TsDHN-2, A Unique Dehydrin Protein from *Thellungiella* and its Role in Salt Tolerance

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in the Department of Biochemistry
University of Saskatchewan
Saskatoon

by

Sarah Catherine Klatt

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ABSTRACT

Salt stress, or salinity, is one of the most common environmental stresses affecting crop yield worldwide. Due to the prevalence of salinity stress, it is not surprising that plants have evolved mechanisms to tolerate osmotic and ionic stress caused by salinity. Dehydrins are intrinsically unstructured proteins that accumulate in photosynthetic organisms under dehydrating conditions, such as salinity, and are thought to confer stress tolerance through the stabilization of cellular membranes. *Thellungiella salsuginea*, a close relative of *Arabidopsis thaliana*, is a halophyte that thrives in the Canadian sub-Arctic (Yukon Territory), that is able to tolerate extreme conditions, including high salinity. TsDHN-2 is a basic dehydrin from *Thellungiella* whose transcript increases over 10-fold in response to salinity treatment. Using RNA interference (RNAi) methodology, TsDHN-2 has been silenced and these lines were used in this study to investigate the role TsDHN-2 may play in the salt tolerance of *Thellungiella*. RNAi line 7-8 presented a 41% reduced expression of TsDHN-2 in comparison to wild-type (WT). Seed of this line showed a 15% germination rate compared to 40% in WT in the presence of 100 mM NaCl. Salinity stress experiments were performed by treating the RNAi lines and WT plants with 300 mM NaCl for up to two weeks. Line 7-8 exhibited a 6.2% greater decrease in photochemical efficiency of photosystem II (PSII) as estimated by the variable to maximal fluorescence ratio (F_v/F_m) and showed 5% greater phenotypic damage than WT when estimated visually. Concentrations of the compatible osmolyte proline increased in response to salt treatment by 3.4-fold in WT and 8.1-fold in line 7-8, suggesting this compound may be a marker for salinity tolerance. Collectively, these data support the notion that TsDHN-2 plays a role in the salinity tolerance mechanisms of *Thellungiella.*

ACKNOWLEDGEMENTS

I learnt many things during my graduate studies. I learnt the value of hard work, the meaning of responsibility, the importance of family and friends, and to appreciate the gift and blessing of good health. But despite all this, the completion of my graduate studies was only made possible due to the support and encouragement of many, and I send my sincere thanks and gratitude to all of those who have helped me along the way.

To my supervisors Dr. Gordon Gray and Dr. Nicholas Low, thank you for your guidance, hard work, patience and expertise.

To Dr. William Roesler, who not only instilled in me a love for biochemistry during my undergrad, but whose guidance and support helped this work come to fruition. Thank you!

To my lab-mates Ze Long Lim, Cody Chytyk, Nitya Khanal, Denise Broersma, Anita Agblor and David McKinnon, thank you for all the laughter, help and support.

To my amazing parents, I can never begin to fully express my love and gratitude. It was your love, support and encouragement that helped me overcome all the obstacles that I faced, and this work was only possible because of you.
I love you.

To my dear sister Christine, thank you for staying up late countless nights to make sure I made it home safe from the lab and for being so supportive and understanding over these past few years. I am so grateful to have a sister like you.
I love you.

To my best friend Heather Ervin, I am truly blessed to have a friend like you. Thank you for the countless hours on the phone, the many cards and notes of encouragement, and your unwavering support. Your selfless nature and constant positivity was a source of inspiration that kept me going through the hard times.
A million thanks!

This project was funded by Advanced Foods and Materials Network (AFMNet) and in part, by the Natural Sciences and Engineering Research Council (NSERC) of Canada.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
CAX	vacuolar Ca^{2+}/H^+ antiporter
CD	circular dichroism
EST	expressed sequence tag
F_m	maximal fluorescence in the dark-adapted state
F_o	minimal fluorescence in the dark-adapted state
F_v	variable fluorescence (F_m-F_o)
F_v/F_m	photochemical efficiency of PSII; variable to maximal fluorescence ratio
GSA	glutamate-semialdehyde
HKT	high affinity K^+ transporter
IDP	intrinsically disordered protein
IUP	intrinsically unstructured protein
LEA	late embryogenesis abundant
NHX	low affinity Na^+/H^+ antiporter
NSCC	non-selective cation channel
P5C	pyrroline-5-carboxylate
P5CR	Δ^1-pyrroline-5-carboxylate reductase
P5CS	Δ^1-pyrroline-5-carboxylate synthase
PCR	polymerase chain reaction
PDH	proline dehydrogenase
PPFD	photosynthetic photon flux density
PSII	photosystem II
RNAi	RNA interference
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
SOS	salt overly sensitive
TAE	Tris-Acetate-EDTA
WT	wild-type

1.0 INTRODUCTION

Soil salinity represents a major abiotic stress limiting crop production worldwide. Salinity imposes, in the short term, an osmotic stress, making it difficult for the plant to extract water from the soil. In the longer term, salinity stress results in an ionic stress due to the accumulation of potentially toxic ions. Plants have evolved various mechanisms to combat these stresses which include the synthesis of stress proteins known as dehydrins (Dure and Chan, 1981; Dure and Galau, 1981; Dure *et al.*, 1981). Dehydrins belong to the D-11 subgroup of late embryogenesis abundant (LEA) proteins that accumulate in all photosynthetic organisms exposed to dehydrating conditions such as salinity, drought, or low temperatures. Dehydrins are intrinsically disordered (unstructured) proteins that are highly hydrophilic and characterized by three conserved sequence motifs designated K-, S-, and Y-segments. While their exact function is unknown, they are proposed to act as chaperones or in some way stabilize cellular or organellar membranes during stress conditions. This is thought to occur by the formation of amphipathic α-helices in the conserved K-segments.

Thellungiella salsuginea (Yukon ecotype) is a crucifer that thrives in the Canadian sub-Arctic where it grows on saline-rich soils and can tolerate salinity as high as 500 mM NaCl, conditions far more extreme than those tolerated by the model organism *Arabidopsis*.
Using a transcriptomic approach to investigate the stress responses of *Thellungiella*, Wong *et al.* (2006) identified a transcript that showed a 3.4-, 31.3- and 10.4-fold increase in ratio of expression in response to cold, drought and salinity respectively. This transcript was identified as an attractive target for further study and identified as an ortholog of a dehydrin RAB18-related protein from *Arabidopsis* (Wong *et al.*, 2006).

This gene from *Thellungiella* was cloned (Barbara Moffatt, unpublished results) and denoted TsDHN-2, a basic dehydrin of the Y_2SK_3-type (Rahman *et al.*, 2010, 2011). Most studies examining dehydrins have utilized an overexpression approach (Brini *et al.*, 2007; RoyChoudhury *et al.*, 2007; Xu *et al.*, 2008). However, few have focused on using plants with reduced dehydrin expression. Several lines silenced in the expression of TsDHN-2 have been generated by Dr. Moffatt's group and were kindly provided for use in this study. While the direct role of TsDHN-2 in membrane stabilization has been investigated (Rahman *et al.*, 2010, 2011), there have been no studies specifically examining the physiological role played by this

dehydrin in response to salinity. This thesis examines the contribution of the *Thellungiella* dehydrin TsDHN-2 in salinity tolerance using RNAi lines with reduced *TsDHN-2* expression.

2.0 LITERATURE REVIEW

2.1 Salinity Stress

Salinity is a major abiotic stress limiting plant growth and development resulting in decreased crop quality and production worldwide. It is currently estimated that 20% of all irrigated agricultural land and 50% of cropland in the world is salt-stressed, with the most obvious effects of salinity in arid and semi-arid regions where rainfall is limited and evaporation is high (Yokoi *et al.*, 2002; Nawaz *et al.*, 2010). The main contributors to soil salinity include environmental factors such as the weathering of parental rocks or the deposition of oceanic salts, and man-made factors such as the use of poor quality water for irrigation and poor drainage (Chen and Jiang, 2010). Saline soil is characterized by having a high concentration of soluble salts, mainly chlorides of sodium, calcium and magnesium, with sodium chloride being the most abundant source of salinity (Munns and Tester, 2008). Furthermore, saline soil is defined as having an equivalency of 40 mM NaCl, with most crop plants being susceptible at lower levels (Chinnusamy *et al.*, 2005).

2.1.1 Osmotic and Ionic Stresses

Adverse effects of salinity on plant growth occur in two phases. The first is a rapid, osmotic phase that inhibits growth of young leaves while the second is an ionic phase that accelerates senescence of mature leaves. Under normal physiological conditions, the osmotic potential in plant cells is higher than that of the soil, thus allowing plants to take up water and essential minerals in root cells. However during salinity stress, the increased concentration of soil solutes disrupts the water potential gradient making it harder for roots to extract water and minerals leading to the reduction of normal cellular activities and eventually plant death (Xiong and Zhu, 2002; Nawaz *et al.*, 2010). Ionic stress is caused when Na^+ and Cl^- accumulate in cells and have direct toxic effects on cell membranes, enzyme activities and the functioning of the photosynthetic apparatus (Chinnusamy *et al.*, 2005; Munns and Tester, 2008). Therefore, to circumvent the consequences of high salinity, plants employ various mechanisms to alleviate both cellular osmotic and ionic disequilibrium. These include the accumulation and/or partitioning of ions, osmotic adjustment through the accumulation of compatible osmolytes and the synthesis of stress proteins such as dehydrins (Zhu, 2002; Munns and Tester, 2008).

2.2 Mechanisms of Salinity Tolerance

2.2.1 Ion Homeostasis

2.2.1.1 Na^+ Influx and Ca^{2+} Signaling

Under normal physiological conditions plants maintain a high K^+/Na^+ ratio in the cytosol, which is essential for normal cellular functions. However, during salinity stress, the sodium electrochemical potential gradient established across the plasma membrane of plant cells favours the passive transport of Na^+ from the environment into the cytosol (Zhu, 2003; Zhang *et al.*, 2010). Sodium gains entry into root cell cytosol through uniporters or ion channel type transporters such as voltage-dependent ion non-selective cation channels (NSCC) and high affinity K^+ transporters (HKTs). Although NSCCs are thought to be the dominant pathway for Na^+-influx, HKTs have also been found to mediate a substantial Na^+-influx in some species (Apse and Blumwald, 2007). In rice, nine HKT homologues (*OsHKT1-9*) have been identified and encode proteins with distinct transport activities, with OsHKT8 being a Na^+-transporter found to mediate salt tolerance by maintaining shoot K^+ homeostasis (Chinnusamy *et al.*, 2005; Apse and Blumwald, 2007). Similarly, in *Arabidopsis,* the *AtHKT1* gene encodes a Na^+-transporter and functions in mediating salt stress tolerance through cytosolic Na^+ detoxification (Berthomieu *et al.*, 2003; Rus *et al.*, 2005). Additionally, other transport proteins that may be involved in regulating Na^+ influx during salinity stress include cation transporters and channels (Zhang *et al.*, 2010).

The increase in extracellular Na^+ sensed by membrane receptors activates intracellular signaling cascades including the generation of secondary messengers, such as Ca^{2+} (Mahajan *et al.*, 2008). This ion plays a fundamental role in plant growth and development under normal physiological conditions, as well as during stress conditions. Under salinity stress, the increase in Ca^{2+} is thought to have an inhibitory effect on the Na^+ entry system, and functions in this stress response leading to salinity tolerance (Yokoi *et al.*, 2002). For example, increase in externally supplied Ca^{2+} is thought to facilitate higher K^+/Na^+ selectivity, thus reducing the toxic effects of NaCl (Zhu *et al.*, 2000). Furthermore, saline conditions also cause increases in cytosolic Ca^{2+}, which is primarily transported from the apoplast and intracellular compartments (Zhu *et al.*, 2000; Mahajan *et al.*, 2008). The increase in cytosolic Ca^{2+} is recognized by Ca^{2+}-sensing proteins, which initiates stress signal transduction leading to salt tolerance.

2.2.1.2 The SOS Pathway and Na^+ Efflux

Zhu and colleagues have identified several *SOS* (salt overly sensitive) genes in *Arabidopsis* that are components of a stress-signaling pathway controlling ion homeostasis and salt tolerance (Liu and Zhu, 1998; Zhu, 2002; Zhu, 2003). The *SOS* pathway helps to reinstate ion homeostasis through the exclusion of excess Na^+ ions out of the cell via the plasma membrane Na^+/H^+ antiporter. The SOS pathway is depicted in Figure 2.1.

SOS3 encodes a Ca^{2+}-binding protein with four Ca^{2+}-binding EF-hands and a N-terminal myristoylation motif (Chinnusamy *et al.*, 2005; Mahajan *et al.*, 2008). The salinity induced increase in cytosolic Ca^{2+} is sensed by SOS3, which transduces the signal downstream by physical interaction with SOS2 (Figure 2.1). SOS2 is a serine/threonine protein kinase with an N-terminal kinase catalytic domain and a unique C-terminal regulatory domain (Zhu, 2003). The C-terminal regulatory domain of SOS2 contains an autoinhibitory FISL/NAF motif, which under normal physiological conditions, interacts with the N-terminal catalytic domain in order to keep the enzyme in the inactive state (Chinnusamy *et al.*, 2005; Mahajan *et al.*, 2008). SOS3 interacts with the SOS2 FISL/NAF motif in a calcium dependent manner resulting in the activation of the substrate phosphorylation activity of SOS2 (Figure 2.1). Deletion of the FISL/NAF motif results in a constitutively active SOS2 that is independent of SOS3 (Zhu, 2003). Furthermore, Halfter *et al*. (2000) analyzed *Arabiopdsis sos2sos3* double mutants and found that there was no additive effect towards salt sensitivity, indicating that *SOS3* and *SOS2* function in the same regulatory pathway (Halfter *et al.*, 2000).

Together SOS3 and SOS2 regulate the expression of SOS1, a plasma membrane Na^+/H^+ antiporter (Figure 2.1). A *sos1* mutant was found to be hypersensitive to salt stress (100 mM NaCl) and demonstrated impaired ionic and osmotic balance (Chinnusamy *et al.*, 2005). *SOS1* is predicted to contain a highly hydrophobic N-terminal region consisting of 12 transmembrane domains and a C-terminal region with a long protruding hydrophilic tail (Mahajan and Tuteja, 2005). The transmembrane domains of SOS1 have substantial similarities with the Na^+/H^+ antiporters isolated from bacteria and fungi, while the long cytoplasmic tail has been proposed to function as a sensor for all solutes that SOS1 transports (Zhu, 2002; Sairam and Tyagi, 2004). Salt stress is perceived by a plasma membrane sensor, which

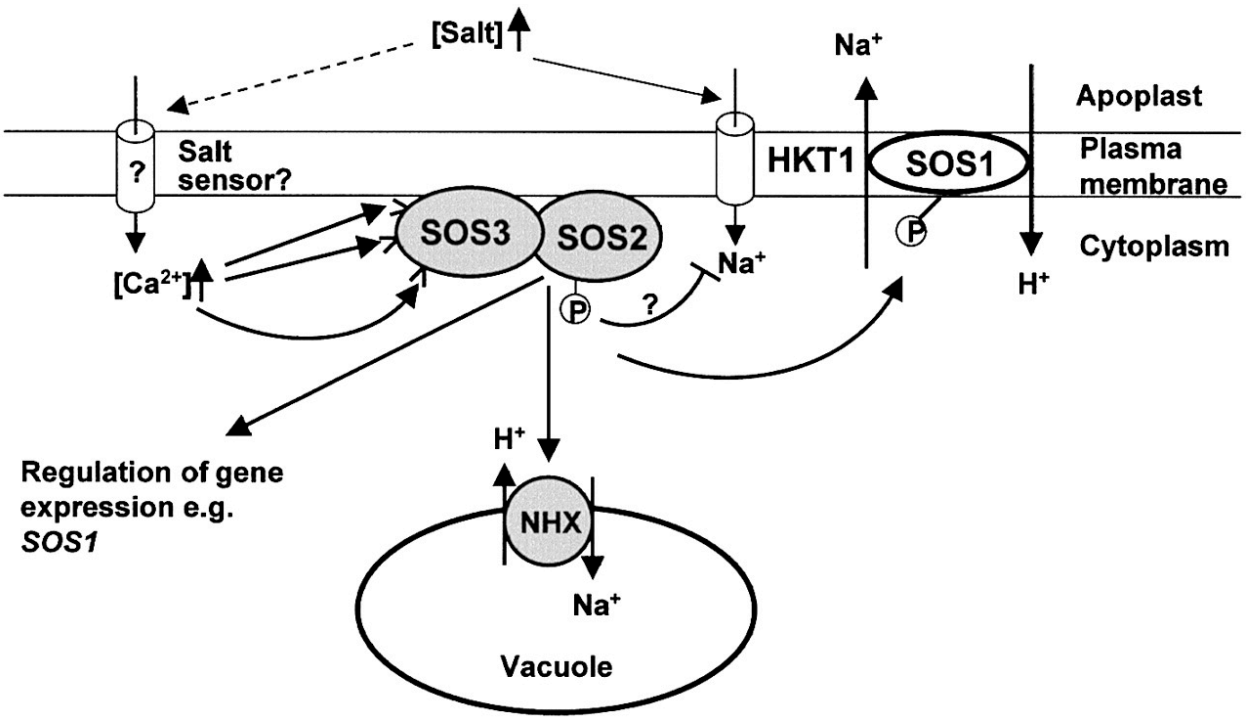

Figure 2.1 Proposed SOS signaling pathway for the maintenance of ion homeostasis during salinity stress

Reproduced from Chinnusamy *et al.* (2004) with permission. SOS, Salt overly sensitive; HKT, High affinity K^+ transporter; NHX, Low affinity Na^+/H^+ antiporter.

elicits an increase in cytoplasmic Ca^{2+}. Perturbation in the cytoplasmic Ca^{2+} level is sensed by SOS3, which interacts with and activates SOS2. The myristoylation motif of SOS3 then recruits the SOS3-SOS2 complex to the plasma membrane where SOS2 phosphorylates and activates the antiporter activity of SOS1 (Zhu, 2002; Mahajan *et al.*, 2008). Cellular ion homeostasis is then restored as excess Na^+ ions are expelled out of the cell (Figure 2.1). Furthermore, the SOS pathway interacts with other regulatory proteins in order to regulate Na^+ ion homeostasis. During salinity stress, the activity of HKT seems to be inhibited by the SOS3-SOS2 complex thus restricting Na^+ entry into the cytosol (Rus *et al.*, 2002; Zhu, 2002; Chinnusamy *et al.,* 2004; Mahajan *et al.*, 2008).

The SOS3-SOS2 complex also functions in regulating vacuolar sequestration of Na^+, which not only lowers cytoplasmic Na^+ concentrations but also contributes to osmotic adjustment by maintaining water uptake (Zhu, 2003; Chinnusamy *et al.*, 2005; Mahajan and Tuteja, 2005). In *Arabidopsis,* Na^+ compartmentation is achieved through the AtNHX1 family of Na^+/H^+ antiporters. Transgenic *Arabidopsis* and tomato plants overexpressing *AtNHX1* exhibited higher salt tolerance at 200 mM NaCl, thus implicating the pivotal role of the AtNHX family in vacuolar Na^+ compartmentation (Yokoi *et al.*, 2002; Xu *et al.*, 2009). Qui *et al*. (2003) compared tonoplast Na^+/H^+-exchange activity originating from AtNHX proteins in wild type and *sos1, sos2,* and *sos3 Arabidopsis* mutants, and found that SOS2 interacts and regulates tonoplast exchange. Additionally, using a yeast two-hybrid assay, it was found that SOS2 regulates the activity of the vacuolar Ca^{2+}/H^+ antiporter (CAX1), resulting in the maintenance of Ca^{2+} homeostasis (Cheng *et al.*, 2004). Furthermore, the activation of CAX1 by SOS2 was independent of the presence or activity of SOS3, suggesting a mechanistic link between Na^+ and Ca^{2+} homeostasis in plants as SOS2 regulates both Na^+ and Ca^{2+} transporters in *Arabidopsis* (Cheng *et al.*, 2004; Gong *et al.*, 2004; Majaham and Tuteja, 2005).

2.2.2 Compatible Osmolytes

A major consequence of high salinity is intracellular water loss. In order to prevent water loss and protect cellular protein, plants accumulate metabolites known as compatible osmolytes. Compatible osmolytes are highly water soluble compounds, have low molecular weights, and generally accumulate in the cytoplasm in order to balance the osmotic pressure that arises as Na^+ and Cl^- are sequestered into the vacuole (Tamayo and Bonjoch, 2001; Sairam

and Tyagi, 2004; Chen and Jiang, 2010). Compatible osmolytes include simple sugars (fructose and inositols), complex sugars (trehalose, raffinose and fructans), quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, proline betaine), tertiary amines (1,4,5,6-tetrahydro-2-metyl-3-carboxy pyrimidine) and sulfonium compounds (choline o-sulfate, dimethyl sulfonium propironate) (Yokoi *et al.*, 2002).

These compounds are thought to protect plants from osmotic stress through several different mechanisms including osmotic adjustment, detoxification of reactive oxygen species (ROS), stabilization of enzymes or proteins, and protection of membrane integrity (Yokoi *et al.*, 2002; Sairam and Tyahi, 2004; Chinnusamy *et al.*, 2005; Chen and Jiang, 2010).

2.2.2.1 Proline

The amino acid proline is one such osmoprotectant thought to stabilize membranes and proteins, buffer cellular redox potential, serve as a storage sink for carbon and nitrogen and also serve as a free-radical scavenger (Tamayo and Bonjoch, 2001; Matysik *et al.*, 2002; Szabados and Savoure, 2010). In organisms ranging from bacteria to higher plants, there is a strong correlation between exposure to abiotic stress and the accumulation of free proline. In bacteria, this correlation is found to be associated with salinity tolerance (Szabados and Savoure, 2010). However, accumulation of free proline does not necessarily confer stress tolerance in all organisms. In salt-sensitive varieties of barley, the accumulation of high levels of proline during salinity stress was not found to confer salt tolerance, but instead was considered to be a symptom of salt-susceptibility (Chen *et al.*, 2007).

Proline is synthesized from either glutamate or ornithine, with glutamate being the primary precursor during osmotic stress (Figure 2.2). Proline synthesis from glutamate occurs in the cytosol and the chloroplasts, and is mediated by Δ^1-pyrroline-5-carboxylate synthase (P5CS) and Δ^1-pyrroline-5-carboxylate reductase (P5CR), with P5CS being a rate-limiting enzyme in this pathway (Tamayo and Bonjoch, 2001; Chen *et al.*, 2007; Szabados and Savoure, 2010). Briefly, P5CS reduces glutamate to glutamate-semialdehyde (GSA), which spontaneously converts to pyrroline-5-carboxylate (P5C). P5CR then reduces the P5C intermediate to proline (Figure 2.2; Szabados and Savoure, 2010). Alternatively, proline can be synthesized in the mitochondria from ornithine, which is first transaminated by ornithine aminotransferase producing GSA and P5C, which is then converted to proline (Tamayo and

Bonjoch, 2001; Szabados and Savoure, 2010). Upon relief from osmotic stress, the catabolism of proline occurs in the mitochondria and is mediated by proline dehydrogenase (PDH; Figure 2.2) (Kishor *et al.*, 2005). Proline catabolism provides electrons for the respiratory chain and therefore contributes energy to resume growth following stress. Furthermore, proline oxidation is an important regulator of cellular ROS balance and influences programmed cell death (Szabados and Savoure, 2010).

2.2.3 Late Embryogenesis Abundant (LEA) Proteins

Late embryogenesis abundant proteins were first identified in seeds during their last stage of maturation when the acquisition of desiccation tolerance occurs in the embryo. They were subsequently found in vegetative organs, especially under water deficit conditions such as cold, drought, or high salinity and in response to abscisic acid (ABA) (Zhang *et al.*, 2007). While their role is not completely understood, LEAs have been suggested to stabilize plasma and organellar membranes, providing a protective role during dehydrative conditions and participation in acclimation and adaptive responses to stress. (Hincha *et al.*, 1990; Dure, 1993b; Bray, 1997; Han *et al.*, 1997; Danyluk *et al.*, 1998; Steponkus *et al.*, 1998; Ismail *et al.*, 1999; Garay-Arroyo *et al.*, 2000; Hoekstra *et al.*, 2001; Puhakainen *et al.*, 2004; Beck *et al.*, 2007; Tolleter *et al.*, 2007; Zhang *et al.*, 2010).

LEA proteins have been separated into at least six different groups on the basis of sequence similarity and expression patterns (Dure *et al.*, 1989; Ingram and Bartels, 1996; Colmenero-Flores *et al.*, 1999; Cuming, 1999; Wise and Tunnacliffe, 2004; Tunnacliffe and Wise, 2007; Battaglia *et al.*, 2008; Hundertmark and Hincha, 2008). The group 2 LEA proteins (D-11 subgroup), also known as dehydrins, are the most widely studied for their role in stress tolerance and over 100 dehydrin genes have been characterized from both angiosperms and gymnosperms (Campbell and Close, 1997; Close, 1997; Garay-Arroyo *et al.*, 2000; Zhu *et al.*, 2000; Allagulova *et al.*, 2003; Puhakainen *et al.*, 2004; Mouillon *et al.*, 2006; Beck *et al.*, 2007; Kosová *et al.*, 2007, 2008; Battaglia *et al.*, 2008).

2.2.3.1 Structural Properties and Classification of Dehydrins

Dehydrins (and most LEA proteins) are part of a broader group of proteins called hydrophilins based on their physicochemical characteristics. Dehydrins are highly hydrophilic

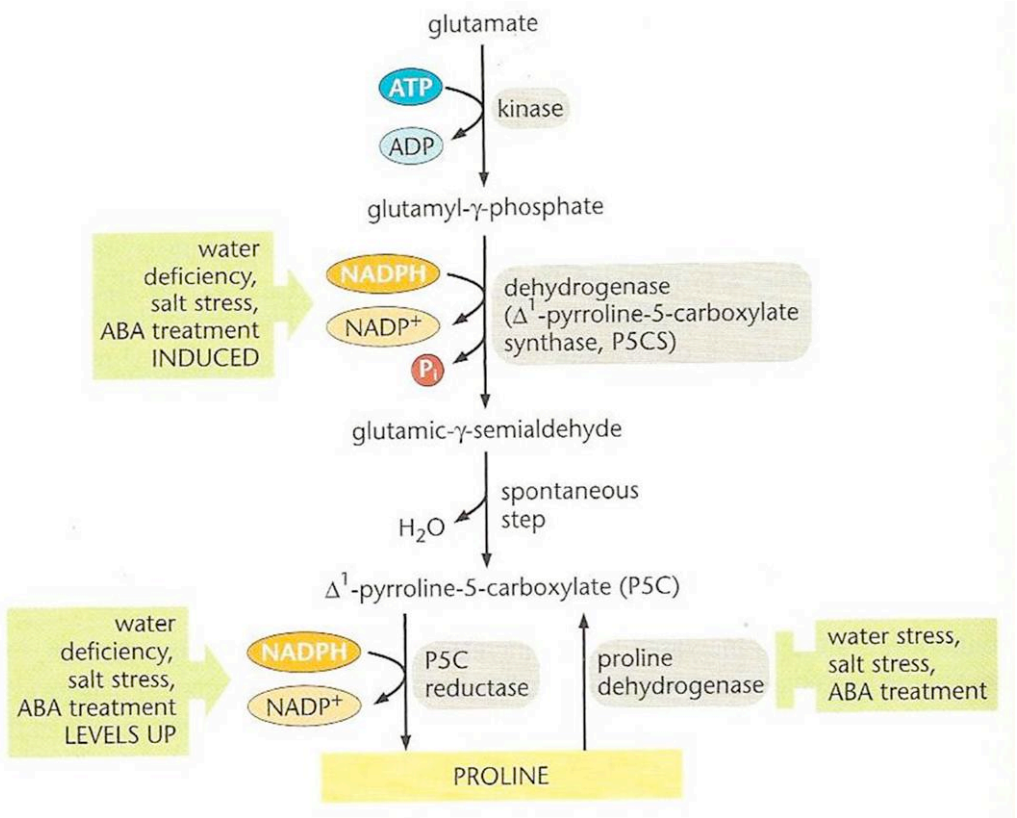

Figure 2.2 Metabolic pathway of proline synthesis and degradation in higher plants
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and are generally enriched with glycine (> 6%), serine, alanine and lysine, and lack cysteine and tryptophan. They are also thermostable, and able to maintain their integrity in aqueous solutions up to 100°C, which is due to their large number of charge and polar amino acids. Dehydrins range in molecular mass from 9 to 200 kD (Allagulova *et al.*, 2003; Zhang *et al.*, 2007). Dehydrins have also been categorized as intrisincally disordered/unstructured proteins (IDPs/IUPs) (Koag *et al.*, 2003; Kovacs *et al.* 2008). IDPs/IUPs lack a defined three-dimensional structure under normal physiological conditions, and may fold into more ordered structures upon interacting with their target molecules (Close, 1997; Bokor *et al.*, 2005; Tompa *et al.*, 2006).

Dehydrins are characterized by three conserved motifs known as the Y-, S- and K-segments. The Y-segment ((V/T)DEYGNP), when present, is found near the N-terminus. This segment shares significant homology with the nucleotide binding site of plant and bacterial chaperones, however nucleotide binding by this segment has yet to be documented (Allagulova *et al.*, 2003). The S-segment, when present, is made up of serine tract repeats and is known to undergo *in vitro* phosphorylation, as has been demonstrated in maize RAB17 and the tomato TAS14 dehydrins (Allagulove *et al.*, 2003). The phosphorylation of dehydrin S-segments has been suggested to promote the ability to bind ligands, such as divalent cationic metal ions, as well as interaction with specific signal peptides involved with nuclear localization (Close, 1996; Campbell and Close, 1997; Heyen *et al.*, 2002; Alsheikh *et al.*, 2003; Zhang *et al.*, 2006; Xu *et al.*, 2008). Finally, the lysine rich K-segment (EKKGIMDKIKEKLPG) is present as one or several copies near the C terminus, and is the only segment found in all dehydrins. The K-segment has been proposed to form an amphipathic α-helix that can associate with membrane surfaces due to electrostatic and hydrophobic interactions (Close, 1997; Campbell and Close, 1997; Allagulova *et al.*, 2003; Bravo *et al.*, 2003; Koag *et al.*, 2003, 2009; Rorat *et al.*, 2006).

The Y-, S- and K-segments are assembled together in a consistent manner with less conserved regions (the Φ-segments) interspersed between the conserved motifs. The Φ-segments are rich in polar amino acids and glycine, and have been proposed to prevent coagulation by interacting with the hydrophobic surfaces of nuclear or cytoplasmic macromolecules (Campbell and Close, 1997). Based upon the number and order of the conserved domains, dehydrins are divided into five subclasses; Y_nSK_y, SK_n, K_n, K_nS and Y_nK_y (Figure 2.3). Y_nSK_y is the most common dehydrin containing one to thirty-five Y-segments,

followed by one S-segment and up to three K-segments. These dehydrins are basic or neutral proteins, which that induced by drought or ABA (Close, 1996; Allagulova *et al.*, 2003). For example, barley dehydrins DHN1, DHN2, DHN3, DHN4, DHN6, and DHN9 are YSK_2 dehydrins that are shown to be up-regulated in seedlings by both dehydration and ABA, but not by cold (Zhang *et al.*, 2007). SK_n dehdyrins contain one S-segment and up to eleven K-segments. These are acidic dehydrins, that are preferentially induced by low temperatures but also respond to other stressers such as salinity, wounding, drought and heavy metals (Allagulova *et al.*, 2003; Zhang *et al.*, 2006). K_n dehydrins are made up of one to 11 K-segments and contain no Y- or S-segments. These are acidic or neutral proteins induced by cold, dehydration and ABA (Allagulova *et al.*, 2003; Zhang *et al.*, 2007). The characteristic feature of K_nS dehydrins is that they contain K-segments that begin with the consensus E(H/Q)KEG rather than EKKG. These dehydrins are induced by chilling and freezing temperatures as is seen in the rice Wsi724 and medic Cas15a and 15b dehydrins (Allagulova *et al.*, 2003). The Y_nK_y contains one to 11 Y-segments and up to four K-segments. These acidic dehydrins are up-regulated by stresses, but do not show any preference to any of the abiotic stresses (Zhang *et al.*, 2006). For example, the chickpea Y_2K dehydrin *cpdhn1* was expressed not only during seed development, but also in leaves during drought, chilling, salinity, and in response to ABA and methyl jasmonate treatment (Bhattarai and Fettig, 2005).

2.2.3.2 Distribution and Function of Dehydrins

Dehydrins accumulate to various cell compartments including the cytoplasm, nucleus, and in the vicinity of the plasma membrane, as well as chloroplasts and mitochondria (Hincha *et al.*, 1990; Danyluk *et al.*, 1998; Tolleter *et al.*, 2007; Carjuzaa *et al.*, 2008). Under normal physiological conditions dehydrins are found to accumulate in a tissue- and cell-type specific manner during plant growth and development. For example, while the *Arabidopsis* dehydrin RAB18 localizes to all parts of the embryo and endosperm of mature seeds, it is only found to accumulate in the stomatal guard cells of stems, leaves and flowers (Nylander *et al.*, 2001). Elevated accumulation of dehydrins is correlated with dehydrating conditions such as high salinity, low temperatures and drought. Under such conditions the expression of dehydrins is more ubiquitous, extending to most cells and tissues (Nylander *et al.*, 2001; Rorat, 2006). Immunohistochemical localization studies of several *Arabidopsis* dehydrins have demonstrated

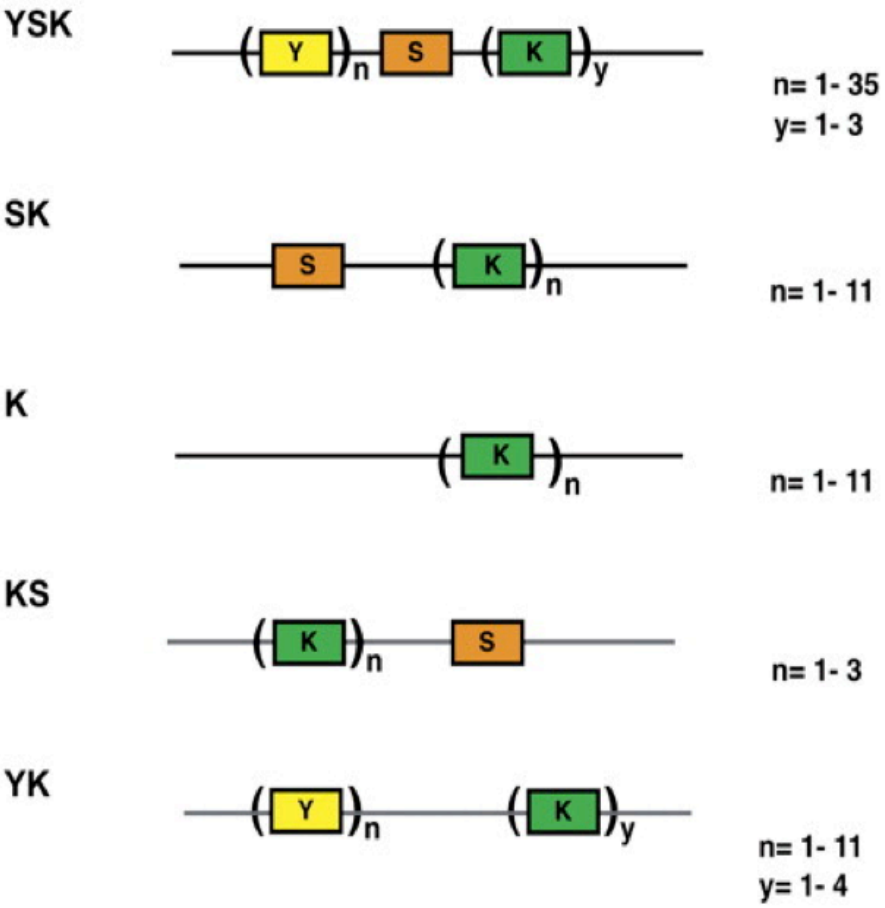

Figure 2.3 Classification of dehydrins (Group 2; D-11 LEA) based on conserved motifs
The five classes Y_nSK_y, SK_n, K_n, K_nS and Y_nK_y are indicated. Y-, S- and K-segments are shown by the yellow, orange and green boxes, respectively. Adapted from Battaglia *et al.* (2008).

high accumulation in the vasculature and surrounding tissues which are the most vulnerable during dehydrating stress conditions (Nylander *et al.*, 2001). While their functional role is not known, dehydrins have been shown to bind proteins and lipids, act as molecular chaperones and cryoprotectants, and to have radical scavenging and metal-binding activity (Hara *et al*., 2001; Heyen *et al.*, 2002; Alsheikh *et al.*, 2003; Bravo *et al.*, 2003; Koag *et al.*, 2003, 2008; Brini *et al.*, 2007; Kovacs *et al.*, 2008; Rahman *et al.*, 2010, 2011). *In vitro* analysis has revealed that each of the five subclasses of dehydrins may display distinct functions (Nylander *et al.*, 2001; Rorat, 2006). For example, the Y_nSK_y-type dehydrins have been found to bind lipids in order to stabilize their structure during dehydrating conditions, while the K_nS-type dehydrins display radical-scavenging and metal-binding activity (Rorat, 2006). SK_n-type dehydrins have also been proposed to have some metal-binding activity, and along with K_n-type dehyrins may participate in protective mechanisms against low temperature stress or are involved in the cold acclimation process (Rorat, 2006). Although no *in planta* evidence has been obtained to date, many *in vitro* studies have suggested possible roles by which dehydrins protect cells against damage caused by dehydration.

Proteins and lipid binding would stabilize vesicles or endomembrane structures and promote protein integrity during stress conditions. This is thought to be accomplished through the K-segment which is predicted to form an amphipathic α-helix with 10 (IMDKIKEKLP) or 12 (GIMDKIKEKLPG) residues of the segment being proposed to form a class A2 amphipathic α-helix, one that has hydrophilic and hydrophobic residues located on opposite faces (Close, 1996). This is analogous to the similar structure found in apolipoproteins, which transport water-insoluble lipids in plasma via the lipid-binding characteristic of the amphipathic α-helices (Close, 1996). Furthermore, similar to apolipoproteins, dehydrins have been shown to increase α-helicity (gain ordered secondary structure) in the presence of helical inducers such as detergents or interaction with lipids (Ceccardi *et al.*, 1994; Ismail *et al.*, 1999; Soulages *et al.*, 2002, 2003; Koag *et al.*, 2003, 2009; Kovacs *et al.*, 2008). This gain of structure indicates that dehydrins may function as an interface between the hydrophobic surfaces of membrane phospholipids and the hydrophilic cytosol in plant cells (Campbell and Close, 1997; Zhang *et al.*, 2006). For example, in maize scutellar parenchyma cells the K-segment of dehydrin DHN1 forms an A2 amphipathic α- helical structure that binds to small lipid vesicles containing acidic phospholipids (Koag *et al.*, 2003; Kovacs *et al.*, 2008). Another analogy can be made with

molecular chaperones, which bind to their target protein via hydrophobic interactions in order to promote proper folding of proteins, prevent protein aggregation and assist in proper refolding of misfolded proteins (Campbell and Close, 1997; Panossian *et al.*, 2009). Therefore, the K-segment of dehydrins may play a critical role in the molecular chaperone activity of dehydrins by interacting with exposed hydrophobic surfaces to prevent protein-protein aggregation during dehydrating or freezing conditions (Campbell and Close, 1997; Zhang *et al.*, 2007). The number of K-repeats is thought to play a critical role in the cryoprotective activity of dehydrins, with most cold-induced dehydrins containing three or more K-repeats (Zhang *et al.*, 2007). During cold stress, dehydrins are proposed to act as cryoprotectants by stabilizing cellular structure and macromolecules, and it is proposed that the amphipathic α-helix formed by the K-segment could interact with exposed hydrophobic patches or lipids in order to prevent further inactivation. The degree of membrane association and putative stabilization is defined by the number of K-segments (Bravo *et al.*, 2003). The citrus dehydrin, CuCOR19, was found to protect catalase and lactate dehydrogenase against freezing inactivation, and the circular dichroism (CD) spectrum for CuCOR19 found the major secondary structure in solution to be a random coil, suggesting that this lack of structure may play an important role in the cryoprotection of enzymes (Hara *et al.*, 2001).

Dehydrins have also been proposed to have radical-scavenging ability and metal-binding activity. Under cold stress, peroxidation and lipid peroxidation causes decreased fluidity in membranes and dehydrins have been proposed to function as radical scavengers in order to protect membrane structures (Matysik *et al.*, 2002; Zhang *et al*., 2007). This has been shown by *in vitro* analysis of transgenic tobacco overexpressing the citrus dehydrin CuCOR19, that enhanced cold tolerance compared to the control due to reduced electrolyte leakage and malondialdehyde production (Zhang *et al.*, 2007). Furthermore, DNA is also considered highly susceptible to radicals, and since dehydrins are known to accumulate in the nucleus, chloroplasts and mitochondria, it is assumed that they protect DNA from oxidative damage under stress conditions (Matysik *et al.*, 2002; Zhang *et al.*, 2007). A metal-binding activity of dehydrins has been proposed to prevent the adverse effects of increasing ionic strength, which occurs due to an increased concentration of metal ions in the cytoplasm from membrane leakage during stress. Several dehydrins capable of binding metal ions have been identified including the citrus dehydrin CuCOR15 which binds copper (Hara *et al.*, 2005), the caster bean

dehydrin ITP which binds iron (Krüger *et al.*, 2002), as well as the celery dehydrin VcaB45 (Heyen *et al.*, 2002) and the *Arabidopsis* dehydrin ERD14 (Alsheikh *et al.*, 2003) that both bind calcium upon phosphorylation. Furthermore, analysis of CuCOR15 found it bound copper through His residues located within a core sequence (HKGEHHSGKDD) found near the N-terminus. This His-X3-His motif has been characterized as a metal-binding site in many metal-binding proteins, and not only do most dehydrins contain a high proportion of His but they also contain the double His sequence and/or the His-X3-His motif, further supporting the notion that dehyrins may be metal-binding proteins (Hara *et al.*, 2005).

In addition, dehydrins form highly stable hydrated gels *in vivo* (Wolkers *et al.*, 2001; Tompa *et al.*, 2006; Mouillon *et al.*, 2008). The intrinsically disordered nature of these proteins allows them to sequester water and sugars in a tightly hydrogen-bonded network to form a gel (Hoekstra *et al.*, 2001; Wolkers *et al.*, 2001; Tompa *et al.*, 2006; Kovacs *et al.*, 2008; Shimizu *et al.*, 2010). For example, nuclear magnetic resonance intensity and differential scanning calorimetry measurements on an *Arabidopsis* dehydrin, ERD10, found it had a high hydration potential and a large ion binding capacity similar to other known IDPs/IUPs (Bokor *et al.*, 2005; Tompa *et al.*, 2006). This suggests that the unstructured nature of dehydrins could aid in preventing water loss and protein denaturation through its ability to bind water and other ions.

2.3 Halophytes

Broadly speaking, a halophyte is a plant that grows in a saline environment. These include semi-deserts, mangrove swamps, marshes, sloughs and seashores. Higher plants in the halophyte category include species of *Atriplex* and *Mesembryanthemum crystallinum*, the salt marsh grass *Spartina alterniflora* (smooth cordgrass) and sea barleygrass (*Hordeum marinum*) to name a few. Relatively few terrestrial plant species, approximately 2%, are halophytes, with the majority of plant species being glycophytes that display a low tolerance to salinity (Radyukina *et al.*, 2007). However, glycophytes are a heterogeneous group and a range of sensitivities can be found in these non-halophyte species.

Halophytes thrive in saline environments because of osmotic adjustment and intracellular compartmentation that partitions otherwise toxic Na^+ and Cl^- ions to the vacuole (Flowers and Clomer, 2008; Ruan *et al.*, 2010). Osmotic adjustment through the accumulation of compatible osmolytes to tolerate the low soil water potential caused by salinity is a common

feature of most glycophytes and halophytes. In fact, halophytes can readily take up Na^+ such that the roots typically have much lower NaCl concentrations than the rest of the plant (Flowers and Clomer, 2008; Ruan *et al.*, 2010). However, halophytes possess a greater capacity to survive salt shock and more readily establish metabolic steady state for growth in a saline environment (Sen and Kasera, 2001). While most plants effectively exclude Na^+ and Cl^- by roots during water uptake, halophytes are able to maintain this exclusion at higher salinities than glycophytes.

The basis of salinity tolerance is still not well understood, despite the fact it has been studied in a variety of glycophytic and halophytic plants (Hasegawa *et al.*, 2000). Unfortunately, most all of the halophytic species are not amenable to genetic analyses. Significant advancements in the area of salinity tolerance have been realized using *Arabidopsis* and the array of genetic resources developed for this genetic model system (Zhu, 2000; Bressan *et al*., 2001). However, these results must be interpreted with caution as *Arabidopsis* is actually a glycophyte which does not exhibit salinity tolerance anywhere near that of a halophyte. *Arabidopsis*, when compared with other species under similar growth conditions, is a salt-sensitive species. Ideally, understanding the exceptional degree of salt tolerance in halophytes requires a genetic model system incorporating the advantages of the *Arabidopsis* model. A close relative of *Arabidopsis* in the genus *Thellungiella* (Bressan *et al.*, 2001; Teusink *et al.*, 2002) satisfies this requirement.

2.3.1 *Thellungiella salsuginea*

Over the past ten years, a small cruciferous plant, commonly known as 'salt cress', has established itself as a new model for research into plant stress tolerance (Bressan *et al.*, 2001; Zhu, 2001; Volkov *et al.*, 2003; Inan *et al.*, 2004; Amtmann *et al.*, 2005; Gong *et al.*, 2005; Wong *et al.*, 2005; Kant *et al.*, 2006; M'rah *et al.*, 2006; Wang *et al.*, 2006; Warwick *et al.*, 2006; Wong *et al.*, 2006). *Thellungiella salsuginea*, synonymous with *Thellungiella halophila* and previously classified as *Arabidopsis halophila*, is a member of the Brassica family and a close relative of *Arabidopsis* (Figure 2.4) (Al-Shebaz *et al.*, 1999).

Thellungiella displays many of the experimental advantages of *Arabidopsis* including a short lifecycle, self-fertility, copious seed production, transformability, small genome (approximately twice the size of the *Arabidopsis* genome) and high sequence similarity to

Arabidopsis (Amtmann, 2009). However, in contrast to *Arabidopsis*, *Thellungiella* is able to grow and reproduce under conditions of extreme cold, drought, and salinity. To date, most studies have utilized the Shandong ecotype of *Thellungiella*, which grows in the high-salinity coastal areas in eastern China, primarily for studies of salinity tolerance mechanisms (Bressan *et al.*, 2001; Inan *et al.*, 2004). The work presented in this thesis was performed with the Yukon ecotype of *Thellungiella*. This ecotype was isolated in the Takhini Salt Flats near Whitehorse in theYukon Territories, Canada, a subarctic and semiarid region (Warwick *et al.*, 2004) characterized by multiple simultaneous abiotic stresses, including cold, drought, and high salinity (Figure 2.4).

Thellungiella (Yukon ecotype) is native to harsh environments, can tolerate salinity as high as 500 mM NaCl and can withstand water losses in excess of 40% of its fresh weight (Inan *et al.*, 2004; Amtmann, 2009), conditions far more extreme than those tolerated by *Arabidopsis*. For instance, prolonged exposure to 100 mM does not allow *Arabidopsis* to complete its life cycle but has no effect on the growth rate of *Thellungiella* (Inan *et al.*, 2004; Kant *et al.*, 2006). The increased salt tolerance of *Thellungiella* over *Arabidopsis* has been attributed to superior ion homeostasis, due in part to the selectivity and regulation of individual ion transporters (Volkov *et al.*, 2003; Kant *et al.*, 2006; M'rah *et al.*, 2006, 2007; Wong *et al.*, 2006; Ghars *et al.*, 2008). In addition, proline increases to higher levels in *Thellungiella* than in *Arabidopsis*, although the mechanistic basis for this between species is still controversial (Inan *et al.*, 2004; Taji *et al.*, 2004; Kant *et al.*, 2006; Ghars *et al.*, 2008).

2.3.2 TsDHN-2

Much work has been performed to investigate the stress responses of *Thellungiella* at the level of gene expression (Taji *et al.*, 2004; Wong *et al.*, 2005, 2006; Kant *et al.*, 2006). Wong *et al.* (2005) compared 6578 ESTs representing 3628 unique genes from cDNA libraries of cold-, drought-, and salinity-stressed plants of the Yukon ecotype and found very little overlap between gene expression in the different conditions. Furthermore, when microarrays spotted with the ESTs were probed with mRNA obtained from stressed plants, a similar pattern was observed (Wong *et al.*, 2006). Out of 154 transcripts that were differentially regulated under conditions of cold, drought and salinity stress, only six of these genes responded to all three stresses. One of these genes was identified as an ortholog of a dehydrin RAB18-related

A.

B.

Figure 2.4 Photographs of the halophytic plant *Thellungiella*
Images are representative of the Yukon ecotype grown in the laboratory (**A.**) or as found in their natural habitat (**B.**). Figure 2.4B reproduced from Amtmann (2009) with permission.

protein from *Arabidopsis* (At5g66400). This unique dehydrin from *Thellungiella*, later named TsDHN-2, showed a 3.4-, 31.3- and 10.4-fold increase in ratio of expression in response to cold, drought and salinity respectively and was identified as a potential target for further study (Wong *et al.*, 2006).

The effect of temperature on interaction of the *Thellungiella* dehydrin TsDHN-2 with membranes has recently been examined using CD and transmission-Fourier transform infrared spectroscopy (Rahman *et al.*, 2010). These investigators used recombinant protein expressed in *Escherichia coli* and demonstrated that ordered secondary structure is induced and stabilized in TsDHN-2 by association with large unilamellar vesicles with similar lipid compositions to that of plant membranes. Low temperatures also seemed to enhance the induced folding supporting a role for TsDHN-2 in membrane stabilization during low temperature stress conditions (Rahman *et al.*, 2010, 2011).

2.4 Thesis Objectives

While the direct role of TsDHN-2 in membrane stabilization has been investigated (Rahman *et al.*, 2010, 2011), there have been no studies specifically examining the physiological role played by this dehydrin in response to salinity. In this thesis, I will examine four silenced RNAi plant lines of the Yukon ecotype of *Thellungiella*, which are thought to have reduced levels of TsDHN-2, to test the hypothesis that this dehydrin confers protection to salinity stress. This will be accomplished by analyzing the level of silencing of *TsDHN-2* and characterizing the phenotypic responses to long-term salinity treatment. Plants will also be assessed for their photosynthetic performance and accumulation of the compatible osmolyte proline. The effect of reduced TsDHN-2 on seed germination in the presence of salt will also be examined. It is hoped that this information will provide an important first step into the elucidation of the role of TsDNH-2 in the stress tolerance mechanisms utilized by *Thellungiella* to thrive under conditions of extreme salinity.

3.0 MATERIALS AND METHODS

3.1 Plant Material

Seeds of WT *Thellungiella salsuginea* (Pall.) O.E. Schulz, Yukon ecotype (Al-Shehbaz *et al.*, 1999; Cody, 2000) and the four *TsDHN-2* RNAi transgenic plant lines (1-1, 5-4, 6-2, and 7-8) were obtained from Dr. Barbara Moffatt (Department of Biology, University of Waterloo) and supplied as T3 homozygote stocks.

3.2 Growth Conditions

Plants of WT *Thellungiella* and four individual *TsDHN2* RNAi transgenic plant lines (1-1, 5-4, 6-2, and 7-8) were grown from seed in controlled environment chambers (Conviron E15; Controlled Environments Ltd., Winnipeg, MB, Canada). Seeds were sown onto the surface in 5 x 15 x 24 plastic trays containing a peat-soil mixture (Sunshine mix; Sun Gro Horticulture, Vancouver, BC, Canada) and grown with a 21/3 h light/dark cycle and day/night temperatures of 22/10°C. Fluorescent lights (Cool White, 215 W, F96T12/CW/VHO; Sylvania, Danvers, MA, USA) provided a photosynthetic photon flux density (PPFD) of 250 μmol photons $m^{-2}s^{-1}$ photosynthetically active radiation. The PPFD was measured at pot height with a Li-Cor (Lincoln, NE, USA) Quantum/Radiometer/Photometer (model LI-189) equipped with a model LI-190SA quantum sensor (Li-Cor). Plants were irrigated every second day with deionized water or nutrient solution (Sommerville and Ogren, 1982). When the plants were 4 weeks old they were subjected to the stress treatment described below.

3.3 Salinity Stress Treatments

Salt-shock treatment was imposed by watering 4-week-old plants with a direct application of 300 mM NaCl once daily which was provided in the irrigation solution. This continued for 14 d. Leaf tissue samples were taken before and during salinity stress treatments, snap frozen in liquid nitrogen and stored at -80ºC until further use.

3.4 Sequence Analyses

The following freely available programs found at the ExPASY Tools homepage were used for analyses: Deduced amino acid sequence was obtained using EMBOSS Transeq

(http://www.ebi.ac.uk/emboss/transeq/; Rice *et al.*, 2000). Primary structure analysis was performed using ProtParam (http://expasy.org/tools/protparam.html; Gasteiger *et al.*, 2005). Amino acid alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/; Chenna *et al.*, 2003; Larkin *et al.*, 2007). The disordered characteristics were examined using the *in silico* prediction method IUPred (http://iupred.enzim.hu/; Dosztanyi *et al.*, 2005a; 2005b)

3.5 Transcript Analysis

3.5.1 RNA Isolation

Total RNA was isolated from 100 mg leaf tissues using the Qiagen RNeasy® Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) following the manufacture's recommendations. Residual DNA was removed by DNase I digestion during RNA purification using an RNase-Free DNase Set (Qiagen) for on-column digestion as described by the manufacturer. Samples were eluted in 30 µL of sterile water. Spectrophotometric quantification of RNA was conducted by measuring the absorbance of the samples at 260 nm with a SmartSpec Plus (Bio-Rad Laboratories, Mississauga, ON, Canada). Samples were stored at -80°C.

3.5.2 cDNA Synthesis

One µg of total RNA was used for first strand cDNA synthesis using the Maxima® First Strand cDNA Synthesis Kit (Fermentas Inc.; Burlington, ON, Canada) as described by the manufacturer. RNA was combined with 4 µL of 5X Reaction Mix, 2 µL Maxima® Enzyme Mix, and brought to a final volume of 20 µL with nuclease-free water. Samples were then heated at 25°C for 10 min followed by 15 min at 50°C. The reaction was terminated by heating samples at 85°C for 5 min. All incubations occurred using a thermocycler (iCycler; Bio-Rad). Samples were stored at -80°C. The single strand cDNA was then used as template DNA in the PCR described below (section 3.5.3).

3.5.3 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Amplification of cDNA was performed using gene-specific primers for *Thellungiella TsDHN-2* (Appendix A; Table 3.1) and the *Arabidopsis* reference gene *ACTIN7* (At5g09810; GenBank accession No. NM_121018; Table 3.1). Primers were synthesized commercially (Alpha DNA; Montreal, QC, Canada). The 50 µL PCR contained: 5 µL cDNA, 25 µL 2X

DreamTaq™ PCR Master Mix (Fermentas), 1 µM of each forward and a reverse primer and RNase free water to volume. The following cycling conditions were used: 1) 94°C for 3 min; 2) 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min and 3) 72°C for 10 min. All steps were carried out in an iCycler thermocycler (Bio-Rad).

3.5.4 Agarose Gel Electrophoresis

PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Samples were mixed with 6X Orange Loading Dye Solution (Fermentas) prior to loading on the gel. The DNA fragments were separated by electrophoresis through a 1% (w/v) agarose gel in 1X TAE buffer (40 mM Tris acetate [pH 8.0], 1 mM EDTA). Electrophoresis was conducted at 100V for 1 h. Ethidium bromide (0.5 µg/mL) was added to the gel, which allowed visualization of the DNA under short-wave UV-B light using a gel documentation system (Gel Doc 2000; Bio-Rad). The images were stored electronically. The sizes of the DNA fragments were estimated by comparing them to standards of known size (O'GeneRuler 1kb DNA Ladder; Fermentas) which were loaded in adjacent lanes. Gel Doc 2000 software (Quantity One, version 4.2.3; Bio-Rad) was used to calculate average band density measurements, which were expressed as the ratio of the target gene product (*TsDHN-2*) band density to the reference gene product (*ACTIN7*) band density.

3.6 Photosynthetic Measurements

Chlorophyll *a* fluorescence imaging was used to determine the photochemical efficiency of PSII ($[F_m-F_o)/F_m] = F_v/F_m$). Images were captured *in planta* at room temperature using a commercially available modulated imaging fluorometer (FluorCam; Photon System Instruments, Brno, Czech Republic), as described in detail previously (Gray *et al.*, 2003; Baerr *et al.*, 2005). Image data were normalized to a false colour scale which resulted in the highest and lowest F_v/F_m values being represented by the red and blue extremes of the colour scale, respectively.

Table 3.1. Oligonucleotide primers used for RT-PCR

Gene	Primer	Sequence (5'→3')	Expected fragment size
TsDHN-2	TsDHN-2F	ATCCGGATCCAGCTCTAGC	474 bp
	TsDHN-2R	CATCGCAGGACGTAGAGAC	
ACTIN7	ACT7F	GATATTCAGCCACTTGTCTGTGAC	211 bp
	ACT7R	CATGTTCGATTGGATACTTCAGAG	

F, forward primer; R, reverse primer.

3.7 Proline Determination

Free proline content was measured according to the method described by Bates *et al.*, (1973) which is specific for the imino group of proline. Leaf tissue (0.5 g) from 4 week control and salt-stressed plants was ground with a motar and pestle in 3% (v/v) aqueous sulfosalicylic acid and the homogenate filtered through Whatman #2 filter paper. The filtrate (2 mL) was added to 2 mL of acid-ninhydrin (1.25 g ninhydrin, 30 mL glacial acetic acid, 20 mL 6 M phosphoric acid) and 2 mL of glacial acetic acid in a glass test tube for 1 h at 100°C, and the reaction was terminated in an ice bath. Toluene (4 mL) was added and the mixture was vortexed for 20 s. The upper aqueous phase containing the chromophore was removed and its absorbance determined at 520 nm (SmartSpec Plus; Bio-Rad). Proline concentration was determined from a standard curve (0 - 250 μg/mL) constructed with L-proline (Sigma-Aldrich; St. Louis, MO, USA) and expressed on a fresh weight basis.

3.8 Seed Germination Studies

3.8.1 Seed Sterilization

Seeds of WT *Thellungiella* and the four RNAi lines were surface sterilized in a microcentrifuge tube containing 5.25% (v/v) sodium hypochlorite (Javex) and 0.05% (v/v) Tween-20). The tubes were vortexed for 30 s and allowed to sit for 7 min. Seeds were then washed 5 times with sterile water.

3.8.2 Germination Tests

Media plates for germination studies were prepared using Murashige and Skoog basal medium (Murashige and Skoog, 1962; Sigma) and 0.7% (w/v) Phytagel (Sigma) in deioized water. Following autoclaving the media was poured into sterile plastic 9-cm Petri dishes. The solidified media plates were stored at 4°C until use. In addition, plates were also prepared and supplemented with a final concentration of 100, 200 and 500 mM NaCl using a 5 M NaCl stock solution.

Following sterilization (section 3.8.1), seeds were plated with sterile toothpicks onto the Petri dishes at a density of 25 seeds per plate and stratified for 2 d in the dark at 4°C in a controlled environment chamber (E8; Conviron). Plates were removed and placed into a Sanyo environmental test chamber (MLR-350HT; Sanyo, Japan) at 20°C with constant light (120

μmol photons m^{-2} s^{-1}) and germination was monitored daily for 14 d. Seeds were considered germinated when the radical had completely penetrated the seed coat. This experiment was replicated three times.

4.0 RESULTS

4.1 Sequence Analysis

The full-length cDNA sequence of *Thellungiella TsDHN-2* was obtained from Dr. Barbara Moffatt (University of Waterloo) (unpublished results) and was generated from overlapping EST sequences previously deposited in GenBank (Wong *et al.*, 2005). The deduced amino acid sequence was obtained using EMBOSS Transeq. These data are presented in Appendix A. Amino acid sequence analysis of TsDHN-2 using ProtParam revealed that, similar to other plant dehydrins, TsDHN-2 is hydrophilic, rich in glycine residues (30.2%) and contains no cysteine or tryptophan. TsDHN-2 contains 215 amino acids and is a basic dehydrin with a theoretical pI of 7.91 and predicted molecular mass of 21.4 kD. The alignment of TsDHN-2 with several previously characterized dehydrins of various classes is shown in Figure 4.1. Comparison to *Arabidopsis* RAB18 revealed a 71% amino acid sequence identity and 74% amino acid similarity of *Thellungiella* TsDHN-2 (Figure 4.1). Furthermore, based on the YSK nomenclature scheme developed by Close and co-workers (Close, 1997), sequence analysis confirmed that TsDHN-2 is a Y_2SK_3 dehydrin with three conserved lysine-rich K-segments (EKKGMMDKIKDKLPG) located near the C-terminus, a single S-segment containing the conserved serine tract repeat, and two Y-segments (DEYGNP) near the N-terminus (Figure 4.1).

4.2 Prediction of Protein Disorder

Many dehydrins are IDPs/IUPs (Koag *et al.*, 2003; Kovacs *et al.*, 2008). TsDHN-2 has been proposed to be an IDP/IUP and this disordered characteristic was supported by *in silico* analysis using the prediction method IUPred (Dosztanyi *et al.*, 2005a; 2005). IUPred is a prediction algorithm for recognizing ordered and disordered regions in proteins based on estimating the capacity of polypeptides to form stabilizing contacts. Presumably, globular proteins are composed of amino acids that have the potential to form a number of favorable interaction, while the amino acid composition of intrinsically unstructured proteins do not, and therefore do not adopt a stable structure (Dosztanyi *et al.*, 2005a; 2005). Proteins that are highly disordered will score above 0.5, which is the threshold separating disordered from ordered regions in protein (Dosztanyi *et al.*, 2005a; 2005).

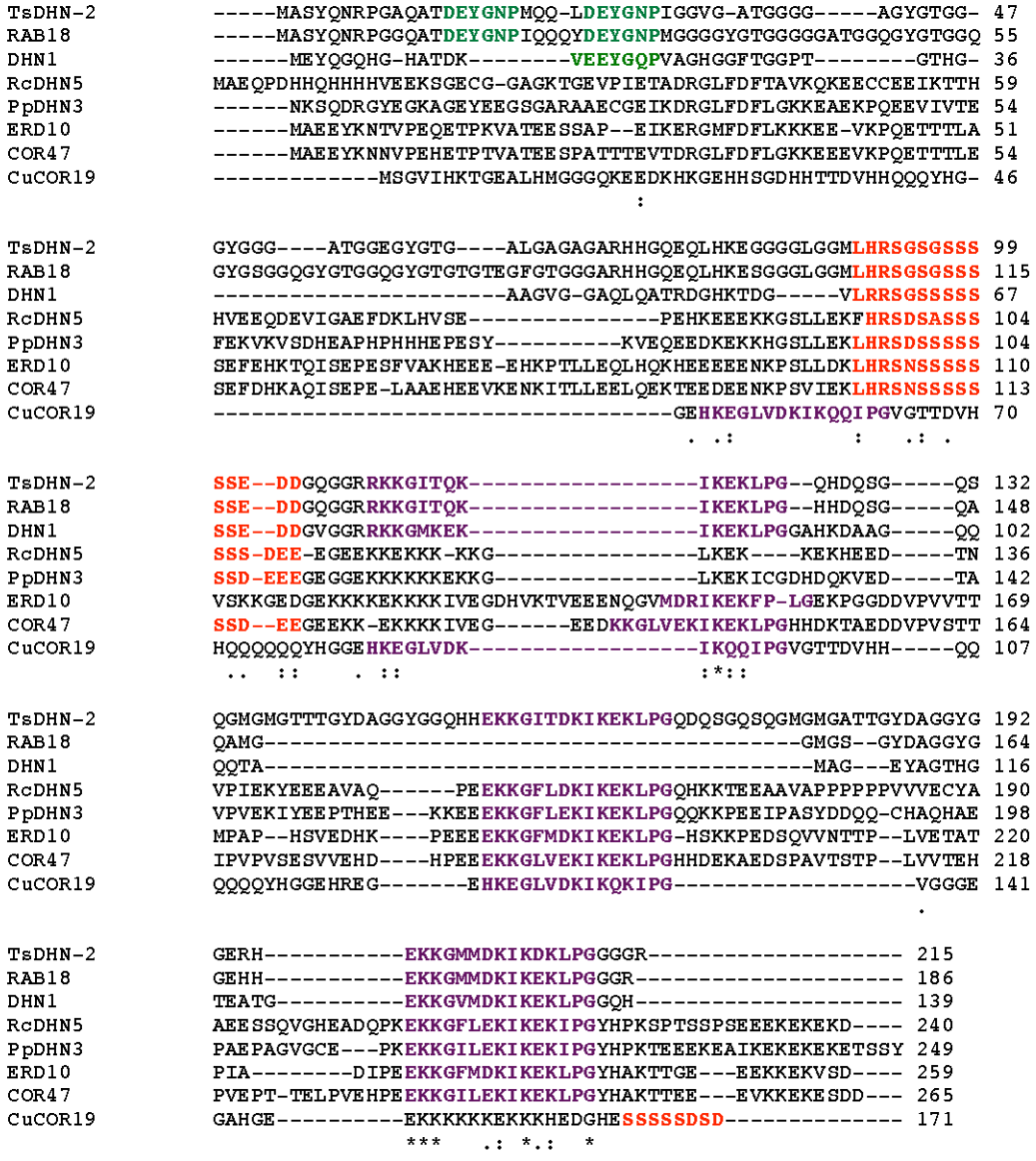

Figure 4.1. Alignment of *TsDHN-2* deduced amino acid sequence
Thellungiella TsDHN-2 (Appendix A) was aligned with the following known dehydrins of various classes; *Arabidopsis* RAB18 (GenBank accession No. CAA48178.1), Barley DHN1 (GenBank accession No. P12951.1), Rhododendron RcDHN5 (GenBank accession No. ACB41781.1), Peach PpDHN3 (GenBank accession No. AAZ83586.1), *Arabidopsis* ERD10 (GenBank accession No. NP_564114.2), *Arabidopsis* COR47 (GenBank accession No. BAA23547.1 and Citrus CuCOR19 (GenBank accession No. BAA74736.1). Conserved Y- (green), S- (red), and K-segments (purple) are indicated. Asterisks (*), colons (:) and periods (.) indicate identical residues, conserved substitutions and semi-conserved substitutions, respectively. Dashes indicated where gaps have been introduced to allow optimal sequence alignment. Sequences were aligned using ClustalW.

As seen in Figure 4.2, *in silico* prediction of TsDHN-2 confirmed the disordered characteristic of this dehydrin, scoring above 0.5 over the entire sequence as did *Arabidopis* RAB18, a known disordered dehydrin used as a positive control (Figure 4.2). In contrast, BSA, a known globular protein used as a negative control scored below 0.5 over its amino acid sequence (Figure 4.2).

4.3 Response of TsDHN-2 to Salinity

4.3.1 Transcript Accumulation

The expression levels of *TsDHN-2* in WT *Thellungiella* and RNAi lines 1-1, 5-4, 6-2, and 7-8 were estimated by RT-PCR performed on leaf tissue before and after salinity stress treatment (Figure 4.3). Under normal growth conditions, expression of *TsDHN-2* was undetectable in WT and all of the RNAi lines (Figure 4.3A). Following salinity treatment for 14 days, the expression of *TsDHN-2* was induced in all of the material examined, with the primers robustly amplifying a distinct DNA product at approximately 474 bp when analyzed by agarose gel electrophoresis (Figure 4.3A). A 211 bp fragment of *ACTIN7* was also amplified and detected in all samples and treatments at approximately the same levels (Figure 4.3B). However, the level of induction of *TsDHN-2* varied with lines 1-1 and 7-8 demonstrating reduced amounts of transcript in comparison to WT and lines 5-6 and 6-2 expressing comparable levels as WT (Figure 4.3A). Based on band density measurements, expressed as the ratio of *TsDHN-2* (Figure 4.3A) to *ACTIN7* (Figure 4.3B), it was estimated that lines 1-1 and 7-8 exhibit a 28 and 42% reduction respectively in *TsDHN-2* in comparison to WT.

4.3.2 Phenotypic Responses

The phenotypic responses the WT and TsDHN-2 RNAi lines were recorded before, and 7 and 14 days after salinity treatment, and representative photographs are shown in Figure 4.4. All material appeared healthy prior to salt treatment (control) and no effects were observed after 24 hours of stress initiation (Figure 4.4). However, symptoms, in the form of chlorotic and necrotic leaves are obvious after 7 days (Figure 4.4). These symptoms progressed and were readily apparent at the 14 day mark (Figure 4.4).

In an attempt to quantify the visual observations, the % viable leaves were calculated at each time point for WT and RNAi lines (Table 4.1). This value was determined as the

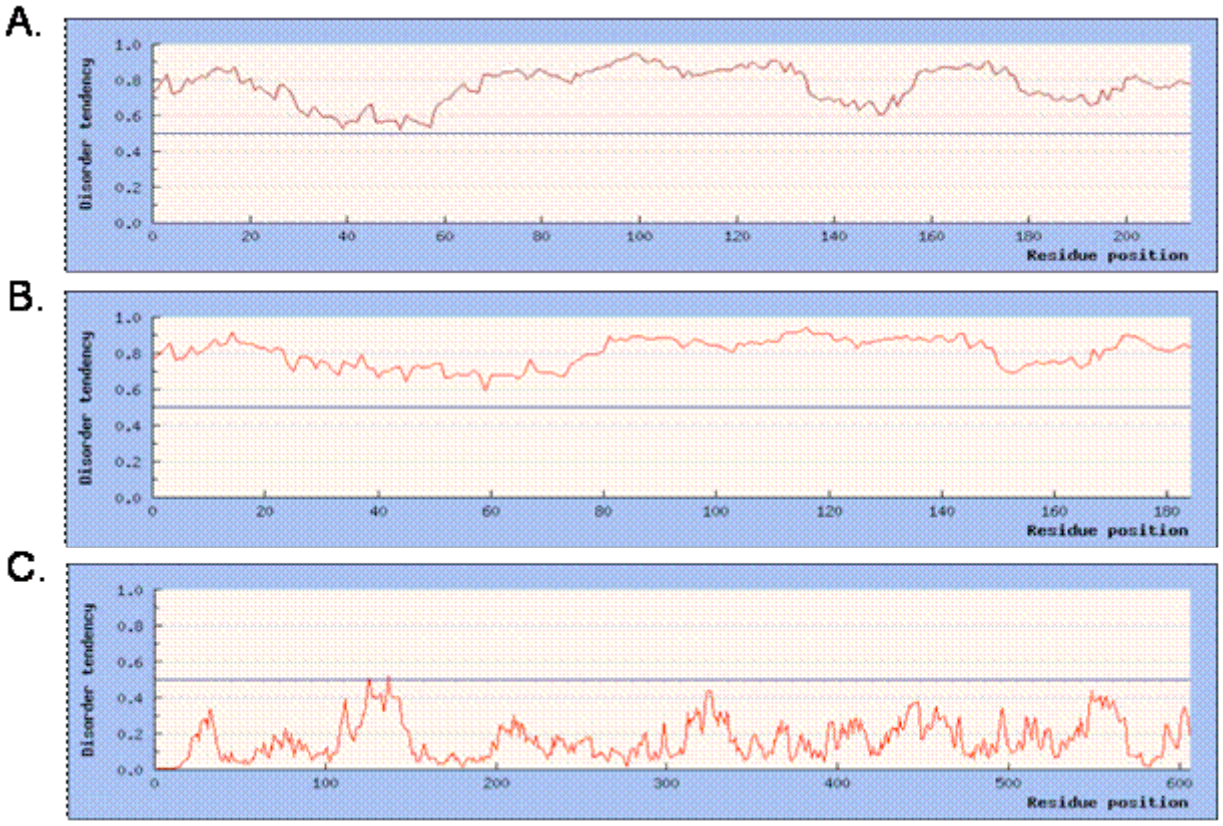

Figure 4.2. Prediction of protein disorder in TsDHN-2
IUPred analysis of the amino acid sequences of TsDHN-2 (**A.**), RAB18 (**B.**) and BSA (**C.**). Scores range from 0 to 1 with values above 0.5 (blue line) suggestive of a disordered structure. Sequences used are: TsDNH-2, Appendix A; RAB18, GenBank accession No. CAA48178.1; BSA, GenBank accession No. AAA51411.1).

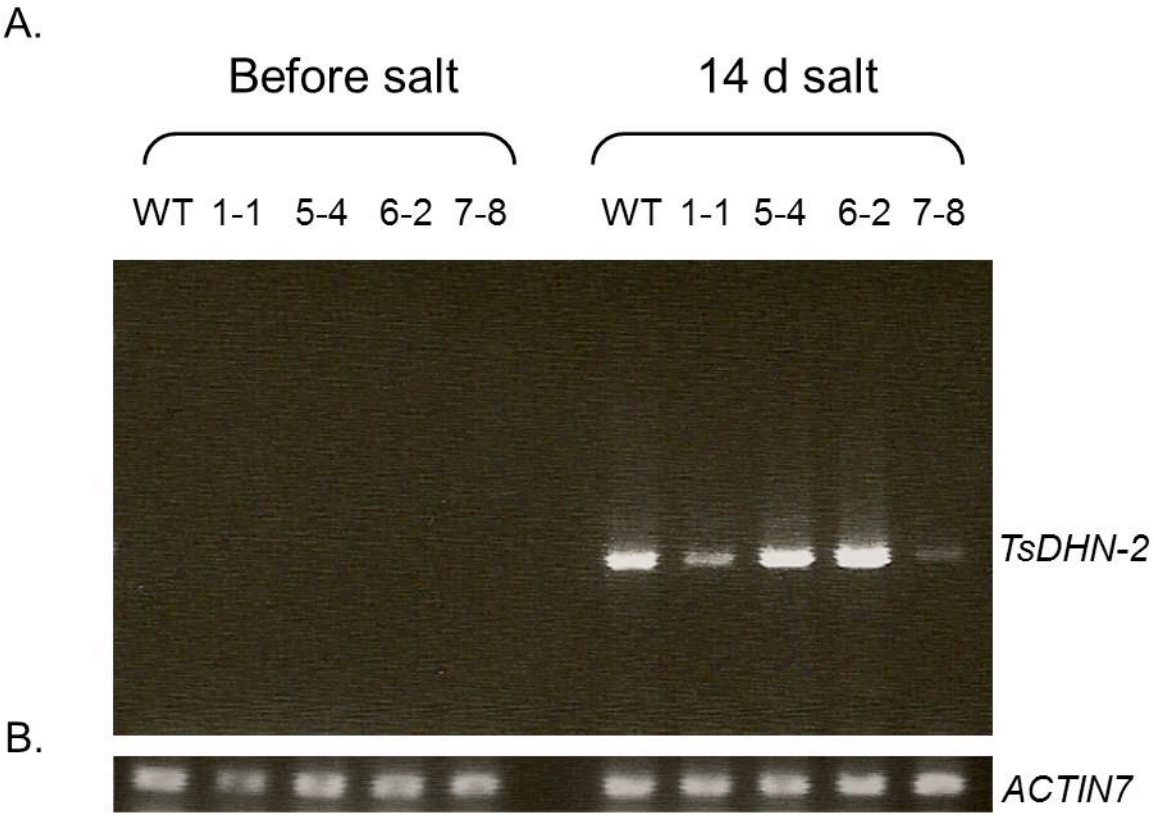

Figure 4.3. Abundance of *TsDHN-2* in leaves of *Thellungiella* in response to salinity stress
Plants of WT *Thellungiella* and TsDHN-2 RNAi lines were subjected to a salt stress of 300 mM NaCl for 14 days as indicated. A 474 bp fragment of *TsDHN-2* (**A.**) was amplified using PCR and analyzed by agarose gel electrophoresis. A 211 bp fragment of *ACTIN7* (**B.**) was also amplified and used for normalization purposes. A representative photo is shown from a minimum of 5 independent measurements.

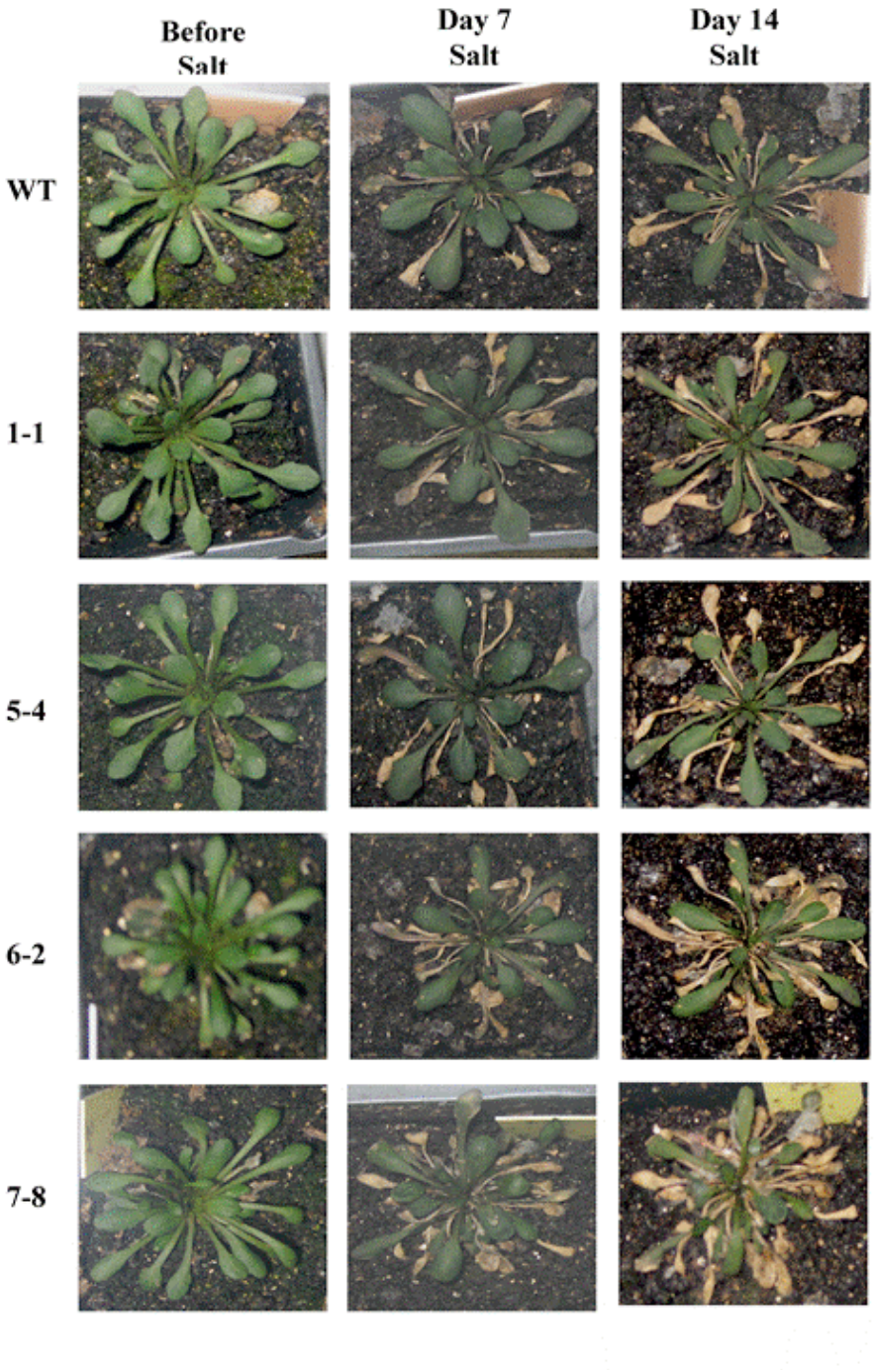

Figure 4.4. Phenotypic responses of *Thellungiella* to salinity stress
Photographs were obtained before and after 7 d and 14 d salt stress in WT *Thellungiella* and TsDHN-2 RNAi lines as indicated. Plants were subjected to 300 mM NaCl for the duration of the experiment. Representative photographs are shown from ten plants for each line and are the exact same plants analyzed in Figure 4.3. All photographs are shown in Appendix B.

Table 4.1. Leaf viability in response to salinity in *Thellungiella*[1]
Plants of WT *Thellungiella* and TsDHN-2 RNAi lines were subjected to 300 mM NaCl for the duration of the experiment. Values represent means ± SD, n = 10.

Viability (%)[2]	Line				
	WT	1-1	5-4	6-2	7-8
Before salt	86.9 ± 3.9	84.3 ± 3.0	82.7 ± 2.0	84.4 ± 2.7	82.3 ± 2.0
7 d salt	64.9 ± 15.0	65.1 ± 21.2	61.2 ± 15.5	60.8 ± 20.8	57.8 ± 15.2
14 d salt	46.9 ± 10.1	45.4 ± 20.2	47.1 ± 10.4	44.1 ± 13.0	41.5 ± 12.8

[1]Values and calculations are based on the data presented in Appendix B.
[2]Viabiliy was calculated as the number of green leaves remaining (> 55% green) and expressed as a percentage of the total leaves present.

number of leaves remaining >55% green as a percentage of the total leaves present. After 7 days of 300 mM salt treatment all of the plants showed a decrease in viability of 19-24% depending on which line was examined (Table 4.1). Viability continued to decrease and at day 14 of the salt treatment the viable leaves represented only 47% of the total in WT and 45, 47, 44, and 42% in the RNAi lines 1-1, 5-4, 6-2 and 7-8 respectively (Table 4.1). These values are consistent with the visual observations in Figure 4.4 and Appendix B.

4.3.3 Photosynthetic Responses

Light absorbed by chlorophyll drives photosynthesis but can also be dissipated as heat or re-emitted as fluorescence. These are competing processes and therefore changes in fluorescence reflect changes in photosynthetic function (Krause and Weis, 1991; Baker, 2008). Chlorophyll fluorescence measurements provide a sensitive, rapid and non-invasive method for the characterization of photosynthetic responses (Bolhàr-Nordenkampf and Öquist, 1993; Schreiber *et al.*, 1994). A useful measurement is the photochemical efficiency of PSII (F_v/F_m). A decrease in F_v/F_m is a reliable indicator of abiotic stresses, which can directly or indirectly, affect the photosynthetic characteristics of the leaves and alter their fluorescence properties (Krause, 1988; Ögren 1991). Furthermore, when combined with a charge-coupled device camera, chlorophyll fluorescence can be imaged, thus allowing the spatial visualization of photosynthetic processes over whole plants (Gray *et al.*, 2003).

The fluorescence images shown in Figure 4.5 and Appendix B were used to generate the values presented in Table 4.2. Prior to salt treatment, the WT and TsDHN-2 RNAi lines all had similar F_v/F_m values ranging from 0.76 ± 0.02 to 0.78 ± 0.01 which are indicative of healthy unstressed plants (Table 4.2; Figure. 4.5; Baker, 2008). Following 24 h and up to 7 days of salinity stress treatment, F_v/F_m remained virtually unchanged (Table 4.2; Figure 4.5). However, 14 days of salinity stress resulted in reductions in F_v/F_m from 6.6 to 15.4% depending on plant line (Table 4.2; Figure 4.5). The greatest reduction was observed in line 7-8 (15.4%) while WT decreased by only 9.2% (Table 4.2; Figure 4.5; Appendix B).

4.3.4 Proline Accumulation

Proline is a compatible osmolyte which frequently accumulates during salt stress in many species, including *Thellungiella* (Tamayo and Bonjoch, 2001; Inan *et al.*, 2004; Ghars *et*

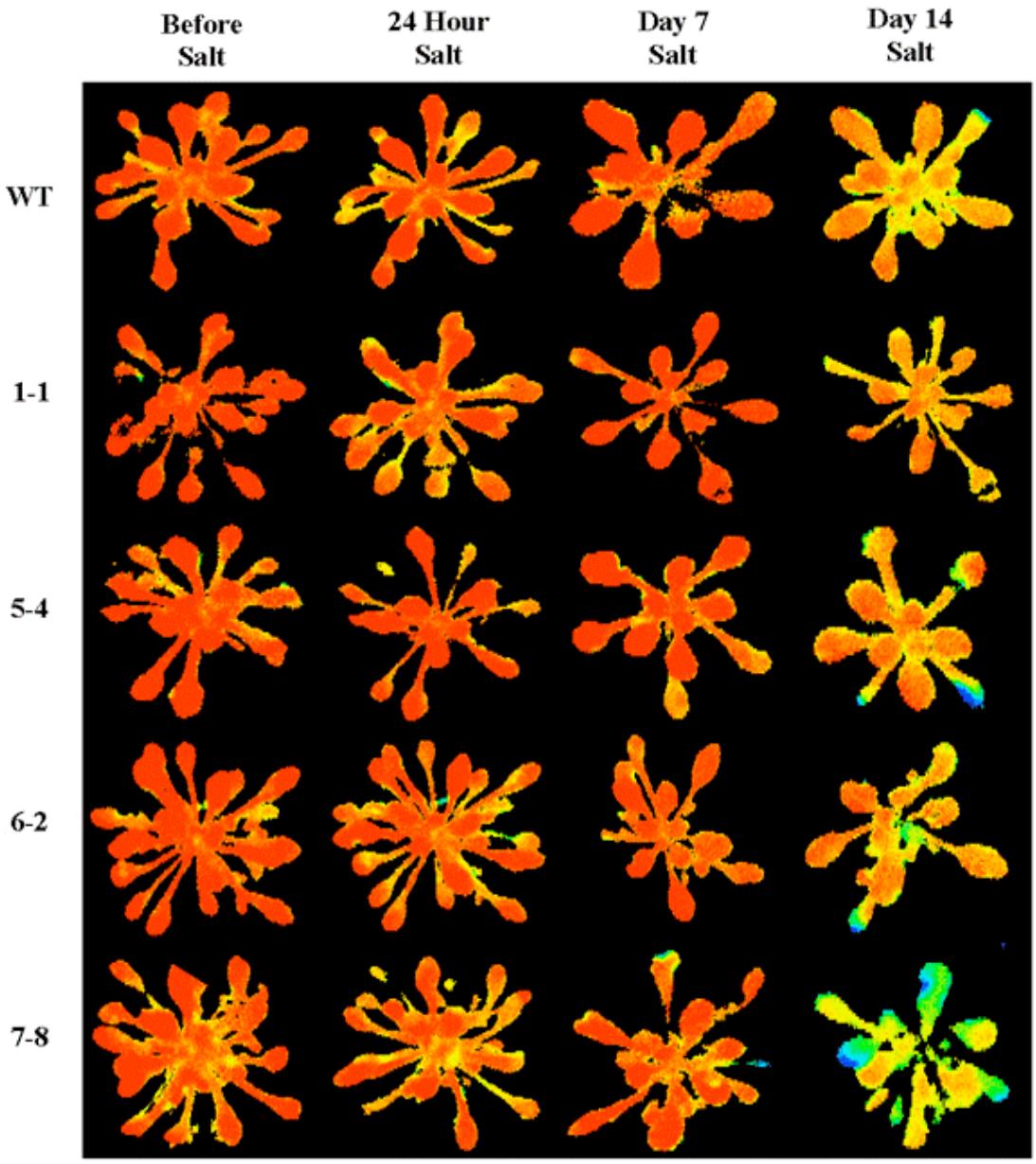

Figure 4.5. Photosynthetic responses to salinity in *Thellungiella*
Images of the photochemical efficiency of PSII (F_v/F_m) were obtained before and after 24 h, 7 d and 14 d salt stress in WT *Thellungiella* and TsDHN-2 RNAi lines as indicated. Plants were subjected to 300 mM NaCl for the duration of the experiment. Representative photographs are shown from ten plants for each line. All images are shown in Appendix B.

Table 4.2. Photosynthetic responses to salinity in *Thellungiella*[1]
Plants of WT *Thellungiella* and TsDHN-2 RNAi lines were subjected to 300 mM NaCl for the duration of the experiment. Values represent means ± SD, n = 10.

F_v/F_m[2]	Line				
	WT	1-1	5-4	6-2	7-8
Before salt	0.76 ± 0.02	0.76 ± 0.02	0.77 ± 0.02	0.77 ± 0.02	0.78 ± 0.01
24 h salt	0.77 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	0.76 ± 0.02
7 d salt	0.76 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	0.77 ± 0.01
14 d salt	0.69 ± 0.03	0.71 ± 0.03	0.67 ± 0.06	0.70 ± 0.02	0.66 ± 0.05

[1]Values and calculations are based on the data presented in Appendix B.
[2]The photochemical efficiency of PSII (F_v/F_m) was determined using chlorophyll fluorescence imaging.

al., 2008; Amtmann, 2009; Szabados and Savouré, 2010). The accumulation of this compound was determined in WT and TsDHN-2 RNAi lines before and after a 300 mM salt treatment for 7 days (Figure 4.6). Prior to salt treatment the RNAi lines presented proline values which were either the same or slightly greater (1.5- to 2-fold) than those observed in WT (Figure 4.6). While WT proline levels increased 3.4-fold in response to salt treatment, accumulation in RNAi lines 1-1, 5-4, and 6-2 was 4.1-, 4.9- and 4.2-fold respectively. The final proline levels attained in these lines ranged from 1.2- 2.7-fold greater than the increase observed in WT (Figure 4.6). The greatest proline accumulation was seen in RNAi line 7-8 with an 8.1-fold increase in response to salt treatment, which was 3.6-fold greater the increased observed than WT (Figure 4.6).

The phenotypic responses of the WT and TsDHN-2 RNAi lines were recorded before, after 24 hours and 7 days of salinity treatment and these photographs are shown in Figure 4.7 and Appendix C. All material appeared healthy prior to salt treatment and no effects were observed after 24 hours of initiating the stress (Figure 4.7). However, symptoms, in the form of chlorotic and necrotic leaves were readily apparent at the 7 day mark (Figure 4.7).

The % viable leaves were calculated at each time point for the WT and RNAi lines (Table 4.3). After 24 h of 300 mM salt treatment, all of the plants showed a minimal decrease in viability (1-8%; Table 4.3). Viability continued to decrease and at day 7 of the salt treatment the viable leaves represented 50% of the total in WT and 47, 44, 45, and 53% in the RNAi lines 1-1, 5-4, 6-2 and 7-8 respectively, with the greated decrease observed in line 7-8. (Table 4.3). These values were consistent with the visual observations in Figure 4.7.

4.4 Seed Germination

In order to establish a baseline germination for WT *Thellungiella*, seeds were plated on media containing 0, 100, 200 and 500 mM NaCl. After 14 days the percentage germination values were 92% in the absence of salt and decreased to 40 ± 4% and 11 ± 2% in the presence of 100 amd 200 mM NaCl, respectively (Figure 4.8). Germination rates assessed at 500 mM were < 2% (data not shown). Based on these data, the germination of the RNAi lines was evaluated at 100 and 200 mM NaCl as further increases in salt concentration essentially proved to be lethal for WT *Thellungiella*.

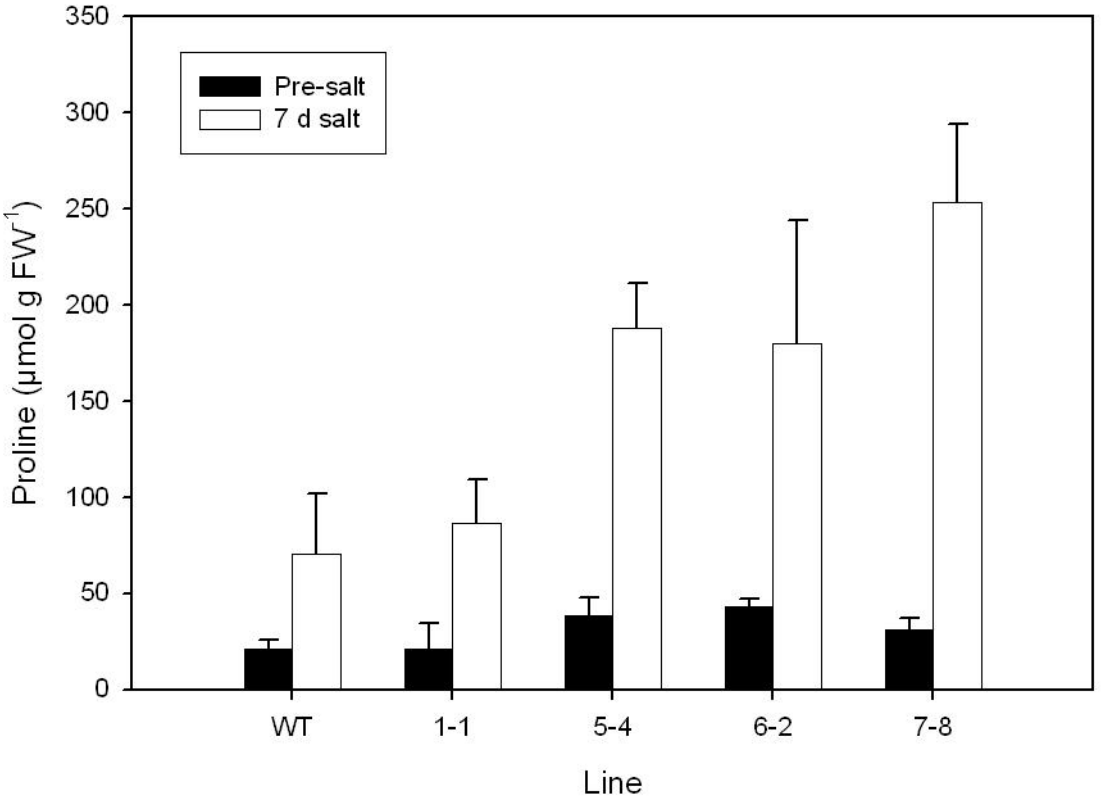

Figure 4.6. Proline accumulation in leaves of *Thellungiella* in response to salinity
Proline was determined before (black bars) and after (white bars) salt stress in WT *Thellungiella* and TsDHN-2 RNAi lines as indicated. Plants were subjected to 300 mM NaCl for 7 days prior to measurement. Values represent means ± SD, $n = 6$.

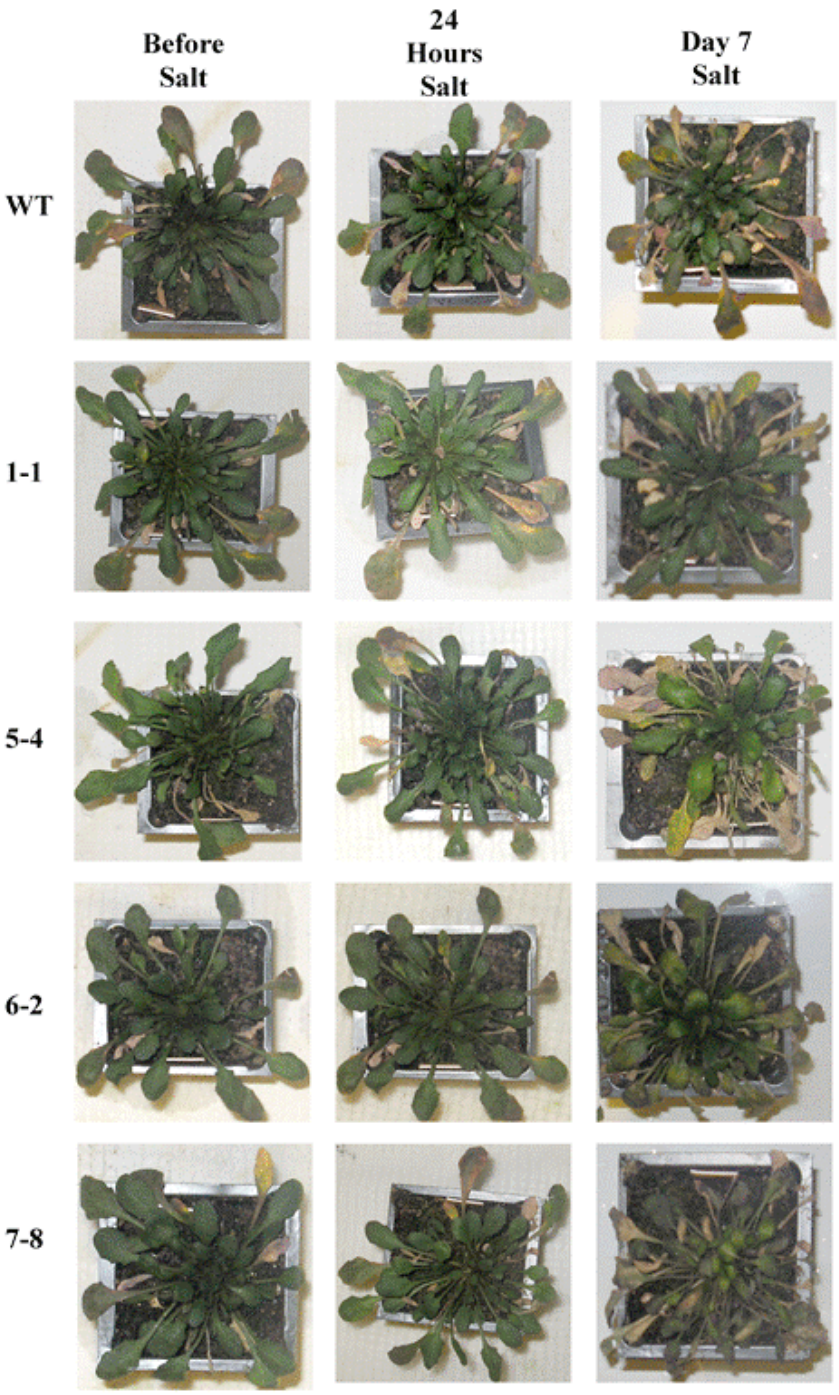

Figure 4.7. Phenotypic responses of *Thellungiella* to salinity during proline experiments Plants of WT *Thellungiella* and TsDHN-2 RNAi lines were subjected to 300 mM NaCl for the durations indicated. Representative photographs are shown from six plants for each line. All photographs are shown in Appendix C.

Table 4.3. Leaf viability in response to salinity in *Thellungiella* during proline experiments[1]

Plants of WT *Thellungiella* and TsDHN-2 RNAi lines were subjected to 300 mM NaCl for the duration of the experiment. Values represent means ± SD, $n = 6$.

Viability (%)[2]	Line				
	WT	1-1	5-4	6-2	7-8
Before salt	74.7 ± 5.4	77.3 ± 4.6	79.7 ± 3.7	78.8 ± 7.5	79.4 ± 6.5
24 h salt	69.6 ± 6.6	72.8 ± 3.8	72.1 ± 5.1	78.4 ± 7.4	74.4 ± 7.0
7 d salt	50.4 ± 5.5	47.0 ± 7.4	43.7 ± 5.5	45.4 ± 4.4	53.4 ± 9.2

[1]Values and calculations are based on the data presented in Appendix C.

[2]Viabiliy was calculated as the number of green leaves remaining and expressed as a percentage of the total leaves present.

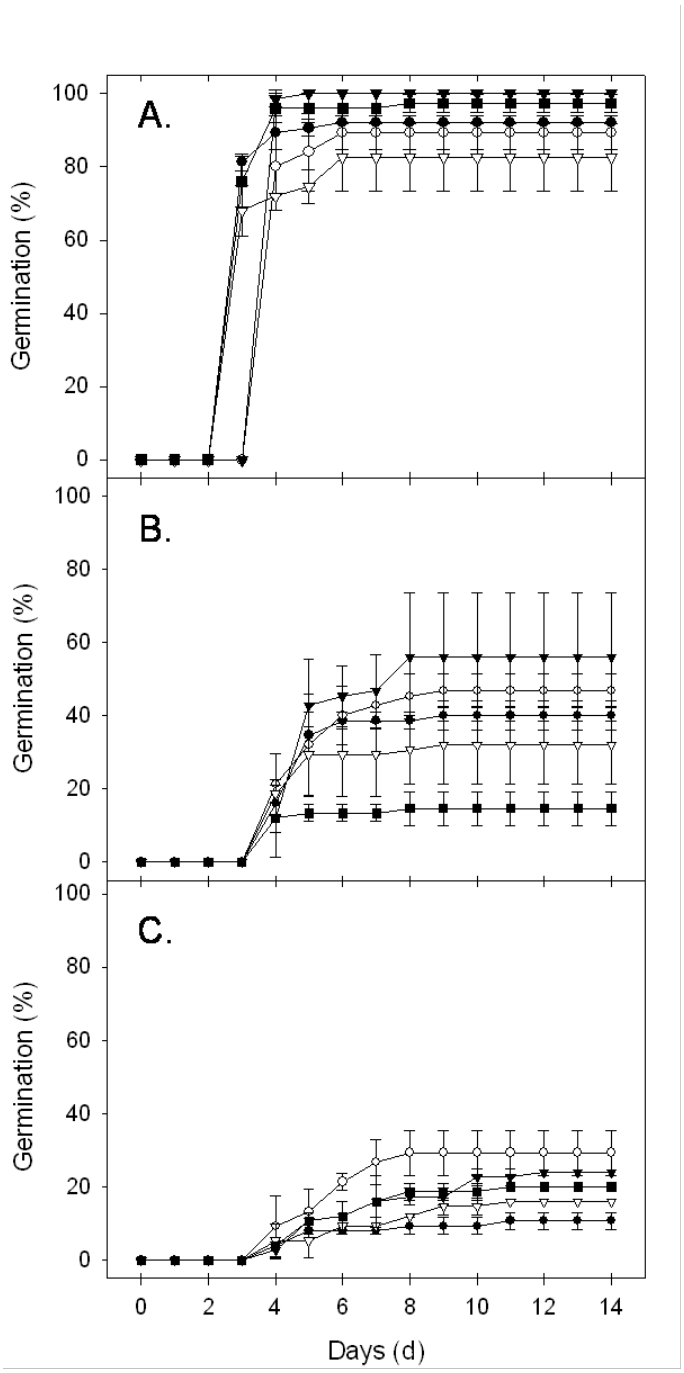

Figure 4.8. Seed germination of *Thellungiella* in response to salinity
Seeds of WT *Thellungiella* (●) and TsDHN-2 RNAi lines 1-1 (○), 5-4 (▼), 6-2 (▽) and 7-8 (■) were sown on MS medium containing 0 (**A.**), 100 (**B.**) or 200 mM (**C.**) NaCl and germination recorded daily. Values represent means ± SD, $n = 3$. When not present error bars are smaller than symbol size.

Under germination conditions free from salt, both WT and all RNAi lines had similar values for percentage germination ranging from 83 to 100% (Figure 4.8A). Interestingly, RNAi lines 1-1 and 5-4 showed a lag in germination by one day (Figure 4.8A). Increasing the concentration of NaCl in the media to 100 and 200 mM resulted in germination of 15 to 56% and 16 to 30% reduction in germination, respectively, for all lines tested (Figure 4.8B and C). In comparison to WT, the RNAi lines showed variable results at each of the NaCl concentrations examined. At 100 mM NaCl the RNAi lines 1-1 and 5-4 had increased germination (1.2- to 1.4-fold respectively), while lines 6-2 and 7-8 had decreased germination (20 and 60% respectively; (Figure 4.8B). In contrast, at 200 mM all RNAi lines had increased germination compared to WT, ranging from 1.5- to 2.6-fold (Figure 4.8C).

5.0 DISCUSSION

This project focused on the role of TsDHN-2 during salinity tolerance in *Thellungiella.* Four silenced lines, created with a RNAi construct against *TsDHN-2*, were obtained from Dr. Barbara Moffatt (University of Waterloo). These lines were obtained as T3 homozygotes for the reduced expression of *TsDHN-2*. Work began immediately to increase seed stock and to verify the reduced expression of *TsDHN-2* in all lines by RT-PCR. Throughout the course of this work, it was discovered that either through seed bulking error or error in the segregation process, only one RNAi line, line 7-8, was deemed to be a true silenced line with reduced *TsDHN-2* expression compared to WT *Thellungiella*. Therefore, this discussion will only focus on the results for WT *Thellungiella* and the RNAi line 7-8 in regards to the role of TsDHN-2 in the salinity tolerance of *Thellungiella.*

5.1 Reduced Expression of *TsDHN-2* Enhances Susceptibility to Salinity

Many studies have reported a positive correlation between the accumulation of dehydrins and salinity tolerance (Xu *et al.*, 1996; Nylander *et al.*, 2001; Du *et al.*, 2011). Furthermore, the over-expression of dehydrins has been found to improve salinity tolerance in transgenic *Arabidopsis* (Brini *et al.*, 2007) and tobacco (RoyChoudhury *et al.*, 2007; Xu *et al.*, 2008). Conversely, reducing the expression of dehydrins has been found to result in salt susceptibility in moss (Saavedra *et al.*, 2006) and *Arabidopsis* (Hundertmark *et al.*, 2011). Transcript analysis revealed that the RNAi line 7-8 demonstrated a 42% reduction in expression of *TsDHN-2* compared to WT *Thellungiella*. Upon treatment with 300 mM NaCl, greater phenotypic damage was observed (wilting, drying of old leaves and necrosis of young leaves) in RNAi line 7-8 indicating that TsDHN-2 plays a role in salinity tolerance. TsDHN-2 is a Y_2SK_3 dehydrin, and this class of dehydrins has been proposed to act by stabilizing membranes (Rorat, 2006; Zhang *et al.*, 2007). Recent work by Ranham *et al.* (2010, 2011) found that TsDHN-2 underwent partial ordering upon association with membranes and this ordered secondary structure is significantly enhanced by further membrane- and/or zinc-association. This suggests that TsDHN-2 may function in *Thellungiella* by interacting and stabilizing cellular membranes in conditions causing dehydration, such as salinity.

5.2 Salinity Decreases Photosynthetic Activity

Reduced growth rates observed in plants subjected to salinity stress is often associated with a decrease in their photosynthetic activity. High soil salinity results in dehydrating conditions, causing plants to close their stomata in order to conserve water. This restricts the entry of CO_2 into the leaf, thus reducing photosynthesis (Sudhir and Murthy, 2004; Munns and Tester, 2008; Hichem *et al.,* 2009; Stepien and Johnson, 2009). Photosystem II is considered to play a key role in the response of photosynthesis in plants to abiotic stresses such as salinity (Baker, 1991), and several studies have reported a link between salinity stress and reduced photochemical efficiency of PSII (Stephen and Klobus, 2006; Siler *et al.,* 2007; Jamil *et al.,* 2007; Hichem *et al.,* 2009). The photochemical efficiency of PSII is measured as F_v/F_m and relates information on the maximum efficiency at which light absorbed by PSII is used to drive photochemistry (Baker, 2008). Recently, a study by Stepien and Johnson (2009) compared the effects of short- and long-term salinity on photoinhibition of PSII in *Arabidopsis* and *Thellungiella.* It was found that short-term salinity did not have any immediate effect on PSII in either species; however, following long-term salinity treatment there was significant photoinhibition to PSII in *Arabidopsis* (seen by a drop in F_v/F_m), while *Thellungiella* showed no sign that the photosynthetic apparatus was stressed (F_v/F_m similar to that of an unstressed plant). These findings support my data for the effects of short- and long-term salinity on PSII in WT *Thellungiella* and the RNAi line 7-8. Prior to salinity stress and during short-term salinity stress (24 hours and 7 days), both WT *Thellungiella* and the RNAi line 7-8 demonstrated similar photochemical efficiencies of PSII. However following long-term (14 days) salinity stress, the F_v/F_m of RNAi line 7-8 demonstrated reduced F_v/F_m in comparison to WT, indicative of the sensitivity to salinity demonstrated in the RNAi line. Rahman *et al.,* (2010; 2011) recently found that under low temperatures TsDHN-2 underwent partial ordering in association with vesicles mimicking the lipid composition of plant plasma and organellar membranes, including chloroplast membranes. This suggests that during salinity stress TsDHN-2 may function by stabilizing chloroplast membranes, which would aid in the maintenance of photosynthetic activity.

5.3 Proline Accumulation as a Marker of Salt Susceptibility

Several roles have been proposed for the accumulation of proline during salinity stress, however the function of proline in salinity tolerance is still a subject of debate. Some studies have reported a positive correlation between proline accumulation and salinity tolerance (Khedr *et al.,* 2003; Kishor *et al.,* 2005; Brini *et al.,* 2007; RoyChoudhury *et al.,* 2007); however, several others have challenged this hypothesis and suggest that proline accumulation is not linked with salinity tolerance but rather is a marker of susceptibility (Liu and Zhu, 1997; Nanjo *et al.,* 2003; Chen *et al.,* 2007; Arbona *et al.,* 2010). For example, Ghars *et al.,* (2008) investigated the role of proline accumulation during salinity stress by comparing *Thellungiella* and *Arabidopsis* to the *eskimo-1* mutant of *Arabidopsis*, which was shown to over-accumulate proline due to both an increase in synthesis and decrease in degradation. It was found that the *eskimo-1* mutant was more salt sensitive than either WT *Arabidopsis* or *Thellungiella* despite accumulating the greatest amount of proline. Furthermore, Claussen (2005) suggested that proline levels could be an indicator of the environmental stress imposed on plants. However, it is not the maximum amount of proline accumulation but the fold increase in proline compared to constitutive proline levels that is important. Additionally, using CD spectra, dehydrin proteins from soybean (Soulages *et al.,* 2003) and *Arabidopsis* (Mouillon *et al.,* 2006) were found to contain a variable content of poly (L-proline)-type II structures and it was suggested that dehydrins may act as reservoirs or buffers for water under dehydrating conditions. Prior to salinity stress, both WT and the RNAi line 7-8 demonstrated similar basal levels of proline. Following salinity stress, both WT and 7-8 demonstrated an increase in proline; with line 7-8 accumulating over three times as much proline compared to WT. Furthermore, line 7-8 also appeared to be more sensitive to salt stress as greater phenotypic damage was observed compared to WT. Therefore, these data support the suggestion that the accumulation of proline itself does not confer salinity tolerance but rather is a marker of salt susceptibility.

5.4 Effects of Salinity on Seed Germination

Several studies have found a correlation between high salinity conditions and impaired halophytic germination (Ungar, 1996; Gulzar and Khan, 2001; Debez *et al.,* 2004; Inan *et al.,* 2004; Hanslin *et al.,* 2005; Orsini *et al.,* 2010; Atia *et al.,* 2011a, 2011b). Salinity could affect the germination of halophytes by osmotic stress (preventing the embryo from taking up water),

ionic stress (toxic effect of ions leading to embryo poisoning), or a combination of the two (Ungar, 1978; Duan *et al.,* 2004; Atai *et al.,* 2011b). Many studies have found that halophytes reach their maximum germination in distilled water, and show a reduction in germination when exposed to salinity (Ungar, 1996; Gulzar and Khan, 2001; Inan *et al.*, 2004). Recently, it was found that in the absence of salinity the germination rates of *Thellungiella* and *Arabidopsis* were close to 100% however, under saline conditions the germination rate of *Thellungiella* was greatly reduced compared to *Arabidopsis* which continued to have a high rate of germination (Inan *et al.,* 2004; Orsini *et al.,* 2010). These data correspond with our findings for the germination rates of WT *Thellungiella* compared to WT *Arabidopsis* at various NaCl concentrations (data not shown). It is believed that under saline conditions, *Thellungiella* enters a state of dormancy characteristic of halophytes. Many halophytic species enter osmotically enforced seed dormancy under saline conditions allowing them to remain viable and germinate when salinity concentrations are reduced, thus ensuring maximal survival (Ungar, 1996; Debez *et al.,* 2004; Inan *et al.*, 2004; Orsini *et al.,* 2010). Dehydrins are known to accumulate during seed maturation and studies have found a positive correlation between the over-accumulation of dehydrins and enhanced germination under saline conditions (Brini *et al.*, 2007). Hundertmark *et al.* (2011) recently found that by reducing the expression of the seed-expressed dehydrins *LEA14, XERO1* and *RAB18* in transgenic *Arabidopsis* plants, this reduced the ability of the plants to germinate under saline conditions, indicating a role for these dehydrins in *Arabidopsis* seed germination.

Under control conditions (no NaCl), both WT *Thellungiella* and the RNAi line 7-8 demonstrated germination rates close to 100% (92 and 97.3% respectively). Germination rates for the WT and line 7-8 were reduced at 100 mM NaCl (40 and 15% respectively), which is in accordance with the literature for *Thellungiella* seed germination (Inan *et al.,* 2004; Orsini *et al.,* 2010). At NaCl concentrations of 200 mM, WT demonstrated a steady decrease in germination to 11%, while the germination rates of line 7-8 actually increased to 20%. The reduced germination rate of line 7-8 compared to WT at 100 mM could be due to the reduced expression of TsDHN-2, indicating a possible role for this dehydrin during *Thellungiella* germination; however, it is uncertain whether TsDHN-2 is playing a role as it is not known whether the seeds used in these experiments were T3 homozygous transformants. It has been found that germination in *Thellungiella* is not uniform with a portion of *Thellungiella* seeds germinating

immediately, while germination of the other portion is spaced out and can extend up to 3 or 4 months after sowing (Inan *et al.,* 2004). Therefore, although it is possible that TsDHN-2 may be playing a role in *Thellungiella* seed germination, it is also reasonable to speculate that the fluctuation in germination patterns seen in line 7-8 can be attributed to the non-uniform germination rates of *Thellungiella* and further testing is required to confirm this notion.

5.5 Conclusions and Future Studies

Dehydrins are intrinsically unstructured proteins that accumulate in photosynthetic organisms under dehydrating conditions and are thought to confer stress tolerance. *Thellungiella salsuginea*, a close relative of *Arabidopsis thaliana*, is a halophyte able to tolerate extreme conditions, such as high salinity. Most dehydrin studies have focused on transgenic plants over-expressing proteins and few have examined their role using transformants with reduced dehydrin expression. This work examined the possible role of a *Thellungiella* dehydrin, TsDHN-2, in salinity tolerance using an RNAi line with reduced *TsDHN-2* expression. It was found that the RNAi plants demonstrated a reduced ability to tolerate salinity stress based on phenotypic observations, photosynthetic determinations, leaf viability and proline accumulation and germination studies. These data suggest that TsDHN-2 plays a role in the salinity tolerance mechanisms of *Thellungiella*.

In order to further elucidate the role(s) TsDHN-2 plays in salinity tolerance it is important to generate additional T3 homozygous lines with reduced dehydrin expression. Analyzing more than one RNAi line with reduced TsDHN-2 expression will validate the results if the same effects are observed in all RNAi lines compared to WT. Since salinity generally reduces leaf water content, better estimators of plant sensitivity to salinity such as plant fresh weight, dry weight and water content could be utilized. Furthermore, *Thellungiella* has demonstrated the ability to tightly control Na^+ accumulation and maintain a high K^+/Na^+ ratio during salinity stress, which is a key feature of salt tolerance (Inan *et al.,* 2004; Wang *et al.,* 2006; Ghars *et al.,* 2008). Therefore, measuring the Na^+ and K^+ ion content in WT and RNAi plants prior to and following salinity stress will give further insight into the salinity sensitivity demonstrated in the RNAi lines as well as further elucidate possible mechanisms by which TsDHN-2 functions in *Thellungiella* salinity tolerance.

Greater proline accumulation in response to salinity stress was demonstrated in the RNAi line compared to WT, which is indicative of a salt susceptibility in the RNAi line. *Thellungiella* is known to accumulate high levels of proline in response to salinity stress, however other compatible osmolytes were also found to accumulate in moderate concentrations (Inan *et al.,* 2004). Therefore, it would be valuable to measure the accumulation of other osmolytes, such as sugar alcohols, in response to salinity stress in order to determine the possible effect of TsDHN-2 on their accumulation.

It is uncertain whether TsDHN-2 plays a role in and during *Thellungiella* germination as sporadic germination rates were seen in line 7-8 at concentrations higher than 100 mM NaCl, and it is also not known whether the seeds used in these experiments were T3 homozygous transformants. Therefore, in order to elucidate the role of TsDHN-2 in and during germination, the assay should be repeated with confirmed T3 homozygous seeds. Furthermore, as *Thellungiella* is known to have nonsynchronous germination and seeds can germinate up to 3 months after sowing, it would be of interest to extend the assay for a longer time period. *Thellungiella* is known to enter a state of dormancy when exposed to saline conditions, thus ensuring that seeds remain viable to germinate once the stress is alleviated. It would be of interest to assess the ability of salt treated seeds to be rescued, indicative of a possible role of TsDHN-2 in the ability to ensure seed viability despite salinity treatment.

Using RNAi methodology, this work examined the role of TsDHN-2, an Y_2SK_3 dehydrin, in the salinity tolerance mechanisms of *Thellungiella*. In response to salinity stress it was observed that RNAi line 7-8 demonstrated a 41% reduction in *TsDHN-2* expression, greater phenotypic damage, decreased photosynthetic activity and increased proline accumulation in comparison to WT. Collectively, these data support the notion of a potential role for TsDHN-2 in *Thellungiella* salinity tolerance.

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Appendix A
TsDHN-2 cDNA and Deduced Protein Sequence

A.

CCCACGCGTCCGAACAGAGATAAATATACTGAAAAGTGTTTGCTTAGA
AGAAGAAAGAGAAGAACAAGATGGCGTCTTACCAGAACCGACCAGGAG
CTCAGGCCACTGACGAGTATGGAAACCCGATGCAACAGTTGGACGAGT
ACGGTAACCCAATTGGCGGTGTAGGAGCGACCGGAGGAGGAGGAGCAG
GTTATGGAACTGGTGGAGGATACGGCGGAGGAGCCACTGGTGGCGAAG
GATACGGAACGGGAGCCTTGGGAGCTGGCGCAGGAGCTAGGCACCACG
GTCAGGAGCAACTCCATAAGGAAGGTGGCGGTGGTTTGGGAGGAATGC
TTCACCGCTCTGGATCCGGATCCAGCTCTAGCTCGGAGGATGATGGAC
AAGGTGGGAGGAGGAAGAAGGGAATAACTCAGAAAATTAAGGAAAAGT
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GAACTACCACCGGTTATGATGCTGGAGGTTACGGCGGCCAACACCACG
AGAAAAAGGGAATAACTGATAAAATTAAGGAAAAGTTGCCAGGTCAAG
ATCAGTCTGGTCAATCCCAAGGGATGGGGATGGGAGCTACCACCGGTT
ATGATGCCGGAGGCTATGGTGGAGAGCGCCATGAGAAGAAGGGGATGA
TGGACAAGATCAAAGATAAACTTCCTGGTGGTGGTGGTCGTTAAGCTG
AATACTATTTAAACTTTGTATACATATAAAATATAAAAATAAAGGAAA
CTCAGTCGTATATGGTCGTTGTACGTTTGCTTTTATGTCTCTACGTCC
TGCGATGTGTTGTATTCGAGTGTGAGAGANGAGTGTGTATGAGCGTGC
AGTTGGCTCTTTTTATGTTCTTGACATGTATTTATTTCTCTACTTGCT
TATCTATGTATACGTATCTTCCTTTTTAACGCTTGTTTCAATTTATCT
TTCTTGTTTTTTTGTGCCTAAAAAAAAAAAAAAAAG

B.

MASYQNRPGAQATDEYGNPMQQLDEYGNPIGGVGATGGGGA
GYGTGGGYGGGATGGEGYGTGALGAGAGARHHGQEQLHKE
GGGGLGGMLHRSGSGSSSSSEDDGQGGRRKKGITQKIKEKLP
GQHDQSGQSQGMGMGTTTGYDAGGYGGQHHEKKGITDKIKE
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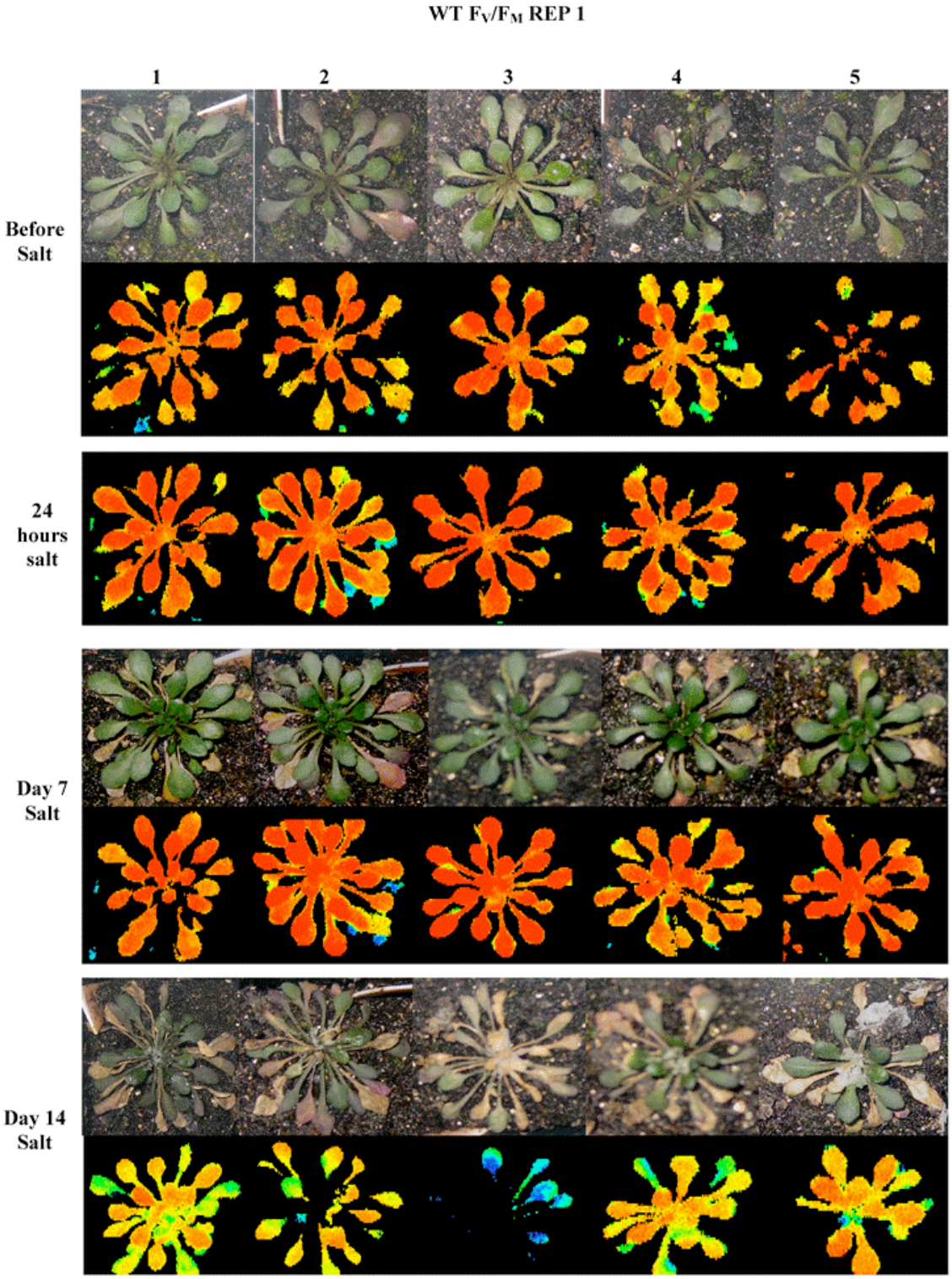
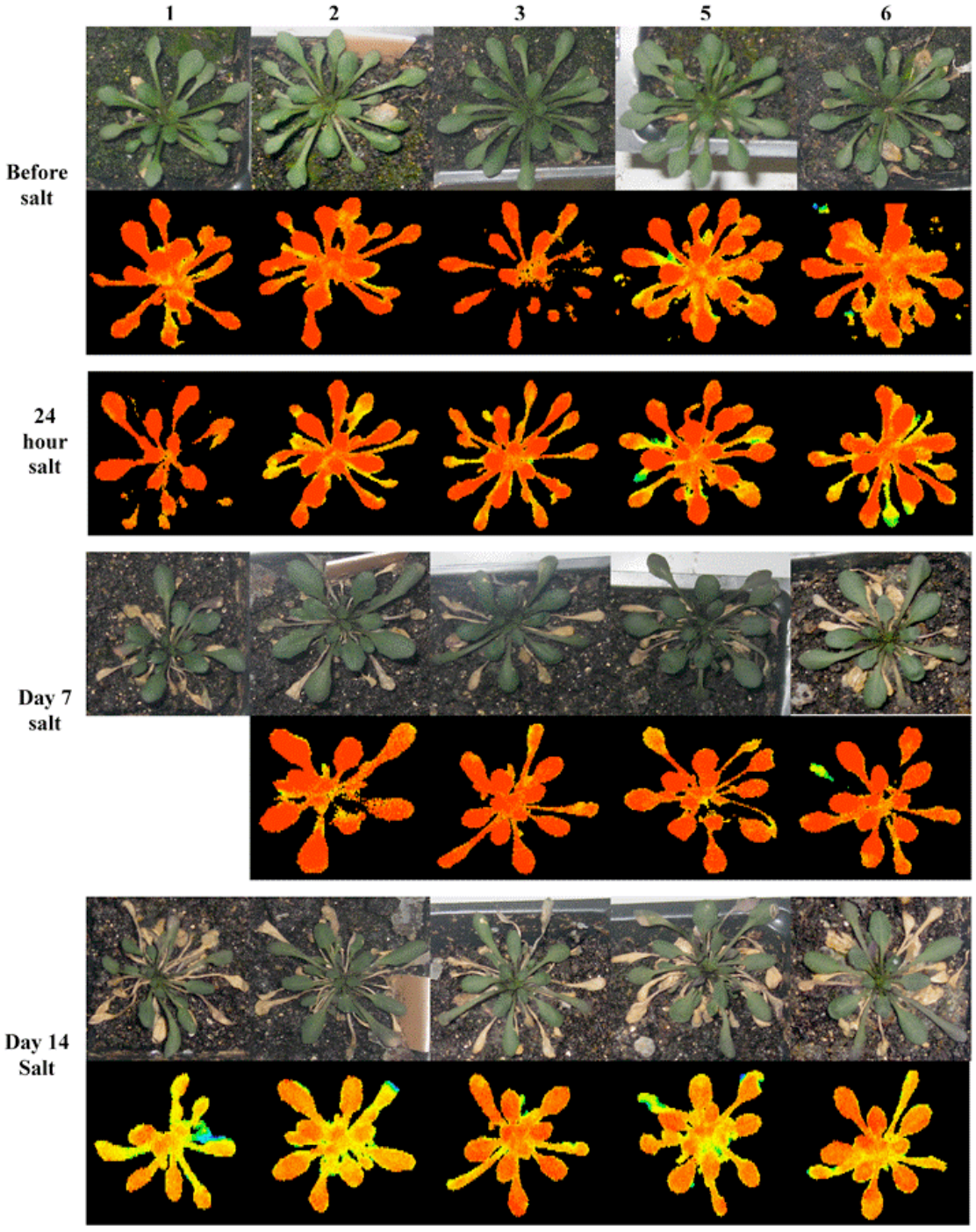
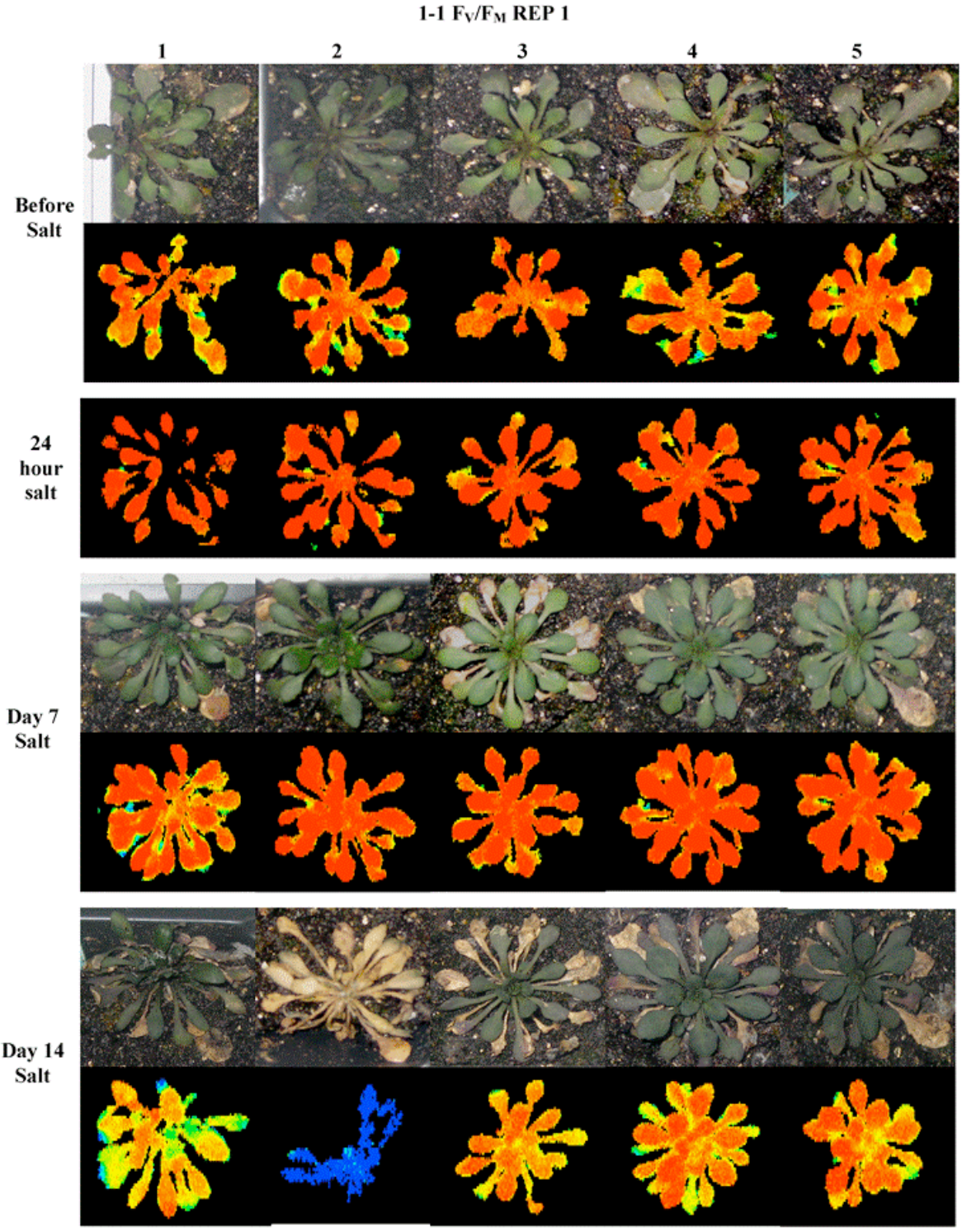
Appendix B

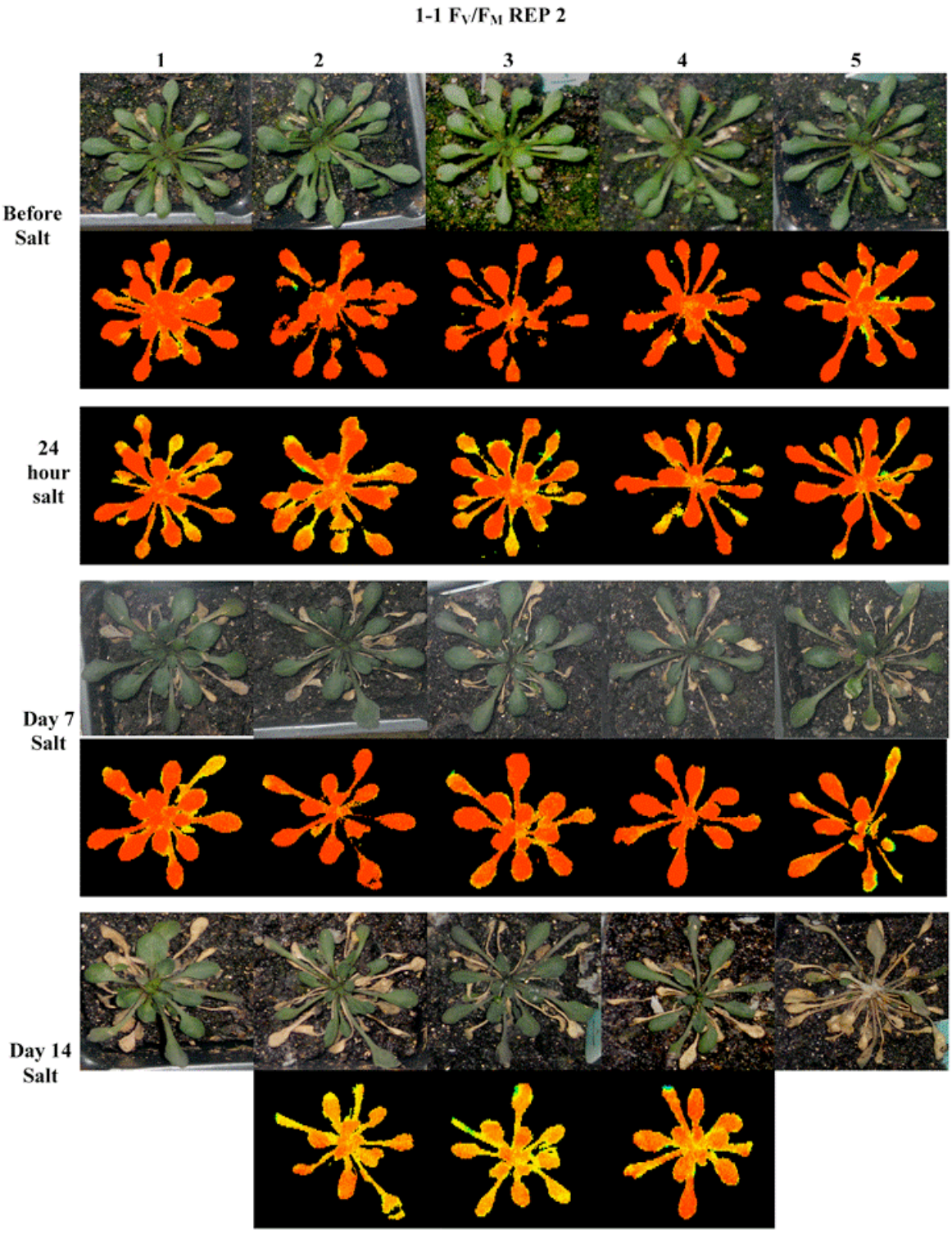
Phenotypic and Photosynthetic Responses - Salinity Experiments

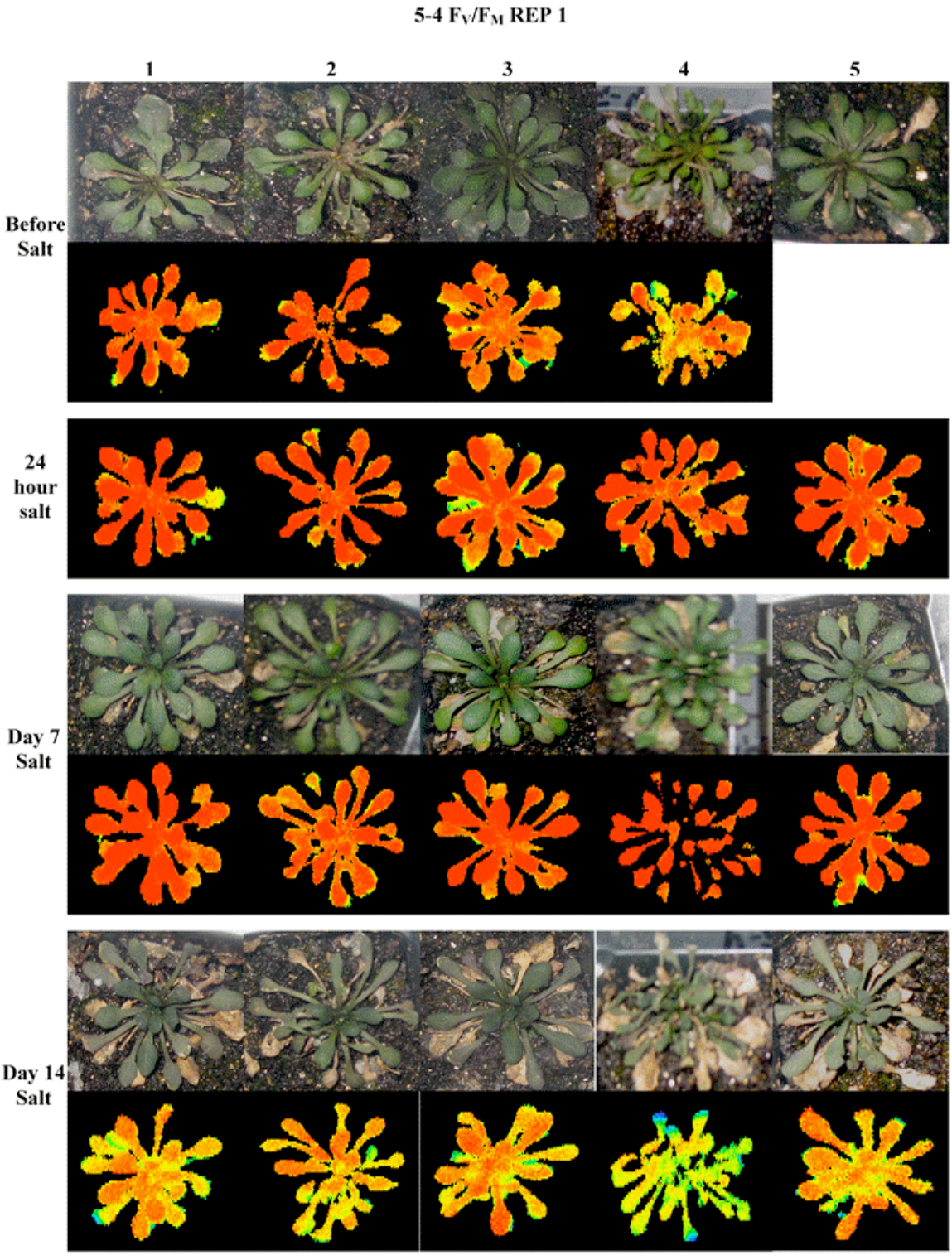


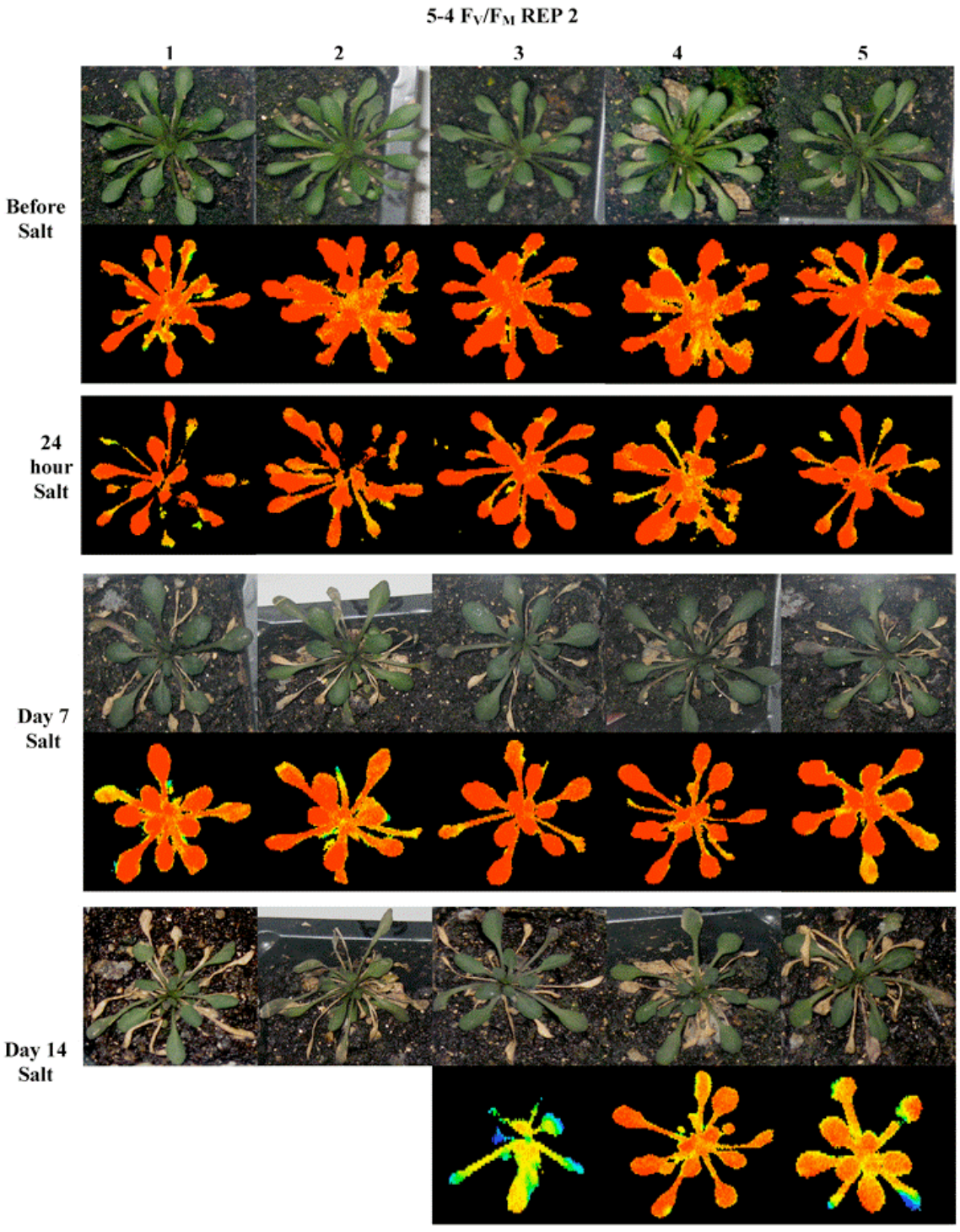
WT F_V/F_M REP 2




1-1 F_V/F_M REP 1
1
2
3
4
5
Before Salt
24 hour salt
Day 7 Salt
Day 14 Salt


1-1 F_V/F_M REP 2
1
2
3
4
5
Before Salt
24 hour salt
Day 7 Salt
Day 14 Salt


5-4 F_V/F_M REP 1
1
2
3
4
5
Before Salt
24 hour salt
Day 7 Salt
Day 14 Salt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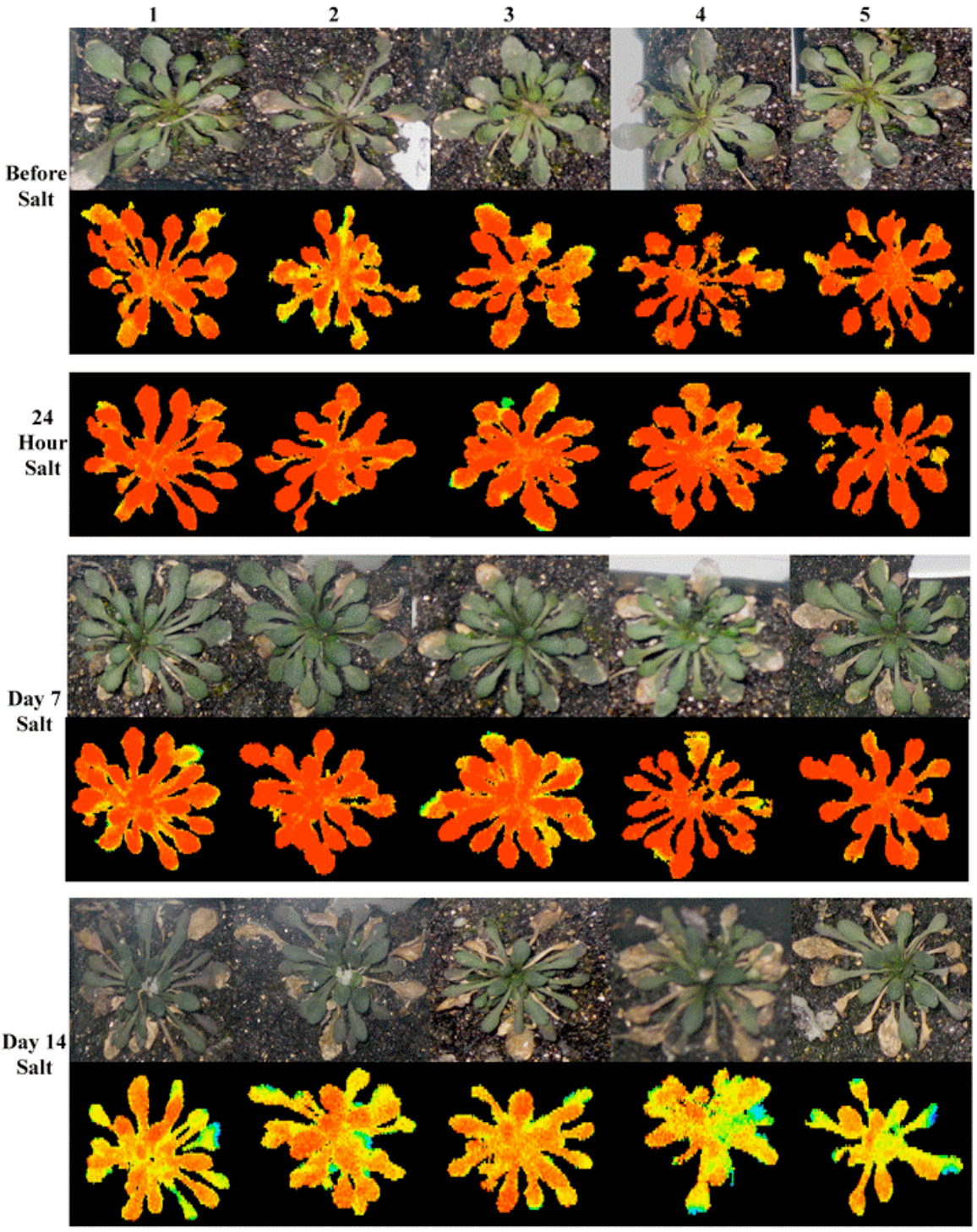
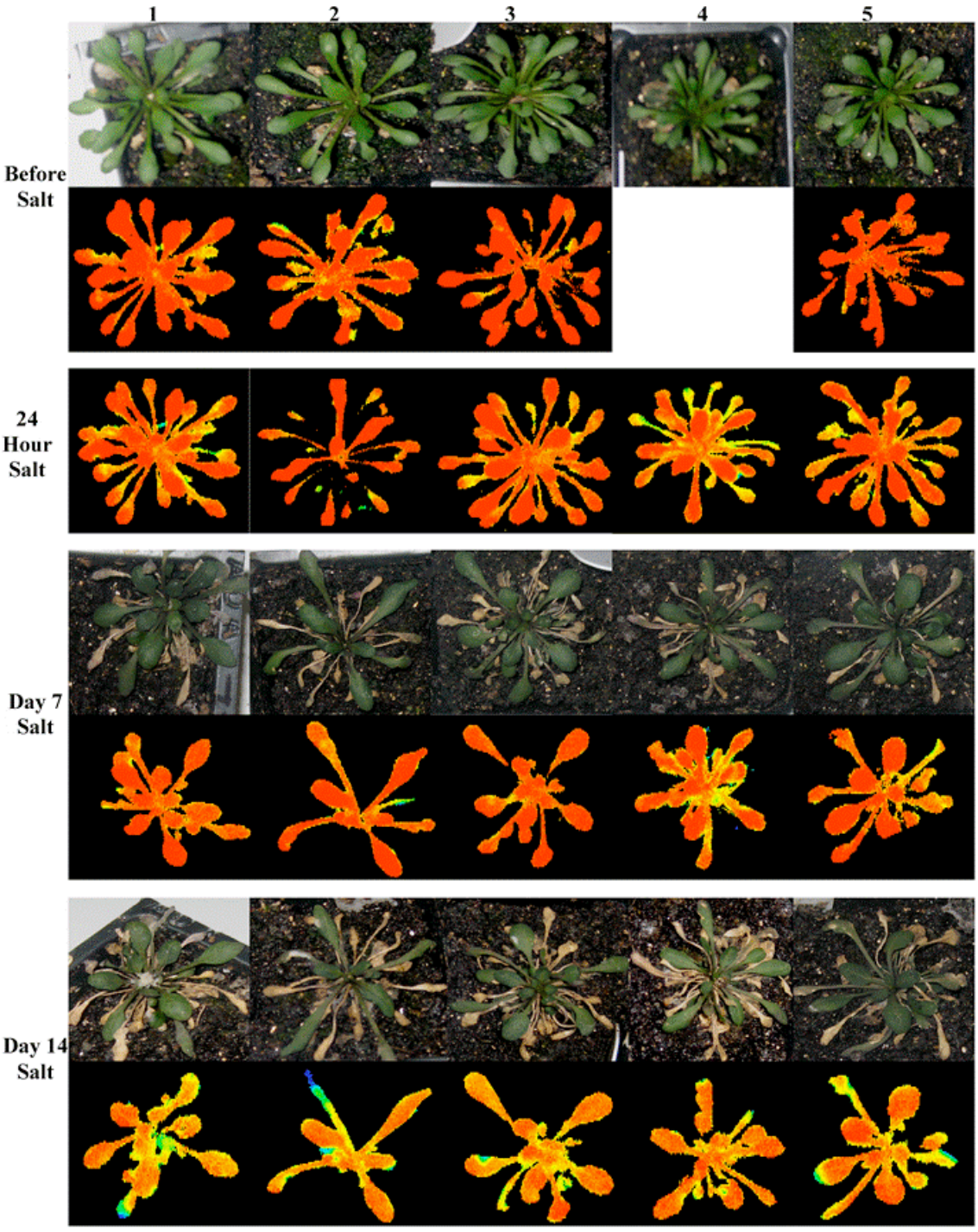
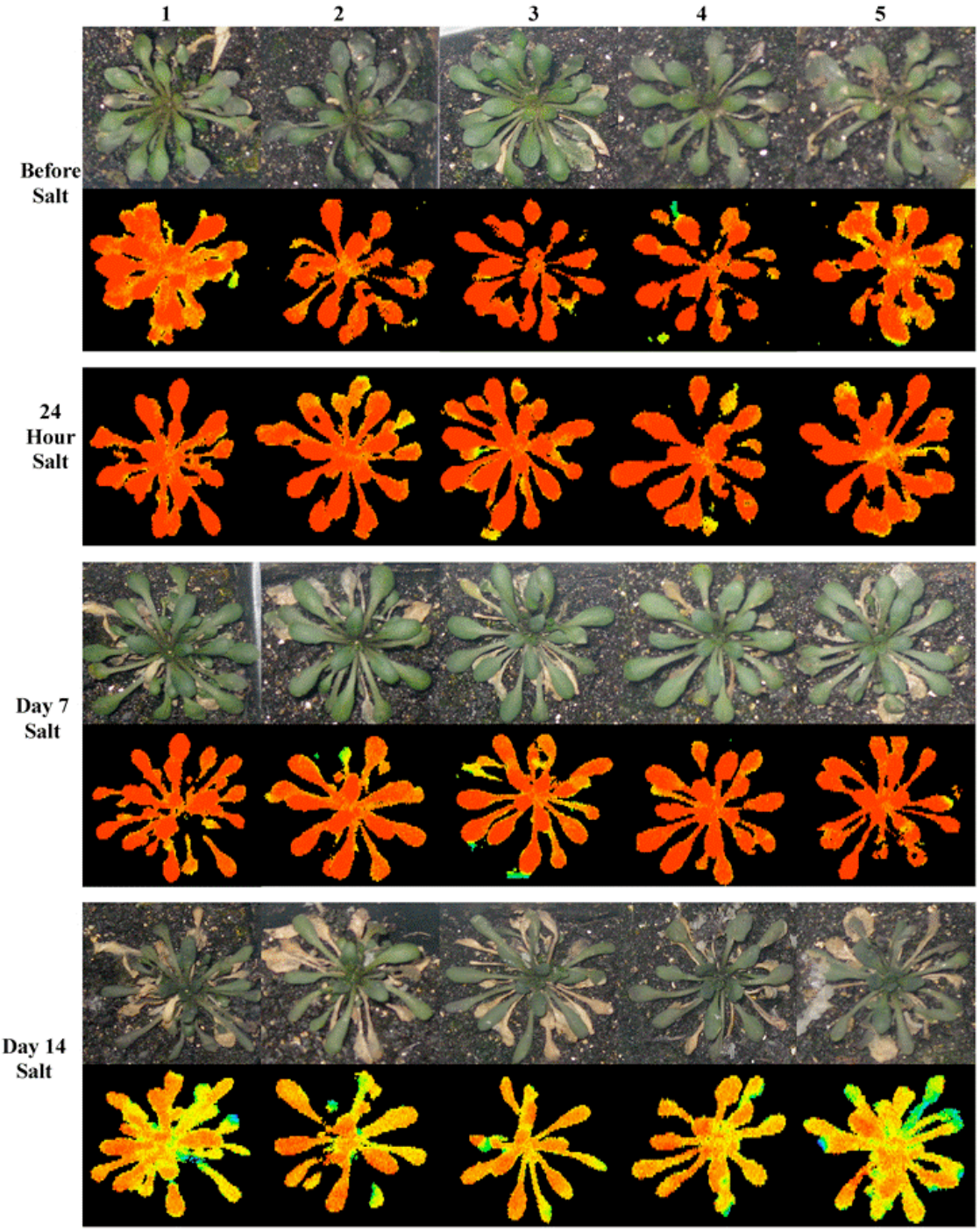
1
2
3
4
5
Before Salt
24 hour Salt
Day 7 Salt
Day 14 Salt


6-2 F_V/F_M REP 1
1
2
3
4
5
Before Salt
24 Hour Salt
Day 7 Salt
Day 14 Salt

6-2 F_V/F_M REP 2



7-8 F_V/F_M REP 1



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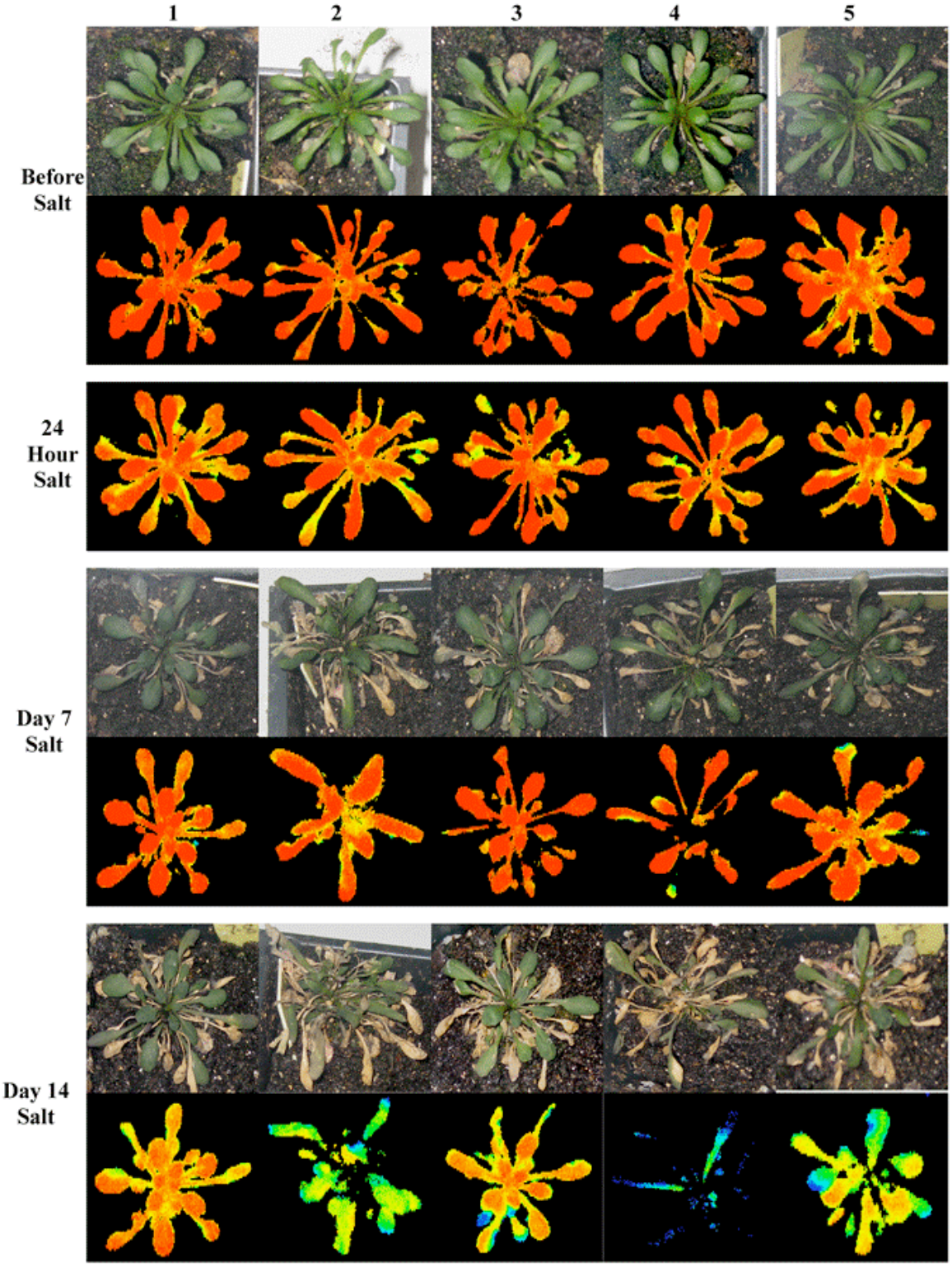
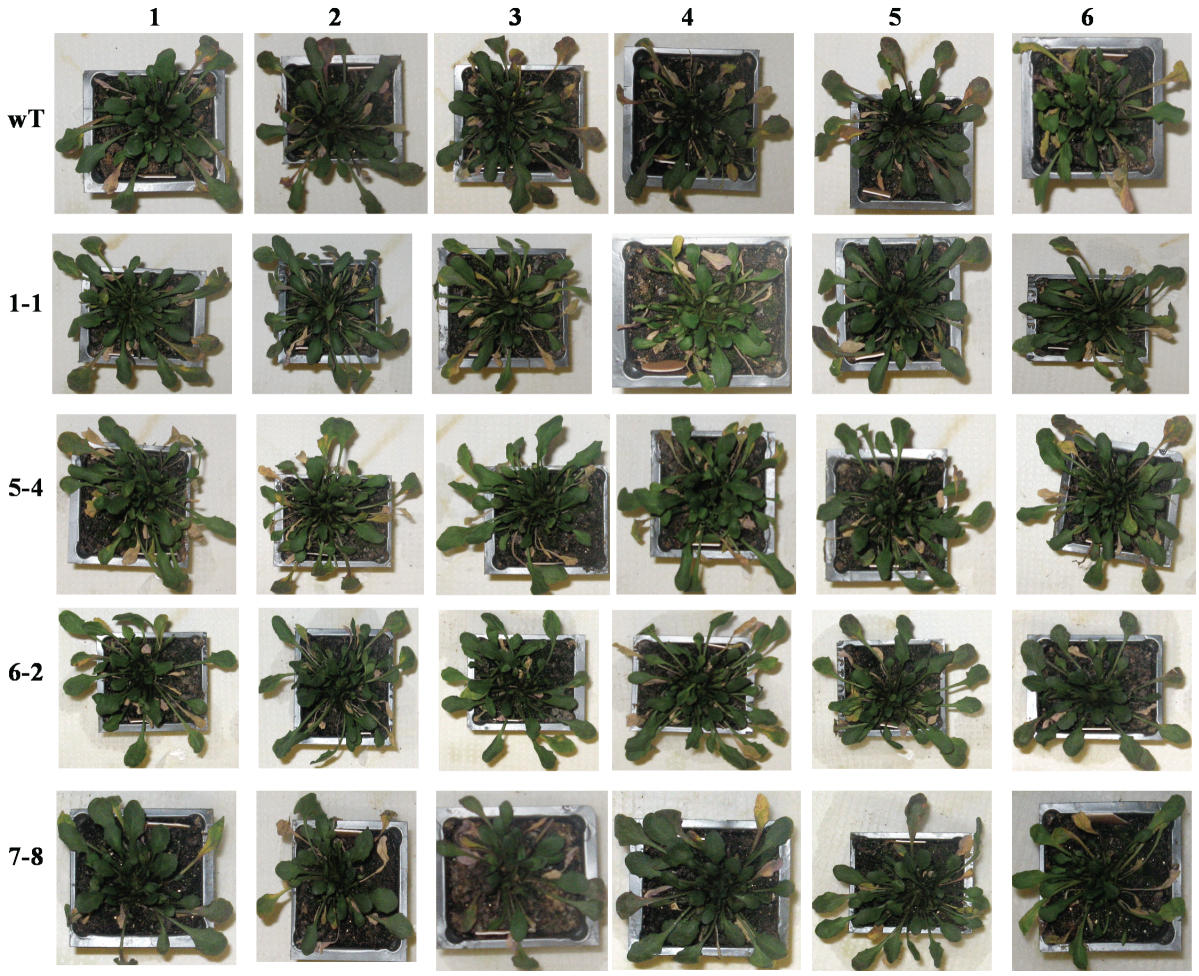
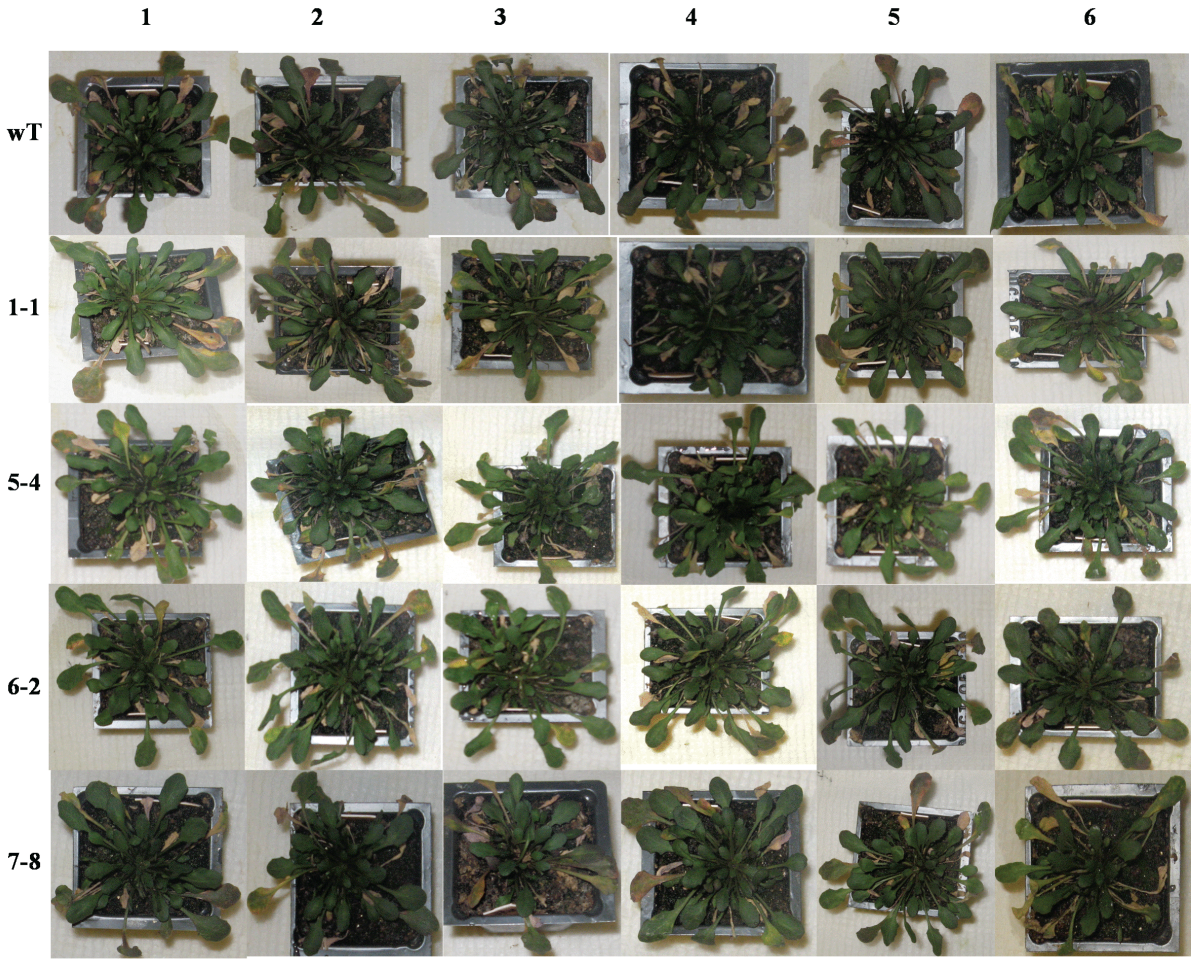


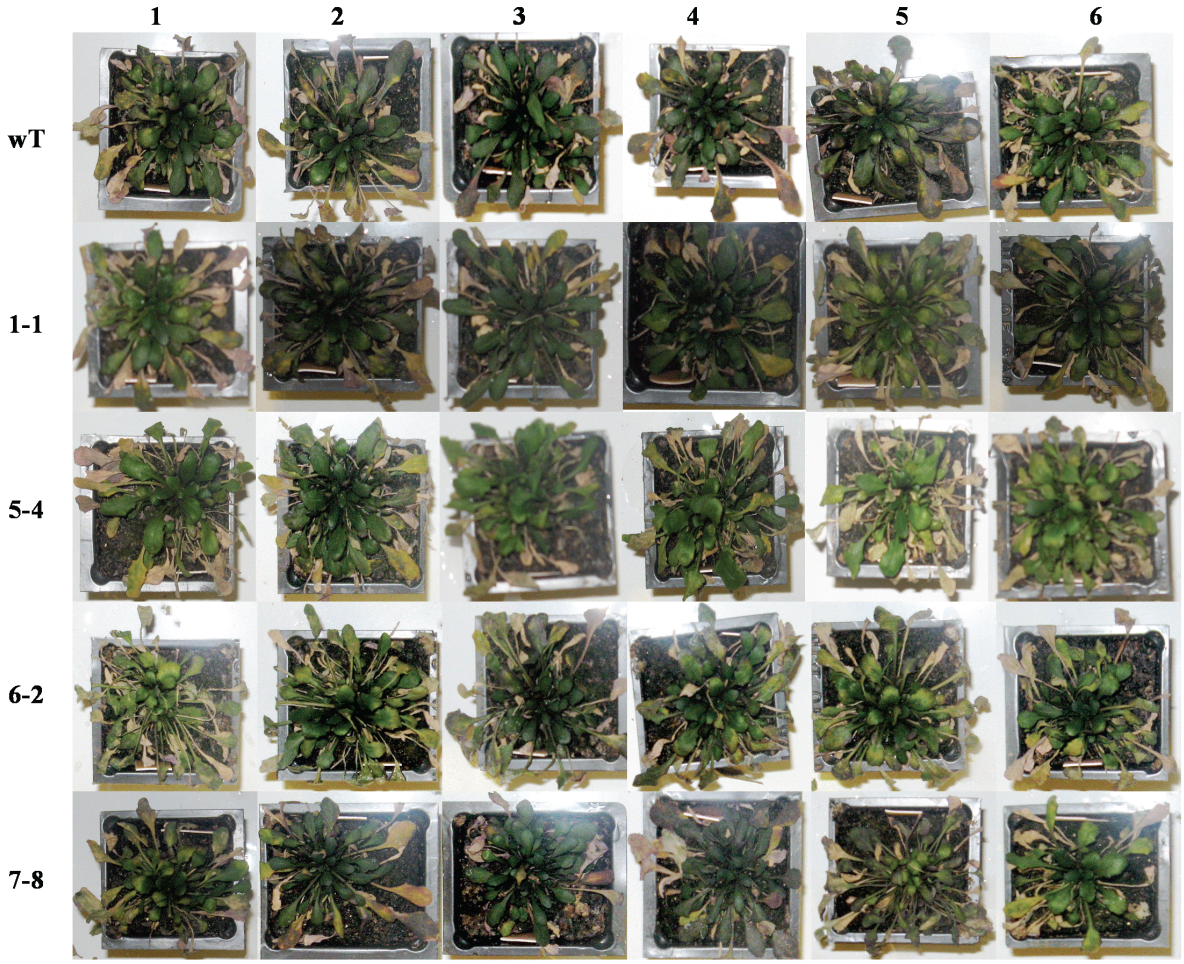
Appendix C
Phenotypic Responses - Proline Experiments



Before salt



24 hours salt



7 day salt