranda and Soria, 2001).

1.3 Salinity and stages of plant development

According to Bernstein and Hayward (1958), plant tolerance or sensitivity to salinity varies from one developmental growth stage to the next. Plants reaching maturity are generally more tolerant to salinity. Also most annual crops show tolerance to salinity at the germinating stage but are sensitive during emergence and the early vegetative stage, as indicated by many studies, though with some exceptions (Läuchli and Epstein, 1990; Maas and Grattan, 1999). This is generally true but it is important to take into consideration the way salt-tolerance is assessed at the different developmental stages. Whereas survival percentage forms the basis of tolerance during the emerging and germinating stages, during the later vegetative growth stages tolerance is assessed on the basis of relative growth reductions (Maas and Hoffman, 1977).

In the view of agronomists and horticulturists, salt tolerance is directly based on the yield of the harvested organs, which can be grain, fibre, fruit, shoot, leaf, root or stem, with reference to that obtained in a non-saline environment. Salt stress adversely affects both vegetative and reproductive stages, leading to intense implications for the harvestable organs. In the vegetative organs salt stress leads to reduction in shoot and root growth with shoot growth being more affected than root growth (Läuchli and Epstein, 1990). In the reproductive organs, it results in reduced number of florets, increased sterility, delayed flowering and maturity in both *Triticum aestivum* (wheat) and *Oryza sativa* (rice) (Maas and Poss, 1989 b; Khatun et al., 1995). Therefore to lessen the salt stress at crucial times, management strategies should be developed with elaborated and proper understanding of effects of salinity at vegetative and reproductive growth developmental stages.

1.3.1. Germination and emergence

Plants are generally more tolerant to moderate salt stress at germinating stage than at later stages with the unavoidable salt effect resulting in delayed seed germination but generally without reducing the number of germinated seeds compared to in absence of salt (Maas and Poss, 1989 b). With increased salinity the germination is not only delayed but also there may be loss of germination depending upon type of species or cultivar. This is highlighted in Figure 1.3.1, which shows the relation between percentage of seed germination and time. According to Carter et al. (2005) germination of the seeds of a commercially grown ornamental flower, *Limonium perezii,* is stimulated with salinity up to 100 mM NaCl, whereas during vegetative stages salinity

up to 60 mM NaCl results in reduced stem length, greatly affecting the quality and marketability of the product. In most circumstances of high salinity, there is a delay in seed germination but also ultimately a lower percentage of seed germination as seen in globe artichoke (Mauromicale and Licandro, 2002). Sugar beet, which is categorized as salt tolerant, is sensitive to salinity to some extent during germination, in contrast to other important crops (Läuchli and Epstein, 1990). Therefore, assessment of salt tolerance trait among plants during germination stage gives incomplete information about salt tolerance of a species. Most studies about salt effects on germination are conducted using petri dishes where seeds are germinated on filter paper saturated with salt solutions or agar containing salt as well as on hydroponic solutions with varying salt concentrations. These conditions make it easier for the observation but these artificial environments can never replicate field conditions (Esechie et al., 2002). In addition inter - crop or inter-stage comparisons can be limited due to various other variables like permeability to water, seed coat pretreatment and seed dormancy, which differ between species.

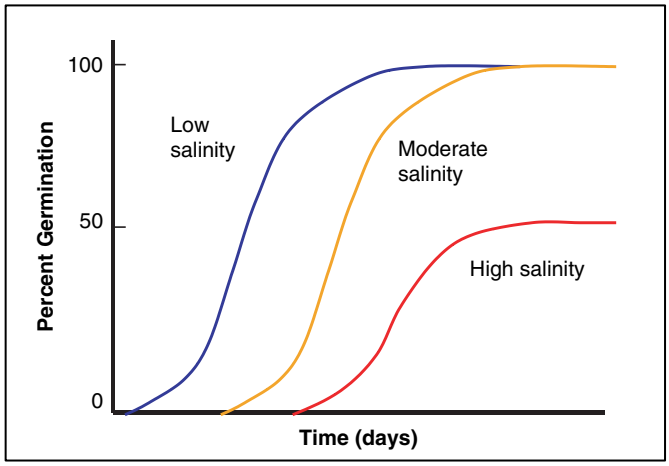


Figure 1.3.1 Generalized relationship between percent germination and time after water addition at low, moderate and high salinity. Although salinity delays germination, high salt conditions will ultimately lower the percentage of germinated seeds, which varies among species and cultivars. (Lauchli and Grattan, 2007).

According to Lauchli and Grattan (2007), most crops at germination are sensitive to salinity during emergence. Various root media have been used under different environmental conditions to assess salinity effect on emergence, which makes the interpretation and comparison of results very complicated, if not impossible. One of the biggest issues during these studies is the use of NaCl as the only salinizing agent, which when used with mineral soil medium leads to sodicity (high sodium relative to calcium plus magnesium). This adds undesirable stresses to the emerging seedling by adversely

affecting the soil physical conditions, with reduced O_2 diffusion rate and increased soil strength (Grattan and Oster, 2003).

Emergence is delayed by salinity and high enough salt stress can lead to compact stand establishment (Maas and Grattan, 1999). Emergence stage tolerance to salinity among different crops varies and like germination stage, crop tolerance at emergence cannot be predicted according to yield production. According to Grattan and Oster (2003), cotton, a salt resistant crop based on lint yields, is especially prone to poor stand development when fields are irrigated with saline-sodic water. This poor stand establishment can lead to reduced lint yields.

In addition to salt stress, under field conditions there are various abiotic stresses faced by the seedling. Water stress (Katerji et al., 1994), diurnal changes in soil surface crusts and soil temperature and evaporation and capillary rise resulting in fluctuating salinities (Pasternak et al., 1979) are the various conditions near the soil surface that the young seedlings are subjected to. According to Vinizky and Ray (1988), salinity is more damaging to the young germinating seedlings when they are outside their optimal range of temperature required for germination. Also, injuries to hypocotyl and cotyledon become more evident for the young seedlings when salinity delays the stages of germination and emergence (Miyamoto et al., 1985; Esechie et al.,, 2002). Percentage of emerged seedlings can reduce drastically due to these unavoidable combinations of stresses under field conditions but at present there is a gap in research regarding salinity tolerance at this level. Integrated research taking into account the different biotic and abiotic stresses can lead to better understanding of seedling tolerance during germination and emergence.

1.3.2. Vegetative growth

In comparison with germination stage, plants are more sensitive to salinity during the seedling and early vegetative growth stages of development. Examples are barley (Ayers et al., 1952), corn (Maas et al., 1983), cotton (Abul-Naas and Omran, 1974), cowpea (Maas and Poss, 1989 b), melon (Botia et al., 2005), New Zealand spinach, red orach (Wilson et al., 2000), rice (Pearson and Ayers, 1966), sorghum (Maas et al., 1986), tomato (Amor et al., 2001), and wheat (Maas and Poss, 1989 a). An experiment conducted in greenhouse conditions with wheat and corn showed that the effect of salinity on total shoot biomass was far less than the overall effect on relative grain yields (Maas et al., 1983; Maas and Poss, 1989 a). But this does not hold true for all the

crops, as it was found that salt tolerance of melon cultivars during early seedling stages directly correlated to the salt tolerance trait measured on the basis of fruit yield (Nerson and Paris, 1984).

1.3.3. Roots

Generally salinity results in a strong reduction of root growth. However, under saline conditions an ample supply of calcium results in reduced shoot growth more than root growth, especially in leaf area (Läuchli and Epstein, 1990). But root growth and membrane functions can be adversely affected within minutes by the presence of inadequate Ca^{2+} concentrations under saline conditions (Epstein, 1961; Läuchli and Epstein, 1970; Cramer et al., 1988). According to Kurth et al. (1986), cotton root cell elongation was favoured over reduced radial cell growth, and cell production rates were maintained when saline medium was supplied with Ca^{2+}. Further studies with cotton roots indicated that supplemental Ca^{2+} inhibited elongation rate but reduction of the root growth zones by salt stress was not restored with supplemental Ca^{2+} (Zhong and Läuchli, 1993). Na^{+} deposition rate was increased due to high salt stress in the growing regions of roots that resulted in decreased selectivity for K^{+} vs. Na^{+} but later was then partially alleviated by supplemental Ca^{2+}, especially in the apical 2 mm region (Zhong and Läuchli, 1994). The conclusion of these studies is that supplemental Ca^{2+} alleviates the inhibitory effect of salt on cotton root growth by maintaining plasma membrane selectivity of K^{+} over Na^{+} (Lauchli and Grattan, 2007).

1.3.4. Shoots

Stunted shoots and reduced leaf area are usually the results of reduction in shoot growth due to salt stress (Läuchli and Epstein, 1990). Both cell elongation and cell division determine the final leaf size. In the case of sugar beet, it was found that cell division was unaffected by salinity whereas leaf extension was sensitive to salinity (Papp et al., 1983). Thus, cell division in leaves of sugar beet appears less salt sensitive than cell elongation. Instead, grass leaves showed reduced cell numbers under saline conditions (Munns and Termaat, 1986). As shown before in roots, supplemental Ca^{2+} can partly lessen the effect of salinity on shoot growth among various species (Läuchli and Epstein, 1990; Cramer, 2002). According to Maas and Grieve (1987), if plants are exposed to high Na^{+}/Ca^{2+} ratios, Ca^{2+}-deficiency in the shoot can be induced, as exhibited in developing corn leaves. In the growing regions of leaves, Ca^{2+} status is especially sensitive to salinity (Läuchli, 1990). This seems to be the result of inhibition of loading of symplastic Ca^{2+} in the xylem in the roots by salinity (Lynch and Läuchli, 1985;

Halperin et al., 1997), which further decreases the Ca^{2+} status in growing region of leaves (Lauchli and Grattan, 2007). The inhibitory effect of high Na^{+} concentrations on growth was counteracted by adding approximately 5 – 10 nM Ca^{2+} to the medium for salinities of 100-150 mM NaCl (Cramer, 2002; Munns, 2002) confirming the observations of LaHaye and Epstein (1971) on the significance of supplemental Ca^{2+} to reduce salt stress effects in the shoot.

Cramer (2002) reported that Ca^{2+} signaling and Na^{+}-Ca^{2+} interactions taking place at the surface of the plasma membrane can be associated with the well-known Na^{+}-Ca^{2+} interactions in plants (Cramer et al., 1985). Ion activities must be used instead of ion concentrations to quantify these Na^{+}-Ca^{2+} interactions (Lauchli and Grattan, 2007). Due to the formation of ion pairs and precipitation as calcite, ion activities for particularly Ca^{2+} are reduced as compared to their concentrations (Cramer and Läuchli, 1986).

In particular, the younger region of the leaf showed reduction in maximal relative elemental growth rate with a 20% decline in the length of the growth zone when leaf growth and development in sorghum was quantitatively studied in response to salt stress (Bernstein et al., 1993 a). Increase in the relative elemental growth rate and length of the growing zone was observed when leaf was supplied with external Ca^{2+} (Bernstein et al., 1993 b). This contradicts the work done on cotton roots where supplemental Ca^{2+} did not shorten the growing zone of the cotton roots (Zhong and Läuchli, 1993). The length of the elongation zone was not affected by salt stress in barley leaves but Ca^{2+} supply to the plant did not differ in this work (Fricke and Peters, 2002). In the growing sorghum leaf, Ca^{2+}, which is partially responsible for leaf growth, is greatly reduced by salt stress (Bernstein et al., 1995). Salt stress does not affect the growth of the leaf when sodium is accumulated in the basal part of the growing zone, which suggests that the growth inhibition was not caused by Na^{+} concentrated in the salt-affected leaf tissue (Bernstein et al., 1995). A similar work on growing wheat leaves has investigated the direct effects of Cl^{-} and Na^{+} toxicity on cell expansion and formation of the leaf cross-sectional area (Hu et al., 2005a). In growing leaves of wheat (Hu et al., 2005b) and sorghum (Baum et al., 2000), the area of proto and metaxylem decreased under salinity, which might be responsible for reducing water movement into the growing part of leaves. In growing leaves, this might indirectly affect the transport of nutrient ions including Cl^{-} and Na^{+}.

1.3.5. Reproductive growth

Numerous investigations have suggested that a plant becomes more tolerant to salinity as it grows older, immediately after the salt-sensitive early-vegetative growth stage (Läuchli and Epstein, 1990; Maas and Grattan, 1999). Work on growth responses of crops to salinity at different developmental growth phases has shown that while some plants do not show any response to salinity during their entire lifespan, some crops show different responses to salinity at different growth stages until harvest. Recirculating sand tanks were used to conduct such studies, which made salinity conditions readily controllable. Crops like sorghum (Maas et al., 1986), cowpea (Maas and Poss, 1989 a) and wheat (Maas and Poss, 1989b) were found to be sensitive in their early reproductive phase while they were less sensitive in their flowering and seed-filling phase.

1.4 Halophytes; how do they differ from glycophytes?

Salt stress to plants results in both hyper-osmotic and hyper-ionic responses that can cause plant death. This is caused by the high concentrations of Na^+ and Cl^- ions in the soil adversely changing the water status of the plant, which brings the initial growth reduction. Later stress results in inhibition of cell division and expansion and due to the sum of all subsequent processes salinity can result in accelerated cell death (Niu et al., 1995; Yeo, 1998). These characteristics hold true in the case of glycophytes (for example *A. thaliana*), plants which are salt-sensitive and are easily damaged by moderate to high salinity (Glenn and Brown, 1999). But on the other hand, halophytes for their optimal growth require an electrolyte (Na^+ or Cl^-) concentration that is much higher than those found in non-saline soils. It is a difficult task to identify how and within what range of NaCl concentrations they respond best (Greenway and Munns, 1980; Glenn and Brown, 1999). Then there are some plants, which are termed salt tolerant non-halophytes and have moderate salt tolerance capabilities. Examples include *Triticum aestivum* (wheat) and *Solanum lycopersicum* (tomatoes). Plants that survive to reproduce in high salt environments (around 200 mM NaCl or more) are called halophytes. *Thellungiella halophila* (salt cress), *Salicornia bigelovii* (dwarf glasswort) and *Mesembryanthemum crystallinum* are a few of the halophytes (Flowers and Colmer, 2008). *Thellungiella halophila* has a short life cycle and is a small winter annual crucifer. It is an extremophile, indigenous to harsh environments. It is capable of abundant seed production in extreme salinity (500 mM [NaCl]) and can withstand cold to −15°C. When compared to *A. thaliana*, stomata of *T. halophila* are distributed on the leaf surface at higher density, but are less open. Plants counter the salt stress by closing them more tightly than *A. thaliana*. Roots in *T. halophila* grow both an extra endodermis and cortex cell layer compared to *A. thaliana* (Inan et al., 2004).

Halophytes survive and flourish in highly saline conditions as osmotic adjustment is possible through intracellular compartmentalization of toxic ions, which keeps them away from the working cytoplasm via energy-regulated transport into vacuoles (Niu et al., 1995; Apse et al., 1999; Glenn et al., 1999; Hasegawa et al., 2000). This process of compartmentalization is not different from many glycophytes and also osmotic adjustment in both happens through accumulation of organic solutes. But some halophytes exclude Na^+ and Cl^- through bladders and glands, which are specialized structures resulting from evolutionary changes that give halophytes an edge over glycophytes in exhibiting higher salt tolerance (Niu et al., 1995; Yeo, 1998). Another

different feature of halophytes is that they readily take up Na^+ ions and roots have much lower ion concentrations than the rest of the plant. On the other hand glycophytes restrict ionic movements to the shoots by tight regulation of the ion influx into the root xylem (Adams et al., 1992; Hasegawa et al., 2000). However, the critical difference between glycophytes and halophytes is the ability of the latter to resist and survive salt shock. In the recent past many studies have examined the responses of halophytes to a sudden change in external salinity. While halophytes take up to 24 to 48 hours to adjust to the sudden change in saline environment, they have the capability to quickly establish a steady state to achieve optimal growth in saline conditions (Hasegawa et al., 2000).

1.5. Salinity Tolerance

1.5.1 Definition of salt tolerance

The inherited capability of plants to face or survive the adverse effects of high salt concentrations in the root zone or on the plant's leaves is referred to as the plant's salt tolerance. To conduct research on salinity two basic guidelines were established: (1) for a particular crop plant, the tolerance levels to salinity will change depending upon the growth stage at which salt stress is applied and also the final level of concentration of salt achieved; (2) the organ of the plant to be commercially used should be considered when stating the salt tolerance values (Lunin et al., 1963). Salinity tolerance is a very complex, quantitative genetic character controlled/regulated by various genes which function in conjunction with other genes involved in general cellular metabolism as well as genes involved in stress responses in general (Shannon and Noble, 1990).

1.5.2 Plants vary in salt tolerance

The majority of the plant species found on earth are glycophytes, which are salt sensitive plants and fairly easily damaged by moderate to high salinity. Only a few plant species (approximately 2%) are halophytes, which can tolerate and might show enhanced growth under high saline conditions (Glenn and Brown, 1999). Observed growth responses in different plants show huge variation in salt tolerance. Barley (*Hordeum vulgare*) is the most tolerant and rice (*Oryza sativa*) is the most sensitive among the different cereals. Durum wheat (*Triticum turgidum ssp. durum*) is less salt tolerant than bread wheat (*Triticum aestivum*), a moderately tolerant species (Munns and Tester, 2008). Among the monocots, a halophytic relative of wheat, *Agropyron elongatum* (tall wheatgrass), is most salt tolerant and shows optimal growth at salinity levels even higher than those of seawater. Among the dicots, the degree of variation exceeds that observed in monocots. Whereas some of the legume species are far more sensitive to salt stress than rice (Lauchli, 1984), a variety of *Medicago sativa* (alfalfa) is very tolerant and halophytes like saltbush can show profound growth at levels of salinity higher than seawater. In some cases, halophytes from dicot species need high concentrations of salt to grow at optimum rate (Flowers et al., 1977). *Arabidopsis thaliana* is less salt tolerant than other plant species grown in similar conditions at high transpiration rates. Analysis of this sensitive species does not expose the unsolved molecular mechanisms underpinning salinity tolerance but when compared with the highly tolerant halophytic *Thellungiella halophila* under similar salt concentration of

100 mM NaCl at high transpiration, key differences between the two species are brought about, providing an invaluable resource for understanding salt-tolerance (Kant et al., 2006; Sickler et al., 2007).

1.6. Salinity Tolerance Mechanisms

Nutritional ailments, osmotic effects and ion toxicity are the various adverse effects imposed by salinity on plants (Lauchli and Epstein, 1990). Taking into account variable factors like salinizing agents, plant age, ionic concentration, genotype and species, the extent of salinity effects varies greatly. These effects make plants suffer various characteristic changes from the salt stress at different developmental stages until maturity (Munns, 2002). Within minutes of exposure to salinity plant cells lose water and shrink due to dehydration and may take a few hours, depending upon species, to regain volume (Munns, 2002). Lower rates of leaf and root growth become apparent within a few days as a consequence of reduced cell elongation and cell division, which may result in fewer leaves and leaves of smaller size. Plants subjected to high salt concentrations may also suffer from visual injuries due to excessive salt uptake. The differences in overall health and growth of stressed and unstressed plants become clear after weeks of exposure to salinity.

To recognize these temporal differences in salt stress responses, the two-phase response to salinity has been established (Munns, 2002; Munns and Tester, 2008), where physiological mechanisms required for tolerance need to be separated for their better understanding. In simple terms, growth reduction suffered by plants due to salinity occurs in two phases: a quick or immediate response to the increased external osmotic pressure (osmotic phase) and a delayed or lengthier response due to accumulation of sodium ions in leaves (toxic/ionic phase) (see Figure 1.4.1). In the first osmotic phase, within minutes from the exposure to salinity, growth reduction happens as an immediate response. It is due to the osmotic effect that alters the cell-water relations in the root cortex. The plant suffers similar stress to water-stress caused by water deficit, where reduced ability to absorb water leads to an initial decrease in leaf growth. After the initial growth reduction, according to the salt concentration outside the roots, a steady growth rate recovery is initiated after several minutes to reach a new stable state (Munns, 2002). In the second phase, salt accumulates to higher toxic levels in older leaves, which stop expanding, therefore cannot dilute the accumulated salts unlike the younger leaves. This salt toxicity takes days, weeks or even months to develop in leaves making it a much slower effect than the osmotic phase. Premature death of leaves reduces the total photosynthetic leaf area, causing reduced production of photosynthates and resulting in overall imbalance of carbon in plants to maintain optimum growth rate (Munns and Tester, 2008). Sodium and chloride ions chiefly

accumulate in the older transpiring leaves for a prolonged period of time that leads to lethal salt toxicity and leaf death. This untimely death due to salinity occurs when the threshold for the salt compartmentation into the vacuoles is reached, resulting in increasing salt concentration to toxic levels in the cytoplasm (Munns and Termaat, 1986; Munns, 2002; Munns, 2005; Munns et al., 2006). Plant survival depends on the difference between the rate at which leaves die, thus reducing the total photosynthetic leaf area, and the rate at which new leaves are produced so there are enough photosynthesizing leaves for plants to flower and produce seeds.

The osmotic phase not only has a quick and immediate effect on plants but a greater effect on growth than the ionic stress. Ionic stress becomes profound much later and more slowly than the osmotic effect and becomes more dominant only during exposure to high/toxic saline concentrations or when plants have reduced ability to transport Na^+. An increased tolerance to both phases will better the chances of a plant to show increased growth rate throughout its life cycle when subjected to salt stress

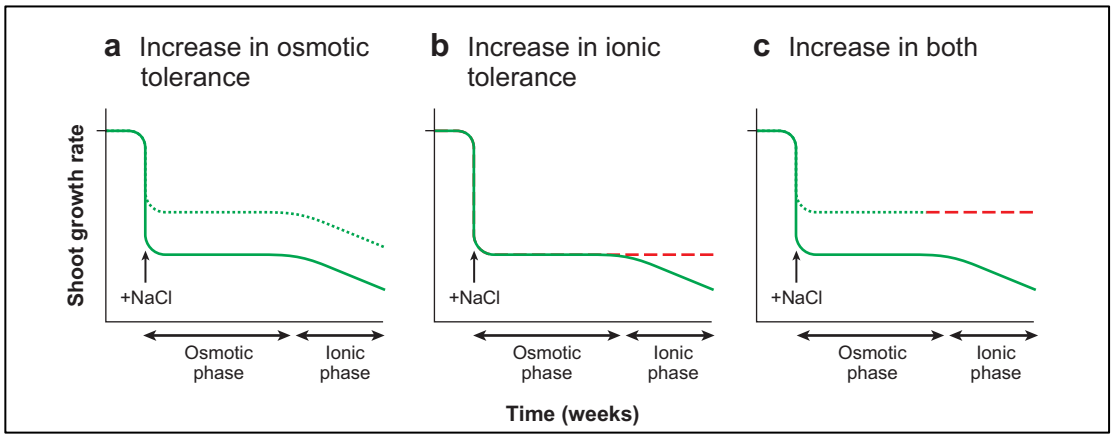


Figure 1.4.1: The two phases of growth responses to salinity stress: the osmotic phase, which is a prompt response to the increase in external osmotic pressure due to salinity, and the ionic phase, which is a delayed response due to the accumulation of sodium ions in leaves. The solid green line is the change in the growth rate after NaCl was added. (*a*) The broken green line is the expected response of a plant with an increased tolerance to the osmotic element of salt stress. (*b*) The broken red line is the expected response of a plant with an increased tolerance to the ionic element of salt stress (*c*) The green-and-red line is the expected response of a plant with increased tolerance to both the osmotic and ionic elements of salt stress. (Munns and Tester, 2008)

1.6.1 Osmotic stress tolerance

Growth

Increase in salinity creates an osmotic effect around the roots, which leads to a decreased rate of growth. Salinity with a moderate amount of stress decreases lateral shoot development over a period of weeks and if continued for over a month can increase adverse effects on the reproductive system. These responses include early flowering and also a reduced number of florets. Younger leaves are produced regularly but accompanied by the death of the older leaves, which makes the changes caused by osmotic effect similar to drought stress. Due to the presence of salt outside the roots, there is a reduced leaf development. To prove this various experiments have been conducted using single salts like KCl (Yeo et al., 1991), nonionic solutes such as PEG and concentrated Hoagland's solution (Termaat and Munns, 1986; Yeo et al., 1991; Sumer et al., 2004), which showed similar damaging effects on plants to that caused by NaCl on leaf expansion.

A subject of debate is that down-regulation of growth rate in leaves and shoots is supposed to be controlled by mechanisms regulated by various hormones or their precursors through long distance signaling because leaf area size is independent of water status and carbohydrate supply (Fricke and Peters, 2002; Munns et al., 2000). Abscisic acid (ABA) accumulates during osmotic stress and acts as an important factor in cellular signaling to regulate growth and stomatal conductance (Zhu, 2002; Davies et al., 2005). This was however contradicted by Makela (2003) and Voisin (2006) who have shown that ABA deficient mutants of maize and tomato had no difference in leaf growth rate when compared to their wild type counterpart when subjected to salt stress (Makela et al., 2003; Voisin et al., 2006). This leads to another key factor that can be involved in limiting the growth, gibberellins (GAs). In their review, Munns & Tester (2008) concluded that it was unclear how ABA affected leaf elongation, but that GA might be involved. Also results from different research on the same issue showed that DELLA proteins, which are negative growth regulators, act as mediators to integrate signals from a range of hormones involved in salinity (Achard et al., 2006). Thus DELLA proteins can hold the key in the coordination for adapting plant growth in different environments.

Stomatal conductance and photosynthesis

Roots in saline soils come under great osmotic effect that readily and most dramatically induces stomatal closure in the whole plant. This immediate effect of salinity on stomatal conductance is divided into two phases: first a transient effect on stomatal closure due to changed water relations followed by that exerted by locally synthesized ABA (Fricke et al., 2004) Then, within hours the tissue levels of ABA return to normal and the new reduced transpiration rate steadies (Fricke et al., 2004; Fricke et al., 2006). Though there is a reduction in stomatal conductance in salt-subjected plants, there is no difference in the rate of photosynthesis per unit leaf area (James et al., 2002). This contradiction is due to the changes in cell anatomy that lead to increases in chloroplast density per unit leaf area with smaller and thicker leaves. But if the rate of photosynthesis is measured on the basis of leaf area, a reduction can be noticed due to salinity. In both events, photosynthesis per plant is always reduced due to the effect of salinity on leaf area. The decreased rate of leaf expansion leads to the build-up of unused photosynthate in the new young growing tissues and may trigger feedback signals to down-regulate photosynthesis. An excessive level of salt can be reached in leaves at high salt concentrations leading to toxic effects on the photosynthetic apparatus, but the precise mechanism of the effect of this established toxicity is still unknown. Salt may dehydrate the cell by building up in the apoplast, changing solute concentration in the cell, but it may lead to direct enzyme inhibition in the cytoplasm with increasing salt in the cytoplasm. Salts may get deposited in chloroplasts and directly exert toxicity on the photosynthetic processes.

Oxidative stress

The decreased rate of photosynthesis results in the rise of accumulation of reactive oxygen species (ROS) and thereby increases the activity of enzymes to detoxify these toxic compounds (Apel and Hirt, 2004; Munns, 2005). ROS exert great oxidative damage to photosystems, therefore plants adjust to the environmental changes that lead to increases in ROS by adjusting leaf morphology and composition of chloroplast pigments to prevent the damage caused by ROS to the photosystems. Photo-inhibition that might increase under salinity stress, particularly under excessive light, is avoided through increased heat dissipation by xanthophyll pigments and electron transfer to oxygen acceptors besides water. Thus to regulate ROS levels, plants up-regulate key enzymes such as superoxide dismutase, ascorbate peroxidases, catalases and various

other peroxidases (Apse et al., 1999; Logan, 2005). To maintain hydrogen peroxide levels for cellular signaling and to keep a balance between the removal and formation of ROS, there should be a strict regulation of the activities of various isoforms of the above antioxidant enzymes in the different cellular compartments. However, the only case where levels of antioxidants may be inadequate is during an oxidative burst induced by biotic stresses such as under pathogen attack leading to programmed cell death (Apse et al., 1999), which might be exacerbated under salinity. The differences found at the genetic level regarding tolerance to salinity are not necessarily associated with the capacity of ROS detoxification (Munns, 2005). However, evidence from various investigations has linked differences in expression or activity levels of ROS detoxifying enzymes with levels of salt tolerance shown by different genotypes. Recently, speculation has risen about manipulation of genes related to anti-oxidative stress reactions to enhance or change the status of tolerance to any abiotic stress (Logan, 2005). A study on Arabidopsis found that mutants lacking one or both of chloroplastic and cytosolic ascorbate peroxidases were more tolerant to salinity, thereby implicating the flexibility of anti-ROS mechanisms in salt-tolerance (Miller et al., 2007).

Cellular signaling

In addition to the direct inhibitory effect of NaCl on growth, long distance signaling from roots to shoot mediated by ABA might also reduce growth. Plant responses to stress by NaCl, mannitol or isosmotic concentrations of PEG were found to be similar (Munns, 2005). However, there are studies that have shown differences in responses of roots to NaCl and sorbitol (Munns, 2005). This shows that roots have to first sense and differentiate between the ionic and osmotic components of Na^+ and rapidly respond accordingly. This response is not only necessary for roots themselves to regulate or maintain the correct uptake of nutrients but also to send the correct signal to shoots to alter or deploy correct functions to face the changes in the external environment due to elevated Na^+. Extracellular Na^+ might be sensed directly by the roots at the plasma membrane or intracellularly in the cytoplasm or in the organelles. It is still not known if a plasma membrane protein may act as sensor of Na^+ or an intracellular sensor exists. There is clearly a knowledge gap in this first and very important step for the deployment of adequate responses to salinity.

However, the increase in cytosolic Ca^{2+} as a consequence of salinity stress induces specific signaling pathways that control different responses to salt (Zhu, 2002). Increase

in Na^+ induces an increase in cytosolic Ca^{2+}, which may be sensed by a calcineurin B-like protein (protein phosphatase 2B, CBL4), also known as SOS3. This physiological increase of cellular Ca^{2+} leads to dimerization of SOS3 followed by interactions with CBL – interacting protein kinase (CIPK24/SOS2) (Halfter et al., 2000). The SOS3/SOS2 complex is associated with the plasma membrane through a myristoyl fatty acid chain that is covalently bound to CBL4/SOS3 (Ishitani et al., 2000), SOS3/SOS2 phosphorylates and thereby activates the plasma membrane Na^+/H^+ antiporter SOS1 (Qiu et al., 2002; Quintero et al., 2011). However, the correspondence between the expression pattern of SOS1 and its function is still unclear and its role in plant salt-tolerance remains still uncertain (Munns, 2005).

There is a need to design and develop screens for salt hypersensitive mutants in salt sensitive and salt tolerant plants to find specific signaling pathways involved in the regulation of salt responses. Largely, plants respond to changes in extracellular Na^+ with various groupings of adjustments – from physiology, growth and development to biochemistry and gene transcription levels (Sunkar and Zhu, 2007). Further work is required to disentangle the complex nature of the myriad of signal transduction networks in plants and also to conduct and perform experiments in real physiological conditions relevant to salinity in the field. This may lead to identifying new processes that may hold the key to salt tolerance.

1.6.2 Control of sodium ion accumulation

After getting into the transpiration stream sodium ions accumulate in the leaves where they cause the most damaging effects, rather than in roots (Munns, 2002). Water lost by transpiration in the leaves is almost 50 times more than what is retained back, which leads to salt-concentration in leaves, therefore exclusion of Na^+ at the root level is a very important process in limiting the amount of salt in shoots. Na^+ accumulated in shoots can only recirculate to the roots through the phloem in very small amounts, thus most of the Na^+ that gets delivered to shoots remain in shoots. Therefore, the processes regulating the accumulation of Na^+ in leaves are mainly the processes directly or indirectly regulating the net movement of Na^+ into the root xylem.

Net Na^+ entry into the exterior half of roots

Movement of sodium ions takes place passively through voltage independent nonselective cation channels (Tester and Davenport, 2003) and also through other high affinity K^+ transporters (*HKT* family) (Haro et al., 2005). There are still incomplete conclusions on the identities of genes encoding non-selective cation channels. Mostly, Na^+ movement into root cells happens through water movement across the root cortex in the direction of the stele, where the sodium ions are removed from the water into the cell and get sequestered into the vacuoles. Vacuoles show reduced concentrations of Na^+ and Cl^- across the cortex, which are lowest in endodermis and highest in epidermis and sub-epidermis as demonstrated by Lauchli et al. (unpublished data cited in Munns and Tester, 2008).

The majority of sodium ions that get into the root cells around the outer parts of roots are pumped back out through plasma membrane Na^+/H^+ antiporters (Tester and Davenport, 2003), a process which consumes a lot of energy evident through measuring large fluxes. If Na^+ efflux active at all times, is required throughout the plant in all the cells, various isogenes encoding Na^+/H^+ antiporters should exist. There is a good chance that other mechanisms or processes for Na^+ efflux, like primary pumping of the ion by Na^+ translocating ATPases, are involved (Munns, 2005). The leftover or remaining Na^+ in the root cells can be sequestered in vacuoles or sent to the shoots. In the case of *A. thaliana*, compartmentation of sodium ions into vacuoles is completed through the Na^+/H^+ exchanger (*NHX*) family of tonoplast Na^+/H^+ antiporters (Pardo et al., 2006). According to Apse et al. (1999), constitutive expression of the *NHX1* gene in *A. thaliana* encoding a vacuolar H^+ translocating pyrophosphatase (*AVP1*) catalyzes the pumping of Na^+ into vacuoles, thereby increasing efficient sequestration and improving tissue tolerance mostly by reducing Na^+ concentration in the cytosol.

Sodium ion loading into and retrieval from the xylem

Sodium ions are removed from the stelar cells into the stelar apoplast from where they move into the xylem in the transpiration stream. Here, the plasma membrane Na^+/H^+ antiporter (*SOS1*) gene is expressed in stellar cells, which may influence the efflux of Na^+ into the xylem. There is also data suggesting that some members of the *HKT* gene family play an important role in the retrieval of Na^+ from xylem (see Figure 1.5.1). In Arabidopsis roots, *AtHKT1* is responsible for retrieval of Na^+ before it moves to the shoots (Sunarpi et al., 2005). This happens also in rice (Ren et al., 2005) and wheat

(Huang et al., 2006) with similar functions of closely related *HKT1.5* gene family members. More closely related genes might be involved in Na^+ exclusion in other species, like the *TmHKT1*-A2 that is involved in Na^+ exclusion in durum wheat (Huang et al., 2006) and insures Na+ exclusion under high leaf K^+/Na^+ ratios. The protein encoded by this gene has a great activity in the leaf sheaths and also in the roots (James et al., 2006). Due to the great diversity of the *HKT* gene family, there has been some confusion about its functions. Therefore, the *HKT* gene family was divided into two distinct subfamilies (Platten et al., 2006): Na^+ selective proteins with serine residue at an important position constitutes subfamily 1, whereas subfamily 2 members have glycine in place of serine at this position and catalyze K^+ transport and also high affinity Na^+ influx (Munns, 2005).

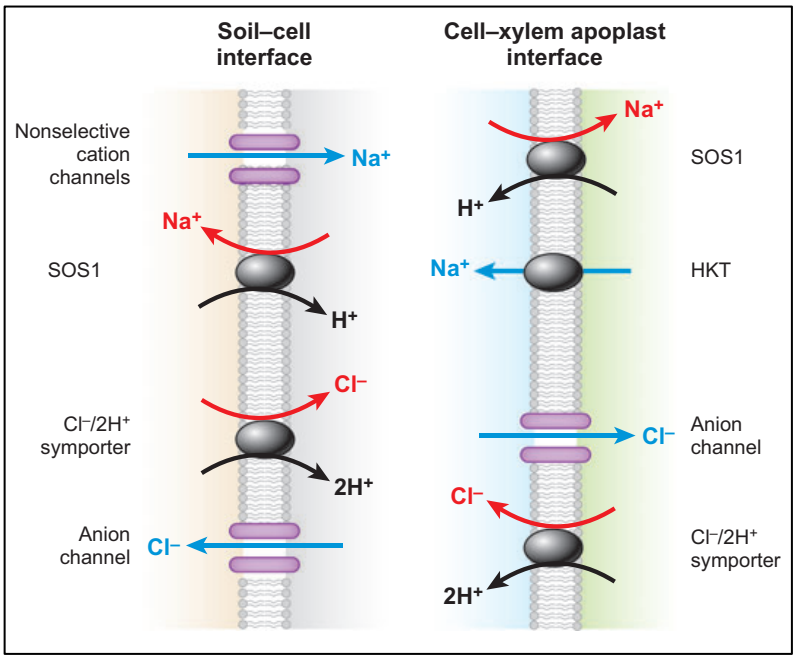


Figure 1.5.1 The expected mechanisms of passive and active Na^+ and Cl^- transport at the soil-root cell and cell-xylem apoplast interfaces (center of the image is intracellular), mediated by ion channels and carriers (uniporters and H^+ -coupled antiporters and symporters) (Munns and Tester, 2008).

1.6.3 Tolerance of tissue to Na^+

At the cellular level, intracellular partitioning and anatomical adaptations help plants to tolerate high levels of absorbed Na^+ and Cl^- ions. Dicot halophytes anatomically adapt by increasing the vacuole size, thereby increasing the cell size, and also use excretion of Na^+ and Cl^- by salt glands or accumulate salts in bladder cells (Flowers et al., 1986).

Salinity effects on intercellular partitioning of ions have mainly been studied in barley, which can tolerate relatively high levels of Na^+ and Cl^- in leaf tissues (Colmer et al., 2005). When barley was salt treated, there was greater accumulation of K^+ and Cl^- in mesophyll cells and epidermal cells respectively. However, there is no data supporting the partitioning of Na+ between different cell types (Munns, 2005).

Intracellular compartmentation of sodium ions

Sodium ions must be partitioned inside the cellular compartments to keep or maintain concentrations in the cytoplasm below toxic levels, around 30 mM. But the exact concentration at which Na^+ becomes toxic is not well known and *in-vitro* research shows that around 100 mM concentration starts to inhibit the activity of key enzymes (Greenway and Osmond, 1972), with some enzymes sensitive at lower concentrations (Flowers and Dalmond, 1992). Also, Cl^- toxicity levels are not well defined but mostly similar to that of Na^+ (Flowers and Dalmond, 1992). Most of the Na^+ and Cl^- ions are sequestered into the cell vacuoles; this is demonstrated by the high concentrations of Na^+ found in leaf tissues of plants with normal functioning. Among different species, differences in level of expression of *NHX1* and *AVP1* (see section 1.5.2) may affect the ability to sequester Na^+ in the vacuoles of leaf cells (Munns, 2005). Overexpressing *NHX* genes (Brini et al., 2007) or the *AtAVP1* gene (Gaxiola et al., 2001) in different species has improved salinity tolerance. Increased vacuolar Na^+ concentration would need a coordinated increase in the osmotic pressure of the other subcellular compartments to sustain their volume. This can be achieved through increased accumulation of K^+ at sub toxic levels and via production of compatible solutes.

Accumulation of compatible solutes

To balance the osmotic pressure exerted by ions in vacuoles, organic solutes that are compatible with metabolic activity even at high concentrations are produced in the different organs and accumulate in the the cytosol and organelles of the cells (Flowers et al., 1977). These accumulated compounds commonly include sucrose (soluble sugar), proline and glycine betaine (amino acids) with various other molecules such as: pinitol, ononitol, and myo-inositol (sugar alcohols), which are species specific (Hasegawa et al., 2000). Various halophytes adjust the osmotic pressure in the cell as a whole by inducing increased production of proline and glycine betaine at high concentrations (40 mM) (Flowers et al., 1977). However, in glycophytes, the concentrations of accumulated solutes are not high, around 10 mM, but if they are partitioned specifically in the

cytoplasm can greatly contribute to the adjustment of osmotic pressure and function as osmolytes. In recent studies, it has been shown that genes regulating the synthesis or metabolism of these compatible solutes have key roles in tolerance towards abiotic stresses including salt stress (Rhodes et al., 2002). In *T. halophila*, lower expression of a gene encoding proline dehydrogenase (PDH) may contribute to the tolerance to salinity by lower proline catabolism when compared with its salt sensitive relative *A. thaliana* (Kant et al., 2006).

However, the synthesis of these compatible solutes comes only with a high energy cost, therefore enforcing a growth penalty on plants. The number of ATP molecules needed for the synthesis or accumulation of one molecule of compatible solute has been estimated to be 34 for mannitol, 41 for proline, 50 for glycine betaine, and almost 57 for sucrose (Raven, 1985) and 4 ATP molecules (plus 2.5 molecules of NADPH) to fix 1 carbon dioxide. The values are based on assumption that 0.5 moles of ATP is produced per mole of photon and nitrate is the source of nitrogen. No doubt the production of osmolytes affects the plant growth rate but they allow plants to tolerate high concentrations of salt and survive with possible recovery. The Na^+ exclusion mechanism makes the plant capable of postponing or in some cases avoiding the setback of ion toxicity but the plant must compensate for the exclusion of Na^+ with the uptake or accumulation of K^+, otherwise it increases the demand for organic solutes for adjusting the osmotic pressure. And as discussed above, synthesis of osmolytes disturbs the energy balance of the plant, therefore causing plants to maintain or keep a check on ion toxicity on the one side and turgor loss on the other throughout salt stress.

1.7 Importance of gene regulation under salt-stress

Salt-stress triggers various biochemical and physiological responses in plants. One of the most important responses is cellular osmotic adjustment via accumulation of compatible solutes, which are osmotically effective metabolites used to lower the osmotic potential to retain/drive water in the cell without affecting metabolism. Salt-tolerant plants have a higher capacity in terms of osmotic adjustment. Therefore, research directed towards genetic engineering of salt-tolerance has focused on the mechanisms of osmotic adjustment (Flowers, 2004). In recent years, metabolomic studies using high throughput methods for analyses of qualitative and/or quantitative changes in metabolites have resulted in a great improvement in the identification of the physiological processes involved in a given biological response. Especially, metabolite fingerprinting and profiling techniques offer access to the vast biological information flow between gene expression and metabolic phenotype (Sanchez et al., 2008). According to Desbrosses et al. (2005), metabolomics can be greatly helpful in comparative research on metabolic phenotypes, which include physiological responses caused by environmental conditions such as salinity. Studies related to physiological responses have shown that sugars such as trehalose, sucrose, sorbitol, raffinose family oligosaccharides (*RFO*), sugar alcohols such as mannitol and inositol, amino acids such as proline and amines like glycine-betaine, accumulate in varied amounts under salt and drought stress in different plant species (Taji et al., 2002). Collectively, these compounds, after accumulation under environmental stresses act as osmolytes, anti-oxidants or scavengers that help plants to tolerate stresses (Bartels and Sunkar, 2005). Change at the cellular level in the concentrations of these metabolites help in maintaining cellular functions and also protecting the structure of cellular components. Plants accumulate different osmoregulators to different levels and this might in part dictate their level of stress-tolerance. This differential accumulation of osmolytes might be caused by the presence and/or the regulation of the genes that encode components of stress targeted metabolic pathways and this is a very promising area of research to enhance salt-tolerance in plants.

Extensive work has been conducted during the last 20 years to understand the basic mechanisms for salt-tolerance in plants. Generally, the development of mechanisms in plants reported to survive extreme environmental conditions including salinity stress is based on three important aspects acting separately or simultaneously: (1) differing gene regulation resulting from differing promoters, transcription factors, or signaling

elements; (2) the evolution of more active forms of gene products conferring tolerance; (3) presence of unique stress response genes. Differential expression of the *SOS1*, *P5CS1* and *PDH* genes (playing important roles under salt-stress) between *A. thaliana* and its salt-tolerant relative, *T. halophila* (*salsuginea*) was shown under salt-stress (Kant et al., 2006), supporting changes in gene regulation as a possible cause of the higher salt-tolerance shown by *T. halophila*. Also, predominance of *P5CS2* transcripts (the *P5CS1* gene is expressed in most plant organs but silent in dividing cells where *P5CS2* gene expression is dominant) in shoots of *T. halophila* under salt-stress might indicate that differences between species at the level of protein structure are part of the evolution of the salt-tolerance trait in *T. halophila*. In addition to that, research conducted by Wong et al. (2005) has shown that some transcripts were detected in *T. halophila* and not in *A. thaliana* under salt-stress suggesting the presence of unique genes for stress-tolerance in *T. halophila*. The relative contribution of these differences to the higher salt-tolerance shown by *T. halophila* is still a matter of debate, but the emerging paradigm suggests that halophytes (salt-tolerant) and glycophytes (salt-sensitive) engage common mechanisms in response to salt-stress and changes in the regulation of a basic set of genes involved in salt-tolerance is hypothesized to be the leading factor for the difference in salt-tolerance levels in plants (Kant et al., 2006). Data supporting this view remain however fragmented and lacking details in terms of what is important in the observed differential response, the timing or the amplitude of the responses or both.

1.8 Hypothesis and Aims

Hypothesis:

The hypothesis upon which this research was based is that differential gene regulation is the leading factor for the differential salt-tolerance shown by different plant species and that difference in the gene regulatory processes including at the promoter level, transcription and post-transcriptional levels as well as signaling level might be the main control of level of salt-tolerance.

Aims:

This PhD project aimed at comparing two different plant species having different salt tolerance levels: *Thellungiella halophila* (salt cress), which shows growth and survival at very high salt concentrations exceeding 500 mM NaCl, to its salt-sensitive close relative *Arabidopsis thaliana*. The project exploited the available genome sequence data for *A. thaliana* to investigate the regulation of salt-responses in *T. halophila*. The research aimed at determining if the same mechanisms were modulated differently to bring about salt-tolerance in these plants or additional mechanisms/genes evolved in *T. halophila*.

Objectives:

(1) To analyze and compare the kinetics of salt-stress responses as well as level (amplitude) of these salt-stress responses exhibited by *A. thaliana* and *T. halophila,* in terms of salt-tolerance through a time course. This analysis involved comparisons at both very early stages of salt-stress (first 48 hours) and after prolonged salt-stress up to 10 days. The analyzed responses included the physiological responses (growth, photosynthesis), metabolic responses (production of osmolytes and accumulation of carbohydrates) and gene responses (*SOS1* and *P5CS1*).

(2) To unravel the mechanisms behind the differential transcript levels including regulation of gene expression shown by the two species (cis-regulation) and posttranscriptional regulation (splicing) using P5CS1 gene to probe these processes in the two species.

1.9. Approaches and organization of the thesis

The aims of this project were followed in four phases, which are described in the thesis in the four following chapters. In Chapter 1 the differential salt stress responses exhibited by *T. halophila* and *A. thaliana* are reported including a comparative analyses of physiological and biochemical responses under salt stress in the two species. The next chapter describes the comparative analysis of changes in transcript levels for genes directly or indirectly involved in salt-responses between *T. halophila* and *A. thaliana*. Then two chapters describe the work about the regulatory modulation of the key processes found to be differentially regulated in the two species (see the first two sections). These two chapters include analysis of the promoter region and splicing of a key stress specific gene (*P5CS1*) and a screening of targeted upstream gene regulatory components (i.e. signaling components, transcription factors).

The end of the thesis provides a comprehensive discussion of the findings with proposals of the possible key mechanisms behind salt-tolerance in plants. Following are the key questions, which will be tried to answer:

- Is there a difference of timing or amplitude or both in responses to salt-treatment observed in *A. thaliana* and *T. halophila*?
- Is there an evolutionary change at the gene level for enhanced salt-tolerant trait in *T. halophila*?
- Is metabolic background important for salt tolerance?

Chapter 2

Time Course Comparison Of the Physiological And Metabolic Responses Of Arabidopsis thaliana And Thellungiella halophila To Salt treatment

2.1 Introduction

High salt concentrations in the soil induce stress in plants in two distinct manners. High soil salinity makes it difficult for roots to extract water from soil and high concentration of salts within the plant result in toxicity. Plants respond to salt-stress in two phases. The first phase is called the osmotic phase where pressure is exerted by the surrounding high salt content of soil on the roots resulting in great reduction in shoot growth. The second phase is called the ion-specific phase, where the plant faces toxic levels of salt accumulation within the cells which might lead to cell death, especially in older leaves. The speed of onset of the above two phases is different. The osmotic stress happens quickly and the plant has to adjust rapidly the osmotic pressure of the cells. The ionic stress progresses slowly with the accumulation of the toxic ions in the cells leading to perturbations of key cellular functions (Munns and Tester, 2008).

Plants deploy three different types of responses to the above-described stress phases. First osmotic adjustment takes place through the closure of stomata and reduced cell expansion in roots and young leaves. Second the process of Na^+ exclusion by roots is engaged, which allows reduced accumulation of Na^+ ions within leaves. The last response is the tissue tolerance of the toxic concentration of accumulated Na^+ ions, which takes place at intercellular and intracellular levels through compartmentalization of salts (Munns and Tester, 2008). There is one more process which significantly helps plants to cope with salt stress contributing to the above three responses: accumulation of organic solutes helps to increase osmotic adjustment during the osmotic phase, and alters the transport mechanisms to limit Na^+ accumulation by acting as compatible solutes in the cytoplasm (Munns and Tester, 2008).

At high salinity when Na^+ or in some cases Cl^- ions are sequestered in the vacuoles, metabolites that do not inhibit metabolic reactions or metabolic activity even at high concentrations accumulate proportionally to change in external osmotic potential, hence called compatible solutes (Brown and Simpson, 1972; Yancey et al., 1982; Ford, 1984). Compatible solutes accumulate in the cytosol and organelles to balance the osmotic pressure exerted by the ions in the vacuole (Jones et al., 1977; Flowers, Troke and Yeo, 1977). Metabolites like sucrose, glycine betaine and proline are the most commonly accumulated compounds however certain species can accumulate other species-specific compounds at higher concentrations (Flowers et al., 1977; Hasegawa et al., 2000; Munns, 2005). As osmolytes, usually accumulated solutes include sucrose and fructose (sugars), methylated inositols and glycerol (sugar alcohols), and trehalose and

fructans as complex sugars. Along these, others include charged ions like K^+ or charged compounds like Dimethylsulfopropionate (DMSP) and glycine betaine, ectoine and proline (Hasegawa et al., 2000). Under high salt concentrations compatible solutes not only lower the inner osmotic potential of the cell to facilitate the osmotic adjustment but also act as osmoprotectants (Delauney and Verma, 1993; Louis and Galinski, 1997). As these solutes are hydrophilic in nature they can easily take the place of water in cells and protect proteins and their complexes and also membranes (Hasegawa et al., 2000). For example, in the case of oxygen-evolving complex of photosystem II (Papageorgiou and Murata, 1995), compatible solutes can limit the inhibition of enzyme activities caused by sodium ions, which can also increase enzymes thermal stability avoiding complex dissociation (Galinski, 1993; Hasegawa et al., 2000). Synthesis of compatible solutes is connected to the main metabolic pathways for basic cellular processes, which have high flux rates (Nuccio et al., 1999; Hasegawa et al., 2000). Also, metabolite synthesis involves energy consumption, which leads to low growth rate. Three and half ATPs are required to accumulate one Na^+, 41 to make one proline and around 52 ATP to make one sucrose (Raven, 1985). These values of ATP requirements are high in comparison with accumulation of one mole of NaCl as osmoticum but metabolites help plants to tolerate salt stress and may help them escape or recover from stress (Munns and Tester, 2008).

In the recent past, extensive work has been conducted in glycophytes and halophytes to understand the relationship between metabolites and salt stress, focusing on their role either as specific or as groups of compatible solutes that are involved in tolerance against salt toxicity. *A. thaliana* and *T. halophila* have largely contributed to the knowledge we possess today about changes in metabolites in general under salinity. Examples include work by Arbona et al. (2010) where GC/MS or LC/MS method used to perform metabolic profiling in the two species to look for common and divergent responses to salt treatment. Kim et al. (2007) conducted a time-course analysis in the first 72 hours of salt treatment in *A. thaliana* cell cultures to identify short term and long term metabolic responses to salt stress.

This chapter compares the growth response and change in photosynthetic capacities in *A. thaliana* and *T. halophila* under salt-treatment. We have measured sodium accumulation because the regulation of uptake and compartmentalization of sodium is a key aspect in salt-tolerance. Uptake of sodium can impact on the uptake of K^+ (a key mineral nutrient) impacting on growth and fitness of the plant. It also describes the differential accumulation of key metabolites including sucrose, fructose,

glucose, malate and proline in the two species subjected to a time-course of salt-treatment at two different NaCl concentrations. Proline produced under salt-stress plays major roles as an osmoregulator and as an anti-oxidant in many plants (Kant et al., 2006). The metabolic pathway for the production of this important amino acid is controlled mainly by the enzyme, delta (1)-pyrroline-5-carboxylate synthetase 1 encoded by the *P5CS1* gene. Therefore it was necessary to check and compare the proline levels in shoots of control and salt-treated plants of *A thaliana* and *T. halophila*. The hypothesis being tested was that the difference in salt tolerance trait between *A. thaliana* and *T. halophila* lies in the differential regulation of the same response mechanisms exhibited by both species.

2.2 Materials & Methods

2.2.1 Stress Treatment

Figure 2.2.1 summarises the experiment plan. *A. thaliana* (Columbia ecotype) and *T. halophila* (Shandong ecotype) seeds were surface sterilized using 70% ethanol, washed three times with sterile water and sown on John Innes soil compost No. 3. The pots (12 cm wide) were placed at 4°C for 72 hours to synchronize germination. The pots were then transferred to a controlled growth room at 23°C with 12/12 hours light/dark periods and light intensity of 150 $\mu mol.m^{-2}.s^{-1}$at plant height. Seven-day-old seedlings were then transferred to smaller pots (2.5 cm wide) containing moist John Innes No. 3 compost with one seedling in each. Then 4-week-old *A. thaliana* and 6-week-old *T. halophila* plants, similar in size and before bolting, were separated into three sets and irrigated with three different NaCl concentrations prepared with normal tap water. *A. thaliana* was watered with 0, 50 and 100 mM [NaCl] and *T. halophila* was watered with 0, 100 and 500 mM [NaCl] (0 mM refers to tap water) at a fixed time (12:00 hours, i.e. 4 hours into the light) every day for 10 days. Shoots and roots were harvested at a fixed time (16:00 hours, i.e. 4 hours before dark) as three plants per sample after 0 hours, 12 hours, 1, 3, 5 and 10 days of the salt treatment, weighed and frozen in liquid nitrogen. Later, the samples were ground in liquid nitrogen and stored at -80°C for various analyses. Before freezing, a part of the each sample was set aside to determine dry weight (DW) through desiccation at 80°C for 24 hours. To assess the impact of salt on water uptake, leaf water content (WC) was determined using the equation: $WC = (FW - DW) / DW$. Three samples were harvested at each time point for each NaCl concentration for both plant species. Control plants were watered with tap water only and harvested in parallel to salt-treated plants.

2.2.2 Determination of growth rate

Two-week-old *A. thaliana* and 3-week-old *T. halophila* plants, similar in size, were separated into three sets and irrigated with three different NaCl concentrations prepared with normal tap water. *A. thaliana* was watered with 0, 50 and 100 mM [NaCl] and *T. halophila* was watered with 0, 100 and 500 mM [NaCl] (0 mM refers to tap water at a fixed time of the day (12:00 hours, i.e. 4 hours into the light) every other day for 4 weeks. Shoot samples were harvested at a fixed time (16:00 hours, i.e. 4 hours before dark) as 3 plants per sample (3 samples per treatment) after each week of the treatment. Dry weight (DW) was determined after desiccation of samples at 80°C for 24 hours and used to assess plant growth.

2.2.3 Measurement of photosynthetic capacities

To assess the effect of salt-stress on photosynthetic capacities, key photosynthetic parameters were measured in *A. thaliana* and *T. halophila* subjected to salt concentrations as explained in section 2.2.1. The measurements were of leaf chlorophyll content and chlorophyll fluorescence. The Fo (minimum fluorescence in the dark) and Fv/Fm (quantum yield at PSII) were the two parameters measured to predict the photosynthetic capacities and extent of oxidative stress under saline conditions. All three parameters, chlorophyll content, Fo and Fv/Fm were measured in three leaves from each of three plants before (T0) and after 12 hours, 1, 3, 5 and 10 days of salinity. Chlorophyll was measured using a chlorophyll content meter (CCM-200, Opti-Science, Inc, USA) and chlorophyll fluorescence was measured using plant efficiency analyzer (PEA-MK2, Hansatech Instruments Ltd, England).

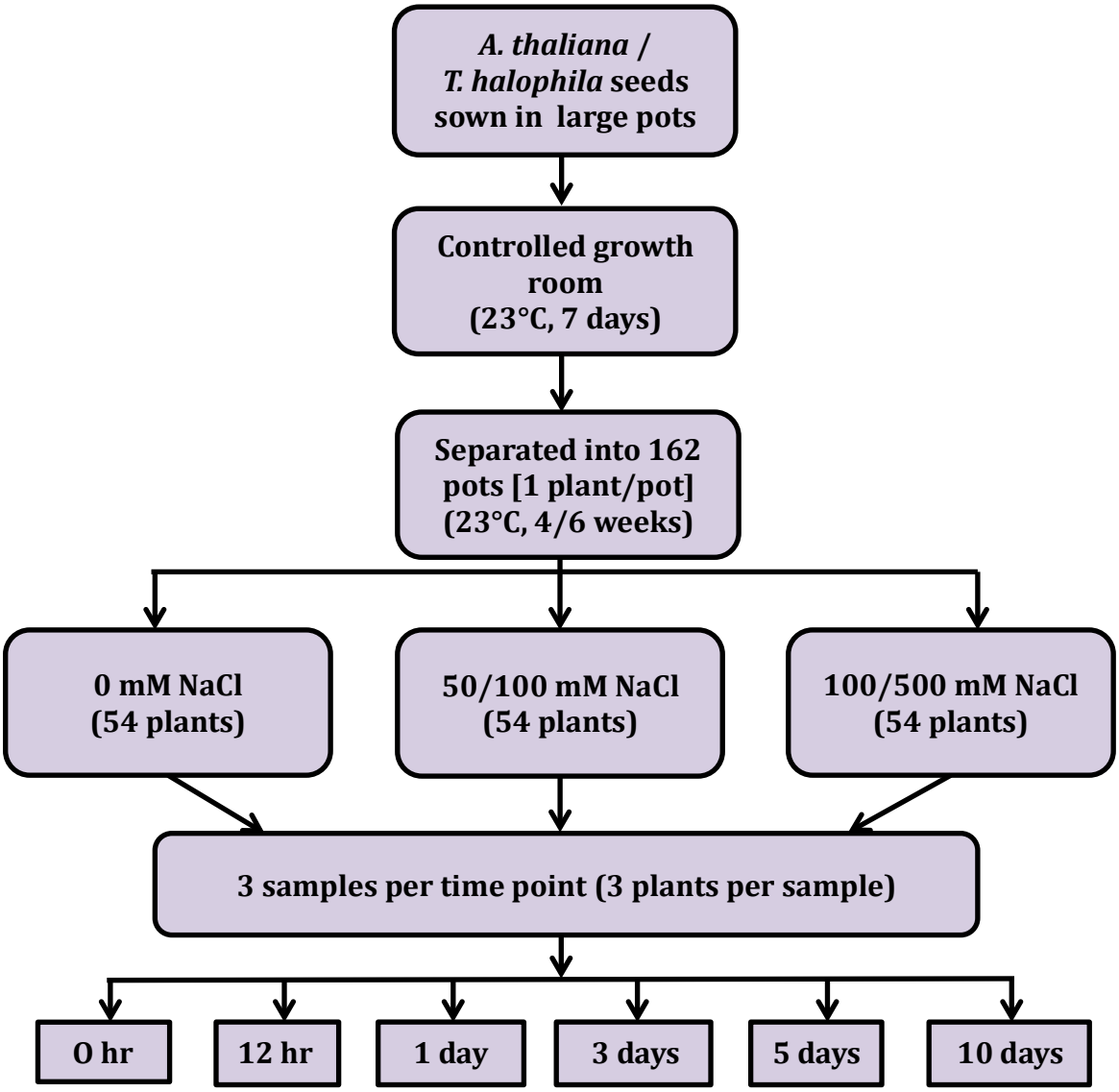


Figure 2.2.1: Schematic representation of the salt treatment experiment and sampling performed on *A. thaliana* and *T. halophila* plants

2.2.4 Determination of Sodium and Potassium contents

The content of Na^+ and K^+ in shoot samples was analyzed by a dry combustion method using hydrochloric acid provided by Prof. Anne Borland. The shoot samples (100 mg /500 mg) previously ground under liquid nitrogen were taken from -80°C and weighed into ceramic crucibles. The samples were ashed overnight in a furnace at 450°C then weighed again. Ashed samples were then damped using a few drops of sterile water and were left to evaporate in a hot steam bath after addition of 2 ml concentrated HCl. After drying, samples were damped again using sterile water and left on the hot steam bath for 1 hour. This was followed by addition of 10 ml 25% HCl and samples were warmed slightly to form the extract, which was filtered through Whatman No. 1 filter paper into a 100 ml volumetric flask. The crucibles were washed many times using 1% HCl and washings were transferred through the same filter paper to the volumetric flask. The extract was allowed to cool down and then was made up to the volume of 100 ml with sterile distilled water. Two sets of standards were prepared each one for sodium and potassium measurements. The standards ranged from 0 to 25 µg, Na^+ and K^+ were measured by flame photometry using appropriate dilutions and expressed as µmol/mg DW.

2.2.5 Determination of Proline content

A colorimetric method adapted from that described in Claussen (2005) was used for measuring proline content. From shoot samples previously ground using a pestle and mortar in liquid nitrogen, 100 mg of sample (one sample consisting of shoots from three different plants) was transferred to a 1.5 ml micro centrifuge tube. To this, 1 ml of 3% (w/v) aqueous sulphosalicylic acid solution was added and the sample vortexed thoroughly. This homogenate was clarified by centrifugation at 10,000 *g* for 3 minutes at room temperature. The supernatant was transferred to a new micro centrifuge to be used for the proline assay. Exactly 500 µl of this supernatant was mixed with glacial acetic acid and acidic ninhydrin reagent (500 µl each) in a new 2 ml micro centrifuge tube. The reaction mixtures in closed tubes were incubated in a boiling water bath for 1 hour then the reaction was stopped by placing the tubes in a water bath at room temperature (19°C - 23°C) for 5 minutes. After terminating the reaction, readings were taken immediately using a spectrophotometer at 546 nm. The concentration of proline was determined against a standard curve produced using commercial L-proline in parallel to the samples and calculated on a dry weight basis (µg proline/mg DW).

2.2.6 HPLC analyses of sugars and sugar alcohols

The levels of sucrose, fructose, glucose and inositol in control and salt-treated plant samples from the two plant species were determined using HPLC adapted from that described in Adams et al. (1992). The analyses were conducted with three biological replicates at each time point. Exactly 500 mg of ground plant tissue was homogenized by vortexing in 5 ml of 80% methanol followed by incubation at 75°C for 40 minutes. The insoluble fraction including debris was removed from the methanol extract by centrifugation at 3500 *g* for 6 minutes at room temperature. Half (2.5 ml) of the methanol extract was dried by evaporation using a sample concentrator overnight and re-suspended in 1 ml of molecular grade (deionized) water. The extract was then desalted using a column of Sigma-Aldrich Dowex® AG50W X4 - 200 (hydrogen form) and Sigma-Aldrich Amberlite® IRA – 67 (free base) in series. For about 120 sample extracts, 30 g of Dowex and Amberlite was used. Dowex was washed with 95% ethanol with one change over 30 minutes to remove the colour and then rinsed with several changes of deionized water. Amberlite was washed with 4 to 5 volumes of 1 M NaOH for 30 minutes and rinsed with deionized water to neutrality. Then the columns were prepared by placing a thin layer of glass wool at the bottom and carefully layered with 0.5 cm^3 of Amberlite then 0.5 cm^3 of Dowex on top. The columns were then washed with molecular grade water multiple times before the extract was desalted. Exactly 400 µl of the extract was passed through the column. To completely collect the desalted extract, the column was washed with 3 ml of molecular grade water. The eluate was then injected (20 µl) into a HPLC column (CarboPac PA100 with guard, Dionex, UK) using isocratic separation with single eluent consisting of 150 mM NaOH to determine the concentrations of sucrose, glucose, fructose and inositol in each sample. Sugars were identified in the separation profiles by retention time in the column based on that of the commercial standards.

2.2.7 Determination of malate content

Malate content was measured in salt-stressed and unstressed plants from both plant species using the Hohorst method (1970) based on an enzymatic assay. L-malate was estimated using L-malate dehydrogenase (MDH) and NAD. Exactly 500 mg of ground plant tissue was homogenized by vortexing in 5 ml of 80% methanol followed by incubation at 75°C for 40 minutes. The insoluble fractions (also debris) were removed from the methanol extract through centrifugation at 10,000 rpm for 3 minutes. Exactly 2.5 ml of methanol extract (supernatant, the other half from HPLC analysis) was

transferred to a 10 ml Falcon tube and labeled. The extracts were dried down overnight by air blowing using a sample concentrator and re-suspended in 2 ml of 200 mM bicine/KOH buffer (pH 7.8). For 100 ml extraction buffer, 7.5 g of glycine, 5.2 g of hydrazine sulphate and 0.2 g of EDTA were suspended in 60 ml sterile water. The buffer was adjusted to pH 9.5 using 4 M KOH and volume increased to 100 ml using sterile water. The extraction buffer in assay reaction was used with a mix of sodium salt of NAD. NAD salt was prepared by taking 0.24 g in 6 ml of sterile water. Buffer plus NAD mix for 120 assays was prepared which included 54 ml of extraction buffer and 6 ml of NAD. The MDH enzyme stock of 10,000 U/ml was reduced to 1 U/µl by diluting 90 µl of stock in 900 µl of sterile water. The reaction was performed at room temperature in 2 ml cuvettes where the reaction consisted of 500 µl of extraction buffer and NAD (3 mM), 500 µl H_2O for blank or 480 µl of H_2O and 20 µl of extract for sample. The reaction was initiated by addition of 10 µl of MDH after 2 minutes of reaching equilibrium and spectrophotometric readings were taken at 340 nm. After the addition, reaction was let to complete for 45 minutes to take the final absorbance. The difference in absorbance was plotted against a calibration curve generated using commercial L-malate run in parallel to samples.

2.3 Results

2.3.1 Effect of salinity on growth of *A. thaliana* and *T. halophila*

As shown in Figure 2.1, salinity showed significant adverse effects on growth of both *A. thaliana* and *T. halophila*. Salinity however had much greater effect on *A. thaliana* than *T. halophila*. The onset of growth reduction began early in *A. thaliana* within the first week of salt treatment at both 50 and 100 mM [NaCl]. In *T. halophila* the impact of salt-treatment on growth became significant after 2 weeks only. Dry weight accumulated in *A. thaliana* at 50 mM [NaCl] was less than half that of control plants and at 100 mM [NaCl] it was less than third of the control plants after 4 weeks of salt-treatment. In *T. halophila* dry weight accumulated after 4 weeks of salt-treatment at 100 mM [NaCl] was about half that accumulated in control plants. *T. halophila* plants completely stopped growth at 500 mM [NaCl], but leaves remained green and alive.

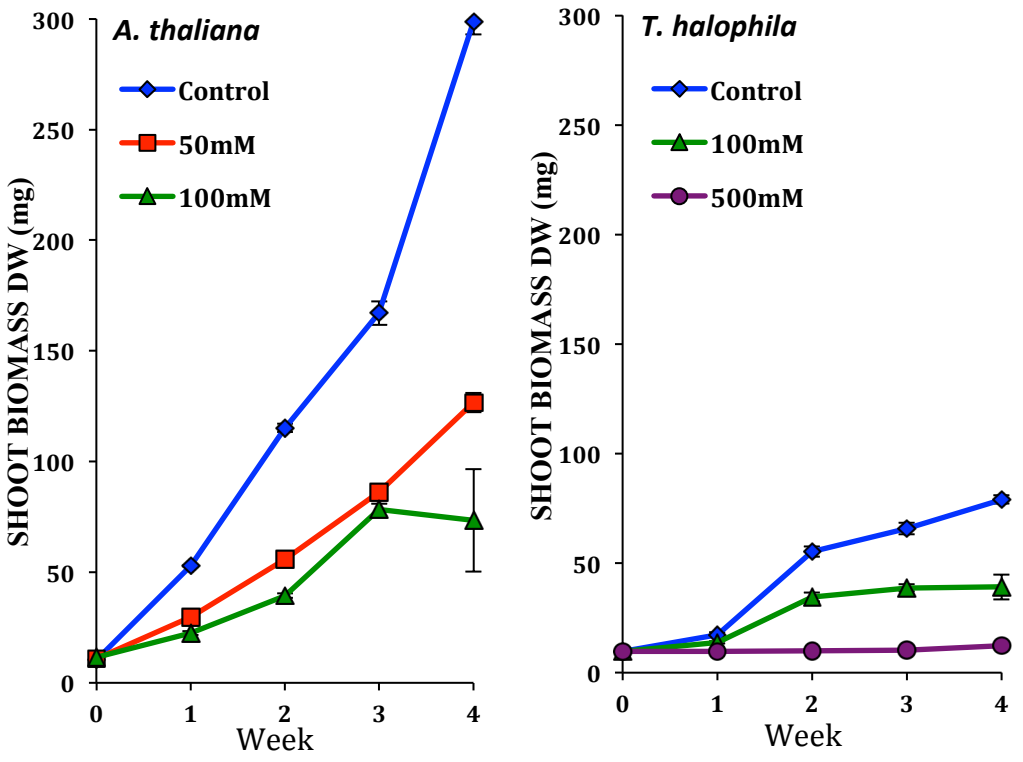


Figure 2.3.1: Effect of NaCl on shoot growth over a period of 4 weeks in *A. thaliana* and *T. halophila*, expressed as dry weight (DW). Each point is a mean of three replicates and standard errors were calculated from the three replicates. Mass is expressed per sample consisting of three shoots.

2.3.2 Water content under salinity

Salt-treatment caused a decrease in the WC of the shoots of both plant species. As shown in Figure 2.3.2, the decrease in WC was higher in *A. thaliana* (33% and 58% decrease at 50 mM and 100 mM [NaCl] respectively after 10 days of salt-treatment) than in *T. halophila* (29% and 44% reduction at 100 mM and 500 mM [NaCl] respectively, after 10 days of salt-treatment). The decrease in WC almost stopped after 3 days of salt-stress in *T. halophila,* whereas it continued over the 10 days of salt-stress in *A. thaliana*. It is worth noting that there was a significant increase in the WC in the control plants of *A. thaliana* and *T. halophila* over the 10-day period.

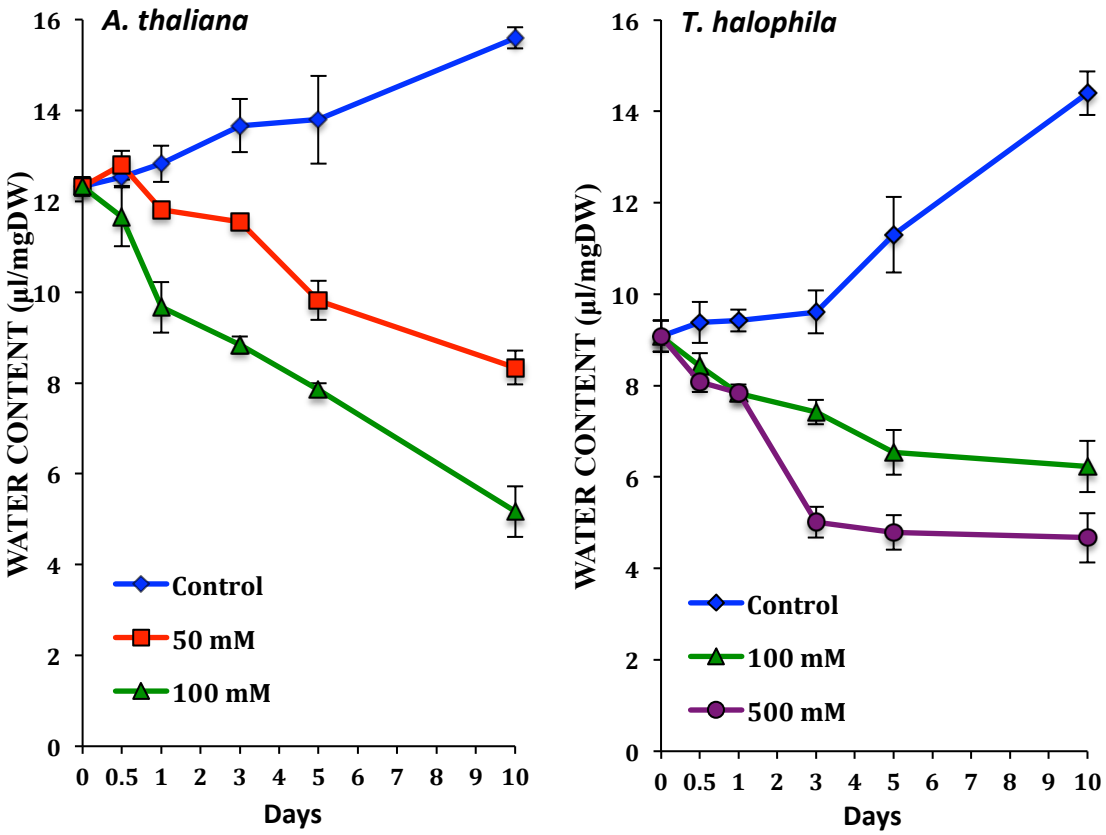


Figure 2.3.2: Change in the water content (WC) levels in shoots of *A. thaliana* and *T. halophila* induced by salt-treatment (NaCl) over a period of 10 days. Each point is a mean of three replicates and standard errors were calculated from the three replicates.

2.3.3 Effect of salinity on photosynthetic capacities

Overall, salt-treatment resulted in a substantial decrease in chlorophyll content in *T. halophila* after a minimal increase during the first 12 hours of salt treatment as shown in Figure 2.3.3 b. In *A. thaliana*, however contrasting results are apparent in Figure 2.3.3 a, Chlorophyll levels increased in the control plants and to a minimal extent in the salt-treated plants with 50 mM [NaCl]. Plants treated with 100 mM [NaCl] showed a rapid and transient increase in chlorophyll levels followed by a decrease to similar levels of the T0 plants. The Fo values which represent the minimal chlorophyll fluorescence showed a substantial increase in both *A. thaliana* and *T. halophila* under salt-treatment at 100mM [NaCl] and 500 mM [NaCl] respectively indicating a reduction in light absorption capacities. This increase in Fo followed a transient decrease in the first 12 hours and 24 hours of salt treatment in the two plant species respectively (Figure 2.3.3 d). In *A. thaliana* there was initially a slight decrease of Fo during the first 12 hours followed by an increase in both the salt-treated plants and the control plants. The Fv/Fm, which measures the efficiency of photosystem II, showed reciprocal changes to those observed for F0, indicating a reduced PSII efficiency in *A. thaliana* and *T. halophila* at 100mM and 500 mM [NaCl] respectively. There was no important difference in Fv/Fm values between the control plants and plants treated with 50 mM [NaCl], for *A. thaliana* (Figure 2.3.4 a) whereas in *T. halophila* plants subjected to 500 mM [NaCl] a substantial decline in Fv/Fm was apparent after 24 hours of salinity (Figure 2.3.4 b). When Fv/Fm is compared at 100 mM [NaCl] between the two species, *A. thaliana* is more affected after 3 days exposure to salinity.

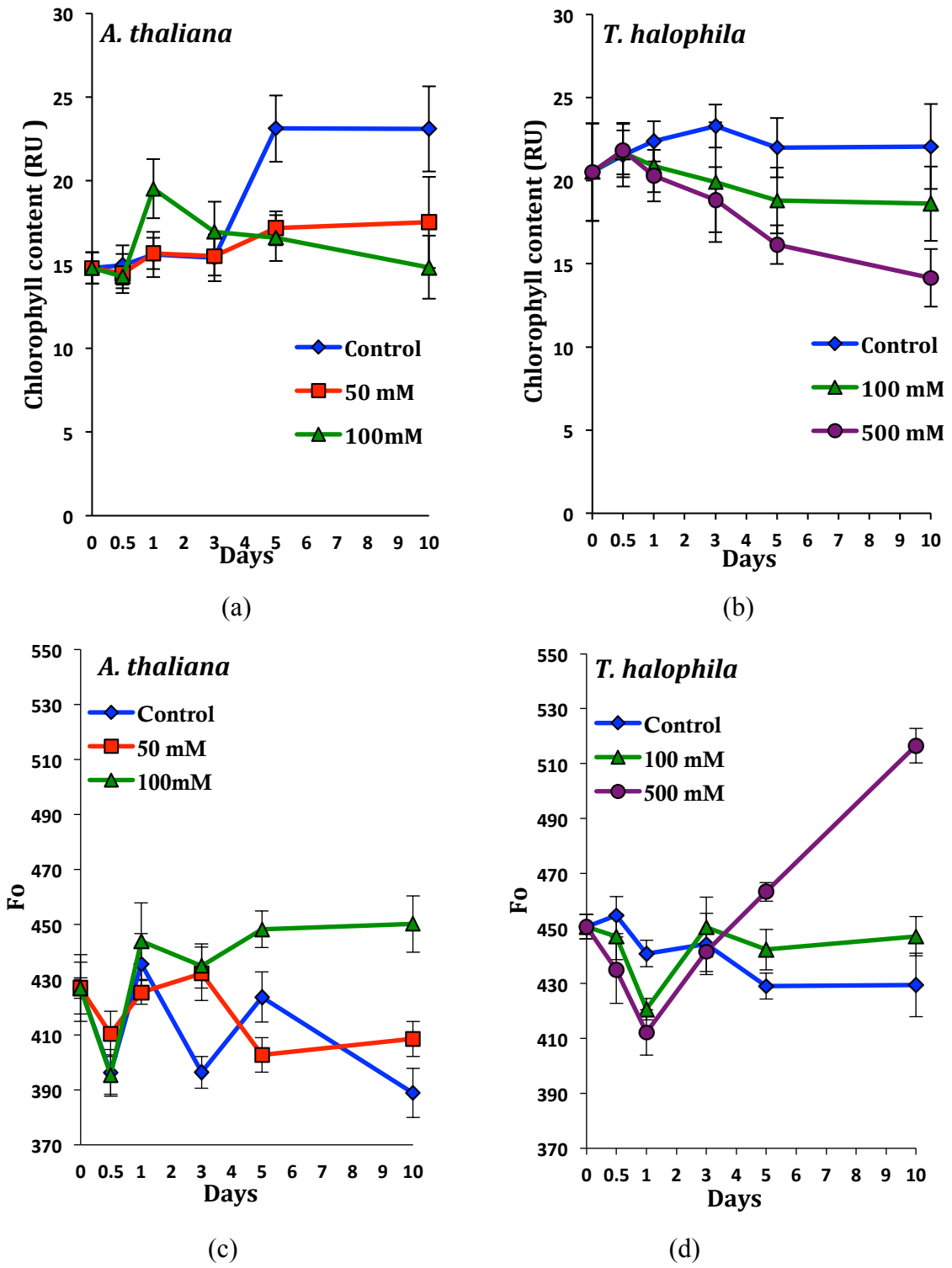
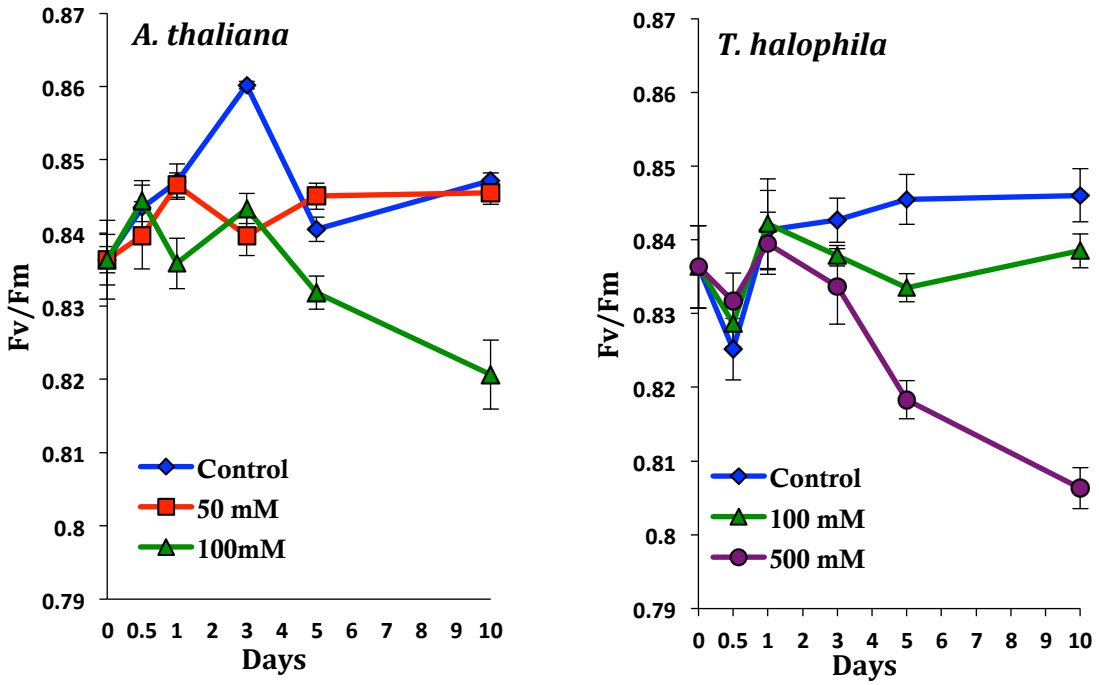


Figure 2.3.3: Chlorophyll content (a & b), Fo (c & d) in *A. thaliana* and *T. halophila* watered with tap-water only (controls) or subjected to salt stress by watering them with NaCl (50 and 100 mM [NaCl] for *A. thaliana*, and 100 and 500 mM [NaCl] for *T. halophila*). Each point is a mean of three replicates and standard errors were calculated from the three replicates. Values are relative units.



(e) (f)

Figure 2.3.4: Fv/Fm in *A. thaliana* (a) and *T. halophila* (b) watered with tap water only (controls) or subjected to salt-treatment by watering them with NaCl (50 and 100 mM [NaCl] for *A. thaliana*, and 100 and 500 mM [NaCl] for *T. halophila*). Each point is a mean of three replicates and standard errors were calculated from the three replicates.

2.3.4 Effect of salinity on ion-uptake: Na^+ and K^+

As shown in Figure 2.3.5, both species showed increased accumulation of sodium ions under salt-treatment. *A. thaliana* accumulated sodium to similar levels under both 50 and 100 mM [NaCl] treatments. After 10 days of salt-treatment, levels of Na^+ in the shoots were about six times the level in the control plants. In *T. halophila* there was however at 100 mM [NaCl] a small rapid transient increase in Na^+ content after 1 day of exposure to salt followed by a decline in Na^+ level in the shoots and finally a slight increase after 5 days of salt-treatment. *T. halophila* plants exposed to 500 mM [NaCl] had restricted Na^+ uptake during the first day of salt-treatment. However, a consistent increase in Na^+ content followed, with Na^+ levels over ten times that in the unstressed plants after 10 days of salt treatment. Together these results suggest that the two plant species have different mechanisms for regulating salt uptake. Although there was a general decrease in the concentration of K^+ (including in the control) plants for both *A. thaliana* and *T. halophila*, the pattern of change in K^+ levels indicated that *T. halophila* increased K^+ uptake to limit Na^+ uptake during the first 48 hours of exposure to salt (see Figure 2.3.6).

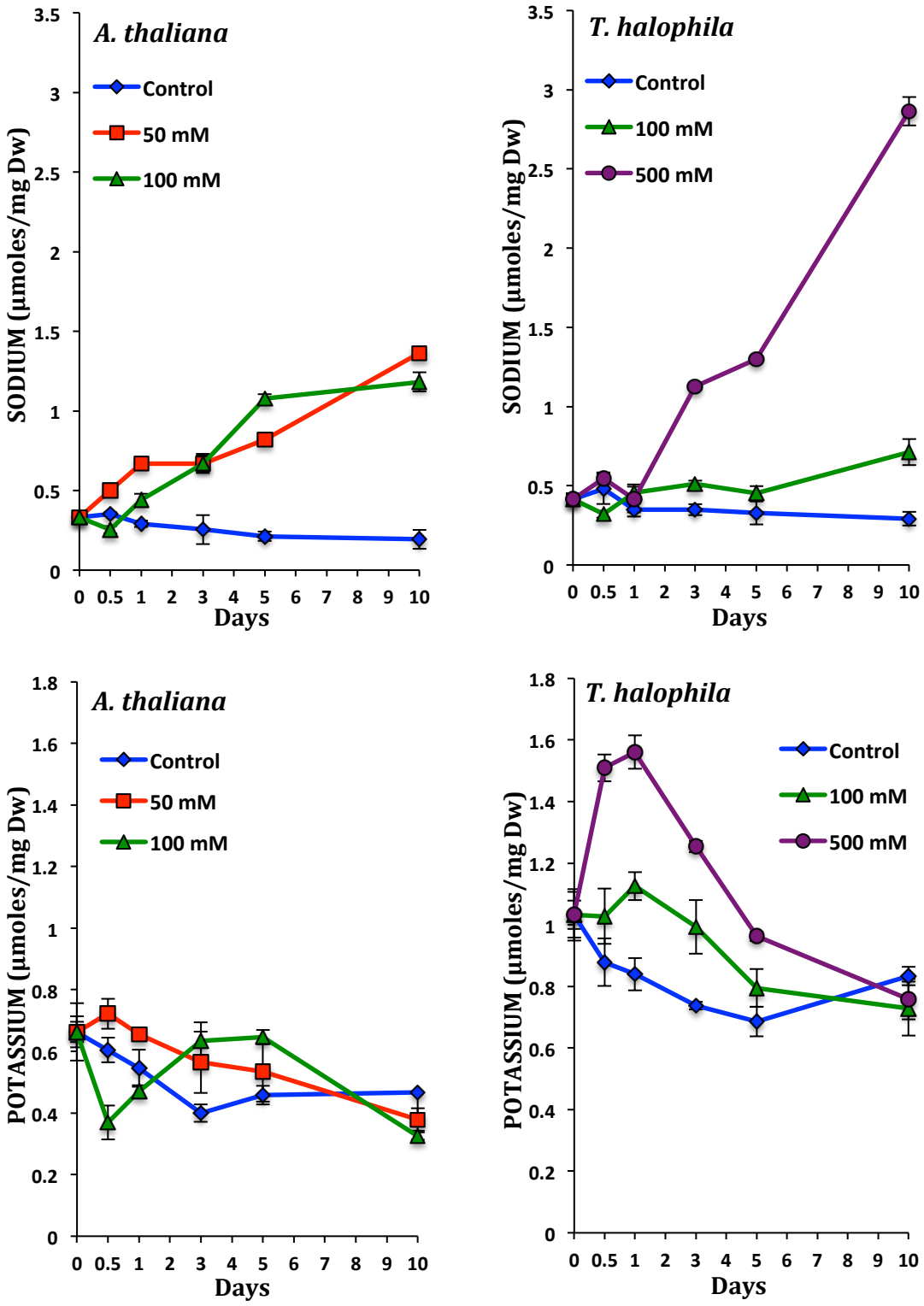


Figure 2.3.5: Levels of Na^+ and K^+ measured in shoots of *A. thaliana* and *T. halophila* over a 10-day period of salt-treatment (NaCl). Each point is a mean of three replicates and standard errors were calculated from the three replicates

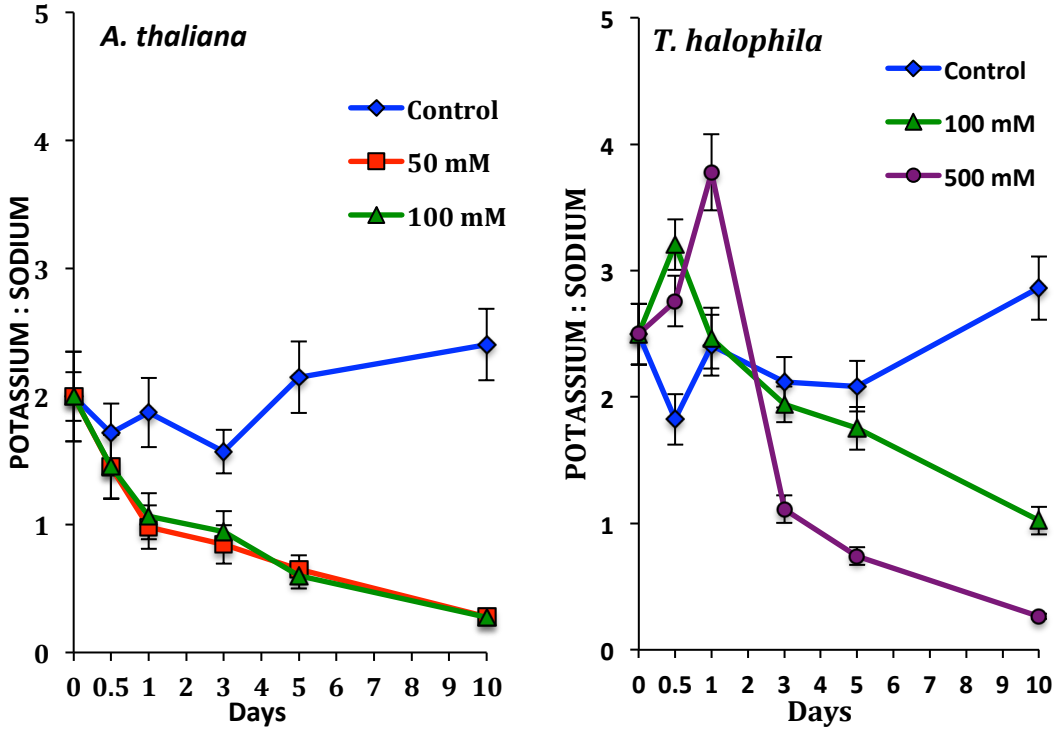


Figure 2.3.6: Potassium (K^+) to Sodium (Na^+) ratio measured in shoots of *A. thaliana* and *T. halophila* over a 10-day period of salt-treatment (NaCl). Each point is a mean of three replicates and standard errors were calculated from the three replicates

2.3.5 Sugars and sugar alcohols

The results of sugar analyses showed an increase in sugars and inositol under salt-treatment compared to the untreated controls in both plant species. In general, clear increases in the levels of sugars and inositol under salt treatment occurred in both species with greater increase seen in *T. halophila* (see Figures 2.3.7, 2.3.8, 2.3.9). At 100 mM [NaCl] there was about four-fold and two-fold more fructose and glucose respectively in *T. halophila* compared to the control, although there was two-fold more sucrose in *A. thaliana* at the end of the stress treatment (see Figure 2.3.8). The basic levels of glucose and sucrose at T0 in *T. halophila* were higher than those in *A. thaliana*. Moreover, the accumulation of glucose in *A. thaliana* increased at a slower rate under salt treatment than in the control. On the other hand, *T. halophila* showed 14-fold higher sucrose accumulation under high salinity compared to controls after 10 days of salt treatment. *T. halophila* maintained higher fructose and glucose levels in both the absence and presence of salt stress than *A. thaliana*. There was a substantial increase in inositol in both plant species under salt treatment. The results suggest an association between inositol accumulation and salt tolerance. *T. halophila* maintained higher levels of inositol in the presence and absence of salt (see Figure 2.3.9).

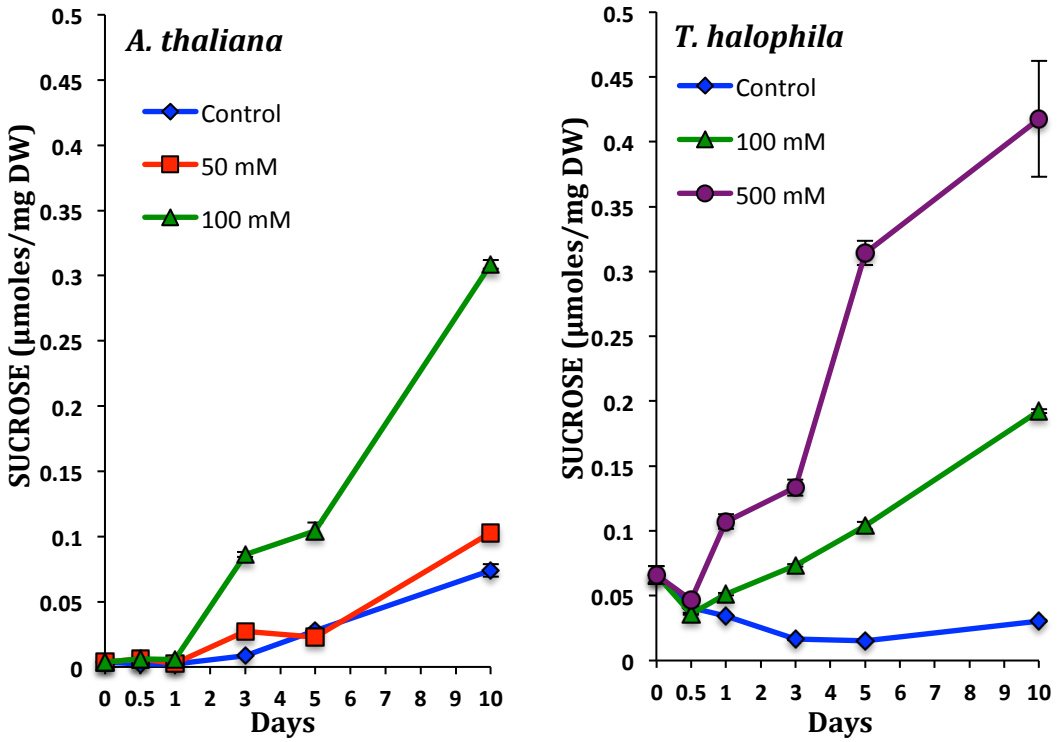
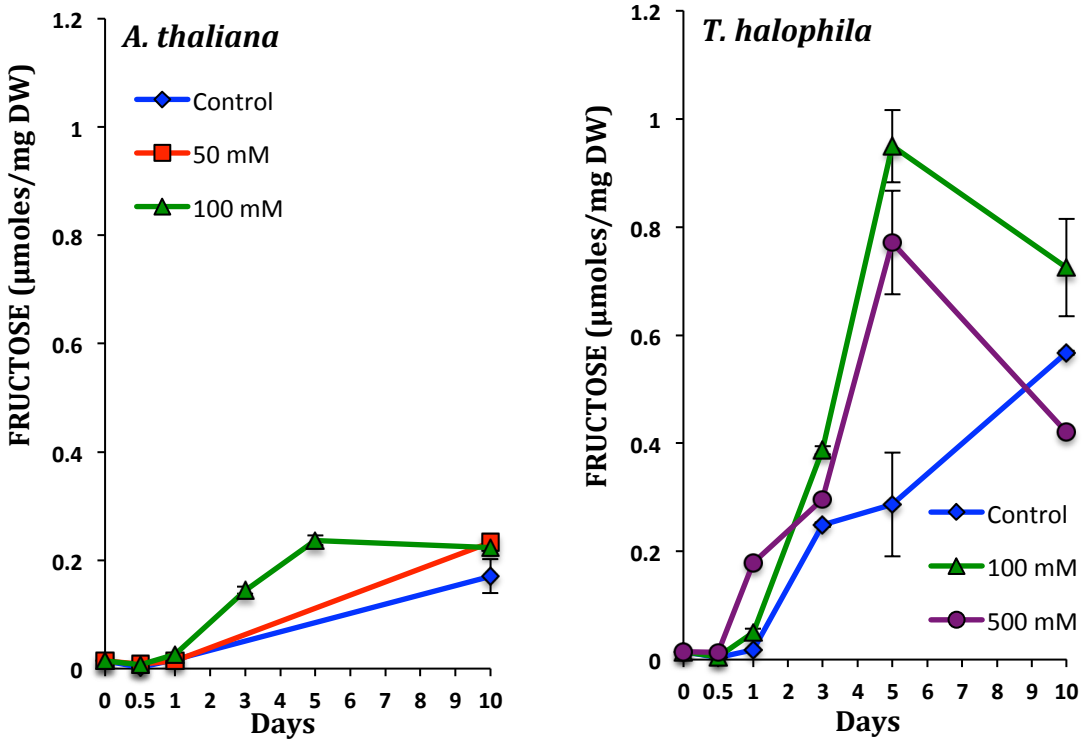


Figure 2.3.7: Levels of sucrose measured in shoots of *A. thaliana* and *T. halophila* over a 10-day period of salt-treatment (NaCl). Each point is a mean of three replicates and standard errors were calculated from the three replicates



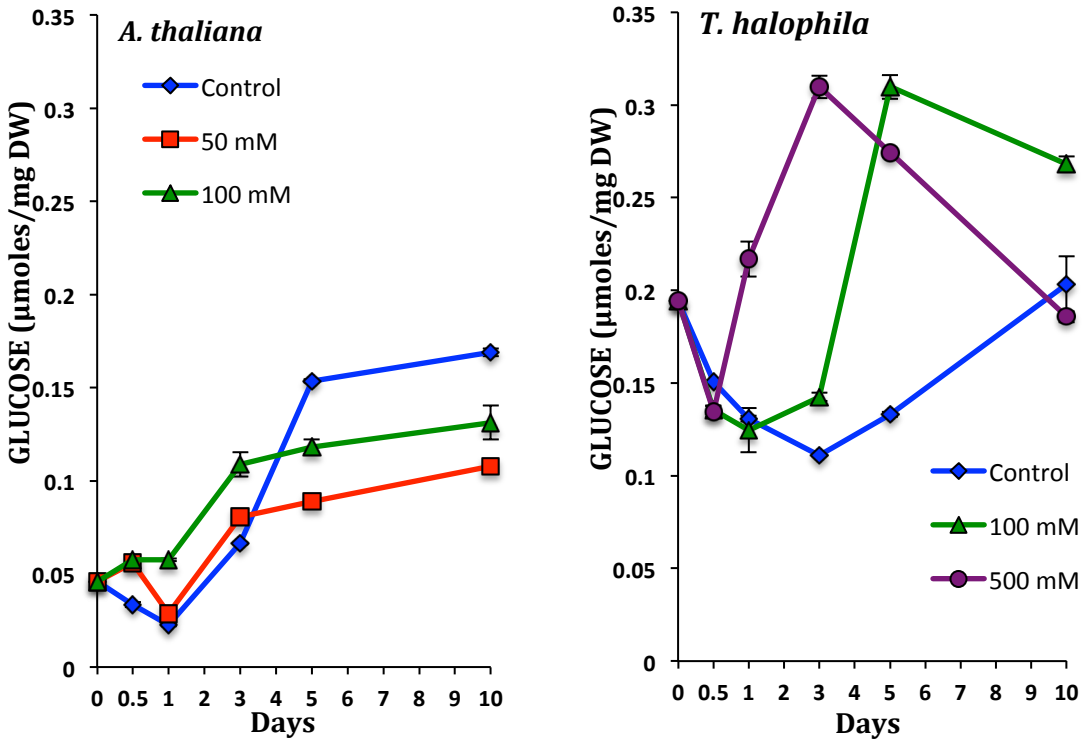


Figure 2.3.8: Levels of fructose and glucose measured in shoots of *A. thaliana* and *T. halophila* over a 10-day period of salt-treatment (NaCl). Each point is a mean of three replicates and standard errors were calculated from the three replicates

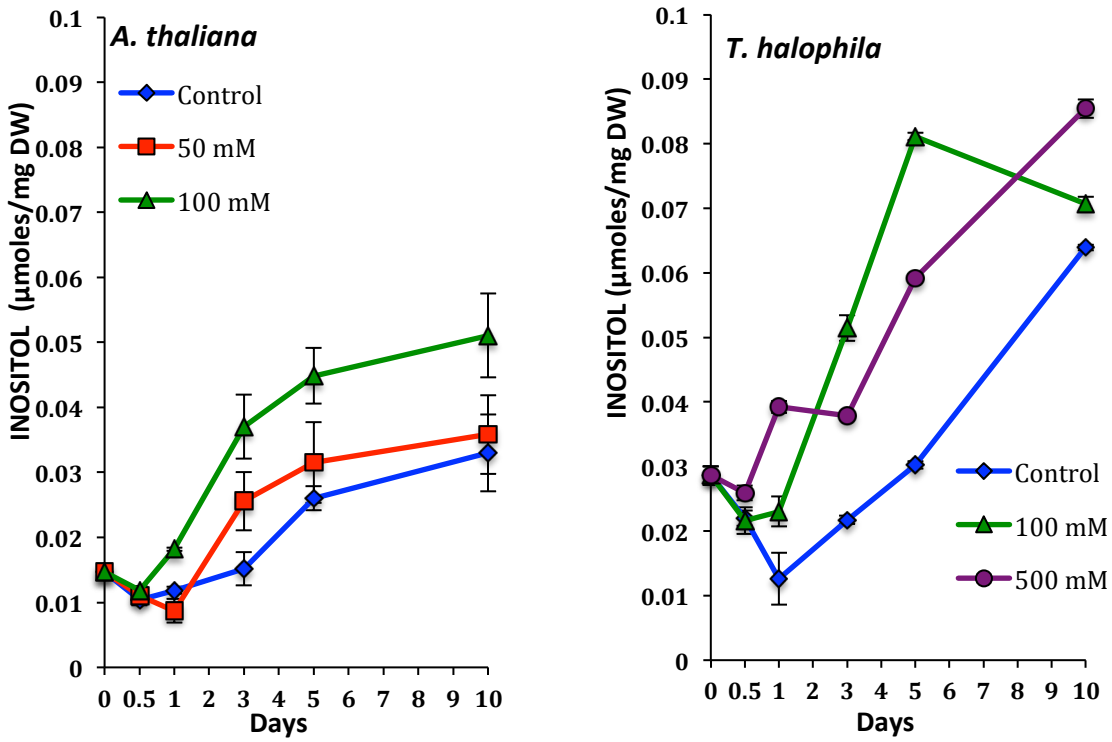


Figure 2.3.9: Levels of inositol measured in shoots of *A. thaliana* and *T. halophila* over a 10-day period of salt-treatment (NaCl). Each point is a mean of three replicates and standard errors were calculated from the three replicates

2.3.6 Malic Acid accumulation

As shown in Figure 2.3.10 salinity had contrasting effects on change in malate content between the two plant species. While there was a small increase in malate in *A. thaliana* under salt stress, there was a substantial decrease in amounts of malate over the 10 days of salt treatment in *T. halophila*. However, even after this decrease, *T. halophila* maintained higher levels of malate in the presence and absence of salt stress compared to *A. thaliana*. These results are in accordance with those reported for *T. salsuginea* (Lugan et al., 2010). In particular, there was a substantial difference between malate levels in the unstressed plants of the two systems. *T. halophila* had four times more malate than *A. thaliana*.

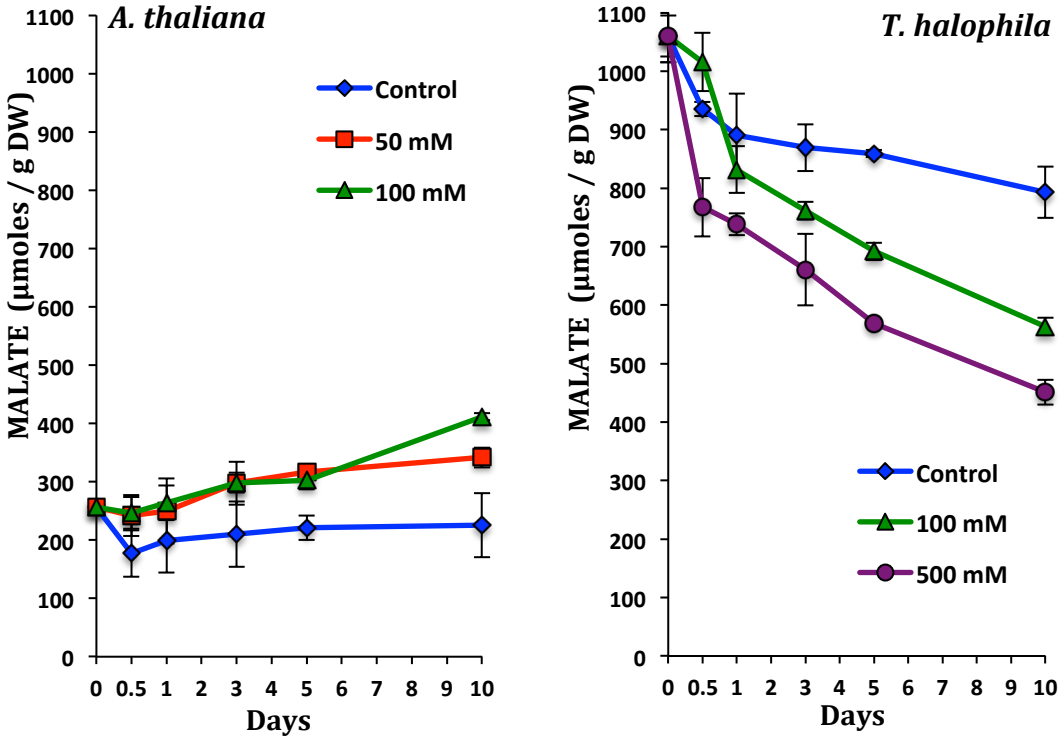


Figure 2.3.10: Change in malic acid levels in shoots of *A. thaliana (left panel) and T. halophila* (right panel) induced by salt-treatment (NaCl) over 10 days. Each point is a mean of three replicates and standard errors were calculated from the three replicates

2.3.7 Proline accumulation

As shown in Figure 2.3.11, large increases in levels of proline were induced by salt-treatment in both *A. thaliana* and *T. halophila*. The level of proline in control plants of *T. halophila* was twice the level in control plants of *A. thaliana.* The pattern of the increase in proline levels in the two plant species under salt-stress was different. While there was a continuous slow accumulation of proline in *A. thaliana*, there was a strong and rapid increase in proline levels in *T. halophila* reaching a maximum after 3 days of salt-treatment followed by a substantial decline. Although there was no difference in the levels of proline in *T. halophila* subjected to100 and 500 mM [NaCl], there was a substantial difference in those measured in *A. thaliana* plants subjected to 50 and 100 mM [NaCl]. It is worth noting that levels of proline in *A. thaliana* and *T. halophila* were similar after 10 days of salt-stress.

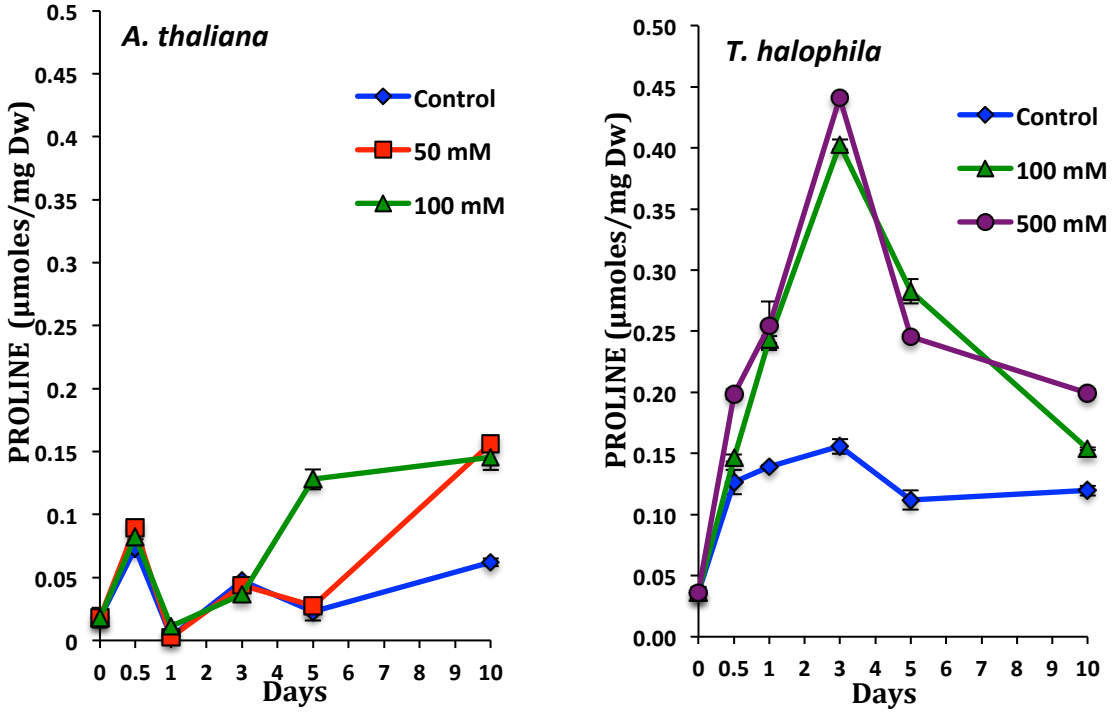


Figure 2.3.11: Level of proline measured in shoots of *A. thaliana* and *T. halophila* over the 10-day period of salt-treatment (NaCl). Each point is a mean of three replicates and standard errors were calculated from the three replicates.

2.4 Discussion

2.4.1 Differential impact of salinity on growth and photosynthetic capacities

As expected, salinity severely reduced the growth of *A. thaliana* and *T. halophila* and growth reduction was higher in *Arabidopsis.* The results suggest that the reduction in growth might be the consequence of the drop in photosynthetic capacities and the deployment of stress-resistance mechanisms, which are large sinks for energy and carbohydrates. The drop in photosynthetic capacities might be a consequence of the deployment of regulatory responses to limit the light energy harvested in the chloroplast to avoid the possible oxidative stress imposed by salinity on the photosynthetic machinery. Even under high salinity (500 mM [NaCl]) *T. halophila* seemed to have resisted oxidative stress by reducing photosynthesis and growth. This helps the plant to survive longer periods of stress and might increase level of photosynthesis and growth if conditions become more favourable.

2.4.2 Ion selectivity: Na^+ and K^+ content under salt stress

A. thaliana and *T. halophila* demonstrated differences in the accumulation of sodium and potassium over the period of 10 days of salt treatment under two different doses of [NaCl]. If only compared under the 100 mM [NaCl], *A. thaliana* showed a gradual increase in the accumulation of Na^+ over the 10 days of stress but *T. halophila* controlled Na^+ uptake after 24 hours of applying salt to a level near to that of the unstressed plants. This differing feature over the time course suggests that *T. halophila* might have a strong capability to restrict uptake of Na^+ at the shoot/root barrier, leading to low uptake of Na^+ from the soil. But this was only true for *T. halophila* when subjected to low salinity (100 mM [NaCl] considered low for many halophytes including *T. halophila*). At higher salt concentration (500 mM [NaCl]) after 24 hours of exposure, the shoot Na^+ content increased drastically in *T. halophila*. In glycophytes, excessive Na^+ content is considered to be highly toxic and has harmful effects on plant growth and acts as a key destructive factor (Niu et al., 1995; Zhu, Liu and Xiong, 1998). Greenway and Munns (1980) supported the argument that an excessive amount of Na^+ in tissue was the main factor behind the level of salt sensitivity of non-halophytes like *A. thaliana.* But in fact, research conducted on the SOS pathway (see Figure 2.4.1 for SOS pathway) genes using *A. thaliana* mutants did not show any correlation between sodium content and salt sensitivity. The *SOS1* mutants in the above study exhibited lower amounts of Na^+ uptake

but did not show any reduction in sensitivity to salt when compared to the wild type (Zhu, Liu and Xiong., 1998). In contrast, the study showed a much closer relation of salt tolerance to the potassium content in the tissues. This was true for the results presented here, which showed higher levels of potassium in shoots of *T. halophila*. Although there was a decrease in levels of K^+ in both species, *T. halophila* maintained higher amounts of K^+ over the period of 10 days of salt treatment than *A. thaliana*. Potassium homeostasis plays an important role in many cellular processes, and small changes in the cellular K^+ content can result in huge differences in plant growth rates (Zhu, Liu and Xiong., 1998). The substantial decrease in K^+ content observed at 500 mM [NaCl] in *T. halophila* can be explained by the direct competition exhibited by Na^+ on root transporters. Na^+ and K^+ are both chemically very similar ions and at very high concentrations external Na^+ has a limiting effect on K^+ uptake by the roots (Ghars et al., 2008). Many different reports have suggested K^+ as an important nutrient during salt stress in plant cell cultures and yeast (Zhu, Liu and Xiong., 1998). Potassium is a very important element that plays critical roles not only in supporting plant growth but also in metabolism and various other cellular processes and is required by the plants in large amounts. Therefore, the ability to take up and maintain high tissue content of K^+ in the presence of excessive amount of external or internal Na^+ is a crucial factor in salt tolerance.

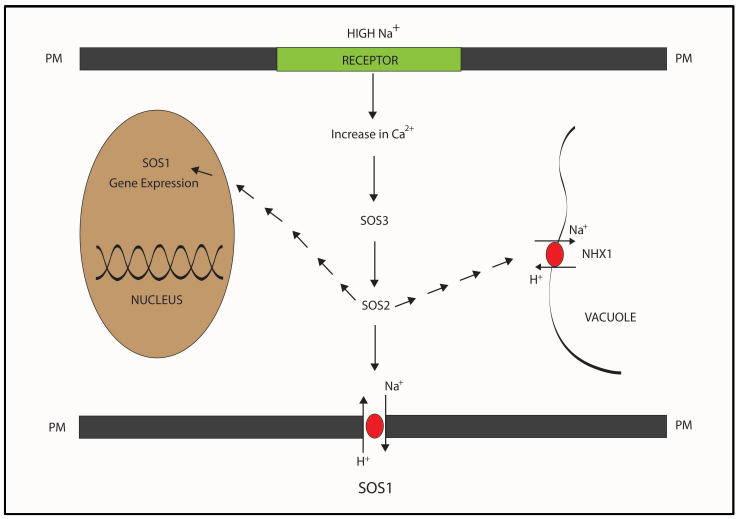


Figure 2.4.1: SOS pathway of Na^+ transport in plants. High external sodium ions lead to increase in cytosolic free Ca^{2+} amounts, which bind to SOS3 that activates the protein kinase SOS2. SOS3 and SOS2 form an activated kinase complex necessary for increased expression of SOS1 under salt stress. Adapted from Zhu (2000).

2.4.3 Regulation of metabolite accumulation

The metabolic responses of *A. thaliana* and *T. halophila* were compared over a time course to identify the difference in accumulation kinetics of key metabolites that may participate in the differential salinity tolerance shown by the two species. The two

species exhibited similar changes in selected metabolites but with differences in the kinetics and amplitude of change under salt treatment. The overall increases observed in sucrose, glucose, fructose, inositol and proline showed a positive correlation with salt-treatment and increased to higher levels in *T. halophila* than *A. thaliana*.

Soluble sugars such as sucrose, glucose, fructose and inositol are the direct products of photosynthesis and components of primary metabolism. They may also result from degradation of starch, which usually increases under stress (Krasensky and Jonak, 2012). From the results it is noticeable that *T. halophila* seemed to accumulate these soluble carbohydrates more effectively at both early and later stages of salt stress. The onset and levels of accumulation of these soluble sugars under 100 mM [NaCl] was much quicker and far greater in *T. halophila*, after just 12 hours compared to more than 24 to 72 hours in *A. thaliana.* There is a possibility that the lower levels of sugars observed after 12 hours of salt-stress are just a direct result of the fact that the samples were taken during the night, when the amounts of sugars are limited due to lack of photosynthesis during this time which would imply that most of these sugars are a direct result of photosynthesis. This (after 12 hours) was the only instance over the period of 10 days where the sampling was done in the night. The amount of these sugars was also seen to be higher in *T. halophila* under control conditions than in *A. thaliana*, especially for glucose. Moreover, the accumulation of glucose in *A. thaliana* was lower in the control than the salt-treated plants. This can be due to the plant favouring the production of fructose over glucose with a limited amount of sucrose (1.5 fold increase) accumulated in *A. thaliana.* The sugar alcohol inositol is a ubiquitous six-carbon cyclohexane hexitol and its derivatives pinitol and D-ononitol are implicated as osmoregulators in various biological systems (Shen et al. 1999). In addition to this, inositol and its methylated derivatives are also implicated in various other cellular functions like regulation of growth, membrane biogenesis, signal transduction and membrane dynamics (Loewus and Murthy 2000).

These differences in carbohydrate accumulation in both species under stressed and non-stressed conditions makes it difficult to assign carbohydrates with the function of primary response to salt stress, as an increase in any one of them may be a result of reactivation of photosynthesis regulated through onset of other defence mechanisms (Gil et al., 2013). Various mechanisms that may regulate metabolic fluxes and signaling pathways all together make a complex network that controls the intracellular levels of these sugars. Regardless of how they accumulate and what their source might be they do accumulate very quickly and to higher levels in *T. halophila* than in *A. thaliana*. This

makes *T. halophila* potentially more effective under salt stress in terms of mobilizing sugars that may move throughout the plant fulfilling roles as major energy sources, precursor for many metabolites, signaling components, osmoregulators and also ROS scavengers (Hare et al., 1998; Gil, et al. 2013). Therefore, sugars might help the plant to maintain high photosynthetic capacities in the continuous presence of salt.

In the case of organic acids, only malate was measured in the two plant systems since it has important roles in most of the plant organelles. Malate is rapidly transferred between the different subcellular compartments (Kalt et al. 1990) due to many transport systems, and the organellar movement of malate has been reported under stress conditions (Renné et al., 2003; Scheibe, 2004). Various biological functions involve malate, as described by Lance and Rustin (1984), including (1) control of cellular pH, (2) support of photorespiration, (3) redox homeostasis, (4) stomatal movement by regulation of osmotic pressure, (5) transport and exchange of reduced equivalents between cellular compartments. From comparing the response kinetics between the two species, two main differences surfaced. First, *A. thaliana* showed an increase and *T. halophila* showed a substantial drop in accumulation upon salt treatment. Second, under unstressed conditions the amount of malic acid throughout 10 days remained at much higher levels in *T. halophila*. This might suggest that *T. halophila* is pre-programmed to tolerate salt-stress, i.e. it has the exclusive feature which is commonly related to halophytes that involves constitutive and adaptive mechanisms making it metabolically ready in the anticipation of stress (Sanchez et al., 2008). And the observed malate decrease may occur because *T. halophila* in the presence of salt stress favours other specialized compounds or even sugars to use as carbon source. In contrast to *A. thaliana* the channeling of energy and carbon for the production of organic solutes that can help sustain the high level of tolerance to salinity is adopted very early and to a higher extent in *T. halophila*.

This key factor can be understood also from results in the different accumulation kinetics of proline between the two species. Proline accumulates in various higher plants, and it is commonly regarded as the main effector response (with hexoses) to salt stress and can contribute to around 50% of the osmotic adjustment (Ashraf and Harris, 2004; Arbona et al., 2013). *T. halophila* seems to favour proline accumulation, particularly in the early stages of salinity, and thus apparently has capable machinery to regulate such a response. In control plants, *T. halophila* again shows its pre-programmed characteristic to face salt-stress with high proline levels maintained compared to *A. thaliana*. These results are in accordance with those reported by Taji et

al. (2004). Proline levels after 72 hours of salt stress were 14 fold and 12 fold higher than at time 0 in *T. halophila* at 100 and 500 mM [NaCl] respectively. But these differences dropped to two-fold at 5 days and to approximately the same level in *T. halophila* at 100 mM as in *A. thaliana* at 50 and 100 mM [NaCl] after 10 days of salt-treatment. This could be because within 24 to 72 hours, *T. halophila* was able to activate/deactivate and increase/decrease different complex mechanisms to defend against salinity and quickly adapt to changed conditions. Increased levels of proline might assist in acclimation to salinity by lessening the effects of salt on cell membranes, regulating the accumulation of available nitrogen protecting enzyme activities and also acting as signaling/regulatory molecule to activate multiple other responses (Ashraf and Harris, 2004). Such responses would increase the plant responsiveness to salt early in the exposure period and channel a range of required acclamatory mechanisms to achieve functional stability throughout the plant body and help the plant survive during extended periods of salinity.

2.5 Conclusion

Results show differential regulation of accumulation of metabolites under salt-stress in the two close relatives, *A. thaliana* and *T. halophila*. *T. halophila* showed faster and stronger responses to salt-stress, with potentially greater osmoregulation and better control over salt uptake and partitioning. These differences in the kinetics and/or amplitude of responses in *T. halophila* compared to *A. thaliana* were observed in the regulation of the accumulation of key compatible metabolites such as sucrose, fructose, inositol and proline for enhanced stress tolerance. The two species have over 90% sequence similarity at the genome level yet they exhibited a striking difference in salinity tolerance. There are various direct comparisons conducted in the past between the two plant systems and these have provided exciting results into which we can have more insight. This chapter supports and backs up the recent emerging paradigm that the higher salt-tolerance exhibited by *T. halophila* is a matter of differential regulation of certain processes and demonstrates that these processes are deployed at a slower rate and to a lower extent in *A. thaliana* under salt-stress compared to *T. halophila*. These results indicate the need to investigate the regulation of gene expression and transcript accumulation in the two species. In the next chapter, the transcript levels of key genes like P5CS1 (production of proline) and SOS1 (movement of Na^+) will be compared and analyzed to identify the key to the differential responses shown by the two species.

Chapter 3

Comparative analysis of changes in transcript abundance for genes directly or indirectly involved in salt-responses in Thellungiella halophila and Arabidopsis thaliana

3.1 Introduction

In response to salinity, salt-tolerant plants (halophytes) and salt-sensitive plants (glycophytes) seem to deploy similar mechanisms, of which some key elements were analyzed and discussed in the previous chapter. The main feature that emerged from this study after comparing *A. thaliana* and *T. halophila* was that the difference in the kinetics of deployment of these common tolerance mechanisms might play a key role in the differential salt-tolerance exhibited by the two species. This difference might be controlled primarily at the gene level via differential expression of basic sets of genes playing key roles in the salt tolerance mechanisms (Taji et al., 2004). Other mechanisms including alternative splicing of specific genes might also play a role in this differential salt-tolerance between the species (Kesari et al., 2012).

Upon salt stress plants induce various biochemical and physiological responses to resist and/or alleviate the negative effects of salt-stress. These responses are underpinned by changes in gene expression. About 13% of *A. thaliana* genes show changes at the transcriptional level under the effect of salt treatment (Kreps et al., 2002; Kant et al., 2006). The protein products of these genes are used in various biological processes including salt stress tolerance mechanisms, with many proteins having unknown functions. Basically, the known products can be classified into two groups: those that are directly involved in protection against salinity and those that regulate signal transduction and gene expression (Hasegawa et al., 2000). Stress-response regulatory networks involve various genes for transcription factors and signaling components like protein kinases, which are up or down regulated to achieve salt resistance (Chen et al., 2002; Kreps et al., 2002; Kant et al., 2006).

In the recent past, the introduction of *T. halophila* as an *A. thaliana* relative model system (ARMS) helped to establish comparisons between closely related species with contrasting levels of salt-tolerance. Differences between the two species in responses to salinity were investigated using various molecular and genetic tools to compare the salt-regulated expression of many genes. For example, *A. thaliana* cDNA microarray analysis was used to compare the transcript levels for nearly 7000 genes in *A. thaliana* and *T. halophila* under salt-treatment (Taji et al., 2004). The analysis concluded that many of the genes induced by salt-stress in *A. thaliana* were expressed to a much higher level in *T. halophila* under unstressed conditions (Taji et al., 2004). Other research, where a microarray of 25000 elements was used to compare transcript profiles between the two species, showed *T. halophila* to have both distinct and shared gene specific

responses with *A. thaliana* under salt-treatment (Gong, Li and Ma, 2005). Both studies concluded that *T. halophila* has stress anticipatory preparedness with constitutive expression of conserved stress mechanisms, which might help it to have higher salt tolerance than *A. thaliana*.

The above two studies with many others have failed to define the basic set of genes controlling salt tolerance and which are responsible for the differential regulation of the key salt-tolerance mechanisms between the two closely related species. This chapter looks into the key results from the previous chapter about differential accumulation of sodium and various metabolites between the two systems and takes a step further to focus on the underlying gene transcript levels which may be responsible for the accumulation differences of the salt ions (Na^+) and metabolites in the two target species. The chapter explores three main mechanisms involved in salt tolerance: (1) Salt accumulation: transcript profile of Salt Overly Sensitive 1 (*SOS1*) gene which codes for a plasma membrane Na^+/H^+ antiporter that retrieves and loads Na^+ ions from and into the xylem and controls sodium accumulation in the plant. The *SOS1* gene plays a critical role in maintaining ion homeostasis during salt stress by controlling the loading and retrieval of Na^+ into and from the xylem stream (Shi et al 2002). (2) Carbohydrates: the transcript profiles of genes involved in metabolism of carbohydrates (sucrose, glucose and fructose), which are required to maintain the structural and nutritional integrity of plants under salt stress and also act as precursors of osmoregulators and play a direct role in osmoregulation themselves. The Sucrose Synthase 3 (*SUS3*) gene is a member of one of the characterized small multi gene families of SUS found in monocot and dicot plants (Komatsu et al., 2002). *SUS3* is one of the six genes encoding the enzyme sucrose synthase involved in sucrose cleavage (Baud et al., 2004). The UDP-Glucose Pyrophosphorylase 2 (*UGP2*) gene product is responsible for producing UDP-glucose, an important precursor for the biosynthesis of cellulose and callose (Park et al., 2010). The Fructose-bisphosphate Aldolase 1 (*FBA1*) gene encodes a key enzyme involved in, glycolysis and gluconeogenesis as well as the pentose phosphate cycle in plants (Fan et al., 2009; Lu et al., 2012). The final selected gene in carbohydrate metabolism was Phosphoenolpyruvate Carboxylase 1 (*PPC1*) gene, which encodes one of the four PEP-carboxylase isoforms in *A. thaliana*. This enzyme plays a key role in the synthesis of oxaloacetate, which is the precursor of malate; PEP-carboxylase is known to catalyse the rate-limiting step of malate production (Sánchez and Cejudo, 2003; Sullivan et al., 2004). (3) Proline: the analysis to compare the transcript profile of the Δ^1-Pyrroline-5-carboxylate synthase 1 (*P5CS1*) gene responsible for the

accumulation of proline, a key compatible solute that accumulates under salt stress (Kant et al., 2006). Δ^1-Pyrroline-5-carboxylate synthase 1 is a rate-limiting enzyme in the biosynthesis of proline. With the help of two-step real time RT-PCR, the transcript levels of the key genes explained above were compared between unstressed and salt-treated plants of *A. thaliana* and *T. halophila*. The aim of this approach is to detect changes in the regulation of the chosen genes under salt stress and to help understand better the key regulatory mechanisms that control the differential responses to salinity exhibited by *T. halophila* and *A. thaliana*.

3.2 Material & Methods

3.2.1 Plant material and stress treatment

Four-week-old *A. thaliana* and 6-week-old *T. halophila* plants, similar in size and before bolting, were divided into three sets and irrigated with 3 different NaCl concentrations prepared with tap water. *A. thaliana* plants were watered with 0, 50 and 100 mM [NaCl] and *T. halophila* plants were watered with 0, 100 and 500 mM [NaCl] (0 mM refers to tap water and represents the control) at a fixed time (12:00) every day for 10 days. Shoots and roots were harvested at a fixed time (16:00) as 2 plants per sample after 12 hours, 1, 3, 5 and 10 days of the treatment (roots were cleaned under running water and dried using paper towel), weighed and frozen in liquid nitrogen. Three samples were harvested at each time point for each NaCl concentration for both plant species (same treatment as explained in section 2.2.2 in chapter 2).

3.2.2 RNA extraction

Total RNA was isolated from the shoots and roots using the Tri-reagent method as described by Taybi and Cushman (1999). Approximately 100 mg of ground tissue was homogenized in 1 ml of Tri-reagent (Helena Bioscience, UK) in a 2 ml RNase/DNase free tube in the fume hood. After 2 minutes, the tubes were hand-shaken thoroughly to mix the suspension. The tubes were then incubated in the fume hood at room temperature for 10 minutes. To the settled suspension, 250 µl of chloroform was added and the sample mixed very well by hand shaking. After 5 minutes at room temperature, the suspension was spun down at 13,000 rpm at 4°C for 10 minutes. The upper phase was transferred to a 1.5 ml RNase/DNase free tube to which 250 µl of 0.8 M Na-Citrate/1.2 M NaCl solution and 250 µl of isopropanol was added. The mixtures were shaken well and spun down at 13,000 rpm at 4°C for 30 minutes. After centrifugation, the supernatant was removed from the white pellet stuck to the tube wall. The pellet was washed with 1 ml of 70% ethanol by vortexing and spinning at 4°C for 5 minutes. The ethanol supernatant was discarded and the pellet left to air dry in the fume hood for no more than 5 to 10 minutes to avoid over drying which may prevent RNA re-suspension. The pellet was suspended in 50 µl of DEPC-water, vortexed and incubated on ice for 1 hour. All the RNA samples were stored at -80°C until use.

3.2.3 DNase I treatment of RNA

To eliminate residual genomic DNA co-extracted with RNA, the RNA samples were treated with DNase I (Invitrogen, UK). DNase I reaction mixture was prepared in a 1.5

ml RNase/DNase free tube where 5 µl of 10x DNase I buffer and 1 µl of DNase I enzyme (1 U) were added per sample and the volume adjusted with DEPC-treated water to 50 µl. After vortexing, the samples were incubated at room temperature for 15 minutes. After incubation, 5 µl of 25 mM EDTA was added to each sample, which was vortexed and incubated at 65°C on a heat block for 10 minutes. After incubation the RNA samples were aliquoted in two batches and stored at -80°C for further use.

3.2.4 RNA quantification

The resulting total RNA was quantified using a NanoDrop spectrophotometer (ND-100; NanoDrop Technologies, Wilington, DE, USA). RNA samples were then diluted to 100 ng/µl aliquoted and stored at -80 °C until use.

3.2.5 Analysis of transcript levels using quantitative RT-Polymerase Chain Reaction

Transcript levels for the genes *P5CS1*, *SOS1*, *SUS3*, *UGP2*, *FBA1*, *PPC1* (targets) and *UBQ10* (Ubiquitin 10 as reference) genes were monitored in the extracted RNA samples using semi-quantitative RT-PCR. Ubiquin was used as reference gene based on early reports showing its stable transcript levels in both *A. thaliana* and *T. halophila* (Kant et al., 2006). A commercial kit based on SYBR Green detection (Agilent Technologies, UK) was used according to the manufacturer's instructions. Exactly 100 ng RNA for all the target genes and 10 ng RNA for the *UBQ10* gene with 100 nM of gene specific primers (see sequences below) were used in 25 µl reactions. QRT-PCR was performed using the following thermal profile: 59°C (*P5CS1*); 53°C (*SOS1*); 55.3°C (*UBQ10*) for 30 min and 95°C for 2 minutes (reverse-transcription) followed by 40 PCR cycles, at 94°C for 15 seconds, 59°C (*P5CS1*); 53°C (*SOS1*); 55.3°C (*UBQ10*) for 30 seconds, plate read and 72°C for 1 min. Melting analysis was performed between 45 and 90 °C at the end of each QRT-PCR run to confirm the specificity of the amplified products from both plant species. Q-RT-PCRs were run in triplicate for each sample and each time point consisted of three samples.

3.2.6 Primer design

The primers were designed using *A. thaliana* sequences available in the GenBank database using AlleleID® 7 software from Premier Biosoft (USA). Reverse transcription was conducted using the same gene-specific reverse primer used subsequently in PCR and the two reactions were run as single tube reactions. The primer pairs used resulted in the amplification of products ranging from 80 bp to 200 bp

depending on the gene. The products amplified from *T. halophila* were examined for similar size and sequence to those of products amplified from *A. thaliana*.

The sequences of each primer pair are as follows:

Sucrose Synthase 3 (*SUS3)*, amplicon size: 188 bp

Forward primer: 5' – GACCAAGACCTGGTGTTTGGG -3'

Reverse primer: 5' – AGACGAACGAGAAGGACGTGG -3'

UDP-Glucose Pyrophosphorylase 2 (*UGP2*), amplicon size: 111 bp

Forward primer: 5' – TCCCTCAGCTCAAATCCGCC -3'

Reverse primer: 5' – CAATGTGCTGGGCTTCACCAC -3'

Fructose bisphosphate aldolase 1 (*FBA1*), amplicon size: 155 bp

Forward primer: 5' – CCGTCAACCTTCCTCTGTCTC -3'

Reverse primer: 5' – CGTTGGACTCATCCATCGCC -3'

Phospho*enol*pyruvate carboxylase 1 (*PpC1*), amplicon size: 200 bp

Forward primer: 5' – GCAGATTGCTTATCGCCGTAG -3'

Reverse primer: 5' – CAGACTGAGTAGGATGAGCAG -3'

Δ^1- Pyrroline-5-carboxylate synthase 1 (*P5CS1*), amplicon size: 80 bp

Forward primer: 5' – GAGCTAGATCGTTCACGTGCTTT-3'

Reverse primer: 5' – ACAACTGCTGTCCCAACCTTAAC-3'

Salt Overly Sensitive 1 (*SOS1*), amplicon size: 130 bp

Forward primer: 5′-CCTTACACTGTCGCTCTTCTCGTTA-3′

Reverse primer: 5' – TTAGCTCCATATTCGAGAGATCCA-3'

Ubiquitin 10 (*UBQ10*), amplicon size: 58 bp

Forward primer: 5'- CTCTCTACCGTGATCAAGATGCA-3'

Reverse primer: 5' – TGATTGTCTTTCCGGTGAGAGTC-3'

3.2.7 Data analysis

Results from the qRT-PCR were analyzed using the $\Delta\Delta$ct method in which the stressed c(t) value for each target gene at each time point was normalized to the c(t) value for the reference gene at the same time point and compared to its respective unstressed c(t) value taking into consideration the reaction efficiency for each primer set (Pfaffl, 2001). This was done for the plants treated with 50 mM and 100 mM [NaCl] and the resultant values gave the differences in transcript abundance as fold difference from the unstressed control. The average values of fold differences to the controls are presented

in the form of line graphs and standard errors calculated from the three replicates are given.

3.3 Results

3.3.1 Comparative analysis of change in transcript levels for genes with potentially direct and/or indirect role in salt-tolerance in *A. thaliana* and *T. halophila*

To better understand the differential gene responses exhibited by *T. halophila* and *A. thaliana*, several candidate orthologous genes were selected from pathways directly or indirectly involved in salt tolerance mechanisms in both species. The targeted mRNA mechanisms were: first the control of Na^+ movement/build up under salt stress (*SOS1*), second the use of carbohydrates under the effect of salt (*SUS3, UGP2* and *FBA1*), and last the production of an important compatible solute and anti-oxidant, proline, as well as malic acid (*P5CS1* and *PPC1*). Due to the presence of high similarity between the genomes of the two selected species (approx. 95%) (Bressan et al., 2001), it was decided to design primers based on *A. thaliana* sequences and use them to quantify and compare salt-induced changes in transcript abundance using real-time RT-PCR in the two species.

3.3.2 Comparative analysis of transcript levels of *SOS1* gene in shoots and roots in *A. thaliana* and *T. halophila*

Figure 3.3.1 shows changes in *SOS1* transcript levels in shoots and roots of *A. thaliana* and *T. halophila* under the effect of salt-treatment over a period of 10 days. There was no significant change in relative transcript levels for the *SOS1* gene in shoots of both plant species under salt-treatment at 50 and 100 mM [NaCl]. Salt treatment at 500 mM NaCl resulted in a transient approximately three-fold increase in transcript level compared to the control at 24 hours of salt-treatment. *SOS1* relative transcript levels declined slightly in the roots during the first 24 hours of salt-treatment. This decrease was transient and a steady increase in *SOS1* relative transcript levels was induced by salt-treatment in both plant species after one day of salt-treatment. This increase continued in *A. thaliana* up to 10 days of salt-treatment and was stronger at 50 mM [NaCl] and reached over twice the T0 value. In *T. halophila SOS1* relative transcript levels reached six times those at T0 in roots of plants treated with 500 mM [NaCl] after 5 days, and over three times higher at 100 mM [NaCl]. Over the next 5 days relative transcript levels at 500 mM [NaCl] came down and met with the levels at 100 mM [NaCl], which were still higher than levels at T0.

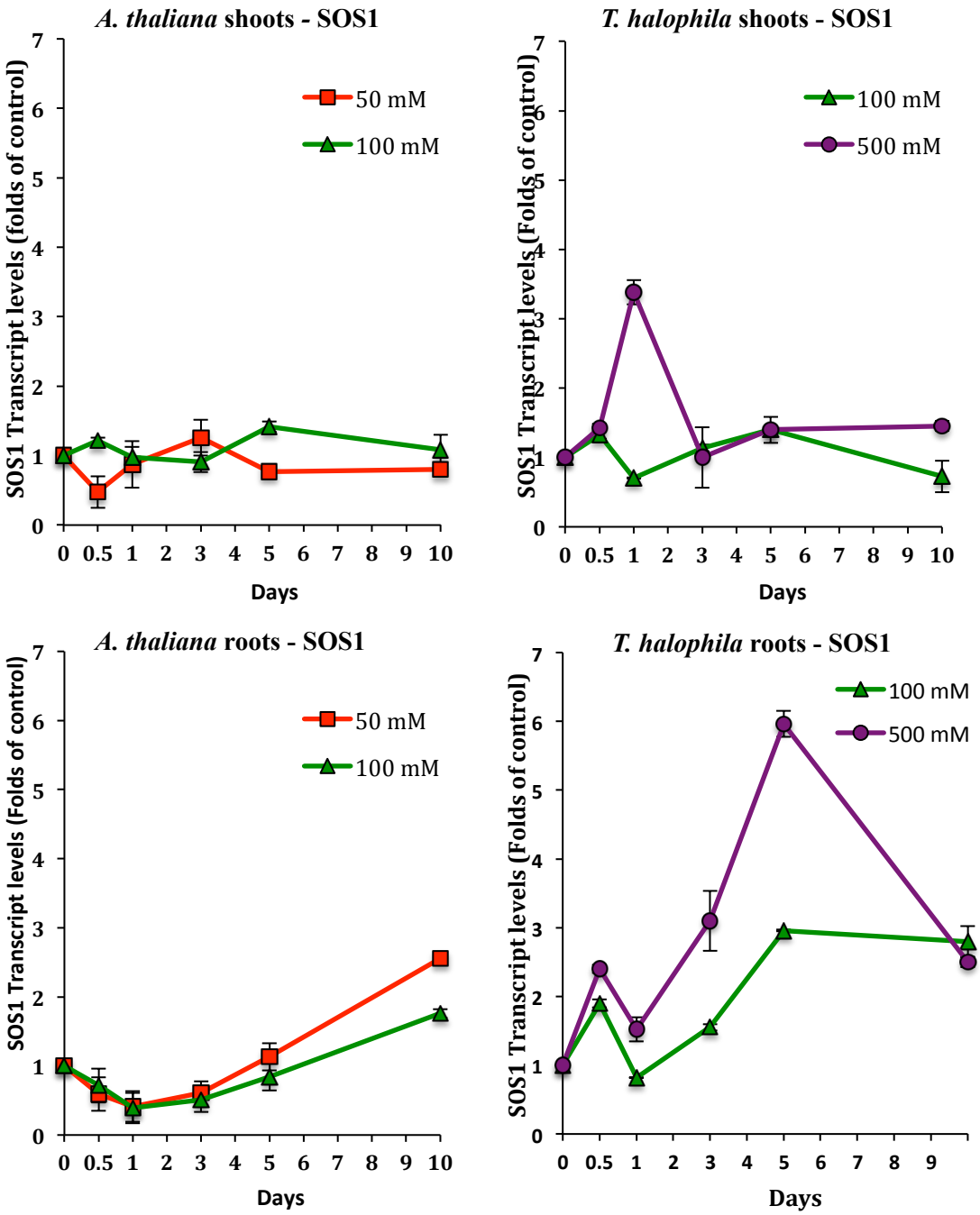


Figure 3.3.1: Transcript levels for SOS1 gene encoding a Na^+/H^+ antiporter under salt treatment (NaCl) over 10 days in *Arabidopsis thaliana* and *Thellungiella halophila*. Fold transcript levels are expressed relative to transcript levels in the water controls (i.e. non salt-treated plants). Each point is a mean of three replicates and standard errors were calculated from the three replicates

3.3.3 Comparative analysis of transcript levels for genes involved in sugar metabolism in the shoots of *A. thaliana* and *T. halophila*

The differential regulation of metabolic activities between *A. thaliana* and *T. halophila* under salt-treatment was investigated at the gene level. Transcript levels of genes from

different parts of carbon metabolism including the genes encoding sucrose synthase 3 (*SUS3*, At4g02280); UDP-glucose pyrophosphorylase 2 (*UGP2*, At5g17310); fructose-bisphosphate aldolase 1 (*FBA1*, At2g21330) and phosphoenolpyruvate carboxylase 1 (*PpC1*, At1g53310) were determined. The transcript abundances of these genes were measured in shoot samples from both species subjected to two concentrations of NaCl stress for 10 days.

As shown in Figure 3.3.2, at 100 mM [NaCl], *A. thaliana* showed a minor increase in relative *SUS3* transcript levels on the 1st day and 5th day of salt-treatment to about 1.5–fold more than control plants before reducing by the 10th day of salt-treatment. On the other hand, in *T. halophila SUS3* transcript levels reached around 1.7-fold higher by increasing slowly till the end of the salt-treatment on the 10th day. But *T. halophila* exhibited a dose response, as at higher [NaCl] of 500 mM the transcript level increased rapidly to 1.7 fold and then 3-fold higher than T0 after 12 hours and 3 days, respectively.

As shown in Figure 3.3.2, at 100 mM [NaCl] *A. thaliana UGP2* relative transcript levels reached 1.5-fold higher than T0 within the first 24 hours of salt-treatment and then fell below the level of the control for the rest of the experiment. In contrast, at the same concentration of salt *T. halophila* showed a delayed and substantial increase in relative transcript levels which took place after 5 days of salt treatment and reached about two-fold higher than the T0 value at the end of the 10 days of salt treatment. In plants treated with 500 mM NaCl relative levels of *UGP2* transcripts decreased during the first 12 hours of salt-treatment and increased afterwards to stabilize at the level of the T0.

As shown in Figure 3.3.3, change in transcript levels for the *FBA1* gene under salt-treatment showed similar trends for the two species. Both species showed a rapid small decrease in relative *FBA1* transcript levels followed by an increase after 12 hours in *A. thaliana* and 1 day in *T. halophila*. This increase reached about 1.5 fold and 1.5-2 fold in *A. thaliana* and *T. halophila* respectively. The trends in *FBA1* transcript change were similar at the two salt concentrations applied to plants. From Figure 3.3.4, it is apparent that *PPC1* relative transcript levels remained unchanged in both *A. thaliana* and *T. halophila plants* over the 10 days of NaCl-treatment.

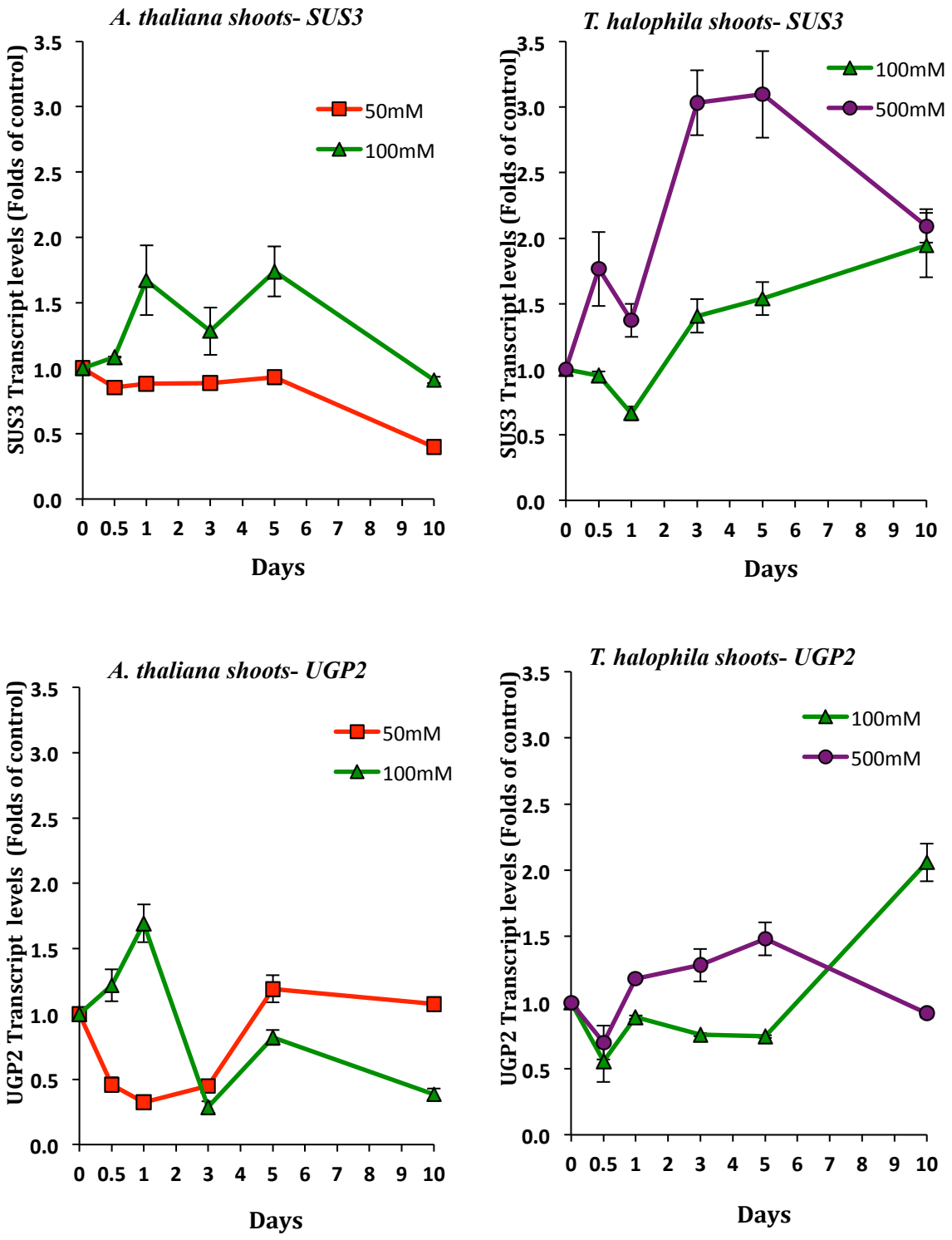


Figure 3.3.2: Change in transcript levels for *SUS3* and *UGP2* genes encoding Sucrose Synthase and UDP-Glucose Pyrophosphorylase responsible for breaking down Sucrose and Glucose-1-phosphate, respectively under salt-treatment (NaCl) over 10 days in *Arabidopsis thaliana* and *Thellungiella halophila*. Transcript levels are expressed as folds to the water controls (i.e. non salt-treated plants). Each point is the mean of three replicates and standard errors were calculated from the three replicates

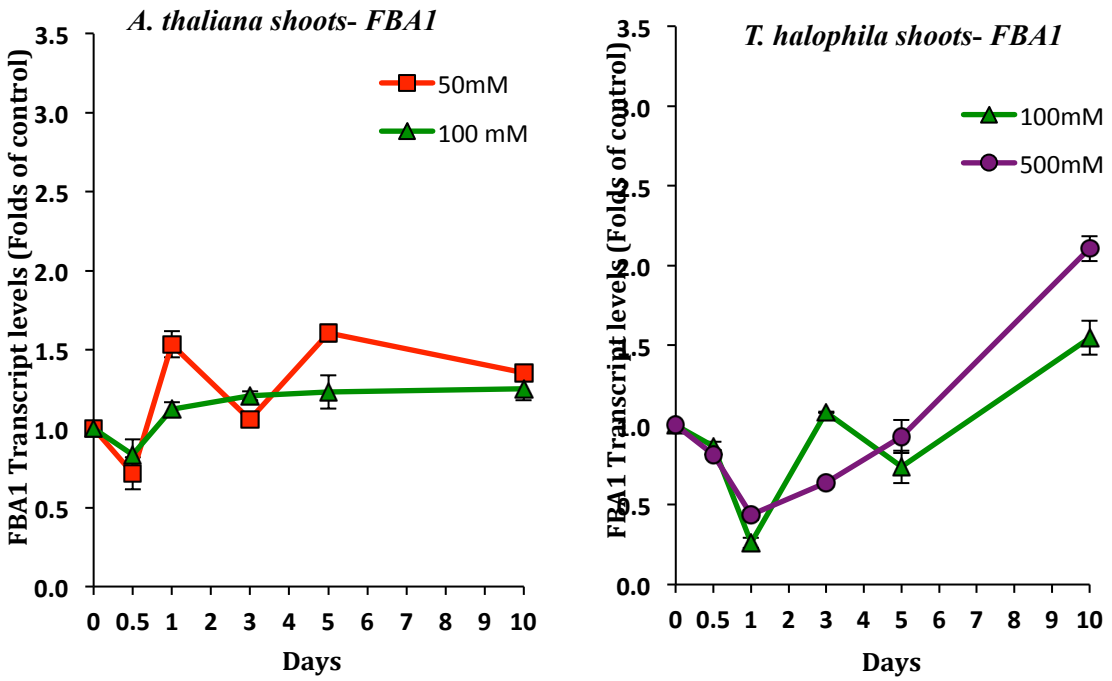


Figure 3.3.3: Change in transcript levels for *FBA1* gene encoding fructose-1, 6-bisphosphate aldolase catalyzing the cleavage of β-fructose-1, 6-phosphate under salt-treatment (NaCl) over 10 days in *Arabidopsis thaliana* and *Thellungiella halophila*. Transcript levels are expressed as folds to the water controls (i.e. non salt-treated plants). Each point is the mean of three replicates and standard errors were calculated from the three replicates

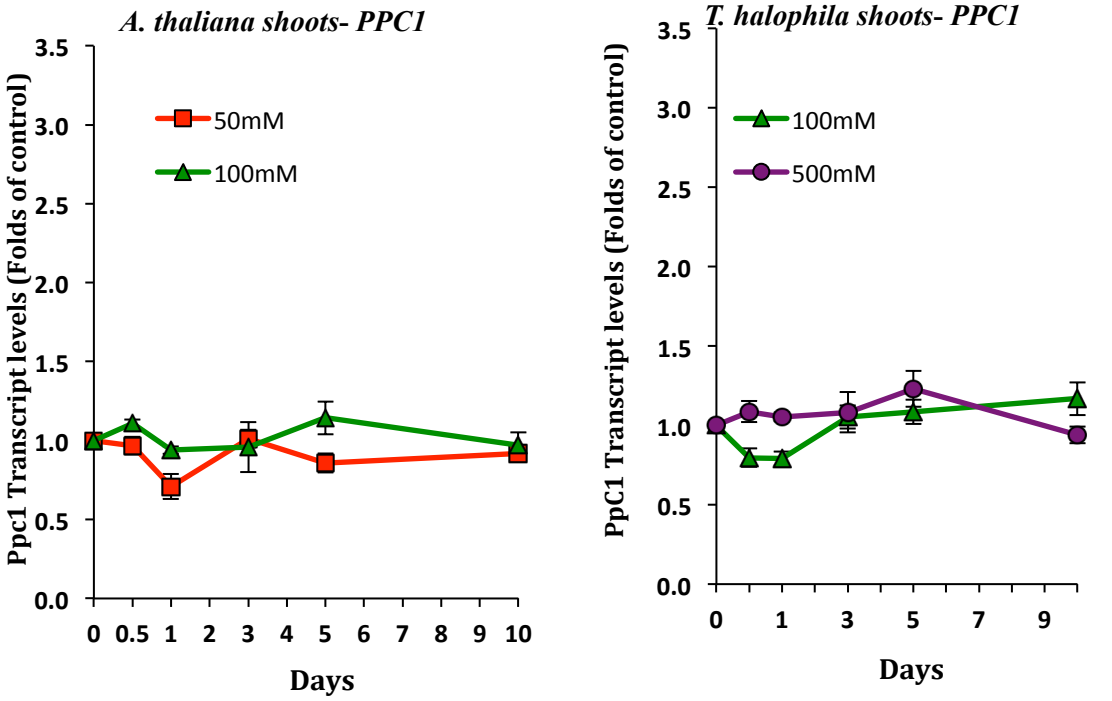


Figure 3.3.4: Change in transcript levels for *PPC1* gene encoding phosphoenolpyruvate carboxylase in *Arabidopsis thaliana* and *Thellungiella halophila* under salt-treatment (NaCl) over 10 days. Fold transcript change is expressed relative to the water controls (i.e. non salt-treated plants). Each point is the mean of three replicates and standard errors were calculated from the three replicates

3.3.4 Comparative analysis of transcript levels for the *P5CS1* gene in shoots and roots of *Arabidopsis thaliana* and *Thellungiella halophila*

The transcript levels of the *P5CS1* gene were monitored in *A. thaliana* and *T. halophila* in control plants and plants subjected to salt-treatment for up to 10 days and expressed relative to the controls. As shown in Figure 3.3.5, different kinetic profiles for the *P5CS1* transcript abundance were observed between the two species and also between the roots and shoots of the same plant species. In shoots, salt-treatment caused a rapid and higher increase in *P5CS1* transcripts in *T. halophila* than in *A. thaliana*. This change was mirrored by the change in accumulation of proline in shoots (data presented in Chapter 2, see Figure 2.3.11, page 51). In shoots, *A. thaliana* showed a maximum of three-fold more transcripts under 100 mM [NaCl] treatment after 5 days with an important drop to the control level after 10 days of salt-treatment. In contrast, *T. halophila* rapidly accumulated *P5CS1* transcripts reaching 5 times the control level after the first 3 days of salt-treatment at 100 mM and 500 mM [NaCl], followed by a decline in transcript levels. There was a higher increase in transcript levels under salt-treatment in the roots of *A. thaliana*, reaching five-fold higher than the T0 level after 5 days of salt-treatment at both 50 mM and 100 mM [NaCl]. There was a similar transcript level of 2 to 3 fold higher in *T. halophila* roots throughout the 10 days of 100 mM and 500 mM [NaCl] treatment. There was no dose effect of salt on *P5CS1* transcript levels in *T. halophila,* whereas treatment with 100 mM [NaCl] resulted in higher relative transcript levels in shoots of *A. thaliana* at 5 days of treatment.

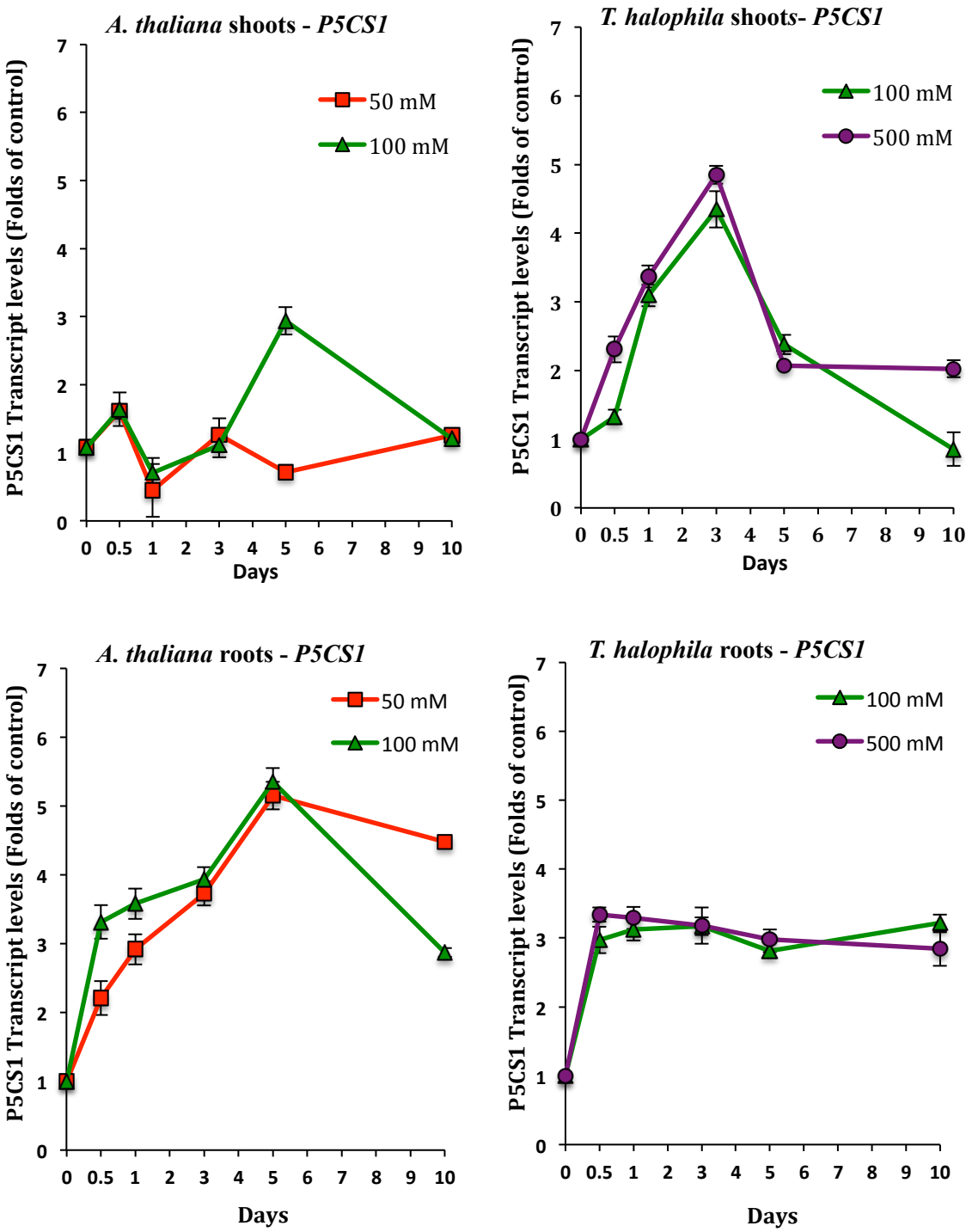


Figure 3.3.5: Transcript levels for *P5CS1* gene encoding Δ^1- pyrroline-5-carboxylate synthase 1, a key enzyme for proline synthesis under salt-treatment (NaCl) over 10 days in roots and shoots of *Arabidopsis thaliana* and *Thellungiella halophila*. Fold increases are expressed relative to those of controls (i.e. non salt-treated plants). Each point is the mean of three replicates and standard errors were calculated from the three replicates

3.3.5 Comparative analysis of gene transcript levels between *A. thaliana* and *T. halophila* at T0

When transcript levels of all the above studied genes were compared in *T. halophila* to *A. thaliana* before the start of the salt treatment (i.e. unstressed controls at T0), it was observed that *T. halophila* had a higher amount of transcripts for each of the genes in unstressed conditions. In Figure 3.3.6, transcript level for each gene in *T. halophila* was normalized to transcript level in *A. thaliana* and also to the reference gene (*UBQ10*) at T0. Relative to the reference genes, *T. halophila* had 2.7 times for *SUS3*, 3 times for *UGP2*, 2.5 times for *FBA1*, 3.2 times for *PpC1*, 3.3 times for *SOS1* and 4.3 times for *P5CS1* more transcripts than *A. thaliana* at T0.

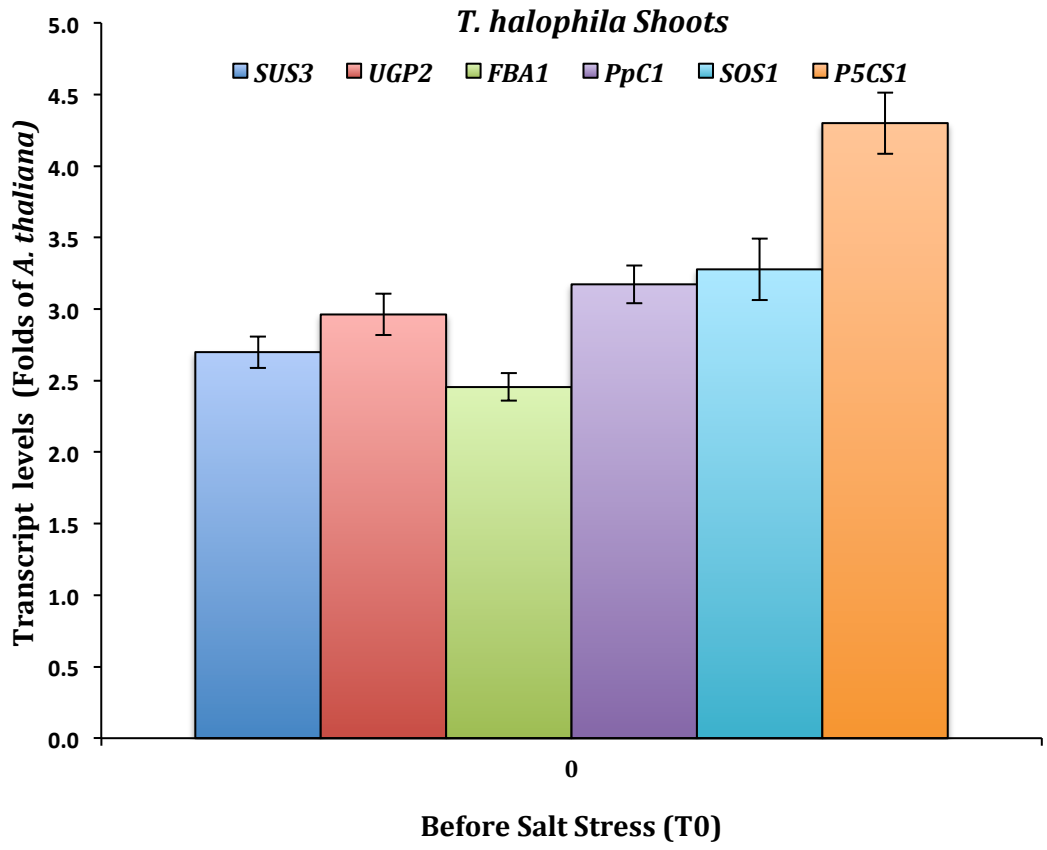


Figure 3.3.6: Transcript levels for ***SUS3, UGP2, FBA1, PpC1, SOS1*** and ***P5CS1*** genes in *Thellungiella halophila* under unstressed conditions before the start of the salt treatment (T0). Fold transcript levels were expressed relative to *Arabidopsis thaliana* controls (i.e. non salt-treated plants at T0). Each point is the mean of three replicates and standard errors were calculated from the three replicates

3.4 Discussion

3.4.1 Differential transcript levels for *SOS1* gene in *A. thaliana* and *T. halophila*

For the normal functioning of plant cells, cation/proton exchangers play a very important role in various functions like cytoplasmic ion homeostasis, regulation of internal pH and turgor of the cell (Venema et al., 2003; Pires et al., 2013). In *A. thaliana* and *T. halophila*, Na^+, K^+/H^+ antiporters are also linked with salt tolerance capabilities as they play a critical role in maintaining ion homeostasis under stress from salt (Kant et al., 2006; Hernandez et al., 2009; Pires et al., 2013). Sodium overly sensitive 1 (*SOS1*) is one of the major exchangers which is localized in the plasma membrane and due to its differential expression may be involved in the difference in the capability of the two closely related species in terms of resisting salinity. *T. halophila* shows adaptation to various environmental stresses by over expressing key mechanisms even in the absence of stress conditions (Taji et al., 2004). In this work *A. thaliana* and *T. halophila* have shown a small increase in relative *SOS1* transcript levels, rapidly after the start of salt-treatment in shoots. This increase was accompanied by a small reciprocal decrease in *SOS1* relative transcript levels in roots in both species during the first 24 hours of salt-treatment followed by an increase during the remaining course of the treatment. This increase was stronger in *T. halophila* than in *A. thaliana*. Despite this increase in relative transcript levels in leaves and roots *A. thaliana* failed to control the uptake and transport of Na^+ to the shoots (see Chapter 2, page 40). *T. halophila* controlled the transport of Na^+ to the shoots when exposed to 100 mM [NaCl]. However, Na^+ accumulated in the shoots of *T. halophila* plants at 500 mM [NaCl], which means that the increase in *SOS1* has failed to limit the uptake and transport of Na^+ to the shoots at high salt concentrations. *T. halophila* seems to up-regulate the transcript levels for this gene to higher levels in roots. In addition to the constitutive preparedness for salt-stress, expression of *SOS1* in *T. halophila* adjusted to saline conditions to higher levels earlier. This capability of *T. halophila* to anticipate stress all the time could makes it more efficient at increasing the Na^+ efflux from within the plant cells, thereby protecting the plant from lethal effects of the ions (Pires et al., 2013). Also, this adjustment in ionic status, which is limited in *A. thaliana*, makes it possible for *T. halophila* to regulate the Na^+/K^+ ratios due to a key function fulfilled by *SOS1* in long distance transport of Na^+, thus keeping the shoots and roots with satisfactory levels of potassium (Pardo et al., 2006). At 100 mM [NaCl], *T. halophila* showed a similar pattern of increase in *SOS1*

transcripts in shoots and roots but to a lower level. The distinct feature of *T. halophila* response at the two salt concentrations is that after 5 days at 100 mM [NaCl] *SOS1* transcripts continued to accumulate at a constant rate till the end of the salt treatment, but at severe 500 mM [NaCl], relative levels of *SOS1* transcripts declined after 5 days. It is likely that this is mostly because of the deleterious effects of sodium ions.

3.4.2 Differential transcript levels for genes involved in carbohydrate metabolism in *A. thaliana* and *T. halophila*

Soluble sugars form the basis of various metabolic functions and components for the structural skeleton of plant cells, which makes them important under various environmental conditions including salt stress. In addition to their metabolic and structural roles sugars play important roles in signaling; they can control specific signaling pathways that might control responses to salinity. Understanding these signaling responses is difficult due to the reversible nature of sugar metabolism: sucrose is broken down to glucose & fructose and they in turn are used to biosynthesize sucrose (Roitsch, 1999). The reversible conversion of sucrose to hexoses and hexoses to sucrose is also greatly affected by unfavorable environmental conditions including salinity (Rosa et al., 2009). To ensure optimal production and usage of carbon resources for energy to regulate optimal metabolism and growth of the plant under salt stress, sugars directly/indirectly affect gene expression and enzyme activities in both source and sink tissues (Stitt and Krappe, 1999). Under the unfavorable saline conditions, changes in source-sink metabolism can lead to differences in the expression of various enzymes and protein products which are involved in carbohydrate metabolism (Rosa et al., 2009). Several candidate genes implicated in the regulation of carbohydrate metabolism under salinity were studied here. They include genes encoding sucrose synthase (SuSy), UDP-glucose pyrophosphorylase (UGPase), fructose-1, 6-bisphosphate aldolase (FBA) and phosphoenolpyruvate carboxylase (PEPC) (see Figure 3.4.1 to understand their involvement in carbohydrate metabolism).

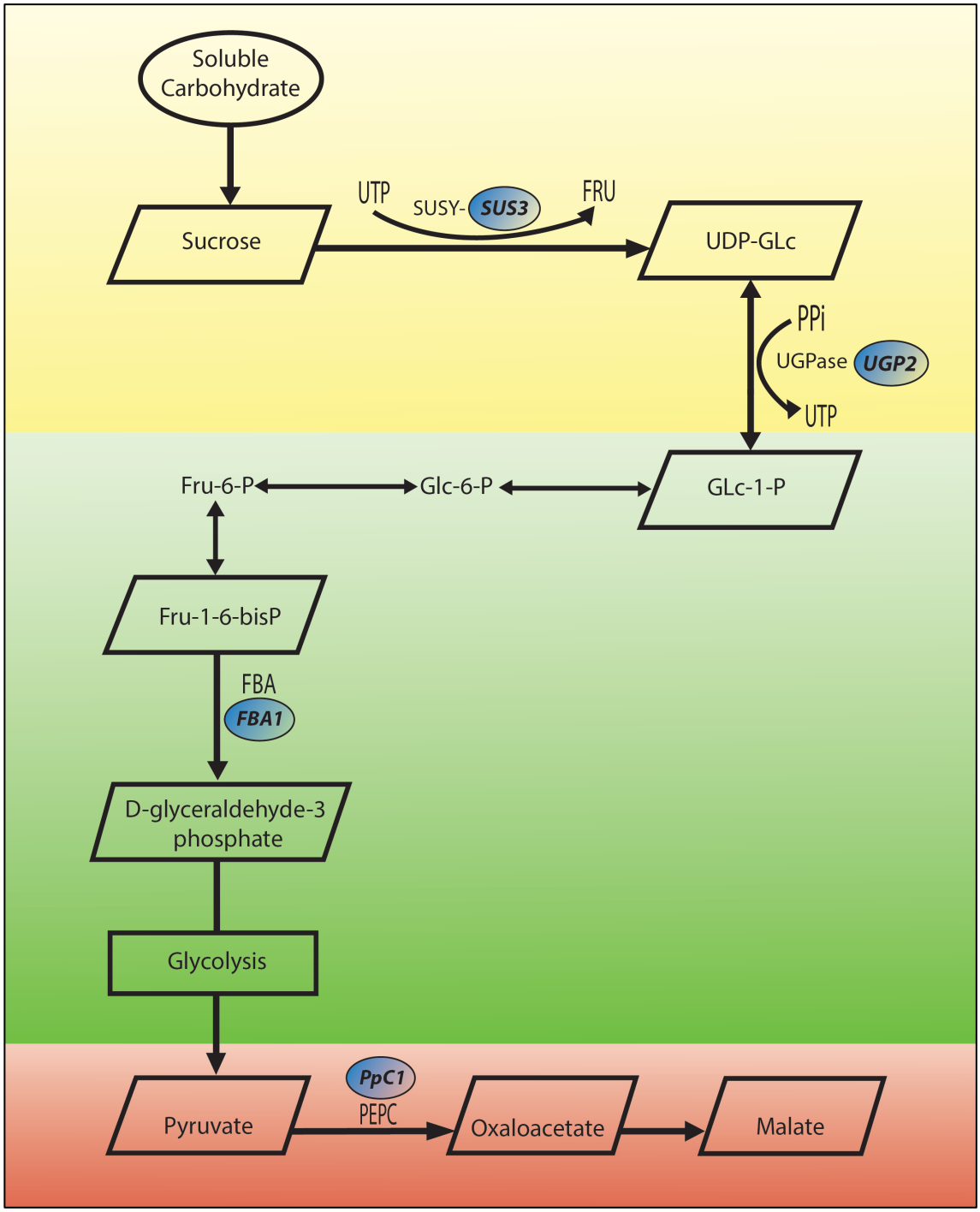


Figure 3.4.1: Simplified pathway of sugar breakdown and C4-carboxylation showing ***SUS3, UGP2, FBA1, and PpC1*** genes with their respective steps in the pathway. SUS3 breaks down sucrose into fructose and UDP-Glucose (UDP-Glc), which then UGP2 further converts it to Glucose-1-phosphate (Glc-1-P), which enters glycolysis and is broken down to Fructose-1, 6-bisphosphatase (Fru-1-6-bisP) which is converted by FBA1 to D-glyceraldehyde-3-phosphate (GBP) and dihydroxyacetone phosphate (DHAP) leading to pyruvate which is converted by PEPc (*PpC1*) to oxaloacetate that serves as precursor of malate.

Sucrose synthase is one of the key enzymes in sucrose metabolism and catalyzes the two-sided reaction of converting sucrose and UTP to UDP-glucose and fructose. The *SUS3* gene is one of the six isogenes in the *A. thaliana* multigene family (Baud et al., 2004). The available data for the six isoforms are still unclear about their differential functional roles within the whole plant (Bieniawska et al., 2007). There are two possibilities, one where all the isoforms have similar cell functions but only work in specific cell types, growth stages and/or in specific environmental conditions. The other is where all the isoforms have totally distinct roles to perform and work together in the same cells (Bieniawska et al., 2007). Expression of the *SUS3* isoform is associated with dehydrating conditions in different organs including leaves and also with osmotic stress caused by factors closely related to salt stress (Bieniawska et al., 2007). It has been suggested that sucrose synthase acts in a main pathway for carbon entry from sucrose in cell metabolism in plants (Bieniawska et al., 2007). Therefore, the higher levels of salt-induced transcripts of *SUS3* suggest that *T. halophila* compared to *A. thaliana* is better able to control the movement of sucrose into different pathways to perform structural, metabolic and storage activities, which are required for normal functioning of the plant cells under severe saline conditions (Baud, Vaultier and Rochat, 2004).

One of the products from *SUS3* activity is UDP-glucose (UDP-Glc), which forms the precursor in the biosynthesis of cell wall components like cellulose and callose (Ruan et al., 2003; Baud et al., 2004; Park et al., 2010). UDP-Glc is broken down into glucose-1-phosphate and enzymes encoded by two genes *UGP1* and *UGP2,* catalyze this reversible reaction. Relative levels of *UGP2* transcripts increased more in response to salinity in *T. halophila* than in *A. thaliana* through 10 days of salt treatment. This might suggest that *T. halophila* not only benefits from the high amounts of UDP-Glc provided via *SUS3* activity but also uses the carbohydrate for other mechanisms under salt stress. Changes in transcript abundance for *UGP2* in *A. thaliana* on the other hand suggested there was an increased breakdown of UDP-Glc early in the stress but this was not extended for prolonged time. The mechanisms described above might imply an increased chance of survival for *T. halophila* under severe salinity. Further along the pathway of carbohydrate metabolism is fructose 1,6-bisphosphate (FBP), which is broken down to D-glyceraldehyde-3-phosphate (GBP) and dihydroxyacetone phosphate (DHAP). This is also a reversible reaction, which is catalyzed by the enzyme fructose-1,6-bisphosphate aldolase (FBA). At present, there are eight known genes in the FBA family in *A. thaliana* and *FBA1* has shown up regulation in *A. thaliana* in response to salinity (Lu et al., 2012). From our data it was seen that *A. thaliana* up-regulated

slightly the transcript levels of this gene but this was not maintained over the entire duration of salinity treatment. In contrast *T. halophila* showed higher up-regulation of transcript levels for this gene under salt-treatment compared to *A. thaliana*. In addition to genes involved in sugar metabolism, the regulation of transcript levels of the phosphoenolpyruvate carboxylase (*PPC1*) gene was also looked into. This enzyme catalyzes the irreversible breakdown of phosphoenolpyruvate (PEP) to inorganic phosphate and oxaloacetate, which is the precursor of malic acid (Wang et al., 2012). Comparison of the *PPC1* transcript levels between the two species showed no difference but when relative transcript levels of *PPC1* were compared in the unstressed control, it was observed that *T. halophila* had greater transcript abundance than *A. thaliana.* This observation might explain the higher malate levels observed in *T. halophila*. However, the changes in malate level in both *A. thaliana* and *T. halophila* under salt-treatment cannot be explained by *PPC1* transcript levels. This phenomenon was true for the other genes involved in sugar metabolism. In *T. halophila UGP2* and *FBA1* showed increased transcripts a little later in stress because they are already up regulated and may thus be preprogrammed to produce higher amounts of protein products. The results gathered here suggest that *T. halophila* has a more flexible and rapid metabolic regulation than *A. thaliana*, in addition to a 'pre-prepared' metabolic background for quick response to salt-stress. The change in transcript levels might not translate into changes in protein levels and enzyme activity, but the overall changes for most gene transcripts seem to have led to changes in enzyme activities as indicated by changes in the levels of metabolites.

3.4.3 Differential transcript levels for the *P5CS1* gene in *A. thaliana* and *T. halophila*

Proline is one of the major compatible solutes that accumulate in some plants under stress conditions (Inan et al 2004). As shown in Chapter 2 (page 51), large increases in levels of proline were induced by salt-treatment in both *A. thaliana* and *T. halophila.* While there was a continuous slow accumulation of proline in *A. thaliana*, there was a strong and rapid increase in proline levels in *T. halophila* reaching a maximum after 3 days of salt-treatment followed by a significant decline. To check for the mechanism behind this differential change, *P5CS1* transcript levels were monitored in both plant species. *P5CS1* is one of the two isoforms present in these two plant species, which is responsible for the synthesis of a key enzyme, Δ^1- pyrroline-5-carboxylate synthase, which catalyzes the production of proline. The activity of this enzyme is a rate limiting

step in proline biosynthesis as it catalyses synthesis of pyrroline-5-carboxylase (P5C) from glutamate and transcription of the *P5CS1* gene is inhibited by the available free proline (Szekely et al., 2008). Although there was no difference in the levels of proline in *T. halophila* subjected to 100 mM and 500 mM [NaCl], there was a significant difference at 5 days in those measured in *A. thaliana* plants subjected to 50 and 100 mM [NaCl] (data shown in Chapter 2). Changes in proline levels in shoots of both plant species mirrored changes in transcript levels for the *P5CS1* gene in shoots. The data obtained in this study is supported by previous work done by Kant et al (2006), which depicted a similar pattern of expression in shoots and roots of both species. *A. thaliana* upon salt treatment increased levels of *P5CS1* transcripts which quickly declined to stay at relatively low levels after 5 days of saline conditions. In roots, the transcript levels of *P5CS1* in *A. thaliana* were up regulated to higher levels than in *T. halophila*, but this was not mirrored by changes in proline levels. As discussed earlier proline levels in *A. thaliana* were generally lower than in *T. halophila*. This suggests that *T. halophila* not only differentially regulates the levels of *P5CS1* transcripts and proline synthesis but the pathway of proline degradation may be regulated differently compared to that in *A. thaliana*. One of the main enzymes involved in proline catabolism is proline dehydrogenase (PDH), which was studied by Kant et al. (2006). This study showed that *PDH* transcripts were not detectable in shoots and were highly suppressed in roots of *T. halophila* but not in *A. thaliana*. With the help of the highly inducible expression levels in shoots and the constitutive over expression in roots throughout 10 days of salinity *T. halophila* maintained higher levels of proline. In turn, this may make *T. halophila* more capable of stabilizing protein structures and efficiently regulating the redox potential inside the cell. Proline could also be used as an antioxidant to negate the harmful effects of free radicals under high salinity (Szekely et al., 2008). The difference in transcript abundance that was shown by *T. halophila* in terms of responding rapidly and strongly to salt-treatment might be a major factor in its higher salt-tolerance than that exhibited by *A. thaliana*.

3.4.4 Is *T. halophila* anticipating salt stress?

From the comparison conducted between *A. thaliana* and *T. halophila* for transcript levels for key genes controlling important metabolic activities, it can be concluded that *T. halophila* can tightly control movement of sodium ions through roots and that it has increased capability to mobilize carbon to have an appropriate metabolic background to support adaptive responses to stresses. That the levels of transcripts for each of the

targeted genes were much higher in *T. halophila* in unstressed conditions strongly suggests the pre-programmed characteristic of *T. halophila* to tackle salt stress. Previously *T. halophila* has been shown to have higher levels of proline in unstressed plants than *A. thaliana,* but here we observed that this phenomenon was not limited to proline or *P5CS1* transcripts. The observations for investigated genes from the sugar metabolism suggest that *T. halophila* is pre-prepared for saline growing conditions using up-regulated basic mechanisms as well as key salt tolerance specific mechanisms.

3.5 Conclusion

The results in this chapter compared the changes in transcript levels of *SOS1, P5CS1, SUS3*, *UGP2, FBA1* and *PPC1* in two closely related plants, *A. thaliana* (a glycophyte) and *T. halophila* (a halophyte) under salinity. The differential observed responses observed might enable *T. halophila* to perform better under salt stress as compared to *A. thaliana* and survive prolonged severe conditions under high salinity. These results strengthen the paradigm that differences in the regulation of salt tolerance mechanisms lead to the development of the salt tolerant trait among the different plant species. The contrasting responses observed in transcript abundance under salinity in the two species might be due to differences in the promoter regions of these genes resulting in diverse expression responses (Kant et al., 2006). Such changes can be introduced artificially and it has been shown that plants are able to accept and cope with changes made to cis-regulatory regions using techniques like sequence insertions and rearrangement (Kant et al., 2006; Wessler et al., 1995). There may also be a possibility of differential regulation of upstream transcription factors and signaling components in the two plant species, which might produce more active forms of the protein products to indirectly control the differential regulation of stress specific responses. Therefore, in the next two chapters this element of the paradigm (evolutionary change of events in the regulation) is studied where the *P5CS1* gene is targeted in *T. halophila* for detailed analysis (promoter and alternative intron splicing). Also *A. thaliana* null mutants of targeted upstream regulatory components (transcription factors and kinases) were screened under salt stress for differential responses comparatively to the wild type *A. thaliana*.

Chapter 4

Comparative Analysis of Δ1- Pyrroline-5-carboxylate synthase 1 (P5CS1) Gene in Thellungiella halophila and Arabidopsis thaliana

4.1 Introduction

Proline is an important osmoregulator, osmoprotectant and antioxidant. Increased levels of proline have been associated with many stresses including drought and salinity (Knight et al., 1997). This increase is controlled at different levels including accumulation of the *P5CS1* transcripts, the gene encoding, Δ1- pyrroline-5-carboxylate synthase 1, a key enzyme in the synthesis of proline. Differential accumulation of *P5CS1* transcripts as well as that of proline between *A. thaliana* and *T. halophila* in response to salt-treatment was demonstrated in the previous chapter. The differential transcript levels of the *P5CS1* gene might be the consequence of modulation of the expression of the gene and/or the consequence of posttranscriptional regulation. The expression of most genes in a given tissue is modulated depending on internal and external conditions of the tissue. This modulation has the function of bringing about adequate levels of proteins necessary to drive basic cellular functions as well as adaptive responses in response to the environment. Differential expression of the same genes or copies of the same genes might bring about differential responses to the same factor in different species. Promoter regions with different cis-regulatory elements (REs) including enhancers and silencers can bring about a desired gene expression according to a specific condition (e.g. salinity) or a particular tissue type. For example, enhancers may bind to a sequence specific site to enhance the gene expression in a particular tissue and silencers may mediate the binding of transcription factors to block the binding of RNA polymerase to stop the gene expression at a specific time or specific conditions (Hoekstra and Coyne, 2007). This regulation can occur through a biochemical product or a protein product that may directly or indirectly drive changes in a pathway of a mechanism. For example, the role of transcription factors (TFs) is to facilitate the spatiotemporal expression of a target gene by binding to a DNA sequence usually upstream of the target gene (Hoekstra and Coyne, 2007). This region upstream (i.e. in 5' direction) from the start codon of the gene is called the promoter sequence that is a non-coding DNA sequence consisting of cis-regulatory elements to which transcription factors bind (see figure 4.1.1). This region controls the expression of the gene (Carroll, 2005a; Carroll, 2005b). These cis-regulatory sequences can be many in number and may lie far from each other in the upstream region and the different cis-regulatory sequences can independently control the transcription initiation of the gene. Therefore, the whole event of expression initiation of gene from an off stage (no or low basal expression) starts when RNA polymerase binds to the promoter region after binding of the transcription factors that may themselves be controlled by other

transcription factors for precise binding (Levine and Tjian, 2003; Wray et al., 2003).

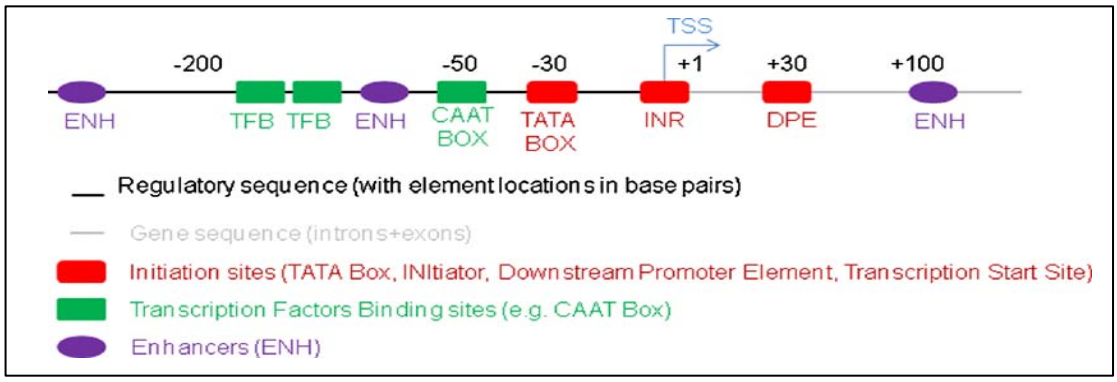


Figure 4.1.1 Structure of a gene cis-regulatory sequence with the core promoter, which is usually located between 0 and −200 bp upstream of the start codon and showing the DNA polymerase binding sites (TATA-box, initiator and downstream promoter element), TF binding sites, and enhancers, situated on the same chromosomes but may be far downstream or upstream of the promoting sequence (Vedel and Scotti, 2011).

The *P5CS1* gene is also controlled by a set of modular cis-regulatory elements. As was observed in Chapters 2 and 3 there was increased accumulation of *P5CS1* transcripts under salt-treatment and this increase was mirrored by higher increase in proline accumulation in *T. halophila* than *A. thaliana.* This salt stress response is of great importance, as the difference between the two species is very big and hence might contribute to the higher salt tolerance of *T. halophila*. This difference in *P5CS1* regulation between the two species may be due to differences in various combinations of cis-regulatory elements working together or independently to mediate complex forms of gene expression differently in the two species. These differences are hypothesized to be a result of evolutionary events taking place in *T. halophila* at the promoter level to adapt to severe salt stress conditions. The evolutionary adaptations may have required the involvement of a new transcription factor to control the gene, which can result in profound co-option of a function (Carroll et al., 2001). However, there is one other possible way to bring about differences in expression besides changes in cis-regulatory elements, which is gene duplication where a duplicated copy or copies of the gene with differences in the promoter structure and/or the 3' un-translated regions appear. This event can lead to a conserved protein with ancestral function while the duplicated copies may evolve to produce new or improved functions. Another aspect of gene duplication is to allow the newly formed gene to have alternative splicing sites to produce a more active form of the protein while still allowing the production of ancestral proteins (Hoekstra and Coyne, 2007).

Hence, it is of great importance to identify and characterize the cis-regulatory elements and their role as key regulators of adaptive responses via the modulation of response mechanisms to given environmental conditions (Priest et al., 2009). Gene duplication is usually the preferred event to result in adapted/enhanced cis-regulation of a gene present in any organism for any specific condition. The work described in this chapter focused on the promoter (5' flanking) region of the *P5CS1* gene and the structure of the *P5CS1* gene in *T. halophila*. These were compared to the orthologous gene present in *A. thaliana,* in an attempt to find out what was behind the differential regulation of the *P5CS1* gene between the two species. Three objectives were followed in this work: (1) isolation of the complete sequence of the *P5CS1* gene(s) in *T. halophila* (2) isolation, analysis and comparison of the 5' and 3' flanking sequences of the *P5CS1* gene(s) in *T. halophila* and *A. thaliana*, (3) Comparison of alternative splicing of the *P5CS1* gene transcripts in *T. halophila* to *A. thaliana*.

4.2 Materials & Methods

4.2.1 *A. thaliana* null mutant growth analysis and stress treatment

A. thaliana ecotype Columbia was used as a wild type, to which growth of *A. thaliana P5CS1* null-mutant was compared under salt-treatment at 50 mM and 100 mM [NaCl]. The null-mutant SALK_142074 (P5CS1) seeds were obtained from NASC European Arabidopsis Stock Centre (Nottingham, UK). Seeds of the *P5CS1* mutant along with the wild type were germinated and the plants grown and treated with salt as explained in Chapter five, section 5.2.2 (see page 125).

4.2.2 *T. halophila* plant material and growth conditions

T. halophila seeds were surface sterilized using 70% ethanol, washed three times with sterile water and sown on John Innes soil compost No. 3. The pots were placed at 4°C for 72 hours to synchronize germination. The pots were then transferred to a controlled growth room with 12 hours light/12 hours dark photoperiod and 23°C/18°C thermo period. Light intensity at plant level was 150 µmol m^{-2} s^{-1}. Seven-day-old seedlings were then transferred to separate pots containing well-moistened John Innes soil compost No. 3 and irrigated with normal tap water. Then 3 weeks later *T. halophila* shoot samples were harvested and either used directly for DNA extraction or frozen in liquid nitrogen and stored at -80°C for later use.

4.2.3 Genomic DNA extraction from *T. halophila*

Invisorb Spin Food Kit II (Invitek, Berlin-Buch, Germany) was used to extract genomic DNA from shoots of *T. halophila*. Exactly 50 to 55 mg of finely ground tissue powder was homogenized in 400 µl of Lysis Buffer P in a 1.5 ml microfuge tube and 20 µl Proteinase K was added to the mix. This was vortexed briefly and placed in a shaking water-bath at 65 °C for 30 min. At the end of the 30 minutes incubation the lysed sample was transferred onto a spin filter placed in a receiver tube and centrifuged at 12000 *g* for 4 minutes at room temperature. After centrifugation, the spin filter was removed and 5 µl of RNase A was added to the filtrate, mixed by vortexing and incubated at room temperature for 5 minutes. Following the incubation 200 µl of Binding Buffer P was added to the solution, which was then vortexed thoroughly. The solution was then transferred onto a spin cartridge placed in a receiver tube, allowed to settle by incubating for 1 minute at room temperature then centrifuged at 12000 *g* for 1 min. The filtrate was discarded and the spin filter was placed back into the same receiver tube. DNA retained by the matrix in the spin cartridge was then washed with

550 µl of Wash Buffer I followed by a wash with 550 µl of Wash Buffer II. After the two washes the spin cartridge was replaced into the receiver tube and spun at 12000 *g* for 1 min at room temperature to remove any traces of residual ethanol from the wash buffers. Finally the spin cartridge was placed in a DNase-free 1.5 ml tube and 100 µl of pre-warmed (55 °C) Elution Buffer D was added on the top of the matrix and incubated for 3 min at room temperature. This was followed by centrifugation at 10000 *g* for 1 min at room temperature to collect DNA in the receiver tube and the spin cartridge was discarded.

4.2.4 Measuring the concentration of DNA and assessing its quality

The extracted genomic DNA was checked for its concentration and quality. This was done using a NanoDrop spectrophotometer. A 1.5 µl to 2.0 µl aliquot of eluted DNA solution was used for measurement. The concentration, the quality and the impurity level of extracted DNA were determined for each sample. The DNA concentration was deduced from absorbance at 260 nm, the ratio of absorbance at 260 and 280 nm wavelengths ($A_{260/280}$) indicated the quality and the ratio of absorbance at 260 and 230 nm wavelengths ($A_{260/230}$) showed the impurity level. Quality readings between 1.6 and 2.4 ranges were considered as good and impurity readings less than 1.0 were considered to be acceptable. The extracted genomic DNA was kept in aliquots of 20 µl volume and frozen at -20 °C. Before the stock aliquots were made the DNA integrity was checked through agarose gel electrophoresis.

4.2.5 Isolation of the 5' and 3' flanking region of *ThP5CS1* gene

The isolation of the 5' flanking region (containing the promoter) and 3' flanking region (containing the terminator) of the *P5CS1* gene was achieved using genome walking PCR based on the Genome Universal™ Walker kit (Clontech, UK, see Figure 4.2.1). Genomic DNA was first digested with four different restriction enzymes then adapters were ligated to the resulting DNA fragments to introduce sites for forward primers for the 5' flanking region and reverse primers for the 3' flanking region. Nested PCR was then performed using gene specific reverse primers and forward primers complementary to the adapter using the four DNA libraries as template. Series of nested PCRs were performed to try to isolate the longest 5' flanking region. The amplified fragments were then cloned and sequenced to confirm that the isolated fragments are indeed part of the *P5CS1* gene sequence. The obtained fragment sequences were aligned together with the coding sequence of the *P5CS1* gene to obtain the promoter region and the 3' flanking region. The details for each step are explained below:

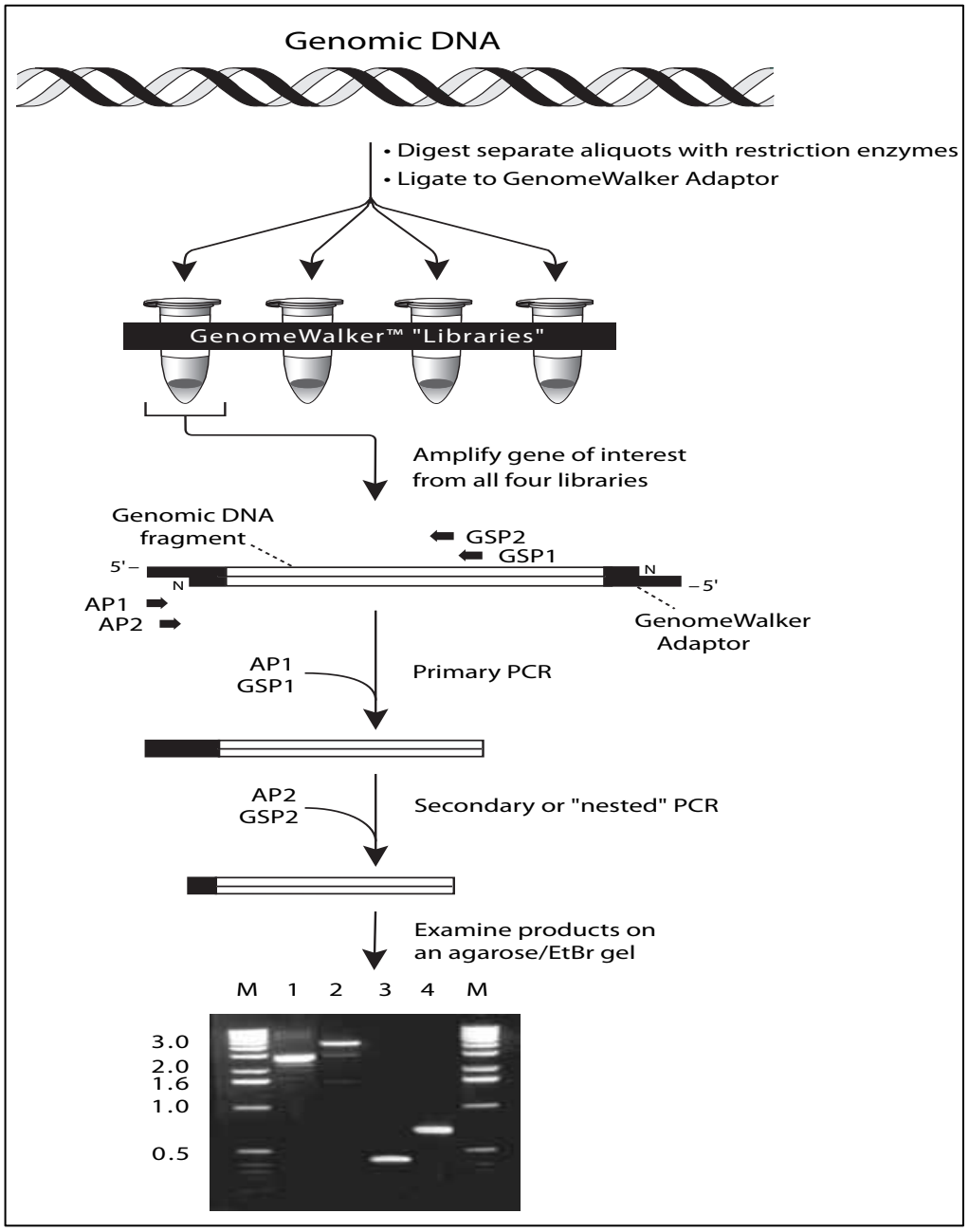


Figure 4.2.1 Flow chart of the steps involved in genome walking. The gel image shows a standard result generated by walking plant genome libraries using gene-specific primers. N is the Amine group that blocks extension of the 3' end of the adaptor-ligated genomic fragments. AP1 and AP2 are the Adaptor primers. GSP1 and GSP2 are the Gene-specific primers. For the subsequent walking, GSP2 products are used as template and re-amplified with GSP3 and so on until pure fragments with desired length of the sequence are obtained. (Image source: Universal GenomeWalker™ Kit User Manual, 2000)

Digestion: Extracted genomic DNA was digested with EcoRV, DraI, PvuII and StuI to obtain DNA fragments of varying sizes. The DNA digestions were performed at 37 ºC overnight in 80 µl reactions using about 1 µg of DNA and 100 U of restriction enzymes. The digested genomic DNA was run on 1% agarose gel to confirm digestion (see

results). The digested DNA was then purified using phenol and chloroform extractions followed by precipitation with 3 M sodium acetate (1/10 of the volume) and 2 volumes of absolute ethanol. The precipitated DNA was washed with 80% ethanol, air dried and re-suspended in 20 µl of TE buffer.

Library construction: Digested DNA from each of the restriction digests was ligated to adaptors using T4 DNA ligase. The reaction mixture consisted of 4 µl digested DNA, 1.9 µl of 25 mM Genome Walker Adaptor (Clontech, Takara Bio Group, France), 1.6 µl 10x ligation buffer and 0.5 µl T4 DNA ligase. The reactions were incubated at 16°C, overnight. The ligation reactions were stopped by incubation at 70°C for 5 minutes. These libraries were carefully aliquoted after addition of 72 µl of TE buffer (pH 7.5) and stored at -20°C for later use.

Genome walker Adaptor:

5'–GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT–3

3'–H2N-CCCGACCA-PO4

PCR amplifications: The libraries were used as template in two serial PCR amplifications to amplify the target sequences. The primary PCR used the outer adaptor primers and outer gene specific primer. The product of the primary PCR was diluted (1/50) and used in the secondary or nested PCR amplification, where the nested adaptor primer and nested gene specific primer were used to amplify the target sequences. PCRs were conducted using Taq DNA polymerase-Pfu (ThermoScientific, UK) mix in a 5:1 ratio to limit the error rates of the PCR. Touchdown PCR was applied for both of the amplifications. PCR reactions were performed in 50 µl final volume consisting of 1x Dream Taq® Buffer, 1.5 mM $MgCl_2$, 1.5 mM $MgSO_4$, 100 µM dNTPs, 400 nM AP1/AP2 forward primer, 400 nM Gene specific reverse primer, Taq/pfu DNA Polymerase mix (1U/0.2U), DNA libraries/primary PCR products and sterile water. Touchdown PCR was run for both of the amplifications and the cycling parameters were 95°C for 2 minutes, 94°C for 15 seconds, 14 cycles of - 0.5°C touchdown from 65°C to 58°C with hold of 30 seconds and 3 minutes of extension time at 72°C, then 34 cycles of 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 3 minutes, followed by final extension for 5 minutes at 72°C. The products were analyzed by 1.5% to 2% agarose gel electrophoresis.

Primers Used:

Adapter primers

AP1:

Forward primer: 5' – GTAATACGACTCACTATAGGG -3'

AP2:

Forward primer: 5' – ACTATAGGGCACGGGTGGT -3'

***P5CS1* gene specific reverse primers (5' flanking region):**

Reverse primer R1: 5' – TCCGCAAGGTGTTCACACAGT -3'

Reverse primer R2: 5' – AACGGACCAAGAGCCAATCTT -3'

Reverse primer R3: 5' – AGTAACAACTGCTGTCCCAAC -3'

Reverse primer R4: 5' – ACGGAAATCAGAAGAGGACAA -3'

***P5CS1* gene specific forward primers (3' flanking region):**

Forward primer F1: 5' – ACAGGTAGGATTCATGCTCGT -3'

Forward primer F2: 5' – TTGAGACTTGAGGAGAGGATG -3'

Forward primer F3: 5' – ACCAAGAATAAGTTACCACTT -3'

Gene specific primers were manually designed from the *P5CS1* gene sequences from *A. thaliana* and *T. halophila* based on guidelines provided by Thein and Wallace (1986).

Cloning and sequencing: After gel electrophoresis the PCR products were cut from the agarose gel and purified using Qiagen Gel Extraction Kit (Qiagen, UK) following the manufacturer's instructions. The purified fragments were then cloned using StrataClone™ PCR cloning kit into the pSC-A vector using the manufacturer's instructions. The positive white colonies were screened for the target sequence by performing colony PCR using gene specific primers. The positive bacterial clones were picked and grown overnight in LB medium supplemented with Kanamycin (50 µg/ml). The amplified plasmid in cell cultures containing putative target insert was extracted using Qiagen MiniPrep Kit (Qiagen, UK) following the manufacturer's instructions. The extracted plasmids were then sent to Geneius Laboratories (Newcastle, UK) for sequencing to identify and confirm the sequence of the target insert using gene specific reverse primers on an Applied Biosytems 3730xl DNA sequencer. The different isolated sequence contigs from the 5' flanking region were aligned using BioEdit sequence

alignment editor to confirm the overlapping sections and also try to deduce any similarities with the orthologous gene from *A. thaliana*.

4.2.6 Qualitative PCR for the isolation of *ThP5CS1*

After the desired 5' and 3' flanking sequences were isolated from *T. halophila*, it was decided to isolate the whole *ThP5CS1* gene including the flanking promoter and terminator regions to compare the sequence and structure of the gene (s) to the *A. thaliana* gene. it was important to analyze the coding region of the *P5CS1* gene and compare the intron sites with the *A. thaliana* orthologous gene. Two approaches were followed to isolate the full gene: (1) Amplifying the whole sequence from 5' to 3' by designing three forward primers in the 5' region and one reverse primer in the 3' region. PCRs were conducted using Taq DNA polymerase-Pfu mix in a 5:1 ratio to limit the error rates of the PCR. PCR reactions were performed in 50 µl final volume consisting of 1x Dream Taq® Buffer, 1 mM $MgCl_2$, 1 mM $MgSO_4$, 150 µM dNTPs, 1200 nM forward primer, 1200 nM reverse primer, Taq/Pfu DNA polymerase mix (1U/0.2U), 1 µl of *T. halophila* genomic DNA (100 ng) and Sterile water. The reactions were subjected to the following thermal cycles: 95°C for 5 minutes, then 39 cycles of 94°C for 25 seconds, 42°C for 30 seconds, 72°C for 6 minutes, followed by final extension for 6 minutes at 72°C. (2) The sequence from 5' to 3' was divided into four sections, which were amplified separately with overlapping regions. The PCR reaction components were the same as those used in the first approach but this time a gradient PCR was performed with the following parameters: 95°C for 5 minutes, then 39 cycles of 94°C for 25 seconds, 49°C-59°C for 30 seconds, 72°C for 5 minutes, followed by final extension for 5 minutes at 72°C. The products were analyzed using 1% agarose gel electrophoresis.

Primers used in the attempt to amplify the full *P5CS1* gene as a single amplicon, expected amplicon size between 5 kb and 6kb:

Forward primer **F1169**: 5' – TATCCTAGGTAATTTACCTCAT -3'

Forward primer **F500**: 5' – TATCCTAGGACGTGAGTTACACAGTTTTAGC -3'

Forward primer **F856**: 5' – TATCCTAGGTCGGACATCAAATCTCCTTTTAAGA-3'

Reverse primer **R480**: 5' – TATGAATTCTCTCTTGTGTGTC -3'

Primers used to amplify overlapping sections of the *P5CS1* gene:

SECTION 1: expected amplicon size – 1200 bp

Forward primer: 5' – GTAAAGTTTATAACCTATGCC -3'

Reverse primer: 5’ – GGTGCACACGCTCATTC -3’

SECTION 2: expected amplicon size – 1700 bp

Forward primer: 5’ – CACTGAGTTAACTCGTTCC -3’

Reverse primer: 5’ – GTCATAACTAAGCGAGCC -3’

SECTION 3: expected amplicon size – 1500 bp

Forward primer: 5’ – TTAGGCGTACTCCTGATTG -3’

Reverse primer: 5’ – CCACTACATAAGAGAGGG -3’

SECTION 4: expected amplicon size – 1100 bp

Forward primer: 5’ – CGGTCGTTCAACTATGAG -3’

Reverse primer: 5’ – GACATTCTACAAGTATCTGG -3’

4.2.7 Qualitative Reverse Transcription PCR to compare splicing of introns in the coding region of *P5CS1* gene between *A. thaliana* and *T. halophila*

To analyze alternative splicing of the *P5CS1* gene under salt treatment, RNA isolated from the two species in the previous 10-day experiment described in Chapters 2 and 3 was used. Reverse transcription PCR was used to detect any change in size of amplicons obtained using RNA from unstressed and stressed samples from *A. thaliana* and *T. halophila* which predicted the presence of alternate splicing due to salt stress. The 25 µl reactions contained: 1x NH_4 Reaction Buffer, 5 mM $MgCl_2$, 10 mM DTT, 100 µM dNTPs, 0.4 units of RNase Out, 1.2 units of MMLV Reverse Transcriptase, 400 nM forward and reverse primers, Taq DNA Polymerase, 10 ng/µl of RNA and DEPC water. PCR was performed using cycling parameters: 42°C for 30 minutes (reverse transcription), 94°C for 2 minutes, 29 cycles of 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 45 seconds, followed by final extension for 5 minutes at 72°C. The products were analyzed using 2% agarose gel electrophoresis.

The sequences of each primer pair based on ThP5CS1a were as follows:

Intron1, amplicon size*: unspliced - 703 bp, spliced - 256 bp*

Forward primer: 5’ – ACGCGCTCACTGACGAAATCC -3’

Reverse primer: 5’ – CGCACCAGATGACACCAAAATC -3’

Intron2, amplicon size*: unspliced - 260 bp, spliced - 140 bp*

Forward primer: 5’ – ATGACAGTAGTTTTAGAGACAAGG -3’

Reverse primer: 5’ – TCGACATCACTCAGAAGAATCAG -3’

Intron3, amplicon size*: unspliced - 418 bp, spliced - 221 bp*

Forward primer: 5’ – TTTGGCGACAAGTCAAGATTAGG -3’

Reverse primer: 5’ – CAACTGCCATGTCACGAGCAG -3’

Intron4, amplicon size*: unspliced - 289 bp, spliced - 190 bp*

Forward primer: 5’ – CCGATCACAGATTCTACTGCTC -3’

Reverse primer: 5’ – CAGCTTCTTGTGCTGCAGATAC -3’

Intron5, amplicon size*: unspliced - 395 bp, spliced - 196 bp*

Forward primer: 5’ – CGACCTGATGCACTTGTACAG -3’

Reverse primer: 5’ – CTTGAAGTCACAAGTCCAATGAG -3’

Intron6, amplicon size*: unspliced - 253 bp, spliced - 95 bp*

Forward primer: 5′- GCAATTCCAGAGACTGTCGGG -3′

Reverse primer: 5’ – TGGCCTAGCACAGGGATTTTTG -3’

Intron7, amplicon size*: unspliced - 284 bp, spliced - 84 bp*

Forward primer: 5’- CTTGATGACGTTATAGATCTTGTG -3’

Reverse primer: 5’ – CATCGCATTACAGGCTGCTGG -3’

Intron8, amplicon size*: unspliced - 323 bp, spliced - 210 bp*

Forward primer: 5’ – GATGGAATCTGTCATGTATATGTC -3’

Reverse primer: 5’ – AGTATTGCACTTGCTCTTGGTC -3’

The sequences of each primer pair based on ThP5CS1b were as follows:

Intron1, amplicon size*: unspliced - 723 bp, spliced - 276 bp*

Forward primer: 5’ – CTTCCCTCACCAGATATTTCC -3’

Reverse primer: 5’ – AGTGCTCCTAAGCGACCAAG -3’

Intron2, amplicon size*: unspliced - 288 bp, spliced - 137 bp*

Forward primer: 5’ – GATTGGCTCTTGGTCGCTTA -3’

Reverse primer: 5’ – GACTAATTGTCTGTATCGAAGC -3’

Intron3, amplicon size*: unspliced - 374 bp, spliced - 206 bp*

Forward primer: 5’ – CTTGCGGAATTAAACTCGGATG -3’

Reverse primer: 5’ – CGAACATAGTCTCGTAATAAGCC -3’

Intron4, amplicon size*: unspliced - 352 bp, spliced - 205 bp*

Forward primer: 5’ – AAGCCTCAGAGTGAACTTGATG -3’

Reverse primer: 5’ – CTCTTCTGGTGCTTATAGCATC -3’

Intron5, amplicon size*: unspliced - 364 bp, spliced - 256 bp*

Forward primer: 5’ – CTCAACTTCTGGTGAATGACAG -3’

Reverse primer: 5’ – GTGTGGATCAACTTTGAGTTAGG -3’

Intron6, amplicon size*: unspliced - 304 bp, spliced - 104 bp*

Forward primer: 5′- CCTAACTCAAAGTTGATCCACAC -3′

Reverse primer: 5’ – CTGAAGCTTTCTGGAACTTTCTC -3’

Intron7, amplicon size*: unspliced - 206 bp, spliced - 110 bp*

Forward primer: 5’- ATAGATAAAGTCCTCCGAGGAC -3’

Reverse primer: 5’ – AAGAGCGTCGGCGATATTATAC -3’

Intron8, amplicon size*: unspliced - 300 bp, spliced - 210 bp*

Forward primer: 5’ – TATAATATCGCCGACGCTCTTG -3’

Reverse primer: 5’ – AAAACACGGCCAATTGGATCTTC -3’

Intron9, amplicon size*: unspliced - 250 bp, spliced -142 bp*

Forward primer: 5’ – AGTTCGTAAGCTAGCCGATATG -3’

Reverse primer: 5’ – CATCAGGTCGGGATTCAAAAAC -3’

Intron10, amplicon size*: unspliced - 210 bp, spliced - 134 bp*

Forward primer: 5’ – GATGGTCTTGTCTTAGAGAAGAC -3’

Reverse primer: 5’ – TCCTTTCCACCCTTCAATAGAAG -3’

Intron11, amplicon size*: unspliced - 275 bp, spliced - 179 bp*

Forward primer: 5’ – ACAGATAGCTTCACTTGCCATC -3’

Reverse primer: 5’ – GAGCAAATCAGGAATCTCTTCTC -3’

Intron12, *amplicon: unspliced- 253 bp, spliced - 168 bp*

Forward primer: 5’ – GCAATTCCAGAGACTGTCGGG -3’

Reverse primer: 5’ – TGGCCTAGCACAGGGATTTTTG -3’

Intron13, *amplicon: unspliced - 284 bp, spliced - 200 bp*

Forward primer: 5′- CTTGATGACGTTATAGATCTTGTG -3′

Reverse primer: 5’ – CATCGCATTACAGGCTGCTGG -3’

Intron14, *amplicon: unspliced - 317 bp, spliced - 219 bp*

Forward primer: 5’- GATGGAATCTGTCATGTATATGTC -3’

Reverse primer: 5’ – AGTATTGCACTTGCTCTTGGTC -3’

Intron15, amplicon size*: unspliced - 287 bp, spliced - 166 bp*

Forward primer: 5’ – GGAAACTCTTCTTGTGCATAAGG -3’

Reverse primer: 5’ – AAGCCTTGGAACAGTACTCATAG -3’

Intron16, amplicon size*: unspliced - 303 bp, spliced - 206 bp*

Forward primer: 5’ – TCACTGTATATGGTGGACCAAG -3’

Reverse primer: 5’ – GAAGGAATAGCTCTGCAACTTC -3’

Intron17, amplicon size*: unspliced - 190 bp, spliced - 111 bp*

Forward primer: 5’ – CACACAGATTGCATTGTGACAG -3’

Reverse primer: 5’ – CCATCTGAGAATCTTGTGCTTG -3’

Intron18, amplicon size*: unspliced - 213 bp, spliced - 125 bp*

Forward primer: 5’ – TTTTCCACAACGCAAGCACAAG -3’

Reverse primer: 5’ – GTAAGTAATCCTTCAACTCCGAC -3’

Intron19, amplicon size*: unspliced - 273 bp, spliced - 145 bp*

Forward primer: 5’ – GTCGGAGTTGAAGGATTACTTAC -3’

Reverse primer: 5’ – TCCTCAAGTCTCAACACACAAC -3’

4.2.8 Agarose gel electrophoresis

To check the integrity of extracted DNA and to analyze the products (amplicons) from PCR amplifications 1% to 2% agarose gel electrophoresis was used. The gels were prepared by dissolving 1 g (2 g for 2%) of agarose (Molecular Biology grade, Web Scientific, UK) in 100 ml of 1xTBE (Tris-boric acid-EDTA) buffer. Agarose was melted in the buffer by heating in a microwave oven at medium power for about 2 to 5 minutes. This heated solution was then left to cool down for about 15 minutes and 6 µl of ethidium bromide (10 mg/ml solution, staining dye) was added to the agarose gel solution and mixed well by swirling. The agarose solution was then poured into the gel-plate fitted with a comb and allowed to set for approximately 30 minutes after which the comb was removed vertically without damaging the wells in the gel. The gel bath was prepared using 1X TBE buffer and the gel was placed in it with the wells side of the gel put at the negative pole side. It was ensured that the gel was fully immersed in the bath for proper movement of DNA in the gel during electrophoresis. To the DNA samples, Loading buffer (5 µl per 30 µl of sample, ThermoScientific, UK) was added then the samples were loaded in the gel. Usually the first well was loaded with a DNA size marker as a reference. Long-range DNA ladder mix or 100 bp ladder (ThermoScientific, UK) were used as size markers. The gels were run at constant voltage of 100 V for 30 minutes to 1 hour depending on the size of the expected fragments and strength of the gel. The gels were then visualized under UV in a gel documentation system (UV Tech, Cambridge, UK). The gel images were captured using the UV proMw software (UV Tech, Cambridge, UK).

4.2.9 Bioinformatic analysis of the 5' flanking regions of the *P5CS1* gene from *T. halophila* and *A. thaliana*

The 5' flanking sequence of the *P5CS1* gene from *A. thaliana* was obtained from the National Center for Biotechnological Information (NCBI) website and the first 1344 bp of the sequence (i.e. starting from the start codon of the *P5CS1* gene until the end of the nearest upstream-located gene) was selected. This sequence along with isolated 5' flanking sequences of *P5CS1* genes from *T. halophila* (see section 4.3.4) were copied separately into the Plant Cis-acting Regulatory DNA Elements (PLACE) database. Using the program SIGNAL SCAN, it was possible to detect and identify cis-acting elements (RE) in the isolated 5' flanking sequences of *P5CS1* genes from *T. halophila* and the *P5CS1* gene of *A. thaliana*. The obtained data is represented in the format of a table showing the number of repeats for each type of RE present in the *P5CS1* 5' flanking sequences of each species. Frequency of REs in each promoter sequence is also calculated by dividing the total number of REs present in the promoter by the length of that promoter.

4.2.10 Statistical analysis

Data were analysed using SPSS (IBM SPSS Statistics 19 64Bit) and graphs were produced using Microsoft Office Excel 2010 and SigmaPlot 11.0. Normal distribution was tested using normality test and significant differences between mean values were verified using LSD ($P < 0.05$) following one-way ANOVA.

4.3 Results

4.3.1 Growth analysis

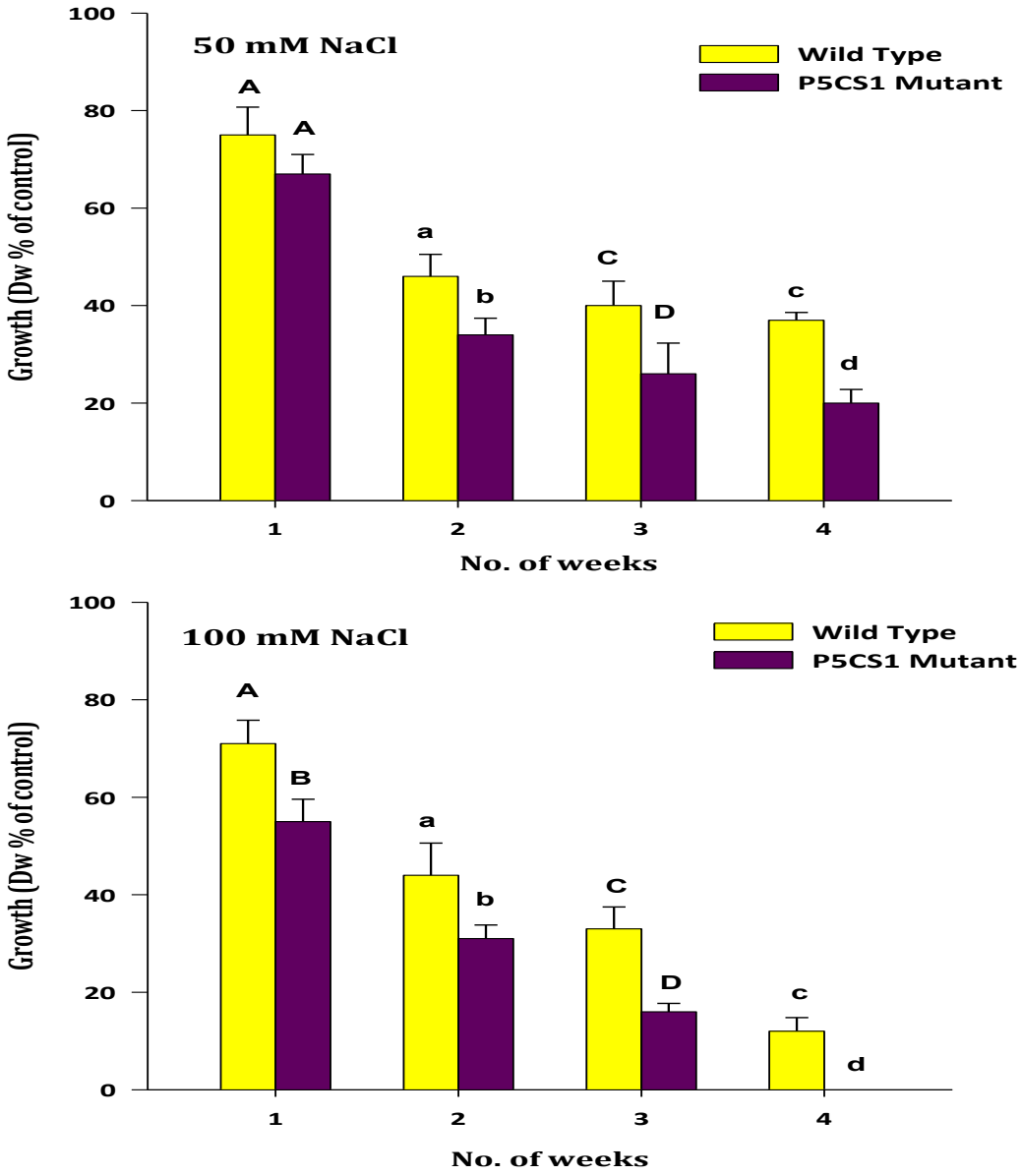


Figure 4.3.1: Growth (DW) analysis at 50 mM and 100 mM [NaCl] of *A. thaliana* P5CS1 null mutant along with wild type, expressed as percentage of their respective unstressed control. Each point is the mean of 3 samples (3 plants per sample) from which the standard errors were calculated. Bars at each time point with different letters are statistically significantly different ($p < 0.05$).

The growth responses exhibited by the *A. thaliana* null mutant for the *P5CS1* gene under the effect of salt treatment were assessed and compared to the wild type. Under unstressed conditions the mutants showed similar growth to the wild type (not shown). As seen in Figure 4.3.1, the *P5CS1 A. thaliana* mutant exhibited reduced growth at both 50 and 100 mM [NaCl] in comparison to the wild type. The biomass analysis showed

the effect of the salt dose as there was a stronger reduction of biomass at 100 mM [NaCl] compared to 50 mM [NaCl]. The significant (p value 0.01) difference between the mutant and wild type was apparent after 1 week at 100 mM [NaCl]. After 3 weeks of salt-treatment at 50 mM [NaCl] the mutant maintained 26% growth (p value, 0.04) while the wild type maintained 40% growth. The growth difference between the mutant and the wild type was even larger under treatment at 100 mM [NaCl]. After 3 weeks of treatment at 100 mM [NaCl] the wild type maintained 33% growth while the mutant maintained 16% only (p value 0.001). Plants of the mutant watered with 100 mM [NaCl] died after 4 weeks of salt-treatment while wild plants kept 12% growth compared to the unstressed control.

4.3.2 Isolation of 5' & 3' flanking sequences of *ThP5CS1*

Isolation of the 5' and 3' flanking regions of the *P5CS1* gene from *T. halophila* was achieved using genome-walking PCR. Figures 4.3.3 (A) and (B) show examples of agarose gel images of second nested PCR products for 5' and 3' flanking sequences of the gene. PCR products were then purified from gels and cloned. Figures 4.3.4 and 4.3.5 show results of colony PCR performed on selected positive colonies to confirm presence of the target sequence. Successful amplification from a colony confirmed the presence of the target sequence. After extraction the plasmids containing inserts were sent for sequencing and the obtained sequences aligned to the coding sequence to obtain the flanking sequences. After sequencing and alignment of genome walking products, two different flanking sequences of the *P5CS1* gene from *T. halophila* were characterized with the first sequence being 1169 bp long 5' flanking sequence (Figure 4.3.6 A) and the second sequence 493 bp long 3' flanking sequence (Figure 4.3.6 B).

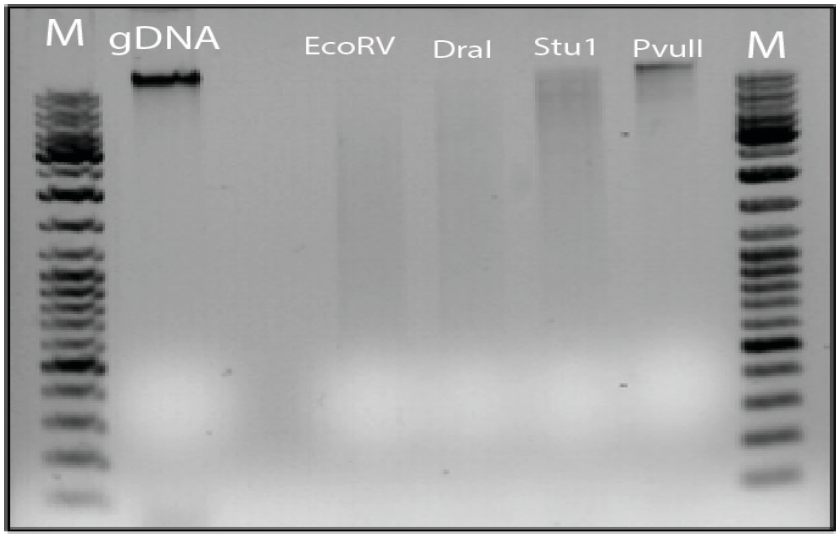


Figure 4.3.2: Agarose gel image of the genomic DNA of *Thellungiella halophila* digested with EcoRV, DraI, PvuII and StuI run against the undigested genomic DNA in lane 1. M: Long range size marker (ThermoScientific, UK)

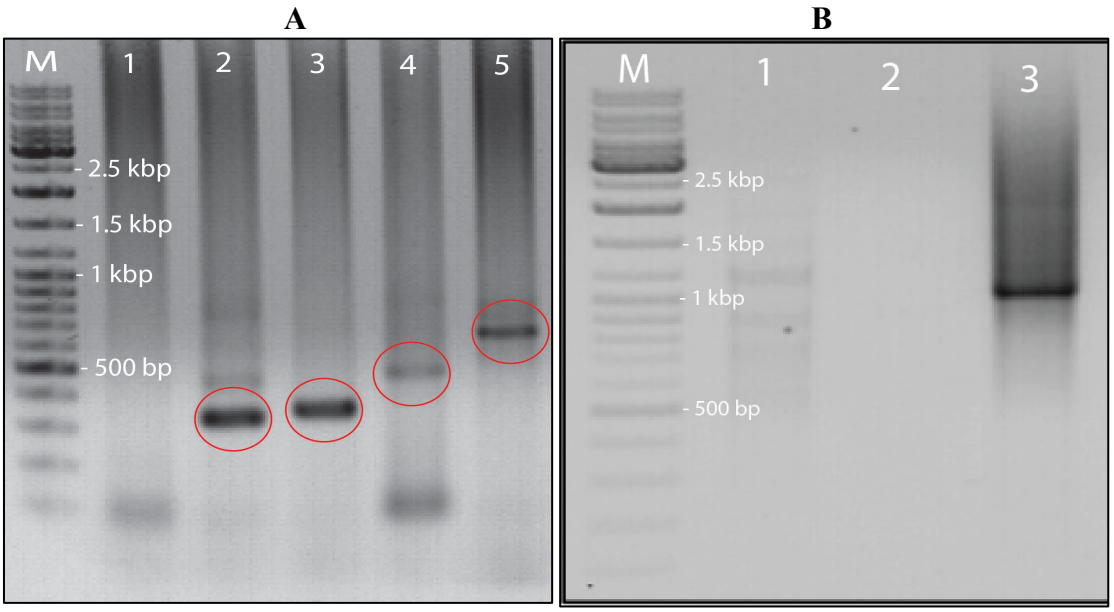


Figure 4.3.3: (A) Gel image of the PCR products for sequence upstream of *P5CS1* gene. Lane 1 is negative control and lane 2, lane 3, lane 4 and lane 5 contain products obtained with amplification of DNA digested with EcoRV, DraI, PvuII and StuI respectively. **(B):** Gel image of the PCR products for sequence downstream of *P5CS1* gene. Lane 3 contains products obtained with amplification of DNA digested with EcoRV. Lane 1 is negative control and lane 2 is empty. M: Long range size marker (ThermoScientific, UK)

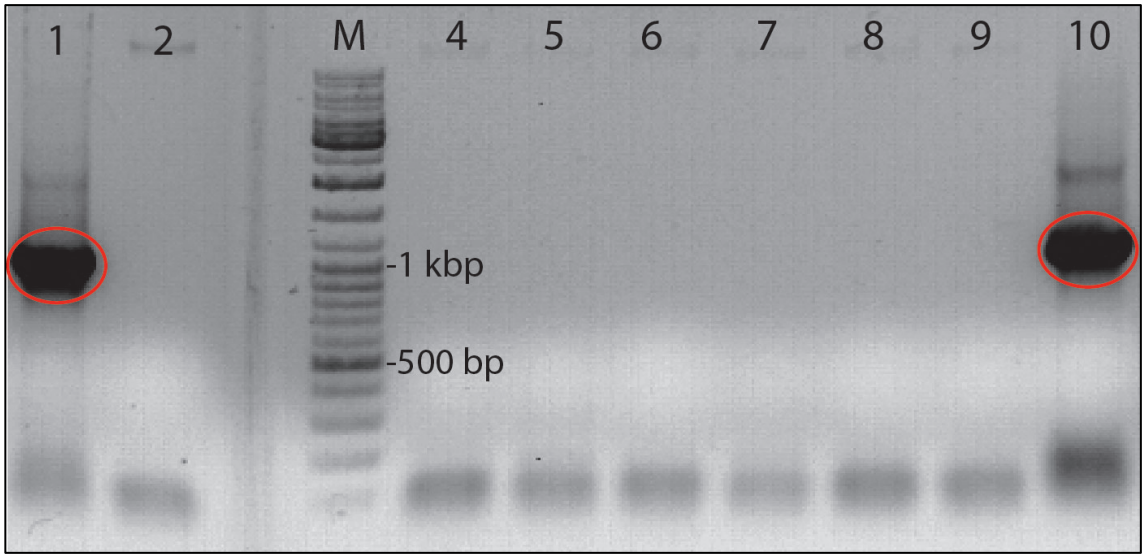


Figure 4.3.4: Gel image showing the products of colony PCR amplifications for confirmation of inserts of putative 5' flanking sequences. PCR products (ringed) for the sequence upstream of *P5CS1* gene are in lane 1 and lane 10, which show positive clones. Lanes 2 and 4-9 represent negative colonies. Plasmids were isolated from representative positive clones and sent for insert sequencing. M: Long range size marker (ThermoScientific, UK)

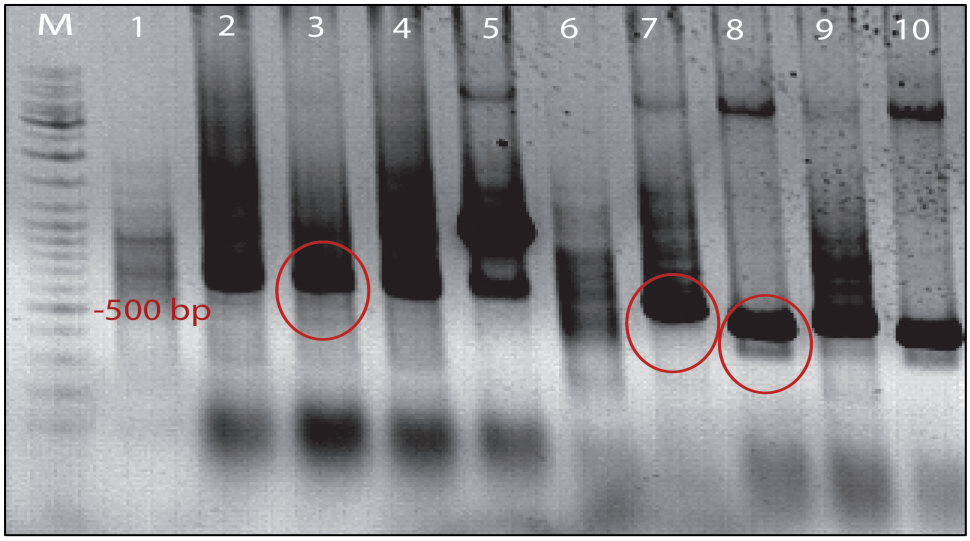


Figure 4.3.5: Gel image showing the products of colony PCR amplifications for confirmation of inserts of 3' flanking sequences (lanes 1-10). Plasmid DNA was isolated from representative positive clones (lanes 3,7 and 8, 'ringed') and sent for insert sequencing. M: Long range size marker (ThermoScientific, UK)

(A)

5'-*TATAGGCTATATATAAGGAAATTTAATTTACCTCATATTATTTCGATTAAATATCAAAAATTATTAGCTGTAAAG*
TTTATAACCTATGCCCTTTAAAGTCATTAAATACTTGTTATGTAATAAATTTATCATAAAAAGAAACAAAAATGTCAT
CCTGGTTTTCATATTAATTATGCGGAGACAAAATTGTTAAGTTGTTTATAAAAGTCCAAATATCAGTAAGATTATTC
AGTATTGATCACTATTAAGTTTATCTTTATATCATCTTTTTTTCTTTTCATTTTATTATATTATCTTTTATTATTTTGTGT
TTTCGGACATCAAATCTCTTTTAAGAGTAAATTGTAATATTTTTGTATATCCTTTCCAAAAGACTATAGAATTTTTTA
GAAGTTTTAGTGACTAACAATATCCTTAAAACATAATCGAACATTACACTTAGACCTTTTAAAAGTTAGATAGATCA
AAATACTTTTATCTATGGTGTGTTCACCAAAAACAAGAACTTATAGAGAATTGTCTAGACATATATGTTATAGAAAA
CAAGAGATATCCAACGCTCTTTCTTTGCAAAACATACAATGTAATCGGTAGGACCTTATGTAAATCACAAAACAAA
GGAAATAGGTTATACTTTTATCGAATAAATGTGAGTTCTTAAAACTACTAAAGTAAGTCTATGTAACATTAGTAACG
TGAGTTATTACACAGTTTTAGCCAAATCTCGCATTATATTTTAATTATATATTTGTTGTTTTACTAATATTTTACAACA
CCGCGGCGTATTTTTTTTTTTCTTCTTCTACCACACTTAAAGGATAAAAAAAGTGATCTCTCTGCAGTAGATTTTAAC
GAAGCGACAGGCGAAAAGTGAACAGGAGAAGACTAAAGGCAAACGGAAACACAACAAGTACAGGTGCCGCGAA
GGAGTCGGCGGCTAAAAAATTAAAGGGTTTTATGGGTGGTGGAGTCATTGATAGAAGACGCTGCGTTGGGTTTA
GGGAAATGCTACGCGGTGGGTTGCTGACGCGCGGTGAAGTAGCTAATCCTAGATTATAAGGTCTATATGGGAATG
AGCGTGTGCACCGCGCAGAACCAAACTATCTTCCTGGACTGAGAGACCACATTCAACACAAATATTTGGGGAAGTA
GAGAAGGAACAACTAGAG-**3'**

(B)

5'-*GTAAGATGAAGAACATTTTCTTAGCTTCTCTTCTTGTTTAAAAAAAACACGTTGTAAGGCTACCTACACCTTTCT*
GATTTATCATTTATCTATATCTTTGGATTTGAGTTTGGACTTCCACTGGGAGTTATACCTTTAATACAAAGTTGCATA
TATGAACTTAAAAAGTCATTACTATTAATTCCCAAGGATCAGCGCAAAATGGTAAACACGTTTGAGTACGTTTGAG
TGCTAAGAATGAACACAAGAGTTCTCATTCTTACATATCTAATTTTTTTCAGGTACTTGAGGAATCGATCTTAACTTT
CATTCTTCCATAAACTTCTTAACATTCTTCAACCACAGCAAGTACTCTCGCTTCACTTTTCTCATCATGTACTCATGCA
AGAAATTCGTTGTGCTTGCTTTCACTCCTCTTATCTCCAGATACTTGTAGAATGTCTTCTTCAGGTTCTCGTCCAAGTC
TCTGCAAAAGTAGAAGACACACAAGAGA-**3'**

Figure 4.3.6: *ThP5CS1 5'* flanking promoter region and 3' terminator sequences obtained from the genome walking experiment. (A) Putative 5' flanking sequence of 1169 bp. Sequences marked in green, blue and pink represent the three forward primers F1169, F856 and F500, respectively. (B) 3' flanking sequence of 493 bp. The part of the sequence marked in yellow represents the one reverse primer R480.

4.3.3 Isolation of the *ThP5CS1* along with flanking regions

After the isolation of 5' and 3' flanking sequences of the *ThP5CS1* gene, it was decided to isolate the complete gene from *T. halophila* including the isolated 5' and 3' flanking regions and the coding region with the exons and the introns. Figure 4.3.7 shows the amplification of the *ThP5CS1* gene using three different combinations of primers: F1169/R480 (lane 3), F500/R480 (lane 5) and F856/R480 (lane7) after agarose gel electrophoresis. Unfortunately none of the three amplicons were successfully sequenced

or could be cloned for isolation. Therefore, various other combinations of primers were designed to achieve the isolation of the target sequence from *T. halophila* by PCR. However, due to the large size of the gene (5 to 6 kbp) all PCRs either failed or gave smaller products than expected.

To overcome this problem, it was decided to isolate the sequence in four different parts by dividing the sequence into four overlapping sections. Each section was amplified separately using gradient PCR. Figure 4.3.8 (A) to (D) shows the results of the gradient PCRs for *ThP5CS1* gene sections (1) to (4), respectively. The expected amplicon size for section (1) was 1200 bp, section (2) was 1700 bp, section (3) was 1500 bp and section (4) was 1100 bp. Except section (1) which contained the 5' flanking region, the other sections were successfully amplified and sequenced. In the meantime, the Phytozome.net online database released an updated version of *T. halophila* draft genome database with unpublished *ThP5CS1* gene sequence, which had about 100 bp of 5' flanking sequence. This 100 bp sequence was not similar to the one we isolated. This suggested that unlike in *A. thaliana P5CS1* might have multiple copies in the genome of *T. halophila*. Hence, it was decided to isolate/extend the 5' flanking sequence of the second *P5CS1* gene through genome walking using the 100 bp sequence available from the database as template. Figure 4.3.9 shows the isolated 501 bp 5' flanking sequence of this gene. Further PCR confirmation was performed for both putative 5' flanking sequences: the 1169 bp *ThP5CS1a* and 501 bp *ThP5CS1b*.

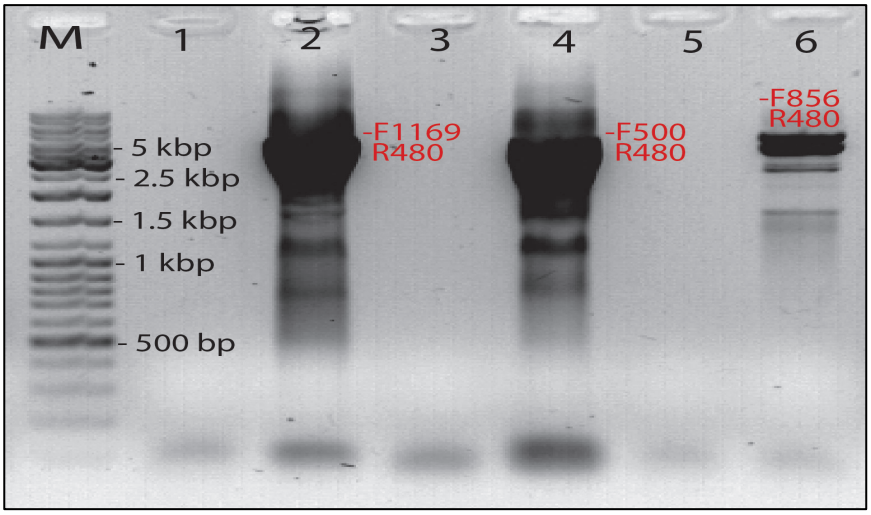


Figure 4.3.7: Agarose gel image of the isolation of *Thellungiella halophila P5CS1* gene by PCR using different sets of primers (see Materials and Methods). Gel image shows the amplification of *ThP5CS1* gene using primers: F1169/R480 in lane 2, F500/R480 in lane 4 and F856/R480 in lane 6 at very low annealing temperature of 42°C. Unfortunately, none of the products could be successfully sequenced from PCRs due the large size of the amplicon and also due to the limited purity of the target amplicon. M: Long range size marker (ThermoScientific, UK)

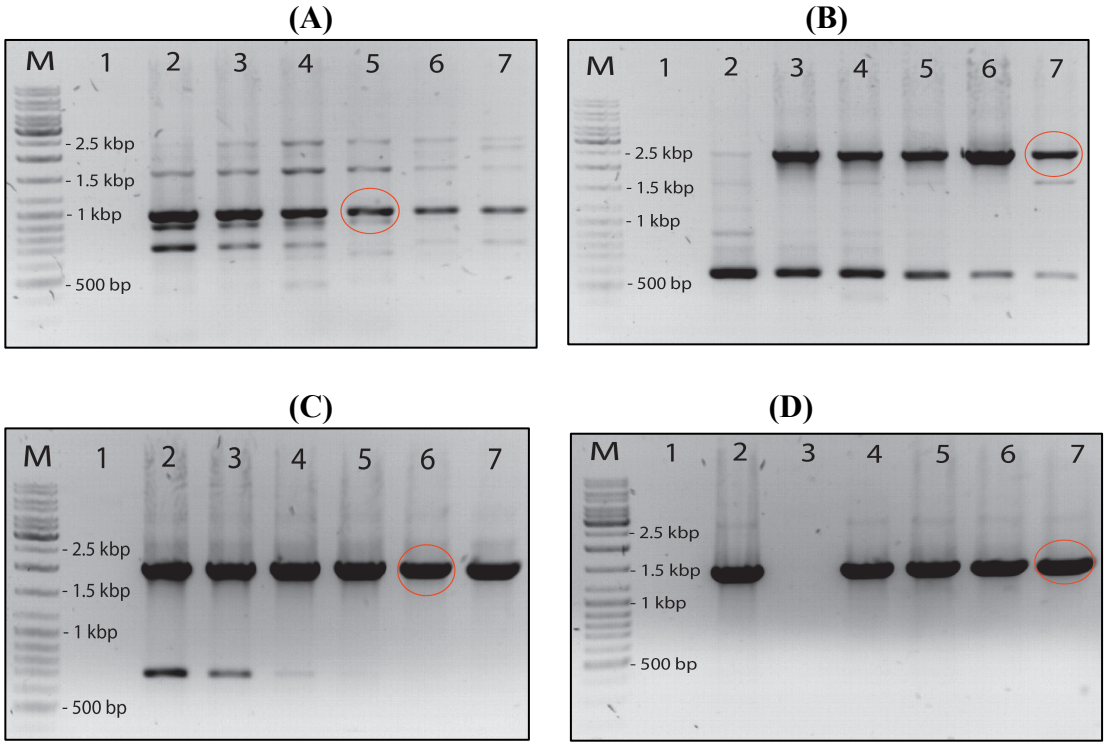


Figure 4.3.8: Agarose gel image of the isolation of *Thellungiella halophila P5CS1* gene by PCR using different sets of primers (see Materials and Methods). Gel images (A) to (D) represent the products of temperature gradient PCR amplifications of *ThP5CS1* gene along with 5' and 3' flanking sequences, divided into 4 sections to ease the process of isolation. Lanes 2, 3, 4, 5, 6 and 7 represent 49°C, 51°C, 53°C, 55°C, 57°C and 59°C annealing temperatures respectively. Products from: Gel (A) lane 5, Gel (B) lane 7, Gel (C) lane 6 and Gel (D) lane 7 were sent for sequencing. M: Long range size marker (ThermoScientific, UK)

5'-*ACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTGACGGTGTGAATTTCCTATTTTTTTCACTAGAGAGACGTCATCTA*
AAATTGCTTCGCTGTCCGCTTTTCACTTGTCCTCTTCTGATTTCCGTTTGCCTTTGTGTTGTTCACGTCCACGGCGCTTCCTCAGCCG
CCGATTTTTTAATTTCCCAAAATACCCACCACCTCAGTAACTATCTTCTGCGACGCAACCCAAATCCCTTTACGATGCGCCACCCAA
CGACTGCGCGCCACTTCATCGATTAGGATCTAATATTCCAGATATACCCTTACTCGCACACGTGGCGCGTCTTGGTTTGATAGAA
GGACCTGACTCTCTGGTGTAAGTTGTGTTTATAAACCCCTTCATCTCTTCCTTGTTGATCTCCACACTTCCCTCACCAGATATTTCCC
TAAACGCGCTCACTGACGAAATCCACCACTGAGTTAACTCGTTCCTTCTCTGGGTTTTGGTAGGCGGCGACA- **3'**

Figure 4.3.9: Putative *ThP5CS1* 5' flanking promoter sequence obtained from the genome walking experiment using the unpublished short length of sequence available from the database.

4.3.4 Comparative analysis of the 5' & 3' flanking sequences of *ThP5CS1*

The two putative 5' flanking sequences isolated from *T. halophila* for the *P5CS1* gene shared very limited similarities. Both 5' flanking sequences obtained were checked for similarities with the corresponding *A. thaliana* 5' flanking sequence of the *P5CS1* gene available in the NCBI database. Figure 4.3.10 (A) & (B) shows the alignment of the *A. thaliana* 1344 bp 5' flanking sequence (*AtP5CS1*) to the isolated 1169 bp (*ThP5CS1*a) and 501 bp (*ThP5CS1*b) *T. halophila* putative 5' flanking sequences. The alignment clearly shows that the three sequences are highly dissimilar from each other with identitiess of 42.3% and 13.8% when *AtP5CS1* was compared to *ThP5CS1*a and *ThP5CS1*b, respectively. And when the two putative *ThP5CS1* 5' sequences were compared to each other they showed only 15.3% identity. Also the isolated *T. halophila* 3' flanking region of the *P5CS1* gene was compared to the corresponding *A. thaliana* 3' flanking sequence of the *P5CS1* gene available in the NCBI database (see Figure 4.3.11). The two 3' flanking sequences from *T. halophila*, on the other hand, showed 100% identity (not shown).

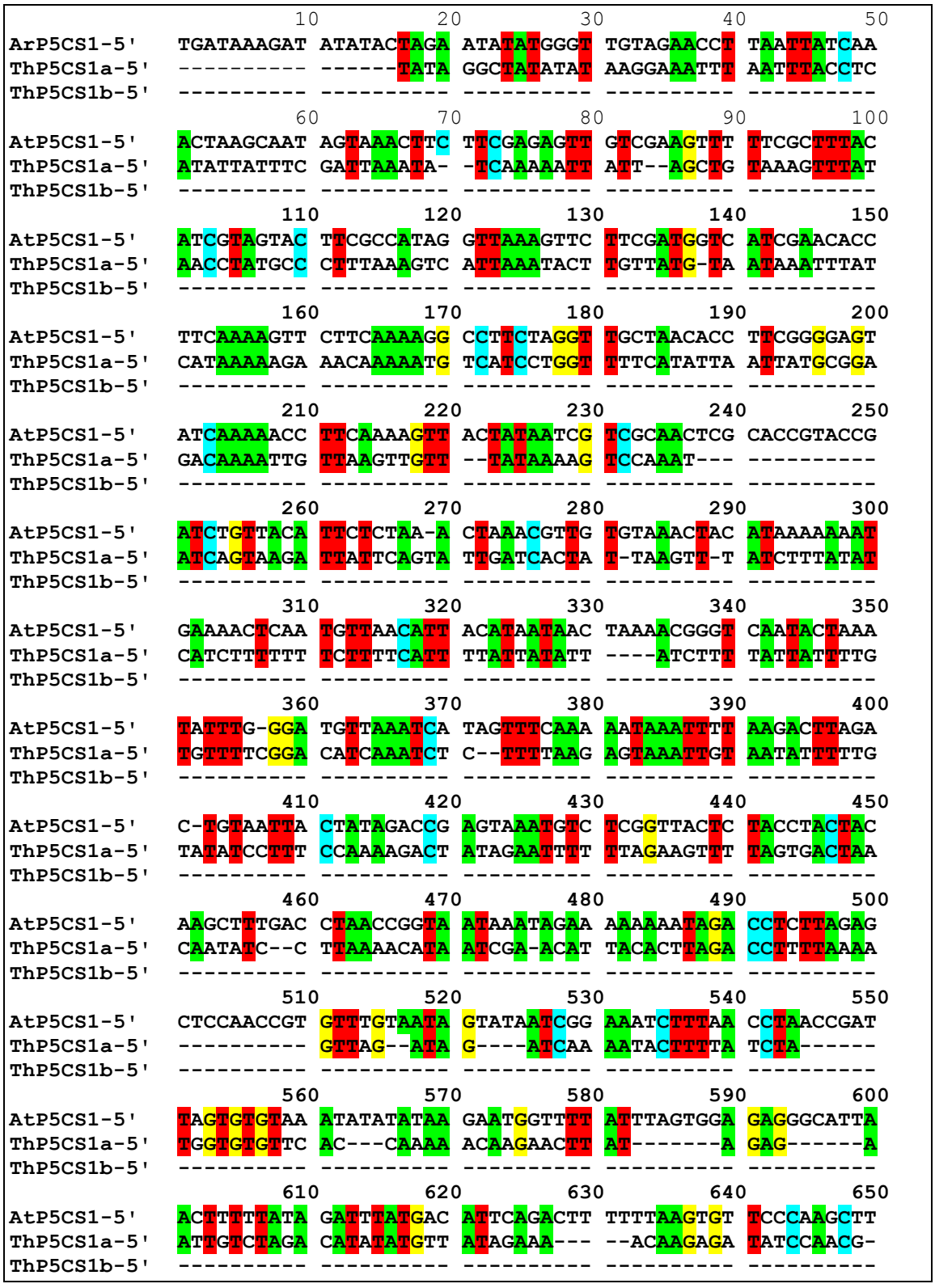


Figure 4.3.10 (A): Alignment of two putative *ThP5CS1* 5' flanking promoter sequences (*ThP5CS1a*-5' and *ThP5CS1b*-5') obtained from the genome walking experiment to the corresponding sequence present in the *A. thaliana* genome (*AtP5CS1*-5') available from NCBI.

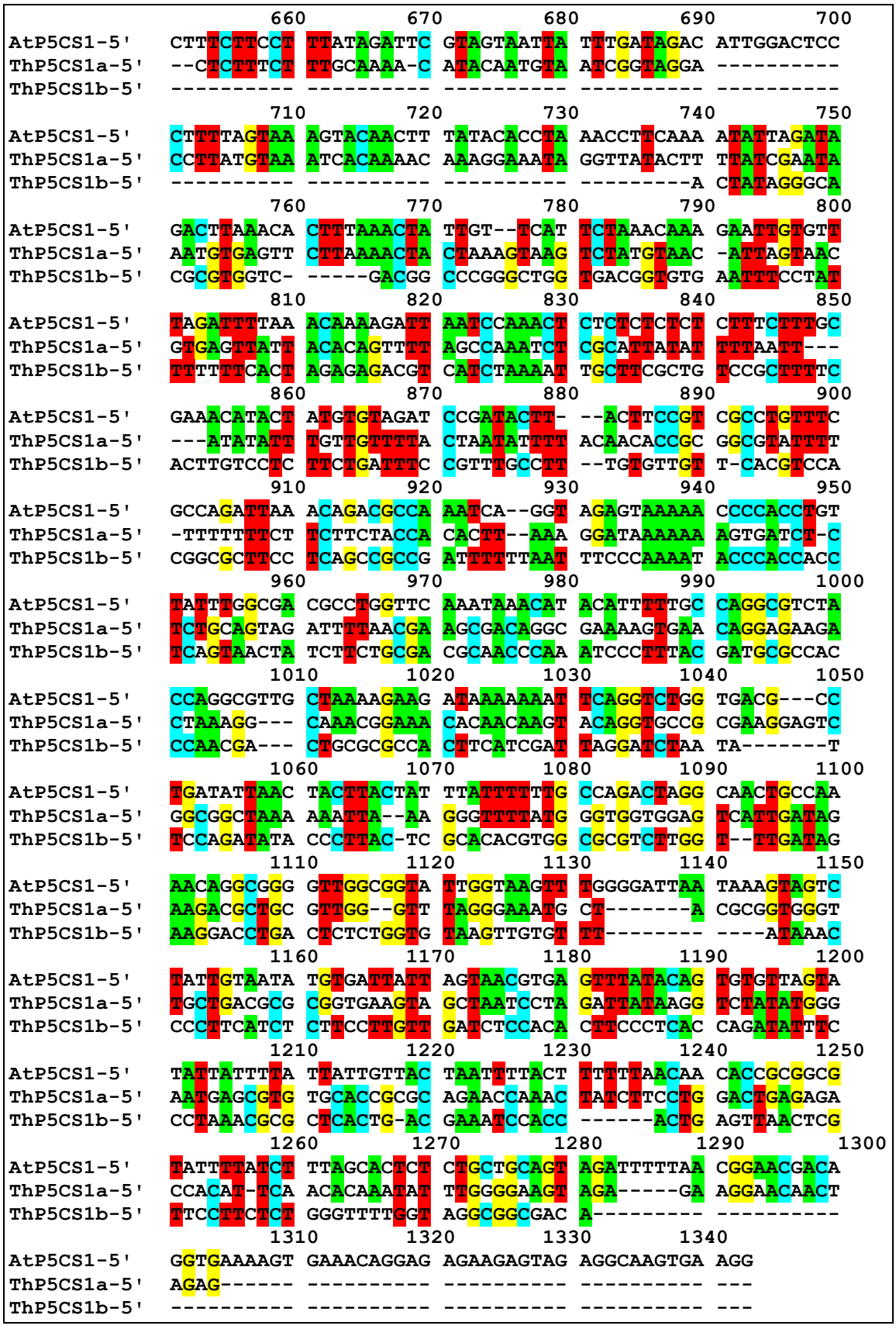


Figure 4.3.10 (B): Alignment of two putative *ThP5CS1* 5' flanking promoter sequences (*ThP5CS1a*-5' and *ThP5CS1b*-5') obtained from the genome walking experiment to the corresponding sequence present in the *A. thaliana* genome (*AtP5CS1*-5') available from NCBI.

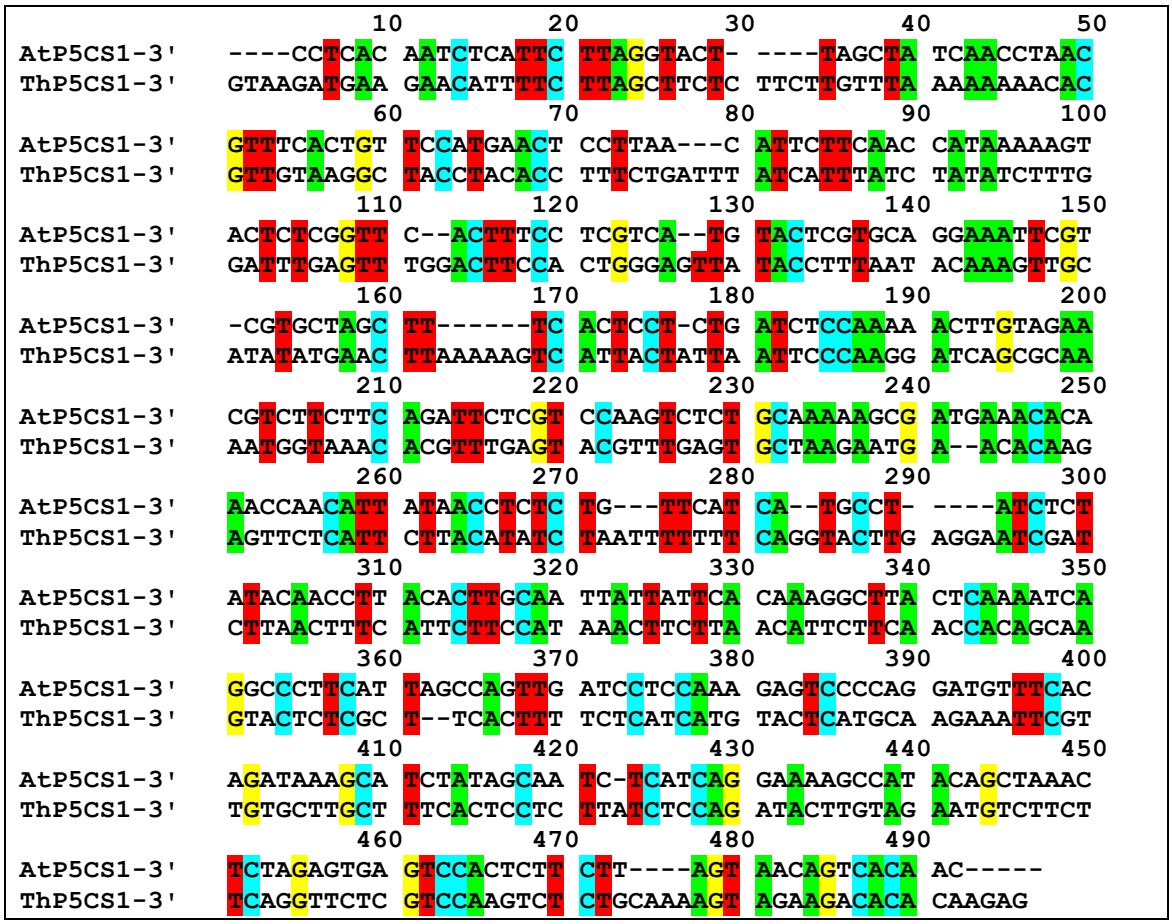


Figure 4.3.11: Alignment of the *ThP5CS1* 3' flanking sequence obtained from the genome walking experiment to the corresponding sequence present in the *A. thaliana* genome (*AtP5CS1*-3') available from NCBI.

4.3.5 Bioinformatic analysis of putative *T. halophila* sequences

The presence of potential response elements (RE) was established in the obtained two putative 5' flanking sequences of *T. halophila P5CS1* genes by searching the PLACE database. The potential REs from the two *T. halophila* sequences were compared to the REs found in the corresponding 5' flanking sequence of *A. thaliana* available from the NCBI gene database. Table 4.3.1 (A) to 4.3.1 (E) shows all the 117 types of REs with their number of repeats and function found in the three sequences. There were a total of 274 REs with frequency of 0.204 REs per base pair in *AtP5CS1* 5' flanking sequence, 267 REs with frequency of 0.228 REs per base pair in *ThP5CS1*a 5' flanking sequence and 127 REs with frequency of 0.253 REs per base pair in *ThP5CS1*b 5' flanking sequence. There were 30 different types of REs present in the *ThP5CS1*a 5' flanking sequence but absent in the *AtP5CS1* 5' flanking sequence and there were 25 different types of REs present in the *AtP5CS1* 5' flanking sequence but absent in the *ThP5CS1*a 5' flanking sequence. Also, there were 16 different types of REs present in the *ThP5SC1*b 5' flanking sequence but absent in both *AtP5CS1* and *ThP5CS1*a 5' flanking sequences.

No.	Code	Bases	Ar	Tha	Thb	Function (Available from PLACE database)
1	-10PEHVPSBD	TATTCT	1	0	0	S000392
2	-300CORE	TGTAAAG	0	1	0	S000001
3	-300ELEMENT	TGHAAARK	2	2	0	S000122
4	5659BOXLELAT5659	GAAWTTGTGA	1	0	0	S000280
5	ABREATCONSENSUS	YACGTGGC	0	0	1	**ABA signaling – increase salt tolerance in Rice**
6	ABREATRD22	RYACGTGGYR	0	0	1	**ABA response elemenet – dehydration response gene (rd22)**
7	ABRELATERD1	ACGTG	0	0	3	**ABRE like sequence – early responsive to dehydration**
8	ABRERATCAL	MACGYGB	0	0	5	**ABRE related – induced by increased cytosolic Ca^{2+}**
9	ACGTABREMOTIFA2OSEM	ACGTGKC	0	0	1	**ABA dependent rd29 expression in response to dehydration**
10	ACGTATERD1	ACGT	2	2	6	**Early response to dehydration**

Table 4.3.1 (A): Comparison of potential Response Elements (RE) present in the upstream region of *P5CS1* gene between *A. thaliana* and *T. halophila*. From Right: column one is RE number, column two is the Code name of the RE, column three is the sequence of bases specific to the RE, columns four, five and six are the RE present in *AtP5CS1*, *ThP5CS1*a and *ThP5CS1*b 5' flanking sequences respectively with their number of repeats and column seven represents the function code of the RE which can be accessed from the PLACE database. RE code names shaded in blue represents their absence in *AtP5CS1*, in green represents their absence in *ThP5CS1*a and in red represents their absence in both *AtP5CS1* & *ThP5CS1*a.

No.	Code	Bases	Ar	Tha	Thb	Function (Available from PLACE database)
11	ACGTCBOX	GACGTC	2	0	2	S000131
12	AGCBOXNPGLB	AGCCGCC	0	1	1	S000232
13	AGMOTIFNTMYB2	AGATCCAA	1	0	0	S000444
14	ANAERO1CONSENSUS	AAACAAA	2	2	0	S000477
15	ANAERO2CONSENSUS	AGCAGC	1	0	0	S000478
16	ANAERO4CONSENSUS	GTTTHGCAA	0	1	0	S000480
17	ARFAT	TGTCTC	0	1	0	S000270
18	ARR1AT	NGATT	22	10	5	ARRI binding elemet (response regulator)
19	ASF1MOTIFCAMV	TGACG	2	1	3	S000024
20	BIHD1OS	TGTCA	2	1	0	S000498
21	BOXIINTPATPB	ATAGAA	3	3	1	S000296
22	BOXIIPCCHS	ACGTGGC	0	0	1	S000229
23	CAATBOX1	CAAT	20	7	1	CAAT promoter consensus sequence
24	CACGTGMOTIF	CACGTG	0	0	2	S000042
25	CACTFTPPCA1	YACT	11	20	8	S000449
26	CARGCW8GAT	CWWWWWWWWG	6	4	0	S000431
27	CBFHV	RYCGAC	0	1	2	Dehydration response element binding proteins (DREBs)
28	CCAATBOX1	CCAAT	5	0	0	S000030
29	CEREGLUBOX2PSLEGA	TGAAAACT	2	0	0	S000033
30	CGACGOSAMY3	CGACG	1	0	2	S000205
31	CGCGBOXAT	VCGCGB	0	9	8	NtER1 gene encoding binding protein (induced through salt stress)
32	CIACADIANLELHC	CAANNNNATC	0	1	0	S000252
33	CPBCSPOR	TATTAG	3	2	1	S000491
34	CRTDREHVCBF2	GTCGAC	0	0	2	S000411
35	CTRMCAMV35S	TCTCTCTCT	3	0	0	S000460
36	CURECORECR	GTAC	0	2	0	S000493
37	DOFCOREZM	AAAG	11	25	4	Dof binding site- enhances transcription from the promoters of a non-photosynthetic PEPC gene

Table 4.3.1 (B): Comparison of potential Response Elements (RE) present in the upstream region of *P5CS1* gene between *A. thaliana* and *T. halophila.* From Right: column one is RE number, column two is the Code name of the RE, column three is the sequence of bases specific to the RE, columns four, five and six are the RE present in *AtP5CS1*, *ThP5CS1*a and *ThP5CS1*b 5' flanking sequences respectively with their number of repeats and column seven represents the function code of the RE which can be accessed from the PLACE database. RE code names shaded in blue represents their absence in *AtP5CS1*, in green represents their absence in *ThP5CS1*a and in red represents their absence in both *AtP5CS1* & *ThP5CS1*a.

No.	Code	Bases	Ar	Tha	Thb	Function (Available from PLACE database)
38	DPBFCOREDCDC3	ACACNNG	0	0	2	S000292
39	DRECRTCOREAT	RCCGAC	0	1	0	**Dehydration response element**
40	EBOXBNNAPA	CANNTG	8	2	4	**S000144**
41	EECCRCAH1	GANTTNC	5	2	2	S000494
42	EMBP1TAEM	CACGTGGC	0	0	1	**Involved ABA mediated stress signaling pathway**
43	EMHVCHORD	TGTAAAGT	0	1	0	S000452
44	EVENINGAT	AAAATATCT	1	0	0	S000385
45	GAREAT	TAACAAR	1	1	0	S000439
46	GATABOX	GATA	17	16	4	**E-box of napA storage-protein gene**
47	GBOXLERBCS	MCACGTGGC	0	0	1	S000041
48	GCCCORE	GCCGCC	0	1	2	S000430
49	GT1CONSENSUS	GRWAAW	12	12	5	**SA- inducible gene expresiion**
50	GT1GMSCAM4	GAAAAA	3	2	1	**GT-1 motif (CaM isoform)- salt induced SCaM-4 gene expression**
51	GT1MOTIFPSRBCS	KWGTGRWAAWRW	0	0	1	S000051
52	GTGANTG10	GTGA	8	9	7	**Motif found in late pollen gene g10**
53	HEXAMERATH4	CCGTCG	0	0	1	S000146
54	HEXMOTIFTAH3H4	ACGTCA	1	0	1	S000053
55	HSELIKENTACIDICPR1	CNNGAANTTCNNG	2	0	0	S000056
56	IBOXCORE	GATAA	7	6	0	**Conserved sequence upstream of light regulated genes**
57	IRO2OS	CACGTGG	0	0	1	S000505
58	INRNTPSADB	YTCANTYY	4	4	0	S000395
59	LECPLEACS2	TAAAATAT	0	2	0	S000465
60	LTRE1HVBLT49	CCGAAA	0	1	0	S000250
61	LTRECOREATCOR15	CCGAC	0	1	0	**Drought induced expression through C/DRE (cor15a gene)**
62	MARTBOX	TTWTWTTWTT	1	3	0	S000067
63	MYB1AT	WAACCA	1	1	1	**MYB recognition site in promoters of dehydration responsive genes**

Table 4.3.1 (C): Comparison of potential Response Elements (RE) present in the upstream region of *P5CS1* gene between *A. thaliana* and *T. halophila.* From Right: column one is RE number, column two is the Code name of the RE, column three is the sequence of bases specific to the RE, columns four, five and six are the RE present in *AtP5CS1*, *ThP5CS1*a and *ThP5CS1*b 5' flanking sequences respectively with their number of repeats and column seven represents the function code of the RE which can be accessed from the PLACE database. RE code names shaded in blue represents their absence in *AtP5CS1*, in green represents their absence in *ThP5CS1*a and in red represents their absence in both *AtP5CS1* & *ThP5CS1*a.

No.	Code	Bases	Ar	Tha	Thb	Function (Available from PLACE database)
64	MYB2AT	TAACTG	1	0	0	AtMYB involved in regulation of genes responsive to water stress
65	MYB2CONSENSUSAT	YAACKG	2	0	0	MYB recognition site in promoters of dehydration responsive genes
66	MYBCORE	CNGTTR	4	0	0	S000176
67	MYBCOREATCYCB1	AACGG	4	1	1	S000502
68	MYBPLANT	MACCWAMC	0	1	0	S000167
69	MYBPZM	CCWACC	2	1	1	S000179
70	MYBST1	GGATA	1	4	0	S000180
71	MYCCONSENSUSAT	CANNTG	8	2	4	CBF3 stress response element
72	NAPINMOTIFBN	TACACAT	1	0	0	S000070
73	NODCON1GM	AAAGAT	1	3	0	S000461
74	NODCON2GM	CTCTT	2	3	2	S000462
75	NTBBF1ARROLB	ACTTTA	0	3	0	S000273
76	OSE1ROOTNODULE	AAAGAT	1	3	0	S000461
77	OSE2ROOTNODULE	CTCTT	2	4	2	S000468
78	P1BS	GNATATNC	0	2	0	S000459
79	POLASIG1	AATAAA	8	4	0	S000080
80	POLASIG2	AATTAAA	0	3	1	S000081
81	POLASIG3	AATAAT	5	4	0	S000088
82	POLLEN1LELAT52	AGAAA	9	5	0	S000245
83	PRECONSCRHSP70A	SCGAYNRNNND	0	1	0	S000506
84	PROLAMINBOXOSGLUB1	TGCAAAG	0	1	0	S000354
85	PYRIMIDINEBOXOSRAMY1A	CCTTTT	0	1	0	S000259
86	RAV1AAT	CAACA	5	4	2	S000314
87	RAV1BAT	CACCTG	0	1	0	S000315
88	RBCSCONSENSUS	AATCCAA	1	0	0	S000127
89	RGATAOS	CAGAAGATA	0	0	1	S000191
90	RHERPATEXPA7	KCACGW	1	1	1	S000512
91	REALPHALGLHCB21	AACCAA	0	1	1	S000362
92	ROOTMOTIFTAPOX1	ATATT	7	14	3	Motif found in in promoters of ro1D
93	SEF1MOTIF	ATATTTAWW	0	1	0	S000006
94	SEF3MOTIFGM	AACCCA	0	2	2	S000115

Table 4.3.1 (D): Comparison of potential Response Elements (RE) present in the upstream region of *P5CS1* gene between *A. thaliana* and *T. halophila.* From Right: column one is RE number, column two is the Code name of the RE, column three is the sequence of bases specific to the RE, columns four, five and six are the RE present in *AtP5CS1*, *ThP5CS1*a and *ThP5CS1*b 5' flanking sequences respectively with their number of repeats and column seven represents the function code of the RE which can be accessed from the PLACE database. RE code names shaded in blue represents their absence in *AtP5CS1*, in green represents their absence in *ThP5CS1*a and in red represents their absence in both *AtP5CS1* & *ThP5CS1*a.

No.	Code	Bases	Ar	Tha	Thb	Function (Available from PLACE database)
95	SEF4MOTIFGM7S	RTTTTTR	5	4	0	S000103
96	SORLIP1AT	GCCAC	2	0	3	S000482
97	SP8BFIBSP8AIB	ACTGTGTA	0	1	0	S000183
98	SREATMSD	TTATCC	0	1	0	S000470
99	SURECOREATSULTR11	GAGAC	1	2	1	S000499
100	T/GBOXATPIN2	AACGTG	0	1	0	S000458
101	TAAAGSTKST1	TAAAG	1	8	1	S000387
102	TATABOX2	TATAAAT	2	0	0	S000109
103	TATABOX3	TATTAAT	2	1	0	S000110
104	TATABOX4	TATATAA	2	2	0	S000111
105	TATABOX5	TTATTT	7	2	0	S000203
106	TATABOXOSPAL	TATTTAA	1	2	0	S000400
107	TATAPVTRNALEU	TTTATATA	1	0	0	S000340
108	TATCCAOSAMY	TATCCA	1	1	0	S000403
109	TBOXATGAPB	ACTTTG	2	0	0	S000383
110	TGACGTVMAMY	TGACGT	1	0	1	S000377
111	TGTCACACMCUCUMISIN	TGTCACA	1	0	0	S000422
112	UP2ATMSD	AAACCCTA	1	0	0	S000472
113	WBOXATNPR1	TTGAC	1	0	0	S000390
114	WBOXHVISO1	TGACT	0	3	1	S000442
115	WBOXNTCHN48	CTGACY	0	0	1	S000508
116	WBOXNTERF3	TGACY	0	3	1	S000457
117	WRKY71OS	TGAC	4	5	4	S000447
	Total		**274**	**267**	**127**	
	Frequency		**0.204**	**0.228**	**0.253**	

Table 4.3.1 (E): Comparison of potential Response Elements (RE) present in the upstream region of *P5CS1* gene between *A. thaliana* and *T. halophila.* From Right: column one is RE number, column two is the Code name of the RE, column three is the sequence of bases specific to the RE, columns four, five and six are the RE present in *AtP5CS1*, *ThP5CS1*a and *ThP5CS1*b 5' flanking sequences respectively with their number of repeats and column seven represents the function code of the RE which can be accessed from the PLACE database. RE code names shaded in blue represents their absence in *AtP5CS1*, in green represents their absence in *ThP5CS1*a and in red represents their absence in both *AtP5CS1* & *ThP5CS1*a. Function column represents the code for each of the respective elements function the promoter region and also mentioning some on the key salt stress related functions found in three sequences.

4.3.6 *P5CS1* gene alternative splicing in *T. halophila*

The isolated whole *ThP5CS1a* sequence revealed the presence of introns that were different from the introns of its *A. thaliana* orthologue. Figure 4.3.12 represents the difference in number and placing of introns and exons in the three *P5CS1* genes. Introns from the isolated *ThP5CS1*a sequence also showed considerable differences from the *ThP5CS1*b sequence recently published in the Phytozome database and whose 5' flanking region was extended in this work. However, the exons of the two *ThP5CS1*

genes were identical. Table 4.3.3 shows the comparison between the introns present in *AtP5CS1* (NCBI) and *ThP5CS1*b (Phytozome) transcripts. There were 21 introns in the *AtP5CS1* gene and 19 in the *ThP5CS1*b gene. The 195 bp intron 6 from *ThP5CS1*b had 67% identity with both introns 6 (84 bp) and 7 (57 bp) from *AtP5CS1*. Identity between the introns from these two genes ranged from 60% to 86.6%. On the other hand, there was a large difference in number of introns found in *ThP5CS1*a and *ThP5CS1*b. There were only 8 introns found in the *ThP5CS1*a gene compared to 19 in *ThP5CS1*b (unpublished gene sequence). Table 4.3.2 shows the comparison between the introns present in *ThP5CS1*a and *ThP5CS1*b. Introns of *ThP5CS1*a showed high identity to corresponding introns of *ThP5CS1b* with varying level of differences but were not 100 % identical.

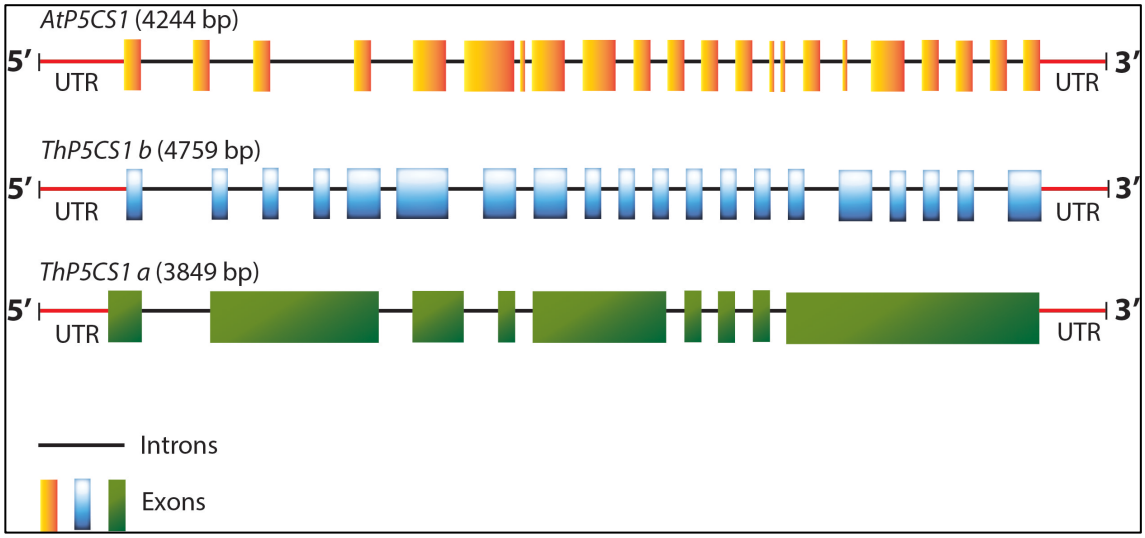


Figure 4.3.12: Comparison of introns and exons present in the *P5CS1* genes between two putative sequences found in *T. halophila* and *A. thaliana P5CS1* gene. There are 21, 19 and 8 introns present in *AtP5CS1*, *ThP5CS1*b and *ThP5CS1*a respectively.

***T. halophila P5CS1*a** Intron Number (Length in bp)	***T. halophila P5CS1*b** Intron Number (Length in bp)	**Identity (%)**
1 (495)	1 (503)	95.3
2 (107)	5 (108)	89.8
3 (197)	6 (195)	92.3
4 (104)	7 (104)	89.4
5 (97)	11 (98)	99.0
6 (85)	12 (85)	100.0
7 (82)	13 (82)	100.0
8 (98)	14 (98)	98.0

Table 4.3.2: Comparison of introns present in the *P5CS1* gene between two putative sequences found in *T. halophila*. Each intron in isolated *ThP5CS1*a is compared to its corresponding intron present in the same postion in *ThP5CS1*b. Comparison is done on the basis of length (bp) and sequence identity (%) of the corresponding introns between the two putative sequences.

A. thaliana P5CS1 Intron Number (Length in bp)	***T. halophila P5CS1*b** Intron Number (Length in bp)	**Identity (%)**
1 (282)	1 (503)	64.3
2 (144)	2 (151)	63.2
3 (212)	3 (168)	65.2
4 (78)	4 (111)	70.0
5 (92)	5 (108)	65.0
6 (84)	**6 (195)**	**67.0**
7 (57)	**6 (195)**	**67.0**
8 (97)	7 (96)	60.0
9 (94)	8 (101)	60.0
10 (128)	9 (108)	69.5
11 (72)	10 (76)	82.2
12 (78)	11 (96)	64.2
13 (118)	**12 (72)**	**83.3**
14 (34)	**N/A**	**N/A**
15 (80)	13 (81)	85.0
16 (151)	14 (96)	70.0
17 (182)	15 (121)	70.6
18 (97)	16 (97)	86.6
19 (83)	17 (79)	80.2
20 (88)	18 (88)	76.1
21 (102)	19 (128)	76.7

Table 4.3.3: Comparison of introns present in the *P5CS1* gene between *A. thaliana* and *T. halophila.* Each intron in *A. thaliana* is compared to its corresponding intron present in the same locus in *T. halophila.* Comparison is done on the basis of length (bp) and sequence identity (%) of the corresponding introns between the two species.

Due to the differences observed in intron composition and sequence in the *P5CS1* gene between *A. thaliana* and *T. halophila,* it was decided to check for salt-dependent alternative splicing in *T. halophila P5CS1* genes. Except for the experiment shown in Figure 4.3.13 C, RNA was from the 5 day time point. Figure 4.3.13 shows the results from the reverse transcription PCR performed with the *T. halophila* mRNA from unstressed (0 mM) and stressed (100 mM and 500 mM [NaCl]) plants using primer sets flanking the introns in *ThP5CS1*a. *T. halophila P5CS1* transcript profiles showed alternative splicing of introns 2, 4 and 5 under 100 mM salt stress (Figure 4.3.13 A). Salt treatment increased level of the transcript with unspliced intron 2, transcript with spliced intron 4 and transcripts with both spliced and unspliced intron 5. When intron 4 was checked over a time course of 100 mM and 500 mM [NaCl] treatment, it was observed that after 1 day of salt treatment, splicing of intron 4 increased with time and that splicing of this intron seemed to be more rapid at the high salt concentration than at the lower one (figure 4.3.13 C). On the other hand, *ThP5CS1* transcript profiles showed

the spliced transcript version at intron 8 under both salt stressed and the unstressed conditions (Figure 4.3.13 B). Figure 4.3.14 shows the result of alternative splicing of *ThP5CS*1 transcript using primer sets designed based on the *ThP5CS1*b sequence. *ThP5CS1* transcripts showed an increase in the spliced version at intron 5 with increase in the concentration of salt (0 mM, 100 mM, 500 mM) (Figure 4.3.14 A). Also *ThP5CS1b* transcripts showed increased transcripts with spliced and unspliced intron 11 with increase in the duration of plant treatment with salt at100 mM [NaCl] (0, 1, 5 and 10 days) (Figure 4.3.14 B).

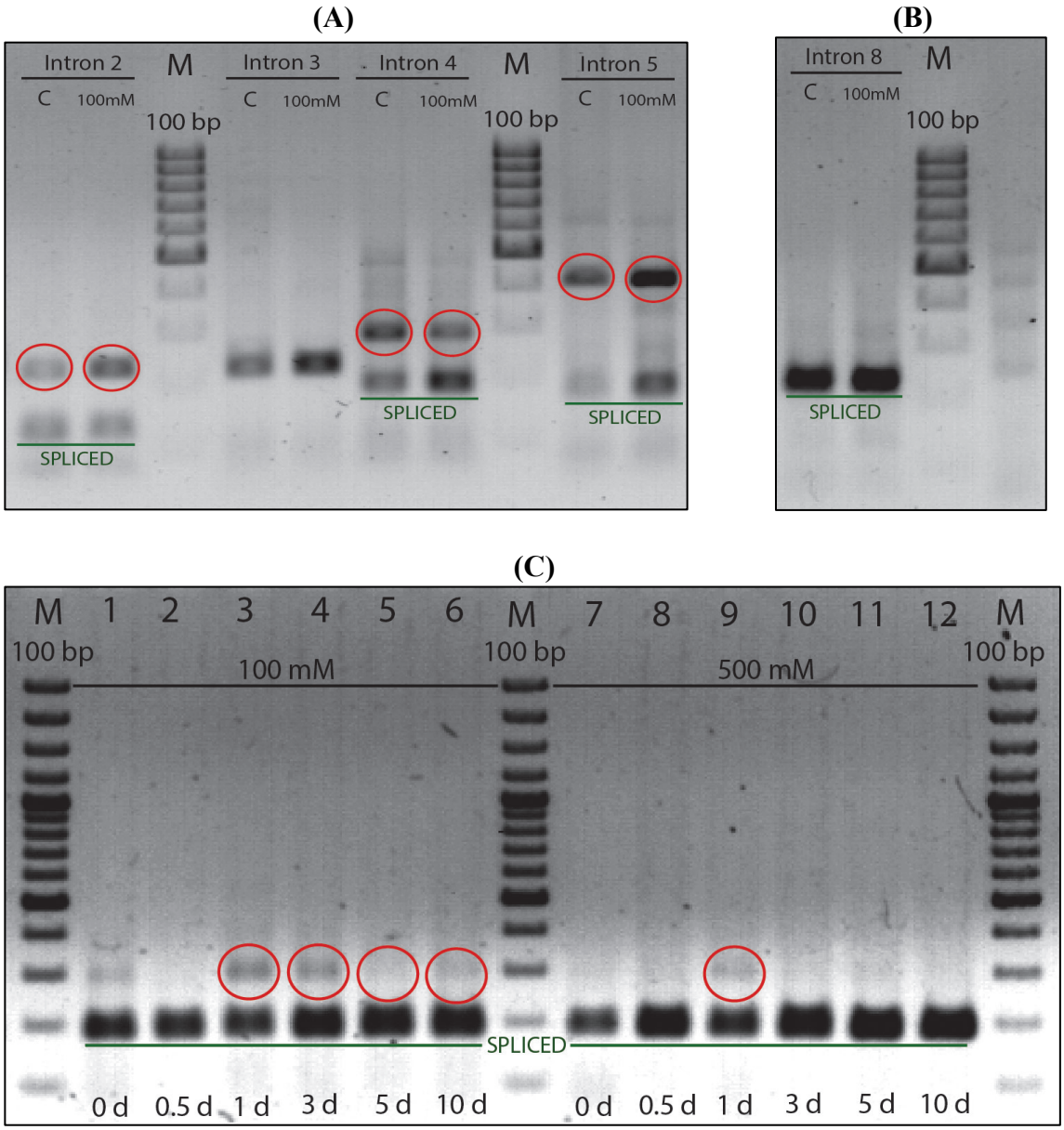


Figure 4.3.13: (A) & (B) Alternative splicing results in the *T. halophila* unstressed (0 mM) and stressed (100 mM) plants assessed using RT-PCR based on primer sets flanking introns from *ThP5CS1*a. (C) Alternate splicing results in the *T. halophila* plants at 100 mM and 500 mM NaCl assessed using RT-PCR based on primer sets flanking intron 4 from *ThP5CS1*a. The amplicons inside the red circles represent the unspliced transcript and amplicons above the green lines represent the spliced transcript products. M: 100 bp size marker (ThermoScientific, UK)

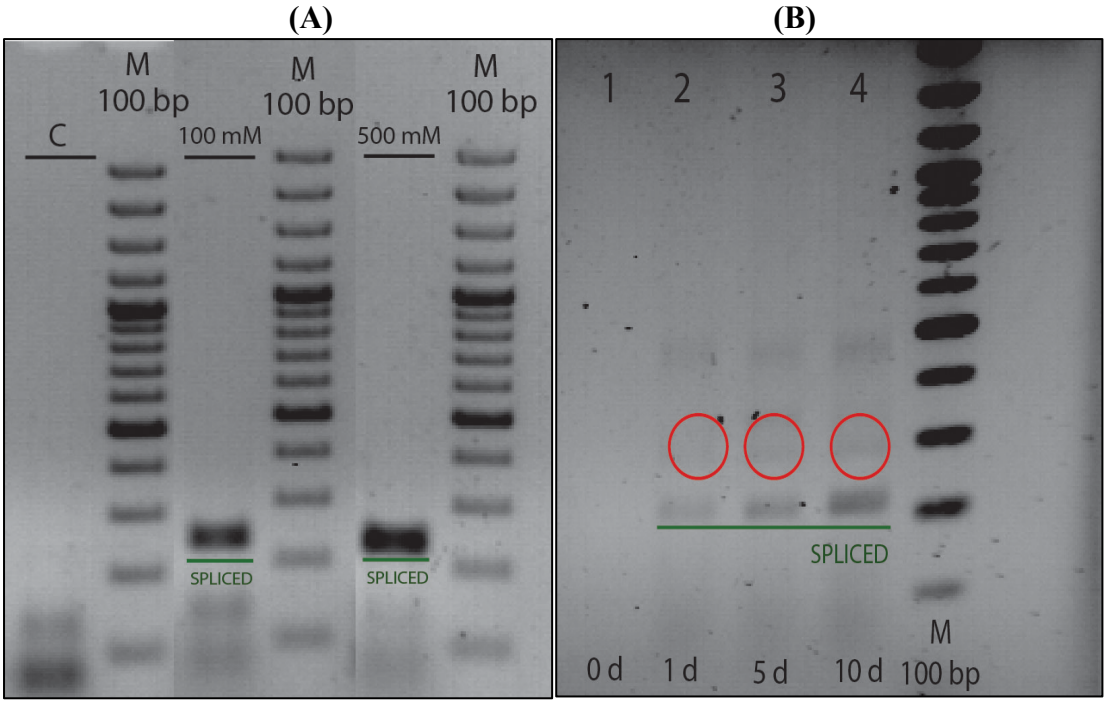


Figure 4.3.14: Agarose gels showing alternative splicing of *ThP5CS1* gene using primers based on *ThP5CS1*b sequence. (A) Alternative splicing results in the *T. halophila* plants at 0 mM, 100 mM and 500 mM (NaCl) assessed using RT-PCR based on primer sets flanking intron 5 from *ThP5CS1*b. (B) Alternative splicing results in the *T. halophila* plants at 100 mM NaCl assessed using RT-PCR based on primer sets flanking intron 11 from *ThP5CS1*b. The amplicons inside the red circles represent the un-spliced transcript and amplicons above the green line represents the spliced transcript. M: 100 bp size marker (ThermoScientific, UK)

4.4 Discussion

Growth analysis of *A. thaliana* wild type and its *P5CS1* null mutant under salt-treatment exhibited clearly the important role of the *P5CS1* gene under saline conditions. From the previous chapters we saw that proline accumulated quickly to higher levels in *T. halophila* than in *A. thaliana* under salt-treatment and proline accumulation mirrored the increase in *P5CS1* transcript abundance. Higher transcript abundance for the *P5CS1* gene under salt-treatment was reported previously in *T. halophila* compared to *A. thaliana* (Taji et al., 2008). The known coding sequences of the gene in the two species have high similarities (exceeding 98% identity) suggesting the very close phylogenetic relationship of these orthologous genes. However, differential regulation of the expression of the gene in the two plant species is very likely to be behind the observed differential transcript levels.

As mentioned earlier, two putative 5' flanking sequences for the *P5CS1* gene (*ThP5CS1*a *& ThP5CS1*b) were isolated from the *T. halophila* genome. The strong dissimilarities between these two sequences (15.3% identity) suggest the presence of more than one copy of the *P5CS1* gene in *T. halophila*. Furthermore, 5' flanking sequences of *ThP5CS1*a and *ThP5CS1*b genes have shown large dissimilarities when compared to the 5' flanking sequence of the orthologue gene in *A. thaliana* (42.3% & 13.8% identity respectively). This might suggest that not only is there a possible duplication event in the *ThP5CS1* gene but also a mutation event took place in the 5' flanking regions.

Bioinformatic analysis of 5' flanking sequences of *ThP5CS1*a and *ThP5CS1*b revealed differences in predicted Response Elements (RE) between themselves as well as with the 5' flanking sequence of *AtP5CS1*. The frequency of predicted REs in *ThP5CS1*a and *ThP5CS1*b (0.228 and 0.253 REs per base pair respectively) was higher than that in *AtP5CS1* (0.204). This indicates that both copies of the *P5CS1* gene in *T. halophila* have more REs potentially responsible for their differential expression, comparatively to *A. thaliana*. There were 30 different types of predicted REs present in the promoter region of *ThP5CS1*a but missing in *AtP5CS1* promoter sequence. On the other hand, there were 16 different types of predictive REs, which were absent in both *ThP5CS1*a and *AtP5CS1* promoter sequences but present in the ThP5CS1b promoter sequence. Therefore, the presence of different REs in *T. halophila* might contribute to the differential expression of *ThP5CS1* genes; four elements, which were found in only the *ThP5CS1*b promoter sequence, belong to the ACGT containing ABA response

elements (ABREs). These elements have been previously shown to regulate *A. thaliana* genes that were differentially expressed under drought and salt conditions. The presence of more REs together with some condition specific REs might result in higher expression of the *ThP5CS1* gene. It should be noted that the transcript levels measured in *T. halophila* (Chapter 3) might be the result of the expression of the two *P5CS1* genes present in *T. halophila,* as the PCR primers do not distinguish between them.

In addition to REs acting on transcription, the expression of a gene could also be controlled by post-transcriptional modifications. The analysis of *ThP5CS1*a and *ThP5CS1*b genes when compared to *AtP5CS1* showed different numbers of introns. There were differences in the identity of the introns between *ThP5CS1a,* b and *AtP5CS1* genes and also between *ThP5CS1*a and *ThP5CS1*b genes. These differences can give rise to alternative splice variants in mature transcripts. Analysis of *P5CS1* transcript variants showed evidence for alternative splicing controlled by salt-treatment. *T. halophila* accumulated transcript variants with spliced and un-spliced introns under salt treatment (100 mM and 500 mM [NaCl]) for both isogenes. *T. halophila* performed alternative splicing according to the concentration of salt and also as per the duration of the salt treatment. Alternative splicing of *P5CS1* transcript has been associated with level of proline in a QTL population of *A. thaliana* with varying degrees of drought-tolerance (Kesari et al., 2012). Sequence variation for introns 2 and 3 led to alternative splicing that resulted in non-functional protein lacking exon 3 in *A. thaliana* Shakdara accession (Sha) in comparison to its counterpart in *A. thaliana* Landseberg erecta (Ler). Moreover, sequence comparison between introns of the two putative *P5CS1* genes in *T. halophila* has revealed that intron 8 from *ThP5CS1*a had the least identity (26.5%) with its corresponding intron 14 of *ThP5CS1*b, even though it is at the same position in the *ThP5CS1* gene. Analysis showed many spliced variants under stressed and unstressed conditions, which might indicate that *ThP5CS1*a transcript is expressed constitutively.

All together the results strongly indicate the presence of more than one *P5CS1* gene in *T. halophila* unlike *in A. thaliana* and *Oryza sativa*. Diverse REs and post-transcriptional modifications might be behind the differential regulation of *ThP5CS1* genes, leading potentially to differential accumulation of proline contributing to the higher salt-tolerance exhibited by *T. halophila*. The fact that *P5CS1* genes from *T. halophila* were very distinct from that of *A. thaliana* by their 5' flanking regions and by their intronic structure might mean specialized functions of the gene have evolved in *T. halophila* with potentially one copy of the gene being associated with tolerance to salt-

stress and one copy with tolerance to cold. Indeed in addition to being a halophyte *T. halophila* is also very tolerant to cold and freezing stress (Gao et al., 2009).

4.5 Conclusion

The results from the chapter clearly show differences in the promoter sequence of *P5CS1* genes in *A. thaliana* and *T. halophila*. The presence of two putative promoter sequences and different intron patterns in *T. halophila* suggests that the *T. halophila* genome contains more than one copy of the *P5CS1* gene. Therefore, the differential expression of the *P5CS1* gene observed earlier in the thesis could be due to combined effects of differential regulation of the gene and regulated alternative slicing leading to transcripts with specialized functions. It is of importance to monitor change in levels of transcripts from the two genes separately under different environmental conditions in the different tissues of the plant to have a better idea about their specific roles.

Chapter 5

Screening for targeted signaling components and transcription factors directly or indirectly involved in the control of responses to salt-treatment using A. thaliana null mutants

5.1 Introduction

Salinity results in serious negative effects on plants. Complete understanding of plants' responses to salinity and their regulation is important but remains challenging despite the recent advancements in technology with exciting new molecular biology techniques and genomic tools. These new methodologies and tools are employed to identify the key factors that might help improve crop tolerance to abiotic stresses. Stress inducible genes in various plants have been investigated under many abiotic stresses. Salt stress is one of the major abiotic stresses and many salt inducible genes have been identified and isolated using various approaches. Transgenic plants overexpressing some of these genes were generated and their effects in terms of improved salt tolerance have been demonstrated (Hare et al., 1998). *Arabidopsis thaliana* has been used as a model because of its available genome sequence and its amenability to transformation, which has significantly facilitated the progress made in this field.

The use of microarray analysis has helped to compare the responses to salt treatment in *Arabidopsis thaliana* and its close relative *Thellungiella halophila*, a halophyte (Taji et al., 2004). This work has identified clusters of genes with differential up-regulation under salt stress in both species. This gave rise to the concept that differential salt responses between different plant species might be a result of differential regulation of basic sets of salt regulated genes present in most plant species. The use of mutant lines to screen the genome for important salt-tolerance genes helped to identify the effect of certain mutations on the expression of salt induced genes, thereby identifying novel genes of the vast regulatory and signaling network that regulates responses to salt-stress (Chinnusamy et al., 2002). These approaches are complementary and have been used for a better understanding of stress responses at the gene level. The genes involved in salt-tolerance can be put into two groups: (1) Genes that encode proteins directly involved in the salt tolerance responses (e.g. proline production) and (2) Genes that encode proteins responsible for the control and regulation of the expression of the genes of group 1 (Shinozaki et al., 2003). These regulators include various signaling components including receptors, protein phosphatases and protein kinases as well as transcription factors. The identification of these different regulators will contribute to the development of a plant system that is able to regulate the cellular mechanisms underpinning acclimation to abiotic stress (Hirayama and Shinozaki, 2010). Considerable efforts around the world have employed different cellular and metabolic strategies to confer increased salt tolerance to plants with only limited success (Flowers, 2004). The most likely to work best is a strategy

based on the modulation of signaling and regulation pathways of stress response mechanisms. Because these factors control complex regulatory networks that control transcription, translation and/or protein degradation, this approach can act on the accumulation of several enzymes/transporters, which could result in the regulation of the processes required for salt tolerance (Golldack et al., 2011).

Emerging developments about the importance of transcription factors in salt or drought responses place them in the centre of approaches for the development of modern robust crops able to give adequate yields even under adverse climatic conditions and which would help to cope with increasing food demands. Recent research has focused on linear pathways using single regulatory transcription factors such as the members of the bZIP transcription factor family and the potential of modifying their expression to confer enhanced stress tolerance has been shown many times (Golldack et al., 2011). For example, a potential target gene would be a key component of the regulation of responses to salt stress identified in *A. thaliana,* bZIP24, which belongs to the F group of bZIP transcription factors (Yang et al., 2009). It is expected that additional key signaling components and transcription factors playing key roles in the regulation of responses to salt stress exist.

The aim of the research presented in this chapter was to investigate the responses to salinity of four *Arabidopsis thaliana* mutants, each with one silenced gene belonging to the network of signaling and regulation systems. The mutants were compared to wild type *A. thaliana* ecotype Columbia (background of mutants) in terms of level of salt-tolerance via the analysis of growth, transcript levels of *P5CS1* and *SOS1*, and levels of key metabolites including sugars, malate and proline. The null mutants used in this part of the work were to study the loss or gain of function of stress response mechanisms by the absence of four regulatory components. The first mutant was a *CRK11* mutant. *CRK11* comes under the sub group of Domain of Unknown Function 26 receptor-like kinases (DUF26 RLKs), also called Cysteine-rich Receptor-like Kinases (CRKs). CRKs play important roles in defence and programmed cell death and the *CRK11* gene has shown increased expression under increased ROS enhancing tolerance to light stress (Wrzaczek et al., 2010). The second mutant is *Rap2.4f* (At4g28140), which is a transcription factor gene belonging to the A-6 subgroup of the DREB subfamily and from the AP2/ERF superfamily (Sakuma et al., 2002; Nakashima et al., 2009). Previous studies have shown its up-regulation in response to various factors including UV, wounding, glucose, salt and drought and it has a major role in leaf senescence (Takahashi et al., 2004; Xu et al., 2010). The third and fourth are *ORG1*

(At5g53450) and *NPK15* (At5g58540). Both have been less studied with regard to their involvement in stress response mechanisms, but along with the first they have shown increased transcript levels in *A. thaliana* and *T. halophila* under salt stress (osmotic stress) with striking differences in terms of transcript increase between the two species (Taji et al., 2004; Tran et al., 2006) *CRK11* (1.95 fold) and *NPK15* (2.31 fold) showed up-regulation in *T*. *halophila* under unstressed conditions when compared to *A*. *thaliana*, whereas *ORG1* was only up-regulated (1.51 fold) in *T*. *halophila* after 2 hours of 250 mM [NaCl] and *Rap2.4f* was only up-regulated (1.66 fold) in *A*. *thaliana* after 2 hours of exposure to 250 mM [NaCl] (Taji et al., 2004). The main objective was to determine the potential direct impacts of these mutations on the amplitude and kinetics of physiological, biochemical and gene responses under salt-treatment and try ultimately to dissect the salt-tolerance mechanisms (if any) in which these genes might be involved.

5.2 Materials and Methods

5.2.1 Selection and sourcing of null mutants

Null mutants produced by T-DNA insertion mutagenesis were selected on the basis of gene expression data identified from the published literature (Taji et al., 2004) including mutants for the GK-142FO7.01 (*ORG1*), JYB578.1 (*Rap2.4f*), SALK_024337 (*NPK15*), and SALK_054879 (*CRK11*) genes. Seeds were obtained from NASC European Arabidopsis Stock Centre (Nottingham, UK). The mutations were confirmed using DNA extracted from seedlings based on PCR detection of T-DNA in the mutated genes. Genomic DNA was extracted from seedlings of the mutants as described in the previous chapter and used in a standard PCR using 2XPCR master mix (Thermoscientific, UK) and the primers below:

The sequences of each primer pair were as follows:

CRK11 - Forward primer: 5' – GAAGCAGAGGAGTTTGTTTTCAG -3'

Rap2.4f - Forward primer: 5' – TTTGACGAGGAGCTAAATCTTTG -3'

ORG1 - Forward primer: 5' – ATGGCACTTTGTGGTTGTTC -3'

NPK15- - Forward primer: 5' – GATTCAATCTCCGAACAGTGTTC -3'

T-DNA specific primer

T-DNA1 Reverse primer: 5' – TGGTTCACGTAGTGGGCCATC -3'

T-DNA2 Reverse primer: 5' – GCGTGGACCGCTTGCTGCAAC -3'

5.2.2 Salt treatment and growth analysis of null mutants

For growth analysis under salt-treatment seeds of wild-type *Arabidopsis thaliana* ecotype Columbia and seeds of homozygous mutants including GK-142FO7.01 (*ORG1*), JYB578.1 (*Rap2.4f*), SALK_024337 (*NPK15*), and SALK_054879 (*CRK11*) were surface sterilized in 70% ethanol for 10 minutes followed by rinsing three times with sterile water. The seeds were germinated in large pots containing soil compost John Innes No.2 in a growth room and after 7 days seedlings were transferred into separate small pots (2.5 inches wide). Plants were grown under a 12 hour photoperiod, 25ºC/18ºC thermo period and were divided into 3 sets. One set was irrigated with 50 mM [NaCl], one set was irrigated with 100 mM [NaCl] solution prepared with normal tap water for salt-treatment and one set was watered with tap water and used as control. For growth analysis, plants were watered at a fixed time of the day (12:00 hours, i.e. 4 hours into the light) every other day for 4 weeks. Shoot samples were harvested at a

fixed time (16:00 hours, i.e. 4 hours before dark) as 3 plants per sample (3 samples per treatment) after each week of the treatment. Dry weight (dw) was determined after desiccation of samples at 80°C for 24 hours and used to assess plant growth. For the analysis of metabolic and gene responses to salt-treatment, four-week-old unstressed *A. thaliana* wild type and mutant plants, similar in size and before bolting, were subjected to similar salt-treatment by watering them with 100 mM [NaCl] solution at a fixed time (12:00 hours) every day for 10 days. Control plants continued to be watered with tap water. Shoots were harvested at a fixed time (16:00 hours) as 3 plants per sample (3 samples per salt concentration) after 12 h, 1 d, 5 d and 10 d of the treatment, frozen in liquid nitrogen and stored at -80°C. Before freezing, a part of each sample was set aside to determine dry weight (DW) through desiccation at 80°C for 24 hours.

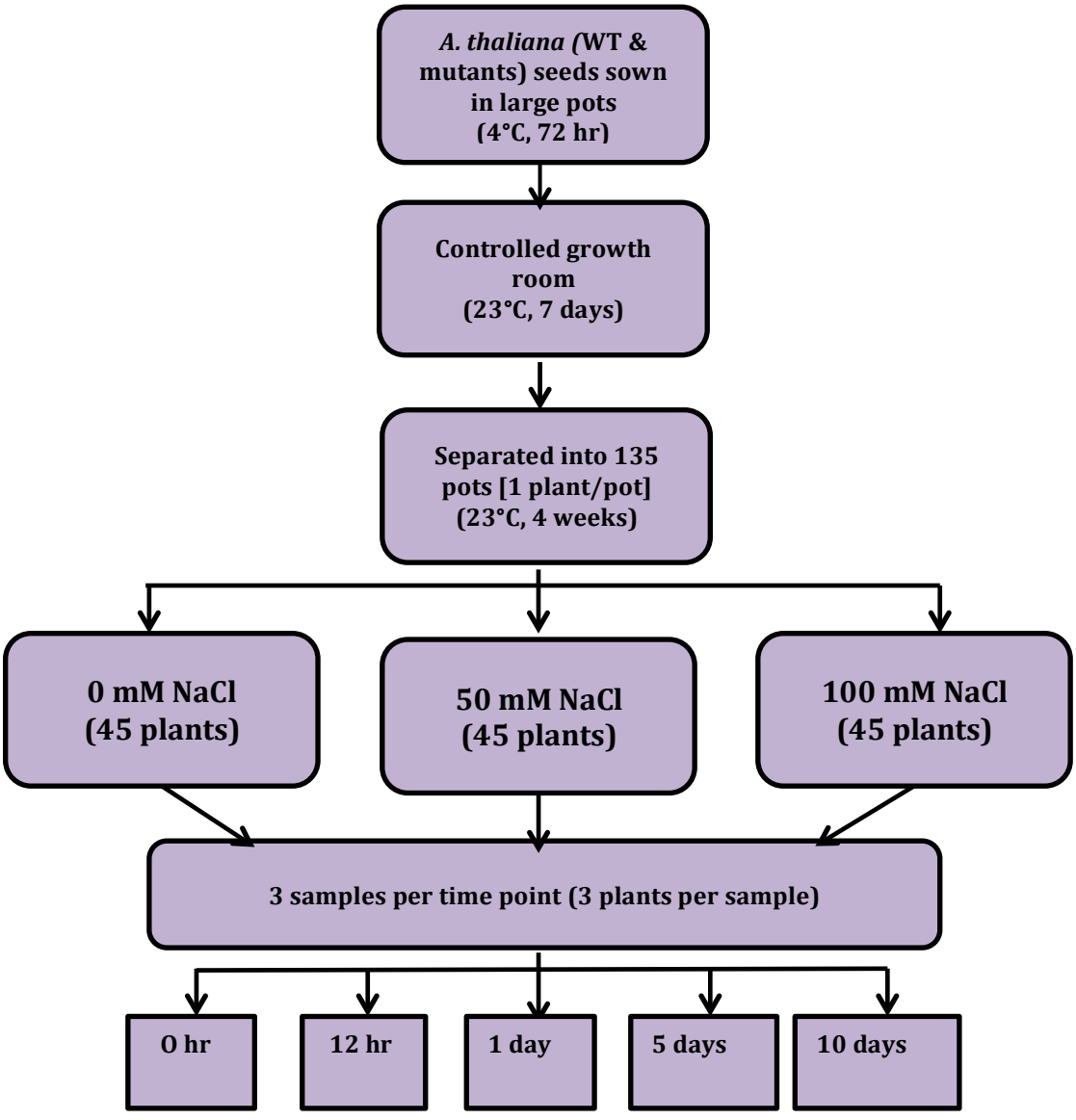


Figure 5.2.1: Schematic representation of the salt treatment experiment and sampling that was performed on *A. thaliana* wild type and the 4 null-mutants.

5.2.3 Analyses of sugars

The levels of sucrose, fructose and glucose in control and salt-treated plants from the wild type and the four null mutants were determined using HPLC. For each replicate, exactly 500 mg of plant tissue was extracted and the content of sucrose, fructose and glucose determined following the procedure described in Chapter 2 (see page 39).

5.2.4 Determination of malate content

Malate content was measured in salt-stressed and unstressed plants from wild type and the four null mutants using the Hohorst method (1970) based on an enzymatic assay. L-malate was estimated using L-malate dehydrogenase (MDH) and NAD. For each replicate, exactly 500 mg of plant tissue was homogenized in 5 ml of 80% methanol followed by incubation at 75°C for 40 minutes and malate measured as described in Chapter 2 (see page 39).

5.2.5 Determination of proline content

Proline levels were measured in 100 mg samples from shoots of control and salt-treated *Arabidopsis thaliana* wild type and the four null mutants. A colorimetric method adapted from that described in Claussen (2005) was used for measuring proline content as described in Chapter 2 (see page 38).

5.2.6 RNA extraction

Total RNA was isolated from the shoots of the unstressed and salt-treated plants of the wild type and the four null mutants using the Tri-reagent method as described by Taybi and Cushman (1999). Approx. 100 mg of ground tissue for each replicate was extracted in the fume hood using 1 ml of Tri-reagent (Helena Bioscience, UK) in 2 ml of RNase/DNase free tubes as described in Chapter 3 (see page 56). Extracted RNA was subjected to DNase I treatment to remove any contaminating DNA co-extracted with RNA and quantified using a Nano-drop spectrophotometer as described in Chapter 3 (see page 62).

5.2.7 Quantitative Reverse Transcriptase Polymerase Chain Reaction

Transcript levels for the *P5CS1* & *SOS1* genes (targets) and Ubiquitin 10 (*UBQ10*) gene (reference) were monitored in the extracted RNA samples using semi-quantitative RT-PCR. A commercial kit based on SYBR Green detection (Agilent Technologies, UK) was used according to the manufacturer's instructions and as described in Chapter 3 (see page 63).

5.3 Results

5.3.1 Growth analysis

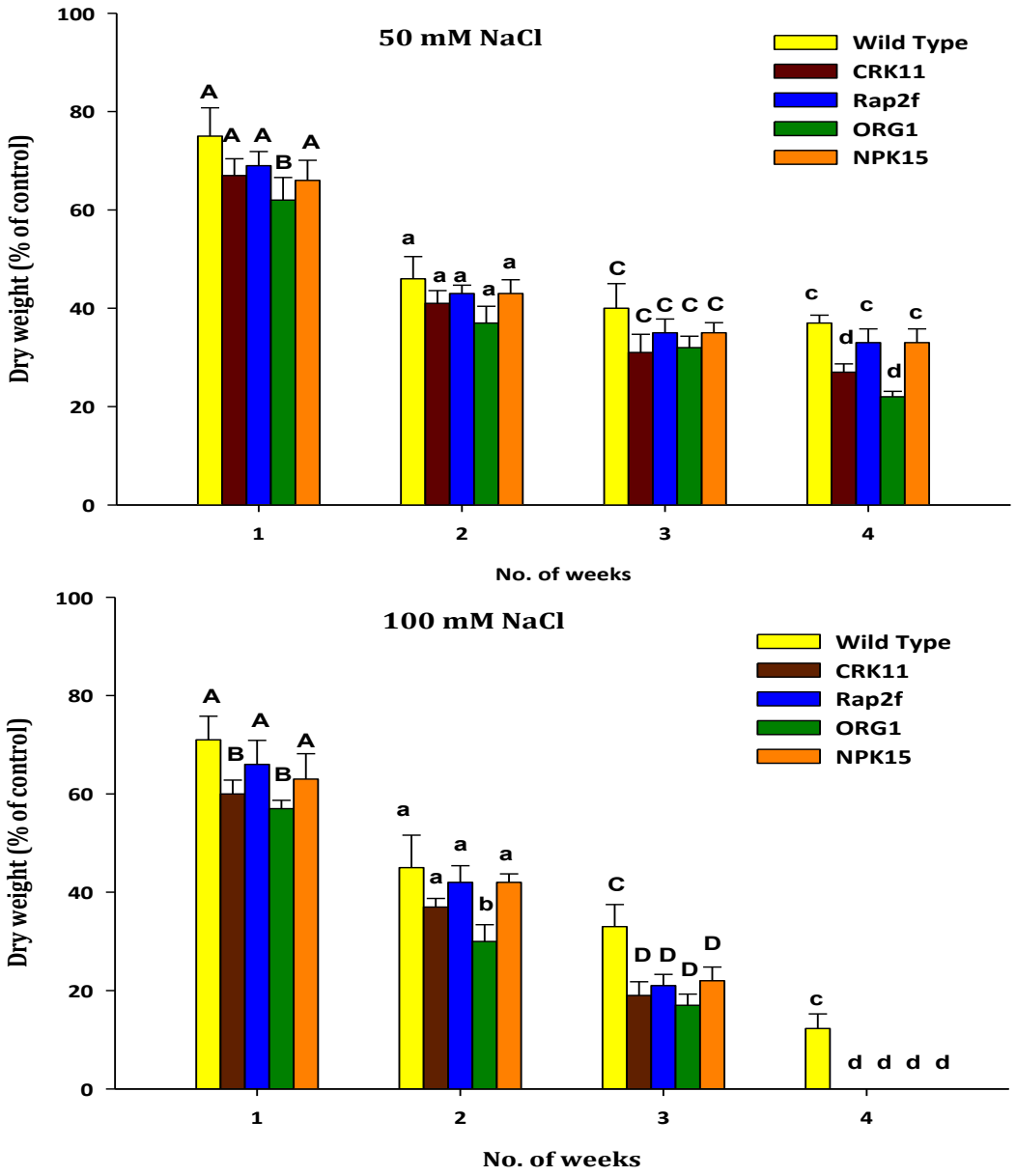


Figure 5.3.1: Growth biomass (DW) analysis of 4 *Arabidopsis thaliana* null mutants *CRK11*, *Rap2.4f*, *ORG1* and *NPK15* genes along with wild type, expressed as percentage of their respective unstressed control. *ORG1* (PKA50) and *CRK11* mutants are most affected under salt treatment among the four mutants. Each point is the mean of 3 samples (3 plants per sample) and standard errors were calculated from the three replicates. Bars at each time point with different letters are statistically significantly different ($p < 0.05$).

The growth responses exhibited by *A. thaliana* null mutants of targeted signaling components and a transcription factor were assessed under the effect of salt treatment and compared to wild type. Under unstressed conditions, the mutant plants' growth was

similar to the wild type plants expect for the *ORG1* mutant, which initially grew slowly but after two weeks there was no difference to the wild type plants (not shown). As observed in Figure 5.3.1, the biomass analysis showed that two mutants, *CRK11* and *ORG1*, were the most affected by the salt treatments. At 50 mM [NaCl], mutants *CRK11* (p value, 0.027) and *ORG1* (p value, 0.001) showed significant differences in growth after 4 weeks of salt treatment from the wild type. The effect of the salt dose was clearly noticeable as there were stronger reductions of biomass at 100 mM [NaCl] than at 50 mM. The difference in biomass between the mutants and the wild type was greatest after 3 weeks at 100 mM [NaCl], when the biomass of all the mutants was significantly lower than the wild-type. Moreover, none of the mutants survived the fourth week of salt-treatment at 100 mM [NaCl], whereas the wild type survived till the fifth week (data not shown). AP2 domain transcription factor (*Rap2f*, JYB578.1, At4g28140) and Ser/Thr specific protein kinase *NPK15* (SALK_024337, At5g58540) mutants exhibited the lowest growth reduction among the *A. thaliana* mutants under salt treatment.

5.3.2 Amount of sugars

Sugar contents were compared between the four mutants of *A. thaliana* and the wild type under treatment with 100 mM [NaCl] and expressed with reference to control plants watered with tap water only. As shown by the results (Figs. 5.3.2, 5.3.4 and 5.3.6) sugar metabolism was differentially affected in all the mutants and levels for sucrose, fructose and glucose changed differently to the wild type under the effect of salt-treatment.

As shown in Figure 5.3.3 sucrose levels increased soon after salt-treatment and reached higher levels in the wild type than in the mutants. Figure 5.3.2 shows the difference in accumulation kinetics of sucrose in the four mutants relative to the wild type. Relative sucrose levels doubled after 12 hours of salt-treatment and reached a maximum of seven times the control level in the wild type.. In the four mutants increases in relative sucrose levels started later to reach about twice the control level after 24 hours of salt-treatment and 2 to 4.5 times the controls after 5 days of salt-treatment. Between 5 and 10 days of salt-treatment, absolute and relative levels of sucrose increased slightly in the *CRK11* mutant but dropped in the *ORG1* mutant. However, both absolute and relative levels of sucrose continued to increase beyond 5 days of salt-treatment in the *Rap2.4f* and *NPK15* mutants.

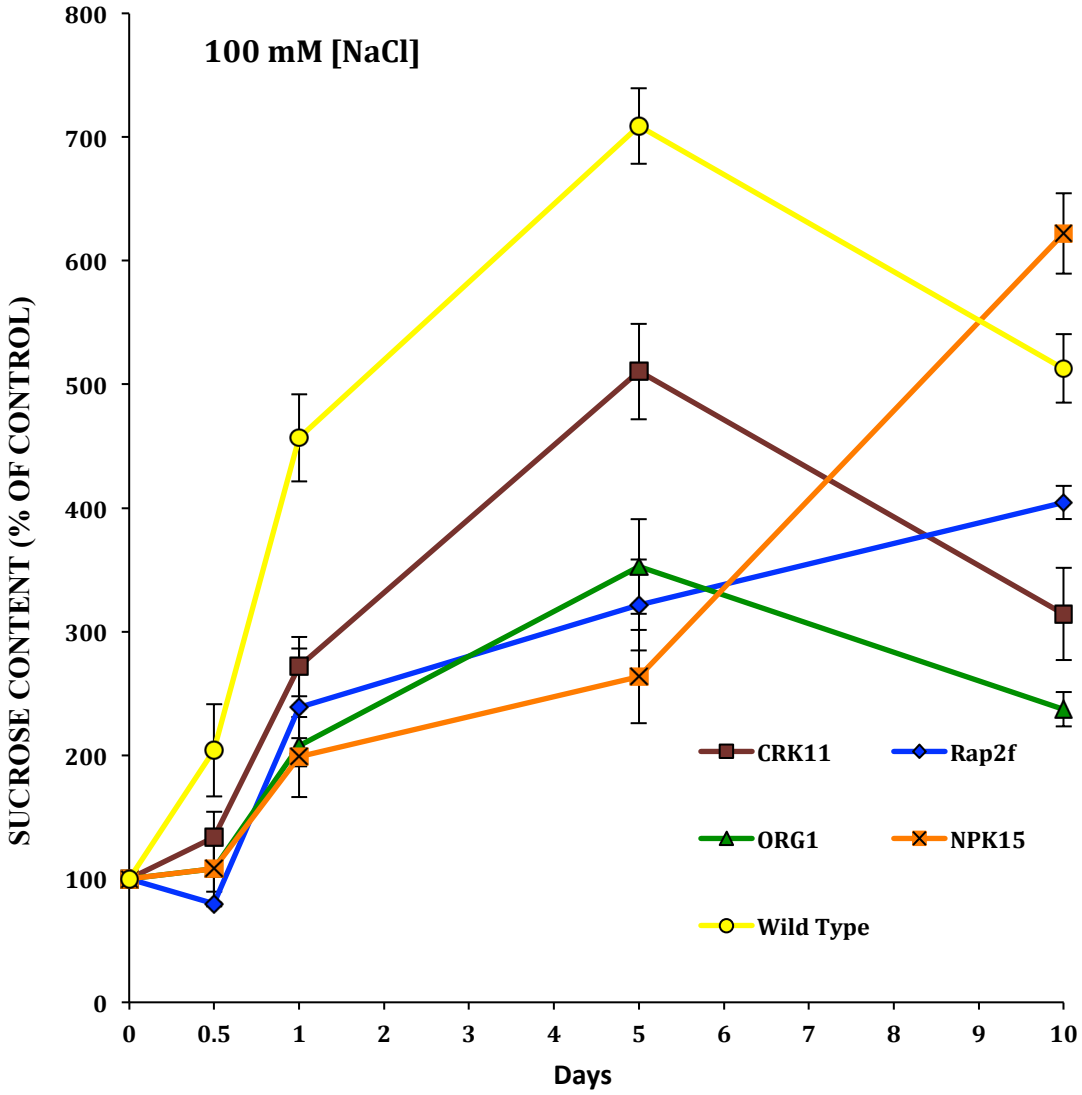


Figure 5.3.2: Sucrose relative levels in null mutants for *CRK11, Rap2.4f, ORG1* and *NPK15* genes and wild type *A. thaliana* plants subjected to salt-treatment (100 mM [NaCl]) over 10 days. Sucrose levels were expressed as percentage of the respective unstressed (0 mM [NaCl]) controls. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

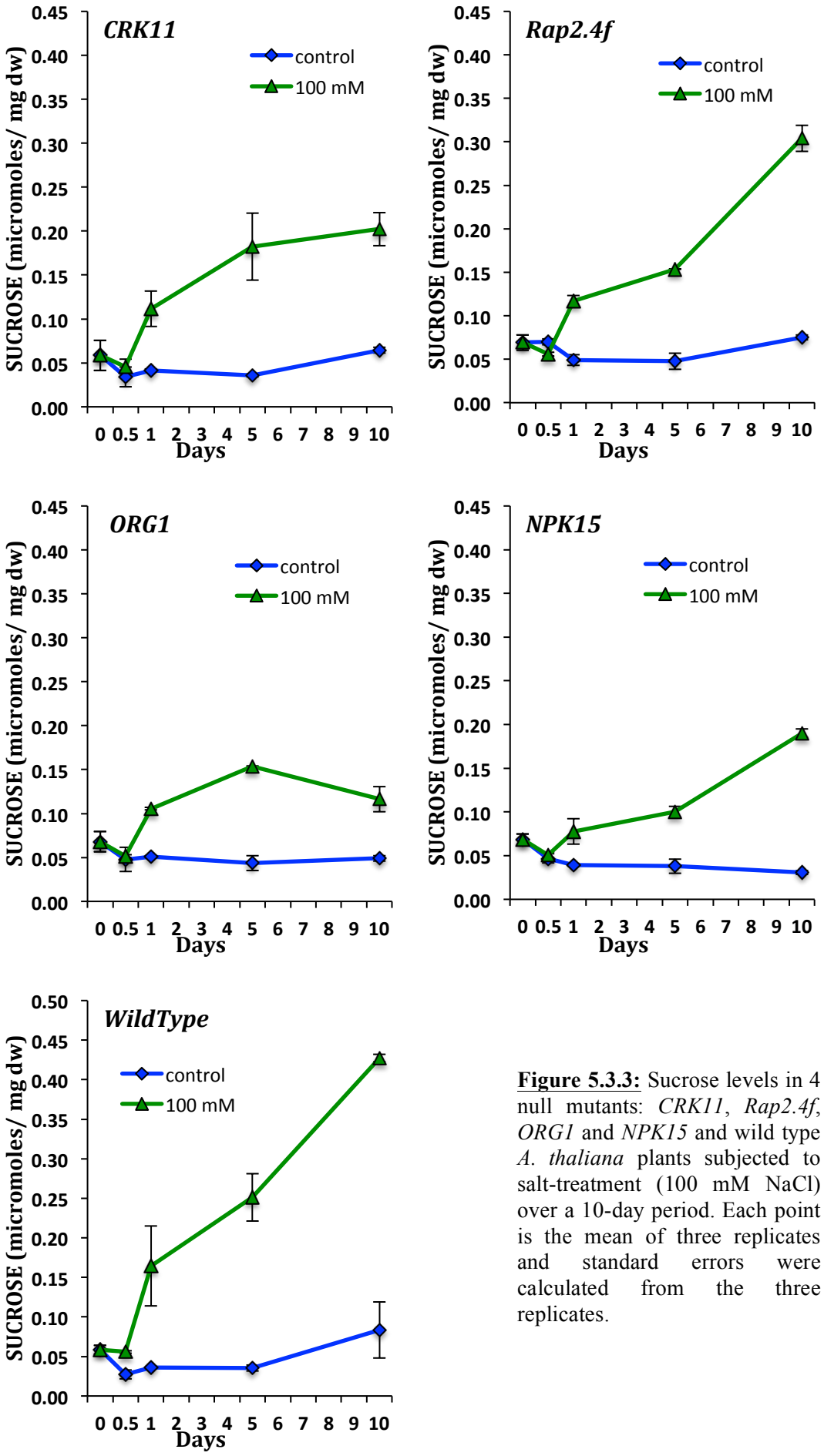


Figure 5.3.3: Sucrose levels in 4 null mutants: *CRK11*, *Rap2.4f*, *ORG1* and *NPK15* and wild type *A. thaliana* plants subjected to salt-treatment (100 mM NaCl) over a 10-day period. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

In the case of fructose, Figure 5.3.5 shows the overall levels of fructose in the mutants were lower than those of the wild type in both unstressed and stressed plants. The wild type *A. thaliana* exhibited a slight drop in the first 12 hours of the salt treatment and a sharp increase after that. As shown in Figure 5.3.4 the level of fructose reached a maximum of 270% of the unstressed wild type after 5 days of salt-treatment. In mutants for the first 12 hours the increase in relative fructose levels was faster except in *CRK11*, in which the relative changes in fructose levels followed a similar pattern to that of the unstressed control. Fructose levels in wild type and mutants showed differences in accumulation kinetics: they increased earlier in mutants than the wild type but the increase failed to reach the high amplitude as seen in the wild type.

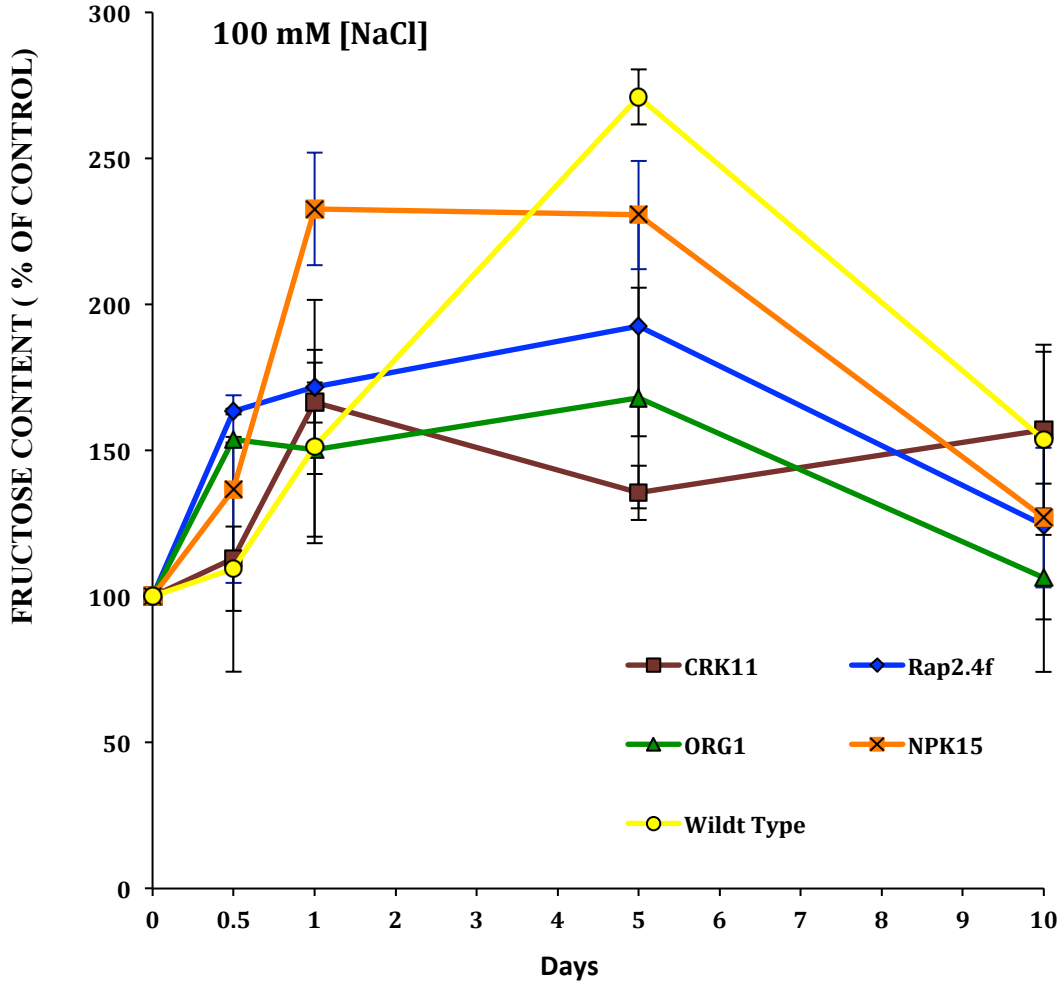


Figure 5.3.4: Fructose levels in null mutants for *CRK11, Rap2.4f, ORG1* and *NPK15* genes and wild type *A. thaliana*, plants subjected to salt-treatment (100 mM [NaCl]) over 10 days. Fructose levels are expressed as percentage of the respective unstressed (0 mM [NaCl]) control. Standard errors were calculated from three replicates for each time point. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

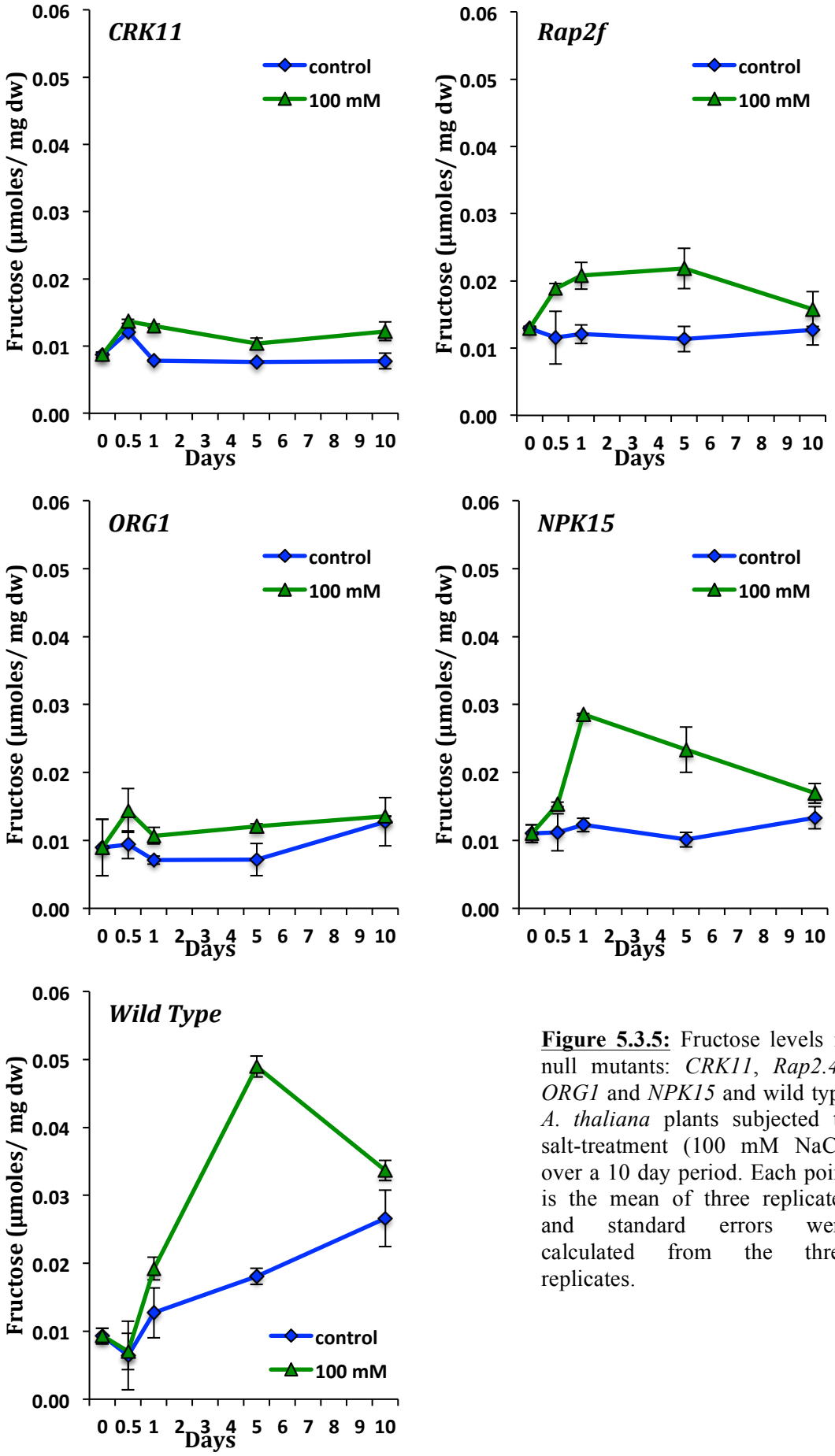


Figure 5.3.5: Fructose levels in null mutants: *CRK11*, *Rap2.4f*, *ORG1* and *NPK15* and wild type *A. thaliana* plants subjected to salt-treatment (100 mM NaCl) over a 10 day period. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

As shown in Figure 5.3.7, glucose levels were very low in all the four mutants in both stressed and unstressed plants when compared to the wild type throughout the 10 days of the treatment. Change in glucose relative levels in wild type *A. thaliana* plants subjected to salt-treatment followed similar patterns to fructose in terms of kinetics and amplitude (Figure 5.3.6). Changes in the mutants were small with the exception of the *ORG1* mutant. Levels of glucose in the *ORG1* mutant increased very rapidly from 12 hours to 24 hours of salt-treatment and reached 385% of the unstressed control value before decreasing and stabilizing at around 150% of the control value.

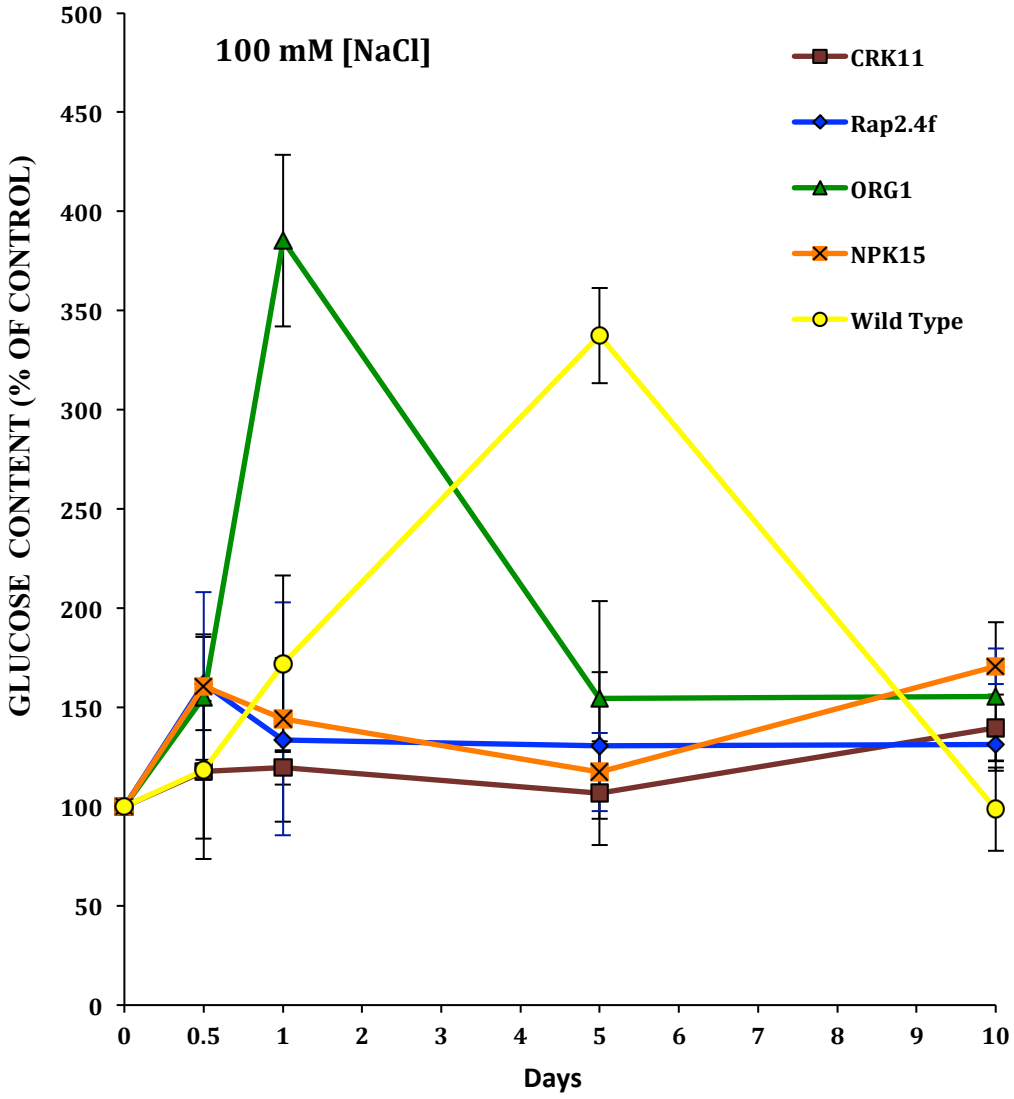


Figure 5.3.6: Glucose levels in null mutants for *CRK11, Rap2.4f, ORG1* and *NPK15* genes and wild type *A. thaliana* plants subjected to salt-treatment (100 mM NaCl) over 10 days. Glucose levels were expressed as percentage of the respective unstressed (0 mM NaCl) controls. Standard errors were calculated from three replicates for each time point. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

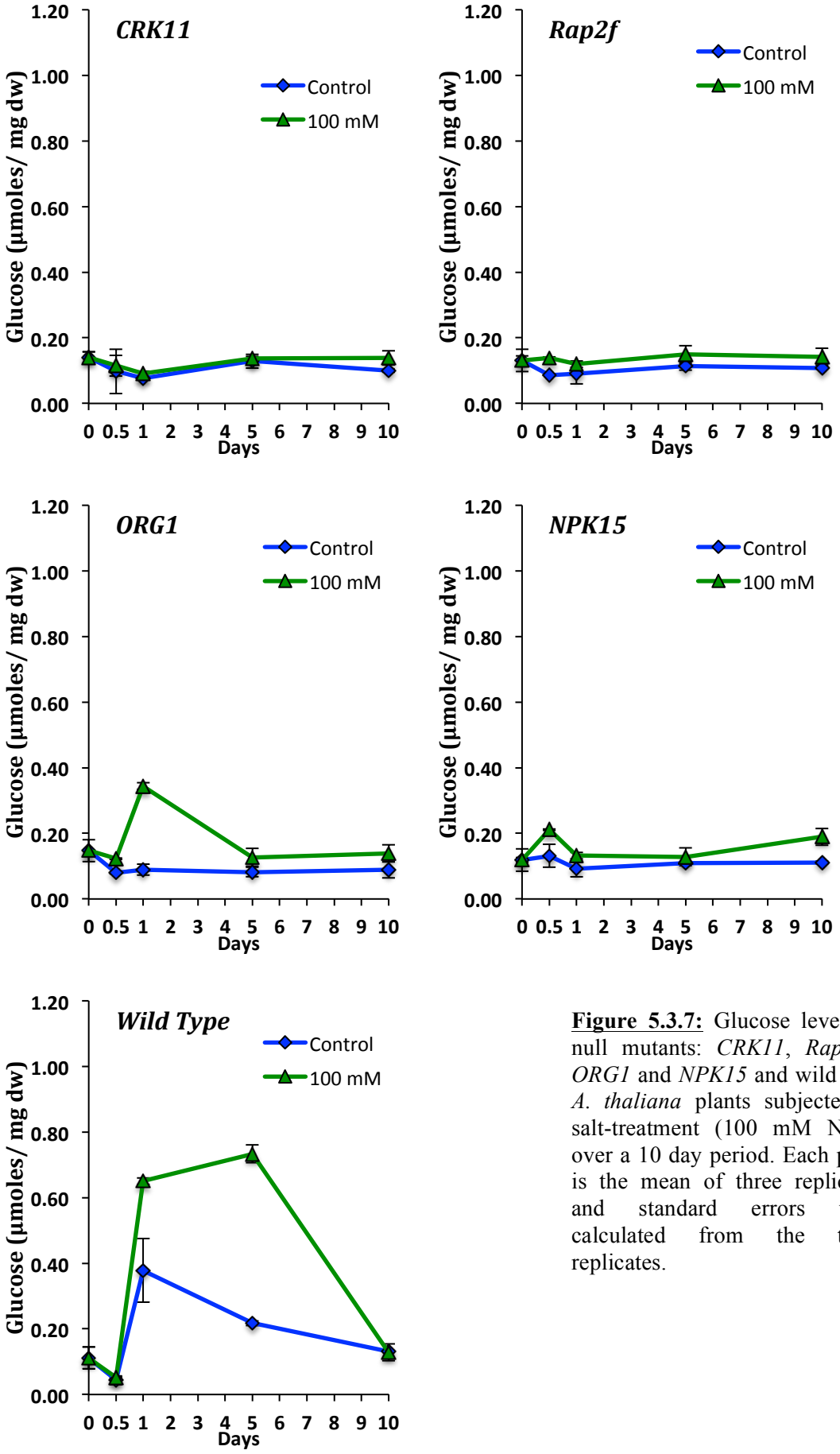


Figure 5.3.7: Glucose levels in null mutants: *CRK11*, *Rap2.4f*, *ORG1* and *NPK15* and wild type *A. thaliana* plants subjected to salt-treatment (100 mM NaCl) over a 10 day period. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

5.3.3 Malic Acid content

As shown in Figure 5.3.8 differential patterns of change in malate relative levels over a 10-day course of salt treatment were apparent between the mutants themselves and between the mutants and the wild type. Overall, all mutants except *NPK15,* showed a rapid increase in malate relative levels within the first 12 hours of salt-treatment. This increase was rapid, strong and transient in the *Rap2.4f* and *ORG1* mutants and peaked at 12 hours of salt-treatment at 100 mM. Figure 5.3.9 shows wild type *A. thaliana* plants showed a slight decrease in malate levels after 12 hours of salt-treatment, which was followed by a sustained increase Levels of malate in the *CRK11* mutant increased during the first 5 days of salt-treatment and started to decline after that, whereas the other three mutants showed a steady increase of malate levels in both stressed and unstressed plants but to lower than the wild type after 10 days of the salt treatment.

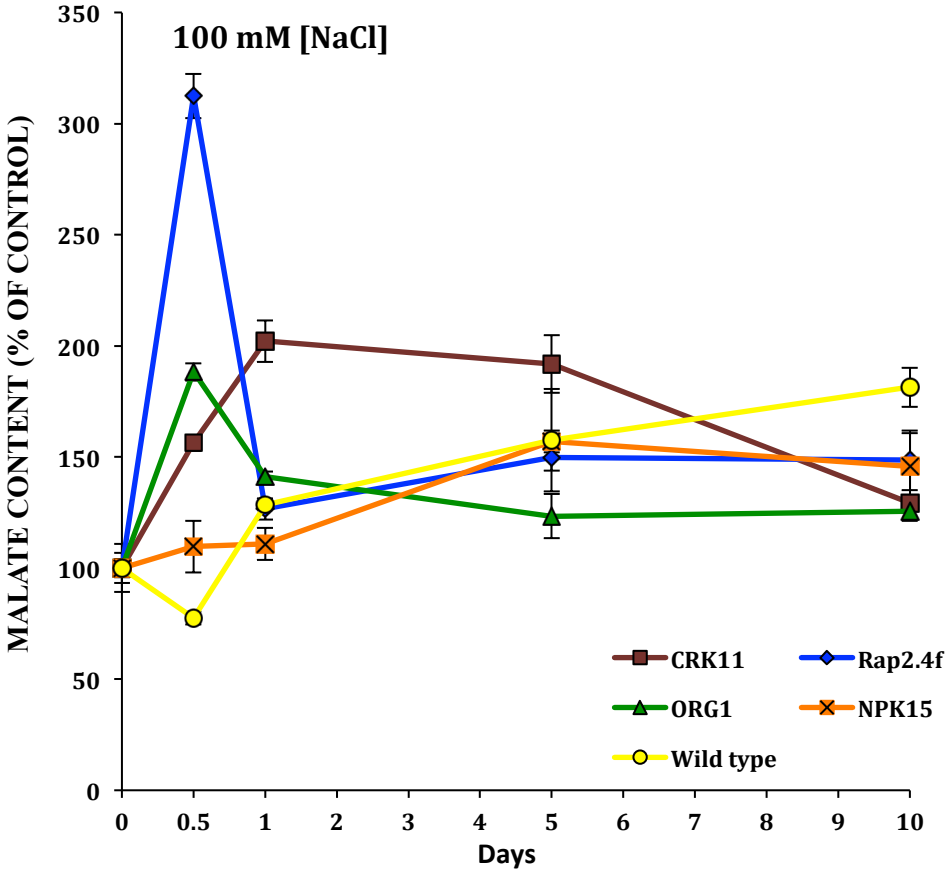


Figure 5.3.8: Malate levels in null mutants: *CRK11*, *Rap2.4f, ORG1* and *NPK15* and wild type *A. thaliana* plants under salt-treatment (100 mM [NaCl]) and expressed as percentage of the respective unstressed (0 mM [NaCl]) controls. Standard errors were calculated from three replicates for each time point. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

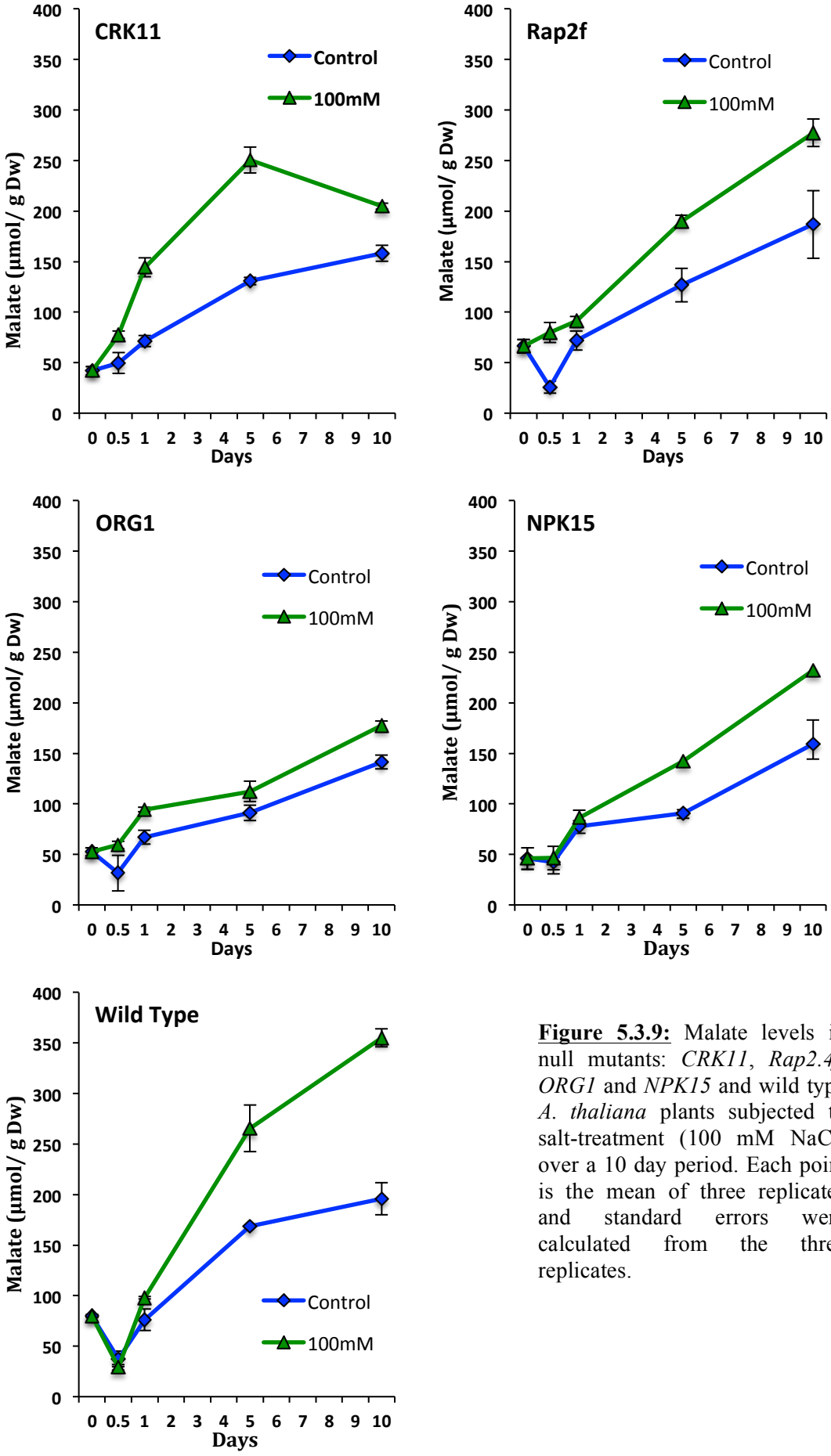


Figure 5.3.9: Malate levels in null mutants: *CRK11*, *Rap2.4f, ORG1* and *NPK15* and wild type *A. thaliana* plants subjected to salt-treatment (100 mM NaCl) over a 10 day period. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

5.3.4 Proline content

Levels of proline were measured in shoots of wild type and mutant plants under salt-treatment (100 mM [NaCl]) and in unstressed control plants. As shown in Figure 5.3.10, increases in levels of proline were differentially induced by salt-treatment in the *A. thaliana* mutants and wild type. Generally levels of proline in mutants remained much lower than those in wild type over the course of salt-treatment. At the end of the experiment, the levels of proline in unstressed wild type *A. thaliana* was around four times the level in unstressed *A. thaliana* mutants, suggesting that the mutations might have affected metabolism at some level that has resulted in lower proline accumulation even under unstressed conditions. Proline should increase with age of the plant as observed in the wild type. When proline accumulation in plants subjected to salt-treatment is presented as percentage of that in unstressed plants (control) for each of the mutants and the wild type *A. thaliana* a clearer picture of the response kinetics is revealed (Figure 5.3.11). Wild type *A. thaliana* had a stronger response in the first 24 hours of salt-treatment in comparison to the four mutants, which started to accumulate proline after the 1st day. *CRK11* and *NPK15* mutants showed a transient increase in relative proline content with highest points of relative accumulation on the 5th day of salt-treatment. In contrast, the other two mutants, *Rap2.4f* and *ORG1,* showed a continuous increase in relative proline content from the 1st until the 10th day of salt-treatment.

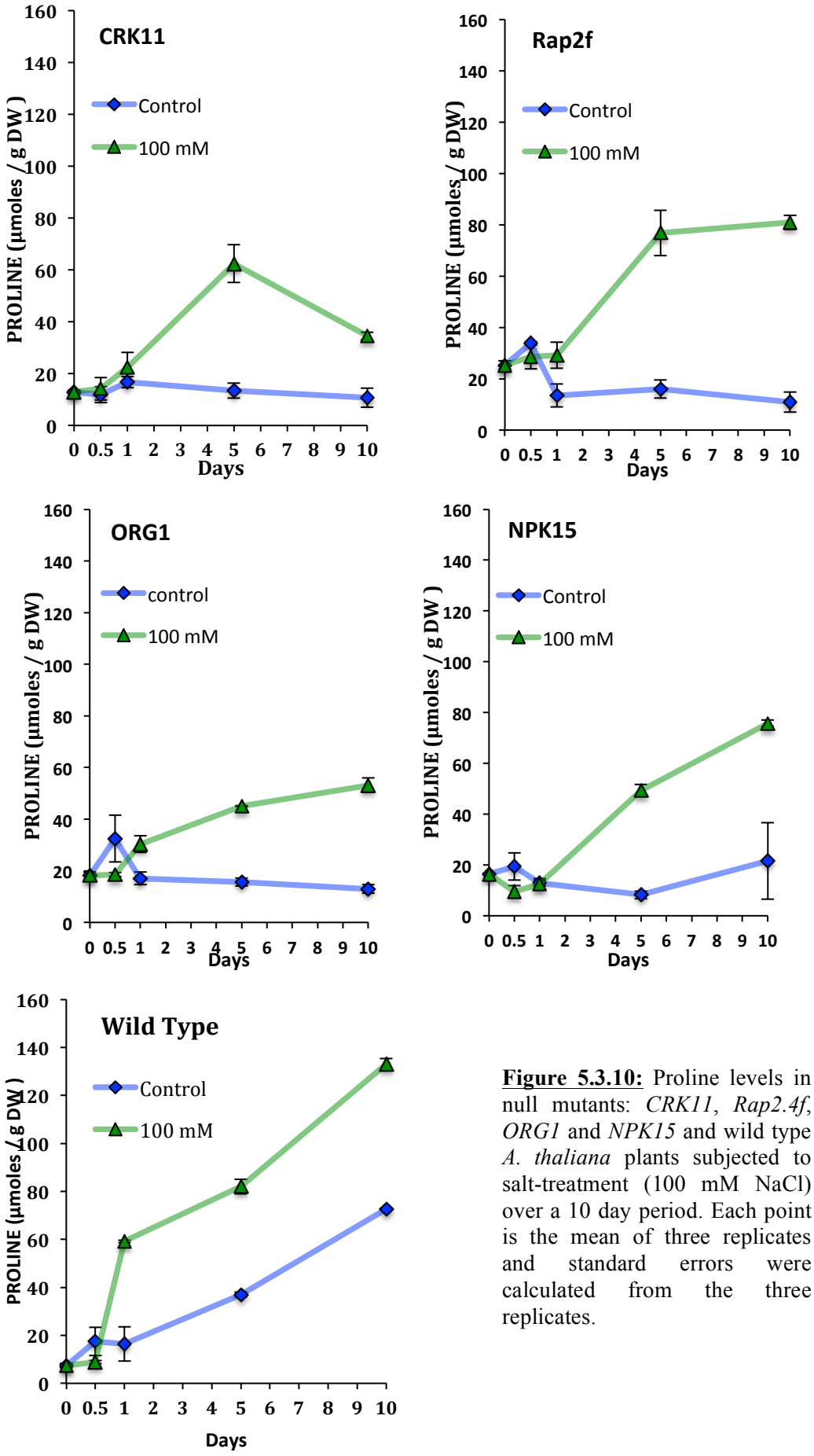


Figure 5.3.10: Proline levels in null mutants: *CRK11*, *Rap2.4f*, *ORG1* and *NPK15* and wild type *A. thaliana* plants subjected to salt-treatment (100 mM NaCl) over a 10 day period. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

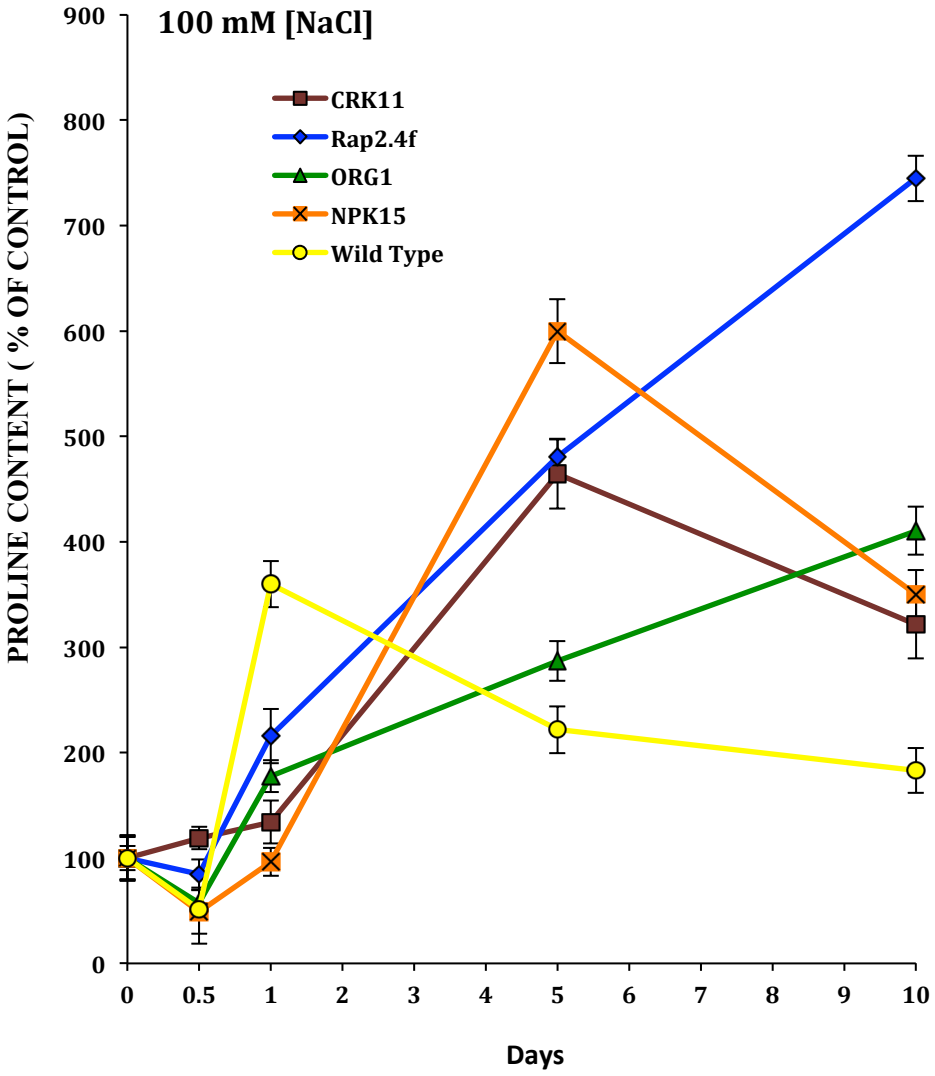


Figure 5.3.11: Proline levels in null mutants for *CRK11, Rap2.4f, ORG1* and *NPK15* genes and wild type *A. thaliana* plants subjected to salt-treatment (100 mM [NaCl]), expressed as percentage of their respective unstressed (0 mM [NaCl]) controls. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

5.3.5 Differences in transcript levels of *P5CS1* and *SOS1* genes in *A. thaliana* mutants

The transcript levels for the *P5CS1* and *SOS1* genes were monitored over the period of 10 days at 100 mM [NaCl] treatment in shoots of the wild type and the four mutants. As shown in Figure 5.3.12 in all the null mutants, the transcript profiles of *P5CS1* and *SOS1* genes did not show any clearly significant differences under salt-treatment when compared to the wild type.

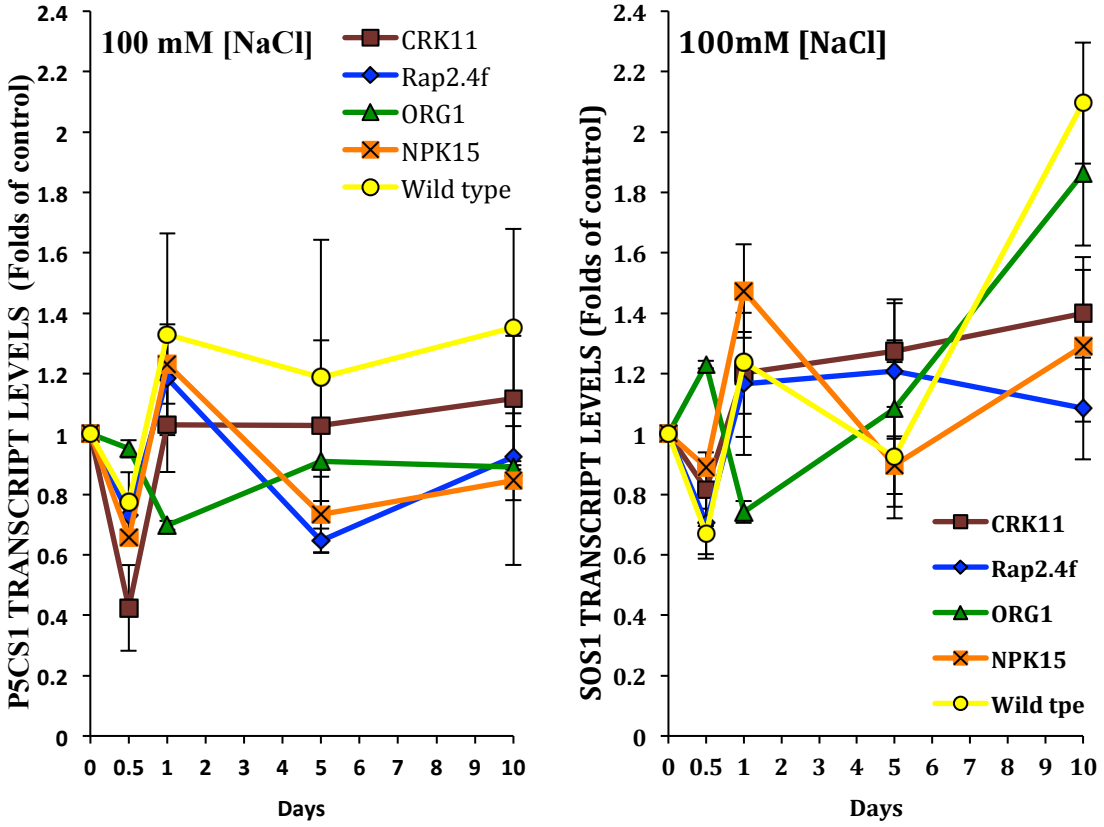


Figure 5.3.12: Change in transcript levels for *P5CS1* and *SOS1* genes in null mutants for *CRK11, Rap2.4f, ORG1* and *NPK15* genes and wild type *A. thaliana* plants subjected to salt-treatment, at 100 mM [NaCl] over a 10-day course. Transcript levels are expressed as fold increases relative to controls (i.e. non-salted plants). *P5CS1* and *SOS1* genes are responsible for encoding enzymes responsible for biosynthesis of proline and Na^+/H^+ antiporter respectively. Each point is the mean of three replicates and standard errors were calculated from the three replicates.

5.4 Discussion

In the previous chapter, the regulation of the *P5CS1* gene in *T. halophila* was examined by analyzing the promoter region and also the intron splicing to uncover possible differences between *A. thaliana* and *T. halophila* in terms of transcriptional and post-transcriptional regulation of the gene. To further understand the regulatory modulation of salt tolerance the work described in this chapter has focused on finding upstream gene regulatory components in *A. thaliana*, with possible involvement in the regulation of key stress responses like the mobilization of carbohydrates, production of osmoregulators including proline and malate as well as Na^+ compartmentalization. Knockout mutants for *CRK11*, *ORG1* and *NPK15*, which code for protein kinases, and *Rap2.4f*, which code for a transcription factor, have been used in this investigation because these genes are up-regulated under salt stress in *A. thaliana* and *T. halophila* (Taji et al., 2004). However, none of these mutants were previously studied to find their link to the regulation of a specific salt tolerance mechanism.

Biomass/growth analysis of the four mutants at 50 mM and 100 mM [NaCl] for four weeks suggested the possible role of the affected genes as regulatory components under salt stress. Growth was significantly reduced in all the four mutants compared to wild type *A. thaliana* under salt-treatment. None of the null-mutants survived after four weeks of salt-treatment at 100 mM [NaCl]. However, wild type plants exhibited 12% growth compared to the unstressed control at four weeks of salt-treatment at 100 mM [NaCl]. The difference in growth between the *ORG1* and *CRK11* mutants and the wild type was apparent even after one week of salt-treatment at 100 mM [NaCl]. The four mutants showed differential impact of salt on their growth with *ORG1* and *CRK11* being the most affected. There was 13% and 10% less growth in *ORG1* & *CRK11*, respectively in comparison to the wild type after one week of salt-treatment at 100 mM [NaCl]. These two mutants continued to be the most affected among the four mutants, with lower biomass accumulated throughout the four weeks of salt-treatment.

SOS1 and *P5CS1* transcript levels were checked within 10 days of 100 mM [NaCl] treatment, and revealed minimal to no difference in transcript accumulation kinetics and amplitude between the wild type and the four mutants. This suggests the mutated genes have no direct control over the accumulation of *SOS1* and *P5CS1* transcripts. However, although production of proline was not totally blocked by the mutations there was clearly a parallel between level of proline accumulated and growth under salt-treatment. The concentration of proline in the *ORG1* and *CRK11* mutants,

which grew the least under salt-treatment, was the lowest. This implies that the two mutations might have affected other genes that play a direct or indirect role in the accumulation of proline.

There were additional differences between the mutants and the wild type *A. thaliana*. Salt-treated mutants followed similar kinetics of sucrose accumulation compared to the wild type but accumulated lesser amounts of sucrose. The amount of sucrose in all the plants is dependent on three factors: (1) amount produced from the hydrolysis of starch, (2) the amount left after metabolism of sucrose in the shoot and (3) the amount of sucrose that is exported to the roots. Wild type plants contained lower amounts of fructose in the early stages of the treatment, which may partly account for the higher amounts of sucrose during the same period (i.e. reduced breakdown of sucrose). On the other hand, mutants appeared to break down sucrose more readily than WT in the early stages of salinity, when levels of fructose and glucose increased. After levels of fructose in the wild type increased above those of the mutants after 24 hours of salt-treatment, relative fructose content then followed similar kinetics to that of the wild type. In contrast, glucose accumulation was severely affected after the first 12 hours of salt-treatment in the mutants. These sugar accumulation characteristics suggest the possibility of the mutants experiencing carbon starvation. This might be due to mutations affecting the amount of starch available and/or the capability of the plant to convert starch into soluble sugars and other metabolites. Another possibility is that the mutants partitioned carbon towards another pathway that has no direct relevance for salt tolerance. The mutants showed broadly similar malate accumulation under salt-treatment compared to the wild type, which might suggest that the mutations did not affect the malate production greatly. The *CRK11* and *ORG1* mutants were most affected by salt and in their sugar and malate contents, respectively. They seem to be the most limited in terms of mobilization of carbohydrates to fuel appropriate responses to salt stress. Accumulation of proline was strongly affected in the four mutants, with a suggestion towards a correlation between level of proline and growth reduction. Proline is a major compatible solute and as explained earlier plays a critical role in defence against salt stress. Possibly carbon starvation might have impacted proline accumulation hence limiting plant tolerance to salt-stress. Although the mutated genes are different and might be involved in different signaling and regulatory events the impact of the mutations suggests that the overall metabolic background of the plant is crucial for salt-tolerance. The deployment of the salt-resistance mechanisms would not be effective if the plant does not have the appropriate supply of carbohydrates to fuel the responses to

salt. It is not possible to pinpoint the exact role of the targeted genes, but their role in salt-tolerance is apparent from the results.

5.5 Conclusion

Various genes have been associated with abiotic stress-responses and their stress-induced expression including under salt stress has been studied using different molecular and genetic tools. Among all the stress-inducible genes there are many which are specific to salt stress and among those are genes which are involved in regulation or signaling networks of salt responses. These genes may encode various products like enzymes for phospholipid metabolism, different protein kinases and many transcription factors. Sometimes more focused research has been conducted on various transcription factors which bind to the same cis-regulatory elements to drive up or down regulation of salt-stress inducible genes. But transcription factors and signaling components are usually part of complex regulatory networks with the possibility of overlapping functions. Identifying a specific signaling pathway to enhance stress responses is an immensely challenging task. This chapter studied four of these regulatory components in *A. thaliana* to look for specific and direct roles of these components in responses to salt-stress. The work has demonstrated that signaling and transcription factors involved in general metabolism might directly impact responses to salinity and hence must be considered for better understanding salt-tolerance.

Chapter 6

General Discussion

6.1 Introduction

This research compared two closely related but different plant species, *A. thaliana,* a glycophyte, and *T. halophila*, a halophyte, with the aim of unraveling the mechanisms underpinning their differences in salt tolerance. To accomplish this, analysis was performed over a period of time where both plant species were subjected to two different salt concentrations (50 mM and 100 mM for *A. thaliana*, and 100 mM and 500 mM [NaCl] for *T. halophila*). This has allowed an insight into the characteristics of the response to salinity in terms of the amplitude and the kinetics of different physiological parameters of the response in the two contrasting plant species. *T. halophila* exhibited enhanced responses in regards to salt partitioning, metabolite accumulation and potentially osmoregulation (e.g. soluble sugars and proline). This was backed by up-regulation of related key genes (e.g. *SOS1* and *P5CS1*). Analysis of transcript accumulation for the *P5CS1* gene together with the isolation of two *P5CS1* isogenes in *T. halophila* and detailed analysis of the promoter regions (cis regulation) together with differential splicing profiles revealed important mechanistic differences between the two species. Selected trans-regulatory components (transcription factors and signaling elements) were indirectly associated with salt-responses via the screening of changes in growth, metabolite levels and transcript levels in *A. thaliana* null mutants under salt treatment. Through all these approaches, the research was able to answer the following questions:

6.2 Is there a difference of timing or amplitude or both in responses to salt-treatment observed in *A. thaliana* and *T. halophila*?

T. halophila was able to deploy responses to salt treatment earlier and to higher levels than *A. thaliana*. Substantial differences were observed in the selectivity for sodium and potassium, and accumulation of sugars, amino acids and organic acids. Unlike *A. thaliana*, *T. halophila* was able to selectively accumulate high amounts of potassium throughout the period of salt treatment. This has helped *T. halophila* to maintain nutrient abundance that fulfilled the needs of cellular metabolism under salinity. *T. halophila* accumulated very high amounts of sucrose, fructose, inositol and proline during the first 24 hours of salt treatment. On the other hand, *A. thaliana* did the same at a slower rate (over 3-5 days) and to a lower extent. The metabolic status of a plant

reflects its biological activities, which change depending on growth, development and environmental conditions. This change in metabolic activities can lead from normal growth under favourable conditions to survival under stress conditions. With regard to this *T. halophila* has shown a larger metabolic plasticity that has allowed the plant to counter the effects of salinity very quickly and adapt its physiology to the changed salt conditions. This gives it the possibility to deploy an even higher response if the saline conditions prevailed for longer durations. With higher levels of metabolites (especially malate) under unstressed conditions *T. halophila* shows its anticipation of stress at all times. Reduction in malate levels through the stress period suggested that *T. halophila* was using carbon from malate possibly for proline production as proline plays a major role as an osmoregulator and also as osmoprotectant and anti-oxidant during salt stress. This observation indicated a better modulation of metabolites and carbon partitioning by *T. halophila* as carbon can be more quickly mobilized from metabolism of malate than starch under stress conditions. Hence, *T. halophila* as a stress anticipatory plant can respond to salinity very quickly and to a higher level than *A. thaliana*. This adapted response shown by *T. halophila* is controlled at the gene level, as shown by the rapidity and amplitude of changes of transcript levels for *SOS1* and *P5CS1* genes as well as genes involved in sugar metabolism.

6.3 Is there an evolutionary change at the gene level for enhanced salt-tolerant trait in *T. halophila*?

There is a wealth of evidence indicating that changes in gene expression occur in plants following an exposure to salinity, consistent with the hypothesis that plants which can naturally tolerate severe saline conditions have either divergent promoter regions or more active forms of gene products. The *P5CS1* gene showed substantial differences in its transcript levels between the two species, therefore the regulation of its expression was investigated at the transcriptional and post-transcriptional levels. It was found that evolutionary changes occurred in the promoter sequence of the gene, which has at least two distinct copies in the genome of *T. halophila.* The two isogenes had not only very distinct promoter regions but also introns spanning the coding sequence were different in terms of their presence and sequence from the only *P5CS1* gene from *A. thaliana*. The *T. halophila* genes not only showed evidence of an increased frequency of REs in their promoter regions, but also showed alternative splicing which could produce more

active forms of protein products. In addition the differences found in the 3' UTR region between *T. halophila* and *A. thaliana* genes could be responsible for increased stability of RNA transcripts in *T. halophila*. The presence of more than one copy of *P5CS1* gene is a very interesting finding, as it has not been observed previously in other plants. These evolutionary changes in the gene in *T. halophila* might be related to salt stress specific responses exhibiting an enhanced pattern of *P5CS1* expression under stressed conditions. If this were true then this would indicate that the evolutionary changes that brought about halophytism in this plant species have involved gene duplication events linked to changes in the regulatory sequences of the gene including promoter regions and intron presence and splicing sites.

Evolution might have affected events upstream of the *P5CS1* gene including at signaling and transcription factor levels. It will be interesting to examine the expression of all the copies of the *P5CS1* gene from *T. halophila* under the control of their native promoter and terminator sequences in an *A. thaliana P5CS1* null mutant. This will allow a better understanding of the evolution of the regulation of *P5CS1* gene because if *A. thaliana* lacks the transcriptional and signaling machinery to regulate the *ThP5CS1* genes, it will not show similar expression profiles as observed in *T. halophila*. But, if the expression profiles are similar, then the difference in the regulation of *P5CS1* gene solely depends on the promoter sequences and splicing. This will help to probe for additional components required for gene regulation under salt-stress in *T. halophila*.

6.4 Is metabolic background important for salt tolerance?

A. thaliana null mutants of protein kinases and transcription factors showed the importance of the metabolic background of the plant for adequate response to salt stress. All the *A. thaliana* null mutants were affected in accumulation of soluble sugars under unstressed conditions as well as under treatment with 100 mM [NaCl]. There were no differences in *P5CS1* transcript levels from the wild type, yet proline level was considerably lower in the mutants than the wild type. This implies that the salt response goes beyond the regulation of basic stress mechanisms and is directly connected to the capability of the plant to successfully regulate metabolism to respond to stress conditions. *A. thaliana* is suspected to not regulate its carbohydrate metabolism efficiently to support rapid deployment of resistance mechanisms against the stress imposed by salinity. Partitioning of carbohydrates under stress conditions in this plant

species might favour growth even in the sink tissues over stress-resistance mechanisms. On the other hand, *T. halophila* seems to regulate and redirect carbohydrate metabolism towards building up resistance mechanisms a lot faster and to higher levels. In addition *T. halophila* seems to be prepared for stress conditions as it shows higher constitutive expression of stress-resistance mechanisms under unstressed conditions. This has a cost in terms of growth, which might explain the lower growth rate shown by *T. halophila* compared to *A. thaliana*.

6.5 Conclusion and future perspective

Effects of environmental stresses like salinity are not only restricted to osmotic and ion imbalances but they affect plants on a broader level that consists of photosynthesis, general metabolic status, energy storage and redox potential. The results indicate that all these mechanisms needed to be regulated to bring about adequate regulation and functioning of stress specific responses. High metabolic plasticity is required for an organism to tolerate substantial amounts of salt for long period of time. Not only changes in the transcriptional regulation of stress specific genes is required for a higher stress tolerance trait but also a metabolic background (carbon source) that can be modulated quickly to support effective establishment and functioning of mechanisms enhancing salt tolerance. To understand the evolution of salt-tolerance it is paramount to focus on the evolution of signaling and regulation modules that regulate metabolic activity as whole in plants adapted to salt-stress. It is in no doubt necessary to uncover the key regulatory elements that act upstream of those directly involved in salt-responses. We need to identify the key signaling elements and transcription factors that act as master regulators or master stimuli of salt-resistance and elucidate their effector pathways that lead to enhanced stress specific responses in halophytes. The use of mixed techniques including microarrays in genome wide approaches, pyrosequencing and RNAi approaches can greatly help to identify and determine the role of the complexes of these regulatory components. For example, the two putative copies of the *P5CS1* gene found in *T. halophila* can be introduced into *A. thaliana P5CS1* null-mutants. This will probe for their upstream components in a system that is not adapted to salt-stress. If *ThP5CS1* genes are not expressed in *A. thaliana* similarly to in *T. halophila* that would mean that *A. thaliana* does not have the necessary transcriptional and signaling machinery to regulate the *T. halophila P5CS1* genes in a way that would be adequate for salt-resistance. However, the *P5CS1* gene is one of the many genes involved in the salt-resistance trait. Our understanding of salt tolerance mechanisms has

taken a big leap forward from the past decade of research, but there are still many unknowns that limit possibilities of breeding salt tolerant or engineering salt-tolerance in plants. Such plants not only have to be successful at the laboratory level but also they have to be successfully transferred to the field.

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Appendices

(A) *Arabidopsis thaliana P5CS1* gene sequence from the NCBI database. Highlighted in green is 5' flanking sequence, in red is start and stop of the CDS, in aqua blue are the exons and in yellow are the introns and in pink is the 3' flanking sequences.

CTTCCACGGCGTTTCCTCAGCCGCCGATTTTATTTATTTCCCAAAATACCCATCACCTATAGC
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CTGAGTCCGACTCAGTTAACTCGTTCCTCTCTCTGTGTGTGGTTTTGGTAGACGACGACGACG
ATAATGGAGGAGCTAGATCGTTCACGTGCTTTTGCCAGAGACGTCAAACGTATCGTCGTTAA
GGTTCGTTGAGATACGTTCGCATTTTCAGATTTTGTTGTTGATGATTAGATTCTTAATTTGTG
ATAATGTGGAAATGAATATTATGTAATTTAAGTGCATCTAAACTCTTTGTTTATTGAATTCGT
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GCATTGTGACAGAGGATCACGAAGTTGCAGAGCTATTCCTTCGCCAAGTGGATAGGTAAAG
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CTTAACCATAAATTCTCAAAGATTGTGCTACATTTGAAAGCTAACAATGAACACAAGAAAA
GAAC

(B) *Thellungiella halophila P5CS1* gene sequence from the Phytozome database (unpublished). Highlighted in green is 5' flanking sequence, in red is start and stop of the CDS, in aqua blue are the exons and in yellow are the introns and in pink is the 3' flanking sequences.

GACACTTCCCTCACCAGATATTTCCCTAAACGCGCTCACTGACGAAATCCACCACTGAGTTA
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TATTCGTTCATCGTGACAATTGCTTTTACAGGTGGCAGATGGTCTTGTCTTAGAGAAGACCTC
ATCCCCATTAGGCGTACTCCTGATTGTTTTTGAATCCCGACCTGATGCACTTGTACAGGTATG
TTAAGAGTCAATGTCCTTTTATCTTCTTAGAATGTGAATTTGCTGAAACCTGTGTTTTATCCA
CAAACCAAACAGATAGCTTCACTTGCCATCCGGAGTGGAAATGGTCTTCTATTGAAGGGTGG
AAAGGAGGCCCGGCGATCAAATGCTATCTTACATAAGGTACAGTGCCTCAGATTTCAGACTC
GGATGTTATCATATATGGCTTCCTCAAAATATGCTGGTTATAATTGATCCATTTAATTTCATT
TTAAATTTCTAGGTGATCACTGATGCAATTCCAGAGACTGTCGGGGGTAAACTCATTGGACT
TGTGACTTCAAGAGAAGAGATTCCTGATTTGCTCAAGGTAAACGGATTTACAAACTTGGAGC
TGCAACAAATCTTTATATCTTGTGTTTAAATGGAAACCACCATTTGCATTTGCGCTACAGCTT
GATGACGTTATAGATCTTGTGATCCCAAGAGGCAGCAACAAGCTTGTTTCCCAGATAAAAAA
TACTACAAAAATCCCTGTGCTAGGCCATGCTGGTACGGTTTCAAGTTTGTTTTTCCATAAAAT
TCTTTAGTTGGATTGTGTTAGAGAGTGACTGTCTTAATTTTGTACTTCCAGATGGAATCTGTC

ATGTATATGTCGACAAGTCATGTAATCTGGATATGGCAAAGCGCATAATTTCCGATGCAAAG
TTGGATTATCCAGCAGCCTGTAATGCGATGGTAAGAGAACTTGTACCAGCCTCTTGAGATTG
GAGTATGCAATGGGCGTATTAATTTCATCCGACTCATTTCACCTTCTCTTTCCTTTGTATTTTC
AGGAAACTCTTCTTGTGCATAAGGATCTAGAGCAGAACGGGCTCAATGAGCTTATTTTTGTG
CTGCAGAGCAATGGTATGTCATAAATGCCGTGTTTGTTGGTCTCTCGTAATCCTGAAGACTTT
TTTTTTGGTTGGTAAAATTAATTCTGAAGACTTGTTTGGAGTAATTTAACTCATGAAGTATTT
TTAACTGCAGGAGTCACTGTATATGGTGGACCAAGAGCAAGTGCAATACTGAACATACCAG
AAGCACGGTCGTTCAACTATGAGTACTGTTCCAAGGCTTGCACCGTTGAAGTTGTAGAAGAC
GTTTACGGTGCTATAGATCACATTCACCGACATGGGAGGTAAAAACTCGATATAACAGACAT
TGAGTTTTGTAATCTTTTTGCCTATGTACTGGAAATGTTCACTCTTTATCTTGTCTTATATTTT
GTTACGAGCAGTGCGCACACAGATTGCATTGTGACAGAGGATACCGAAGTTGCAGAGCTAT
TCCTTCGCCAAGTGGACAGGTAAAATACCGGATCATGAACTTGTTTAGTGGCTGTCTTTGAT
TATGTTGGTAACTGACTGTAAGATGTACGTCCTTGAACAGCGCTGCTGTTTTCCACAACGCA
AGCACAAGATTCTCAGATGGGGCTCGATTTGGACTTGGTGCCGAGGTAAGTGAGAGACATA
CAAATAATCCTATTTATCAAACAGGGAAAAGAGGGAAGAGTGAGTGATGAAGTAAGTTTTG
GTTGGTTATACATAGGTGGGAATAAGCACAGGTAGGATTCATGCTCGTGGCCCAGTCGGAGT
TGAAGGATTACTTACAACAAGATGGTACCATTTTACTTACTTGAAACACCATTGTTGTTATGT
CGATATATCCTCGCAATAAGCTTTTTCTTCTTAGCTTTATTTGTAAATTTTCATGGTGAAATG
GTTTGAAGTATGAGTGATGGTGGTTGCAGGTTAATGAGAGGAAAAGGACAAGTTGTTGATG
GAGACAATGGGATTGCTTACACCCATCAAGACATTCCCATCCAATCTTAGAAGACTGTTGTG
TGTTGAGACTTGAGGAGAGGATGGGCTTTTTGTTTCCTCTCTGCTAATATCATATCCTATTAT
TATTGTTATTGAAACCCTCTCTTATGTAGTGGTTTTGATTTAGGAATTAGGGATTGCACCAAG
AATAAGTTACCACTTGGTCTTGCTCATAAGTAAGATGAAGAACATTTTCTTAGCTTCTCTTCT
TGTTTAAAAAAAACACGTTGTAAGGCTACCTACACCTTTCTGATTTATCATTTATCTATATCT
TTGGATTTGAGTTTGGACTTCCACTGGGAGTTATACCTTTAATACAAAGTTGCATATATGAAC
TTAAAAAGTCATTACTATTAATTCCCAAGGATCAGCGCAAAATGGTAAACACGTTTGAGTAC
GTTTGAGTGCTAAGAATGAACACAAGAGTTCTCATTCTTACATATCTAATTTTTTTCAGGTAC
TTGAGGAATCGATCTTAACTTTCATTCTTCCATAAACTTCTTAACATTCTTCAACCACAGCAA
GTACTCTCGCTTCACTTTTCTCATCATGTACT

(C) *Thellungiella halophila P5CS1* gene sequence which was isolated (unpublished). Highlighted in green is isolated 5' flanking sequence, in red is start and stop of the CDS, in aqua blue are the exons and in yellow are the introns and in pink is the isolated 3' flanking sequences.

TATAGGCTATATATAAGGAAATTTAATTTACCTCATATTATTTCGATTAAATATCAAAAATTA
TTAGCTGTAAAGTTTATAACCTATGCCCTTTAAAGTCATTAAATACTTGTTATGTAATAAATT
TATCATAAAAAGAAACAAAAATGTCATCCTGGTTTTCATATTAATTATGCGGAGACAAAATT
GTTAAGTTGTTTATAAAAGTCCAAATATCAGTAAGATTATTCAGTATTGATCACTATTAAGTT
TATCTTTATATCATCTTTTTTTCTTTTCATTTTATTATATTATCTTTTATTATTTTGTGTTTTCGG
ACATCAAATCTCTTTTAAGAGTAAATTGTAATATTTTTGTATATCCTTTCCAAAAGACTATAG
AATTTTTTAGAAGTTTTAGTGACTAACAATATCCTTAAAACATAATCGAACATTACACTTAG
ACCTTTTAAAAGTTAGATAGATCAAAATACTTTTATCTATGGTGTGTTCACCAAAAACAAGA
ACTTATAGAGAATTGTCTAGACATATATGTTATAGAAAACAAGAGATATCCAACGCTCTTTC
TTTGCAAAACATACAATGTAATCGGTAGGACCTTATGTAAATCACAAAACAAAGGAAATAG
GTTATACTTTTATCGAATAAATGTGAGTTCTTAAAACTACTAAAGTAAGTCTATGTAACATTA
GTAACGTGAGTTATTACACAGTTTTAGCCAAATCTCGCATTATATTTTAATTATATATTTGTT
GTTTTACTAATATTTTACAACACCGCGGCGTATTTTTTTTTTTCTTCTTCTACCACACTTAAAG
GATAAAAAAAAGTGATCTCTCTGCAGTAGATTTTAACGAAGCGACAGGCGAAAAGTGAACAG
GAGAAGACTAAAGGCAAACGGAAACACAACAAGTACAGGTGCCGCGAAGGAGTCGGCGGC
TAAAAAATTAAAGGGTTTTATGGGTGGTGGAGTCATTGATAGAAGACGCTGCGTTGGGTTTA
GGGAAATGCTACGCGGTGGGTTGCTGACGCGCGGTGAAGTAGCTAATCCTAGATTATAAGG
TCTATATGGGAATGAGCGTGTGCACCGCGCAGAACCAAACTATCTTCCTGGACTGAGAGACC
ACATTCAACACAAATATTTGGGGAAGTAGAGAAGGAACAACTAGAGGACACTTCCCTCACC
AGATATTTCCCTAAACGCGCTCACTGACGAAATCCACCACTGAGTTAACTCGTTCCTTCTCTG
GGTTTTGGTAGGCGGCGACAATGGAGGAGCTAGATCGTTCACGCGCTTTTGCCAAAGACGTC
AAGCGTATCGTCGTTAAGGTTCTCGTCTCTCTCTCTCTCTCTTTGTATCTGTTTGTTTATCT
CCTTATCCGTGTTTCGTTGAGAAACGTCCGCATTCTCAGATTTTGATTTGATTATCGACTGTT
TTTGGCTTAATTGCTGATTTCGATTTTTTTTTGTTTTTTTTTCTCTGCGTTCGTCTGAATCTGTG
AAGTGTTCGTCGTCGTTGGTTGTCGATGTGGATTGGGTTTAGTGTGTTTTTTAATTTCATTTTA
AGCTGTTTTTTGCGGCTGAGTGAAATCTGCGGTAATGTGAAAAATCGAATATTATATGATTT
AACGTGCATCTGAATATTTTTTGTTTGTCTCTGTTATTGAAAAGCTCTCAACGGAAAAGTTTC
TCGAATCTGAATACCATTTGTCTCGGAAAAATTAAACCTCTCGTAATCACGCTTATGAATCTT
AATCTGCATGTCATCAGAGAGTGATGAAGAATCAGAATATTCGGATAATTAATATTCTGTTT
TTTTTTTTTTGTAAATATGGGACCGCTGTTGTTACTGGGAAAGGTGGAAGATTGGCTCTTGGT
CGCTTAGGAGCACTGTGTGAACAGCTTGCGGAATTAAACTCGGATGGATTTGAGGTGATTTT
GGTGTCATCTGGTGCGGTTGGCCTTGGCAGGCAAAGGCTTCGATACAGACAATTAGTCAATA
GCAGCTTTGCGGATCTTCAGAAGCCTCAGAGTGAACTTGATGGGAAGGCTTGCGCTGGTGTT
GGACAAAGCAGTCTTATGGCTTATTACGAGACTATGTTCGACCAGCTGGATGTGACGGCGGC
TCAACTTCTGGTGAATGACAGTAGTTTTAGAGACAAGGATTTCAGAAAGCAACTTAATGAAA
CTGTCAAGTCGATGCTTGATTTGAGGGTTATTCCGATTTTCAATGAGAATGATGCTATAAGC
ACCAGAAGAGCCCCATATCAGGTTTGTGACTATCTTAGTCCCTTTGGAATGAGTACTCCTTG
AAATTAGCTGCTTCCTATGAATCTCGTAGTCTAATATGTTCAAGTTCAATGGCATTTCAATAT
ACGCAGGGATTCCTCTGGCATCTTTTGGGATAACGACAGCTTAGCTGCTCTACTGGCGCTGG
AACTGAAAGCTGACCTTCTGATTCTTCTGAGTGATGTCGAAGGTCTTTACACAGGCCCTCCC
AGTGATCCTAACTCAAAGTTGATCCACACATTTATTAAGGAAAAACATCAAGATGAGATTAC
ATTTGGCGACAAGTCAAGATTAGGAAGAGGTGGCATGACTGCAAAAGTCAAAGCTGCAGTG
AATGCAGCATATGCTGGGATTCCTGTCATCATAACCAGGTGTGGGCCCTTTTACATTCATTGT
GCATAATTAATACGCTTTCCAAATTTGTCAAGTGTTCTTGATCTCGCTTTCAGTTATGACCCT
GAATATCATCATCTCAATTCCTCCGAAACACCCCAATTTACGTTTGATTGAGAAATATTCGA
AAGTTATTTAGTGGGATAGAAAGTTGATACTCTTCATGCTTCCGAAGTGGGTATTCAGCTGA
AAACATAGATAAAGTCCTCCGAGGACTGCGTGTTGGAACCTTGTTCCATCAAGATGCTCGTC
AATGGGCTCCGATCACAGATTCTACTGCTCGTGACATGGCAGTTGCTGCAAGAGAAAGTTCC
AGAAAGCTTCAGGTACTGCTAGATCAAGCTTGGGTGTTTTTTTTCCACCATTCTGATGTGAGA
AATCTTTTCTTTCTCGGTAGAGATGTATTTAAACTGCTGGTAATTTGCTACAGGCCTTATCTT
CAGAAGATAGGAAACAAATTCTGTATAATATCGCCGACGCTCTTGAAGCAAATGAAAAAAC
AATCAGAGATGAGAATGAATTAGATGTATCTGCAGCACAAGAAGCTGGATTTGAAGAGTCA
TTGGTGGCTCGCTTAGTTATGACACCTGCAAAGATCTCAAGCCTTGCAGCTTCAGTTCGTAA
GCTAGCCGATATGGAAGATCCAATTGGCCGTGTTTTAAAGAAAACTGAGGTGGCAGATGGT
CTTGTCTTAGAGAAGACCTCATCCCCATTAGGCGTACTCCTGATTGTTTTTGAATCCCGACCT
GATGCACTTGTACAGATAGCTTCACTTGCCATCCGGAGTGGAAATGGTCTTCTATTGAAGGG
TGGAAAGGAGGCCCGGCGATCAAATGCTATCTTACATAAGGACAGTGCCTCAGATTTCAGA

CTCGGATGTTATCATATATGGCTTCCTCAAAATATGCTGGTTATAATTGATCCATTTAATTTC
ATTTTAAATTTCTAGGTGATCACTGATGCAATTCCAGAGACTGTCGGGGGTAAACTCATTGG
ACTTGTGACTTCAAGAGAAGAGATTCCTGATTTGCTCAAGGTAAACGGATTTACAAACTTGG
AGCTGCAACAAATCTTTATATCTTGTGTTTAAATGGAAACCACCATTTGCATTTGCGCTACAG
CTTGATGACGTTATAGATCTTGTGATCCCAAGAGGCAGCAACAAGCTTGTTTCCCAGATAAA
AAATACTACAAAAATCCCTGTGCTAGGCCATGCTGGTACGGTTTCAAGTTTGTTTTTCCATAA
AATTCTTTAGTTGGATTGTGTTAGAGAGTGACTGTCTTAATTTTGTACTTCCAGATGGAATCT
GTCATGTATATGTCGACAAGTCATGTAATCTGGATATGGCAAAGCGCATAATTTCCGATGCA
AAGTTGGATTATCCAGCAGCCTGTAATGCGATGGAAAGAGAACTTGTACCAGCCTCTTGAGA
TTGGAGTATGCAATGGGCGTATTAATTTCATCCGACTCATTTCACCTTCTCTTTCCTTTGTATT
TTCAGGAAACTCTTCTTGTGCATAAGGATCTAGAGCAGAACGGGCTCAATGAGCTTATTTTT
GTGCTGCAGAGCAATGGAGTCACTGTATATGGTGGACCAAGAGCAAGTGCAATACTGAACA
TACCAGAAGCACGGTCGTTCAACTATGAGTACTGTTCCAAGGCTTGCACCGTTGAAGTTGTA
GAAGACGTTTACGGTGCTATAGATCACATTCACCGACATGGGAGTGCGCACACAGATTGCAT
TGTGACAGAGGATACCGAAGTTGCAGAGCTATTCCTTCGCCAAGTGGACAGCGCTGCTGTTT
TCCACAACGCAAGCACAAGATTCTCAGATGGGGCTCGATTTGGACTTGGTGCCGAGGTGGG
AATAAGCACAGGTAGGATTCATGCTCGTGGCCCAGTCGGAGTTGAAGGATTACTTACAACA
AGATGGTTAATGAGAGGAAAAGGACAAGTTGTTGATGGAGACAATGGGATTGCTTACACCC
ATCAAGACATTCCCATCCAATCTTAGAAGACTGTTGTGTGTTGAGACTTGAGGAGAGGATGG
GCTTTTTGTTTCCTCTCTGCTAATATCATATCCTATTATTATTGTTATTGAAACCCTCTCTTAT
GTAGTGGTTTTGATTTAGGAATTAGGGATTGCACCAAGAATAAGTTACCACTTGGTCTTGCT
CATAAGTAAGATGAAGAACATTTTCTTAGCTTCTCTTCTTGTTTAAAAAAAACACGTTGTAA
GGCTACCTACACCTTTCTGATTTATCATTTATCTATATCTTTGGATTTGAGTTTGGACTTCCAC
TGGGAGTTATACCTTTAATACAAAGTTGCATATATGAACTTAAAAAGTCATTACTATTAATT
CCCAAGGATCAGCGCAAAATGGTAAACACGTTTGAGTACGTTTGAGTGCTAAGAATGAACA
CAAGAGTTCTCATTCTTACATATCTAATTTTTTTCAGGTACTTGAGGAATCGATCTTAACTTT
CATTCTTCCATAAACTTCTTAACATTCTTCAACCACAGCAAGTACTCTCGCTTCACTTTTCTC
ATCATGTACTCATGCAAGAAATTCGTTGTGCTTGCTTTCACTCCTCTTATCTCCAGATACTTG
TAGAATGTCTTCTTCAGGTTCTCGTCCAAGTCTCTGCAAAAGTAGAAGACACACAAGAGA

(D) Forward primer positions on *Arabidopsis thaliana* mutants, which were used to confirm the presence of T-DNA insertion.

1. ORG1 (At5g53450) Tm: 66 °C

ATGGCACTTTGTGGTGTTTGTTC

ATGGCACTTTGTGGTGTTTGTTCGACTCCAAATCTGCCGAATTTACAAGTGTTTCGTTCTGTTAGGAACT
CGAGCATTGGTTATAAACGAAACCATAGTCTATGGCAGCTCAGAAGTTCATCATTTCGTGCTAAATCTGT
GATATTTCATTGTTCATCGTCCTTAAGACAGTCACCATCTAATGTGGAGGAGATAGATGATAATCCTTCG
GTATCATTAGAGGATGAATCAGCACATGTGATGCAGTTCAAGTGGTCTGATTTTAGGATTCTTGATCGTG
TTAGCATTGGTCATGGCGGCAGGGCTGATGAGCTTGTGTTTGAAGCTATAGTTCAGGTTCCAGATAGCCC
TTTGTTTAACCAAGGAGTTGTTCTTCGGAAATTGAATACCACTCGAGCTCAAAGACGAGGAAGAAGAGCT
ATAGAAGTTTTTAAGAAGCTAGTTCGTCGGAGACTTCTCTACCATTCTTACTCAATGCAAGTTCACGGTT
ATATCACCAATAACTTAAGCGATGATCAGTACTCATTTACCCTTGTACATGGGTGCCATGGAAGTTTCTC
GATTAGGCACTGGCTTCAACAATCTGATTGGATTCCAACATTAGAAGCTACTCTTGCATTAGATGAAGAA
TCCTTTCGAAGGGTGGGTGATGATACTACTGGAGGGCCTGCAGTTTCAAGGCAGTTAAGACTAATCCGTA
CACTAATGAGGGATATTTTGATCGGAGTCAATTACTTGCACAGCCATGGTCTGGCTCACACAGAACTGAG
ATTGGAAAATGTGCATATTAGCCCTGTGGATAGACATATCAAAGTAGGGATTCTCGGAAATGCTGCTGAC
TTTAACGGGGATGTTCCAAGTACTAGCAACGCTTACAGTACCATGGACAGACGACAAATGATGATAGCAT
TTGACATGAGATGTGTTGGATTCATGATGGCAAAAATGGTACTTCAAGAATTGATGGATCCATTAATCTT
TGCGAAATTGAAGTCTTTCCTGGCAAAGGGGAATGATCCTTCCTCGCTGCGGGAGTTTTTTGTGACGACG
CTCAATACAAACTCTGAATCTGGAAATACCGGAGTGCAAATACTTGATAGAAACTGGGGAGCAGGTTGGC
ACCTTTTATCTTTGTTAATTGCTACCAGACCTTCTGAAAGAATAAGTTGCTTGGATGCTCTTAAGCATCC
CTTTCTATGTGGACCAAGATGGCGTGTTGCCCCATCAATGGATATCATCAGATGGGGTCTTGGATCAACC
GCAGTAAAGATTTCAGAAGAATACATTTACCGCATGCCTCAGCGCCAAAGACTTGCCCACTTCATTGGAC
TGATGGAGATGCTAAATCCTTATCCAAAGCCAAACTGTTGGTTGGAGCTTTTACCGGGAAGATGGCGTCT
TTTATACTCAACTGGAAAGCACATAGGTCTAACTCTGCGTCAGCCTTCCACACGTGCCTTAATAGGCAAC
GTTCACTTAACGATAACTCGAGCTTCAGAATCCATAAACAACACTTCACTTTCCTTTACCTCTGATATAC
GCTTCACTGCCATAACCAGCAAAGACTGGCCACACAACAAAATCGGAGCTGCTGGGAAATTACAAACGCT
CTCTCAATTCAGACTAATAGCTGGAAAAAGACTTTACCTCAAAGAAGAGAAAAAGAACATTGGTAAGTTC
TCAATGGGTGAACCAGATGCTGAAGAAGGTTTAGCCGAGAAGCTTGAAACCGAGAAATGGAAAAAAGTCG
TGCCCTTCAAGGAGTTTCCGTCGAGTCTTCCTGTAGCAAAACTCGTCTCTGGAGAAATCGAAGTGACGAT
GAACATGAATGATCATATAGATTCACCTGGGAGTGTGATTGGAGAAGTTAGAAAGCAAATTCCGCCGGAA
ATGTTCGATCTTTCTAAGCTTGTGTGTGGGACTTATATAGACAGTAGGTTACTTGTACTTAGGTGTGTAA
ATGGTTCAGCATTGTTGTTCACAAGGTCCAGCTTGGACCATAAGTCTATGTAG

2. Rap2.4f (At4g28140) Tm: 64 °C TTTGACGAGGAGCTAAATCTTTG

ATGGACTTTGACGAGGAGCTAAATCTTTGTATTACGAAAGGTAAAAATGTTGATCATTCTTTTGGAGGAG
AAGCTTCTTCCACGTCCCCAAGATCTATGAAGAAAATGAAGAGTCCTAGTCGTCCTAAACCCTATTTCCA
ATCCTCTTCTTCTCCTTATTCGTTAGAGGCTTTCCCTTTTTCTCTCGATCCAACACTTCAGAATCAGCAA
CAACAACTCGGATCATACGTTCCGGTACTTGAGCAACGACAAGACCCGACAATGCAAGGCCAGAAGCAAA
TGATCTCCTTTAGTCCTCAACAACAACAACAGCAGCAGCAGTATATGGCCCAGTACTGGAGTGACACATT
GAATCTGAGTCCAAGAGGAAGAATGATGATGATGATGAGCCAAGAAGCTGTTCAACCTTACATCGCAACG
AAGCTGTACAGAGGAGTGAGACAACGTCAATGGGGAAAATGGGTCGCAGAGATCCGTAAGCCACGAAGCA
GGGCACGTCTTTGGCTTGGTACCTTTGATACAGCTGAAGAAGCTGCCATGGCCTACGACCGCCAAGCCTT
CAAATTACGAGGCCACAGCGCAACACTGAATTTCCCGGAGCATTTTGTGAATAAGGAAAGCGAGCTGCAT
GATTCAAACTCGTCGGATCAGAAAGAACCTGAAACGCCACAGCCAAGCGAGGTTAACTTGGAGAGCAAGG

AACTACCGGTGATTGATGTTGGGAGAGAGGAAGGTATGGCTGAGGCATGGTACAATGCCATTACATCGGG
ATGGGGTCCTGAAAGTCCTCTTTGGGATGATTTGGATAGTTCTCATCAGTTTTCATCAGAAAGCTCATCT
TCTTCTCCTCTCTCTTGTCCTATGAGGCCTTTCTTTTGA

3. NPK15 (At5g58540) Tm: 66 °C GATTCAATCTCCGAACAGTGTTC

ATGAGTTCCAAGAGACGGCGGAGATTCAATCTCCGAACAGTGTTCTCCATCATCTTCCTCACCTTTC
TTCCTCTGAATCTTAACTCTCAAGAGATTGTAGAGGTCTTTGATTCTTCTCAAGATCACTTCTTGAT
CCAATCCCGAGTTTATGCGAACCATCGCAGCCTTATCGATACACCTCTTCCTGGCAAAGACCCTGCC
CTTGATGCCTCTCCGCCATCTCCTGAATCCGCTATCCTCAAAGATCCATTGCTGCCTCCGCCACCAC
CAGAAGGCAACGAAACCCCGAGCCCTCCTCGAAGTGGTGTGCCAACACAAACACCAGAGACCCCACC
TGCTATCACTCCCCTGCCTGTACCACTGGCTCCAGCTCCGTCTCCGTCTCCTCCTGTGTCTCCAGGA
ACTACAAAGAAGTCTCCCAAAGTTTATATGATCGTTGGCATAGTCGGTGGGGTATTCACAGTCTCGG
TAGCATTAATCATCATCTTTCTTATCCTCACTCGAAAGATTCCAATCAAGCCTTGGACCAACAGTGG
CCAGCTTCGCGATGATCTTATCACAGATGTTCCGAGGCTGCAGCTATCTGAGCTACAAGCAGCCTGC
GAAGATTTCAGTAATGTCATAGGATCTTTCTCAGACGGCACCATTTATAAAGGAACTTTGTCCACTG
GTGCTGAAATCGCTGTGGTGTCTATTGTGGCCGGTTCTCGTTCAGACTGGTCCACCACCATGGACAC
ACAGTTGCTACAAAAGATGCATAATTTATCCAAAGTGGATCACAAGAACTTTTTGAATGTGATCGGT
TATTGCCTTGAGGAAGAGCCCTTCAAGCGAATGCTGGTTTTTGAATACGCTCCCAATGGATCACTCT
CCGAGCATCTGCACTCTCAATACGTGGAGCACTTGGACTGGCCTACCAGACTCAGAATCGTCATGGG
AATAGCTTACTGTCTAGAGCACATGCACAATCTTAACCCACCCATCTTGCTCTCCAATTTGGACTCC
TCTTCTGTGTACTTGACTGAAGACAACGCCGCCAAAGTCTCTGACTTTTCTGTCATCAACTCCATAT
TTCCCTCTAAGGAGGGTTCCTCGAGCAAGAACCTTCTAGAACCCTCGTTACTTGATCCCCATACCAA
CGTCTTTAACTTTGGTGCCGTTTTATTCGAAATCATCAGTGGAAAATTACCAGACCCGGATTCTATG
CTTCTCGAACCCAAGCCCACAAGAGATATTGTGGACCCGACACTGAAAACATTTCAGGAAAATGTTG
TTGAGAGACTGTTGGAGGTGGTTAGGCAGTGTTTGAATCCATACTCAGATCAGCGGCCGACAATGAG
AGAGGTTGTGGTGAAATTGAGAGAGATAACTGGAATAGAAGCTGACGCAGCAATGCCGAGGCTGTCT
CCACGGTGGTGGACAGAGCTGGAGATCATATCCACAGAAGGAAACTAA

4. CRK11 (At4g23190) Tm: 66 °C GAAGCAGAGGAGTTTGTTTTCAG

ATGAAGCAGAGGAGTTTGTTTTCAGTCCTCTGTTTTTTCTTCATAAGTTTTGGTGTTGCTTCAGTTT
CAGCACAAACATGCACGACGGACAAGGGGACTTTCAGACCCAACGGTACTTACGACGTAAATCGCCG
TCTCATCCTCTCTTCTCTTCCTTCAAATGTCACGGACCAAGACGGCTTATACTACAACGGTTCCATA
GGACAACAACCGAACCGTGTCTACGCAATAGGGATGTGCATCCCAGGATCAACTTCAGAAGACTGTT
CTGATTGTATCAAGAAAGAGTCTGAATTTTTTTTAAAGAATTGTCCTAACCAAACAGAGGCGTATTC
ATGGCCAGGTGAGCCAACGCTTTGCTATGTGCGCTACTCCAACACTTCTTTCTCAGGATCTGCTGAT
CTGAACCCGCGAAATTGGCTCACCAACACTGGAGACCTAGACTCAAATCTAACAGAGTTTACGAAAA
TATGGGAAGGATTAATGGGTCGTATGATTTCTGCAGCTTCCACAGCAAAAAGCACACCTTCTTCAAG
TGATAACCATTACTCAGCTGATTCAGCAGTCTTGACACCTCTCCTGAATATATATGCATTGATGCAA
TGCACGCCGGATCTTTCCTCCGGTGATTGTGAAAACTGTCTGCGACAAAGCGCAATTGACTATCAGT
CGTGCTGTAGCCAGAAGCGAGGAGGTGTTGTTATGCGGCCAAGCTGCTTTTTGCGGTGGGATTTGTA
TACATATTCTAACGCTTTTGATAATCTTACGGTGGCTTCTCCTCCTCCAGAACCTCCTGTGACTGTG
CCACAACCTGCAGGTGATCAGGACAACCCGACCAACAATGATAGCAAAGGAATCTCAGCTGGAGTTG
TTGTGGCGATCACCGTTCCCACTGTTATTGCCATCTTGATACTGCTGGTTTTAGGATTTGTTCTTTT
CCGGAGAAGAAAATCCTACCAAAGAACTAAGACTGAATCTGAAAGTGATATTTCAACTACAGATTCA
TTGGTATACGATTTTAAGACAATTGAAGCCGCAACTAACAAGTTTTCAACAAGTAATAAGCTTGGTG
AAGGTGGATTCGGTGCGGTTTACAAGGGTAAGCTTTCTAACGGAACTGATGTAGCTGTGAAGCGACT
GTCGAAAAAGTCAGGACAAGGCACAAGGGAGTTCAGGAACGAGGCTGTTCTTGTGACAAAACTTCAA
CATAGGAATCTGGTTAGACTTCTTGGATTCTGTTTGGAAAGAGAGGAACAGATTCTGATCTATGAAT
TTGTCCACAACAAAAGCCTTGACTACTTTCTTTTCGACCCGGAAAAGCAAAGTCAGCTAGACTGGAC
CCGGCGATACAAGATCATTGGAGGAATTGCTCGAGGGATTCTATATCTTCATCAAGATTCACGGCTC
AAAATCATACATCGTGACCTCAAAGCCAGCAACATTCTCTTAGATGCAGACATGAACCCAAAAATTG
CAGATTTTGGATTGGCAACTATTTTTGGAGTGGAGCAAACTCAAGGAAACACGAACAGAATTGCTGG
AACCTACGCTTACATGTCTCCCGAGTATGCGATGCATGGTCAATACTCCATGAAATCTGACATTTAT
AGCTTTGGAGTCTTAGTTCTTGAGATTATAAGCGGCAAGAAAAACAGCGGCGTCTACCAGATGGATG
AAACTAGTACTGCAGGAAACTTGGTCACTTATGCTTCGAGGCTTTGGAGAAACAAGTCACCATTAGA
GCTGGTGGATCCAACTTTTGGAAGGAATTATCAGAGTAATGAAGTCACTAGGTGCATCCATATCGCG
CTGTTATGTGTTCAAGAAAATCCAGAAGACCGTCCAATGTTATCAACAATCATCTTAATGCTGACTA

GTAACACAATTACTCTACCAGTGCCTCGCCTACCGGGATTTTTCCCACGAAGCAGGCAACTGAAACT
GGTATCTGAAGGATCAGAGTCTGATCAGTATACAAGCAAGTCTTCCTCATTTAGTTCGTAA

(E) Forward and reverse primers used in the Sybr Green based QPCR assay; primer positions used to check the transcript abundance between the two species of the selected genes. The sequence marked in yellow is forward primer and sequence marked in green is reverse primer.

1. PpC1

ATGGCGAATCGGAAGTTAGAGAAGATGGCATCGATTGATGTTCATCTTCGTCAACTGGTTCCTGGCAAAG
TTAGTGAAGACGACAAGCTTGTTGAGTATGATGCTTTGCTTCTAGATCGGTTTCTCGATATCCTCCAGGA
TTTGCACGGTGAAGATCTCCGTGAAACTGTTCAAGAGCTTTATGAGCATTCTGCAGAATACGAAGGGAAG
CATGAACCTAAGAAGCTAGAGGAGCTAGGGAGTGTGCTAACGAGTTTAGATCCAGGAGATTCCATTGTTA
TCGCTAAAGCTTTCTCTCATATGCTTAACTTAGCCAATTTGGCTGAGGAAGTGCAGATTGCTTATCGCCG
TAGGATCAAGAAGCTGAAGAAAGGTGATTTTGTTGATGAGAGCTCTGCTACTACTGAATCTGATCTTGAA
GAAACTTTCAAGAAGCTTGTTGGAGATCTGAACAAGTCTCCTGAAGAGATCTTTGATGCTCTCAAGAATC
AGACTGTGGATTTGGTTTTGACTGCTCATCCTACTCAGTCTGTGAGAAGATCATTGCTTCAGAAACATGG
GAGGATAAGAGACTGTCTGGCTCAACTATATGCTAAGGATATTACTCCTGATGACAAGCAAGAGCTCGAT
GAGGCTCTTCAGAGAGAGATTCAAGCTGCATTCCGAACAGATGAAATCAAAAGAACACCACCTACTCCTC
AAGATGAGATGAGAGCGGGAATGAGTTATTTCCATGAAACTATCTGGAAAGGTGTTCCTAAGTTTCTGCG
CCGTGTTGACACGGCTTTGAAAAACATAGGGATCGAAGAACGTGTTCCATATAATGCTCCATTGATTCAG
TTCTCTTCTTGGATGGGTGGTGATCGTGACGGTAACCCAAGGGTTACACCTGAAGTCACCAGAGATGTTT
GCTTGTTAGCTAGAATGATGGCTGCTACTATGTACTTTAACCAAATCGAAGATCTTATGTTTGAGATGTC
TATGTGGCGTTGCAATGACGAGCTGCGTGCGCGAGCTGATGAAGTTCATGCAAATTCGAGGAAAGATGCT
GCAAAACATTACATAGAATTCTGGAAGTCAATTCCTACAACTGAGCCATACCGTGTGATTCTTGGCGATG
TAAGGGACAAGCTTTATCACACACGTGAACGCGCTCATCAACTGCTCAGCAATGGACACTCTGATGTCCC
TGTAGAGGCTACTTTCATTAACTTGGAACAGTTCTTGGAACCTCTTGAGCTCTGTTACCGATCTCTGTGT
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TCTCTCTTGTGAGACTTGACATAAGGCAAGAATCTGACCGCCACACTGATGTATTGGATGCTATCACCAC
GCATTTAGATATCGGATCCTACAGAGAGTGGTCTGAAGAACGCCGCCAAGAATGGCTTTTATCTGAGCTA
AGTGGCAAACGTCCGCTTTTCGGTTCTGATCTTCCTAAAACCGAAGAAATAGCTGATGTTCTGGACACGT
TTCATGTCATAGCCGAGCTACCAGCAGATAGCTTTGGTGCTTACATTATCTCTATGGCAACTGCACCTTC
TGATGTATTAGCTGTTGAGCTTTTACAGCGTGAATGCCGAGTGAAACAGCCTTTGAGAGTTGTTCCGCTC
TTTGAGAAGCTAGCAGATCTGGAAGCAGCTCCTGCTGCAGTTGCTAGGCTCTTTTCTGTTGATTGGTACA
AAAACCGAATTAACGGTAAGCAAGAGGTTATGATTGGTTATTCGGATTCAGGAAAAGATGCTGGACGGTT
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CTTTAGAGTCACCATTGATCTAATCGAAATGGTGTTCGCTAAAGGAGATCCTGGTATTGCTGCTTTGTAC
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2. FBA1

ATGGCGTCAAGCACTGCGACTATGCTCAAAGCCTCACCGGTGAAATCTGATTGGGTTAAGGGACAGAGTC
TTCTCCTCCGTCAACCTTCCTCTGTCTCAGCTATTCGCAGCCACGTGGCACCTTCCGCTCTCACCGTCCG
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GCCAAGGACTTGACGGTTTAGCCTCTCGCACCGCTGCTTACTACCAACAAGGTGCTCGTTTCGCCAAATG
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GGCACCGAACCCATGGCACGTGTCCTTCTCCTACGCACGTGCCTTGCAGAACACTTGCTTGAAGACATGG
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CTCAGCTCGGGAAATACACTGGAGAAGGCGAGTCTGAGGAAGCCAAGGAGGGTATGTTTGTAAAAGGCTA
CACCTACTAA

3. UGP2

ATGGCTGCCACCGCAACCGAGAAGCTCCCTCAGCTCAAATCCGCCGTCGATGGACTTACTGAGATGAGCG
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GAGCAAGATCCAGACACCTACTGATGAAATTGTTGTTCCTTATGATAAAATGGCTAACGTCTCTGAAGAT
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AGAGGATCTGTGA

4. SUS3

ATGGCAAACCCTAAGCTCACTAGGGTTCTAAGCACAAGGGATCGCGTGCAAGACACGCTTTCCGCTCACC
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