# RESPONSES OF *THELLUNGIELLA* TO WATER DEFICITS

# PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF YUKON AND SHANDONG *THELLUNGIELLA* TO WATER DEFICITS

By

JEFF DEDRICK, BSc

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2007)

(Biology)

McMaster University

Hamilton, ON

| | |
|---|---|
| TITLE: | Physiological and biochemical responses of Yukon and Shandong *Thellungiella* to water deficits |
| AUTHOR: | Jeff Dedrick, BSc (McMaster University) |
| SUPERVISOR: | Dr. Elizabeth A. Weretilnyk |
| NUMBER OF PAGES: | xi, 150 |

**ABSTRACT**

*Thellungiella salsuginea* (also known as *T. halophila*), is an emerging model species for studies of plant tolerance to osmotic stress. Currently, *Thellungiella* plants originating from two geographical locations are being developed for research. Yukon *Thellungiella* is native to the saline and alkaline soils of the Yukon Territory, Canada. The seeds in this study were collected at the Takhini Salt-Flats near Whitehorse, YT, a sub-arctic and semi-arid region. Plants from the Yukon have been proposed to be a suitable species to study plant tolerance to salinity, cold temperatures, and water deficits. Shandong *Thellungiella* is native to the saline coast of north-eastern China in Shandong Province where the climate is temperate and affected by summer monsoons. This plant has been proposed as an ideal model for the study of salt tolerance mechanisms but is reported to show little drought tolerance.

An objective of this study was to compare Yukon and Shandong *Thellungiella* plants with respect to their ability to withstand water deficits. Plants were grown simultaneously in controlled environment chambers where watering was withheld until plants visibly wilted. Wilting occurred at a leaf relative water (RWC) content of about 50-60% and then turgor was restored by re-watering. In a second experiment plants allowed to wilt and recover once were then subjected to a second wilting and recovery cycle. The third experimental approach tested the survival capacity of plants after experiencing a wilting episode where leaf RWC dropped to about 30%.

With the first drought simulation treatment, both Yukon and Shandong plants took about 6 days to wilt when watering was stopped. After re-watering and recovery, the

Yukon plants subjected to a second drought episode took almost two days longer to wilt while the similarly treated Shandong plants showed no change in the days taken to wilt. This indicates that Yukon plants show improved tolerance to stress after a single exposure to a water deficit. Yukon plants were also able to grow and complete their life cycle following exposure to severe water deficit treatment whereas Shandong plants died. Measurements of solute potential showed that the Yukon plants re-established turgor at a lower solute potential of -2.06 +/- 0.03 MPa following recovery from a second drought treatment suggesting that this plant can undergo osmotic adjustment. No evidence of osmotic adjustment was observed for Shandong *Thellungiella*.

Gas chromatography/mass spectrometry (GC/MS) was used to identify the metabolites associated with *Thellungiella* leaves recovering from water deficits relative to those from unstressed, well-watered controls. For comparison, metabolite profiles were also prepared from leaves of plants harvested at a Yukon field site during a dry year (2003) and a year of higher than normal rainfall (2005). The data was analyzed to identify treatment/sample-specific patterns using ANOVA to test for significance among quantitative and qualitative changes for individual metabolites. Significant changes were then subjected to hierarchical cluster analysis (HCA) and principal component analysis (PCA). Using ANOVA and HCA, we were able to identify the most likely metabolite candidates contributing to the superior tolerance of *Thellungiella*, and their linkages between broad spectrums of metabolites. Using PCA we were able to assign clusters to the individual plant treatments for each plant source, and identify the most important components contributing to these clusters. Of the ca 289 components detected, only a

small subset of components underwent statistically significant changes in abundance. Most of the drought-stress related changes were attributed to sugars: hexoses and disaccharides. Sugars accumulating in the more drought-tolerant Yukon plants and in a dry field season included fructose, glucose and galactose. Of the sugar alcohols, only *myo*-inositol showed patterns of interest in view of its enrichment in tissues showing superior tolerance to low water conditions. Similar patterns were also shown by the organic acid, threonic acid.

A complementary approach was used to characterize metabolic traits associated with exposure to cold temperatures. In this study, a higher content of proline and citrate distinguished plants exposed to cold temperatures irrespective of whether the plants were in cabinets or in the field. Proline content, however, did not show drought-responsive accumulation under any drought treatment tested. As such, by comparison with the drought-stress data we can identify possible stress-specific signatures among metabolites undergoing changes. The study of stress-responsive traits could help develop a better understanding of plant systems and their response to specific environmental conditions.

## ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Elizabeth Weretilnyk. Thank you for your invaluable guidance and support throughout the past three years of my research. I am very grateful to have been a part of your laboratory.

I extend a special thanks to Dr. Peter Summers. Thank you for your assistance and knowledge, making my job as a researcher much easier and a great learning experience.

To Dave Guevara, thank you for being a great mentor and research partner. Your research has paved the way for my thesis and many to follow.

Also I'd like to thank Amber Gleason, Chris Wang, Mike BeGora and Mitch MacLeod. Thank you all for being there to lend a hand. Working with all of you was a lot of fun.

Lastly, I would like to thank Dr. Robin Cameron and Dr. Susan Dudley for your support as my thesis committee.

## TABLE OF CONTENTS

## LIST OF FIGURES, TABLES AND APPENDICES

### Figures

**Tables**



**Appendices**

## ABBREVIATIONS

| | |
|---|---|
| AMDIS | Automated Mass Spectral Deconvolution and Identification System |
| amu | atomic mass unit |
| ANOVA | analysis of variance |
| ca | circa |
| DW | dry weight |
| FW | fresh weight |
| GASP | gas chromatography/mass spectrometry data analysis software package |
| GC/MS | gas chromatography / mass spectrometry |
| HCA | hierarchical cluster analysis |
| MPa | mega pascals |
| $N_2$ | nitrogen gas (g) and liquid (l) |
| PCA | principal component analysis |
| ROS | reactive oxygen species |
| RI | retention index |
| RRF | relative response factor |
| RT | retention time |
| RW | re-water |
| RW1 | plants wilted once then re-watered (Y=Yukon, S=Shandong) |
| RW2 | plants wilted twice with re-watering before and after second wilt |
| RWC | plant relative water content |

| | |
|---|---|
| SD | Shandong Province |
| TIC | total ion chromatogram |
| TW | turgid weight |
| YT | Yukon Territory |
| $\Psi p$ | turgor potential |
| $\Psi s$ | solute potential |
| $\Psi w$ | water potential |

## LITERATURE REVIEW

Drought is a major environmental condition limiting plant growth and crop production (Zhu, 2001). Meteorologically, drought can be defined as a long period of exceptionally low rainfall in which rainfall totals fail to match the potential evaporation for a specific area (Passioura, 1996). Due to its deleterious effects on meeting plant water requirements, drought adversely affects the potential growth and yield of plants (Passioura, 1996). Models predicting future global climate change suggest an increased incidence of extreme weather events, including drought in many areas of the globe (Palmer and Rälsänen, 2002; Ackerly, 2003; Diffenbaugh et al., 2005). Uneven distribution of rainfall coupled with ever increasing global water shortage makes understanding the genetic and molecular basis of drought resistance vital towards sustaining or improving crop yields in the future (Luo and Zhang, 2001).

### Water status

Plants typically grow under conditions covering a specific range of water content (Xiong and Zhu, 2002). Within a plant, the amount of water in leaf tissue relative to the amount of water the tissue can hold when turgid is referred to as the relative water content (RWC). Leaves from well-watered plants typically have a RWC of 85-95% (Hsiao and Bradford., 1980). During a period of drought, the concentration of solutes in soil increases as water is lost by evapotranspiration. Eventually little water is available in soil for use by the plant to replenish water lost by transpiration. The loss of water content in

the plant causes a gradual decrease in RWC. It has been shown that most crop plants do not survive after their leaf RWC drops below 60-70% (Barr and Weatherley, 1962).

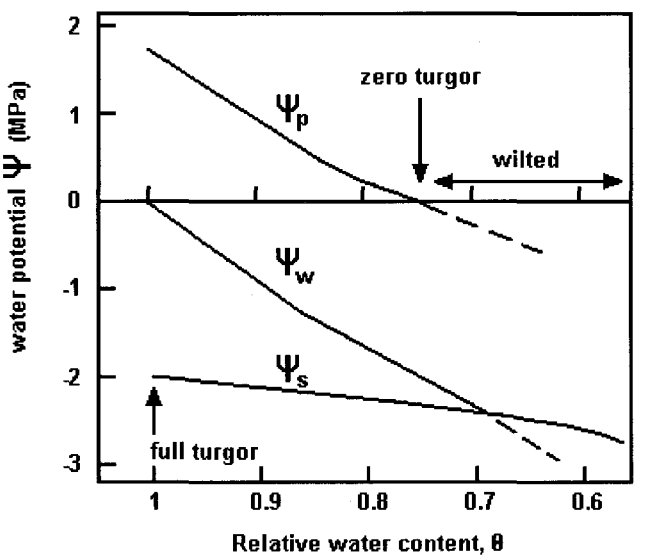
**Free energy of water**

Water status of plants can also be described on the basis of thermodynamics. In this regard, water movement in plants occurs in response to a gradient of water potential ($\Psi w$) from high to low $\Psi w$. The term $\Psi w$ is based upon the chemical potential of water and so is a measure of the free energy of water inside the cell and is largely the sum of two major components: osmotic potential ($\Psi s$), and turgor potential ($\Psi p$) (Hsiao, 1973; Hanson and Hitz, 1982).

The term $\Psi s$ is a measure of the solute effect on the free energy of water. The term $\Psi w$ is greatest when no solutes are present. As solute concentration increases, $\Psi s$ becomes more negative and therefore decreases $\Psi w$. $\Psi p$ is a measure of the pressure effect on water potential. If water is under pressure, $\Psi p$ becomes more positive in a plant cell and as a result increases $\Psi w$. Positive $\Psi p$ is essential for the expansion of plant cells (Frensch and Hsiao, 1994).

Pure water has no solutes present to increase disorder of the system and so has the most free energy available and a $\Psi w$ of zero. In biological systems solutes are always dissolved in water and so cellular $\Psi w$ is always less than pure water and hence negative. For example, the water potential of a leaf from a well-watered plant ranges between –0.9 to –1.3 MPa (Taiz and Zeiger, 1991). Upon conditions of water stress when plants are unable to replenish water lost by transpiration, $\Psi w$, $\Psi s$ and $\Psi p$ drop over time and when

$\Psi w = \Psi s$, $\Psi p$ reaches zero, the leaf is wilted. The drop in $\Psi s$ occurs because solutes are passively concentrated when water is lost from the cell (Hanson and Hitz, 1982). The level of $\Psi_W$ at which water stress sets in will vary among plants due to evolutionary and physiological adaptations to different environments (Kramer, 1983). The associated changes in $\Psi_W$, $\Psi_S$ and $\Psi_P$ described above with respect to the onset of water deficits are summarized by the classical Höfler diagram as follows:




(Jones, 1992)

**Adverse effects of drought on plants**

Following prolonged dehydration, the decrease in volume of a plant cell leads to an increased concentration of cytoplasmic components including ions such as $Cl^-$ and $NO_3^-$ (Hartung et al., 1998). As cytoplasmic components begin to crowd, the cellular

matrix becomes increasingly viscous. In turn, the chance for incompatible or perturbing molecular interactions to form between cellular components increases potentially leading to protein denaturation and membrane fusion (Hoekstra et al., 2001). Drought stress can also lead to the production of reactive oxygen species (ROS). ROS are harmful molecules generated by disruption of the electron transport system (Munne-Boche et al., 2001). Under normal conditions, ROS molecules are scavenged by different antioxidative defenses found within a plant cell. However under increasing water stress, the equilibrium between production and scavenging of ROS may be disrupted (Alscher et al., 1997). When the antioxidative defense capacity of a plant is overwhelmed the accumulation of ROS can lead to severe damage of essential cellular components. Peroxidation of lipids causing membrane leakages, denaturation of essential proteins, and destruction of DNA are all possible outcomes of ROS accumulation (Bowler et al., 1992).

**Adaptive strategies to water deficits**

Plants can employ many strategies to mitigate damage due to water deficits including developmental, physiological, and metabolic modifications at the cellular or organism level (Cellier et al., 1998). These adaptive survival strategies have been classically divided into three categories: escape, avoidance and tolerance of water deficit (Ludlow, 1989). These strategies are not mutually exclusive in that more than one can be used concurrently by the plant (Ludlow, 1989).

*Drought escape* is a strategy used by annual plants to evade the negative consequences associated with periods of dehydration by completing their life cycles

prematurely. This allows the plant to successfully reproduce before the onset of water deficit (Ludlow, 1989).

*Drought avoidance* describes the ability of a plant to retain relatively high turgidity in its tissues during periods of water deficit (Ludlow, 1989). Plants can accomplish high water retention through enhanced uptake of water from the soil and reduced water loss by transpiration. Enhanced water uptake can occur in plants from an increase of rooting depth and improved hydraulic conductance (Lauenroth et al., 1987). Reduced water loss can occur through the reduction of stomatal and epicuticular conductance, reduced absorption of solar radiation by leaf rolling or folding, reduced evaporation surface area, development of smaller and thicker leaves with waxy cuticles, and higher stomatal concentration on the bottom leaf surfaces to reduce exposure to sunlight (Smith and Nobel, 1977; McDonald et al., 2003).

*Drought tolerance* is achieved through the co-ordination of physiological and biochemical changes within a plant cell (Seki et al., 2001). Strategies of drought tolerance serve to stabilize and protect cellular structures and reduce disruption to metabolism. Some of these strategies include osmotic adjustment, reduction in cell size for newly enlarging cells, higher cell elasticity and tolerance of the protoplasm to low water potentials (Turner and Jones, 1980).

**Osmotic Adjustment**

The synthesis and accumulation of solutes or "osmotic adjustment" is an important mechanism exhibited by plants for the retention of water and maintenance of

cell turgor (Flowers et al., 1977; Greenway and Munns, 1980; Bohnert and Jensen, 1996). During a period of water stress plants can accumulate solutes in the cytoplasm to establish a $\Psi_w$ gradient to promote water uptake by roots thereby maintaining cellular turgor (Hanson and Hitz, 1982). Solute accumulation can arise by passive accumulation through desiccation or as a consequence of active uptake and/or synthesis by plants. In the latter case, the plant is turgid and is said to have undergone osmotic adjustment (Bray et al., 2000). These solutes, also referred to as "compatible solutes", can be accumulated at high concentrations without hindering cell function (McNeil et al., 1999; Xiong and Zhu, 2002). Examples of compatible solutes include sugars (sucrose, fructose, glucose), polyols (galactinol, myo-inositol, pinitol, mannitol, sorbitol) and amino acids (proline) or amino acid derivatives such as glycine betaine (Popp and Smirnoff, 1995; Xiong and Zhu, 2002). In addition to osmotic adjustment, compatible solutes can also protect cells against oxidative damage by free radicals and stabilize proteins and membranes (McNeil et al., 1999; Hare et. al., 1998).

Studies of grain development have shown that accumulation of compatible solutes reduced the effect of drought on yield loss (Morgan et al., 1986; Sobrado, 1986; Santamaria et al., 1990; Morgan et al., 1991; Rodríguez Maribona et al., 1992; Moustafa et al., 1996). Therefore, the identification of compatible solutes contributing to drought tolerance may offer insight for improving crop growth and yield under water stress conditions.

**Technological advancements and the study of drought-stress tolerance**

Advances in plant molecular genetics, plant transformation technologies, and metabolomics have opened new avenues for investigating the regulation of solute accumulation in plants (Hare et. al., 1998). These technologies offer the potential for a broad-based examination of the physiological, biochemical, and molecular aspects of stress tolerance. This knowledge will not only help our understanding of the remarkable flexibility of plant metabolism but will also aid in devising more effective strategies for enhancing the stress tolerance capabilities of stress-sensitive crop plants.

To date, model plants used in genetic and molecular studies have not been highly tolerant to osmotic stress thereby limiting our ability to determine the molecular basis of osmotic stress tolerance (Tripathy et al., 2000; Zhu, 2001). It has been argued that further characterization of the response mechanisms to osmotic stress will require use of a highly stress tolerant genetic model plant amenable to molecular genetic analysis. In this regard, extremophile plants that have an innate ability to grow and reproduce in extreme habitats could serve as important models for the study of abiotic stress tolerance mechanisms (Inan et al., 2004). Research into the physiology and metabolism of extremophiles will contribute to a better understanding of the evolutionary processes behind plant adaptation to adverse environmental conditions. Ultimately, these findings will have economic benefits through agricultural biotechnology.

**Using *Thellungiella* as a model plant for the study of osmotic stress tolerance**

*Thellungiella* plants vary with respect to their natural distribution and are found in areas of high salinity, freezing temperatures, and/or prolonged periods of water deficits

including drought (Warwick et al., 2004; Wong et al., 2006). *Thellungiella* also shares similar morphological and developmental attributes to the closely related genetic model plant *Arabidopsis* (Bressan et al., 2001; Wong et al., 2006). Some of these characteristics include small size, comparatively short life cycle length, abundant seed yield, small genome, and the capability of being transformed. These features are all essential characteristics for rapid genetic analysis (Bressan et al., 2001; Wong et al., 2006). In addition to possessing all the qualities of a genetic model plant, the close phylogenetic relationship of *Thellungiella* to *Arabidopsis* enables one to use the genetic resources developed for *Arabidopsis* in order to study *Thellungiella* (Taji et al., 2002; Wong et al. 2005; Wong et al., 2006).

In Canada, the distribution of *Thellungiella* extends from the salt-flats of the Yukon, through British Columbia and across the Prairie region (Scoggan, 1978). It has also been reported in the United States, specifically Colorado, and in Eastern Europe extending to Asia (Scoggan, 1978). Currently, lines derived from two geographically distinct *Thellungiella* populations are being developed for research.

*Thellungiella salsuginea* (hereafter referred to as "Yukon *Thellungiella*") is a rare plant native to the saline and alkaline soils of the Yukon. Seeds we are using in our study originated from the Takhini Salt Flats near Whitehorse, Yukon, Canada, which is a sub-arctic and semiarid region (Warwick et al., 2004). Its ability to grow and reproduce under highly saline soils, cold including freezing temperatures, and long periods of low rainfall makes this plant an ideal model for the study of metabolic, physiological and

developmental traits underlying plant environmental stress tolerance mechanisms (Wong et al., 2005).

*Thellungiella halophila* (hereafter referred to as "Shandong *Thellungiella*") is native to the saline coast of northeastern China in Shandong Province and has been reported to be an ideal model for the study of salt tolerance mechanisms (Bressan et al., 2001; Inan et al., 2004). The Shandong *Thellungiella* is not particularly tolerant to prolonged periods of soil desiccation (Inan et al., 2004). It is possible that its poor ability to tolerate drought may be a reflection of its natural environment, a region featuring a temperate climate characterized by abundant rainfall (Song et al., 1989).

To date there has not been a study that compares the relative capacities of Yukon and Shandong *Thellungiella* to withstand dry conditions. Therefore, the objective of this study was to characterize and compare the response of Yukon and Shandong *Thellungiella* to simulated drought stress and their capacity to recover from water deficits using physiological and metabolic measurements as a basis for comparison. Simulated drought stress was carried out using plants in controlled environment cabinets and the treatment consisted of withholding watering until plants visibly wilted. Recovery from this treatment was monitored once watering was resumed and occasionally plants were subjected to more severe drought treatment and/or repeated cycles of drought followed by re-watering. Observations recorded included days to onset of wilting, leaf relative water content (RWC), and leaf water and solute potential ($\Psi w$ and $\Psi s$) measurements. Plant tissue was also harvested in each experiment for subsequent metabolite profiling.

**Metabolic profiling**

The term "metabolome" refers to the full set of metabolites present in an organism (including metabolic intermediates, hormones and other signalling molecules and secondary metabolites) (Fiehn, 2001; Raamsdonk and Teusink, 2001). Metabolic profiling is a tool that involves the rapid, high-throughput characterization of metabolites needed for the survival and growth of an organism (Fiehn et al., 2000). The ultimate goal of metabolic profiling is to quantify, characterize, and identify all metabolites in an organism at a given biological state. Gas chromatography coupled with mass spectrometry (GC/MS) allows for the simultaneous identification of compounds including sugars (mono-, di-, and trisaccharides), sugar alcohols, amines, amino acids, organic acids and phosphorylated compounds (Adams et al., 1998). In this study GC/MS was used to compile "snapshots" of the polar metabolite present in leaves of *Thellungiella* in order to determine how this plant responds to water deficits. Each GC/MS profile can show in excess of three hundred polar compounds extracted from leaves and multiple runs are required to compare and identify reproducible and statistically significant treatment-specific differences between chromatographic profiles from multiple samples/sources (Fiehn, 2000).

In this work the software used to analyze GC/MS metabolite profiles is GASP (Gas chromatography-mass spectrometry data analysis software package, Paulo Nuin et al., 2004). GASP extracts and aligns data produced from multiple GC/MS runs into a format that can be analyzed by packages such as "R" (Ihaka and Gentlemen, 1996) in order to identify metabolites undergoing statistically significant changes between stressed

and unstressed plants. The dataset representing metabolites undergoing changes can then be subjected to multivariate data-mining tools including principal component (http://www.statsoft.co.uk/mrinfo6.html) and hierarchical clustering (Cluster: http://rana.lbl.gov/EisenSoftware.htm, and Java Treeview: http://jtreeview.sourceforge.net) analyses. Principal component analysis (PCA) helps identify similarities among clustering behaviour of datasets for each stress treatment and also distinguishes which compounds contribute to the greatest variance between these clusters. Hierarchical cluster analysis (HCA) allows a comprehensive analysis of the metabolite distribution patterns of changes between given metabolites for different samples under study. In the literature, analysis of metabolic profiling data using PCA and HCA statistical methods has provided an effective means for biochemical phenotyping a diverse array of biological systems, including genetically or environmentally modified plant systems (Roessner et al., 2000), plant ecotypes and mutants (Fiehn et al., 2000) and plant-microbe interactions (Desbrosses et al., 2005).

**Field analysis**

To date, there are few studies relating cellular activities monitored under controlled environmental conditions to those undergone by plants in their natural habitats (eg. Lajtha and Barnes, 1991; Cheikh and Brenner, 1992; Külheim et al., 2002). Moreover, most studies involving metabolic profiling are carried out using plants grown in controlled environments. These experiments have proven useful for identifying stress-specific responses; however, they do not take into consideration that plants growing in

their native environment are exposed to multiple, simultaneous stresses. A more useful comparison would be provided by comparing the metabolic response of plants grown under controlled environmental conditions to the response of plants growing in their natural habit.

In order to identify whether specific traits are essential for the tolerance of *Thellungiella* to drought or cold stress, it is useful to know whether metabolites undergoing stress-responsive changes in plants in growth cabinets are also present in plants experiencing comparable stresses under field conditions. In this study, tissue was harvested from *Thellungiella* field plants exposed to low to no precipitation in 2003, to higher than average rainfall in 2005 and to cool conditions of spring in 2006. The addition of cold-stressed field plants will allow for a comparison with plants responding to cold treatments in cabinets. This comparative genomics approach adds a new dimension in our studies towards the assessment of metabolic traits associated with the cold tolerance of *Thellungiella.*

In closing, the field data, in conjunction with data from calibrated stress treatments in cabinets, should make it possible to find stress-responsive traits that are expressed under one or more environmental condition. We believe this approach has the potential to identify critical metabolic traits underlying increased tolerance of crop plants to environmental stresses, conditions likely to contribute to yield losses with greater frequency under the growing problems associated with global warming.

## MATERIALS AND METHODS

### Plant material

*Thellungiella salsuginea* (Yukon *Thellungiella*) seed stock was collected from Tahkini Salt-Flats in Whitehorse, Yukon, Canada, by Dr. Bruce Bennett, a botanist working with Yukon Wildlife. *Thellungiella halophila* (Shandong *Thellungiella*) seed stock was provided by Dr. Ray Bressan (Department of Horticulture and Landscape Architecture, Purdue University, Indiana). Seeds were stored at room temperature in 10 mg portions in 1.5 mL microfuge tubes containing drierite desiccant (W. A. Hammond Drierite, Xenia, OH). Seeds were sterilized using a vapour-phase (gas) technique following a procedure devised for use with *Arabidopsis thaliana* seed (Bent, 2007). Seeds were placed in open microfuge tubes in a desiccation bell jar set in the fumehood. A volume of 100 mL of 10% (v/v) sodium hypochlorite (Javex bleach) was placed in a 500 mL beaker inside the bell jar containing the seeds. After the total unit was placed in a fume hood, 3 mL of concentrated HCl was added to the bleach solution. The bell jar was quickly sealed shut and allowed to sit for 1 h before the tubes containing the seeds were removed from the jar, capped, and stored at room temperature until use.

### Plant growth

The potting soil mixture consisted of 6 parts Promix soil (Premier Horticulture, Rivière-du-Loup, QC), 1 part Turface (Profile Product LLC, Buffalo Grove, IL) and 3 parts deionized, distilled water in a w/w/v mixture (the deionized water, prepared using a Barnstead Nanopure II system, was the source of water used for everything in this study

including watering plants and preparing reagents and will hereafter be referred to as "water" in the remainder of the thesis where reagent preparation and plant care at McMaster is concerned). The potting mixture was distributed evenly into 2-inch square pots for each plant. 1.0 mL of 1.5% (w/v) agar media (Difco$^{TM}$ Agar, Granulated, catalogue no. 214530) was placed in a 1.5 mL microfuge tube and heated in a Kenmore microwave for 30 s on high power until liquefied. The agar was allowed to cool but was still molten when the contents of 1 tube of seeds (0.25 mL volume) were added. A 200 µL Pipetman was modified by cutting off the narrow tip and the broader opening made it easier to dispense 3-4 seeds directly onto the potting soil mixture. Each 0.25 mL batch of seeds produced about 60 plants. The trays (flats) containing the pots were covered with a transparent lid and left in the dark at 4°C for two days (Yukon plants) or seven days (Shandong plants). Seeds were transferred to an AC 60 Econair plant growth cabinet (Winnipeg, MB) with 21 h light / 3 h dark photoperiod and photon flux density of 250 $\mu$moles $m^{-2}$ $s^{-1}$. The AC 60 Econair growth cabinet was equipped with Sylvania 4100K 59W fluorescent lamps (Wesco International, Inc., catalogue no. 04613529815) and Philips 120V 60W incandescent bulbs (Wesco International, Inc., catalogue no. 78667713530). Day and night temperatures were set at 22°C and 10°C, respectively. The plants were watered for the first week by misting once daily using a spray bottle. Germination normally took place three days following transfer of the trays to the growth chamber. Seven days after germination the transparent lid was removed and plants were carefully thinned to one plant per pot. Plants were watered once daily at a time-point 4 h

into the light period, and once every seven days with 1 g/L 20-20-20 nitrogen-phosphate-potassium fertilizer.

**Drought simulation treatment**

28-day-old *Thellungiella* plants were randomly divided into three groups of equal numbers for drought simulation experiments. The first group of plants was watered as needed and comprised the control group. The leaf RWC for these plants remained in the range of 80-85%. For the second group, water was withheld and leaves were monitored several times each day for signs of wilting. Visible wilting of leaves corresponded to a leaf RWC in the range of 50-60%. After wilting, the plants from this group were re-watered and allowed to recover for two days (RW1) before sampling. A subset of these plants was randomly selected to undergo a second episode of drought and leaves were monitored several times each day for signs of wilting. After wilting, these plants were re-watered and allowed to recover for two days (RW2) before sampling. The third group of plants underwent a prolonged drought treatment. In this drought simulation protocol plants were left un-watered past the point of first signs of wilting until the leaf RWC approached 30%. With this severe water deprivation leaves took on a pale green almost chlorotic appearance and became dry and brittle to the touch. Plants reaching a leaf RWC of 30% were re-watered every other day and monitored for recovery.

Measurements taken at periodic intervals for well-watered control plants and plants subjected to drought stress included leaf relative water content (RWC), and leaf water and solute potentials ($\Psi w$ and $\Psi s$). For plants where water was withheld, the time

taken to the onset of visible leaf wilting was recorded. Plant tissue was also harvested for subsequent analysis by GC/MS. 200 mg of leaf tissue from individual plants was pre-weighed into microfuge tubes that were flash frozen by immersion in $N_2(l)$. Frozen tissue samples were stored at -80°C until further processing.

**Leaf water relations**

*Leaf relative water content (Leaf RWC)*

A disc of 7 mm diameter was excised from the most recent, fully expanded rosettes using a sharp cork bore. Fresh weight (FW) was measured immediately and then the disc was placed on 1 mL of water for 24 h of hydration in the dark. The fully turgid disc was then blotted dry and weighed to determine turgid wt (TW) before being dried at 75°C for 24 h to determine leaf tissue dry weight (DW). RWC is expressed as a percentage using the equation below:

$$\text{RWC (\%)} = \frac{\text{(FW - DW)}}{\text{(TW - DW)}} \text{ x } 100$$

(Hewlett and Kramer, 1962)

*Leaf tissue water and solute potential*

Measurements of leaf water content were synchronized and taken at a time-point of 6 h into the photoperiod, which was 2 h after watering. These measurements were done to coincide with plant watering and not pre-dawn measurements as is normally done. A Wescor Dew Point Microvoltmeter (model HR-33T) fitted with C-52 chambers (Wescor Corp., Logan, UT) was used to measure leaf water potential (Ψw leaf, MPa) of 4

mm diameter leaf discs removed from the most recent, fully expanded rosettes. Leaf solute potentials (Ψs leaf, MPa) were measured using leaf discs of identical dimension that were flash-frozen in $N_2(l)$ and allowed to thaw and come to room temperature before placement in the C-52 chamber. The Wescor C-52 chamber allows the vapour pressure of air inside the chamber to come into equilibrium with the water vapour pressure in the leaf disc. Water vapour pressure developed over the leaf tissue is directly related to its water potential. An electrical current passing through the chamber allows for the thermocouple to cool causing condensation to collect on thermocouple junction. This point of moisture acts as a wet bulb. As the condensation evaporates, the junction is cooled below ambient temperature and the difference of temperature between the wet bulb and dry bulb is calculated. The temperature difference is a direct function of the vapour pressure of the air in the chamber. The thermocouple output (μV) is divided by -7.5 to convert μV to units of pressure (MPa) (Campbell et al., 1973). Turgor potential (Ψp) values for leaf tissues were calculated using the following equation: $\Psi w = \Psi s + \Psi p$, where both Ψw and Ψs were determined empirically using the Wescor psychrometer and Ψp calculated by difference.

**Sample preparation**

Plants subjected to drought treatment were harvested either at the approximate point of visible wilting or two days after watering was resumed. The most recent, fully expanded rosette tissue from control plants was harvested at the same time-points as the

plants undergoing drought/re-watering treatments were harvested. Chamber conditions were as described previously.

Cauline leaf tissue of plants growing in the field was also harvested from the same field sites on different years in the Yukon over the course of several visits (N 60° 55.928', W 135° 10.249', elevation: 647 m). *Thellungiella* leaf tissue was harvested by Sandra Little from a population of plants growing on a salt-flat located 5 km north of Whitehorse, YT on July 13th, 2003 (Fig. 1). According to the Meteorological Service of Canada national precipitation survey, precipitation totals for the 2003 growing season were 20-30% lower than the long-term normals, and by definition, this is considered a drought year (Hogg and Wein, 2005). Ten days prior to leaf tissue harvest only 1.8 mm of rainfall was recorded for the area, with no rain in the four days immediately preceding her visit. The rainfall pattern for the 2003 growing season was typical of an arid region such as Tuscon, Arizona (Table I). Average high and low temperatures for the ten-day period prior to harvest was 22°C and 15°C, respectively. On the day of harvest, soil temperature was 17°C, air temperature was 24°C and the light intensity was 1500 $\mu$moles $m^{-2}$ $s^{-1}$. On June 23rd, 2005, David Guevara and I harvested *Thellungiella* leaf tissue from plants at the same locations as those sampled in 2003 (Fig. 1). For the ten-day period leading up to harvest, 60 mm of rainfall was recorded for the area with 50% of this rainfall occurring four-days before sampling. Rainfall totals for the 2005 growing season were much higher than the seasonal average recorded for Whitehorse, YT from 1971-2000 (Table I). Average high and low temperatures for the ten-day period before harvest in 2005 were 18°C and 7°C, respectively. On the day of harvest soil temperature was 18°C, air

**Figure 1. Climatic data.** Precipitation (■), maximum (—) and minimum (- - -) temperature values for Whitehorse, YT for May, June and July of the years 2003, 2005 and 2006. Leaf tissue was harvested on July 13th, 2003 by Sandra Little, and harvested by Jeff Dedrick and David Guevara on June 23rd, 2005, and May 20th, 2006. The meteorological data was obtained from was collected from the Government of Canada Weather Office (2007).

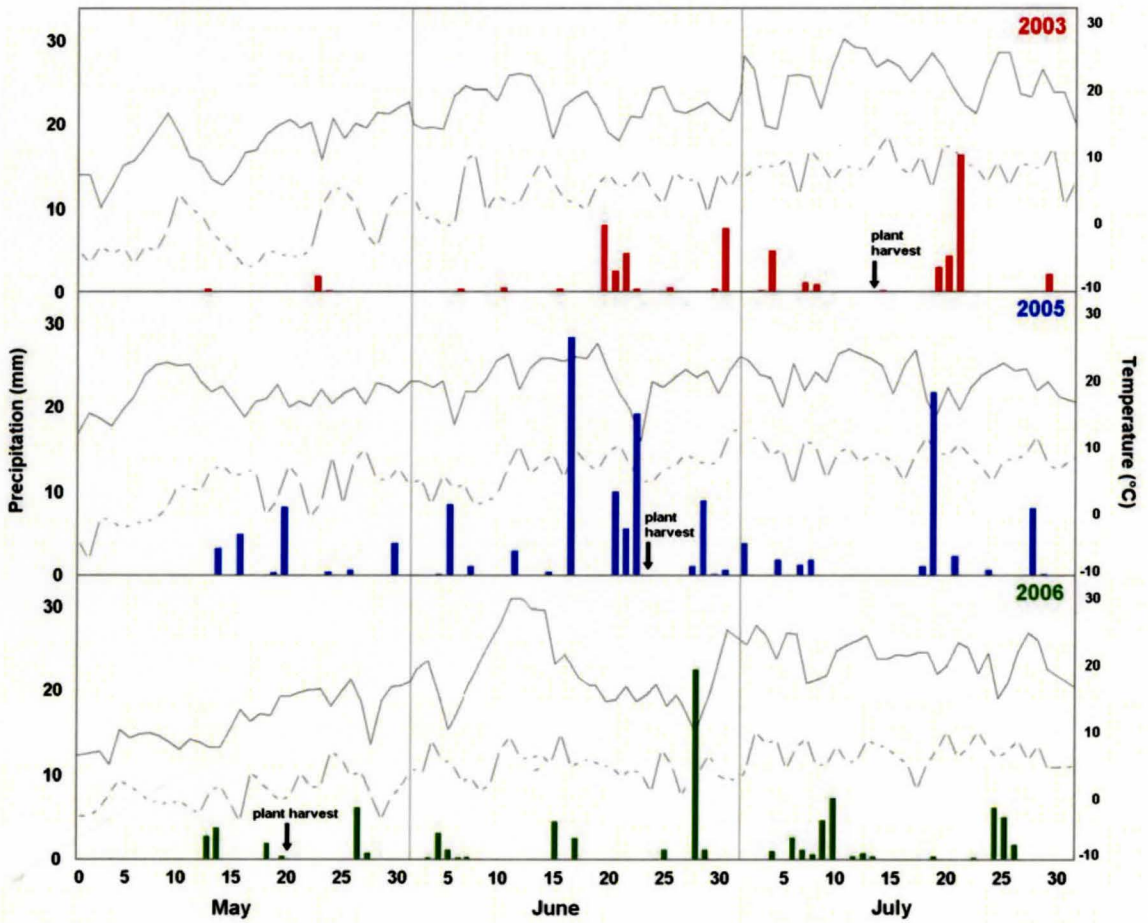

2003
2005
2006
plant harvest
plant harvest
plant harvest
Precipitation (mm)
Temperature (°C)
May
June
July

**Table I. Precipitation totals.** Comparison of precipitation totals (mm) recorded for Whitehorse, YT, Quindao, SD, Hamilton, ON and Tucson AZ. Climate data for Whitehorse, YT and Hamilton, ON were collected from the Government of Canada Weather Office (2007). Precipitation values for Quingdao, Shandong, China were collected from the National Climatic Data Center (2007). Precipitation values for Tucson, Arizona were collected from the Arizona Meteorological Network (2007).

| | | Whitehorse YT, Canada | | | Quindao SD, China | Hamilton ON, Canada | Tuscon AZ, U.S.A. |
|---|---|---|---|---|---|---|---|
| | 2003 | 2005 | 2006 | 1971-2000 | 1886-1989 | 1971-2000 | 1998-2005 |
| **April** | < 1 | 19.2 | 21.6 | 1.3 | 33.6 | 68.7 | 14.4 |
| **May** | 2.3 | 20.3 | 6.8 | 13 | 40.5 | 81.8 | 2.7 |
| **June** | 24.6 | 82.2 | 38 | 29.7 | 86.4 | 71.6 | 11.4 |
| **July** | 34.4 | 41.4 | 33.4 | 41.4 | 168.7 | 74.9 | 57.8 |
| **TOTAL** | **62.3** | **163.1** | **99.8** | **85.4** | **329.2** | **297** | **86.3** |

temperature was 24°C and the light intensity was 1433 $\mu$moles $m^{-2}$ $s^{-1}$. On May 20$^{th}$, 2006, David Guevara and I harvested *Thellungiella* leaf tissue from plants at the same field location as those in 2003 and 2005 (Fig. 1). Rainfall totals for the 2006 growing season were typical of the norms for the Whitehorse, YT region (Table I). In the ten-day period preceding sampling there was a total of 8 mm of rainfall and 0.6 mm of snowfall, with 2 mm of precipitation occurring the day before leaf tissue was harvested. Average high and low temperatures for the ten-day period preceding harvest were 10°C and -1°C, respectively. On the day of harvest, soil temperature was 4°C, air temperature was 9°C and the light intensity was 1427 $\mu$moles $m^{-2}$ $s^{-1}$.

Climate data for Whitehorse, YT and Hamilton, ON were collected from the Government of Canada Weather Office (2007). For comparison, precipitation values for Quingdao, Shandong, China are also shown in Table I (National Climatic Data Center, 2007). Precipitation values for Tucson, Arizona were collected from the Arizona Meteorological Network (2007).

Approximately 200 mg of leaf tissue was harvested at the same relative time-point each year. This time-point corresponded to 6 h after dawn. This tissue was transferred to a pre-weighed 2 mL CryoVial® Cryogenic Storage Vials (catalogue no. 5000-0012) and quickly flash frozen by immersion into $N_2$(l) at the field site. $N_2$(l) used in the field was provided by Jacobs Industries Ltd.(Whitehorse, YT). Leaf tissue from the field sites was stored and shipped back to McMaster University using a charged Vapour Shipper (model no. XC 20/3V) supplied by the Population Health Research Institute affiliated with

McMaster University and the Hamilton Health Sciences Corporation (arrangements were made by contacting Ms. Rose Mayhew).

Unstressed "control" tissue from field sites is not available. As such, cauline leaves taken from mature Yukon *Thellungiella* plants grown in controlled environment chambers was used for metabolite extraction. This tissue was prepared and sent to us by Dr. Annie Wong, Biology Department, U. of Waterloo.

The protocol reported by Fiehn et al. (2000) was used to extract the metabolites present in the polar and lipid phases prepared from leaves of *Thellungiella* plants. Previously frozen or freshly harvested leaf tissue was ground in a chilled mortar with a pestle and 700 µL of 100% HPLC grade methanol (Caledon Laboratories Ltd, catalogue no. 6701-7). 50 µL of 2 mg/mL ribitol was added to the slurry as an internal recovery standard. This slurry was transferred to a 15 mL Corex© tube and placed in a 70°C water bath and shaken for 15 min. The tubes were then centrifuged at 10,000 *g* for 3 min at 4°C using a Beckman Avanti$^{tm}$ J-25 centrifuge fitted with a Beckman JA-20 rotor. The supernatant was transferred to a 16 x 100 mm disposable culture tube (Fisherbrand®, catalogue no. 14-961-27) and 700 µL of chloroform was added to resuspend the pellet. The resuspended pellet was heated at 37°C for 5 min and then centrifuged as above and the second supernatant was pooled with the first. A volume of 1.4 mL water was added to the pooled supernatant and this mixture was centrifuged at 3,000 *g* for 15 min at room temperature using an IEC Clinical centrifuge. The upper, polar phase was removed and 250 µL aliquots were dispensed into Wheaton 1-mL V-vials (catalogue no. 986254) and evaporated to dryness under a stream of $N_2$(g) in an N-evap analytical evaporator

(Organomation Associates Inc., Westbury, NY). Once dried, each sample was closed with a Teflon-lined screw cap and stored with desiccant at -20°C until subjected to further processing. Alternatively, 250-µL aliquots of the polar phase were combined with 250 µL of water and flash frozen by immersion in $N_2(l)$. The frozen samples were freeze-dried in a microfuge tube sealed with a cap containing three ventilation holes (1 mm in diameter) overnight using a Freezone Plus 6 lyophiliser (Labconco, Kansas City, MO). Freeze-dried samples were stored at -20°C until subjected to further processing.

**Fatty acid standards**

A volume of 50 µL of fatty acid standard mix (dissolved in HPLC grade tetrahydrofuran, Caledon Laboratories Ltd, catalogue no. 8901-7) was added to the dried sample and then samples with fatty acid standards were evaporated to dryness under $N_2(g)$ before derivatization. The composition of the fatty acid standard mix was the same as described by Roessner et al. (2000). The mixture consisted of nine fatty acid standards (all from Sigma): heptanoic acid ($C_7$) 3.7% v/v, nonanoic acid ($C_9$) 3.7% v/v, undecanoic acid ($C_{11}$) 3.7% v/v, tridecanoic acid ($C_{13}$) 3.7% v/v, pentadecanoic acid ($C_{15}$) 3.7% v/v, nonadecanoic acid ($C_{19}$) 7.4% v/v, tricosanoic acid ($C_{23}$) 7.4 % v/v, heptacosanoic ($C_{27}$) acid 22.2% v/v and hentriacontanoic acid ($C_{31}$) 55.5% v/v. The fatty acid standards were dissolved in tetrahydrofuran to a final concentration of 10 µg/µL. These standards were used to calculate retention time indices (RTIs) using the following equation (Roessner et al., 2000):

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

In this equation, $C_n$ represents the number of carbons present in the fatty acid that elutes before compound x, $C_{n+1}$ represents the number of carbons present in the fatty acid that elutes after compound x and $RT_x$ denotes the retention time of compound x.

**Derivatization**

The method of derivatization used follows the protocol described by Fiehn et al. (2000). In a fume-hood, dried samples were dissolved in 50 µL of methoxyamine (20 mg/mL prepared in pyridine; Pierce Chemical, Rockford, IL) and the tubes were closed and inclubated in a water bath at 30°C for 90 min. After methoxymation, chemical components were converted to volatile trimethylsilyl (TMS) derivatives with 80 µL of 100% N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA; Pierce Chemical, Rockford, IL) at 37°C for 30 min. After 30 min at 37°C samples were transferred to 150-µL Q-sert vials with PTFE/Silicone/PTFE Non-bonded septa (National Scientific Company, catalogue no. C4000-53B) for analysis by GC/MS.

**Gas Chromatography/Mass Spectrometry (GC/MS)**

Immediately before analysis by GC/MS, derivatized samples were diluted 25-fold and mixed by the Gerstel MPS2 autosampler. A volume of 48 uL hexane was added to 2 uL of sample in a 350-µL Target microsampling vial fitted with a Target 10 mm screw cap. A volume of 1 µL diluted sample was injected into the splitless injection port of a GC/MS set at 230°C. Samples were analyzed with a TRACE Gas Chromatograph coupled to a DSQ Mass Spectrometer controlled by Thermo Finnigan data system

software (Thermo Fisher Scientific, Inc.). A Rtx®-5M5 column (Restek Co., RK12623127) with a 5% v/v diphenyl / 95% v/v dimethyl polysiloxane stationary phase, 0.25 μm film thickness, 30 m length, 0.25 mm diameter, and a 5 m built-in guard column was used. Helium was the carrier gas at a column head pressure of 90 kPa and 1 mL/min flow rate. The instrument settings included: mass transfer line at 275°C, the ion source was 200°C, and the multiplier voltage of the MS was about 1100 V. Perfluorotributylamine was used to tune and calibrate the TRACE DSQ. A scan rate of 2004 amu/s was used in the range of 50-650 amu. For each run the GC oven was set at 50°C initial temperature which was held for 2.5 min then increased at 7.5°C/min to 70°C at which point it is increased at 5°C/min to 310°C where it was held for 6 min.

**Statistical Analysis**

Data retrieved from the GC/MS was analysed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) with a scan direction set from low to high. Deconvolution sensitivity, deconvolution resolution and shape requirement was set at low, medium and low, respectively. GC/MS Data Analysis (GASP) was used to correct variability in GC/MS column performance by converting retention time of peaks to a retention index (RI). Peaks with the same RI values were aligned from multiple GC runs using GASP software (Nuin, 2004). RRF values of 0 were replaced with the threshold of detection 0.00005. The aligned metabolic profiles were subjected to multivariate analyses: HCA and PCA to search for statistically significant patterns between chromatographic components from different plant samples and in response to

drought stress treatments. PCA analysis was performed using Statistica 6.1 (http://www.statsoft.co.uk/mrinfo6.html). HCA analysis was performed using the software packages Cluster (http://rana.lbl.gov/EisenSoftware.htm) and Java Treeview 1.1.0 (http://jtreeview.sourceforge.net). A $\log_{10}$ transformation was applied to the data prior to the multivariate analyses for normalization. Normalization is important for reducing redundancies and inconsistencies caused by systematic or sporadic changes in relative abundance measurements leading to the loss of the database integrity. One-way ANOVA using a function from "R" (http://www.r-project.org/) called from the program GASP was performed on metabolite relative response factors and changes in metabolite content with $P \leq 0.05$ were considered to be statistically significant. Venn diagrams were constructed using the number of metabolites undergoing statistically significant changes ($P \leq 0.05$) between treated and untreated plants (Venn, 1880). Multi-factorial ANOVA was performed using "R" and Duncan's Multiple Range Test was a described by Steel and Torrie (1980).

## RESULTS - PART 1

### Native habitat of Yukon and Shandong *Thellungiella*

Yukon *Thellungiella* plants used in this study were grown from seeds originally collected at field sites near Whitehorse, Yukon and propagated for bulk seeds at McMaster University. A map of the field locations where the plants have been found is shown in Figure 2. The native habitat of the Yukon plants is characterized by a semi-arid, sub-arctic climate with a very short growing season (Warwick et al., 2004). The permafrost soils are saturated with NaCl, $MgSO_4$ and $CaHCO_3$, and are deficient in essential macronutrients (personal communication from Dr. Bruce Bennett, Wildlife Viewing Biologist, Yukon Department of Environment). The soil profile consists of an organic layer that is approximately 20 cm deep that overlays a clay sub-soil. The soil surface has white to gray-white encrustations of concentrated salt crystals over areas comprising the salt flats (Fig. 3). The saline and alkaline soils found at the Yukon salt-flats are the result of the accumulation of dissolved minerals from rocks carried by water flowing down from the nearby mountains (Bennett, 2000). With a layer of permafrost below the salt-flats, leaching of minerals to groundwater and movement away from the site does not occur. Rather, dissolved minerals are concentrated at the soil surface by evaporation during the hot, dry summers typical for the area, eventually forming the salt flats (Fig. 3; Bennett, 2000).

Seeds of the Shandong *Thellungiella* plants used in this study originated from the seacoast of north-eastern China near the mouth of the Yellow River where highly saline

**Figure 2. Aerial map of field sites.** Dillabough's Grazing Lease field site relative to Whitehorse, YT (N 60° 55.928', W 135° 10.249', elevation: 647 m). Figure was drawn by Jeff Dedrick.

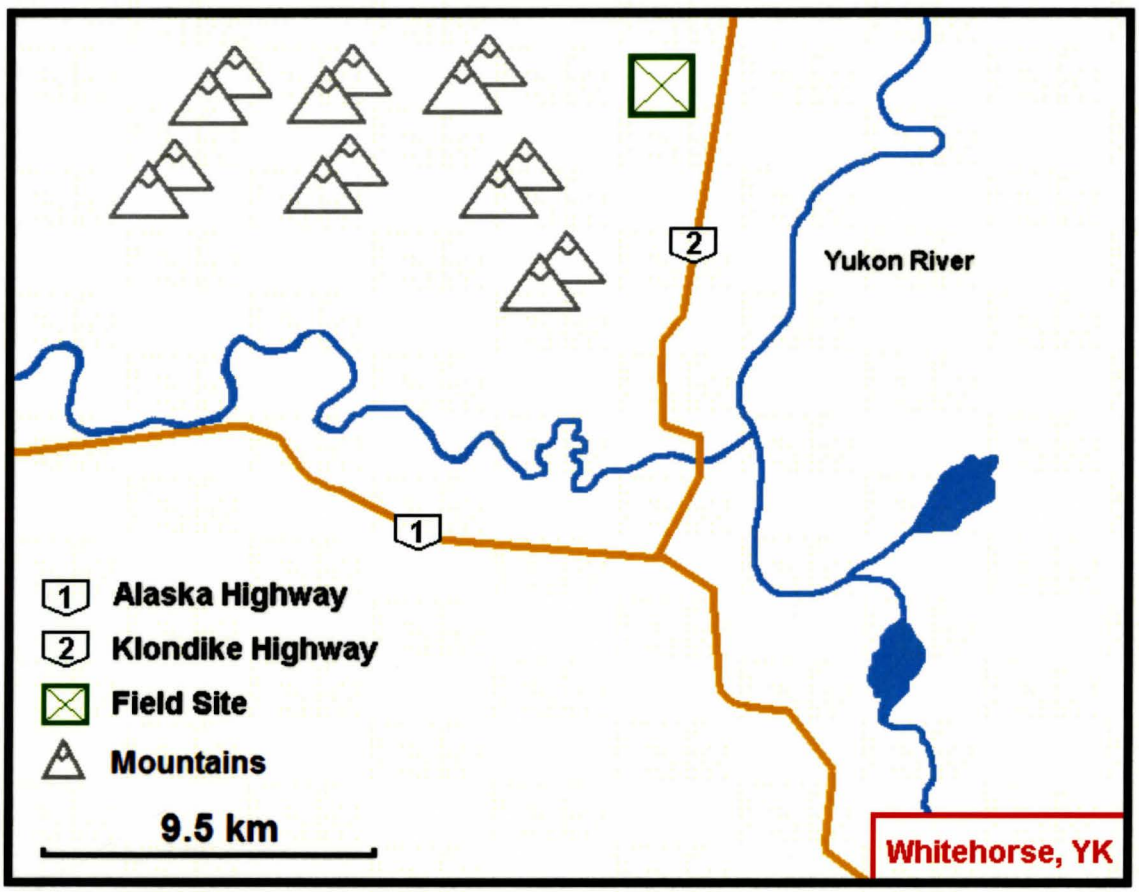

Yukon River
2
1
1 Alaska Highway
2 Klondike Highway
Field Site
Mountains
9.5 km
Whitehorse, YK

**Figure 3. *Thellungiella* growing in the field.** Photos showing field site at Dillabough's Grazing Lease, YT (N 60° 55.928', W 135° 10.249', elevation: 647 m). **A**, Population of *Thellungiella* plants identified by the white flowers in the center of the photo. **B,** Asterisk (*) identifies a single *Thellungiella* plant growing in the field site on soil with surface encrusted by salt crystals. Photos were taken on June 23rd, 2005 (A) and on May 17, 2006 (B) by Jeff Dedrick.


A


B
*

soils predominate (Inan et al., 2004). They were propagated to provide bulk seed by Dr. Ray Bressan of Purdue University and again at McMaster University to ensure sufficient seeds were available for this study. The native habitat of the Shandong *Thellungiella* has a temperate climate characterized with abundant rainfall (Koornneef et al., 1998).

**Yukon and Shandong *Thellungiella* have distinct phenotypes**

In their vegetative stage (28-day-old plants), Yukon *Thellungiella* plants grown in controlled environment chambers resemble Arabidopsis plants in having a prominent basal rosette composed of 25 to 35 rosette leaves. The rosette leaves are ovate (2 cm long, 1 cm wide) and radiate from the base of the plant by a petiole that is 1 – 1.5 cm long (Fig. 4). Vegetative Shandong *Thellungiella* plants grown under the same conditions have a similar arrangement of rosette leaves as described for the Yukon plants. However, the Shandong *Thellungiella* has elongated, serrated rosette leaves (2 cm long, 0.5 cm wide) that radiate from the stem at the base of the plant by a petiole that is 2 – 3 cm long (Fig. 4). Flowers on Yukon plants first emerge within the rosette and this is followed by the appearance of a bolt bearing terminal flowers (Fig. 5, A) and eventually siliques bearing seeds.

Flowering Yukon *Thellungiella* plants found in the field are phenotypically different from plants grown in controlled environment chambers. Basal rosettes are usually absent or, if present, they are smaller having fewer leaves of reduced size than those found in chamber-grown plants (Fig. 5, B, C and D). In lieu of rosette leaves, flowering plants in the field have cauline leaves that are slightly larger (2.5 cm long and

**Figure 4. Yukon and Shandong *Thellungiella* phenotypes.** Yukon and Shandong *Thellungiella* plants are distinguished by different leaf phenotypes. These 4-wk-old plants were grown under identical controlled environment conditions (see Methods and Materials for details). Leaves of Yukon plants are typically ovate while Shandong leaves are serrated. (Scale: white bar = 2 cm).

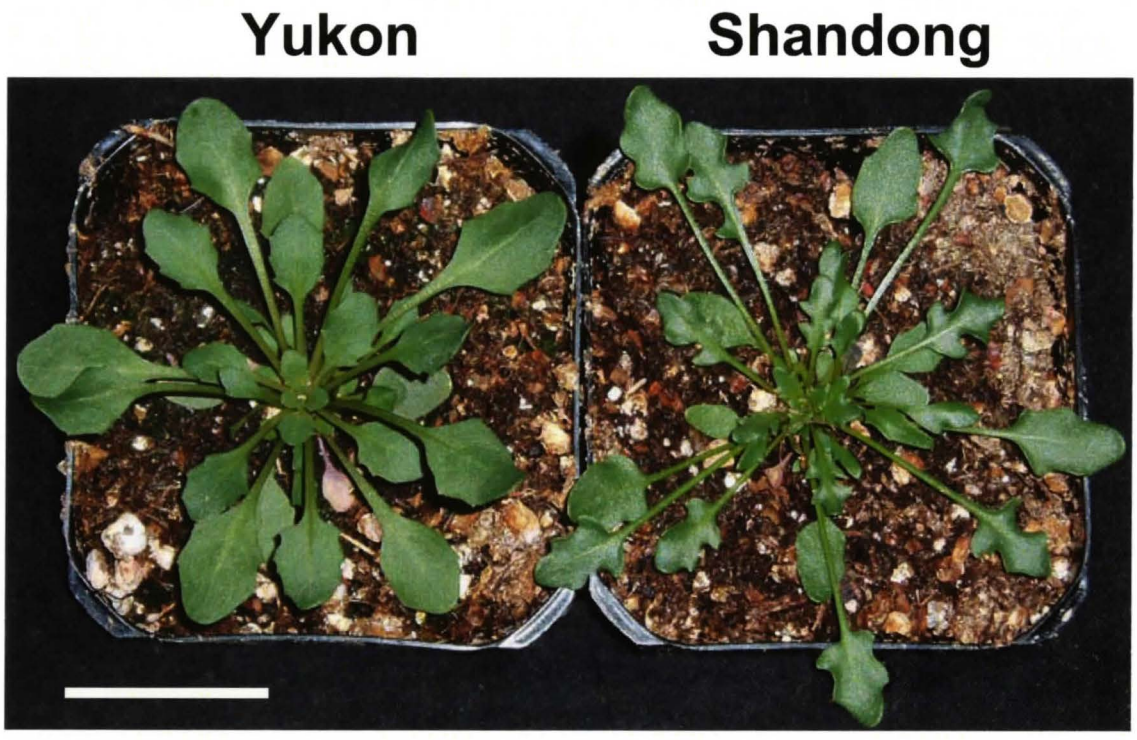
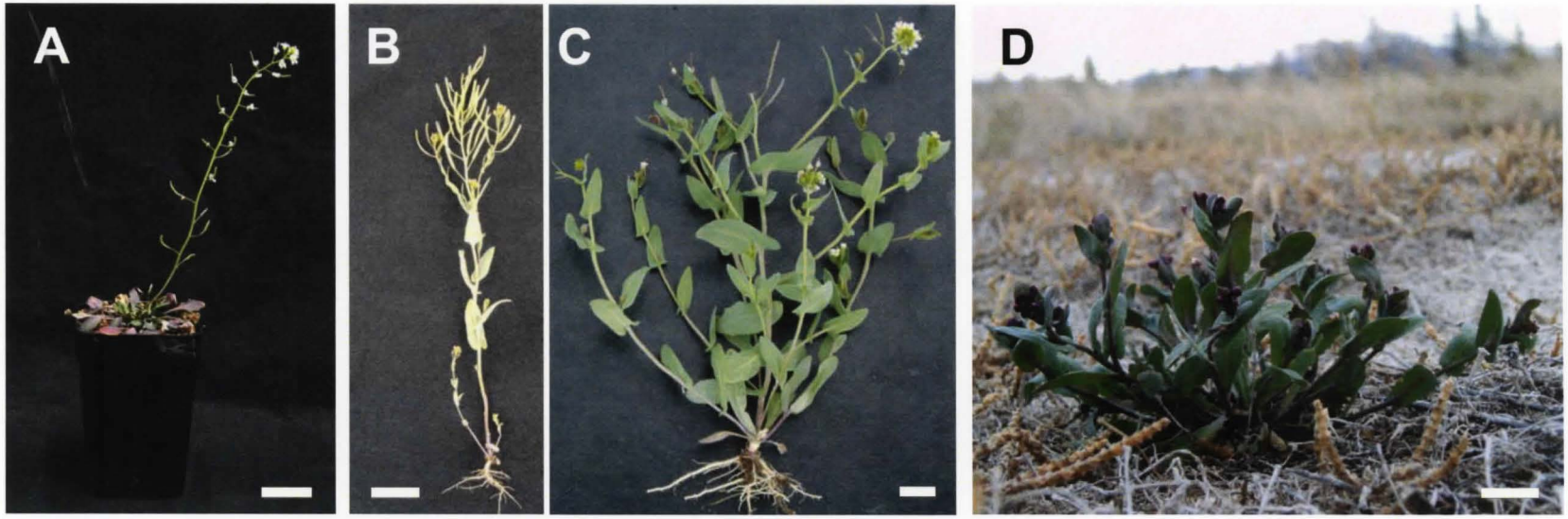

Yukon
Shandong

**Figure 5. Flowering *Thellungiella*.** Flowering Yukon *Thellungiella* plants display different phenotypes under varying environmental conditions. **A**, Typical Yukon plant at 10-weeks post germination grown under controlled environment cabinet conditions have white flowers and prominent basal rosette leaves. B and C, Mature field plants with white flowers, cauline leaves, negligible basal rosette present and considerable variation in size between individual plants growing at the same site near Whitehorse YT in June, 2005. **D**, Plants in May, 2006 had purple flowers and leaves tinged by purple coloration. Photos were taken by Jeff Dedrick. (Scale: white bar = 2 cm).


A
B
C
D

1.5 cm wide) than the typical rosette leaf of plants in growth chambers (Fig. 5, B, C, and D). Plants found on the salt-flats usually have one main stem or bolt with many terminal flowers or siliques but larger plants can have more than one auxiliary stem. The height of the stem ranged from 5 to 20 cm. Small plants (5 to 10 cm tall, one main stem) have between 5 to 10 cauline leaves (Fig. 5, B) whereas large plants (> 10 cm tall, > 1 stem) could have as many as 40 cauline leaves (Fig. 5, C).

**Comparison of the drought stress response for Yukon and Shandong plants**

28 day old Shandong and Yukon plants were left unstressed (controls) or were subjected to a single episode of simulated drought in controlled chamber conditions. Leaf relative water content (RWC) as well as the time elapsed until the first visible signs of wilting after watering was stopped were recorded (Fig. 6 and Table II). The average RWC for 28-day-old well-watered plants of the Shandong and Yukon plants averaged 85.9 +/- 1.6% and 81.0 +/- 1.9%, respectively. After water was withheld the leaves showed signs of wilting at average leaf RWCs of 52.5 +/- 2.0% for the Shandong plants (Fig. 7, A and C) and 54.8 +/- 0.8% for the Yukon plants (Fig. 7, B and D). There were no significant differences observed in the average time until visible leaf wilting for the two types of *Thellungiella* following a first exposure to simulated drought: 6.3 +/- 0.1 days and 6.2 +/- 0.1 days for the Shandong and Yukon plants, respectively (Fig. 6). After wilting, a subset of plants were re-watered and allowed to recover for two days (Fig. 8, A, B, C and D). Among these plants, 75% of the RW1 Shandong *Thellungiella* recovered full turgor (leaf RWC of 82.1 +/- 1.4%) and the remainder remained flaccid and

**Figure 6. Norm of reaction.** A representative Norm of Reaction for Yukon and Shandong plants subjected to one or two successive simulated drought exposures shows that only Yukon plants develop increased tolerance to water deficits after one episode of drought. In this experiment 4-wk-old well-watered plants were left un-watered and the time recorded until the leaves became visibly wilted (Drought Episode 1). Wilted plants were then watered to re-establish turgor and then left un-watered until the plants wilted a second time and the length of time taken for leaves to visibly wilt was recorded (Drought Episode 2). Data used in analysis was $n = 30 \pm$ standard error. Experiment was replicated three times with similar results. See Methods and Materials for experimental details.

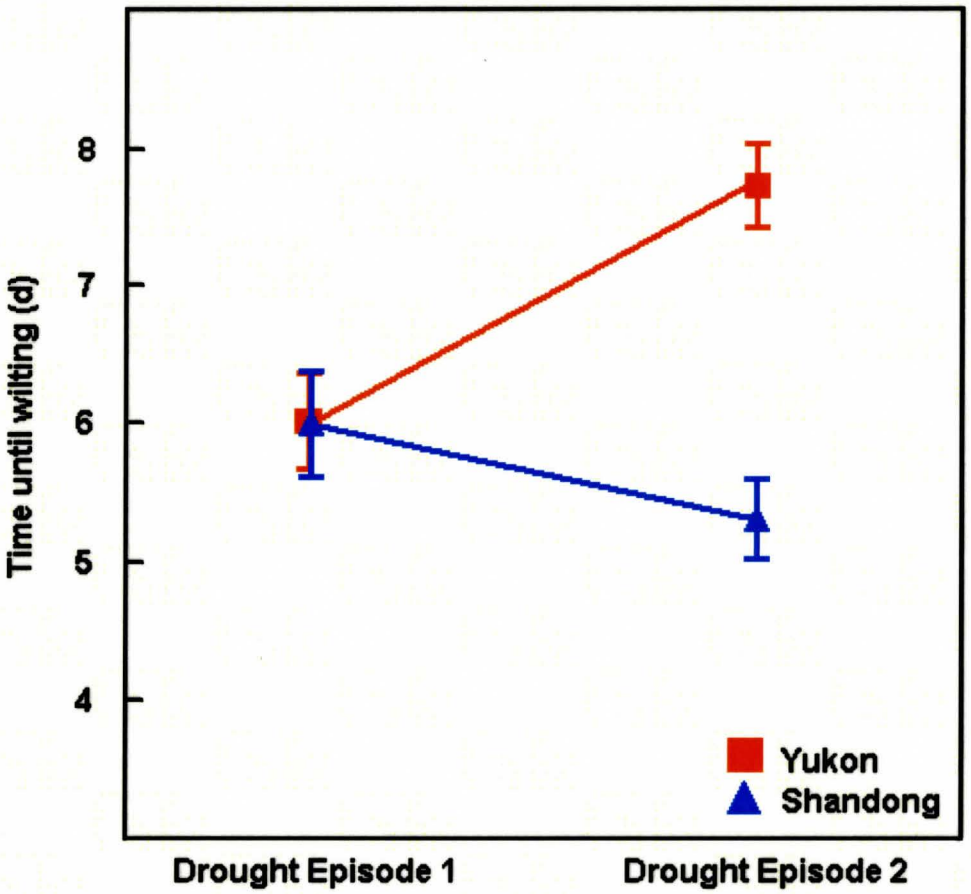
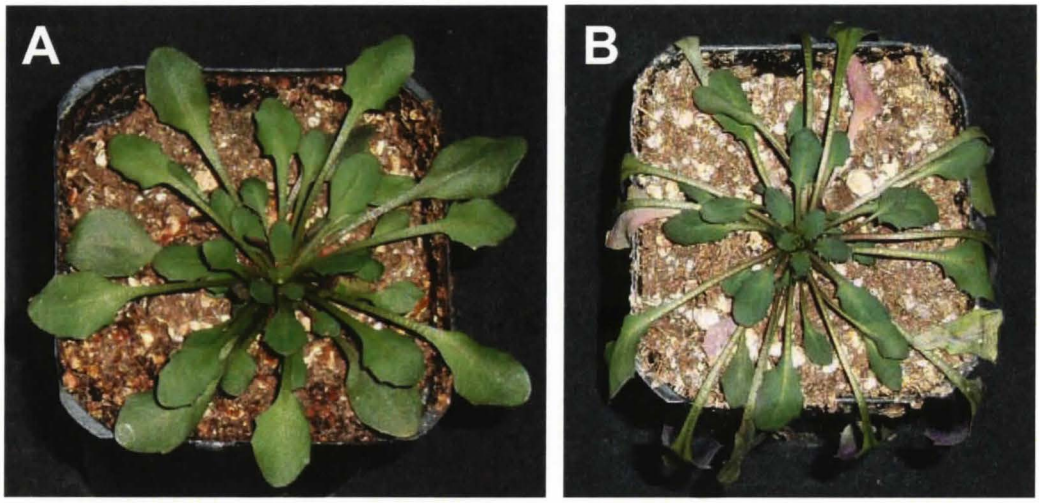

Time until wilting (d)
8
7
6
5
4
Yukon
Shandong
Drought Episode 1
Drought Episode 2

**Table II. Leaf water status.** Leaf water status for two types of *Thellungiella* (Yukon and Shandong) plants growing under controlled environmental conditions. Measurements for well-watered plants were recorded at 6-weeks post germination. Plants were exposed to two consecutive drought episodes separated by a two-day recovery period when plants were watered. Values measured include relative water contents (RWC), water ($\psi w$), solute ($\psi s$) and turgor ($\psi p$) potentials (n is a minimum of 9). Data shown is the mean +/- the standard error. Plant type by Treatment interaction means for each of the four measurements that do not bear the same letter differ ($P<0.05$) as determined by Duncan's Multiple Range Test (Steel and Torrie, 1980). See Appendix D for ANOVA tables corresponding to this data.

| | | Well-watered | Wilted | RW1 | RW2 |
|---|---|---|---|---|---|
| RWC (%) | | | | | |
| | Shandong | 85.86 ± 0.98*b* | 52.47 ± 1.33*e* | 82.08 ± 0.12*cd* | 84.49 ± 1.69*bc* |
| | Yukon | 80.98 ± 0.27*d* | 54.82 ± 0.75*e* | 83.92 ± 0.46*bc* | 91.91 ± 0.51*a* |
| Ψw (MPa) | | | | | |
| | Shandong | -1.19 ± 0.04*a* | -2.63 ± 0.18*c* | -1.16 ± 0.02*a* | -1.17 ± 0.02*a* |
| | Yukon | -1.20 ± 0.06*a* | -2.82 ± 0.07*d* | -1.09 ± 0.03*a* | -1.66 ± 0.02*b* |
| Ψs (MPa) | | | | | |
| | Shandong | -1.49 ± 0.04*a* | -2.54 ± 0.19*c* | -1.49 ± 0.02*a* | -1.48 ± 0.03*a* |
| | Yukon | -1.60 ± 0.04*a* | -2.77 ± 0.07*d* | -1.48 ± 0.06*a* | -2.06 ± 0.03*b* |
| Ψp (MPa) | | | | | |
| | Shandong | 0.31 ± 0.03*c* | 0*d* | 0.33 ± 0.04*bc* | 0.32 ± 0.04*c* |
| | Yukon | 0.39 ± 0.03*abc* | 0*d* | 0.43 ± 0.04*a* | 0.42 ± 0.02*ab* |

**Figure 7. *Thellungiella* well-watered and visibly wilted.** Plants used in this study include Yukon (A, B) and Shandong (C, D) *Thellungiella* control plants that were watered once daily (A, C) or plants subjected to water deficits and allowed to visibly wilt (B, D). Typical leaf RWC values were 80-85% for controls and 55-60% for wilted plants. Controlled chamber conditions included a 21 h light / 3 h dark photoperiod and a photon flux density of 250 $\mu$M $m^{-2}$ $s^{-1}$. Day and night temperatures were set at 22°C and 10°C, respectively. (Scale: white bar = 2 cm).

## Yukon




## Shandong

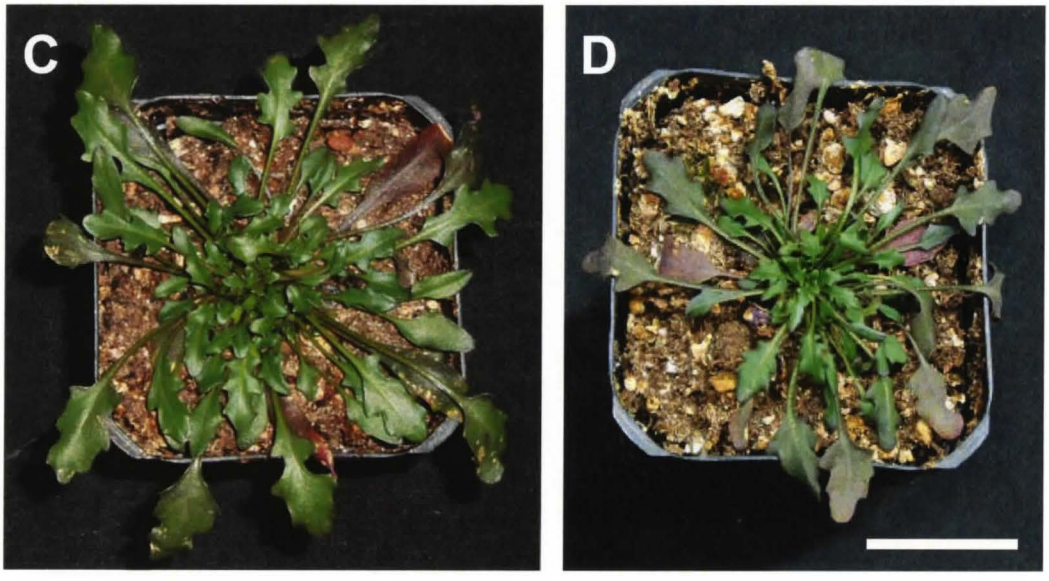
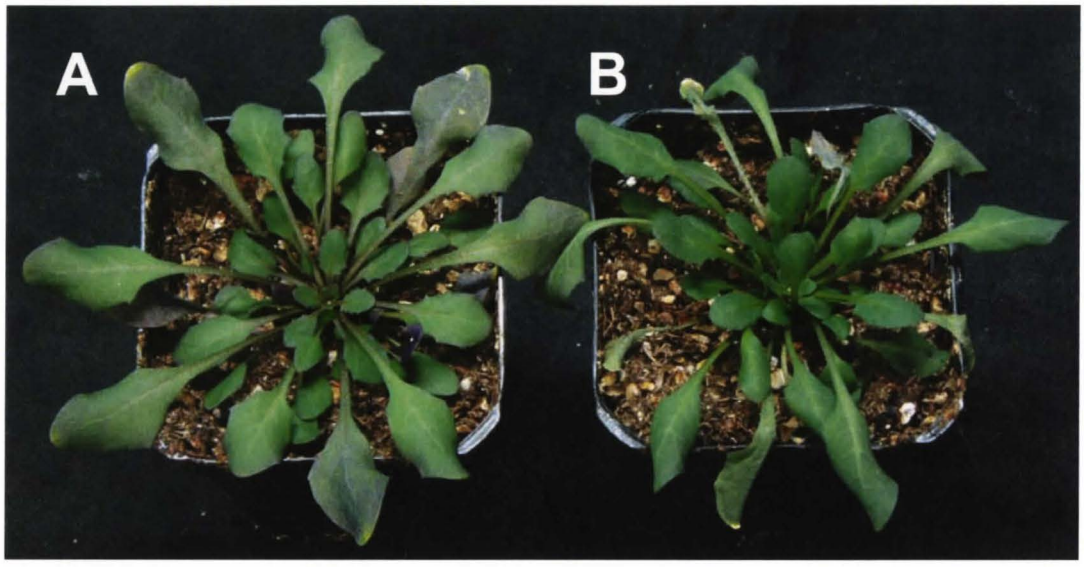
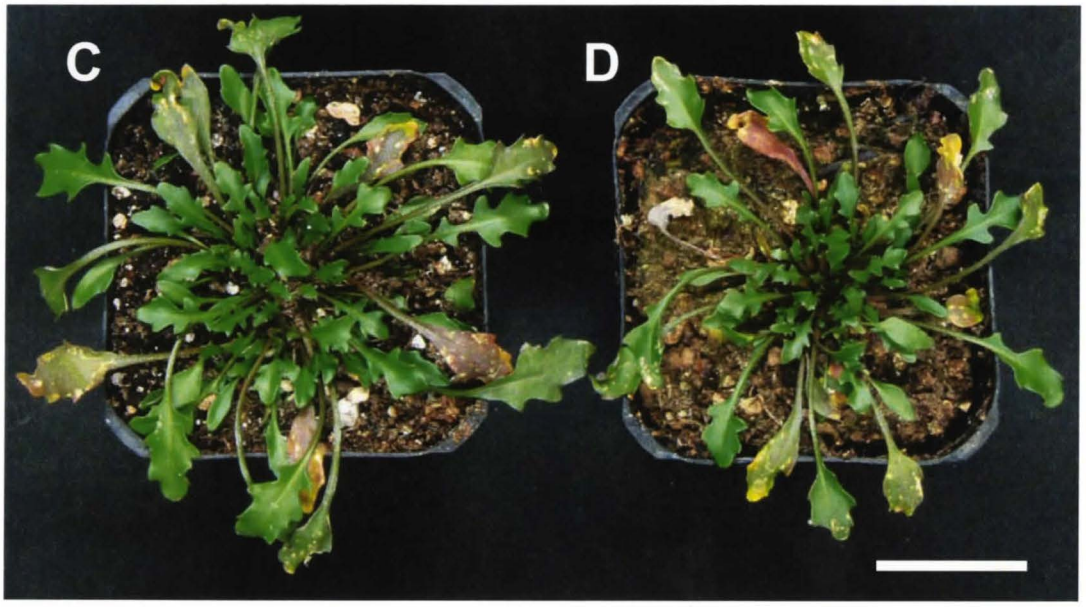


**Figure 8.** ***Thellungiella*** **well-watered and re-watered.** Yukon and Shandong plants recover turgor within two days following watering after a simulated drought treatment. Representative well-watered plants (A, C) are shown along with plants watered and allowed to recover after one episode of drought (B, D). Plants that experience drought followed by re-watering and recovery are phenotypically indistinguishable from their respective well-watered control plants. Plants were grown under identical controlled growth chamber conditions (see Materials and Methods). (Scale: white bar = 2 cm).

Yukon




Shandong



eventually died while under the same conditions 100% of the RW1 Yukon plants recovered full turgor (leaf RWC of 83.9 +/- 0.7%).

In order to estimate the impact of a second, subsequent exposure to drought on plant survival a subset of recovered RW1 plants underwent another simulated drought episode followed by re-watering and recovery. The recovered plants are designated as RW2 in figures relating the outcome of these experiments. Leaves of RW2 plants showed average leaf RWCs of 84.5 +/- 1.9% and 91.9 +/- 1.3% for the Shandong and Yukon *Thellungiella*, respectively. A significant difference in drought-response behaviour was observed between the two types of *Thellungiella* with respect to time taken for leaves of RW2 plants to wilt. The norm of reaction data comparing the time elapse before the first sign of visible wilting as a result of this second drought exposure is shown in Figure 6. In a typical experiment 72% of the Shandong plants exposed to a single period of water deficit decreased their time required to wilt by one or more days, with a mode decrease of 1 day (n = 24) during a subsequent drought simulation. The remaining 28% of the Shandong plants showed no change in their time required to wilt between RW1 and RW2 plants. In contrast, on average 93% of the Yukon plants that experienced a single exposure to drought and recovery required one or more additional days without water in order to wilt, with a mode increase of two days (n = 30), compared to plants experiencing a first exposure to water deprivation. The remaining 7% of the Yukon plants showed no change in their time required to wilt between RW1 and RW2 plants. This is consistent with the majority of Yukon plants developing an increased tolerance to drought following a single episode of water deprivation while Shandong plants became less tolerant under the same experimental conditions.

The long term impact of two drought episodes was evaluated for both plants. RW2 plants were watered as needed for a further 10 weeks and >95% of the drought-stressed Yukon plants went on to flower and produce viable seeds before dying while all of the drought-stressed Shandong plants died before flowering.

In order to assess the extent to which both types of *Thellungiella* can withstand a more prolonged period of drought plants were left un-watered past the point of visible leaf wilting until the average leaf RWC approached 30%. This period of drought simulation lasted about 7-9 days. With this severe degree of water deprivation the leaves took on a pale green, chlorotic appearance and became dry and brittle to the touch (Fig. 9, B and E for Yukon and Shandong plants, respectively). Plants reaching a leaf RWC of approximately 30% were then re-watered every other day and observations were taken to evaluate their capacity to recover. After two weeks, Shandong plants showed no signs of recovery (Fig. 9, F); however, Yukon plants eventually showed emergence of new leaf tissue (Fig. 9, C) from which plants developed that went on to flower and produce viable seeds.

**Yukon and Shandong plants show similar wilting response but distinctive recovery patterns as assessed by water, solute and turgor potential measurements**

Leaf water ($\Psi w$), solute ($\Psi s$) and turgor ($\Psi p$) potential values were measured during the exposure of plants to two, consecutive episodes of drought (Fig. 10, Table II). A typical control leaf $\Psi s$ value for *Thellungiella* ranges from -1.5 to -1.6 MPa. When *Thellungiella* plants are stressed by a drought simulation the leaf $\Psi s$ undergoes a decrease to $-2.77 \pm 0.07$ MPa and $-2.54 \pm 0.19$ MPa for the Yukon and Shandong plants,

**Figure 9. Prolonged drought treatment.** Sequence of photos shows the typical response of Yukon and Shandong plants to prolonged drought treatment. Four-week old well-watered plants had a leaf RWC of 80-85% (A, D are Shandong and Yukon, respectively). These plants were left un-watered until leaves reached a RWC of 30% (B, E) whereupon plants were re-watered and allowed to recover for 2 weeks (C, F). The figure follows a single plant of each type through each stage of the drought/recovery experiment. This figure shows that the leaves for both types of *Thellungiella* did not recover turgor after this severe drought exposure. However, while Shandong plants died (F), Yukon plants survived as indicated by the emergence of new leaves(C). (Scale: white bar = 2 cm).

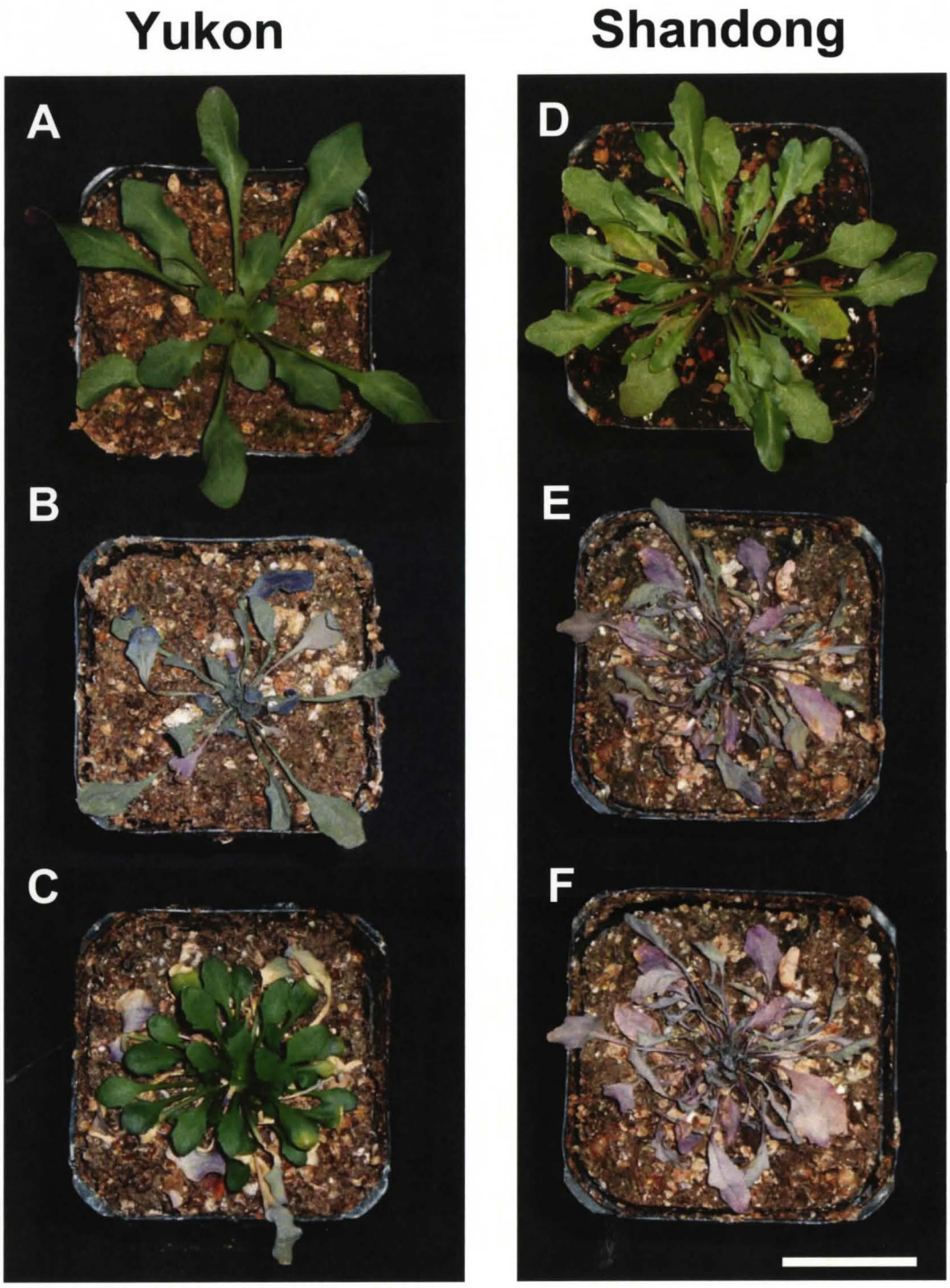
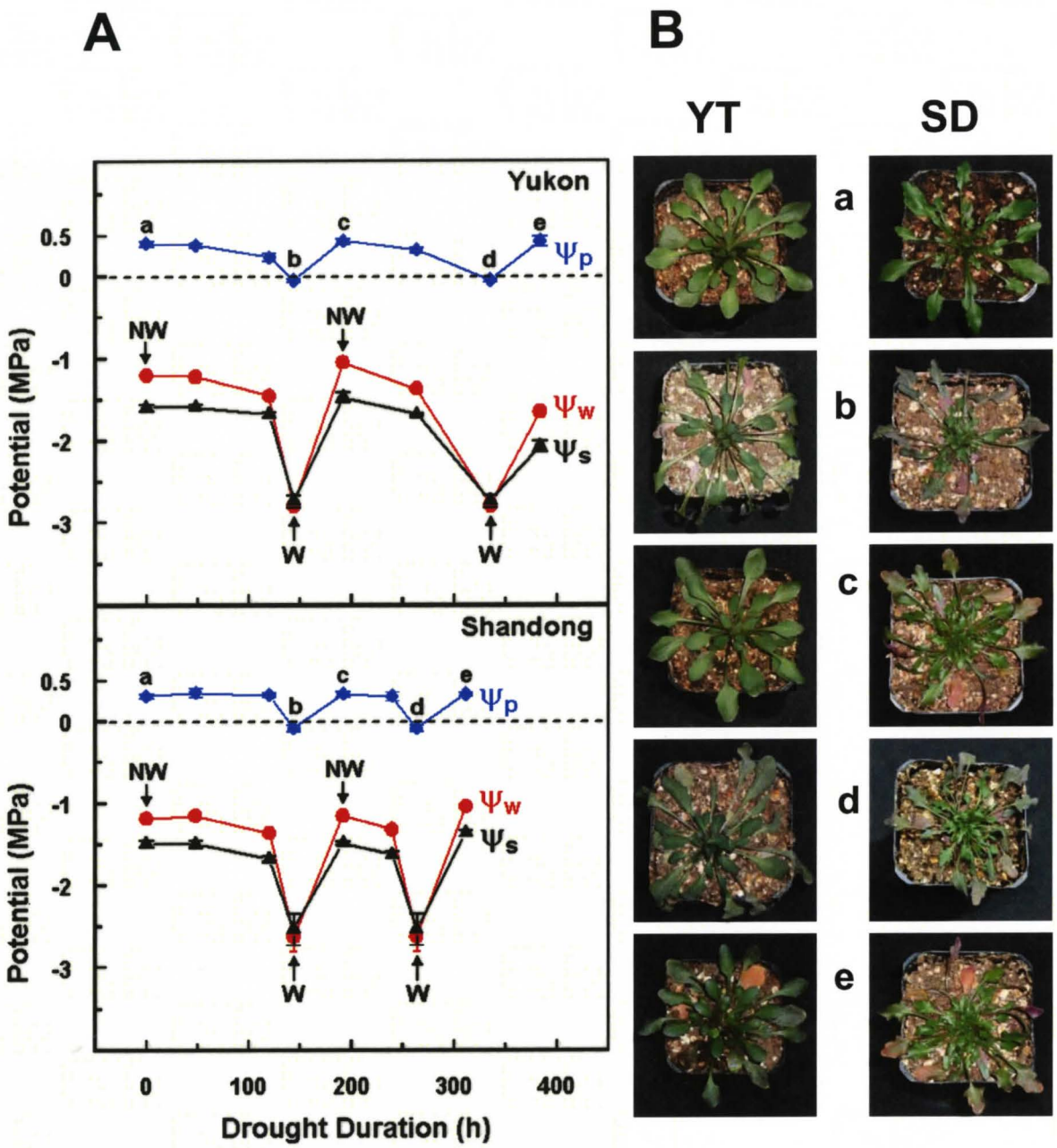

Yukon
Shandong
A
B
C
D
E
F

**Figure 10. Water, solute, and turgor potential values.** The water ($\psi_w$), solute ($\psi_s$) and turgor ($\psi_p$) potentials for the Shandong (SD) and Yukon (YT) plants were recorded over two subsequent drought episodes (n = 9 ± standard errors). Time 0 represents onset of the experiment when 4-week old plants were deprived of water (NW). Re-watering (W) took place upon visible wilt which was equivalent to day 6 & 11 for Shandong plants, and on day 6 & 14 for Yukon plants. After re-watering, plants were allowed to recover for 48-h and then watering was withheld. Representative plants at each stage are shown in panel B: time 0 (a), first wilt (b), re-watered after first wilt (c), second wilt (d), and re-watered after second wilt (e).


A
B
YT
SD
Yukon
Shandong
Potential (MPa)
0.5
0
-1
-2
-3
a
b
c
d
e
$\Psi_p$
$\Psi_w$
$\Psi_s$
NW
W
0
100
200
300
400
Drought Duration (h)

respectively. When the wilted plants are watered and allowed to recover (RW1) leaves fully re-hydrate and Ψs values return to pre-drought levels. When Yukon plants are deprived of water a second time the leaf Ψs reached following the two-day recovery period (RW2 plants) is significantly lower than Shandong plants stressed similarly (Fig. 10, Table II). Thus Shandong RW2 plants do not appear to accumulate solutes with a second episode of drought while Yukon RW2 plants do.

Phenotypically, both types of *Thellungiella* shared similar appearance at each stage of measurement. Representative plants for these stages are shown in Figure 10, panel B.

**Leaf Ψs measurements for Yukon *Thellungiella* from field sites**

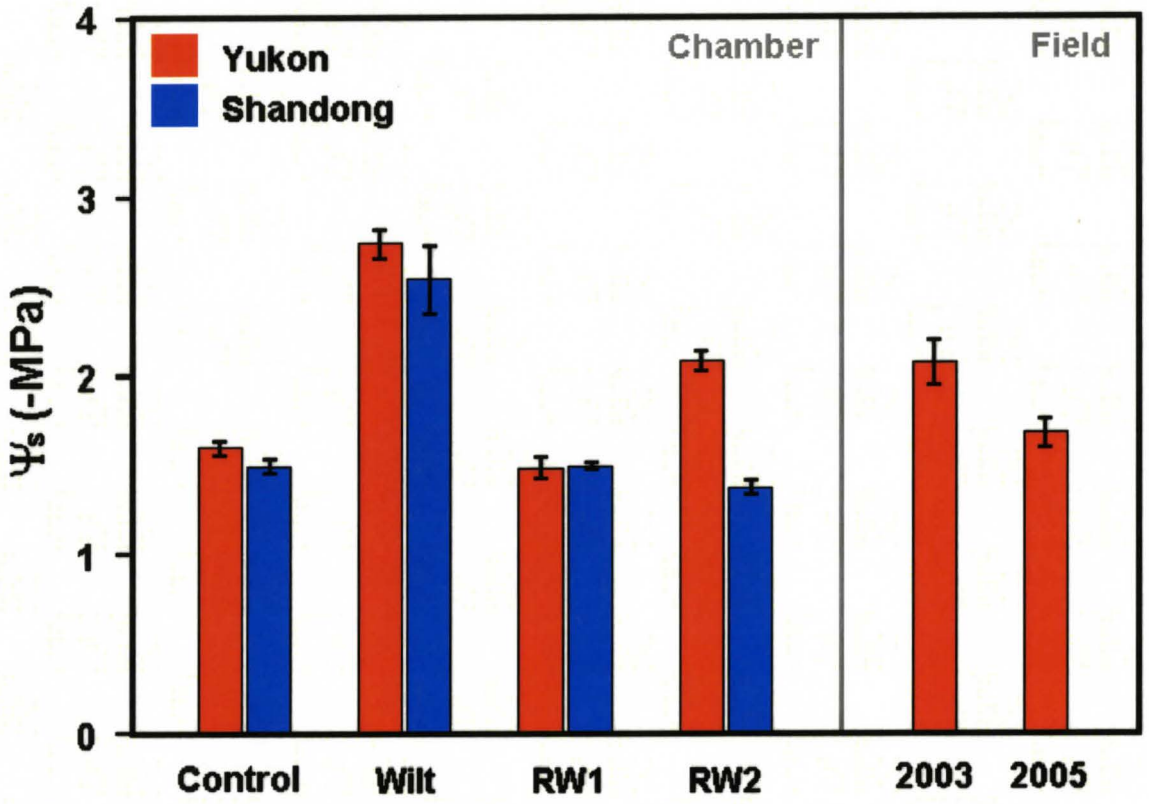
For comparison, leaf Ψs measurements of *Thellungiella* growing in the field during 2003 and 2005 are shown in Figure 11. Plants growing in the field in the 2003 season have significantly lower leaf Ψs values than plants growing at the same field site in 2005. Interestingly, the Ψs leaf values for plants from the dry growing season (2003) are the same as the leaf Ψs values measured for the Yukon plants after turgor recovery following two successive drought episodes in a growth cabinet (RW2), while leaf Ψs values for field plants from 2005 were more similar to the leaf Ψs of a well-watered control plants regardless of whether they are from Shandong or Yukon plants (Fig. 11).

**Metabolite profiling of *Thellungiella* leaves**

*GC/MS analysis of metabolites and data output*

Qualitative and quantitative changes in leaf metabolite levels in response to

**Figure 11. Solute potential values of *Thellungiella*.** Solute potential ($\Psi_s$) measurements for Yukon *Thellungiella* plants exposed to varying growth conditions. 'Control' plants were well-watered throughout the experiment, 'Wilt' plants were left unwatered until visible leaf wilting (55-60% RWC). 'RW1' were plants re-watered after wilting and 'RW2' was a subset of plants that had undergone one episode of drought and recovery and then randomly selected to undergo a second episode of drought followed by re-watering treatment. For plants subjected to drought and re-watering, leaf tissue Ψs was measured two days after re-watering. For field plants, Ψs values were measured by David Guevara for '2003' plants during a growing season characterized with low rainfall and long periods of drought. Ten-days leading up until time of harvest in 2003 there was only 1.8 mm of rainfall, including four straight days of no precipitation prior to the day of harvest. Ψs values '2005' refers to plants measured during a growing season with rainfall totals much higher than the seasonal average for Whitehorse, YT. Ten-days leading up until time of harvest in 2005 there was a total of 60 mm of rainfall, with 50% of this rainfall occurring within four-days of the time of harvest. n is a minimum of 5 measurements from independent plant samples ± standard error. See Table 2 for values of significance.

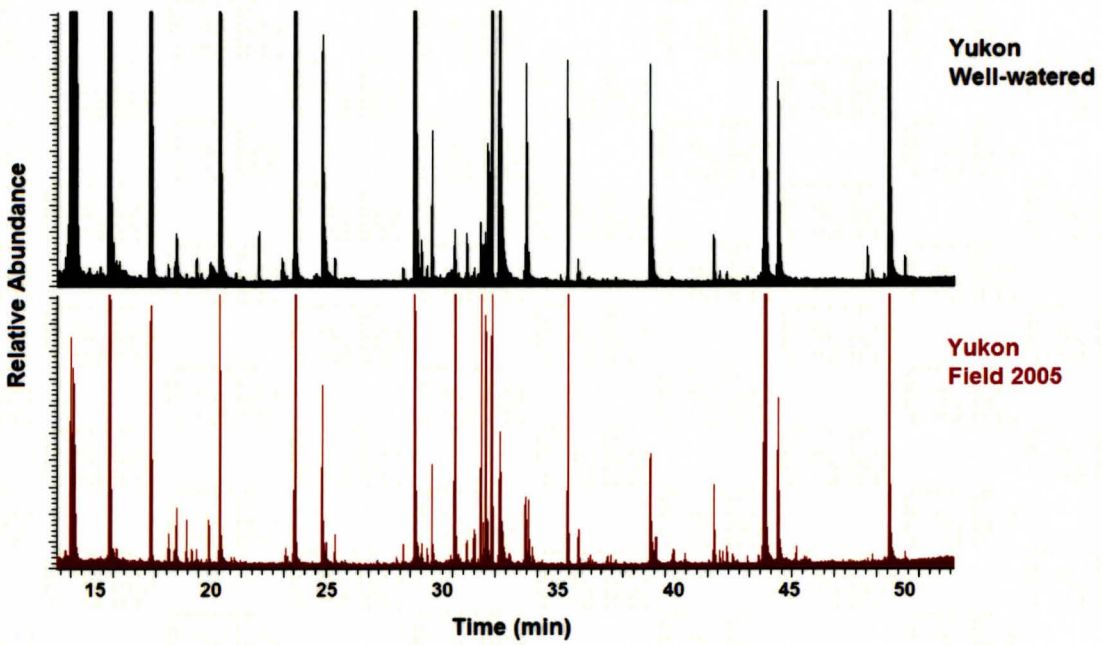

Yukon
Shandong
Chamber
Field
Ψs (-MPa)
0
1
2
3
4
Control
Wilt
RW1
RW2
2003
2005

simulated drought exposure were evaluated by GC/MS (see Materials and Methods for details on plant material and methodology). The classes of compounds separated during a single analytical run include sugars (mono-, di-, and trisaccharides), sugar alcohols, amines, amino acids, organic acids and phosphorylated compounds. Each analysis yields a chromatogram showing polar chemical components resolved by the GC. Comparisons made between chromatograms from plants exposed to various conditions can be used to identify stress-responsive changes that are reproducible and statistically significant. I used this approach to identify drought-responsive differences between the Shandong and Yukon *Thellungiella* plants with respect to polar metabolites.

Figure 12 shows representative chromatograms (TIC or total ion chromatogram) of components present in leaf polar-phase extracts of Yukon plants grown under different conditions. In parallel studies, profiles were produced from Shandong plants as well as Yukon *Thellungiella* harvested at a field site near Whitehorse, YT. As this figure suggests, many peaks are found in chromatograms for each sample and any of these peaks could be expected to differ between various samples (including replicates). It is, therefore, important to identify reproducible profiles for specific plants and/or treatments before one can try to identify a "typical" metabolic phenotype let alone one associated with stress tolerance.

In any comparative study it is important to determine the reproducibility of the methodology used. For a GC/MS analysis, peak areas for any or indeed all components may change between runs. For example, in this study GC/MS chromatograms detected $289 \pm 7$ ($n = 40$) components. Since each analysis involves many peaks, accounting for

**Figure 12. GC/MS chromatograms.** Representative GC/MS chromatograms of polar fractions of *Thellungiella* leaf extracts from plants that were grown in controlled environmental conditions: **well-watered**, and from a saline **field site** in the Yukon. The metabolite profiles shown here show a wide array of comparable peaks present in the polar phase of leaf extracts. Over 300 peaks can be detected in a single run and components are associated with various classes of compounds such as amino acids, organic acids, sugars (mono-, and disaccharides) sugar alcohols, amines, and phosphorylated compounds.


Yukon
Well-watered
Yukon
Field 2005
Relative Abundance
15
20
25
30
35
40
45
50
Time (min)

experimental variation is important. This variation can result from a number of sources: 1) inherent but natural biological variability with respect to the amount of a component originally present in a tissue extracted from the same or different plants, 2) variable extraction efficiencies sample to sample with global losses potentially arising at multiple steps of a lengthy preparatory protocol or 3) some components may be inherently more unstable than others and recoveries may be a function of extraction efficiency and component stability.

To assess the overall recovery of components between successive extractions we standardized the height of each peak to the peak height of an internal standard, ribitol, that is added when samples are initially ground. The relative response factor (RRF) associated with the peak for ribitol in each chromatogram of a complex leaf extract is compared to an RRF for ribitol run alone to minimize processing and optimize ribitol recovery; we then assume peak height in this sample represents 100% recovery. The two RRF values are compared yielding a recovery correction factor that is then applied to each component in the chromatogram of the plant sample containing the ribitol internal standard. Thus, quantitative changes detected between the heights of particular peaks between runs are based on differences between RRFs corrected for recovery each time a sample is analyzed. This approach assumes that all components behave as ribitol during the extraction procedure and will undergo the same extent of losses. This is a simplistic assumption in that different components have different physical and chemical properties and are likely to be extracted with different efficiencies but offers, nonetheless, a pragmatic solution to estimating recovery losses for a chemically complex sample when many metabolites present cannot be identified. Moreover, the reproducibility of

extraction associated with each component is of greater importance in this approach. That is, if a component extracts equally well each time but with lesser or greater efficiency of ribitol we should still be able to detect statistically significant differences associated with the relative abundance of a metabolite that can be correlated with specific growth conditions.

Several factors can affect the sensitivity by which reproducible differences are detected. One source is that a single peak often represents the product of co-eluting compounds. It is important to determine how each component contributes to the height of a single peak and whether the composition of each peak changes reproducibly between samples. In this study peaks were deconvoluted (resolved from each other) on the basis of their characteristic mass spectra using AMDIS software and then the peak area for each co-eluting component was integrated manually. The second factor involves run-to-run variation with respect to retention times (RTs). We used GASP (Gas chromatography-mass spectrometry data analysis software package, Nuin, 2004) to align peaks associated with identical components between multiple GC runs. GASP extracts instrument output then converts and aligns data generated from multiple GC/MS runs into a spreadsheet format. The spreadsheet data can be statistically tested by analysis of variance (ANOVA) approaches to identify metabolites undergoing statistically significant changes ($P \leq 0.05$) between treated and untreated plants. GASP aligns identical peaks between runs automatically. The software compares mass spectral patterns iteratively to check and self-correct the positioning of each component until a unique line of data is produced representing the RRFs of a single component. The positioning of components in multiple alignments is then checked manually to ensure that the program has optimized

output and some manual fine-tuning is routinely completed before the statistical tests are performed. Another source of variation is run-to-run variation in column performance that can lead to slight changes in retention time of components resolved by the GC. Slight shifts in RTs can influence the efficiency by which GASP identifies and aligns the same component from multiple runs done either consecutively on a single day or runs performed on different days or even months. Correction for these shifts is done by the inclusion of odd-chain length fatty acid standards that produce peaks at regular time intervals during a run. Peaks arising from these standards are aligned first before the peaks of other components are matched by GASP and are used to convert variable RTs for each component between runs to an invariable RI (retention index) term. In the end, for each unique RI position that describes a given component GASP aligns associated RRF values arising from multiple runs.

**Drought exposure leads to differences among metabolites in *Thellungiella***

*Drought exposure in cabinet-grown plants: Shandong versus Yukon Thellungiella*

We hypothesize that components essential to stress tolerance for plants subjected to simulated drought in growth cabinets would also be present in plants experiencing drought under field conditions. Thus plants grown under both conditions were used to prepare extracts for GC/MS analysis.

The polar fractions of RW1 and RW2 leaf extracts prepared from plants of the Yukon and Shandong plants were compared to their respective well-watered controls and to each other. ANOVA was used to assess the statistical significance of differences in the distribution of components contributing to the chromatographic spectra. Of the ca

289 components detected, statistically significant ($P \leq 0.05$) changes in the abundance of 36 and 41 were found in response to drought stress for the Shandong and Yukon *Thellungiella*, respectively (reported in detail in Appendix A, B).

In order to identify the component (or at least the chemical nature of the component) giving rise to mass spectral peaks, two strategies were utilized. First, the mass spectra of each component were compared with NIST, a commercially available electron impact mass spectrum library (Gaithersburg, MD, available: http://www.nist.gov/srd/). Secondly, identification was performed using our own reference library created by Chris Wang, containing both the RI and corresponding mass spectrum of over four hundred standard compounds. Identification of components was based on the best match using these databases. In addition, as part of his doctoral research, David Guevara co-injected a known amount of authentic standards (including fructose, glucose, proline and galactose) to prepared samples of *Thellungiella* to raise the level of certainty for their identification in GC/MS analyses done in our lab.

Mass spectral comparisons of the 50 components undergoing changes in abundance due to the imposition of drought stress suggest that five are sugars (includes fructose, glucose, sucrose, erythrose, galactose) and twelve are unidentified but likely sugars,, five are organic acids (includes citric acid, succinic acid, malic acid, threonic acid, RT 33.83) three are unidentified but likely organic acids, two are amino acids (valine and threonine), one is a sugar alcohol (*myo*-inositol), one is a hydroxyl acid (ribonic acid), one is a nitrogenous compound (ethanolamine), one is phosphate, and 19 could not be associated with a particular chemical class. Figure 13 shows Venn diagrams

**Figure 13. Drought-responsive changes of metabolites in cabinets.** Venn diagram depicting the significant changes in component abundance for the Yukon and Shandong *Thellungiella* during one and two successive episodes of drought. Upon onset of visible wilting corresponding to leaf RWC of 55-60%, plants were re-watered and tissue was harvested two days later (RW1). Following re-watering, a subset of plants was randomly selected to undergo a second episode of drought. As before, these plants were re-watered and tissue was harvested two days later (RW2).

**Increasing Abundance:**

**Shandong**

**RW1**: N/A
**RW2**: glucose, malate, organic acid (RT 33.75), sugar (RT 46.56), unknown (RT 28.80, 43.18, 46.68)
**RW1 & RW2**: fructose, sugar (RT 30.13, 31.64, 32.92, 34.13), ribonic acid, unknown (RT 18.65, 29.87, 30.03, 30.70, 35.08)

**Yukon**

**RW1:** unknown (RT 42.28, 49.55)
**RW2:** ethanolamine, *myo*-inositol, organic acid (RT 33.75, 33.83), sugar (RT 27.63, 30.13, 36.33, 42.89, 46.56, 49.85), threonic acid, unknown (RT 18.65, 29.93, 35.08. 36.65, 43.18)
**RW1 & RW2:** fructose, galactose, glucose, phosphate, organic acid (RT 31.42), sugar (RT 31.64, 32.92, 34.13, 36.07), ribonic acid, unknown (RT 28.80, 29.87, 49.79), valine.

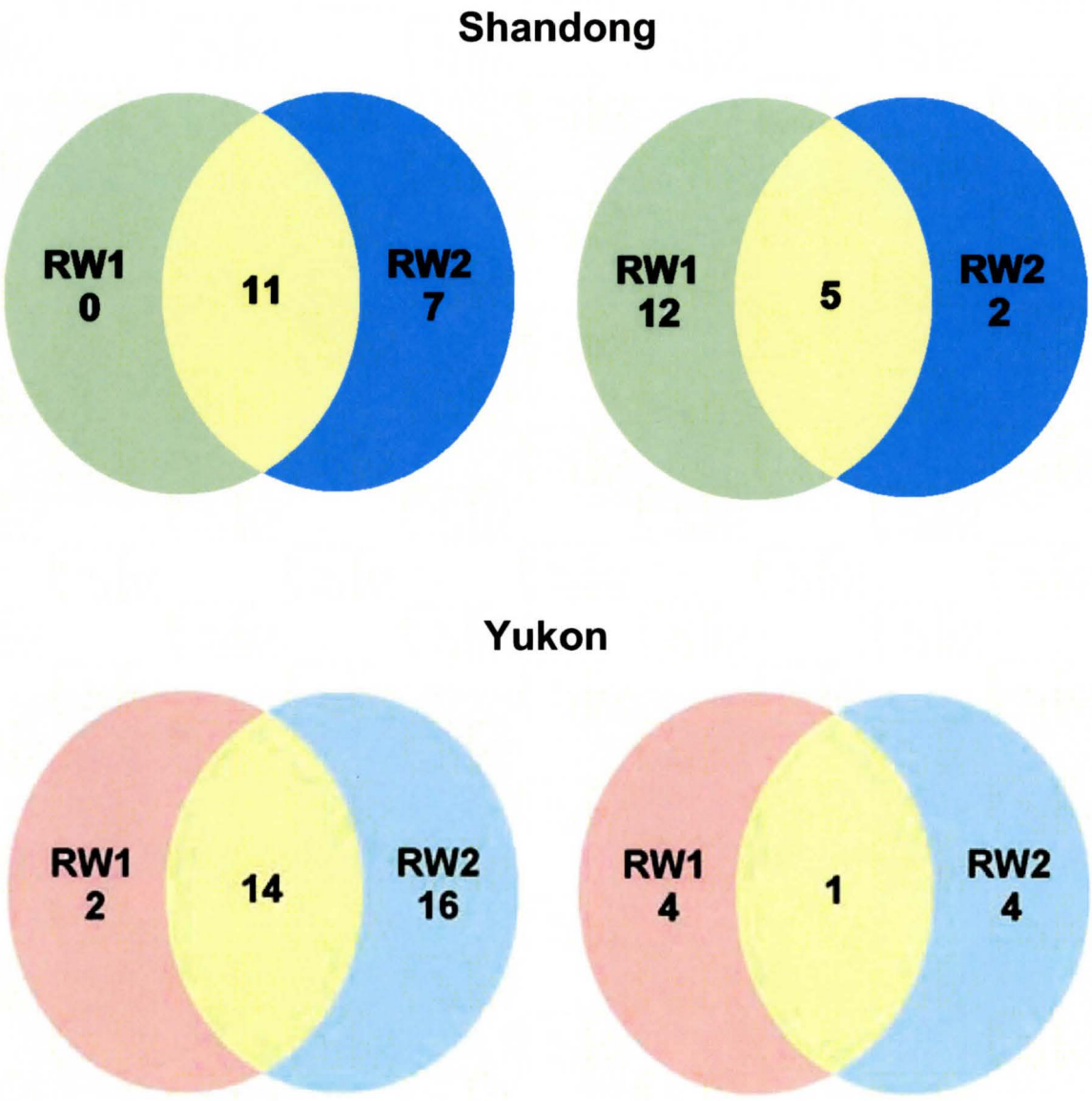
**Decreasing Abundance:**

**Shandong**

**RW1**: galactose, malate, sugar (RT 36.07, 36.33, 49.85, 51.32), succinic acid, threonic acid, threonine, unknown (RT 14.12, 42.28, 49.79)
**RW2**: organic acid (RT 29.25), unknown (RT 37.65)
**RW1 & RW2**: citric acid, erythrose, phosphate, unknown (RT 29.00, 29.39)

**Yukon**

**RW1:** sugar (RT 42.94), succinic acid, threonine, unknown (RT 22.15)
**RW2:** erythrose, unknown (RT 15.00, 37.65, 46.68)
**RW1 & RW2:** citric acid


Shandong
RW1
0
11
RW2
7
RW1
12
5
RW2
2
Yukon
RW1
2
14
RW2
16
RW1
4
1
RW2
4
Increased
Abundance
Decreased
Abundance

describing the number of metabolites undergoing significant changes in abundance between RW1 and RW2 leaf extracts for the Yukon and Shandong plants. Out of the 36 components identified as undergoing changes ($P \leq 0.05$) for Shandong *Thellungiella*, 18 increased and 18 decreased in abundance relative to their respective well-watered controls. Of these, several components were present in both RW1 and RW2 samples: 11 components increased and 5 components decreased in abundance. For the Yukon *Thellungiella*, 32 components increased and 9 decreased in abundance with an exposure(s) to wilting; of 15 components present in both RW1 and RW2 samples, 14 increased and only one decreased in abundance.

**Use of field and cabinet-grown plants to identify drought tolerance traits**

In order to identify metabolic traits important in the tolerance of *Thellungiella* to drought stress it is necessary to determine whether metabolites enriched or detected only in plants stressed in growth cabinets are also present in field plants experiencing water deficits. *The hypothesis tested is that traits essential to stress tolerance should be expressed in Thellungiella plants experiencing water deficits regardless of where they are growing.* Thus a growing season with low and/or infrequent episodes of rainfall would be a suitable period to harvest. As Figure 1 shows, these conditions were prevalent in the spring and early summer of 2003. In contrast, plants growing in 2005 received frequent rainfall in a year characterized by higher than average precipitation for a semi-arid zone.

In this study polar fractions of metabolites were prepared from *Thellungiella* leaves of field plants harvested during the 2003 and 2005 growing seasons (see Materials

and Methods for details on sample harvest, storage and shipping and Fig. 1 for field meteorological conditions). Polar fractions were also prepared from leaves of well-watered and drought-stressed Yukon plants grown in controlled environmental conditions. The chemical components detected in these extracts were compared and the results are summarized by Venn diagram. Figure 14 shows the number of components increasing or decreasing in abundance in leaves of Yukon plants exposed to a simulated drought episode relative to well-watered controls and relates these changes to those components undergoing comparable changes in abundance in the leaves of 2003 and 2005 field plants. Of the 57 components that are more abundant in the 2003 field plants relative to a cauline leaf control, only 12 are also enriched in drought-stressed chamber-grown Yukon plants. Of these 12 components, 6 overlap with both the RW1 and RW2 treatments indicating that one or two consecutive drought-treatments result in their increased abundance. Four of the six elevated components common to all sources of tissue (RW1, RW2, 2003) were not elevated in leaf extracts of field plants harvested during the growing season of above-average precipitation (2005) and, of these peaks, two were not elevated in drought-stressed Shandong plants. One of the two peaks has been identified as galactose while the second peak (RT 36.07) is likely a monosaccharide based upon its retention time and mass spectral fingerprint including the mass fragments 147, 160, 205, 217, 229 and 319. In contrast, 55 components were present in lower abundance in the leaves of 2003 field plants relative to cauline leaf controls; three of these also decreased in Yukon plants exposed to drought relative to the well-watered controls. None of the three components were overlapping in samples prepared from tissue of RW1 and RW2 treatments (Fig. 14).

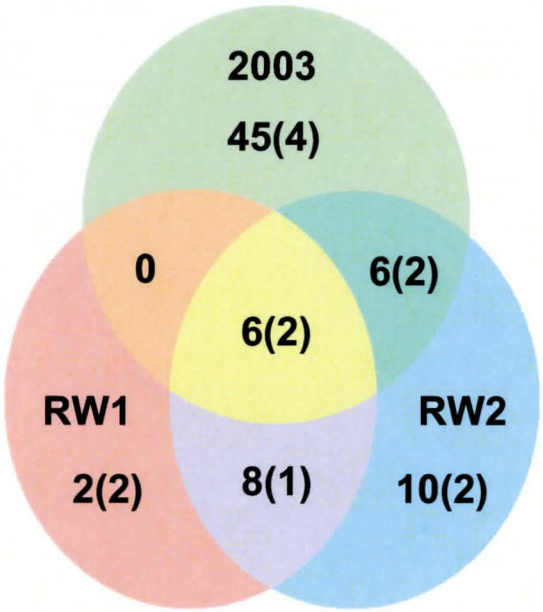
**Figure 14. Commonalities in components between cabinet and field plants.** Venn diagram showing commonalities in components that increase or decrease in plants of the Yukon *Thellungiella* in response to one (RW1) and two (RW2) successive episodes of drought. An analysis of field tissue (2003) for the Yukon plants was performed using tissue harvested during a dry growing season as a consequence of drought. Numbers in brackets represent the number of peaks that are elevated or lower in field plants during a wet growing season (2005).

**Increasing Abundance:**

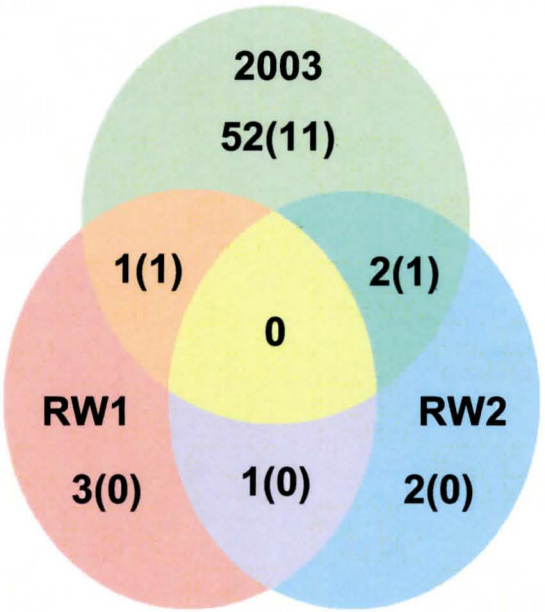
| | |
|---|---|
| **RW1**: | unknown (RT 42.28, 49.95) |
| **RW2**: | organic acid (RT 33.75, 33.83), possible sugar (27.63, 30.13, 42.89, 46.56, 49.85), unknown (18.65, 35.08, 36.65) |
| **2003**: | data not shown |
| **RW1 & RW2:** | phosphate, organic acid (RT 31.42), sugar (RT 31.64, 32.92), unknown (RT 28.80, 29.87, 49.79), valine |
| **RW1 & 2003:** | N/A |
| **RW2 & 2003:** | ethanolamine, *myo*-inositol, sugar (RT 36.33), threonic acid, unknown (29.93, 43.18) |
| **RW1, RW2 & 2003:** | fructose, galactose, glucose, possible sugar (RT 34.13, 36.07), ribonic acid |

**Decreasing Abundance:**

| | |
|---|---|
| **RW1**: | possible sugar (RT 42.94), succinic acid, unknown (RT 22.15) |
| **RW2**: | erythrose, unknown (RT 15.80) |
| **2003**: | data not shown |
| **RW1 & RW2:** | citric acid |
| **RW1 & 2003:** | threonine |
| **RW2 & 2003:** | unknown (37.65, 46.68) |
| **RW1, RW2 & 2003:** | N/A |




Increased Abundance




Decreased Abundance

**Use of Principal Component Analysis to identify quantitative and qualitative differences among chemical components of different leaf extracts**

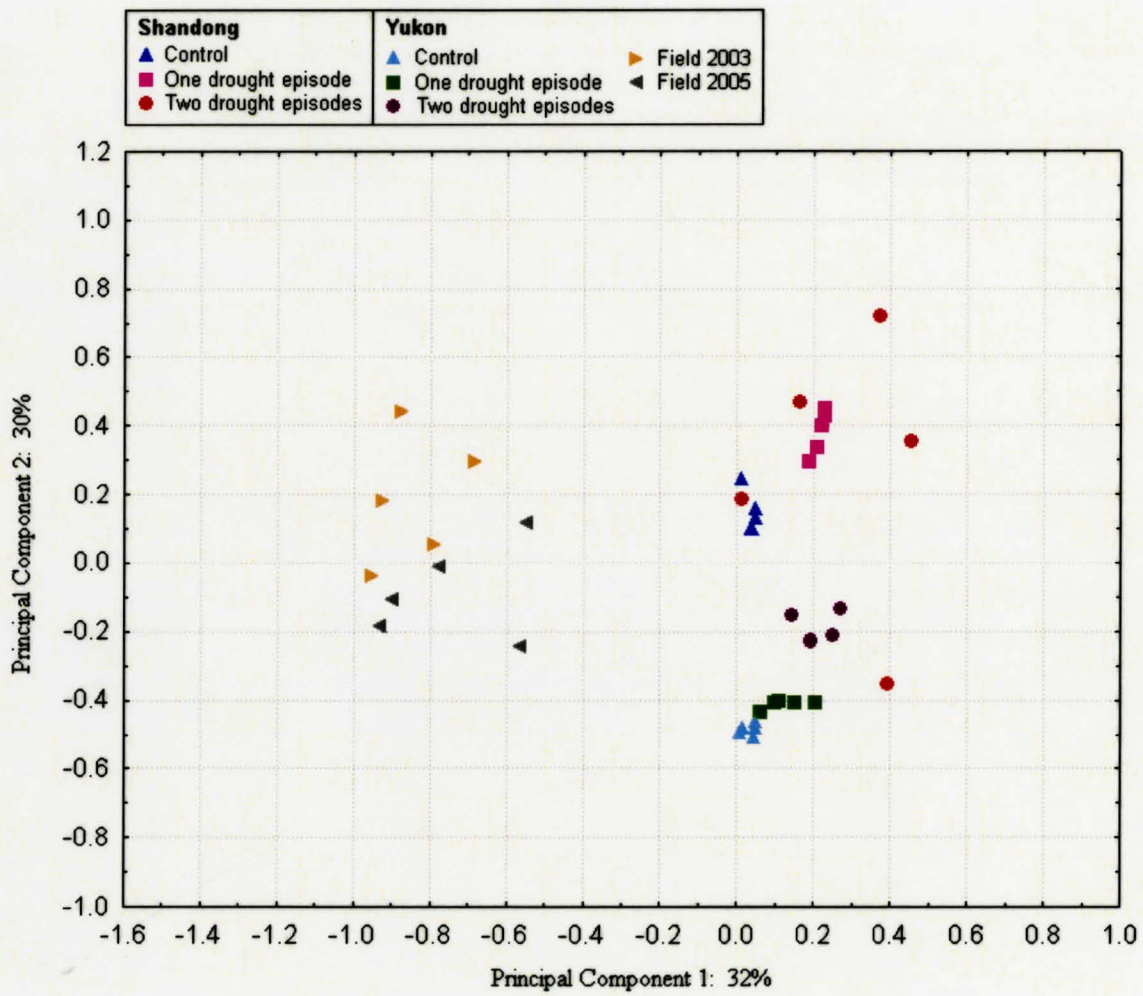
Principal component analysis (PCA) was used to describe differences and similarities among the components found in polar extracts of leaves from Shandong and Yukon plants grown in controlled cabinets and Yukon plants found in the field. PCA is an unsupervised method used for examining relationships of a large set of quantitative variables and determines a linear relationship among multivariate data (Fernandez, 2002). Samples that assemble into a distinct cluster can be considered as sharing a specific metabolic phenotype (Fiehn et al., 2000; Roessner et al., 2000; Sumner et al., 2003).

In this study, PCA analysis showed that the two highest ranking principal components accounted for 62% of the total variance within the dataset. Figure 15 is a plot showing distinct metabolic phenotypes for each treatment. The first principal component accounted for 32% of the variance and separated plants into two main groups: those growing in the field and those growing in growth cabinets. The second principal component accounts for a further 30% of the variance and groups the Shandong and Yukon plants into separate clusters above and below 0 on the y axis, respectively. Together, principal components 1 and 2 led to distinct groupings of profiles from well-watered, RW1 and RW2 plants growing in growth cabinets.

*Principal component loadings help identify metabolites that influence PCA grouping*

The contribution of individual metabolites for each principal component is determined by the loading value derived from PCA analysis. The outcome of this

**Figure 15. Metabolic phenotype clustering: cabinet vs field.** Clusters found after PCA of log-scaled polar metabolite data of 40 samples originating from the Yukon and Shandong plants exposed to two consecutive drought episodes in growth chambers, and also *Thellungiella* field plants growing in Whitehorse, Yukon during a wet (2005) and dry (2003) growing season. Each point represents the entire complement of polar metabolites present in leaves from an individual plant sample. Principal component vectors 1 ($x$ axis) and 2 ($y$ axis) describe 32% and 30%, respectively, of total sample variance.


Shandong
Control
One drought episode
Two drought episodes
Yukon
Control
One drought episode
Two drought episodes
Field 2003
Field 2005
Principal Component 2: 30%
Principal Component 1: 32%
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.6
-1.4
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

analysis is shown in Figure 16. Metabolites with the highest loading value have the strongest influence on the respective position or co-ordinates where sub-groupings cluster relative to each other. Conversely, metabolites with the least influence on the positioning of sub-groups will cluster more closely to the 0,0 position in Figure 16. Principal component one was influenced most by the content of sucrose, citrate, and an unknown chemical (RT 31.64) while principal component two was influenced most by fructose content.

**Hierarchical Cluster Analysis identifies patterns among changes in the relative abundance of chemical components detected in extracts of tissues undergoing drought-responsive changes**

The ANOVA application in GASP was used to identify chemical compounds showing statistically significant differences between treatments and/or field years versus their respective controls. Components undergoing significant changes relative to controls were then subjected to hierarchical cluster analysis (HCA) and the results are shown graphically as a heat-map in Figure 17. Metabolic profiles from treated plants were expressed as the fold-change relative to respective well-watered controls for growth chamber grown plants or, in the case of field plants, controls for comparison were cauline leaves from mature, flowering chamber-grown plants. The heat-map shows contiguous patches of colour representing groups of metabolites found in multiple samples that share similar fold-changes in response to multiple conditions. Six, separate clusters were identified and are labelled in the dendrogram in Figure 17. Identification of each component was performed using mass spectral libraries as described previously.

**Figure 16. Metabolite impacts on clustering results.** The contribution of individual polar metabolites to the PCA vector calculation is computed by linear combination. The closer to zero, the less influence of a metabolite on linear combination is found. Examples of metabolite identity are numbered: 1 = fructose; 2 = sucrose; 3 = citric acid; 4 = unknown (RT 31.64); 5 = *myo*-inositol; 6 = malate; 7 = proline; 8 = glucose; 9 = phosphate; 10 = unknown (RT 29.87); 11 = unknown (RT 30.03); 12 possible sugar (RT 30.13); 13 = unknown (RT 29.00).

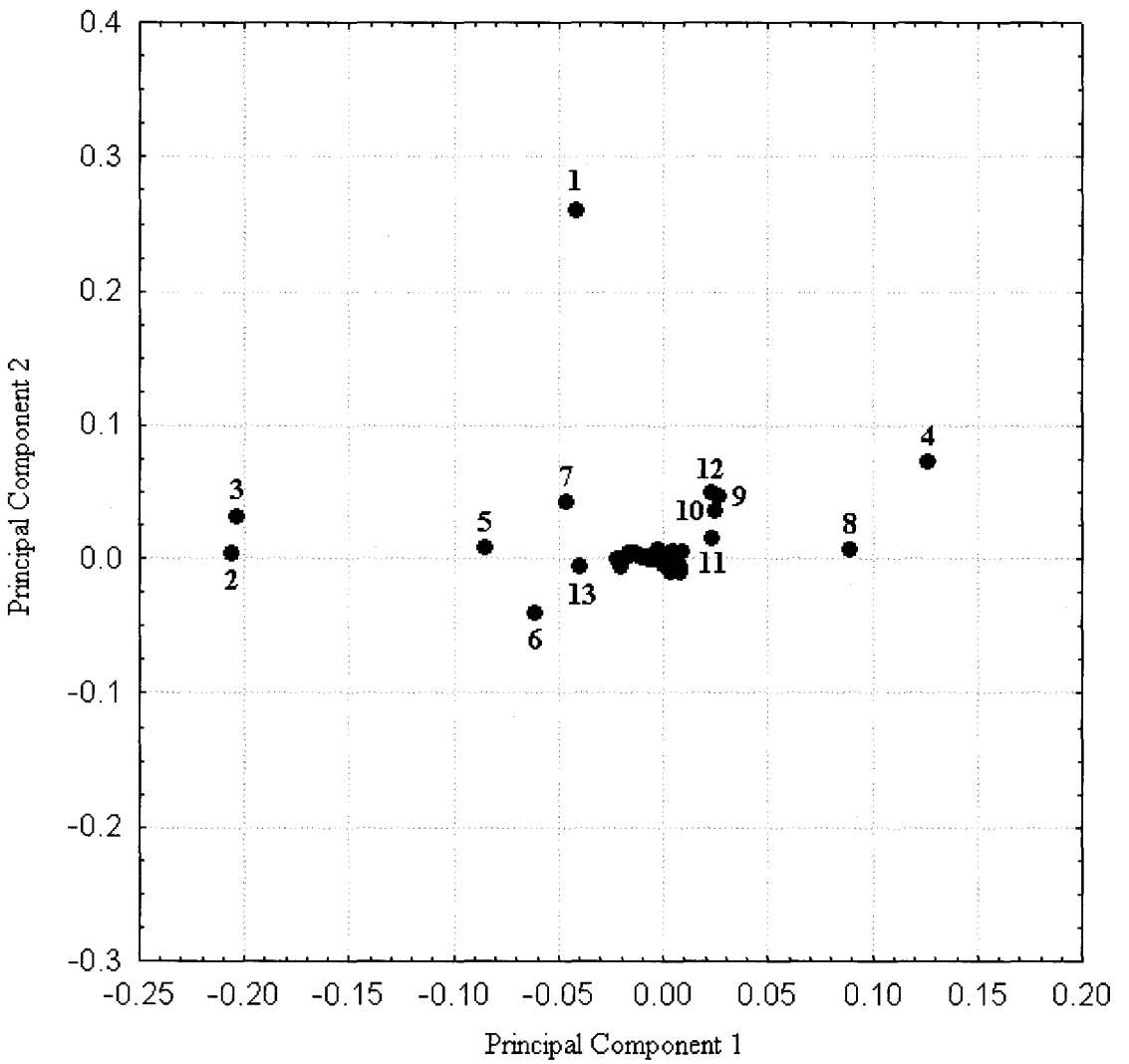
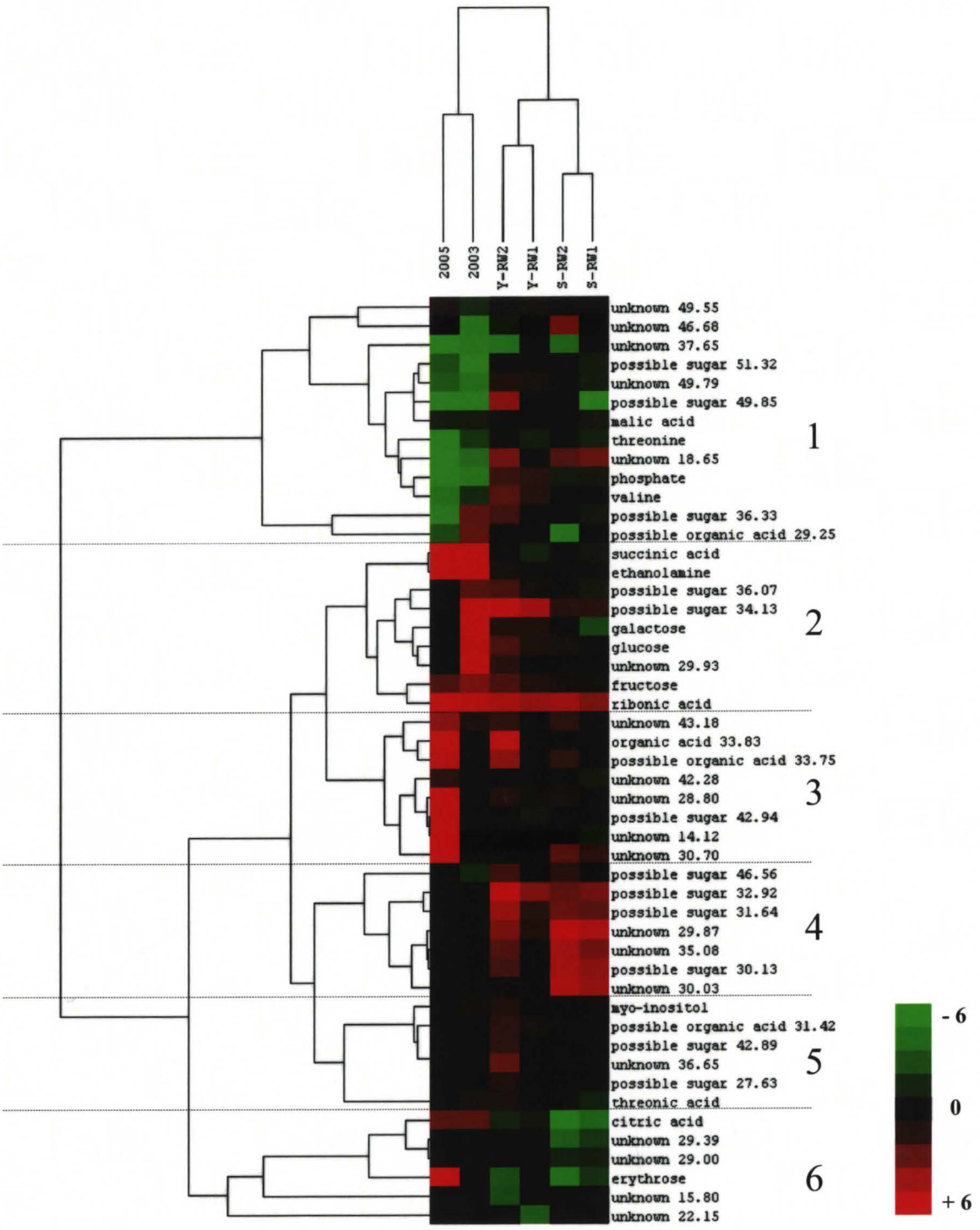

0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.25
-0.20
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
0.20
Principal Component 2
Principal Component 1
1
2
3
4
5
6
7
8
9
10
11
12
13

**Figure 17. Heat map grouping of metabolites.** Heat map grouping of metabolites based on significant differences in relative abundance of specific components in extracts prepared from leaves harvested from the Shandong and Yukon *Thellungiella* plants exposed to two simulated drought episodes. Plants were re-watered once after wilting following the first drought episode and allowed to recover for 2 days before harvest (Yukon = Y-RW1, Shandong = S-RW1). After a 2-day recovery, plants were exposed to a second drought treatment. Upon wilting for the second time, plants were re-watered and allowed to recover for 2 days before harvest (Yukon = Y-RW2, Shandong = S-RW2). Leaf tissue extracts were also prepared from field plants growing in Whitehorse, Yukon after a period of high rainfall accumulation (2005) and after a spring of low rainfall and then 4 days of no rain prior to harvest (2003). Mass spectra and retention times of each component were compared using two electron impact mass spectrum libraries, including: NIST (Gaithersburg, MD, available: http://www.nist.gov/srd/) and our own reference library created by Chris Wang, containing both the retention index and corresponding mass spectrum of over four hundred standard compounds assumed to be present in detectable amounts in plant tissues. Identification of components was based on the best match using these databases. In addition, David Guevara co-injected a known amount of authentic standards for compounds including fructose, glucose, proline and galactose to prepared samples of *Thellungiella* to raise the level of certainty for identification. Threshold of detection was 0.00005.


2005
2003
Y-RW2
Y-RW1
S-RW2
S-RW1
unknown 49.55
unknown 46.68
unknown 37.65
possible sugar 51.32
unknown 49.79
possible sugar 49.85
malic acid
threonine
unknown 18.65
phosphate
valine
possible sugar 36.33
possible organic acid 29.25
succinic acid
ethanolamine
possible sugar 36.07
possible sugar 34.13
galactose
glucose
unknown 29.93
fructose
ribonic acid
unknown 43.18
organic acid 33.83
possible organic acid 33.75
unknown 42.28
unknown 28.80
possible sugar 42.94
unknown 14.12
unknown 30.70
possible sugar 46.56
possible sugar 32.92
possible sugar 31.64
unknown 29.87
unknown 35.08
possible sugar 30.13
unknown 30.03
myo-inositol
possible organic acid 31.42
possible sugar 42.89
unknown 36.65
possible sugar 27.63
threonic acid
citric acid
unknown 29.39
unknown 29.00
erythrose
unknown 15.80
unknown 22.15
1
2
3
4
5
6
- 6
0
+ 6

The majority of metabolites making up cluster 1 were detected at relatively lower levels in 2003 and 2005 field plants as compared to leaves of chamber-grown plants. Valine, threonine, malic acid, and phosphate were members of this cluster. Cluster 2 contained metabolites showing greater abundance in leaves of 2003 field plants and many of these components were present at higher levels in Yukon plants following one or two drought/recovery treatments (Y-RW1, Y-RW2, respectively). Components of cluster 2 that were identified included galactose, glucose, fructose, ribonic acid, two possible sugars (RT 36.07 and 34.13) and one component of unknown chemical class (RT 29.93). Cluster 3 includes metabolites whose content is found, in large part, elevated in leaves of the 2005 Yukon field plants. In contrast, cluster 4 was comprised of metabolites showing similar content in field plants during both years but whose levels were elevated in Shandong plants following one or two drought/recovery treatments (S-RW1, S-RW2, respectively) and in Yukon plants but only after a second drought stress/recovery treatment (Y-RW2). Cluster 5 metabolites exhibited higher levels in S-RW2 Yukon plants and three of these components were also detected at elevated levels in 2003 field plants relative to cauline leaf controls. These components included *myo*-inositol, threonic acid, and a possible organic acid (RT 31.42). Cluster 6 was comprised of six members, including four metabolites showing lower levels in S-RW1 and S-RW2 plants relative to Shandong control plants. The most notable feature of cluster 6 components is that their levels tended to be higher in the field as opposed to the growth cabinet-grown plants.

**Chemical classes of components undergoing changes in abundance offer links to changes in metabolism**

*Amino acids*

We detected two amino acids undergoing statistically significant changes in abundance (using a 95% confidence level). Valine and threonine content showed the greatest decrease in 2005 field plants and a slight decrease in 2003 field plants relative to cauline leaves from mature plants grown in cabinets (Fig. 17). Threonine was also lower in plants exposed to one drought/recovery treatment for the Shandong and Yukon plants relative to unstressed controls. Valine content was higher in plants exposed to one and two drought/recovery treatments for the Yukon *Thellungiella* relative to unstressed controls and remained unchanged in the comparably treated Shandong plants.

*Organic acids*

Organic acids undergoing statistically significant changes in content included threonate, succinate, citrate and malate (Appendix A, B). These organic acids were present at significantly lower levels or remained unchanged in leaves of drought-stressed Shandong plants relative to unstressed controls. Of these organic acids, threonate was of most interest, with elevated levels in 2003 field plants and in Y-RW2 plants, relative to unstressed controls. Citrate was elevated in 2003 and 2005 field plants, and remained unchanged or was significantly lower relative to unstressed controls in Shandong and Yukon plants following one or two drought/recovery treatments in controlled environments. Levels of malate were present at slightly lower levels in both field years and remained unchanged in plants exposed to drought/recovery in growth cabinets, relative to unstressed controls.

*Sugars and sugar alcohols*

Sugars exhibited variable treatment-related distribution patterns (Fig. 17). Fructose content was significantly higher in drought-stressed cabinet-grown Shandong and Yukon plants and in field plants. Interestingly, the fold-increase in fructose content relative to controls was two- and four- fold greater for Yukon plants experiencing one (Y-RW1) and two (Y-RW2) episode(s) of drought and recovery, respectively, compared to the fold-increases for comparably treated Shandong plants. Levels of glucose were significantly higher in drought-stressed cabinet-grown Shandong and Yukon plants and in 2003 Yukon field plants. The relative fold-increase for glucose was four-fold greater in Y-RW2 plants relative to the fold-increase in S-RW2 plants. Content of galactose and a likely monosaccharide (RT 36.07) were significantly higher in both Y-RW1 and Y-RW2 plants as well as in 2003 Yukon field plants. Levels of these two metabolites were not significantly higher in drought-stressed Shandong plants or in 2005 Yukon field plants. The component identified as a likely monosaccharide (RT 36.07) was more abundant in only two tissue sources: Y-RW2 and 2003 Yukon field plants. This pattern of occurrence suggests an association between this chemical and the repeated or continuous exposure of Yukon *Thellungiella* to conditions of low water availability

Among sugar alcohols present only *myo*-inositol was identified as undergoing statistically significant changes in abundance in the tissues analyzed. Levels of *myo*-inositol were more abundant only in Y-RW2 and 2003 Yukon field plants relative to control tissues. Thus this component was present in the tissue sources showing the greatest drought-tolerance in our study.

**Many components of interest undergoing changes in abundance cannot be identified**

As mentioned previously, in this study we identified components on the basis of retention time (RT) and mass spectral patterns using two electron impact mass spectrum libraries, including: NIST (Gaithersburg, MD, available athttp://www.nist.gov/srd/) and our own reference library. Identification of components was based on the best match using these databases. Co-injection with authentic standards was occasionally used when additional verification was required. Unfortunately, some components offered no clues as to their identity and 19 of the compounds identified by ANOVA as undergoing statistically significant changes in treated or field tissues relative to their controls were not identified with respect to chemical class. Five of these compounds were elevated in drought-stressed cabinet-grown Yukon plants and not in comparably treated Shandong plants. Of these, one component was also elevated in 2003 Yukon field plants (RT 29.93).

## RESULTS – PART 2

### Comparison of plant material obtained from the field in 2003, 2005 and 2006

On May 20th, 2006, Mr. David Guevara and I went to the Yukon and harvested *Thellungiella* leaf tissue from a population of plants that were at the same field site sampled in 2003 and 2005. Rainfall totals for the 2006 growing season were similar to the norms reported for southern Yukon (Table II). In comparison to the leaf tissue harvested in 2003 and 2005, 2006 field plants were harvested much earlier in the growing season when temperatures were much cooler (Fig. 1). The average high and low temperatures for the 10-day period before harvest were 10°C and -1°C, respectively. On the day of harvest our measurements showed the soil temperature to be 4°C, and the air temperature 9°C.

### Physiological differences of plants growing in the field in different years

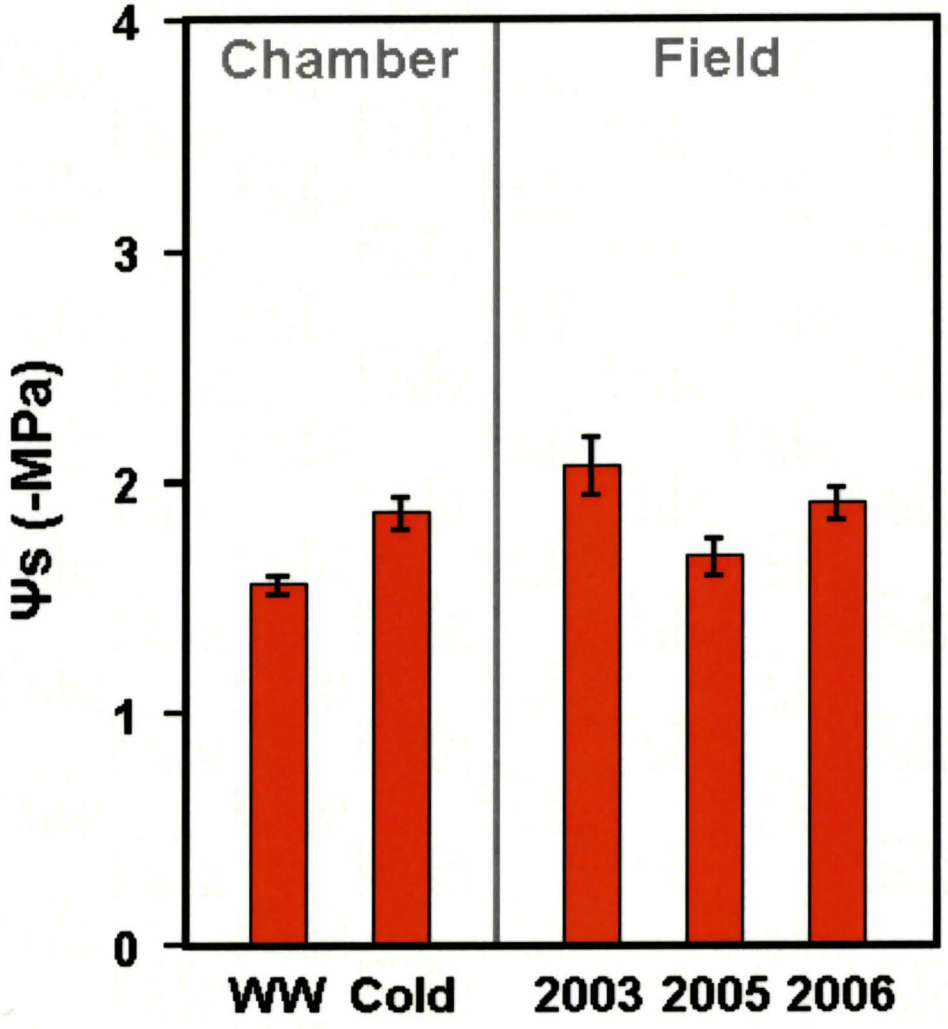
The phenotype of 2006 field plants was different compared to those found in 2003 and 2005. In 2006 we found *Thellungiella* plants that were growing closer to the ground almost prostrate in appearance with prominent cauline leaves and little to no basal rosette of leaves present. The majority of 2006 field plants also were of smaller size in comparison to the majority plants studied during the 2003 and 2005 field years. Leaves of the 2006 plants were dark green and appeared almost purple (Fig. 5, D) while leaves of the majority of 2003 and 2005 plants were lighter green (Fig. 5, B and C). Some plants found in 2005 also bore leaves with purple coloration and judging by the number of mature siliques, these were probably plants that emerged early in spring or over-wintered

(data not shown). Leaves from these plants were not analyzed for this study. Another distinctive feature of the plants found in 2006 was associated with the flowers. The flowers on the 2006 field plants were distinctive in having a dark purple colour which contrasts with the white flowers found on the 2003 and 2005 field plants.

**Solute accumulation by Yukon *Thellungiella* plants in the field varies with growth conditions**

Leaf Ψs of *Thellungiella* plants were measured for samples obtained from growth cabinets and field sites in 2003, 2005, and 2006 by Jeff Dedrick and Dave Guevara (Fig. 18). The average leaf Ψs value during the spring of 2006 season was significantly lower than the values determined for the plants during 2005. This indicates that leaves of plants in 2006 had a higher internal solute concentration than those in 2005. While 2006 was characterized by cool temperatures and adequate precipitation, 2005 was significantly warmer on average but also had above average precipitation (Government of Canada Weather Office, 2007, Fig. 1). This raises a question whether exposure to cooler temperatures can lead to higher solute content in leaves. To address this question, leaf Ψs values from field-grown plants were compared to those determined by Dave Guevara for well-watered and cold stressed Yukon plants growing in controlled environment cabinets (Fig. 18). The Ψs leaf values determined for field plants in the spring of 2006 were not significantly different from those measured for the Yukon plants exposed to cold (Fig. 18). In contrast, well watered plants in the growth cabinet had the same leaf Ψs values as those found in the field in 2005 indicating that these leaves (WW and 2005 field) did not accumulate as high a content of solutes as 2006 field plants.

**Figure 18. Comparison of Yukon *Thellungiella* leaf solute potentials.** 'WW' plants were well-watered throughout the experiment. 'Cold' plants were exposed to 5°C for 3 weeks starting at 4-weeks post germination (A. Wong and M. Griffith, U. Waterloo). '2003' plants were measured during a growing season characterized with low rainfall and long periods of drought. Average high and low temperatures for the 10-day period before the 2003 harvest were 22°C and 15°C. '2005' refers to plants measured during a growing season with rainfall totals much higher than the seasonal average for Whitehorse, YT. Average high and low temperatures for the 10-day period before the 2005 harvest were 18°C and 7°C. '2006' plants were measured during a growing season with typical rainfall for Whitehorse, YT. Average high and low temperatures for the 10-day period before harvest were 10°C and -1°C, much cooler than the days preceding the 2003 and 2005 harvest. n is a minimum of 5 measurements from independent plant samples ± standard error. Measurements were taken by Jeff Dedrick and Dave Guevara.


Chamber
Field
4
3
2
1
0
Ψs (-MPa)
WW
Cold
2003
2005
2006

**Quantitative changes in metabolites for *Thellungiella* plants growing in the field**

Qualitative and quantitative changes in leaf metabolites in leaf samples from plants in 2003, 2005 and 2006 were assessed by GC/MS. Each GC/MS chromatogram of the polar fraction of leaf extracts had 294 ± 9 (n = 15) components (or unique mass spectra) present. Of the ca 294 components detected, 57, 26 and 62 components were enriched and 55, 64 and 63 were reduced in 2003, 2005 and 2006 field plants, respectively, relative to a cauline leaf from a mature, chamber-grown Yukon plant (Fig. 19). Of the components found at increased abundance in response to these environments only eight were common to all three field years and another four were common to plants from the field in 2005 and 2006. In contrast, of the components found at lower abundance, 39 were found in all three field years sampled while 11 were present in at least two field years.

**Principal Component Analysis indicates that leaf metabolite levels in plants growing in the field varied between years.**

PCA was used to examine any differences and similarities between the leaf metabolic profiles in plants harvested during each of the three field years (Fig. 20). This analysis showed that the two highest ranking principal components accounted for 78% of the total variance within the dataset. The first principal component reported on the x-axis

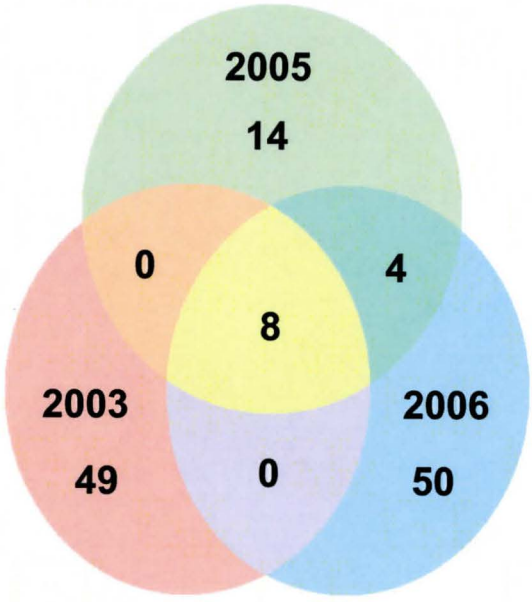
**Figure 19. 2003, 2005, and 2006 field plant metabolite commonalities.** Venn diagram showing commonalities in components that increase or decrease in leaves of Yukon *Thellungiella* plants growing in the field during a dry (2003), wet (2005) and cold (2006) growing season (see Materials and Methods for details).

**Increasing Abundance:**

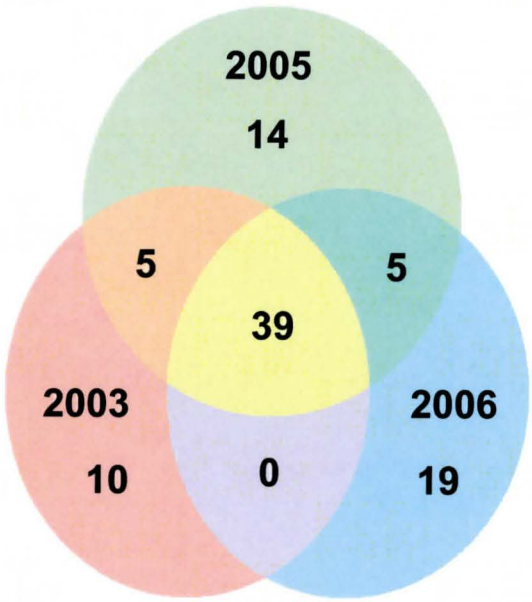
| | |
|---|---|
| **2003:** | data not shown |
| **2005:** | data not shown |
| **2006:** | data not shown |
| **2003 & 2005:** | N/A |
| **2003 & 2006:** | N/A |
| **2005 & 2006:** | sugar (RT 37.31), unknown (28.55, 32.70, 33.33) |
| **2003, 2005 & 2006:** | amino acid (RT 30.26), citramalic acid, citric acid, ethanolamine, fructose, glycine, succinic acid, sucrose |

**Decreasing Abundance:**

| | |
|---|---|
| **2003:** | data not shown |
| **2005:** | data not shown |
| **2006:** | data not shown |
| **2003 & 2005:** | amino acid (RT 24.40), sugar (RT 42.69, 51.28), quinic acid, unknown (RT 21.79) |
| **2003 & 2006:** | N/A |
| **2005 & 2006:** | amino acid (RT 28.15, 29.25), proline, unknown (RT 40.85, 42.54) |
| **2003, 2005 & 2006:** | amino acid (RT 28.28), aspartic acid, galactinol, glucose-6-P, glutamic acid, glutamine, glycine, fumaric acid, lysine, malate, phosphate, phosphorylated sugar (RT 38.21), pyroglutamic acid, sugar (34.12, 36.88, 43.83, 45.56, 45.78, 47.18, 48.50, 49.79, 49.85, 50.39, 50.80, 50.84), raffinose, serine, threonine, unknown (RT 17.58, 22.96, 25.77, 34.90, 37.57, 40.13, 41.06, 41.73, 47.86, 52.89), valine |

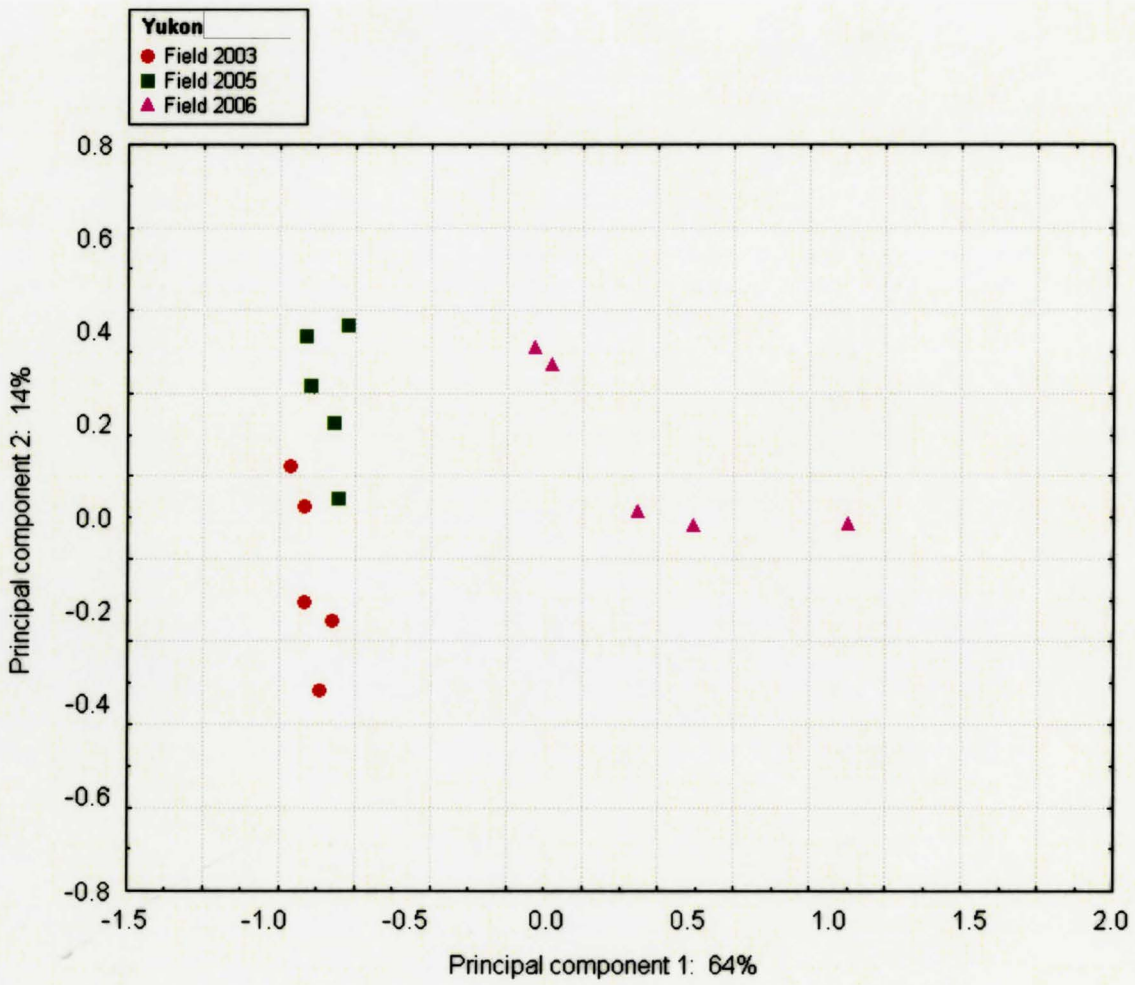



Increased Abundance




Decreased Abundance

**Figure 20. Metabolic phenotype clustering: 2003, 2005, 2006 field plants.** Clusters found after PCA of log-scaled polar metabolite data of 15 samples originating from *Thellungiella* field plants growing in Whitehorse, Yukon during a dry (2003), wet (2005) and cold (2006) growing season. Principal component vectors 1 ($x$ axis) and 2 ($y$ axis) describe 64% and 14%, respectively, of total sample variance.


Yukon
Field 2003
Field 2005
Field 2006
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Principal component 2: 14%
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Principal component 1: 64%

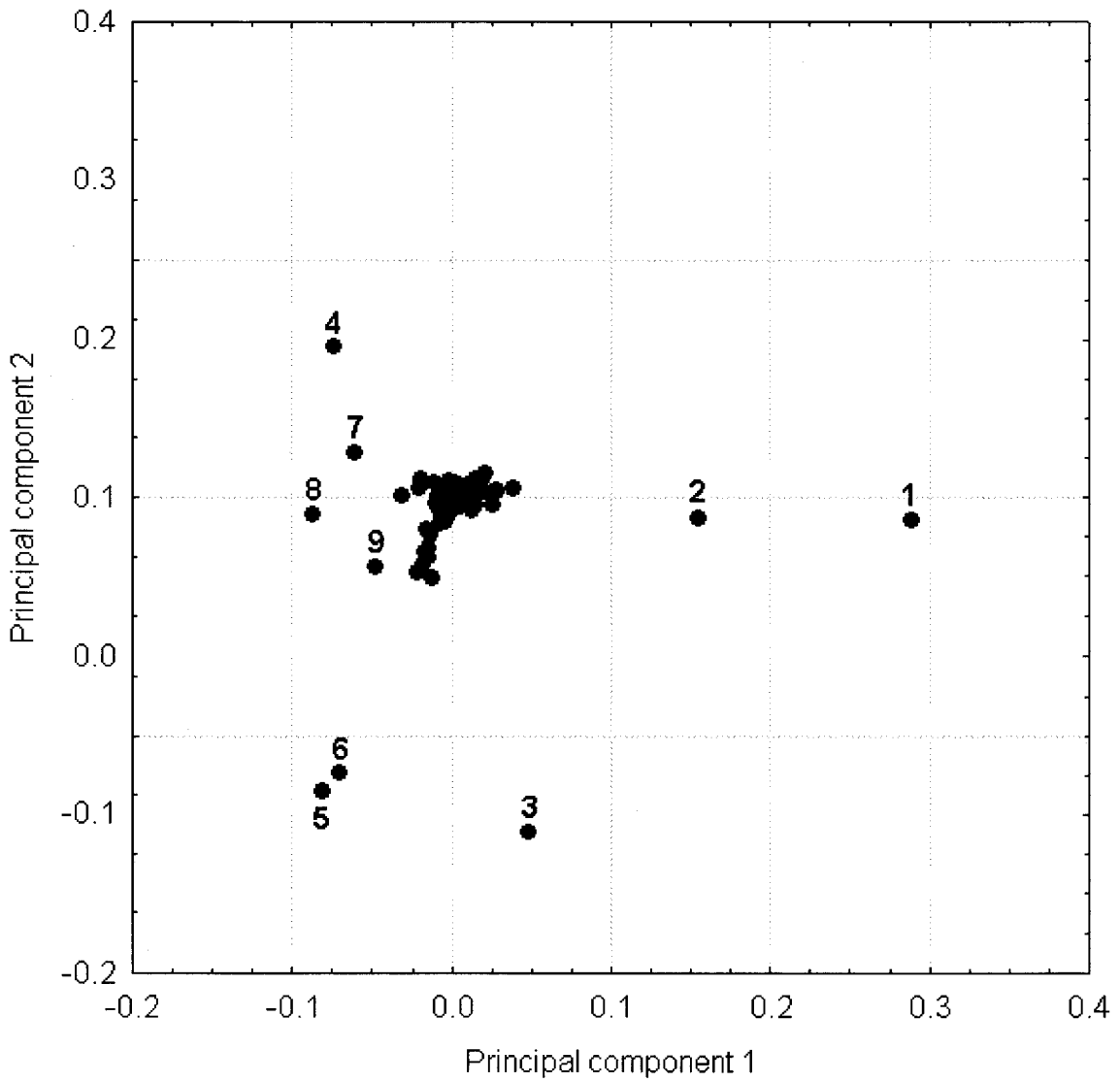
accounted for 64% of the variance among the data and was responsible, in large part, for separating points on the figure associated with the 2003 and 2005 field plants from the plants harvested during the 2006 growing season. A probable sugar (RT 34.38) and a compound of unknown chemical class (RT 29.80) contributed most strongly towards the clustering of data along the x-axis and separating the 2006 data sets away from those associated with the 2003 and 2005 field plants. Other chemical compounds contributing towards principal component 1 included sucrose, proline, malate, glucose and citric acid. In contrast, the second principal component which accounts for 14% of the variance does not clearly separate the data associated with the 2003, 2005 and 2006 field plants. All of the polar chemical components contributing to this vector calculation are shown in Figure 21 and include fructose, proline, glucose and malate.

**Metabolic profiles exhibited by *Thellungiella* field plants and cold-stressed cabinet-grown plants differ**

*ANOVA identifies statistically significant changes associated with 30 chemicals*

ANOVA was used to assess the statistical significance of qualitative and quantitative differences among the peaks contributing to the chromatographic spectra of cold-stressed plants grown in controlled environmental conditions (data of D. Guevara, unpublished) versus field conditions. Statistically significant ($P \leq 0.05$) changes in the abundance of 30 chemicals contributing to the profiles were found between control (22°C day/10°C night) and *Thellungiella* cold-stressed by exposure to a constant 4°C growth temperature (primary data used in this comparison providing by D. Guevara). Comparisons between mass spectra and retention times between chemical

**Figure 21. Metabolite impacts on clustering results.** The contribution of individual polar metabolites to the PCA vector calculation is computed by linear combination. Examples of metabolite identity are numbered: 1 = possible sugar (RT 34.38); 2 = unknown (RT 29.80); 3 = fructose; 4 = malate; 5 = proline; 6 = glucose; 7 = citric acid; 8 = sucrose; and 9 = *myo*-inositol.


0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
Principal component 2
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
Principal component 1
1
2
3
4
5
6
7
8
9

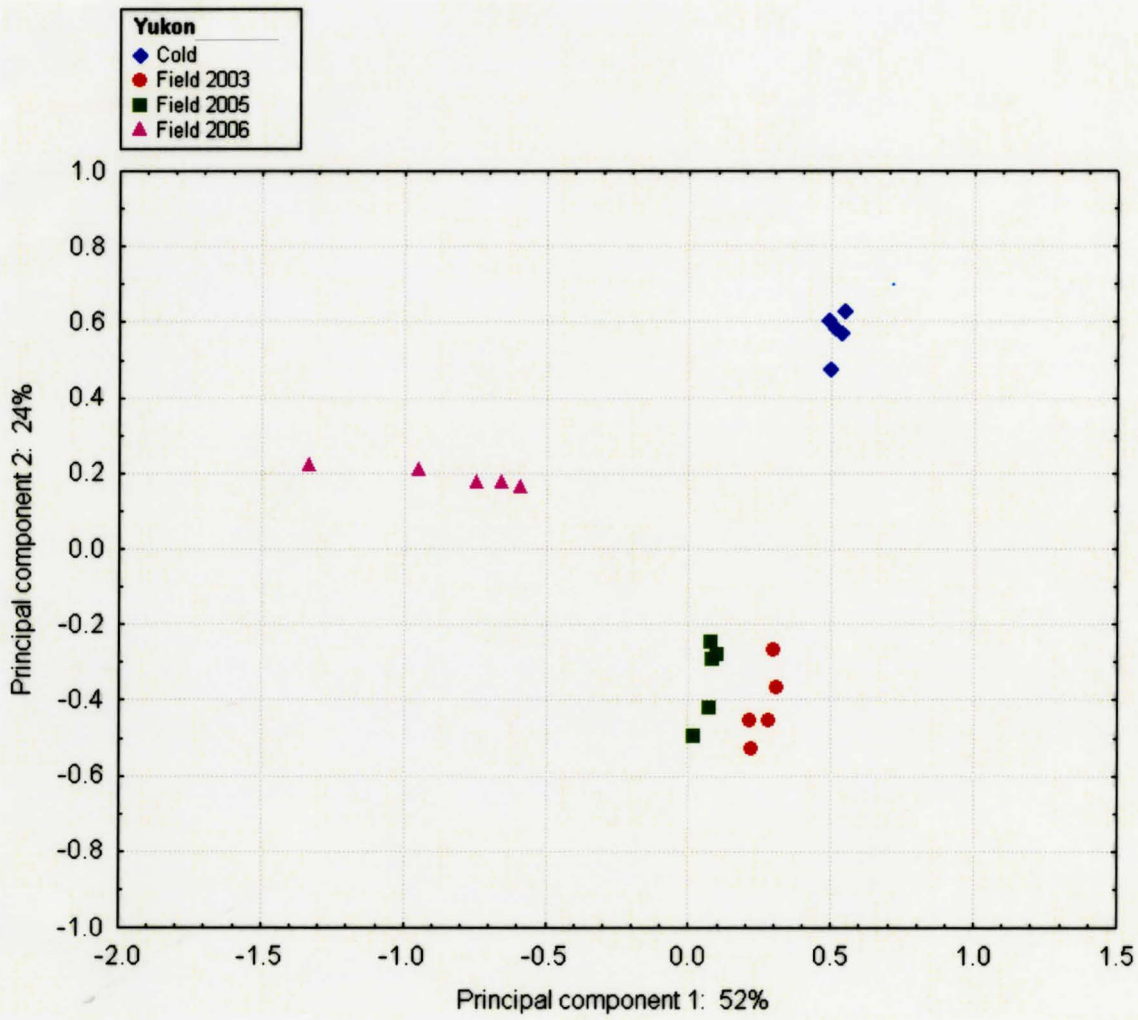
components in samples and libraries of standards were used to help identify metabolites undergoing significant changes in abundance. In summary, metabolites that vary in abundance between samples include: eight sugars (fructose and glucose were identified along with six components that were not identified but have spectra consistent with sugars including RT 32.99, 34.29, 34.38, 37.31, 49.85, and 50.80), six organic acids (including quinnate, citramalate, fumarate, citrate, malate, and threonate), eight amino acids (including aspartate, glutamate, glutamine, glycine, proline, pyroglutamate, serine, threonine, and two unidentified but likely amino acids including RT 24.40 and 28.15), phosphate, a phospho-sugar (RT 31.21), an unidentified but probable sugar alcohol (RT 36.13), and three chemical components that could not be identified even with respect to chemical class (RT 28.55, 29.80, and 33.33).

*PCA distinguishes metabolites associated with leaf tissue exposed to cold temperatures*

Tissues were collected during three different field conditions (Fig. 1); two field visits were made during summer months characterized by warm days often exceeding 20°C maxima (June 2003 and July 2005) and one was made in May 2006 when temperatures were low with daily maxima usually below 15°C and minima were frequently below 0°C. With respect to rainfall, 2003 was a summer of lower than average precipitation compared to 2005 or 2006 when average or higher than average precipitation was recorded.

PCA was used to identify differences between the leaf metabolite profiles produced by leaves of *Thellungiella* growing in the field with cold-stressed Yukon plants

**Figure 22. Metabolic phenotype clustering: field vs control.** Clusters found after PCA of log-scaled polar metabolite data of 20 samples originating from the Yukon plants exposed to cold temperatures (5°C), and also *Thellungiella* field plants growing in Whitehorse, Yukon during a dry (2003), wet (2005), and cold (2006) growing season. Principal component vectors 1 ($x$ axis) and 2 ($y$ axis) describe 52% and 24%, respectively, of total sample variance.


Yukon
Cold
Field 2003
Field 2005
Field 2006
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Principal component 2: 24%
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Principal component 1: 52%

growing in controlled cabinets (Fig. 22). PCA showed that the two highest ranking principal components accounted for 76% of the total variance within the dataset. The first principal component shown on the x-axis accounted for 52% of the variance and separated the different datasets into distinct clusters. Metabolites influencing the first principal component vector calculation most strongly included a possible sugar (RT 34.38), proline, and galactose (Fig. 23). The second principal component contributed towards 24% of the data variance and was largely responsible for separating the 2003 and 2005 field plant data from the cold-stressed cabinet-grown and 2006 field plants. Metabolites contributing to the clustering pattern associated with the second principal component are shown in Figure 23 and include the organic acid citrate and amino acid proline

*HCA identifies patterns among changes in metabolite abundance in field and chamber-grown plants*

Heat-map signatures of all 30 components undergoing changes in cold-stressed plants grown in controlled environment cabinets were compared to their relative abundance in field plants harvested in 2003, 2005, and 2006. Two clusters identified by this analysis are labelled in the dendrogram of Figure 24. The first cluster was largely comprised of metabolites present at relatively low levels in all the field plants. These same components were detected at higher or lower levels in cold-stressed chamber plants relative to chamber grown plants that were not exposed to cold conditions. The majority of amino acids were characteristic of this cluster. Cluster 2 was of greater interest as it

**Figure 23. Metabolite impacts on clustering results.** The contribution of individual polar metabolites to the PCA vector calculation is computed by linear combination. Examples of metabolite identity are numbered: 1 = proline; 2 = citric acid; 3 = possible sugar (RT 34.38); 4 = galactose; 5 = unknown (RT 29.80); 6 = sucrose; 7 = fructose; 8 = glucose; 9 = glutamic acid; 10 = aspartic acid; 11 = serine; 12 = malate; 13 = possible sugar (RT 32.99); 14 = possible sugar (RT 42.69); and 15 = *myo*-inositol.

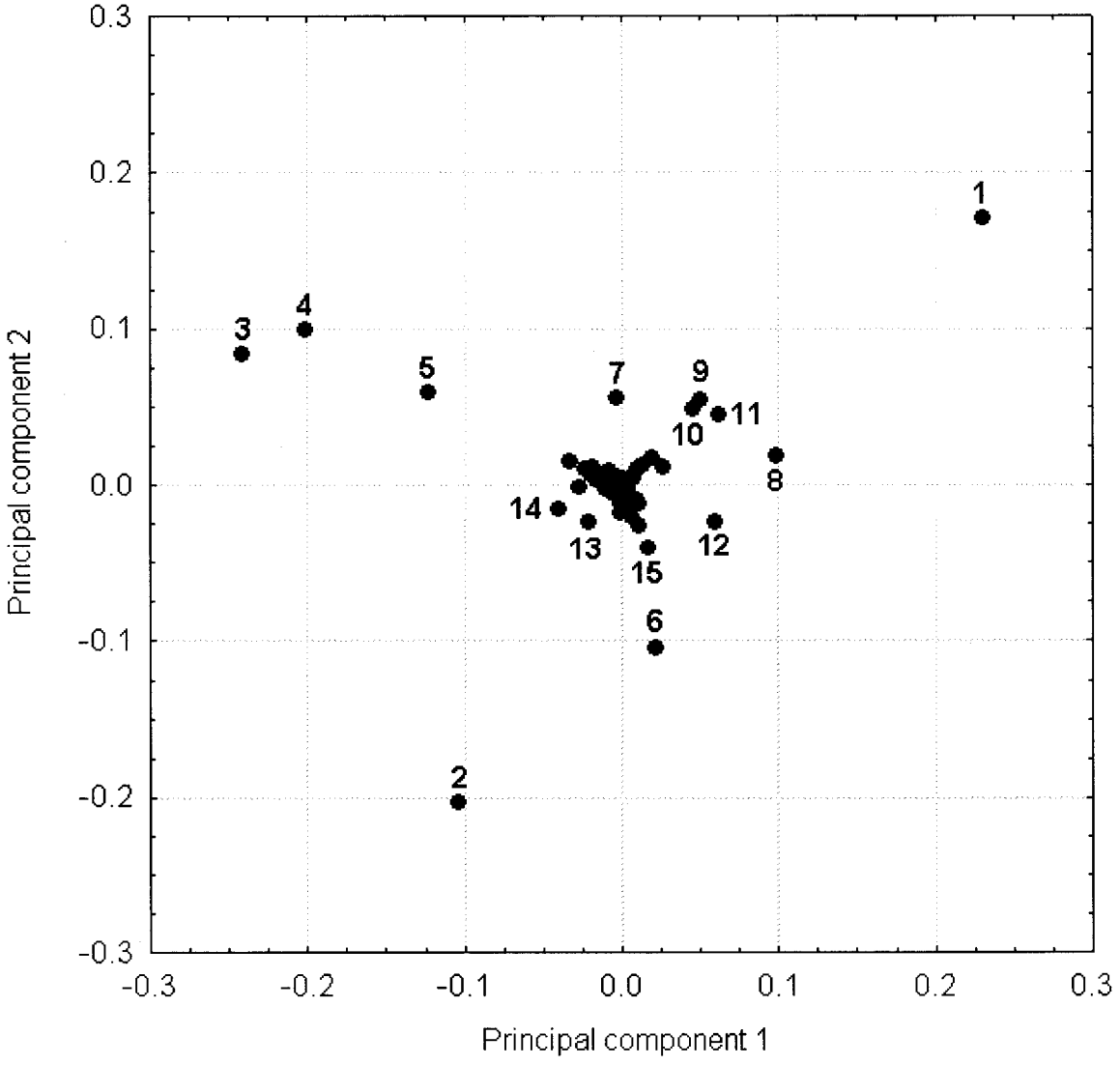
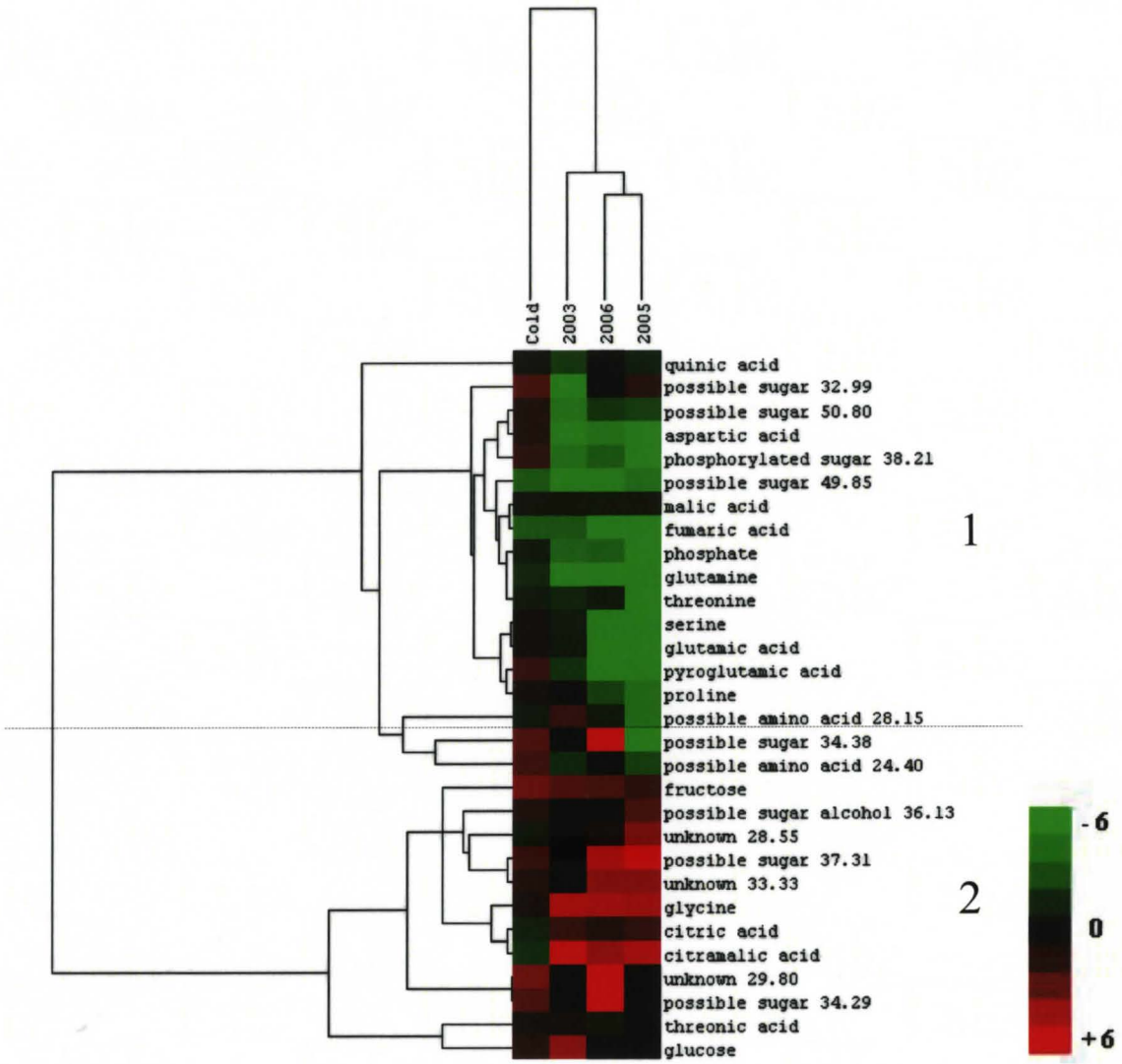

0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
Principal component 2
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
Principal component 1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

**Figure 24. Heat map grouping of metabolites.** Grouping of metabolites based on significant differences in relative abundance of specific components in extracts prepared from leaves harvested from *Thellungiella* field plants growing in Whitehorse, Yukon after a 4-day period of drought (2003), after a period of high rainfall accumulation (2005) and during a period of sub-zero temperatures (2006). Leaf tissue extracts were also prepared from leaves harvested from Yukon plants exposed to cold temperatures (5°C) in controlled environment conditions. The threshold for detection was 0.0005.


Cold
2003
2006
2005
quinic acid
possible sugar 32.99
possible sugar 50.80
aspartic acid
phosphorylated sugar 38.21
possible sugar 49.85
malic acid
fumaric acid
phosphate
glutamine
threonine
serine
glutamic acid
pyroglutamic acid
proline
possible amino acid 28.15
possible sugar 34.38
possible amino acid 24.40
fructose
possible sugar alcohol 36.13
unknown 28.55
possible sugar 37.31
unknown 33.33
glycine
citric acid
citramalic acid
unknown 29.80
possible sugar 34.29
threonic acid
glucose
1
2
-6
0
+6

includes metabolites whose abundance was generally higher in the field compared to cold-stressed chamber grown plants and the fold-changes showed variation among the samples used. Three metabolites of particular interest included those whose abundance was higher in both 2006 field plants and cold-stressed cabinet-grown plants and these included an unknown chemical with a RT of 29.80 and two possible sugars, RT 34.29 and RT 34.38. Another type of variation shown by the dendrogram is exemplified by citrate and citramalic acid. The fold-differences for the content of these components between 2006 field plants or cold-exposed plants and their respective controls suggests that these metabolites are present at lower abundance in these sources than in 2003 or 2005 field plants. A common environmental condition between the 2006 field plants and the cold-stressed plants in cabinets is exposure to a prolonged period of low temperature. Distinct variation also shown by the dendrogram was exhibited by aspartic acid, pyroglutamic acid and three unidentified compounds including a likely amino acid (RT 24.40), sugar (RT 50.80), and phosphorylated sugar (RT 38.21). The fold-differences for the content of these components between cold-exposed plants and their respective controls suggest that these metabolites are present at higher abundance in these cold-exposed cabinet-grown plants than in any of the field-grown plants.

## DISCUSSION

Plant tolerance to water deficit has been studied in a number of ways. One way researchers have studied drought tolerance is by measuring the stability of cellular structures such as the cell membrane in response to soil desiccation (Premachandra et al., 1991; Tripathy et al., 2000). Other researchers have chosen to study drought tolerance using a measure of plant productivity and yield during water deficits (Morgan et al., 1986; Sobrado, 1986; Richards, 1996). A third approach used by researchers involves trying to identify morphological and physiological phenotypic traits associated with drought tolerance (Jones and Turner, 1978; Farris, 1984; Guralnick and Ting, 1978; Davies and Zhang, 1991; Tuinstra et al., 1997). In large part this study exemplifies the third approach in that the research reported in this thesis describes how *Thellungiella* responds to water deficits produced under defined conditions in controlled environment chambers and experienced by plants in the field.

The overall objective of this thesis research is to determine the extent to which *Thellungiella* tolerates drought. This study involves comparing the water deficit tolerance of *Thellungiella* plants originating in two areas of the globe that feature different climates, namely Shandong Province in China and the Yukon Territory in Canada. I have applied comparative physiology approaches in order to determine whether Yukon and Shandong *Thellungiella* plants possess different innate capacities to withstand drought exposure and then tried to identify physiological or metabolic traits associated with tolerance and recovery from water deficits. For plants from the Yukon, an additional approach was taken by examining plants at a field site to determine if any stress- and recovery-responsive traits identified for plants in growth cabinets were also

expressed in plants in the field under different meteorological conditions of precipitation and temperature. The hypothesis tested here was the expectation that traits essential to survival under extreme conditions of water deficit should be expressed in plants experiencing this type of stress regardless of where they are growing.

**Physiological response of Shandong and Yukon *Thellungiella* to simulated drought stress**

The results of this study showed distinct differences in the drought response exhibited between Shandong and Yukon *Thellungiella*. The Yukon plants displayed a greater tolerance to prolonged water deficits than the Shandong plants by several criteria:

*1) Recovery of Thellungiella following water deficit*

Hoekstra et al. (2001) reported that one indication of drought tolerance is the ability of plant cells to re-hydrate successfully following cellular dehydration. By this criterion, the capacity of wilted plants to regain turgor quickly upon re-watering would be an important indication of drought tolerance. In my study, plants that were left un-watered underwent wilting and their recovery was monitored for two days after they were re-watered (designated RW1 plants). Only 75% of the RW1 Shandong plants recovered full turgor and the rest remained flaccid and eventually died without recovering turgor. In contrast, under the same experimental conditions 100% of the RW1 Yukon plants recovered full turgor and survived. By this measure of recovery from exposure to simulated drought, the Shandong plants did not display drought tolerance to the same degree as the Yukon plants.

*2) Recovery of Thellungiella pre-exposed to water deficit*

The ability of plants to maintain leaf turgor during a period of water deficit is an important adaptation to soil desiccation and is recognized as one indicator of drought tolerance (Hsiao et al., 1976). In wheat, plants that maintained turgid leaves for long periods of time during water deficit were selected for breeding in order to create a line of plants less susceptible to soil desiccation (Shimshi et al., 1982; Schonfield et al. 1988). In my study I determined the length of time taken for plants to wilt once watering was stopped in the case of plants that had already experienced a similar simulated drought exposure. The outcome of this experimental approach is shown in Figure 6 as a norm of reaction plot. In this figure, data comparing the time elapsed before signs of visible leaf wilting shows that Yukon plants retained water longer and hence took longer to wilt during a second drought if they experienced a previous exposure to drought. In contrast, similarly treated Shandong plants wilted more quickly when exposed to a second water deficit. This is consistent with Yukon plants developing an increased resistance to wilting following pre-exposure to drought while Shandong plants became less tolerant under the same experimental conditions.

The ability of Yukon *Thellungiella* to develop increased resistance to wilting as a result of pre-exposure to water deficit is an example of drought acclimation. Acclimation to water deficit is a trait shown by many drought-tolerant plant species (Clayton-Greene, 1983; Myers and Neales, 1986; Mulkey et al., 1991; White et al., 2000; Bogeat-Triboulot et al., 2007). In a study by Evans et al. (1990), authors predicted that re-hydration-induced changes as a result of acclimation would be more likely to occur in plants native

to arid environments than in plants native to mesic environments. This hypothesis was further developed by Tognetti et al. (1998) in a study using European beech from two Italian populations: plants native to southern Italy representing a xeric population and plants native to central Italy representing a mesic population. In this study, plants native to xeric climates had a superior potential to acclimate to dry conditions as compared to plants native to mesic climates. These findings are consistent with the results exhibited in my study in that Yukon plants native to the semi-arid, sub-artic region were able to undergo an acclimation to drought treatment while the Shandong plants that are native to a temperate region with much higher precipitation showed little to no capacity to undergo drought acclimation.

Teulat et al. (2003) proposed that drought tolerance can be measured quantitatively and that RWC is an appropriate measure to screen for tolerance to water deficits. Yukon plants preconditioned through exposure to drought (designated RW2 plants) had a higher leaf RWC than well-watered controls that had not experienced drought or non-preconditioned plants (RW1) by about 11 and 8%, respectively (Table II). By way of comparison, drought preconditioned Shandong plants (RW2), well-watered controls, and non-preconditioned plants (RW1) showed little difference (1 to 3%) with respect to leaf RWC values (Table II). Therefore, drought preconditioning treatment that led to a superior drought tolerance exhibited by the Yukon plants involves increased water retention as shown by a higher leaf RWC.

*3) Prolonged water deficits*

A subset of plants underwent prolonged water deficits to allow plants to reach a leaf RWC around 30%. When these plants were re-watered every second day for two weeks, the Shandong *Thellungiella* showed no signs of survival or recovery (Fig. 9, F) but the Yukon plants survived the treatment and re-grew through the emergence of new leaf tissue (Fig. 9, C). Most crop plants do not survive after their leaf RWC drops below 60-70% (Barr and Weatherley, 1962); however, plants adapted to xeric climates known as "resurrection plants" can tolerate almost entire water loss from their vegetative tissues and 'resurrect' following re-watering (Vicré et al., 2004). The capacity of plants to recover from extreme dehydration of its tissues would enable them to survive low rainfall including extended periods of no rainfall in their native habitat. In semi-arid environments, such as the southern Yukon region, the ability to 'resurrect' following prolonged dehydration could be essential for plant survival.

Mechanisms responsible for the recovery of plants following extreme dehydration as measured by low leaf RWC have been extensively studied. Accumulation of sugars has been proposed as a key response that resurrection plants require in order to protect dehydrated cells from dessication-related injury (Ingram and Bartels, 1996). Sugars such as sucrose are believed to protect desiccated cells in resurrection plants by stabilizing membranes and proteins, and preserving the lipid bilayer thereby maintaining cell structure (Ingram and Bartels, 1996; Crowe et al., 1998).

*4) Long term impact of water deficits*

In order to determine the long-term impact of drought, RW2 plants were watered once daily and then observed for 10 weeks. Greater than 95% of the RW2 Yukon plants

went on to flower and produce viable seeds before dying while all of the RW2 Shandong plants died before flowering. Thus Yukon plants were able to complete their annual life cycle following exposure to water deficit while Shandong plants could not. Successful reproduction following periods of low rainfall or drought is essential for plants in arid regions because persistence of these species is associated with a copious seed bank (Kemp and Culvenor, 1994). Without successful reproduction and the production of viable seeds under adverse conditions, plants in drought-prone areas cannot proliferate and the species would quickly die out. With this consideration in mind, Shandong *Thellungiella* would be predicted to be less able to proliferate and sustain its population numbers in xeric climates, such as the semi-arid climate of the southern Yukon region.

**Drought tolerance in Shandong and Yukon Thellungiella**

The pronounced difference in drought tolerance shown between Shandong and Yukon plants described above likely has a genetic basis. Genotypic variability is known to exist with respect to various plant responses related to drought tolerance. Some of these responses include stomatal sensitivity (Henzell et al., 1976; Radin et al., 1994), osmotic adjustment (Morgan, 1983), cell membrane stability (Tripathy et al., 2000), and epicuticular wax load (Nazam Uddin and Marshall, 1988). In my study, I focused upon solute accumulation and the role it plays through osmotic adjustment as a drought response of adaptive value to plants experiencing water deficits

*Osmotic adjustment of chamber grown plants*

Plant survival during periods of water deficit is associated with osmotic adjustment and prolonged positive turgor maintenance (White et al., 1992). In this study, the contribution of osmotic adjustment to drought-stressed *Thellungiella* plants from the Yukon and Shandong Province was investigated (Fig. 11). When water was withheld, $\Psi s$ values for *Thellungiella* decreased until visible leaf wilting occurred and this point usually corresponded to a leaf RWC of 50-60%. Two days after wilted plants were re-watered (RW1), leaf $\Psi_S$ was restored to pre-treatment levels. After a second drought episode plants recovered turgor within two days of re-watering (RW2); however, the Yukon plants re-established turgor at a significantly lower leaf $\Psi_S$ than did Shandong plants (Fig. 11 and Table II). By comparison, Shandong plants showed no treatment-responsive changes with respect to leaf $\Psi_S$ values. Several studies have reported that pre-exposure of plants to water deficit results in decreased osmotic potential during subsequent drought exposure, and that this behaviour results in increased plant tolerance to stress (Bennett and Sullivan, 1981; Abrams, 1988; Mulkey et al., 1991). This is consistent with the capacity of Yukon plants to develop an increased ability to withstand drought following a single episode of water deprivation and may explain why Shandong plants became less tolerant under the same experimental conditions.

*Osmotic adjustment of field plants*

In the field, the degree of osmotic adjustment was greater in 2003 field plants than in the plants measured in 2005 (Fig. 11). This understanding is supported by a significantly lower leaf $\Psi_S$ for 2003 plants than the leaf $\Psi_S$ measured for leaves of 2005 field plants. In both cases no differences were noted with respect to leaf turgor in the

field plants that might account for the difference in leaf $\Psi_S$ values but we did not have an instrument to directly measure leaf $\Psi_p$ at the field site. In a study by White et al. (2000), inherently low $\Psi_S$ was reported to confer advantages for Eucalyptus growing in low-rainfall environments typical of the Mediterranean. Leaf $\Psi_S$ values for the 2003 and 2005 field plants were also compared to values determined for drought-stressed *Thellungiella* growing in growth cabinets. Interestingly, leaf $\Psi_S$ values for plants of a dry growing season (2003) were the same as the leaf $\Psi_S$ values measured for the Yukon plants that had regained turgor, following two successive drought episodes in a growth cabinet (RW2 plants). In contrast, leaf $\Psi_S$ values determined for leaves of 2005 field plants was more similar to the leaf $\Psi_S$ of a well-watered control plant (Yukon or Shandong). Therefore, a similar response in water-deficit induced solute accumulation and hence osmotic adjustment was shown for Yukon *Thellungiella* in plants growing at field sites and controlled environment chambers.

**Osmotic adjustment via compatible solutes**

Decreased osmotic potentials signal solute accumulation (Oosterhuis and Wullschleger, 1987). The accumulation of solutes as part of "osmotic adjustment" in response to osmotic stress is an important physiological mechanism exhibited by plants for the retention of water and maintenance of cell turgor (Hsiao et al., 1976; Turner and Jones, 1980). These solutes, also referred to as "compatible solutes", are chemically diverse, typically low molecular mass compounds that can be accumulated at high concentrations without hindering metabolism (Yancey et al. 1982; McNeil et al., 1999; Xiong and Zhu, 2002). During periods of water stress, these compatible solutes

accumulate in the cytoplasm and help establish a $\Psi_w$ gradient to promote water uptake by roots thereby maintaining cellular turgor (Hanson and Hitz, 1982). Compatible solutes can also protect cells against oxidative damage by free radicals and stabilize proteins and membranes within the cytosol (McNeil et al., 1999; Hare et. al., 1998).

Studies on crop plants including wheat, sorghum, maize, chickpea and pea have often shown a positive correlation between the accumulation of compatible solutes and a reduced impact of drought on grain yield loss (Morgan et al., 1986; Sobrado, 1986; Santamaria et al., 1990; Morgan et al., 1991; Rodríguez Maribona et al., 1992; Moustafa et al., 1996). Therefore, the identification of compatible solutes contributing to the superior tolerance of the Yukon plants to drought could offer insight for improving crop growth and yield under water stress conditions.

**Metabolic profiling by Gas Chromatography / Mass Spectrometry**

Plants exposed to water deficit exhibit a wide range of responses including stress-induced changes in metabolism that result in the synthesis and accumulation of compatible organic solutes (Pattanagul and Madore, 1999, Xiong and Zhu). One way to identify and quantify the stress-induced changes of metabolites present in *Thellungiella* is by metabolic profiling. Metabolic profiling is an approach used to identify and quantify all the metabolites present in a biological sample (Weckwerth and Fiehn, 2002). Metabolic profiling applied to functional genomics, termed metabolomics, is an important tool for unraveling functions of uncharacterized genes (Trethewey, 2001). In addition, metabolic profiling is being used to study biochemical pathways and their regulation in a non-biased, broad manner (Fiehn et al., 2000). In my thesis research I

have used metabolic profiling by GC/MS coupled with multivariate statistical methods in order to identify treatment-responsive changes among metabolites for Shandong and Yukon *Thellungiella* plants grown in controlled environmental conditions.

*Data analysis of GC/MS instrumentation output for drought-stressed cabinet plants*

GC/MS was used to analyze chemical components present in the polar phase of extracts prepared from leaves of RW1, RW2 and control plants for both Shandong and Yukon *Thellungiella.* GC/MS allowed the detection of many components in each extract (typically 289 ± 7; n = 40). Three statistical methods were applied to characterize the metabolic phenotype of Shandong and Yukon plants in response to drought-stress:

*1. ANOVA of metabolic component distribution*

The identification of similarities and differences among peaks detected between chromatograms of various samples was the first step of data analysis. ANOVA assessed the statistical significance ($P \leq 0.05$) associated with these differences in response to a given condition such as drought stress or recovery from drought. Of the ca 289 components, statistically significant ($P \leq 0.05$) changes in the relative abundance of 36 and 41 components detected in leaves of Shandong and Yukon plants, respectively, were found in response to simulated drought treatment (see Appendix A, B). A Venn diagram was constructed to show the number of metabolites undergoing significant changes between RW1 and RW2 leaf extracts of the Shandong and Yukon plants (Fig. 13). Given the greater tolerance to drought shown by Yukon plants, components of particular interest were those common to both RW1 and RW2 Yukon plants but either undetected or

showing no stress-responsive changes in drought-stressed Shandong plants. Metabolic profiles showed 14 components whose abundance was elevated in RW1 and RW2 Yukon plants, eight of these were also enriched in drought-stressed Shandong plants. Therefore, the metabolites of interest included the remaining six components that were detected only in drought-stressed Yukon plants, and these included galactose, phosphate, valine, a possible organic acid (RT 31.42), a possible sugar (RT 36.07), and a compound of unknown chemical class (RT 49.79).

We hypothesized that changes in the levels of critical stress-responsive compounds, including compatible solutes, would happen whether plants were exposed to simulated drought stress in growth cabinets or experiencing drought under field conditions. The Venn diagram in Figure 14 summarizes the number of components increasing or decreasing in simulated drought-stressed Yukon plants relative to well-watered controls as compared to the number of components shown to undergo changes in extracts prepared from leaves of 2003 and 2005 field plants. A total of six components were elevated among RW1, RW2 and 2003 plants, and four of these were not elevated in 2005 plants. These four components were identified as galactose, glucose, and two possible sugars (RT 34.13 and 36.07). Interestingly, levels for galactose and the possible sugar with the RT 36.07 were not significantly different in drought-stressed Shandong plants. No components showing significant decreases in abundance were found to overlap between RW1, RW2 and 2003 Yukon plants.

A subset of components that were also targeted for special attention included those elevated in RW2 Yukon plants. These compounds were of interest because they may have contributed to the significantly lower $\Psi_S$ values measured for RW2 plants of

Yukon *Thellungiella* and, as discussed earlier, these plants have undergone changes that are consistent with drought-acclimation. Of the 16 elevated components detected only in extracts of RW2 Yukon plants, four were also present at higher levels in 2003 field plants but absent in 2005 plants. This suggests a role for these four compounds in drought-acclimation or drought tolerance in the field. These four components include *myo*-inositol, a possible sugar (RT 36.33), threonic acid and a compound of unknown chemical class (RT 29.93).

*2. Principal Component Analysis (PCA) clustering of metabolic change in response to water deficit*

Another data mining tool, PCA, provided a means to help identify important treatment-responsive patterns among metabolites by distinguishing the compounds that exhibit the greatest variance within treatments/genotypes and ranking these differences according to their contribution to the formation of each distinct cluster (Fig. 15). Interestingly, distinct clusters were not observed for RW2 Shandong *Thellungiella*. This may be a result of their decreased tolerance following an initial exposure to water deficit.

Principal component 1 accounted for 32% of the variance and allowed clear separation of plants growing in the field versus plants growing in growth cabinets. Key metabolites contributing to this separation includes sucrose, citrate and an unknown compound (RT 31.64) (Fig. 16). Other metabolites contributing, albeit to a lesser extent towards this separation, are glucose, *myo*-inositol and malate. Principal component 2 comprised 30% of the variance and it was the primary component separating Shandong from Yukon *Thellungiella* plants. Among the metabolites detected, fructose was the

primary contributor distinguishing these genotypes (Fig. 16).

*3. Hierarchical Cluster Analysis (HCA) of metabolic change in response to drought*

HCA was applied to the drought-responsive components previously identified by ANOVA as undergoing statistically significant changes in order to sort and classify metabolites according to their relative abundance as compared to their respective levels in well-watered controls (Fig. 17). HCA reaffirmed that the metabolic response of Shandong and Yukon *Thellungiella* plants to drought-stress was different. Six clusters were identified on the basis of contiguous patches of colour representing groups of metabolites grouped by similar fold-changes in abundance over multiple conditions. For the purpose of this analysis we were particularly interested in clusters containing metabolites showing elevated levels in drought-stressed, cabinet-grown Yukon plants and 2003 Yukon field plants but either lower or constant levels in drought-stressed Shandong plants and 2005 Yukon field plants. This clustering, we believed, would contain metabolites present in the samples prepared from plants that were exposed to osmotic stress and most tolerant of these conditions. Two clusters shown on Figure 17 included metabolites that met this criterion:

(i) Cluster 2 includes six metabolites of interest: galactose, two possible sugars (RT 34.13 and 36.07), an unknown compound (RT 29.93), and two metabolites that were not identified previously using the Venn diagram method: fructose and glucose.

(ii) Cluster 5 contained two metabolites of interest, and both of these were identified previously: *myo*-inositol and threonic acid.

*Possible roles of metabolites and their contribution to drought-stress tolerance in Yukon Thellungiella*

Using ANOVA, PCA and HCA statistical methods we were able to identify and short-list a number of compounds with possible roles in the drought tolerance of Yukon *Thellungiella*. In addition, these statistical methods identified metabolites that have been previously reported in the literature as having an important role in drought-tolerance. These metabolites have been grouped according to their respective chemical class and discussed below.

**Amino acids**

The response detected among amino acids for Shandong and Yukon leaves was attributed to differences in the levels of threonine and valine. Levels of the amino acid threonine were lower in RW1 Shandong and Yukon *Thellungiella* plants, as well as plants growing in the field. There appears to be no association with heightened stress tolerance and so this metabolite was not studied further. However, valine shows a three-fold increase in RW1 and six-fold increase in RW2 Yukon plants and remained unchanged in drought-stressed Shandong plants. In *Thellungiella* plants harvested from the field, valine levels were not higher in 2003 relative to 2005 and, as such, a compelling association with dry conditions and hence a role in drought tolerance of Yukon *Thellungiella* is not supported. The reason for the drought-associated changes under controlled environments is interesting but unclear at this point. It may be, however, that valine is a storage compound for carbon and nitrogen, a role that has been suggested for

other nitrogen containing amino acids such as proline (Keller and Ludlow, 1993). Given that cabinet-grown plants were fertilized weekly it may be that the N provided is not fully utilized and temporary storage in the form of valine may be a useful strategy for plants whose growth is slowed by stress.

The accumulation of amino acids by plants in response to osmotic stress has been observed and reported by several authors to contribute to osmoregulation in leaf tissues (Munns et al., 1979; Elhaak et al., 1993; Gzik, 1996). In a study using the crucifer canola (*Brassica napus*), a close relative of *Thellungiella*, the majority of amino acids accumulated in response to drought declined rapidly upon re-hydration (Good and Zaplachinski, 1994). Deviating from this pattern was proline, which was the most actively accumulated amino acid. Accumulation of proline is one of the most common responses of plants (and other organisms) to water deficits and is reported to contribute to osmotic adjustment as a compatible solute (Yancey et al. 1982), as an osmoprotectant (Wyn Jones and Storey, 1978), and as a detoxification agent of reactive oxygen species (Floyd and Nagy, 1984). Interestingly, in our studies proline did not exhibit stress-responsive increases in abundance in RW1 and RW2 Yukon or Shandong plants growing in controlled environments or in plants harvested in the field during the 2003 growing season. This suggests that proline is not an important compatible solute in drought-stressed *Thellungiella*.

**Organic acids**

Shandong and Yukon *Thellungiella* plants were also distinguished by different amounts of organic acids detected in their leaves. In the literature, organic acids have

been identified as having an important role in the metabolic and physiological response of plants to water deficit (Timpa et al., 1986). In this study, threonic acid exhibited stress-responsive changes that were associated with elevated levels in Yukon RW2 plants and 2003 field plants. In contrast, the level of threonic acid was lower or showed no significant change in drought-stressed Shandong plants and 2005 field plants compared to their respective controls. This pattern of response suggests that threonic acid could contribute to the osmotic adjustment of Yukon *Thellungiella* in response to drought or that it plays a role in metabolism underway in stressed plants.

Other organic acids were shown to undergo stress-responsive changes with exposure to water deficits including succinate, citrate, and malate. Both citrate and succinate levels decreased in cabinet plants exposed to simulated drought relative to unstressed controls but their levels were many-fold higher in leaf tissues harvested from plants of both field years relative to cauline leaves of cabinet-grown controls. In the PCA shown in Figure 16, *Thellungiella* grown under controlled environmental conditions versus field conditions was primarily distinguished by different amounts of citrate and to a lesser extent, malate.

Organic acids can account for a large proportion of leaf dry weight (Eaton and Engle, 1949). For example, malate and citrate, two key metabolites in the tricarboxylic acid cycle, accounted for upwards of 10% of the dry weight of leaf tissue in cotton (Eaton and Engle, 1949). In the same study, the levels of these two organic acids were noticeably affected by the imposition of drought: malate increased two-fold and citrate decreased two-fold relative to unstressed controls.

A study by Cutler and Rains (1978) implicated malate as an important contributor to osmotic adjustment in response to drought stress. One drought-responsive property of malate is as a counter-ion, balancing positive charges imposed by cations and therefore playing an important role in osmotic adjustment (Henry et al., 2007).

The chelation of alkaline materials by plants that hyper-accumulate citrate has been well documented (Pich et al., 1994; Brown et al., 1998; Chen et al., 2003). A study of alkali grass showed that the accumulation of citrate is a physiological response to alkali salt stress in that it enables plants to maintain constant pH levels (Shi et al., 2002; Shi et al., 2003). The ability of plants to accumulate citrate as a chelator and maintain constant pH could be roles accounting for the elevated levels of citrate in *Thellungiella* field plants native to the alkaline salt-flats of the southern Yukon region and could account for their apparently unchanging levels in leaves under controlled environment conditions.

**Sugars and sugar alcohols**

Most of the drought-stress related differences in metabolite levels shown by *Thellungiella* were attributed to changes in the amount of hexoses and disaccharides, a large number of which were only tentatively identified or could not be identified with any certainty. One sugar which was identified and deemed to be of interest was fructose. Fructose was elevated in all *Thellungiella* plants stressed in growth cabinets relative to controls, as well as in the field, particularly in 2003. HCA showed that fructose levels underwent a fold-increase that was two times greater for Yukon RW1 plants, and four times greater for Yukon RW2 plants relative to the fold-increase in drought-stressed

Shandong RW1 and RW2 plants, respectively. In the field, fructose showed a two-fold higher level in 2003 plants relative to controls than the 2005 plants. Glucose, another sugar of interest, was present at levels significantly higher in drought-stressed *Thellungiella* grown in controlled environments and in 2003 field plants. Interestingly, the relative fold-increase for glucose was four times greater in leaves of RW2 Yukon plants relative to the increase found in Shandong RW2 plants. Both fructose and glucose also were predominant contributors in the distribution of clusters using PCA. Fructose was the primary contributor to the separation of clustering among differentially treated plants grown in growth cabinets (Fig. 14 and 15), while glucose contributed to the separation of field plants from growth cabinet plants. Galactose, another monosaccharide undergoing stress-responsive changes was present at significantly higher levels in RW1, RW2 and 2003 Yukon plants, and was not significantly higher in drought-stressed Shandong plants or in 2005 Yukon field plants. Of the disaccharides, sucrose was identified using PCA as having an important contribution to the separation of field plants from chamber plants but the content of this metabolite did not undergo stress-responsive changes in drought-stressed *Thellungiella* grown in controlled environments.

The differences between Shandong and Yukon *Thellungiella* with respect to the accumulation of monosaccharides may be of physiological importance towards the superior tolerance of the Yukon plants to periods of water deficit and contribute to their recovery after the stress is relieved. In the literature, several studies have reported that the capacity of drought-adapted plants to maintain turgor at lower leaf $\Psi w$ values was associated with levels of specific leaf carbohydrates, including glucose, fructose and sucrose (Ackerson, 1981; Schwab and Gaff, 1986; Kameli and Losel, 1993; Geigenberger

et al., 1997). In response to drought-stress, sugars have been identified as compatible solutes or osmoprotectants contributing to the osmotic adjustment of plant cells. As compatible solutes, sugars can act to protect desiccated cells by stabilizing membranes and proteins, and preserving the lipid bilayer and therefore maintain cell structure (Ingram and Bartels, 1996; Crowe et al., 1998). The accumulation of sugars has also been reported to be necessary for the development of desiccation tolerance in embryo cells (Blackman et al., 1992). In addition to protective roles, sugars can also function as storage reserves during the onset of drought-stress and support re-growth upon re-hydration (Hanson and Hitz, 1982; Volaire et al., 1998).

Glucose is one sugar of specific interest in its perceived role in drought tolerance. In a study by Ackerson (1981), glucose was shown to be accumulated to higher levels in cotton plants pre-exposed to drought stress as compared to plants with no previous drought exposure. Interestingly, these cotton plants pre-adapted to drought displayed a superior tolerance to drought than non-adapted plants. Another study evaluating the role of glucose found that accumulation of this sugar in leaves of Populus species exposed to water deficit was negatively correlated with ion leakage of membranes and positively correlated with drought tolerance (Pelah et al., 1997)

Sucrose levels were only higher relative to controls in leaves of 2003 and 2005 field plants (personal ommunication from David Guevara: Dept. of Biology, McMaster University). Sucrose is the dominant carbohydrate to accumulate in all resurrection plants and has been extensively studied in view of this property (Ingram and Bartels, 1996; Scott, 2000). In our study, the lack of drought-response for this sugar in either Shandong or Yukon *Thellungiella* suggests that sucrose accumulation does not contribute

towards adjustment to drought-stress for RW1 and RW2 plants. However, a possible role for sucrose and other metabolites in the recovery of Yukon *Thellungiella* from extreme water deficits (< 30% leaf RWC), somewhat comparable to the severe conditions of water loss by a resurrection plant, has not yet been determined.

The only response with respect to changes in the levels of sugar alcohols for Shandong and Yukon plants was associated with *myo*-inositol. *Myo*-inositol content was significantly higher in RW2 plants and 2003 Yukon field plants but remained unchanged relative to their respective controls in all other treatments; a pattern of interest in view of its enrichment in tissues showing superior tolerance to low water conditions . In the literature, *myo*-inositol has been identified as a compatible solute with excellent hydroxyl radical scavenging properties to protect enzymes from denaturing during periods of drought (Smirnoff and Cumbes, 1989). *Myo*-inositol is also a precursor to other compatible solutes including D-pinitol, whose accumulation was also observed in response to drought in pine (Nguyen and Lamant, 1988) and in pigeon pea (Keller and Ludlow, 1993), raffinose, accumulated by Arabidopsis in response to drought and cold stress (Taji et al., 2002) and stachyose, accumulated in response to drought in yellow lupin (Zalewski et al., 2001). These metabolites were either not detected (eg, stachyose) or not found to be significantly higher in RW1 or RW2 Shandong and Yukon *Thellungiella* plants.

**Evaluating Yukon *Thellungiella* as a model for osmotic stress tolerance**

To date, most studies involving metabolic profiling are carried out using plants grown in controlled environments. Experiments done under these conditions have proven

useful for identifying stress-specific responses. However, the adaptive value of processes identified under these unnatural conditions is difficult to judge when one takes into consideration that plants growing in their native environment are exposed to multiple, simultaneous stresses. Biotic and abiotic stresses, diurnal changes in ambient light (directionality, quality, and quantity), temperature and wind are some of the conditions that cannot be simulated faithfully in a cabinet (Kulheim et al., 2002; Rizhsky et al,. 2004). To address this deficiency I have compared the drought responsive changes of plants in growth cabinets to those found in plants growing under different prevailing meteorological conditions in the field. This approach has yielded a comparatively small subset of metabolites of ten from about 300 original components that merit further study in order to determine whether any of them play an essential role in the survival and growth of *Thellungiella* in the native habitat of this plant.

The ability of Yukon *Thellungiella* to grow and reproduce under comparatively extreme environmental conditions involving multiple, simultaneous abiotic stresses, including high salinity, freezing, and drought, renders it an ideal model for the study of metabolic, physiological and developmental traits underlying plant environmental stress tolerance mechanisms (Wong et al., 2005). Thus far in my discussion I have described how leaf tissue harvested from *Thellungiella* field plants exposed to lower than average rainfall (2003) and higher than average rainfall (2005) can be used in conjunction with drought experiments conducted in controlled environments for the identification of drought-responsive metabolic traits. A third dimension to our study involved using *Thellungiella* leaf tissue harvested during the beginning of the 2006 growing season when temperatures were much cooler and frequently dropped below 0°C. The addition of cold-

stressed field plants opens an opportunity to identify traits underlying the cold tolerance of *Thellungiella* (Griffith et al., 2007). In the following discussion I will briefly describe how this material can be used towards identifying metabolic traits conferring plant tolerance to cold temperatures.

*Analysis of Thellungiella field plants harvested during the 2003, 2005 and 2006 growing seasons*

In this study, the physiological and metabolic responses shown by Yukon plants harvested during field trips in 2003, 2005 and 2006 were compared. The lower leaf $\Psi_S$ observed for plants exposed to drought (2003) and cold (2006) field conditions compared to plants in 2005 or well-watered controls is an indication that *Thellungiella* accumulates solutes in response to the prevailing growth conditions (Fig. 17). For comparison, leaf $\Psi_S$ of *Thellungiella* exposed to continuous cold temperatures in controlled environment conditions are also given. Interestingly, the $\Psi$s leaf values for plants exposed to cold and freezing temperatures in the field (2006) are the same as the leaf $\Psi$s values measured for the Yukon plants exposed to cold in growth cabinets, and both are significantly lower than their respective, unstressed plants. The accumulation of solutes during cold acclimation has been documented in many species (Yelenosky and Guy, 1989; Koster and Lynch, 1992; Gilmour et al., 2000).

Qualitative and quantitative changes in leaf metabolite levels detected in the 2003, 2005 and 2006 field tissues were monitored using the GC/MS as described previously. Each GC/MS chromatogram of the polar fraction of leaf extracts had 294 ± 9 (n = 15) components (or unique mass spectra) present. Figure 18 shows that a subset of these metabolites showed reproducible patterns in the various tissues: of the ca 294

components detected, 57, 26 and 62 components showed greater abundance in 2003, 2005 and 2006 field plants, respectively, compared to a cauline leaf control. Conversely, the content of 55, 64 and 63 components was lower in 2003, 2005 and 2006 field plants, respectively, compared to a cauline leaf control (Fig. 18).

Of the components found accumulating in response to the field conditions, only eight were common to all three field years and four to a combination of two field years (Fig. 18). For compounds showing a decrease in content, 39 were common to all three field years and 11 were common to a combination of two field years. This finding suggests that the more frequent response to stressful field conditions is a reduction in the level of phytochemicals as opposed to an increase in their abundance.

The data was analyzed further using PCA, which simultaneously compared the metabolic signatures of 2003, 2005 and 2006 field plants and identified major sources of variance within the data set that led to clustering of each sample (Fig. 18). PCA showed that the two highest ranking components accounted for 78% of the total variance within the dataset and yielded distinct clusters distinguishing each field treatment. Principal component 1 accounted for 64% of this variation and separated 2006 field plants from 2003 and 2005 field plants. Primary contributors to this component included a possible sugar (RT 34.38) and an unknown compound (RT. 29.80), and secondary contributors included sucrose, proline, malate, glucose and citrate. Principal component 2 accounted for 14% of the variation and the separation between the clusters associated with 2003, 2005 and 2006 field plants showed overlap.

*Comparison of 2006 field plants with plants cold-stressed under controlled conditions*

In my discussion thus far, I have shown how GC/MS analysis together with software programs including chromatogram alignment tools (GASP), deconvolution programs (AMDIS), and statistical tools such as ANOVA, HCA and PCA, have enabled the simultaneous and comparatively unbiased analysis of hundreds of metabolites from *Thellungiella* plants stressed in controlled environments and in the field. These tools offer an opportunity to speculate about metabolic traits required for survival under sub-optimal or adverse conditions. In this part of my study I describe how I applied multivariate methods of analysis, PCA and HCA, and an ANOVA to study *Thellungiella* plants growing in the field and, in particular, during the cold conditions of 2006. Since freezing conditions can invoke cellular dehydration (Bray et al., 2000), it is very likely that leaves exposed to freezing temperatures accumulate compatible solutes in a manner analogous to plants stressed by water deficits or salinity.

When PCA was applied to the data set, samples from individual plants were separated into four distinct clusters corresponding to their respective treatment (Fig. 21). Principal component one accounted for 52% of the variation and allowed for the separation of each plant treatment into respective groups along the x-axis. Metabolites making the largest contribution to this separation included: proline, a possible sugar (RT 34.38), and galactose (Fig. 22). Principal component two accounted for 24% of the variation and separated 2006 field plants and cold-stressed plants grown in cabinets from the 2003 and 2005 field plants. Metabolites making the largest contribution to this separation include citrate and proline (Fig. 22).

ANOVA was used to assess the statistical significance of differences in the relative abundance of each metabolite. Statistically significant ($P \leq 0.05$) differences

were found for 30 components in leaves of cold-stressed *Thellungiella*. Heat-map signatures of all 30 components undergoing changes in cold-stressed plants from growth cabinets were compared to their relative abundance in field plants harvested in 2003, 2005, 2006 (Fig. 23). Two separate clusters were identified and are labeled in the dendrogram shown in Figure 23. Components of particular interest in this study included those present in greater abundance in plants exposed to cold in growth cabinets and 2006 field plants as both were exposed to cold temperatures although cabinet-grown plants had not been exposed to freezing conditions. Using these criteria, three components were identified and included an unknown compound, RT 29.80, and two possible sugars, RT 34.29 and RT 34.38.

## CONCLUSION

A comparison of physiological and biochemical responses of two genotypes of *Thellungiella* shows that different populations of this plant display a varying capacity to withstand water deficits. Plants whose seeds were found in the Yukon proved to be more tolerant of water deprivation than plants whose seeds originated in Shandong Province. Yukon plants were able to grow and flower following drought treatments that led to the death of Shandong plants. Yukon *Thellungiella* also showed a capacity to improve its innate drought tolerance upon exposure to a single drought and recovery treatment.

GC/MS analysis of leaf metabolite extracts coupled with statistical tools of data analysis proved to be an excellent method to study metabolic response to environmental stress. An important and useful comparison in this respect was provided by monitoring metabolites in plants grown under controlled environmental conditions and comparing their abundance to that of Yukon *Thellungiella* growing in its natural habitat. While many phytochemicals can be detected in a single GC/MS run, comparatively few undergo statistically significant stress-responsive changes. Further characterization of the metabolites undergoing changes may offer important insights into stress responses required for survival and growth of plants under field conditions.

**Appendix A. Metabolite levels for Shandong *Thellungiella*.** Levels of metabolites shown as expressing statistically significant ($P \leq 0.05$) changes in their abundance for Shandong *Thellungiella* control (SC), RW1 (SD), and RW2 (SD2) samples. Values are expressed as average RRF ± standard error.

| metabolites | SC | SE | SD | SE | SD2 | SE |
|---|---|---|---|---|---|---|
| citric acid | 0.003 | ± 0.001 | 0.000 | ± 0.000 | 0.000 | ± 0.000 |
| fructose | 2.563 | ± 0.114 | 3.127 | ± 0.136 | 3.690 | ± 0.136 |
| galactose | 0.037 | ± 0.005 | 0.008 | ± 0.004 | 0.018 | ± 0.004 |
| glucose | 2.002 | ± 0.116 | 1.933 | ± 0.351 | 3.269 | ± 0.351 |
| malic acid | 2.054 | ± 0.058 | 1.576 | ± 0.133 | 1.630 | ± 0.133 |
| phosphate | 0.340 | ± 0.026 | 0.219 | ± 0.023 | 0.180 | ± 0.023 |
| ribonic acid | 0.000 | ± 0.000 | 0.001 | ± 0.000 | 0.002 | ± 0.000 |
| succinic acid | 0.019 | ± 0.001 | 0.015 | ± 0.001 | 0.027 | ± 0.001 |
| sucrose | 2.151 | ± 0.113 | 2.129 | ± 0.045 | 2.468 | ± 0.045 |
| threonic acid | 0.015 | ± 0.002 | 0.007 | ± 0.000 | 0.012 | ± 0.000 |
| threonine | 0.015 | ± 0.002 | 0.007 | ± 0.002 | 0.016 | ± 0.002 |
| possible organic acid 29.25 | 0.001 | ± 0.000 | 0.001 | ± 0.000 | 0.000 | ± 0.000 |
| possible organic acid 33.75 | 0.001 | ± 0.000 | 0.001 | ± 0.000 | 0.002 | ± 0.000 |
| possible sugar 30.13 | 0.020 | ± 0.004 | 0.237 | ± 0.035 | 0.361 | ± 0.035 |
| possible sugar 31.64 | 0.136 | ± 0.044 | 0.779 | ± 0.049 | 0.983 | ± 0.049 |
| possible sugar 32.92 | 0.000 | ± 0.000 | 0.001 | ± 0.000 | 0.001 | ± 0.000 |
| possible sugar 34.13 | 0.005 | ± 0.001 | 0.013 | ± 0.001 | 0.013 | ± 0.001 |
| possible sugar 36.07 | 0.005 | ± 0.000 | 0.003 | ± 0.000 | 0.006 | ± 0.000 |
| possible sugar 36.33 | 0.003 | ± 0.000 | 0.002 | ± 0.000 | 0.003 | ± 0.000 |
| possible sugar 46.56 | 0.001 | ± 0.000 | 0.000 | ± 0.000 | 0.002 | ± 0.000 |
| possible sugar 49.85 | 0.002 | ± 0.000 | 0.000 | ± 0.000 | 0.006 | ± 0.000 |
| possible sugar 51.32 | 0.008 | ± 0.001 | 0.005 | ± 0.001 | 0.013 | ± 0.001 |
| unknown 14.12 | 0.013 | ± 0.001 | 0.007 | ± 0.001 | 0.014 | ± 0.001 |
| unknown 18.65 | 0.000 | ± 0.000 | 0.001 | ± 0.000 | 0.001 | ± 0.000 |
| unknown 28.80 | 0.013 | ± 0.001 | 0.014 | ± 0.000 | 0.022 | ± 0.000 |
| unknown 29.00 | 0.048 | ± 0.006 | 0.026 | ± 0.002 | 0.022 | ± 0.002 |
| unknown 29.39 | 0.002 | ± 0.000 | 0.000 | ± 0.000 | 0.000 | ± 0.000 |
| unknown 29.87 | 0.011 | ± 0.005 | 0.226 | ± 0.041 | 0.298 | ± 0.041 |
| unknown 30.03 | 0.019 | ± 0.008 | 0.261 | ± 0.040 | 0.401 | ± 0.040 |
| unknown 30.70 | 0.003 | ± 0.001 | 0.010 | ± 0.002 | 0.019 | ± 0.002 |
| unknown 35.08 | 0.000 | ± 0.000 | 0.003 | ± 0.001 | 0.006 | ± 0.001 |
| unknown 37.65 | 0.001 | ± 0.000 | 0.000 | ± 0.000 | 0.000 | ± 0.000 |
| unknown 42.28 | 0.003 | ± 0.000 | 0.002 | ± 0.000 | 0.004 | ± 0.000 |
| unknown 43.18 | 0.000 | ± 0.000 | 0.000 | ± 0.000 | 0.001 | ± 0.000 |
| unknown 46.68 | 0.000 | ± 0.000 | 0.000 | ± 0.000 | 0.000 | ± 0.000 |
| unknown 49.79 | 0.003 | ± 0.000 | 0.002 | ± 0.000 | 0.002 | ± 0.000 |

**Appendix B. Metabolite levels for Yukon *Thellungiella*.** Levels of metabolites shown as expressing statistically significant ($P \leq 0.05$) changes in their abundance for Yukon *Thellungiella* control (YC), RW1 (YD), and RW2 (YD2) samples. Values are expressed as average RRF ± standard error.

| metabolites | YC | | SE | YD | | SE | YD2 | | SE |
|---|---|---|---|---|---|---|---|---|---|
| citric acid | 0.112 | ± | 0.003 | 0.078 | ± | 0.007 | 0.047 | ± | 0.013 |
| ethanolamine | 0.019 | ± | 0.002 | 0.013 | ± | 0.001 | 0.031 | ± | 0.001 |
| fructose | 0.051 | ± | 0.004 | 0.120 | ± | 0.012 | 0.234 | ± | 0.022 |
| galactose | 0.024 | ± | 0.003 | 0.055 | ± | 0.006 | 0.051 | ± | 0.008 |
| glucose | 0.976 | ± | 0.042 | 2.065 | ± | 0.139 | 4.536 | ± | 0.192 |
| myoinositol | 0.207 | ± | 0.013 | 0.240 | ± | 0.015 | 0.483 | ± | 0.071 |
| organic acid 33.83 | 0.000 | ± | 0.000 | 0.000 | ± | 0.000 | 0.004 | ± | 0.001 |
| phosphate | 0.033 | ± | 0.007 | 0.092 | ± | 0.027 | 0.119 | ± | 0.031 |
| ribonic acid | 0.000 | ± | 0.000 | 0.001 | ± | 0.000 | 0.001 | ± | 0.000 |
| succinic acid | 0.020 | ± | 0.002 | 0.015 | ± | 0.002 | 0.028 | ± | 0.005 |
| sucrose | 2.552 | ± | 0.124 | 2.112 | ± | 0.089 | 3.272 | ± | 0.380 |
| threonic acid | 0.022 | ± | 0.002 | 0.020 | ± | 0.001 | 0.040 | ± | 0.007 |
| threonine | 0.001 | ± | 0.000 | 0.001 | ± | 0.000 | 0.004 | ± | 0.001 |
| valine | 0.002 | ± | 0.000 | 0.005 | ± | 0.000 | 0.010 | ± | 0.002 |
| possible organic acid 31.42 | 0.026 | ± | 0.003 | 0.038 | ± | 0.003 | 0.075 | ± | 0.004 |
| possible organic acid 33.75 | 0.000 | ± | 0.000 | 0.000 | ± | 0.000 | 0.002 | ± | 0.000 |
| possible sugar 27.63 | 0.004 | ± | 0.001 | 0.007 | ± | 0.001 | 0.009 | ± | 0.001 |
| possible sugar 30.13 | 0.002 | ± | 0.001 | 0.002 | ± | 0.001 | 0.009 | ± | 0.002 |
| possible sugar 31.64 | 0.062 | ± | 0.030 | 0.168 | ± | 0.042 | 0.878 | ± | 0.217 |
| possible sugar 32.92 | 0.000 | ± | 0.000 | 0.001 | ± | 0.000 | 0.003 | ± | 0.000 |
| possible sugar 34.13 | 0.000 | ± | 0.000 | 0.003 | ± | 0.001 | 0.025 | ± | 0.002 |
| possible sugar 36.07 | 0.003 | ± | 0.001 | 0.004 | ± | 0.000 | 0.013 | ± | 0.003 |
| possible sugar 36.33 | 0.002 | ± | 0.000 | 0.002 | ± | 0.000 | 0.006 | ± | 0.001 |
| possible sugar 42.89 | 0.004 | ± | 0.001 | 0.004 | ± | 0.000 | 0.013 | ± | 0.002 |
| possible sugar 42.94 | 0.003 | ± | 0.001 | 0.002 | ± | 0.000 | 0.002 | ± | 0.001 |
| possible sugar 46.56 | 0.000 | ± | 0.000 | 0.000 | ± | 0.000 | 0.002 | ± | 0.000 |
| possible sugar 49.85 | 0.001 | ± | 0.000 | 0.001 | ± | 0.000 | 0.013 | ± | 0.004 |
| unknown 15.80 | 0.001 | ± | 0.000 | 0.000 | ± | 0.000 | 0.000 | ± | 0.000 |
| unknown 18.65 | 0.000 | ± | 0.000 | 0.001 | ± | 0.001 | 0.003 | ± | 0.001 |
| unknown 22.15 | 0.001 | ± | 0.000 | 0.000 | ± | 0.000 | 0.002 | ± | 0.000 |
| unknown 28.80 | 0.009 | ± | 0.000 | 0.012 | ± | 0.001 | 0.023 | ± | 0.002 |
| unknown 29.87 | 0.001 | ± | 0.000 | 0.002 | ± | 0.000 | 0.009 | ± | 0.002 |
| unknown 29.93 | 0.003 | ± | 0.001 | 0.003 | ± | 0.001 | 0.008 | ± | 0.001 |
| unknown 35.08 | 0.001 | ± | 0.000 | 0.001 | ± | 0.000 | 0.006 | ± | 0.002 |
| unknown 36.65 | 0.000 | ± | 0.000 | 0.001 | ± | 0.000 | 0.002 | ± | 0.001 |
| unknown 37.65 | 0.002 | ± | 0.001 | 0.001 | ± | 0.000 | 0.000 | ± | 0.000 |
| unknown 42.28 | 0.044 | ± | 0.004 | 0.044 | ± | 0.004 | 0.069 | ± | 0.008 |
| unknown 43.18 | 0.001 | ± | 0.000 | 0.001 | ± | 0.000 | 0.002 | ± | 0.000 |
| unknown 46.68 | 0.001 | ± | 0.001 | 0.000 | ± | 0.000 | 0.002 | ± | 0.000 |
| unknown 49.55 | 0.024 | ± | 0.002 | 0.037 | ± | 0.002 | 0.028 | ± | 0.007 |
| unknown 49.79 | 0.003 | ± | 0.001 | 0.006 | ± | 0.000 | 0.005 | ± | 0.001 |

**Appendix C. Metabolite levels for *Thellungiella* field plants.** Levels of metabolites shown as expressing statistically significant ($P \leq 0.05$) changes in their abundance for *Thellungiella* field plants harvested during the 2003 and 2005 growing season as compared to the same cauline leaf control. Values are expressed as average RRF ± standard error.

| metabolites | Control | | SE | 2003 | | SE | 2005 | | SE |
|---|---|---|---|---|---|---|---|---|---|
| citric acid | 0.691 | ± | 0.085 | 3.654 | ± | 0.532 | 3.668 | ± | 0.701 |
| ethanolamine | 0.006 | ± | 0.002 | 0.056 | ± | 0.008 | 0.034 | ± | 0.010 |
| fructose | 0.285 | ± | 0.095 | 2.468 | ± | 0.392 | 1.292 | ± | 0.216 |
| galactose | 0.002 | ± | 0.000 | 0.076 | ± | 0.020 | 0.002 | ± | 0.000 |
| glucose | 0.967 | ± | 0.21 | 2.273 | ± | 0.247 | 1.165 | ± | 0.238 |
| malic acid | 5.513 | ± | 0.276 | 3.582 | ± | 0.324 | 3.348 | ± | 0.197 |
| myoinositol | 1.035 | ± | 0.056 | 1.454 | ± | 0.116 | 0.997 | ± | 0.075 |
| phosphate | 0.126 | ± | 0.026 | 0.006 | ± | 0.001 | 0.044 | ± | 0.014 |
| ribonic acid | 0.000 | ± | 0.000 | 0.003 | ± | 0.001 | 0.004 | ± | 0.001 |
| succinic acid | 0.015 | ± | 0.006 | 0.009 | ± | 0.003 | 0.039 | ± | 0.004 |
| sucrose | 4.055 | ± | 0.129 | 9.114 | ± | 0.953 | 14.248 | ± | 1.221 |
| threonic acid | 0.116 | ± | 0.014 | 0.198 | ± | 0.018 | 0.094 | ± | 0.009 |
| threonine | 0.215 | ± | 0.005 | 0.058 | ± | 0.002 | 0.002 | ± | 0.001 |
| valine | 0.032 | ± | 0.001 | 0.011 | ± | 0.001 | 0.002 | ± | 0.001 |
| organic acid 33.83 | 0.000 | ± | 0.000 | 0.000 | ± | 0.000 | 0.002 | ± | 0.001 |
| possible organic acid 29.25 | 0.002 | ± | 0.001 | 0.003 | ± | 0.001 | 0.006 | ± | 0.003 |
| possible organic acid 33.75 | 0.001 | ± | 0.000 | 0.002 | ± | 0.001 | 0.020 | ± | 0.002 |
| possible sugar 34.13 | 0.000 | ± | 0.002 | 0.006 | ± | 0.001 | 0.000 | ± | 0.000 |
| possible sugar 36.07 | 0.007 | ± | 0.003 | 0.016 | ± | 0.003 | 0.017 | ± | 0.004 |
| possible sugar 36.33 | 0.001 | ± | 0.001 | 0.008 | ± | 0.002 | 0.000 | ± | 0.003 |
| possible sugar 42.94 | 0.017 | ± | 0.001 | 0.006 | ± | 0.002 | 0.022 | ± | 0.004 |
| possible sugar 46.56 | 0.020 | ± | 0.001 | 0.022 | ± | 0.004 | 0.021 | ± | 0.005 |
| possible sugar 49.85 | 0.004 | ± | 0.001 | 0.002 | ± | 0.001 | 0.011 | ± | 0.004 |
| possible sugar 51.32 | 0.024 | ± | 0.003 | 0.002 | ± | 0.001 | 0.015 | ± | 0.005 |
| unknown 14.12 | 0.020 | ± | 0.008 | 0.000 | ± | 0.000 | 0.029 | ± | 0.003 |
| unknown 18.65 | 0.006 | ± | 0.001 | 0.000 | ± | 0.000 | 0.000 | ± | 0.000 |
| unknown 28.80 | 0.051 | ± | 0.004 | 0.130 | ± | 0.010 | 0.027 | ± | 0.004 |
| unknown 29.93 | 0.000 | ± | 0.000 | 0.007 | ± | 0.002 | 0.004 | ± | 0.002 |
| unknown 30.70 | 0.000 | ± | 0.000 | 0.053 | ± | 0.005 | 0.084 | ± | 0.016 |
| unknown 37.65 | 0.624 | ± | 0.037 | 0.024 | ± | 0.003 | 0.002 | ± | 0.001 |
| unknown 42.28 | 0.097 | ± | 0.006 | 0.116 | ± | 0.013 | 0.267 | ± | 0.072 |
| unknown 43.18 | 0.002 | ± | 0.000 | 0.001 | ± | 0.000 | 0.020 | ± | 0.003 |
| unknown 46.68 | 0.007 | ± | 0.000 | 0.003 | ± | 0.000 | 0.013 | ± | 0.005 |
| unknown 49.55 | 0.122 | ± | 0.030 | 0.005 | ± | 0.003 | 0.015 | ± | 0.008 |
| unknown 49.79 | 0.115 | ± | 0.009 | 0.006 | ± | 0.001 | 0.007 | ± | 0.003 |

## Appendix D. Summary of ANOVA values reported in Table II.

**Analysis of Variance for Water Potential**

| Source | *df* | Sum Squares | Mean square | *F* |
|---|---|---|---|---|
| Replicate | 2 | 0.404 | 0.202 | 4.451* |
| Ecotype | 1 | 0.4917 | 0.492 | 10.822** |
| Treatment | 3 | 31.600 | 10.533 | 231.809*** |
| EcoxTreat | 3 | 0.924 | 0.308 | 6.777*** |
| Error | 70 | 3.181 | 0.045 | |

**Analysis of Variance for Solute Potential**

| Source | *df* | Sum Squares | Mean square | *F* |
|---|---|---|---|---|
| Replicate | 2 | 0.256 | 0.128 | 2.276 |
| Ecotype | 1 | 0.963 | 0.963 | 17.109*** |
| Treatment | 3 | 16.420 | 5.473 | 97.269*** |
| EcoxTreat | 3 | 1.012 | 0.337 | 5.996** |
| Error | 70 | 3.939 | 0.056 | |

**Analysis of Variance for Turgour Pressure**

| Source | *df* | Sum Squares | Mean square | *F* |
|---|---|---|---|---|
| Replicate | 2 | 0.069 | 0.0.34 | 3.731 |
| Ecotype | 1 | 0.102 | 0.102 | 10.983** |
| Treatment | 3 | 1.819 | 0.606 | 65.450*** |
| EcoxTreat | 3 | 0.034 | 0.011 | 1.220 |
| Error | 70 | 0.648 | 0.009 | |

**Analysis of Variance for Relative Water Content**

| Source | *df* | Sum Squares | Mean square | *F* |
|---|---|---|---|---|
| Replicate | 2 | 0.007 | 0.003 | 3.037 |
| Ecotype | 1 | 0.003 | 0.003 | 0.082 |
| Treatment | 3 | 1.371 | 0.457 | 416.421*** |

$P < 0.1$; * $P < 0.05$; ** $P < 0.01$; $P < 0.001$.

## REFERENCES


**Abrams MD** (1988) Sources of variation in osmotic potentials with special reference to orth American tree species. Forest Sci **34**(4): 1030-1046.

**Ackerly D** (2003) Canopy gaps to climate change—extreme events, ecology and evolution. New Phytol **160**: 2-4.

**Ackerson RC** (1980) Stomatal responses to water stress and abscisic acid as affected by water stress history. Plant Physiol **65**: 455–459.

**Ackerson RC** (1981) Osmoregulation in cotton in response to water stress. Plant Physiol **67**: 489-493.

**Adams MA, Chen Z, Landman P, Colmer TD** (1998) Simultaneous determination by capillary gas chromatography of organic acids, sugars, and sugar alcohols in plant tissue extracts as their trimethylsilyl derivatives. Anal Biochem **266:** 77–84.

**Alscher RG, Donahue JH, Cramer CL** (1997) Reactive oxygen species and antioxidants: relationships in green cells. Physiol Plant **100**: 224–233.

**Arizona Meteorological Network** (2007) Tucson Monthly Precipitation. [http://ag.arizona.edu/azmet/01.htm]. College of Agriculture and Life Sciences. Accessed July 15, 2007.

**Barr HD, Weatherley PE** (1962) A re-examination of the relative turgidity technique for estimating water deficit in leaves. Aust J Biol Sci **15**: 413-428.

**Bennett B** (2000) The Yukon's own salt flats. Botanical Electronic News **249**: 3-5.

**Bennett JM, Sullivan CY** (1981) Effect of water stress preconditioning on net photosynthetic rate of grain Sorghum. Photosynthetica **15**(3): 330-337.

**Bent A** (2007) Vapor-Phase Sterilization of Arabidopsis Seed [http://plantpath.wisc.edu/~afb/vapster.html]. University of Wisconsin, Madison. Accessed: July 18, 2007.

**Blackman SA, Obenderf RL, Leopold AC** (1992) Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. Plant Physiol **100**: 225-230.

**Bogeat-Triboulot MB, Broshe M, Renaut J, Jouve L, Le Theic D, Fayyaz P, Vinocur B, Witters E, Laukens K, Teichmann T, Altman A, Hausman JF, Polle A, Kangasjarvi J, Dreyer E** (2007) Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performace in Populus euphratica, a poplar growing in arid regions. Plant Physiol **143**: 876-892.

**Bohnert HJ, Jensen RG** (1996) Metabolic engineering for increased salt tolerance, the next step. Aust J Plant Physiol. **23**: 661–666.

**Bowler C, Van Montagu M, Inze D** (1992) Superoxide dismutase and stress tolerance. Annu Rev Plant Physiol Plant Mol Biol **43**: 83-116.

**Bray EA, Bailey-Serres J, Weretilnyk EA** (2000) Responses to abiotic stress. In: W. Gruissem, B. Buchannan and R. Jones (Eds.), Biochemistry and Molecular Biology of Plants, American Society of Plant Physiologists, Rockville, MD, pp. 1158–1249.

**Bressan RA, Zhang C, Zhang H, Hasegawa PM, Bohnert HJ, Zhu JK** (2001) Learning from the Arabidopsis experience. The next gene search paradigm. Plant Physiol **127**: 1354–1360.

**Brown DA, Sawicki JA, Sherriff BL** (1998) Alteration of microbially precipitated iron oxides and hydroxides. Amer Min **83**: 1419-1425.

**Campbell EC, Campbell GS and WK Barlow** (1973) A dewpoint hygrometer for water potential measurement. Agric Meteorol **12**: 113–121.

**Cellier F, Conéjéro G, Breitler JC, Casse F** (1998) Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. Accumulation of dehydrin transcripts correlates with tolerance. Plant Physiol **116**: 319-328.

**Cheikh N, Brenner ML** (1992) Regulation of key enzymes of sucrose biosynthesis in soybean leaves. Plant Physiol **100**: 1230-1237.

**Chen YX, Lin Q, Luo YM, He YF, Zhen SJ, Yu YL, Tain GM, Wong M** (2003) The role of citric acid on the phytoremediation of heavy metal contaminated soil. Chemosphere **50**: 807-811.

**Clayton-Greene KA** (1983) The tissue water relationships of *Callitris columellaris*, *Eucalyptus melliodora* and *Eucalyptus microcarpa* investigated using the pressure-volume technique. Oecologia **57**: 368–373.

**Crowe JH, Carpenter JF, Crowe LM** (1998) The role of vitrification in anhydrobiosis. Annual Rev of Physiol **60**: 73-103.

**Culter JM, Rains DW** (1978) Effects of water stress and hardening on the internal water relations and osmotic constituents of cotton leaves. Plant Physiol **42**: 261-268.

**Davies WJ, Zhang J** (1991) Root signals and the regulation of soil and plant water potentials, mechanical constraints and ABA concentration in the xylem sap. Plant Cell Environ **14**: 121-124.

**Desbrosses GG, Kopka J, Udvardi MK** (2005) *Lotus japonicus* metabolic profiling. Development of gas chromatography-mass spectrometry resources for the study of plant-microbe interactions. Plant Physiol **137**: 1302-1318.

**Diffenbaugh NS, Pal JS, Trapp RJ, Giorgi F** (2005) Fine-scale processes regulate the response of extreme events to global climate change. Proc Natl Acad Sci *USA* **102**: 15774-15778.

**Eaton FM, Engle DR** (1949) Organic acids of the cotton plant. Plant Physiol **24**: 373-386.

**Elhaak MA, Sharaf El-Din A, Sammour RH** (1993) Response of *Phragmites australis* to water stress from flooding to drought. Pakistan J Bot **25**(1): 41-46.

**Evans RD, Black RA, Link SO** (1990) Rehydration-included changes in pressure-volume relationships of *Artemisia tridentate* Nutt. Ssp. *Tridentate*. Plant Cell Environ **13**: 455-461.

**Farris MA** (1984) Leaf size and shape variation associated with drought stress in Rumex acetosella L. (Polygonaceae). Amer Midland Natur **111**(2): 358-363.

**Fernandez G** (2002) Principal component analysis, a powerful un-supervised learning technique. CRC Press NY.

**Fiehn O, Kopka J, Doormann P, Altmann T, Trethewey RN, L Willmitzer** (2000) Metabolic profiling for plant functional genomics. Nature Biotechnol **18**: 1157–1161.

**Fiehn O** (2001) Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. Comp Func Genom **2**(3): 155-168.

**Flowers TJ, Troke PF, Yeo AR** (1977) The mechanism of salt tolerance in halophytes. Annu Rev Plant Physiol **28**: 89–121

**Floyd RA, Nagy ZS** (1984) Formation of long lived hydroxyl free radical adducts of proline and hydroxy-proline in a Fenton reaction. Biochim Biophys Acta **790**: 94-97.

**Ford CW, Wilson JR** (1981) Changes in levels of solutes during osmotic adjustment to water stress in leaves of four tropical pasture species. Aust J Plant Physiol **8**: 77-91.

**Frensch J, Hsiao TC** (1994) Transient responses of cell turgor and growth of maize roots as affected by changes in water potential. Plant Physiol **104**: 247-254.

**Geigenberger P, Reimholz R, Geiger M, Merlo L, Canale V, Stitt M** (1997) Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit. Planta **201**: 502-518.

**Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF** (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol **124**: 1854-1865.

**Good AG, Zaplachinski ST** (1994) The effects of drought stress on free amino acid accumulation and protein synthesis in *Brassica napus*. Physiol Plant **90**: 9-14.

**Government of Canada Weather Office** (2007) Canadian Climate Normals or Averages 1971-2000 [http://www.climate.weatheroffice.ec.gc.ca/climate_normals/index_e.html]. Environment Canada. Accessed July 14, 2007.

**Greenway H, Munns R** (1980) Mechanisms of salt tolerance in nonhalophytes. Annu Rev Plant Physiol **31**: 149-190.

**Griffith M, Timonin M, Wong ACE, Gray GR, Akhter SR, Saldanha M, Rogers MA, Weretilnyk EA, Moffatt B** (2007) *Thellungiella*: an Arabidopsis-related model plant adapted to cold temperatures. Plant Cell Environ **30**: 529-538.

**Guralnick LJ, Ting IP** (1987) Physiological changes in *Portulacaria afra* (L.) Jacq. during a summer drought and rewatering. Plant Physiol **85**: 481-486.

**Gzik A** (1996) Accumulation of proline and pattern of ∞-amino acids in sugar beet plants in response to osmotic, water and salt stress. Environ Exp Bot **36**(1): 29-38.

**Handa S, Bressan RA, Handa AK, Carpita NC, Hasegawa PM** (1983) Solutes contributing to osmotic adjustment in cultured plant cells adapted to water stress. Plant Physiol **73**: 834-843.

**Hanson A, Hitz W** (1982) Metabolic responses of mesophytes to plant water deficits. Annu Rev Plant Physiol **33**: 163-203.

**Hare PD, Cress WA, Van Steden J** (1998) Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ **21**: 535-553.

**Hartung W, Schiller P, Karl-Josef D** (1998) Physiology of poikilohydric plants. Prog Bot **59**: 299-327.

**Henry A, Doucette W, Norton J, Bugbee B** (2007) Changes in crested wheatgrass root exudation caused by flood, drought, and nutrient stress. J Environ Qual **36**: 904-912.

**Henzell RG, McCree KJ, Van Bavel CHM, Schertz KF** (1976) Sorghum genotype variation in stomatal sensitivity to leaf water deficit. Crop Sci **16**: 660-662.

**Hewlett JD, Kramer PJ** (1962) The measurement of water deficits in broadleaf plants. Protoplasm **57**: 381–391.

**Hoekstra PA, Golovina EA, Buitink J** (2001) Mechanisms of plant dessication tolerance. Trends Plant Sci **6**: 431-438.

**Hogg EH, Wein RW** (2005) Impacts of drought on forest growth and regeneration following fire in southwestern Yukon, Canada. Can J For Res **35**: 2005.

**Hsiao TC** (1973) Plant responses to water stress. Annu Rev Plant Physiol **24**: 519-570.

**Hsiao TC, Acevmo E, Fitsasa E, Henderson DW** (1976) Water stress, growth and osmotic adjustment. Philos Trans R Soc Lond B Biol Sci **273**: 479-500.

**Hsiao TC, Bradford KJ** (1980) Physiological consequences of cellular water deficits. In HM Taylor, WR Jordan, TR Sinclair, eds. Limitations to efficient water use in crop production. ASA-CSSA-SSSA, Madison pp 227-265.

**Ihaka R, Gentleman R** (1996) R: A language for data analysis and graphics. J Comp Graphical Stat **5**(3): 299-314.

**Inan G, Zhang Q, Li PH, Wang ZL, Cao ZY, Zhang H, Zhang CQ, Quist TM, Goodwin SM, Zhu JH, Shi HH, Damsz B, Charbaji T, Gong QQ, Ma SS, Fredricksen M, Galbraith DW, Jenks MA, Rhodes D, Hasegawa PM, Bohnert HJ, Joly RJ, Bressan RA, Zhu JK** (2004) Salt cress. A halophyte and cryophyte Arabidopsis relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. Plant Physiol **135**(3): 1718–1737.

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

**Jones H** (1992) Plants and microclimate: a quantitative approach to environmental plant physiology (2nd edn). Cambridge University Press.

**Jones MM, Turner NC** (1978) Osmotic adjustment in leaves of sorghum in response to water deficits. Plant Physiol **61**: 122-126.

**Kameli A, Losel DM** (1993) Carbohydrates and water status in wheat plants under water stress. New Phytol **125**(3): 609-614.

**Keller F, Ludlow MM** (1993) Carbohydrate metabolism in drought-stress leaves of pigeon pea. J of Exp Bot **44**(265): 1351-1359.

**Kemp DR, Culvenor RA** (1994) Improving the grazing and drought tolerance of temperate perennial grasses. New Zealand J Agr Res **37**: 365-378.

**Koornneef M, Alonso-Blanco C, Peeters AJM, Soppe W** (1998) Genetic control of flowering time in Arabidopsis. Annu Rev Plant Physiol Plant Mol Biol **49**: 345–370.

**Koster KL and Lynch DV** (1992) Solute accumulation and compartmentation during the cold acclimation of puma rye. Plant Physiol **98**: 108-113.

**Kramer PJ** (1983) Water Relations of Plants. Academic Press, New York. pp. 489.

**Külheim C, Agren J, Jansson S** (2002) Rapid regulation of light harvesting and plant fitness in the field. Science **297**: 91-93.

**Lajtha K, Barnes FJ** (1991) Carbon gain and water use in pinyon pine-juniper woodlands of northern New Mexico: field versus phytotron chamber measurements. Tree Physiol **9**: 59-67.

**Lauenroth WK, Sala OE, Milchunas DG, Lathrop RW** (1987) Root dynamics of *Bouteloua gracilis* during short-term recovery from drought. Func Ecol **1**: 117-124.

**LeRudulier D, Strom AM, Dandekar AM, Smith LT, Valentine RC** (1984) Molecular biology of osmoregulation. Science **224**: 1064-1068.

**Ludlow MM** (1989) Strategies of response to water stress In: Structural and Functional Responses to Environmental Stresses (eds Kreeb KH Richter H Hinckley TM), pp. 269–281. SPB Academic, The Hague.

**Luo LJ and QF Zhang** (2001) The status and strategy on drought resistance of rice (*Oryza sativa* L.). Chinese J Rice Sci **15**: 209–214.

**McDonald PG, Fonseca CR, Overton JM, Westoby M** (2003) Leaf-size divergence along rainfall and soil-nutrient gradients: is the method of size reduction common among clades? Func Ecol **17**(1): 50-57.

**McNeil SD, Nuccio ML, Hanson AD** (1999) Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. Plant Physiol **120**(4): 945-50.

**Morgan, JM** (1983) Osmoregulation as a selection criterion for drought tolerance in wheat. Aust J of Agric Res **34**(6): 607-614.

**Morgan JM, Hare RA, Fletcher RF** (1986) Genetic variation in osmoregulation in bread and durum wheats and its relationship to grain yield in a range of field environments. Aust J Agric Res **37**: 449–457.

**Morgan JM, Rodríguez Maribona B, Knights EJ** (1991) Adaptation to water-deficit in chickpea breeding lines by osmoregulation: Relationship to grain-yield in the field. Field Crops Res **27**: 61–70.

**Moustafa MA, Boersma L, Kronstad WE** (1996) Response of four spring wheat cultivars to drought stress. Crop Sci **36**: 982–986.

**Mulkey SS, Wright JS, Smith AP** (1991) Drought acclimation of an understory shrub (*Psychotria limonensis*; Rubiaceae) in a seasonally dry tropical forest in Panama. Amer J Bot **78**(4): 579-587.

**Munne-Bosch S, Jubany-Mari T, Alegre L** (2001) Drought-induced senescence is characterized by a loss of antioxidant defences in chloroplast. Plant Cell Environ **24**: 1319-1327.

**Munns RM, Brady CJ, Barlow EWR** (1979) Solute accumulation in the apex and leaves of wheat during water stress. Aust J Plant Physiol **6**: 379-389.

**Myers BA, Neales TF** (1986) Osmotic adjustment, induced by drought, in seedlings of three Eucalyptus species. Aust J Plant Physiol **13**: 597–603.

**National Climatic Data Center** (2007) NOAA Satellite and Information Service. [ftp://ftp.ncdc.noaa.gov/pub/data/normals]. U.S. Department of Commerce. Accessed: July 18, 2007.

**Nazam Uddin M, Marshall DR** (1988) Variation in epicuticular wax content in wheat. Euphytica **38**: 3-9.

**Nguyen A, Lamant A B** (1998) Pinitol and myo-inositol accumulation in water-stressed seedlings of maritime pine. Phytochemistry **27**(11): 3423-3427.

**Nuin P, Weretilnyk E, Summers P, Guevara D, Golding G** (2004) GASP: GC/MS Analysis Software Package. [http://www.flintbox.com/technology.asp?sID={D99C74B6-235B-4CEE-A21A-4C93A2669945}&page=685]. McMaster University. Accessed: July 24, 2007.

**Oosterhuis DN, Wullschleger SD** (1987) Osmotic adjustment in cotton (*Gossypium hirsutum* L.) leaves and roots in response to water stress. Plant Physiol **84**: 1154-1157.

**Orthen B, Popp M, Smirnoff N** (1994) Hydroxyl radical scavenging properties of cyclitols. Proc R Soc Edin Sect B (Biol) **102**: 269–272.

**Palmer TN, and J Rälsänen** (2002) Quantifying the risk of extreme seasonal precipitation events in a changing climate. Nature **415**: 512-514.

**Passioura JB** (1996) Drought and drought tolerance. Plant Growth Reg **20**: 79-83.

**Pattanagul W, Madore MA** (1999) Water deficit effects on raffinose family oligosaccharide metabolism in Coleus. Plant Physiol **121**: 987-993.

**Pelah D, Wang W, Altman A, Shoseyov O, Bartels D** (1997) Differential accumulation of water stress-related proteins, sucrose synthase and soluble sugars in Populus species that differ in their water stress response. Physiol Plant **99**: 153-159.

**Pharr DM, Stoop JMH, Williamson JD, Studer Feusi ME, Massel MO, Conkling MA** (1995) The dual role of mannitol as osmoprotectant and photoassimilate in celery. Hortscience **30**: 1182–1188.

**Pich A, Scholz G, Stephan UW** (1994) Iron-dependent changes of heavy metals, nicotianamine, and citrate in different plant organs and in the xylem exudate of two tomato genotypes. Nicotianamine as possible copper translocator. Plant Soil **165**(2): 189-196.

**Popp M, Smirnoff N** (1995) Polyol accumulation and metabolism during water deficit In, Smirnoff N, eds, Environment and Plant Metabolism. BIOS Scientific Publishers Ltd. Oxford, pp 199-215.

**Premachandra GS, Saneoka H, Kanaya M, Ogata S** (1991) Cell membrane stability and leaf surface wax content as affected by increasing water deficits in maize. J Exp Bot **42**(235): 167-171.

**Raamsdonk ML, Teusink B** (2001) A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. Nat Biotechnol **19**: 45–50.

**Radin JW, Zhenmin L, Percy RG, Zeiger E** (1994) Genetic variability for stomatal conductance in Pima cotton and its relation to improvements of heat adaptation. Proc Natl Acad Sci USA **91**: 7217-7221.

**Richards RA** (1996) Defining selection criteria to improve yield under drought. Plant Growth Reg **20**: 157-166.

**Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R** (2004) When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. Plant Physiol **134**: 1683–1696.

**Rodríguez Maribona B, Tenorio JL, Conde JR, Ayerbe L** (1992) Correlation between yield and osmotic adjustment of peas (Pisum sativum L.) under drought stress. Field Crops Res **29**: 15–22.

**Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR** (2001) Metabolic profiling allows a comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell **13**: 11–29.

**Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer** L (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography/mass spectrometry. Plant J **23**: 131-142.

**Santamaria JM, Ludlow MM, Fukai S** (1990) Contribution of osmotic adjustment to grain yield in Sorghum bicolor(L.) Moench under water-limited conditions. I. Water stress before anthesis. Aust J Agric Res **41**: 51–65.

**Schonfield MA, Johnson RC, Carver BF, Mornhinweg DW** (1988) Water relations in winter wheat as drought resistance indicators. Crop Sci **28**: 526–531.

**Schwab KB, Gaff DF** (1986) Sugar and ion content in leaf tissues of several drought tolerant plants under water stress. Plant Physiol **125**: 257-265.

**Scoggan HJ** (1978) The Flora of Canada. Ottawa : National Museum of Natural Sciences, National Museums of Canada : Available from National Museums of Canada Marketing Services, 1978-1979.

**Scott P** (2000) Resurrection plants and the secrets of eternal leaf. Ann Bot **85**: 159-166.

**Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki** K (2001) Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell **13**: 61-72.

**Shi DC, Yin SJ, Yang GH, Zhao KF** (2002) Citric acid accumulation in an alkali-tolerant plant *Puccinellia tenuiflora* under alkaline stress. Acta Bot Sin **44**: 537–540.

**Shi H, Lee BH, Wu SJ, Zhu JK** (2003) Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana. Nat Biotech **21**: 81–85.

**Shimshi D, Mayoral ML, Atsman D** (1982) Responses to water stress in wheat and related wild species. Crop Sci **22**: 123–128.

**Smirnoff N, Cumbes QI** (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry **28**: 1057–1060.

**Smith WK, Nobel PS** (1977) Influences of seasonal changes in leaf morphology on water-use efficiency for three desert broadleaf shrubs. Ecology **58:** 1033–1043.

**Sobrado MA** (1986) Tissue water relations and leaf growth of tropical corn cultivars under water deficit. Plant Cell Environ **9**: 451–457.

**Song Y, Simelton E, Chen D, Dong W** (1989) Influence of climate change on winter wheat growth in north China during 1950-2000. Acta Meteorologica Sinica. **19**: 501-510.

**Steel RGD, Torrie JH** (1980) Principles and procedures of statistics. 2nd ed. McGraw-Hill, Toronto.

**Sumner LW, Mendes P, Dixon RA** (2003) Plant metabolomics: large scale phytochemistry in the functional genomics era. Phytochemistry **62**: 817–836.

**Taji T, Oshumi C, Luchi S, Seki M, Kasuga M, kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K** (2002). Important roles of drought- and cold inducible genes for galactinol synthase in stress tolerance in Arabidopsis thaliana. Plant J. **29**: 417-426.

**Teulat B, Zoumarou-Wallis N, Rotter B, Ben Salem M, Bahri H, This D** (2003) QTL for relative water content in field-grown barley and their stability across Mediterranean environments. Theor Appl Gen **108**: 181–188.

**Taiz L, Zeiger E** (1991) Plant Physiology: Water and Plant cells. The Benjamin/Cummings Publishing Co. Inc., Menlo Park, California, pp 61-81.

**Timpa JD, Burke JJ, Quisenberry JE, Wendt CW** (1986) Effects of water stress on the organic acid and carbohydrate composition of cotton plants. Plant Physiol **82**: 724-728.

**Tognetti R, Johnson JD, Michelozzi** M (1995) The response of European beech (Fagus sylvatica L.) seedlings from two Italian populations to drought and recovery. Trees **9**: 348-354.

**Trethewey N** (2001) Gene discovery via metabolic profiling. Curr Opin Biotech **12**: 135-138.

**Tripathy JN, Zhang J, Robin S, Nguyen TT, Nguyen HT** (2000) QTLs for cell-membrane stability mapped in rice (Oryza sativa L.) under drought stress. Theor Appl Genet **100**: 1197-1202.

**Tuinstra MR, Grote EM, Goldsbrough PB, Ejeta G** (1997) Genetic analysis of post-flowering drought tolerance and components of grain development in *Sorghum bicolor* (L.) Moench Molec Breeding **3**: 439-448.

**Turner NC, Jones MM** (1980) Turgor maintenance by osmotic adjustment: A review and evaluation. pp. 87–103. In N.C. Turner and P.J. Kramer (eds). Adaptation of plants to water and high temperature stress. John Wiley & Sons, New York.

**Tyree MT, Engelbrecht DMJ, Vargas G, Kursay TA** (2003) Desiccation tolerance of five tropical seedlings in Panama. Relationship to a field assessment of drought performance. Plant Physiol **132**: 1439-1447.

**Venn J** (1880) On the diagrammatic and mechanical representation of propositions and reasonings, The London, Edinburgh, and Dublin Philosophical Magazine and J of Sci **9**: 1-18.

**Vicré M, Farrant JM, Driouich A** (2004) Insights into the cellular mechanisms of desiccation tolerance among angiosperm resurrection plant species. Plant Cell Environ **27**(11): 1329-1340.

**Volaire F, Thomas H, Lelievre, F** (1998) Survival and recovery of perennial forage grasses under prolonged Mediterranean drought. New Phytol **140**: 439-449.

**Warwick SI, Francis A, Mulligan GA** (2004) Brassicaceae of Canada (contribution no. 981317.1225). Agriculture and Agri-Food Canada, Eastern Cereal and Oilseed Research Centre, Ottawa, ON, Canada.

**Weckwerth W, Fiehn O** (2002) Can we discover novel pathways using metabolomic Analysis? Current Opin Biotech **13**: 156-160.

**White DA, Turner NC, Galbraith JH** (2000) Leaf water relations and stomatal behavior of four allopatric Eucalyptus species planted in Mediterranean southwestern Australia. Tree Physiol **20**: 1157–1165.

**White RH, Engelke MC, Morton SJ, Ruemmele BA** (1992) Competitive turgor maintenance in tall fescue. Crop Sci **32**: 251-256.

**Wong CE, Li Y, Labbe A, Guevara D, Nuin P, Whitty B, Diaz C, Golding GB, Gray GR, Weretilnyk EA, Griffith M, Moffatt BA** (2006) Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in *Thellungiella*, a close relative of Arabidopsis. Plant Physiol **140**: 1437-1450.

**Wong CE, Li Y, Whitty BR, Diaz-Camino C, Akhter SR, Brandle JE, Golding GB, Weretilnyk EA, Moffatt BA, Griffith M** (2005) Expressed sequence tags from the Yukon ecotype of *Thellungiella* reveal that gene expression in response to cold, drought and salinity show little overlap. Plant Mol Biol **58**: 561-574.

**Wyn Jones RG, Storey R** (1978) Salt stress and comparative physiology in the Gramineae. Glycine betaine and proline accumulation in two salt and water stressed barley cultivars. Aust J Plant Physiol **5**: 817–829.

**Xiong L, Zhu JK** (2002) Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ **25**: 131-139.

**Yancey P, Clark M, Hand S, Bowlus R, Somero G** (1982) Living with water stress: evolution of osmolyte systems. Science **217**: 1214-1222.

**Yelenosky G, Guy CL** (1989) Freezing tolerance of citrus, spinach, and petunia leaf tissue. Plant Physiol **89**: 444-451.

**Zalewski K, Lahuta LB, Horbowicz M** (2001) The effect of soil drought on the composition of carbohydrates in yellow lupin seeds and triticale kernels. Acta Physiol Plant **23**: 73-78.

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