CHARACTERIZATION OF DROUGHT RESPONSE STRATEGIES IN *EUTREMA SALSUGINEUM* USING COMPARATIVE PHYSIOLOGY AND TRANSCRIPTOME SEQUENCING

By

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ABSTRACT

The drought response of the extremophile *Eutrema salsugineum* (*Thellungiella salsuginea*) was studied using an experimental protocol involving two progressive drought exposures separated by a recovery period. Accessions from the Yukon Territory, Canada, and Shandong Province, China, were distinguished with respect to their responses to the initial drought, their recovery from wilting, and their response to a subsequent drought following recovery. *Eutrema* cauline leaves and rosettes were sampled at different stages of the drought treatment for water status and biomass measurements and this information guided tissue selection for transcriptome sequencing by RNA-Seq.

For Yukon plants, the initial drought led to a 46% reduction in stomatal conductance (from 122.3 to 66.3 mol $m^{-2}s^{-1}$) and 25% reduction in rosette water loss relative to unstressed control plants, evidence of drought avoidance to conserve water. Yukon leaf solute potentials decreased to -1.83 MPa compared to -1.54 MPa for Shandong leaves indicating that more solutes accumulated in Yukon leaves in response to drought. Upon wilting, Yukon plants re-established turgor at significantly lower leaf solute potentials than the level for well-watered Yukon plants consistent with osmotic adjustment. In contrast, leaf solute potentials in re-watered Shandong plants returned to pre-drought levels (-1.6 MPa). During the second drought exposure, leaf water content and specific leaf area measurements were significantly higher in Yukon plants compared to plants experiencing the initial drought and wilting was delayed relative to Shandong plants.

At the transcriptional level, the initial drought exposure resulted in over 2000 differentially expressed genes in leaves of Yukon plants compared to only two in

Shandong plants. Following exposure to a second drought only 45 genes were differentially expressed in leaves of Yukon plants while Shandong plants underwent substantial transcriptional re-programming with nearly 500 genes showing differential expression.

Studies of *Eutrema* grown under controlled conditions were supplemented by physiological measurements made using *Eutrema* plants found on saline soils in the Yukon. The average stomatal conductance for field plants was 84.8 mol $m^{-2}s^{-1}$, a rate similar to that of drought-treated Yukon plants in the cabinet. Leaf solute potentials of field plants ranged from -2.0 MPa to -3.5 MPa. RT-qPCR showed the relative expression of four dehydrin-encoding genes, *EsRAB18*, *EsRD22*, *EsRD29A*, and *EsERD1*, was high in the field plants and levels of expression were comparable to drought-stressed cabinet plants.

In summary, *Eutrema salsugineum* has a naturally high tolerance to water deficits. Between the two accessions studied, Yukon plants have a superior capacity to withstand drought relative to Shandong plants. The heightened capacity for Yukon plants to recover from drought and tolerate repeated drought exposures makes this accession a particularly valuable model for studying many mechanisms underlying innate and inducible plant tolerance to drought.

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ABBREVIATIONS

µL	Microlitre(s)
µM	Micromolar
µmol	Micromole
Ψ_w	Water potential
Ψ_s	Solute potential
Ψ_p	Pressure potential
A	Net assimilation
ABA	Abscisic acid
ABRE	ABA-binding responsive element
ANOVA	Analysis of Variance
bZIP	Basic leucine zipper transcription factor
bp	Base pairs
C	Celsius
Ci	Sub-stomatal carbon dioxide
CO_2	Carbon dioxide
cm	Centimetre
CRT	C-repeat element
CRWL	Cut rosette water loss
CW	Cut weight
d	Day(s)

D1	Drought period one
D2	Drought period two
DEGs	Differentially expressed genes
DPG	Days post germination
DRE	Dehydration-responsive element
DW	Dry Weight
E	Transpiration
FTSW	Fraction of transpirable soil water
FU	Frequency units
FW	Fresh Weight
g	Gram(s)
g_1	Stomatal conductance
Gb	Gigabytes
GO	Gene ontology
GC-MS	Gas chromatography and mass spectrometry
h	Hour(s)
HCA	Hierarchical cluster analysis
I	Irradiance
JA	Jasmonic acid
IRGA	Infrared gas analyzer
L	Litre(s)

LEA	Late-embryogenesis abundant protein
LWC	Leaf water content
m	Metre
mg	Milligram
min	Minute
mm	Millimetre
mL	Millilitre(s)
mM	Millimolar
mmol	Millimole
mol	Mole
MPW	Measured pot weight
MST	Mass spectral tag
ng	Nanogram
OD	Optical density
PAR	Photosynthetically active radiation
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PLC	Parkinson leaf cuvette
ppm	Parts per million

RT-qPCR	Real-time reverse transcription polymerase chain reaction
RPKM	Reads per kilobase per million mapped reads
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	Relative water content
RRF	Relative response factor
s	Second(s)
SLA	Specific leaf area
sHSP	Small heat shock protein
TW	Turgid weight
SPW	Mean pot weight at soil water holding capacity
W	Watt
WGCNA	Weighted gene co-expression network analysis
WPW	Mean pot weight at wilting
WT	Wild-type
WW	Well-watered

APPENDICES

CHAPTER ONE

Literature Review

Introduction

In plants, water deficit occurs when the demand for water exceeds the available supply to the plant. Water deficit is a major constraint on productivity for all plants including crop species where yield losses from water deficit likely exceed all other forms of crop loss (Kramer, 1980; Boyer, 1982). Water deficits can result from freezing temperatures, saline soils or low soil water availability due to meteorological drought. Drought alone results in more than 50% of annual crop loss globally (Boyer, 1982).

Drought response strategies: escape, avoidance, and tolerance

All plants respond to drought by reducing growth and respiration rates, diverting more sugars to storage, and reducing water loss from transpiration by closing their stomates (Iljin, 1957). However, different species of plants tend to institute these changes and other adaptive responses to varying degrees. Different strategies for coping with water deficit can be divided into three categories: drought escape, drought avoidance, and drought tolerance (Levitt, 1980). Plants can use strategies associated with all three categories when responding to drought and to different degrees depending on factors such as the duration and intensity of the water deficit (Maes et al. 2009).

Drought escape

Drought escape strategies are observed in plants that are exposed to very severe periods of water deprivation that occur in a recurring and predictable pattern (Levitt, 1980). The

strategies associated with escape involve adapted life history traits that are fixed in populations. For example, many plants that escape drought exhibit rapid phenological development meaning they complete stages of their life cycles quickly when conditions are favourable (Turner, 1979). Drought escape is a strategy often employed by desert annuals that are able to complete a very short life cycle when water becomes available (Eppel et al. 2014). As such, drought escape traits are not particularly suitable for transfer to agricultural crops in efforts to improve plant yield with low water availability. In contrast, both drought avoidance and drought tolerance strategies involve traits that exhibit plasticity in response to water deficits. As a result, efforts to improve yield under drought conditions have focused on avoidance (Laporte et al. 2002) and tolerance strategies (Babu et al. 2004).

Drought avoidance strategies

Drought avoidance strategies can be alternatively thought of as dehydration postponement (Turner, 1979). The traits that characterize avoidance strategies include mechanisms that help maintain adequate internal water levels as soil water becomes unavailable to the plant. Low soil water content due to drought or extreme draws on water due to long days or heat resulting in excessive evapotranspiration (Ludlow, 1989) can trigger drought avoidance responses.

Reducing gas exchange

Plant responses that reduce water loss from leaves are classified as drought avoidance strategies (Levitt, 1980). Among the initial responses to drought is the closure of stomata to reduce evapotranspiration (Cowan, 1977). However, even when stomata are closed, water can evaporate from the leaf surface (Kosma et al. 2009). One avoidance strategy to combat evaporative water loss from leaves is the development of a thick and/or waxy cuticle that prevents a water vapour diffusion gradient from forming across the plasma membrane of epidermal cells (Xu et al. 2014). A waxy (glaucous) cuticle that reflects a larger portion of incident light can reduce water loss due to heat (Teusink et al. 2002).

Reducing growth

Another way in which plants can conserve water is by slowing growth and associated metabolic activities (Mathews et al. 1984). In plants exposed to drought resulting in significantly lower values of stomatal conductance, ribulose-1,5 bisphosphate carboxylase/oxygenase (RuBisCO) activity is inhibited resulting in reduced long term growth limited by impaired carbon fixation (Flexas and Medrano, 2002). However, reduced growth during a water deficit is not necessarily a consequence of low carbon availability. Hummel et al. (2010) showed that the expansion rate of *Arabidopsis* rosettes subjected to mild drought decreased more than photosynthesis resulting in more available carbon in the form of starch, sugars, and organic acids. Moreover, Baerenfaller et al. (2012) found that *Arabidopsis* plants grown in soil containing 40% less water than soil for

control plants showed a 34% reduction in leaf area and a 19% reduction in leaf thickness. However, transcripts associated with enzymes involved in carbon fixation were more abundant in drought-treated plants. Baerenfaller et al. (2012) concluded that the reduction in growth they observed in the drought-treated *Arabidopsis* plants was not due to decreased amounts of fixed carbon but rather an adaptive growth reduction response to avoid acute dehydration during water deficit.

Acquiring previously unavailable water resources

A second class of drought avoidance strategies includes mechanisms that allow plants to access previously unavailable water (Kramer, 1980). This can be accomplished through increased access to existing water stored in plant tissues or in the soil. Many succulent species store large amounts of water in the stem that can be redistributed during a water deficit. For example the drought-tolerant tree species *Jatropha curcas* has a succulent stem that can act as a buffer against low soil Ψ_w by moving water from the stem to leaves and roots during periods of water deficit (Maes et al. 2009). Alternatively, an increased allocation of photosynthate to root growth has been documented in several species, particularly when drought conditions reduce leaf growth yet allow for continued photosynthetic activity (Doss et al. 1960; Malik et al. 1979; Sharp and Davies, 1979).

Drought tolerance strategies

Drought tolerance mechanisms are required when the plant can no longer take up or retain enough water to meet the needs of the plant for this resource. At this point, cells become dehydrated which can result in the accumulation of reactive oxygen species that damage membranes, DNA, and proteins (Jubany-Marí et al. 2010). Moreover, cell metabolism is adversely affected when there is not enough water to allow for the proper movement of molecules within the cell or between compartments of the cell. Dehydration tolerance strategies/adaptations can mitigate the negative impacts of water deficit (Levitt, 1980).

Osmotic adjustment

The water status of a plant can be determined by measuring water potential (Ψ_w) (Boyer and Knipling, 1965). This is a thermodynamic measure that describes the reduction in the chemical potential of water as a function of dissolved solutes (solute or osmotic potential; Ψ_s) and positive pressure exerted by water against the cell wall (pressure or turgor potential; Ψ_p). The equation relating these factors to each other in leaves is: $\Psi_w = \Psi_s + \Psi_p$ (Scholander et al. 1964). Pure water has a Ψ_w of zero but all Ψ_w values in plants are negative due to the inevitable presence of dissolved solutes. Water movement in plants is governed by gradients of Ψ_w with water flowing from areas with higher Ψ_w (less negative) to areas with lower Ψ_w (more negative). During a water deficit, the water content of the soil will drop as will the soil Ψ_w. To encourage the uptake of water, plants must reduce the root Ψ_w relative to the soil Ψ_w and this is achieved by accumulating solutes to reduce

the Ψ_s component of root Ψ_w. Some plants can maintain a favorable Ψ_w gradient between the root and the water-deprived soil that helps promote water uptake (Hsiao et al. 1976). The active synthesis and accumulation of solutes accompanying water deficit stress that lowers cell Ψ_s (and hence, Ψ_w) to promote water uptake and maintain turgor is referred to as osmotic adjustment (Jones and Turner, 1978; Morgan, 1980).

A well-studied drought tolerance mechanism used by plants involves osmotic adjustment through the accumulation of non-perturbing organic solutes (Hsiao et al. 1976). Organic solutes that can be present at high concentrations in the cell without deleterious effects on metabolism are said to be non-perturbing or "compatible". Osmotic adjustment has been a target in attempts to improve drought tolerance especially in agricultural crops where osmotic adjustment already occurs naturally such as pigeonpea (Flower and Ludlow, 1986), sorghum (Jones and Turner, 1978), wheat (Morgan, 1980), and maize (Westgate and Boyer, 1985). Osmotic adjustment involves the simultaneous accumulation of multiple solutes (Jones et al. 1980) and some have been implicated in conferring other benefits to the plant beyond their function as osmotica. For example, glycine betaine serves as a chemical chaperone to protect proteins including RuBisCO from damage due to salinity (Incharoensakdi et al. 1986) while proline can act as a direct scavenger of peroxide radicals (Signorelli et al. 2014).

Accumulation of protective proteins

In addition to compatible organic solutes, some proteins, including many members of the late embryogenesis abundant (LEA) family, accumulate during drought (Hundertmark and Hincha, 2008). Most LEAs are intrinsically disordered proteins (Mouillon et al. 2008) that have been hypothesized to sequester water and/or sugars to form a gel during embryogenesis or in cells under water stress (Hoekstra et al. 2001). The LEA family is very large comprising about one third of the *Arabidopsis* proteome and consisting of at least six different groups (Hundertmark and Hincha, 2008). The Group Two LEAs are also known as the dehydrins. Dehydrin proteins contain three conserved sequences known as the K-, S-, and Y-segments and the number and combination of these segments determines how the dehydrins are grouped (Campbell and Close, 1997). The accumulation of dehydrin-encoding transcripts during water deficit is associated with drought-tolerant but not drought-susceptible lines of sunflower (Cellier et al. 1998). The dehydrin CDeT11-24, isolated from the resurrection plant *Craterostigma plantagineum,* protects the enzymes citrate synthase and lactate dehydrogenase from damage due to desiccation *in vitro* (Petersen et al. 2012). Several recent papers have investigated TsDHN-1 and TsDHN-2, homologues of the dehydrin RAB18 in the halophyte *Eutrema salsugineum* (*Thellungiella salsuginea)*. These two dehydrins can associate with membranes via the K-segment (Rahman et al. 2010). In a follow up study, Rahman et al. (2013) showed that TsDHN-1 isolated from *Eutrema* helps to maintain the fluidity of a

lipid monolayer with a lipid composition similar to the outer mitochondrial membrane at cold temperatures.

Small heat shock proteins (sHSPs) comprise another large family of proteins that are ubiquitous and conserved (Jakob et al. 1993). In plants sHSPs accumulate in plants during heat stress but also in response to water deficits (Sun et al. 2001). These proteins have an evolutionarily conserved role as molecular chaperones, preventing protein aggregation and maintaining native conformation of cytosolic proteins (Jakob et al. 1993). Transgenic *Arabidopsis* plants that over-expressed *HSP17.6A* survived drought and salinity treatments that were lethal to wild-type control plants (Sun et al. 2001).

Changing the elastic modulus of the cell wall

The elastic modulus describes the elasticity of the cell wall (Radin, 1983). Depending on the plant, the elastic modulus can increase or decrease during drought exposure. Clifford et al. (1998) showed that cell wall rigidity increases 68% during drought exposure in the shrub *Ziziphus mauritiana*. Increasing the rigidity of cell walls results in lower Ψ_w values than a cell with the same volume with more elastic cell walls (Radin, 1983). Thus, decreasing the elasticity of the cell wall can help a plant maintain a favourable Ψ_w gradient between plant and soil to facilitate water uptake (Clifford et al. 1998). Conversely, a more elastic cell wall enables turgor to be maintained over a greater range of water contents such that a greater drop in water potential is required before turgor is

lost. For example, increased cell wall elasticity allowed for maintained turgor and growth in durum wheat under drought (Kikuta and Richter, 1986).

Osmotic adjustment, the accumulation of protective proteins, and alterations to the elastic modulus do not represent the only drought tolerance mechanisms employed by plants. Mittler et al. (2001) reported temporary plant dormancy in *Retama raetam.* This process involved repression of photosynthetic genes during drought that was quickly alleviated when water returned. Moreover, many of the reversible changes observed in resurrection plants during desiccation, including the accumulation of dehydrins and the sugar 2-octulose, fall into the drought tolerance category (Bianchi et al. 1991). One of the features shared by these tolerance mechanisms is that they either allow for the maintenance of metabolism in plants during water deficit or they enable the rapid resumption of normal metabolism after water is returned to the system (Levitt, 1980).

Molecular responses to drought

Drought sensing by plants

In order for a plant to respond to drought it must first perceive that there is reduced water availability or insufficient internal water to allow for normal cell metabolism. Plant cells respond to hydraulic signals (changes in Ψ_w) in leaves, roots, or the xylem (Christmann et al. 2007). Changes in Ψ_w result in alterations in turgor that have been shown to precede the accumulation of the phytohormone abscisic acid (ABA) (Pierce and Raschke, 1980). The link between changes in turgor and ABA accumulation is not fully understood

although it is believed that either changes in Ψ_s or mechanical changes in the plasma membrane or cell wall are involved (Christmann et al. 2007). ABA has many downstream effects including the closure of stomates when ABA accumulates in the guard cells (Schulze 1986). Upon exposure to drought, levels of ABA also increase in roots and leaves where ABA regulates the expression of a number of drought-responsive genes including those encoding LEAs and dehydrins discussed above (Hundertmark and Hincha, 2008). Using a whole genome tiling array to study the transcriptional response of *Arabidopsis* to drought as well as ABA, Huang et al. (2008) found that 66.5% of drought-responsive genes were also differentially expressed under increased ABA concentrations. Although results of this nature suggest that ABA plays a large role in determining the molecular response to water deficit, there are genes that respond to drought before ABA accumulates (Shinozaki and Yamaguchi-Shinozaki, 1996).

After a water deficit has been sensed by the plant a coordinated genetic response is initiated (Shinozaki and Yamaguchi-Shinozaki, 2000). Since these responses range from changes in metabolism related to organic solute synthesis to modification of cell walls, ideally stress tolerant plants must appropriately match the extent of their response to the degree of stress. In *Arabidopsis*, genes expressed in response to drought have been shown to depend on both the duration and the severity of the imposed water deficit (Bray, 2004). For example, Harb et al. (2010) reported that the expression of *NCED3,* a gene encoding an ABA biosynthesis gene, was induced 4-fold more by a progressive 10 d drought that resulted in wilting than under a moderate 10 d drought exposure wherein plants were

given 70% less water than controls but did not wilt. In *Brachypodium distachyon,* Verelst et al. (2013) profiled gene expression via tiling array and found that only 73 genes were differentially expressed in response to both moderate or severe drought treatments compared to 131 and 677 genes that were only affected by moderate and severe drought, respectively. Thus there is merit in profiling the genetic response to drought by examining drought-responsive gene expression under a variety of conditions over an extended period of time to more fully understand how a gene and its product may serve the plant in stress tolerance.

Drought-responsive gene expression

The genes that are expressed in response to drought have been grouped into two categories, namely ABA-dependent and ABA-independent, based on the timing of their expression relative to the accumulation of ABA (Shinozaki and Yamaguchi-Shinozaki, 2000). As the designation implies, the ABA-dependent pathway involves genes that are regulated downstream of ABA accumulation and their expression is regulated via the action of transcription factors that bind to an ABA-responsive (ABRE) element (Bray, 1994). The basic leucine zipper (bZIP) transcription factors AREB and ABF bind to ABRE elements resulting in cis-activation of ABRE-dependent gene expression in the presence of ABA (Kang et al. 2002). Yoshida et al. (2010) identified *AREB1, AREB2,* and *ABF3* as master regulators of ABA-dependent gene expression in *Arabidopsis.* These researchers produced an *areb1 areb2 abf3* triple knockout line that was drought sensitive

relative to the wild-type line and this outcome implicated multiple LEA and regulatory genes as downstream targets of these three transcription factors (Yoshida et al. 2010).

While the ABRE element is found in the promoter sequence of ABA-dependent pathway genes, the upstream regulatory region of ABA-independent genes contain the DRE (dehydration-responsive element)/CRT (C-repeat) element (Yamaguchi-Shinozaki and Shinozaki, 1994). Not only is the DRE/CRT element found upstream of genes involved in drought stress but it is also found upstream of genes responsive to low temperature and salinity (Shinozaki and Yamaguchi-Shinozaki, 2000). During water deficit the transcription factor DREB2 accumulates and binds to the DRE/CRT element controlling the expression of ABA-independent genes (Shinozaki and Yamaguchi-Shinozaki, 1996). Interestingly, the *DREB2A* promoter has both the ABRE and the DRE/CRT elements (Kim et al. 2011) which the authors believe may allow for enhanced control over gene expression during osmotic stress. Other regulatory proteins including RD29A (Yamaguchi-Shinozaki and Shinozaki, 1994) and the chromatin modifier ATX1 (Ding et al. 2011) may regulate the expression of genes in both the ABA-dependent and ABA-independent pathways. There are other regulatory elements and transcription factors that have roles in drought response including those associated with the MYB, MYC and NAC families of transcription factors (reviewed by Shinozaki and Yamaguchi-Shinozaki al. 2007). The cross-talk between the ABA-dependent and -independent pathways and the variety of drought-responsive transcription factors that have been identified underscores

the need for technologies that can determine the expression of many genes from multiple pathways simultaneously.

Omics approaches

The advent of transcript profiling (transcriptomics) has allowed researchers to take a "snap shot" of all of the transcripts present in a particular source (cell, tissue or organ) at a single time point during the response of a plant to water deficit (Rodriguez et al. 2010; Kang et al. 2011; Guevara et al. 2012; Sharma et al. 2013). When transcript profiling is combined with metabolic profiling, the operation of entire pathways in response to water deficit can be monitored (Kang et al. 2011; Sharma et al. 2013). Technical advances, particularly next-generation sequencing, have made "omics" approaches viable for many non-model species such as *C. plantagineum* (Rodriguez et al. 2010) and emerging models like *Eutrema salsugineum* (Wong et al. 2006; Champigny et al. 2013) and *Brachypodium distachyon* (Verelst et al. 2013). Furthermore, "omics" approaches can be used to study drought escape, avoidance, and tolerance strategies at the molecular level (Rodriguez et al. 2010; Zhou et al. 2014).

Approaches to studying plant response to water deficits

As sequencing and microarray technologies have become easier and more affordable, studies applying genomics approaches to study plant response to drought have become more common (Wong et al. 2006; Harb et al. 2010; Baerenfaller et al. 2012; Des Marais

et al. 2012; Rengel et al. 2012; Verelst et al. 2013). Drought response genomics can be combined with a more complete characterization of the physiological response that the plants undergo in response to water deficits (Baerenfaller et al. 2012; Des Marais et al. 2012). For example, Des Marais et al. (2012) showed that leaf nitrogen content increased in *Arabidopsis* during drought treatment and this increase was correlated with the expression of genes involved in histone modification known to be involved in epigenetic regulation of some drought-responsive genes (Ding et al. 2012). Also using *Arabidopsis*, Baerenfaller et al. (2012) showed that reduced growth due to prolonged water deficit was correlated with reduced drought-responsive gene expression leading the authors to conclude that the plants had acclimated to the drought treatment. While many drought response studies have capitalized on the use of *Arabidopsis*, this plant is not particularly drought tolerant (Lugan et al. 2010) and may not show the full breadth of plant responses to drought desirable in a drought model plant. Bouchabke et al. (2008) showed that, despite variation in leaf physiological responses to drought, 21 out of the 24 genetically distinct natural accessions of *Arabidopsis* tested used drought avoidance to cope with water deficits. These considerations mean that other species must be used for additional insights into the various strategies used by plants under drought.

Drought response in drought-tolerant species

Plants that exhibit exceptional drought tolerance make for interesting and informative systems in which to study drought response. Roche 454 pyrosequencing was used to

generate transcriptomes of the resurrection plant *C. plantagineum* during dehydration, desiccation, and after re-watering (Rodriguez et al. 2010). Gene ontology (GO) enrichment analysis showed that the expression of ABA-responsive genes was enriched in desiccated (dried) but not dehydrated (wilted) plants suggesting that ABA may only accumulate under very severe drought conditions in this species (Rodriguez et al. 2010). In the drought-tolerant grass model *B. distacyhon*, a custom tiling array was developed to profile the molecular response of this plant to moderate and severe drought (Verelst et al. 2013). Three leaf/blade developmental zones were delineated and profiled separately to determine if the zones responded differently to drought treatment. The authors found that each leaf zone responded differently involving little overlap in drought-responsive gene expression in the three zones in response to severe drought. Recently, transcriptome sequencing and metabolic profiling were performed in switchgrass in response to drought and re-watering. Meyer et al. (2014) showed that the expression of some drought-responsive genes was tightly correlated to the abundance of metabolites in drought-treated and re-watered tissues. These associated genes and metabolites appear to respond only after specific physiological thresholds are reached (Meyer et al. 2014). For example the expression of a group of 27 genes, which was significantly enriched for the GO term monosaccharide metabolism, was not altered by drought until the efficiency of photochemical quenching fell below a threshold. Below the threshold value the expression of these 27 genes was significantly lower.

The study of drought responses using naturally drought-tolerant species such as *C. plantagineum* (Rodriguez et al. 2010) and *B. distachyon* (Verelst et al. 2013) is emerging as a tool for identifying novel drought tolerance traits. Combining exploitable natural variation in plants that are adapted to dry environments may offer new insights into the molecular and physiological basis of drought tolerance (Luo et al. 2011).

Exploiting natural variation to study drought tolerance

Many ecotypes/natural accessions of *Arabidopsis* that originate from regions of contrasting climates are now available (Aguirrezabal et al. 2006). Bouchabke et al. (2008) compared the response of 24 *Arabidopsis* accessions to water deficit with plants monitored by measures of plant water status. The authors showed that several accessions of *Arabidopsis* could tolerate lower leaf water content during water deficit and still continued to grow. Juenger et al. (2010) compared two "climactically extreme accessions" of *Arabidopsis* with "putatively locally adapted phenotypes" in order to determine if these accessions had adapted drought responses commensurate to the conditions found in their original habitats. This group found that mild soil water deficits had only minimal impacts on physiology between the accessions in the form of small differences in relative water content (RWC) but they found large differences in gene expression between the accessions with 352 genes exhibiting differences between the accessions as well as multiple sequence polymorphisms (Juenger et al. 2010). In the case of *B. distachyon*, a comparison of 57 natural populations identified three populations that were more drought

tolerant than the others tested (Luo et al. 2011). Plants from the three most tolerant populations exhibited delayed wilting and maintained higher leaf water and chlorophyll content when water was withheld for one week. The natural variation in *B. distachyon* allowed for the development of six divergent recombinant inbred lines that were used to identify over 300 genes with genotype-dependent drought-responsive gene expression that can now be further characterized to determine potential involvement in drought tolerance (Gordon et al. 2014).

Eutrema salsugineum: A model for studying drought response strategies

Eutrema salsugineum (aka *Thellungiella salsuginea*) is an extremophile crucifer, belonging phylogenetically to the tribe *Eurtemeae* (Koch et al. 2013), that can tolerate saline soil, survive exposure to freezing temperatures, and recover from water losses exceeding 40% of its fresh weight (Wong et al. 2006). There are two well-studied natural accessions of *Eutrema* that have evolved under contrasting natural habitats allowing for comparative approaches. The Yukon accession originates from the semi-arid, sub-arctic Yukon Territory in Canada while the Shandong accession originates from the more temperate coastal Shandong Province in China (Guevara et al. 2012).

Much of the research on *Eutrema* has focused on its very high natural tolerance to salinity stress (Inan et al. 2004; Gong et al. 2005; Oh et al. 2010). Both salinity stress and drought reduce the availability of water to plant cells so we expect to find overlap between the mechanisms underlying *Eutrema's* response to salinity and drought. *Eutrema*

(Shandong accession) is better able to control water and ion fluxes than *Arabidopsis* and, as a result, is more efficient in its use of water (Gong et al. 2005). Shandong *Eutrema* also has a higher stomatal density than *Arabidopsis* as well as higher levels of some epicuticular waxes (Teusink et al. 2002). A reinforced root endodermis and thicker layer of leaf pallisade cells may also help prevent water loss in *Eutrema* (Inan et al. 2004; Gong et al. 2005).

Basal levels of the compatible solute proline were reported to be 3.2-fold higher in Shandong *Eutrema* than in *Arabidopsis* (Arbona et al. 2010). The researchers postulated that higher basal levels of proline found in the leaves of Shandong plants may be an example of *Eutrema* anticipating stress, mitigating the early deleterious effects of salinity (Arbona et al. 2010). Microarray analysis of Shandong plants indicated that they do not have as many salt stress-responsive transcripts as *Arabidopsis* and that a number of the genes that are salt-responsive in *Arabidopsis* are constitutively expressed in Shandong *Eutrema* (Taji et al. 2004; Gong et al. 2005). Lugan et al. (2010) found that the total solute contents of *Arabidopsis* and *Eutrema* were mostly unchanged after salt treatment indicating osmotic adjustment did not occur in the Shandong accession under the stress conditions imposed by the authors.

Eutrema responses to drought/dehydration

Arbona et al. (2010) showed that *Arabidopsis* leaves exposed to water stress by detachment accumulated more of the stress-related hormones ABA and jasmonic acid

(JA) than Shandong leaves treated similarly. Leaf detachment also had a greater effect on metabolite composition in *Arabidopsis* leaves with 115 differentially-regulated mass features responding to drought compared to only 40 in Shandong leaf samples. The authors postulated that *Eutrema* undergoes fewer stress-responsive changes than *Arabidopsis* because it is more prepared for stress due to constitutive expression of some genes and the abundance of stress-associated metabolites such as the osmoprotectant proline.

Lugan et al. (2010) exposed *Eutrema* and *Arabidopsis* to simulated dehydration by treatment with polyethylene glycol (PEG). They found that *Eutrema* leaves had lower water content prior to treatment with PEG and that *Eutrema* leaves were able to lose more water than *Arabidopsis* during the treatment while maintaining turgor. *Eutrema* leaves had a lower Ψ_s than *Arabidopsis* which the authors attributed to the passive accumulation of solutes due to the reduced water content of *Eutrema* leaves (Lugan et al. 2010). Both the Arbona et al. (2010) and Lugan et al. (2010) studies are consistent with the conclusion that *Eutrema* is better equipped to withstand water deficits than *Arabidopsis.* However, in these studies only the Shandong accession was used. Although both accessions of *Eutrema* have innate tolerance to water stress, the drought response shown by the two accessions was only compared recently (Xu et al. 2014). This study looked closely at the composition of the cuticular lipids in Yukon and Shandong plants and at the role of the cuticle in preventing water loss in *Eutrema*. Analysis of leaf wax content showed that Yukon leaves had eight-fold higher fatty acid wax content than Shandong leaves.

Although Xu et al. (2014) provided new insights into how water loss is prevented in *Eutrema*, these authors did not delve into many aspects of plant response to drought nor did they consider how *Eutrema* recovers from water deficit exposure.

Context of my research

The overall objective of my PhD research was to better understand how plants respond to drought using *Eutrema salsugineum* as the model plant. My hypothesis was that Yukon and Shandong plants would be differentially tolerant to water deficits. This premise was based upon two considerations: 1) A preliminary project carried out in the lab showed that the two accessions could be distinguished based upon response to drought and their capacity to recover (J. Dedrick, unpublished) and 2) the biogeographical background of the two accessions is different in that the Shandong plants are found in an area of higher precipitation than the Yukon plants.

The study of J. Dedrick was also very interesting in that it suggested that Yukon plants naive to stress behaved differently than Yukon plants that had wilted while Shandong plants did not show this trait. Although this was a very interesting observation, the Yukon wilting phenotype was difficult to replicate initially making it difficult to discern how or if the two accessions actually differed with respect to this trait. As a consequence, the first objective of my thesis was to devise a protocol that could more precisely monitor plants during the phases when water was withheld. I found that monitoring the water status of the potting medium enabled me to select stages at which

Yukon and Shandong plants could be matched on the basis of water availability allowing me to test whether they diverged with respect to drought behaviour using physiological measurements. The inclusion of a synchronized recovery stage followed by a second drought treatment that was also monitored on the basis of pot weight was an important innovation in that it offered a means to test the hypothesis that Yukon plants would show a different, stronger recovery following exposure to water deficits relative to Shandong plants. Given that Yukon plants are exposed to sporadic rainfall where they are found and Shandong plants are native to an area of consistently higher rainfall (Guevara et al. 2012), I expected that Yukon plants would be more drought tolerant than Shandong plants. Extending the drought-treatment assay by a second, consecutive drought exposure enabled me to definitively show that an exposure to drought reproducibly differentiated the accessions with Yukon plants showing a superior capacity to recover.

With the physiological data as a foundation, the next objective of my work was to characterize drought and drought recovery-responses at the level of gene expression for Yukon and Shandong plants. For this objective, the synchronized drought assay was used to identify time-points that were roughly equivalent for the two accessions with respect to their water availability and then 16 RNA-Seq libraries were generated. For this study, I selected *Eutrema* plants during an initial water deficit, shortly after recovery, and following a second imposed water deficit. These libraries were used to identify Yukon and Shandong-specific changes in gene expression and for comparisons between the accessions. As a resource, these libraries will provide unique insights into the molecular

basis of the drought response traits expressed in *Eutrema* at key stages of water deficit exposure and recovery from deficits.

The third objective of my thesis was to better characterize the physiological features of Yukon plants in their natural field environment. Our group and others have shown that physiological traits and gene expression are different between plants in the field and those in cabinets (Guevara et al. 2012; Champigny et al. 2013). This is not surprising given the complexity of environmental variables in the field versus the tightly controlled conditions in a cabinet. Thus my thesis includes an assessment of water status and photosynthetic measurements of field plants in order to provide a foundation for making more meaningful conclusions about how Yukon *Eutrema* acclimates to water deficits in the field. This information would ideally only be a beginning of an ongoing effort to compile many physiological "snapshots" to more fully understand how molecular traits shown by this plant translate to metabolic and physiological phenotypes in the field.

CHAPTER TWO

Materials and Methods

Materials

Chemicals and enzymes were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON) unless otherwise stated. All solutions were prepared with de-ionized water purified by a NANOpure II water purification system (SYBRON / Barnstead, Dubuque, IA). All primers were synthesized by The Institute for Molecular Biology and Biotechnology (MOBIX) (http://www.science.mcmaster.ca/mobix/) or Integrated DNA Technologies (IDT) (www.idtdna.com). Drought treatment refers to the deliberate stress imposed by withholding water from plants. Physiological measurements were made on fully-expanded leaves unless otherwise noted.

Plant growth and drought-treatment conditions

Seed source and plant growth conditions

The Yukon *Eutrema* seeds used were bulked as a pooled seed source from descendents of wild populations collected in the Yukon (location: 60° 55.928'N, 135° 10.249'W; elevation 647 m). Shandong *Eutrema* seeds were bulked from seeds originally supplied by Dr. R. Bressan, Purdue University, and was the same single-seed decent source used by Champigny et al. (2013).

Seeds were sterilized in 1 mL of a solution containing 30% (v/v) bleach, 50% (v/v) ethanol and 0.1% (v/v) Triton X-100 (T8787) for 10 min. Seeds were rinsed with sterile water until all traces of detergent and bleach were removed. The sterile seeds were suspended in 0.1% (w/v) Phytagel (P8169) and placed in the dark at 4°C for 24 h. For

planting, 205 to 210 g of a moistened and autoclaved soil mixture containing six parts Promix BX (Premier Horticulture, Rivière-du-Loup, PQ), three parts water and one part Turface (Profile Products LLC, Buffalo, NY) was added to individual 5 x 5 x 7 cm pots (The Lerio Corp, Mobile, AL). To prevent the soil from hardening during drought treatment the wetting agent Aquagro 2000 (Aquatrols, Paulsboro, NJ) was added to the soil prior to potting but after the soil mix had been sterilized and allowed to cool. For every litre of soil 10 mL of 10-fold diluted Aquagro was added. Seeds (5 to10 per pot) were pipetted directly on to the soil surface using a 200 μL Pipetman modified by cutting off the narrow tip to produce a broader opening. Pots were transferred to a 4°C cold room for either 4 d (Shandong) or 7 d (Yukon). Following cold-treatment the pots were transferred to a growth chamber (AC 60 Econair, Winnipeg, MB) with a 21 h, 22°C light: 3 h, 10°C dark cycle). The irradiance (I) was 250 μmol m^{-2} s^{-1}. Up to 32 plants were kept in a flat under a clear plastic lid for 7 to 10 d to maintain high humidity during germination. After the lids were removed plants were watered as needed and fertilized once per week with 1 g L^{-1} 20-20-20 (N-P-K) fertilizer (Plant Products Co. Ltd., Brampton, ON). Plants were thinned to one plant per pot at seven days post germination (DPG). At 27 DPG all plants were watered thoroughly and the soil allowed to drain freely until no further water drained.

Drought treatment protocol

The drought treatment was imposed at 28 DPG by withholding water from half of the plants but continuing to water the remaining plants daily. Following the onset of the treatments, well-watered plants were no longer fertilized once per week because the drought-treated plants would not be receiving fertilizer while drying down. The loss of water was monitored gravimetrically and individual pot weights were converted to fraction of transpirable soil water remaining (FTSW). According to Sinclair and Ludlow (1986), at 0% FTSW the relative transpiration rate of plants undergoing water deficits is 10% of the average daily water loss of well-watered control plants. Under our growth conditions, well-watered control plants had an average daily water loss of 20 to 25 g of water. For ease of reference, we converted FTSW to units of percent where a FTSW value of 1 is 100%. We found that FTSW was approximately 0% on the day plants visibly wilted, with leaves hanging flaccid over the edge of the pot. The mean pot weight at which wilting occurred (WPW) was determined using all the pots in an experiment and was typically 65 to 75 g. Likewise, the mean pot weight at soil water holding capacity (SPW) was determined for each experiment (usually 220 to 230 g). During a drought exposure pot weights were measured daily (MPW) (between 4 and 5 h into the day light cycle and used, along with the average WPW and SPW for that experiment, for calculating FTSW according to the formula: FTSW = 100((MPW-WPW)/(SPW-WPW) (Sinclair and Ludlow 1986).

Water was withheld from the drought-treated plants until the first signs of visible wilting which occurred 8 to 12 d after water was withheld. On the day of wilting, each plant was re-watered to 50% of the soil water holding capacity initiating the recovery period. The following day, both the drought-treated and well-watered plants were brought up to the soil water holding capacity with the fertilizer solution described above. After allowing the re-watered plants to recover for 48 h, water was withheld from drought-treated plants marking the onset of the second drought treatment. Plants were again assessed for wilting which occurred from 6 to 10 d following the onset of the second drought. The sequential treatments were designated Drought 1 (or D1) and Drought 2 (or D2).

Physiological measurements

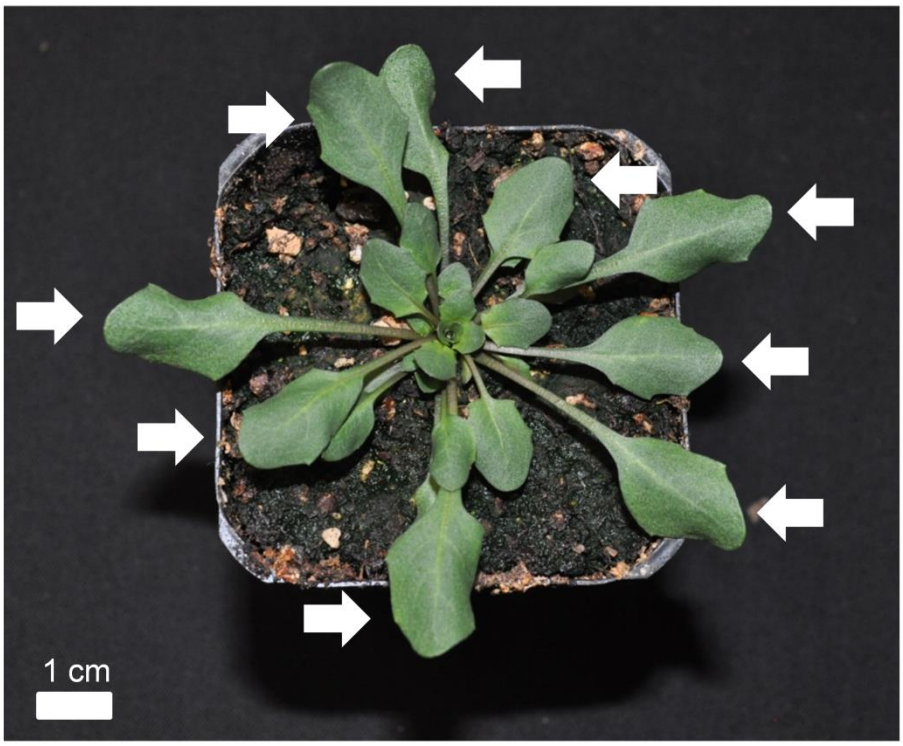
Psychrometry and relative water content measurements

Plants were harvested at eight time points during the drought assay: 1) Day 0 or the day prior to the onset of water deficit (WW1), 2) 40% FTSW during D1 (D1-40%), 3) 10% FTSW during D1 (D1-10%), 4) 0% FTSW when the plants exposed to D1 wilted (D1-0%), 5) two days after wilted plants were re-watered (WW2), 6) 40% FTSW during D2 (D2-40%), 7) 10% FTSW during D2 (D2-10%), and 8) 0% FTSW after plants had wilted with D2 exposure (D2-0%).

For harvest, four randomly selected plants, one from each treatment, were removed from the growth chamber: Yukon well-watered control (YWW), Shandong well-watered

control (SWW), Yukon drought-treated (YD), and Shandong drought-treated (SD). First, 100-200 mg of fully-expanded leaf tissue (Fig. 2.1) was harvested, flash-frozen and stored at -80°C for later nucleic acid extraction and gene expression analysis. Next, 6 mm diameter discs were excised from mature, fully-expanded leaves for leaf water (Ψ_w) and solute (Ψ_s) potential measurements using an HR33T psychrometer fitted with a C52 chamber (Wescor Inc., Logan, UT) as described by Weretilnyk et al. (1995). For Ψ_w determinations, leaf discs were sealed in the sample chambers and allowed to equilibrate for 30 min. Measurements reported for each treatment were based upon three plants using the average values from two leaves from each plant. To determine the Ψ_s for each plant, two leaves were flash-frozen in a sealed tinfoil packet along with two filter paper discs that were made using a standard three-hole punch and Whatman filter paper, Grade 1 (GE Healthcare, Burlington, ON. Cat. No. 1001125). The filter paper discs were positioned between the two leaves. Prior to measurement the leaves were thawed and the liquid contents were forced out of the leaves into the filter paper discs. For each individual plant the Ψ_s was the average of the measurements made from each of the two filter paper discs. Measurements were performed on three individual plants per treatment at each drought stage. Packets were stored at -80°C until the end of the drought experiment when all the leaf Ψ_s measurements were taken on the same day. Leaf turgor (Ψ_p) pressure was estimated as the difference between Ψ_w and Ψ_s measurements: $\Psi_w = \Psi_s + \Psi_p$ (Scholander et al. 1964).

Figure 2.1 - A well-watered cabinet-grown Yukon *Eutrema salsugineum* plant. The fully-expanded leaves are indicated with white arrows.


1 cm

After leaf tissue had been removed from the plants for Ψ_w and Ψ_s measurements, leaf tissue was taken for relative water content (RWC) determinations. An 8 mm diameter leaf disc was excised

from three different, fully-expanded leaves of each plant. These discs were pooled and weighed immediately to determine fresh weight (FW). The same discs were floated on water at room temperature under laboratory lights for 18 h in a 60 x 15 mm petri dish (Fisher Scientific, Nepean, ON, Cat. No. 875713A). Rehydrated leaf discs were blotted dry with a Kimwipe (Kimtech Science, Roswell, GA) to remove surface water and then the discs were weighed to determine turgid weight (TW). Turgid leaf discs were placed in 1.5 ml microfuge tubes and dried with the tube lids open for 72 h in a 65°C oven. The dried leaf discs were weighed to determine dry weight (DW). RWC was determined using the equation RWC = 100 (FW-DW/TW-DW) as outlined by Barrs and Weatherly (1962).

Water loss, leaf area, and leaf water content measurements

To ensure there was enough tissue for all downstream analyses, control and drought-treated plants other than those used for water status measurements (described above) but grown at the same time, were harvested for water loss and biomass measurements.

Twelve plants (three from each treatment) were removed from the chamber and immediately photographed. Approximate above-ground surface area was determined for each plant using Adobe Photoshop 8.0 (O'Neal et al. 2002). The rosette was severed from the root system with a razor blade, residual soil was removed from the bottoms of leaves

and the stem of the rosette was then placed in a small plastic weigh boat (Fisher Scientific, Cat. No. 02-202-100). An initial weight was taken for each rosette approximately 30 sec after separation from the roots; this measure represents the total above ground FW used for the leaf water content (LWC) determination. The rosettes were left on the lab bench at laboratory conditions of light and temperature then weighed again to determine cut-weight (CW) at 180 min. Following the CW measurements, rosettes were transferred to a 65°C oven and dried for 72 h. The dried rosettes were weighed to obtain the dry weight (DW) needed for the LWC equations. CRWL was determined using the formula: CRWL = 100-(100/FW)CW as described by Bouchabke et al. (2008). Leaf water content (LWC) was determined using the formula: LWC = (FW-DW)/DW (Juenger et al. 2010).

Measurement of plant water status from field tissue samples

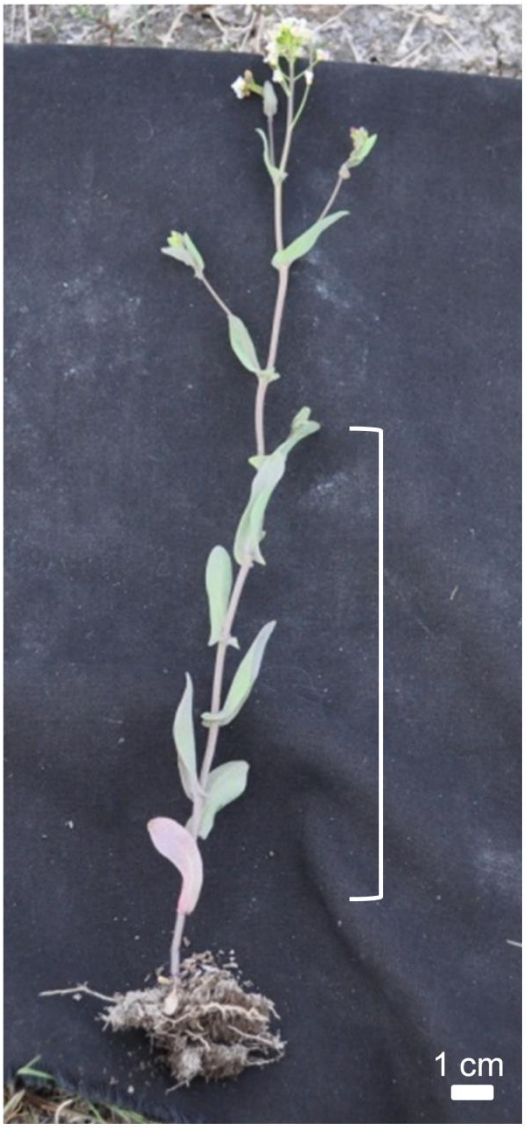
Measurements of leaf Ψ_w on plants growing at Yukon field locations were made on site in June 2011. This required the preparation of standard curves for calibration of the sample chambers at the field site under the same conditions as the Ψ_w measurements made on leaf tissue. Salt solutions with concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 M NaCl were prepared prior to going out to the field. Taking a measurement for the standard curve involved immersing a filter paper disc in a single concentration of salt solution and then placing it in the sample chamber. Psychrometry is sensitive to temperature and so all

measurements were made inside an enclosure that blocked wind and kept the chambers out of direct sunlight.

For leaf Ψ_w measurements larger cauline leaves closer to the base of the inflorescence were selected (Fig. 2.2). The selection of larger leaves allowed for multiple leaf discs to be taken from a single leaf to allow for replicate measurements. Leaf discs were allowed to equilibrate for 30 min in sealed sample chambers and Ψ_w readings from three individual leaves were averaged to determine the Ψ_w for a field plant.

Measurements of Ψ_s were made at McMaster on leaf tissue harvested from Yukon field sites in 2011. In the field, tissue was flash frozen in 2.0 mL Nalgene cryovials by submerging the tube in a slurry of isopropanol and dry ice prior transfer to a charged vapour MVE XC20/3V shipper (Jencons Scientific Inc, Bridgeville, PA) where samples were kept frozen at -150°C for transport. For the plants harvested in the field, a minimum of three fully-expanded cauline leaves were sampled for Ψ_s measurements. Plant tissue for Ψ_s determination was also harvested in the field from vegetative plants. For these much smaller plants, 5-7 individuals were pooled together in a single tube. When the vapour shipper arrived at McMaster the sample vials were transferred to -80°C for storage until Ψ_s determinations could be performed using the method with filter paper discs described above.

Figure 2.2 - A representative Yukon *Eutrema salsugineum* field plant found near Whitehorse, YT showing the larger cauline leaves used for physiological measurements (indicated by a rectangular bracket).


1 cm

Gas exchange measurements

Gas exchange measurements were performed with a CIRAS-1 infrared gas analyzer (PP Systems, Amesbury, MA) with a Parkinson broad leaf cuvette (PLC-4B) fitted with a halogen light unit (PP Systems, Bulb specifications: M35 12 V 20W G4). Unless otherwise stated, measurements of transpiration (*E*), stomatal conductance (g_1), net assimilation (*A*), and sub-stomatal CO_2 (*Ci*) were made on leaves detached from well-watered plants. Leaves were allowed to equilibrate inside the cuvette for a minimum of 2 min prior to a gas exchange measurement. A CO_2 flow rate of 200 mL min^{-1} was used for all measurements. The leaf temperature determination setting was set to "EBC" as per the manufacturer's instructions. Boundary layer resistance was kept constant at 0.30 $m^2 s^{-1}mole^{-1}$ while the transmission coefficient was set at 0.14 when the halogen light unit was used and 0.17 when plant stand lights were used (sun and sky mode). CO_2 levels were set to ambient (385 ppm) and water vapor was set to 70% relative humidity. The CIRAS-1 combined with the PLC- 4(B) cuvette measures rates of gas exchange by assuming a leaf area that fills the cuvette of ≥ 2.5 cm^2. However, most rosette leaves of cabinet-grown *Eutrema* did not fill the entire cuvette. In order to correct the measurements made on smaller leaves, the area of each leaf was determined using Photoshop (O'Neal et al. 2002). The actual leaf areas along with the recorded values for gas exchange were used to calculate values for *E*, g_1, *A*, and *Ci* using the calculation spreadsheet available on the manufacturer's website (http://ppsystems.com/ciras1-portable-photosynthesis-system).

Base line measurements were made on Yukon and Shandong plants under low and high light conditions. To take gas exchange readings under low light conditions, the light unit was switched off and the PLC-4(B) internal PAR sensor indicated that ≤ 10 μmol m^2s^{-1} reached the leaf surface. After recording gas exchange values under low light conditions, the light unit was switched on and the light level was set to maximum to make high light readings at approximately 800 μmol m^2s^{-1}.

Producing light response curves for Yukon and Shandong plants

Light response curves were made for well-watered Yukon and Shandong plants by measuring net assimilation (*A)* while varying the amount of light (*I).* The completion of all measurements for a light response curve required gas exchange readings to be taken on a single leaf for more than 30 min. In order to have leaves that were large enough to work with and extended beyond the margins of the pot, *Eutrema* plants were specifically grown to have large leaves and long petioles. To do so, only 12 plants were grown in a single flat and pots were spaced well apart so that rosette growth was uninhibited by contact between neighbouring plants.

Leaves were allowed to equilibrate at the maximum *I* (≥800 μmol m^2s^{-1}) for 5 min. Subsequently, *I* was reduced step-wise allowing for 2 min equilibration before each reading. The PAR increments tested were: 800, 600, 300, 150, 75, 50, 25, and 10 μmol m^2s^{-1}. *A* measurements were plotted as a function of *I* for the light response curve.

The light response curve data from three Yukon and Shandong plants was used for calculating the light compensation point, quantum yield and light saturation point. To calculate the light compensation point only the linear portion of the curve was used (Long and Hällgren 1993) which included all values of *A* made from 0 to 200 μmol m^2s^{-1}. A linear regression was performed using STATISTICA 7 (StatSoft) to determine the x-intercept and hence the light compensation point. The slope of the linear regression line was used to calculate quantum yield, which is defined as the amount of CO_2 that can be assimilated per photon of light absorbed by the leaf. The light saturation point was estimated from the values of *A* and *I* where the light response curve reaches a plateau. To estimate the light saturation point, rectangular parabolic curves were fit to the data collected from Yukon and Shandong plants using GraphPad Prism Version 5.02 (GraphPad software Inc.).

Gas exchange measurements on drought-treated plants

Plants subjected to drought treatments were removed from the cabinet to perform gas exchange measurements at D1-40% FTSW and D1-10% FTSW. The plants were first photographed and then moved to a stand equipped with 6 54 W cool white fluorescent lights (FP54/841/HO/ECO Sylvania Mississauga, ON) supplying 250 μmol m^2s^{-1} to match cabinet growth conditions. Measurements were made in "sun and sky" mode without using the cuvette light unit. All gas exchange measurements were made between 7 and 10 h of the light cycle.

Measuring gas exchange in the field

All IRGA field measurements were made by Amanda Garvin in June 2014. Measurements were taken under fluctuating natural light levels. Light readings were recorded at the time of measurement using the internal PAR sensor on the PLC4(B) cuvette. Detached, fully expanded cauline leaves (Fig. 2.2) were used for all measurements.

Data analysis for metabolite profiling

Polar metabolite extraction and analysis by gas chromatography and mass spectrometry (GC-MS) were performed by Mr. Jeff Dedrick using well-watered and drought-treated Yukon and Shandong *Eutrema*. Using his data, a one-way analysis of variance (ANOVA) was performed for the mass spectral tags (MST) relative response factors (RRF) values between profiles of well-watered control and drought-treated plants. MSTs with a RRF significance value $P < 0.05$ were selected and the values were normalized by a $\log_{10}$ transformation then used for principal component analysis (PCA) with the Bioconductor package in R (http://bioconductor.org/) and hierarchical clustering analysis (HCA) with GENE CLUSTER 3.0 (Eisen et al. 1998). The HCA clusters were formed based on the complete linkage method using uncentered correlation as the similarity metric and a heat map was generated using TreeView (Saldanha 2004).

Assessing relative transcript abundance using RT-qPCR

Total RNA extraction

Approximately 125 mg of leaf tissue was harvested from individual plants grown in cabinets and the tissue was flash-frozen and stored at -80°C. All RNA extractions were performed using a modified Trizol/hot borate method (Wan and Wilkins 1994) as modified by Champigny et al. (2013). A 1 μL aliquot of isolated RNA was assessed for quality and integrity using an RNA Nano 6000 chip (5067-1511, Agilent) on a Bioanalyzer 2100 instrument (Agilent, Santa Clara, CA). Electropherograms for each RNA sample were inspected for DNA contamination and degraded RNA with high quality RNA showing sharp18S and 25S rRNA peaks with a relatively flat fluorescence base line. The quality and quantity of the RNA was determined spectrophotometrically using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE). A quality score of ~2.0 was used for the OD A260/280 and A260/230 ratios and the quantity of total RNA was determined by the OD A260 (1 OD = 40 $\mu g\ ml^{-1}$).

The extraction of total RNA from Yukon plants harvested from our field sites was performed with the Norgen Total Plant RNA isolation kit (Cat. No. 25800, Norgen Biotek, Thorold, ON) following the manufacturer's protocol. For RNA extraction, 75 mg of leaf tissue, or approximately one large cauline leaf was used. Measures of RNA quality control and quantification were the same as described above.

Primer design

Primer-BLAST® (Ye et al. 2012; http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to identify reference and gene-specific primers based on *Eutrema* genomic sequences downloaded from Phytozome (http://phytozome.jgi.doe.gov). The primer specificity check was enabled in the Primer-BLAST settings using the *Eutrema salsugineum* (taxid ID: 72664) Refseq mRNA database. Melting structures of putative PCR amplicons were elucidated using the mfold® DNA folding form (http://mfold.rna.albany.edu/). Amplicon sequences with unwanted secondary structures (loops, hairpins) within 50 bp of primer binding sites at 60°C were excluded. Sequences of all primers used in this study are detailed in Table 2.1.

cDNA synthesis

First-strand cDNA synthesis was carried out using 2 µg of total RNA as template for a 40 µL reaction volume. Final concentrations of 1 µM oligo dT_{20} primer (MOBIX), 0.25 mM dNTP mix (DNTP100-1KT) as well as 80 units of ribonuclease inhibitor (R1158) and 400 units of M-MLV reverse transcriptase (M1302) were used for the first-strand synthesis reaction using the conditions recommended by the manufacturer of the reverse transcriptase (Sigma). A 5 µL aliquot of each cDNA sample from a biological replicate was pooled together, diluted 5-fold in sterile water, and used for primer validation and the preparation of standard curves with the remainder stored at -20°C for later use.

Table 2.1 - Primer sequences used for amplification of qPCR targets

Gene name	Eutrema ID	Forward Primer	Reverse Primer	Product size
EsRAB18	Thhalv10004906m	TGGACGAGTACGGTAACCCA	CGTTCCGTATCCTTCGCCAC	116
EsRD22	Thhalv10004736m	CTACTGCCACAAGGCGATGA	TAGGAACAGTGCCAGGCTTC	164
EsRD29A	Thhalv10015427m	TGGAGGAGGAGGAATGGTGG	CACAGCTCCGGTACCACTTC	199
EsERD1	Thhalv10012591m	CGTCATTTTCCGACAGCTCG	GCAAGAAAGGCTTCGCTGAG	225
EsEF1ά	Thhalv10013526m	TTGACGCACCTGGACATCGTGA	ACAGCACAATCGGCCTGGA	70
EsUBQ	Thhalv10006290m	CTTCGAGCTGCTTTCCGGCGAA	GGAGGTCGAGAGTTCTGACACCA	110
EsYL8S	Thhalv10014963m	TGACCAACCCACGACCCTTCCT	ACGAGCTGTACGATCCCTGAACGG	173

RT-qPCR

All RT-qPCR was performed using 10 µL reaction volumes composed of 4 µL diluted cDNA, 200 nM of each primer (forward and reverse) and 1X LuminoCt® SYBR® Green qPCR ReadyMix™ (L6544, Sigma). Three technical replicates were prepared for each sample and reactions were prepared for RT-qPCR on Bio-Rad optical 96-well clear reaction plates (Cat. No. MLL9601) sealed with Bio-Rad Microseal 'B' adhesive seals (Cat. No. MSB1001). The RT-qPCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). For each primer pair, an optimum annealing temperature was established by RT-qPCR using an annealing temperature gradient. The efficiency of each set of primers was determined using an eight-point standard curve using a four-fold serial dilution of pooled cDNA. Primers were only used if they had an efficiency between 90 and 110%.

RT-qPCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) on the Bio-Rad CFX Manager 3.0 platform. Transcript levels of *EsRAB18* (Thhalv10004906m), *EsRD22* (Thhalv10004736m), *EsRD29A* (Thhalv10015427m) and *EsERD1* (Thhalv10012591m) were measured relative to reference transcripts of genes encoding *EsEF1α* (Thhalv10013526m), *EsUBQ10* (Thhalv10006290m) and *EsYL8S* (Thhalv10014963m). These three reference genes were selected from a list of many reference genes that had been previously used with *Arabidopsis*. *EsEF1α, EsUBQ10, a*nd *EsYL8S* were selected because they had the most stable transcript levels across transcriptomes from *Eutrema* plants under different growth conditions.

RNA-Seq

Total RNA extraction for RNA-Seq

Approximately 500 mg of frozen leaf tissue was collected between 14:00 and 16:00, approximately 10 h into the day cycle under our cabinet conditions. Once harvested, the leaf tissue was flash-frozen in tinfoil packets using liquid N and then transferred to a freezer for long term storage at -80°C. The frozen leaf tissue was used for RNA extraction using the modified Trizol/hot borate method described by Champigny et al. (2013). RNA quantity and integrity was assessed using RNA Nano 6000 chips on a Bioanalyzer 2100 instrument as described above. The yield of total RNA required to proceed with mRNA purification was 1.5 μg μL^{-1}.

Purification of mRNA

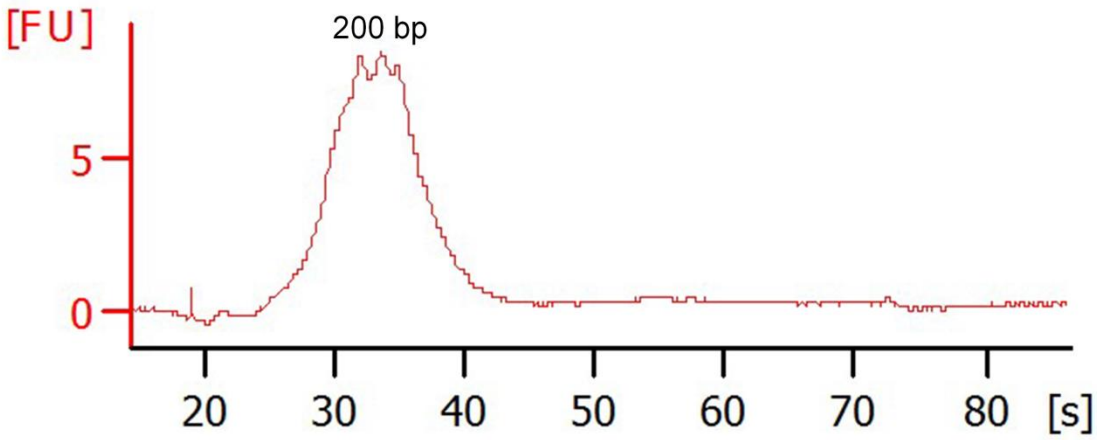
The mRNA purification was performed using the Genelute mRNA miniprep kit (Cat. No. MRN10, Sigma). At least 150 μg of total RNA extracted above was subjected to on-column purification. The manufacturer's protocol was followed until the final elution step. Purified mRNA was eluted three times in 85 μL of elution buffer. The entire purification procedure was repeated twice using 250 μL of the eluate as the input for each successive purification. The purified mRNA was analyzed using RNA Pico chips (Cat. No. 5067-1513, Agilent) and the Bioanalyzer 2100. The electropherograms of high quality mRNA have a normal distribution around a peak at 200 nucleotides with relatively little background noise and no other major peaks representing contaminating rRNA and tRNA

as shown in Fig. 2.3 (Kuschel 2000). The effluent from the sequential passages of RNA through the Genelute columns showed that the total RNA after the first purification was approximately 80 to 90% free of contaminating tRNA and rRNA, a second passage provided 95 to 98% contaminant free RNA and the third purification provided mRNA that was >99% free of contaminating rRNA and tRNA. The concentration of the purified mRNA determined by the Bioanalyzer was used to determine mRNA inputs for cDNA library synthesis.

cDNA library synthesis

Approximately 50 ng of purified mRNA was used as an input for the library construction. For typical mRNA samples (with a peak height of 5 fluorescence units), 18 μL of mRNA was used. For more concentrated mRNA samples the amount of input mRNA was decreased proportionally in that an mRNA sample with peak size of 10 fluorescence units would only require 9 μL of mRNA. Preparation of cDNA libraries was performed using the NEBNext multiplex cDNA synthesis kit for Illumina (Cat. No. E7335, New England Biolabs, Ipswich, MA) according to the manufacturer's protocol (Protocol #E6110) with the random hexamers included in the kit. Instead of performing the cleanup step to remove fragmented RNA via the recommended on-column or ethanol precipitation step, the purification was performed with Agencourt AMPure XP Beads (Cat. No. A63987, Beckman Coulter, Mississauga, ON) according to the manufacturer's protocol. For each library, one of six barcoding index sequences was added using NEBNext Multiplex

Figure 2.3 - Example of an electropherogram produced from a high quality sample of mRNA. The size of the peak at 200 bp in frequency units (FU) is proportional to the quantity of mRNA in the sample.


[FU]
200 bp
5
0
20
30
40
50
60
70
80
[s]

Oligos (Cat. No. E7335S). The barcoding index sequence that was added to each library is listed in Table 2.2.

Transcriptome sequencing

Quality control, amplification, and sequencing of the 16 cDNA libraries was conducted at the sequencing facility of the Farncombe Family Digestive Health Research Institute (McMaster University) using the Illumina Hi-Seq 2000 platform. A total of five sequencing runs were performed to sequence all of the cDNA libraries (Table 2.2). Two of the sequencing runs were performed in "High Output" mode and the other three runs used "Rapid" mode. For both sequencing modes a read length of 100 bp was used. The multiplex index IDs that were added to the cDNA libraries allowed for multiple libraries to be run in a single sequencing lane (Table 2.2). Libraries were named in such a way as to denote accession and drought stage, to simplify the nomenclature the FTSW at which the plants were harvested was not included in the ID. Using this nomenclature the libraries that were sequenced were YWW1-1/2, YD1-1/2, SWW1-1/2, SD1-1/2, YWW2-1/2, SWW2-1/2, YD2-1/2, and SD2-1/2. In general, two libraries were sequenced together on a single Illumina flow cell although several libraries were multiplexed in groups of three with related or unrelated samples (Table 2.2).

Table 2.2 - Technical details on the sequencing of 16 cDNA libraries

Tissue description	ID	Lane usage	Multiplexed with	Barcoding Index	Sequencing mode	Flow cell ID
Yukon well-watered	YWW-1	0.33	YD1-1, YWW2-1	NEBNext Index 6	High output	C15U7ACXX
	YWW-2	0.50	YD1-2, YWW2-2, SWW1-2	NEBNext Index 2	Rapid	H0E3UADXX
Yukon first drought stress	YD1-1	0.50	YWW1-1, YWW2-1	NEBNext Index 6	High output	C15U7ACXX
	YD1-2	0.50	YWW1-2, YWW2-2, SWW1-2	NEBNext Index 4	Rapid	H0E3UADXX
Yukon re-watered	YWW2-1	0.50	YWW1-1, YD1-1	NEBNext Index	High output	C15U7ACXX
	YWW2-2	0.50	YWW1-2, YD1-2, SWW1-2	NEBNext Index 6	Rapid	H0E3UADXX
Yukon second drought stress	YD2-1	0.50	YD2-2, SD2-1, SD2-2	NEBNext Index 2	Rapid	H0EFFADXX
	YD2-2	0.50	YD2-1, SD2-1, SD2-2	NEBNext Index 4	Rapid	H0EFFADXX
Shandong well-watered	SWW-1	0.33	SD1-1, SD1-2	NEBNext Index	High output	D1N72ACXX
	SWW-2	0.50	YWW1-2, YD1-2, YWW2-2	NEBNext Index 12	Rapid	H0E3UADXX
Shandong first drought stress	SD1-1	0.33	SWW1-1, SD1-2	NEBNext Index 4	High output	D1N72ACXX
	SD1-2	0.33	SWW1-1, SD1-1	NEBNext Index 6	High output	D1N72ACXX
Shandong re-watered	SWW2-1	0.50	SWW2-2	NEBNext Index 5	Rapid	H0E3VADXX
	SWW2-2	0.50	SWW2-1	NEBNext Index 7	Rapid	H0E3VADXX
Shandong second drought stress	SD2-1	0.50	YD2-1, YD2-2, SD2-2	NEBNext Index 6	Rapid	H0EFFADXX
	SD2-2	0.50	YD2-1, YD2-2, SD2-1	NEBNext Index 12	Rapid	H0EFFADXX

Determination of transcript abundance from sequencing data

Sequencing data was collected and processed for transcriptome assembly by Mr. Wilson Sung. The sequencing reads obtained were trimmed with Cutadapt v1.1 (Martin 2011) using default parameters. After trimming and quality control, reads were mapped to the JGI *Thellungiella halophila* genome (http://www.phytozome.net/thellungiella) with STAR v2.30e (Dobin et al. 2013) using default parameters with the exception of –alignIntronMax and –alignMatesGapMax, which were both set to 8000 bp. The number of reads that mapped uniquely to each gene were counted using the script htseq-count from HTSeq v0.5.3p3 (Anders et al. 2014) using the intersection-nonempty overlap resolution mode (http://www-huber.embl.de/users/anders/HTSeq) and summarizing counts at the gene level. Transcript abundance is reported as the number of reads per kilobase per million mapped reads (RPKM), a determination accounting for both mRNA length and library size (Mortazavi et al. 2008). Differentially expressed genes (DEGs) were called between pair-wise library comparisons using the DESeq Bioconductor package (http://bioconductor.org/packages/release/bioc/html/DESeq) with a false discovery threshold of 0.05 (Champigny et al. 2013).

CHAPTER THREE

Exposure of two *Eutrema salsugineum* (*Thellungiella salsuginea*) accessions to water deficits reveals different coping strategies in response to drought

Preface

This chapter describes the contrasting responses of the Yukon and Shandong accessions to a controlled water deficit assay. Under my supervision, some plant material was grown and collected by Patrick Pearce and Amanda Garvin as undergraduate summer students, their technical contribution is acknowledged in the manuscript. Claire Ashton performed some of the RNA extractions, cDNA preparation and RT-qPCR work that is shown in Fig. 7. Metabolite profiling was performed by Jeff Dedrick as part of his Master's work (Dedrick, 2007) but was reanalyzed by me with the help of Wilson Sung. The PCA biplots shown in Fig. 6A were prepared by Wilson Sung. The transcript abundance data given in Appendix S3 was taken from a data set published by Champigny et al. (2013). With the guidance of Elizabeth Weretilnyk, I developed and carried out the remainder of the experiments. I wrote the first draft of the manuscript and subsequent drafts were prepared with editing input by Drs. Marc Champigny and Elizabeth Weretilnyk. This research has been accepted for publication by Physiologia plantarum. All supplementary files (Appendix S1-S4) referred to in this chapter are available online: http://onlinelibrary.wiley.com/doi/10.1111/ppl.12316/suppinfo

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Exposure of two *Eutrema salsugineum* (*Thellungiella salsuginea*) accessions to water deficits reveals different coping strategies in response to drought

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Abstract

Eutrema salsugineum is an extremophile related to *Arabidopsis.* Accessions from Yukon, Canada and Shandong, China were evaluated for their tolerance to water deficits. Plants were exposed to two periods of water deficit separated by an interval of re-watering and recovery. All plants took the same time to wilt during the first drought exposure but Yukon plants took one day longer than Shandong plants following the second drought treatment. Following re-watering and turgor recovery, solute potentials of Shandong leaves returned to pre-drought values while those of Yukon leaves were lower than pre-drought levels consistent with having undergone osmotic adjustment. Polar metabolites profiled in re-watered plants showed that different metabolites are accumulated by Yukon and Shandong plants recovering from a water deficit with glucose more abundant in Yukon and fructose in Shandong leaves. The drought-responsive expression of dehydrin genes *RAB18, ERD1, RD29A*, and *RD22* showed greater changes in transcript abundance in Yukon relative to Shandong leaves during both water deficits and recovery with the greatest difference in expression appearing during the second drought. We propose that the initial exposure of Yukon plants to drought renders them more resilient to water loss during a subsequent water deficit leading to delayed wilting. Yukon plants also established a high leaf water content and increased specific leaf area during the second deficit. Shandong plants undergoing the same treatment regime do not show the same beneficial drought tolerance responses and likely use drought avoidance to cope with water deficits.

Abbreviations

CRWL, cut rosette water loss; FTSW, fraction of transpirable soil water; GC-MS, gas chromatography/mass spectrometry; HCA, hierarchical cluster analysis; LWC, leaf water content; MST, mass spectral tags; PCA, Principal component analysis; RWC, relative water content; SLA, specific leaf area

Introduction

Water deficits lead to significant reductions in plant productivity. Approximately 50% of annual crop yield loss due to adverse abiotic conditions is attributable to drought (Boyer 1982). Recent climate models predict that rising temperatures will result in increased drought in many areas of the globe over the next century thereby placing food supply at increased risk of falling short of meeting the nutritional needs for a growing world population (Dai 2012, Wheeler and von Braun 2013). Producing drought-tolerant crops is one strategy to combat the potential impact of climate change on global food security. However, plant responses to water deficits are complex and our understanding of beneficial responses is incomplete making it difficult to identify suitable traits amenable to developing drought-tolerant crops (Juenger et al. 2013). Comparative studies between drought-tolerant species and closely related drought susceptible plants have proven useful in identifying adaptive traits associated with tolerance to water deficits (Rampino et al. 2006). This approach has been enabled by the advent of economical high-throughput sequencing and metabolite profiling technologies. These platforms have created

opportunities to open comparisons to non-model plants including extremophiles that are particularly well adapted to tolerating extreme environmental conditions including severe short and long term water deficits (Rodriguez et al. 2010, Verelst et al. 2013).

The extremophile crucifer, *Eutrema salsugineum* (also known as *Thellungiella salsuginea*), is closely related to the genetic model *Arabidopsis* and several agronomically important *Brassica* species. *Eutrema* shoots are reported to contain less water than *Arabidopsis* shoots and yet can lose more water without losing turgor when exposed to polyethylene glycol-induced dehydration (Lugan et al. 2010). This dehydration response enables drought-tolerant species to establish water gradients that improve water uptake (Hoekstra et al. 2001). However, controlling water loss from leaves is also an important tolerance mechanism for plants experiencing low water availability. For example, the wax composition of the cuticle can be altered to reduce the rate of passive water loss through the cuticle in *Arabidopsis* exposed to water deficit (Kosma et al. 2009). Recently Xu et al. (2014) compared two natural accessions of *Eutrema* plants, one originating from Shandong, China and the other from Yukon Territory, Canada, and showed that Yukon plants lose less water from leaves than do Shandong *Eutrema* plants when exposed to water deficits. They detected significant qualitative and quantitative differences in cuticular waxes between the accessions and proposed these differences may explain the differential tolerance to low water availability between these accessions. While both the Yukon and Shandong habitats feature saline soils, the Shandong region is temperate and monsoonal while the Yukon is semi-arid and sub-Arctic. Despite the fact that both

accessions thrive on saline soil, their origins in areas of contrasting patterns and rate of precipitation likely explain their different adaptive capacities to tolerate water deficits.

The molecular response of Yukon *Eutrema* subjected to water deficits has been analyzed at the level of transcript abundance through microarrays and metabolite profiles. Using a microarray chip representing 3628 unigenes, Wong et al. (2006) detected 101 drought-responsive changes in gene expression for plants that were left un-watered under cabinet conditions. Only six of these genes were also differentially expressed in plants subjected to cold temperature or salinity (Wong et al. 2006). The lack of overlap among genes responsive to the stress treatments used is consistent with *Eutrema* having distinct molecular responses to different sources of abiotic stress. This study was extended to a comparison between plants exposed to actual drought conditions at a Yukon field location. Many, but not all genes that were found to be drought-responsive in controlled environment cabinet conditions were also drought-responsive in the field (Guevara et al. 2012). Collectively, the studies described above indicate that the Yukon and Shandong *Eutrema* accessions show a differential tolerance to water deficits and that controlled drought treatments under cabinet conditions can elicit responses shared by plants tolerating stress in the field.

The way that plants respond to drought is dependent upon the severity and duration of the stress (Bray 2004). For Yukon plants, sporadic and light rainfall interspersed with dry periods is a feature of the natural habitat. Thus in this work we undertook to develop a controlled drought treatment regime that incorporated a re-watering and recovery phase

followed by a second progressive drought treatment. This experimental regime was paired with multiple physiological and molecular measurements during drought progression to obtain greater insight into how plants respond to a progressive exposure to drought stress. Our work corroborates the findings of Xu et al. (2014) in that Yukon *Eutrema* shows greater tolerance to drought than does the Shandong accession. We establish that the *Eutrema* accessions differ with respect to solutes accumulated with exposure to water deficit and show temporal differences in drought-responsive gene expression.

Materials and methods

Plant growth conditions and drought simulation assay

Yukon *Eutrema* seeds were bulked as a pooled seed source as descendants of wild populations collected in the Yukon (location: 60°55.928'N, 135° 10.249'W; elevation 647 m; Champigny et al. 2013). Shandong *Eutrema* seeds were bulked from seeds originally supplied by Dr. R. Bressan, Purdue University (Champigny et al. 2013). Sterile seeds suspended in Phytagel (Sigma) were applied by pipette onto a moistened soil mixture of six parts Promix BX® and one part Turface® in individual 5 x 5 x 7 cm pots. The pots were transferred to 4°C for either 4 days (Yukon) or 7 days (Shandong) to synchronize germination. Pots were transferred to an AC 60 Econair controlled environment growth cabinet set to a 21 h day with a light intensity of 250 μmol $m^{-2}s^{-1}$ and 22°C/10°C day/night temperature regime (Wong et al. 2005).

Four weeks after germination, water was withheld to initiate the drought treatment. Soil water loss was monitored gravimetrically and the fraction of transpirable soil water remaining (FTSW) was determined using the method of Sinclair and Ludlow (1986). For ease of reference, we converted FTSW to units of percent where a FTSW ratio of 1 is 100%. According to Sinclair and Ludlow (1986) at 0% FTSW the relative transpiration rate of plants undergoing water deficits in pots is 10% of the average daily water loss of well-watered control plants. Under our growth conditions, FTSW was 0% on the day plants visibly wilted. Re-watering treatment began on the day a plant wilted by restoring the water content of the soil to 50% full capacity and then adding more water the following day to bring the soil to 100% of its water holding capacity. After completion of a first drought treatment (D1) followed by a 48 h recovery period from the time wilted plants were first re-watered, water was withheld from plants marking the onset of the second drought treatment (D2). A set of plants watered daily to replace transpiration loss served as controls for plants undergoing the drought simulation assay.

Characterization of Physiological Response to Water deficit

Drought-treated plants were harvested at six time points to assess leaf Ψ_w, Ψ_s, Ψ_p, and relative water content (RWC): 1 d prior to the onset of the drought treatment (WW1), at 40% FTSW (D1 and D2) and 10% FTSW (D1 and D2), and 48 h after wilted plants were re-watered between the two drought treatments (WW2). Well-watered control plants were harvested at the same time-points. On the date of harvest, leaf Ψ_w and Ψ_s measurements

were performed using a HR33T psychrometer fitted with a C52 chamber (Wescor® Inc.) using a 6 mm diameter disc excised from a mature, fully expanded leaf as previously described (Weretilnyk et al. 1995). Leaf Ψ_p was estimated as the difference between Ψ_w and Ψ_s. An 8 mm leaf disc was excised from fully expanded leaves for RWC determinations. The protocol described by Barrs and Weatherley (1962) was used to determine RWC.

Approximate total leaf area was measured using Adobe Photoshop 8.0 (O'Neal et al. 2002). For cut rosette water loss (CRWL) assays the rosette was severed from the root and the initial fresh weight (FW) was measured then rosettes were re-weighed after 180 min to determine the cut weight (CW). The severed rosettes were then dried for 72 h at 65°C (DW). CRWL values were determined using the formula: CRWL = 100-(100/FW)CW. (Bouchabke et al. 2008). Static leaf water content (LWC) of the entire rosette was determined using the formula: LWC = (FW-DW)/DW (Juenger et al. 2010). Specific leaf area (SLA) was calculated as the ratio of total above ground leaf area (m^2) over rosette DW (g).

Anthocyanin content was measured spectrophotometrically according to the method described by Kim et al. (2003). Approximately 100 mg of fresh leaf tissue (1-2 fully expanded leaves) was used and absorbance was measured at 530 and 657 nm with a Uvikon 530 spectrophotometer. The amount of anthocyanin was calculated using the formula: $(A_{530} - 0.33*A_{657})$ /g FW^{-1} .

Metabolite Profiling

Metabolite extraction and analysis by GC-MS were performed according to the method described by Guevera et al. (2012). Polar metabolites for profiling by GC-MS were obtained from 200 mg of frozen *Eutrema* leaf tissue taken from an individual plant. Automated mass spectral deconvolution and identification system (AMDIS) software was used to extract peak abundance and mass spectra information for each mass spectral tag (MST). The retention index (RI) was calculated as described by Roessner et al. (2000) and the relative abundance for each MST was determined relative to the recovery of the internal standard ribitol and expressed as a relative response factor (RRF). The chemical identity of an MST was determined by comparing its RI and spectra to authentic standards analyzed using the same instrument and experimental conditions. MSTs that shared m/z ratios with authentic standards but had different RIs were classified according to predicted chemical classes such as sugars or organic acids.

A one-way ANOVA was performed on MST RRF values between profiles of well-watered control and drought-treated plants with a $P < 0.05$ significance cut off for inclusion in PCA. A $\log_{10}$ transformation was applied to normalize the data prior to multivariate analyses. Five plants were used from each treatment. Hierarchical cluster analysis (HCA) was performed on the same data used for PCA with the software GENE CLUSTER 3.0 (Eisen et al. 1998) and visualized using TreeView (Saldanha 2004).

Gene expression analysis

Tissue was harvested from well-watered control and drought-treated plants prior to the onset of the drought treatment and at: D1-40%, D1-10%, WW2, D2-40% and D2-10% FTSW. Approximately 125 mg of fully-expanded leaf tissue was harvested from individual plants, flash-frozen and stored at -80°C. Total RNA was isolated from frozen tissue using a modified Trizol/hot borate method as described by Champigny et al. (2013). Relative RT-qPCR was performed with LuminoCt® SYBR® Green qPCR ReadyMix using cDNA generated with M-MLV reverse transcriptase and oligo dT_{20} primer using conditions specified by the manufacturer (Sigma). Reactions were run in optical 96-well reaction plates on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System and data were analyzed using Bio-Rad CFX Manager 3.0 software. Transcript levels of *EsRAB18* (Thhalv10004906m), *EsRD22* (Thhalv10004736m), *EsRD29A* (Thhalv10015427m) and *EsERD1* (Thhalv10012591m) were measured relative to reference transcripts of genes encoding *EsEF1α* (Thhalv10013526m), *EsUBQ10* (Thhalv10006290m) and *EsYL8S* (Thhalv10014963m). All oligonucleotide sequences were selected using NCBI Primer-BLAST based on *Eutrema salsugineum* sequences available at Phytozome (phytozome.net) and are listed in Appendix S4 and Table 2.1. Relative normalized expression was determined for well-watered and drought-treated plants at each stage of drought treatment. The data for relative gene expression that is reported is the net value corrected for the normalized gene expression determined for well-watered control plants. This was done to correct for any change in gene

expression with development over time. This experiment was repeated three times and each RT-qPCR determination used two individual plants selected at random and each assay included three technical replicates.

Statistical Analysis

For physiological measurements, statistical analysis was performed using STATISTICA software (Version 7.0; StatSoft Inc.). All data were subjected to factorial analysis of variance (ANOVA) to determine the effect of treatment (well-watered, drought), drought stage (D1/D2-100, 40, 10, 0% FTSW) and accession (Yukon, Shandong) on physiological response variables. For significant interactions a post-hoc test using Fisher's least significant difference was performed ($P < 0.05$).

Results

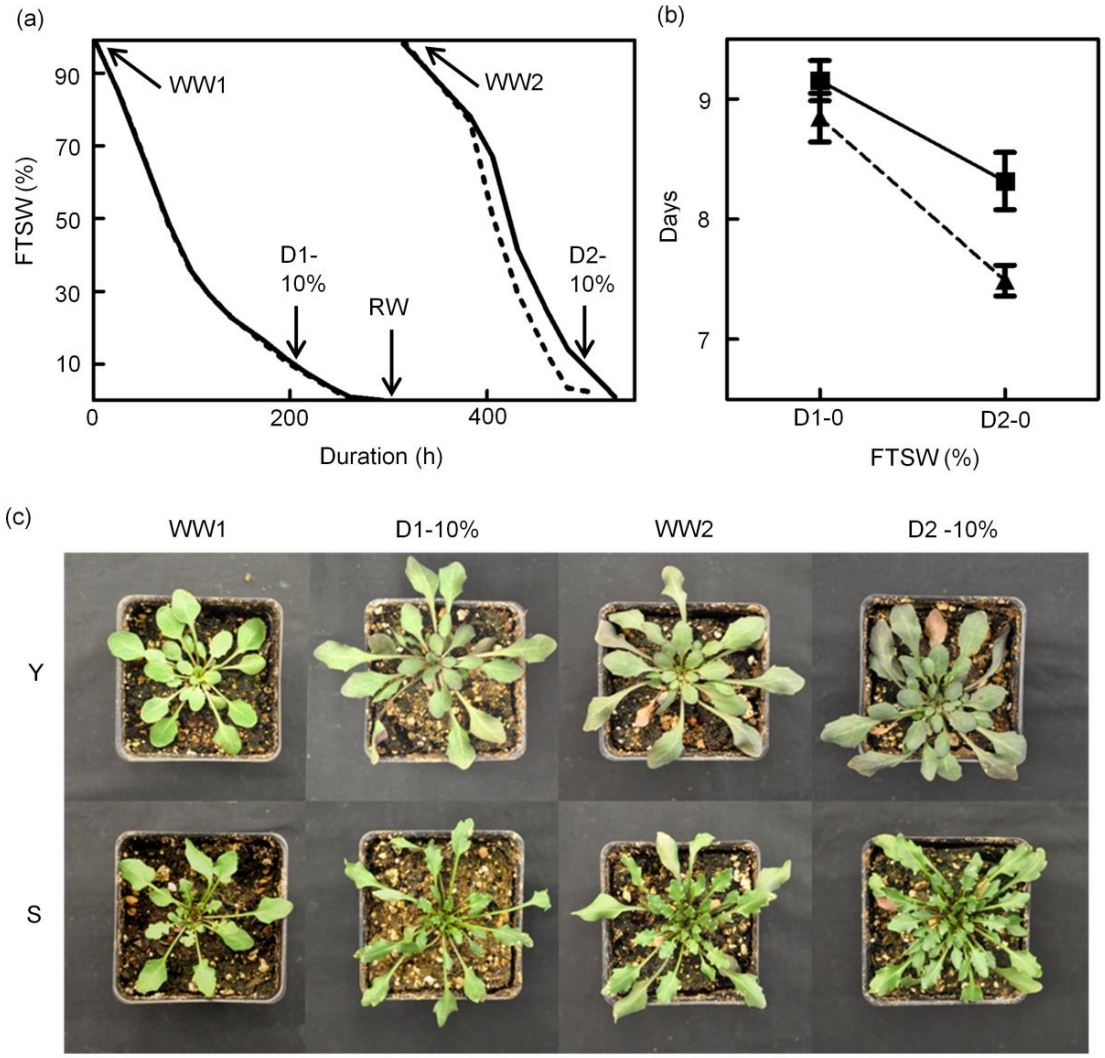
Progression of the water deficit

Four-week-old Shandong and Yukon *Eutrema* plants were subjected to a water deficit protocol that included two successive drought treatments separated by a 48 h recovery period. Before starting the drought treatment, all pots were watered to soil water holding capacity, approximately 3 g $H_2O \cdot g^{-1}$ dry soil. We tracked the progression of water loss gravimetrically from individual pots during the course of the experiment and converted those values to ones describing fraction of transpirable soil water (FTSW) remaining. Fig.

3.1A shows the relationship between the duration of the water deficits and FTSW where values of FTSW approach 100% for well-watered plants and wilting coincides with 0% FTSW. During the first drought treatment (D1), the slope relating the drop in FTSW with time is similar for plants of both accessions and leaves of Shandong and Yukon plants wilted at the same time. After re-watering, turgor of leaves was recovered within one hour, well before the end of the 48 h recovery period. While the rate of water loss from the pots was similar at the beginning of D2, the slopes diverged at about 72 h following re-watering with the Shandong plants wilting earlier than the Yukon plants. This difference in drought-responsive behaviour was reproducible with at least 150 plants tested for each accession. A norms of reaction plot (Fig. 3.1B) shows that plants of both accessions took the same length of time to wilt during the first drought treatment whereas during the second drought treatment the Yukon plants took, on average, one day longer than Shandong plants before they wilt. The difference in FTSW and wilting behaviour during the second drought suggests that the Yukon plants are able to mitigate their water deficit stress response following their first exposure to drought treatment while the Shandong plants do not respond similarly.

Representative Yukon and Shandong plants undergoing various stages of the drought treatments are shown in Fig. 3.1C. Given the high natural tolerance of *Eutrema* plants to salinity (Gong et al. 2005, Wong et al. 2005), it is not surprising that both *Eutrema* accessions coped similarly well with the osmotic challenge presented by the

Fig. 3.1. The progression of consecutive water deficits for plants from two *Eutrema* accessions reported as a function of fraction of soil water (FTSW). (a) Water was withheld from Yukon (Y, —) and Shandong (S, - - -) plants until wilting when FTSW equalled 0%. Plants were subjected to a first drought treatment (D1), they were then re-watered (RW) following wilting and then plants were allowed to recover for 48 h when water was again withheld for a second drought treatment (D2). (b) Norm of reaction plot showing days to wilting for Yukon (■, —) and Shandong (▲, - - -) *Eutrema* plants exposed to consecutive periods of water deficit. Bars represent the mean ± SE of 4 biological replicates, total n across all replicates was a minimum of 150 plants for each accession. The interaction between drought treatment and accession was significant by two-way ANOVA (P <0.05). (c) Photos of plants from a representative experiment are shown at different stages during the drought assay.

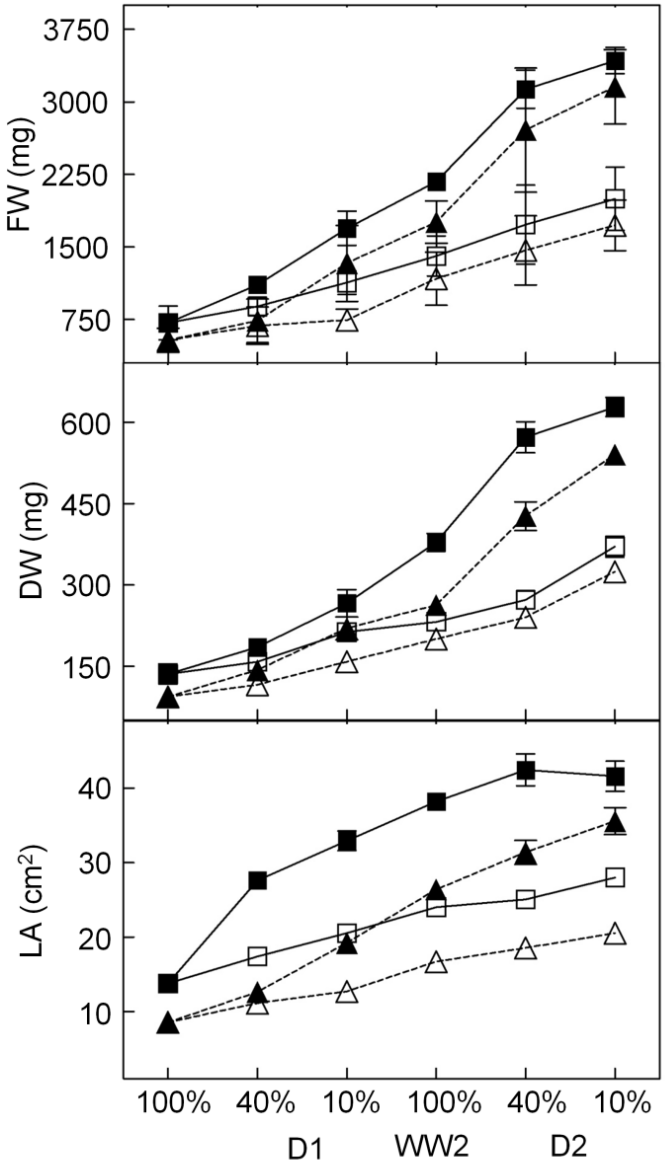

(a)
WW1
WW2
D1-
10%
RW
D2-
10%
FTSW (%)
90
70
50
30
10
0
200
400
Duration (h)
(b)
Days
9
8
7
D1-0
D2-0
FTSW (%)
(c)
WW1
D1-10%
WW2
D2 -10%
Y
S

water deficits. Fig. 3.1C shows that the plants recovered quickly when re-watered after wilting and showed few to no signs of senescence or leaf loss even after a second drought exposure. However, Yukon plants took on a purple coloration when the FTSW was approximately 40% having 8.6-fold more anthocyanin $g \cdot FW^{-1}$ than Shandong plants at D1-40% (Fig. 3.1C) although a purple coloration was evident at wilting (0% FTSW).

Drought response physiology in *Eutrema*

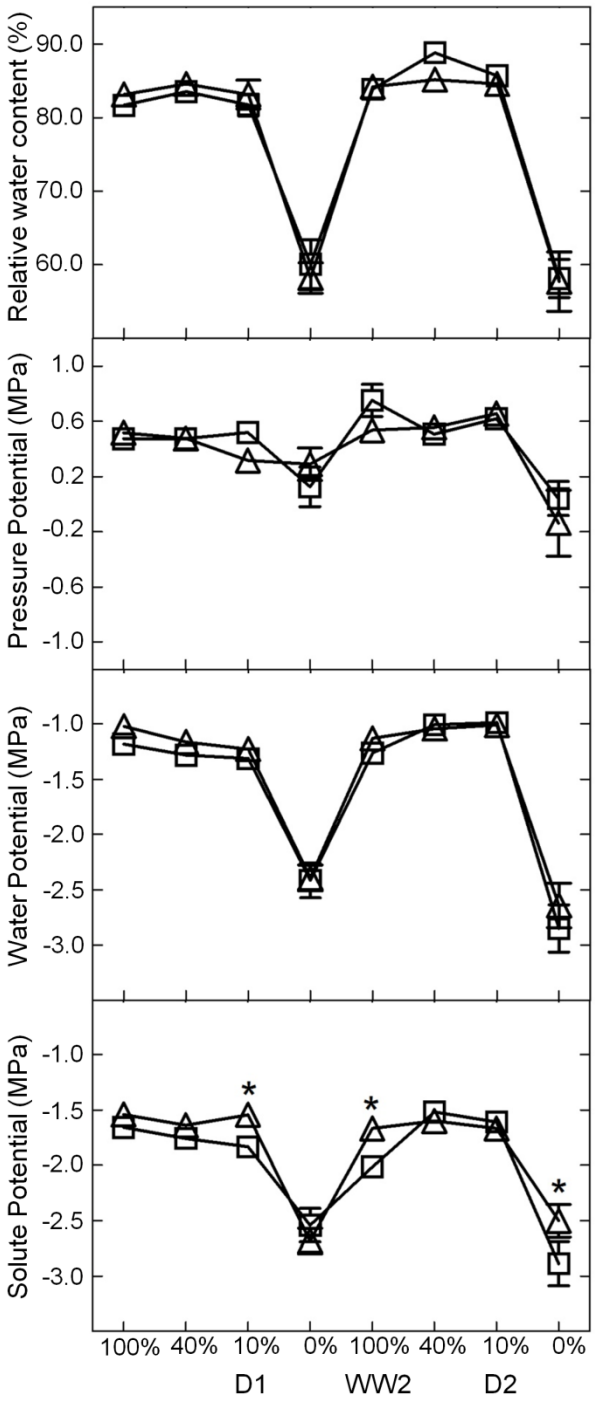
An early adverse effect of water deficits on plants is a cessation of leaf expansion and reduction in overall growth (Boyer 1970). Fresh and dry mass determinations and leaf area estimates were taken at time points corresponding to the FTSW stages shown in Fig. 3.1A. The measurements shown in Fig. 3.2 establish that the plants continued to increase in fresh and dry biomass over the course of the experiment with the greatest incremental change in dry matter for both accessions happening during the second drought exposure. However, the treatment did reduce growth in that following the second drought treatment, the fresh and dry biomass measurements for plants of both accessions were only 55 to 60% of the values for their corresponding well-watered control plants. Leaf area also increased in plants undergoing the two successive drought treatments but the accessions showed differences with respect to this parameter. Leaf areas of Yukon and Shandong plants were reduced by 33% and 42% relative to well-watered controls, respectively, indicating that the water stress reduced leaf expansion for Shandong plants

Fig. 3.2. Measurements of leaf growth of *Eutrema* plants subjected to consecutive water deficits. Fresh weight (FW), dry weight (DW) and leaf area (LA) were measured for well-watered Yukon (■, —) and Shandong (▲, - - -) plants as well as drought-treated Yukon (□,—) and Shandong △,- - -) plants at 100, 40, 10 and 0% fraction of transpirable soil water (FTSW) during both drought periods. Bars represent the mean ± SE of 4 biological replicates (n = 12). Error bars that are not visible are within the symbol.


FW (mg)
3750
3000
2250
1500
750
DW (mg)
600
450
300
150
LA (cm²)
40
30
20
10
100%
40%
10%
100%
40%
10%
D1
WW2
D2

to a greater extent. Thus while the water deficit treatments were progressive and severe enough to result in wilting, they were sufficiently mild to enable the plants to recover and grow despite experiencing two periods of water deficit (Figs. 3.1 and 3.2). To address the difference in stress responsive behaviour between the accessions we measured the RWC and components of leaf ψ_w at specific FTSW stages representing well-watered plants (WW1 at 100% FTSW), a moderate water deficit early in the drought treatment (D1 and D2 at 40% FTSW), late stress but before leaves show any visible signs of wilting (D1 and D2 at 10% FTSW), wilted plants (D1 and D2 at 0% FTSW) and after two days recovery following re-watering of wilted D1 plants (WW2 at 100% FTSW) (Fig. 3.3). At comparable FTSW points the RWC measurements for Yukon and Shandong plants were similar. The impression that the water status for plants of both accessions undergoes the same pattern of change with the treatment was corroborated by the similar leaf ψ_w and ψ_p values that were tracked as a function of FTSW. However, leaf ψ_s measurements did show a significant difference between Yukon and Shandong plants at three points during the two-stage drought treatment protocol (Fig. 3.3). The Yukon plants first showed a significantly lower leaf ψ_s at D1-10% FTSW indicating that drought-treated Yukon plants accumulated more solutes during the first drought treatment than did Shandong plants at the same stage. There was also a significant difference in solute potential at WW2 when Yukon plants retained more solutes than Shandong plants when turgor was re-established after watering. An interesting and significant difference between the two accessions was

Fig. 3.3. Leaf water status measurements of *Eutrema* plants subjected to consecutive water deficits. Leaf relative water content (RWC), pressure potential, water potential and solute potential were measured for Yukon (□) and Shandong (△) plants at 100, 40, 10 and 0% fraction of transpirable soil water (FTSW) during both drought periods. Bars represent the mean ± SE of 4 biological replicates (n =12). Error bars that are not visible are within the symbol. Asterisks indicate statistically significant differences between accessions (Fisher's LSD, P <0.05)

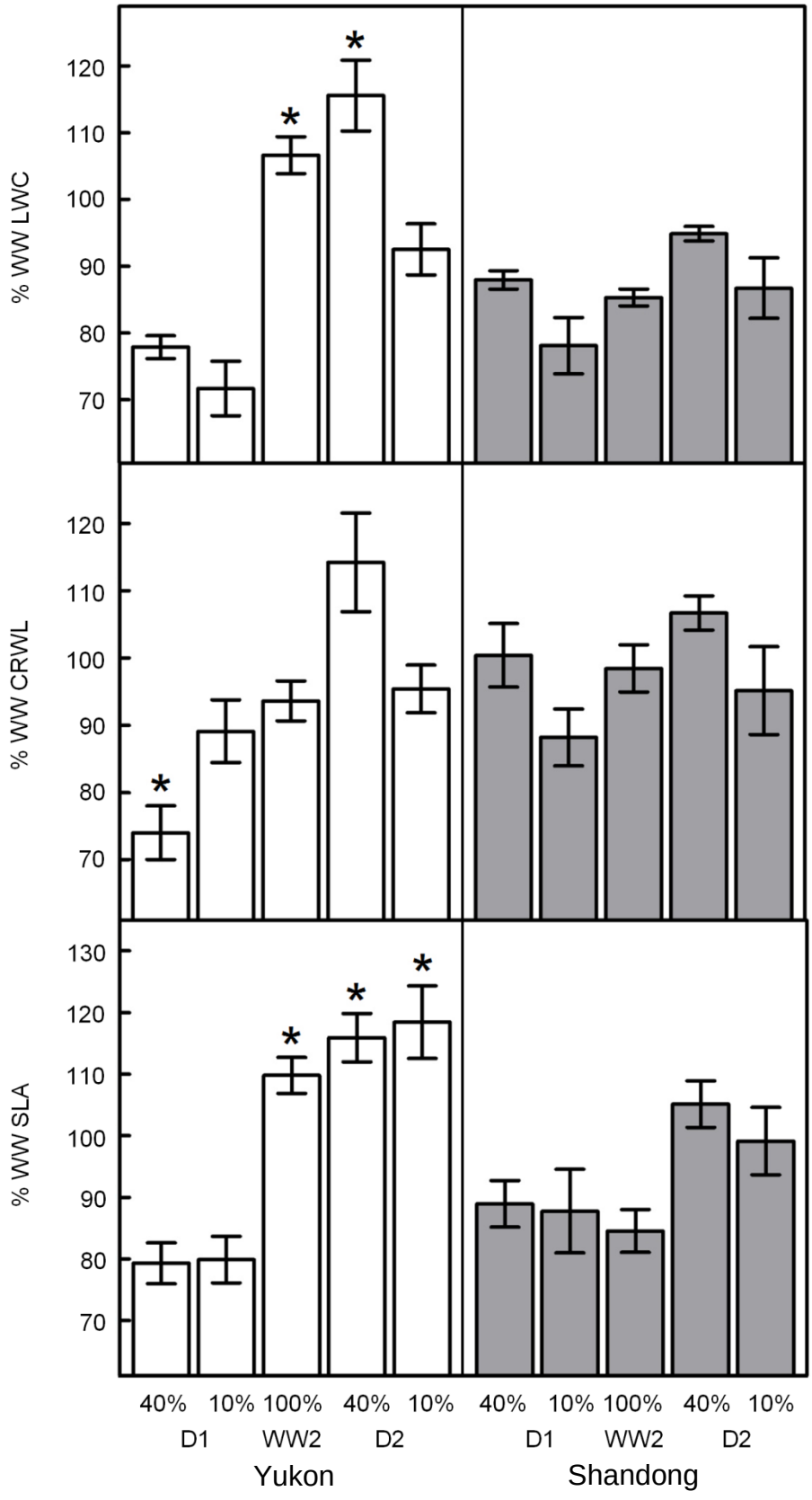

Relative water content (%)
90.0
80.0
70.0
60.0
Pressure Potential (MPa)
1.0
0.6
0.2
-0.2
-0.6
-1.0
Water Potential (MPa)
-1.0
-1.5
-2.0
-2.5
-3.0
Solute Potential (MPa)
-1.0
-1.5
-2.0
-2.5
-3.0
*
*
*
100% 40% 10% 0% 100% 40% 10% 0%
D1
WW2
D2

found when the plants wilted for the second time (D2-0% FTSW). Yukon leaves showed a significantly lower leaf ψ_s at D2-0% FTSW relative to leaves of Shandong plants at this same time point. The ψ_s value reached by the Yukon leaves during D2-0% FTSW was also significantly lower than the value reached by Yukon plants wilted at D1-0% FTSW ($P = 0.0009$). These changes in leaf ψ_s values are consistent with Yukon plants accumulating solutes before a discernible decrease in leaf RWC or ψ_w during their initial exposure to drought. Moreover, the recovery from D1 wilting in Yukon plants was, in contrast to Shandong plants, accompanied by an elevated solute content relative to unstressed plants.

The similarities in leaf water status along with the lack of response in terms of leaf Ψ_w and RWC are consistent with Yukon and Shandong plants responding comparably to water deficits. However, as described above, differences in wilting and measurements of solute content demonstrate that the strategy for coping with water deficits is not the same for the two accessions.

Yukon plants showed indications of an osmotic adjustment mechanism that is primed or improved by a previous exposure to stress (Fig. 3.1B). In addition to changes in solutes, we expected that Yukon and Shandong plants would differ in how they managed water loss during stress and the extent to which they would be adversely impacted by water limitations. This prediction was based, in part, on the differences in precipitation patterns the plants experience in their native habitats. To further characterize their responses to water loss we tracked Leaf Water Content (LWC), Cut Rosette Water Loss (CRWL) and Specific Leaf Area (SLA) during the drought treatment protocol (Fig. 3.4). Again, the Yukon and Shandong plants

Fig. 3.4. Water content, biomass and leaf area for *Eutrema* plants subjected to consecutive water deficits. Cut rosette water loss (CRWL), static leaf water content (LWC), and specific leaf area (SLA) were taken at 40% and 10% FTSW for Yukon (open bars) and Shandong (grey bars) plants during both drought periods (D1 and D2) and when plants were re-watered (RW) between droughts. Measurements were made on whole rosettes and are shown as a % of well-watered controls. Bars represent the mean ± SE of 3 biological replicates (n =12). Asterisks indicate statistically significant differences between accessions (Fisher's LSD, $P < 0.05$)


% WW LWC
% WW CRWL
% WW SLA
120
110
100
90
80
70
130
40%
10%
100%
40%
10%
D1
WW2
D2
Yukon
Shandong

displayed significant differences, in this case with respect to their management of water. Predictably, Yukon and Shandong plants showed a reduction in LWC relative to well-watered plants during the first drought exposure (D1-40 and D1-10; Fig. 3.4). This reduction in LWC was also observed in Shandong plants after re-watering (WW2) and during the second drought treatment. However, in contrast, Yukon plants had a LWC that returned to the level of a well-watered control plant at the WW2 stage and this LWC remained high until D2-10% FTSW when LWC only dropped to 90% of the level recorded for well-watered Yukon control plants. With respect to the CRWL assay, Yukon rosettes only exhibited a significant difference with respect to water retention relative to well-watered controls when drought-stressed plants were severed at the early, D1-40% FTSW stage of the protocol. This difference in CRWL represents the earliest physiological response to water deficit for either accession. At D2-40%, Yukon plants showed no difference in water loss relative to controls, again indicating that the initial drought exposure alters the way in which Yukon plants respond to a subsequent drought stress.

During the first drought treatment (D1 at 40% and 10% FTSW), SLA was reduced relative to well-watered controls for both accessions. However, after re-watering and recovery, the SLA measurements for both accessions increased with values for Yukon plants meeting and even exceeding measurements of SLA for well-watered control plants. Thus although the two accessions share a high capacity to tolerate salinity (Inan et al. 2004, Wong et al. 2005), they show important differences in their physiological responses to water deficits with the differences more apparent during a second exposure to drought.

Changes in polar metabolites in *Eutrema* in response to drought

In view of the significantly different leaf ψ_s measurements between the two accessions (Fig. 3.3), we profiled polar metabolites present in leaves of Yukon and Shandong *Eutrema* plants to determine whether the accessions were accumulating the same or different solutes in response to water deficits. We chose to compare leaves sampled from the WW1 and WW2 plants because Yukon and Shandong plants showed significantly different leaf ψ_s values at these points of the treatment (Fig. 3.3) and these are stages where the relative abundance of solutes in the plants would not be confounded by a passive increase in concentration due to water loss (as would be the case at the 10% or 40% FTSW points). The GC-MS analysis resolved about 300 MSTs but only 51 metabolites were found to show statistically significant changes in abundance between the drought-treated and well-watered control plants (T-test, $P < 0.05$; Appendix S2) and these were used for HCA (Fig. 3.5). For Yukon plants, 67% of the 51 MSTs increased in abundance in drought-treated relative to well-watered plants compared to only 41% between drought-treated and control Shandong plants. The direction and/or magnitude of change in relative abundance of MSTs were not always the same for both accessions. For example, the abundance of several sugars as well as phosphate increased in drought-treated Yukon plants but not in Shandong plants.

We also performed a principal component analysis using the relative abundance data for the 51 MSTs and compared the scores and loadings biplots (Fig. 3.6A; solid and open icons) produced by the 51 observations from the four samples. PC (Principal Component) 1 explained approximately 79% of the variance in the dataset and, together, PC2 through PC5 explained essentially all of the remaining variance.

Fig. 3.5. HCA of MSTs showing differential abundance in leaves of drought-treated *Eutrema* plants. Illustrated is a heat map of an HCA using 51 MSTs showing differential abundance between leaves of well-watered and drought-treated *Eutrema* plants. The mean MST abundance is expressed as the fold-difference between drought-treated and well-watered control levels for Shandong and Yukon plants, respectively. Those ratios were log10 transformed before being subjected to HCA to generate the heat map. Euclidean distance was used to calculate the distance matrix and a complete linkage method was used for HCA of MSTs.

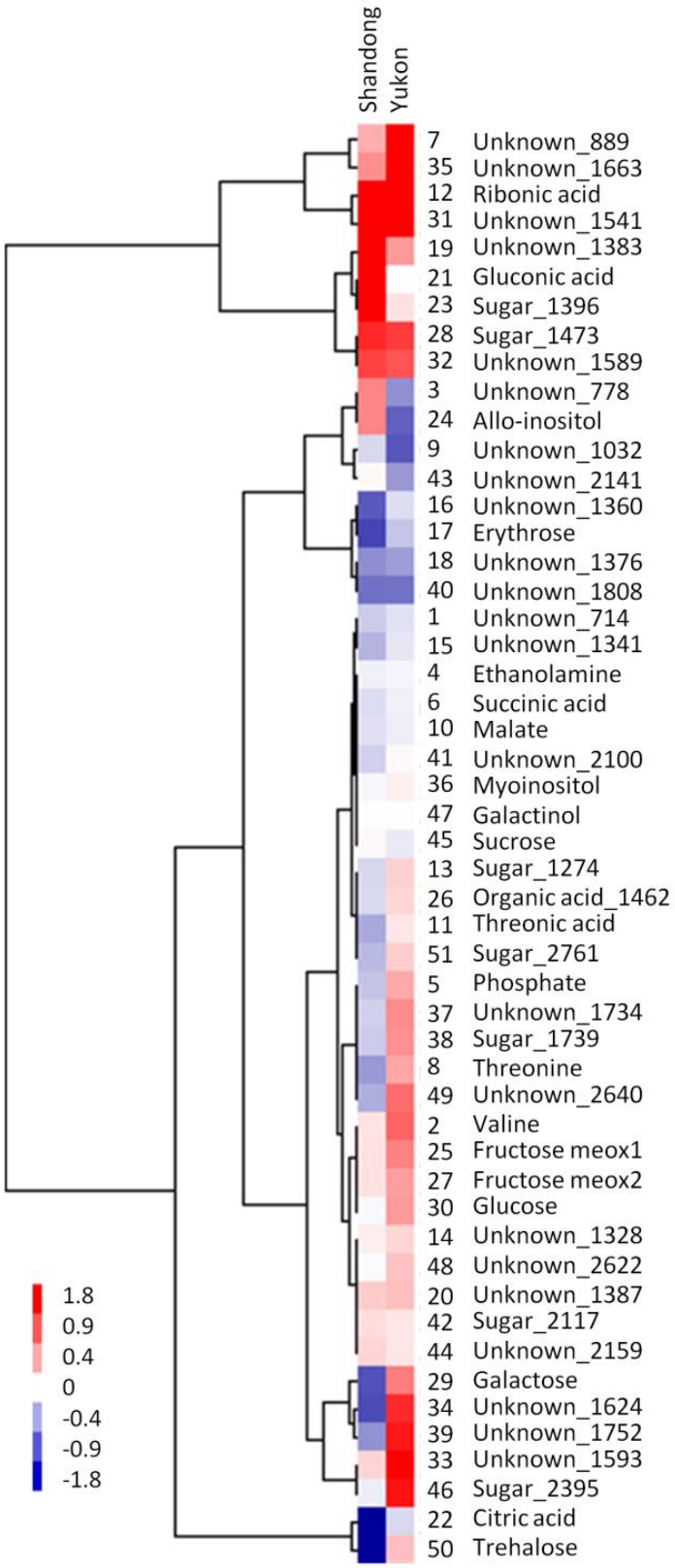
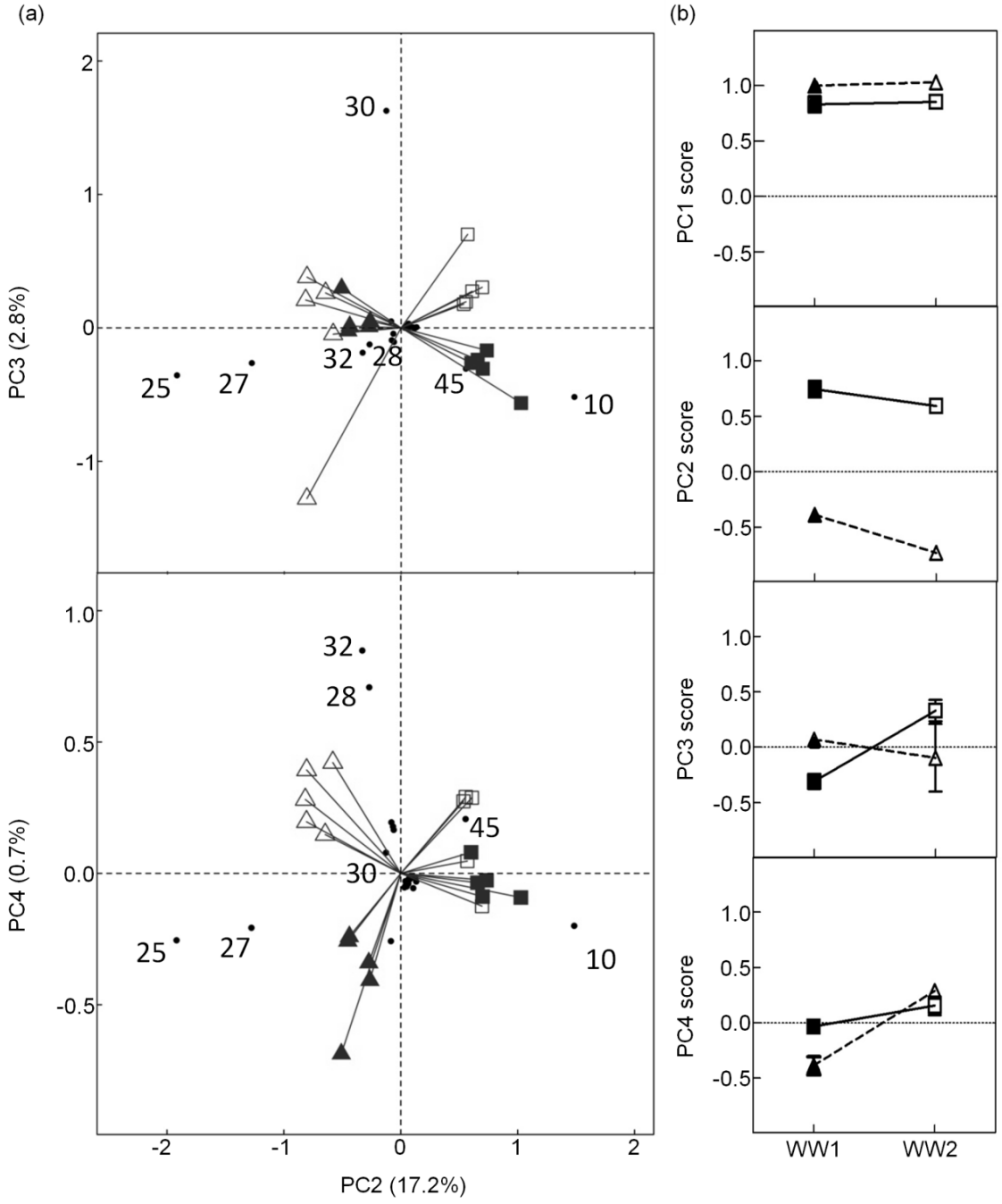

Shandong
Yukon
7 Unknown_889
35 Unknown_1663
12 Ribonic acid
31 Unknown_1541
19 Unknown_1383
21 Gluconic acid
23 Sugar_1396
28 Sugar_1473
32 Unknown_1589
3 Unknown_778
24 Allo-inositol
9 Unknown_1032
43 Unknown_2141
16 Unknown_1360
17 Erythrose
18 Unknown_1376
40 Unknown_1808
1 Unknown_714
15 Unknown_1341
4 Ethanolamine
6 Succinic acid
10 Malate
41 Unknown_2100
36 Myoinositol
47 Galactinol
45 Sucrose
13 Sugar_1274
26 Organic acid_1462
11 Threonic acid
51 Sugar_2761
5 Phosphate
37 Unknown_1734
38 Sugar_1739
8 Threonine
49 Unknown_2640
2 Valine
25 Fructose meox1
27 Fructose meox2
30 Glucose
14 Unknown_1328
48 Unknown_2622
20 Unknown_1387
42 Sugar_2117
44 Unknown_2159
29 Galactose
34 Unknown_1624
39 Unknown_1752
33 Unknown_1593
46 Sugar_2395
22 Citric acid
50 Trehalose
1.8
0.9
0.4
0
-0.4
-0.9
-1.8

Fig. 3.6. Principal component analysis of the relative abundance of polar metabolites in *Eutrema* plants exposed to water deficit. Metabolites were profiled in well-watered (WW1; Yukon ■; Shandong ▲) and drought-treated plants that had wilted and were subsequently re-watered and allowed to recover for 2 d (WW2; Yukon □; Shandong △). Relative abundance of metabolites was measured in 5 individuals for each treatment. (a) biplots of PC2 vs. PC3 and PC2 vs. PC4. Points represent factor loadings for each of the 51 MSTs used in the analysis. MSTs with factor loadings with an absolute value ≥ 0.5 are numbered. These numbers correspond to the numbering of MSTs in Fig. 3.5 and Appendix S2. (b) Norm of reactions plots of PC scores for PC1-4 as a function of treatment. Bars represent the mean ± SE (n = 5).


(a)
(b)
PC3 (2.8%)
PC4 (0.7%)
PC2 (17.2%)
30
32
28
25
27
45
10
PC1 score
PC2 score
PC3 score
PC4 score
WW1
WW2

We re-plotted the PC scores as norms of reaction figures to help identify associations between treatment and genotype (Fig. 3.6B). The norms of reaction figure of PC1 scores corresponding to WW1 and WW2 MSTs of Shandong and Yukon plants are similar. The similar scores indicate that MSTs with high factor loadings were the more abundant ones in all four samples and, conversely, the MSTs with low factor loadings were the least abundant ones in all four samples. The sources of variance captured by PC2 through PC4 are more informative in that the scores of the different observations clustered into discrete patterns in the bi-plots and norms of reaction plots shown in Fig. 3.6. PC2 scores for WW1 and WW2 plants are positive for Yukon *Eutrema* with respect to PC2 but negative for Shandong along this axis. Thus variance along PC2 distinguished the two genotypes but not the treatment observations. The scores of PC3, however, separate treatment effects for WW1 and WW2 plants but only for the Yukon accession where scores for Yukon WW1 are negative and those for Yukon WW2 are positive along this axis. In contrast, while PC4 explained only 0.7% of the total variance and offers no insight into sources of variance for Yukon plants, this axis clearly separates the positive scores for Shandong WW2 plants from the negative scores of Shandong WW1 plants. The norms of reaction plots for PC3 and PC4 reiterate this interpretation that these axes together reveal significant interactions between accessions and imposed treatment.

The MSTs that showed a significant difference between treatments are listed in Appendix S2 along with their estimates of relative abundance and PC factor loadings. Only seven of 51 MSTs had factor loadings with an absolute value ≥ 0.5 and hence have influenced the separation of MST scores for well-watered and drought-stressed

Yukon and Shandong plants (Fig. 3.6A). These seven MSTs are numbered on the PC biplots (Fig. 3.6A) using the same numbering as the HCA heat map shown in Fig. 3.5 and the tabular data given in Appendix S2. Some notable associations between factor loadings and sample scores were observed and many of these associations involved carbohydrates. Fructose had a high, negative factor loading along PC2 positioning it near the Shandong scores while malic acid and sucrose had high positive factor loadings closer to the Yukon scores along PC2. The MSTs with the highest factor loading for PC3 was identified as glucose and this metabolite accumulated in WW2 Yukon plants to a much greater extent than in the Shandong plants (Appendix S2). The MSTs with the highest positive factor loadings for PC4 were both unknowns with retention indices and MS fragmentation patterns resembling other monosaccharides in our profiles. These MSTs were more abundant in the leaves of WW2 Shandong plants that had experienced drought but not the Yukon WW2 plants. Fructose and phosphate had the most negative factor loadings associated with PC4. Both metabolites had a higher relative abundance among the leaf metabolites of Shandong WW1 plants and were detected at lower levels in leaves of Shandong WW2 plants that experienced a single exposure to drought (Appendix S2). Thus PCA analysis discerned accession-specific differences among MSTs as well as changes in MSTs that were both drought-treatment associated and accession-related.

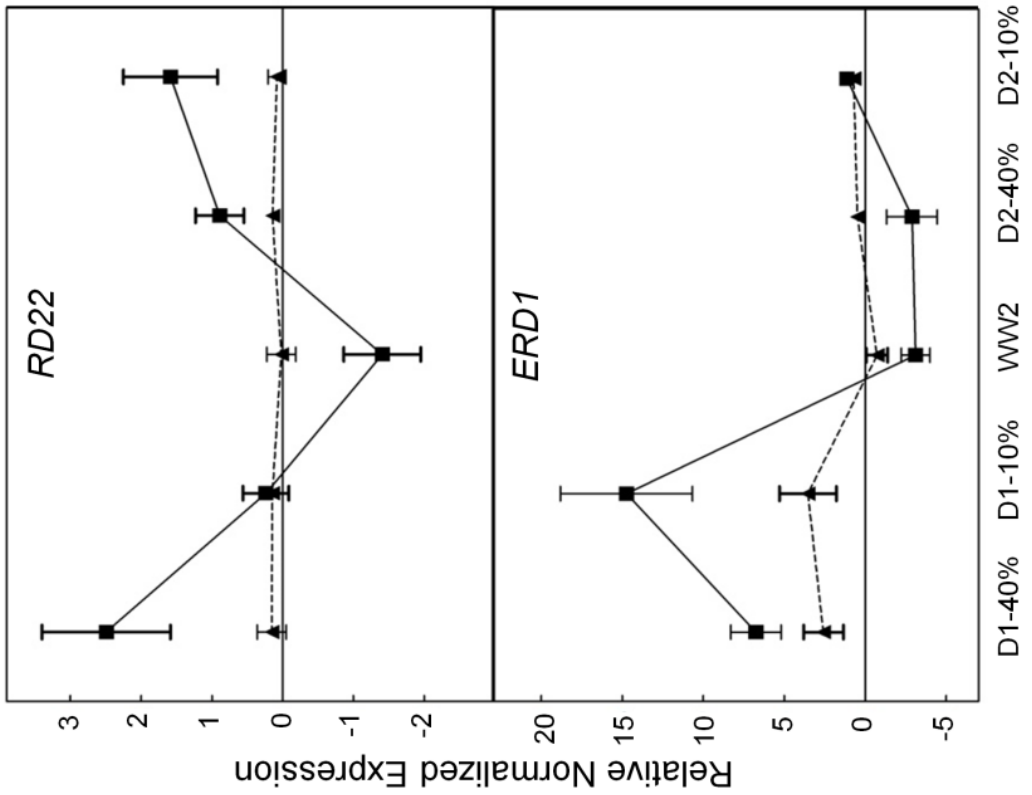
Changes in steady-state transcript abundance of dehydrin genes in response to drought

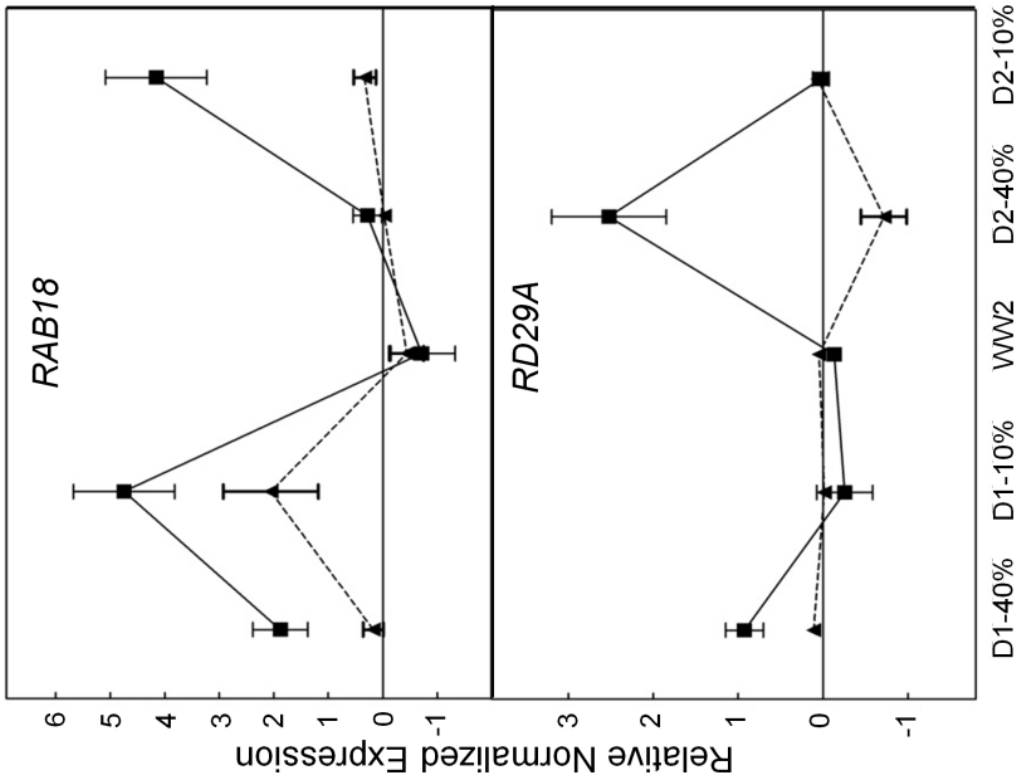
There were several early indications that the Yukon plants were stressed before Shandong plants. These changes included anthocyanin accumulation in drought-

stressed Yukon leaves (Fig. 3.1C) and a significant difference between Yukon and Shandong plants with respect to % CRWL values at D1-40% (Fig. 3.4). Thus we hypothesized that the Yukon plants would show earlier and/or a greater amplitude in drought-responsive gene expression during D1 than the Shandong plants. For this expression comparison we selected four genes encoding dehydrins that are reported to be induced in response to dehydration stress in *Arabidopsis* and other plants including *Eutrema* (Ding et al. 2012, Shinozaki and Yamaguchi-Shinozaki 2007, Wong et al. 2006). For ease of reference, we refer to the four *Eutrema* genes using the names of their reciprocal best BLAST hit to *Arabidopsis*: *AtRAB18, AtRD22, AtRD29A,* and At*ERD1*. *Eutrema* RNA-Seq transcriptome profiles (Champigny et al. 2013) confirm that the genes we selected are expressed in four-week old, Yukon and Shandong plants grown in cabinets and in Yukon field plants (Appendix S3) making these genes good candidates for cabinet and field-related comparisons.

RT-qPCR analysis using RNA from leaves of well-watered control and drought-stressed plants showed that expression of *EsRAB18, EsRD22, EsRD29A,* and *EsERD1* did not respond similarly to drought in the two accessions (Fig. 3.7). The accession-differences in expression of these drought-associated genes were particularly evident during D2. For example, the pattern of increased *EsRAB18* expression is similar for both accessions over D1 and D2 but the amplitude of the change is different in that the drought-responsive fold-changes were typically higher for Yukon plants. Furthermore, *EsRD22* transcript levels changed in a drought-responsive manner for Yukon plants but that was not true for Shandong plants subjected to the same treatments. Among other notable gene expression changes, at D1- 40% FTSW the relative expression of all four genes was higher in leaves of Yukon plants yet only *EsERD1* showed

Fig. 3.7. Selected dehydrins show different patterns of expression during the imposed water deficit. Transcript levels of *EsRAB18, EsRD22, EsRD29A, and EsERD1* are shown for different stages of water deficit treatments in Yukon (■, —) and Shandong (▲, - - -) *Eutrema* plants. Relative expression values at each stage are corrected for the normalized gene expression values of well-watered control plants. RT-qPCR data are the mean ± SE of three biological replicates (n = 6).


RD22
ERD1
Relative Normalized Expression
D1-40%
D1-10%
WW2
D2-40%
D2-10%


RAB18
RD29A
Relative Normalized Expression
D1-40%
D1-10%
WW2
D2-40%
D2-10%

increased expression in Shandong plants. Later in the D1 (at 10% FTSW), *EsRAB18* and *EsERD1* expression remained high in Yukon and Shandong plants relative to well-watered controls while *EsRD22* and *EsRD29A* expression in Yukon plants decreased to levels resembling control plants. Re-watering the drought-stressed plants restored turgor (Fig. 3.3) and this was accompanied by changes in the level of expression of *EsRAB18* and *EsERD1* for both accessions and *EsRD22* in Yukon plants (Fig. 3.7). While *EsERD1* expression remained largely unchanged for both accessions during D2, for Yukon plants the increases in *EsRAB18* expression was observed later and for *EsRD29A* the increased relative expression was greater during D2 compared to D1.

The relative differences in dehydrin expression observed for the cabinet experiment (Fig. 3.7) raises a question about absolute transcript abundance and whether Shandong and Yukon plants have similar constitutive transcript levels of these genes. For example, if well-watered Shandong plants have a higher baseline level of transcripts encoding dehydrins than Yukon plants there may be no need for additional induction of these genes to generate sufficient products for stress protection. To address this concern, we examined the absolute transcript levels reported by Champigny et al. (2013) for the genes selected in this study. The Yukon and Shandong *Eutrema* subjected to RNA-Seq by Champigny et al. (2013) are roughly equivalent to the WW1 plants used in this study with respect to age and growth conditions. We found that baseline transcript levels were either approximately equivalent in Yukon and Shandong plants or higher in Yukon (Appendix S3). Leaves of unstressed Yukon plants grown in cabinets have an almost 10-fold higher level of *EsRAB18* and *EsRD29A* transcripts compared to unstressed Shandong plants. Thus the

drought-responsive changes seen for *EsRAB18* and *EsRD29A* are all the more noteworthy given their higher baseline level in unstressed Yukon relative to Shandong plants.

Discussion

We predicted that the *Eutrema* plants used in this study would tolerate drought well given their capacity to withstand the osmotic stress posed by their highly saline natural habitat. However, Yukon plants inhabit an area where precipitation is sporadic and typically of short duration during the growing season while Shandong plants are native to an area where precipitation is, on average, both more abundant and frequent (Guevara et al. 2012). Xu et al. (2014) reported that Shandong plants wilted after an eight day water deficit while Yukon plants remained turgid. To further test this response, we developed a protocol to simulate Yukon field conditions where successive, progressive water deficits separated by re-watering and a brief recovery period would not be unusual. We then subjected Yukon and Shandong plants to this protocol in order to compare their capacities to tolerate water deficits. Reflecting upon the outcome, the incorporation of a second drought treatment was particularly insightful in that it led to the first noteworthy difference in water deficit responses between the accessions which was the time taken for plants to wilt after watering was stopped. For D1, both accessions took the same length of time to wilt but for D2, Yukon plants responded in a significantly different manner in taking about a day longer to wilt (Fig. 3.1B). This response is consistent with Yukon plants developing an improved capacity to retain and/or take up water relative to Shandong plants as a consequence of the drought exposure earlier in the protocol. In their discussion Xu et

al. (2014) remarked upon an increased time to wilting with a second drought for both accessions. They attributed this response to reduced cuticle permeability elicited by the first drought. Our findings differ in that we found no indication that Shandong plants took longer to wilt with a second exposure and while we did not test cuticular wax, we observed several physiological and molecular changes during D2 that would contribute to the enhanced capacity of Yukon plants to resist wilting following an initial drought exposure.

In addition to an offset in time to wilting during D2, our findings show that Yukon plants accumulated solutes during the initial drought exposure (Fig. 3.3) and this change was concomitant with improved water status as evidenced by the increase in LWC observed in Yukon plants at WW2 and at D2-40% FTSW (Fig. 3.4). In Yukon plants, glucose was prominent among the solutes showing an increased relative abundance as a consequence of drought but it was not among the solutes that increased in abundance in Shandong plants with drought exposure (Fig. 3.5; Appendix S2). Guevara et al. (2012) also found glucose was more abundant in Yukon plants exposed to drought in both the cabinet and under field conditions relative to well-watered cabinet plants and field plants collected during a year of greater than average precipitation. There is, however, evidence for plasticity in the metabolic responses of *Eutrema* to osmotic stress (Guevara et al. 2012, Kazachkova et al. 2013). For example, Kazachkova et al. (2013) found glucose and fructose were more abundant in Shandong *Eutrema* grown on soil when effects of salinity on plants grown on nutrient media and in soil were compared. How this plasticity may affect the tolerance of plants to osmotic stress is not known but the nature of solutes accumulated likely has functional significance (Miller et al. 2010). Kazachkova et al. (2013) postulated that

glucose accumulation in the cytosol along with fructose may replenish malate which is more abundant in *Eutrema* than in *Arabidopsis*. Our PCA identified malate as the metabolite with the highest positive factor loading along PC2 (Appendix S2). Malate was present at levels 1.6 and 1.8-fold higher in both YWW1 and YWW2 leaves relative to SWW1 and SWW2 leaves, respectively. However, malate was not one of the solutes that showed a drought-responsive increase in relative abundance for Yukon or Shandong plants (Fig. 3.5) suggesting that the glucose accumulated under drought may not be used for replenishing malate or that any malate that is produced is turned over. Glucose has been shown to have hydroxyl scavenging activity *in vitro* (Nishizawa et al. 2008) and so it may also have a protective function in Yukon plants during drought when free radicals and reactive oxygen species are expected to be more abundant (Miller et al. 2010).

Mechanisms broadly classified as escape, avoidance, and tolerance are strategies used by plants and considerable diversity exists in nature with respect to the contribution each mechanism makes in how plants respond to water availability (Juenger 2013). Plants that complete their life cycle before serious water deficits escape the consequences of drought, those that maintain high water status despite diminishing water in the environment employ avoidance strategies, and plants that tolerate water deficits mitigate the damage that occurs to cellular contents during dehydration (Nishizawa et al. 2008). The accumulation and maintenance of protective solutes is classified as a drought tolerance strategy (Levitt 1980) and the higher solute content of Yukon plants at WW2 is one significant feature that distinguishes Yukon from Shandong plants. Retention of solutes should confer an adaptive advantage to Yukon plants exposed to consecutive periods of water deficit in their native habitat.

There are other subtle but significant differences in the physiological responses of Yukon and Shandong plants with respect to water deficits. Reducing water lost through leaf stomata is classified as a drought avoidance strategy and it is observed in most plants at some point during drought stress (Cowan 1977). Using cut rosettes, we observed that Yukon plants have reduced water loss relative to well-watered plants early in D1 but showed no reduction in water loss during D2 when Yukon plants took longer to wilt than Shandong plants (Fig. 3.4). Thus Yukon plants appear to conserve water during D1 but resume normal rates of water loss through stomata and recover the water content of their leaves (LWC) to levels meeting and even exceeding those of unstressed control plants during WW2 and D2. Indeed, the higher values for specific leaf area (SLA) of drought-treated Yukon plants relative to well-watered controls during D2 indicate that the influx of water is sufficient to allow for growth despite a water deficit (Fig. 3.4). The consequence for the Yukon accession is that these plants have more biomass and leaf surface area than Shandong plants by the conclusion of D2 and they have lost less biomass and leaf area relative to their well-watered controls than have the Shandong plants (Fig. 3.2). These results are consistent with the conclusion that Yukon plants tolerate consecutive water deficits by a combination of drought tolerance and avoidance strategies.

Differences in the drought response strategies employed by plants of the two accessions were also observed at the molecular level and the data in Appendix S3 leads to interesting predictions related to the drought profiles for gene expression (Fig. 3.7). Leaves of unstressed Shandong plants have almost 10-fold fewer *EsRD29A* and *EsRD22* transcripts than do unstressed Yukon plants. Shandong plants subjected to drought show negligible changes in the expression of these genes even at D1-10% or

D2-10%, the stages of water deficits just prior to wilting. In many respects, the distinct accession-related differences in organic solutes are recapitulated in the gene expression observations. That is, Yukon plants respond to water deficits in the cabinet in a manner that shows altered transcript abundance with a first or second exposure to stress even for genes that are naturally abundant while Shandong plants either do not alter the expression of these dehydrin-encoding genes or only show a modest capacity to modulate their expression, even after a first exposure to drought. Although the basal expression of the drought genes studied was higher in the Yukon accession for *EsRAB18, EsRD22,* and *EsRD29A*, it is clear that the expression of these drought-associated transcripts is not simply constitutive but also regulated to be stress-responsive.

Eutrema is frequently referred to as an extremophile plant, a classification based upon its capacity to withstand highly saline soils (Gong et al. 2005). In this report we show that the Shandong and Yukon *Eutrema* accessions are not equivalent and that despite being plants with a high innate tolerance to osmotic stress, they can be distinguished based upon different response mechanisms to water deficits. Yukon plants exhibit signs of early response to water deficit in terms of both physiological and molecular responses as evidenced by early stress-inducible solute accumulation and dehydrin-encoding transcript abundance. However, what truly distinguishes the drought response of the two accessions is that Yukon plants respond differently to a second water deficit while the Shandong plants largely repeat their D1 response during D2 with little to no change at either the physiological or molecular level. These results indicate a degree of plasticity in how Yukon plants respond to the two-stage drought treatment that we used and the same plasticity is not found in Shandong

plants. Thus while both accessions show tolerance to water deficits in that they continue to grow despite having no water provided for two of the six weeks that they were grown, we show that they employ different strategies with the Yukon accession more suitably equipped for coping with chronic soil water deficits by a suite of adaptive metabolic, physiological, and molecular tolerance traits.

Author contributions

MJRM and CA performed the RT-qPCR determinations, plant growth and physiological measurements, JD performed the GC-MS analyses. WWS performed the PCA. MJRM, MJC and EAW designed experiments, performed data analysis and prepared the manuscript.

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Supporting Information

The following supporting information is available for this article online:

http://onlinelibrary.wiley.com/doi/10.1111/ppl.12316/suppinfo

Appendix S1- Complete set of growth and water content measurements

Appendix S2- Abundance of metabolites in Yukon and Shandong drought-treated plants

Appendix S3- Levels of dehydrin transcript abundance in Yukon and Shandong plants

Appendix S4- Primer sequences used for qPCR

CHAPTER 4

Comparative transcriptome sequencing of two natural accessions of *Eutrema salsugineum* at different stages of drought treatment

Preface

The work in this chapter builds upon the physiological differences identified in Chapter 3 using RNA-Seq to generate transcriptomes from plants harvested during the drought-treatment assay. I prepared all plant tissue samples from harvest to the point of mRNA purification. Eight of the 16 cDNA libraries used for RNA-Seq were prepared by Dr. Marc Champigny and he supervised me in the production of the remaining libraries. Mr. Wilson Sung performed the transcriptome assembly and DESeq analysis and prepared the PCA biplot shown in Figure 4.1. I collaborated with Caitlin Simopoulos on the WGCNA work and she generated the heat map shown in Figure 4.4. Tissue selected for sequencing and other analyses was performed by the author under the guidance of Dr. Elizabeth Weretilnyk. This chapter has been formatted as a journal article to be submitted to BMC Plant Biology.

Comparative transcriptome sequencing of two natural accessions of *Eutrema salsugineum* at different stages of drought treatment

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Abstract

Background

Our approach to identifying genes responsive to drought benefits from the use of the extremophile crucifer *Eutrema salsugineum* (*Thellungiella salsuginea*), a species adapted to severe environmental conditions in its natural habitat. We previously developed a two-stage drought treatment protocol that delineates the drought response and recovery from drought for two natural accessions found, namely Yukon and Shandong *Eutrema* plants. Based upon their physiological responses to drought, Yukon plants show superior coping strategies including a heightened capacity to accumulate solutes relative to Shandong plants. In this study we generated and compared 16 transcriptomes for RNA-Seq analysis using leaf samples from plants of both accessions subjected to the same progressive water deficit protocol in order to develop a better mechanistic understanding of the gene expression underlying their differential drought tolerance strategies.

Results

Over 1.1 billion high-quality reads were produced providing read support for >99% of the predicted coding capacity of the *Eutrema* genome. The response to an initial drought led to the differential expression between leaves of well-watered and drought-stressed plants of more than 2000 genes in Yukon plants compared to only two genes in Shandong plants. The analysis of transcriptomes from plants harvested during the recovery phase and following a second, successive drought treatment provided a different outcome. We identified relatively few differentially expressed genes in Yukon plants after they were re-watered and allowed to recover but re-watered Shandong plants were found to undergo a substantial transcriptional response

involving the differential expression of more than 500 genes. Gene ontology enrichment analysis showed that genes associated with secondary metabolism are up-regulated during drought-response in Yukon plants while genes involved in photosynthesis and translation were down-regulated. A weighted gene correlation network analysis revealed clusters of co-expressed genes that responded similarly to the two drought treatments in Yukon plants but not Shandong plants.

Conclusions

The transcriptional response of the two accessions was divergent. This is consistent with previous reports on differences in physiology between the two accessions. While Shandong plants responded more strongly at the transcriptional level to re-watering and the second water deficit, Yukon plants showed a more muted transcriptional response relative to the one observed after the initial drought. We speculate on the relationship between these changes at the level of gene expression and how they may be related to previously observed physiological differences between the accessions.

Background

Crop losses due to limited soil water availability brought on by periods of drought exceed losses to all other abiotic and biotic stressors (Boyer 1982). Climate change may exacerbate these losses in the near future and has already reduced the productivity of crops globally (Zhao and Running 2010). Understanding how plants respond to, and recover from, drought is vital to improving global crop yields (Mittler and Blumwald 2010).

Although plant responses to drought are complex and variable, our understanding of this subject has advanced nonetheless, in part through the benefits

accrued from using different experimental approaches. A plant's response to water deficit brought on by drought can be examined from the whole plant level (Juenger et al. 2005) down to specific molecular responses (Ryu et al. 2010). The transcriptional response to water deficit has been assessed in genetic model plants (Baerenfäller et al. 2012), valuable agricultural plants (Hayano-Kanashiro et al. 2009), and more recently new model plants with exceptional drought tolerance (Meyer et al. 2014).

The plant tissue used for gene expression profiling is an important consideration in light of how varied the transcriptional response to drought can be depending on the severity and duration of an imposed treatment (Bray 2004). For example, Verelst et al. (2013) examined the transcriptional response to both moderate and severe drought in *Brachypodium distachyon* and found little overlap in the transcriptional response as only 10% of the 884 differentially expressed genes they uncovered were detected under both moderate and severe droughts. In selecting the stage at which plant tissue is harvested for gene expression profiling it is important to consider the physiological response to the drought-treatment (Harb et al. 2010; Des Marais et al. 2012). The selection of tissue from plants that have responded physiologically to the imposed stress means that correlations between the physiological and transcriptional response to drought can be considered. This approach was used by Meyer et al. (2014), who showed that in switchgrass some genes only respond to drought-treatment exposures that extended beyond critical physiological thresholds for measurements such as water potential and photochemical quenching. In some cases, sequential drought treatments produce plants that display altered responses to subsequent exposures to water deficits. For example, the transcriptional response to repeated drought exposures is distinct from the response to a single water deficit (Hayano-Kanashiro et al. 2009,

Ding et al. 2012). When *Arabidopsis* seedlings grown on media plates were exposed to repeated dehydration stresses the relative expression of several drought-responsive genes showed evidence of "training", a phenomenon also referred to as drought memory. A genome-wide RNA-Seq approach helped resolve four distinct classes of drought memory genes in *Arabidopsis* that reflect their broad strategic roles in protecting plants from the deleterious aspects of drought (Ding et al. 2013). Despite progress made on the subject of drought tolerance and training, our understanding of the transcriptional response to multiple drought exposures and recovery from drought, especially in naturally drought tolerant plants, is lacking. Assessing drought response at the transcriptional level in these tolerant species, plants that are adapted to severe environmental conditions in their natural range, is expected to provide new insights into the mechanisms responsible for drought tolerance (Rodriguez et al. 2010).

In this report we describe the transcriptional responses of the extremophile crucifer *Eutrema salsugineum* to drought. *Eutrema* is a crucifer and halophyte that experiences periods with little precipitation in parts of its natural range. MacLeod et al. (2015) reported that two natural accessions of *Eutrema* respond differently to a drought treatment that includes two periods of water deficit separated by a brief recovery period. Plants of the Yukon accession, native to a more arid environment, show a heightened capacity to withstand repeated drought-exposures, accumulate solutes in response to drought, and maintain leaf expansion during water deficits relative to plants of the Shandong accession that are native to a region where precipitation is, on average, more abundant. A difference in response to repeated drought exposures was also observed at the level of gene expression with three of four dehydrin-encoding genes tested showing divergent patterns of gene expression

between the two accessions even during the first exposure to drought when their physiological phenotypes were similar (MacLeod et al. 2015).

This work extends the physiological responses to water deficit and recovery reported by MacLeod et al. (2015) to include comparative transcriptome sequencing of these climactically distinct accessions of *Eutrema*. We show, using *Eutrema* cDNA libraries from leaves collected at specific stages of an imposed water deficit, that the Yukon accession undergoes a strong transcriptional response upon first exposure to drought with comparatively fewer genes showing altered expression during recovery and a subsequent drought whereas the converse is true for the Shandong accession that displays drought avoidance strategies (MacLeod et al. 2015). These results suggest that following a first exposure to water deficit stress, mechanisms underlying drought tolerance for *Eutrema* requires early and strong transcriptional re-programming and these changes confer long term beneficial impacts whereas transcriptional changes accompanying drought avoidance are comparatively weak and are only augmented with recovery and subsequent exposures to stress.

Methods

Plant growth conditions and drought simulation assay

Shandong and Yukon *Eutrema* plants were grown in a controlled environment growth chamber and subjected to a drought simulation assay consisting of two periods of water deficit separated by a two-day recovery period (MacLeod et al. 2015). Water was withheld from four-week-old plants to initiate the first drought treatment (D1). The progress of the drought treatment was monitored gravimetrically and the fraction of transpirable soil water remaining (FTSW) was determined. FTSW was maintained

at approximately 100% for well-watered, control plants and for plants undergoing drought treatment, the FTSW was 0% on the day plants visibly wilted. The re-watering treatment was started on the day a plant wilted and FTSW was restored to 100% within 48 h (WW2). After 48 h, water was again withheld from plants to begin the second drought treatment (D2). A set of plants was watered daily over the course of the entire experiment and served as well-watered control plants.

Selection of plant tissue for transcriptome profiling

Only fully-expanded leaves were harvested from both Yukon and Shandong plants. Leaf samples for RNA extraction were collected between 14:00 and 16:00 approximately 10 h into the day cycle under our cabinet conditions. Once harvested, the leaf tissue was flash-frozen in liquid N and then transferred to a freezer for long term storage at -80°C.

Leaves for RNA extraction were collected at three stages of the water deficit protocol. Plants were harvested during the initial water deficit (D1-10% FTSW), during recovery (WW2-100% FTSW), and during the second water deficit (D2-10% FTSW). In addition, leaf tissue was harvested from two, well-watered control plants with one plant harvested when drought-treated plants had reached D1-10% FTSW and the second control plant at the WW2-100% stage of the drought protocol. The difference in age between these control plants was 5 d.

RNA extraction and cDNA library construction

Total RNA was extracted using a modified hot borate method (Wan and Wilkins 1994) as described by Champigny et al. (2013). RNA quantity and integrity was

assessed using RNA Nano 6000 chips on a Bioanalyzer 2100 instrument. mRNA purification was performed by three successive on-column purifications using the Genelute mRNA miniprep kit (Cat. No. MRN10, Sigma). Preparation of cDNA libraries was performed with the NEBNext multiplex cDNA synthesis kit for Illumina using random hexamers (Cat. No. E7335, New England Biolabs, Ipswich, MA). The cleanup of fragmented RNA was performed with Agencourt AMPure XP Beads (Cat. No. A63987, Beckman Coulter, Mississauga, ON) following the manufacturer's protocol.

Transcriptome sequencing and determination of transcript abundance

Quality control, amplification, and sequencing of the 16 cDNA libraries was conducted at the sequencing facility of the Farncombe Family Digestive Health Research Institute (McMaster University, ON, Canada). A combination of two high-output and three rapid paired-end sequencing runs using a 100 bp read length were performed using the Illumina Hi-Seq 2000 platform (Table 2.2). The libraries are identified by accession (S or Y), drought stage (WW1, D1, WW2, or D2), and plant number (1 or 2). Using this identification system, the libraries were: YWW1-1 and -2, YD1-1and -2, SWW1-1and -2, SD1-1 and -2, YWW2-1 and -2, SWW2-1and -2, YD2-1 and -2, and SD2-1and -2.

Following sequencing, the reads obtained were trimmed of adaptor sequences and low quality reads (quality > q20) with Cutadapt v1.1 (Martin 2011). Only reads ≥ 36 bp after trimming were mapped to the JGI *Thellungiella halophila* genome (http://www.phytozome.net/thellungiella) using STAR v2.30e (Dobin et al. 2013) with –alignIntronMax and –alignMatesGapMax both set to 8000 bp to reflect the intron

size distribution in *Arabidopsis* (Hong et al. 2006) which is closely related to *Eutrema* and has a similarly compact genome (Yang et al. 2013). The number of reads that mapped uniquely to each gene were counted using the script htseq-count from HTSeq v0.5.3p3 (Anders et al. 2014) using the intersection-nonempty overlap resolution mode (http://www.huber.embl.de/users/anders/HTSeq) and summarizing counts at the gene level. Transcript abundance is reported as the number of reads per kilobase per million mapped reads (RPKM), a determination accounting for both mRNA length and library size (Mortazavi et al. 2008).

Multivariate analysis and identification of differentially expressed genes

Statistical analyses on gene expression data was performed using R v2.14.1 (R Development Core Team 2011) using the RPKM values shifted by a constant of 0.5 to allow the data to be $\log_{10}$ transformed in order to normalize for the disparity in transcript abundance for genes with very low (eg. 0.01 RPKM) or very high (eg. 10000 RPKM) expression. Principal component analysis (PCA) was performed on the covariance matrix for all genes detected across the 16 libraries to explore variation within and between the transcriptomes with regards to the abundance of transcripts using a custom R script based on the "bpca" R package (Champigny et al. 2013). RPKM values for 26352 genes were treated as the variables while each of the 16 cDNA libraries that were sequenced were treated as observations. Differentially expressed genes were called using the DESeq Bioconductor package (http://bioconductor.org/packages/release/bioc/html/DESeq) using a false discovery threshold of 0.05 (Champigny et al. 2013).

GO enrichment analysis of differentially expressed genes

Sets of DEGs identified by DESeq were uploaded to the agriGO Gene Onotology (GO) term enrichment analysis tool (http://bioinfo.cau.edu.cn/agriGO website, Du et al. 2010). The Singular Enrichment Analysis tool was used to identify significantly enriched GO terms based on the Plant GO Slim term annotation from *Arabidopsis*. A customized reference list of *Arabidopsis* loci IDs was uploaded consisting of the *Arabidopsis* gene models that were best-hit matches for the 26252 genes with read support from the 16 transcriptomes described above. Fisher and Yekutieli false discovery statistical tests were selected with a 0.05 significance level (Benjamini and Yekutieli, 2001).

Network analysis of co-expressed differentially expressed genes

Weighted gene co-expression network analysis (WGCNA) was performed on sets of DEGs using the WGCNA R package (Langfelder and Horvath 2008) according to the protocol outlined by Coneva et al. (2014).

Results and Discussion

RNA-Seq of *Eutrema* accessions during drought and recovery

We prepared and analysed leaf transcriptomes of Yukon and Shandong plants subjected to a two-stage drought treatment protocol described by MacLeod et al. (2015). A total of 16 cDNA libraries were prepared with 8 from each genotype corresponding to plants at WW1 (100% FTSW), severe drought at D1 (10% FTSW), recovering from drought WW2 (100% FTSW), and subjected to a second drought at D2 (10% FTSW). Sequencing produced a total of 212 gigabytes (GB) of sequence

data with the resulting files for each library being, on average, more than 13 GB in size (Table 4.1). The data-file size (amount of sequence data generated) was not correlated with how many samples were multiplexed in a lane as the SD1-2 library was one of the largest libraries despite also being among the most multiplexed (Table 4.1; Table 2.2). After trimming the transcript reads for all libraries, an average of 86.9% of the over 1.1 billion high-quality reads were mapped to the JGI *Thellungiella halophila* reference genome (http://www.phytozome.net/thellungiella) and used for assembling the transcriptome and determining differential gene expression (Anders et al. 2014). In summary, the sequencing data was of high quality with less than 2% of the sequence data trimmed and over 85% of reads uniquely mapped to the reference genome.

From our RNA-Seq analysis we found read support for 26352 genes that are included in the JGI *Thellungiella halophila* genome (http://www.phytozome.net/thellungiella). This result compares with an estimated protein-coding capacity for the *T. halophila* reference genome of 26531 genes reported by Yang et al. (2013). Thus among the 16 sequenced transcript libraries we have read support for 99.3% of the predicted genes in the *Eutrema salsugineum* genome. For both accessions, the average number of expressed genes detected was approximately 22000 per library (Table 4.2). Using a Roche 454 FLX sequencer, Champigny et al. (2013) detected transcripts for approximately 19000 expressed genes per Yukon and Shandong leaf cDNA library sequenced. Thus due to the greater sequencing depth provided by the Illumina HiSeq platform (Liu et al. 2012), the 16 libraries described here include transcript abundance data for a number of genes that were previously undetected in *Eutrema.*

Table 4.1 - Size and quality assessments for the sequenced cDNA libraries

Tissue Description	ID	Size	Raw reads	Trimmed Reads		Mapped Reads	
		GB	*No.*	*No.*	*%* [a]	*No.*	*%* [b]
Yukon well-watered	YWW-1	14	78,872,323	78,619,442	99.7	68,086,770	86.6
	YWW-2	14	71,967,370	71,678,655	99.6	61,042,347	85.2
Yukon first drought stress	YD1-1	13	76,404,759	76,109,450	99.6	67,175,992	88.3
	YD1-2	14	75,229,666	75,051,120	99.8	64,099,314	85.4
Yukon re-watered	YWW2-1	14	77,170,213	76,998,858	99.8	66,144,106	85.9
	YWW2-2	13	68,518,931	68,197,487	99.5	59,220,161	86.8
Yukon second drought stress	YD2-1	13	70,512,179	70,422,180	99.9	61,600,491	87.5
	YD2-2	13	68,505,814	68,467,096	99.9	59,673,877	87.2
Shandong well-watered	SWW-1	9	52,341,901	51,632,966	98.6	45,544,767	88.2
	SWW-2	14	70,558,682	70,386,042	99.8	60,274,855	85.6
Shandong first drought stress	SD1-1	13	73,137,873	71,784,266	98.1	62,998,977	87.8
	SD1-2	15	83,184,148	81,525,059	98.0	71,515,067	87.7
Shandong re-watered	SWW2-1	15	74,751,096	74,598,367	99.8	64,806,383	86.9
	SWW2-2	14	69,700,866	69,509,225	99.7	60,174,930	86.6
Shandong second drought stress	SD2-1	13	69,744,235	69,714,945	100.0	61,836,469	88.7
	SD2-2	11	57,695,534	57,663,232	99.9	50,017,089	86.7

[a] Percentage of raw reads remaining after trimming

[b] Percentage of raw reads counted

Table 4.2 - Total number of genes detected for each of the 16 sequenced cDNA libraries.

Sample	Accession	
	Yukon	Shandong
	No. of genes (RPKM > 0)	
WW1-1	21569	21644
WW1-2	21751	21751
D1-1	21922	21823
D1-2	22762	21652
WW2-1	21676	22542
WW2-2	22259	22528
D2-1	21569	22498
D2-2	22272	21726
Average[a]	21972	22020

[a] Average number of genes detected for each accession over all libraries

We identified 19530 genes for which transcripts were detected in all 16 cDNA libraries (Table 4.3); a value representing 73.6% of the predicted coding capacity for *Eutrema*. A small number, 83 (0.3%) and 20 (0.08%) genes, were detected as expressed only in leaves of Shandong and Yukon plants, respectively. Thus the expression of less than 1% of the reference genome annotated as encoding proteins was found to be accession-specific. This outcome was surprising in that in a comparison between leaf transcriptomes of Yukon and Shandong plants, Champigny et al. (2013) found more genes (2.8%) to be expressed in Shandong but not Yukon plants. Again, the discrepancy between the two estimates for accession-specific differences in expression is likely due to the different sequencing platforms used in the two studies with the improved depth given by Illumina here enabling the detection of rare transcripts. We also detected a small number of genes that were only expressed in the leaf libraries of plants from the SWW2 and YWW2 treatments (Table 4.3). To be included in this small group, transcripts associated with these genes had to be found in libraries sequenced from duplicate plants. The number of uniquely expressed genes detected in WW2 plants was 37 for Shandong and 7 for Yukon plants representing nearly half of the total number of genes detected as accession-specific.

In summary, despite improved sequencing depth relative to previously published transcriptomes (Champigny et al. 2013), we found comparatively few genes that were expressed in an accession- and treatment-stage specific manner (Table 4.3). Based on their relatively small number, it seems unlikely that accession-specific genes expressed during only one stage of the drought protocol can explain all of the physiological differences observed between accessions with regards to drought response reported by MacLeod et al. (2015) and described in Chapter 3.

Table 4.3 - Number of genes common to all libraries versus those common to select treatment and/or accession-specific libraries.

Sample	Number of genes detected (RPKM >0)	% Total protein-coding genes[a]
All libraries	19530	73.60
SWW1, SD1, SWW2, SD2	83	0.30
YWW1, YD1, YWW2, YD2	20	0.08
SWW2	37	0.14
YWW2	7	0.03

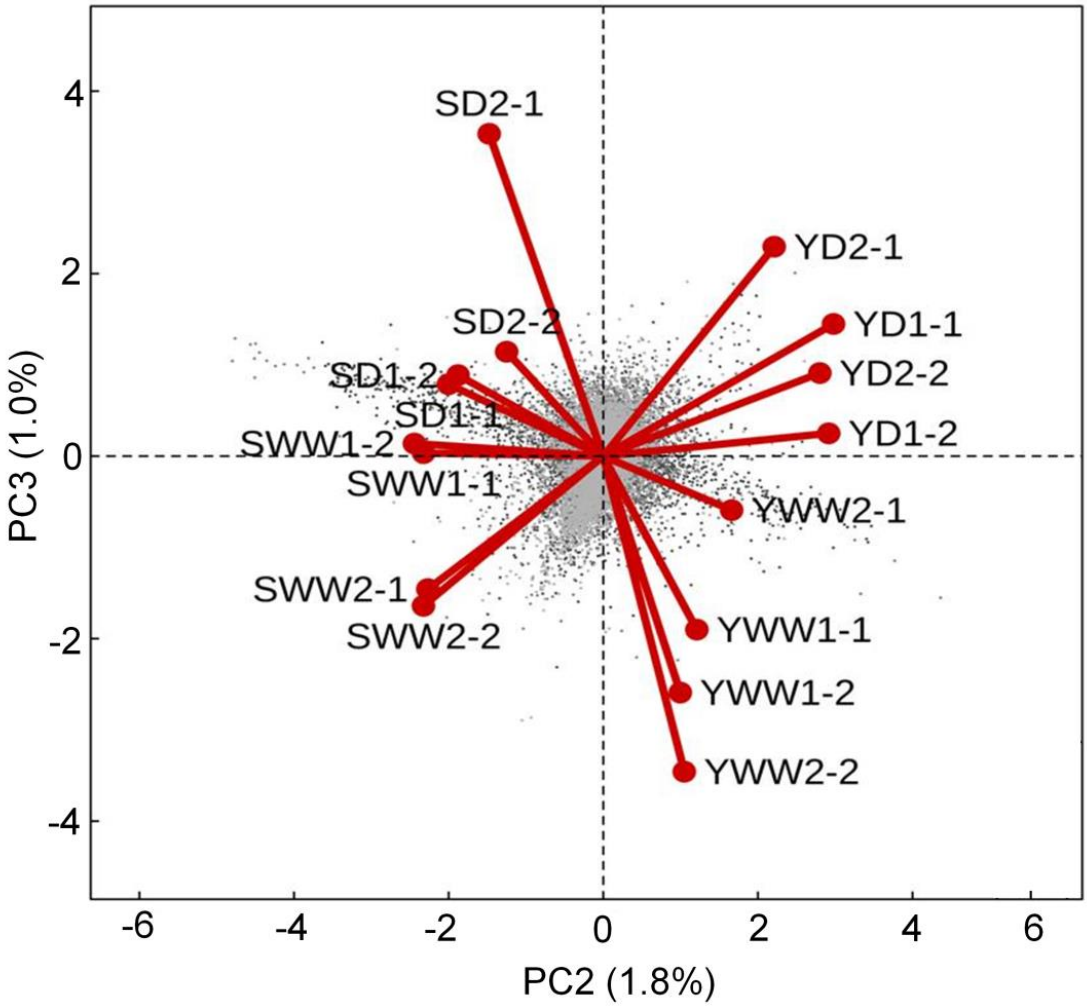
[a] Protein coding capacity predicted to be 26531 (Yang et al. 2013)

Principal component analysis of transcript abundance

Principal component analysis (PCA) was used to explore the variance in transcript abundance for the 16 plant cDNA libraries sequenced. PCA produces uncorrelated axes that each account for a portion of the variance in the data set and comparisons made between axes can be used to reveal patterns in the data. In the PCA bi-plot shown in Figure 4.1, the grey dots indicate the factor loadings for an individual gene (variable). The factor loadings show the extent to which the expression of each gene is correlated to a given PC axis. The biplot also shows the scores for each of the 16 libraries sequenced (observations) as red points. The scores for each plant library represent the average factor loadings for every gene in that plant for which transcripts were detected. Thus the close grouping of plant scores for each pair of repeat libraries is consistent with good agreement in the expression levels for all of the genes detected from those sources (Figure 4.1). For example, although the replicate libraries sequenced from well-watered controls (YWW1-1 and YWW1-2 or SWW1-1 and SWW1-2) were collected five days apart (see Methods), the relatively close positioning of the YWW1 and SWW1 scores for each pair of plants in this biplot suggests that the replicate libraries for each treatment were similar.

PC1 accounted for 95.5% of the variance in the data but a vertical alignment of scores was produced (data not shown) indicating that all plants sequenced had similar scores along the PC1 axis. This result was expected as Champigny et al. (2013) reported that a similar PCA analysis using *Eutrema* transcriptome data showed that PC1 only differentiated genes by the quantity of expression (i.e. high versus low expression). Although PC2 and PC3 accounted for far less variance than PC1, the PC2 and PC3 scores were differentiated by both accession and water availability (Figure

Figure 4.1 - Principal component analysis of transcript abundance for 16 cDNA libraries using RNA-Seq. Scores for individual plants (red vectors) as well as factor loadings for all genes (grey dots) are shown on a biplot of PC2 and PC3. The percentage of variance explained by a given axis is shown.


SD2-1
YD2-1
SD2-2
YD1-1
SD1-2
YD2-2
SD1-1
YD1-2
SWW1-2
SWW1-1
YWW2-1
SWW2-1
SWW2-2
YWW1-1
YWW1-2
YWW2-2
PC3 (1.0%)
PC2 (1.8%)
4
2
0
-2
-4
-6
-4
-2
0
2
4
6

4.1). To wit, PC2 distinguished the libraries of Yukon and Shandong plants while PC3 discerned drought-treated from well-watered or re-watered plants.

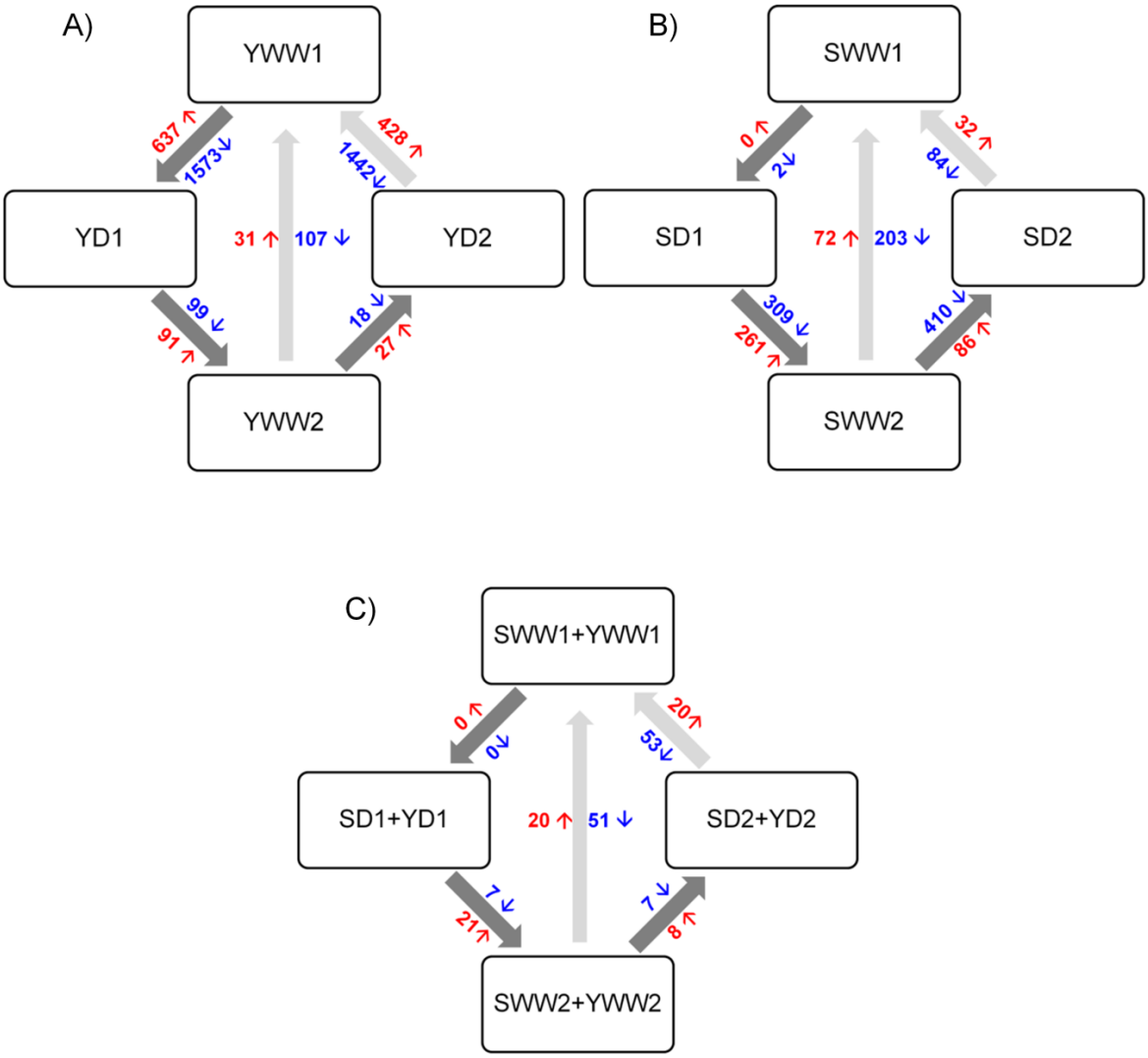
PCA was also used by Champigny et al. (2013) to explore differences in gene expression between the transcriptomes of well-watered, cabinet-grown Yukon and Shandong plants (library sources similar to the ones from WW1 plants sequenced here) with transcriptomes from field-grown Yukon plants. These authors also found that PC2 and PC3 explained far less of the variance between the three groups of plant cDNA libraries sequenced relative to PC1. While PC1 explained the majority of variation (>89%), the alignment of all library scores along the same position of PC1 means that the libraries are indistinguishable on this basis. The variation explained by PC1 is attributed to transcript abundance where highly expressed genes were the same between all libraries as were the same rarely transcribed genes. Rather, PC2 and PC3 were associated with the most meaningful biological comparisons in that accession and field versus cabinet differences could be distinguished (Champigny et al. 2013). Des Marais et al. (2012) used PCA to explore gene expression data from multiple natural accessions of *Arabidopsis* to identify "suites" of genes with high factor loadings for different PC axes. The authors identified physiological measurements that correlated with the expression of these different suites of genes. Thus a future avenue for the use of the cDNA libraries prepared for this project would be to use a bioinformatic approach involving correlation analysis with groups of genes in *Eutrema* that had high factor loadings for PC3 and the physiological measurements described by Macleod et al. (2015). Ideally, gas exchange

data should be included in these correlations given the relationship between photosynthesis and drought, competing activities that may be managed better by the Yukon as opposed to Shandong plants under drought stress (Figure 5.3).

Detecting differential transcript abundance using DESeq

Using DESeq, we compiled a list of 4317 differentially expressed genes (DEGs) from pair-wise comparisons between transcriptomes of plants sampled at the four stages of the drought assay described above (WW1, D1, WW2, and D2). An overview of the DEGs identified in the comparisons between the 16 transcriptomes is shown for each accession separately in Figures 4.2A and B and overlapping DEGs shared by both accessions are summarized in Figure 4.2C. The 4317 DEGs identified by this study represents a substantial increase in the number of drought-responsive transcripts identified in Yukon *Eutrema* as a previous study using a customized microarray chip only identified 101 drought-responsive genes (Wong et al. 2006). Overall, a striking impression instilled by Figure 4.2 is that more genes show up as DEGs for Yukon plants (3137 total DEGs) subjected to drought than for Shandong plants (981 total DEGs) exposed to the same treatments. Moreover, even in comparisons between transcriptomes of Shandong plants where several hundred DEGs are found (eg, 261 up- and 309 down-regulated genes between SD1 and SWW2), there are only 21 up- and 7 down-regulated genes that are common to the transcriptomes for both accessions that show the same direction of change (Figure 4.2C). The finding that Yukon plants exhibited a stronger transcriptional response

Figure 4.2 - Graphic representation of differentially expressed genes (DEGs) between transcriptomes of Yukon and Shandong *Eutrema* plants at different stages of a progressive drought treatment. Comparisons were made between *Eutrema* leaf cDNA libraries of (A) Y, Yukon and (B) S, Shandong plants at four stages of the drought treatment: well-watered (WW1), during an initial drought (D1), following re-watering after the initial drought (WW2), and during a second drought (D2). (C) Shows the number of overlapping DEGs between Yukon and Shandong expression data. Two cDNA libraries were sequenced at each drought stage. Dark grey arrows delineate comparisons between DEGs corresponding to the experimental progression of the drought assay. Light grey arrows correspond to comparisons for DEGs that were made *in silico* and are not a product of an experimental shift that was empirically tested. Red and blue numbers indicate the number of expressed genes up-regulated and down-regulated, respectively. Genes were identified as differentially expressed using DESeq software with a 0.05 false discovery rate (FDR) cutoff (see Materials and Methods).


A)
YWW1
YD1
YD2
YWW2
637
1573
428
1442
31
107
99
91
18
27
B)
SWW1
SD1
SD2
SWW2
0
2
32
84
72
203
309
261
410
86
C)
SWW1+YWW1
SD1+YD1
SD2+YD2
SWW2+YWW2
0
0
20
53
20
51
7
21
7
8

to drought treatment, in having a greater number of DEGs than Shandong plants, is not consistent with predictions based upon an analogous comparison performed in *Arabidopsis.* Juenger et al. (2010) compared the transcriptional response of two natural *Arabidopsis* accessions to water deficit and found that the accession originating from the more arid climate had fewer DEGs than the accession from a more temperate region. Thus, one could reasonably predict that the Yukon plants, from the semi-arid Yukon Territory, would show fewer DEGs than the Shandong accession, a genotype originally sourced from a more temperate environment featuring greater precipitation (Guevara et al. 2012). However, the converse was found (Figure 4.2A and B) when well-watered Shandong and Yukon plants were exposed to the initial drought treatment. One reason for this apparent reversal between the number of DEGs found for Yukon and Shandong plants with drought exposure may relate to underlying differences in drought tolerance thresholds between *Eutrema* and *Arabidopsis.* Specifically, *Eutrema* can lose more water than *Arabidopsis* while still maintaining turgor (Lugan et al. 2010) and *Eutrema* plants withstand repeated exposure to progressive water deficits (Xu et al. 2014, Macleod et al. 2015). With respect to *Eutrema* accessions, Yukon plants show a greater capacity to tolerate repeated water deficits relative to Shandong plants (Xu et al. 2014, Macleod et al. 2015). These reports have shown that *Arabidopsis* and Shandong *Eutrema* plants mainly employ drought avoidance strategies in response to water deficits (MacLeod et al. 2015, Bouchabke et al. 2008) while Yukon plants employ aspects of both avoidance and tolerance strategies (MacLeod et al. 2015). Thus the greater number of genes undergoing

changes in expression with drought for Yukon plants is consistent with the proposal that mechanisms underlying drought tolerance require a greater recruitment of diverse gene products to elicit a positive response compared to mechanisms responsible for drought avoidance.

Exploring biological processes associated with response to water deficit

An important objective of RNA-Seq is to identify DEGs and use this information to provide insight into beneficial biological processes underlying stress tolerance. In this regard, the number of DEGs detected using next-generation sequencing approaches can vary considerably. For example, a recent study of switchgrass sampled at multiple stages of drought identified more than 10000 DEGs (Meyer et al. 2014) whereas a study of drought response in two varieties of quinoa identified fewer than 500 DEGs (Raney et al. 2014). Thus the approximately 4000 DEGs identified here is neither exceptionally high nor low. However, studying a set of 4000 genes is not trivial and any means of identifying genes that are over-represented among those undergoing expression changes can offer insights into the biological changes underway. Accordingly, a GO enrichment analysis was performed comparing the proportion of GO terms among the lists of DEGs identified in Figure 4.2 to the proportion expected for all annotated genes in the predicted genome. A full list of significantly enriched GO terms is shown in Appendix 1 and four specific comparisons are highlighted below.

First drought response stage: WW1 to D1

The most marked difference between the accessions identified through DESeq analysis was in the number of genes undergoing a significant quantitative change between the WW1 and D1 stages. Analysis by DESeq led to the discovery of over 2000 DEGs in drought-treated Yukon plants, indicative of a strong transcriptional response in Yukon plants at this stage, compared with only two DEGs in drought-treated Shandong plants (Figure 4.2A and B). We compared the list of 2210 DEGs identified in this study using DESeq for Yukon plants to the 101 drought-responsive genes that were identified for the same accession by Wong et al. (2006), and found 64 overlapping genes including the orthologues of well-known drought-responsive genes such as *LIPID TRANSFER PROTEIN 4 (LTP4)* and *ABA-INSENSITIVE 2 (ABI2)*. Notably, Macleod et al. (2015) showed that by D1-10%, the same stage when leaves were harvested for RNA extraction and transcriptome sequencing, solutes had already accumulated in drought-treated Yukon but not Shandong plants (Figure 3.3). Thus the active accumulation of solutes observed in Yukon plants and strong transcriptional response are two ways by which the drought response strategy exhibited by Yukon and Shandong plants differ.

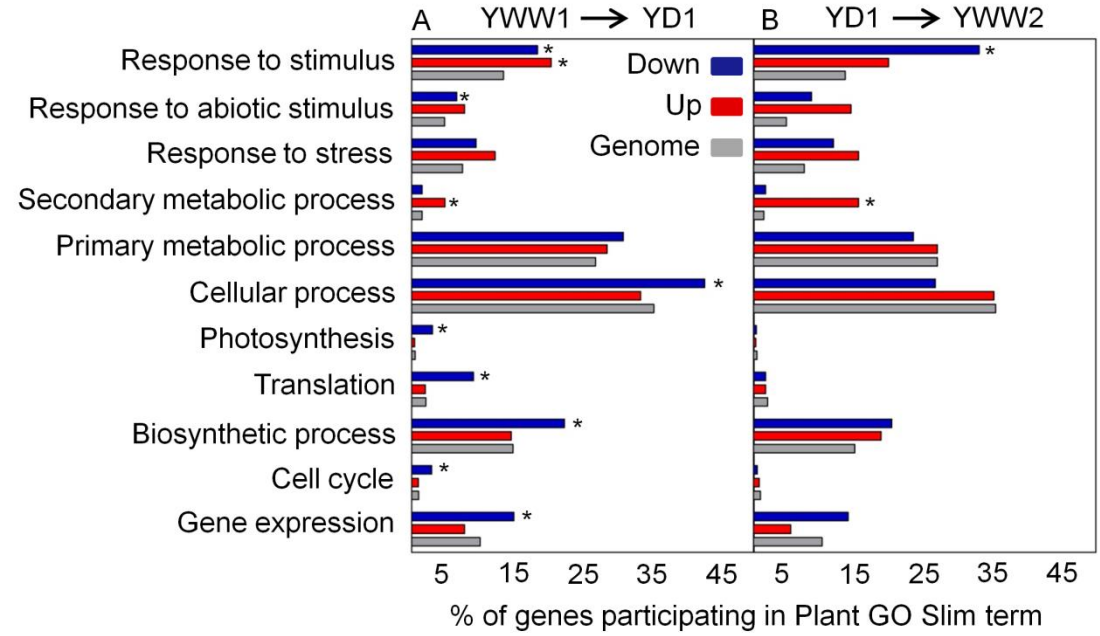
A GO analysis of the set of genes with altered expression between YWW1 and YD1 transcriptomes revealed that the terms "secondary metabolic process" and "response to stimulus" were both enriched among the 637 DEGs that were up-regulated by drought treatment. The enrichment of genes associated with secondary metabolism is not surprising given that drought exposure is reported to increase the content of products of

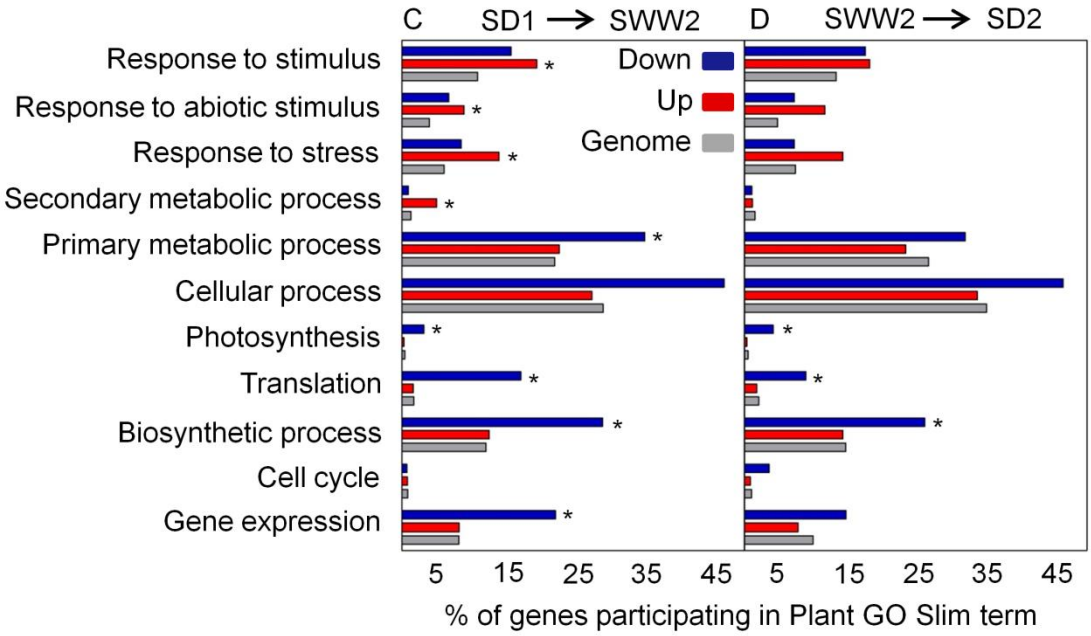
secondary metabolism including terpenoids, alkaloids, and sulphur containing glucosinolates (Gershenzon 1984). Additionally, the terms "translation" and "photosynthesis" were enriched among the 1573 DEGs that were down-regulated by drought treatment (Figure 4.3A). Similar GO-enrichment results involving the up-regulation of stress-response pathways and down-regulation of processes associated with plant growth have been reported in both drought-tolerant species such as *Craterostigma plantagineum* (Rodriguez et al. 2010) and less drought-tolerant species such as *Arabidopsis* (Harb et al. 2010). A parallel comparison of Shandong plants using SWW1 and SD1 was not performed because of the low number of DEGs (two). From the comparison made between WW1 and D1 stages it is clear that YD1 plants undergo a more robust transcriptional response than SD1 plants involving many genes that have been previously associated with stress-responses.

Second drought response stage: D1 to WW2

Comparisons were made between D1 and WW2 libraries in order to identify DEGs between plants in the midst of a water deficit compared to plants that have regained turgor and are recovering from drought exposure. As discussed earlier, only two DEGs were identified from comparisons of SWW1 and SD1 libraries and hence we expected the transcriptional response to re-watering to remain minimal in Shandong plants and more pronounced in recovering Yukon plants. Instead, we found that the transition from drought to recovery was associated with a total of 570 DEGs in Shandong plants

Figure 4.3 - Gene Ontology (GO) enrichment analysis of differentially expressed genes involved in drought response in *Eutrema.* GO enrichment was performed on sets of differentially expressed genes (DEGs) identified from pair-wise comparisons (see Fig. 4.2) for A) drought-treated (YD1) and well-watered (YWW1) Yukon plants, B) drought-treated (YD1) and re-watered (YWW2) Yukon plants, C) drought-treated (SD1) and re-watered (SWW2) Shandong plants and D) re-watered (SWW2) and subsequently drought-treated (SD2) Shandong plants. GO categories associated with DEGs for each panel were identified. The direction of comparisons made is shown by an arrow where genes were identified as up-regulated (red bars) or down-regulated (blue bars) between the treatment stages. The proportion of genes expected for each category is based upon their abundance predicted from the whole genome annotations (grey bars). An asterisk (*) highlights categories where participating DEGs are significantly greater (FDR < 0.05) than the proportion expected from the genome.


A YWW1 → YD1
B YD1 → YWW2
Down
Up
Genome
Response to stimulus
Response to abiotic stimulus
Response to stress
Secondary metabolic process
Primary metabolic process
Cellular process
Photosynthesis
Translation
Biosynthetic process
Cell cycle
Gene expression
5 15 25 35 45 5 15 25 35 45
% of genes participating in Plant GO Slim term


C SD1 → SWW2
D SWW2 → SD2
Down
Up
Genome
Response to stimulus
Response to abiotic stimulus
Response to stress
Secondary metabolic process
Primary metabolic process
Cellular process
Photosynthesis
Translation
Biosynthetic process
Cell cycle
Gene expression
5 15 25 35 45 5 15 25 35 45
% of genes participating in Plant GO Slim term

compared to only 190 DEGs in Yukon plants (Figure 4.2A and B) with only 28 genes in common (Figure 4.2C). Thus, Shandong plants underwent a greater transcriptional response upon re-watering following drought than during the initial drought exposure.

A GO-enrichment analysis of the 261 DEGs that were up-regulated in SD1 relative to SWW2 plants showed that the terms "response to stimulus", "response to abiotic stimulus", "response to stress", and "secondary metabolic process" were all significantly enriched. Among the 309 DEGs that were down-regulated in SD1 plants were "primary metabolic process", "photosynthesis", "translation", " biosynthetic process", and "gene expression" (Figure 4.3C). Thus many biological processes typically affected by drought such as secondary metabolism and photosynthesis (Harb et al. 2010, Rodriguez et al. 2010) are altered in SD1 plants relative to SWW2 plants. The larger transcriptional response of SD1 plants when considered relative to SWW2 (570 DEGs) compared to SWW1 plants (2 DEGs) suggests that upon re-watering, the expression of many drought-associated genes is changed resulting in stress-response genes being detected as differentially expressed for the first time. For example, genes associated with the GO term "secondary metabolic process" were not differentially expressed in Shandong plants as they progressed from SWW1 to SD1. However, genes associated with secondary metabolism were differentially expressed after SD1plants were re-watered (SWW2). Thus, the expression of genes associated with secondary metabolism must be repressed in SWW2 plants in order for them to be identified in comparisons of SD1 and SWW2 plants but not SWW1 and SD1 plants. Based on the number of DEGs identified through

comparisons of SD1 and SWW2 plants, and the GO analysis of this same set of genes, it appears that Shandong plants quickly revert to processes associated with growth once water is available.

Third drought response stage: WW2 to D2

DESeq analysis was also used to identify genes involved in the transcriptional response to D2. MacLeod et al. (2015) reported that the initial exposure to water deficit (D1) altered the way that Yukon plants responded to the second water deficit (D2). For example, both leaf water content and leaf Ψ_s were different for Yukon plants during D2 compared to D1 and there was a reproducible increase in the length of time taken before Yukon leaves wilted during D2 relative to Shandong plants (Figure 3.1, 3.3 and 3.4). In contrast, Shandong plants responded similarly to the two drought exposures with no discernible changes in leaf water content or leaf Ψ_s. Based on these physiological outcomes, we expected the transcriptional response of Yukon plants to be distinctive between D1 and D2 while D1-associated changes for Shandong plants were expected to be repeated during D2. The experimental results, however, were not entirely consistent with this prediction.

As expected, the transcriptional response of YD2 plants was distinct from that of YD1 plants in terms of the number of DEGs identified in the pair-wise comparisons shown in Figure 4.2A. While the initial drought treatment (YWW1 vs. YD1) resulted in the differential expression of more than 2000 genes, the second drought treatment (YWW2 vs. YD2) resulted in only 45 DEGs with 40% of the genes down-regulated.

Although only 45 genes are differentially expressed between YWW2 and YD2 plants, if we compare YD2 and YWW1 transcriptomes, 1870 DEGs distinguish plants that started the experiment from those that completed the second drought exposure. A total of 1193 (223 up, 970 down) or 64 % of these DEGs were present in both the YD2 and YD1 transcriptomes as differentially expressed relative to well-watered Yukon plants (YWW1). The fact that only 45 DEGs distinguished YWW2 and YD2 transcriptomes is consistent with gene expression in YWW2 plants being more similar to YD2 plants than YWW1 plants. One conclusion of this outcome is that for Yukon plants drought-responsive genes are up-regulated with D1 and then expression remains, at least somewhat, unchanged through recovery and D2.

Our prediction of a transcriptional response for SD2 that resembled the one observed in SD1 plants was not supported by the DESeq results (Figure 4.2B). While only two genes showed differential expression in response to the initial drought (SWW1 versus SD1), 496 DEGs were identified between re-watered SWW2 plants subjected to drought during D2 (SD2) with 83% of these genes being down-regulated as plants transitioned from a well-watered to water-deprived state.

With the lack of drought-responsive DEGs for the D1 treatment of Shandong plants, the inclusion of WW2 and D2 treatments were important additions in that they allowed for the detection of 979 drought-responsive DEGs that would not have been found in Shandong comparisons otherwise (Figure 4.4B). Analysis of the 410 DEGs that were down-regulated in SD2 plants identified three terms: "biosynthetic process" as well as

"photosynthesis" and "translation"(Figure 4.3D). The terms associated with photosynthesis and translation were also the most significantly enriched among the down-regulated, drought-responsive DEGs found between comparisons of SD1 and SWW2 transcriptomes. Thus for SD1 and SD2 plants, there is overlap in the behaviour of genes with similar predicted GO term functions in that genes associated with translation, biosynthetic processes, and photosynthesis are down-regulated.

Drought response studies: Approaches and interpretations

A WW1 and WW2 comparison is a natural product of microarray experiments where a pair-wise comparison usually involves a control and “treated” sample (Hayano-Kanashiro et al. 2009, Kang et al. 2011). Our approach is different in that the data we generated are not relative expression values enabling us to establish quantitatively how the expression levels of different genes changed between drought-treated and re-watered plants. Moreover, few studies take a progressive approach where recovery from drought is followed by a successive drought treatment (Galle et al. 2011).

Studies of progressive drought using alfalfa (Kang et al. 2011) and switchgrass (Meyer et al. 2014) both show that fewer genes are differentially expressed during recovery than during a severe water deficit. Thus the finding that the number of DEGs (Figure 4.2A and B) associated with YWW2 relative to YWW1 plants, at 138, being lower than the number of DEGs associated with YWW1 versus YD1 plants at 2210 is in agreement with these reports. However, the number of DEGs for SWW2 plants compared

to SWW1 at 275 is much higher than the 2 DEGs between SWW1 vs SD1, an outcome that is inconsistent with both the Yukon results and the previously published reports. One potential explanation for this difference may be found in how Shandong plants perceive the initial drought. Specifically, we found that none of the physiological measurements made on Shandong plants during D1 were different than ones for well-watered control plants (Figure 3.3 and 3.4). This infers that Shandong plants were not stressed or did not sense stress at D1-10% FTSW possibly explaining why only two DEGs were identified.

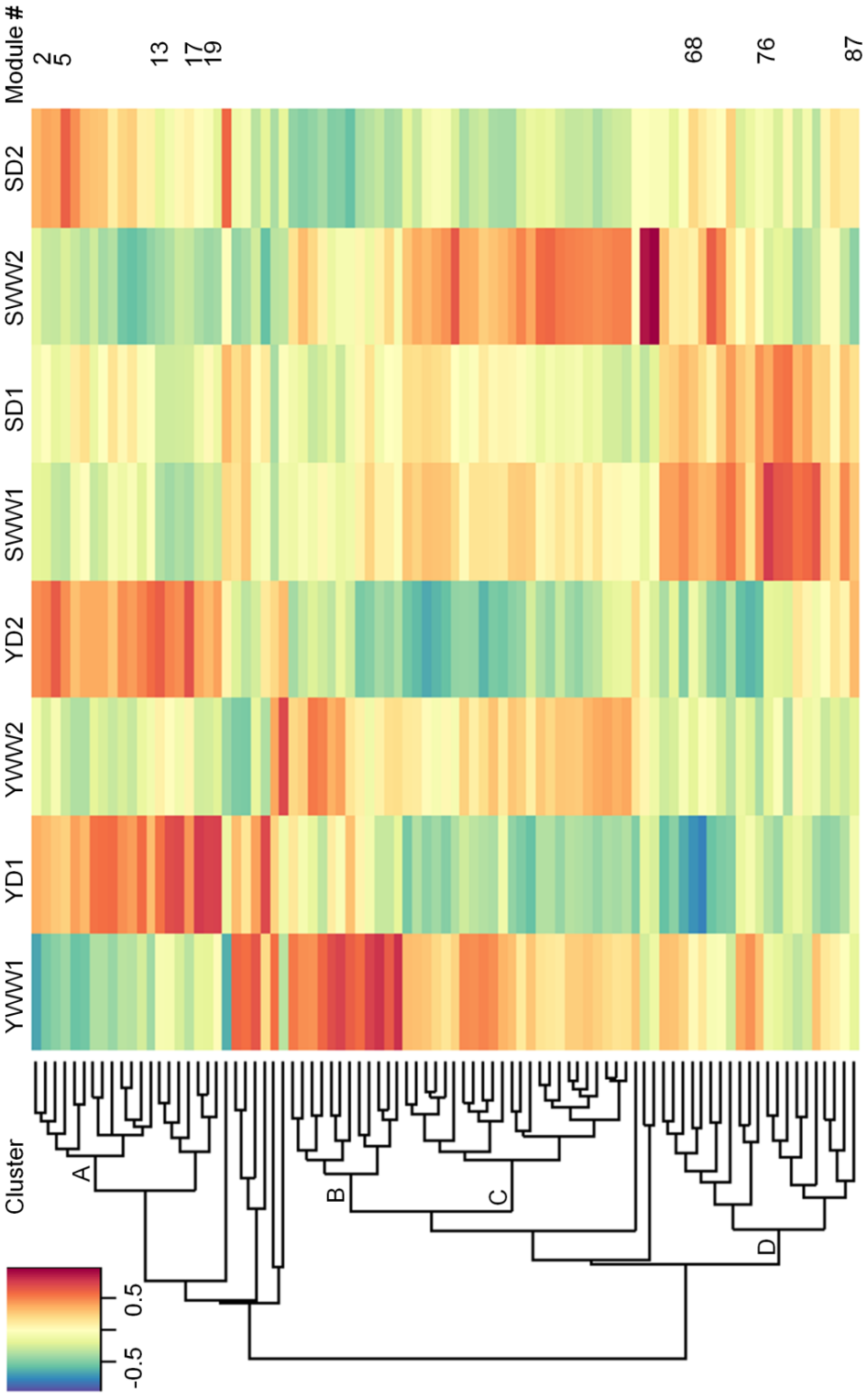
Discerning patterns among drought-responsive genes using weighted gene correlation network analysis (WGCNA)

As discussed above, the finding that only 45 DEGs were identified when YWW2 plants were subjected to a second drought (YD2) was surprising and stands in contrast to the higher number of DEGs (496) found for the Shandong accession in the same comparison (Figure 4.2A and B). What is difficult to discern from these quantitative comparisons are patterns of correlated gene expression changes among the DEGs. For example, a Yukon plant undergoing D1 treatment may show the highest number of genes undergoing changes in expression (2210; Figure 4.2A) but how many of those genes remain up- or down- regulated in the YWW2 and/or YD2 transcriptomes? One approach to addressing this question is through weighted gene correlation network analysis (WGCNA). WGCNA is used to identify genes with similar changes in expression as a function of treatment(s) where genes that are co-responsive and potentially co-regulated occupy the same module

(Langfelder and Horvath 2008). Modules can be grouped together by hierarchical clustering to identify clusters of modules with similar correlation scores. As genes in common signalling or biochemical pathways may be co-responsive to similar stimuli, WGCNA has been used to identify genes associated with related biological roles through "guilt by association" inferences drawn from genes occupying the same module or cluster (Coneva et al. 2014).

Figure 4.4 is a correlation heat map for the WGCNA produced from the 4317 DEGs identified using the 16 cDNA libraries. The correlation-based heat map shows how correlated the expression of genes within a module are to each other. Horizontal bands of a similar colour indicate tight co-expression between modules with variable colour between modules in a row showing differential expression relative to the treatments. A list of the correlation scores for each module is shown in Appendix 2. Overall, the patterns of correlation between modules of Yukon plants produce vertical bands that show a more consistent pattern for the pairs of well-watered (YWW1/2) or drought-treated (YD1/2) plants. This suggests that there is, on average, a good consensus of co-expressed genes that respond to the presence of adequate water or drought. In contrast, there is almost no consistent vertical or horizontal pattern of coloration for the same modules generated from the transcriptome data for Shandong plants. For example, clusters A and C contain modules that have more similar correlation scores for YD1 and YD2 than is true for SD1 and SD2. In this figure, modules comprised of genes showing

Figure 4.4 - Heatmap representing the correlation strength between modules of differentially expressed genes and different stages of a water deficit treatment in *Eutrema*. Weighted gene correlation network analysis (WGCNA) was used to identify modules of co-expressed genes in Yukon (Y) and Shandong (S) *Eutrema.* Pearson's correlation coefficient describes the correlation between modules and well-watered controls (WW1; 100% FTSW), plants experiencing a drought (D1 at 10% FTSW), recovering from wilting (WW2 at 100% FTSW), and experiencing a subsequent drought (D2 at 10% FTSW). Modules containing genes with positively correlated (increased) expression under a particular treatment are red while modules containing genes that are more negatively correlated (decreased) expression are blue. The correlation scores for all modules are provided in Appendix 2. Hierarchical clustering was used to group modules with genes showing similar correlation in expression patterns. Four clusters containing similar correlation patterns originating from the indicated nodes were identified for ease of reference in the Results and Discussion section.


Module #
2
5
13
17
19
68
76
87
SD2
SWW2
SD1
SWW1
YD2
YWW2
YD1
YWW1
Cluster
A
B
C
D
0.5
-0.5

increased expression with drought are on the red end of the scale and modules of genes showing reduced expression with drought are blue. Figure 4.4 has more modules that are blue and hence comprised of genes whose co-expression shows reduced expression with drought treatment, an expected visual outcome because more than 50% of the DEGs identified in Yukon and Shandong plants were down-regulated with drought. The heat map also shows some interesting accession-specific patterns among the modules of co-expressed genes. For example, cluster A (modules 1-20) contains genes that are up-regulated during both D1 and D2 in Yukon plants but the genes in this clusters are, for the most part, not highly correlated to the expression of the same genes in SD1 transcriptomes. Interestingly, while little agreement in co-expression is found with SD1, the genes in some of these modules such as module 5 are highly correlated between YD1, YD2 and SD2 (Figure 4.4). The modules of co-expressed genes in clusters B and C contain genes that are co-expressed under well-watered conditions and down-regulated, to varying degrees, for Yukon and Shandong plants exposed to drought. The modules in cluster D provides an example where co-expressed genes are largely down-regulated once well-watered Yukon plants are exposed to drought but very contradictory impressions are given by the corresponding groups of co-expressed genes for unstressed or drought-stressed Shandong plants (see modules 68, 76 or 87 for examples).

The four dehydrin-encoding genes profiled by RT-qPCR by MacLeod et al. (2015 ; Figure 3.7) belong to modules that comprise cluster A with *EsRAB18* and *EsRD29A* in module 19, *EsRD22* in module 17, and *EsERD1* in module 2 (Figure 4.4). The transcripts

associated with *EsRAB18* and *EsRD29A* were significantly less abundant in DESeq comparisons of SWW2 relative to both SD1 and SWW1 libraries. The repression of *EsRAB18* and *EsRD29A* expression after Shandong plants were re-watered likely explains why these genes were identified as differentially expressed in the comparison between SD2 and SWW2 transcriptomes but not the SD1 and SWW1 comparison.

The WGCNA correlation heat map also offers clues as to why fewer DEGs were detected in the comparisons involving the YWW2 and YD2 transcriptomes relative to the DEGs identified between the YWW1 and YD1 transcriptomes (Figure 4.2). Many of the gene modules with highly co-expressed genes for YD1 and YD2 showed weaker evidence of co-expression in YWW2 compared to YWW1. The reduced expression of many genes in re-watered Yukon plants following D1 indicates that expression does not return to pre-stress levels for many genes and this lack of return to the status quo may benefit Yukon plants in terms of increased tolerance following drought. MacLeod et al. (2015) reported that Yukon plants better tolerate repeated drought exposure relative to Shandong plants with benefits seen in solute accumulation and a longer time to turgor loss (Figure 3.1). Furthermore, Griffith et al. (2007) showed that exposure to an initial drought improved freezing tolerance in Yukon plants from -19°C to -21°C. It is possible that by not reverting to pre-stress levels, the expression of stress-responsive genes enables the plant to retain a complement of gene products that serve as a "molecular buffer" for prolonged stress protection in Yukon plants or more rapidly harness the products they encode should the stress return. Modules that have a correlation pattern consistent with genes encoding

products comprising a "molecular buffer" include modules 3, 13 and 17 where genes are up-regulated in both YD1 and YD2 plants and the baseline expression is higher in YWW2 relative to YWW1. The average normalized expression levels for all the genes in modules 3, 13 and 17 are shown in Appendix 3. Module 17 contains *EsRD22*, a gene that responds uniquely to drought in Yukon plants (Wong et al. 2006). Although the expression of the genes in module 17 is reduced following re-watering, the average normalized expression levels of all the genes in module 17 at YWW2 are nearly 2-fold higher than prior to the initial drought (YWW1). In comparison, the average normalized expression levels for all genes in module 17 at SWW2 and SWW1 were more similar. Thus, some level of expression is maintained in YWW2 plants after the initial drought exposure but expression returns to pre-drought levels in SWW2 plants.

Conclusions

The analysis of the 16 transcriptomes shows that Yukon and Shandong plants subjected to progressive drought treatments have divergent transcriptional responses to water deficit and following recovery from water deficit. The divergent transcriptional response between the accessions to drought treatment is noteworthy and it underscores the importance of employing a comparative genomics approach in arriving at a more meaningful interpretation of transcriptomic data. That is, had we only used the Shandong accession for this study our conclusion could well have been that drought does not induce

a strong transcriptional response in *Eutrema* as a species given the 2 DEGs that we detected. Instead, through a comparison using two accessions we have found evidence that the drought response in Yukon *Eutrema* plants involves a robust transcriptional response involving over 2000 DEGs. Moreover, had we only used a single drought treatment we would not have discovered that the comparatively strong transcriptional response of Yukon plants to a first drought exposure would be followed by a far more modest level of change among genes expressed during a subsequent drought treatment. In contrast, for Shandong plants the response to a second drought exposure led to a comparatively stronger transcriptional response (Figure 4.2). The addition of a re-watering and recovery step was particularly informative. Identifying DEGs in drought-treated Shandong plants (SD1 or SD2) relative to plants that were re-watered (SWW2) and recovered from an initial drought revealed hundreds of DEGs in Shandong plants that would have otherwise been missed (Figure 4.2).

Transcriptome profiling has also enabled us to identify an interesting difference between how *Eutrema* responds to two sources of osmotic stress, namely drought and salinity. The well-supported salinity tolerance paradigm for this halophyte involves a pattern of higher basal expression with little induction for genes associated with many salinity tolerance traits (Inan et al. 2004, Wong et al. 2006; Lugan et al. 2010, Guevara et al. 2012). If this paradigm held for drought, we would expect to find a relatively small number of genes responding to the imposed water deficit in *Eutrema,* particularly the more drought-tolerant Yukon accession. That expectation did not hold. Instead the

transcriptional response to drought is much more dynamic and that holds true for both *Eutrema* accessions. We also found no evidence that drought-tolerance associated genes are constitutively up-regulated in either accession and we found evidence that gene regulation differs between the accessions.

One objective of this research was to identify DEGs encoding products associated with the differential physiological responses displayed by the two accessions in response to water deficit (Macleod et al. 2015). The use of correlated changes in gene expression that can then be mapped to associated changes in physiological measurements offers a feasible route towards identifying traits and gene products underlying the natural tolerance that *Eutrema* displays to water deficits. Using WCGNA we found that some drought-responsive genes, including those encoding dehydrin family proteins such as *EsRD22*, are induced in Yukon plants during D1 and remain up-regulated thereafter (Figure 4.4). Of the two accessions, Yukon plants have a heightened capacity to cope with water deficits following an initial exposure to drought (Figure 3.1) so the drought-responsive induction and residual expression of some genes in Yukon plants (YWW2) that have recovered turgor suggests that Yukon plants may generate a stress-protective complement of gene products that remain even after the immediate stress is removed by re-watering. Conceivably, this complement could provide a "molecular buffer" that enables Yukon plants to respond to subsequent drought exposures more rapidly and in a manner requiring fewer changes at the transcriptional level (Figure 4.2). DEGs encoding products conferring this level of protection would include attractive target genes for

improving plant yields under water deficit, particularly if their beneficial roles can be validated and their functions elucidated.

Authors' contributions

MJRM performed the plant growth and prepared the RNA. WWS performed the assembly, PCA and DESeq analysis. MJC and MJRM prepared the cDNA libraries. CM performed the WGCNA. MJRM and EAW designed the experiments, performed data analysis and prepared the manuscript.

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CHAPTER FIVE

Gas exchange, water status, and gene expression using natural populations and cabinet-grown plants of the Yukon accession of *Eutrema salsugineum*

Preface

This work includes data that was collected from two visits to Yukon field sites (2011 and 2014). The first trip was in June 2011 and the research was conducted by Mr. Philip Carella and myself. I performed all of the plant water status measuremets and we both made tissue collections and photographs. The second visit to the Yukon in June, 2014 was made by Ms. Amanda Garvin and Mr. Carella. The gas exchange measurements were taken on this trip by Amanda after we discussed the logistics of measuring gas exchange under field conditions and the type of samples to select for her measurements. Amanda also performed the experiment and gas exchange measurements that are presented in Fig. 4 under my supervision as a summer student in 2013. All other experiments as well as the analysis and preparation of this chapter were performed by the author under the supervision of Elizabeth Weretilnyk. This chapter has been formatted as a manuscript following the guidelines for Physiologia Plantarum.

Gas exchange, water status, and gene expression using natural populations and cabinet-grown plants of the Yukon accession of *Eutrema salsugineum*

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Abstract

Eutrema salsugineum in the Canadian Yukon must tolerate saline soils, high light levels, hot or freezing cold temperatures, and infrequent precipitation. In light of the exceptional capacity of Yukon *Eutrema* to tolerate multiple abiotic stresses, we performed gas exchange and water status measurements of field plants to provide basic physiological information about these plants in their natural environment. We then compared this data to that from plants grown under controlled environment conditions exposed to a simulated water deficit. The field plants used ranged in development from vegetative rosettes to reproductively mature plants setting seeds while all cabinet plants were vegetative rosettes. At all stages, water and solute potentials for field plants averaged below -2.0 and -2.4 MPa, respectively; values significantly lower than the most water-stressed cabinet-grown plants. Stomatal conductance rates from field-grown plants averaged 84.8 mol $m^{-2}s^{-1}$, and rates similar to those found for cabinet-grown plants that experienced water deficits. In contrast, assimilation rates were more variable for field plants ranging from 2–8 μmol m^{-2} s^{-1} compared to 4–6 μmol m^{-2} s^{-1} for cabinet grown plants. RT-qPCR was used to measure the steady-state transcript levels of four dehydrin-encoding genes (*ERD1, RAB18, RD22,*and *RD29A*) in the rosette and cauline leaves of several randomly selected field plants. Transcript levels for these genes were found to be consistent between the field plants sampled, an outcome that was not anticipated given their variable water status and gas exchange values and the natural variation in environmental conditions. This study shows that despite the poor accessibility to *Eutrema* in its habitat, it is feasible

nonetheless to harvest tissue for nucleic acids and perform measurements of water status and gas exchange in situ at Yukon field sites. The data obtained from Yukon field plants is consistent but often qualitatively and quantitatively different than that provided by control or deliberately stressed cabinet-grown plants. Thus a deeper understanding of how this extremophile has adapted to its environment will likely only transpire when research with this plant routinely includes field research in its natural habitat complemented by cabinet studies.

Abbreviations

TSW, fraction of transpirable soil water; IRGA, infrared gas analyzer; PAR, photosynthetically active radiation; PPFD, photosynthetic photon flux (area) density

Introduction

Studying plant responses to water deficits under cabinet or greenhouse conditions allows researchers to control the duration and the severity of an imposed water deficit. The ability to control these experimental parameters has allowed researchers to study different stages in the response of a plant to drought (Harb et al. 2010) and to compare how plants respond to a range of water deficit severity (Kim et al. 2012, Rengel et al. 2012). However, the challenges encountered by plants exposed to simulated water deficits by withholding water are not the same as those faced by plants during water deficit brought on by limited precipitation in the field. Response to a heterogeneous mixture of abiotic

and biotic stresses in the field requires a response tailored to the specific combination of conditions encountered by the plant at any given time (reviewed by Mittler and Blumwald, 2010). For example, using *Arabidopsis*, Sewelam et al. (2014) showed that the transcriptional response to a combination of heat, mannitol, and salinity was distinct from the response to any of those stresses individually. These researchers observed that genes encoding protective proteins such as late embryogenesis abundant (LEA) and small heat shock proteins (sHSP) were expressed at higher levels under multiple stress conditions compared to conditions where plants were exposed to the individual stresses. Thus approaches that can identify traits that are important to plants responding to not only water deficit but also other stresses concurrent with drought are desirable (Tester and Bacic 2005). However, there are a number of practical challenges associated with studying plant response to multiple stresses under field conditions (Jubany-Marí et al. 2010). These include the inability to control for variability in temperature and to isolate the plants under study from biotic interactions with pests or pathogens that could confound results.

The extremophile plant *Eutrema salsugineum* is an interesting model for studying response to multiple concurrent stresses as the Yukon accession of *Eutrema* grows naturally in the Canadian Yukon where plants are exposed to a semi-arid and sub-arctic climate as well as highly saline soils (Guevara et al. 2012). The Shandong accession of *Eutrema* also grows in highly saline soils but precipitation is more abundant and temperatures are more temperate (Champigny et al. 2013). In addition to limited water

availability, the growing season for Yukon *Eutrema* is marked by extremely long photoperiods, including near full days of sunlight. The unique combination of continuous exposure to osmotic stress and high levels of irradiance makes Yukon *Eutrema* an interesting plant in which to compare the traits expressed under field conditions with those expressed in cabinet-grown Yukon and Shandong plants. Guevara et al. (2012) compared the responses of cabinet-grown Yukon plants exposed to controlled drought, cold, or salinity stresses to the responses of field-grown Yukon plants. The natural variation in precipitation at the Yukon field site between two field seasons (2003 and 2005) was exploited to compare the transcriptional and metabolic response to unusually dry and high-precipitation periods. The relative abundance of glucose, galactose, and fructose increased in both field and cabinet-grown Yukon plants exposed to drought relative to field and cabinet plants that were not exposed to water deficits. Thus Guevara et al. (2012) showed that although the combination stresses encountered by field-grown plants are variable, there are similarities in how field and cabinet-grown plants respond to abiotic stress and in this case, water deficits.

There are many ways of identifying abiotic stress tolerance traits in plants, from exposing plants to conditions generated entirely in cabinets (Kasuga et al. 2004), to those comparing the changes of plants in cabinets to those found in the field (Nelson et al. 2007). The difficulty, however, remains in distinguishing plant responses that are simply perturbations due to poor conditions from those critical changes underlying acclimation and tolerance to stress. In addressing this challenge, we hypothesize that the physiological

and molecular traits that overlap between those expressed by field and cabinet-grown plants are most likely to include ones that are essential for tolerance to stress. Otherwise stated, stress responses that are observed only in cabinet-grown plants exposed to a given stress and not observed in similarly stressed field plants are unlikely to be traits essential for tolerance. At first approximation, however, *Eutrema* plants in the field bear little resemblance to cabinet plants. Field plants feature inflorescences comprised of bolts terminating in flowers and bolts display prominent cauline leaves whereas cabinet plants are characterized by a prominent rosette and bolts largely void of cauline leaves (Guevara et al. 2012). This raises a question about how dissimilar field and cabinet plants are with respect to other, less visible phenotypic features such as gas exchange and water status.

The objective of this work was to better characterize *Eutrema* plants in the field with respect to their gas exchange properties, water status (Ψ_w and Ψ_s), and expression of a select group of four drought-responsive dehydrin-encoding genes (MacLeod et al. 2015; Fig. 3.7). We compared measurements made on Yukon plants grown in cabinets or found in their natural habitat to the Shandong accession of *Eutrema* plants, where rates of stomatal conductance and assimilation have already been reported (Inan et al. 2004, Stepien and Johnson 2009, Eppel et al. 2014). To determine how gas exchange and water status properties were altered by stress, a state endemic to plants in the field, plants in cabinets were either well-watered or subjected to a water deficit. With respect to rates of assimilation and solute potential measurements we found variability, particularly between field plants. Our gene expression results were, by comparison, far more consistent than

we would have predicted given the range in the physiological determinations from field plants. The consistency in dehydrin gene expression for different individuals in the field suggests that relative to physiological means, transcript profiling of field plants offers a meaningful platform from which to compare the degree and nature of long term stress experienced by plants in natural environments.

Materials and methods

Field site

Cauline leaf tissue was harvested from mature, flowering Yukon *Eutrema* at three field sites near Whitehorse, Yukon: Site 1 (location: 60.847560°N, 135.699222°W), Site 2 (location: 60.931687°N, 135.173909°W), and Site 3 (location: 60.81098°N, 136.79213°W). Although tissue was harvested and used from three sites for the measurements below, the field-site of origin was not used as a treatment in the analysis. Tissue harvested from field plants was classified according to morphology as shown in Fig. 5.6: Phenotype 1: Vegetative plants dominated by rosette leaves, Phenotype 2: Plants lacking rosette leaves but displaying bolts with flowers but lacking mature siliques, and Phenotype 3: Plants lacking rosette leaves but displaying bolts bearing mature siliques. The field plants were of indeterminate age as our visit to the field sites was of short duration (about one week). Tissue was harvested and flash-frozen at the field site then shipped from the Yukon in a vapour shipper for subsequent analyses according to the sampling procedures described by Guevara et al. (2012).

Gas exchange analysis of Yukon *Eutrema*

Gas exchange measurements of transpiration (*E*), stomatal conductance (g_1), net assimilation (*A*), and sub-stomatal CO_2 (*Ci*) were performed with a CIRAS-1 infrared gas analyzer (IRGA) (PP Systems Intl.) fitted with a PLC-4 broad leaf cuvette. CO_2 levels were set to ambient (385 ppm) and water vapor was set to 70% relative humidity. Leaves were frequently less than the 2.5 cm^2 surface area assumed by the instrument. As such, a leaf area correction was applied to the data. The area of the leaf that was placed inside the cuvette was determined using Adobe Photoshop 8.0 software (O'Neal et al. 2002). The measurements used for generating the light response curves were made on intact leaves of well-watered, cabinet-grown Yukon and Shandong *Eutrema* plants. Plants used for these determinations were grown in flats where the pots could be spaced to allow the plants to produce long petioles that allowed leaves to be sealed in the cuvette while remaining attached to the plant. All of the remaining gas exchange measurements were performed on detached leaves. Unless otherwise stated, detached leaves were allowed to equilibrate in the cuvette for no longer than four minutes. The calculations used for determining baseline gas exchange in Yukon and Shandong plants are given in Chapter 2.

Gas exchange measurements were performed at Yukon Field Site 2 in June 2014 near solar noon (approx. 1400 h). Two leaves from 15 individual plants were sampled at either low (30-300 μmol m^{-2} s^{-1}) or high (301-1000 μmol m^{-2} s^{-1}) irradiance. The same instrumentation and settings were used as described above.

Ψ_w and Ψ_s measurements of field-grown Yukon *Eutrema*

An HR33T psychrometer fitted with a C52 chamber (Wescor® Inc.) was used to determine leaf Ψ_w. Six mm diameter discs were excised from a mature, fully expanded leaf as previously described (Weretilnyk et al. 1995). Ψ_w measurements were performed in the field while Ψ_s values were determined in the lab using leaf discs flash-frozen in the field using the protocol outlined in Chapter 2 (MacLeod et al. 2015). Ψ_p was calculated for plants from which both Ψ_w and Ψ_s measurements were taken using the equation $\Psi_w = \Psi_s + \Psi_p$ (Boyer 1968). The psychrometric measurements of Phenotype 1 plants were performed on a pool of approximately 10 small rosette plants, growing within an area 1 m^2, in order to amass enough tissue for both Ψ_s measurements and RT-qPCR analysis.

RT-qPCR analyses of field-grown Yukon *Eutrema*

Approximately 75 mg of rosette tissue from multiple individual plants was pooled for Phenotype 1 plants or cauline leaf tissue from a single, individual plant was used for Phenotype 2 or 3 plants. Total RNA was extracted using the Plant/Fungi Total RNA Purification Kit (Norgen BioTek, Thorold, Ontario, CAN) following the manufacturer's instructions. Transcript levels of *EsRAB18* (Thhalv10004906m), *EsRD22* (Thhalv10004736m), *EsRD29A* (Thhalv10015427m) and *EsERD1* (Thhalv10012591m) were measured relative to reference transcripts of genes encoding *EsEF1α* (Thhalv10013526m), *EsUBQ10* (Thhalv10006290m) and *EsYL8S* (Thhalv10014963m).

Preparation of cDNA and RT-qPCR analysis was performed as per MacLeod et al. (2015). The primers used are listed in Table 2.1.

Statistical analysis

For the light response curves, rectangular parabolic curves were fit to the data collected using GraphPad Prism (Version 5.02; GraphPad Software Inc.) A linear regression was performed on the net assimilation (*A)* data of light intensities from 0 to 200 μmol m^{-2} s^{-1} using STATISTICA (Version 7.0; StatSoft Inc.) in order to determine the x-intercept (light compensation point) and the slope of the line (quantum yield) as described by Long and Bernacci (2003). All other statistical analysis was performed using STATISTICA. All data were subjected to either one-way or factorial analysis of variance (ANOVA). If ANOVA showed significant interactions, a post-hoc test using Fisher's least significant difference was performed ($P < 0.05$).

Results

Comparison of gas exchange in *Eutrema* plants

Gas exchange measurements for attached leaves of Yukon and Shandong *Eutrema* plants growing under controlled environment conditions are reported in Table 5.1. As expected, rates of transpiration (*E)*, stomatal conductance (g_1) and net CO_2 assimilation (*A*) are highest with illumination while the internal CO_2 pool (C_i) decreases as plants overcomes

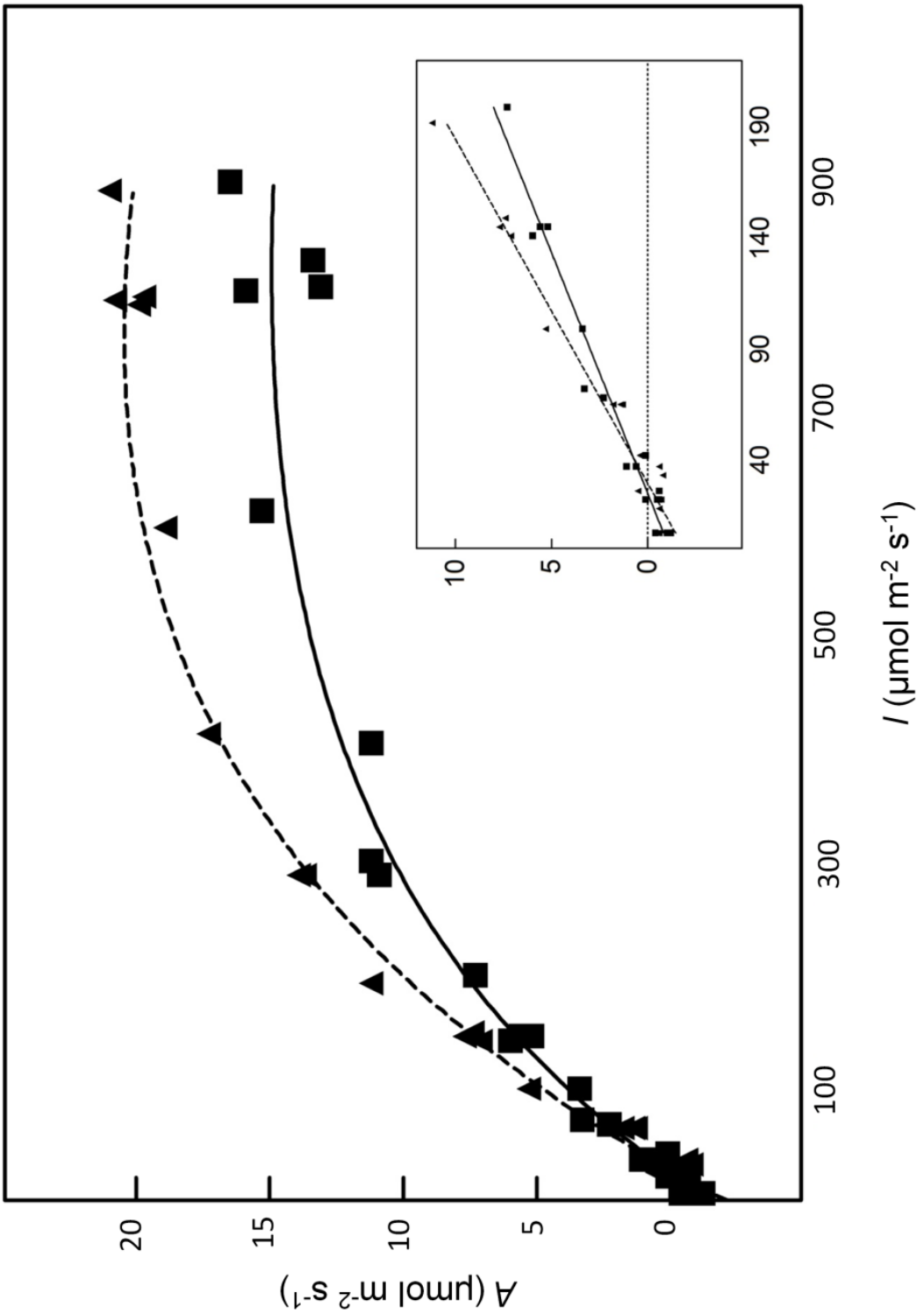
dark-mediated respiratory CO_2 losses. However, there are significant differences between the accessions with respect to these determinations. *A* was more negative in low light in Shandong plants indicating rates of dark respiration are higher relative to Yukon plants. In general, plants that have higher rates of dark respiration often show higher rates of gas exchange (Boyer 1970). The fact that Shandong plants had significantly higher values for *E*, g_1, and *A* relative to Yukon plants is consistent with this generalization. Eppel et al. (2014) plotted *A* and g_1 as a function of light and using similar irradiance levels (600 μmol m^{-2} s^{-1}) and estimates for Shandong plants of 20 μmol CO_2 m^{-2} s^{-1} and 250 mol H_2O m^{-2} s^{-1}, respectively, are obtained from their data. These values for *A* and g_1 are in close agreement to those obtained in Table 5.1 at 19.3 μmol CO_2 m^{-2} s^{-1} and 261.8 mol H_2O m^{-2} s^{-1}, respectively.

To determine other baseline parameters, light response curves were generated for well-watered Yukon and Shandong plants (Fig. 5.1). Both accessions grown under cabinet conditions had light saturation points of approximately 600 μmol $m^{-2}s^{-1}$ although the value of *A* at the light saturation point was 1.4-fold higher in Shandong compared to Yukon leaves. Shandong plants also had a higher light compensation point at 29 μmol $m^{-2}s^{-1}$ compared to the Yukon plants grown under the same conditions at 25 μmol $m^{-2}s^{-1}$ (inset Fig. 5.1). Given the higher rates of gas exchange for Shandong plants relative to Yukon plants (Table 5.1), it is not surprising that Shandong plants have a higher

Table 5.1. Gas exchange measurements of Yukon and Shandong *Eutrema* leaves. Data is the mean +/- SE for two biological replicates where n = 10 plants for each accession. Numbers in bold are significantly different between the accessions (Fisher LSD, $p < 0.05$).

Parameter	Irradiance			
	Yukon Eutrema		Shandong *Eutrema*	
	≤10 µmol m^2 s^{-1}	≥750 µmol m^2 s^{-1}	≤10 µmol m^2 s^{-1}	≥750 µmol m^2 s^{-1}
E mmol H_2O m^{-2} s^{-1}	**1.66** ± 0.07	**1.83** ± 0.07	2.42 ± 0.26	2.48 ± 0.19
g_1 mol H_2O m^{-2} s^{-1}	**164.4** ± 10.8	**194.5** ± 10.5	246.0 ± 29.8	261.8 ± 22.6
A µmol CO_2 m^{-2} s^{-1}	-1.17 ± 0.11	**13.74** ± 0.38	-1.87 ± 0.45	19.31 ± 2.02
C_i µmol CO_2 mol^{-1}	414.2 ± 1.6	260.8 ± 4.4	414.8 ± 2.0	263.5 ± 6.6

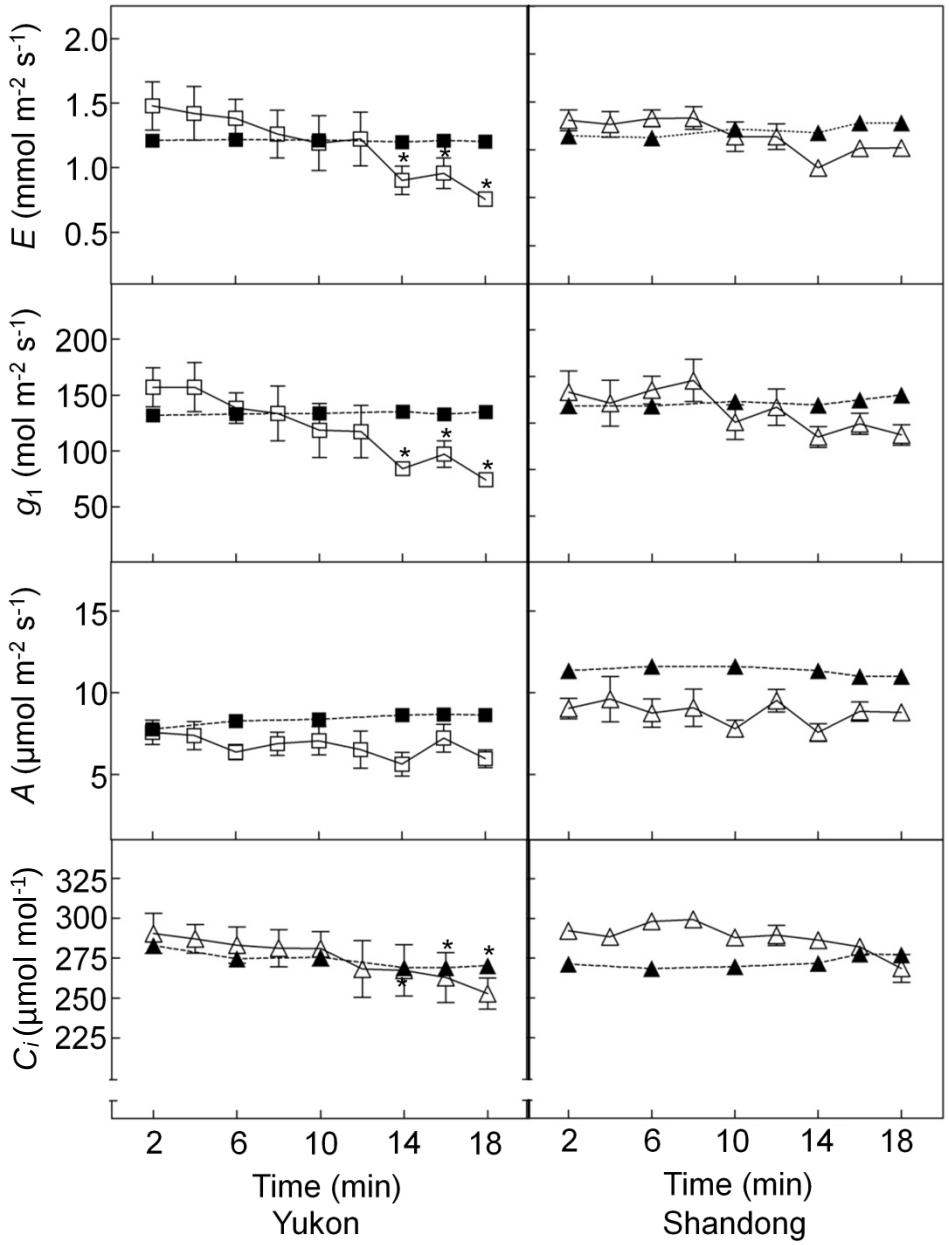
Fig. 5.1. Light and CO_2 response curves for leaves of cabinet-grown Yukon and Shandong *Eutrema* plants. Net assimilation of CO_2 (*A*) as a function of photosynthetically active irradiance (*I*). Inset: portion of the light response curve from 0-200 μmol m^2 s^{-1} when net assimilation of CO_2 and photosynthetically active irradiance (*I*) have a linear relationship. All measurements were made on intact leaves of Yukon (■, —) and Shandong (▲, ---) plants. A non-linear least-squares-curve was fit to data collected from three plants from each accession. A linear regression analysis was performed on inset curves: Yukon - $R^2 = 0.96$ $y = 0.046x - 1.15$, Shandong - $R^2 = 0.96$ $y = 0.064x - 1.90$.


A (μmol m-2 s-1)
0
5
10
15
20
100
300
500
700
900
I (μmol m-2 s-1)
10
5
0
40
90
140
190

quantum yield but need more light to overcome respiratory loss of carbon to achieve positive values for *A*.

The data in Table 5.1 and Fig.5.1 shows that the two accessions differ with respect to gas exchange as a function of irradiance but we were most interested in whether the plants would show altered rates of gas exchange in response to water deficit. However, unlike the unstressed plants used for baseline determinations, drought-stressed plants develop smaller leaves and petioles making them inaccessible to the IRGA cuvette while still attached to the rosette. Excised leaves have been used successfully to determine gas exchange (Chang et al. 2004, Zeeman et al. 2004) and they have been used to provide information about changes in gas exchange over time as leaves undergo dehydration (Zeeman et al. 2004). Thus we compared gas exchange over time using intact and detached leaves of Yukon and Shandong plants to determine if a) measurements on intact and detached leaves were comparable and b) to determine if detachment led to differences in gas exchange properties between the two accessions. Fig. 5.2 shows *E*, g_1, *A*, and *Ci* measured over time in detached and intact leaves of *Eutrema* from well-watered plants. In the case of intact leaves the data for a single, representative leaf is plotted and as these determinations show, *E*, g_1, *A*, and *Ci* estimates for any given leaf made over the 18 min time-course of the experiment were similar to the first determination made at 2 minutes. However, Fig. 5.2 also shows that in detached relative to intact Yukon leaves there is a decline in these gas exchange parameters over time. This outcome is not particularly

Fig. 5.2. Leaf gas exchange measurements over time for detached leaves of Yukon and Shandong *Eutrema* plants. Transpiration (*E*), stomatal conductance (g_1), net assimilation of CO_2 (*A*), and sub-stomatal level of CO_2 (C_i) were measured. Gas exchange was determined at irradiance levels similar to cabinet conditions (250 μmol m^{-2} s^{-1}). Measurements made on detached Yukon (□, —) and Shandong (△,—) leaves are the mean ± SE of two biological replicates where n = 5 plants for each accession. Data shown for a single representative intact Yukon (■,---) and Shandong (▲,---) leaf. Asterisks indicate statistically significant differences between accessions for detached leaves (Fisher's LSD, P <0.05).


E (mmol m-2 s-1)
2.0
1.5
1.0
0.5
g1 (mol m-2 s-1)
200
150
100
50
A (µmol m-2 s-1)
15
10
5
Ci (µmol mol-1)
325
300
275
250
225
2
6
10
14
18
Time (min)
Yukon
Time (min)
Shandong

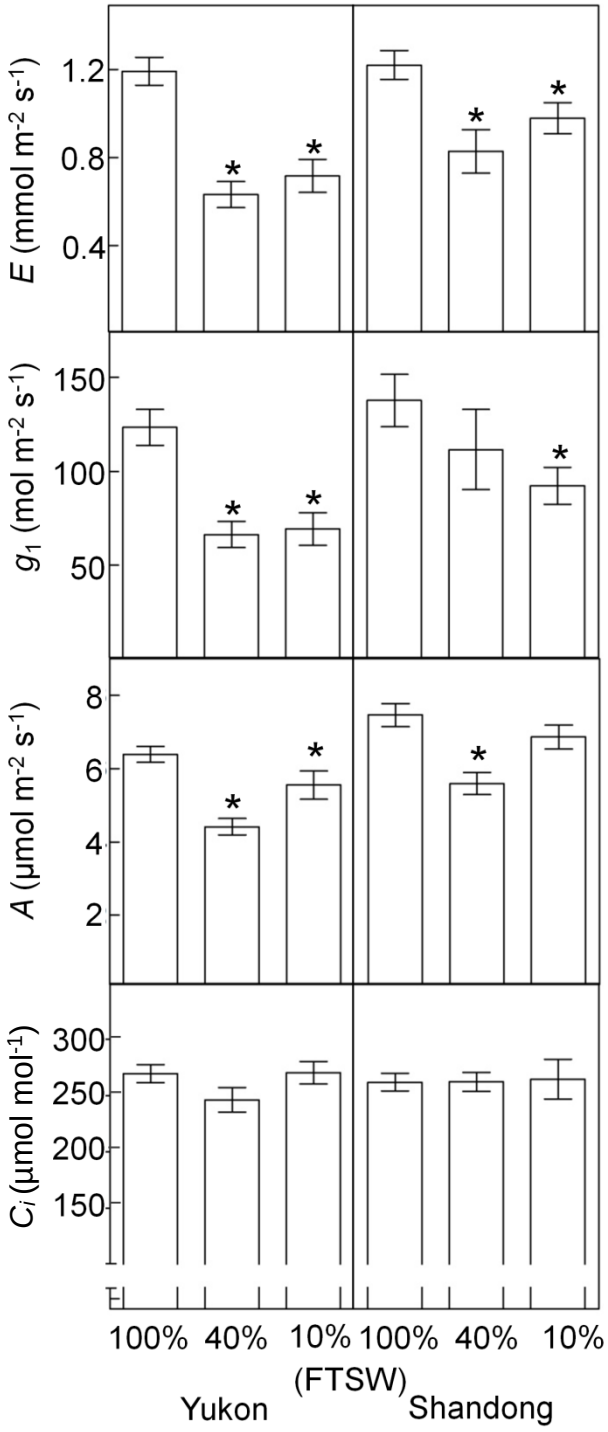
surprising as leaf detachment can provoke a water deficit response that leads to stomatal closure and hence reduced transpiration and CO_2 assimilation (Zeeman et al. 2004). Notably, the decline in *E*, g_1, and *A* values did not occur until nearly 10 minutes after leaves were detached. Interestingly, detached Shandong leaves showed relatively stable *E*, g_1, *A*, and *Ci* values over the same time period when similarly treated Yukon leaves showed declining values. Thus the data in Fig. 5.2 is evidence that reproducible experimental measurements can be made on detached Yukon and Shandong leaves and the leaves are sufficiently stable for a time that allows for the four minute period used for a detached leaf to come into equilibrium in the IRGA cuvette. Moreover, Fig. 5.2 shows that data from intact and detached leaves led to similar conclusions. For example, estimates of *A* based upon intact or detached leaves leads to the same conclusion that the accessions differ with respect to this parameter. We next applied the same strategy to determine whether the two accessions diverged with respect to gas exchange characteristics when the plants from which the leaves were removed had been drought-stressed.

Eutrema plants were exposed to a single, progressive, water deficit equivalent to the first part of the two-stage drought protocol described by MacLeod et al. (2015) (Fig. 3.1). Gas exchange was measured using detached leaves taken from rosettes of the two accessions at 40% and 10% FTSW corresponding to a moderate and severe drought treatment. It is important to note that the leaves used were not wilted, the phenotype reached at 0% FTSW (MacLeod, et al, 2015). The measurements for gas exchange values

for detached leaves from plants under water deficit stress are shown in Fig. 5.3 with a comparison provided from a well-watered control plant grown at the same time. We selected an irradiance that matched cabinet conditions at 250 μmol m^2 s^{-1} PPFD as these were the conditions that both accessions had been grown under. No significant differences were found for any of the measurements made using the detached leaves of well-watered Yukon and Shandong leaves. However, at the 40% FTSW stage there was a significant reduction, approximately 50%, in *E* and g_1 in drought-treated Yukon plants that was not observed in Shandong plants. While the g_1 values remained low in Yukon plants at the 10% FTSW stage, at this stage of drought the g_1 values also decreased significantly for Shandong plants. A comparison between the data of Figs. 5.2 and 5.3 shows an interesting similarity in the behaviour of the Yukon plants with respect to gas exchange responses. The *E*, g_1, *A*, and *Ci* values for a detached leaf from a well-watered, Yukon plant determined 18 minutes following its removal from the plant is roughly equivalent to the same values for a detached leaf from a plant stressed to 40 or 10% FTSW measured within four minutes of being severed from the plant. In summary, Yukon plants respond to water deficits by reducing transpiration and stomatal conductance in that rates of *E* and g_1 fell by almost half yet values of *A* were only reduced by about 10 to 20% while Shandong plants had, on average, higher rates for all of these processes and were less adversely affected by water stress.

The cabinet conditions are very different from those found in the natural habitat for *Eutrema*. For example, precipitation is variable in the semi-arid Yukon and the plants are

Fig. 5.3. Gas exchange for cabinet-grown *Eutrema* plants exposed to adequate water and moderate or severe water deficits. Transpiration (*E*), stomatal conductance (g_1), net assimilation of CO_2 (*A*) and sub-stomatal level of CO_2 (C_i) were measured using detached leaves of Yukon well-watered (YWW), Yukon drought-treated (YD), Shandong well-watered (SWW) and Shandong drought-treated (SD) plants. Drought-treated plants were exposed to either moderate (40% FTSW) or severe (10% FTSW) water deficits. Gas exchange was evaluated in leaves at irradiance levels similar to cabinet conditions (250 μmol m^{-2} s^{-1}). Data are the mean ± SE of three biological replicates where n = 9 plants for each accession. Asterisks indicate statistically significant differences between accessions (Fisher's LSD, P <0.05).


E (mmol m-2 s-1)
1.2
0.8
0.4
g1 (mol m-2 s-1)
150
100
50
A (µmol m-2 s-1)
8
6
4
2
Ci (µmol mol-1)
300
250
200
150
100% 40% 10% 100% 40% 10%
(FTSW)
Yukon
Shandong

found on highly saline, alkaline soils. Furthermore, in contrast to our cabinet-grown plants, *Eutrema* growing in the field is exposed to higher and constantly changing light intensities (Guevara et al. 2012). In order to compare the gas exchange measurements of field plants with those of cabinet plants we performed measurements on 15 individual plants from the field and the cabinet under either low (30-300 μmol m^{-2} s^{-1}) or high (301-1000 μmol m^{-2} s^{-1}) irradiance. Box-whisker plots showing the range of the measurements obtained are shown in Fig. 5.4. The values of E and g_1 for field and cabinet plants had similar maximum values under low light conditions but the minimum observed values for E and g_1 from field plants were more than two-fold lower than those from cabinet plants. Under both high and low light conditions field plants had lower average values for E, g_1, and A. In contrast, Ci levels were similar in field and cabinet plants under high and low light.

We compared the gas exchange values from field-grown plants with the values obtained from cabinet-grown Yukon plants undergoing water deficit (Fig. 5.5). This meta-analysis of data used to generate Figs. 5.3 and 5.4 showed that values of E and g_1 for drought-stressed cabinet plants and field-grown plants (the latter measured under high or low irradiance) were more similar to each other and less than the values obtained from well-watered cabinet-grown plants. In contrast, A values were significantly lower in field-grown plants exposed to lower light levels but as high as the rates of well-watered cabinet plants when the higher irradiance range was used for measurements. Correspondingly, the Ci measurement is highest in field plants under low irradiance due to less available CO_2

Fig. 5.4. Box-whisker plots of gas exchange measurements for Yukon field and cabinet-grown *Eutrema* plants. Measurements were made over two different ranges of irradiance (150-300 μmol m^{-2} s^{-1} or ≥ 300 μmol m^{-2} s^{-1}). Transpiration (*E*), stomatal conductance (g_1), net assimilation of CO_2 (*A*) and sub-stomatal CO_2 (*Ci*) were assessed in leaves from *Eutrema* plants growing at a field site near Whitehorse in June 2014 (Field) and in cabinet-grown Yukon plants (Cabinet). The bottom of the box is the 25th percentile and the top is the 75th. The whiskers extend to the highest and lowest values. A minimum of fifteen Yukon field or cabinet-grown plants were measured for each range of irradiance values.

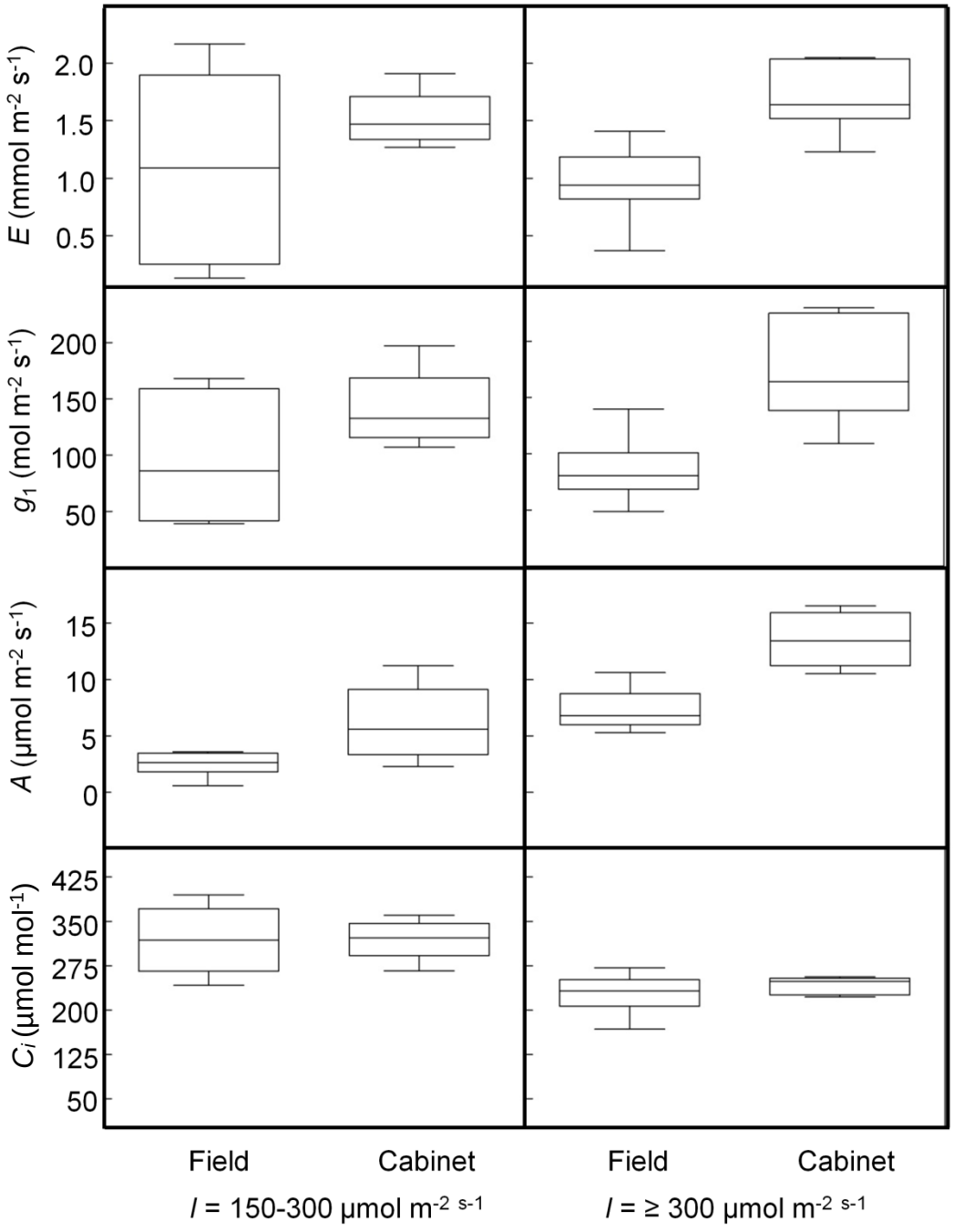
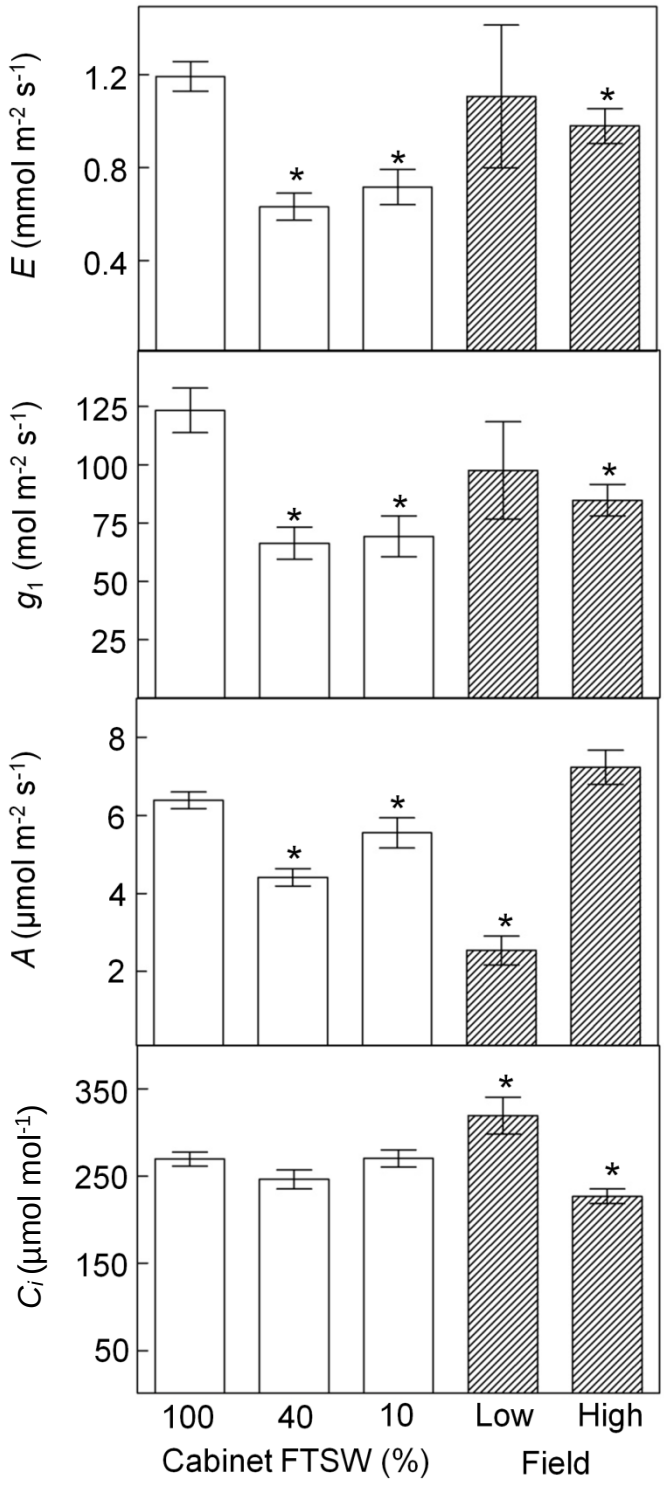

E (mmol m-2 s-1)
2.0
1.5
1.0
0.5
g1 (mol m-2 s-1)
200
150
100
50
A (µmol m-2 s-1)
15
10
5
0
Ci (µmol mol-1)
425
350
275
200
125
50
Field
Cabinet
Field
Cabinet
I = 150-300 µmol m-2 s-1
I = ≥ 300 µmol m-2 s-1

Fig. 5.5. Comparison of gas exchange measurements in field-grown and drought-stressed cabinet-grown Yukon plants. Transpiration (*E*), stomatal conductance (g_1), net assimilation of CO_2 (*A*) and sub-stomatal CO_2 (*Ci*) were assessed in detached leaves of Yukon well-watered (YWW, 100% FTSW) or Yukon drought-treated (YD) plants. Drought-treated plants were exposed to either moderate (40% FTSW) or severe (10% FTSW) water deficits. Gas exchange measurement for cabinet plants were made at irradiance levels matched to cabinet conditions (250 μmol m^{-2} s^{-1}). Data shown for cabinet-grown Yukon plants are the mean ± SE of three biological replicates where n = 9 plants for each treatment. Gas exchange measurements using *Eutrema* plants growing at a field site near Whitehorse in June 2014 were taken under low (150-300 μmol m^{-2} s^{-1}) and high (≥ 300μmol m^{-2} s^{-1}) irradiance; data are the mean ± SE of 15 individuals. Asterisks indicate statistically significant differences between treatments (Fisher's LSD, P <0.05).

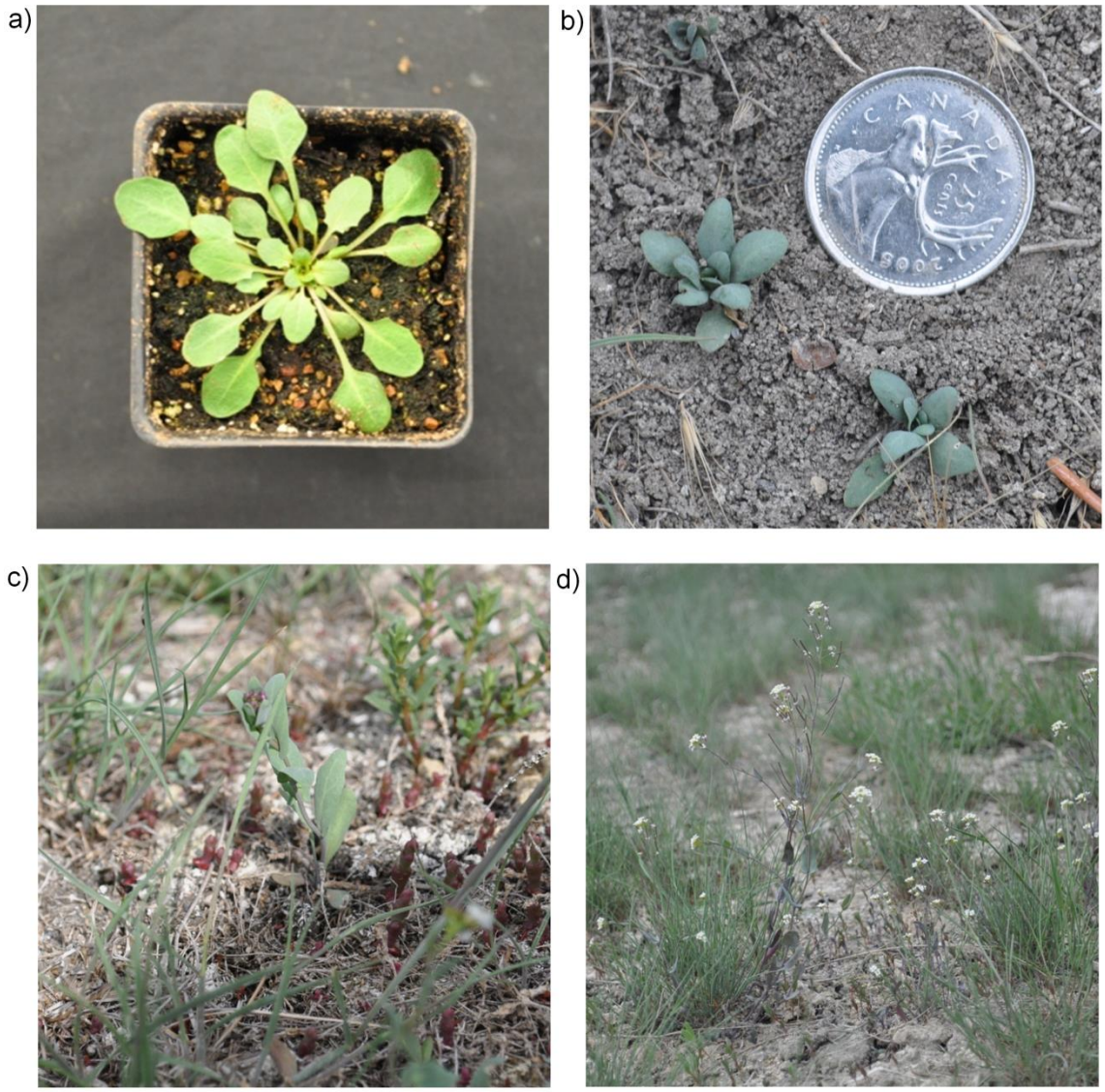

E (mmol m-2 s-1)
1.2
0.8
0.4
gl (mol m-2 s-1)
125
100
75
50
25
A (µmol m-2 s-1)
8
6
4
2
Ci (µmol mol-1)
350
250
150
50
100
40
10
Low
High
Cabinet FTSW (%)
Field

being assimilated and significantly lower under the high irradiance regime and associated higher rate of CO_2 assimilation.

Water status of field-grown Yukon plants

Psychrometric measurements were taken of field plants growing in the Yukon. Due to the difference in leaf type (cauline or rosette) and size among the plants found in the field the plants were grouped into three categories based upon above-ground phenotype as shown in Fig. 5.6. Phenotype 1 was comprised of plants that were rosettes at the time of harvest and these plants were typically very small (Fig. 5.6A). Phenotype 2 and 3 plants were inflorescent plants with cauline leaves and lacking rosette leaves. Phenotype 2 plants lacked mature siliques (Fig. 5.6B) whereas Phenotype 3 plants had mature siliques and were, on average, larger than Phenotype 2 plants (Fig. 5.6C). Leaf Ψ_w and Ψ_s measurements were made on cauline leaves of Phenotype 2 and 3 plants but only Ψ_s values are available for the rosette leaves of Phenotype 1 plants as the leaves were too small to reliably use for Ψ_w determinations. The plants were harvested from two Yukon field sites in June 2011 (see Materials and methods) and the Ψ_w and Ψ_s show that there was no difference in water status measurements between cauline leaves of Phenotype 2 and Phenotype 3 plants at the two sites (Table 5.3). In contrast, Phenotype 1 plants had significantly lower Ψ_s values in rosette leaves indicating that solutes are more concentrated in these leaves compared to cauline leaves of the older Phenotype 2 and 3 plants. Leaf Ψ_p of field plants was estimated from the leaf Ψ_w and Ψ_s measurements of

Fig. 5.6. Variability among Yukon *Eutrema* phenotypes found in the cabinet and field. (A) Well-watered, cabinet-grown Yukon plant and (B) Field Phenotype 1 vegetative plants dominated by rosette leaves, (C) Field Phenotype 2 plants featuring cauline leaves, lacking rosette leaves, and displaying bolts with flowers and immature siliques, and (D) Field Phenotype 3 plants that lack rosettes but have cauline leaves and bolts bearing mature siliques.


a)
b)
c)
d)
CANADA
2006

three individual plants (Appendix 4). The plant sampled with the lowest Ψ_w also had the lowest Ψ_s and the lowest estimate of Ψ_p suggesting that P3-3 was experiencing a greater water deficit than P3-1 or P3-2. It is notable that while all three plants had very low Ψ_s values, all Ψ_p values were positive and therefore the plants were turgid.

In general, water status measurements of field plants were more variable than those made using leaves of plants in our controlled environment cabinets (Fig. 5.7). The range of Ψ_w and Ψ_s values for field plants is shown beside values compiled for cabinet-grown Yukon plants from well-watered and drought-treated plants (no values were used from wilted plants) during the drought exposure described by MacLeod et al. (2015). Despite using measurements from many cabinet-grown plants including those exposed to prolonged water deficit, the range of Ψ_w values for cabinet-grown and plants in the field does not overlap. In this respect, while progressive drought treatments in the cabinet may elicit many physiological changes (MacLeod et al. 2015), they fail to induce the same level of osmotic adjustment as found for *Eutrema* plants growing in their natural habitat.

Determination of dehydrin expression in field-grown Yukon *Eutrema*

The expression of four dehydrin-encoding genes (*EsERD1*, *EsRAB18*, *EsRD22* and *EsRD29A*, see Chapter 3) was assessed by RT-qPCR analysis using leaves of field plants and expressed relative to their expression in well-watered, cabinet-grown Yukon plants (YWW1). For comparison, the expression of the dehydrin genes in cabinet-grown Yukon plants exposed to a single (YD1) or two consecutive drought treatments (YD2) are given

Table 5.3. Water potential (Ψ_w) and solute potential (Ψ_s) measurements made on Yukon *Eutrema* plants from two field sites. Plants were classified as one of three phenotypes according to morphological observations made in the field. Ψ_w and Ψ_s data are the mean ± SE of three individuals with three technical replicates per plant.

		Mean Potential ± SE (MPa)	
Phenotype	Field Site	Ψ_w[a]	Ψ_s
1	1	n/a	-4.50 ± 0.38
	2	n/a	-4.34 ± 0.12
2	1	-2.28 ± 0.78	-2.60 ± 0.13
	2	-2.02 ± 0.22	-2.49 ± 0.15
3	1	-2.29 ± 0.66	-2.62 ± 0.09
	2	-2.27 ± 0.19	-2.63 ± 0.09

[a] n/a = not available

Fig. 5.7. Differences in morphological phenotype and water status between cabinet and field-grown Yukon *Eutrema*. (A) Representative images of cabinet-grown (left) and field-grown (right) Yukon plants. (B) Box-whisker plots of the range of water potential (Ψ_w) and solute potential (Ψ_s) measurements of cabinet and field-grown plants. The bottom of the box is the 25th percentile and the top is the 75th. The whiskers extend to the highest and lowest values. Ψ_w and Ψ_s measurements were made on 12 individual cabinet-grown plants at different stages of drought treatment. Measurements from field plants are those shown in Tables 1 and 2 (excluding Phenotype 1). All measurements were made on turgid plants.

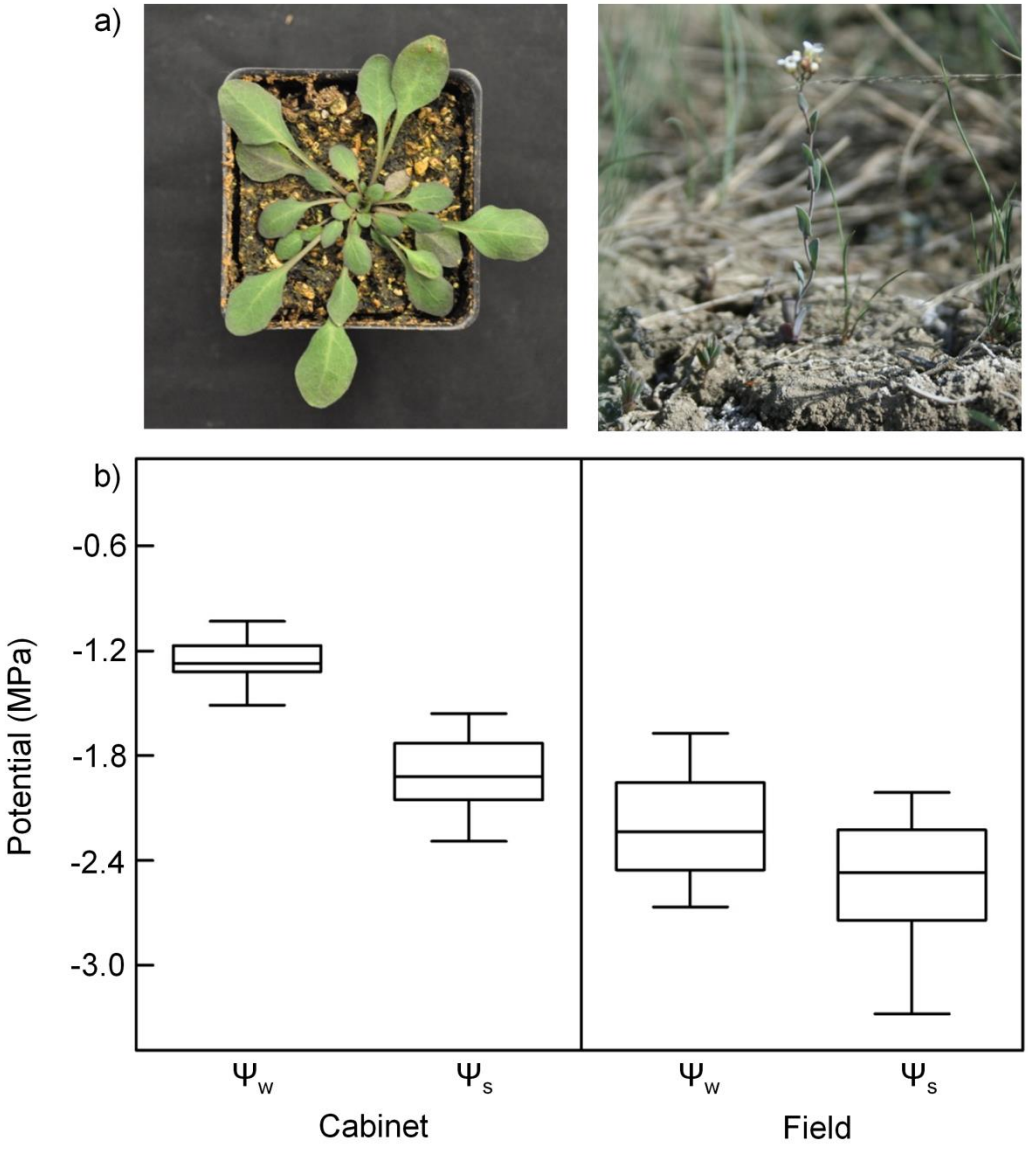
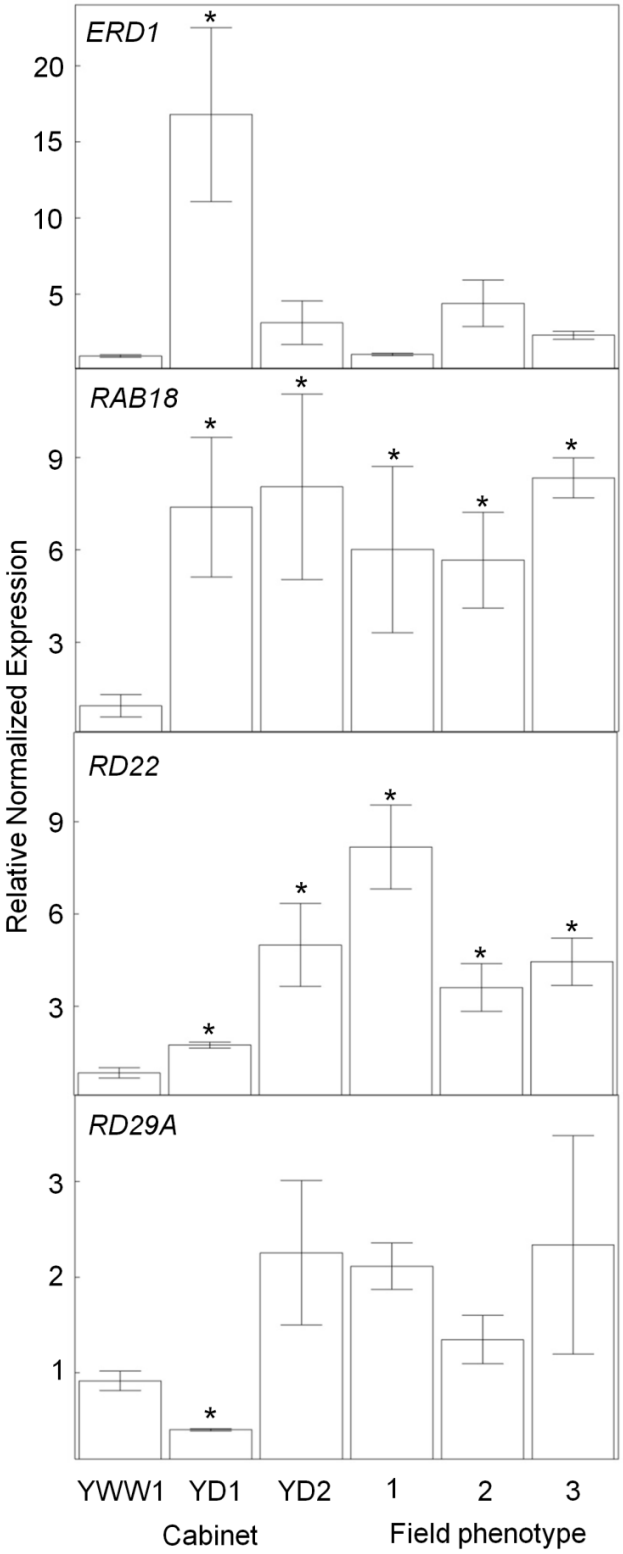

a)
b)
-0.6
-1.2
-1.8
-2.4
-3.0
Potential (MPa)
Ψw
Ψs
Ψw
Ψs
Cabinet
Field

Fig. 5.8. Expression of dehydrin-encoding genes in field-grown Yukon *Eutrema* plants compared to cabinet-grown plants. The relative expression of *EsERD1, EsRAB18, EsRD22* and *EsRD29A* were determined for field and drought-treated cabinet-grown plants at two stages of drought (D1-40% and D1-10% FTSW). qRT-PCR data for field grown plants are the mean ± SE of three plants from each phenotype and for cabinet-grown plants are the mean ± SE of two biological replicates where n = 4 plants for each treatment. Asterisks indicate statistically significant differences between treatments and the YWW1 control (Fisher's LSD, $P < 0.05$).


ERD1
RAB18
RD22
RD29A
Relative Normalized Expression
YWW1
YD1
YD2
1
2
3
Cabinet
Field phenotype

(Fig. 5.8). The relative expression of the four dehydrin-encoding transcripts was surprisingly consistent in field plants with no significant difference in relative expression between the three field phenotypes. The more frequent outlier determinations with respect to transcript abundance was for Yukon YD1 plants. Indeed, the most significant difference in relative expression was for *EsERD1* in the cabinet-grown YD1 plants. The relative expression values for *EsRAB18* and *EsRD22* were significantly higher in the field samples and both YD1and YD2 samples compared to YWW1 plants while the relative expression of *EsRD29A* was consistently high in the field plants sampled and YD2 but not the YD1 cabinet plant samples. Overall, the relative expression values of the four dehydrin genes indicates that their transcript abundance in field plants more closely resembles the values obtained from cabinet YD2 plants as opposed to the YD1 plants.

Discussion

The ability to study naturally stress-adapted populations in growth cabinets and in their native environment is a tool that can help elucidate the traits allowing these plants to withstand harsh natural environments. Yukon *Eutrema* are particularly interesting due to the combination of severe abiotic stresses the plants must endure in their natural habitat with the most consistent one being exposure to salt and a less frequent stress arising from low precipitation. While previous studies have used metabolomics and transcriptomics to compare Yukon field and cabinet plants, relatively little is known about photosynthesis and water relations in these natural populations (Guevara et al. 2012, Champigny et al.

2013). In that regard, the results presented here offer novel insights into both gas exchange and water status of Yukon *Eutrema* plants found in the cabinet and in the field.

In this study we first compared gas exchange properties between Yukon and Shandong plants. Gas exchange parameters have not been reported for Yukon *Eutrema* whereas Shandong plants have been characterized in terms of gas exchange physiology (Inan et al. 2004, Stepien and Johnson 2009, Eppel et al. 2014). As a halophyte, Shandong *Eutrema* naturally have gas exchange and photosynthetic rates that are lower relative to its glycophyte relative *Arabidopsis* (Inan et al. 2004). However, even under optimized cabinet conditions, we found that transpiration, stomatal conductance and net assimilation were all lower in Yukon relative to Shandong plants (Tables 5.1 and 5.2). This comparatively low rate of gas exchange in Yukon relative to Shandong plants is interesting in light of the finding that Yukon plants also have more wax associated with the cuticle than Shandong plants (Xu et al. 2014). Both reduced gas exchange and waxy cuticles are mechanisms used to reduce water loss (Boyer et al. 1997) and both of these traits appear to be constitutive in Yukon plants as they are expressed even under optimal growth conditions.

With the knowledge that baseline levels of gas exchange were lower in Yukon plants compared to Shandong plants, we set out to determine if drought treatment affected gas exchange differently in the two accessions. Our protocol for imposing drought treatment conditions for potted plants in cabinets (MacLeod et al. 2015) enabled us to control the duration and severity of the stress and this was advantageous in helping us

establish how gas exchange values for *Eutrema* plants change when exposed to water deficits. There is negligible published information on gas exchange in *Eutrema* exposed to drought but work has been published describing the impacts of salinity on gas exchange for Shandong plants. Salinity and drought are examples of osmotic stress and there is overlap in plant response to these conditions (Chaves et al. 2009). Both Stepien and Johnson (2009) and Inan et al. (2004) reported that in Shandong plants there is a modest decrease in stomatal conductance after prolonged exposure to salinity, a response that we also observed in the more severe drought treatment when Shandong plants reached 10% FTSW (Fig. 5.3). However, earlier in their salinity treatment Stepien and Johnson (2009) reported that there was no significant difference in stomatal conductance between Shandong plants exposed to 500 mM NaCl compared to untreated controls. We also found no significant difference in stomatal conductance between unstressed Shandong control plants and plants exposed to the moderate water deficit (Fig. 5.5). In contrast, even under moderate water deficit stomatal conductance was significantly reduced in Yukon plants compared to unstressed control plants. Furthermore, compared to Shandong *Eutrema,* Yukon plants exhibited both lower constitutive levels of gas exchange under more optimal conditions (Table 5.1) and reduced stomatal conductance earlier in response to water deficit (Fig. 5.3).

Our Yukon field populations were used to determine if the same conservative approach to gas exchange was expressed in natural populations where temperatures, precipitation, irradiance, and day length are all variable (Guevara et al. 2012).

Measurements of evaporation and stomatal conductance from field plants were similar to drought-treated Yukon cabinet plants, a condition we did not expect given that the field plants experience higher irradiance levels that cannot be attained in the cabinet that we used (Fig. 5.5). The similarity in transpiration and stomatal conductance between field and drought-treated cabinet plants is likely due to partial or complete closure of stomates in order to reduce water loss (Cowan 1977). A consequence of reduced evapotranspiration through stomates is that it likely restricts the movement of Na^+ ions from saline soil water into the shoots of field plants by limiting the need for water uptake (Lovelock and Ball 2002). The reduced rates of evaporation and stomatal conductance in drought-treated cabinet plants indicates that Yukon plants are more responsive to acute water stress than Shandong plants and it would be interesting if this difference could be discerned in response to water deficits under field conditions.

Overall gas exchange in field plants is low relative to well-watered cabinet plants which would appear to be due to more stressful environmental conditions in the field. Measurements of leaf water status varied from plant to plant (Fig. 5.7B) but showed that Ψ_w and Ψ_s values for field plants were consistently lower than those of even the most water-stressed cabinet plants (Fig. 5.6). Especially low leaf Ψ_s values were found in Phenotype 1 plants that were juvenile and comprised of rosettes (Table 5.3). Oh et al. (2010) investigated the halophytic nature of *Eutrema* and found that *Eutrema* behaves as a true halophyte by exhibiting controlled accumulation of Na^+. Tissue harvested from field-grown Yukon plants is enriched in Na^+ (Guevara et al. 2012), providing an

explanation for the disparity between Ψ_w and Ψ_s values between field plants and drought-stressed cabinet-grown Yukon plants. If properly sequestered, Na^+ can help maintain a Ψ_w gradient between plants roots and saline soils (Oh et al. 2010).

Even though gas exchange and water status values were lower for Yukon field plants relative to cabinet-grown plants, we observed surprisingly consistent gene expression for the selected dehydrin-encoding genes in field plants despite different above-ground phenotypes (Fig. 5.8). The consistency of expression patterns of the dehydrin-encoding genes agrees with the findings of Champigny et al. (2013) in that gene expression appears relatively stable in randomly selected individuals sampled simultaneously from natural populations. The expression of *EsRAB18, EsRD22*, and *EsRD29A* in plants of all three field phenotypes was similar to drought-stressed cabinet-grown plants. This was expected for *EsRAB18* as it was identified as a gene that was significantly upregulated by drought, cold and salinity treatment (Wong et al. 2006). The only gene tested that was expressed at much higher levels in drought-stressed cabinet plants relative to field plants was *EsERD1.* Interestingly, the expression of *EsERD1* was shown to be up-regulated only in response to an initial drought exposure in a cabinet plant and not a second drought treatment (Fig. 3.7). This result suggests that *EsERD1* may only be expressed in naive Yukon plants experiencing a water deficit for the first time. In general, the expression of these dehydrin-encoding genes was responsive to the severity of an imposed water deficit in cabinet-grown plants (Fig. 3.7). Thus, we expected that their expression would be higher in the Phenotype 1 plants sampled due to their

significantly lower mean Ψ_s values (Table 5.3). However, only *EsRD22* was significantly up-regulated in Phenotype 1 plants relative to both Phenotype 2 and 3 field plants. The fact that only one (*EsRD22*) out of the four dehydrins tested was up-regulated in Phenotype 1 plants, which on average had lower Ψ_s values, indicates that gene expression may not be as responsive to stress severity in field plants.

In order to fully understand how a particular trait is involved in improving survival of Yukon plants in the field it is essential to understand how that trait will be expressed under the complex combination of conditions encountered in the field. In this regard, the consistency we observed in the relative expression of the selected dehydrin-encoding genes is particularly promising. Further studies of these natural populations of Yukon *Eutrema* can investigate the effects of either natural or imposed conditions on gene expression with confidence that interplant variability is relatively low. Ideally, information gained by transcript profiling of field plants should be combined with physiological measurements including gas exchange and water status as described here as this will provide a more complete picture of how this extremophile copes with the complex and dynamic stresses of its challenging habitat.

Authors' contributions

MJRM and AG performed the physiological measurements, MJRM performed the RT-qPCR determinations. MJRM and EAW designed experiments, performed data analysis and prepared the manuscript.

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CONCLUSIONS

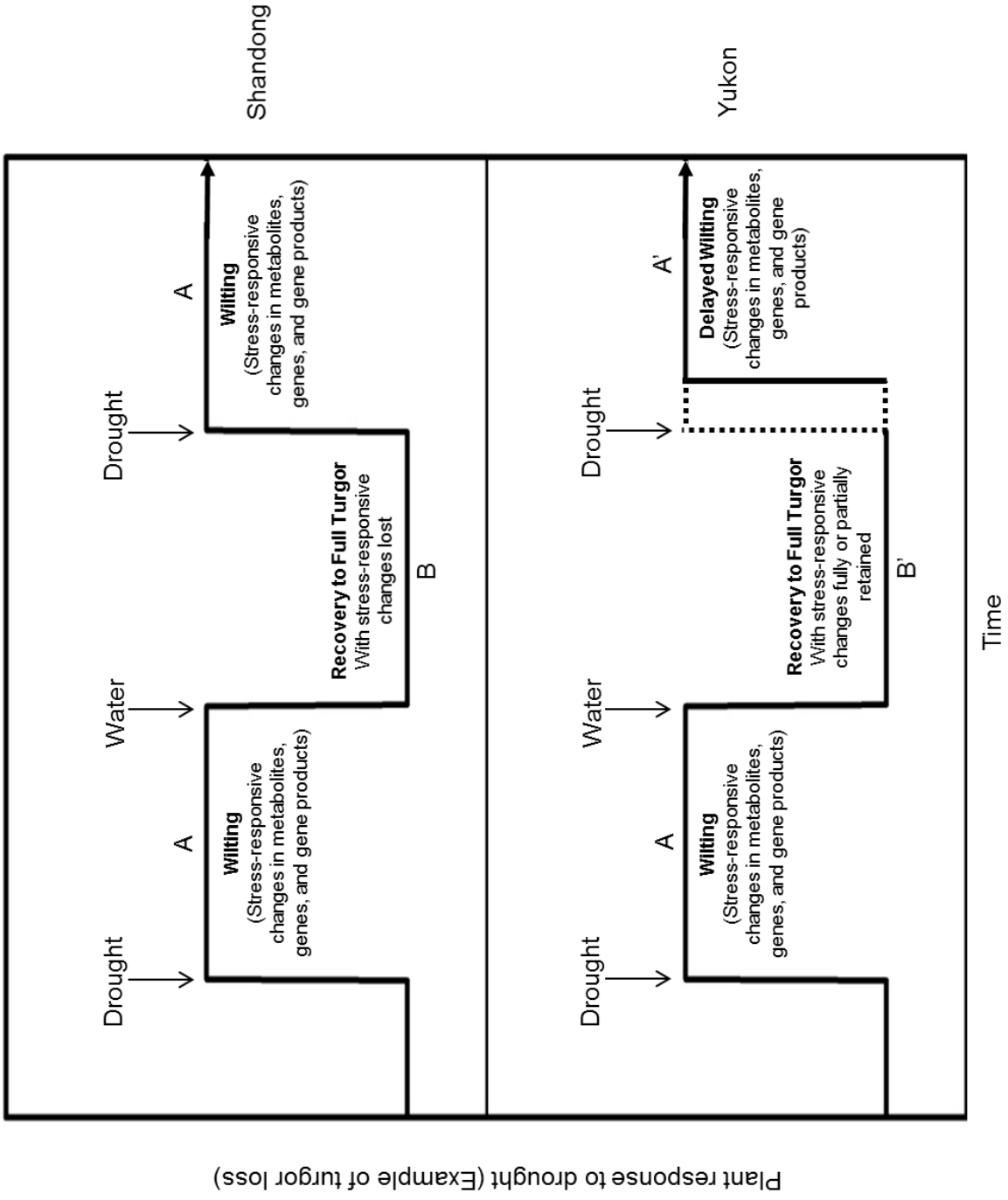
Our understanding of how plants respond to drought continues to increase through research on established and emerging plant models. For example, *Arabidopsis* is a popular model for studying drought response as approximately 50% of published studies on plants that have been genetically modified to improve drought tolerance have used *Arabidopsis* (Lawlor, 2013). Furthermore, natural variation in drought tolerance among different accessions of *Arabidopsis* has been exploited to identify adaptations to drier climates (Bouchabke et al. 2008; Verslues and Juenger, 2011). A different approach has been to study drought response in stress-tolerant species such as *Arabidopsis lyrata* (Sletvold and Ågren, 2012) and *Eutrema salsugineum* (Xu et al. 2014) that are closely related to *Arabidopsis thaliana* but can withstand more severe water deficits. This work utilizes natural variation between accessions by comparing drought response in two accessions of the extremophile *Eutrema salsugineum* at multiple levels from transcript abundance to whole plant productivity.

One of the unique aspects of this research is the drought treatment, used in Chapters 3 and 4, which includes both a recovery period and a second drought exposure. Neither the recovery period nor the second drought exposure are commonly used in other studies of drought (Galle et al. 2011). However, both the ability to recover from water deficit and withstand multiple drought exposures are vital to plants in natural environments (Hayano-Kanashiro et al. 2009; Galle et al. 2011). Without both the recovery period and subsequent drought we would not have observed that the initial drought exposure significantly alters how Yukon plants respond, both physiologically and transcriptionally,

to the subsequent drought. Conversely, for Shandong plants the responses to the two drought exposures were similar in terms of physiological but not transcriptional outcomes. The altered response to the second drought in Yukon plants raises the question of whether a unique form of abiotic stress memory is present in Yukon plants. In a previous study, Griffith et al. (2007) demonstrated that exposing Yukon *Eutrema* plants to drought improved freezing tolerance, as opposed to cold acclimation alone, from -18.5°C to -21°C. In Chapter 3, I showed that Yukon plants experiencing a second drought maintained higher LWC and SLA than during the initial drought (Fig. 3.4). This response meant that the plants were in a better physiological state to continue growth despite sustained exposure to water deficit (Fig. 3.2). In Yukon plants at the molecular level, the initial drought exposure resulted in the differential expression of more than 2000 genes while the subsequent drought exposure led to fewer than 100 genes that were differentially expressed compared to the re-watered stage (Fig. 4.2). The net result of the physiological and transcriptional responses that occur in Yukon plants is that they can withstand the second water deficit for a longer period of time than Shandong plants (Fig. 3.1).

The dissimilar responses to repeated water deficits described above for Yukon and Shandong plants led to the development of a hypothetical model to explain how Yukon plants respond positively to repeated drought exposures while the response of Shandong plants is unchanged by repeated drought exposures (Fig. 6.1). The model uses turgor as an example of a drought response that is initially the same for both Yukon and Shandong

Figure 6.1 - A model depicting the response of Shandong and Yukon plants to repeated drought exposure using turgor as an example. After the initial drought exposure both Shandong and Yukon plants respond by undergoing physiological and biochemical changes equating to level A with the eventual result being wilting. When water is returned to Shandong plants they recover turgor equating to level B. For Shandong plants, the onset of the second drought results in a return to level A and wilting. However, Yukon plants retain some or all of the stress-responsive changes that occurred during the first drought exposure. Unlike Shandong plants, this altered state, distinguished by level B', continues into the second drought leading to a delay in wilting or level A' in the model.


Shandong
Yukon
Drought
Water
Drought
A
Wilting
(Stress-responsive changes in metabolites, genes, and gene products)
Recovery to Full Turgor
With stress-responsive changes lost
B
A'
Delayed Wilting
(Stress-responsive changes in metabolites, genes, and gene products)
Recovery to Full Turgor
With stress-responsive changes fully or partially retained
B'
Time
Plant response to drought (Example of turgor loss)

plants and is designated level A. After drought is alleviated, Shandong plants recover turgor but most or all of the drought-responsive changes are lost, shown as level B in Fig. 6.1. The subsequent drought exposure again results in wilting and Shandong plants return to level A. In Yukon plants, the initial drought exposure instigates long-term adjustments resulting in a new drought-response baseline-level A' after water is returned to Yukon plants. The drought-response baseline-level A' is heightened relative to pre-drought baseline levels consistent with the existence of a "memory" of the drought stress leaving the plants "primed" for better tolerating subsequent exposures unlike Shandong plants. After a repeated drought exposure, only modest changes are required for Yukon plants to reach the heightened drought-response level labelled B' (Fig. 6.1). This concept of a "memory" has been proposed to explain, in part, the response of *Arabidopsis* to desiccation and has been demonstrated in *Arabidopsis* in terms of the transcription of genes related to the ABA-responsive pathway including *RD22* and *RAB18* (Goh et al. 2003; Ding et al. 2012). Stress memory at the molecular level can be maintained through epigenetic modifications (Chinnusamy and Zhu, 2009; Thellier and Lüttge, 2013), particularly the methylation of lysine residues on histones that would allow a modification to persist longer than DNA methylation epigenetic marks (Liu et al. 2014). It is particularly interesting that, using the drought treatment devised here, the initial drought treatment alters how Yukon plants respond to the second drought treatment whereas Shandong plants respond similarly to both droughts. Future studies could exploit this difference between the accessions to identify drought memory traits in *Eutrema.* In

Chapter 4, I invoked the concept of a "molecular buffer" to explain how the expression of some genes in Yukon plants were altered by the initial drought treatment (Fig. 4.4). These Yukon genes, which do not return to pre-stress expression levels even after re-watering, are good drought-stress memory candidate genes in Yukon plants.

Comparative approaches that contrast accessions or varieties with different capacities to tolerate water deficits have become an important tool in studying plant tolerance to drought (Rampino et al. 2006; Juenger et al. 2010; Sharma et al. 2013). The comparison of Yukon and Shandong plants does not fit the typical tolerant versus susceptible paradigm used for this type of work in that both Yukon and Shandong plants are well equipped to withstand osmotic stress (Inan et al. 2004; Wong et al. 2006; MacLeod et al. 2015). As such, teasing out the distinct osmotic stress tolerance mechanisms for two extremophiles represents an uncommon comparative approach. As natural accessions of an extremophile halophyte species, we did not expect to find so many significant differences in physiology and gene expression between the accessions with regards to drought and recovery. Many of the quantitative physiological measurements made on Shandong plants showed that these plants did not respond to drought treatment like Yukon plants, instead the Shandong plants seemingly "ignored" the drought treatment until the more severe stages of water deficit were reached (Fig. 3.4, Fig. 3.7, Fig. 4.4, Fig. 5.3). Although this behaviour resulted in diminished tolerance of Shandong relative to Yukon plants under the controlled environment water deficit conditions we used, it is possible that the capacity of plants to ignore shorter periods of

drought would be advantageous in terms of productivity under a more moderate drought regime. For example, if experimental conditions included just a single drought period where plants reached only the 40% FTSW threshold then the ability to maintain water status (Fig. 3.4) and transcription (Fig. 3.7, Fig. 4.2), as the Shandong plants did, may yield long term benefits relative to Yukon plants that reduced water loss and increased expression of *ERD1,RAB18, RD22,* and *RD29A* presumably at some fitness cost. The differences in the drought responses between the two accessions raises an interesting question albeit one that was unresolved by this study: Why do Shandong plants show negligible transcriptional response to drought with the first treatment? One possibility is that Shandong plants do not sense a decrease in soil water status with the same sensitivity or as quickly as Yukon plants. Another potential explanation is that Shandong plants do respond to more moderate drought exposures but this response occurs predominantly in the root tissue, which we did not analyse in this study.

The research reported in this thesis advances our knowledge of how plants respond to repeated stresses and recovery, conditions that are often encountered in a field setting. Although our drought protocol is a better approximation of natural drought conditions than most, future efforts must still be made to study the behaviour of plants in the field. Ideally, we would also like to compare the knowledge that is gained from controlled experiments with data acquired from more testing in the field. The availability of natural populations of Yukon *Eutrema* are thus invaluable and could be used for just such a purpose. Phenotypically, field plants and cabinet-grown plants are distinct (Guevara et al.

2012; Fig. 5.6) and field plants must grow and reproduce at much lower Ψ_w and Ψ_s than cabinet-grown plants (Fig. 5.7). However, rates of transpiration and stomatal conductance were similarly low in both field plants and drought-treated cabinet-grown Yukon plants relative to well-watered cabinet-grown Yukon plants (Fig. 5.5) demonstrating that both field and drought-stressed cabinet conditions result in low rates evaporative water loss. The reproducible patterns of gene expression reported by Champigny et al. (2013) were also found for the field plants used in this study (Fig. 5.8) suggesting that future experiments conducted using these natural populations of Yukon plants could be used to investigate the response of gene expression to water availability in the field. For example, transcriptomes from plants used in irrigation trials performed on plants from delineated field plots could be compared to cabinet plants that have been re-watered after undergoing a water deficit. The literature is lacking this type of approach as most complimentary field and cabinet trials focus on the response to drought and not the recovery from drought. Natural populations of *Eutrema* also offer an interesting set of plants to test the "molecular buffer" hypothesis. If certain genes are always expressed after an initial drought exposure in cabinet-grown plants then it follows that these genes should be expressed constitutively in field plants and perhaps at levels correlated with the degree of external osmotic stress as evaluated by leaf ψ_w and ψ_s values

One of the greatest challenges to applying the knowledge about drought stress responses gained from a combination of experimental approaches is integrating their results. In this study, gene expression, metabolic response, and whole plant physiology

data from field and cabinet-grown plants were generated and analysed. For example, Chapter 4 describes how the differences in drought response physiology between Yukon and Shandong plants were used to determine the best stages of the drought treatment to harvest plants for RNA-Seq analysis. Many of the physiological responses, particularly in Yukon plants, were concomitant with transcriptional changes such as the up-regulation of genes associated with secondary metabolism in drought-treated Yukon plants undergoing the accumulation of solutes during drought treatment (Fig. 3.3; Fig. 4.3). Future work could provide more in-depth transcriptome profiling using plants at stages of the drought treatment that were not sampled here to provide deeper genomic comparisons to accompany the physiological data. This in-depth approach was used by Meyer et al. (2014) to propose that there are discrete thresholds for physiological traits at which there are abrupt changes in gene expression, a proposal that could be tested with the particularly drought-tolerant Yukon plant.

In describing the drought response for two accessions of *Eutrema* I have shown that the Yukon accession is tolerant to water deficits making it a valuable model in which to study plant response to drought. The question of what makes the Yukon accession more tolerant than Shandong plants is an interesting one and the answer likely resides in several traits. Firstly, the Yukon accession is by nature more "conservative" in terms of gas exchange. This is based on the lower baseline values of both transpiration and stomatal conductance for Yukon plants (Table 5.1) and by reduced cut rosette water loss values during the initial drought exposure (Fig. 3.4). It is perhaps more difficult to ascertain what

constitutes a "conservative" approach where drought-responsive gene expression is concerned but it is clear that Yukon plants respond earlier to the imposed drought at the transcriptional level (Fig. 3.7) and this response likely confers or instigates a program that confers a protective advantage. The up-regulated expression of these genes does not fully return to pre-drought levels in re-watered Yukon plants (Fig. 4.4; Fig. 6.1) and this may serve to mitigate the impact or in effect “buffer” recovering plants from the adverse effects of subsequent drought exposure. The physiological difference that most likely confers the observed advantage in surviving repeated drought exposures is the drought-responsive accumulation of solutes during the initial drought in Yukon plants (Fig. 3.3). Elevated solute levels remain after re-watering and turgor restoration is indicative of a role for these solutes in osmotic adjustment. At the genome level, analysis of the transcriptomes of drought-treated Yukon plants revealed that genes encoding detoxifying enzymes, dehydrins and sHSPs as well as other protective proteins are up-regulated and their expression patterns are consistent with a role in helping the plants cope with the intracellular consequences of water deficit.

In conclusion, many traits likely contribute to the innate drought tolerance exhibited by Yukon *Eutrema*. My work also raises important questions about the mechanisms involved and so the research tools including transcriptomes that have been developed as part of my doctoral research can be used to further enhance our understanding of how *Eutrema* copes with water deficits. Finally, the availability of the Shandong accession that responds differently to drought treatment, Yukon in-bred lines as

well as different Yukon natural populations are all valuable research tools making *Eutrema* an ideal model plant to study how plants respond to drought in the cabinet and under field conditions.

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Appendix

Appendix 1. Significantly enriched GO terms for all DESeq comparisons

Appendix 2. Correlation scores for all modules from WGCNA

Appendix 3. Mean expression data for modules 3, 13, and 17 from the WGCNA

Appendix 4. Water potential (Ψ_w), solute potential (Ψ_s) and pressure potential (Ψ_p) for individual Yukon *Eutrema* field plants.

Appendix 1. Gene ontology (GO)-enriched terms from sets of differentially expressed genes (DEGs) identified from pair-wise comparisons made using DESeq of Yukon (Y) or Shandong (S) plants that were: well-watered (WW1), drought-treated (D1), re-watered (WW2), or re-watered and subsequently drought-treated (D2). GO terms were significantly enriched if they had a false discovery rate (FDR) <0.05.

DESeq comparison	Set of DEGs used for analysis	Enriched GO terms	FDR
YD1 → YWW1	Up in YD1	secondary metabolic process	1.80E-05
		response to stimulus	2.20E-03
	Down in YD1	translation	6.70E-45
		photosynthesis	1.10E-21
		cellular macromolecule biosynthetic process	3.80E-11
		macromolecule biosynthetic process	3.80E-11
		cellular biosynthetic process	1.30E-10
		biosynthetic process	2.90E-10
		cell cycle	3.20E-08
		cellular protein metabolic process	9.50E-08
		gene expression	7.30E-07
		protein metabolic process	9.30E-06
		response to stimulus	1.80E-05
		DNA metabolic process	1.10E-04
		cellular metabolic process	1.40E-04
		cellular process	4.20E-04
		generation of precursor metabolites and energy	1.90E-03
		metabolic process	4.10E-03
		cellular macromolecule metabolic process	1.20E-02
		response to biotic stimulus	2.20E-02
		macromolecule metabolic process	3.00E-02
		response to abiotic stimulus	3.50E-02

Appendix 1. Continued

DESeq comparison	Set of DEGs used for analysis	Enriched GO terms	FDR
YD1 → YWW2	Up in YD1	secondary metabolic process	1.60E-07
		cellular amino acid and derivative metabolic process	7.40E-04
	Down in YD1	response to stimulus	4.50E-03
YD2 → YWW2	Up in YD2	response to abiotic stimulus	3.10E-02
		response to stress	4.30E-02
		response to stimulus	4.60E-02
	Down in YD2	none	n/a[a]
YD2 → YWW1	Up in YD2	photosynthesis	4.00E-26
		cell cycle	4.80E-10
		translation	9.00E-06
		generation of precursor metabolites and energy	2.00E-05
		DNA metabolic process	1.60E-04
	Down in YD2	response to stress	3.20E-05
		response to stimulus	2.50E-03
YWW2 → YWW1	Up in YWW2	response to biotic stimulus	2.60E-02
	Down in YWW2	none	n/a
SD1 → SWW1	Up in SD1	none	n/a
	Down in SD1	none	n/a

[a] not applicable as no terms were enriched

Appendix 1. Continued

DESeq comparison	Set of DEGs used for analysis	Enriched GO terms	FDR
SD1 → SWW2	Up in SD1	response to stress	1.80E-03
		secondary metabolic process	3.30E-03
		response to biotic stimulus	1.20E-02
		response to stimulus	1.20E-02
		response to abiotic stimulus	1.50E-02
		response to external stimulus	1.80E-02
SD1 →SWW2	Down in SD1	translation	1.00E-27
		cellular protein metabolic process	6.40E-09
		cellular biosynthetic process	9.20E-09
		gene expression	1.10E-08
		biosynthetic process	1.10E-08
		cellular macromolecule biosynthetic process	3.80E-08
		macromolecule biosynthetic process	4.20E-08
		protein metabolic process	3.90E-07
		cellular metabolic process	2.60E-06
		cellular macromolecule metabolic process	1.20E-04
		photosynthesis	1.20E-04
		cellular process	3.20E-04
		metabolic process	6.70E-04
		macromolecule metabolic process	1.50E-03
		primary metabolic process	1.90E-03
		generation of precursor metabolites and energy	4.80E-03
		multi-organism process	2.50E-02
		embryonic development	2.80E-02
		homeostatic process	4.10E-02
		cellular homeostasis	4.30E-02

Appendix 1. Continued

DESeq comparison	Set of DEGs used for analysis	Enriched GO terms	FDR
SD2 → SWW2	Up in SD2	none	n/a
	Down in SD2	translation	1.40E-08
		DNA metabolic process	5.90E-08
		photosynthesis	1.10E-07
		cellular biosynthetic process	6.30E-05
		biosynthetic process	7.50E-05
		cellular macromolecule biosynthetic process	2.20E-03
		macromolecule biosynthetic process	2.30E-03
		cell cycle	5.30E-03
		cellular metabolic process	1.90E-02
SWW2 → SWW1	Up in SWW2	photosynthesis	6.70E-08
		cellular metabolic process	3.80E-02
		translation	3.80E-02
	Down in SWW2	response to biotic stimulus	7.20E-04
		multi-organism process	2.70E-03
		response to stimulus	2.70E-03
		response to stress	2.70E-03
		response to external stimulus	4.80E-02

Appendix 2. Correlation scores for modules of co-expressed genes identified by WGCNA performed on 4317 DEGs identified from transcriptomes of Yukon (Y) or Shandong (S) plants that were: well-watered (WW1), drought-treated (D1), re-watered (WW2), or re-watered and subsequently drought-treated (D2). The significance of teach correlation is indicated by a *p*-value

	Correlation score and *p-value*															
Mod. #	YWW1		YD1		YWW2		YD2		SWW1		SD1		SWW2		SD2	
33	0.74	*0.00*	-0.05	*0.87*	0.42	*0.10*	-0.15	*0.58*	-0.14	*0.61*	0.29	*0.29*	-0.06	*0.81*	-0.48	*0.06*
34	0.64	*0.01*	0.31	*0.24*	0.15	*0.59*	-0.27	*0.32*	-0.11	*0.68*	0.75	*0.75*	-0.07	*0.81*	-0.57	*0.02*
35	0.58	*0.02*	0.06	*0.82*	0.08	*0.76*	-0.52	*0.04*	0.09	*0.74*	0.88	*0.88*	0.05	*0.85*	-0.39	*0.14*
36	0.74	*0.00*	-0.09	*0.74*	-0.11	*0.69*	-0.48	*0.06*	0.24	*0.37*	0.52	*0.52*	-0.13	*0.63*	-0.35	*0.19*
37	0.80	*0.00*	-0.29	*0.28*	0.09	*0.74*	-0.39	*0.13*	0.09	*0.74*	0.75	*0.75*	0.09	*0.75*	-0.30	*0.26*
38	0.64	*0.01*	-0.29	*0.28*	0.18	*0.51*	-0.44	*0.09*	0.09	*0.74*	0.99	*0.99*	0.21	*0.43*	-0.40	*0.13*
39	0.81	*0.00*	-0.17	*0.53*	0.20	*0.46*	-0.37	*0.16*	-0.02	*0.95*	0.95	*0.95*	-0.06	*0.82*	-0.37	*0.15*
40	0.32	*0.23*	-0.52	*0.04*	0.13	*0.63*	-0.54	*0.03*	0.29	*0.27*	0.47	*0.47*	0.29	*0.27*	-0.17	*0.53*
41	0.32	*0.23*	-0.35	*0.18*	0.12	*0.67*	-0.60	*0.01*	0.24	*0.37*	0.50	*0.50*	0.38	*0.14*	-0.29	*0.28*
42	0.29	*0.27*	-0.46	*0.07*	0.03	*0.92*	-0.65	*0.01*	0.32	*0.23*	0.42	*0.42*	0.34	*0.20*	-0.08	*0.76*
43	0.21	*0.44*	-0.40	*0.12*	-0.06	*0.82*	-0.61	*0.01*	0.30	*0.25*	0.46	*0.46*	0.40	*0.12*	-0.04	*0.88*
43	0.15	*0.57*	-0.48	*0.06*	0.04	*0.88*	-0.57	*0.02*	0.29	*0.28*	0.56	*0.56*	0.46	*0.07*	-0.05	*0.84*
44	0.24	*0.38*	-0.37	*0.16*	-0.06	*0.81*	-0.45	*0.08*	0.21	*0.44*	0.99	*0.99*	0.65	*0.01*	-0.22	*0.40*
45	0.49	*0.06*	-0.27	*0.31*	0.25	*0.36*	-0.44	*0.09*	0.04	*0.89*	0.81	*0.81*	0.39	*0.14*	-0.38	*0.14*
46	0.47	*0.07*	-0.33	*0.22*	0.10	*0.72*	-0.46	*0.08*	0.16	*0.55*	0.95	*0.95*	0.38	*0.15*	-0.30	*0.25*
47	0.51	*0.04*	-0.36	*0.17*	0.19	*0.47*	-0.61	*0.01*	0.16	*0.55*	0.66	*0.66*	0.26	*0.33*	-0.27	*0.31*
48	0.48	*0.06*	-0.38	*0.15*	0.30	*0.26*	-0.51	*0.04*	0.15	*0.57*	0.88	*0.88*	0.30	*0.26*	-0.38	*0.14*
49	0.36	*0.17*	-0.10	*0.70*	0.13	*0.63*	-0.54	*0.03*	0.14	*0.59*	0.81	*0.81*	0.35	*0.19*	-0.40	*0.12*
50	0.31	*0.25*	-0.45	*0.08*	0.29	*0.28*	-0.47	*0.06*	0.26	*0.33*	0.84	*0.84*	0.40	*0.12*	-0.39	*0.13*
51	0.13	*0.63*	-0.50	*0.05*	0.25	*0.35*	-0.44	*0.09*	0.27	*0.31*	0.93	*0.93*	0.50	*0.05*	-0.23	*0.39*
52	0.29	*0.27*	-0.57	*0.02*	0.10	*0.72*	-0.25	*0.35*	0.25	*0.36*	0.80	*0.80*	0.40	*0.12*	-0.15	*0.57*
53	0.16	*0.55*	-0.36	*0.17*	0.28	*0.30*	-0.38	*0.15*	0.04	*0.87*	0.58	*0.58*	0.58	*0.02*	-0.19	*0.49*
54	0.14	*0.61*	-0.35	*0.18*	0.21	*0.43*	-0.44	*0.08*	0.08	*0.77*	0.77	*0.77*	0.60	*0.01*	-0.15	*0.58*
55	0.12	*0.66*	-0.44	*0.09*	0.29	*0.28*	-0.29	*0.28*	0.15	*0.58*	0.65	*0.65*	0.54	*0.03*	-0.25	*0.36*
56	0.26	*0.33*	-0.34	*0.19*	0.28	*0.29*	-0.40	*0.12*	0.09	*0.74*	0.71	*0.71*	0.52	*0.04*	-0.30	*0.25*
57	0.26	*0.33*	-0.37	*0.16*	0.27	*0.31*	-0.46	*0.08*	0.16	*0.55*	0.81	*0.81*	0.50	*0.05*	-0.31	*0.25*
58	0.29	*0.28*	-0.38	*0.14*	0.35	*0.19*	-0.36	*0.17*	0.07	*0.80*	0.55	*0.55*	0.49	*0.05*	-0.28	*0.29*
59	0.24	*0.36*	-0.44	*0.09*	0.38	*0.14*	-0.32	*0.23*	0.16	*0.56*	0.65	*0.65*	0.47	*0.06*	-0.38	*0.15*
60	0.20	*0.46*	-0.38	*0.14*	0.41	*0.12*	-0.21	*0.45*	0.03	*0.91*	0.35	*0.35*	0.50	*0.05*	-0.30	*0.26*
61	0.16	*0.55*	-0.40	*0.13*	0.36	*0.17*	-0.16	*0.54*	0.04	*0.88*	0.36	*0.36*	0.51	*0.04*	-0.27	*0.32*

	Correlation score and *p-value*															
Mod. #	YWW1		YD1		YWW2		YD2		SWW1		SD1		SWW2		SD2	
1	-0.67	*0.00*	0.38	*0.14*	-0.08	*0.77*	0.47	*0.07*	-0.19	*0.49*	0.72	*0.72*	-0.16	*0.55*	0.34	*0.19*
2	-0.53	*0.03*	0.33	*0.21*	-0.24	*0.37*	0.50	*0.05*	-0.19	*0.48*	0.96	*0.96*	-0.29	*0.27*	0.40	*0.12*
3	-0.47	*0.06*	0.27	*0.30*	-0.07	*0.80*	0.64	*0.01*	-0.30	*0.26*	0.56	*0.56*	-0.30	*0.26*	0.38	*0.14*
4	-0.41	*0.11*	0.25	*0.35*	-0.27	*0.30*	0.47	*0.06*	-0.32	*0.23*	0.67	*0.67*	-0.23	*0.40*	0.63	*0.01*
5	-0.55	*0.03*	0.42	*0.11*	-0.37	*0.16*	0.32	*0.23*	-0.10	*0.72*	0.68	*0.68*	-0.29	*0.27*	0.47	*0.07*
6	-0.54	*0.03*	0.34	*0.20*	-0.36	*0.17*	0.39	*0.14*	0.01	*0.96*	0.49	*0.49*	-0.35	*0.18*	0.33	*0.22*
7	-0.37	*0.16*	0.56	*0.03*	-0.18	*0.50*	0.39	*0.14*	-0.29	*0.28*	0.61	*0.61*	-0.27	*0.31*	0.30	*0.26*
8	-0.36	*0.17*	0.57	*0.02*	-0.24	*0.37*	0.39	*0.14*	-0.25	*0.36*	0.97	*0.97*	-0.40	*0.13*	0.29	*0.27*
9	-0.43	*0.10*	0.58	*0.02*	-0.29	*0.27*	0.29	*0.28*	-0.06	*0.82*	0.54	*0.54*	-0.35	*0.18*	0.11	*0.69*
10	-0.33	*0.21*	0.47	*0.07*	-0.24	*0.37*	0.48	*0.06*	-0.11	*0.68*	0.86	*0.86*	-0.53	*0.03*	0.23	*0.40*
11	-0.34	*0.19*	0.42	*0.10*	-0.29	*0.27*	0.43	*0.09*	-0.04	*0.88*	0.63	*0.63*	-0.57	*0.02*	0.26	*0.33*
12	-0.25	*0.36*	0.58	*0.02*	-0.15	*0.59*	0.51	*0.04*	-0.20	*0.46*	0.90	*0.90*	-0.53	*0.03*	0.07	*0.79*
13	-0.41	*0.11*	0.32	*0.23*	-0.09	*0.74*	0.58	*0.02*	-0.02	*0.94*	0.95	*0.95*	-0.45	*0.08*	0.09	*0.75*
14	-0.07	*0.80*	0.56	*0.02*	0.08	*0.76*	0.63	*0.01*	-0.36	*0.17*	0.30	*0.30*	-0.41	*0.11*	-0.17	*0.54*
15	-0.08	*0.77*	0.70	*0.00*	0.01	*0.97*	0.50	*0.05*	-0.41	*0.11*	0.31	*0.31*	-0.37	*0.16*	-0.08	*0.75*
16	-0.24	*0.37*	0.72	*0.00*	-0.09	*0.74*	0.47	*0.07*	-0.36	*0.17*	0.34	*0.34*	-0.28	*0.29*	0.05	*0.86*
17	-0.33	*0.21*	0.45	*0.08*	0.07	*0.80*	0.69	*0.00*	-0.40	*0.12*	0.35	*0.35*	-0.30	*0.25*	0.08	*0.76*
18	-0.18	*0.51*	0.78	*0.00*	-0.26	*0.34*	0.40	*0.13*	-0.28	*0.30*	0.70	*0.70*	-0.38	*0.15*	0.01	*0.96*
19	-0.18	*0.51*	0.74	*0.00*	-0.24	*0.36*	0.33	*0.21*	-0.24	*0.38*	0.89	*0.89*	-0.42	*0.11*	-0.04	*0.88*
20	0.02	*0.95*	0.76	*0.00*	-0.20	*0.46*	0.42	*0.10*	-0.28	*0.30*	0.64	*0.64*	-0.40	*0.12*	-0.19	*0.49*
21	-0.64	*0.01*	-0.13	*0.63*	-0.41	*0.11*	0.06	*0.82*	0.21	*0.43*	0.21	*0.21*	-0.02	*0.93*	0.60	*0.01*
22	0.60	*0.01*	0.32	*0.22*	-0.50	*0.05*	-0.22	*0.42*	0.16	*0.56*	0.48	*0.48*	-0.44	*0.09*	-0.11	*0.68*
23	0.56	*0.02*	0.15	*0.57*	-0.51	*0.04*	-0.32	*0.23*	0.29	*0.28*	0.29	*0.29*	-0.39	*0.14*	-0.07	*0.80*
24	0.66	*0.01*	0.42	*0.10*	-0.16	*0.56*	-0.25	*0.35*	-0.10	*0.71*	0.95	*0.95*	-0.26	*0.32*	-0.33	*0.21*
25	0.20	*0.45*	0.72	*0.00*	-0.36	*0.17*	0.12	*0.65*	-0.06	*0.83*	0.64	*0.64*	-0.57	*0.02*	-0.18	*0.49*
26	0.57	*0.02*	0.24	*0.37*	0.40	*0.13*	0.22	*0.42*	-0.34	*0.20*	0.14	*0.14*	-0.33	*0.21*	-0.37	*0.15*
27	-0.33	*0.21*	-0.11	*0.68*	0.72	*0.00*	0.33	*0.22*	-0.10	*0.71*	0.93	*0.93*	-0.31	*0.24*	-0.17	*0.52*
28	0.54	*0.03*	0.14	*0.60*	0.21	*0.42*	-0.40	*0.13*	-0.12	*0.66*	0.71	*0.71*	0.17	*0.52*	-0.45	*0.08*
29	0.48	*0.06*	-0.08	*0.77*	0.26	*0.34*	-0.29	*0.27*	-0.05	*0.86*	0.58	*0.58*	0.32	*0.22*	-0.49	*0.06*
30	0.46	*0.07*	-0.16	*0.55*	0.54	*0.03*	-0.22	*0.42*	-0.10	*0.72*	0.28	*0.28*	0.21	*0.43*	-0.45	*0.08*
31	0.60	*0.01*	-0.31	*0.24*	0.51	*0.05*	-0.27	*0.31*	0.04	*0.87*	0.36	*0.36*	0.08	*0.77*	-0.40	*0.13*
32	0.70	*0.00*	0.11	*0.69*	0.37	*0.16*	-0.33	*0.22*	-0.07	*0.80*	0.58	*0.58*	-0.14	*0.59*	-0.49	*0.05*

	Correlation score and *p-value*															
Mod. #	YWW1		YD1		YWW2		YD2		SWW1		SD1		SWW2		SD2	
62	0.17	*0.53*	-0.36	*0.18*	0.39	*0.13*	-0.15	*0.57*	-0.02	*0.94*	0.26	*0.26*	0.52	*0.04*	-0.26	*0.34*
63	0.29	*0.27*	-0.49	*0.05*	0.15	*0.58*	0.22	*0.42*	-0.02	*0.95*	0.59	*0.59*	0.01	*0.98*	-0.01	*0.97*
64	-0.24	*0.36*	-0.16	*0.55*	0.06	*0.84*	-0.06	*0.84*	-0.16	*0.56*	0.23	*0.23*	0.90	*0.00*	-0.01	*0.96*
65	-0.12	*0.65*	-0.24	*0.37*	-0.11	*0.68*	-0.23	*0.40*	-0.02	*0.94*	0.48	*0.48*	0.96	*0.00*	-0.04	*0.87*
66	0.31	*0.24*	-0.57	*0.02*	-0.26	*0.32*	-0.38	*0.15*	0.42	*0.11*	0.41	*0.41*	0.30	*0.25*	-0.05	*0.87*
67	0.30	*0.26*	-0.47	*0.07*	-0.24	*0.38*	-0.18	*0.50*	0.42	*0.11*	0.34	*0.34*	0.11	*0.70*	-0.19	*0.49*
68	0.24	*0.38*	-0.65	*0.01*	-0.05	*0.84*	-0.49	*0.06*	0.49	*0.06*	0.20	*0.20*	0.13	*0.63*	-0.01	*0.98*
69	0.03	*0.90*	-0.73	*0.00*	-0.18	*0.50*	-0.11	*0.68*	0.40	*0.13*	0.27	*0.27*	0.09	*0.75*	0.21	*0.43*
70	0.14	*0.60*	-0.78	*0.00*	-0.03	*0.92*	-0.27	*0.32*	0.33	*0.21*	0.48	*0.48*	0.30	*0.26*	0.12	*0.67*
71	-0.06	*0.83*	-0.56	*0.02*	-0.06	*0.83*	-0.45	*0.08*	0.36	*0.17*	0.78	*0.78*	0.64	*0.01*	0.06	*0.83*
72	-0.04	*0.90*	-0.50	*0.05*	-0.22	*0.41*	-0.50	*0.05*	0.48	*0.06*	0.30	*0.30*	0.49	*0.05*	0.02	*0.95*
73	-0.26	*0.33*	-0.48	*0.06*	-0.34	*0.20*	-0.33	*0.21*	0.56	*0.03*	0.09	*0.09*	0.18	*0.51*	0.24	*0.37*
74	0.38	*0.14*	-0.15	*0.59*	-0.13	*0.63*	-0.57	*0.02*	0.41	*0.12*	0.28	*0.28*	-0.01	*0.98*	-0.23	*0.39*
75	0.46	*0.07*	-0.07	*0.79*	-0.21	*0.43*	-0.62	*0.01*	0.18	*0.51*	0.41	*0.41*	0.17	*0.52*	-0.14	*0.62*
76	0.31	*0.24*	-0.22	*0.40*	-0.39	*0.14*	-0.57	*0.02*	0.47	*0.07*	0.07	*0.07*	0.00	*0.99*	-0.05	*0.84*
77	-0.12	*0.66*	-0.35	*0.19*	-0.19	*0.49*	-0.17	*0.52*	0.77	*0.00*	0.14	*0.14*	-0.24	*0.37*	-0.09	*0.75*
78	-0.17	*0.52*	-0.42	*0.11*	-0.03	*0.91*	-0.19	*0.48*	0.67	*0.00*	0.03	*0.03*	-0.18	*0.51*	-0.23	*0.40*
79	-0.24	*0.37*	-0.20	*0.45*	-0.38	*0.15*	-0.22	*0.42*	0.65	*0.01*	0.02	*0.02*	-0.21	*0.43*	0.04	*0.87*
80	-0.31	*0.24*	-0.13	*0.62*	0.05	*0.84*	0.16	*0.57*	0.56	*0.02*	0.19	*0.19*	-0.42	*0.11*	-0.26	*0.34*
81	-0.25	*0.35*	-0.24	*0.37*	-0.13	*0.64*	0.06	*0.82*	0.59	*0.02*	0.12	*0.12*	-0.36	*0.17*	-0.09	*0.75*
82	0.30	*0.26*	-0.39	*0.13*	-0.08	*0.76*	-0.09	*0.74*	0.67	*0.00*	0.40	*0.40*	-0.29	*0.28*	-0.33	*0.21*
83	0.14	*0.61*	-0.46	*0.07*	-0.28	*0.29*	0.02	*0.96*	0.28	*0.30*	0.37	*0.37*	0.02	*0.94*	0.05	*0.84*
84	0.09	*0.75*	-0.44	*0.09*	-0.20	*0.46*	0.16	*0.56*	0.17	*0.53*	0.91	*0.91*	0.02	*0.95*	0.18	*0.52*
85	-0.02	*0.96*	-0.35	*0.18*	-0.27	*0.31*	0.07	*0.80*	0.48	*0.06*	0.28	*0.28*	-0.30	*0.26*	0.10	*0.71*
86	-0.18	*0.51*	-0.08	*0.76*	-0.19	*0.47*	0.33	*0.20*	0.29	*0.28*	0.51	*0.51*	-0.46	*0.07*	0.11	*0.68*

Appendix 3. Transcript abundance for selected groups of genes correlated to drought treatments in Yukon *Eutrema* according to WGCNA. The values reported are the mean ± SE of the expression (RPKM) values for all genes in modules 3, 13, and 17.

Module #	Mean expression (RPKM) ± SE							
	YWW1	YD1	YWW2	YD2	SWW1	SD1	SWW2	SD2
3	8.68 ± 2.01	28.81 ± 6.05	16.04 ± 4.26	40.95 ± 9.4	11.23 ± 3.26	15.05 ± 4.70	10.73 ± 2.77	28.19 ± 6.91
13	6.26 ± 1.29	18.40 ± 3.14	11.42 ± 2.51	30.45 ± 6.68	10.92 ± 2.01	11.61 ± 2.10	6.80 ± 1.46	19.33 ± 4.78
17	7.66 ± 1.56	37.54 ± 9.54	14.71 ± 3.46	41.20 ± 10.11	7.32 ± 1.92	9.33 ± 1.97	8.15 ± 2.08	20.03 ± 4.48

Appendix 4. Water potential (Ψ_w), solute potential (Ψ_s) and pressure potential (Ψ_p) for individual Yukon *Eutrema* field plants. Ψ_w and Ψ_s data are the mean ± SE of three technical replicate measurements per plant.

Field sample	Mean Potential ± SE (MPa)		
	Ψ_w	Ψ_s	Ψ_p[a]
P3-1	-1.67 ± 0.03	-2.35 ± 0.06	0.68 ± 0.07
P3-2	-1.81 ± 0.10	-2.60 ± 0.06	0.79 ± 0.12
P3-3	-2.66 ± 0.01	-2.93 ± 0.03	0.27 ± 0.03

[a] Ψ_p given ± propagated SE of Ψ_w and Ψ_s measurement