IDENTIFICATION OF ABIOTIC STRESS RELATED MIRNAs IN *THELLUNGIELLA HALOPHILA* ECOTYPES

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ABSTRACT

IDENTIFICATION OF ABIOTIC STRESS RELATED MIRNAs IN *THELLUNGIELLA HALOPHILA* ECOTYPES

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Environmental stress with climate change currently contributes to approximately 50% of crop loss occurring world-wide. *Thellungiella halophila* is a plant that is native to highly saline and semiarid environments and exhibits an exceptional ability to tolerate abiotic stress. Primarily, the determination of relatively high GSH/GSSG ratios (glutathione assay), elevated proline accumulation, less MDA (lipid peroxidation) production and increased anthocyanin levels and less reduced RWC of the *T. halophila* (Shandong and Yukon ecotypes) after exposure to increasing concentrations of NaCl allowed us to support that *Thellungiella* tolerate environmental stress conditions. Genomic studies have shown that differences in stress tolerance arise not only from the presence or absence of stress-associated genes but also from their regulation. In plants, miRNAs are the key gene regulators that have been shown to control various developmental processes and responses to environmental stresses. In order to assess the expression profiles of miRNAs in

abiotic stress tolerance of *T. halophila* ecotypes, salt, cold and drought treatments were carried out. As a result, we identified the changes in the expression profiles of seven conserved miRNAs under 250 mM NaCl, 4°C and 200 mM mannitol using Northern-Blot analysis and TaqMan qRT-PCR assays. Four of the stress related miRNAs (miR156, miR169, miR319 and miR393) were found to be downregulated and three (miR159, miR398 and miR169) were found to be upregulated during exposure to salt, cold and drought stress. Based on the conservation of mature miRNA sequences, potential target genes for miRNAs were also predicted by using conservation of miRNA target sites among species. The target mRNA expression analysis were done by RT-qPCR after exposure to salt stress. RT-qPCR results showed decrease in the expression of miR156, miR169, miR319 and miR393 with a corresponding upregulation of their putative target mRNA transcripts (*SPL10*, *NF-YA5*, *ITD1* and *WRKY33*, respectively). In addition, the induction of miR159 was accompanied by the down regulation of its probable target mRNA (*HEC1*). Taken together, these findings suggest that a number of conserved miRNAs may function in *Thellungiella halophila* ecotypes tolerance to environmental stresses.

Keywords: *Thellungiella halophila*, miRNA, abiotic stress, Taqman assay, target mRNA

ÖZ

THELLUNGİELLA HALOPHİLA EKOTİPLERİNDE ABİYOTİK STRESLE İLİŞKİLİ MİRNA'LARIN BELİRLENMESİ

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Ekim 2015, 93 sayfa

İklim değişikliği ile birlikte çevresel stres faktörleri dünya çapında meydana gelen ekin kayıplarına yaklaşık olarak %50 oranında katkıda bulunmaktadır. *Thellungiella halophila* yüksek derecede tuzlu ve yarı kurak ortamlarda doğal olarak yetişen bir bitkidir ve olağanüstü bir abiyotik stres toleransına sahiptir. Öncelikle, artan NaCl konsantrasyonlarına maruz kaldıktan sonra *T. halophila* ekotiplerinde ölçülen GSH/GSSG oranlarındaki (glutatyon deneyi) nispi artış, yüksek prolin birikimi, daha az MDA (lipit peroksidasyonu) üretimi, antosiyanin seviyelerindeki artış ve daha az azalmış bağıl su içeriği tayini, *Thellungiella* bitkisinin çevresel stres koşullarına karşı dirençli olduğunu belirlememize katkı sağladı. Genomik çalışmalar, stres toleransındaki farklılıkların sadece stresle bağlantılı genlerin varlığı veya yokluğu ile ilgili değil, aynı zamanda bu genlerin düzenlemeleriyle de ilgilli olduğunu göstermiştir. Bitkilerde, miRNA'lar çeşitli gelişim süreçlerini ve çevresel strese tepkileri kontrol ettiği gösterilmiş olan temel gen regülatörleridir. *T. halophila*

ekotiplerinin abiyotik stres toleransında etkili miRNA'ların ekspresyon profillerini belirlemek için tuz, soğuk ve kuraklık uygulamaları yapılmıştır. Sonuç olarak, yedi korunmuş miRNA'nın ifadesindeki değişiklikler 250 mM NaCI, 4°C ve 200 mM mannitol stresine maruz bırakılarak Northern blot analizi ve TaqMan qRT-PCR deneyleri kullanılarak tespit edilmiştir.

Tuz, soğuk ve kuraklık stresine maruz kalma sırasında stres ile ilgili miRNA'ların dördünün (miR156, miR169, miR319 ve miR393) ifadesinde azalma ve diğer üçünün (miR159, miR398 ve miR169) ifadesinde ise artış olduğu tespit edilmiştir. Olgun miRNA dizilerinin korunması prensibine dayanarak, miRNA'lar için potansiyel hedef genler türler arasındaki miRNA hedef bölgelerin korunması durumuna uygun olarak belirlenmiştir. Hedef mRNA'larının ifade analizi tuz stresine maruz kaldıktan sonra RT-qPCR yapıldı. RT- qPCR sonuçları hedef mRNA'lar oldukları varsayılan transkriptlerin (sırasıyla *SPL10*, *NF-YA5*, *ITD1* ve *WRKY33*) ifadesindeki azalmaya karşılık, miR156, miR169, miR319 ve miR393 ekspresyonunda bir upregülasyon olduğunu gösterdi. Buna ek olarak, miR159 indüksiyonuna da muhtemel hedef mRNA'nın (*HEC1*) ifadesindeki azalma eşlik etti. Tüm bu bulgular birlikte ele alındığında, korunmuş miRNA'ların *Thellungiella halophila* ekotiplerinin bir dizi çevresel stres toleransı esnasında işlev görebildiğini ortaya koymaktadır .

Anahtar Kelimeler: *Thellungiella halophila*, miRNA, abiyotik stress, Taqman analizi, hedef mRNA

Dedicated to my family,

For their endless help, love and support

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LIST OF ABBREVIATIONS

miRNA	MicroRNA
ANOVA	Analysis of Variance
DNase I	Deoxyribonuclease I
dNTP	Deoxyribonucleotide triphosphate
mRNA	Messenger RNA
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pri-miRNA	Primary microRNA
Pre-miRNA	Precursor microRNA
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse Transcription Polymerase Chain Reaction
DEPC	Diethylpyrocarbonate
Glutathione	Glutathione
ROS	Reactive oxygen species
TF	Transcription factor
C	Control

CHAPTER I

INTRODUCTION

Higher plants evolve various adaptations to almost all diverse environments. Due to their immobile nature, plants are continuously exposed to abiotic and biotic stress factors which negatively affect plant growth, development and reproduction (Khraiwesh et al., 2012). The impact of unfavorable conditions on the ecosystem is increasing at an alarming rate due to rapid increase in population, destruction of vegetation through excessive grazing and incorrect irrigation management (Mantri et al., 2012). Understanding the traits of plants living in extreme environments is a powerful tool to study suitable adaptive responses coping with stress conditions. Extremophiles are such plants surviving in the most compelling situations such as high amount of salt and lack of water, low temperatures, toxic ions, desiccation and flooding (Inan et al., 2004).

1.1 Thellungiella: a Halophytic Model Plant

Thellungiella (salt-water cress) is a small member of Brassicaceae (crucifer) family with a short life cycle (Inan et al., 2004). Both *Thellungiella* and *Arabidopsis* which are closely related genera of the family have been used as model plants for plant biology experiments due to several reasons such as relatively small plant size, small genome size, rapid life cycle, high seed production and ease of transformation. *Thellungiella* species (*T. halophila* and *T. parvula*) are also salt-tolerant and endemic to several other resource-

poor environments (ecotypes showing a range of stress responses) making them good models for identifying the plant adaptation mechanisms to extreme environmental conditions. The genome of *T. halophila* is about twice as large of *Arabidopsis*. Both *T. halophila* and *T. parvula* are highly tolerant to salt, drought and cold conditions which make them extremophiles (Orsini et al., 2010, Wu et al., 2012). These adaptations are compatible with their natural habitats. *T. halophila* ecotypes (Shandong and Yukon) are inhabitants of stress-prone environments that have been used as model halophyte (Griffith et al., 2007, Wong et al., 2006) and they are also tolerant to related osmotic stress as well as oxidative stress (Wong et al., 2006). *T. halophila* is a small rosette plant (Figure 1). Flower, shoot and leaf structures are highly similar to *Arabidopsis*. For germination of the seeds and synchronized flowering after formation of shoots and roots, nearly 3 weeks of vernalization is needed (Lugan et al., 2010, Volkov and Amtmann, 2006, Volkov et al., 2004).

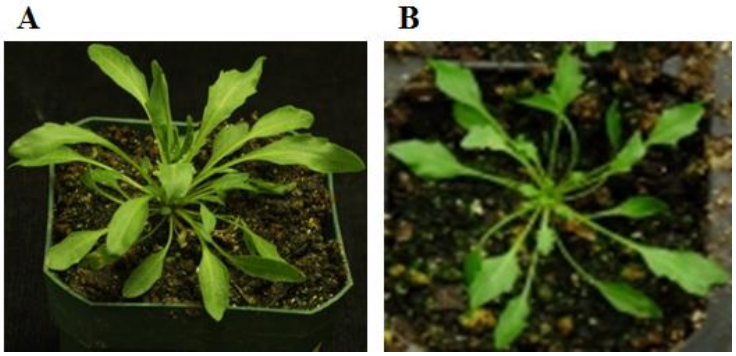

Figure 1 Rosette structure of *Thellungiella halophila* ecotypes. **A** Yukon ecotype **B** Shandong ecotype.

Gene expression patterns appear to be more stress-specifically altered in *Thellungiella* than in *Arabidopsis*. Additionally, the expression of some universal genes that are responsible for the production of stress responses is high

in *Thellungiella* even in the absence of extreme stress conditions. Others are induced only at much higher levels of stress than their isologs in *Arabidopsis* (Oh et al., 2010). So the usage of *Thellungiella* rather than *Arabidopsis* especially in stress physiology studies is appropriate for better understanding of the stress tolerance mechanisms in plants. Preliminary results indicate that salt cress, like many other halophytes, has substantially better water-use efficiency than crop plants. Therefore, it may be possible to use salt cress for the genetic examination of water-use efficiency and osmotic stress situations derived from salinity, drought and cold stresses. Salt cress has the capacity to reproduce even after exposure to extremely high salt concentrations or low temperatures. Furthermore, 90% sequence identity in the genome (derived from cDNAs) with Arabidopsis assist orthology-based cloning of stress-responsive genes in *Thellungiella* (Amtmann, 2009, Bressan et al., 2001, Inan et al., 2004, Taji et al., 2004).

Due to the stationary lifestyle of plants, they need more protection which led them to evolve a wide range of extraordinary molecular mechanisms to cope with environmental and climate changes that cause stress (Rao et al., 2006, Sunkar, 2010).

1.2 Abiotic Stress

Detrimental and irreversible variations in a plant normal physiology, development and function are identified as stress in biological terms (Ahmad and Prasad, 2011, Cramer et al., 2011). Any environmental factor that imposes harmful situations for the favorable functioning of an organism is best defined as abiotic stress. Abiotic stress is commonly caused by water deficit, high salt, heat, cold and freezing, excessive light, nutrient deficiency, toxins etc. either individually or in combination. Abiotic stress factors cause reversible or irreversible changes on plant metabolism causing damaging impacts on plant growth, development and productivity. In order to handle the negative impacts of

the stress, plants are able to change their physiologic responses, metabolic activities, expression of stress-associated genes and developmental processes. Therefore, plants have various complicated and unusual mechanisms for abiotic stress tolerance (Cramer et al., 2011, Rao et al., 2006, Wang et al., 2004). Stress adaptation of a plant associated with its survival and reproduction capacity under unfavorable conditions. Stress tolerant plants have better tolerance, resistance, protection and acclimation strategies for survival under harsh circumstances where stress sensitive plants cannot survive. However, the degree of the tolerance is directly related to the type of plant and the intensity of the stress (Mantri et al., 2012, Wang et al., 2003). The activation of defense-related genes in response to stress is one of the central processes in plant stress tolerance responses. Accordingly, the signal transduction pathways detecting the severe environmental conditions are highly important for the induction of plant stress tolerance (Sunkar and Zhu, 2004, Yordanov et al., 2000).

5% of world's cultivated land and 50% of crop productivity are affected every year due to abiotic stresses. If the effects of abiotic stress are severe, plants become sterile and productivity is completely lost. Most abiotic stress factors are interconnected and cause a series of morphological changes (white leaf tip followed by tip burning, leaf browning and necrosis, poor root growth, leaf rolling, change in flowering time, stunted plant growth and low grain yield and quality), physiological and biochemical changes (increase in the compatible solutes and polyamine levels, lower fresh and dry weights of shoots and roots, lower level of relative water content, increase in lipid peroxidation) leading to the death of plants (Kumari et al., 2014, Rodríguez et al., 2005, Wang et al., 2003).

Severe metabolic alterations originated from abiotic stress factors individually or together induce the production of reactive oxygen species (ROS). The formation of reactive oxygen species (ROS) as strong oxidants at cellular and molecular level causes serious damage to membrane systems and DNA. Both enzymatic (superoxide dismutases, catalase, peroxidases, phenol oxidase, and ascorbic acid oxidase) and non-enzymatic antioxidative systems (compounds that are strong reductants such as glutathione, phenols,

flavonoids, and polyamines) are indispensable for the balance and prevention of the oxidative damage (Gechev et al., 2006, Inze and Van Montagu, 2003). ROS production is involved in signaling cascades during cell growth, spatial regulation of development and organogenesis (Gapper and Dolan, 2006). Given the importance of stress on plant growth, individual stress factors and their specific physiological effects on plants will be discussed in the following sections.

1.2.1 Salt Stress

The amount of dissolved mineral salts found in the soil and water is defined as salinity. A mixed constitution of cation and anion electrolytes are expressed as dissolved mineral salts. The major cations in saline soils are Na^{+}, Ca^{+2}, Mg^{+2} and K^{+} and the major anions are Cl^{-}, SO_4^{-2}, HCO_3^{-}, $CO_3^{-2}$and NO_3^{-} (Volkmar et al., 1998). An elevated concentration of dissolved salts is the main abiotic stress that has sadverse effects on crop quality and productivity. Harmful levels of chlorides and sulphates of sodium is the main characteristic of saline soils. Salinity is a general problem in arid and semi-arid areas due to salt accumulation via over-irrigation, inappropriate drainage and usage of seawater for irrigation (Chinnusamy and Zhu, 2004).

Salt stress shows its negative effects on plants in two major ways; elevated amounts of salt within the soil disturb the root water extraction capacity and increased amount of salt accumulation inside the cells can be harmful to plants. These can finally inhibit several biochemical, molecular, and physiological processes such as uptake of nutrients and absorption, cell signaling pathways like synthesis of active metabolites for osmoprotection, synthesis of stress-specific proteins, and specific free radical scavenging enzymes (Parvaiz and Satyawati, 2008). Furthermore, salinity may cause osmotic stress, toxic ion accumulation, nutritional deficiencies, oxidative stress, changes in metabolic

processes, changes in membrane organization, reduced amount of cell division and expansion (Munns and Tester, 2008, Zhu, 2001).

Salinity decreases the uptake of essential elements such as phosphorus, potassium, nitrate and calcium. High levels of salt accumulation lead to ion cytotoxicity and osmotic stress that are detrimental to plant growth and development. Ions like Na^+ and Cl^- diffuse the hydration shells of proteins under salinity and inhibit the function of these proteins. Metabolic instability and oxidative stress are secondary constraints of salinity caused by ion toxicity, water stress, and nutritional defects. Ionic and osmotic adjustment, repair and detoxification for stress damage control and regulation of plant growth are the main salt tolerance mechanisms for plants. Better understanding of the molecular basis of salt tolerance is indispensable for the success of breeding programs aiming to improve crop productivity (Parida and Das, 2005, Wang et al., 2003).

Prevention of cellular death and formation of physiological and biochemical steady states are crucialfor plant growth and completion of the life cycle after establishing the ionic and osmotic balance upon salt stress(Bohnert et al., 1995). Inhibition of the salt accumulation in the plant especially in photosynthetic tissues via reducing the salt uptake and decreasing the concentration of salt inside the cells are the two main types of salt tolerance mechanisms in plants (Munns, 2002). Corresponding stress avoidance and stress tolerance are two major adaptive strategies for plants to tolerate high salinity. The adverse effects of stress are improved by the help of utilizing various physical, physiological and/or metabolic barriers in stress avoidance. Otherwise several adaptation mechanisms like salt exclusion, salt excretion and intracellular ion compartmentation are enabled for successful survival of the plant despite the internal effects of stress for stress tolerance (Touchette et al., 2009).

Based on salt stress tolerance, all plants can be divided into two major groups: halophytes and glycophytes (non-halophytes). Halophytes can resist even 20% of salts in the soil and glycophytes exhibit limited growth in the presence of salts (Flowers and Colmer, 2008).

Plant growth and final productivity are limited by soil and water salinity. The ability of plants to survive under saline environments varies among different species of glycophytes and halophytes. Most of the halophytes survive at 200-300 mM NaCl concentrations by adjusting some adaptation mechanisms. The glycophytes develop two adaptive responses to salt: (a) osmotic response which is a rapid response increasing with the amount of the external salt and (b) ionic response which is slower than osmotic response characterized with the accumulation of Na^+ ions within vacuoles (Flowers and Colmer, 2008, Glenn et al., 1999). It is clear that the regulation of Na^+ absorption and Na^+ transportation across the plasma membranes and vacuolar membrane are of great importance for determining the plant cell response to salt stress (Blumwald, 2000).

Salt stress has negative impacts on the germination of the seeds and seed vigor. Increased amount of salt interferes with the seed germination while lower level of salinity promotes a state of dormancy (Ungar, 1996). Throughout the germination process salinity changes the water uptake by seeds due to lower osmotic potential, causes ion toxicity compelling the activity of enzymes, alters the metabolism of proteins, leads to hormonal imbalances and decreases the usage of seed reserves (Ahmad et al., 2012). The low germination efficiency is followed by the reduction in the growth rate. Salt stress can affect plant growth in several ways. First, the soil salinity reduces the uptake of water due to the osmotic effect of the soil solution containing salt. Secondly, on the other hand, higher levels of salt leading to osmotic and ionic toxicity in the plants due to the accumulation of salt in leaves. Finally, elevated amount of salt uptake and accumulation result in the death of leaves and reduce photosynthetic activity of the leaves and ultimately affect the yield. Depending on the salinity level, exposure time and the type of plant, the growth of a plant affects from salinity in various ways (Carillo et al., 2011, Munns, 2002, Munns and Tester, 2008).

Fundamental secondary effects of salinity stress include K^+ acquisition disturbances, improper functioning of the membranes, defects in the mechanism of the photosynthesis and other biochemical processes, programmed cell death and formation of ROS. Salt

stress causes stomata to close, which reduces the availability of CO_2 in the leaves and interferes with the fixation of carbon, subjecting chloroplasts to excessive excitation energy which in turn induce the oxidative stress increasing the formation of ROS such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen (Chinnusamy et al., 2006, Chinnusamy and Zhu, 2004, Parida and Das, 2005). On the other hand, as a result of salt stress, water deficit also leads to the formation of ROS. Salinity often activates reactive oxygen species (ROS) detoxification mechanisms as a fundamental defense mechanism against salt stress (Parvaiz and Satyawati, 2008).

1.2.2 Drought Stress

80-95% of the all living matter of non-woody plants is comprised of water which is the principle molecule in all physiological processes. Drought emerges when there is a deficiency of water in the atmosphere and soil. Water deficit is not only caused by lack of water but also by harmful environmental conditions such as low temperature or high salinity, thus it is not surprising that stress responses share many molecular mechanisms (Bray, 1997, Cattivelli et al., 2008).

Plants suffer from a number of cellular changes during drought. These variations result in stomatal closure, decline in transpiration, inhibition of photosynthetic activity and the growth of the cell. Plants accumulate compatible solutes (proline), abscisic acid (ABA) and also produce new stress tolerating proteins (Cattivelli et al., 2008, Shinozaki and Yamaguchi-Shinozaki, 1996). Damage prevention and neutralization of the detrimental effects of the damage are the two central strategies for drought tolerance improvement. The synthesis of protective molecules during dehydration process generate defense against dehydration damage. Moreover, the regulation of the repair-based mechanisms

that induce the rehydration is crucial for alleviation of the negative consequences during the damage (Shao et al., 2008).

Water stress is one of the major environmental factors that constrain productivity and stability of plants. Water deficit affects plants on several situations. Initially, plant growth and development decline under water stress. Afterwards, the rate of photosynthesis is adversely affected with progressive water deficit. Finally, membranes and proteins can be damaged by dehydration on the cellular level and ROS production gradually increases due to dysfunction of biochemical and molecular pathways (Araus et al., 2002, Shinozaki and Yamaguchi-Shinozaki, 1996). Plants possess several adaptation mechanisms such as reduction of water loss, protection of photosynthetic machinery and osmotic adjustment to overcome and tolerate the damaging impacts of the drought (Shinozaki and Yamaguchi-Shinozaki, 1997). The plants' drought response is accompanied by the activation of drought-responsive genes involved in the stress perception and stress signal transmission. Some of these genes encode regulatory proteins that further adjust stress signal transduction and regulate gene expression (Seki et al., 2003). Others encode proteins that protect the cells from the effects of desiccation. They control the compatible solute accumulation, membranetransportation, transport of water that require energy, and preservation of cell structures from drying process and damage caused by ROS formation (Cattivelli et al., 2008, Seki et al., 2003).

Drought stress leads to several biochemical and physiological responses and transcription of a number of drought responsive genes. It is important to figure out the functions of stress-inducible genes not only for further understanding of stress response mechanisms of higher plants at molecular level but also for further improvement of stress tolerance of crops (Umezawa et al., 2006).

The signaling pathways induced by drought are either ABA-dependent or ABA-independent. ABA (Abscisic acid) is a plant hormone functions in many developmental processes that is produced during shortage of water. Most plants become more adaptive

and tolerant to low water and high salt under the influence of ABA. Several genes induced by drought and low temperature stress also get induced by exogenous administration of ABA, revealing ABA-dependent signal transduction during stress perception and response (Yamaguchi-Shinozaki and Shinozaki, 2006, Zhu, 2001). Based on the microarray results, almost 40% of drought-inducible genes were also found to be induced by cold stress. Furthermore, approximately all the genes expressed during high salinity and ABA treatment were also induced by water deficit stress. These findings definitely show that there is a significant strong cross-talk between the signaling pathways initiated by a range of environmental stresses. According to their biological functions, the drought-responsive genes can be divided into two groups. The first group of genes encodes functional proteins involved in membrane and protein stabilization and cellular homeostasis such as late embryogenesis-abundant (LEA) proteins that encodes metabolic components. The second group includes genes that function in signaling pathways in response to stress such as MYB transcription factors that suppress ABA or jasmonic acid (JA)-induced genes. Therefore, better understanding of the drought tolerance mechanisms is in accordance with the identification of the expression patterns of functional and regulatory protein encoding genes (Boominathan et al., 2004, Yamaguchi-Shinozaki and Shinozaki, 2006).

1.2.3 Cold Stress

Cold (low temperature) is the other highly general environmental stress affecting the growth and development of plants and crop yield in an unfavorable manner. The process of cold acclimation is to expose non-tolerant plants to the chilling or non-freezing temperatures for increasing their tolerance against freezing temperatures. It is important for the induction of certain cold-responsive gene expression. Elucidation of the sensory and regulatory mechanisms of plants against cold via transcriptomic studies helps to

understand how the plants develop cold acclimation response (Chinnusamy et al., 2007b, Thomashow, 1999).

Cold stress triggers the alterations in the lipid composition of the cell membranes and leads to reconstruction of membrane structure both physically and biochemically by inducing other non-enzymatic proteins (Sharma et al., 2005). Most of the early cold responsive genes encode transcription factors that finally activate the late-induced cold-responsive genes. Therefore, cold stress triggers multiple transcriptional cascades. Additionally, cold stress also functions in the regulation of the functional genes involved in the biosynthesis or signaling of plant hormones. Moreover, the DNA replication machinery, spliceosome and mismatch repair pathways are associated with cold stress (Boominathan et al., 2004, Chinnusamy et al., 2007b).

Stabilization of membranes and cryopreservation are the main strategies for plant tolerance to cold. Membrane stabilization via phase transitions is the key step for cold stress tolerance. Increase in the amount of minor lipid components such as phosphatidylinositol, glycolipids and cholesterol in the membrane or alternatively failure to enclose crucial intrinsic membrane proteins into the cell membrane is critical for consolidating the membrane stability. Finally, it can be assumed that changes in lipid composition have been proposed to augment the membrane stability against low temperature stress (Mahajan and Tuteja, 2005, Thomashow, 1999). Cryopreservation is the protection of cell membranes and organelles from cold with the help of cryoprotective function of soluble sugars and other compatible solutes (osmolytes) (Nayyar et al., 2005).

Plants are sessile organisms and develop control systems to adjust genetic networks for accurate and proper functioning of the organism to handle with the negative effects of environmental stresses. The regulatory mechanisms underlying the gene expression in response to water deficit, low temperature and high salt are not well-understood (Cramer et al., 2011). Although abiotic stresses induced protein synthesis is known, regulatory mechanisms remain unknown.

1.3 MicroRNAs (miRNAs): Regulatory Molecules for Plants

miRNAs are a class of endogenous small non-coding RNAs (~21 nt) that regulate the protein-coding gene expression post-transcriptionally by either binding directly to mRNAs for degradation, or by repressing the protein translation (Frazier et al., 2010). In plants, miRNAs are highly evolutionarily conserved from lower mosses to higher flowering plants. They have roles in growth regulation, development, timing of development, hormone signaling, organ formation (formation of leaf, shoot and root) and abiotic stress responses (Sunkar, 2010). Plant miRNAs was firstly identified in *Arabidopsis thaliana* in 2002 (Reinhart et al., 2002) and then, computational and experimental methods have been utilized to discover miRNAs in several plant species, such as soybean, cotton, maize, potato, rice, apple, poplar and switchgrass according to the conservation of mature miRNA sequences (Zhang et al., 2006, Zhang et al., 2007).

mRNA cleavage (Bartel, 2004) or translational repression (Brodersen et al., 2008) are the two mechanisms for regulation of gene expression post-transcriptionally by miRNAs in plants. Most of the early miRNAs were studied and identified in plants grown under unstressed normal conditions. In the recent studies miRNAs have been predicted and identified from the plants exposed to different stress conditions. Thus, the direct relation of the microRNAs with the regulation of the stress-related genes was clearly discovered (Lu and Huang, 2008, Sunkar, 2010, Sunkar et al., 2007, Sunkar and Zhu, 2004). For example, the expression of miR398 is downregulated transcriptionally by oxidative stress, and this is important for accumulation of CSD1 and CSD2 mRNAs posttranscriptionally and oxidative stress tolerance (Sunkar et al., 2006). Another miRNA, miR172 regulates expression of a small group of *AP2*-like transcription factors and functions in regulating the transitions between developmental stages (Zhu and Helliwell, 2010).

A great number of plant miRNA targets are transcription factors functioning in plant growth and development (Rhoades et al., 2002) and genes involved in responses to abiotic stress (Sunkar and Zhu, 2004). The complete removal of the corresponding target mRNA and the reduction or increase in the target mRNA expression via the induction of a specific miRNA cause changes in the cell-fate. Almost all plant miRNAs play crucial roles in various developmental processes, such as leaf and flower development, root, shoot and stem formation, control of flowering time, hormone signaling and nutrient homeostasis (Sunkar et al., 2007, Wang et al., 2007).

1.4 miRNA Biogenesis

The *MIRNA* genes (defined genetic loci) found predominantly within intergenic regions in plants give rise to miRNAs. An immature miRNA primary transcript (pri-miRNA) arises from a *MIRNA* locus by RNA polymerase II (Pol II) transcription (Figure 2). pri-miRNA is having the capacity of forming a distinctive fold-back hairpin (stem-loop) structure. This imperfect dsRNA is identified and processed by a Dicer Like enzyme1 (DCL1)-containing complex to produce a miRNA/miRNA* duplex. DCL1 plays dual roles corresponding to the roles of Drosha and Dicer in animals. The formation of the complex is a multi-step process involving a stem-loop intermediate (pre-miRNA). HYPONASTIC LEAVES1 (HYL1) is a double-stranded RNA binding protein that helps the DCL1 making the first cut on the primary miRNA (pri-miRNA) at the stem side. DCL1 interaction with HYL1 is a critical step for effective and consistent processing of pri-miRNAs. This first cut is essential for the production of precursor-miRNA (pre-miRNA) in the process. After that following second cut is done by the same complex to produce mature miRNA duplex (Chen, 2005, Reinhart et al., 2002, Voinnet, 2009). This duplex has two nucleotide overhangs at the 3’ end on the both strands. The 3’ end of the miRNA/miRNA* duplex is then methylated by the small RNA-specific methyl

transferase (a small RNA methylase), HUA ENHANCER1 (HEN1). Methylation is required for the protection of miRNAs from exonucleases that target the 3' end of the miRNAs. It is also crucial for plant argonaute (AGO) proteins to recognize miRNAs. The methylation process is unique to the plants and such mechanism is absent in animal miRNA biogenesis (Figure 2) (Chinnusamy et al., 2007a, Yu et al., 2005).

The biogenesis of miRNAs, formation of pre-miRNA and processing, and also the arrangement of miRNA complexes are thought to happen in the nuclear dicing bodies within the nucleus of the plant cell (Zhu 2008). The transportation of the processed mature miRNA duplexes from nucleus to the cytoplasm is then carried out by a nuclear exporting protein HASTY (HST) (Voinnet, 2009). HASTY is an ortholog of Drosophila Exportin-5 protein in plants (Du and Zamore, 2005).

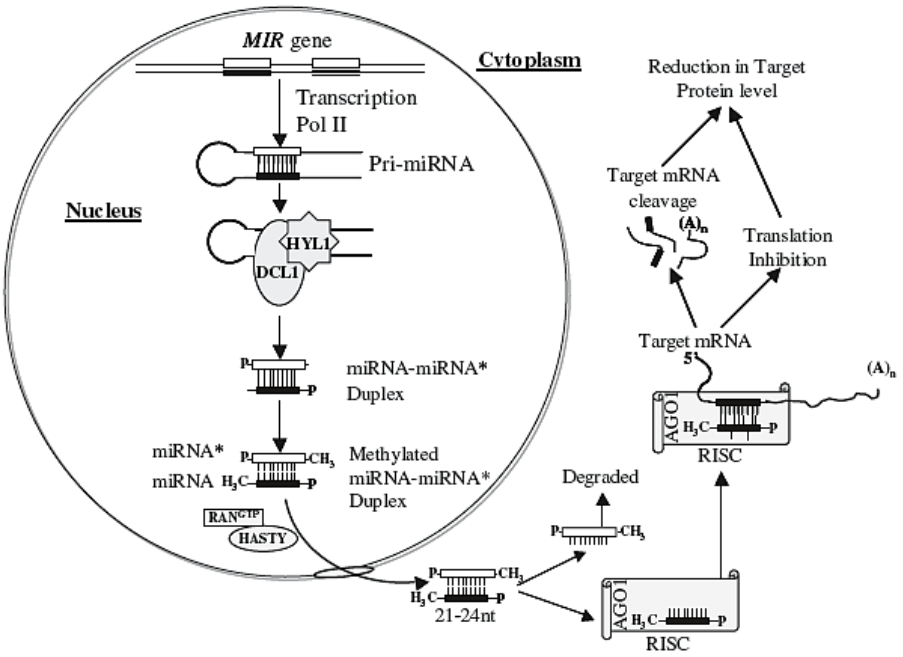

Figure 2 A model for Biogenesis of miRNAs and Post-transcriptional Gene Regulation in Plants. Pri-miRNA is transcribed from miRNA gene (MIR gene) by RNA polymerase II. The pri-miRNA transcript forms a stem-loop structure cleaved by DCL1 protein. DCL1 interacts with HYL1 for recognition and cleavage of pri-miRNA into miRNA-miRNA* duplex. The duplex is then methylated by HEN1 at the 3' end. Methylated miRNA-miRNA* duplex is exported to cytoplasm by HASTY (HST) and unwound into single stranded mature miRNA. The then enters the RNA induced silencing complex (RISC) containing Argonaute (AGO) family protein, AGO1. RISC then cleaves the target mRNA or inhibits translation (Chinnusamy et al., 2007a).

The thermodynamic features of the duplex appear to determine the fate of each strand in a miRNA/miRNA* duplex. In the duplex, the miRNA strand is called guide strand that is then enters to enter to RNA-induced silencing complex (RISC) and forms miR-RISC complex. The miRNA* strand is called passenger strand that is degraded by an unknown mechanism (Chen, 2005). That is, the miRNA strand is selectively incorporated into AGO-containing RISC, whereas the miRNA* strand is often short-lived. After being transported to the cytoplasm the 3' overhang of the mature miRNA bind to the PAZ domain of Argonaute (AGO) protein. AGO is an essential component of the RISC and

also found at the catalytic center of the RISC. The PIWI domain of AGO shows RnaseH activity involved in target cleavage (Voinnet, 2009). Mature plant miRNAs are typically 21-nt in length and negatively regulate expression of specific genes by guiding target recognition and cleavage. The miRNA containing RISC binds to the target mRNA in a sequence specific manner. The complex either binds to target mRNA and cleaves it (Bartel, 2004), or prevents the translation of the target gene product (Brodersen et al., 2008). In plants, the cleavage of the target mRNA is the predominant way of post-transcriptional gene regulation (Vaucheret, 2006). In plants, the cleavage and rapid degradation of "target" mRNAs by miRNAs almost always induce via the formation of base-pairing interactions (Jackson and Standart, 2007).

The majority of miRNAs exhibit unique tissue-specific expression (Pandey et al., 2014, Sood et al., 2006). Most of them have development-related (Jover-Gil et al., 2005) and stress-induced expression (Lu and Huang, 2008). Detailed expression profiling of individual miRNAs is required for better understanding of miRNA function. However, it is really challenging to obtain an accurate expression pattern for specific miRNAs due to the short length of the mature miRNAs, the availability of highly similar miRNA family members and sometimes the presence of precursors including the miRNA sequence (Wang and Yang, 2010).

1.5 Plant and Animal miRNAs

Plant and animal miRNAs exhibit functional similarities so that both play essential role in development and control regulatory genes. Despite the similarities of both plant and animal miRNAs show their control over gene expression fundamentally in different ways. In plants, DCL1 in concert with HYL1 involves in the first step of miRNA biogenesis whereas Drosha, an RNaseIII enzyme, with double-stranded RNA-binding

domain protein Pasha are the key regulators for processing pre-miRNA from pri-miRNA in animals (Saikumar and Kumar, 2014, Voinnet, 2009). Both plant miRNA genes and most animal miRNA genes are transcribed by RNA polymerase II (Figure 3). But a specified group of animal miRNAs (C19MC) are transcribed by RNA polymerase III (Faller and Guo, 2008, Voinnet, 2009).

Pre-miRNA itself is exported from nucleus to cytoplasm with the help of HASTY protein in plants and processing of the pre-miRNA transcript for formation of the miRNA-miRNA* duplex happens in the cytoplasm. However, processing of the pre-miRNA transcript for formation of the miRNA-miRNA* duplex occurs in the nucleus and miRNA-miRNA* duplex is just transported from nucleus to cytoplasm with help of exportin-5 protein in animals as seen in Figure 3 (Axtell et al., 2011, Park et al., 2005). Unlike from most animal mature miRNAs, plant mature miRNAs are methylated at the 3' end by HEN1 (Yu et al., 2005).

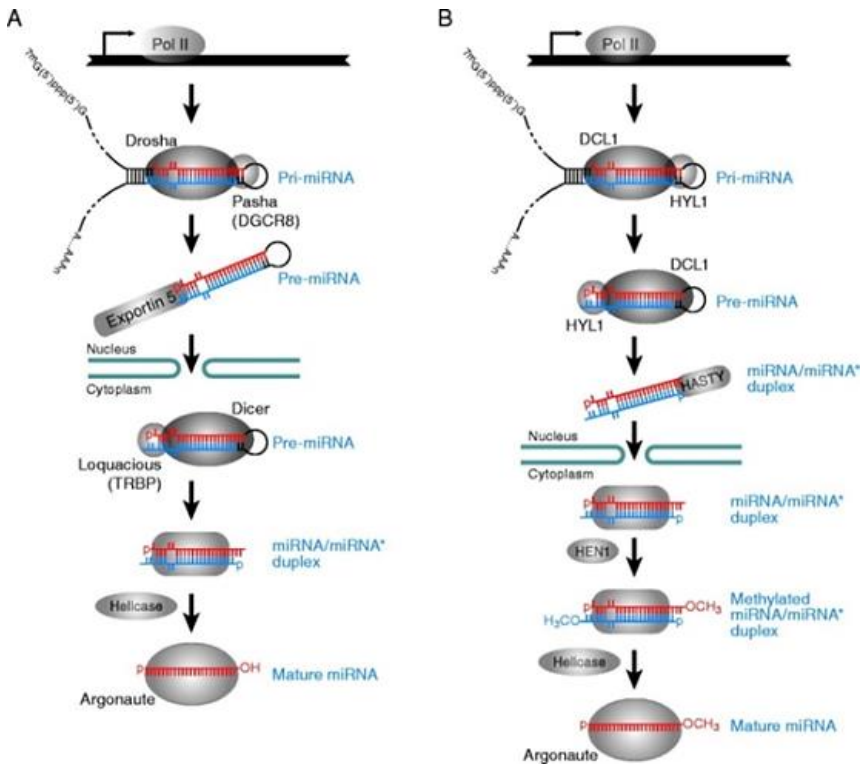

Figure 3 The biogenesis of miRNAs, **A** in Animals (In animals, two processing steps yield mature miRNAs. Each step is catalyzed by an RNase III endonuclease (Drosha) together with a double-stranded RNA-binding protein partner. First, Drosha cleaves the flanks of pri-miRNA to form an ~70 nucleotide stem loop, the precursor miRNA (pre-miRNA). Accurate and efficient pri-miRNA processing by Drosha requires a dsRBD protein, known as Pasha in *Drosophila* and DGCR8 in mammals. The pre-miRNA is then exported from nucleus to cytoplasm by Exportin 5. In the cytoplasm, Dicer makes a pair of cuts on the mature miRNA, producing a ~21-nucleotide RNA duplex. From the miRNA/miRNA* duplex, one strand, the miRNA, preferentially enters the protein complex that represses target gene expression, the RNA-induced silencing complex (RISC). Whereas, the miRNA* strand is degraded. The choice of strand relies on the thermodynamic stability of the duplex.) and **B** in Plants (In animals, two processing steps yield mature miRNAs. Each step is catalyzed by an RNase III endonuclease (Drosha) together with a double-stranded RNA-binding protein partner. First, Drosha cleaves the flanks of pri-miRNA to form an ~70 nucleotide stem loop, the precursor miRNA (pre-miRNA). Accurate and efficient pri-miRNA processing by Drosha requires a dsRBD protein, known as Pasha in *Drosophila* and DGCR8 in mammals. The pre-miRNA is then exported from nucleus to cytoplasm by Exportin 5. In the cytoplasm, Dicer makes a pair of cuts on the mature miRNA, producing a ~21-nucleotide RNA duplex. From the miRNA/miRNA* duplex, one strand, the miRNA, preferentially enters the protein complex that represses target gene expression, the RNA-induced silencing complex (RISC). Whereas, the miRNA* strand is degraded. The choice of strand relies on the thermodynamic stability of the duplex.) (Du and Zamore, 2005).

Both plant and animal miRNA genes mainly exist in the intergenic regions. However, significant number (~25%) of animal miRNAs is found in the introns of pre-mRNAs. (Millar and Waterhouse, 2005). Plant miRNAs substantially regulate their target genes by directly cleaving mRNAs whereas animal miRNAs generally act by the repression of translation (Sunkar, 2010).

Plant miRNAs have their own promoters. Although dicer-independent miRNA pathways are not found in plants (Voinnet, 2009), they are found in animals such as mirtron pathway and characterization of pre-miRNA ends by splicing instead of Drosha cleavage. Plant miRNA targets have a single target site and are regulated by just one miRNA. But animal miRNA target genes have conserved target sites for different miRNAs (i.e., one target is controlled by many miRNAs) (Axtell et al., 2011).

In plants, miRNAs show perfect or near perfect complementarity to their targets and lead to mRNA degradation, and to some extent they lead to translational repression. However, miRNAs often guide the translational repression, and sometimes the process ends up with the decay of target mRNAs in animals, by means of partial complementarity base pairing to the 3′ untranslated regions (UTRs) (Faller and Guo, 2008). Length of pre-miRNA is highly variable in plants, ranging from 70 to hundreds of nucleotides. On the other hand, the length of pre-miRNAs in animals is more consistent, mostly 55-70 nucleotides (Axtell et al., 2011).

1.6 miRNAs and Plant Development

Discovery of detection and cloning methods used for identification of mRNAs in the *Arabidopsis* opened a new era for the posttranscriptional gene regulation by miRNAs in plants. A set of this early cloned miRNAs are involved in the growth and development of the plant by targeting various transcription factors and hormone-signaling related genes that are abundantly expressed in the whole plant (Reinhart et al., 2002). Cloning of miRNAs from *Arabidopsis* subsequently open a new path for the plant miRNA world and the plant miRNA research has improved with the cloning of numerous miRNAs from several plant species under diverse environmental conditions (Lu and Huang, 2008, Sunkar and Zhu, 2004, Xu et al., 2012). Since, miRNA targets the mRNA in a sequence specific manner, it is possible that the miRNAs have a similar functional role across different plant species (Axtell, 2008, Li and Mao, 2007). Most of the miRNA target genes are involved in leaf, shoot, stem and root development, floral identity, development of flowers and indication of time for flowering, hormone signaling and vascular tissue development (Axtell and Bowman, 2008). miRNAs are said to be involved in various metabolic processes in plants and they also promote the vegetative phase to reproductive phase transition (Lauter et al., 2005). Either miRNA or the target gene overexpression studies displayed the roles of miRNAs in plant growth and development (Wang et al., 2007). Some of the miRNAs functioning in the processes in the scope of this study are summarized in Table 1.

Table 1 miRNAs involved in plant growth and development through targeting various transcription factors in different plant species.

miRNA	Target	Function	Plant Species
mir156	Squamosa-promoter binding-like proteins (*SPL*s)	Developmental timing (transition of plant growth from vegetative phase to reproductive phase), shoot and leaf development	*Arabidopsis* and rice (Wu and Poethig, 2006, Xie et al., 2012) Switchgrass (Fu et al., 2012) *Triticum aestivum* (Yin et al., 2010)
miR159	*MYB* and *TCP* (Teonsite branched 1-Cycloidea-PCF) transcription factors	Floral organ development and leaf morphogenesis Hormone signaling during seedling development, seed germination Anther development	*Arabidopsis* (Allen et al., 2007) *Arabidopsis* (Reyes and Chua, 2007) *Arabidopsis* (Millar and Gubler, 2005) Strawberry and tomato (Csukasi et al., 2012, Karlova et al., 2013)
miR166	*HD-ZIP* (Homeo domain leucine zipper) transcription factors CCAAT-box binding transcription factor family member (Nuclear factor Y)	Leaf development, leaf polarity, vascular tissue development, shoot apical meristem and floral development Root and nodule development Regulation of ABA (Abscisic acid)-responsive elements	*Arabidopsis* (Jung and Park, 2007) *Medicago truncatula* (Boualem et al., 2008) Soybean and populus (Song et al., 2011, Ko et al., 2006)
miR169	Nuclear factor Y (*NF-Y*) transcription factors *HAP2* (Heme Activator Protein2)	Early flowering Abiotic stress response	*Arabidopsis* (Li et al., 2008), tomato (Zhang et al., 2011) and soybean (Ni et al., 2013) Populus (Potkar et al., 2013)
miR319	*TCP* and *NAC* (NAM (No Apical Meristem), ATAF (*Arabidopsis* Transcription Activation Factor) and CUC (CUp-shaped Cotyledon)) family of transcription factors	Formation of shoot apical meristem, floral organs and lateral shoots Control of senescence Abiotic stress response Leaf morphogenesis (cell division and cell differentiation in leaves)	*Arabidopsis* and tomato (Schommer et al., 2012) *Arabidopsis* (Schommer et al., 2008) Sugarcane and creeping bentgrass (Thiebaut et al., 2012, Zhou et al., 2013) *Arabidopsis* and rice (Gonzalez et al., 2012, Yong et al., 2013)
miR393	F-box auxin receptors *TIR1* (Transport Inhibitor Response Protein 1) and *AFB1* (Auxin Signaling F-box Protein 1)	Signaling pathways, regulate plant metabolism via signal transduction Regulation of normal plant development Abiotic stress response	*Arabidopsis* (Mallory et al., 2005) *Arabidopsis* (Chen et al., 2011) Rice and tobacco (Gao et al., 2011, Feng et al., 2010)

1.7 miRNAs and Plant Abiotic Stress

Plants develop certain regulatory mechanisms used for tolerating severe environmental conditions and stress tolerance of plants is a complicated process resulting in the synthesis of proteins to eliminate the damaging impacts imposed by stress conditions and it is linked with the gene regulation simultaneously. Gene products involved in the biosynthesis of the detoxification enzyme systems and osmoprotectant compounds are essential components for generating an effective response to abiotic stresses. The significance of transcriptional gene regulation pathways in response to abiotic stresses in plants is well-known. However, the involvement of post-transcriptional gene regulation through miRNAs during stress is an emerging field to understand as well as to explore the strategies for stress tolerance (Bartels and Sunkar, 2005, Vaucheret, 2006). miRNAs directly or indirectly involved in abiotic stress responses were listed in Table 2.

Table 2 miRNAs involved in plant abiotic stress responses through targeting various transcription factors in different plant species.

miRNA	Abiotic Stress	Target	Plant Species
mir156	Drought stress Salt stress Cold stress	Squamosa-promoter binding-like protein (*SPL*)	Barley (Kantar et al., 2010) *Arabidopsis* (Liu et al., 2008) Cassava (Zeng et al., 2009)
miR159	Drought stress Salt stress	*TCP* and *MYB* (MYB33, MYB65, MYB101 and MYB104) transcription factors	*Arabidopsis* (Reyes and Chua, 2007) and *Phaseolus vulgaris* (Arenas-Huertero et al., 2009) *Arabidopsis* (Liu et al., 2008)
miR166	Drought stress Cold stress	*HD-ZIP III* transcription factor family	Barley (Kantar et al., 2010) and *Triticum dicoccoides* (Kantar et al., 2011) *Arabidopsis* (Zhou et al., 2008)
miR169	Drought stress Salt stress Cold stress	*NF-YA* transcription factor CCAAT-box binding transcription factor	Rice (Zhao et al., 2009, Zhao et al., 2007) and *Medicago truncatula* (Saini et al., 2012) *Arabidopsis* (Liu et al., 2008) and rice (Zhao et al., 2009) *Arabidopsis* (Zhou et al., 2008) and *Brachypodium* (Zhang et al., 2009)
miR319	Drought stress Salt stress Cold stress	*TCP*, *NAC* (NAM (No Apical Meristem), ATAF (*Arabidopsis* Transcription Activation Factor) and CUC (CUp-shaped Cotyledon)) transcription factors *PCF5* and *PCF6* (members of TCP family)	*Arabidopsis* (Schommer et al., 2012, Liu et al., 2008) *Arabidopsis* (Liu et al., 2008) Sugarcane (Thiebaut et al., 2012)
miR393	Drought stress Salt stress Cold stress	*TIR1* (Transport Inhibitor Response 1) (Auxin receptor)	*Arabidopsis* and rice (Sunkar and Zhu, 2004, Zhao et al., 2007) *Phaseolus vulgaris* (Arenas-Huertero et al., 2009) Zea mays (Luo et al., 2014) *Arabidopsis* (Liu et al., 2008) *Arabidopsis* (Zhou et al., 2008)
miR398	Oxidative stress Drought stress Salt stress Cold stress	*CSD1* and *CSD2* (Cu-Zn Superoxide Dismutases) (Detoxification of ROS)	*Arabidopsis* (Jones-Rhoades and Bartel, 2004) *Medicago truncatula* (Saini et al., 2012) *Arabidopsis* (Jagadeeswaran et al., 2009) *Populus tremula* (Jia et al., 2009) Tobacco (Fraizer et al., 2011) *Arabidopsis* (Sunkar and Zhu, 2004)

Salt and drought stresses as well as cold stress induce miRNA expression via various mechanisms, such as the regulation of oxidative stress or plant growth inhibition. Most of the target genes of drought-induced miRNAs are transcription factors (Xiong et al., 2002). The activity of transcription factors regulate the gene expression that encode proteins functioning in salt tolerance or enzymes functioning in the protection and repairing pathways. All of these proteins serve as regulators to restore homeostasis of cells and normal functioning of the system under salt stress(Flowers, 2004, Munns, 2005).

1.8 Aim of the Study

The stress-tolerant characteristics of *Thellungiella* species make them suitable models for the progression of plant adaptation to extreme environments. However, miRNA functions' in abiotic stress conditions of *Thellungiella* remain poorly understood. Therefore, in this study, evolutionarily conserved miRNAs in *Thellungiella* were investigated in terms of their expression profiles and their potential targets upon salt, cold and drought stresses. The information of how stress induced miRNA expression changes the expression of potential mRNA targets is of importance to better understand the salt, cold and drought tolerance in *Thellungiella halophila* plants not only to better examine the underlying biology but also for future development of abiotic stress tolerant transgenic plants.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant Material and Plant Growth Conditions

Throughout the study the seeds of *Thellungiella halophila* (Shandong and Yukon ecotypes) were used. For experiments, the seeds were potted in 1:3, sand:soil mixture containing plastic pots and the pots were kept in a cold room (4ºC) for vernalization. After two weeks of vernalization, the pots were incubated, which provides a germination and growth environment for seeds, at 24 ± 2°C under fluorescent light with a 16/8-h (light/dark) period. Subsequent six weeks period, the pots were watered with half-strength liquid Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal media for normal growth of plants and synchronized flowering. Plants were watered regularly, not allowing soil to dry completely until all flowering is finished and siliques begin to ripen and then the watering was gradually decreased. At this stage, ripening seeds were harvested within seed about 1 to 2 weeks after termination of watering. Fresh *Thellungiella* seeds were surface sterilized in 20% (v/v) sodium hypochlorite (NaOCl) including 0.05% (v/v) Sodium Dodecyl Sulfate (SDS). Then they were rinsed with sterile distilled water. Seeds were sown into MS medium supplemented with 3% sucrose and 0.8% plant agar containing boxes and the boxes were put at 4ºC for two weeks of vernalization. Afterwards seeds were germinated and plants were grown in a growth chamber at 24 ± 2°C under fluorescent light with a 16/8-h (light/dark) period.

2.2 Abiotic Stress Treatments

Plants grown in boxes containing MS medium supplemented with 3% sucrose and 0.8% plant agar were used as controls. However, after 4 weeks of germination and growth in a growth chamber, plants (for salt and drought stresses) or the boxes (for cold stress) were transferred to stress conditions. For salt stress treatments, plants were transferred to MS medium supplemented with 3% sucrose and 0.8% plant agar and gradually increased concentrations of NaCl (0, 100, 150, 200 and 250 mM) containing boxes. Normally grown plants were also transferred to MS medium containing 200 mM mannitol for drought stress treatment. Some of the plants were transferred to boxes containing fresh MS medium and stored at 4°C for cold exposure of the plants. At the end of the each stress treatment, shoots of control and treated seedlings were excised. Harvested plant materials were frozen immediately in liquid nitrogen and preserved at -80°C.

2.3. Physiological Parameters for Identification of Salt Tolerance

2.3.1 Determination of Glutathione (GSH-GSSG Assay)

Glutathione act as a regulator of gene expression in plants and plays an important role in defense of plants and other organisms against oxidative stress. The reduced tripeptide form (GSH) exists interchangeably with the oxidized form (glutathione disulphide, GSSG). The spectrophotometric method for glutathione (GSH) involves oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm. The glutathione disulfide (GSSG) formed can be recycled to GSH by glutathione reductase in

the presence of NADPH. The method is simple, convenient, sensitive and accurate. Total GSH and GSSG measurements were done according to a revised version of a protocol prepared by Owen and Butterfield (Owen and Butterfield, 2010).

Control and salt-stress treated plant tissue samples were ground with liquid nitrogen after sampling. Then, ~0.1 g of ground sample was homogenized in cold 5% (w/v) sulfosalisylic acid containing 1 mM EDTA and 2% (w/v) triethanolamine (Sigma-Aldrich) for extraction and neutralization. The tubes were gently mixed by vortexing and centrifuged at 15.000 g for 10 min at 4 ºC for collecting the supernatant containing glutathione. The reaction starts by the addition of 50 µl of plant extract (supernatant) into the tubes containing 750 µl of the reaction buffer composed of phosphate buffer (100 mM, pH=7.5), 3 mM DTNB (Sigma-Aldrich), 1 mM EDTA and 0.5 U/ml glutathione reductase. Subsequently, the tubes were incubated for 5 min at room temperature and 50 µl of NADPH (Sigma) solution (mg/ml) was added into the tubes. The change in absorbance (A412) was monitored at 2 min intervals for 10 min in comparison with an assay mixture containing no plant extract. The concentration of oxidized glutathione (GSSG) was determined in the same reaction buffer after the addition of 2 µl of 2-vinylpyridine (Sigma-Aldrich) in 100 µl of plant extract. The reaction for the deactivation of reduced glutathione was last for 1 hour at room temperature. The amount of reduced glutathione was calculated as the difference between total and oxidized glutathione (GSH = Total glutathione - GSSG). The amount of total glutathione and oxidized glutathione was calculated using a standard curve with GSH standard solution of known concentrations (0.125-4 µM) and the concentrations were expressed as µmols GSH or GSSG g-1 fresh weight (FW).

2.3.2 Proline Concentration Determination

Colorimetric detection of proline was determined (Bates et al., 1973) based on proline's reaction with ninhydrin. Approximately, 0.2 g of shoots were homogenized and then extracted in 1 ml of 3% sulphosalycylic acid. The extracts were transferred into eppendorf tubes. 1:1:1 ratio of acid ninhydrin: glacial acetic acid: 3% sulphosalycylic acid (200 µl from each) was added into tubes containing 200 µl of supernatant obtained from tissue extraction step. The tubes were then incubated at 96°C for 1 hour for the complete hydrolysis of proteins. Proline concentration was calculated by a constructed standard curve. Proline concentration was defined as µmol/g FW. Data represent the average of three experiments with three replicates, and data were analyzed via paired-samples t-test.

2.3.3 Determination of Lipid Peroxidation

MDA (malondialdehyde) is produced by the disruption of polyunsaturated fatty acids of cellular membranes and accumulates in cells due to abiotic stresses. Lipid peroxidation was determined by calculating the MDA content (Ohkawa et al., 1979) in 0.2 g fresh weight (FW) of shoots under salt stress conditions. Tissues were homogenized in liquid nitrogen and suspended in 2 ml of 0.1% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10000 g for 15 min at room temperature. After centrifugation, 0.5 ml of supernatant from each sample was taken and 0.5 ml 0.1 M Tris/HCl buffer (pH=7.6) and 1 ml of freshly prepared TCA-TBA (thiobarbituric acid)-HCl (1:1:1) solution were added into the tubes. Samples were incubated at 95°C for 1 hour. After incubation, tubes were immediately placed into ice until reaching to room temperature. The concentration of MDA was calculated using 532 nm absorbance and the

extinction coefficient of 155 mM^{-1} cm^{-1}. Unspecific turbidity correction was done by subtracting the 600 nm absorbance value. Data represent the average of three experiments with three replicates, and data were analyzed via paired-samples t-test.

2.3.4 Relative Anthocyanin Determination

Anthocyanin preserve plants against reactive oxygen species and used as an indicator of oxidative stress. For relative anthocyanin determination (Mancinelli, 1990, Neff and Chory, 1998), plant tissues were ground in liquid nitrogen with a pestle. This powder was incubated overnight in 150 μL of methanol acidified with 1% HCl at 20°C in a dark refrigerator. Following the addition of 100 μL of distilled water, anthocyanins separated from chlorophylls with the addition of 250 μL of chloroform to each tube. The tubes were centrifuged at the highest speed for 5 min. and the supernatant was placed in a new tube. It is important not to take any white plant tissue or organic bottom solution. The tube volume was completed to 1 ml by adding a mixture of 60% acidified methanol with 1% HCl and 40% distilled water. Total anthocyanins were determined by measuring the absorbance at 530 and 657 nm of the aqueous phase. By subtracting the absorbance at 657 nm from the absorbance at 530 nm, the relative amount of anthocyanin per seedling was calculated. Data represent the average of three independent experiments with three replicates, and data were analyzed via paired-samples t-test.

2.3.5 Determination of Relative Water Content

The relative water content (RWC) is a useful indicator of the water balance status of a plant under normal growth conditions and stress conditions. RWC is a measure of the relative cellular volume showing the changes in cellular volume that could be affecting interactions between macromolecules. In order to measure the RWC (González and González-Vilar, 2001), the eppendorf tubes were weighed and labelled. The freshly excised rosette plants with relatively same size were taken and put into the eppendorf tubes. The tubes were sealed and weighed immediately to give a measurement for tissue fresh weight (FW). Each tube was then filled with same amount of (1.2 ml) cold deionised water and placed in a fridge for 4 hours to allow the tissue taking up water. The plants were taken away from the water and put on to a tissue paper to remove carefully the excess of water on the leaf surface. Every sample was transferred to tubes and reweighed. This measurement was given the fully turgid fresh weight (TFW). Finally, the samples were carefully taken from the tubes and put on to dry sections of filter paper and placed in an oven at 70°C at least 48 hours. After drying, the tissues were reweighed and recorded as a measurement for dry weight (DW). Relative water content (RWC) was then calculated using the following equation:

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100$$

2.4 Total RNA Isolation

All equipments (mortars, pestles, eppendorf tubes and pipette tips), solutions, other glass- and plastic-wares and distilled water used in RNA extraction procedure should be free of any contaminating RNases. Therefore, all materials were immersed into 0.1% (v/v) Diethylpyrocarbonate (DEPC) (Sigma-Aldrich) containing distilled water for decontamination. All DEPC-treated equipment was then autoclaved at 121°C for 20 min.

TRIzol reagent (Invitrogen) was used to perform total RNA isolation from plant material (Chomczynski and Sacchi, 1987). TRIzol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Approximately, 0.1-0.2 g of freshly weighed shoot samples were ground in liquid nitrogen in pre-cooled mortars using pestles to obtain a fine powder. The half of the pre-cooled 2 ml eppendorf tubes were filled with the tissue powder and immediately put into the liquid nitrogen. Powdered tissue samples were suspended in 1.5 ml Trizol reagent. The tubes were incubated with occasional shaking at least 15 minutes in a vortex mixer at room temperature until all plant material was fully dissolved. Samples were then centrifuged at 21.000 g for 5 min to precipitate insoluble plant material. Approximately, 0.1-0.2 g of freshly weighed shoot samples were ground in liquid nitrogen (grinding step was crucial) in pre-cooled mortars using pestles to obtain a fine powder. The half of the pre-cooled 2 ml eppendorf tubes were filled with the tissue powder and immediately put into the liquid nitrogen. Powdered tissue samples were suspended in 1.5 ml Trizol reagent. The tubes were incubated with occasional shaking at least 15 minutes in a vortex mixer at room temperature until all plant material was fully dissolved. Samples were then precipitated by centrifugation at 21.000 g for 5 min. Almost 900 µL of the upper phase was transferred to new eppendorf tubes and 200 µL of chloroform was then added to the tube content. The solution was mixed vigorously by vortexing for 15 sec and incubated for 3 min. Samples were then centrifuged at 21.000 g for 15 min at 4°C for phase separation. 450-500 µL of the

supernatant was put into a clean Eppendorf tube. RNA precipitation was performed by adding equal volume of pre-chilled isopropanol and the tubes were gently mixed by inverting. After incubation at -80°C for 30 min, the samples were thawed and then centrifuged at 21.000 g for 15 min at 4°C in a refrigerated centrifuge. The upper aqueous phase was thrown away carefully. Samples were mixed briefly and again centrifuged at 21.000 g for 5 min at 4°C. Finally, the formed pellet of RNA was dissolved in 40 µL of DEPC-treated ultrapure water. Dissolved RNA samples were kept at -80°C for further use.

2.5 RNA Quantification by Spectral Absorption

RNA concentration and purity were determined by using absorption of light at 260 nm and 280 nm (A260/A280) on a spectrophotometer and the fluorescence was recorded by using NanoDrop 3300 Fluorospectrometer (Thermo Scientific). An A260 reading of 1.0 corresponds to ~40 µg/ml for single-stranded RNA. An A260/A280 ratio of 1.8-2.1 is an indicator of purified RNA.

2.6 DNase Treatment for Clean-up RNA Samples

The contamination of DNA in the samples of RNA was eliminated by using RNase-free DNase I (Thermo Scientific) as described by the manufacturer. In a total 50 µL of the reaction mixture, nearly 10 µg of total RNA was incubated at 37°C for 30 min with 5U DNaseI. 5 µL of 10X reaction buffer was added and the reaction was ended by the suplementation of 5 µL 50 mM EDTA (ethylenediaminetetraacetic acid) and the final mixture was incubated at 65°C for 10 min. Ethanol-sodium acetate precipitation was

performed immediately after DNase I treatment to remove impurities and concentrate the RNA samples. RNA quality and quantity were determined by using absorption of light at 260 nm and 280 nm (A260/A280) on a spectrophotometer (NanoDrop 3300 Fluorospectrometer (Thermo Scientific)) as described in the previous RNA Quantification section.

2.7 Northern Blot Analysis of Selected miRNAs

Seven abiotic stress-associated miRNAs (miR319, miR156, miR159, miR398, miR166, miR169 and miR393) were selected from the microRNA database **(**mirBASE) (http://www.mirbase.org) according to the identified miRNAs from *Arabidopsis.* The sequences of oligonucleotide probes specific for selected miRNAs (miR319, miR156, miR159, miR398, miR166, miR169 and miR393) were given in Table 3.

Table 3 Oligonucleotide probe sequences used for Northern Blots of selected miRNAs.

Probe ID	Sequence (5'-3')
miR156	5'-ctgacagaagagagtgagcaca-3'
miR159	5'-gagctccttgaagttcaatggag-3'
miR166	5'-ggactgttgtctggctcgaggac-3'
miR169	5'-gcagccaaggatgacttgccgat-3'
miR319	5'-ttggactgaagggagctccctct-3'
miR393	5'-gaatcatgctatctctttggatt-3'
miR398	5'-agggttgatatgagaacacacg-3'

For Northern Blot experiments, initially 40 to 60 µg of total RNA of *Thellungiella halophila* (Shandong and Yukon ecotypes) plants, grown in plastic pots and exposed to stress conditions, was heat-denatured at 95°C for 5 min and immediately chilled on ice for another 5 min. ¼ volume of loading buffer (50% glycerol, 50 mM Tris-HCl pH=7.7, 5 mM EDTA, 0.03% Bromophenol Blue) was added on to RNA samples. Total RNA was loaded on a 15% polyacrylamide gel (prepared by using 40% Acryl/Bisacryl 19:1 (Bio-Rad) solution, 5X TBE buffer and 42% urea) containing 10% Ammonium persulfate (APS) and N,N,N',N'-Tetra Methyl Ethylene Diamine (TEMED) (Bio-Rad). The gel was run for 4 h at constant voltage (80 V) in 0.5X TBE buffer. Later the gel electrophoresis apparatus was dismantled and the gel was washed in the 0.5X TBE buffer. RNAs was transferred to a Hybond-NX membrane (GE Healthcare Amersham-Fisher Bioscientific) using Trans-Blot Semi-Dry Electrophoretic Transfer Cell apparatus (Bio-Rad) under the transfer conditions of constant 6 V voltage for overnight in 0.5X Tris-Borate EDTA (TBE) buffer. RNAs were cross-linked to membrane via using a UV cross-linker (Stratalinker 1800) and the membrane was dried in an oven for an hour at 50°C. Afterwards, the membrane was transferred into a rotating glass tube in a hybridization oven (HB-1000, Hoefer). Prehybridization was performed by adding 15 mL of PerfectHyb plus Hybridization Buffer (Sigma-Aldrich) at 42°C for at least an hour while overnight prehybridization was preferentially applied. DNA oligonucleotide probes complementary to the miRNA sequence of interest were end-labelled with ^{32}P-ATP using T4 polynucleotide kinase (PNK) (New England Biolabs, Beverly, MA) as described in Table 4. The 3' termini of small RNAs contain a hydroxyl group, thus the small RNAs can be 3' end-labeled by ligation to ^{32}P-pCp. The incubated probe solution was purified by a Sephadex G-25 (GE-Healthcare) column. This probe solution was directly added into a rotating glass tube containing membrane already prehybridized with Perfect Hyb plus buffer (Sigma) for 1 h at 42°C. Hybridization was performed overnight at 37°C in a hybridization oven. At the end of the hybridization process, the membrane was washed three times with 20 mL of washing buffer composed of SDS 0.1% and 2X Saline-Sodium Citrate (SSC) for 20 min at 42°C. Finally, the washed membranes were kept in a

PhosphorImager screen cassette (GE Healthcare) overnight for miRNA detection. The screen was scanned using Typhoon 9400 (GE Healthcare) image scanner and signals were quantified via ImageJ (http://imagej.nih.gov/ij/), image processing program. The same membranes were stripped with stripping buffer (0.1X SSC and 0.5% SDS) three times and used for hybridization with end labeled internal control (U6). The quantified data of individual Northern blots were normalized with respective U6 internal controls and bar charts were plotted via using MicroSoft Excel program.

Table 4 Components of end-labeling reactions.

Reagent	**Reaction Volume (ml)**
miRNA probe (single-strand) (20 pmol/ml)	1 µl
10X T4 polynucleotide kinase buffer	2 µl
^{32}P-pCp	2 µl
T4 polynucleotide kinase (20u/ ml)	1 µl
Sterile ultra-pure water	14 µl
Total	20 µl
The reaction carried out at 37°C for 1 hour.	

2.8 Verification of miRNAs Using Quantitative Real Time PCR (qRT-PCR)

The expression of (7 abiotic stress-related) mature miRNAs in *Thellungiella* plants were detected and verified by Applied Biosystems TaqMan® miRNA assays. In addition, an assay for *snoR41Y* was used as mature miRNA control. RNAs isolated (as stated in RNA isolation part) from control and abiotic stress (salt, drought and cold) applied plants grown in boxes were directly used for TaqMan assays. First, reverse transcription PCR (RT-PCR) was performed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers included in the assay kit. Briefly, approximately 2.5 µg of total RNA was utilized for the generation of single-stranded miRNA cDNA for

each of the miRNAs listed in Table 5. RT-PCR conditions were given according to the manufacturer's protocol in Table 6.

Table 5 Mature miRNA sequences of selected abiotic stress-related miRNAs.

Selected miRNAs	Mature miRNA Sequence
miR156	UGACAGAAGAGAGUGAGCAC
miR159	UUUGGAUUGAAGGGAGCUCUU
miR166	UCGGACCAGGCUUCAUUCCCC
miR169	CAGCCAAGGAUGACUUGCCGA
miR319	UUGGACUGAAGGGAGCUCCC
miR393	UCCAAAGGGAUCGCAUUGAUC
miR398	UGUGUUCUCAGGUCACCCCUU

Table 6 RT-PCR components and conditions for the synthesis of cDNAs.

Reagent	Reaction Volume (µl)
100 mM dNTPs	0.15 µl
Reverse transcription enzyme (50 u/µl)	1.00 µl
10x Buffer	1.50 µl
RNase inhibitor (20u/ ml)	0.19 µl
Ultra-Pure Water	4.16 µl
Total	7.00 µl
Mix the reaction components and place it on ice.	
Add 5 µl of RNA (250 ng) into each reaction tube. Mix them and spin the tubes. Then, add 3 µl of 5X RT primers specific for each miRNA.	
Place the tubes on ice and stand for 5 min. Start the reaction with given cycling conditions; 40 min at 16°C, 45 min at 42°C, 5 min at 85°C and storage at 4°C.	
Use freshly synthesized cDNAs for the following qRT-PCR experiments.	

Finally, miRNA specific primers and synthesized cDNAs were used for quantitative real time PCR (qRT-PCR) for the verification of the 7 selected miRNAs' expression levles. Reaction mixtures were prepared as stated in Table 7 and cycling conditions were also given in this table.

Table 7 Reaction components and thermal cycling conditions for TaqMan qRT-PCR.

Reagent	**Reaction Volume (µl)**
TaqMan assay (20X)	0.5 µl
TaqMan Universal PCR Master Mix (2X)	5.0 µl
cDNA (from reverse transcription)	1.0 µl
Ultra-Pure Water	3.5 µl
Total	10 µl
Start the reaction with given cycling conditions; 10 min incubation at 94°C for once and 40 cycles of 15 sec at 42°C and 1 min at 94°C.	

All the qRT-PCR reactions were run on Rotor Gene 6000 (Corbett, Qiagen) cycler. Following qRT-PCR, the reaction efficiency incorporated $\Delta\Delta C_t$ formula was used for relative quantification (Fleige et al., 2006). GraphPad Prism (California, USA) was used to perform one-way ANOVA with Tukey's multiple comparison post-test to assess normalized values against the reference gene (internal control).

2.9 Prediction of Potential miRNA Target Genes

miRU, a potential plant mRNA target finder was used to predict the possible miRNA target mRNAs and the updated version of this database is psRNATarget (http://plantgrn.noble.org/psRNATarget/). psRNATarget was used for the selection of putative targets of some selected abiotic stress-related miRNAs based on identified

miRNAs from *Arabidopsis*. Expression levels of potential candidates were determined by SYBR Green Mastermix (Roche Applied Science) using the Rotor Gene 6000 (Corbett, QIAGEN) cycler.

2.9.1 Synthesis of Single-Stranded cDNA

Initially, RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) was used for cDNA synthesis from 2.5 µg of DNase-treated RNA samples. Lack of DNA contamination was confirmed by PCR. All RT-qPCR reactions were performed in a volume of 20 µL with 300 nM of specific primer pairs listed in Table 6. Fold change for miRNA target levels were normalized against the reference gene *18S rRNA* (Brunner et al., 2004*)* amplified with the primer sets shown in Table 8.

Table 8 Primer pairs used for amplification of target sequences

Primer Name	Sequence (5'-3')	Product Size
ITD1-F	5'-actaggccgcaagaaccaag-3'	140 bp
ITD1-R	5'-agagagccaccttcagtcca-3'	
HEC1-F	5'-catggcagctatgcgagaga-3'	198 bp
HEC1-R	5'-ttcgtcccaccaggaacaag-3'	
WRKY33-F	5'-cagagcaccacaggattcgt-3'	143 bp
WRKY33-R	5'-cgtaaccaaaaggcccggta-3'	
NFYA5-F	5'-gagtgccattgcctcatcac-3'	145 bp
NFYA5-R	5'-tgaaggtacggtttacggca-3'	
SPL10-F	5'-cgtcgtttctgccaacagtg-3'	105 bp
SPL10-R	5'-gagaaagacgtttgcggcag-3'	
18S rRNA-F	5'-aaacggctaccacatccaag-3'	243 bp
18S rRNA-R	5'-cccatcccaaggttcaacta-3'	

2.9.2 RT-qPCR Analysis of Selected Targets

Following reaction conditions were used for *ITD1*, *HEC1*, *WRKY33* and *SPL10*; incubation at 95ºC for 10 minutes, 40 cycles of 94ºC for 15 seconds, 59ºC for 40 seconds, and 72ºC for 30 seconds and also for *NFYA5* and *18S rRNA*; incubation at 95ºC for 10 minutes, 40 cycles of 94ºC for 15 seconds, 58ºC for 40 seconds, and 72ºC for 30 seconds. Reaction efficiency incorporated $\Delta\Delta C_t$ formula was used for performing the relative quantification. Three independent biological replicates with 3 technical replicas per experiment were utilized for each qRT-PCR.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Plant Material, Growth Conditions and Preliminary Stress Treatments

Thellugiella halophila, ecotype Shandong and Yukon seeds were used throughout the study for abiotic stress treatments and unstressed control conditions. Seeds of both ecotypes were sown into MS medium supplemented with 3% sucrose and 0.8% plant agar containing boxes. The boxes were then stored at 4ºC for two weeks of vernalization to synchronize germination. Afterwards the boxes were kept in a growth chamber at 24 ± 2°C under fluorescent light with a 16/8-h (light/dark) period for seed germination and plant growth. After 4 weeks of germination and growth in normal growth media (MS), plants (for salt and drought stresses) or the boxes (for cold stress) were transferred to stress conditions.

A preliminary evaluation of ecotype responses to NaCl and mannitol were carried out for identification of salt and drought stress treatments for the experimental conditions. For identification of the LD_{50} NaCl (NaCl concentration in the nutrient medium that is lethal to 50% of the population) value, 8 salt concentrations were applied (0, 50, 100, 150, 200, 250, 300 and 350 mM NaCl) by gradual increase of 50 mM NaCl. For the determination of the LD_{50} mannitol, 6 mannitol treatments (0, 50, 100, 150, 200 and 250 mM) were tested by the gradual increase of 50 mM mannitol. At the end of the treatments, plants that survived were counted from pool of 15 plants/ecotype/treatment. The survival rate of both ecotypes is given as % in Table 9. The treatments were repeated three times

independently and their average results are presented in the Table 9. The NaCl and mannitol lethal dose to 50% of the population (LD_{50}) was identified to evaluate the survival rate of the plants against stress.

Table 9 Survival rates of *Thellungiella* ecotypes at various salt and mannitol concentrations. Each value represents the mean ± SD (standard deviation) of three independent treatments (The red circled conditions were selected as abiotic stress conditions for Northern blots and qRT-PCR experiments).

	% Survival Rate													
	Salt Concentrations (mM NaCl)								**Drought Concentrations (mM Mannitol)**					
	0	**50**	**100**	**150**	**200**	**250**	**300**	**350**	**0**	**50**	**100**	**150**	**200**	**250**
Shandong ecotype	97.2 ± 0.81	94.4 ± 0.58	88.9 ± 1	83.3 ± 1	77.8 ± 1,53	69.4 ± 0.58	50 ± 1	22.2 ± 0.58	97.2 ± 0.58	83.3 ± 0.81	77.8 ± 1,15	69.4 ± 0.58	61.1 ± 1.15	27.8 ± 1.58
Yukon ecotype	94.4 ± 0.58	91.7 ± 1	86.1 ± 0.58	75 ± 1	63.8 ± 0.58	58.3 ± 1	33.3 ± 1.15	19.4 ± 1.53	97.2 ± 0.58	86.1 ± 0.58	75 ± 1	63.8 ± 0.58	58.3 ± 1.15	22.2 ± 1.58

As expected, the survival rate of both Shandong and Yukon ecotypes under no/low salt conditions (0, 50, 100 and 150 mM NaCl concentration) were high ranging from 97.4-83.3% and 94.4-75%, respectively. The sharp decrease in the survival rate was observed in both ecotypes at 300 mM NaCl (~ 20% for Shandong and 25% for Yukon) and 250 mM NaCl was selected as the highest concentration of salt for plant survival as presented in Table 9. 200 mM mannitol concentration was selected for drought treatments after evaluation of LD_{50} mannitol for both ecotypes because of the 61.1% and 58.3% surviving amounts.

Moreover, morphology changes were taken into consideration for evaluating the stress responses. As expected, there was no morphology changes were observed in no/low salt conditions (0, 50, 100 and 150 mM NaCl concentration). However, some morphological differences such as color change (from pale green to yellow) and reduction in leaf size and number were seen especially with increasing salt concentrations at 200 and 250 mM NaCl (Figure 4). At the concentrations of 300 and 350 mM NaCl, plants became wilted followed by necrosis on all leaves (data not shown).

Figure 4. Untreated (A and C) and 250 mM NaCl (B and D) treated *Thellugiella halophila* ecotypes. **A** Untreated Shandong ecotype **B** 250 mM NaCl-treated Shandong ecotype **C** Untreated Yukon ecotype **D** 250 mM NaCl-treated Yukon ecotype.

At the beginning of the study, for salt-stress response of plants reduced (GSH) and oxidized (GSSG) glutathione levels, proline, malondialdehyde (MDA) and anthocyanin accumulation and relative water content (RWC) were determined for *Thellungiella* ecotypes.

3.2 Characterization of Physiological Responses in *Thellungiella* Ecotypes to Gradually Increased Salt Stress

In the step which followed the determination of LD_{50} values, *Thellungiella* plants were subjected to stepwise increased salt concentrations for 30 days to assess physiological changes during stress. The plant shoots were used as a tissue source for identification of physiological parameters.

3.2.1 Reduced (GSH) and Oxidized (GSSG) Glutathione Levels and GSH/GSSG as a Marker of Abiotic Stress

In the current study, GSH and GSSG levels were measured as an indicator of the redox status of Shandong and Yukon ecotypes of *Thellungiella*. The degree of increase in GSH levels showed more variation under high salt concentrations (200-250 mM NaCl) between two ecotypes (Figure 5A), nearly 4.5-fold increase was seen in Shandong ecotype and 3-fold increase was recorded in Yukon ecotype upon salt exposure. Almost a similar situation was available for GSSG levels (Figure 5B). Approximately, 5-fold increase was measured in Shandong ecotype and 2.5-fold increase was observed in Yukon ecotype at high salt concentrations (200-250 mM NaCl). However, the

GSH/GSSG ratio (Table 10) was nearly the same for both ecotypes under 200 mM NaCl stress and a relatively less decrease was noticed at the highest salt concentration (250 mM NaCl). More or less the same GSH/GSSG ratio of untreated and salt-treated plants suggesting that better maintenance of this ratio confers tolerance against salt stress (Sairam et al., 2005, Tausz et al., 2004). The reduced tripeptide form (GSH) exists interchangeably with the oxidized form (GSSG) with the assistance of glutathione reductase. Most salt sensitive plants have low GSH/GSSG values with increasing salt concentrations. Higher GSH/GSSG ratios are representative of better ROS scavenging (especially for hydrogen peroxide) in stress adapted plants (Benina et al., 2013, REDDY and RAGHAVENDRA, 2006). Reduced form of glutathione (GSH) protects the cell from salt-induced oxidative damage based on its redox buffering action and abundance in the cell. Salt-tolerant genotypes of rice exhibited a higher reduced versus oxidized glutathione ratio (GSH/GSSG). Higher glutathione concentration in a salt resistant rice cultivar in comparison to its susceptible counterpart further supports the role of glutathione in salt tolerance (El-Shabrawi et al., 2010). Exactly the opposite situation was observed in salt-sensitive pea cultivars exposed to long-term salt stress, the GSH/GSSG ratio was remarkably low in salt-treated plants (Hernandez et al., 2000). The relatively similar GSH/GSSG ratios for unstressed (basal levels) and salt-stress treated Shandong and Yukon ecotypes further strengthens the involvement of glutathione in salt tolerance mechanism.

Table 10 GSH/GSSG ratios as a marker of oxidative stress and an indicator of cellular health in *Thellungiella halophila* ecotypes exposed to increasing concentrations of NaCl.

	GSH/GSSG Ratio of Salt-treated *Thellungiella halophila* Ecotypes				
	0 mM NaCl	**100 mM NaCl**	**150 mM NaCl**	**200 mM NaCl**	**250 mM NaCl**
***T. halophila* (S)**	1.03 ± 0.0119	0.96 ± 0.0167	0.90 ± 0.0567	0.98 ± 0.0481	0.79 ± 0.0202
***T. halophila* (Y)**	1.17 ± 0.0236**	1.11 ± 0.0251**	0.99 ± 0.0134**	0.94 ± 0.0103*	0.87 ± 0.0392*

Each value represents the mean ± SEM of three biological replicates. * and ** indicates a significant difference of *T. halophila* Yukon ecotype with *T. halophila* Shandong ecotype according to paired samples student's t-test).

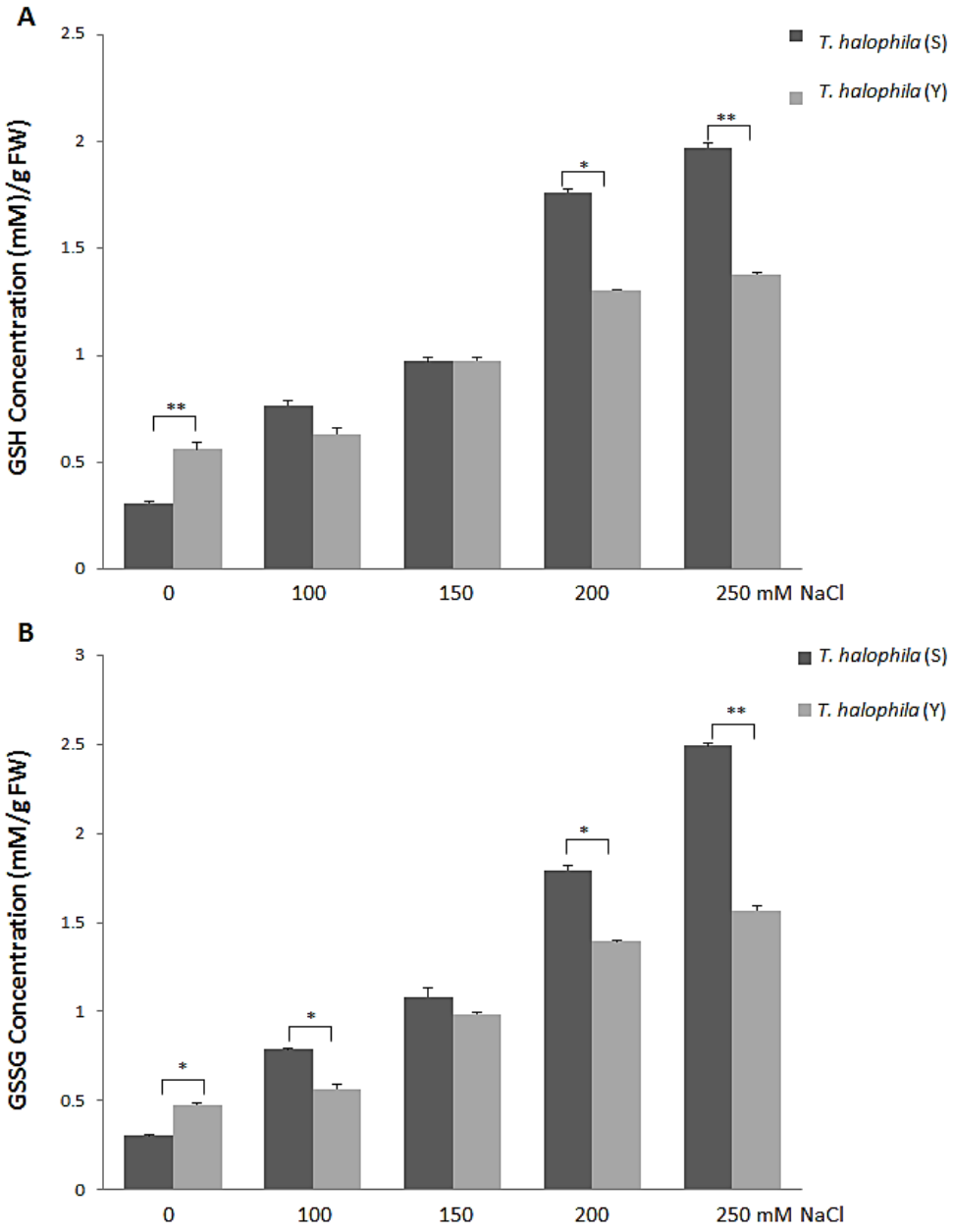

Figure 5 A Reduced glutathione (GSH) and **B** Oxidized glutathione (GSSG) levels in *T. halophila* ecotypes (Shandong ecotype - abbreviated as S, Yukon ecotype - abbreviated as Y) exposed to salt stress with increasing concentrations of NaCl. Plants grown at optimal conditions at 24 ± 2 °C under fluorescent light with a 16/8 h (light/dark) period in boxes. GSH and GSSG are expressed as mM per gram fresh weight (FW). ~ 0.1 g of untreated and salt-stress treated plant tissue samples were used for measurements. Asterisks indicate significant difference between *T. halophila* ecotypes (* ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); unmarked columns are not significant ($p > 0.05$), student's t-test). Error bars indicate the standard error of the mean (SEM). The data are means ± SEM of three biological replicates.

3.2.2 Determination of Proline, MDA, Anthocyanin amount and RWC in *T. halophila* as Physiological Responses to Salt Stress

To further confirm the salt tolerance of *Thellungiella* plants, proline accumulation was measured as an osmoprotectant and an indicator of stress adaptation. As expected, salt stress caused substantially high amount of proline accumulation in both ecotypes. The gradual increase in the proline content was detected depending on the gradual increase in the NaCl concentration and untreated plants accumulated proline to a lesser extent. The proline accumulation of Shandong ecotype was about 1.5-2 times higher than Yukon ecotype at all concentrations of NaCl (Figure 6A). The ability of *Thellungiella* plants to accumulate proline was also detected in response to osmotic stress and salt stress-sensitive relative, *Arabidopsis* did not show any significant variation in proline content (Arbona et al., 2010).

The formation and the accumulation of osmoprotective compounds are absolutely necessary for plants to respond for long- and short-term salinity stress. These organic molecules can reach to high concentrations in cells without affecting any metabolic processes. Proline is one of the important compatible solute that balances the water potential hence helps turgor maintenance of the cells (Carillo et al., 2011, Soshinkova et al., 2013). Other known traits of proline, such as enzyme protection s from denaturation, interaction with membrane systems, regulation of acidity within the cytosol, free radical scavenging, adjusting the $NADH/NAD^+$ balance and acting as a energy source may be more important for the total health of the plants under osmotic stress (Kumar et al., 2013). The elevated levels of proline effectively reduce free radical levels caused by salinity stress. This was determined by measuring the levels of malondialdehyde (MDA), a marker for lipid peroxidation and membrane damage due to free radicals (Yazici et al., 2007).

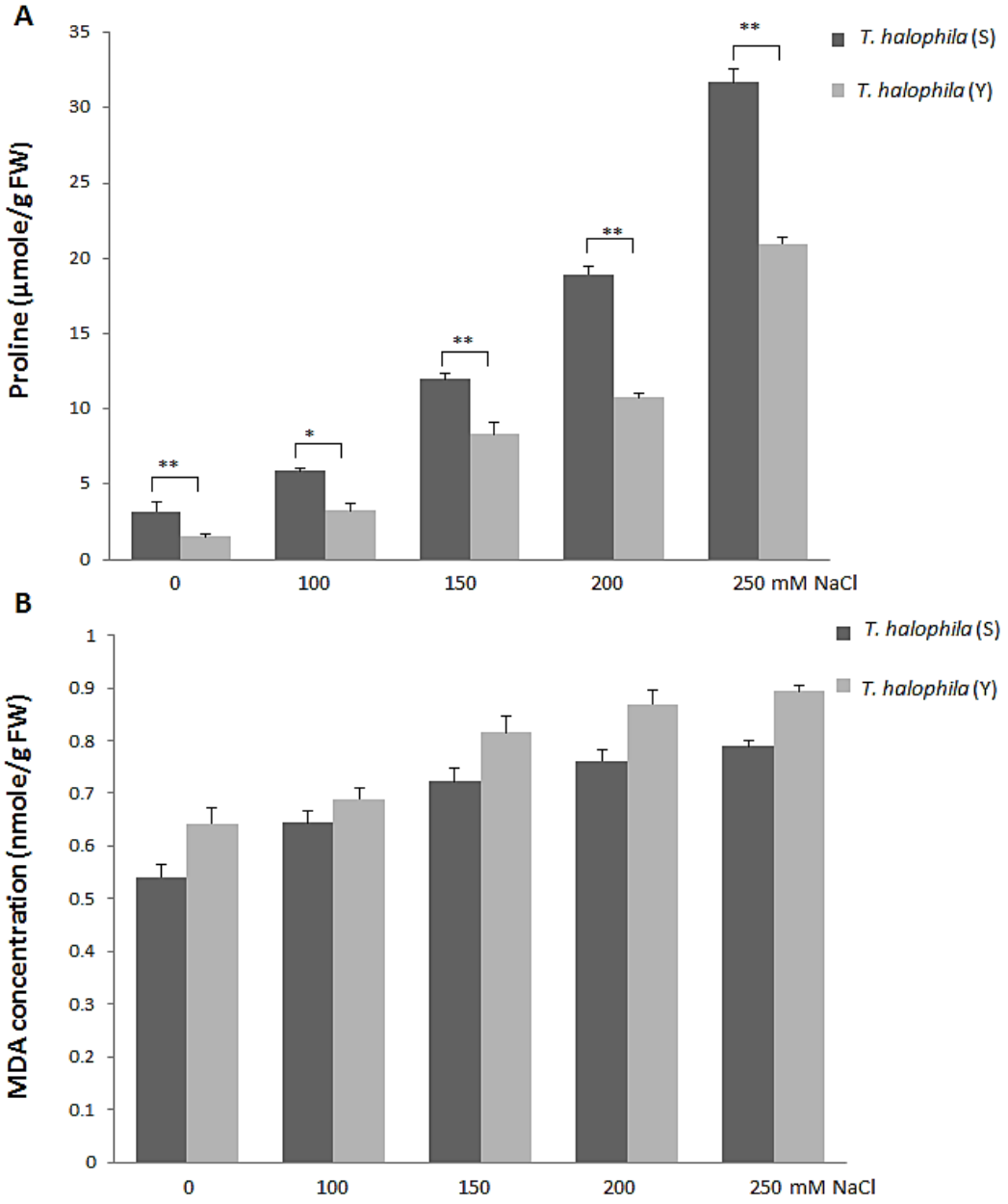

Figure 6 Effect of increasing concentrations of NaCl on the **A** Proline and **B** Malondialdehyde (MDA), content of *T. halophila* ecotypes (Shandong ecotype- abbreviated as S, Yukon ecotype- abbreviated as Y) as physiological indicators of stress adaptation. Plants grown at optimal conditions at 24 ± 2°C under fluorescent light with a 16/8-h (light/dark) period in boxes. Proline is expressed as µmole per gram fresh weight (FW) and MDA is expressed as nmole per gram fresh weight (FW). Means ± SEM from three biological replicates are used to test the significancy of the data of the data according to the paired samples student's t-test (* ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); unmarked columns are not significant ($p > 0.05$)). Error bars indicate the standard error of the mean (SEM).

The amount of MDA as a response to increasing NaCl concentrations in both ecotypes remained almost the same with untreated plants. The MDA levels of Yukon ecotype were higher than that of Shandong ecotype at no salt and 150, 250 mM salt concentrations (Figure 6B). The unaltered MDA levels of salt tolerant *Thellungiella* plants were consistent with others that showed lipid peroxidation was enhanced only in salt-stressed leaves of the salt-sensitive genotype of maize. However, in salt-tolerant genotype, the MDA content was not affected by salinity (de Azevedo Neto et al., 2006). High GSH/GSSG (Table 2) levels and elevated amount of anthocyanins (Figure 6A) might compensate for the adverse effects of the ROS production and contribute to the low levels of MDA (Figure 6B). Cell membrane stability is a discriminating factor for stress tolerant and intolerant plant species. MDA is a lipid breakdown product produced by the disruption of polyunsaturated fatty acids of membranes and accumulates in cells due to abiotic stress (Sharma et al., 2012). Elevated MDA levels leads to the acceleration of lipid peroxidation. Cell membrane stability has been widely used to differentiate stress-tolerant and susceptible cultivars of some crops and in some cases higher membrane stability could be correlated with abiotic stress tolerance (Agrawal et al., 2015).

Anthocyanins are powerful antioxidant flavonoid molecules that reduce highly oxidizing free radicals and ameliorating the negative effects of these molecules at cellular and molecular level. Salt-tolerant species possess enhanced amount of anthocyanin in order to protect from oxidative damage (Kytridis et al., 2008). The higher levels of anthocyanins in 200 and 250 mM salt-treated *Thellungiella* ecotypes (Shandong and Yukon) compared to unstressed plants (3- and 4-fold increase, respectively) (Figure 7A) may be a part of the adaptation strategy to stress conditions. According to the data obtained from the experiment, anthocyanin production positively correlated with elevated NaCl treatments in the plants (Figure 7A). Anthocyanins plays a key role in the regulation of ROS levels and lipid peroxidation product accumulation within the cells (Kreslavski et al., 2013) Support evidence comes from a study related with the oxidative stress-tolerant *Arabidopsis* mutant that was shown to have much higher levels of anthocyanins than the

wild type (Gechev et al., 2013). Furthermore, salt-tolerant maize genotypes showed higher percentages of anthocyanins than salt-sensitive genotypes (Chutipaijit et al., 2011).

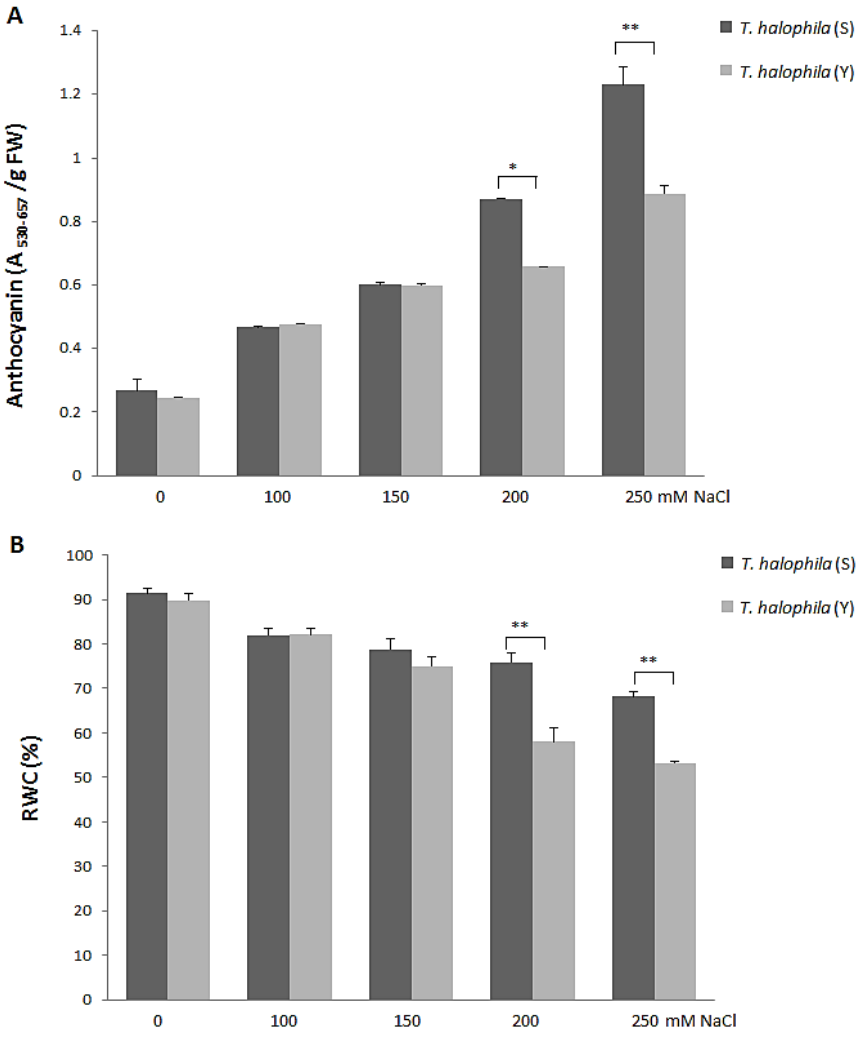

Figure 7 Effect of increasing concentrations of NaCl on the **A** Anthocyanin amount and **B** Relative water content (RWC) of *T. halophila* ecotypes (Shandong ecotype- abbreviated as S, Yukon ecotype- abbreviated as Y). Anthocyanins are presented as the difference in the absorbances between 530 and 657 nm per g fresh weight (FW). Means ± SEM from three biological replicates are used to test the significancy of the data according to the paired samples student's t-test (* ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); unmarked columns are not significant ($p > 0.05$)). Error bars indicate the standard error of the mean (SEM).

The relative water content (RWC) is a functional marker for the water balance of a plants and a measure of the relative cellular volume showing the changes that could be affecting from the interactions between macromolecules and organelles. RWC levels below 80% produce metabolic changes in the cells by failing the photosynthetic machinery, rising the respiration and increasing the proline accumulation (González and González-Vilar, 2001). In this study, it was showed that salt stress decreased the RWC of both ecotypes and the effect increased with salinity level (200-250 mM NaCl). The RWC at 250 mM NaCl was declined from 90% to 72% and from 88% to 60% in Shandong and Yukon ecotypes, respectively (Figure 7B). However, the decrease in the RWC was fairly low compared with the results of a study revealed that the RWC of stress-sensitive *Arabidopsis* plants subjected to abiotic stress was sharply declined to 18% (Gigon et al., 2004). Yukon ecotype showed greater reduction in the RWC than Shandong ecotype at 200 and 250 mM salt concentrations (Figure 7B). The results suggest that Shandong ecotype had the ability to avoid the water stress induced by salinity than Yukon ecotype. Our results are in agreement with a study reporting a greater decrease in the RWC of salt sensitive cultivar of wheat as compared to tolerant one under salinity stress (Sairam et al., 2005).

Finally, high GSH/GSSG values, increased proline accumulation as well as increased anthocyanin levels and less reduction in RWC may contribute to salt tolerance mechanisms in *Thellungiella* plants. These parameters were primarily linked to mechanisms of stress adaptation. The physiological characterization of abiotic stress responses were considered to be an important prerequisite for pioneering the genetic studies related to stress-responsive features of plants (Guevara et al., 2012, Orsini et al., 2010)

3.3 Identification of abiotic stress responsive *Thellungiella* miRNAs

Because miRNAs are evolutionarily conserved from species to species across the plant kingdom, it is possible to detect and predict conserved miRNAs from any plant species with the existing miRNA sequences from different plant species (Axtell and Bartel, 2005). Based on this principle, 7 abiotic stress responsive miRNAs (miR319, miR156, miR159, miR398, miR166, miR169 and miR393) were selected based on the identified *Arabidopsis* miRNA sequences assembled in the miRNA database **(**mirBASE) (http://www.mirbase.org) for identification and validation of the relation between abiotic stress and miRNAs in *Thellungiella halophila* (ecotype Shandong and Yukon) plants. *T. halophila* is a natural inhabitant of highly saline and water-poor environments and exhibits an exceptional ability to tolerate various abiotic stresses. Thus, the selection of *Thellungiella* as a model plant for stress physiology studies is a practical step to develop strategies for stress tolerance due to its ability to complete its life cycle under severe environmental conditions (Amtmann, 2009).

In the present study, miRNA expression was studied under three different abiotic stress conditions (salt, cold and drought) in the shoots of *Thellungiella halophila* plants using Northern Blot analysis and TaqMan® microRNA assays. All of the selected miRNAs have known functions in the plant and play key roles in the growth, development, and response to environmental stresses. Therefore, it is important to quantify the expression amount of the miRNAs to understand the relations between miRNAs and stress responses. Abotic stress treatments and plant growth conditions are stated in Table 11.

Table 11 Abiotic stress conditions for Northern blot analysis and TaqMan qRT-PCR assays.

		Abiotic Stress Treatments		
		Salt Stress	**Drought Stress**	**Cold Stress**
Northern blot and qRT-PCR Experiments	**Control conditions for *Thellungiella* ecotypes Shandong and Yukon**	MS+3% sucrose+0.8% plant agar containing boxes	MS+3% sucrose+0.8% plant agar containing boxes	MS+3% sucrose+0.8% plant agar containing boxes
	Treatment conditions for *Thellungiella* ecotypes Shandong and Yukon	MS+3% sucrose+0.8% plant agar+250 mM NaCl containing boxes (for 15 and 30 d of treatment)	MS+3% sucrose+0.8% plant agar+200 mM Mannitol containing boxes (for 10 and 20 d of treatment)	MS+3% sucrose+0.8% plant agar containing boxes were kept in a cold room (4ºC) (for 7 and 14 d of treatment)

miRNA probes and assays were designed and selected based on the known abiotic stress responsive miRNA sequences from *Arabidopsis*. The miRNA signals and relative expression levels were quantified and normalized with respect to internal controls (U6 used for Northern Blots and *snoR41Y* was used as mature microRNA control for qRT-PCR). miRNA expression profiles of Shandong and Yukon ecotypes were very similar to each other under three abiotic stress conditions (salinity, low temperature and drought).

3.3.1 Salt Stress

According to the quantified signals obtained from Northern blot analysis, four of the stress inducible miRNAs (miR156, miR169, miR319 and miR393) were downregulated and three (miR159, miR166 and miR398) were upregulated under 30 days of salt stress treatment when compared to untreated plants of Shandong and Yukon ecotypes (Figure 8A and 8B, Figure 9A and 9B). The highest amount of upregulation was observed in miR398 in both ecotypes. miR398 expression was 3.24-fold higher than untreated plants of Shandong ecotype (Figure 8B) and in Yukon ecotype the increase was 2.69-fold

(Figure 9B). miR159 and miR166 were expressed with fold increase in expression of 1.46, 1.89 in Shandong ecotype and 1.63, 2.48 in Yukon ecotype, respectively, as compared to the expression of the reference gene, U6 (Figure 8B, Figure 9B). In Yukon ecotype miR166 was 1.4 times more expressed than Shandong ecotype. In the salt-stressed Shandong ecotype, miR169 was expressed the least out of all miRNAs tested with a fold change as compared to U6 of 0.36 and miR156 showed the lowest expression in Yukon ecotype under salt stress with a fold change of 0.28. miR156 was also one of the least expressed miRNAs after miR169 in Shandong ecotype with a fold change of 0.54 (Figure 8B).

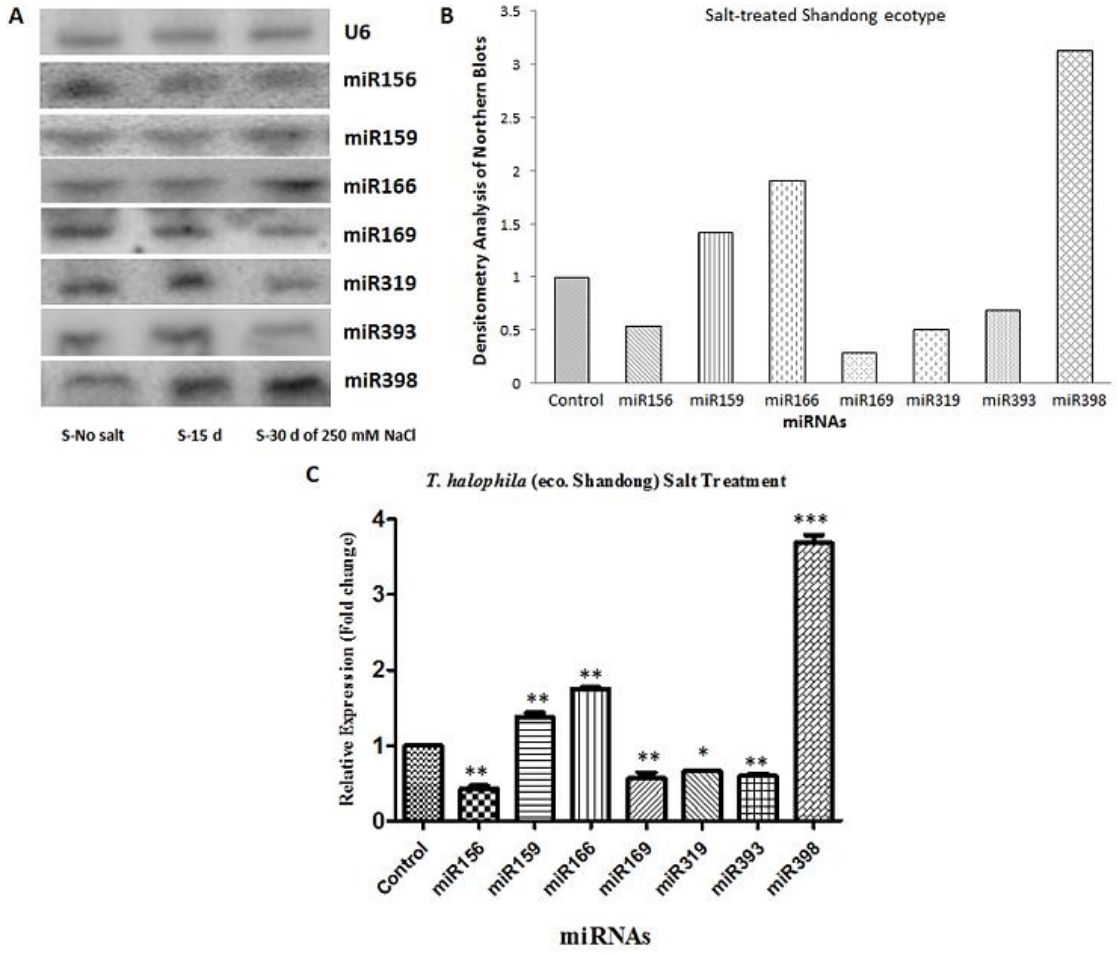

Figure 8 A Northern blot analysis of 7 abiotic stress-associated miRNAs in *Thellungiella halophila* (Shandong ecotype- abbreviated as S) plants grown in plastic pots after 15 and 30 days of 250 mm NaCl treatment. Total RNAs isolated from the untreated and salt-treated plants were analyzed by northern hybridizations using probes of miRNAs for validation. The same blots were hybridized with the probe of U6 used as a loading control and standard for quantification. **B** The relative accumulation levels of miRNAs to U6 are shown in histograms. The signals were quantified via ImageJ, image processing program. The level of each miRNA was normalized by comparison with their expression in the untreated samples, which was set to 1.0. **C** Relative expressions of mature miRNAs during 30 days of salt stress treated *Thellungiella* plants grown in boxes. Mature miRNA expressions' were detected by Applied Biosystems TaqMan® microRNA assays. The expression of miRNAs were normalized by comparison with *Arabidopsis snoR41Y* mature miRNA control and expression levels of each miRNA were then normalized by comparison with their expression in the untreated plants, which was set to 1.0. The reaction efficiency incorporated $\Delta\Delta C_t$ formula was used for relative quantification. Three independent biological replicates with 3 technical replicas per experiment were carried out for each miRNA. GraphPad Prism (California, USA) was used to perform one-way ANOVA with Tukey's multiple comparison post-test to assess normalized values against the reference gene. Error bars represent standard error of the mean (statistically significant results are marked with * ($p< 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)).

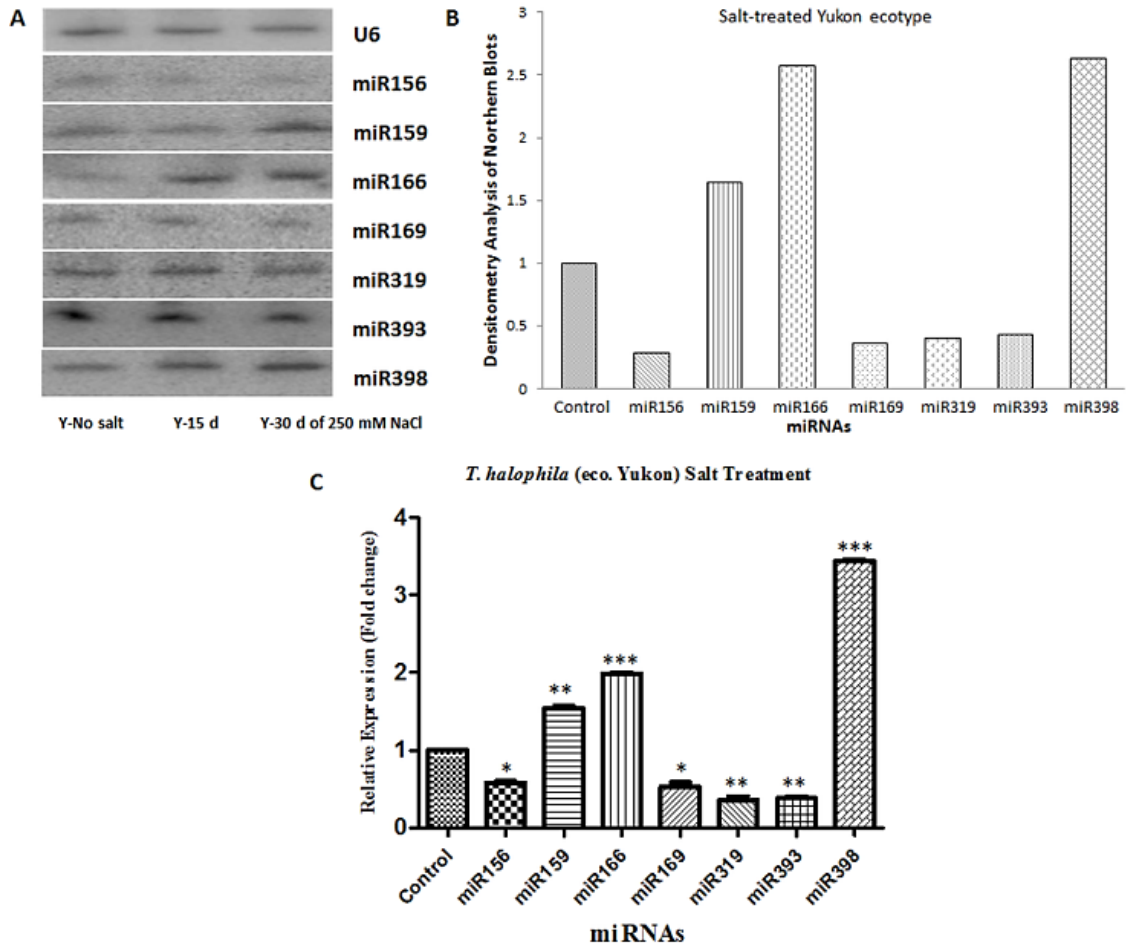

Figure 9 A Northern blot analysis of 7 abiotic stress-associated miRNAs in *Thellungiella halophila* (Yukon ecotype- abbreviated as Y) plants grown in plastic pots after 15 and 30 days of 250 mm NaCl treatment. Total RNAs isolated from the untreated and salt-treated plants were analyzed by northern hybridizations using probes of miRNAs for validation. The same blots were hybridized with the probe of U6 used as a loading control and standard for quantification. **B** The relative accumulation levels of miRNAs to U6 are shown in histograms. The signals were quantified via ImageJ, image processing program. The level of each miRNA was normalized by comparison with their expression in the untreated samples, which was set to 1.0. **C** Relative expressions of mature miRNAs during 30 days of salt stress treated *Thellungiella* plants grown in boxes. Mature miRNA expressions' were detected by Applied Biosystems TaqMan® microRNA assays. The expression of miRNAs were normalized by comparison with *Arabidopsis* *snoR41Y* mature miRNA control and expression levels of each miRNA were then normalized by comparison with their expression in the untreated plants, which was set to 1.0. The reaction efficiency incorporated $\Delta\Delta C_t$ formula was used for relative quantification. Three independent biological replicates with 3 technical replicas per experiment were carried out for each miRNA. GraphPad Prism (California, USA) was used to perform one-way ANOVA with Tukey's multiple comparison post-test to assess normalized values against the reference gene. Error bars represent standard error of the mean (statistically significant results are marked with * ($p< 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)).

qRT-PCR experiments was performed to confirm the results of the Northern blots and the validation of *Thellungiella* miRNAs were in agreement with the Northern blot data. By analyzing the relative expression of each miRNA via $\Delta\Delta C_t$ method, miR156, miR169, miR319 and miR393 were expressed at lower levels and miR159, miR166 and miR398 were expressed at higher levels in comparison with the normalized reference gene at salt stress conditions in Shandong and Yukon ecotype. miR156, miR169, miR319 and miR393 were expressed the least out of all miRNAs tested with fold changes of 0.53, 0.61, 0.74, 0.68 in Shandong plants and 0.61, 0.44, 0.25, 0.36 in Yukon plants. miR166 expression increased in both ecotypes with fold changes of 1.82 and 1.9. The increased expression profile of miR398 and miR159 was detected in Shandong ecotype with fold changes of 3.57 and 1.53, respectively and in Yukon ecotype with fold changes of 3.21 and 1.49 (Figure 8C, Figure 9C).

3.3.2 Cold Stress

In Northern blot experiments, the expression of three miRNAs (miR159, miR166, and miR398) was upregulated and four was downregulated (miR156, miR169, miR319 and miR393) in response to 14 days of cold stress in both ecotypes likewise at salt stress conditions (Figure 10A, Figure 11A). These downregulated miRNAs had average fold changes of 0.53, 0.65, 0.3 and 0.48 in Shandong ecotype (Figure 10B) and 0.66, 0.54, 0.27 and 0.3 in Yukon ecotype (Figure 11B). miR159, miR166, and miR398 had increases in fold change of expression with 1.48, 1.75, 2.15 values for Shandong plants and 1.53, 2.16, 1.8 values for Yukon plants. The highest expression for miR166 was detected in signals obtained from Northern blot analysis of cold-treated Yukon ecotype (2.15) (Figure 11B). miR159, miR166 and miR398 were significantly upregulated with fold changes in the qRT-PCR experiments of cold treated Shandong ecotype of 1.79, 1.55, and 1.84, respectively. In cold-treated Yukon plants the fold changes of these three

were 1.44, 1.87 and 1.84. 14 days of cold exposure decreased the expression levels of miR156, miR169, miR319 and miR393 in *Thellungiella* ecotypes. Of all significantly downregulated miRNAs, miR393 showed the minimum fold changes in Shandong and Yukon ecotype with the values of 0.21 and 0.22 (Figure 10C, Figure 11C).

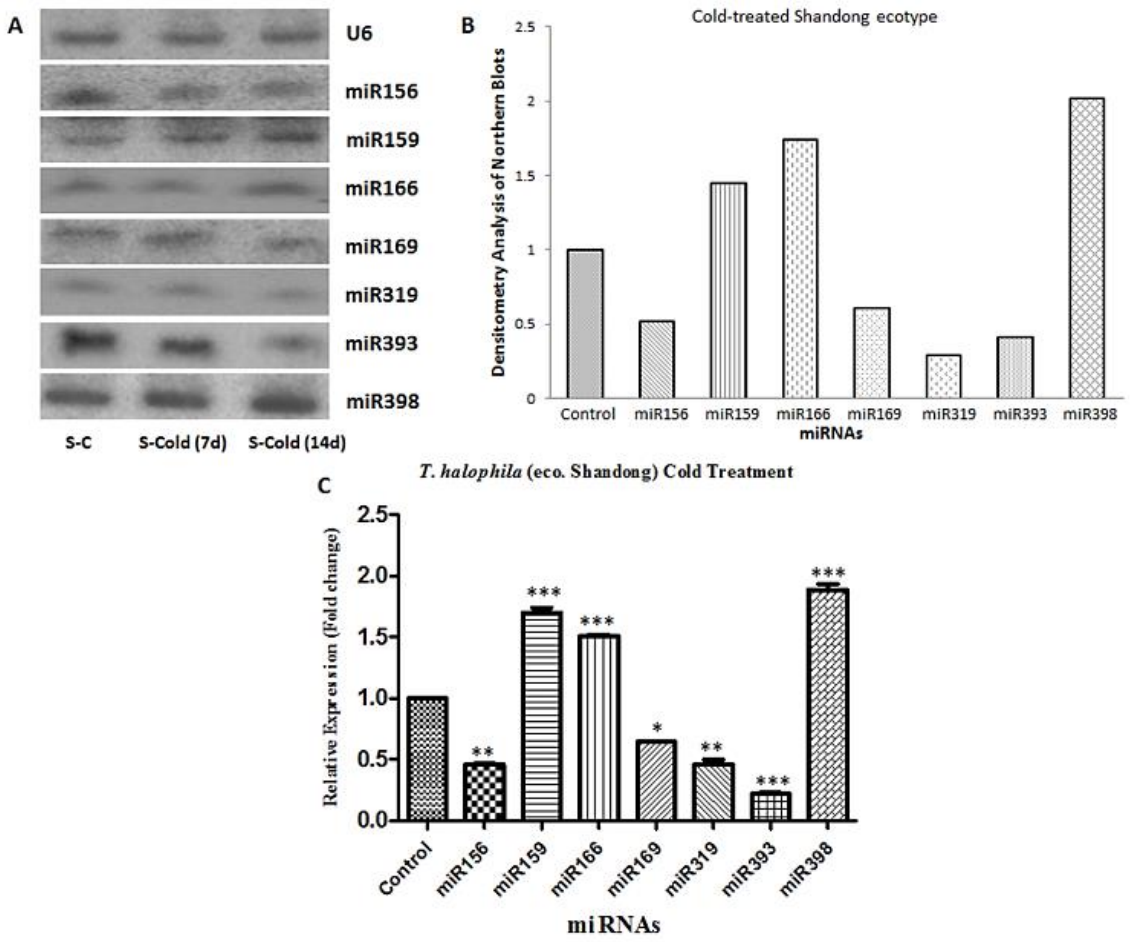

Figure 10 A Northern blot analysis of 7 abiotic stress-associated miRNAs in *Thellungiella halophila* (Shandong ecotype- abbreviated as S) plants grown in plastic pots after 7 and 14 days of cold treatment. Total RNAs isolated from the untreated and salt-treated plants were analyzed by northern hybridizations using probes of miRNAs for validation. The same blots were hybridized with the probe of U6 used as a loading control and standard for quantification. **B** The relative accumulation levels of miRNAs to U6 are shown in histograms. The signals were quantified via ImageJ, image processing program. The level of each miRNA was normalized by comparison with their expression in the untreated samples, which was set to 1.0. **C** Relative expressions of mature miRNAs during 14 days of cold stress treated *Thellungiella* plants grown in boxes. Mature miRNA expressions' were detected by Applied Biosystems TaqMan® microRNA assays. The expression of miRNAs were normalized by comparison with *Arabidopsis snoR41Y* mature miRNA control and expression levels of each miRNA were then normalized by comparison with their expression in the untreated plants, which was set to 1.0. The reaction efficiency incorporated $\Delta\Delta C_t$ formula was used for relative quantification. Three independent biological replicates with 3 technical replicas per experiment were carried out for each miRNA. GraphPad Prism (California, USA) was used to perform one-way ANOVA with Tukey's multiple comparison post-test to assess normalized values against the reference gene. Error bars represent standard error of the mean (statistically significant results are marked with * ($p< 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)).

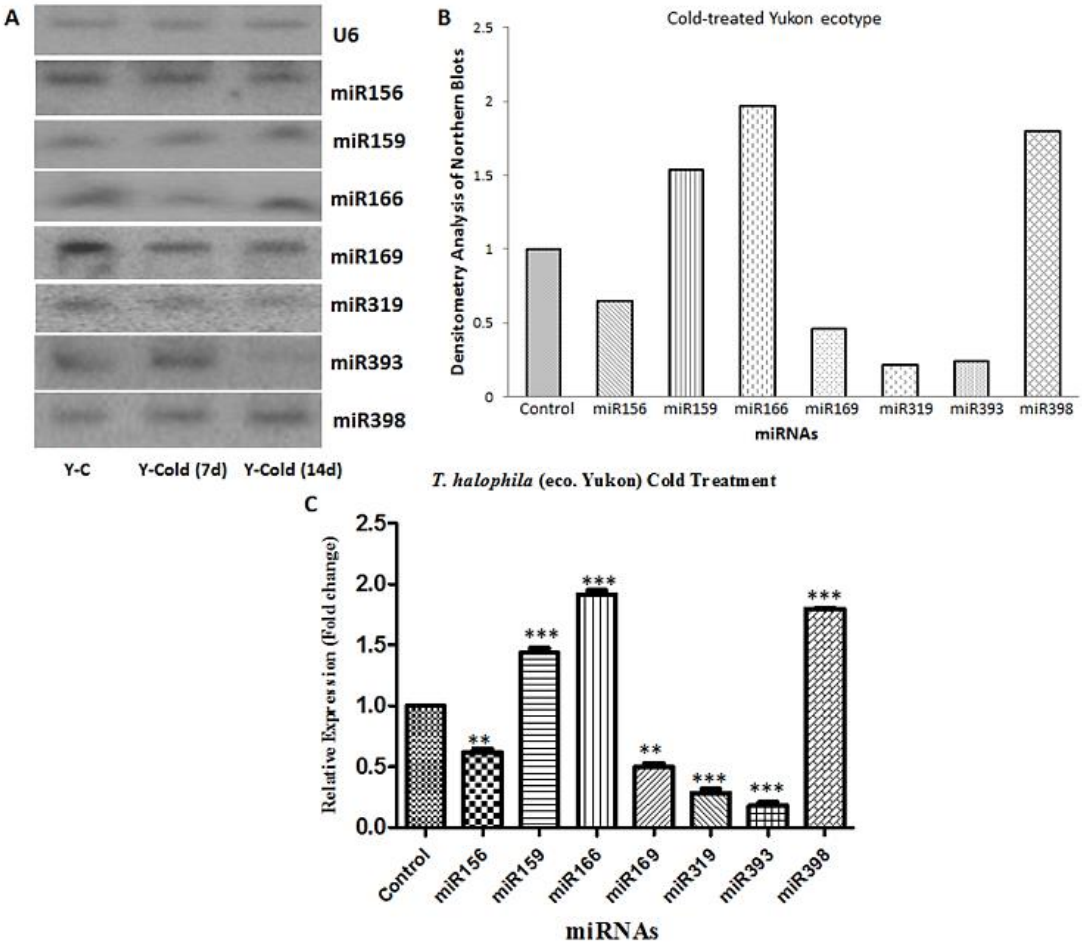

Figure 11 A Northern blot analysis of 7 abiotic stress-associated miRNAs in *Thellungiella halophila* (Yukon ecotype- abbreviated as Y) plants grown in plastic pots after 7 and 14 days of cold treatment. Total RNAs isolated from the untreated and salt-treated plants were analyzed by northern hybridizations using probes of miRNAs for validation. The same blots were hybridized with the probe of U6 used as a loading control and standard for quantification. **B** The relative accumulation levels of miRNAs to U6 are shown in histograms. The signals were quantified via ImageJ, image processing program. The level of each miRNA was normalized by comparison with their expression in the untreated samples, which was set to 1.0. **C** Relative expressions of mature miRNAs during 14 days of cold stress treated *Thellungiella* plants grown in boxes. Mature miRNA expressions' were detected by Applied Biosystems TaqMan® microRNA assays. The expression of miRNAs were normalized by comparison with *Arabidopsis snoR41Y* mature miRNA control and expression levels of each miRNA were then normalized by comparison with their expression in the untreated plants, which was set to 1.0. The reaction efficiency incorporated $\Delta\Delta C_t$ formula was used for relative quantification. Three independent biological replicates with 3 technical replicas per experiment were carried out for each miRNA. GraphPad Prism (California, USA) was used to perform one-way ANOVA with Tukey's multiple comparison post-test to assess normalized values against the reference gene. Error bars represent standard error of the mean (statistically significant results are marked with * ($p< 0.05$); ** ($p < 0.01$); *** ($p < 0.001$).

3.3.3 Drought Stress

The results of Northern blot and qRT-PCR experiments after 20 days of drought treatment exhibited a similar status of expression profile with salt and cold stress treatments in Shandong and Yukon ecotype. We detected, by both Northern blots and qRT-PCR, that miR156 was downregulated significantly in both ecotypes upon drought stress (Figure 12B, Figure 13B). miR169, miR319 and miR393 also had decreased expression in Shandong and Yukon ecotypes due to drought. On the other hand, miR159, miR166 and miR398 expression levels significantly increased upon drought stress with the fold changes of 2.13, 1.88 and 1.82 for Shandong ecotype (Figure 12C) and 1.77, 1.49 and 1.8 for Yukon ecotype (Figure 13C).

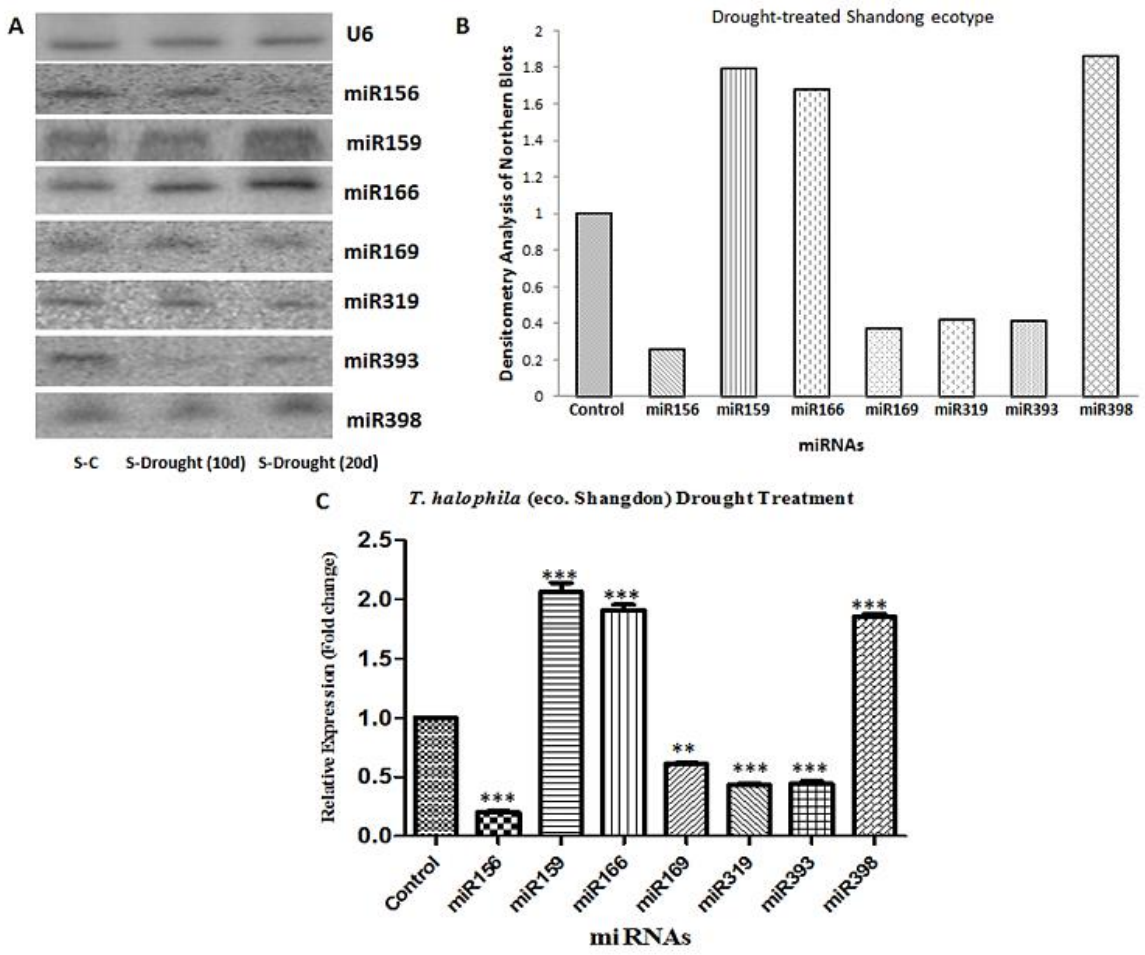

Figure 12 A Northern blot analysis of 7 abiotic stress-associated miRNAs in *Thellungiella halophila* (Shandong ecotype- abbreviated as S) plants grown in plastic pots after 10 and 20 days of drought treatment. Total RNAs isolated from the untreated and salt-treated plants were analyzed by northern hybridizations using probes of miRNAs for validation. The same blots were hybridized with the probe of U6 used as a loading control and standard for quantification. **B** The relative accumulation levels of miRNAs to U6 are shown in histograms. The signals were quantified via ImageJ, image processing program. The level of each miRNA was normalized by comparison with their expression in the untreated samples, which was set to 1.0. **C** Relative expressions of mature miRNAs during 20 days of drought stress treated *Thellungiella* plants grown in boxes. Mature miRNA expressions' were detected by Applied Biosystems TaqMan® microRNA assays. The expression of miRNAs were normalized by comparison with *Arabidopsis snoR41Y* mature miRNA control and expression levels of each miRNA were then normalized by comparison with their expression in the untreated plants, which was set to 1.0. The reaction efficiency incorporated $\Delta\Delta C_t$ formula was used for relative quantification. Three independent biological replicates with 3 technical replicas per experiment were carried out for each miRNA. GraphPad Prism (California, USA) was used to perform one-way ANOVA with Tukey's multiple comparison post-test to assess normalized values against the reference gene. Error bars represent standard error of the mean (statistically significant results are marked with * ($p< 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)).

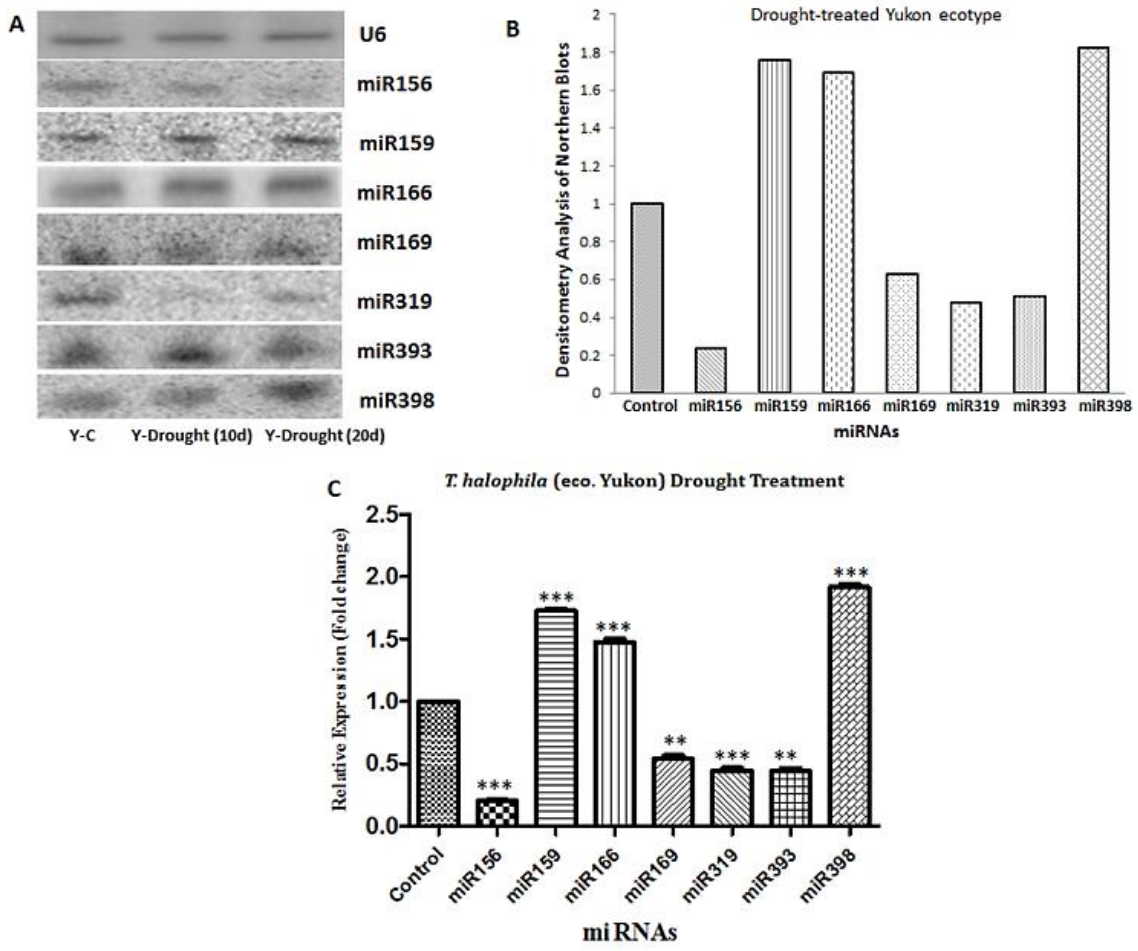

Figure 13 A Northern blot analysis of 7 abiotic stress-associated miRNAs in *Thellungiella halophila* (Yukon ecotype- abbreviated as Y) plants grown in plastic pots after 10 and 20 days of drought treatment. Total RNAs isolated from the untreated and salt-treated plants were analyzed by northern hybridizations using probes of miRNAs for validation. The same blots were hybridized with the probe of U6 used as a loading control and standard for quantification. **B** The relative accumulation levels of miRNAs to U6 are shown in histograms. The signals were quantified via ImageJ, image processing program. The level of each miRNA was normalized by comparison with their expression in the untreated samples, which was set to 1.0. **C** Relative expressions of mature miRNAs during 20 days of drought stress treated *Thellungiella* plants grown in boxes. Mature miRNA expressions' were detected by Applied Biosystems TaqMan® microRNA assays. The expression of miRNAs were normalized by comparison with *Arabidopsis snoR41Y* mature miRNA control and expression levels of each miRNA were then normalized by comparison with their expression in the untreated plants, which was set to 1.0. The reaction efficiency incorporated $\Delta\Delta C_t$ formula was used for relative quantification. Three independent biological replicates with 3 technical replicas per experiment were carried out for each miRNA. GraphPad Prism (California, USA) was used to perform one-way ANOVA with Tukey's multiple comparison post-test to assess normalized values against the reference gene. Error bars represent standard error of the mean (statistically significant results are marked with * ($p< 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)).

In summary, the selected miRNAs exhibited identical responses to salt (Figure 14A), cold (Figure 14B) and drought (Figure 14C) stresses in both ecotypes. This resemblance might have been originated from osmotic stress as a final effect, which indicated the existence of significant crosstalk between salt, drought and cold stress signaling pathways in plants (Zhao et al., 2009). Both *Thellungiella halophila* Shandong and Yukon ecotype are tolerant to harsh environmental conditions and inhabits in stress-prone locations. Shandong ecotype is a native inhabitant of maritime China (Shandong Province) that naturally grows on highly saline soils. Both Yukon that naturally inhabits in sub-arctic Canada (Yukon Territory) and Shandong ecotypes not only show highly salt tolerant characteristics but also adaptive to cold and semi-arid regions as well (Guevara et al., 2012, Wong et al., 2006). Because of this, the expression profiles of selected miRNAs in *T. halophila* ecotypes were nearly similar after subjected to salt, cold and drought stress conditions (Figure 14).

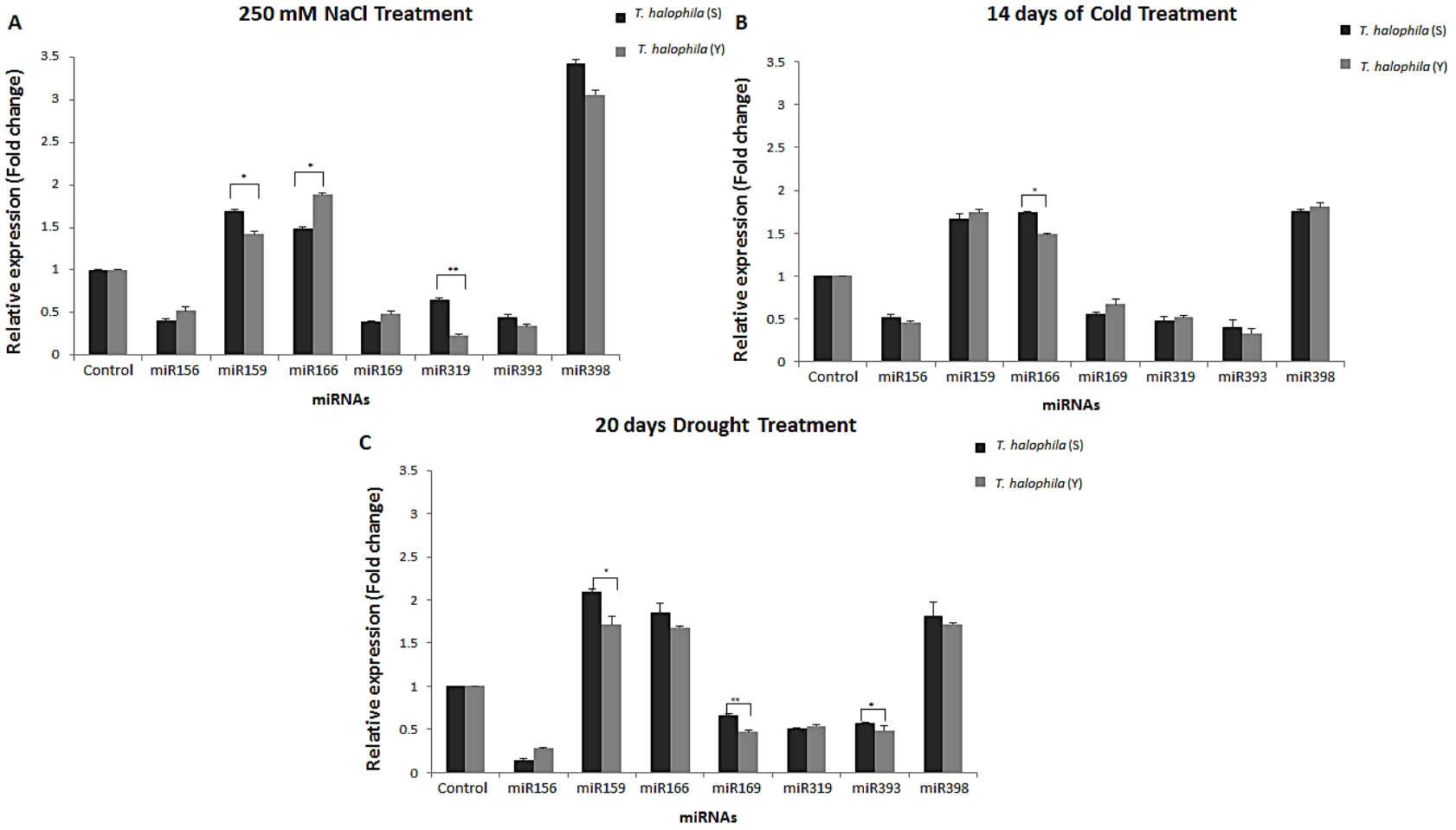

Figure 14 Comparison of the relative expression levels of selected mature miRNAs in two *T. halophila* ecotypes (Shandong ecotype- abbreviated as S, Yukon ecotype- abbreviated as Y) upon abiotic stress. **A** Salt stress **B** Cold stress **C** Drought stress. *Arabidopsis snoR41Y* was used as an internal control. The results shown are the averages ± SEM of three biological replicates. Error bars indicate the standard error of the mean (SEM). Student's *t*-tests were performed; statistically significant results are marked with * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); unmarked columns are not significant ($p > 0.05$).

We found that the expression miR169 was decreased and the expression miR166 was increased in Shandong and Yukon ecotypes at salt, cold and drought stress conditions. Similarly, miR169 downregulation in *Arabidopsis* and *Medicago truncatula* (Li et al., 2008, Trindade et al., 2010) and miR166 upregulation in barley leaves (Kantar et al., 2011) has been shown in response to drought stress. The result that miR398 upregulation at salt, cold and drought stresses in *Thellungiella* plants is also supported by several studies with different plant species under different abiotic stress conditions (Jia et al., 2009, Kantar et al., 2011, Trindade et al., 2010). However, miR398 showed differential regulation in *Populus tremula* (Jia et al., 2009) and *Arabidopsis* (Jagadeeswaran et al., 2009). This is due to the fact that most of the conserved plant miRNAs are differentially regulated by abiotic stress in different plant species due to extent and duration of the stress and the unique characteristics of the species (Ding et al., 2013, Wang et al., 2011).

Another miRNA selected for the present study, miR159, was shown to be induced in Shandong and Yukon plants due to salt, cold and drought stress treatments. Consistent with our findings, this miRNA has also been shown to be up-regulated under drought stress in *Arabidopsis* (Reyes and Chua, 2007) and has been implicated to provide plant tolerance to environmental stress by functioning through hormone and abiotic stress signaling networks (Phillips et al., 2007). mir156, miR319 and miR393 are the additional miRNAs that was shown to be differentially expressed under abiotic stress conditions (salt, cold and drought) in *Thellungiella* ecotype Shandong and Yukon plants (salt, cold and drought). Similar results have been noted that miR393 expression level was altered under salinity and cold (Liu et al., 2008) as well as under drought conditions (Zhao et al., 2007). In the present study, we also found that miR393 was downregulated after exposure of *Thellungiella* ecotypes to NaCl, cold and drought. Likewise, miR393 down-regulation identified in *T. halophila* by constructing small RNA libraries (Zhang et al., 2013). miR393 is speculated to inhibit plant growth and development during times of environmental stress in stress-sensitive plants by targeting TIR1, a positive regulator of plant growth (Shukla et al., 2008). miR319 was found to be downregulated after exposure to NaCl, 4 °C and mannitol in the current study. Similar results was obtained from studies in

maize hybrid lines during salt and drought stress (Kong et al., 2010) and studies related with the hormone (abscisic acid, gibberellin and jasmonate) signaling pathways (Liu and Chen, 2010) as well.

It is interesting to know that the upregulated (miR159, miR166 and miR398) and downregulated (miR156, miR169, miR319 and miR393) miRNAs in the current study are directly implicated in the abiotic stress response (Lu and Huang, 2008, Mallory and Vaucheret, 2006, Sunkar and Zhu, 2004). Recent studies have shown that the expression of miRNAs as gene regulators is altered after abiotic stress treatments (Ding et al., 2009, Zhou et al., 2010, Zhou et al., 2008) and they play important regulatory roles in plant growth and development (Jones-Rhoades et al., 2006, Lu and Huang, 2008). However, most of these studies have been performed in glycophytic model organisms such as Arabidopsis and rice. In this study, we investigated the changes in miRNA expression levels after exposure to stress in halophytic *Thellungiella*, an important candidate for adaptation studies to harsh conditions including, high salinity, low temperatures and prolonged periods of water deficit (Griffith et al., 2007).

3.4 Prediction of potential *Thellungiella* miRNA targets

To begin understanding the consequences of altered miRNA expression due to abiotic stress conditions, we focused on the possible miRNA targets. Based on the complementarities between miRNAs and their target mRNAs, we employed a target search using psRNATarget, a plant small RNA target analysis server, for the selection of putative targets of some selected abiotic stress-related miRNAs based on identified miRNAs from *Arabidopsis*. Basically, target prediction depends on sequence similarity between target mRNA and miRNA. Complementarity scores are given according to sequence similarity (Dai and Zhao, 2011). Table 12 showed the predicted targets, target accessibility and alignment positions of the selected miRNAs.

Table 12 Target prediction results for selected miRNAs (The red circled bases are the uncomplimentary bases between miRNA and predicted target mRNA).

miRNA	Target	Target Accessibility	Alignment	Target Description	Inhibition
ath-miR156	AT1G27370	12.3	miRNA 20 CACGAGUGAGAGAAGACAGU 1 : Target 2368 GUGCUCUCUCUCUUCUGUCA 2387	Symbol: SPL10 (Squamosa promoter binding protein-like 10), Length=2727	Cleavage
ath-miR159	AT5G67060	13.45	miRNA 20 UUUGGAUUGAAGGGAGCUCU 1 : Target 1126 AAACCUGUCUUCCCUCGAGA 1145	Symbol: HEC1 (Hecate1) Length=1427	Cleavage
ath-miR169	AT1G54160	18.91	miRNA 20 GCCGUUCAGUAGGAACCGAC 1 : Target 1285 CGGGAAGUCAUCCUUGGCUA 1304	Symbol: NF-YA5 (Nuclear factor Y, subunit A5), Length=1462	Cleavage
ath-miR319	AT4G00940	18.1	miRNA 20 UCCCUCGAGGGAAGUCAGGUU 1 : Target 882 AGAGAGCCACCUUCAGUCCAA 902	Symbol: ITD1 (Dof domain zinc finger), Length=970	Cleavage
ath-miR393	AT2G38470	12.87	miRNA 20 UAGUUACCGCUAGGGAAACCU 1 : Target 716 AUCAGUCUGAUUCCUUUGGG 735	Symbol: WRKY33 (DNA-binding protein), Length=1902	Cleavage

Our prediction of target genes for the *T. halophila* miRNAs is in line with the knowledge that more than one gene is usually regulated by an individual miRNA and is similar to the recent findings in other plant species (Jones-Rhoades and Bartel, 2004, Mendes et al., 2009). The number of predicted targets for miR156, miR159, miR169, miR319 and miR393 are listed as 23, 6 19, 9 and 16, respectively. The alignment of binding sites between miRNAs (miR156 (Figure 15A), miR159 (Figure 15B), miR169 (Figure 15C), miR319 (Figure 15D) and miR393 (Figure 15E)) and the predicted target mRNAs are illustrated as follows.

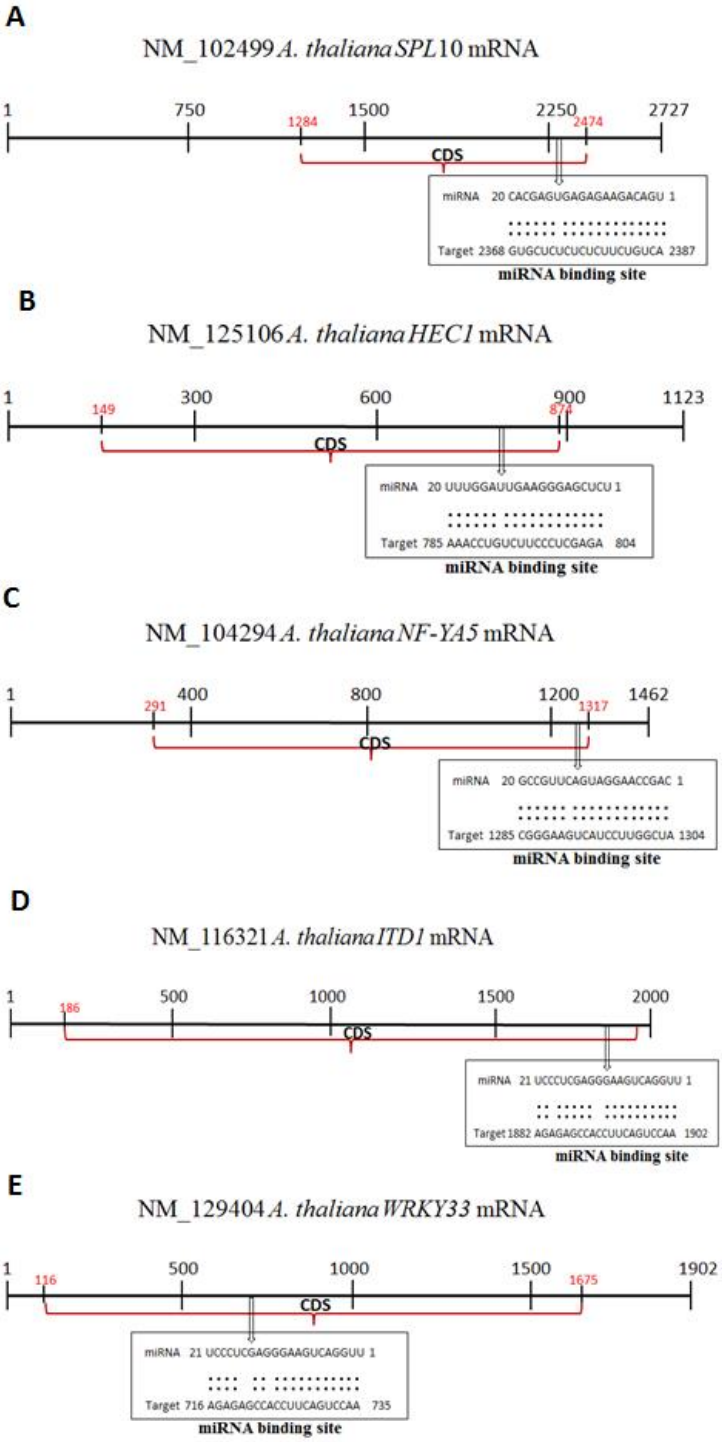

Figure 15 The binding sites between target mRNAs and the corresponding miRNAs were predicted by psRNA Target (**A** Binding site between *SPL10* and miR156 **B** Binding site between *HEC1* and miR159 **C** Binding site between *NF-YA5* and miR169 **D** Binding site between *ITD1* and miR319 **E** Binding site between *WRKY33* and miR393).

To understand whether predicted genes were actual miRNA targets, we examined the expression of five putative targets, *ITD1* (Intercellular trafficking DNA-binding with One Finger) (Dof-type zinc finger DNA-binding family protein) (predicted target of miR319), *HEC1* (HECATE1) (Basic helix-loop-helix transcription factor) (predicted target of miR159), *WRKY33* (transcription factor, DNA-binding protein) (predicted target of miR393), *NF-YA5* (transcription factor, nuclear factor-Y subunit alpha) (predicted target of miR169) and *SPL10* (transcription factor, squamosa promoter binding protein-like) (predicted target of miR156)) in the current study during salt stress conditions. The majority of the plant miRNA targets encode regulatory factors (transcription factors) known to be involved mainly in plant growth and developmental patterns. The remaining contained targets encoding a range of different proteins implicated in various metabolic processes, hormone responses, stress defense and signaling (Carrington and Ambros, 2003, Jones-Rhoades and Bartel, 2004).

qRT-PCR analysis of *ITD1* mRNA showed a significant 2.2-fold change increase in the expression during salt stress treatment of *T. halophila* Shandong ecotype plants (Figure16A) and a negative correlation with the corresponding miR319 expression was observed (Figure 16B). Dof transcription factors are defined as transcription factors containing the Dof (DNA-binding one finger) domain and they are specific to plants whose actions are related to biological processes unique to plants such as formation of vascular tissue during leaf development, the expression of photosynthetic genes and genes responsive to plant hormones and/or stress signals (Le Hir and Bellini, 2013, Yanagisawa, 2000). The water stress as a consequence of other abiotic stresses, (Krasensky and Jonak, 2012) induces the upregulation of Dof1 (Huerta-Ocampo et al., 2011) which contributes to prevent water loss by transpiration. The decrease in the expression of miR319 (0.43 fold) (Figure 16A) and parallel upregulation of *ITD1* (Figure 16B) may be connected with the slower decrease in the relative water content (RWC) of the salt-treated plants (Figure 7B).

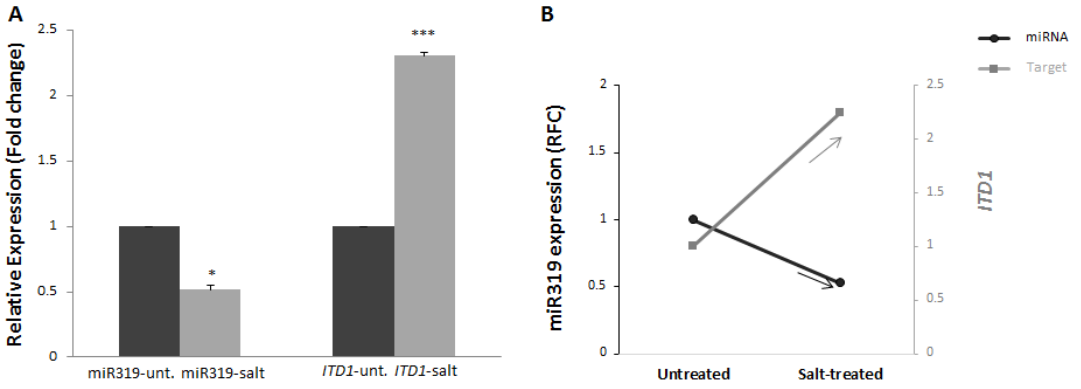

Figure 16 Expression level of the selected putative target gene (*ITD1*) of miR319 in *T. halophila* (Shandong ecotype) plants**. A** Relative expression levels in comparison with the expression levels of corresponding miRNAs. Total RNA from untreated and 250 mM NaCl treated plants were extracted, and RT-qPCR was used to analyze the relative expression of the selected target by using SYBR Green Mastermix (Roche Applied Science). *18S rRNA* was used as an internal control. The results given are averages ± SEM of three biological replicates with three technical replicas. Error bars indicate the standard error of the mean. **B** Increase or decrease in the expression levels of target mRNAs are inversely correlated with the increase or decrease in the expression levels of corresponding miRNAs (↗ stands for the increase in the expression level, ↙ stands for the decrease in the expression level).

The miR156 and miR169 family members were predicted as well as validated to target SPL (squamosa promoter-binding protein-like) and NF-Y (nuclear factor-Y) family gene transcripts (Li et al., 2008, Wu and Poethig, 2006), respectively. Nuclear factor-Y overexpression involved in increased drought tolerance, delayed senescence and recovery after stress treatment in maize plants (Nelson et al., 2007). To investigate whether this *NF-YA5* gene was in fact regulated by miR169, we further quantified the mRNA level after salinity treatment and we found that *NF-YA5* mRNA level was increased 2.1 fold (Figure 17B) in connection with the 0.6 fold decrease in the miR169 expression (Figure 17A). Given that the NF-Y complex potentially regulates so many genes (Covarrubias and Reyes, 2010), we speculated that the role of miR169 under abiotic stress could be involved in extensive physiological processes.

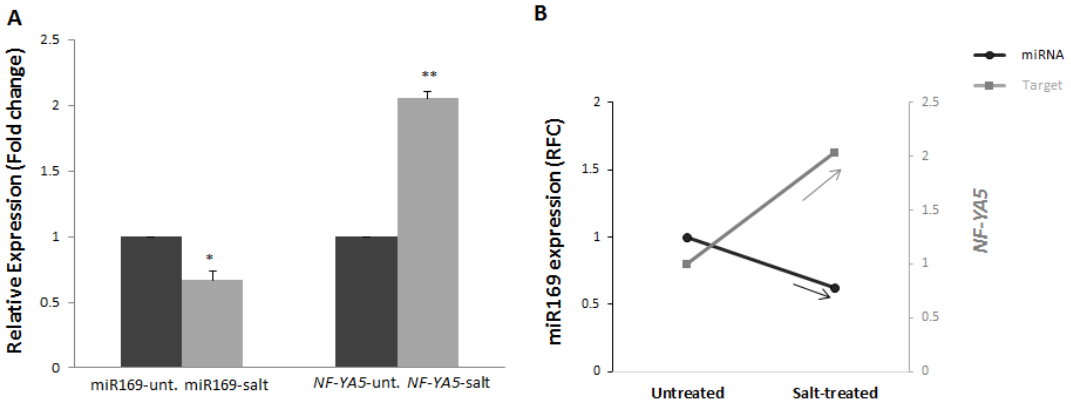

Figure 17 Expression level of the selected putative target gene (*NF-YA5*) of miR169 in *T. halophila* (Shandong ecotype) plants**. A** Relative expression levels in comparison with the expression levels of corresponding miRNAs. Total RNA from untreated and 250 mM NaCl treated plants were extracted, and RT-qPCR was used to analyze the relative expression of the selected target by using SYBR Green Mastermix (Roche Applied Science). *18S rRNA* was used as an internal control. The results given are averages ± SEM of three biological replicates with three technical replicas. Error bars indicate the standard error of the mean. **B** Increase or decrease in the expression levels of target mRNAs are inversely correlated with the increase or decrease in the expression levels of corresponding miRNAs (↗ stands for the increase in the expression level, ↙ stands for the decrease in the expression level).

qRT-PCR analysis of putative miR156 target mRNA (*SPL10*), showed a corresponding 2.4 fold increase in the target mRNA in salt-treated Shandong plants compared to untreated ones (Figure 18A). SPL family members are plant-specific transcription factors. miR156 and its target SPL gene regulation play important roles in diverse aspects of plant development including phase transition from vegetative phase to reproductive phase, embryonic patterning and anthocyanin biosynthesis (Rubio-Somoza and Weigel, 2011, Silva et al., 2014). Current study shows a high level of expression of miR156 target mRNA (Figure 18B), hence it might be possible that miR156 might be involved in rapid flowering of plants under stress (data not shown) and especially increased amount of anthocyanin at high salt concentrations (Figure 7A).

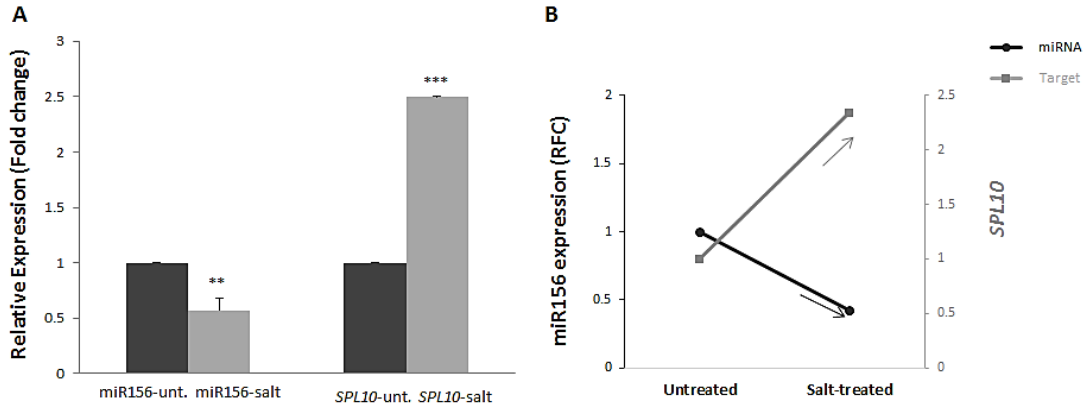

Figure 18 Expression level of the selected putative target gene (*SPL10*) of miR156 in *T. halophila* (Shandong ecotype) plants**. A** Relative expression levels in comparison with the expression levels of corresponding miRNAs. Total RNA from untreated and 250 mM NaCl treated plants were extracted, and RT-qPCR was used to analyze the relative expression of the selected target by using SYBR Green Mastermix (Roche Applied Science). *18S rRNA* was used as an internal control. The results given are averages ± SEM of three biological replicates with three technical replicas. Error bars indicate the standard error of the mean. **B** Increase or decrease in the expression levels of target mRNAs are inversely correlated with the increase or decrease in the expression levels of corresponding miRNAs (↗ stands for the increase in the expression level, ↙ stands for the decrease in the expression level).

miR393 showed a 0.2 fold downregulation during salt-stress treatments indicating its potential role in abiotic stress tolerance (Figure 19A). Taking into consideration decrease in the miR393 expression, we analyzed the expression of *WRKY33* mRNA in salt-treated *Thellungiella* Shandong ecotype using qRT-PCR (Figure 19B). The *WRKY33* mRNA expression was 3.3 fold high in salt-treated Shandong plants in contrast to an expected negative correlation with the miR393 level, which might be due to the posttranscriptional regulation. WRKY transcription factors regulate transcriptional reprogramming associated with multiple plant processes such as biotic and abiotic stress, seed development, seed germination and leaf senescence. Overexpression of a WRKY gene in rice resulted in enhanced salt and drought tolerance (Rushton et al., 2010). The increased amount of seed production of *Thellungiella* plants under abiotic stress (data not shown) might be correlated with the overexpression of the WRKY gene.

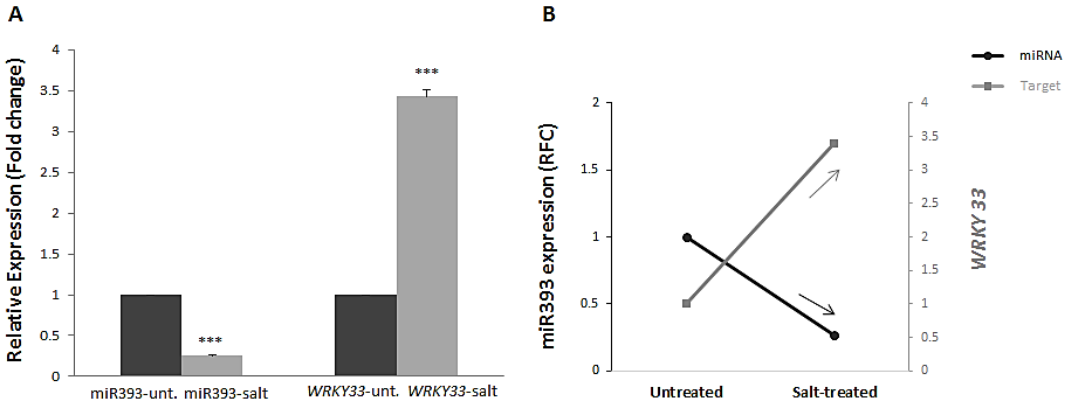

Figure 19 Expression level of the selected putative target gene (*WRKY33*) of miR393 in *T. halophila* (Shandong ecotype) plants**. A** Relative expression levels in comparison with the expression levels of corresponding miRNAs. Total RNA from untreated and 250 mM NaCl treated plants were extracted, and RT-qPCR was used to analyze the relative expression of the selected target by using SYBR Green Mastermix (Roche Applied Science). *18S rRNA* was used as an internal control. The results given are averages ± SEM of three biological replicates with three technical replicas. Error bars indicate the standard error of the mean. **B** Increase or decrease in the expression levels of target mRNAs are inversely correlated with the increase or decrease in the expression levels of corresponding miRNAs (↗ stands for the increase in the expression level, ↙ stands for the decrease in the expression level).

Another stress responsive miRNA, miR159 1.6 fold overexpression (Figure 20A) was accompanied with the 0.58 fold decrease in its putative target, *HEC1* mRNA level (Figure 20B). The function of *HEC1* in stress signaling pathways remains to be unknown. Despite this, it was known that *HEC1* functions in hormone signaling pathways (Schuster et al., 2014). The relation between miR159 and its putative target mRNA needs to be identified by confirming the role of *HEC1* in abiotic stress responses.

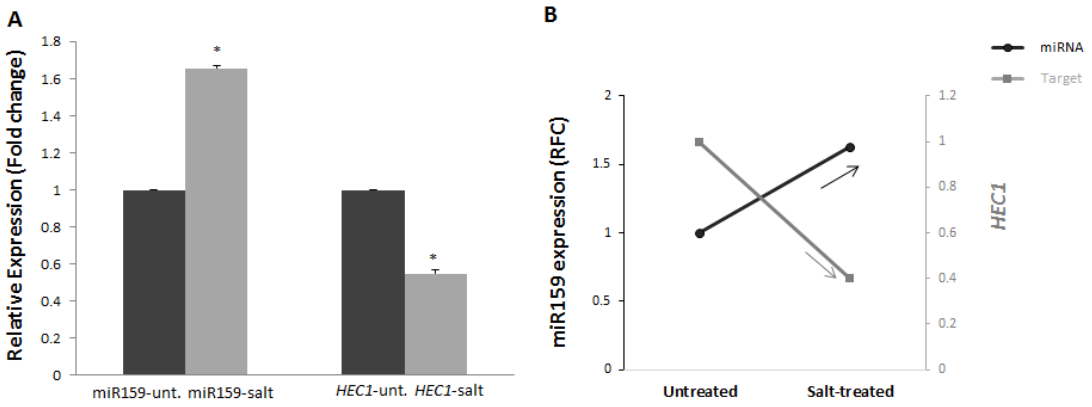

Figure 20 Expression level of the selected putative target gene (*HEC1*) of miR159 in *T. halophila* (Shandong ecotype) plants**. A** Relative expression levels in comparison with the expression levels of corresponding miRNAs. Total RNA from untreated and 250 mM NaCl treated plants were extracted, and RT-qPCR was used to analyze the relative expression of the selected target by using SYBR Green Mastermix (Roche Applied Science). *18S rRNA* was used as an internal control. The results given are averages ± SEM of three biological replicates with three technical replicas. Error bars indicate the standard error of the mean. **B** Increase or decrease in the expression levels of target mRNAs are inversely correlated with the increase or decrease in the expression levels of corresponding miRNAs (↗ stands for the increase in the expression level, ↙ stands for the decrease in the expression level).

The miRNA target gene identification is an important step for understanding the role of miRNAs in gene regulatory networks. Plant miRNAs are a perfect or near-perfect match to their target mRNAs and help regulate post-transcriptional gene expression by binding to mRNAs (Bartel, 2004). Most plant miRNAs that bind to mRNAs lead to transcript cleavage (Jones-Rhoades et al., 2006).

CHAPTER IV

CONCLUSION

The study of miRNAs in plants started around 2002 (Reinhart et al., 2002), after the discovery of a new class of RNA in nematodes (Lee et al., 1993). The new RNA class turned out to be what we recognize today as miRNA. Indeed, we now know that all higher life forms use small RNAs to regulate the expression of specific genes. Plants were among the few model organisms that helped elucidate miRNA functions. One of the reasons for this being that plant miRNAs are highly complementary to conserved target mRNAs, which allows for the fast and confident prediction of miRNA targets in silico before applying costly biochemical and molecular methods. miRNAs target protein coding genes through post transcriptional gene silencing, then prior knowledge of target gene biology can help to elucidate on the reasons why miRNAs silence particular targets in particular conditions at particular times (Jones-Rhoades and Bartel, 2004, Jones-Rhoades et al., 2006).

The goal of our research was to investigate several abiotic stress associated miRNAs and their expression profiles in the stress-tolerant model plant *Thellungiella halophila* ecotypes (Shandong and Yukon) at salt, cold and drought stress conditions to elucidate the role of miRNAs in the context of plant stress. We predicted some miRNA targets in silico and the expression patterns of 5 putative targets under salt stress were detected by qRT-PCR. At the beginning of the study, the physiological parameters such as GSH/GSSG value, proline, MDA and anthocyanin levels and relative water content, related to abiotic stress were quantified for the identification of the tolerance to gradually increased salt stress in *Thellungiella halophila* plants. High GSH/GSSG values, increased proline and anthocyanin levels, low MDA production and less reduction in RWC indicated the salt tolerance of *Thellungiella* plants. Thus, we started to detect miRNA expression

under various abiotic stresses (salt, cold and drought). Out of the 7 miRNAs, we found that all of them were differentially expressed at stress conditions in Shandong and Yukon ecotypes. We found that miR159, miR166 and miR398 exhibited increased fold change in expression and miR156, miR169, miR319 and miR393 showed a decrease in the expression during salt, cold and drought treatments. The expression profiles of mir156, mir159 and mir169 after exposure to salt in salt-tolerant cultivar of cotton have been shown to have similar patterns (Yin et al., 2011) as in salt-tolerant *Thellungiella* plants in the current study. This may indicate the potential functional role of these miRNAs under salt stress conditions. miR156 and miR169 were downregulated by salt stress in salt-tolerant cultivar, but were not affected in salt-sensitive one. Additionally, miR159 was down-regulated in salt-sensitive cultivar, but was more expressed in salt-tolerant one (Yin et al., 2011). mir393 expression was low in drought-resistance cultivar of sugarcane during drought stress as found in our study. However, the expression of mir393 was upregulated in drought-sensitive cultivar after exposure to drought (Ferreira et al., 2012). The predicted targets of miR156, miR159, miR169, miR319 and miR393 are the transcription factors that have important roles during plant growth and development, hormone biosynthesis and abiotic stress responses. The negative correlation between miRNAs and their putative targets mRNA expression profiles might reveal that the selected miRNAs play roles in the regulation of the target gene expression. However, this needs to be confirmed in further studies.

Future studies should focus on the functional and mechanistic aspects of altered miRNA expressions we detected in this study. Collectively, it will be important to understand the stress tolerance mechanisms of plants in terms of crop yield and crop quality.

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PUBLICATIONS

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Ferhunde AYSİN, A. Elif ERSON-BENSAN, Füsun EYİDOĞAN, Hüseyin Avni ÖKTEM (2015). Generating salt-tolerant *Nicotiana tabacum* and identification of stress-responsive miRNAs in transgenics. **Turk J Bot.**, 39, 757-768.

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PROJECTS

TUBITAK 108O786. ''NAC tipi transkripsiyon faktörleri kullanılarak abiyotik stress dirençli transgenik buğday çeşitlerinin geliştirilmesi ve elde edilen bitkilerde abiyotik stres koşullarında gen ifade profillerinin mikroarray yöntemiyle incelenmesi''. Yücel M, Gürel E, Çelikkol Akçay U, Kavas M, Öz MT, Kalemtaş G, Kayıhan C, Ercan O, Aysin F, Aksoy E, Baloğlu MC.