PHYSIOLOGICAL AND METABOLIC RESPONSES OF *THELLUNGIELLA SALSUGINEA* TO OSMOTIC STRESS

PHYSIOLOGICAL AND METABOLIC RESPONSES OF *THELLUNGIELLA SALSUGINEA* TO OSMOTIC STRESS

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ABSTRACT

Abiotic stresses such as extreme temperatures, drought and high salinity severely compromise plant productivity, and have placed selective pressure for the acquisition of traits enabling plants to adjust to and recover from these unfavorable environmental conditions. *Thellungiella salsuginea* is a plant that is native to highly saline and semi-arid environments and exhibits an exceptional ability to tolerate abiotic stress. In this thesis, I report on laboratory and field studies aimed at identifying traits that allow *Thellungiella* to tolerate harsh environmental conditions. It was found that *Thellungiella* accumulates organic solutes in response to abiotic stress. Transcript and metabolite profiling approaches were used to identify metabolic pathways important for the accumulation of compatible organic solutes in *Thellungiella* in response to sub-optimal environmental conditions. The relative abundance of transcripts encoding enzymes associated with the biosynthesis of compatible organic solutes such as proline or galactinol showed stress-responsive increases in cabinet-grown material and these metabolites were accumulated in salt or drought treated plants, respectively. However, proline and galactinol were found to be of low relative abundance in leaves of field plants. In contrast, several carbohydrates including sucrose, glucose, and fructose made a greater relative contribution to the field plant profiles suggesting that carbohydrates play an important role in plant abiotic stress tolerance during growth under field conditions. The identification of stress-specific metabolic changes can be used to identify important biochemical traits underlying environmental stress tolerance in *Thellungiella*. This

information can be used to improve the tolerance of stress – sensitive crops (including a related crucifer species, canola) that are grown in areas where persistent droughts, saline soils and early or late frosts frequently occur.

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My interest in plants all started when I was a toddler roaming in the maize fields of rural El Salvador where my grandfather, Papa Tino, taught me about his farm and the importance this crop has played for the economy and throughout civilization. I thank my parents, Jose and Ana, who left everything behind for us to pursue our dreams and I am fortunate to have found mine; the love of my life, Rovena, and plant science. I thank my brothers Nestor and Herb, and sister Carolyn for their love and encouragement throughout my journey. It's actually difficult to put in words the gratitude I have for the unconditional love and encouragement that Rovena provided to me throughout my studies. Her patience, love and support throughout my studies motivated me to step up to the challenges.

This work is dedicated to our families who depend on agriculture for a living and I have made it my personal mission to improve crop yields during a time when we are more than ever dependant on plant products due to explosive growth in the world population, but are most vulnerable as a result of the unpredictability in climatic conditions which compromises our food resources.

OVERVIEW

This sandwich thesis contains five chapters: Chapter One (Literature review); Chapter Two (Materials and Methods); Chapters Three and Four (Enclosed as separate manuscripts to be submitted); and Chapter Five (General discussion). My contributions and the contribution from the co-authors will be described in a preamble to chapters three and four. The final chapter will include a general discussion and future directions.

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LIST OF ABBREVIATIONS USED

ABA	Abscisic acid
AMDIS	Automated Mass Spectral Deconvolution Information System
d	Day
ELIP	Early light induced protein
FW	Fresh weight
GASP	Gas chromatography/Mass spectrometry Analysis Software Package
GC/MS	Gas chromatography/Mass spectrometry
HSP	Heat shock protein
LEA	Late embryogenesis abundant
LTP	Lipid transfer protein
m/z	mass-to-charge ratio
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
MST	Mass spectral tags
MPa	Megapascal
NaCl	Sodium chloride
HCA	Hierarchical cluster analysis
ANOVA	Analysis of variance
PCA	Principal component analysis
ROS	Reactive oxygen species
ψ_w	Water potential

ψ_S	Solute potential
ψ_P	Pressure/Turgor potential
RI	Retention index
RT	Retention time
RWC	Relative water content
TIC	Total ion chromatogram
TMS	Trimethylsilyl

Chapter One

Literature Review

1.1 Abiotic stress decreases plant growth and productivity

Plants growing in their native habitats are confronted with ever-changing environmental factors that may limit their growth and productivity. Some of these factors include prolonged periods of water deficit (drought), excessively high or low temperatures, or high soil salinity. These conditions reduce the quantity of water that can be taken up from the soil by plant roots and so represent factors contributing to osmotic stresses. Unfortunately, most major crop species are poorly adapted and vulnerable to osmotic stress (Boyer, 1982). It is estimated that osmotic stresses are responsible for decreasing major crop yields by about 69%, depending on the crop (Boyer, 1982; Bray et al, 2000). To some extent, the impact of water deficit can be partially offset through irrigation of crops to produce higher yields in agricultural areas prone to recurrent drought episodes during the growing season. However, irrigation of agricultural lands with poorly drained soils can lead to the buildup of salts in the soil to levels that can be toxic to plants (Schoups et al., 2005). The Intergovernmental Panel on Climate Change (IPCC) has predicted an increased incidence of more intense and longer drought in the future as a result of increased soil drying due to higher temperatures and decreased land precipitation patterns (IPCC, 2008). Consequently, drought effects on crop yield will

become more severe and lead to reduced food production. The current position held by many plant scientists is that we must enhance the tolerance of stress-sensitive crops so that they achieve higher yields despite exposure to sub-optimal environmental conditions (Flowers and Yeo, 1995). This is especially important since higher yields will be required to meet the increased demand for plant products to sustain the growing human population (Flowers and Yeo, 1995; Trewavas, 2001). However, in order to improve the productivity of crops grown under sub-optimal environmental conditions we must develop a more complete understanding of the mechanisms underlying osmotic stress tolerance in plants (Flowers and Yeo, 1995; Tester and Bacic, 2005).

1.2 Plant responses to osmotic stress

1.2.1 Water status

Plant cell growth generally occurs within a narrow range of water content (Hsiao et al., 1976). There are two parameters that are widely used to determine the water status of a plant: the relative water content and the water potential.

1.2.1.1 Relative water content

The relative water content (RWC) refers to the amount of water in the tissue relative to the amount the tissue can hold when fully turgid. A leaf from a well-watered

plant will have a RWC in the range of 85-95 %, and decreases when plants are exposed to osmotic stress (Hsiao, 1973). Although the RWC is a useful parameter to describe the effect water deficits have on cell volume and water status of a plant (Sinclair and Ludlow, 1985), one short-coming is that it does not provide information on water movement (Kramer, 1988). A more suitable parameter to help describe water movement in the cell is the water potential.

1.2.1.2 Water potential

Plant water status can be monitored by measuring the free energy state of water molecules inside cells or tissues, a parameter that is estimated by plant physiologists using the term water potential (Ψ_w). Ψ_w is comprised of two major components: the solute (or osmotic) potential (Ψ_s) which represents the effect of dissolved solutes on Ψ_w, and the pressure (or turgor) potential (Ψ_p) which describes the effect of pressure on the energy status of water. The presence of solutes reduces the free energy of water, and thus decreases Ψ_w, whereas a positive Ψ_P increases the Ψ_w. Pure water has the highest capacity to do work and is frequently represented by a Ψ_w of zero megapascal (MPa). In biological systems, water has less potential energy than pure water due to the effect of dissolved solutes that are inevitably present in a cell and thus Ψ_w values are always negative. Well hydrated plants maintain a leaf Ψ_w of – 0.85 to – 1.0 MPa (Flowers and Ludlow, 1986). Plants can take up water from their environment through the roots only when their root Ψ_w is lower than the surrounding soil Ψ_w.

1.2.1.3 Impact of osmotic stress on water relations

When plants are subjected to soil water deficits water lost by transpiration can exceed uptake by roots. Under these conditions, plant Ψ_W decreases with the passive accumulation of solutes (Acevedo et al., 1979; Flower and Ludlow, 1986). The decrease in root Ψ_w must be sufficient to maintain a $\Delta\Psi$w gradient between the root Ψ_w and soil Ψ_w to ensure continued water uptake by the root. If this gradient is not established, plant growth ceases as a first consequence of water deficit (Nonami and Boyer, 1989) with turgor loss (or wilting) when the Ψ_p reaches 0 Mpa.

Drought, extreme temperatures, or high soil salinity affect plant water status in different ways, but all of these environmental conditions can ultimately lead to cellular dehydration. In the field, drought and high temperature stress significantly reduce water content in the soil as a result of evaporation leading to decreased water uptake by plants and reduced water content of plant tissues (Ristic et al., 1991). Soil salinity, on the other hand, need not be accompanied by a shortage of water. Water moves down a Ψ_W gradient from high (less negative) to low Ψ_W. As such, the presence of high salts can impede the ability of a plant to take up water unless the plant can overcome the reduction in soil Ψ_W caused by the presence of high levels of dissolved solutes in the soil (Yeo et al., 1991). Chilling temperature (5°C) leads to lower water content in plant tissues as a result of decreased water absorption by the roots and water transport to the shoot (Burchett et al., 2006). Finally, freezing temperatures (<0°C) lead to the formation of ice

in the intercellular spaces and the resulting movement of water out of the cell during extracellular freezing causes cellular dehydration (Yelenowsky and Guy, 1989).

Inadequate water supply to plant tissues during osmotic stress triggers physiological responses aimed at minimizing transpirational water loss, an objective achieved by stomatal closure (Ackerson and Krieg, 1977) when the leaf RWC decreases to 70 to 75% (Chaves et al., 2002) . However, stomatal closure during water deficits leads to lower CO_2 intake for photosynthesis (Meyer and Genty, 1999). Drought-induced reduction of photosynthesis limits plant growth and development because fewer resources are available to support cell expansion. Therefore, in general, water deficits produce plants of decreased stature and this reduction in plant size can severely compromise yield potential (Blum and Sullivan, 1997).

1.2 2 Osmotic adjustment

Some plants have the ability to maintain full turgor of the leaves during water deficits by actively accumulating non-toxic (compatible) organic solutes. It has been proposed that compatible organic solute accumulation serves to decrease the plant Ψ_S in order to establish a $\Delta\Psi w$ gradient between the plant and drying or saline soil that promotes water uptake (Hsiao et al., 1976). Plants exposed to saline environments passively accumulate the Na^+ ions dissolved in the water absorbed by their tissues. To offset the deleterious effects of high Na^+ concentrations on metabolic processes, plants sequester potentially perturbing ions in the vacuole (Matoh et al., 1987). However, this

response can lead to an osmotic imbalance within the cell (Matoh et al., 1987; Binzel et al., 1988). Thus, it is generally accepted that the balance in the osmotic pressure between the cytoplasm and the vacuole is achieved primarily via the accumulation of compatible solutes in the cytoplasm (Binzel et al., 1988). This active net accumulation of solutes by plants in response to water deficit or salinity helps them remain turgid under water deficits and the response is called osmotic adjustment. Osmotic adjustment has been proposed to be an important adaptive process conferring osmotic stress tolerance in plants (Flowers and Ludlow, 1986). This is believed to be the case because plants that can osmotically adjust are better able to acclimate to adverse environmental conditions (Acevedo et al., 1979; Nonami and Boyer, 1989) than plants that close their stomata during exposure to soils with low ψ_W (Turner, 1974).

Two extreme examples of highly desiccation and salt tolerant plants include the 'resurrection plant' *Craterostigma plantagenium* and the 'common ice plant' *Mesembryanthemum crystallinum*. *C. plantagenium* is capable of recovering from complete vegetative tissue dehydration (Bianchi et al., 1991) whereas *M. crystallinum* can grow in the presence of sea-water strength salinity (Paul and Cockburn, 1989). *C. plantagenium* and *M. crystallinum* are able to thrive under osmotic stress due, at least in part, to their ability to osmotically adjust in response to exposure to extreme environmental conditions. *C. plantagenium* accumulates sucrose in response to prolonged periods of desiccation (Bianchi et al., 1991) while the pinitol content of *M. crystallinum* increases when exposed to sea-level strength salinity (Paul and Cockburn, 1989). The capacity for osmotic adjustment is not restricted to 'extremophile' plants but

is believed to be a process that many organisms including many plant species undergo when experiencing osmotic stress (Yancey 2005; Yancey et al., 1982).

The compatible organic solutes involved in osmotic adjustment are chemically diverse but essentially fall into five main classes of compounds: 1) sugars, including trehalose (Garg et al., 2002) and sucrose, (Bianchi et al., 1991; Rizhsky et al., 2004), 2) sugar alcohols, like galactinol, inositol, mannitol, sorbitol, and pinitol,(Paul and Cockburn, 1989; Popp and Smirnoff, 1995; Pattanagul and Madore, 1999, Taji et al., 2002), 3) N-containing solutes such as the amino acid proline (Kishor et al., 1995), or quaternary ammonium species like proline betaine, β-alanine betaine and glycine betaine (Hanson et al., 1991), 4) tertiary sulfonium compounds including 3-dimethylsulfoniopropionate and choline-O-sulfate (Trossat et al., 1998). The capacity to accumulate these compatible solutes differs between plant species. For example, stress-responsive increases in proline content occurs broadly among plant species (Delauney and Verma, 1991) whereas other classes of compatible solutes such as glycine betaine are accumulated in plants of select families such the Chenopodiaceae and Poaceae (Storey and Jones, 1977).

Many crop plants lack the ability to synthesize and accumulate compatible solutes that are found in osmotic stress tolerant native plants. Therefore, genes encoding enzymes involved in the synthesis of compatible solutes have been introduced into non-accumulating species in an effort to genetically engineer crop plants that are more stress tolerant. For example, transgenic tobacco plants engineered to over-express a gene encoding the *M. crystallinum* myo-inositol *O*-methyltransferase were more drought and

salt tolerant than wild-type tobacco plants (Sheveleva et al., 1997). These transgenic tobacco plants were found to contain higher levels of ononitol compared to wild-type tobacco plants, an observation that led the authors to conclude that ononitol plays a role in osmotic adjustment. This approach of metabolic engineering to increase compatible solute biosynthesis by plants has contributed towards increased stress tolerance in non-accumulating, stress-sensitive crops such as rice (Zhu et al., 1998; Garg et al., 2002), tobacco (Kishor et al., 1995; Sheveleva et al., 1997), and wheat (Abebe et al., 2003). However, despite these successes, frequently the observed levels of compatible solutes accumulated by the transgenic plants were not high enough to explain their protective role through their contribution towards osmotic adjustment. This observation is consistent with the conclusion that compatible solutes may increase the tolerance of plants through protective mechanisms other than simply osmotic adjustment. Flowers (2004) has also raised the concern that most of these studies were performed under artificial, controlled environmental conditions and it is uncertain whether transgenic plants with an increased capacity for compatible solute biosynthesis have an increased tolerance to abiotic stress under field conditions.

An example of the difficulty in associating stress tolerance with solute accumulation is given by sucrose. The accumulation of sucrose in many plants experiencing water deficits is widespread and found among osmotic stress tolerant and sensitive species. However, the significance of sucrose accumulation during osmotic adjustment is uncertain due to the difficulty in demonstrating conclusively that sucrose accumulated in response to stress actually contributes towards osmotic adjustment

(Munns and Weir, 1981). In fact, accumulation of organic solutes like sucrose may represent the outcome of impairments in metabolism and thus may be indicators of cell damage as opposed to providing protective functions (Paul and Cockburn, 1989). More recently the adaptive value of organic solute accumulation and their contribution to osmotic adjustment for abiotic tolerance in plants has been challenged. A comprehensive analysis of solute accumulation in *Limonium latifolium* subjected to NaCl treatments shows that the major contributors to osmolarity were inorganic solutes, while organic solutes such as sucrose, hexoses, β-alanine betaine, and glycine betaine only accounted for approximately 25% of the osmolarity (Gagneul et al., 2007). Interestingly, compartmental analysis of proline and β-alanine betaine in salt-shocked *L. latifolium* found that both of these compatible solutes were localized in the vacuoles rather than the cytosol raising questions as to whether these metabolites are involved in osmotic adjustment in this species (Gagneul et al., 2007). Therefore, future work must be directed towards delineating the significance of compatible organic solute accumulation during exposure to osmotic stress conditions if this trait is to be used in long-term selection strategies to develop crops showing greater tolerance to osmotic stress.

The failure to genetically engineer plants with greater osmotic stress tolerance through an increased capacity to produce a given organic solute reveals that the manipulation of a single gene involved in compatible solute synthesis is likely insufficient to yield a more stress tolerant crop (Ramanjulu and Bartels, 2002). This may be due to the multigenic nature of osmotic stress tolerance in plants with an innate ability to thrive under sub-optimal environmental conditions (Ramanjulu and Bartels, 2002). As

a consequence, current efforts are focused on gaining an appreciation for higher order controls that serve to coordinate cellular functions at multiple levels using genomic approaches. Approaches that afford a better appreciation of global regulatory networks include technologies that allow the unbiased detection of many simultaneous changes among gene products that may occur in response to an abiotic challenge. These approaches include quantifying products at the transcript, protein and metabolite level. Changes occurring at each of these levels in response to abiotic stress compared to unstressed conditions should include those that are essential for enabling plants to adjust to and recover from exposure to sub-optimal environmental conditions. Recent technological advancements in genomics approaches now permit the correlation of physiological responses in plants subjected to osmotic stress with multiple, concurrent changes among specific gene products allowing for the development of deeper insights into the cellular processes associated with osmotic stress tolerance.

1.3 Using genomics approaches to search for osmotic stress tolerance mechanisms

The availability of the full genome sequence of the genetic model plant *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) has led to the development of tools and resources that can be used to gain insights into the genetic basis of plant abiotic stress tolerance (Somerville and Somerville, 1999; The *Arabidopsis* Genome Initiative, 2000). One noteworthy technological advance in identifying the molecular basis underlying plant abiotic stress tolerance has been the development of genome wide

expression profiling methods (Somerville and Somerville, 1999). The high-throughput analysis of gene expression (transcriptomics) using DNA microarrays has greatly helped in the large-scale identification of genes that are stress-responsive.

Transcriptomics is performed using DNA microarrays containing 1,000 to 10,000 cDNA sequences per square centimeter deposited onto a solid surface (a glass slide). These arrayed sequences are hybridized with fluorescently labeled probes derived from mRNA samples of different tissue types (Wisman and Ohlrogge, 2000) allowing the simultaneous analysis of thousands of genes in a single microarray experiment. For some plant species including *Arabidopsis* and rice (*Oryza sativa*), whole genome DNA microarray chips are commercially available (www.affymetrix.com). A recent advance in transcript profiling methodologies is RNA sequencing (RNA-Seq). RNA-Seq approaches enable the analysis of the entire transcriptome in a high-throughput manner with greater sensitivity and a higher level of reproducibility compared with DNA microarrays (Wang et al., 2009). The use of RNA-Seq methodologies will enable the accurate quantification of transcriptional responses to abiotic stress in many non-model plants including halophytes, as no prior knowledge on genome sequence is required to quantify gene expression levels in an unbiased fashion (Wang et al., 2009).

Plant responses to water deficits are modulated by altered gene expression that is triggered by sub-optimal environmental conditions. As such, transcriptomics has been used to elucidate the molecular mechanisms underlying osmotic stress tolerance. For example, transcriptomics has been used to monitor the gene expression patterns exhibited by *Arabidopsis* exposed to osmotic stress (Seki et al., 2002; Kreps et al., 2002). These

studies showed that hundreds of genes were differentially expressed in *Arabidopsis* upon short-term exposure to abiotic stress conditions by studying 1, 2 up to 24 h time-points following stress exposure (Seki et al., 2002; Kreps et al., 2002). Among the genes showing increased transcript abundance following exposure to abiotic stresses were those encoding proteins associated with: the synthesis of compatible solutes or phytohormones, the protection against ROS, dehydrin proteins, HSP proteins, LEA proteins, LTP proteins, transcription factors, carbohydrate metabolism, and others of unknown function. The precise role for many of these gene products remains elusive, and this is particularly true for the comparatively well studied dehydrins, LEAs and LTPs that over many years have been well documented to increase in a stress-responsive manner (Seki et al., 2002). However, the over-expression of LEA (Xu et al., 1996) in stress-sensitive crops, for example, has led to enhanced tolerance confirming their role for abiotic stress tolerance. Interestingly, transcriptomics results show that different sets of genes are involved in the response to cold, drought, or salinity stress (Seki et al., 2002). This observation provides evidence that responses to a given stress are specific at the molecular level (Seki et al., 2002). This specificity offers targets for a narrower range of products to test *in planta*. For example, associating survival during long-term exposure to abiotic stress under natural conditions in the field with the presence or absence of a given gene product would provide compelling evidence for the role of a specific gene in abiotic stress tolerance.

The information afforded by transcriptome profiling is extremely useful in associating metabolic pathways that are altered in plants subjected to sub-optimal environments. However, transcriptome data alone cannot establish whether the increased

abundance of transcripts encoding a given enzyme actually leads to more enzyme and enzyme activity in the cell (Fiehn et al., 2000). Consequently, large-scale analysis of metabolites present in an organism should be used to help establish or define the metabolic phenotype that results from the altered expression of genes in response to adverse environmental conditions.

The metabolome is the entire metabolite complement of a biological organism and as such, is complex. For the plant kingdom it is estimated that over 200,000 chemically diverse metabolites exist (Weckwerth, 2003) with an estimated number of about 5,000 metabolites in a single plant cell (Krishnan et al., 2005). Current methodologies permit the simultaneous analysis of over 300 metabolites using gas chromatography-mass spectrometry (GC/MS) (Fiehn et al., 2000) and up to 5,200 putative metabolites using Fourier transform ion cyclotron mass spectrometry (FTMS) (Aharoni et al., 2002). Of these technologies, the GC/MS approach has been the preferred metabolomics method due to its amenability for high-throughput analysis (methodologies reviewed by Allwood et al., 2008). To date no technology has been developed that allows one to study the complete metabolome of an organism. Studying the complete metabolome remains a challenge because no single approach suitably handles the wide dynamic range in metabolite abundance in tissues where, without enrichment, a large proportion of metabolites are present at levels below the current limit of detection. No instrumentation exists that can simultaneously resolve and detect all of the chemical components that are found in the plant kingdom (Sumner et al., 2003). Despite these inherent shortcomings, metabolomic approaches have been used to identify

stress-induced changes in the metabolism of plants including *Arabidopsis* subjected to drought or heat (Rizhsky et al., 2004), cold temperatures (Kaplan et al., 2004; Cook et al., 2004; Gray and Heath, 2005) and salinity (Kim et al., 2007). In these studies distinct metabolic phenotypes could be associated using the profiles of chemical components extracted from *Arabidopsis* cell cultures or plants subjected to stress-treatments when compared to profiles from unstressed controls. For example, *Arabidopsis* subjected to cold temperatures undergoes a substantive re-organization of the metabolome compared to unstressed controls (Kaplan et al., 2004; Cook et al., 2004, Gray and Heath, 2005). *Arabidopsis* exposure to cold temperatures leads to the accumulation of sugars and proline, and unexpectedly, other metabolites including putrescine, ornithine, and citrulline that implicate polyamines as contributing towards improved stress tolerance upon exposure to low temperatures (Cook et al., 2004). Rizhsky et al. (2004) reported that *Arabidopsis* exposed to drought accumulates proline, whereas exposure to a combination of drought and heat stress resulted in the accumulation of sucrose instead of proline. *Arabidopsis* cell cultures exposed to 100 mM NaCl over a period of 72 h showed that salt treatment leads to the co-induction of glycolysis and sucrose metabolism suggesting that sucrose accumulation may serve to promote water uptake and a potential source of energy to sustain cellular metabolism during exposure to salinity stress (Kim et al., 2007). Current metabolomics methodologies may not be unbiased or comprehensive but they are sufficient to uncover novel changes among metabolites across multiple metabolic pathways. Improvements in this technology could increase our capacity to detect changes among chemical components in plants subjected to abiotic stress and will

no doubt increase our capacity to define essential cellular networks needed by plants to respond to and recover from sub-optimal environmental conditions.

1.4 Field studies

The advent of genomic tools has offered important insights into identifying gene products associated with plant response to cold temperature, drought or salinity. In general, these environmental conditions induce the expression of many different genes or different members of gene families. Although these studies have identified traits associated with abiotic stress tolerance, the adaptive value and contribution of these traits to long-term acclimation of plants experiencing sub-optimal environments remains difficult to judge (Kulheim et al., 2002; Rizhsky et al., 2004). This difficulty may be due to the relatively low number of studies focusing on traits expressed in plants exposed to abiotic stress conditions in their natural environment. In fact, most studies aimed at identifying stress-specific responses have been performed using plants subjected to artificial calibrated treatments imposed in controlled environmental growth chambers. Extrapolating plant responses in the laboratory to their response in the field is difficult given that growth chambers cannot mimic the complexity of a natural environment where traits that are important for long-term survival and productivity under challenging conditions are needed. Comparative studies reporting on plants subjected to abiotic stress under controlled environments and under field conditions have been published (Miyazaki et al., 2004; Dhanaraj et al., 2006). Although overlap is found between transcripts

expressed in plants subjected to stress in growth chambers and those in the field, major differences were also found. Key to this latter observation is that caution should be exercised when extrapolating results based on laboratory experiments alone to form predictions or expectations of plant responses in the field (Dhanaraj et al., 2007).

1.5 *Thellungiella*: a valuable model for the study of abiotic stress tolerance

The adoption of *Arabidopsis* as a model organism has greatly improved our understanding of the genetic mechanisms that control plant growth and development (Bevan and Walsh, 2005). While much progress has been made in identifying mechanisms by which plants respond and adapt to cold temperatures, high salinity, drought, and other stresses, our understanding of the molecular basis of abiotic stress tolerance has been impeded, in part, by the lack of an appropriate genetic model with an innate ability to withstand stress. *Arabidopsis* displays little to no inherent tolerance to abiotic stress (Bressan et al., 2001). However, a close relative of *Arabidopsis*, *Thellungiella salsuginea* (also known as *Thellungiella halophila* or salt-lick mustard, hereafter referred to as *Thellungiella*) is native to areas with highly saline soils. *Thellungiella* has been reported as a suitable genetic model plant to study stress tolerance due to its ability to complete its life cycle under harsh environmental conditions (Zhu 2001; Xiong and Zhu, 2002; Inan et al., 2004, Taji et al., 2004, Wong et al., 2005, 2006; Griffith et al., 2007). *Thellungiella* shares many traits of *Arabidopsis* including a small size, short life cycle, copious seed production, a relatively small genome and it can be

genetically transformed (Bressan et al., 2001). *Thellungiella* coding DNA also has a high degree of sequence similarity to homologous *Arabidopsis* genes (90 – 95%); a feature that facilitates gene identification in this species. This similarity in genome content enables one to apply the abundant genetic resources developed for the study of *Arabidopsis* to the study *Thellungiella*'s response to abiotic stress.

Several natural accessions of *Thellungiella* have been identified that are specialized for diverse habitats worldwide (Scoggan, 1978). In Canada, *Thellungiella*'s distribution extends from south western Yukon and northern British Columbia to the Prairie Provinces, regions with semi-arid conditions (Scoggan, 1978). *Thellungiella* is also found in the western United States, Maritime China, Russia (Scoggan, 1978). Thus, unlike *Arabidopsis*, *Thellungiella* natural distribution exemplifies a species that has had to adapt to areas featuring cold including freezing temperatures, high salinity, and prolonged periods of water deficit. The molecular basis conferring the capacity of extremophiles like *Thellungiella* to withstand harsh environments is poorly understood and provides an exciting research opportunity to gain insights into the evolution of adaptive abiotic stress tolerance traits in plants using readily available approaches and methodologies.

1.6 Research objectives

Identifying metabolic pathways needed by plants stressed under field conditions will provide information that can be used to produce more stress tolerant crops. To date,

no studies have compared traits expressed in extremophiles, such as *Thellungiella* plants, exposed to stress under controlled environmental conditions and in their natural habitat. In this thesis, I report comparisons of transcript and metabolite profiles of *Thellungiella* plants grown in the laboratory and at a Yukon field site. The objective of this comparative study is identifying abiotic stress tolerance traits in the Yukon ecotype of *Thellungiella*. During my studies, I addressed the following questions:

1) Is there evidence for osmotic adjustment in Yukon *Thellungiella* subjected to abiotic stress treatments in growth chambers and plants in the field?
2) Is there overlap among transcripts or metabolites that accumulate in Yukon *Thellungiella* growing at the Yukon field site and plants subjected to calibrated stress treatments in controlled growth chambers?
3) Can comparisons between transcript and metabolite profiles of plants exposed to field and growth cabinet conditions identify biochemical traits needed for survival and growth under abiotic stress?

My hypothesis is that a critical trait(s) needed for survival under osmotic stress should be expressed by the plant regardless of whether the plant experiencing stress is growing in the cabinet or a remote field site.

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Chapter Two

Methods and Materials

2.1 Yukon field site location and plants

In 2002, 2003 and 2005, cauline leaf tissue was harvested from mature, flowering Yukon *Thellungiella* at a field site near Whitehorse, Yukon (location: 60° 55.928'N, 135° 10.249' W; elevation = 647m), located 20 km north of Whitehorse (Fig. 1). Soil samples were collected from the top 18 cm depth at the field site where Yukon *Thellungiella* was harvested. Soil pH, electrical conductivity, and chemical content were determined and the elemental composition of soil samples, field and chamber leaf tissues were analyzed (Ping et al., 1998). Five to six cauline leaves, weighing approximately 200 mg FW were harvested from at least ten different plants 7 h from sunrise in the Yukon, and then tissue was transferred quickly to individual 2-mL Nalgene cryovials. The cryovials containing the samples were immediately flash frozen in liquid nitrogen (Jacob's Industries Limited, Whitehorse, YT), and then transferred to a charged MVE XC20/3V vapour shipper (Jencons Scientific Inc., Bridgeville, PA) where samples were kept frozen at –150°C for transport. Vials were transferred to –80°C for storage pending analysis.

2.2 Controlled environment plant growth conditions

Seeds of Yukon *Thellungiella* were sterilized using a vapour-phase gas technique (Clough and Bent, 1998) and then mixed with 0.1% (w/v) Phytagel (Sigma, Oakville, ON) and pipetted onto a moistened soil mixture containing six parts Promix BX (Premier Horticulture, Rivière-du-Loup, PQ) and one part Turface (Profile Products LLC, Buffalo, NY) in individual 5 x 5 x 7 cm pots. The pots containing seeds were stratified for 2 d at 4°C before transfer to growth chambers (AC 60 Enconair, Winnipeg, MB) set with a 21h/3h day/night cycle (light intensity of 250 μmol $m^{-2}s^{-1}$) and 22°C/10°C day/night temperature regime. Plants were watered daily with de-ionized distilled water prepared using Barnstead NANO Pure II system (Barnstead International, Dubuque, IA) and fertilized once per week with 1 g L^{-1} 20-20-20 (N-P-K) fertilizer.

Cauline leaves from mature, flowering, 12-week-old Yukon *Thellungiella* plants grown in Enconair chambers served as control, unstressed tissue in microarray and metabolite profiling experiments in comparisons involving the cauline leaves from field-grown plants (Prepared by Mr. Yong Li under the supervision of Dr. Barbara Moffatt, University of Waterloo). Basal rosette leaves of unstressed control plants served as the basis of comparison for metabolite profiles in the case of cold, drought, drought/re-watered and saline plant samples.

2.3 Stress treatments

For drought experiments, 4-week-old Yukon *Thellungiella* plants were randomly divided into two groups: a control group that was well-watered and a drought group where water was withheld until plants were visibly wilted. Approximately one-half of

the wilted plants in the drought treatment were re-watered by watering the soil. The re-watered plants were allowed to recover and regain turgor for 48 h prior to harvest. To impose salinity stress, 4-week-old Yukon *Thellungiella* plants were salinized with 50 mM NaCl for three days and the salinity level increased in increments of 50 mM NaCl every three days to a final concentration ranging from 100 to as high as 500 mM NaCl. Plants were irrigated with the final NaCl concentration for 3 d prior to harvest. Leaf tissue was harvested and flash-frozen with liquid nitrogen and then stored at –80°C for subsequent GC/MS analysis. Cold temperature experiments were performed by Dr. Marilyn Griffith's lab at the University of Waterloo. Four-week-old Yukon *Thellungiella* plants grown at 22°C were randomly divided into two groups: a control group that was grown at 22°C and a group that was shifted to 5/4°C for 3-weeks. Upon completion of the stress treatments a portion of the leaf tissue was flash-frozen in liquid nitrogen and stored at -80°C until it was processed for transcript or metabolite profiling

2.4 Water status and physiological response measurements

Relative water content (RWC)

For RWC measurements, 6 mm diameter discs were removed from leaves using a cork borer and weighed to obtain their FW and then allowed to float on 2 mL of de-ionized, distilled water in covered transparent Falcon® plastic wells (Becton Dickinson Labware, Franklin Lakes, NJ) for 24 h. The discs were blotted dry and weighed to obtain the turgid weight. The dry weight was then obtained after by taking the leaf discs and

drying them in an oven set at 70°C for 24 h in pre-weighed 1.5 mL microfuge tubes. The RWC was determined using the following equation:

RWC (%) = [(Fresh weight – Dry weight)/(Turgid weight – Dry weight)]

Dewpoint psychrometry

Leaf water potential was measured using a HR 33 T psychrometer (Wescor Inc. Logan, UT). A disc was excised from a fully expanded leaf using a 6 mm diameter cork borer and then was placed in a C52 leaf chamber. Discs were allowed to come to equilibrium with the air in the chamber for 20 min. Leaf solute potential was determined by submerging the leaf disc in liquid nitrogen for a few seconds. The frozen leaf disc was allowed to thaw and come to equilibrium for 20 min with the air in a C52 leaf chamber. Leaf turgor pressure was estimated as the difference between the water potential and osmotic potential (Nonami and Boyer, 1989). A calibration standard curve was performed using NaCl concentrations in the range of 0 to 1000 μM before taking leaf ψ_W measurements.

Infrared gas analysis

A CIRAS-1 infrared gas analyzer (IRGA) (PP Systems, Haverhill, MA) was used to determine rates of photosynthesis, transpiration, and stomatal conductance using excised leaves. The leaves were left to equilibrate in the leaf chamber for 60 s before taking a measurement. The leaf boundary-layer resistance was determined by following

the operator's manual (CIRAS-1 Version 2). A calibration curve estimating resistance as a function of leaf area was determined and used to calculate rates of photosynthesis and stomatal conductance. Given that the leaves were typically less than 2.5 cm^2 and so did not cover the area in the leaf chamber of the instrument, it was necessary to correct rates using leaf areas. Leaf areas were determined using an AM100 leaf area meter (Analytical Development Company Ltd., Herts, UK). For photosynthesis rate measurements the light intensity was set to 300 $\mu mol\ m^{-2}s^{-1}$ and the CO_2 concentration was maintained at 450 ppm. The instrument data was imported to a statistical analysis software (SAS) Version 7 (SAS Institute, Cary, NC) and rates of photosynthesis, transpiration and stomatal conductance were calculated with the corrected leaf area using PS_EQN_CIRAS equation (Appendix 1), which is saved in the SAS program (the PS_EQN_CIRAS equation was created by Jon Sleeman, former M.Sc. student of Dr. S. Dudley).

2.5 Metabolite analysis of Yukon *Thellungiella*

Polar metabolite extraction of Yukon *Thellungiella* leaf tissue

At harvest, 200 mg of leaf tissue was weighed and then flash frozen in liquid nitrogen and stored in a -80°C freezer until samples were processed. For processing, the tissue was crushed to a powder with a mortar and pestle that was chilled with liquid nitrogen prior to grinding. The leaves were ground to a powder in liquid nitrogen and after the nitrogen evaporated 1.4 mL of 100% HPLC grade methanol was added (Caledon Laboratories Limited, Georgetown, ON). 50 µL of 2 mg mL^{-1} ribitol (Sigma) was added

as an internal recovery standard and 58 µL of 1 M NaCl was added to improve the polar/non-polar phase separation of the slurry. This slurry was transferred to a 15 mL Corex© tube. The Corex© tube containing the brei was covered with aluminum foil with a punctured hole and was placed in a 70°C water bath and shaken for 15 min. The tubes were then centrifuged for 3 min at 14000 *g* at 4°C using a Beckman Coulter Avanti J-25 Centrifuge equipped with a JA-20 rotor (Fullerton, CA). The supernatant was removed and transferred to a 16 x 100 mm test tube (VWR) and 1.4 mL of de-ionized, distilled H_2O was added. To the remaining pellet in the Corex© tube, 750 µL of chloroform were added, the tube was shaken for 5 min at room temperature and then centrifuged for 3 min at 14000 *g* as above. The supernatant was removed and combined with the methanol water phase in the 16 x 100 mm test tube and the pooled mixture was vortexed and centrifuged for 15 min (International Clinical Centrifuge Model CL, Needham, MA) at the step 5 speed setting. The polar (upper) phase was transferred to a new 16 x 100 mm test tube and vortexed again. After vortexing, 250 µL of polar extract was removed and transferred to a 1 mL reaction vial (Wheaton, Millville, NJ). This polar fraction was dried down with nitrogen gas using an N-evap (Meyer N-evap organomation model no. 111 Berlin, MA) with an airflow pressure of 20 psi and the bottom of the tubes were suspended in a water bath set at 40°C. A 250 µL aliquot of the polar phase was combined with 250 µL of de-ionized distilled water in a microfuge tube with 1 mm holes in the lids. These tubes were then flash frozen in liquid nitrogen and lyophilized using a freeze dry system (Labconco Freezone Plus 6, Kansas City, MO) overnight and then the tubes were stored in desiccant at -20°C.

Oxime derivatization

Direct trimethylsilyl derivatization of reducing sugars leads to different peaks related to cyclic and linear forms for the same compound (Fiehn et al., 2000b). One way to prevent the formation of the cyclic conformation of sugars is to treat them with methoxyamine in order to reduce and stabilize the carbonyl moiety (Roessner et al., 2000). Therefore, 50 µL of 20 mg mL^{-1} methoxyamine hydrochloride (Sigma) dissolved in biotech grade ≥ 99.9% pyridine (Sigma) was added to the dried leaf extract and the mixture was incubated at 30°C in a Baxter temperature block heater (Lab-line instruments, Inc. Melrose Park, IL) for 90 min prior to trimethylsilyl derivatization.

Trimethysilyl derivatization

After methoxymation, the polar metabolites were converted into volatile trimethylsilyl derivatives by using the derivatization agent N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA). MSTFA (Chromatographic Specialties Inc. Brockville, ON) is the preferred trimethylsilyl reagent due to its ability to derivatize a broad range of chemical compound classes (Fiehn et al., 2000a; Roessner et al., 2000). The samples were derivatized with 80 µL of 100% MSTFA at 37°C for 30 min. After incubation, the derivatized samples were placed in a desiccator jar containing anhydrous calcium sulphate (Drierite) for at least 2 h prior to analysis by GC/MS.

GC/MS analysis

Derivatized plant extracts were analyzed using a Trace DSQ GC-MS system (Thermo Finnigan, Austin, TX) operated in the positive ion electron impact (EI^+) full scan mode. Samples were diluted 25-fold using HPLC-grade hexane and then 1-μL was injected into the Trace DSQ GC-MS system using the MPS 2 autosampler (Gerstel GmbH & Co., Mülheim, Germany). Chromatography was performed using a Restek Rtx-5MS integra column (crossbond 5% diphenyl – 95% dimethyl polysiloxane; Cat.#12623 – 127, Chromatographic Specialties Inc.) with a length of 30 m, a column ID of 0.25mm and a film thickness of 0.25μm. The column was fused with a 10 m guard column that had the same composition as the column. The injection temperature was 230°C, and the ion source was kept at 200°C. The carrier gas was high purity (>99.999%) helium (VitalAire, Hamilton, ON) at a constant flow rate of 1 mL min^{-1}. The oven temperature program was initially set at 50°C isocratic for 2.5 min, the temperature was increased first at 7.5°C min^{-1} to 70°C, and then followed by a 5°C min^{-1} ramp to a final temperature of 310°C that was isocratic for 6 min. The system was allowed to return to 50°C for 5 min before the next injection. Mass spectra were recorded at three scans s^{-1} with an m/z 50 – 650 scanning range.

Criteria used to monitor GC column performance

GC column performance was monitored by injecting a polycyclic aromatic hydrocarbon (PAH) calibration standard solution mix #5 (Restek Co., Bellefonte, PA). This standard solution contained the benzofluoranthenes, a group of PAHs with identical mass spectrum that can only be resolved chromatographically. Therefore, GC column

performance was monitored by ensuring that the peaks for benzofluoranthene (b) and (k) were successfully resolved. If these PAHs were not resolved, the removal of 50 to 70 cm of the retention gap would restore the GC column performance that was checked by re-running the PAH calibration standard solution prior to metabolite profiling of plant extracts.

GC/MS data analysis

GC/MS chromatograms or metabolite profiles are quite complex revealing the presence of several hundred peaks. Figure 2 shows representative GC/MS chromatograms generated for polar extracts prepared from Yukon *Thellungiella* subjected to different stress treatments or from plants harvested from the field site near Whitehorse, YT. The metabolite profiles show the presence of major and minor peaks. The enlarged portion of Figure 2 shows a comparison between the metabolite profiles generated for cold-treated Yukon *Thellungiella* plants and a plant that was grown under unstressed conditions. While a number of peaks are not different between Yukon *Thellungiella* plants treated with cold temperatures relative to an unstressed plants (a), a number of peak are higher (b) or lower (c) in the metabolite profiles from treated Yukon *Thellungiella* relative to the unstressed control plant. The main objective from these metabolite profiling studies was to identify metabolites undergoing statistically significant differences in abundance in *Thellungiella* subjected to stress treatments compared with respective controls. Therefore, it is necessary to extract information on

the areas of peaks present in the metabolite profiles of control and treated *Thellungiella* plants in a reproducible and efficient fashion.

Deconvolution of mass spectral components

High-throughput approaches require efficient and reproducible extraction of data. As such, visual peak-by-peak comparison between multiple GC/MS analyses was not feasible. Although the majority of peaks could be resolved by peak area integration using the Xcalibur software, some peaks co-eluted posing a challenge for precise peak abundance determination. For example, Figure 3 shows a sample raw chromatogram (a) with panel (b) showing irregularities in peak shape due to the presence of more than one peak component. Upon careful examination, two peaks were found to have different mass spectra within this major peak, as shown in panels c and d. Therefore, it was necessary to extract the pure mass spectra to distinguish between closely eluting peaks along with their accurate peak abundance information. The automated mass spectral deconvolution of identification system (AMDIS) was used to extract peak abundance and pure mass spectral component information from the complex chromatograms generated for leaf metabolite profiles. With AMDIS it is possible to separate the mass spectra of overlapping, co-eluting peaks rapidly, a process commonly referred to as deconvolution (Stein, 1999). AMDIS creates an eluent file (*.elu) in a tab-delimited format that contains information on peak abundance and mass spectrum of an individual component. Unfortunately, the absolute retention times (RTs) for identical peaks from multiple successive GC/MS analyses were not identical making direct comparisons for these peaks

difficult. An additional step is necessary to align identical peaks from multiple GC/MS analyses prior to statistical analysis.

Peak alignment using the GC/MS data analysis software package (GASP)

The eluent tab-delimited data files generated from GC/MS using AMDIS are complex and difficult to compare. Variation is found in the absolute RTs of identical peaks in chromatograms from multiple analyses done on different days or even different times on the same day. Correcting for shifting RTs was not possible due to the non-linearity of changes across the chromatogram. To correct for shifting RTs, Roessner et al. (2000) added fatty acid standards of different molecular weights (non-branched) to each sample prior to derivatization and GC/MS analysis. In this approach, a retention index (RI) value for a peak can be calculated that describes peak position relative to the two flanking fatty acid standard peaks in the chromatogram (Roessner et al., 2000). Thus all peaks are designated by their RI and peaks from multiple runs that share the same RI can then be aligned.

I used the exact method by Roessner et al. (2000) to calculate the RIs for peaks from GC/MS runs. In order to calculate the RI for peaks, a fatty acid standard mix was added to extracts. The fatty acid standard mix was prepared by adding 9 fatty acid standards that had variable lengths in hydrocarbon chain (C_7, C_9, C_{11}, C_{13}, C_{15}, C_{19}, C_{23}, C_{27}, C_{31}) dissolved in tetrahydrofuran in a Kimble vial and expressed as a % v/v: heptanoic acid 3.7%, nonanoic acid 3.7%, undecanoic acid 3.7%, tridecanoic acid 3.7%, pentadecanoic acid 3.7%, nonadecanoic acid 7.4%, tricosanoic acid 7.4 %, heptacosanoic

acid 22.2% and hentriacontanoic acid 55.5% (Sigma). The fatty acid standards were dissolved in tetrahydrofuran to a final concentration of 10 $\mu g\ \mu L^{-1}$. To the dried leaf extract, 25 µL of a 10 $\mu g\ \mu L^{-1}$ fatty acid standard mix dissolved in tetrahydrofuran was added and the mixture was dried down using nitrogen gas prior to methoxymation and derivatization.

All the peaks present in the GC/MS files were deconvoluted using AMDIS to create the tab-delimited eluent file. This eluent file was then converted into a GC/MS universal (*.gmu) file format using the GC/MS data analysis software package (GASP) (Nuin et al., 2004). The GC/MS universal file contains all the peak abundance information as well as the associated RT and mass spectrum information for an individual component. In order to calculate the RI, the exact RT for each fatty acid standard needs to be entered into GASP (available at: www.flintbox.com) and then the RIs for all the peaks present in the GC/MS universal file are calculated using the following equation:

$$\mathbf{RI_x = [(100)x(C_n) + (((100)x(C_{n+1}) - (100)x(C_n))x((RT_x - RT_{Cx-1})/(RT_{Cx+1} - RT_{Cx-1})))]}$$

C_n represents the number of carbons present in the fatty acid that elutes before the compound x

C_{n+1} represents the number of carbons present in the fatty acid that elutes after the compound x

RT_x denotes the RT of compound x

RT_{X+1} denotes the RT of fatty acid standard that elutes after compound x

RT_{X-1} denotes the RT of fatty acid standard that elutes before compound x

In addition to creating a RI for each peak present in the GC/MS universal file, the peak area is divided by the peak area of the internal recovery standard, ribitol, and hence only this normalized peak area is compared between multiple analyses. Once the RI and normalized peak areas have been determined for each GC/MS analysis, peaks sharing the same RI from multiple GC/MS universal files from multiple analyses are aligned using GASP, and a GC/MS alignment format (*.gma) file is created. This GC/MS alignment format file will contain three columns: a column containing the normalized peak area, a column containing the original RT and a column containing the calculated RI. This file can be subjected to a variety of statistical test that are available within GASP as this software interfaces with the statistical package "R" (www.r-project.org) or it can be exported to Excel or any other software package for data mining.

Data mining

The strategy for data mining of metabolomic data is outlined in Figure 4. After peak deconvolution using AMDIS and alignment using GASP, the data was subjected to at least three different statistical methods routinely used for metabolomic data mining. This included using a combination of unsupervised methods such as principal component analysis (PCA) and hierarchical cluster analysis (HCA), and more stringent methods such as analysis of variance. These methods are designed to visualize and differentiate

between large datasets obtained from metabolite profiles (Sumner et al., 2003; Fiehn, 2002).

Principal component analysis

One of the first questions to answer during the exploration of metabolomic data is whether or not the data acquired groups into distinct groups based on differences in metabolite concentrations (Fiehn, 2002). For example, if distinct metabolite profile groups (also referred to as metabolic phenotypes, Fiehn et al., 2000a) emerge when comparing the entire dataset generated for wild-type and mutant or treated and control samples, this would signify that differences exist in their metabolite composition. PCA is an important multivariate technique used to determine whether groups exist among metabolite profiles. PCA transforms the large dataset (hundreds of peaks from multiple GC/MS analyses) into a smaller set of uncorrelated variables, called principal components that are determined by linear combinations using the peak abundance data (Fiehn et al., 2000a; Taylor et al., 2002). The contribution of each peak on this principal component (referred to as a principal component loading) is used to determine whether it plays an important role in the observed grouping patterns. Normally, the top three principal components are plotted against one another to determine whether distinct groups are evident in the overall dataset (Sumner et al., 2003). Once the existence of distinct groups are established, classical statistical methods such as Student's *t*-tests or analysis of variance (ANOVA) can be used to identify metabolites whose levels are statistically significantly different between the groups (Fiehn, 2002).

PCA analysis of metabolite profiles obtained for leaf polar extracts prepared from *Thellungiella* plants was done using the STATISTICA Version 6.0 software package (StatSoft, Inc., Tulsa, OK). The peak abundance data was $\log_{10}$ transformed due to the observed dynamic range of concentrations in metabolite abundance. This transformation allows a better comparison of large and small numbers (Roessner et al., 2001). A covariance matrix was used to determine the principal components from the dataset.

Analysis of variance

One-way ANOVA was used to identify peaks that were present at statistically significantly different levels in *Thellungiella* plants subjected to stress treatments relative to respective controls. This approach enabled the comparison of the means of two or more independent groups with the assumption that the means follow a normal distribution with equal variances (Sokal and Rohlf, 1995). The null hypothesis that the means of all groups are equal, was rejected when a threshold for significance of $P<0.05$ was obtained as determined using STATISTICA Version 6.0 software package (StatSoft, Inc., Tulsa, OK) or GASP (Nuin et al., 2004). At a $P<0.05$, at least one pair of means was found to be different during multiple means comparisons. Given that several hundred variables were compared between groups, the expected number of incorrect null hypotheses rejections was 15 (false positives). However, ANOVA yielded considerably more peaks undergoing statistically significant differences between treatment and control (58 for the "salt" data) or field and chamber (109 for "field") comparisons than the expected number of false positives. Means of statistically significantly different peaks between groups

were further explored using hierarchical cluster analysis (HCA) (described in the next section). This was performed to group peaks undergoing similar expression patterns in response to stress treatments. These results were then related to the literature for biological interpretation instead of applying multiple test corrections such as the Bonferroni correction to decrease the false discovery rate, as the Bonferroni correction is generally too conservative (Sokal and Rohlf, 1995).

Hierarchical cluster analysis

HCA methods are used to assess the similarity between metabolite profiles (Fiehn, 2002). The most commonly used distance metric for metabolomic data analysis is the Euclidean distance (Taylor et al., 2002). The result of HCA is visualized as a dendrogram or a tree where the branch lengths are proportional to the distances between groups (Sumner et al., 2003). HCA can also be used to group peaks undergoing similar expression patterns. The first step was to identify statistically significantly different peaks between *Thellungiella* plants subjected to abiotic stress treatments compared with their respective controls. The next step was to express the mean peak abundance as a fold-ratio of the mean for treated and control samples. These fold-ratios were $\log_{10}$ transformed and then subjected to HCA to group peaks undergoing similar expression patterns. Peaks that were not detected in either treated or control samples were assigned a threshold for detection value of 0.00005 since their true value is between zero and the detection limit. A Euclidean distance was used to calculate the matrix of all the $\log_{10}$ transformed peak fold ratios and the average-linkage method was used to perform the

HCA either using STATISTICA Version 6.0 software package or the Gene Cluster software program (available at http://rana.lbl.gov, Eisen et al., 1999) to construct heatmaps. The output of the Gene Cluster software program is a *.cdt file that can be opened with the Java Treeview program (available at: http://jtreeview.sourceforge.net, Saldanha, 2004) to construct heatmaps. On the heatmap white indicates no change, and red or blue indicates a peak that is present at higher or lower levels, respectively, for data of *Thellungiella* subjected to calibrated stress treatments imposed in growth cabinets relative to respective controls or field grown relative to chamber grown controls.

In summary, using a combination of PCA, HCA and ANOVA, peaks that help distinguish between metabolic phenotypes can be identified. The next step is to identify the chemical structure for these peaks in order to relate them to metabolic pathways and, in doing so, gain insights into the biochemical changes associated with response to abiotic stress.

2.6 Criteria for identification of metabolites

Mass spectral library

A mass spectral library created by Dr. Chris Wang using authentic standards has been prepared using the same methodology and instrumentation as that used for the leaf samples. The first step to identify peak components was to find the closest matching mass spectrum in the library for a given peak of interest. The next step was to compare the RI for the unknown peak to that of an associated mass spectrum. Therefore, all peaks

that were positively identified had an identical mass spectrum and RI to that obtained for an authentic standard present in the library.

Co-injection using authentic standards

Wherever possible, further confirmation for the identity of an unknown component was obtained by co-injection. In this approach, addition of a known amount of authentic standard to the extract helps identify whether the peak height associated with the target unknown increases as a result of the addition of the authentic standard. The first step is to estimate the relative amount of the peak so that the addition of the authentic standard to the extract does not overwhelm the initial peak amplitude. Ideally, the peak amplitude of the extract should double (at most) upon addition of the authentic standard. The response of the authentic standard was determined by injecting an equivalent of 5 ng and diluting appropriately prior to its addition to the extract. This approach is only feasible if the authentic standard is commercially available and there is some certainty as to its chemical identity.

Galactosidase/glucosidase hydrolysis of complex sugars

Peaks with mass spectra that resembled disaccharides (presence of m/z 361) were assayed to determine whether they had specific linkage bonds connecting monomers. A β-glucosidase (E.C 3.2.1.21; Sigma) purified from almonds was used to cleave β-glucose linkages from complex sugars present in the polar extracts. The protocol used to perform the β-glucosidase hydrolysis reaction was developed by Mr. David Guevara and Dr. Peter

Summers in conjunction with Mr. Jeff Malins. In total, only MST 2137 classified as a C12 sugar alcohol/disaccharide was demonstrated to undergo hydrolysis by β-glucosidase using this approach.

Briefly, to a 1 mL microfuge tube that contains the re-suspended polar extract in 125 μL of de-ionized distilled water at a concentration of 7.2 mg FW equivalent mL^{-1}, 25 μL of 0.05 Units μL^{-1} of the β-glucosidase enzyme was added and the reaction was allowed to proceed for 4 h in a water bath set to 37°C. To stop the reaction, 50 μL of the extract subjected to β-glucosidase hydrolysis was transferred into a new microfuge tube on ice, then 350 μL of cold 100% HPLC grade methanol was added and the mixture vortexed. To remove the enzyme, 407.5 μL of de-ionized distilled water and 188 μL of chloroform were added to this microfuge tube. This microfuge tube was vortexed and centrifuged for 1 min at 3000 *g* at room temperature. The polar (upper) phase was placed into a new microfuge tube and an aliquot of 467 μL was transferred into a 1 mL Wheaton reaction vial. This polar fraction was dried down with 20 psi of N_2 (g) using an N-evap. The bottoms of the tubes were suspended in a water bath set at 40°C. The dried extract was then derivatized and analyzed by GC/MS using the same methods described previously.

2.7 Biological variability of metabolite profiles

The initial challenge was to determine whether reproducible metabolite profiles could be obtained using an experimental size of 5 plants per treatment. A sample size of five is a manageable size since samples can be harvested rapidly to avoid changes due to

time effects. With larger sample sizes manipulations can take more time thereby risking the degradation of samples or the possible formation of artifacts. This was an important step since baseline variability in metabolite profiles needed to be established before further treatment comparisons could be made since Yukon *Thellungiella* seeds used for experiments were not from single seed descent.

Ten individual plants grown together under identical conditions in a growth chamber were analyzed. A Student's *t*-test was performed to determine whether the peak areas of the first 5 samples run were the same as the component areas from the subsequent 5 samples. Out of 275 peak components detected using AMDIS, only 10 were different at a significance of $P<0.05$, a number that is lower than the 14 expected incorrect null hypotheses rejections (false positives) for this dataset. This indicates that the metabolite profiles between the two groups representing 10 individual plants were similar. Furthermore, only 3 of the 10 significantly different peaks were present at levels 2-fold higher or lower between these groups. One reason to explain the variability in these 3 peaks is that these peaks represent chemicals whose abundance was low and near the detection limit for the detector. However, metabolites can be expected to exhibit variations in their levels between different plants since biochemical networks are not static due to oscillations that are part of maintaining plant cellular homeostasis (Weckwerth, 2003). These findings are in keeping with other studies that have found metabolite profiles to be highly reproducible, with a small number of metabolites that exhibit higher variations in their levels even though plants are grown under the same conditions (Fiehn et al., 2000b; Roessner et al., 2000). Based on these results, we

determined that N=5 is a suitable number of replicates to detect changes between metabolite profiles at a significance cut-off of $P<0.05$.

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Figure 1. Natural distribution of *Thellungiella* in the Yukon Territory.

Red dot denotes locations where Yukon *Thellungiella* populations were found by our research group. The purple asterisk indicates the field site location referred to as Dillabough's grazing lease, where tissue was harvested from Yukon *Thellungiella* growing on the field in the years 2002, 2003 and 2005. The Yukon Territory map was accessed from http://www.yukoncommunities.yk.ca/communities.

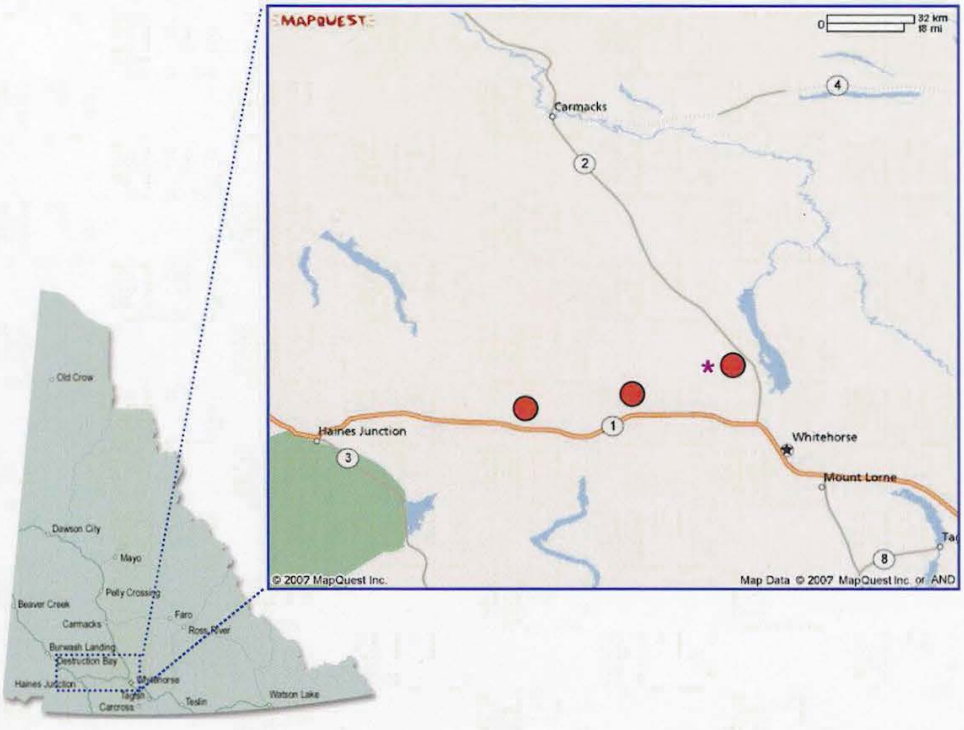

MAPQUEST
0
32 km
18 mi
Carmacks
Haines Junction
Whitehorse
Mount Lorne
© 2007 MapQuest Inc.
Map Data © 2007 MapQuest Inc. or AND
Old Crow
Dawson City
Mayo
Pelly Crossing
Beaver Creek
Carmacks
Faro
Ross River
Burwash Landing
Destruction Bay
Haines Junction
Whitehorse
Teslin
Watson Lake
Carcross

Figure 2. Metabolomic analysis of *Thellungiella*.

A) Representative total ion chromatogram of the polar phase of leaf extracts prepared from *Thellungiella* plants subjected to various abiotic stress treatments in growth cabinets or harvested from a salt flat near Whitehorse, YT. The metabolite profiles are complex and show the presence of several hundred peaks.

B) Inset shows differences between peaks of treatment and controls; a = no change, b = higher in treated, c = lower in treated.

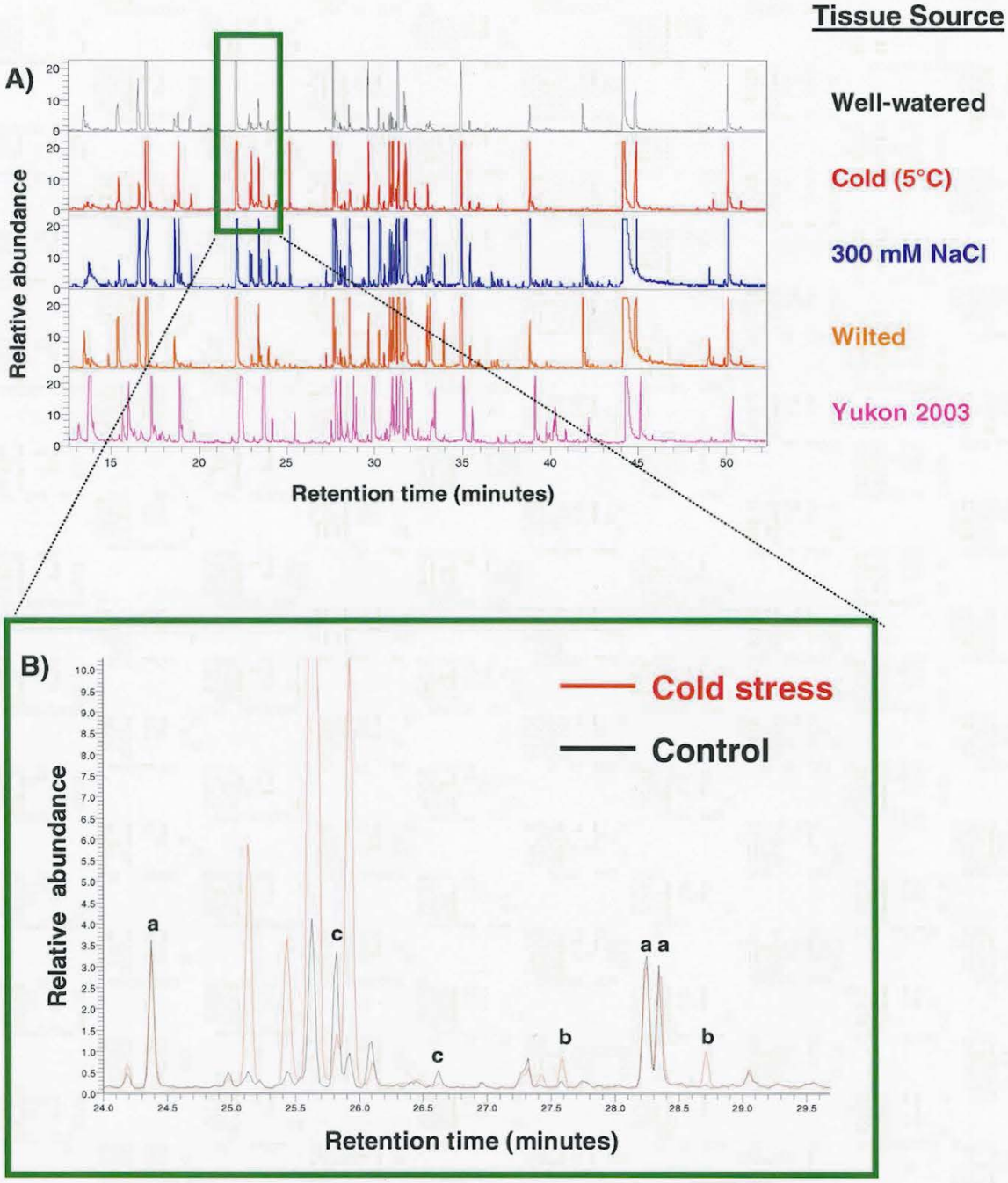

A)
Relative abundance
Retention time (minutes)
Tissue Source
Well-watered
Cold (5°C)
300 mM NaCl
Wilted
Yukon 2003
B)
Cold stress
Control
Relative abundance
Retention time (minutes)
a
c
c
b
a a
b

Figure 3. Peak deconvolution from complex GC/MS chromatograms.

A) Chromatogram of a polar extract prepared from *Thellungiella* leaves.

B) Co-eluting peaks can be separated (deconvoluted) using AMDIS to show unique mass spectral components with unique ion traces such as m/z 319 (blue) and m/z 173 (red). Mass spectra for co-eluting peaks i & ii are given in C & D, respectively. Mass spectral information as well as RIs calculated for mass spectral components were used to align peaks from multiple chromatograms.

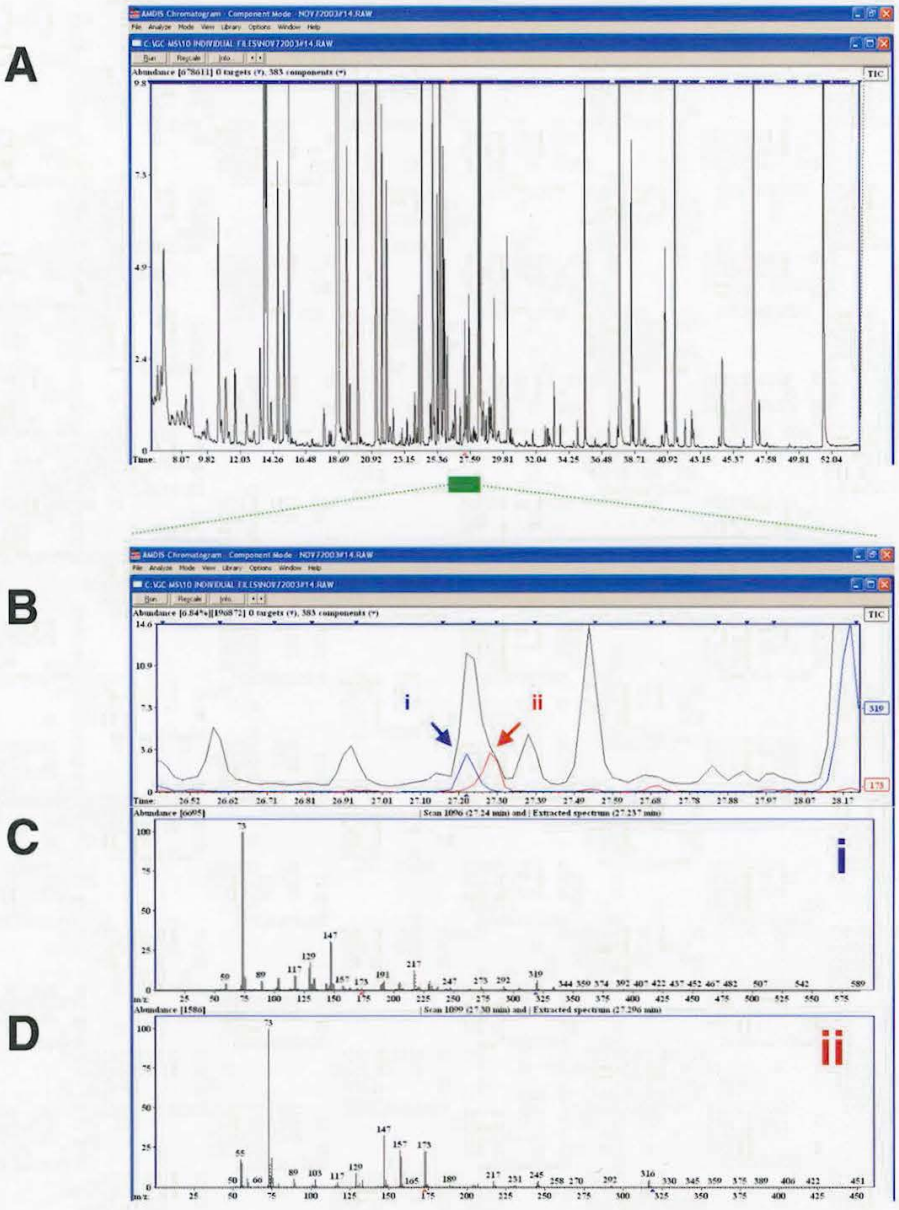
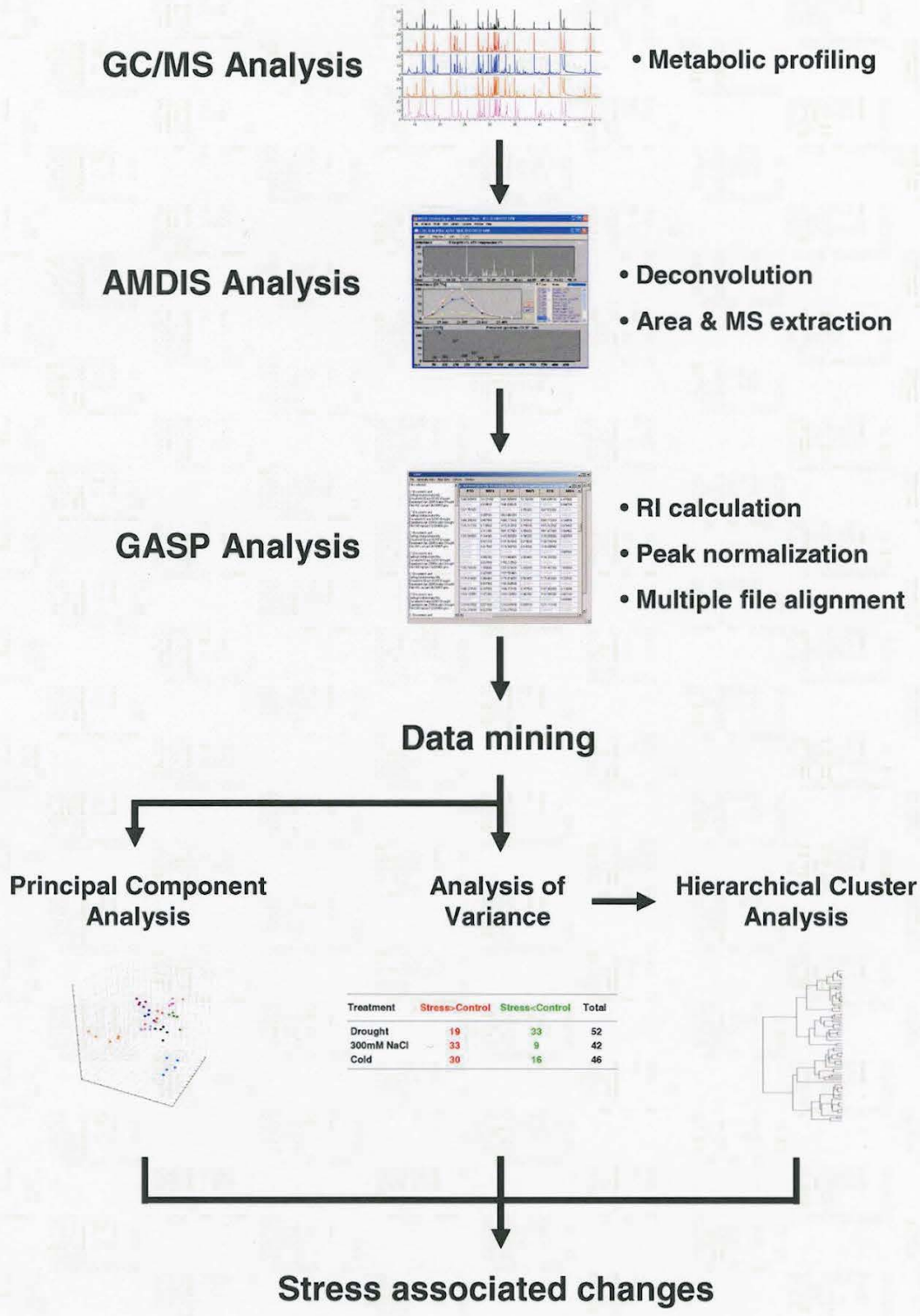

A
B
C
D
i
ii

Figure 4. Strategy for data mining of metabolomic data to identify metabolic traits associated with abiotic stress responses.


GC/MS Analysis
• Metabolic profiling
AMDIS Analysis
• Deconvolution
• Area & MS extraction
GASP Analysis
• RI calculation
• Peak normalization
• Multiple file alignment
Data mining
Principal Component Analysis
Analysis of Variance
Hierarchical Cluster Analysis
Treatment Stress>Control Stress<Control Total
Drought 19 33 52
300mM NaCl 33 9 42
Cold 30 16 46
Stress associated changes

Chapter Three

"Physiological and metabolic responses of the halophytic plant, *Thellungiella salsuginea*, to salinity."

3.0 PREFACE: Author contributions and acknowledgements for manuscript to be submitted for publication.

David Guevara: Performed salinity stress experiments (water status & physiology, metabolomic analysis and statistical analysis); compiled figures; wrote first draft of the manuscript.

Dr. Sajjad Akhter: Performed seed germination and root growth experiments.

Dr. Brian McCarry: Provided suggestions for metabolomic analysis.

Dr. Susan Dudley: Provided CIRAS-1 equipment and suggestions for stomatal conduction and photosynthesis analysis.

Dr. Peter Summers: Critical reading and revision of the manuscript.

Dr. Elizabeth Weretilnyk: Supervised the project and oversaw revisions of this and all the drafts.

RUNNING HEAD: **Yukon *Thellungiella* response to salinity**

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Research category

Environmental Stress and Adaptation

TITLE:

Physiological and metabolic responses of the halophytic plant, *Thellungiella salsuginea*, to salinity.

AUTHORS:

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3.1 **ABSTRACT**

Thellungiella salsuginea Yukon ecotype is a subarctic crucifer native to habitats with highly saline soils and displays an exceptional ability to tolerate sea-strength salinity. Yukon *Thellungiella* plants were subjected to NaCl treatments to identify traits associated with salinity tolerance. Yukon *Thellungiella* seeds can germinate on agar media supplemented with up to 100 mM NaCl and once germinated seedlings maintain 60% the root elongation rates of seedlings grown in the absence of NaCl. Plants salinized gradually to 300 mM NaCl on soil showed rates of stomatal conductance and photosynthesis that were 48% and 64%, respectively, of rates for well-watered, unsalinized, control plants. Leaf water and solute potential measurements from Yukon *Thellungiella* irrigated with NaCl show that leaf solute content increases in a salt-responsive manner. Polar solutes from leaves were profiled using gas chromatography/mass spectrometry. Of the 300 mass spectral tags (MSTs) detected in metabolite profiles, 25, 27 and 48 MSTs were found to be significantly different in Yukon *Thellungiella* subjected to 100 mM, 300 mM or 500 mM NaCl, respectively, relative to unsalinized controls. Among the metabolites consistently more abundant in Yukon *Thellungiella* subjected to 300 mM NaCl and 500 mM NaCl were glucose, proline, pyroglutamic acid, sucrose, valine, and six sugar-like compounds. Our results suggest that part of the mechanism enabling Yukon *Thellungiella* to tolerate salinity involves the accumulation of proline and carbohydrates.

3.2 INTRODUCTION

Soil salinity negatively impacts plant growth and productivity. Unfortunately, the accumulation of salt in arable lands as a result of poor irrigation poses serious threats to agriculture (Shani et al., 2005). Therefore, it will be necessary to improve the salinity tolerance of crops in order to meet the demands for food production. The family Brassicaceae includes a number of agronomically important species that are negatively affected by high salinity (Ashraf and McNeilly, 2004), while at least thirty of its members are halophytes (Flowers et al., 1986). Among these halophytic species is *Thellungiella salsuginea* (also known as salt cress, *Thellungiella halophila*, hereafter referred to as *Thellungiella*) which is capable of completing its lifecycle when grown under sea-water strength salinity (Zhu 2001; Gong et al., 2005; Kant et al., 2006). *Thellungiella*'s close similarity to *Arabidopsis thaliana* (>90% nucleotide identity; Zhu, 2001) permits the use of the genetic resources and tools amassed for the study of Arabidopsis to unravel the molecular mechanism(s) underlying salinity tolerance of *Thellungiella* (Bressan et al., 2001; Zhu 2001). The use of *Thellungiella* as a genetic model species for the study of salinity tolerance will provide invaluable information that can be used to develop crops that have a greater ability to tolerate saline soils.

Plants growing on saline soil have a reduced ability to take up water due to the high concentrations of dissolved salts in the soil water that lowers the soil water potential (ψ_W) relative to that of the roots (Rodriguez et al., 1997). Some plants are able to lower their root ψ_W by accumulating non-toxic "compatible" solutes such as amino acids,

sugars and straight chain or cyclic polyols in order to create a ψ_W gradient that allows for water uptake from saline soils (Jefferies et al., 1979). Natural accessions of *Thellungiella* native to maritime China (Shandong Province) and sub-arctic Canada (Yukon Territory) grow on highly saline soils (Inan et al., 2004; Guevara et al., 2009) and it has been shown that they accumulate solutes in response to abiotic stress (Inan et al., 2004; Wong et al., 2006). Shandong *Thellungiella* plants exposed to 250 mM NaCl for 24 h were shown to accumulate sugars such as galactinol, glucose, inositol, trehalose, and a trehalose-like sugar and the amino acids glutamate and proline (Gong et al., 2005). The observation that Shandong *Thellungiella* accumulates a number of carbohydrates and amino acids in response to salinity suggests that this plant uses chemically diverse compatible solutes to promote water uptake during exposure to saline conditions. This interpretation finds further support in the reports that transcripts encoding enzymes involved in the synthesis of sugars and amino acids are enriched in both the Shandong and Yukon ecotypes of *Thellungiella* exposed to abiotic stress (Inan et al., 2004; Taji et al., 2004; Gong et al., 2005; Wong et al., 2006).

Proline accumulation, a response associated with stress tolerance in many plant species (Delauney and Verma, 1993), has been proposed to be an important trait for salinity tolerance in Shandong *Thellungiella* (Gong et al., 2005; Inan et al., 2004; Kant et al., 2006). However, the roles organic solutes such as sugars or sugar-like metabolites play in salinity tolerance in *Thellungiella* subjected to saline environments is unknown. In this study, Yukon *Thellungiella* plants were subjected to a step-wise increase in salt to identify physiological and metabolic traits that are present during plant acclimation to

NaCl. The role of proline and soluble sugars in promoting water uptake in Yukon *Thellungiella* exposed to saline environments is discussed.

3.3 MATERIALS AND METHODS

Plant growth and stress treatments

For seed germination and primary root growth studies, *Thellungiella* seeds were surface sterilized in 0.6% (v/v) sodium hypochlorite for 10 min, rinsed twice with 95% ethanol and then rinsed extensively with sterile, distilled water. Sterile seeds were sown on 0.7% (w/v) agar or media supplemented with NaCl. The seeds were stratified for 2 d in the dark at 4°C, and then the plates were transferred to 20°C under continuous light (50 μmol $m^{-2}s^{-1}$) for 10 d. The emergence of a 2 mm radicle from the seed coat defined the time of germination and primary root length measurements were recorded daily.

Thellungiella seeds of the Yukon ecotype were sown on a moistened soil mixture containing six parts Promix BX (Premier Horticulture, Rivière-du-Loup, PQ) and one part Turface (Profile Products LLC, Buffalo, NY) in individual 5 x 5 x 7 cm pots, as described in Wong et al. (2006). The seeds were stratified for 2 d at 4°C and then the pots were transferred to growth chambers (AC60 Enconair, Winnipeg, MB) set for a 21-h/22°C d with an irradiance of 250 μmol $m^{-2}s^{-1}$ and a nighttime temperature of 10°C. For salinity treatments, four-week-old plants were watered with 50 mM NaCl for 3 d and the salinity level of the irrigating solution was increased in increments of 50 mM NaCl every

3 d to final concentrations of 100mM, 300 mM, and 500 mM NaCl. Plants were watered for 3 d at their final salinity level prior to physiological analysis and harvest of salinized and respective unsalinized controls at each salt step.

Water potential measurements

Leaf water (ψ_W) and solute (ψ_S) potential measurements were performed using a HR33T psychrometer fitted with a C52 chamber (Wescor Inc., Logan, UT) using a 6 mm diameter disc excised from a mature fully expanded leaf (Wong et al., 2006). Leaf turgor (ψ_P) pressure was estimated as the difference between the water and solute potential measurements (Nonami and Boyer, 1989).

Photosynthesis measurements

A CIRAS-1 infrared gas analyzer (IRGA) (PP Systems, Haverhill, MA, USA) was used to measure rates of photosynthesis and stomatal conductance. Excised leaves were quickly placed in the leaf chamber and left to equilibrate for 60 s before taking a measurement. The light intensity was set to 300 μmol $m^{-2}s^{-1}$ and the CO_2 concentration was 450 ppm. A calibration curve plotting resistance as a function of leaf area was performed to determine the leaf boundary-layer resistance, as outlined in the CIRAS-1 Version 2 operator's manual to correct rates of photosynthesis and stomatal conductance for leaf area. Leaf area was measured using an AM100 leaf area meter (Analytical Development Company Ltd., Hoddesdon,UK).

Metabolite analysis

The procedure followed for polar metabolite extraction and analysis by gas chromatography/mass spectrometry was essentially that described by Fiehn et al. (2000). A 200-mg sample of leaf tissue to which 50 μL of the internal standard ribitol (2 mg mL^{-1}) was added was ground and the polar metabolites were extracted using 1.4 mL of methanol, 1.4 mL of de-ionized distilled water and 0.75 mL of chloroform. The methanol/water fraction containing the polar metabolites was dried under a stream of N_2 (g), a 25 μL aliquot of RT standard mixture containing odd-chained fatty acids (Roessner et al., 2000) was added and the mixture dried as before. To the residue 50 μL of methoxyamine (20 mg mL^{-1} in pyridine) was added and the contents incubated at 30°C for 90 min. Samples were derivatized with 80 μL of N-methyl-N-trimethylsilyl trifluoroacetamide and incubated at 37°C for 30 min.

The samples were diluted 25-fold in hexane and then 1 μL was injected using a MPS 2 autosampler (Gerstel GmbH & Co., Mülheim, Germany) into a Trace DSQ GC/MS system (Thermo Finnnigan, Austin TX) operated in the positive ion electron impact (EI^+) full scan mode. Chromatography was performed using a 30 m x 0.25 mm I.D. and 0.25 μm film thickness Restek Rtx-5MS integra column (Chromatographic Specialties Inc., Brockville, ON) equipped with a 10 m guard column of the same composition. The injection temperature was 230°C, and the ion source was kept at 200°C. The carrier gas was helium at a flow rate of 1 mL min^{-1}. The temperature was initially set at 50°C for 2.5 min then 7.5°C min^{-1} to 70°C followed by 5°C min^{-1} to a final

temperature of 310°C where it was maintained for 6 min. Mass spectra were recorded at 3 scans s^{-1} with a mass-to-charge (m/z) scanning range of 50 to 650.

The automated mass spectral deconvolution and identification system (AMDIS) was used to extract peak abundance and mass spectral information for each trimethylsilyl (TMS) derivative component. This information was imported into the GC/MS Data Analysis Software Package (GASP; Available at: www.flintbox.com, Nuin et al., 2004) where peak area was normalized to that of the internal standard ribitol, and the RI calculated according to Roessner et al. (2000). Each TMS component was labeled with a RI. The RI and mass spectrum (MS) are used to distinguish different mass spectral tags (MSTs) as defined by Kopka (2006). The putative chemical identity for MSTs was obtained by querying a library generated using authentic standards that were analyzed using the same instrument.

Data analysis

Principal component analysis (PCA) and analysis of variance (ANOVA) were performed using the STATISTICA software package Version 6 (StatSoft Inc., Tulsa, OK) with the abundance information for each MST deconvoluted using AMDIS. For PCA, the abundance data was $\log_{10}$ transformed and then a co-variance matrix was used to calculate the principal components. The abundance information of MSTs that were significantly different between treated and control tissue at a $P<0.05$ as determined by ANOVA were expressed as a $\log_{10}$ transformed fold-ratio of the means between treated and control groups. This fold-ratio was then subjected to hierarchical cluster analysis

(HCA) to group metabolites according to their similarity in pattern of expression at each salt step. Euclidean distance was used to calculate the distance matrix using the Cluster program (http://rana.lbl.gov, Eisen et al., 1998). The complete-linkage method was used for data clustering and heatmaps were constructed using the JavaTreeview program (Saldanha, 2004).

3.4 RESULTS

Thellungiella growth on NaCl

The germination efficiency of Yukon *Thellungiella* seeds on agar media was not affected by exposure to 50 or 100 mM NaCl. However, Figure 1 shows that seedlings exposed to the agar medium containing 50 mM or 100 mM NaCl resulted in a reduced root growth relative to unstressed controls. After 10 d, roots of seedlings on 50 mM and 100 mM NaCl were 17% and 26% shorter, respectively, than roots of unsalinized controls. The germination efficiency of seeds on media supplemented with 150 mM NaCl was reduced to 80% and was only 30% when seeds were exposed to 200 mM NaCl (data not shown).

Yukon *Thellungiella* plants were grown on soil and watered with a solution containing increasing concentrations of NaCl. Figure 2A shows representative Yukon *Thellungiella* plants watered with a solution lacking added NaCl and plants watered with

100 and 300 mM NaCl. All plants eventually flowered and set seed. *Thellungiella* plants watered with a final concentration of 100 mM NaCl showed rates of stomatal conductance and photosynthesis that were not significantly different from rates observed for unsalinized control plants (Fig. 2B). However, *Thellungiella* plants salinized to 200 mM NaCl or 300 mM NaCl showed 60% and 48% the rates of stomatal conductance observed in unsalinized controls, respectively. Plants exposed to 300 mM showed 64% of the rates of photosynthesis recorded for the corresponding controls (Fig. 2B).

Thellungiella plants accumulate solutes in response to NaCl

Unstressed Yukon *Thellungiella* plants have a leaf ψ_W of – 0.9 MPa and leaf ψ_W decreases with exposure to successively higher concentrations of NaCl (Fig. 3). Similarly, the leaf ψ_S of plants decreases in a salt-responsive manner. Leaf ψ_S decreased by 36% upon exposure to 100 mM NaCl, and up to a 300% decrease in leaf ψ_S was observed when plants were subjected to 500 mM NaCl compared to an unsalinized plant (Fig. 3). However, even when leaf ψ_S values were found to be as low as – 4.0 MPa the leaves showed a positive ψ_P. Therefore, the drop in leaf ψ_s shows that solute content of *Thellungiella* leaves increased as a function of exposure to higher concentrations of NaCl.

Metabolite profiling of Yukon _Thellungiella_ subjected to salt treatments

The polar organic solutes contributing to the drop in leaf ψ_s were profiled by GC/MS analysis of leaves from plants exposed to 100 mM NaCl ("low"salt), 300 mM

NaCl ("medium"salt) or 500 mM NaCl ("high"salt). All metabolite profiling included comparisons made with control, unsalinized plants of the same age grown under identical conditions. The metabolite profiles derived using GC/MS were complex and were composed of several hundred TMS derivatives including amino acids, amines, organic acids, sugars, and polyols.

Principal component analysis (PCA) was used to investigate differences between the metabolite profiles of Yukon *Thellungiella* plants irrigated with salt and their respective controls. PCA is an unsupervised multivariate data analysis approach used to determine whether groups exist in metabolomic data through visual interpretation (Fiehn, 2002). PCA can also be used to calculate the relative contribution of an individual metabolite towards the observed grouping pattern through the examination of the principal component (PC) loadings. We performed PCA using the complete data comprised of 30 metabolite profiles representing 300 MSTs. Of the total metabolite variance detected, 63% can be accounted for by the first three PCs (PC1 = 41%, PC2= 12%, PC3= 9%). Figure 4A shows PC1 and PC2 plotted against each other giving rise to separate groups for the metabolite profiles of polar extracts prepared from leaves of Yukon *Thellungiella* plants watered with NaCl or not. Four main groups are shown by the treatments and these distinguish three groups representing treatments for metabolite profiles of plants exposed to 100 mM, 300 mM or 500 mM NaCl and the treatment for all of the controls grouped as a fourth group (Fig. 4A). PC loading analysis revealed that the variables citrate, galactose, glucose, malate, proline, and sucrose all contribute to the observed grouping (Fig 4B).

An ANOVA performed using the same data shows 58 MSTs to differ significantly with respect to relative abundance ($P<0.05$) among the different treatments. PCA was performed using the abundance of these MSTs only. Of the total metabolite variance detected, 90% can be accounted for by the first three PCs (PC1 = 58%, PC2= 17%, PC3= 15%). The main groups observed were comparable to those observed using the entire data (Fig. S1A). The variables galactose, glucose, malate, proline, and sucrose were also found to exert the greatest influence in the observed grouping patterns (Fig. S1B). To determine the relative influence of each of these variables on the overall grouping pattern observed for all 58 MSTs, MST relative abundance for galactose, glucose, malate, proline or sucrose were removed from the data in turn and PCA analysis was carried out on the remaining data representing the remaining 57 MSTs. It was found that only malate (Fig. S2E) and proline (Fig. S2F) led to a change in the grouping pattern observed for the analysis performed using the abundance of the 58 statistically significant MSTs. By contrast, the removal of glucose (Fig. S2B), galactose (Fig. S2C), or sucrose (Fig S2D) from the dataset did not change the overall grouping pattern. This provides evidence for malate and proline as playing prominent roles in generating the grouping patterns observed in the PC1 and PC2 biplots. Therefore, the formation of distinct groups from by distinct treatments provides evidence that differences among metabolite abundance and/or composition distinguishes samples prepared from Yukon *Thellungiella* subjected to saline conditions compared to unsalinized controls.

The mean abundance for each of the 58 statistically different MST was expressed as a ratio between salt-treated and unsalinized control, the ratio was $\log_{10}$ transformed,

then subjected to hierarchical cluster analysis (HCA) (Fig. 5). Of these MSTs, 23 were identified by comparison of their RI and MS to those of authentic standards analyzed using the same GC/MS instrument while 15 MSTs could only be categorized according to their chemical class based on their MS pattern (i.e., sugar, sugar alcohol, phosphorylated sugar). A total of 20 MSTs could not be classified to their chemical class and are labeled as "unknown". A total of 25, 27 and 48 MSTs were found to be significantly different in Yukon *Thellungiella* subjected to 100 mM, 300 mM or 500 mM NaCl, respectively, relative to unsalinized controls (Fig. 5). Only 5 of the 25 MSTs (20%) were present at higher levels in Yukon *Thellungiella* plants irrigated with 100mM NaCl compared to unsalinized controls and they were asparagine, fumaric acid, proline, pyroglutamate and serine (Fig. 5). By contrast, for plants salinized to 300 mM NaCl or 500 mM NaCl almost 80% of the MSTs showing statistically significant differences were ones that underwent increases in abundance relative to controls (Fig. 5). Among the metabolites consistently more abundant in *Thellungiella* subjected to 300 mM NaCl and 500 mM NaCl were glucose, proline, pyroglutamic acid, sucrose, valine, six sugar-like compounds and six MSTs labeled as unknowns (Fig. 5). Some MSTs did not show a consistent pattern of increase with salt exposure. For example, glucose, sucrose, and several other sugar-like MSTs were lower in leaves of *Thellungiella* exposed to 100 mM NaCl as compared to control plants of the same age. Only proline was found to consistently increase in abundance with successively higher exposure of plants to saline conditions (Fig. 5, 6). MSTs that underwent only an increase in abundance in leaves of *Thellungiella* subjected to 500 mM NaCl were ascorbic acid, galactose, glutamine, quinic

acid, and three sugar-like compounds. Figure 5 shows that the organic acids ascorbate, citramalate, fumarate and quinate are more abundant in samples from salt-stressed plants while citrate, malate, maleate, succinate and threonate are present in lower abundance.

3.5 DISCUSSION

The salt level of soils in Yukon *Thellungiella*'s natural habitat is estimated to be roughly equivalent to 160 mM NaCl (Guevara et al., 2009). Under these conditions, our in vitro germination trial suggests that Yukon *Thellungiella* seeds should germinate at about 80% efficiency if there is little to no dilution of salts by spring run-off or precipitation. Neither the Shandong nor Yukon ecotypes show any decreased capacity to germinate when media is not supplemented by NaCl so there is no apparent need for salt to promote germination (Inan et al., 2004). However, Inan et al (2004) show that Shandong *Thellungiella* seeds fail to germinate on any MS agar media supplemented with NaCl as low as 100 mM. This response has been observed in other halophytes presumably as a means to prevent NaCl induced injury to the seedling (Flowers et al., 1986). Figure 1 shows that root growth of Yukon *Thellungiella* seedlings on agar containing NaCl is slower than for seedlings without added salt. However, at 100 mM the rate of root elongation after 6 d exposure to salt is 1.4 mm d^{-1} which is 60% of the rate determined for plants on NaCl-free media. This observation suggests that Yukon *Thellungiella* can germinate and grow, albeit more slowly than controls, under the levels of NaCl that approximate those found at Yukon field sites.

Stomatal conductance of Yukon *Thellungiella* decreases in response to salinity but rates of photosynthesis were not reduced to the same extent (Fig. 2B). Shandong *Thellungiella* undergoes a decrease in stomatal transpiration during exposure to saline conditions (Volkov et al., 2003; Inan et al., 2004). This decrease in stomatal transpiration, however, was not specific to Na^+, but also occurred during treatments with elevated K^+ concentrations (Volkov et al., 2003). Flowers et al. (1986) states that transpiration rates expressed on the basis of fresh weight generally show a decline with salt exposure but cautions that interpretation of these data may be confounded by other factors including the basis used for their expression. Our data, corrected for leaf area, suggests that Na^+ ion influx into the shoot via the transpiration stream is ongoing in *Thellungiella* but decreased stomatal conductance should reduce the salt accumulated in the mature, fully expanded leaves of the salinized plants used for our measurements. Control of transpiration and mechanisms impacting water uptake by *Thellungiella* requires further work to delineate the mechanism(s) underlying salt-induced stomatal closure and to determine its significance for salinity tolerance in this plant.

Yukon *Thellungiella* shows a decrease in leaf ψ_S with increasing external salt showing that this plant accumulates solutes in a salt-responsive manner (Fig. 3). The estimated leaf ψ_p remains positive, an outcome consistent with a plant able to actively accumulate solutes in order to take up water from potting media that is undergoing increasing salinization. Shandong *Thellungiella* (Inan et al., 2004) plants also undergo a decrease in leaf ψ_S and both accessions show decreases to as low as -4.0 MPa when exposed to 500 m M NaCl. Thus, a general mechanism for the halophile *Thellungiella*

appears to be an ability to accumulate solutes in order to establish a ψ_W gradient to promote water uptake under saline conditions (Inan et al., 2004).

We compared leaf metabolite profiles of Yukon *Thellungiella* plants subjected to saline conditions in order to identify organic solutes undergoing changes in abundance with increasing salt exposure. The 58 statistically significant changes among MSTs led to distinct grouping of treatments representing Yukon *Thellungiella* plants exposed to different NaCl concentrations (Fig. S1A). Fewer metabolites underwent an increase in abundance in leaves of Yukon *Thellungiella* plants exposed to 100 mM NaCl (Fig. 5). Among the metabolites showing a higher content in Yukon *Thellungiella* irrigated with 100 mM NaCl was proline and pyroglutamate (Fig. 5). The increase in proline content for this accession is salt-responsive, a finding that is consistent with that reported for Shandong *Thellungiella* (Inan et al., 2004; Gong et al., 2005; Kant et al., 2006). Positively correlated changes among MST relative abundance with increasing salt are noteworthy as so few MSTs showed this pattern. For example, only proline was found to undergo an increased abundance that correlated positively with increasing salinity (Fig. 5 and 6). Proline serves diverse roles in plant stress tolerance including osmotic adjustment, a N-storage molecule, scavenging ROS, maintaining $NADP^+$/NADPH ratios during stress, all of which can contribute towards important strategies for abiotic stress tolerance (For a review, see Kishor et al., 2005).

Although proline accumulated in a manner that was positively correlated with increasing salinity at all salt-levels tested, proportionately more compounds were present at higher levels in Yukon *Thellungiella* salinized to 300 mM or 500 mM compared with

100 mM NaCl treated plants, relative to their respective, unsalinized controls. For example, we found 13 MSTs (5 unknown, 7 sugars, and valine) to be present at lower abundance in leaves of Yukon *Thellungiella* plants watered with 100 mM NaCl relative to controls and the converse to be true for plants stressed to 500 mM NaCl (Fig. 5). For a halophyte like *Thellungiella*, lowering of ψ_W at a salt level of 100 mM NaCl or even higher could be accomplished simply by accumulating inorganic solutes such as Na^+ and Cl^-. In support of this suggestion, Na^+ and Cl^- concentrations can be accumulated to ca 100 mM in the cytoplasm of cells adapted to 428 mM NaCl (Binzel et al., 1988). For example, the halophytic plant *Limonium latifolium* accumulates proline, sucrose, cyclitols and inorganic ions in response to salinity (Gagneul et al., 2007). The contribution of organic solutes towards ψ_S was estimated to be only about 25% while inorganic ions were a major contributor, accounting for 75% towards ψ_S (Gagneul et al., 2007). The accumulation of readily available inorganic ions to lower the ψ_W during exposure to low concentrations of salt may provide an energetically less expensive means to promote water uptake (Gagneul et al., 2007).

The redirection of carbon towards accumulated sugars represents a redirection of photosynthate away from growth and maintenance metabolism. Nonetheless, significant increases in the total soluble sugars have been reported for Shandong *Thellungiella* plants subjected to saline conditions compared to unsalinized controls (Inan et al., 2004). We also observed an accumulation of sugars and sugar-like MSTs when Yukon *Thellungiella* plants were exposure to 300 or 500 mM NaCl (Fig. 5). We also found that content of several organic acids were lower in Yukon *Thellungiella* exposed to saline conditions

relative to the unsalinized control plants. Decreases in citrate, malate, and succinate content were observed in leaves of plants subjected to 300 or 500 mM NaCl treatments (Fig. 5). Sanchez et al. (2008b) propose that organic acids contribute to salt tolerance by restoring ion balance under stress and by contributing to nitrogen assimilation for amino acids and the synthesis of compatible solutes. All of these roles could redirect organic acids and reduce their total content in leaves and conceivably other organs. The decreases that we find for the leaf content of several organic acids is consistent with the roles proposed by Sanchez et al (2008b) in directing carbon into amino acids and sugars for Yukon *Thellungiella* exposed to saline conditions. Some exceptions to the observed depletion of organic acids in plants exposed to saline conditions included quinic acid and ascorbic acid, both of which were found to be more abundant in Yukon *Thellungiella* exposed to 500 mM NaCl relative to unsalinized controls (Fig. 5). These metabolites may be involved in the protection against oxidative damage that can occur in plants exposed to high salinity, and may not directly contribute towards the synthesis of organic solutes (Apel and Hirt, 2004). For example, quinic acid is an important precursor for the synthesis of antioxidants (Niggeweg et al., 2004) that are implicated in protection against oxidative stress (Apel and Hirt, 2004) and ascorbic acid is capable of scavenging reactive oxygen species (Conklin et al., 1996; Apel and Hirt, 2004).

3.6 CONCLUSION

The ability of *Thellungiella* to thrive under saline soils is likely due, in part, to its ability to osmotically adjust (Inan et al., 2004). In this study, we found that Yukon *Thellungiella* plants accumulate proline and sugars in response to salinity. The higher of amino acid and sugar content in Yukon *Thellungiella* subjected to saline conditions relative to unsalinized controls was accompanied by lower levels of organic acids. This suggests that organic acids provide a potential carbon source to support the increased synthesis of compatible organic solutes that are accumulated in Yukon *Thellungiella* in response to salinity. For *Thellungiella*, proline has been proposed to be a major compatible solute when plants are salt-stressed (Inan et al., 2004). However, the salinity-induced increases in the abundance of sugars such as glucose and sucrose in Yukon *Thellungiella* strongly suggests that in addition to proline, carbohydrates also play a prominent role in lowering ψ_S to promote water uptake from sea-level salinity conditions. The role these metabolites play for *Thellungiella* adaptation to the highly saline soils typically found in its natural habitat in the Yukon requires further investigation.

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Figure 1. Effect of salinization on the root growth of *Thellungiella* seedlings.

Data represent the mean of ten seedlings at each time point for three independent experiments ± SEM.

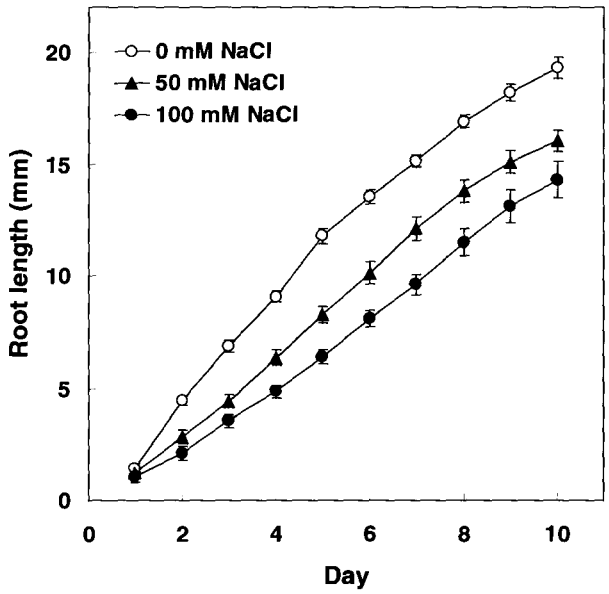

0 mM NaCl
50 mM NaCl
100 mM NaCl
Root length (mm)
Day
0
5
10
15
20
0
2
4
6
8
10

Figure 2. Performance of *Thellungiella* plants subjected to salt treatments.

A) *Thellungiella* plants following treatment with 0 mM, 100mM or 300mM NaCl. The photograph was taken on the third day of exposure to NaCl treatment by Dr. Peter Summers. Bar = 1cm.

B) Stomatal conductance and photosynthetic rates of Yukon *Thellungiella* exposed to NaCl expressed as the % of rates for unsalinized, control plants at each salt step. Values are the mean determination of rates for mature fully expanded leaves from six individual plants ± SEM. Stomatal conductance for control plants were 84±6.5, 102±3.5, and 89±5.4 mmol m^{-2} s^{-1} at 100, 200, or 300 mM NaCl treatments, respectively. Photosynthetic rates for control plants were 7.1±0.5, 6.9±0.8, and 3.6±1.6 μmol m^{-2} s^{-1} at 100, 200, or 300 mM NaCl treatments, respectively. An asterisk (*) denotes that values of salt-treated plants are significantly different from controls at $P<0.05$; Student's *t* test.

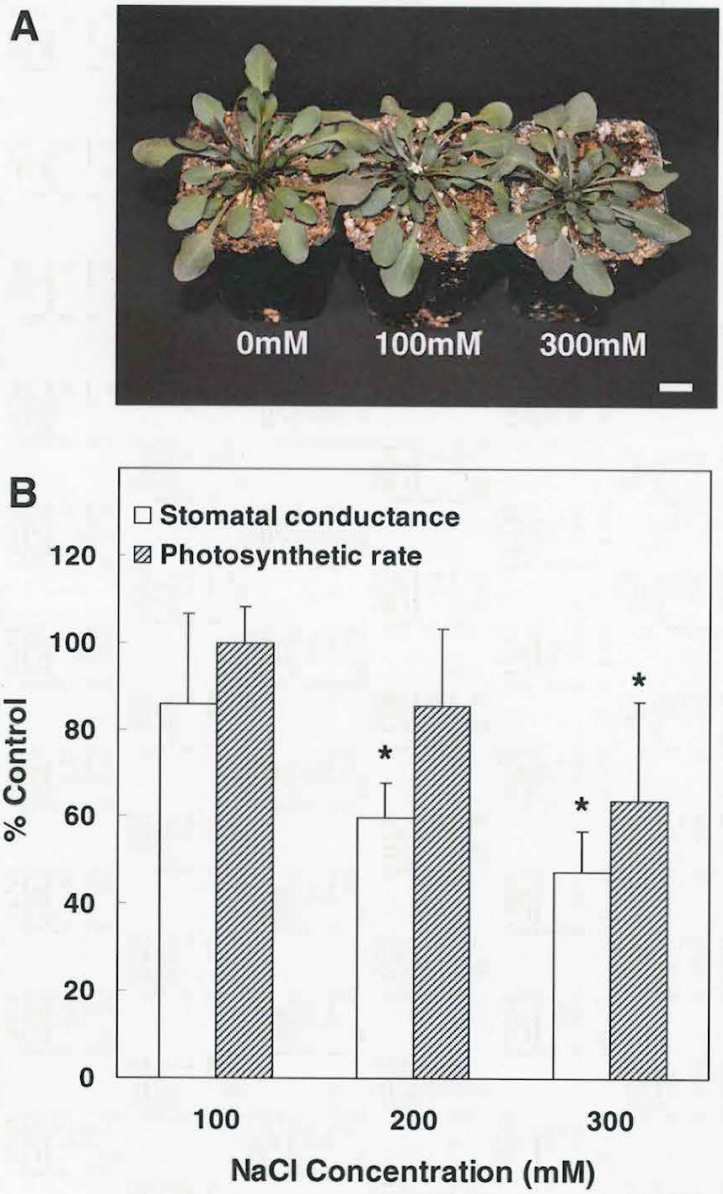

A
0mM
100mM
300mM
B
Stomatal conductance
Photosynthetic rate
% Control
120
100
80
60
40
20
0
100
200
300
NaCl Concentration (mM)
*
*
*

Figure 3. Water relations of Yukon *Thellungiella* exposed to NaCl.

Plants were salinized step-wise by increasing the concentration of NaCl in water by 50 mM increments. Leaf ψ_W and ψ_S potential measurements were taken after 3 d of acclimation to each salt concentration. The ψ_P was estimated as the difference between ψ_W and ψ_S. Values are means of six individual plants ± SEM from two different experiments.

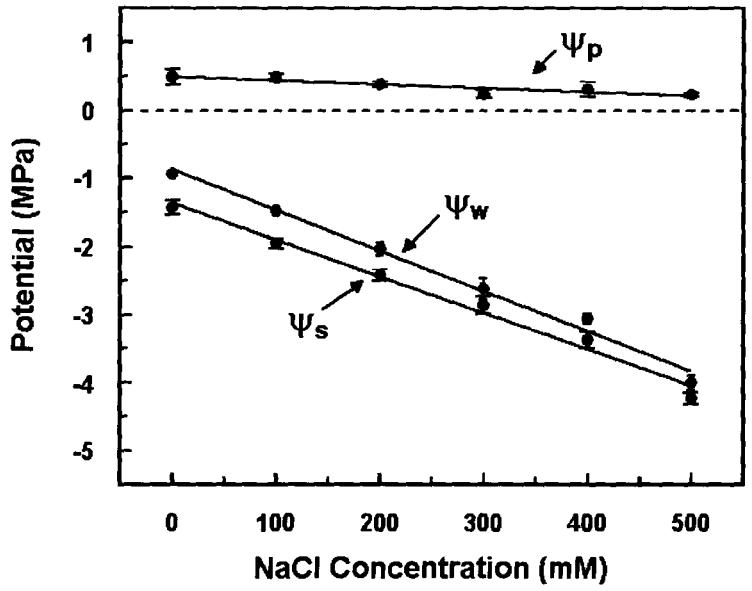
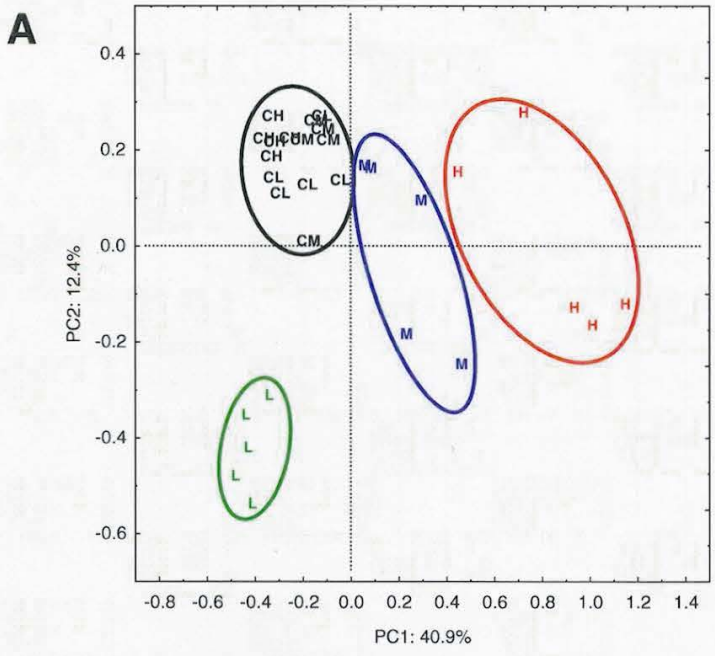

Potential (MPa)
1
0
-1
-2
-3
-4
-5
Ψp
Ψw
Ψs
0
100
200
300
400
500
NaCl Concentration (mM)

Figure 4. PCA of MST data for Yukon *Thellungiella* plants show grouping as a function of salt exposure.

A) The PC1 and PC2 were plotted against each other giving rise to distinct groups. Each letter represents the entire complement of 300 MSTs present in leaves from an individual Yukon *Thellungiella* plant that either received no salt (Controls for each salt-step: "100mM NaCl"=CL; "300mM NaCl"=CM; "500mM NaCl"=CH) or varying concentrations of salt: 100 mM=L, 300 mM=M and 500 mM=H. The metabolite profiles from five individual plants were determined at each salt step as well as for five corresponding unsalinized control plants.

B) PC loading plot for PC1 and PC2. MSTs with highest absolute PC loadings were identified and are labeled: Cit, citrate, Gal, galactose, Glc, glucose, Mal, malate, Pro, proline, Suc, sucrose.


A
PC2: 12.4%
PC1: 40.9%
CH
CL
CM
M
H
L

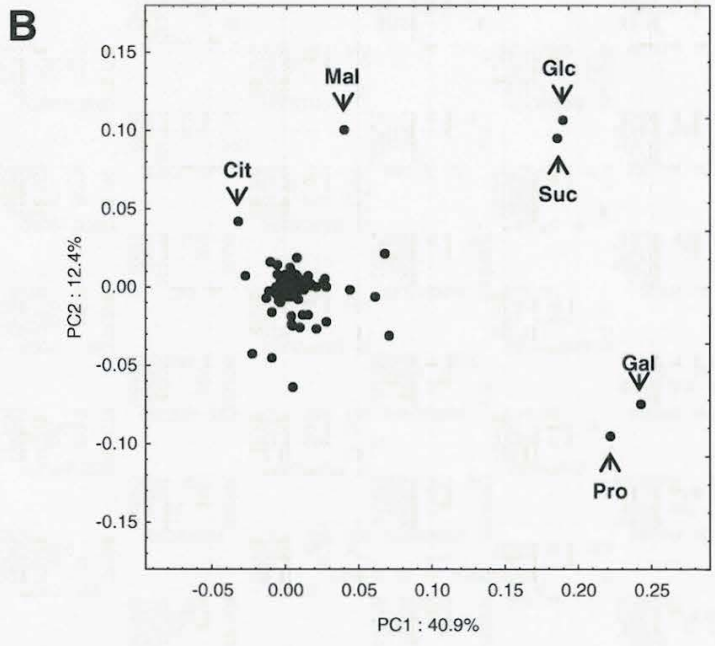

B
PC2 : 12.4%
PC1 : 40.9%
Mal
Glc
Cit
Suc
Gal
Pro

Figure 5. Hierarchical cluster analysis of salt-responsive MSTs.

Heat map showing MSTs found to be statistically significantly different in abundance in leaves of Yukon *Thellungiella* subjected to 100 mM, 300 mM or 500 mM NaCl. White indicates no difference in MST ratio between treatment and respective control while red or blue indicates that MST abundance was higher (positive number) or lower (negative number), respectively, relative to control. Data shown is reported as relative to MST content in their respective controls that received no NaCl. Wherever possible, each MST was labeled with the RI number. Chemical structure of putative matches were made by comparing MS patterns to those of authentic standards analyzed using the same instrument. Proline and glucose were identified by co-injection. Data are based on MST levels from five individual plants at each salt treatment as well as respective unsalinized controls.

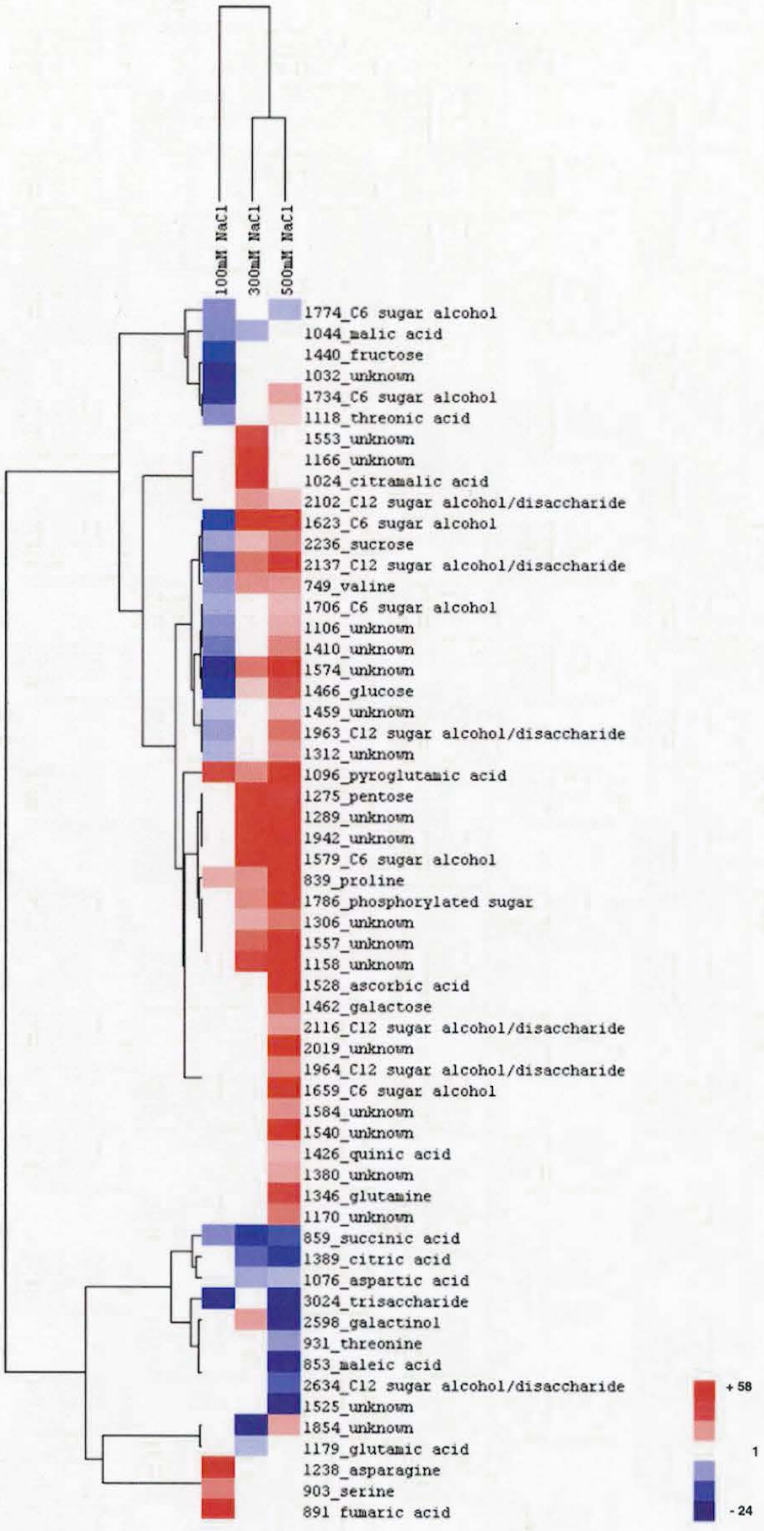

100mM NaCl
300mM NaCl
500mM NaCl
1774_C6 sugar alcohol
1044_malic acid
1440_fructose
1032_unknown
1734_C6 sugar alcohol
1118_threonic acid
1553_unknown
1166_unknown
1024_citramalic acid
2102_C12 sugar alcohol/disaccharide
1623_C6 sugar alcohol
2236_sucrose
2137_C12 sugar alcohol/disaccharide
749_valine
1706_C6 sugar alcohol
1106_unknown
1410_unknown
1574_unknown
1466_glucose
1459_unknown
1963_C12 sugar alcohol/disaccharide
1312_unknown
1096_pyroglutamic acid
1275_pentose
1289_unknown
1942_unknown
1579_C6 sugar alcohol
839_proline
1786_phosphorylated sugar
1306_unknown
1557_unknown
1158_unknown
1528_ascorbic acid
1462_galactose
2116_C12 sugar alcohol/disaccharide
2019_unknown
1964_C12 sugar alcohol/disaccharide
1659_C6 sugar alcohol
1584_unknown
1540_unknown
1426_quinic acid
1380_unknown
1346_glutamine
1170_unknown
859_succinic acid
1389_citric acid
1076_aspartic acid
3024_trisaccharide
2598_galactinol
931_threonine
853_maleic acid
2634_C12 sugar alcohol/disaccharide
1525_unknown
1854_unknown
1179_glutamic acid
1238_asparagine
903_serine
891_fumaric acid
+58
1
-24

Figure 6. Leaf proline content of Yukon *Thellungiella* subjected to NaCl treatments and their respective, unsalinized control plants.

The absolute concentration of leaf proline levels were determined by using an eight-point calibration standard curve prepared from known quantities of proline (156 pg μL^{-1} to 20 ng μL^{-1}, correlation coefficient, R^2=0.994). Numbers on the x-axis denote NaCl concentration (mM). Open bars, unsalinized controls; solid bars, salinized plants. Values are means ± SE from five individual plants.

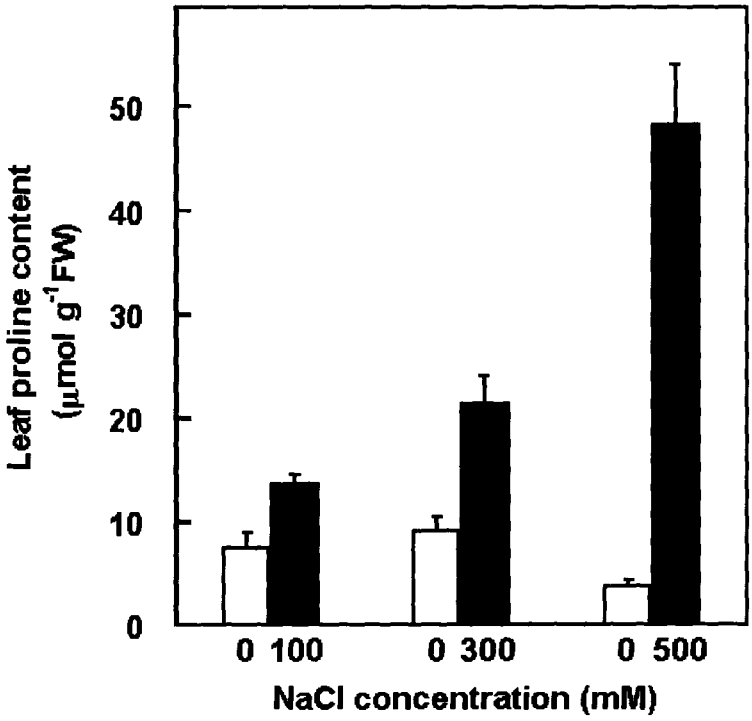

Leaf proline content
(μmol g-1 FW)
50
40
30
20
10
0
0 100
0 300
0 500
NaCl concentration (mM)

Figure S1. PCA of the 58 statistically significant MSTs.

A) The PC1 and PC2 were plotted against each other giving rise to distinct groups. Each letter represents the 58 MSTs present in leaves from an individual Yukon *Thellungiella* plant that either received no salt (Controls for each salt-step: "100mM NaCl"=CL; "300mM NaCl"=CM; "500mM NaCl"=CH) or varying concentrations of salt: 100 mM=L, 300 mM=M and 500 mM=H. The metabolite profiles from five individual plants were determined at each salt step as well as for five corresponding unsalinized control plants.

B) PC loading plot for PC1 and PC2. MSTs with highest absolute PC loadings were identified and are labeled: Cit, citrate, Gal, galactose, Glc, glucose, Mal, malate, Pro, proline, Suc, sucrose.

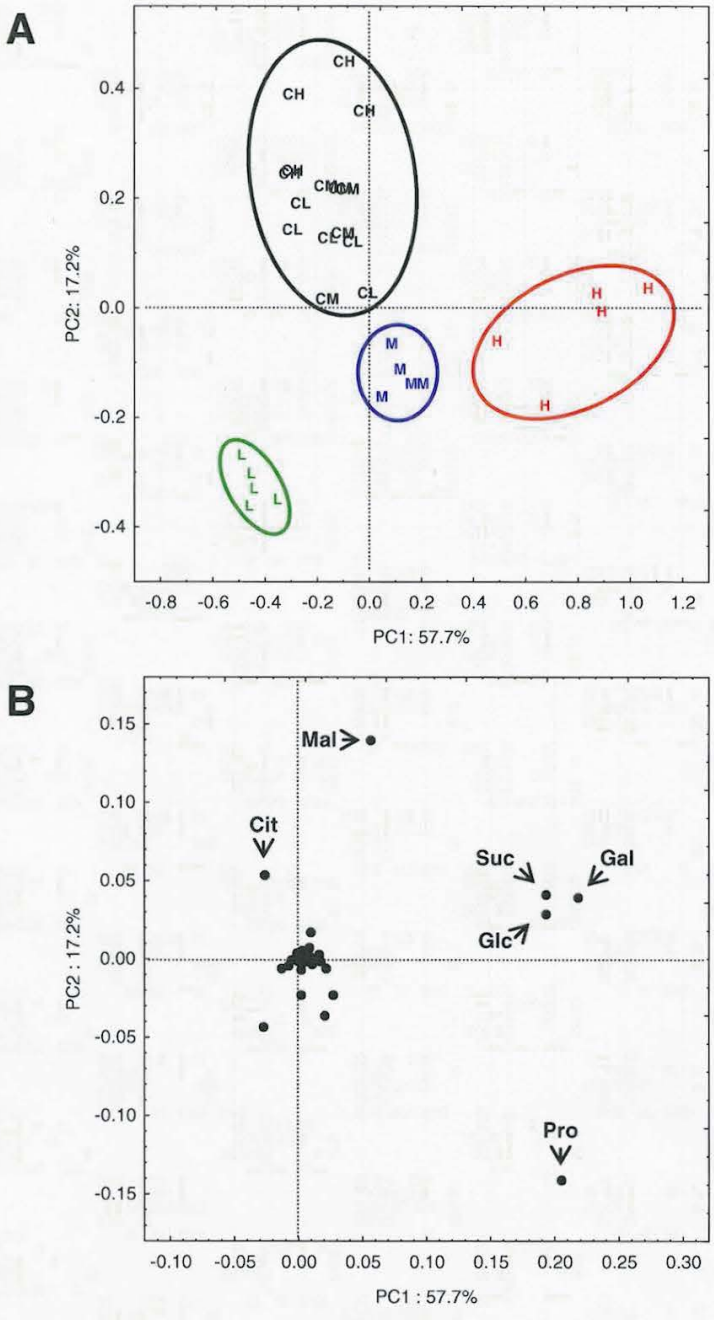
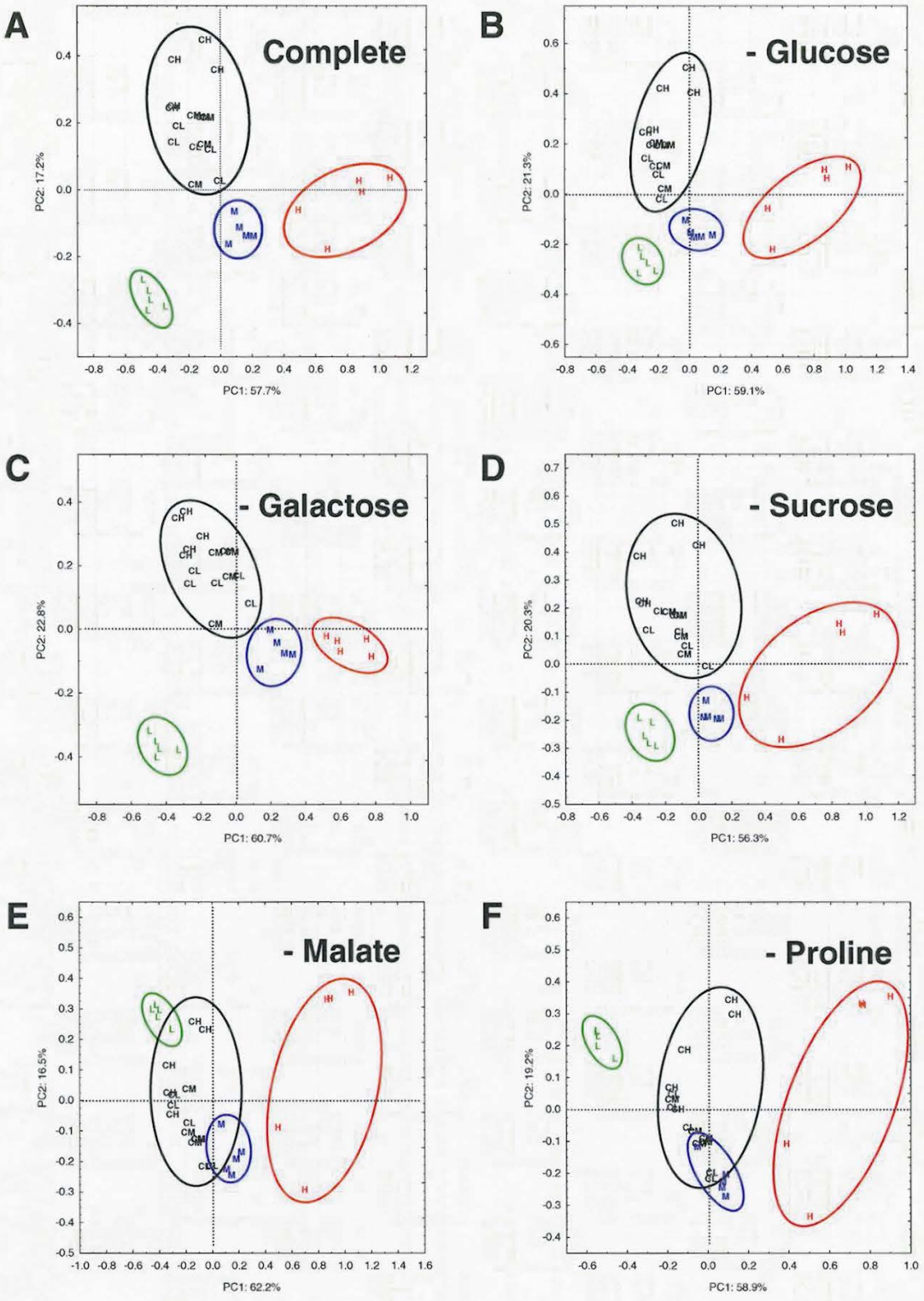

A
PC2: 17.2%
PC1: 57.7%
B
Mal
Cit
Suc
Gal
Glc
Pro
PC2 : 17.2%
PC1 : 57.7%

Figure S2. Analysis of the contribution of MSTs with highest absolute PC loadings on metabolite grouping patterns observed using 58 statistically significant MSTs.

A) The PC1 and PC2 were plotted against each other giving rise to distinct groups using MST abundance for the 58 statistically significant MSTs. Glucose (B), Galactose (C), Sucrose (D), Malate (E) and Proline (F) were individually removed from the dataset and performed PCA on the remaining 57 MSTs to determine the effect of removal on the original grouping pattern.


A
Complete
PC2: 17.2%
PC1: 57.7%
B
- Glucose
PC2: 21.3%
PC1: 59.1%
C
- Galactose
PC2: 22.8%
PC1: 60.7%
D
- Sucrose
PC2: 20.3%
PC1: 56.3%
E
- Malate
PC2: 16.5%
PC1: 62.2%
F
- Proline
PC2: 19.2%
PC1: 58.9%

Chapter Four

"Transcriptomic and metabolomic analysis of Yukon *Thellungiella* plants growing under cabinet and field conditions shows overlapping and distinct responses to environmental conditions"

4.0 PREFACE: Author contributions and acknowledgements for manuscript to submitted for publication.

David Guevara: Performed metabolomic analysis of Yukon *Thellungiella* subjected to cold, drought and salinity treatments in growth cabinets and of plants harvested in 2003 from the Yukon; performed all statistical analysis of metabolome data and meta-analysis of transcriptome data from "growth chamber" and "field" microarray experiments; compiled figures 3 to 6 and supplemental tables; wrote the first draft of the manuscript.

Jeff Dedrick: Compiled Figure 2; Performed metabolomic analysis of Yukon *Thellungiella* harvested from the Yukon in 2005.

Dr. Annie Wong & Yong Li: Performed microarray experiments for Yukon *Thellungiella* harvested from field sites in the Yukon in 2003 & 2005.

Dr. Aurelie Labbe: Performed statistical analysis of microarray experiments.

Dr. Chien-Lu Ping: Performed Yukon soil analysis

Dr. Chris Wang: Created the library of TMS-metabolite derivatives database

Dr. Brian Golding: Designed platform for storage and retrieval of microarray experiments.

Dr. Brian McCarry: Provided suggestions for metabolomic analysis.

Dr. Peter Summers: Critical reading and revision of the manuscript.

Dr. Barbara Moffatt: Supervised microarray experiments.

Dr. Elizabeth Weretilnyk: Supervised the project, compiled Figure 1, wrote final version of the discussion, and oversaw the revisions of the first draft and all subsequent drafts.

RUNNING HEAD: **Transcriptome and metabolite analysis of Yukon *Thellungiella***

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Transcriptomic and metabolomic analysis of Yukon *Thellungiella* plants growing under cabinet and field conditions shows overlapping and distinct responses to environmental conditions

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4.1 ABSTRACT:

Thellungiella salsuginea has a high natural tolerance to abiotic stresses including salt, cold and water deficits. Microarray studies have shown that this extremophile plant when grown under controlled environment conditions undergoes many stress-responsive changes in transcript abundance. However, cabinet-grown plants have prominent rosettes with few cauline leaves while the opposite is true for plants found in the field. To determine whether stress-responsive changes in cabinet-grown plants are also found in field plants we analyzed leaf transcript and metabolite profiles of *Thellungiella* found at its native Yukon habitat during two years with contrasting meteorological conditions. Of 673 genes showing differential expression between field and chamber-grown plants, 27% are categorized as stress-associated by GO Biological Process with several more highly expressed in the year with below average precipitation. We found 63 differentially expressed genes in field-grown plants whose patterns of expression were different in plants stressed in growth cabinets. In general, the relative abundance of metabolites in leaves of field plants was lower than plants in chambers. The relative abundance of serine, proline, galactinol and raffinose showed stress-responsive increases in cabinet-grown material but they were found to be of low relative abundance in leaves of field plants. In contrast, several carbohydrates including sucrose, glucose, galactose and fructose made a greater relative contribution to the field plant profiles than to plants in growth cabinets. Comparisons between plants responding to stress in cabinets and the field implicates the involvement of many common products but site-specific differences

may offer important targets for improving crops that must respond appropriately to multiple, concurrent stresses.

4.2 INTRODUCTION

Abiotic stresses such as drought, extreme temperatures, and high salinity decrease plant growth and productivity and are responsible for major losses in crop yield (Boyer, 1982). *Thellungiella salsuginea* (also known as salt-lick mustard, saltwater cress, *Thellungiella halophila*, herein referred to as *Thellungiella*) is an emerging physiological and genetic model for the study of abiotic stress tolerance in plants. An important factor underlying this development is *Thellungiella*'s significant natural ability to tolerate adverse environmental conditions including, water deficits, freezing temperatures, and high salinity (Inan et al., 2004; Taji et al., 2004; Wong et al., 2005; 2006; Griffith et al., 2007). Of additional importance is the close relationship between *Thellungiella* and *Arabidopsis* (Al-Shehbaz et al., 2006). This relatedness between the two species allows one to apply the knowledge and genetic tools already developed for the study of *Arabidopsis* towards increasing our understanding of molecular mechanisms underlying stress tolerance in *Thellungiella* (Bressan et al., 2001). *Thellungiella* shares some advantageous properties with *Arabidopsis* including a comparatively small genome size (approximately two-fold the genome size of *Arabidopsis*), small stature, prolific seed production and a relatively short life cycle of six to eight weeks (Inan et al., 2004). In many respects, *Thellungiella* is ideally suited to studies designed to identify traits underlying stress tolerance of plants with an objective to improving the productivity of stress-sensitive crops.

Transcript profiling was used to study stress-induced changes in gene expression for *Thellungiella* genotypes originating in Shandong Province, China and Yukon Territory, Canada. The gene expression pattern shown by Shandong *Thellungiella* subjected to salinity stress treatments in controlled environments has been studied using *Arabidopsis* microarray slides (Inan et al., 2004; Taji et al., 2004; Gong et al., 2005). Microarrays showed six genes undergoing salt-responsive changes in expression in Shandong *Thellungiella* compared to 40 genes in *Arabidopsis* plants exposed to the same salt stress conditions (Taji et al., 2004). Interestingly, among the genes identified as stress-responsive in *Arabidopsis* were genes associated with higher constitutive expression in unstressed *Thellungiella* (Taji et al., 2004). Together these observations suggest that many genes associated with salinity are constitutively expressed in *Thellungiella* producing a plant with a heightened innate resilience towards stress.

Gene expression patterns following response to cold, drought or salinity in Yukon *Thellungiella* was assessed using a microarray derived from cDNA libraries enriched for genes associated with stress (Wong et al., 2006). Exposure to salt stress resulted in the differential expression of 22 genes, a number that is low compared to cold stress (76 genes) or drought stress (101 genes). However, little overlap was found among the genes whose expression changed with exposure to cold, drought or salinity leading to the conclusion that *Thellungiella* has rather specific responses to these stresses (Wong et al., 2006).

Metabolite profiling has been used to describe plant phenotype as a function of genotype or genotype and environment interaction (Messerli et al., 2007; Li et al., 2008).

Since metabolite profiling provides an overview of the complement of metabolites present in an organism at a set time-point, this data complements transcript profiling and offers a means to link the potential for a plant to express a stress-specific response to an actual metabolic phenotype. In this way, comparisons made between unstressed plants and plants experiencing sub-optimal environmental conditions can provide valuable insights into traits conferring tolerance (Rizhsky et al., 2004). One such study combines transcript and metabolite profiling methodologies to monitor Shandong *Thellungiella*'s response to salinity under controlled environmental conditions (Gong et al., 2005). Shandong *Thellungiella* was shown to have higher levels of proline, and sugar alcohols, in plants subjected to salinity stress relative to unsalinized controls and this outcome was associated with the enhanced expression of transcripts encoding enzymes involved in their synthesis (Gong et al., 2005).

The study of plant responses to stress frequently uses plants grown under controlled environments in cabinets and the application of treatments intended to simulate adverse field conditions. This approach has been used successfully in identifying stress responses and genes whose products are involved in stress tolerance (for a review see Bartels and Sunkar, 2005). However, it is impossible to faithfully reproduce the complexities of a natural environment in a growth cabinet. Several studies using microarrays have highlighted this important consideration by comparing phenotypes observed in controlled conditions to those expressed in the field (Dhanaraj et al., 2007; Miyazaki et al., 2004). In these comparisons, the association and value of traits

observed in controlled environment studies and stress tolerance can find support or be questioned depending on their expression under field conditions.

In this study we harvested leaves from Yukon *Thellungiella* plants growing on salt flats near Whitehorse, YT, Canada. Microarray and metabolite profiling was completed using leaves harvested during two years of contrasting meteorological conditions. In 2003, the Yukon experienced below average precipitation and in 2005 the rainfall was above average. Analysis of Yukon *Thellungiella* during the different sampling years allowed us to compare the profile of transcripts and metabolites for field plants with comparable profiles prepared from plants grown and stressed under growth cabinet conditions. In our study we found many drought-responsive changes in the expression of gene products identified in simulated drought experiments in growth cabinets to be also represented among transcripts showing differential expression in plants harvested in 2003, a dry year. However, some changes were field or cabinet specific, raising questions about the essential nature of various gene products for stress protection. As such, comparative genomic analysis involving field studies can offer an important companion strategy to the study of chamber-grown plants in the identification of critical traits underlying abiotic stress tolerance.

4.3 MATERIALS AND METHODS

Yukon field site location and plants

In 2002, 2003 and 2005 cauline leaf tissue was harvested from mature, flowering Yukon *Thellungiella* at a field site near Whitehorse, Yukon (location: 60° 55.928'N, 135° 10.249' W; elevation = 647m). Leaf tissue was transferred quickly to 2-mL Nalgene cryovials, immediately flash frozen in liquid nitrogen, and then transferred to a charged MVE XC20/3V vapour shipper (Jencons Scientific Inc , Bridgeville, PA) where samples were kept frozen at -150°C for transport. Vials were transferred to -80°C for storage pending analysis.

Controlled environment plant growth conditions and stress treatments

Seeds of the Yukon genotype were sterilized using a vapour-phase gas technique (Clough and Bent, 1998) and then mixed with 0.1% (w/v) Phytagel (Sigma) and pipetted onto a moistened soil mixture containing six parts Promix BX (Premier Horticulture, Rivière-du-Loup, PQ) and one part Turface (Profile Products LLC, Buffalo, NY) in individual 5 x 5 x 7 cm pots. The pots containing seeds were stratified for 2 d at 4°C before transfer to growth chambers (AC 60 Enconair, Winnipeg, MB) set with a 21-h d and irradiance of 250 μmol $m^{-2}s^{-1}$ and 22°C/10°C day/night temperature regime. Plants were watered daily as needed and fertilized one time per week with 1 g L^{-1} 20-20-20 (N-P-K) fertilizer.

Cauline leaves obtained from mature, flowering, 12-week-old *Thellungiella* plants grown in controlled environment chambers served as the source of control, unstressed tissue in microarray and metabolite profiling experiments in comparisons involving the cauline leaves from field-grown plants. Basal rosette leaves of unstressed control plants

served as the basis of comparison for metabolite profiles in the case of cold, drought, drought/re-watered and saline plant samples (Wong et al., 2006). The growth conditions, stress treatments and nature of tissue harvested were as described by Wong et al. (2006). Upon completion of the stress treatments a portion of the leaf tissue was flash-frozen in liquid nitrogen and stored at -80°C until it was processed for metabolite profiling.

Soil and plant analysis

Soil samples were collected from the top 18 cm depth at the field site where Yukon *Thellungiella* was harvested. Soil pH, electrical conductivity, and chemical content were determined and the elemental composition of soil samples, field and chamber leaf tissues were analyzed (Ping et al., 1998).

Solute potential measurements

Leaf solute potential measurements were made using a HR33T psychrometer (Wescor Inc., Logan, UT). A frozen leaf disc 6 mm in diameter was allowed to thaw and come to equilibrium with the air in the chamber for 20 min in a C52 leaf chamber.

Microarray analysis

Total RNA extraction and microarray hybridization conditions were as reported by Wong et al. (2006) except the mRNA was first amplified using the Ambion MessageAmp aRNA Amplification kit (Applied Biosystems) and the cDNA was primed with a random primer mix (Invitrogen). The cDNA microarrays were hybridized with

Cy3 and Cy5 fluorescently labeled probe pairs prepared using RNA extracted from untreated cauline leaves of chamber grown *Thellungiella* and cauline leaves of *Thellungiella* harvested at the Yukon field site. Three biological replicates with dye swap as a technical replicate were used for leaf tissue harvested from the field in 2003 and 2005 and statistical analysis was based on a total of six replicates per field year (three biological replicates plus three technical replicates). ScanArray confocal scanning system and QuantArray data acquisition software (Perkin-Elmer) were used to obtain data, and after normalization, differentially expressed genes between growth chamber and field-grown Yukon *Thellungiella* were detected using a Bayesian model as previously described (Wong et al., 2006). Transcripts in leaves of field plants showing differential expression relative to those in chamber-grown plants were annotated and classified using the TAIR9 gene ontology functional categorization tool (http://www.arabidopsis.org/tools/bulk/sequences/index.jsp).

Metabolite analysis

Polar metabolites for profiling by gas chromatography/mass spectrometry (GC/MS) were obtained from frozen leaf tissue (200 mg) to which 50 μL of ribitol (2 mg mL^{-1}) was added and then ground together to a fine powder in liquid nitrogen. The protocol followed for metabolite extraction using methanol and chloroform was as described by Fiehn et al. (2000) and the fraction in the methanol phase containing polar metabolites was retained for this study. The final steps of preparation included the addition of a mixture of fatty acid standards so that RTs could be converted to RIs

(Roessner et al., 2000). Methoxymation and derivatization were as reported by Roessner et al. (2000).

Samples were analyzed using a Trace DSQ GC-MS system (Thermo Finnigan, Austin, TX) operated in the positive ion electron impact (EI^+) full scan mode. Samples were diluted 25-fold with hexane and then 1-μL was injected using a MPS 2 autosampler (Gerstel GmbH & Co., Mülheim, Germany). Chromatography was performed on a 30 m long, 0.25 mm ID and 0.25 μm film thickness Restek Rtx-5MS integra column (crossbond 5% diphenyl – 95% dimethyl polysiloxane; Chromatographic Specialties Inc., Brockville, ON) fused with a 10 m guard column of the same composition. The injection temperature was 230°C, and the ion source was kept at 200°C. The carrier gas was helium (>99.999% purity; VitalAire, Hamilton, ON) delivered at a constant flow rate of 1 mL min^{-1}. The temperature program was initially set at 50°C for 2.5 min then temperature was first increased at a rate of 7.5°C min^{-1} to 70°C followed by 5°C min^{-1} ramp to a final temperature of 310°C where it remained for 6 min. Mass spectra were recorded at three scans per second with a scanning range of 50 to 650 mass-to-charge (m/z) ratio.

The automated mass spectral deconvolution and identification system (AMDIS) software was used to extract peak abundance and mass spectral (MS) information for each trimethylsilyl derivative resolved in GC/MS chromatograms (Stein, 1999). This information was imported in tab-delimited format to the GC/MS Analysis Software Package (GASP) (available at: www.flintbox.com, Nuin et al., 2004) where the RI was calculated as described by Roessner et al. (2000) and the relative abundance for each

component was adjusted to reflect the estimated recovery of the internal standard ribitol. The term mass spectral tag (MST) refers to an individual trimethylsilyl derivative identified by a characteristic RI and MS (Kopka, 2006). Identical MSTs from multiple GC/MS analyses were aligned with GASP and subjected to statistical tests to identify those associated with specific samples and/or treatments. An arbitrary threshold detection limit for relative response factors associated with MSTs of 0.00005 was used in determining abundance and fold-differences among chemical components from different samples.

The chemical identity of an MST was determined by comparing its RI and MS parameters to those obtained for authentic standards analyzed using the same instrument and experimental conditions. MSTs that shared m/z ratios with authentic standards that had different RIs were classified according to predicted chemical classes such as sugars or sugar phosphates.

Statistical analysis of data

Transcripts found differentially expressed in the field relative to cabinet-grown plants were compared to those showing stress-related changes in abundance reported previously by Wong et al. (2006). The Wong et al. (2006) dataset is comprised of 147 transcripts showing statistically significant stress-responsive changes in leaves of Yukon *Thellungiella* plants grown in cabinets and subjected to cold, drought, salinity and drought followed by re-watering treatments. Transcripts showing a 1.5-fold or greater difference in expression in *Thellungiella* harvested from the field compared to growth

chamber grown plants ($P<0.01$) were subjected to multivariate analysis. MSTs whose abundance was significantly different ($P<0.05$) between treated and control or field and control plants were expressed as a ratio, and were then $\log_{10}$ transformed before being subjected to multivariate analysis. Euclidean distance was used to calculate the distance matrix and a complete linkage method was used for hierarchical clustering MSTs and genes using the program, Cluster (Eisen et al., 1998). Heat maps were constructed using the Java Treeview program (http://jtreeview.sourceforge.net, Saldanha, 2004) where white indicates no difference between transcript or MST abundance between the sample and its respective control while red or blue indicates a gene or MST is detected at higher or lower levels, respectively, relative to a control.

4.4 RESULTS

Yukon field site conditions

The season for plant growth in the Yukon is typically short and for *Thellungiella* this extends from early May to late July (Bruce Bennett, personal communication). We visited the field site over one to two week periods in late May 2002, early July 2003 and late June 2005. Sampling in 2002 was largely carried out to select a suitable field location and establish a sampling protocol suitable for preserving the RNA and metabolite content of specimens during transport from the remote Yukon location.

Specimens harvested in 2003 and 2005 were subjected to both transcript and metabolite profiling.

At the field site we selected *Thellungiella* plants growing on soil encrusted by salt deposits. Soil at the site is composed of an upper mineral-organic layer of approximately 20 cm that overlays clay. Chemical analysis of the upper layer (Supplemental Table S1) shows the soil to be highly alkaline with a pH of 8.3 and saline (soil electrical conductivity of 15.7 dS m^{-1} is roughly equivalent to 157 mM NaCl; Munns, 2005). This soil is high in magnesium, sodium and sulfates, a feature shared by other saline soils in the area (Day, 1962). With respect to other nutrients, the soil has adequate levels of potassium and phosphorus (236 and 26 mg kg^{-1}, respectively), whereas the total nitrogen content is very low (0.26%; Martens and Lindsay, 1990). The Ca/Mg ratio is less than one while a typical soil usually has a Ca/Mg ratio exceeding one (Brady et al., 2005).

For the Whitehorse, Yukon area, the average temperature for the 2003 and 2005 growing seasons was approximately 20° C (Fig. 2). In 2003, average high and low temperatures for the ten-day period prior to harvest were 22°C and 15°C, respectively. At the field site on the day of harvest, soil temperature was 17°C, the air temperature was 24°C, and the light intensity was 1500 μmoles m^{-2} s^{-1}. In 2005, the average high and low temperatures for the ten-day period before harvest was, 18°C and 7°C, respectively. At the field where plants were collected in 2005, the soil temperature was 18°C, the daytime air temperature was 24°C, and the light intensity was 1433 μmoles m^{-2} s^{-1}.

The average cumulative rainfall for this region is 84.1 mm during the growing season for *Thellungiella* (May through July) classifying this area as semi-arid

(Environment Canada climate normals 1971 – 2000; http://www.climate.weatheroffice.ec.gc.ca/climate_normals). In 2003, however, total precipitation was 28% lower than the long-term norms and, as such, can be considered a drought year even for a semi-arid region (Fig. 2, Hogg and Wein, 2005). In a ten day period leading up to our visit to the field site in 2003 only 1.8 mm of rainfall was recorded for the area.

Thellungiella growing in the field and in growth chambers have different phenotypes

The natural accession of *Thellungiella* found in the Yukon displays a variable phenotype under different growth conditions. A prominent difference in a mature, flowering plant is the distribution of leaves between the basal rosette and aerial stems bearing flowers. In growth cabinets set to mimic the temperature range and summer day-length of the Yukon, plants develop multiple (60 to 80 by 8 weeks) basal rosette leaves (Fig. 1A) with flowers first appearing at about 4 to 5 weeks as a small cluster within the rosette. Plants usually bolt at about six weeks after germination and flowers are borne on a main stem. At about 8 weeks, auxiliary stems bearing flowers appear and cauline leaves develop on the main and auxiliary stems with the basal rosette leaves remaining a prominent feature. In contrast, *Thellungiella* at field sites have prominent cauline leaves that clasp around the main and auxiliary flowering stems and either lack or have only a few, diminutive rosette leaves (Fig. 1B, C, and D). The chemical analysis of leaf tissue show N, Ca^{++}, and Mg^{++} contents to be similar for cabinet and field-grown plants, but the

Na^+ content of leaves harvested in the field is over six-times higher than that of the cabinet-grown control plants (Supplemental Table S1).

The plants collected in 2003 had experienced below-average precipitation (32.4 mm) in a 60 d period before harvest, in the same timeframe the plants collected in 2005 had experienced more frequent episodes of rainfall leading to above-average cumulative precipitation (92.2 mm). The contrasting precipitation patterns allowed us to compare the field plants exposed to water deficits in 2003 with those experiencing above-normal cumulative precipitation in 2005. Figure 1C shows the grass surrounding *Thellungiella* to be brown and either dead or dying in 2003. The *Thellungiella* plants were small (8 to 18 cm tall) but green and either flowering or setting seed. In contrast, at the same site in 2005 the above-average precipitation patterns contributed to an abundance of green vegetation around the *Thellungiella* plants who were themselves almost 2 to 3-fold larger than the plants found in 2003 (Fig. 1B and D). The transcript and metabolite composition of these plants were then compared to plants subjected to more calibrated stress treatments in controlled environment chambers so as to identify shared and site-specific stress-responsive traits.

Transcript profiling of *Thellungiella* growing under field conditions

We used a cDNA microarray containing 3628 unique sequences derived from libraries of stress-induced cDNAs as described in Wong et al. (2006) to profile transcripts present in the leaves of plants obtained from the Yukon field site. Of the gene products represented on the microarray, 673 (19%) were found to be differentially expressed in

cauline leaves of *Thellungiella* growing in the field (Supplemental Table S2). In 2003, transcripts associated with 216 cDNAs were differentially expressed with 132 and 84 being present at higher or lower levels, respectively, relative to control cauline leaves from cabinet-grown plants. For 2005, transcripts associated with 548 genes were found to be differentially expressed with 301 and 247 being present at higher or lower levels, respectively, relative to cauline leaf controls. Of the 673 genes differentially expressed in field samples, only 7% were expressed at higher levels over controls in both years and 6% were expressed to a lesser extent in both field years relative to cabinet-grown controls. Only two gene products were found to be up-regulated in one year and down-regulated in the other year sampled.

We classified the genes showing differential expression between 2003 and 2005 by the biological processes and molecular functions encoded by their products according to the Gene Ontology (GO) annotations (www.arabidopsis.org) and summarized this analysis in pie charts (Fig. 3). The GO Biological Process categorizes 180 of the 673 (~27%) genes showing differential expression in the field plants relative to chamber plants as being associated with stress (Fig. 3A; Supplemental Table S2). Few stress-associated genes were detected in both years with only twelve found to be more highly expressed and fourteen repressed in both 2003 and 2005. Gene products classified as stress-responsive by GO comprised a two-fold greater proportion of the transcripts showing enhanced expression in 2003 as compared to 2005 (Fig. 3A).

The GO Slim analysis by molecular function (Fig. 3B) shows that 20% of the genes expressed at higher levels in 2003 encode products of unknown function compared

to 11% in 2005. Interestingly, proteins with kinase activity contribute <1% towards the complement of products with increased expression in 2003 whereas 8% are repressed that year. In contrast, products encoding protein kinases have expression levels that are similar in control tissue and in leaves harvested in 2005.

We compared the transcripts showing differential expression in field plants with those previously reported as undergoing changes in abundance in rosette leaves of *Thellungiella* subjected to cold, drought, drought recovery or salinity treatments in growth cabinets (Wong et al., 2006). From this meta-analysis we found 63 genes that were differentially expressed in leaves of field-grown plants that were not changed in expression in leaves of plants stressed in growth cabinets. An additional 85 genes show differential expression in the field and in response to at least one growth cabinet stress condition. The fold-differences associated with these 148 gene products were then compared using HCA (Fig. 4). The HCA comparison does not include genes whose transcripts were identified as undergoing differential expression in leaves of field plants from only one year (2003 or 2005) unless the gene product was also reported to undergo stress-associated changes following treatments in the growth cabinet. All of the genes whose products showed a change in relative abundance in 2003 and/or 2005 are listed in Supplemental Table S2.

HCA groups the two field datasets into a single cluster that has greater similarity to the data for cold and drought treated cabinet-grown plants than data obtained from plants subjected to salt stress or recovering from drought (Fig. 4). From the HCA heat map, some generalizations can be made about differences among transcript abundance

with respect to sample source. The top half of the figure includes genes whose transcripts are down-regulated in the field relative to control, cauline leaves of cabinet-grown plants while the bottom half includes genes found to be more highly expressed in the field. Differential expression for some genes was only observed in leaf samples obtained from the field site. In total there were 28 genes showing higher expression and 30 genes showing lower expression in leaves of 2003 and 2005 field plants relative to cauline controls and for which no differential expression was observed in leaves of stressed plants from cabinets.

Of the genes shown in the HCA comparison (Fig. 4) about 60% are associated with a stress-responsive change in expression previously reported for cabinet grown plants subjected to cold, salinity, drought, or drought followed by re-watering treatments (Wong et al., 2006). For example, the first six genes at the top of the figure show comparatively strong stress-responsive expression in growth cabinets with exposure to cold or drought treatments but none were detected at higher abundance in the field for either year sampled. This contrasting pattern of gene expression makes it difficult to conclude whether these genes play a role in plant stress response under field conditions. Other genes show changes in expression that supports a role in stress acclimation in field and cabinet grown plants. By way of example, a cluster of seven genes at the base of the HCA heat map showed increased expression in 2005 and in leaves of plants re-watered following drought treatment in cabinets but they showed no increased expression in 2003 or following drought, cold or salinity treatments in cabinets. This group would appear to be comprised of genes whose expression is down-regulated with stress irrespective of

where the plants are growing and are more likely associated with plants recovering from stress or growing under more optimal field conditions such as those of 2005.

Patterns of differential expression associated with a number of the genes in the heat map are strongly suggestive of a stress-responsive role that is not influenced by where the plants are growing. For example, transcripts associated with 15 genes showed higher abundance in 2003 plant samples and in plants that had undergone a drought treatment in growth cabinets but their transcript abundance for 2005 was no different than for cauline leaves of well-watered controls in growth cabinets. Transcripts associated with five of the 15 genes (At4g16190, At5g66400, At1g20440, At1g20450 and At5g59310) also showed increased expression after a cold treatment in cabinet conditions. Enhanced expression with salinity is shown for a total of four genes, three of which are also up-regulated under field conditions. One of these salt-responsive products (At5g52300) shows higher transcript abundance in the field but more so for 2003. The broad stress responsive behaviour for At5g52300 (annotated as Responsive to Desiccation 29B) in cabinet grown plants and elevated transcript levels for field plants distinguishes this product from others in the heat map. Nine genes showed decreased expression in 2003 field samples but no differential expression relative to controls in 2005. Of these genes, six are also repressed in drought-exposed chamber-grown plants with four (At4g02380, At2g06850, At3g01500 and At2g10940) also showing increased expression in drought-stressed plants that are re-watered and recovering from stress.

Of the gene products showing differential expression in the heat map (Fig. 4), only 49 were categorized by GO-Slim analysis to be associated with stress. Among gene

products showing enhanced expression following stress treatments in cabinets and in field plants, 13 genes have transcripts that increase in response to drought imposed on plants in cabinets and six of these are more abundant in leaf samples from plants harvested in 2003. Interestingly, the six genes associated with these drought-responsive transcripts are not categorized by GO Biological Functions as responsive to stress (including abiotic or biotic stress). The genes associated with these transcripts are At3g09390, At4g33550, At5g42050, At5g42800, At5g53870, and At5g60360. Indeed, two from this list (At5g42050 and At5g53870) were not among the products used to compile the GO Biological Process shown in Figure 3A.

Two genes encoding expressed proteins of unknown function (At5g66860 and At2g15890) showed no apparent stress-responsive pattern in plants exposed to cold, salt or drought stress in cabinets but were found to show higher expression in the field in 2003, the year of prolonged water deficits at the field site. Furthermore the expression levels for these products were repressed in 2005 field plants, a year of adequate rainfall.

Metabolite profiling of *Thellungiella* growing under field conditions

The GO annotation analysis of Figure 3 shows that transcripts associated with metabolism comprise one of the largest categories of differentially regulated genes in *Thellungiella* field plants. In order to gain greater insight into the biochemical activities of these plants we extracted and profiled polar metabolites present in samples of the same leaf tissues used for microarray experiments. Using this approach, it was possible to simultaneously monitor over 300 MSTs corresponding to chemically diverse compounds.

We found 109 MSTs in leaf extracts prepared from plants collected in both 2003 and 2005 that showed statistically significant differences in abundance relative to extracts from cauline leaf controls (Supplemental Table S3). Of these components, the majority of MSTs showed reduced levels in the field (62) or showed a difference in abundance for only one year (48) compared to controls. Only two MSTs, (RI 1320 and RI 1377) showed differential abundance between the two years with higher content in 2003 samples.

In parallel to the transcript HCA, we compared MSTs found to undergo changes in abundance in 2003 and 2005 field plants (Supplemental Table S3) with those undergoing stress-responsive changes in abundance in cabinet-grown plants subjected to stress. This HCA (Fig. 5) includes MSTs showing a change in relative abundance in field material for both 2003 and 2005. Also included were 15 MSTs found at a level different from controls in one year (either 2003 or 2005) and in at least one cabinet stress treatment. The most striking impression given by HCA is that most components are less abundant in leaves of field plants than cauline leaves from a growth cabinet control plant. Leaves harvested from *Thellungiella* growing on saline field sites in 2003 and 2005 had a higher content of citrate, unknown RI 1429, succinate, ethanolamine, Gly, citramalate, sucrose and fructose compared to cauline leaf controls. None of the laboratory stress conditions led to an increased relative abundance of the first four MSTs in this list. Carbohydrates, including several sugars (sucrose, glucose, galactose and fructose) and sugar alcohols, also show increased abundance in field samples relative to well-watered cauline control leaves. Finally, a number of components were of low relative abundance

in the field samples but showed a significant change in content in leaves of plants stressed in growth cabinets. These MSTs include several amino acids (Ser, Glu, Pro, Ile, and Val), galactinol and, raffinose.

The content of plant metabolites can show diurnal changes in abundance (Gibon et al., 2006). We took two measures to address the impact of time of harvest on MST composition. In the first measure we grew laboratory plants under a Yukon day-length regime and synchronized leaf removal from cabinet-grown and field plants. Harvest time approximated the same number of hours (7 h) from sunrise in the Yukon or "lights on" in the growth cabinet. A second measure involved harvesting leaf tissue from field plants at three time-points corresponding to 2, 7 and 12 h after sunrise. Measurements of MST abundance at any given time point were expressed as an average fold-change of the three time points selected for tissue analysis and Figure 6 shows an HCA for the group of 29 MSTs that were found to undergo significant changes in abundance. The majority of the compounds that underwent changes in content showed maximum abundance in the samples obtained 7 h after sunrise. This group includes *myo*-inositol, raffinose, galactinol, quinic acid, fructose, sucrose, and 16 unidentified MSTs. A smaller group whose abundance was highest in early morning (2 h) samples included phosphate, citrate, Gly and unknown RI 1320 while one MST, unknown RI 966, was most abundant in the late time-point sample (12 h after sunrise). Given the difficulty of sampling at a remote field site and the pattern of highest abundance tending to be near midday, we selected a single time-point corresponding to 7 h after sunrise for routine sampling and synchronized this sampling time for comparisons with plants in growth cabinet material.

Our finding agrees with those reported by Gibon et al. (2006) who reported that metabolite abundance is relatively low early in the day and increases over the course of the day.

4.5 DISCUSSION

Many features distinguish a model organism and among them is their ease of propagation under laboratory conditions. While the study of model plants such as *Arabidopsis* under these conditions has provided an abundance of genetic and physiological information, applying this knowledge to plant performance under more complex, natural environmental conditions has lagged behind (Ungerer et al., 2008). In this study we have applied functional genomic approaches to the study of a related crucifer extremophile growing under laboratory conditions with a population of the same species found in its native Yukon habitat. The Yukon Territory of Canada offers a very challenging environment for plants. With a relatively short growing season, typically cold and frequently dry conditions, and a highly saline and alkaline soil medium, the field site we selected for our study is ideally suited for studying plants requiring traits needed for survival under adverse conditions.

Given the diversity of concurrent environmental stresses possible under Yukon field conditions, a highly plastic phenotype could be advantageous in developing a plant more suitably structured for a prevailing environment. In July 2003 the only green and flowering vegetation among dead and dying grass at the field site was *Thellungiella* (Fig.

1C). These plants were small in stature compared to those found in 2005 and aside from this morphological indication of stress, leaf solute potential (Ψ_s) measurements also distinguished these Yukon *Thellungiella* plants. A well-watered plant in the growth cabinet has a cauline leaf Ψ_s of -1.50 ± 0.05 MPa, a comparatively low value for an unstressed plant (Morgan, 1984). In comparison, visibly turgid cauline leaves from field plants were -2.07 ± 0.13 MPa and -1.63 ± 0.07 MPa in 2003 and 2005, respectively. These leaf Ψ_s values show the most solute-rich leaves as being from plants experiencing the 2003 drought. Solute accumulation by plants under water deficits is a beneficial physiological response that can improve water uptake to maintain or restore turgor (Hanson and Hitz, 1982; Shao et al., 2009).

Analysis of transcripts by biological process corroborates the extent of stress experienced by plants under field conditions. The theoretical expectation for transcripts classified by GO Biological Process for "Response to stress or biotic stimulus" and "Response to stress" given the coding potential for the *Arabidopsis* genome would be approximately 8.4% (TAIR; http://www.arabidopsis.org/tools/bulk/go/) and for the unigenes represented on our microarray chip about 8% (Wong et al., 2005). However, we found our observed and expected frequencies with respect to transcripts classified by these categories to diverge. The observed frequency of stress-associated transcripts was over two-fold higher at 19% for *Thellungiella* leaf tissue collected in 2005, a year of above-normal precipitation. In 2003 the corresponding value is just over 38% or almost four times as high as the theoretical expectation showing that stress-responsive gene expression made an even greater relative contribution towards the transcripts found in

leaves of 2003 field plants. This observation suggests that gene products undergoing changes in relative abundance uniquely in this year could be associated with survival under extreme water deficit conditions.

The gene products found to undergo an increase in transcript abundance in leaves of field plants of 2003 but not 2005 are given in supplemental Table S2E. Of the 78 genes found to undergo a 2-fold or greater increase in expression, 14 (18 %) were reported to be stress responsive in growth cabinet treated plants by Wong et al. (2006) and the remaining 64 products only showed increased relative expression in tissue from 2003 field plants. Only half of the 64 gene products are categorized by GO Annotation to be associated with stress although their enrichment in this tissue source would suggest otherwise. With a focus on the 15 most highly expressed gene products in 2003, all but seven showed increased abundance in tissue subjected to stress, including simulated drought in cabinets. These seven transcripts encode genes for heat shock protein 17.6 kDa class II (At5g12020), a lipid transfer protein family member (At1g62510), heat shock protein 81-1 (At5g52640), beta-amylase 8 (BMY8, At4g17090), a putative lipocalin (At5g58070), ferretin 1 (At5g01600), and cold, circadian rhythm, and RNA binding 2 (At2g21660). The heat-shock products, putative lipocalin, BMY8 and lipid transfer protein share a common feature in that all have a reported association with exposure to high temperature (Chi et al., 2009; Kaplan and Guy, 2004; Rizhsky et al., 2004), consistent with the prevailing hot and dry weather conditions for 2003 (Fig. 2).

A combination of heat and drought stress in the field is not uncommon and usually leads to a more significant adverse impact on crop productivity than either stress

alone (reviewed by Mittler, 2006). Plants subjected to heat stress and water deficits display characteristic physiological changes including a combination of high respiration and low photosynthesis and these responses are associated with stress-specific changes among gene products related to carbon metabolism (Rizhsky et al., 2004). Although light and circadian rhythms are two factors regulating starch metabolism, environmental conditions of temperature stress (heat or cold) and drought can also influence starch synthesis and degradation (reviewed in Lu and Sharkey, 2006). Among the highly expressed transcripts only found in 2003 leaves were those encoding two β-amylases namely BMY8 (At4g17090) which was one of the ten most highly expressed products, and BMY7 (At3g23920), and an α- amylase-like 2 product (At1g76130). The BMYs are of particular interest as they are believed to play a key role in starch catabolism, particularly under controlled environment conditions (Fulton et al., 2008). In *Arabidopsis* the expression of genes encoding BMYs are regulated by many factors including light, phytohormones and various stress conditions with BMYs 7 and 8 associated more specifically with heat and cold stress (Kaplan and Guy, 2004; Kaplan et al., 2006). BMY8 is considered the most important enzyme in the breakdown of leaf starch but a very specific functional overlap between BMYs 7 and 8 has been revealed through a particularly severe, starch-accumulation phenotype of a double mutant (Fulton et al., 2008). BMYs are involved in starch degradation and the maltose produced can protect photosynthetic proteins and membranes from stress-associated damage (Kaplan and Guy, 2004). This relief may be transitory until other compatible solutes including sugars can accumulate as part of a long-term stress acclimation strategy. In our case we

detected and verified the presence of maltose among our chemical components but found no evidence that the maltose content was significantly higher in leaf tissue from 2003 compared to cauline leaf controls produced in our growth cabinets or in comparison to field plants harvested in 2005. The lack of accumulated maltose would suggest that this organic solute is not used in long-term adjustment for drought and heat stress protection. Since levels of maltose are typically low during the day (Weise et al., 2005) and the field plants are exposed to a daylength in excess of 20 h during the summer, it is possible that maltose cannot accumulate in field plants under these conditions. The heightened capacity for starch degradation including the production of glucose, sucrose and fructose could explain the elevated levels of these sugars in 2003 leaf tissue (Fig. 5).

Of 2,806 drought and/or heat responsive changes among transcripts, Rhizsky et al. (2004) found over 770 (41%) that were unique to *Arabidopsis* plants exposed to a simultaneous heat and drought treatment. These unique changes extended to the metabolome where drought and heat-specific metabolites, particularly sugars, were found to accumulate with sucrose content increasing by almost 24-fold over unstressed controls (Rizhsky et al., 2004). In field plants where most metabolites were found at levels well below those found in plants grown in growth cabinets, we found statistically significant higher contents associated with sucrose and a number of other sugars. In our case, however, the highest fold-increase in sucrose was found in plants exposed to the more favorable 2005 conditions as opposed to the dry and hot 2003 season. In agreement with their study, however, we found other sugars including glucose, fructose and galactose to undergo significant increases in 2003 tissues as compared to plant tissues obtained in

2005. For these latter sugars in particular, levels frequently increased with other stress treatments in cabinets but in each case drought elicited the highest-fold accumulation.

Not all of the heat stress and drought-related changes represented a positive accumulation. Rather, the study by Rizhsky et al. (2004) reports proline levels to be increased by drought but suppressed under a combination of drought and heat stress, an observation that seems at variance with the purported stress-protective role of proline (Szekely et al., 2008 and reviewed by Verbruggen and Hermans, 2008). Our comparative study between the field and cabinet stress treatments also shows factors contributing to proline accumulation to be at least as complex for Yukon *Thellungiella* plants.

Proline accumulation under stress results from its increased synthesis and decreased turnover (Delauney and Verma, 1993) and a salt stress-responsive increase in proline content for Shandong *Thellungiella* plants has been well documented (Inan et al., 2004; Kant et al., 2006). Wong et al. (2006) reported that transcripts associated with *delta-1-pyrroline-5-carboxylate synthetase* (*P5CS A*, At2g39800 and *P5CS B*, At3g55610) were expressed at higher levels in drought and cold-treated plants relative to controls. Based upon these expression patterns we expected proline content to be higher in cold and drought-stressed chamber-grown plants. However, Figure 5 shows that proline content was highest in cold and salt-stressed plants and not in leaves of plants subjected to drought treatment, an observation showing poor agreement to predictions based upon the relative abundance of *P5CS* transcripts. For the field plants, the transcript profiles for leaves of 2003 plants show no enrichment for *P5CS A* or *B* transcripts and proline content was, at best, no different than the level found in a well-watered cauline

leaf found in the growth cabinet. Thus whether drought was simulated in a growth cabinet or in combination with salt and heat stress in the field as was the case in 2003, proline did not accumulate in Yukon *Thellungiella* under water deficits. Moreover, while a salt stress treatment imposed by irrigating plants in growth chambers with saline solutions led to increased proline content, plants growing under non-drought conditions that are exposed to salt in the field (2005) also had low levels of proline compared to a well-watered cabinet-grown plant. Together these observations suggest that Yukon *Thellungiella* plants show metabolic plasticity with respect to their capacity to accumulate proline. It may be that any benefits conferred by proline under a controlled, cabinet salt stress treatment are not realized during water deficits or when plants are subjected to the multiple, concurrent stresses experienced in the field. Our findings support the conclusions of Gagneul et al. (2007) who found proline levels in the halophyte *Limonium latifolium* to respond modestly but positively to salt up-shifts but did not display strong indications of a being an osmolyte in comparison to other solutes.

Galactinol was also present at higher relative abundance in *Thellungiella* leaves from plants subjected to drought conditions in growth cabinets but was found at lower levels in the leaf tissue of field plants compared to well-watered, chamber-grown *Thellungiella* (Fig. 5). Galactinol synthase catalyzes a key step in galactinol synthesis and the expression of the gene encoding this enzyme (At1g56600) was up-regulated in *Thellungiell*a harvested from the field during both years relative to cabinet grown controls (Fig. 4). This observation suggests that the capacity for galactinol accumulation by these plants should not be limited by the absence of this enzyme. However, this data

is based upon a static snapshot of metabolites, and it is possible that galactinol fails to accumulate because it is used in other synthetic or catabolic reactions although raffinose, one such end-product, also did not accumulate in field plants (Fig. 5). Galactinol content was found to change over the course of a day with the highest level found at the 7 h (mid-day) time-point (Fig. 6). This pattern of changing abundance is consistent with a role in carbohydrate transport and both galactinol and galactinol synthase have known roles in phloem loading in other plant species (McCaskill and Turgeon, 2007).

A genome-wide analysis of *Arabidopsis* revealed that 1310 out of 1969 (~67%) genes that were differentially expressed in *Arabidopsis* subjected to drought were also significantly up or down-regulated by exogenously applied abscisic acid (ABA) or an analog of ABA (Huang et al., 2007, 2008). Our study shows 15 genes whose transcript levels were higher in leaf tissue from 2003 and in plants subjected to drought treatments in cabinets. Expression of these genes was suppressed in plants recovering from drought treatment and showed no increased expression in 2005 plant tissue relative to well-watered cabinet plants. With one exception (At5g60360), these genes were classified by Huang et al. (2007) as ABA-responsive. Among the 2003-specific changes, the gene product encoded by At2g46680 is an ABA-responsive homeodomain Leu zipper transcription factor (ATHB7) that has been reported to be upregulated by drought, salinity, and white light (Söderman et al., 1996; Henriksson et al., 2005) . Transcripts associated with *ATHB7* were over 3-fold more abundant in leaves of 2003 *Thellungiella* field plants relative to well-watered control plants and in a previous study were not found to be more abundant in drought-treated plants in growth cabinets (Wong et al., 2006).

Thus water deficits simulated in growth cabinets did not elicit the same response with respect to expression of *ATHB7* as a prolonged drought in the field.

The capacity of a plant to withstand an environmental extreme will probably not reside with one metabolite, protein or transcript but is more likely to be a combination of many control levels working in concert (Mazzucotelli et al., 2008). If this is true then it is likely that an extremophile may be an organism that can successfully orchestrate its response to maximize the advantages that various response pathways may confer. One dramatic albeit descriptive indication of *Thellungiella*'s capacity to show flexibility toward its environment is displayed by the contrasting field and cabinet phenotypes. It is perhaps not surprising that plants with such striking and distinct architectures should also have underlying differences in transcriptome and metabolome profiles. Nonetheless, we found many traits to be expressed by plants in the field and in growth cabinets. As such, some traits are expressed by plants experiencing stress regardless of where they are growing and it is tempting to propose that these will be the genes and gene products that offer the greatest potential for crop improvement.

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Figure 1. Phenotype of *Thellungiella* grown in controlled environments and at the Yukon field site

A) 9-week old *Thellungiella* grown in controlled environment cabinets showing seed-bearing siliques and a prominent rosette (scale: white or black bar =2 cm).

B) *Thellungiella* found on salt flats have cauline leaves borne on multiple stems that terminate in flowers and siliques, but lack the prominent rosette observed in plants grown in growth cabinets. The inset shows an example of *Thellungiella* found on salt flats with one main stem.

C) *Thellungiella* growing on Yukon field site in 2003 amongst dried up vegetation.

D) *Thellungiella* growing on Yukon field site in 2005

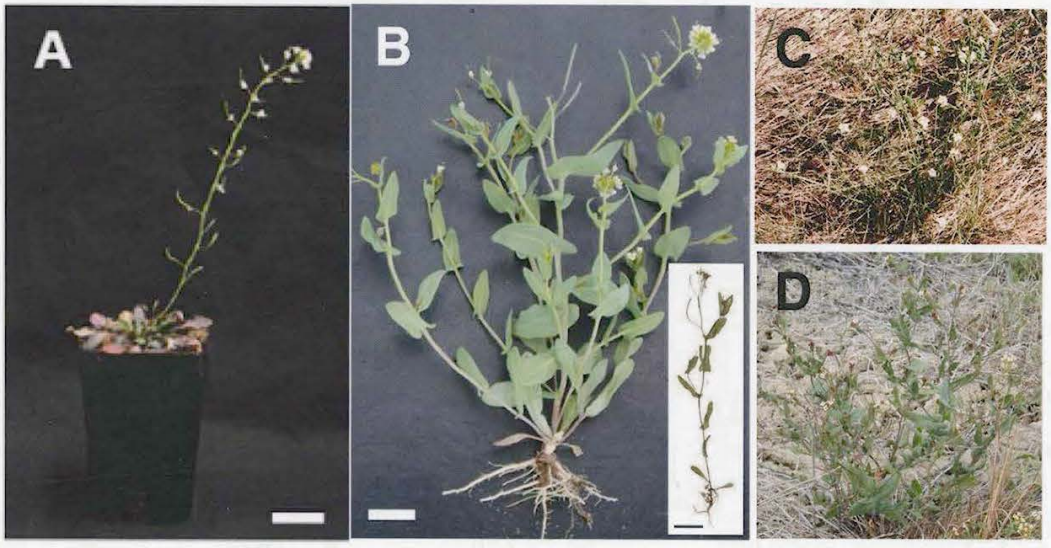

A
B
C
D

Figure 2. Meteorological conditions near Yukon field site

Data were taken from Environment Canada for the Whitehorse site and the months of May to July for 2003 and 2005 (www.climate.weatheroffice.ec.gc.ca/climate_normals/). Asterisks denote dates on which tissue was harvested from *Thellungiella* plants growing in the field.

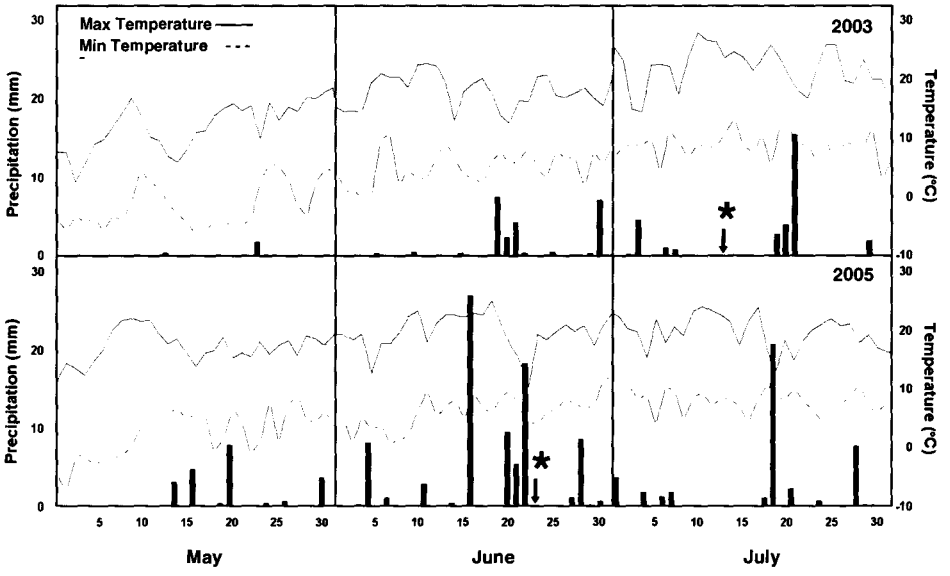
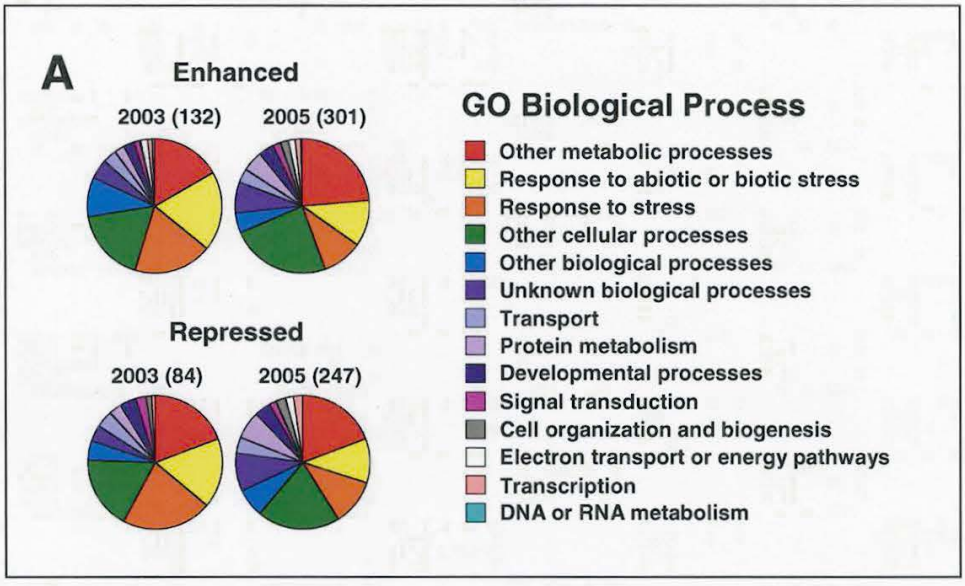

Max Temperature
Min Temperature
2003
2005
Precipitation (mm)
Temperature (°C)
May
June
July

Figure 3. Gene ontology classification of transcripts differentially expressed in *Thellungiella* growing at the Yukon field site.

The transcripts enhanced or repressed in Yukon *Thellungiella* harvested from the field in 2003 and 2005 relative to growth chamber grown plants were classified into the (A) biological processes and (B) molecular function categories using the TAIR9 gene ontology categorization (www.arabidopsis.org/tools/bulk/go/index.jsp). The number of transcripts that are differentially expressed in Yukon *Thellungiella* harvested from the field in 2003 or 2005 relative to growth chamber grown plants are indicated in brackets.


A
Enhanced
2003 (132)
2005 (301)
Repressed
2003 (84)
2005 (247)
GO Biological Process
Other metabolic processes
Response to abiotic or biotic stress
Response to stress
Other cellular processes
Other biological processes
Unknown biological processes
Transport
Protein metabolism
Developmental processes
Signal transduction
Cell organization and biogenesis
Electron transport or energy pathways
Transcription
DNA or RNA metabolism

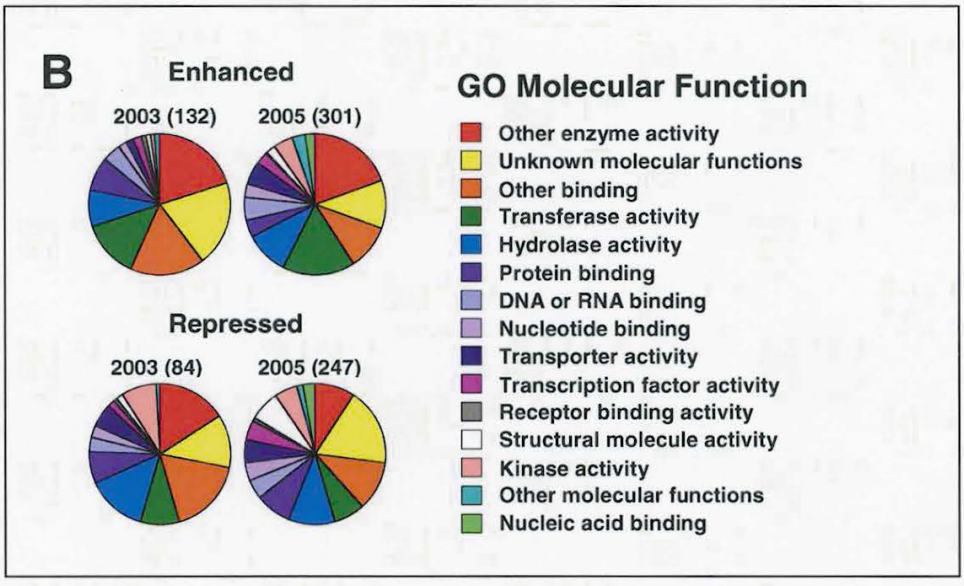

B
Enhanced
2003 (132)
2005 (301)
Repressed
2003 (84)
2005 (247)
GO Molecular Function
Other enzyme activity
Unknown molecular functions
Other binding
Transferase activity
Hydrolase activity
Protein binding
DNA or RNA binding
Nucleotide binding
Transporter activity
Transcription factor activity
Receptor binding activity
Structural molecule activity
Kinase activity
Other molecular functions
Nucleic acid binding

Figure 4. Hierarchical cluster analysis of genes differentially expressed in *Thellungiella* harvested from the Yukon field site in 2003 and 2005 and *Thellungiella* exposed to abiotic stresses in growth cabinets

Transcript expression levels were converted to a ratio of the treatment over the appropriate control. Ratios greater or less than the controls by at least 1.5 fold were then $\log_{10}$ transformed before being subjected to HCA and illustrated as a heatmap. White indicates no difference in gene expression between the sample and its respective control while red or blue indicates that gene expression was higher (positive number) or lower (negative number), respectively, relative to a control. Values in legend are fold-differences compared to the appropriate control.

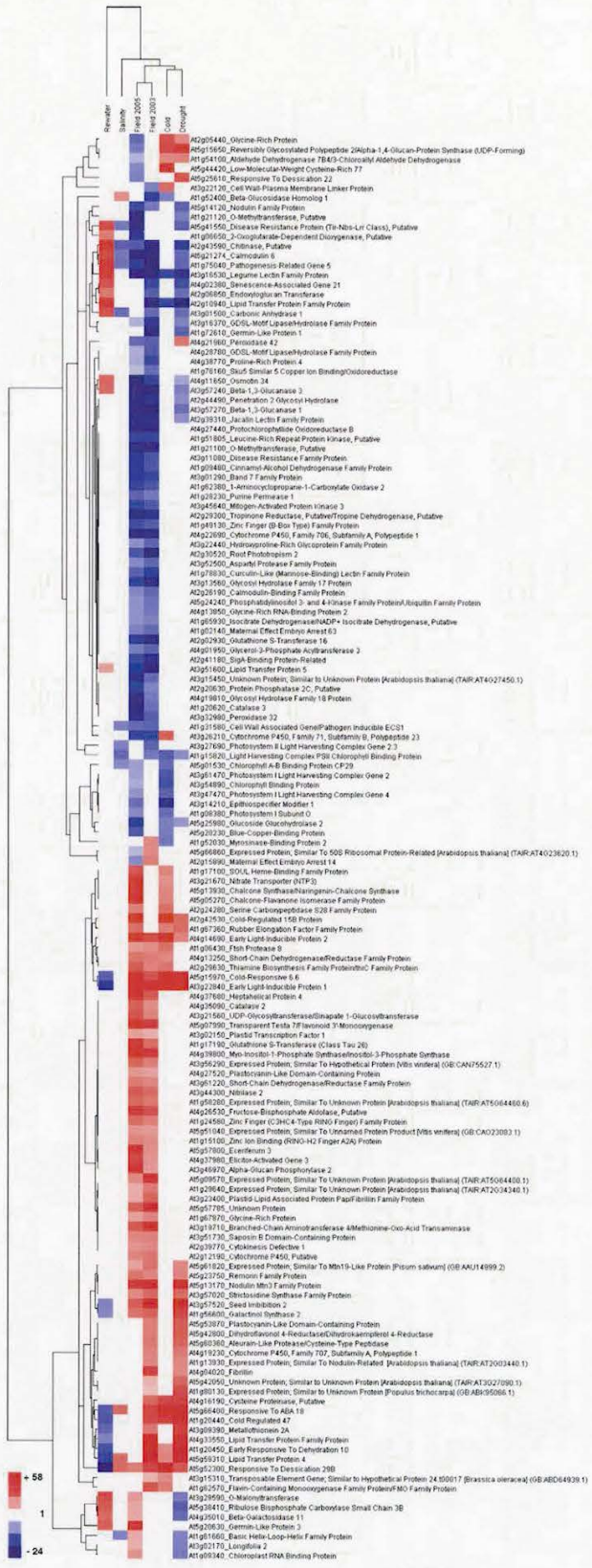

Rewater
Salinity
Field 2005
Field 2003
Cold
Drought
At2g05440_Glycine-Rich Protein
At5g15650_Reversibly Glycosylated Polypeptide 2/Alpha-1,4-Glucan-Protein Synthase (UDP-Forming)
At1g54100_Aldehyde Dehydrogenase 7B4/3-Chloroallyl Aldehyde Dehydrogenase
At5g44420_Low-Molecular-Weight Cysteine-Rich 77
At5g25610_Responsive To Dessication 22
At3g22120_Cell Wall-Plasma Membrane Linker Protein
At1g52400_Beta-Glucosidase Homolog 1
At5g14120_Nodulin Family Protein
At1g21120_O-Methyltransferase, Putative
At5g41550_Disease Resistance Protein (Tir-Nbs-Lrr Class), Putative
At1g06650_2-Oxoglutarate-Dependent Dioxygenase, Putative
At2g43590_Chitinase, Putative
At5g21274_Calmodulin 6
At1g75040_Pathogenesis-Related Gene 5
At3g16530_Legume Lectin Family Protein
At4g02380_Senescence-Associated Gene 21
At2g06850_Endoglucan Transferase
At2g10940_Lipid Transfer Protein Family Protein
At3g01500_Carbonic Anhydrase 1
At3g16370_GDSL-Motif Lipase/Hydrolase Family Protein
At1g72610_Germin-Like Protein 1
At4g21960_Peroxidase 42
At4g28780_GDSL-Motif Lipase/Hydrolase Family Protein
At4g38770_Proline-Rich Protein 4
At1g76160_Blus5 Similar 5 Copper Ion Binding/Oxidoreductase
At4g11650_Osmotin 34
At3g57240_Beta-1,3-Glucanase 3
At2g44490_Penetration 2 Glycosyl Hydrolase
At3g57270_Beta-1,3-Glucanase 1
At2g39310_Jacalin Lectin Family Protein
At4g27440_Protochlorophyllide Oxidoreductase B
At1g51805_Leucine-Rich Repeat Protein Kinase, Putative
At1g21100_O-Methyltransferase, Putative
At3g11080_Disease Resistance Family Protein
At1g09480_Cinnamel-Alcohol Dehydrogenase Family Protein
At5g01290_Band 7 Family Protein
At1g62380_1-Aminocyclopropane-1-Carboxylate Oxidase 2
At1g28230_Purine Permease 1
At3g45640_Mitogen-Activated Protein Kinase 3
At2g29300_Tropinone Reductase, Putative/Tropine Dehydrogenase, Putative
At1g49130_Zinc Finger (B-Box Type) Family Protein
At4g22690_Cytochrome P450, Family 706, Subfamily A, Polypeptide 1
At3g22440_Hydroxyproline-Rich Glycoprotein Family Protein
At2g30520_Root Phototropism 2
At3g52500_Aspartyl Protease Family Protein
At1g78830_Curculin-Like (Mannose-Binding) Lectin Family Protein
At3g13560_Glycosyl Hydrolase Family 17 Protein
At2g26190_Calmodulin-Binding Family Protein
At5g24240_Phosphatidylinositol 3- and 4-Kinase Family Protein/Ubiquitin Family Protein
At4g13850_Glycine-Rich RNA-Binding Protein 2
At1g65930_Isocitrate Dehydrogenase(NADP+ Isocitrate Dehydrogenase, Putative
At1g02140_Maternal Effect Embryo Arrest 63
At2g02930_Glutathione S-Transferase 16
At4g01950_Glycerol-3-Phosphate Acyltransferase 3
At2g41180_SigA-Binding Protein-Related
At3g51600_Lipid Transfer Protein 5
At3g15450_Unknown Protein; Similar to Unknown Protein [Arabidopsis thaliana] (TAIR:AT4G27450.1)
At2g20630_Protein Phosphatase 2C, Putative
At4g19810_Glycosyl Hydrolase Family 18 Protein
At1g20620_Catalase 3
At3g32960_Peroxidase 32
At1g31580_Cell Wall Associated Gene/Pathogen Inducible ECS1
At3g26210_Cytochrome P450, Family 71, Subfamily B, Polypeptide 23
At3g27690_Photosystem II Light Harvesting Complex Gene 2.3
At1g15820_Light Harvesting Complex PSII Chlorophyll Binding Protein
At5g01530_Chlorophyll A-B Binding Protein CP29
At5g01470_Photosystem I Light Harvesting Complex Gene 2
At3g54890_Chlorophyll Binding Protein
At3g47470_Photosystem I Light Harvesting Complex Gene 4
At3g14210_Epithiospecifier Modifier 1
At1g08380_Photosystem I Subunit O
At5g25980_Glucoside Glucohydrolase 2
At5g20230_Blue-Copper-Binding Protein
At1g52030_Myrosinase-Binding Protein 2
At5g66860_Expressed Protein; Similar To 50S Ribosomal Protein-Related [Arabidopsis thaliana] (TAIR:AT4G23620.1)
At2g15890_Maternal Effect Embryo Arrest 14
At1g17100_SOUL Heme-Binding Family Protein
At3g21670_Nitrate Transporter (NTP3)
At5g13930_Chalcone Synthase/Naringenin-Chalcone Synthase
At5g05270_Chalcone-Flavanone Isomerase Family Protein
At2g24280_Serine Carboxypeptidase S28 Family Protein
At3g47520_Cold-Regulated 15B Protein
At1g67360_Rubber Elongation Factor Family Protein
At4g14690_Early Light-Inducible Protein 2
At1g06430_Ftsh Protease 9
At4g13250_Short-Chain Dehydrogenase/Reductase Family Protein
At2g29630_Thiamine Biosynthesis Family Protein/ThiC Family Protein
At5g15970_Cold-Responsive 6.6
At3g22840_Early Light-Inducible Protein 1
At4g37680_Heptahelical Protein 4
At4g25090_Catalase 2
At3g21560_UDP-Glycosyltransferase/Sinapate 1-Glucosyltransferase
At5g07990_Transparent Testa 7/Flavonoid 3'-Monooxygenase
At3g02150_Plastid Transcription Factor 1
At1g17190_Glutathione S-Transferase (Class Tau 26)
At4g39800_Myo-Inositol-1-Phosphate Synthase/Inositol-3-Phosphate Synthase
At3g56290_Expressed Protein; Similar To Hypothetical Protein [Vitis vinifera] (GB:CAN75527.1)
At4g27520_Plastocyanin-Like Domain-Containing Protein
At3g61220_Short-Chain Dehydrogenase/Reductase Family Protein
At3g44300_Nitrilase 2
At1g58280_Expressed Protein; Similar To Unknown Protein [Arabidopsis thaliana] (TAIR:AT5G64460.6)
At4g26530_Fructose-Bisphosphate Aldolase, Putative
At1g24580_Zinc Finger (C3HC4-Type RING Finger) Family Protein
At5g51040_Expressed Protein; Similar To Unnamed Protein Product [Vitis vinifera] (GB:CAO23083.1)
At1g15100_Zinc Ion Binding (RING-H2 Finger A2A) Protein
At5g57800_Exordium 3
At4g37980_Elicitor-Activated Gene 3
At3g46970_Alpha-Glucan Phosphorylase 2
At5g09570_Expressed Protein; Similar To Unknown Protein [Arabidopsis thaliana] (TAIR:AT5G64400.1)
At1g29640_Expressed Protein; Similar To Unknown Protein [Arabidopsis thaliana] (TAIR:AT2G34340.1)
At3g23400_Plastid-Lipid Associated Protein Pap/Fibrillin Family Protein
At5g57785_Unknown Protein
At1g67870_Glycine-Rich Protein
At3g19710_Branched-Chain Aminotransferase 4/Methionine-Oxo-Acid Transaminase
At3g51730_Saposin B Domain-Containing Protein
At2g39770_Cytokinesis Defective 1
At2g12190_Cytochrome P450, Putative
At5g61820_Expressed Protein; Similar To Mtn19-Like Protein [Pisum sativum] (GB:AAU14999.2)
At5g23750_Remorin Family Protein
At5g13170_Nodulin Mtn3 Family Protein
At3g57020_Strictosidine Synthase Family Protein
At3g57520_Seed Imbibition 2
At1g56600_Galactinol Synthase 2
At5g53870_Plastocyanin-Like Domain-Containing Protein
At5g42800_Dihydroflavonol 4-Reductase/Dihydrokaempferol 4-Reductase
At5g60360_Aleurain-Like Protease/Cysteine-Type Peptidase
At4g19230_Cytochrome P450, Family 707, Subfamily A, Polypeptide 1
At1g13930_Expressed Protein; Similar To Nodulin-Related [Arabidopsis thaliana] (TAIR:AT2G03440.1)
At4g04020_Fibrillin
At5g42050_Unknown Protein; Similar to Unknown Protein [Arabidopsis thaliana] (TAIR:AT3G27090.1)
At1g80130_Expressed Protein; Similar to Unknown Protein [Populus trichocarpa] (GB:ABK95066.1)
At4g16190_Cysteine Proteinase, Putative
At5g66400_Responsive To ABA 18
At1g20440_Cold Regulated 47
At3g09390_Metallothionein 2A
At4g33550_Lipid Transfer Protein Family Protein
At1g20450_Early Responsive To Dehydration 10
At5g59310_Lipid Transfer Protein 4
At5g52300_Responsive To Dessication 29B
At3g15310_Transposable Element Gene; Similar to Hypothetical Protein 24.t00017 [Brassica oleracea] (GB:ABD64939.1)
At1g62570_Flavin-Containing Monooxygenase Family Protein/FMO Family Protein
At3g29590_O-Malonyltransferase
At5g38410_Ribulose Bisphosphate Carboxylase Small Chain 3B
At4g35010_Beta-Galactosidase 11
At5g20630_Germin-Like Protein 3
At1g61660_Basic Helix-Loop-Helix Family Protein
At3g02170_Longifolia 2
At1g09340_Chloroplast RNA Binding Protein
+ 58
1
- 24

Figure 5. Hierarchical cluster analysis of metabolites in *Thellungiella* harvested from the Yukon field site in 2003 and 2005 and chamber grown plants exposed to abiotic stresses.

MSTs that were significantly ($P<0.05$) higher or lower in *Thellungiella* growing at the Yukon field site relative to unstressed growth cabinet-grown controls are compared to similar values for *Thellungiella* subjected to abiotic stress treatments imposed in growth cabinets. If an MST was only significantly higher or lower in one field year, it was not included in the analysis. The mean MST abundance was expressed as the fold ratio of treated or field over appropriate control levels. Those ratios that were higher or lower by a factor of 1.5-fold were then $\log_{10}$ transformed before being subjected to HCA and illustrated as a heatmap. White indicates no difference in MST abundance between the sample and its respective control while red or blue indicates that the MST is detected at higher (positive number) or lower (negative number) levels, respectively, relative to a control. The values in the legend are fold differences compared to the appropriate control.

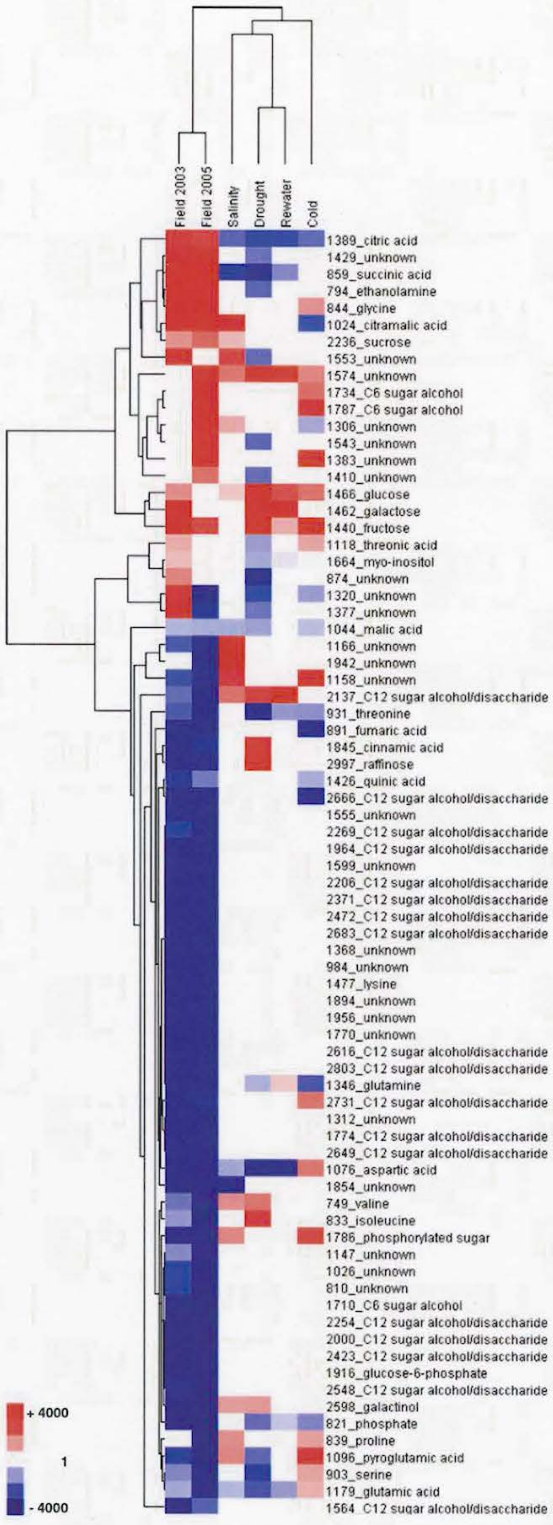
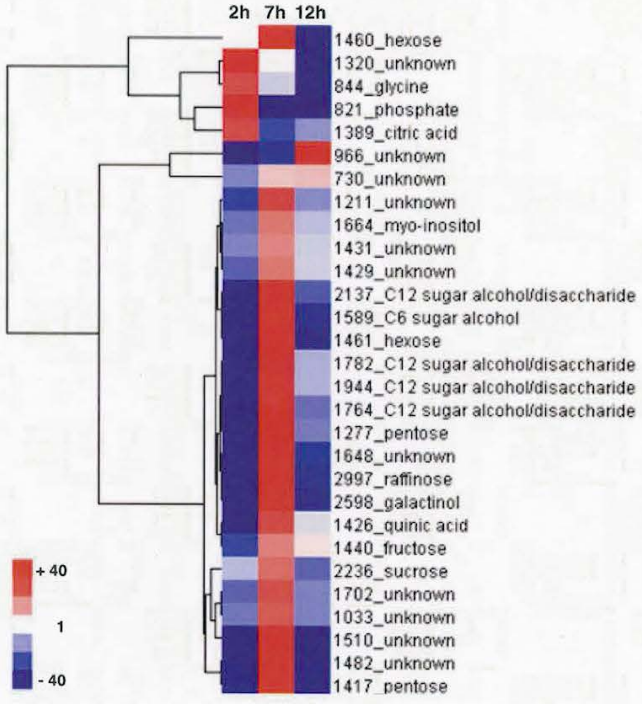

Field 2003
Field 2005
Salinity
Drought
Rewater
Cold
1389_citric acid
1429_unknown
859_succinic acid
794_ethanolamine
844_glycine
1024_citramalic acid
2236_sucrose
1553_unknown
1574_unknown
1734_C6 sugar alcohol
1787_C6 sugar alcohol
1306_unknown
1543_unknown
1383_unknown
1410_unknown
1466_glucose
1462_galactose
1440_fructose
1118_threonic acid
1664_myo-inositol
874_unknown
1320_unknown
1377_unknown
1044_malic acid
1166_unknown
1942_unknown
1158_unknown
2137_C12 sugar alcohol/disaccharide
931_threonine
891_fumaric acid
1845_cinnamic acid
2997_raffinose
1426_quinic acid
2666_C12 sugar alcohol/disaccharide
1555_unknown
2269_C12 sugar alcohol/disaccharide
1964_C12 sugar alcohol/disaccharide
1599_unknown
2206_C12 sugar alcohol/disaccharide
2371_C12 sugar alcohol/disaccharide
2472_C12 sugar alcohol/disaccharide
2683_C12 sugar alcohol/disaccharide
1368_unknown
984_unknown
1477_lysine
1894_unknown
1956_unknown
1770_unknown
2616_C12 sugar alcohol/disaccharide
2803_C12 sugar alcohol/disaccharide
1346_glutamine
2731_C12 sugar alcohol/disaccharide
1312_unknown
1774_C12 sugar alcohol/disaccharide
2649_C12 sugar alcohol/disaccharide
1076_aspartic acid
1854_unknown
749_valine
833_isoleucine
1786_phosphorylated sugar
1147_unknown
1026_unknown
810_unknown
1710_C6 sugar alcohol
2254_C12 sugar alcohol/disaccharide
2000_C12 sugar alcohol/disaccharide
2423_C12 sugar alcohol/disaccharide
1916_glucose-6-phosphate
2548_C12 sugar alcohol/disaccharide
2598_galactinol
821_phosphate
839_proline
1096_pyroglutamic acid
903_serine
1179_glutamic acid
1564_C12 sugar alcohol/disaccharide
+ 4000
1
- 4000

Figure 6. Hierarchical cluster analysis of metabolites present at different levels throughout the day in *Thellungiella* harvested from the Yukon field site.

The mean of measurements from five individual plants harvested at 2 h, 7 h and 12 h from the beginning of the light period were expressed as a ratio relative to the mean abundance for all three timepoints). The fold ratios were then $\log_{10}$ transformed subjected to HCA and illustrated as a heatmap. MSTs whose levels were significantly different ($P<0.05$) at any time-point from the average of the day are illustrated. White indicates no difference between the mean MST abundance at a given time point and the daily average while red or blue indicates that the MST is present at significantly higher (positive number) or lower (negative number) levels, respectively, compared to the daily average. The values in the legend are fold differences compared to the average.


2h
7h
12h
1460_hexose
1320_unknown
844_glycine
821_phosphate
1389_citric acid
966_unknown
730_unknown
1211_unknown
1664_myo-inositol
1431_unknown
1429_unknown
2137_C12 sugar alcohol/disaccharide
1589_C6 sugar alcohol
1461_hexose
1782_C12 sugar alcohol/disaccharide
1944_C12 sugar alcohol/disaccharide
1764_C12 sugar alcohol/disaccharide
1277_pentose
1648_unknown
2997_raffinose
2598_galactinol
1426_quinic acid
1440_fructose
2236_sucrose
1702_unknown
1033_unknown
1510_unknown
1482_unknown
1417_pentose
+ 40
1
- 40

Supplemental Table S1. *Soil properties and mineral composition of Yukon Thellungiella harvested from the experimental field site near Whitehorse, YT, and from growth chamber grown plants.*

	Soil Properties		*Thellungiella* Mineral Composition		
Parameter	Field Site 1	Field Site 2	Field Site 1	Field Site 2	Chamber
pH	8.3	8.3			
E.C. (dS m^{-1})	15.68	15.47			
Concentration (ppm)					
NH_4^+	1	1			
NO_3^-	10	60			
Total C	26100	100500			
Total N	2600	10700	31900	26500	42000
P	26	47	2800	2900	9200
K	236	131	22300	22200	43700
Ca	521	469	15800	14000	10400
Na	2224	2076	13630	7100	2058
Mg	1456	1583	6000	6100	4200

Supplemental Table S2. *Transcripts differentially expressed in Yukon Thellungiella harvested from the field in 2003 and 2005.*

Values represent fold-ratio between field (F) and chamber grown Yukon *Thellungiella*, where a positive or negative number represents an enhancement or repression of transcript expression in field-grown, respectively, compared to cabinet grown plants. Column E denotes whether annotated gene is associated with stress based on gene ontology categorization (Yes=stress associated). Column F denotes whether the annotated gene is associated with abscisic acid signaling (S) or metabolism (M), or responsive to abscisic acid stimulus (R) based on gene ontology categorization. Column G denotes whether the annotated gene is also stress-responsive (indicated with "1") based on experiments performed in growth chambers by our research group as reported in Table 2 in Wong et al. (2006).

Transcripts down-regulated (P<0.01) in Yukon Thellungiella harvested from the field in 2003 and 2005

A	B	C	D	E	F	G
AGI code	Annotation	F 2003 / Chamber	F 2005 / Chamber			
At2g43590	Chitinase, Putative	-23.7	-3.7			1
At3g57270	Beta-1,3-Glucanase 1	-18.6	-14.3			1
At3g57240	Beta-1,3-Glucanase 3	-14.7	-20.0	Yes		1
At1g75040	Pathogenesis-Related Gene 5	-10.9	-5.3	Yes		1
At2g30520	Root Phototropism 2	-10.7	-4.0	Yes		
At3g51600	Lipid Transfer Protein 5	-10.3	-5.6			1
At5g21274	Calmodulin 6	-9.3	-10.0			1
At4g27440	Protochlorophyllide Oxidoreductase B	-9.0	-9.1			
At1g21100	O-Methyltransferase, Putative	-8.8	-7.7			
At3g13560	Glycosyl Hydrolase Family 17 Protein	-8.2	-5.0			
At3g16530	Legume Lectin Family Protein	-8.0	-12.5			1
At2g02930	Glutathione S-Transferase 16	-7.6	-5.6			
At4g22690	Cytochrome P450, Family	-7.1	-9.5			
At2g39310	Jacalin Lectin Family Protein	-7.0	-7.1			1
At2g44490	Penetration 2 Glycosyl Hydrolase	-6.5	-8.3	Yes		1
At2g41180	SigA-Binding Protein-Related	-6.4	-4.5			
At2g29300	Tropinone Reductase, Putative	-6.3	-8.3			
At3g16370	GDSL-Motif Lipase/Hydrolase	-6.0	-1.8			1
At1g78830	Curculin-Like Lectin Family Protein	-5.2	-3.4			
At4g11650	Osmotin 34	-5.1	-21.4	Yes		1
At3g26210	Cytochrome P450, Family 71	-4.9	-5.0			1
At3g01290	Band 7 Family Protein	-4.8	-5.6			
At1g51805	Leucine-Rich Repeat Protein Kinase	-4.7	-4.8			
At4g38770	Proline-Rich Protein 4	-4.6	-1.8			
At3g11080	Disease Resistance Family Protein	-3.9	-5.6	Yes		
At1g09480	Cinnamyl-Alcohol Dehydrogenase	-3.8	-5.6			
At1g62380	ACC Oxidase 2	-3.8	-4.9	Yes		
At3g45640	Mitogen-Activated Protein Kinase 3	-3.7	-4.8	Yes	S	
At4g28780	GDSL-Motif Lipase/Hydrolase	-3.7	-1.7			
At4g21960	Peroxidase 42	-3.6	-3.2	Yes		1
At1g49130	Zinc Finger Family Protein	-3.5	-4.3			
At3g32980	Peroxidase 32	-3.5	-6.7	Yes		
At4g01950	Glycerol-3-Phosphate Acyltransferase 3	-3.4	-2.7			
At1g31580	Pathogen Inducible ECS1	-3.3	-3.1	Yes		1
At3g22440	Hydroxyproline-Rich Glycoprotein	-3.3	-2.2			
At3g52500	Aspartyl Protease Family Protein	-3.2	-2.4			
At2g26190	Calmodulin-Binding Family Protein	-3.2	-2.5			
At1g02140	Maternal Effect Embryo Arrest 63	-3.1	-2.6			
At1g76160	Sku5 Oxidoreductase	-3.0	-1.5			
At3g15450	Unknown Protein	-3.0	-7.0			
At1g20620	Catalase 3	-2.9	-9.7	Yes		
At5g24240	Phosphatidylinositol 3- and 4-Kinase	-2.9	-2.2			
At4g13850	Glycine-Rich RNA-Binding Protein 2	-2.8	-2.2	Yes		

At5g14120	Nodulin Family Protein	-2.8	-4.5			1
At4g19810	Glycosyl Hydrolase Family 18 Protein	-2.8	-7.7			
At1g28230	Purine Permease 1	-2.7	-3.3			
At1g65930	Isocitrate Dehydrogenase, Putative	-2.6	-2.2	Yes		
At2g20630	Protein Phosphatase 2C, Putative	-2.5	-5.0			

Transcripts up-regulated (P<0.01) in Yukon Thellungiella harvested from the field in 2003 and 2005

A	B	C	D	E	F	G
AGI Code	Annotation	F 2003 / Chamber	F 2005 / Chamber			
At3g02150	Plastid Transcription Factor 1	1.6	1.9			
At4g27520	Plastocyanin-Like Protein	1.9	2.1			
At3g46970	Alpha-Glucan Phosphorylase 2	2.1	5.9	Yes		
At5g51040	Expressed Protein	2.1	2.2			
At4g37980	Elicitor-Activated Gene 3	2.2	4.6	Yes		
At4g35090	Catalase 2	2.3	3.7	Yes		
At5g57800	Eceriferum 3	2.3	5.5			
At2g39770	Cytokinesis Defective 1	2.4	2.2	Yes		
At1g06430	FtsH Protease 8	2.4	2.1			1
At1g15100	Zinc Ion Binding Protein	2.4	2.6		S	
At5g23750	Remorin Family Protein	2.5	3.0			1
At3g61220	Short-Chain Dehydrogenase/Reductase	2.5	2.9	Yes		
At1g67870	Glycine-Rich Protein	2.5	1.9			
At2g29630	Thiamine Biosynthesis Family Protein	2.6	3.8			1
At3g51730	Saposin B Domain-Containing Protein	2.6	2.6			
At3g21560	Sinapate 1-Glucosyltransferase	2.6	4.1			
At1g24580	Zinc Finger Family Protein	2.7	3.4			
At3g56290	Expressed Protein	2.7	3.6			
At1g29640	Expressed Protein	2.7	1.8			
At4g13250	Short-Chain Dehydrogenase/Reductase	2.7	3.1			1
At2g12190	Cytochrome P450, Putative	2.8	2.6			
At1g58280	Expressed Protein	2.8	3.5			
At5g61820	Expressed Protein	3.1	1.9			1
At1g17190	Glutathione S-Transferase	3.2	5.3	Yes		
At3g44300	Nitrilase 2	3.2	4.0	Yes		
At3g57020	Strictosidine Synthase Family Protein	3.3	2.4			1
At3g23400	Plastid-Lipid Associated Protein	3.5	1.8			
At1g56600	Galactinol Synthase 2	3.6	3.1			1
At4g37680	Heptahelical Protein 4	3.8	8.5			
At5g15970	Cold-Responsive 6.6	4.1	6.4	Yes	R	1
At4g26530	Fructose-Bisphosphate Aldolase	4.2	5.5			
At5g52300	Responsive To Dessication 29B	4.4	2.7	Yes	S	1
At4g14690	Early Light-Inducible Protein 2	4.6	29.0	Yes		1
At5g07990	Flavonoid 3'-Monooxygenase	5.2	10.2	Yes		
At4g39800	Myo-Inositol-1-Phosphate Synthase	5.3	9.2			
At5g09570	Expressed Protein	5.3	2.5			
At3g19710	Methionine-Oxo-Acid Transaminase	5.8	4.0			
At5g13170	Nodulin Mtn3 Family Protein	5.9	5.3			1
At5g57785	Unknown Protein	6.1	3.7			
At3g57520	Seed Imbibition 2	8.2	3.9			1
At3g22840	Early Light-Inducible Protein 1	13.0	52.9	Yes		1

Transcripts up-regulated (P<0.01) in Yukon Thellungiella harvested from the field in 2003, down-regulated (P<0.01) in 2005

A	B	C	D	E	F	G
AGI code	Annotation	F 2003 / Chamber	F 2005 / Chamber			
At2g15890	Maternal Effect Embryo Arrest 14	2.8	-2.4			
At5g66860	Expressed Protein	2.4	-1.6			

Transcripts down-regulated (P<0.01) in Yukon Thellungiella harvested from the field in 2003 only

	B	C	D	E	F	G
AGI code	Annotation	F 2003 / Chamber	F 2005 / Chamber			
At1g72610	Germin-Like Protein 1	-20.5				1
At1g56340	Calreticulin 1	-11.2		Yes		
At2g10940	Lipid Transfer Protein Family Protein	-10.6				1
At3g16470	Jacalin Lectin Family Protein	-9.3		Yes		
At5g41750	Disease Resistance Protein	-8.9		Yes		
At5g22580	Expressed Protein	-7.5				
At1g74710	Isochorismate Synthase 1	-6.3		Yes		
At1g52400	Beta-Glucosidase Homolog 1	-5.0				1
At4g31500	Cytochrome P450 Monooxygenase	-4.9		Yes		
At1g71695	Peroxidase 12	-4.7		Yes		
At1g66980	Glycerophosphoryl Phosphodiesterase	-4.7				
At4g02380	Senescence-Associated Gene 21	-4.6		Yes		1
At1g80440	Kelch Repeat-Containing F-Box	-4.3				
At1g26770	Expansin A10	-4.2				
At2g45470	Arabinogalactan Protein 8	-4.2				
At2g21650	Maternal Effect Embryo Arrest 3	-4.1				
At3g25020	Disease Resistance Family Protein	-3.8		Yes		
At2g06850	Endoxyloglucan Transferase	-3.5		Yes		1
At5g14320	30S Ribosomal Protein S13	-3.4				
At3g27690	PS II Light Harvesting Complex	-3.3				1
At4g03210	Xyloglucan Endotransglucosylase	-3.3				
At2g05070	PS II Light Harvesting Complex	-3.2				
At2g28190	Copper/Zinc Superoxide Dismutase 2	-3.2		Yes		
At5g37600	Glutamate-Ammonia Ligase	-3.2				
At1g08450	Calreticulin 3	-3.2				
At3g01500	Carbonic Anhydrase 1	-3.1		Yes		1
At1g48300	Unknown Protein	-2.9				
At4g23190	Cysteine-Rich Rlk11 Kinase	-2.9		Yes		
At3g62630	Expressed Protein	-2.9				
At1g14870	Expressed Protein	-2.8		Yes		
At1g15820	PSII Chlorophyll Binding Protein	-2.8		Yes		1
At1g59870	Pleiotropic Drug Resistance 8 Protein	-2.7		Yes		
At3g22120	Plasma Membrane Linker Protein	-2.6				1
At1g44446	Chlorophyllide A Oxygenase	-2.6				
At5g40780	Lysine Histidine Transporter 1	-2.5				
At3g17390	S-Adenosylmethionine Synthase 3	-2.3		Yes		

Transcripts up-regulated (P<0.01) in Yukon Thellungiella harvested from the field in 2003 only

A	B	C	D	E	F	G
AGI code	Annotation	F 2003 / Chamber	F 2005 / Chamber			
At1g80130	Expressed Protein	1.5		Yes		1
At3g15310	Transposable Element Gene	1.6				1
At1g22370	UDP-Glucosyl Transferase 85A5	1.6				
At3g55430	Beta-1,3-Glucanase, Putative	1.6				
At5g42050	Unknown Protein	1.6				1
At1g27770	Autoinhibited Ca2+ -ATPase 1	1.7				
At1g47510	Endonuclease/Exonuclease/Phosphatase	1.7			R	
At2g34640	Plastid Transcriptionally Active 12	1.8				
At1g09070	Soybean Regulated by Cold 2	1.9				
At2g16890	UDP-Glucosyl Transferase	1.9				
At3g23920	Beta-Amylase 1	2.0				
At4g23600	Coronatine Induced 1, JA Responsive 2	2.1		Yes	R	
At1g64370	Unknown Protein	2.1				
At4g38740	Peptidyl-Prolyl Cis-Trans Isomerase	2.1				
At3g08510	Phospholipase C 2	2.1				
At5g09590	Heat Shock Protein 70	2.1		Yes		
At1g63810	Nrap Family Protein	2.1				
At2g33590	Cinnamoyl-CoA Reductase Family	2.2				
At3g56310	Alpha-Galactosidase/Melibiase, Putative	2.2				
At2g17695	Expressed Protein	2.3				
At3g10985	Wound-Induced Protein 12/SAG20	2.3		Yes		
At1g80530	Nodulin Family Protein	2.3				
At5g48180	Kelch Repeat-Containing Protein	2.3				
At1g30360	Early-Responsive To Dehydration 4	2.3				
At4g35460	NADPH-Thioredoxin Reductase 1	2.3				
At1g76130	Alpha-Amylase-Like 2	2.3				
At1g05340	Expressed Protein	2.3				
At1g13930	Expressed Protein	2.4		Yes		1
At1g29330	ER Lumen Retaining Receptor 2	2.4				
At2g43100	Aconitase Containing Protein	2.5				
At1g21680	Expressed Protein	2.5				
At2g01150	Zinc Ion Binding Protein	2.5				
At1g52030	Myrosinase-Binding Protein 2	2.5		Yes		1
At2g37220	RNA-Binding Protein Cp29, Putative	2.5		Yes		
At5g42980	Thioredoxin H-Type 3 Protein	2.5		Yes		
At4g34950	Nodulin Family Protein	2.5				
At1g31230	Aspartate Kinase	2.6				
At5g52310	Cold Regulated 78	2.6		Yes	R	
At5g60600	4-OH-3-Mebut-2-en-1-yl DiP Synthase	2.6		Yes		
At4g19230	Cytochrome P450	2.6		Yes	M	1
At1g17870	Yellow-Green 3	2.6		Yes		
At2g29450	Glutathione S-Transferase	2.6		Yes		
At5g53870	Plastocyanin-Like Protein	2.7				1
At1g68050	Flavin-Binding Kelch Domain Protein	2.7		Yes		
At1g78380	Glutathione Transferase 8	2.7		Yes		
At1g19570	Dehydroascorbate Reductase	2.7				
At5g42800	Dihydroflavonol 4-Reductase	2.7				1
At4g01610	Cathepsin B-Like Cysteine Protease	2.7				
At5g16970	2-Alkenal Reductase	2.7		Yes		
At2g38230	Pyridoxine Biosynthesis 1.1	2.8		Yes		
At5g56600	Profilin 3	2.8				
At3g62830	UDP-Glucuronic Acid Decarboxylase 2	2.8				
At4g39730	Lipid-Associated Family Protein	2.8				
At1g55490	Chaperonin 60 Beta	2.8		Yes		
At3g51780	Bcl-2-Associated Athanogene 4	2.8		Yes		
At3g09390	Metallothionein 2A	2.8				1

At1g01470	Late Embryogenesis Abundant 14	2.8		Yes		
At5g60360	Aleurain-Like Protease	2.9				1
At2g34810	FAD-Binding Protein	2.9		Yes		
At1g66390	Production Of Anthocyanin Pigment 2	2.9				
At5g23010	2-Isopropylmalate Synthase 3	2.9				
At2g40300	Ferritin 4	2.9		Yes		
At1g09240	Nicotianamine Synthase, Putative	2.9				
At3g56090	Ferritin 3	3.0		Yes		
At2g17840	Early-Responsive To Dehydration 7	3.0		Yes		
At1g16410	Cytochrome P450	3.0				
At1g16850	Unknown Protein	3.1		Yes		
At3g17800	UV-B Responsive MEB5.2 Gene	3.2		Yes		
At2g46680	Homeobox 7 Transcription Factor	3.2		Yes	S	
At3g15840	Post-Illumination Chlorophyll Protein	3.3				
At3g48510	Expressed Protein	3.3				
At5g21100	L-Ascorbate Oxidase, Putative	3.3				
At4g16190	Cysteine Proteinase, Putative	3.4				1
At2g40080	Early Flowering 4	3.5		Yes		
At1g07040	Expressed Protein	3.6				
At2g21660	Cold, Circadian Rhythm Protein	3.6		Yes		
At1g62570	Flavin-Containing Monooxygenase	4.0				1
At5g01600	Ferretin 1	4.1		Yes		
At5g58070	Lipocalin, Putative	4.2		Yes		
At1g20440	Cold Regulated 47	4.3		Yes	R	1
At5g66400	Responsive To ABA 18	4.5		Yes	R	1
At4g17090	Beta-Amylase 3, Beta-Amylase 8	4.9		Yes		
At4g04020	Fibrillin	4.9		Yes	R	1
At5g52640	Heat Shock Protein 81-1	5.8		Yes		
At1g62510	Lipid Transfer Protein Family Protein	6.2				
At5g12020	17.6 kDa Class II Heat Shock Protein	6.3		Yes		
At5g59310	Lipid Transfer Protein 4	8.1			R	1
At4g33550	Lipid Transfer Protein Family Protein	8.4				1
At1g20450	Early Responsive To Dehydration 10	8.7		Yes	R	1

Transcripts down-regulated (P<0.01) in Yukon Thellungiella harvested from the field in 2005 only

A	B	C	D	E	F	G
AGI code	Annotation	F 2003 / Chamber	F 2005 / Chamber			
At1g78850	Curculin-Like Lectin Family Protein		-13.3			
At1g21120	O-Methyltransferase, Putative		-11.1			1
At5g41550	Disease Resistance Protein		-8.3	Yes		1
At3g51000	Epoxide Hydrolase, Putative		-7.4			
At1g06650	2-OG-Dependent Dioxygenase, Putative		-5.9			1
At5g55450	Lipid Transfer Protein Family Protein		-5.9	Yes		
At5g61210	Synaptosomal-Associated Protein 33		-5.6	Yes		
At5g25980	Glucoside Glucohydrolase 2		-5.5			1
At4g16500	Cysteine Protease Inhibitor		-5.0			
At2g38530	Lipid Transfer Protein 2		-5.0			
At2g05530	Glycine-Rich Protein		-4.7			
At1g76790	O-Methyltransferase Family 2 Protein		-4.5			
At2g05520	Glycine-Rich Protein 3		-4.4	Yes	R	
At2g41430	Early Responsive To Dehydration 15		-4.3	Yes		
At5g20950	Glycosyl Hydrolase Family 3 Protein		-4.3			
At1g15670	Kelch Repeat Family Protein		-4.2			
At3g51920	Calmodulin 9		-4.0	Yes	R	
At5g20230	Blue-Copper-Binding Protein		-3.8	Yes		1
At2g20960	pEARLI4		-3.8			
At3g03870	Hypothetical Protein		-3.8			

At4g29190	Zinc Finger Family Protein		-3.5			
At5g35525	Unknown Protein		-3.4			
At5g39670	Calcium-Binding EFHand Protein		-3.3			
At3g14990	4-Me-5-OH-Me-Thiazole P Protein		-3.3			
At2g05440	Glycine-Rich Protein		-3.3			1
At2g39210	Nodulin Family Protein		-3.2			
At1g13260	ABI3/Vp1 1 Transcription Factor		-3.2			
At2g33830	Auxin Associated Family Protein		-3.1			
At5g48380	Leucine-Rich Repeat Family Protein		-3.1			
At5g61530	Small G Protein Family Protein		-3.1			
At3g14210	Epithiospecifier Modifier 1		-3.1	Yes		1
At1g56220	Auxin Associated Family Protein		-3.0			
At3g11130	Clathrin Heavy Chain, Putative		-2.9			
At1g54030	GDSL-Motif Lipase, Putative		-2.9			
At4g24190	Shepherd ATP Binding Protein		-2.9	Yes		
At5g21940	Unknown Protein		-2.9			
At5g11090	Serine-Rich Protein		-2.9			
At1g22710	Sucrose-Proton Symporter 2		-2.9			
At2g29320	Tropinone Reductase, Putative		-2.9			
At3g28930	AvrRpt2-Induced Gene 2		-2.8	Yes		
At5g61020	Evolutionary Conserved C-Terminal 3		-2.8			
At4g35480	Zinc Ion Binding Protein		-2.8			
At5g15650	Alpha-1,4-Glucan-Protein Synthase		-2.7	Yes		1
At5g21090	Leucine-Rich Repeat Protein, Putative		-2.7			
At4g05070	Unknown Protein		-2.7			
At5g24550	Glycosyl Hydrolase Family 1 Protein		-2.6	Yes		
At5g01750	Unknown Protein		-2.6			
At5g25610	Responsive To Dessication 22		-2.6	Yes	R	1
At3g14230	APETALA2 2 Transcription Factor		-2.6			
At5g18490	Unknown Protein		-2.6			
At2g30250	WRKY DNA-Binding Protein 25		-2.6	Yes		
At3g10020	Unknown Protein		-2.5	Yes		
At4g10450	60S Ribosomal Protein L9		-2.5			
At2g47470	Maternal Effect Embryo Arrest 30		-2.5			
At3g06500	Beta-Fructofuranosidase, Putative		-2.5			
At3g56510	TBP-Binding Protein, Putative		-2.5			
At5g20290	40S Ribosomal Protein S8		-2.4			
At4g26120	Ankyrin Repeat Family Protein		-2.4			
At5g58375	Unknown Protein		-2.4			
At5g44420	Low-Molecular-Weight Cys-Rich 77		-2.4	Yes		1
At1g08380	Photosystem I Subunit O		-2.4			1
At5g20010	Ras Related Nuclear Protein		-2.4	Yes		
At5g57887	Unknown Protein		-2.4			
At4g19160	Unknown Protein		-2.3			
At5g56000	Heat Shock Protein 81-4		-2.3			
At1g14710	HO-proline-Rich Glycoprotein		-2.3			
At2g17720	2OG-Fe(II) Oxygenase Family Protein		-2.3			
At1g58983	40S Ribosomal Protein S2, Putative		-2.3			
At5g27850	60S Ribosomal Protein L18 (Rpl18C)		-2.3			
At1g67970	Heat Shock Transcription Factor A8		-2.3			
At1g53280	DJ-1 Family Protein		-2.3			
At4g39670	Glycolipid Transporter		-2.3			
At3g48530	SNF1-Related Kinase		-2.3			
At4g19200	Proline-Rich Family Protein		-2.3			
At3g52800	Zinc Finger Family Protein		-2.3			
At1g67430	60S Ribosomal Protein L17		-2.2			
At3g47470	PS I Light Harvesting Complex		-2.2			1
At4g14270	PAM2 Motif Containing Protein		-2.2			
At1g14790	RNA-Dependent RNA Polymerase 1		-2.2	Yes		
At4g10970	Unknown Protein		-2.2			
At4g34670	40S Ribosomal Protein S3A		-2.2			
At3g02230	Reversibly Glycosylated Polypeptide 1		-2.2	Yes		
At1g12240	Vacuolar Invertase		-2.1			

At2g41530	S-Formylglutathione Hydrolase		-2.1			
At2g36160	40S Ribosomal Protein S14		-2.1			
At2g37190	60S Ribosomal Protein L12		-2.1	Yes		
At3g24830	60S Ribosomal Protein L13A		-2.1			
At3g51840	Acyl-CoA Oxidase 4		-2.1			
At3g25890	AP2 Transcription Factor, Putative		-2.1			
At2g44060	Late Embryogenesis Abundant Protein		-2.1	Yes		
At4g05150	Octicosapeptide/Phox/Bem1P Protein		-2.1			
At4g18630	Unknown Protein		-2.1			
At1g47540	Trypsin Inhibitor, Putative		-2.1	Yes		
At5g23860	Tubulin Beta-8		-2.1	Yes		
At1g50010	Tubulin Alpha-2 Chain		-2.1	Yes		
At5g50900	Armadillo/B-Catenin Repeat Protein		-2.0			
At3g14310	Pectin Methylesterase 3		-2.0	Yes		
At1g02130	Responsive To Abscisic Acid 1B		-2.0			
At3g61460	Zinc Ion Binding Protein		-2.0			
At3g21140	FMN Binding Protein		-2.0			
At5g53460	NADH-Glutamate Synthase 1 Gene		-2.0			
At1g73680	Pathogen-Responsive a-Dioxygenase		-2.0	Yes		
At3g26090	Regulator Of G-Protein Signaling 1		-2.0	Yes	R	
At4g20890	Tubulin Beta-9 Chain		-2.0			
At3g17410	Serine/Threonine Protein Kinase		-2.0			
At4g29410	60S Ribosomal Protein L28		-2.0			
At1g21310	Extensin 3		-2.0			
At5g01530	Chlorophyll A-B Binding Protein CP29		-2.0			1
At3g45860	Receptor-Like Protein Kinase, Putative		-2.0	Yes		
At5g12250	Beta-6 Tubulin		-2.0	Yes		
At1g61820	Beta-Glucosidase 46		-2.0			
At1g30230	Elongation Factor 1-Beta/Ef-1-Beta		-2.0			
At3g58660	60S Ribosomal Protein-Related		-1.9			
At5g60640	Protein Disulfide Isomerase-Like 1-4		-1.9	Yes		
At5g10360	40S ribosomal protein S6-2		-1.9			
At4g02350	Exocyst Complex Subunit Sec15-Like		-1.9			
At3g61470	PS I Light Harvesting Complex		-1.9			1
At2g17480	Mildew Resistance Locus O 8		-1.9	Yes		
At3g16640	Translation-Controlled Tumor Protein		-1.9	Yes		
At5g61190	Zinc Finger Related Protein		-1.9			
At4g34180	Cyclase Family Protein		-1.9	Yes		
At4g13940	Adenosylhomocysteinase		-1.9			
At2g20780	Mannitol Transporter, Putative		-1.9			
At5g09810	Actin 7		-1.9	Yes		
At1g09690	60S Ribosomal Protein L21		-1.9			
At5g39590	Unknown Protein		-1.9			
At2g34420	PS II Light Harvesting Complex		-1.9			
At3g44340	Clone Eighty-Four Transporter Protein		-1.8			
At3g17520	Late Embryogenesis Abundant Protein		-1.8			
At2g41400	Unknown Protein		-1.8			
At5g24150	Squalene Monooxygenase 1		-1.8			
At1g29920	Chlorophyll A/B-Binding Protein 2		-1.8			
At5g11740	Arabinogalactan Protein 15		-1.8			
At1g77460	Binding Protein		-1.8			
At3g54810	Blue-Micropylar Transcription Factor		-1.8			
At1g30580	GTP Binding Protein		-1.8			
At5g10010	Unknown Protein		-1.8			
At4g22670	Heat Shock Protein 70		-1.8			
At5g54900	RNA-Binding Protein 45A		-1.8			
At3g27740	Carbamoyl Phosphate Synthetase A		-1.8			
At5g02500	Heat Shock Cognate Protein 1		-1.8	Yes		
At3g54890	Chlorophyll Binding Protein		-1.8			1
At1g72150	Patellin 1		-1.8			
At1g01820	Peroxisomal Biogenesis Factor 11c		-1.8			
At4g15030	Unknown Protein		-1.8			
At1g22882	Unknown Protein		-1.8			

At1g35780	Unknown Protein		-1.8			
At3g27090	Unknown Protein		-1.8			
At3g17650	Yellow Stripe Like 5 Transporter		-1.8			
At3g07790	DiGeorge Syndrome Related Protein		-1.7			
At5g60390	Elongation Factor 1-Alpha/EF-1-Alpha		-1.7			
At4g01070	UDP-Glucosyltransferase		-1.7	Yes		
At1g55460	DNA-Binding Protein-Related		-1.7			
At4g39280	Phenylalanyl-tRNA Synthetase		-1.7			
At1g72160	Sec14 Cytosolic Factor Family Protein		-1.7			
At5g19510	Elongation Factor 1B Alpha-Subunit 2		-1.7	Yes		
At3g55620	Translation Initiation Factor		-1.7			
At1g26630	Translation Initiation Factor		-1.7	Yes		
At1g54100	3-Chloroallyl Aldehyde Dehydrogenase		-1.7	Yes	R	1
At2g39805	Integral Membrane Yip1 Family Protein		-1.7			
At2g30740	Serine/Threonine Protein Kinase		-1.7			
At3g52140	Tetratricopeptide Repeat Protein		-1.7			
At1g51680	4-Coumarate CoA Ligase 1		-1.7	Yes		
At5g26940	Exonuclease Family Protein		-1.6			
At5g62620	Galactosyltransferase Family Protein		-1.6			
At2g44450	Glycosyl Hydrolase Family 1 Protein		-1.6			
At3g02200	Proteasome Family Protein		-1.6			
At5g19450	Calcium-Dependent Protein Kinase 19		-1.6			
At4g02120	UTP--Ammonia Ligase, Putative		-1.6			
At1g63980	G-Patch Domain-Containing Protein		-1.6			
At3g25990	DNA-Binding Protein GT-1-Related		-1.6			
At4g23250	Embryo Defective 1290/Kinase		-1.6			
At3g14100	Oligouridylate-Binding Protein, Putative		-1.6			
At1g05850	Chitinase		-1.6	Yes		
At1g13160	SDA1 Family Protein		-1.6			
At3g05690	Homolog 2B/Transcription Factor		-1.6			
At4g21660	Pro-Rich Spliceosome-Associated		-1.6			
At3g51890	Unknown Protein		-1.6			
At5g11500	Unknown Protein		-1.6			
At2g21390	Coatomer Protein Complex, Putative		-1.6			
At1g18950	Aminoacyl-tRNA Synthetase Family		-1.6			
At5g26751	Shaggy-Related Kinase 11		-1.6			
At2g16400	Bell1-Like Homeodomain 7		-1.6			
At3g10800	bZIP28 Transcription Factor		-1.6	Yes		
At5g39450	F-Box Family Protein		-1.6			
At3g27700	RNA Recognition Motif Protein		-1.6			
At4g10170	Synaptobrevin-Related Family Protein		-1.6			
At1g04190	Tetratricopeptide Repeat Protein		-1.6			
At3g11830	Chaperonin, Putative		-1.5			
At5g26710	Glutamate-tRNA Ligase, Putative		-1.5			
At1g78040	Extensin Family Protein		-1.5			
At2g43320	Unknown Protein		-1.5			
At1g06960	Ribonucleoprotein Spliceosomal Protein		-1.5			
At5g66730	Zinc Finger Family Protein		-1.5			
At5g57630	Cbl-Interacting Protein Kinase 21		-1.5			
At4g28510	Prohibitin 1		-1.5	Yes		
At5g43010	Regulatory Particle Triple-A 4A		-1.5			

Transcripts up-regulated (P<0.01) in Yukon Thellungiella harvested from the field in 2005 only

A	B	C	D	E	F	G
AGI code	Annotation	F 2003 / Chamber	F 2005 / Chamber			
At3g62910	Translation Release Factor		1.5			
At5g08410	Thioredoxin Reductase Subunit A		1.5			
At5g44650	Unknown Protein		1.5			
At3g60690	Auxin-Responsive Family Protein		1.5			
At2g18915	Ubiquitin-Protein Ligase		1.5			
At5g03420	Dentin Sialophosphoprotein		1.5			
At1g60990	Aminomethyltransferase		1.5			
At4g32260	ATP Synthase Family		1.5	Yes		
At3g25690	Chloroplast Unusual Positioning 1		1.5			
At5g38520	A/B Fold Family Protein/Hydrolase		1.5			
At1g33720	Cytochrome P450		1.5			
At4g26500	Transcription Regulator		1.5			
At3g46690	UDP-Glucosyl Transferase		1.6			
At5g13690	Alpha-N-Acetylglucosaminidase		1.6			
At3g25530	Phosphogluconate Dehydrogenase		1.6	Yes		
At1g03680	Thioredoxin M-Type 1		1.6	Yes		
At5g27380	Glutathione Synthetase 2		1.6			
At2g31040	ATP Synthase Protein I -Related		1.6			
At1g49970	Endopeptidase CLP		1.6			
At1g14920	GA Insensitive/Transcription Factor		1.6	Yes	R	
At3g44680	Histone Deacetylase 9		1.6			
At5g58950	Protein Kinase Family Protein		1.6			
At1g60600	1,4-OH-2-Naphthoateprenyltransferase		1.6			
At3g08730	P70 Ribosomal S6 Kinase		1.6	Yes		
At4g18010	Inositol PolyP 5-Phosphatase II		1.6		R	
At5g63330	DNA-Binding Protein		1.6			
At4g13220	Unknown Protein		1.6			
At1g49880	Erv1/Alr Family Protein		1.6			
At4g04640	ATP Synthase Gamma Chain 1		1.6			
At5g57360	Zeitlupe Ubiquitin-Protein Ligase		1.7	Yes		
At1g67090	Ribulose-Bisphosphate Carboxylase		1.7	Yes		
At3g21690	Mate Efflux Family Protein		1.7			
At2g01570	Repressor Of GA Transcription Factor		1.7	Yes	R	
At2g40480	Unknown Protein		1.7			
At1g76520	Auxin Efflux Carrier Family Protein		1.7			
At2g32950	Constitutive Photomorphogenic 1		1.7	Yes		
At5g54080	Homogentisate 1,2-Dioxygenase		1.7			
At2g20860	Lipoic Acid Synthase 1		1.7			
At3g63490	Ribosomal Protein L1 Family Protein		1.7			
At1g19835	Unknown Protein		1.7			
At5g67530	Peptidyl-Prolyl Cis-Trans Isomerase		1.7			
At2g47590	Photolyase/Blue-Light Receptor 2		1.7			
At4g34190	Stress Enhanced Protein 1		1.7	Yes		
At1g20225	Unknown Protein		1.7			
At1g21065	Unknown Protein		1.7			
At2g38270	Protein Disulfide Oxidoreductase		1.7			
At3g27300	Glucose-6-Phosphate Dehydrogenase 5		1.7			
At4g37510	Ribonuclease III Family Protein		1.7			
At3g27570	Unknown Protein		1.7			
At1g44920	Unknown Protein		1.7			
At1g16240	Syntaxin Of Plants 51		1.7			
At1g52510	Hydrolase, A/B Fold Family Protein		1.7			
At1g61660	Basic Helix-Loop-Helix Family Protein		1.7			1
At4g09680	Unknown Protein		1.7			
At3g04870	Zeta-Carotene Desaturase		1.7		M	
At3g24170	Glutathione-Disulfide Reductase		1.7			

At5g22640	Embryo Defective 1211		1.8			
At4g01310	Ribosomal Protein L5 Family Protein		1.8			
At5g48300	ADP Glucose Pyrophosphorylase		1.8	Yes		
At2g27680	Aldo/Keto Reductase Family Protein		1.8			
At1g55920	Serine Acetyltransferase 1		1.8	Yes		
At5g50920	Heat Shock Protein 93-V		1.8			
At3g02170	Longifolia 2		1.8			1
At1g50020	Unknown Protein		1.8			
At2g31370	bZIP Transcription Factor		1.8			
At5g54960	Pyruvate Decarboxylase-2		1.8	Yes		
At1g74730	Unknown Protein		1.8			
At5g03880	Unknown Protein		1.8			
At5g40500	Unknown Protein		1.8			
At5g67030	ABA Deficient 1/Zeaxanthin Epoxidase		1.8	Yes	M	
At4g35010	Beta-Galactosidase 11		1.8			1
At1g64970	Gamma-Tocopherol Methyltransferase		1.8			
At5g06260	Nucleolar Protein-Related		1.8			
At1g16880	Uridylyltransferase-Related Protein		1.8	Yes		
At3g22370	Alternative Oxidase 1A		1.8	Yes		
At1g68830	Stt7 Homolog Stn7/Protein Kinase		1.8			
At1g11650	RNA Binding Protein		1.8			
At4g34350	4-HO-3-Mebut-2-En-1-DiP Reductase		1.8			
At5g07370	Inositol Polyphosphate Kinase 2 Alpha		1.8			
At5g52200	Protein Phosphatase Inhibitor		1.8			
At1g64150	Unknown Protein		1.8			
At3g26085	Amino Terminal Protease		1.8			
At5g58060	Unknown Protein		1.8			
At4g13430	Aconitase Family Protein		1.8			
At4g11600	Glutathione Peroxidase 6		1.8	Yes		
At1g78960	Lupeol Synthase 2		1.8			
At3g13180	Nol1/Nop2/Sun Family Protein		1.9			
At1g22600	Unknown Protein		1.9			
At5g38410	Ribulose Bisphosphate Carboxylase		1.9			1
At1g44575	Nonphotochemical Quenching		1.9	Yes		
At2g42130	Plastid-Lipid-Associated Protein 13		1.9			
At3g11560	Unknown Protein		1.9			
At3g07220	Transcriptional Activator, Putative		1.9			
At4g00895	ATP Synthase Delta Chain-Related		1.9			
At4g11175	Translation Initiation Factor IF-1		1.9			
At4g20360	Translation Elongation Factor		1.9			
At4g13010	Zinc-Binding Dehydrogenase		1.9			
At3g52230	Unknown Protein		1.9			
At3g51180	Zinc Finger Family Protein		1.9			
At5g26570	Phosphoglucan Water Dikinase		1.9			
At5g54870	Unknown Protein		1.9			
At2g46900	Unknown Protein		1.9			
At4g32770	Vitamin E Deficient 1		1.9	Yes		
At1g43670	Fructose-1,6-Bisphosphatase, Putative		1.9			
At4g20060	Embryo Defective 1895/Binding		1.9			
At2g28900	P-P-Hydrolysis-Driven Transporter		1.9	Yes		
At1g28140	Unknown Protein		1.9			
At1g53520	Chalcone-Flavanone Isomerase		1.9			
At5g57040	Glyoxalase I Family Protein		1.9			
At3g47570	Leucine-Rich RepeatKinase, Putative		1.9			
At2g37770	Aldo/Keto Reductase Family Protein		2.0			
At1g15690	Vacuolar-Type H+-Pyrophosphatase		2.0	Yes		
At4g37930	Serine Hydroxymethyltransferase 1		2.0	Yes		
At5g05200	ABC1 Family Protein		2.0			
At4g13310	Cytochrome P450		2.0			
At2g22830	Squalene Epoxidase 2		2.0			
At3g12780	Phosphoglycerate Kinase 1		2.0	Yes		
At4g35250	Vestitone Reductase-Related		2.0			
At1g36160	Acetyl-Coenzyme A Carboxylase 1		2.0			

At1g64850	Calcium-Binding Family Protein		2.0			
At5g16540	Zinc Finger Protein 3		2.0			
At2g18710	P-P-Bond-Hydrolysis-Driven Protein		2.1			
At1g64860	Sigma Factor A Transcription Factor		2.1			
At2g46910	Fibrillin Family Protein		2.1			
At4g09650	ATP Synthase Delta Chain		2.1	Yes		
At5g52420	Unnknown Protein		2.1			
At2g03390	uvrB/uvrC Motif-Containing Protein		2.1			
At3g03250	UDP-Glucose Pyrophosphorylase		2.1	Yes		
At5g14970	Amine Oxidase		2.1			
At1g12090	Extensin-Like Protein/Lipid Binding		2.1			
At4g37000	Accelerated Cell Death 2		2.1	Yes		
At3g29590	O-Malonyltransferase		2.1			1
At1g70730	Phosphoglucomutase, Cytoplasmic		2.1			
At3g17930	Unknown Protein		2.1			
At5g55990	Calcineurin B-Like Protein 2		2.2			
At1g79270	Evolutionarily Conserved C-Terminal 8		2.2			
At3g46610	Pentatricopeptide Repeat-Protein		2.2			
At5g65720	Cysteine Desulfurase/Transaminase		2.2			
At1g22850	Unknown Protein		2.2			
At3g63410	Albino Or Pale Green Mutant 1		2.2			
At4g09750	Short-Chain Dehydrogenase		2.2			
At5g26820	Ferroportin-Related Protein		2.2			
At5g56860	N-/C-Metabolism-Transcription Factor		2.2			
At2g24090	Ribosomal Protein L35 Family Protein		2.2			
At2g33250	Unknown Protein		2.2			
At2g13360	Alanine:Glyoxylate Aminotransferase		2.2			
At1g22070	TGA1A-Related Transcription Factor		2.2	Yes		
At5g55620	Unknown Protein		2.2			
At1g05190	Embryo Defective 2394		2.2			
At4g39970	Haloacid Dehalogenase		2.2			
At2g37540	Short-Chain Dehydrogenase		2.2			
At1g48030	Dihydrolipoamide Dehydrogenase 1		2.2	Yes		
At1g80380	Phosphoribulokinase		2.3			
At5g58040	RNA Binding Protein		2.3			
At5g28840	GDP-D-Mannose 3',5'-Epimerase		2.3			
At1g44000	Unknown Protein		2.3			
At5g04140	Fd-Dependent Glutamate Synthase 1		2.3	Yes		
At1g09340	Chloroplast RNA Binding Protein		2.3	Yes		1
At5g20280	Sucrose Phosphate Synthase 1F		2.3			
At4g32190	Centromeric Protein-Related		2.3			
At1g68570	Oligopeptide Transport Family Protein		2.3			
At4g36390	Radical SAM Protein		2.3			
At4g17560	Ribosomal Protein L19 Family Protein		2.3			
At3g16250	Ferredoxin-Related Protein		2.3			
At1g52870	Peroxisomal Membrane Protein-Related		2.4			
At3g01060	Unknown Protein		2.4			
At3g10180	Kinesin Motor Protein-Related		2.4			
At4g25170	Unknown Protein		2.4			
At4g11570	Haloacid Dehalogenase		2.4			
At3g59300	Unknown Protein		2.4			
At1g79600	ABC1 Family Protein		2.4			
At4g13770	Cytochrome P450 83A1		2.4	Yes		
At1g48350	Ribosomal Protein L18 Family Protein		2.4			
At1g05560	UDP-Glucosyl Transferase 75B1		2.4		M	
At5g20380	Transporter-Related Protein		2.4			
At5g42270	ATP-Dependent Metallopeptidase		2.4			
At2g04520	Translation Initiation Factor 1A		2.4			
At1g27370	Squamosa Promoter-Binding Protein		2.4			
At4g01080	Unknown Protein		2.4			
At3g55120	Chalcone Isomerase		2.5	Yes		
At3g23700	S1 RNA-Binding Protein		2.5	Yes		
At5g30510	RNA Binding Ribosomal Protein S1		2.5			

At2g37240	Antioxidant Oxidoreductase		2.5			
At1g57770	Amine Oxidase Family		2.5			
At3g16910	Acetate-coA Ligase		2.5			
At4g34135	UDP-Glucosyltransferase		2.5	Yes		
At5g34850	Purple Acid Phosphatase 26		2.6			
At4g17610	tRNA/rRNA Methyltransferase		2.6			
At4g34240	3-Chloroallyl Aldehyde Dehydrogenase		2.6	Yes	R	
At3g27830	Structural Constituent Of Ribosome		2.6			
At1g52590	DCC Family Protein Precursor		2.6			
At1g66330	Senescence-Associated Family Protein		2.6			
At1g29390	Cold Regulated 314		2.6	Yes		
At1g60950	Ferredoxin 2		2.6	Yes		
At4g04770	Nucleosome Assembly Protein 1		2.7	Yes		
At3g63140	mRNA-Binding Protein, Putative		2.7			
At4g14040	Embryo Sac Development Arrest 38		2.7			
At3g14770	Nodulin MtN3 Family Protein		2.7			
At2g24280	Serine Carboxypeptidase S28		2.7			1
At4g13930	Serine Hydroxymethyltransferase 4		2.8			
At1g73990	Signal Peptide Peptidase		2.8	Yes		
At3g63520	Carotenoid Cleavage Dioxygenase 1		2.8	Yes	M	
At5g13650	Elongation Factor Family Protein		2.8			
At1g50250	FtsH Protease 1 Metallopeptidase		2.8			
At3g02830	Zinc Finger Protein 1		2.8			
At4g04350	Aminoacyl-tRNA Ligase		2.9			
At1g64680	Unknown Protein		2.9			
At5g17170	Enhancer Of SOS3-1		2.9			
At5g67360	Subtilase		2.9			
At5g15410	Rectifier Potassium Channel		2.9	Yes		
At2g48070	Unknown Protein		2.9	Yes		
At2g30490	Cinnamate 4-Hydroxylase		3.0	Yes		
At1g32080	Membrane Protein, Putative		3.0			
At1g62780	Unknown Protein		3.0			
At3g53260	Phenylalanine Ammonia-Lyase 2		3.0	Yes		
At4g12320	Cytochrome P450		3.0			
At1g04350	2-OG-Dependent Dioxygenase, Putative		3.0			
At5g24460	Hydrolase		3.0			
At2g38740	Haloacid Dehalogenase		3.1			
At4g33010	Glycine Decarboxylase P-Protein 1		3.1			
At2g35840	Sucrose-Phosphatase 1		3.2			
At2g42810	Protein Ser/Thr Phosphatase 5		3.2	Yes		
At5g60540	Pyridoxine Biosynthesis 2		3.2			
At5g10170	Inositol-3-P Synthase, Putative		3.2			
At1g67360	Rubber Elongation Factor Protein		3.2			1
At3g25410	Bile Acid:Na Symporter Family Protein		3.3			
At1g16080	Unknown Protein		3.3			
At1g78070	WD-40 Repeat Family Protein		3.4			
At4g31870	Glutathione Peroxidase 7		3.4	Yes		
At5g20070	Nudix Hydrolase Homolog 19		3.4			
At5g53970	Aminotransferase, Putative		3.5			
At4g36530	Hydrolase, A/B Fold Family Protein		3.5			
At1g78510	Solanesyl Diphosphate Synthase 1		3.5			
At5g52250	WD-40 Repeat Family Protein		3.5	Yes		
At5g05580	Omega-3 Fatty Acid Desaturase		3.6	Yes		
At2g17290	Calcium-Dependent Protein Kinase 6		3.7		S	
At1g45230	Defective Chloroplasts Protein-Related		3.7			
At2g26080	Glycine Decarboxylase P-Protein 2		3.7			
At5g46800	A Bout De Souffle Binding Protein		3.7			
At1g62750	Translation Elongation Factor		3.7			
At5g24120	RNA Polymerase Sigma Subunit E		3.8	Yes		
At3g21890	Zinc Finger Family Protein		3.8	Yes		
At3g21250	Multidrug Resistance-Associated 6		3.9			
At1g17100	SOUL Heme-Binding Family Protein		4.2			1
At4g15530	Pyruvate Orthophosphate Dikinase		4.4			

At2g04039	Unknown Protein		4.5			
At4g15480	Sinapate 1-Glucosyltransferase		5.0	Yes		
At4g37990	Elicitor-Activated Gene 3		5.1	Yes		
At1g02205	Eceriferum 1		5.2			
At5g20630	Germin-Like Protein 3		5.5	Yes		1
At5g13930	Naringenin-Chalcone Synthase		6.0	Yes		1
At3g21670	Nitrate Transporter (NTP3)		6.4			1
At2g38240	2OG-Fe(II) Oxygenase Family Protein		6.5			
At2g42530	Cold-Regulated 15B Protein		6.7	Yes		1
At5g05270	Chalcone-Flavanone Isomerase		7.1			1
At3g44990	Xyloglucan:Xyloglucosyl Transferase 8		7.1			
At2g37040	Phenylalanine Ammonia-Lyase		7.6	Yes		
At2g18230	Pyrophosphorylase 2		7.7			
At4g26850	Vitamin C Defective 2		9.4	Yes		
At1g62710	Vacuolar Processing Enzyme		10.5			

Supplemental Table S3. *Metabolites present at statistically significantly different levels in Yukon Thellungiella harvested from the field in 2003 and 2005.*

Values represent fold-ratio between field and growth chamber grown Thellungiella, where a positive or negative number represents a metabolite present at higher or lower level in field-grown plants, respectively, relative to cabinet grown plants.

Metabolites present at significantly lower levels (P<0.05) in Yukon Thellungiella harvested from the field in 2003 and 2005

ID	Field 2003 / Chamber	Field 2005 / Chamber
1076_aspartic acid	-12326.0	-880.4
1346_glutamine	-10508.4	-10508.4
2206_C12 sugar alcohol/disaccharide	-1686.8	-1405.7
2803_C12 sugar alcohol/disaccharide	-1179.0	-471.6
1312_unknown	-1025.2	-28.2
984_unknown	-978.8	-978.8
1564_C12 sugar alcohol/disaccharide	-858.0	-3.5
2683_C12 sugar alcohol/disaccharide	-580.4	-580.4
2997_raffinose	-366.0	-1683.7
2666_C12 sugar alcohol/disaccharide	-316.5	-46.4
1599_unknown	-278.4	-696.0
2649_C12 sugar alcohol/disaccharide	-220.0	-27.5
1770_unknown	-196.0	-78.4
1854_unknown	-175.5	-280.8
2371_C12 sugar alcohol/disaccharide	-138.0	-115.0
1368_unknown	-123.2	-123.2
2472_C12 sugar alcohol/disaccharide	-95.2	-95.2
1477_lysine	-91.2	-91.2
1894_unknown	-68.0	-68.0
2731_C12 sugar alcohol/disaccharide	-64.9	-7.3
1774_C12 sugar alcohol/disaccharide	-64.2	-19.0
1786_phosphorylated sugar	-55.2	-286.8
1555_unknown	-50.3	-201.2
1956_unknown	-45.2	-45.2
821_phosphate	-24.3	-120.8
2616_C12 sugar alcohol/disaccharide	-22.7	-16.0
2423_C12 sugar alcohol/disaccharide	-22.7	-254.0
1964_C12 sugar alcohol/disaccharide	-17.4	-48.8
2000_C12 sugar alcohol/disaccharide	-16.3	-130.0
2254_C12 sugar alcohol/disaccharide	-16.2	-368.8
891_fumaric acid	-15.8	-120.4
1845_cinnamic acid	-15.6	-7.0
1916_glucose-6-phosphate	-12.7	-56.0
1710_C6 sugar alcohol	-8.3	-675.2
2548_C12 sugar alcohol/disaccharide	-7.6	-21.8
1426_quinic acid	-6.2	-2.6
1096_pyroglutamic acid	-5.6	-951.2
2269_C12 sugar alcohol/disaccharide	-5.6	-9.8
810_unknown	-4.8	-137.0
1026_unknown	-4.7	-166.8
1158_unknown	-4.2	-7.7

1166_unknown	-3.8	-25.6
931_threonine	-3.7	-104.8
2137_C12 sugar alcohol/disaccharide	-3.0	-37.3
749_valine	-2.8	-19.5
2598_galactinol	-2.5	-5.4
903_serine	-2.5	-845.0
1147_unknown	-2.3	-123.2
833_isoleucine	-2.1	-188.8
1179_glutamic acid	-1.8	-5747.3
1044_malic acid	-1.5	-1.6

Metabolites present at higher levels (P<0.05) in Yukon Thellungiella harvested from the field in 2003 and 2005

ID	Field 2003 / Chamber	Field 2005 / Chamber
2236_sucrose	2.2	3.5
1389_citric acid	5.3	4.8
1429_unknown	6.1	9.6
1440_fructose	8.7	4.5
844_glycine	46.2	36.2
1024_citramalic acid	69.9	44.7
859_succinic acid	863.6	276.6
794_ethanolamine	1118.0	688.0

Metabolites present at higher levels (P<0.05) in Yukon Thellungiella harvested from the field in 2003, and lower levels (P<0.05) in 2005

ID	Field 2003 / Chamber	Field 2005 / Chamber
1320_unknown	5.3	-42.3
1377_unknown	6.1	-6.6

Metabolites present at higher levels (P<0.05) in Yukon Thellungiella harvested from the field in 2003 only

ID	Field 2003 / Chamber
1664_myo-inositol	1.5
1118_threonic acid	1.7
1466_glucose	2.4
874_unknown	2.6
1062_unknown	21.3
1141_unknown	27.0
1042_unknown	45.7
1462_galactose	49.5
1415_unknown	100.8
1559_C6 sugar alcohol	114.4
1553_unknown	120.8
1079_gamma amino butyric acid (GABA)	218.9
1364_unknown	260.8

1727_C6 sugar alcohol	313.6
1315_unknown	468.4
1344_unknown	611.3
1443_unknown	638.4
815_glycerol	743.2
1495_pentose	892.4
1565_unknown	1549.6
1778_C12 sugar alcohol/disaccharide	1568.4

Metabolites present at lower levels (P<0.05) in Yukon Thellungiella harvested from the field in 2005 only

ID	Field 2005 / Chamber
1522_C6 sugar alcohol	-1189.6
1569_C12 sugar alcohol/disaccharide	-730.4
1241_asparagine	-609.0
794_unknown	-338.8
1979_C12 sugar alcohol/disaccharide	-285.2
1260_unknown	-97.2
839_proline	-43.4
1221_unknown	-37.5
611_alanine	-28.6
1942_unknown	-14.0
1193_phenylalanine	-3.5

Metabolites present at higher levels (P<0.05) in Yukon Thellungiella harvested from the field in 2005 only

ID	Field 2005 / Chamber
1410_unknown	3.1
1734_C6 sugar alcohol	7.2
1306_unknown	17.5
1574_unknown	22.6
929_unknown	64.0
2205_C12 sugar alcohol/disaccharide	73.6
1913_C12 sugar alcohol/disaccharide	107.2
1978_C12 sugar alcohol/disaccharide	107.9
1383_unknown	113.0
2240_unknown	130.0
1543_unknown	221.0
1774_C6 sugar alcohol	343.2
2141_C12 sugar alcohol/disaccharide	445.6
1330_unknown	542.0
1108_unknown	1183.2
1975_C12 sugar alcohol/disaccharide	1206.8

Chapter Five

General Discussion

In the field, the soil water content can vary within a single day and throughout the growing season (Acevedo et al., 1979; Hanson and Hitz, 1982). Plants must therefore constantly 'fine-tune' physiological processes to ensure continued growth and development under fluctuating water availability (Hanson and Hitz, 1982). However, prolonged exposure to water deficits severely compromises plant growth and productivity and consequently, a lack of water is the single most important factor limiting crop production (Boyer, 1982). A better understanding of the molecular basis of plants that are naturally tolerant to abiotic stress holds great promise for improving the tolerance of stress-sensitive crops using genetic engineering approaches (Bressan et al., 2001). However, to date, the genes enabling naturally stress tolerant plants to thrive in their harsh habitats are unknown.

The identification of traits associated with abiotic stress tolerance has typically been performed by subjecting plants to calibrated stress treatments imposed under controlled growth chamber conditions. Under field conditions, however, plants are exposed to multiple stresses simultaneously leading to altered physiological responses that are not necessarily predicted based on experiments performed in artificial laboratory conditions (Rizhsky et al., 2004). Comparisons made between traits expressed by plants subjected to abiotic stress in their natural habitats and those exposed to growth chamber

experiments could identify critical mechanisms enabling plants to acclimate to abiotic stress.

Thellungiella is exceptionally tolerant to cold temperatures, drought and salinity and is therefore an emerging genetic model for the study of abiotic stress tolerance mechanisms (Inan et al., 2004; Taji et al., 2004; Gong et al., 2005; Griffith et al., 2007; Wong et al., 2006). The highly saline soils in the Yukon where *Thellungiella* populations were sampled combined with the prevailing precipitation patterns during the periods plants were harvested enabled us to compare the traits expressed in *Thellungiella* experiencing drought and relatively well-watered conditions in the field (Guevara et al., 2009b). Our research group has demonstrated that Yukon *Thellungiella* accumulates organic solutes in response to drought and salinity treatments imposed in controlled environmental chambers (Wong et al., 2006, Guevara et al., 2009a) and in plants experiencing water deficits in the field (Guevara et al., 2009b), relative to unstressed chamber grown plants. Compatible organic solute accumulation in response to drought or salinity has been observed in other naturally tolerant plants (Bianchi et al., 1991; Paul and Cockburn, 1989), although the precise role compatible organic solute accumulation plays for stress tolerance is still under debate (Gagneul et al., 2007). Therefore, it will be an important objective to demonstrate the importance for organic solute accumulation for stress tolerance in *Thellungiella* grown under field conditions.

Proline has been proposed to be a major compatible solute in Shandong *Thellungiella* subjected to saline environments (Inan et al., 2004; Kant et al., 2006). Proline levels were similar between Yukon *Thellungiella* harvested from the field during

drought and growth chamber grown controls (Guevara et al., 2009). Moreover, we found no evidence for altered expression of genes encoding enzymes involved in proline biosynthesis or degradation in Yukon *Thellungiella* harvested from salt flats in the Yukon (Guevara et al., 2009b). This outcome was not anticipated as we previously reported on genes encoding enzymes associated with proline metabolism such as delta-1-pyrroline-5-carboxylate synthetase (P5CS1, At2g39800; P5CS2, At3g55610) which were enriched in *Thellungiella* subjected to drought or cold stress treatments imposed in growth cabinets , relative to unstressed controls (Wong et al., 2006). The lack of proline accumulation in Yukon *Thellungiella* plants growing under highly saline environments in the field exposed to drought conditions raises important questions about the role of this solute in abiotic stress tolerance. One possible explanation for the lack of proline accumulation in *Thellungiella* subjected to abiotic stress in the field may be the result of the low nitrogen (N) content in the soil at the Yukon field site (Supplemental Table S1 in Chapter 4). Insufficient N in the soil leads to plant N-deficiency (Kant et al., 2008), and this could limit the amount of N that can be diverted towards the synthesis of N-containing compatible solutes, including proline (Stewart and Lee, 1974). For example, Yukon *Thellungiella* experiencing water deficits under N limitation may accumulate polyols or sugars rather than N-containing metabolites such as proline, to ensure sufficient N pools are present for the synthesis of macromolecules such as chlorophyll, nucleotides and proteins (Tattersall, 2009). It will also be important to determine the effects light and temperature have on Yukon *Thellungiella*'s ability to accumulate proline during growth under field conditions, as these environmental conditions can be quite variable during the

day. Further work will be required to demonstrate whether glucose and sucrose accumulation trutly contributes towards stress tolerance in Yukon *Thellungiella* growing in its naturally harsh habitat. For example, a recent study revealed that these two sugars can accumulate in *Arabidopsis* in response to potassium deficiency (Armengaud et al., 2009). The authors concluded that shoot glucose and sucrose accumulation is caused by impaired sugar metabolism in roots of potassium deficient plants (Armengaud et al., 2009). Thus, it will be important to study Yukon *Thellungiella*'s response to abiotic stress under full nutrient and nutrient deficient conditions to distinguish between general stress symptoms and traits associated with stress tolerance.

One striking observation was the drastic difference in morphology exhibited by Yukon *Thellungiella* from field sites in the Yukon compared to plants grown under growth chambers. Yukon *Thellungiella* harvested from the field sites had prominent cauline leaves in lieu of larger rosette leaves typically observed in growth chamber grown plants (Guevara et al., 2009). It has been demonstrated that the investment in vegetative biomass in wild barley was plastic and sensitive across water and nutrient stress treatments (Volis et al., 2004). This plasticity enabled an enhancement in fitness as a result of early onset flowering during water deficits (Volis et al., 2004). The significance of the apparent morphological differences observed between field grown and growth chamber grown Yukon *Thellungiella* for abiotic stress tolerance is unknown. It is likely that the phenotypic plasticity exhibited by Yukon *Thellungiella* may represent an important strategy enabling this plant to thrive in a highly variable environment.

The validation of the importance of genes for survival under harsh environmental conditions will require the use of both forward and reverse genetic approaches to determine the effect of gene manipulation on stress tolerance. For example, a recent functional gene-mining approach where a cDNA expression library from Shandong *Thellungiella* was constructed and used to transform *Arabidopsis*, generating a transgenic seed library which was screened for salt-tolerant *Arabidopsis* lines (Du et al., 2008). Among the salt-tolerant lines isolated, it was conclusively demonstrated that the enhancement in tolerance to salinity for the line ST6-66 was as a result of an unknown gene (At1g13930), that at the amino acid level was 67% similar to the *Arabidopsis* homologue with an as of yet, unknown function (Du et al., 2008). Intriguingly, this transcript was also elevated in Yukon *Thellungiella* harvested from the salt flats in 2003, the year when plants experienced drought conditions and in Yukon *Thellungiella* exposed to drought treatments in growth chambers. The next task would be to test the effect over-expression of this gene has for plant abiotic stress tolerance in stress-sensitive crops such as canola in highly saline environments in the field.

The response of *Arabidopsis* and *Thellungiella* to calibrated stress treatments imposed in controlled environmental conditions has been compared (Inan et al., 2004; Taji et al., 2004; Gong et al., 2005; Kant et al., 2006), and this work has led to several hypotheses regarding the special attributes responsible for conferring *Thellungiella*'s superior ability to tolerate harsh environments. For example, *Thellungiella*'s innate ability to tolerate sub-optimal environments to a greater extent than *Arabidopsis* may be due to: **1)** novel genes (Inan et al., 2004); **2)** the expansion of gene complement in

Thellungiella through gene or partial genome duplications which may have led to the evolution of paralogous genes that encode more active forms of enzymes (Inan et al., 2004; Gong et al., 2005; Kant et al., 2006); **3)** divergent promoter structures in *Thellungiella* compared to *Arabidopsis* that permit diverse patterns of gene expression giving rise to halophytic characteristics (Inan et al., 2004); **4)** an altered sensitivity to external factors or internal hormonal signals (Gong et al., 2005). A comparison between *Arabidopsis*, *Brassica* and *Thellungiella* responses to abiotic stress should help identify traits giving rise to *Thellungiella*'s 'extremophile'-like characteristics that distinguish it from its less tolerant close relatives. The entire genome sequence of *Arabidopsis thaliana* is already available and whole genome sequencing efforts are currently underway or planned for other crucifers such as *Arabidopsis lyrata*, *Capsella rubella*, *Brassica rapa*, and Shandong *Thellungiella* (Schranz et al., 2007). Thus, through comparative genomics between *Arabidopsis* and *Thellungiella* whole genome sequence, it will be possible to identify changes within the genome structure, discover novel genes, and reveal modifications in the sequence of homologous genes, as well as the identification of differences in promoter structures between these halophyte and glycophyte species.

CONCLUSION

The combination of transcript and metabolite profiling of Yukon *Thellungiella* harvested from its harsh habitat enabled us to identify metabolic pathways associated

with abiotic stress tolerance under field conditions. This study revealed that the enrichment in sugar levels of field-grown Yukon *Thellungiella* experiencing drought conditions compared to unstressed controls is brought about by the higher level of expression of genes encoding enzymes associated with carbohydrate metabolism. This was in contrast to amino acids levels which were present at significantly lower levels in leaf tissues from Yukon *Thellungiella* harvested from salt flats in the Yukon, compared to unstressed growth chamber grown plants. Interestingly, proline, and its precursor glutamate, did not accumulate in Yukon *Thellungiella* harvested from the field in either year or in plants subjected to water deficit in growth chambers raising questions regarding proline's importance for stress tolerance in Yukon *Thellungiella* when grown under field conditions. The altered gene expression in Yukon *Thellungiella* subjected to drought in the field compared to unstressed, chamber grown plants is likely regulated by both ABA and the drought-responsive transcription factor AtHB7. It will be important to manipulate the expression of *AtHB7* in order to determine its importance for abiotic stress tolerance in extremophile plants such as *Thellungiella* grown under field conditions. This information can then be used for improving the tolerance of stress-sensitive crops plants using biotechnological approaches.

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APPENDIX

CIRAS-1 Equation to Correct for Leaf Area to Calculate Stomatal Conductance and Photosynthesis Rate Measurements of Yukon *Thellungiella*

```
PROC IMPORT OUT= WORK.photosyn
DATAFILE= "C:\Users\Dave\ps\irgacom1.xls"
DBMS=EXCEL97 REPLACE;
GETNAMES=YES;
RUN;

Proc sort data=photosyn; by date;
Run;

*-----Procedures to calc PS and SC------*;
data ps; set photosyn;



*--Sec 4-----------------------------------------------------*
vapor pressure at saturation(es) and in the cuvette(eo)
measured in bars (may not need)  {in bars}
*-----------------------------------------------------------*;
MBRa=MBR/1000;
rha=rh/1000;

es=(6.13753e-3)*(EXP((CAT*(18.564-(CAT/254.4)))/(CAT+255.57)));

eo=(MBRa+rha);

*-----------------------------------------------------------*
correction of co2 readings for the broadening of the
bands by water vapor -assumes dry air before air is passed
over leaf (MBRa is zero).  {in ppm = micromol/mol}
*-----------------------------------------------------------*;

cout=(CO2rf+diff);

*-----------------------------------------------------------*
```

```
estimation of boundary layer resistance from a regression of
boundary layer resistance on filter paper 'leaves' of differing
sizes. The regression may be linear or quadratic.
Change to fit your leaves  {in m2 s/mol?} rb=0.20-> set on CIRAS IRGA
OLD rb~0.36=1/(5.94871+(-2.32984*LAR)+(0.508707*LAR*LAR)+(-
).037207*LAR*LAR*LAR))
----------------------------------------------------------*;

if lfincuv then LAR=lfincuv;
rb=((0.102*lfincuv)+0.218);
625

*----------------------------------------------------------*
{GOOD} conversion of mass flow rate (mls/min) to molar flow rate per unit
leaf area (mols/m2/s) (manual lacks 1/60 minute/second conversion)
      {in mols/m2/s}
*----------------------------------------------------------*;

W=(FLOW/1000)*(1/22.41)*(273/293)*(1/60)*(1/1.013)*(10000/lfincuv);

*----------------------------------------------------------*
{GOOD} calculation of transpiration rate (Ea)
Manual uses mmol/m2/s
*----------------------------------------------------------*;

Ea=(W*(eo-MBRa)/(1.013-eo))*1000;

*----------------------------------------------------------*
{GOOD} calculation of leaf temperature from energy balance equation
PARM is micromol/m2/s. Manual uses W/m2 -> divide PAR by 1.895?
*----------------------------------------------------------*;

deltan=((PARM*0.16)-((Ea/1000)*(45064.3-(CAT*42.9))));
deltad=(((0.93*28.97*1.012)/rb) + (4.639+(0.0583*CAT)));
delta=deltan/deltad;

lftemp=CAT+delta;

*--Sec 4----------------------------------------------------*
calculation of leaf vapor pressure from leaf temp {in bars}
*----------------------------------------------------------*;

el=6.13753e-3*EXP((lftemp*(18.564-(lftemp/254.4)))/(lftemp+255.57));
```

```
*-------------------------------------------------------*
calculation of leaf stomatal conductance
Manual uses mmol/m2/s
*-------------------------------------------------------*;

Ga=(1/(((el-eo)/((Ea/1000)*1.013))-rb))*1000;

*-------------------------------------------------------*
calculation of photosynthetic rate
Manual uses micromol/m2/s
*-------------------------------------------------------*;

Aa=-(W*(cout-CO2rf)+(cout*(Ea/1000)));

proc print;
var date TLTL lftemp EVAP Ea GSGS Ga PNN Aa;
run;
```