CHLOROPHYLL FLUORESCENCE, CELL DEATH, AND
TRANSCRIPTION PROFILE OF LONG TERM MODERATELY HIGH
TEMPERATURE HEAT STRESSED *ARABIDOPSIS THALIANA*,
*BOECHERA ARCUATA*, AND *BOECHERA DEPAUPERATA*

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with a Concentration in
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by
Abdullah Jamali

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The Undersigned Faculty Committee Approves the

Thesis of Abdullah Jamali:

Chlorophyll Fluorescence, Cell Death, and Transcription Profile of Long Term
Moderately High Temperature Heat Stressed Arabidopsis thaliana, Boechera
arcuata, and Boechera depauperata

Elizabeth R. Waters, Chair
Department of Biology

Ricardo Zayas
Department of Biology

Faramar Valafar
Bioinformatics and Medical Informatics Program

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Approval Date
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ABSTRACT OF THE THESIS

Chlorophyll Fluorescence, Cell Death, and Transcription Profile of Long Term Moderately High Temperature Heat Stressed Arabidopsis thaliana, Boechera arcuata, and Boechera depauperata

by
Abdullah Jamali
Master of Science in Biology with a Concentration in Molecular Biology
San Diego State University, 2014

Most studies of plant responses to heat stress examine basal thermotolerance and short-term acquired thermotolerance to high heat. In nature plants experience moderately high temperatures (30°C-38°C) for long periods of time. The goal of this study is to find the effects of long term moderately high temperature heat stress on the two native California species, Boechera arcuata and Boechera depauperata, and compare it to a model organism Arabidopsis thaliana. We measure chlorophyll fluorescents (CF), cell death, and transcriptional fold change for APX2, HSFA2, HSP101, and HSP18.1 to heat stress at 35°C and 38°C. We asked the following three questions: How is the efficiency of Photosystem II (PSII) affected by moderately high temperature heat stress in A. thaliana, Boechera arcuata, and Boechera depauperata? Is cell death under heat stress different between A. thaliana and the two Boechera species? Is gene expression under heat stress different between A. thaliana and the two Boechera species? B. depauperata is the most thermotolerant of the three species; at both heat stress temperatures, PSII does not lose conformation and continues to absorb light and fixate carbon. Ion leakage is minimized and there is very little cell death. Upregulation of APX2, HSFA2, and HSP18.1 only occur after 72 hours of continuous heat stress at 38°C in B. depauperata. HSP101 is upregulated at 1 hour at 35°C, but decays after that, and at 72 hours at 38°C. A. thaliana can withstand 35°C heat stress, but 38°C results in PSII efficiency loss, and cell death. Transcription of APX2, HSFA2, HSP101, and HSP18.1 is upregulated substantially more than the other two species at both heat stress temperatures. B. arcuata is the least thermotolerant of the three species studied. Both 35°C and 38°C have an effect on PSII efficiency and cell death, but 38°C has a larger affect. Transcription of APX2, HSFA2, and HSP101 are upregulated early at 1 hour, but decay and back to the same levels as the control by hour 12. HSP18.1 is not upregulated at 35°C and upregulated at 38°C after 48 hours of continuous stress. All three species handle moderately high temperature heat stress differently. B. depauperata is the most thermotolerant, A. thaliana is the second most thermotolerant, and B. arcuata is the least thermotolerant. These findings can help us better understand and predict the effects of global rise in temperatures on plant species and it can lead to designing better crops to withstand abiotic stress.
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INTRODUCTION

OVERVIEW

The aim of this project is to understand the effects of moderately high temperatures on plant species *Arabidopsis thaliana*, *Boechera arcuata*, and *Boechera depauperata*. Heat stress response (HSR) on a molecular level is complex, our goals are to better understand the effects of heat stress on chlorophyll fluorescents (CF) and cell death, HSR, and thermotolerance pathways (Ahuja et al., 2010). We measured cell death and membrane integrity by measuring ion leakage. We looked at the differences in efficiency of photochemical energy conversion to fixate carbon by Photosystem II (PSII). This project identified differentially expressed genes between temperatures and in each species.

Most studies of plant responses to stress examine basal thermotolerance and short-term acquired thermotolerance to high heat. In nature plants are exposed to moderately high temperatures (30°C-38°C) for 12 hours or more in some places. Plants experience moderately high temperatures more often than extreme high temperatures. In a laboratory experiment all conditions are controlled except for one variable. This allows us to associate one heat stress to a cell death. The goal of this study is to find the effects of moderately high temperatures on the two native California species and compare it to a model organism.

While there are very detailed studies of the stress responses of model species there is much less known about the stress responses of non-model species. It is important to note that plant responses to stress due to the environment are complex and include changes in gene expression, protein production and metabolic rates (Ahuja et al., 2010). Stress response is regulated by interacting pathways, and this project sheds light on the specific responses of the thermotolerance pathways in relation to stress. Plants potential to adapt to environmental conditions depends on many interacting mechanisms, such as genetic diversity, demographic factors, evolutionary history, interaction of genes with the environment, and ranges of temperatures in their current and historical environment (Alonso-Blanco et al., 2009). Species that are near their lethal thermal limit have little room to adapt to oncoming higher temperatures (Somero, 2010). Changes in gene expression due to heat are different for basal
heat stress events compared to long term moderately high temperature stress (Mittler et al., 2012).

Elevated temperatures or heat stress can cause proteins to denature, leading to loss of function and cell death. Chaperone proteins are a group of proteins that fold nascent proteins into their final form, refold denatured proteins and prevent protein aggregation (Eyles and Gierasch, 2010). Plants are sessile organisms which must acclimate rapidly to changing environmental conditions to prevent death (Yeh et al., 2012). During heat stress, plants are able to turn on cell machinery to prevent cell death (Xue et al., 2012). How plants are able to survive fluctuating temperatures is not well understood (Mittler et al., 2012). There are numerous studies on short term heat stress and plant acclimation to heat stress, but little is known about continuous heat stress to moderately high temperatures (Yeh et al., 2012).

The aim of this project was to identify the mechanisms leading to long term moderate heat (35°C and 38°C) tolerance in *A. thaliana*, *B. arcuata*, and *B. depauperata*. *A. thaliana* is a model organism, and there are many tools already available for it. Previous studies in our lab of *Boechera* species have shown *B. arcuata* to be heat sensitive and *B. depauperata* to be very heat tolerant. This project revealed species-specific and temperature differences by comparing phenotype, genotype, and protein expression levels.

Previous studies have focused on short term heat stress or acquired heat stress. Heat stress is important because planet Earth is warming up (Horvath et al., 2012). Historical records from NASA have shown Earth to have increased about three degrees Celsius, what affects does another three degrees change have on native California plant species? In this study two native California plants and one model organism were heat stressed at 35°C and 38°C for up to 96 hours. Photosynthesis was measured using Pulse-amplitude modulated (PAM) junior, cell death was measured using ion leakage to check for membrane integrity, and RT-qPCR was performed to measure transcript levels for heat shock response genes.

**HEAT STRESS RESPONSE IN PLANTS**

Plants are sessile organisms in a constantly changing environment. Heat stress can significantly affect cellular homeostasis, denaturing proteins and loss of membrane stability. Proteins can aggregate and lose conformation at temperatures as low as 35°C (Deridder et al,
In the real world, plants are not just stressed by heat, but a combination of heat, light, drought, salt, UV light, and other stresses.

In order to deal with heat-associated types of damage, plants have developed mechanisms to detect and respond to temperature change (Tyedmers et al., 2010). Before an appropriate cellular response to heat stress can be activated, the plant must first be able to detect that it is exposed to a temperature high enough to cause cellular damage. Five mechanisms have been proposed for how plants detect and activate cellular responses to temperature change. The first proposed mechanism is that the physical structure of the cell membrane changes at higher temperature by becoming more fluid, releasing a substrate that interacts with the DNA, leading to activation of heat shock response (HSR) (Mittler et al., 2012). The second model (Figure 1) is that heat shock increases membrane permeability to calcium, and that a resulting increase in intercellular calcium triggers HSPs induction (Horvath et al., 2012). The third method of how cells sense temperature is through chromatin changes leading to DNA-protein interaction and the displacement of histones leading to HSR. The fourth way is through the buildup of toxic byproducts from incomplete enzymatic reactions, such as the production of reactive oxygen species (ROS) leading to HSR (Miller et al., 2008). The fifth way cells are able to sense temperature is through RNA unfolding, miRNA interaction and loss of spliceosome function (Mittler et al., 2012).

Figure 2 shows the different ways HRS can be turned on once a plant is exposed to heat (Mittler et al., 2012).

**HSR Genes**

Heat shock proteins (HSPs) are chaperone proteins that are found in all living organisms (Renner and Waters, 2007). HSPs transport proteins across membranes into organelles, fold newly translated proteins, and repair aggregated proteins (Haslbeck et al., 2005). It has been established that HSPs are molecular chaperones that bind to denatured proteins and prevent irreversible aggregation when plants are under heat stress (Eyles and Giersachs, 2010). Heat shock proteins are not only required for thermotolerance but they have also been shown to play a role at ambient temperatures as housekeeping genes (Haslbeck et al., 2005).
Figure 1. HSR. When a cell starts to experience heat, the membrane becomes fluid and there is calcium influx into the cell, proteins denature, and reactive oxygen species (ROS) are made. The influx of calcium, denatured proteins, and ROS turn on HSR.

There are different types of heat stress. In basal heat stress, samples are moved from 22°C directly to 44°C-45°C. In acquired thermotolerance, there is a pretreatment at 38°C for an hour followed by 22°C for an hour before being exposed to 44°C-45°C heat stress (Yeh et al., 2012). Figure 3 shows the different type of heat stress regimens being studied. Acquired thermotolerance is the ability to acclimate to heat stress. Acclimation allows plants to survive longer under heat stress compared to non-acclimated plants (Larkindale and Vierling, 2008). Plants that have a temperature threshold before HSR is activated and different species have different minimum temperature for induction of HSR (Amano et al., 2012).
Figure 2. Schematic model of temperature sensing in plants.

Figure 3. Different types of heat stress regimens.
**Species Studied**

*Arabidopsis thaliana* is an annual plant in the *Brassicaceae* family that can be found in Europe and central Asia. *Arabidopsis* is a model organism studied by many labs, because it is a small plant that has a short vernalization time of 3 days, it matures quickly, it produces a lot of seeds, has a small genome, the whole genome has been sequenced, and the plant has been studied for over 50 years (Arabidopsis Genome Institute, 2000; Meyerowitz, 1989). There are many tools available for *Arabidopsis thaliana*, for example the SALK institute has tDNA lines that can be ordered, with tDNA insertion in each specific known gene, for gene knockout studies. In our studies, we use *Arabidopsis thaliana* ecotype Columbia, as a control to compare our non-model organism species to.

The genus *Boechera* is also in the *Brassicaceae* family, and it is widely distributed throughout North America (Beck et al., 2012). They are a close relative to *Arabidopsis thaliana*, and many of the *Boechera* species are endemic to California. They live in diverse habitats with different temperatures and precipitation ranges (Beck et al., 2012). Many of the molecular, biochemical, and genetic tools available for *Arabidopsis* can also be used in *Boechera* studies. The accession of *B. “depauperata”* was collected in Yosemite National Park in California at 11,150 feet elevation. *B. depauperata* is a polyploidy. The accession of *B. arcuata* is from the Theodore Payne foundation, was collected from Los Angeles River in Little Tujunga, CA. *B. arcuata* is limited to southern and western California. These two *Boechera* accessions come from two very different environments, one from high elevation with winter freezing, and the other from coastal low elevation without winter freezing.

**CF and PSII Efficiency**

PSII is the enzyme responsible for absorbing light energy which later is used to fixate carbon. After absorbing light, PSII oxidizes water through the transfer of electrons, and then electrons are transferred to plastoquinone (Goussias et al., 2002). When a cell is under stress conditions such as salt stress or heat stress, there is a decrease in quantum yield of PSII electron transfer on both the receiver and donor ends of PSII (Lu and Vonshak, 2002). Extreme temperatures induce a reduction in CO2 fixation rate with a constant increase in ROS production, leading to leaf senescence and yield loss (Pinto-Marijuan and Munne-Bosch, 2014).
Photosynthesis is essential for plants, but excess light can be damaging. Plants have evolved different methods to combat excessive light, by reducing leaf surface area, or growth of trichomes, production of wax, or changing leaf angle to the sun (Murata et al., 2007). Figure 4 shows when light energy is absorbed by a plant, it can go down three pathways; Production of heat, fluorescence, or photochemistry to fixate carbon. Light not utilized for photosynthesis can be damaging to the plant. Plants have biochemical photoprotective mechanisms to reduce excess light energy within the plant to prevent absorbed light from causing damage by dissipation of light energy as heat (Murata et al., 2007). When light begins to exceed the capacity of the photochemical machinery, the xanthophyll cycle begins by dissipating excess light energy using carotenoids to dissipate excess light energy (Murata et al., 2007). Plants grown in high light have more carotenoid pigments compared to low-light grown plants (Murata et al., 2007).

![Figure 4. CF pathway. The chlorophyll is excited from absorbing light energy, the energy absorbed energy can then go down three different paths of, fluorescence, dissipation as heat, or carbon fixation.](image)

When the plant is under heat stress, the internal mechanisms may stop working, excess light energy is not converted to heat, but instead reactive oxygen species (ROS) are made (Murata et al., 2007). The production of ROS can lead to further photo-inhibition and photo damage. Photoinhibition results in the decrease of light energy conversion to photochemical energy by the photosystems, especially PSII (Murata et al., 2007). Light is essential for photosynthesis, but excess light can be harmful for a plant due to oxidative stress and the production of ROS (Rossel et al., 2006). ROS can damage proteins, enzymes, and lipids. Non-photochemical quenching and changes in gene expression are used by the plant to reduce the impact of excess light (Kimura et al., 2003; Rossel et al., 2006). Heat
shock proteins are also expressed in plants during light stress, independently of heat stresses (Knight and Ackerly, 2003).

**ION LEAKAGE MEMBRANE INTEGRITY AND CELL DEATH**

When an plant is under heat stress, the membrane can become leaky, which can lead to cell death (Yeh et al., 2012). The buildup of toxic molecules and release of enzymes from vacuoles due to heat stress induced membrane collapse can lead to programmed cell death (Kwon et al., 2013). Irreversible injury to plants is characterized by loss of membrane integrity that results in swelling of the cytoplasm and release of cellular content (Van Breusegem and Dat, 2006). In heat stressed plants lipid peroxidation occurs when ROS levels reach a threshold that damages both cellular and organelle membranes (Van Breusegem and Dat, 2006). The cell is lysed, and the contents are leaked out (Van Breusegem and Dat, 2006). We used ion leakage to measure plant damage and cell death.

**RT-qPCR**

Reverse transcription (RT) followed by polymerase chain reaction (PCR) is a very powerful tool to detect trace amounts of mRNA (Fleige et al., 2006). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a method to measure the abundance of a gene with high sensitivity, high specificity and good reproducibility, while being cost effective as well (Pfaffl, 2001). RT-qPCR makes it easy to study many genes or many samples in parallel. This method builds upon PCR by binding a fluorescent dye to double-stranded DNA. When the amplification of a gene reaches a predetermined threshold of fluorescence, the point is saved as a threshold cycle (Ct). RT-qPCR is performed on cDNA made from total RNA or messenger RNA (mRNA). By normalizing this data one can determine relative expression of a gene of interest in various samples (Pfaffl, 2001).

The RT-qPCR data needs to be normalized to accurately determine abundance of a transcript. First the total RNA in any given sample can vary, therefore by using the same amount of total RNA to synthesize cDNA we can normalize for total RNA (Vandesompele, 2002). To compare gene expression between samples an internal control gene must be used to normalize the amount of mRNA (Vandesompele, 2002). This internal control gene is called a reference gene, and genes used as reference should have the same level of expression
regardless of different experimental conditions (Hruz et al., 2011). Finding one reference
gene that is good is difficult and so multiple reference genes are recommended (Hellemans et
al., 2007). RNA integrity is critical in obtaining meaningful gene expression data, and good
quality RNA must be extracted (Fleige et al., 2006).

There are two methods to analyze data from RT-qPCR, absolute quantification and
relative quantification (Livak and Schmittgen, 2001). Absolute quantification determines the
input copy number by a standard curve, while relative quantification relates the PCR signal
of one sample to a control sample (Livak and Schmittgen, 2001). We are interested in the
relative expression of a gene, and not the absolute quantification of a transcript. To determine
the relative expression of a gene, we used $2^{-\Delta\Delta CT}$ equation but modified for multiple
reference genes as described (Hellemans et al., 2007). Change in expression was calculated
by normalizing the data to two reference genes, and compared to the control. Samples were
run in triplicates to minimize technical errors (Tichopad et al., 2009). The two reference
genes that we used are SAND and ACT2, and the three genes of interest are APX2, HSFA2,
HSP101, and HSP18.1. Further information can be found on the genes of interest below.

**Reference Genes**

The first reference gene we used is actin 2, ACT2 (AT3G18780). This gene is
involved in many cellular housekeeping functions, and often used as a reference gene in plant
species for RT-qPCR (Chandna et al., 2012; Pellino et al., 2011). The second reference gene
is the SAND family protein (At2g28390), and is found across many developmental stages in
Arabidopsis (Lamesch et al., 2012). SAND has stable expression under many conditions,
including heat stress (Wang et al., 2014). Using ACT2 and SAND, we should be able to
normalize our gene expression data.

**ROS: APX2**

In plants reactive oxygen species (ROS) is produced continuously as byproduct of
metabolic pathways and under stress there is a burst of ROS production (Apel and Hirt,
2004). Heat stress disrupts cell metabolism, resulting in the production of (ROS) (Miller et
al., 2008). Figure 5 shows PSII harvesting light and using it to free an electron to make ATP.
Figure 5. PSII light harvesting electron pathway.

When a plant is under stress, this pathway can be disrupted, and the free electron produces ROS instead of ATP. Besides being a toxic by-product of aerobic metabolism during plant stress, ROS also play a role in signaling transduction (Miller et al., 2008). Plants have evolved sophisticated acclimation and defense mechanisms that are activated in primary tissue exposed to stress as well as in distal portions not directly exposed to stress (Baxter et al., 2014). When a plant is stressed, there is an ion influx across the plasma membrane, increased Ca2+ levels in the cytosol, activation of MAPKs, and production of ROS (Baxter et al., 2014). If ROS levels are elevated, the mitochondria is damaged and the whole cell can be damaged, so it is important to prevent high levels of ROS in the cells (Moller, 2001). ROS levels in plants are controlled by a large network of genes which include H2O2-scavenging enzymes cytosolic ascorbate peroxidase (APX1 and 2) (Suzuki et al., 2013). A. thaliana plants lacking APX2 has been shown to be more sensitive to heat stress at the seedling stage, but more tolerant to heat stress at the reproductive stage (Suzuki et al., 2013). We measured APX2 levels in the plant during long term heat stress using RT-qPCR.

**HSFA2**

HSR is regulated by heat shock transcription factors (HSF) family. HSFs bind heat shock elements in the promoter region of heat shock proteins and other heat shock related elements when a plant is under heat stress (Amano et al., 2012). HSFA2 is responsible for upregulation of *HSP18.1, HSP25.3*, and HSA32 (Charng et al., 2007; Schramm et al., 2008). Plants have more than 20 HSF family members, while *Drosophila* and yeast have only
one, and vertebrates have four (Amano et al., 2012). It has been shown that HSFA2 is not needed for normal growth and development, and is strongly induced by heat stress (Amano et al., 2012; Charng et al., 2007; Liu et al., 2011; Yoshida et al., 2011). Plants knocked-out with HSFA2 display reduced basal and acquired thermotolerance (Charng et al., 2007). HSFA2 is essential for extending the duration of thermotolerance, but not in initiation of thermotolerance (Charng et al., 2007). During heat stress, HSFA2 is responsible for nuclear localization of ROF1-HSP90.1 complex (Meiri and Breiman, 2009). HSFA2 is the strongest expressed member of the HSF family under heat stress conditions (Schramm et al., 2006). Using RT-qPCR, we measured HSFA2 transcript levels in A. thaliana, B. arcuata, and B. depauperata. We expect B. arcuata to have the greatest fold change in transcription, and B. depauperata to have the lowest fold change in transcript.

**HSP101**

Heat Shock Protein 101 (HSP101) is part of HSP100/ClpB heat shock protein family and it is required for high temperature survival (Tonsor et al., 2008). HSP101 has been shown to be essential in thermotolerance in bacteria, yeast, and plants (Hong and Vierling, 2000). HSP101 is an emergency high-temperature tolerance mechanism (Tonsor et al., 2008). HSP101 in association with HSA32 has been shown to prolong thermotolerance in rice seedlings (Lin et al., 2014). HSP101 enhances the translation of HSA32 during recovery after heat treatment, and HSA32 retards the decay of HSP101 (Wu et al., 2013). HSP101 is essential in acquired thermal tolerance, but it also needed under normal growth conditions (Tonsor et al., 2008; Wu et al., 2013). Knocking out HSP101 decreases fruit production, days to germination, days to bolting, total dry mass, number of inflorescences, increased transpiration rate and allocation to root mass (Hong and Vierling, 2000; Tonsor et al., 2008). Using RT-qPCR, we measured HSP101 transcript levels in A. thaliana, B. arcuata, and B. depauperata.

**HSP18.1: Small Heat Shock Proteins**

Small heat shock proteins (sHSPs) are crucial components of the plant heat shock response (Waters et al., 2008a). sHSPs are chaperones which maintain protein homeostasis by binding proteins in non-native conformations, there by maintain protein folding capacity (Haslbeck et al., 2005). sHSPs protect cells from protein losses or toxicity caused by
aggregation, but do not fold proteins themselves (Eyles and Gierasch, 2010). It has been shown that plants accumulate sHSPs while under heat stress and peak accumulation occurs at different temperatures according to how heat tolerant a plant is (Knight and Ackerly, 2003). HSP26, a small heat shock protein, has been found to protect PSII and lower electrolyte leakage when exposed to heat stress (Kim et al., 2012). HSP21, another sHSP, has been shown to protect PSII from temperature-dependent oxidative stress (Neta-Sharir et al., 2005).

All small heat shock proteins function as large oligomers, share a conserved α-crystallin domain of about 100 amino acid residues, and have a compact β-sheet sandwich structure (Waters et al., 2008a). Using RT-qPCR, HSP18.1 transcript levels were measured in A. thaliana, B. arcuata, and B. depauperata samples. HSP18.1 is upregulated early in heat stress by HSFA1 and later in heat stress by HSFA2 (Li et al., 2010).

### RNA-SEQUENCING

*RNA-sequencing analysis:* RNA-seq technology is advancing rapidly, and we are expecting to have 100bp Illumina short reads. Illumina High-Seq can do 120+ million reads, and allowing us to run 6-12 samples per flow tube, giving us 10-20 million reads (Pellny et al., 2012, Sultan et al., 2012). The RNA-seq for Arabidopsis thaliana were aligned against TAIR10\_gff3 from www.arabidopsis.org (Schmitz et al., 2011), and Boechera depauperata RNA-seq was aligned against Boechera depauperata transcriptome previously assembled in Dr. Waters’ lab. To filter the experiment results, a minimum of 50 reads per kilobase of exon model per Million mapped reads (RPKM), and a fold change of 2 or greater were used (Dugas et al., 2011).

To align sequence reads to a reference genome, TopHat is used. TopHat uses Bowtie as an alignment engine (Trapnell et al., 2010). When quantifying transcription, splice variants of each gene needs to be correctly aligned to the genome; Cufflinks assembles individual transcripts from the RNA-seq reads, and reports the transcript abundance (Trapnell et al., 2010). To find differential gene analysis, Cuffdiff (part of Cufflinks), calculates expression in two or more samples (Trapnell et al., 2010). To visualize the differential gene expression from the Cuffdiff output files, CummeRbund is used, a tool in part of R statistical computing environment (Trapnell et al., 2010).
Besides doing heat stress experiments, we started a single seed descent (SSD) for all of the plant species we were in our lab. SSD is a method of inbreeding to segregating genetic diversity in a population (Snape and Riggs, 1975). SSD can be done by self-pollinating or cross pollinating plants. SSD is used to evaluate individual lines and inbreeding the population is done to remove genetic diversity. From a mixed population of seeds, one ‘founder’ plant is selected to self-pollinate, and the offspring are self-pollinated also, until the 4th generation. This single seed line represents a genetically uniform line that is representative of one genotype from the original collected population.

Plants flower in response to many varied cues, such as temperature, photoperiod, and age (Zhou et al., 2013). In winter annuals and perennials a plant flowering is typically blocked in the first growing season. Exposure to prolonged cold is required to alleviate this block and permit flowering (Sung and Amasino, 2004). A few weeks of cold is often sufficient to promote flowering, and longer periods of cold treatment can accelerate flowering to a greater extent, until vernalization response becomes saturated (Trevaskis et al., 2007). Longer winters accelerated flowering, while elevated ambient temperatures delayed flowering (Anderson et al., 2011).

Single seed decent (SSD) is a breeding method to produce offspring that are genetically homozygous for all genes, by self-pollinating plants. Having plants that are genetically homozygous for all locus reduces variability that may arise from genetic diversity. Seed banks keep two bulk lines; one is the ‘mixed’ population that comes from the original seed collection that maintains variation. SSD lines are genetically uniform line that is representative of one genotype from the original collected population.

Not all plants flower once they mature; they need different cues, such as temperature, photoperiod, and age (Zhou et al., 2013). Genetic and environmental factors influence flower production (Anderson et al., 2011). Some of the Boechera species flower once they mature, and many of them do not. The seed supply in our lab is limited, and we must produce more seeds to continue to do experiments. To make many of the species studied in our laboratory flower, we had to find the right conditions to put them in once adulthood is reached. We looked at the materials and methods of two research papers (Bergonzi et al., 2013, Zhou et al., 2013), we found that they vernalized adult plants to induce flowering and seed
production. We made our own vernalization protocol to induce flowering, by reducing the temperature and decreasing day length.
SIGNIFICANCE

Currently the world population is over 7 billion people and it is expected to peak at 10 billion by 2050. With a growing population, limited arable land, and a fluctuating climate, crop loss due to prolonged periods of heat could be catastrophic. Additionally, heat stress can also have a detrimental effect on wild populations of plant species, which would be particularly damaging for endangered species where only a small number of individuals remain. Considering that global temperatures are predicted to increase, the strong possibility exists that loss due to heat stress may become a serious problem.

Change in membrane fluidity, denatured proteins, Ca2+ fluxes, toxic byproduct buildup, and RNA unfolding are the possible ways heat is sensed by plants (Horvath et al., 2012; Miller et al., 2008; Mittler et al., 2012). ROS levels increase linearly with stress (Apel and Hirt, 2004; Miller et al., 2008; Suzuki et al., 2013). Photosynthesis has been shown to be negatively affected by heat stress (Maxwell and Johnson, 2000; Pinto-Marijuan and Munne-Bosch, 2014). In this thesis we looked at the impact of long-term stress on photosynthetic efficiency, membrane integrity and gene transcription. Previous research from our lab has shown B. arcuata to be more sensitive to basal heat stress than A. thaliana and B. depauperata to be more heat tolerant than A. thaliana. We would like to know if these three species behave identically under moderately high temperature heat stress as basal heat stress.
GOALS, HYPOTHESES AND PREDICTIONS

The aim of this project is to understand the effects of moderately high temperatures on plant species *Arabidopsis thaliana*, *Boechera arcuata*, and *Boechera depauperata*. I would like to document the effects of 35°C and 38°C heat stress on PSII and membrane integrity, and gene expression of *APX2*, *HSFA2*, *HSP101*, and *HSP18.1*.

Question 1: How is the efficiency of PSII affected by moderately high temperature heat stress in *A. thaliana*, *Boechera arcuata*, and *Boechera depauperata*?

- Hypothesis 1A: PSII is protected and all three plant species have identical ability in protecting PSII from heat stress. The plant species have not evolved unique ability to deal with moderately high temperature heat stress, they use the same methods to protect PSII, and PSII loses efficiency equally in all three species.
- Hypothesis 1B: The three plant species cannot protect PSII equally. One species is better at protecting PSII than the other two. Each plant species has a unique method in protecting PSII, and the efficiency of PSII in each species should be different.
- Hypothesis 1C (null): PSII efficiency is not affected by moderately high temperature heat stress in these species. 35°C and 38°C heat stress does not affect PSII efficiency.

Question 2: Is cell death under heat stress different between *A. thaliana* and the two *Boechera* species?

- Hypothesis 2A: The three plant species have identical cell death percentage. All three species are able to protect cell from 35°C and 38°C heat stress equally.
- Hypothesis 2B: The percentage of cell death is different between the species. Each species has a different amount of cell death from 35°C and 38°C heat stress.
- Hypothesis 2C (null): Membrane integrity is not affected by moderately high temperature heat stress in these species. 35°C and 38°C heat stress does not cause cell death.

Question 3: Is gene expression under heat stress different between *A. thaliana* and the two *Boechera* species?

- Hypothesis 3A: The three plant species have identical transcription of *APX2*, *HSFA2*, *HSP101*, and *HSP18.1*. All three species have the same heat shock response and equally upregulate *APX2*, *HSFA2*, *HSP101*, and *HSP18.1*.
- Hypothesis 3B: The three plant species use distinct HSR and transcription levels for *APX2*, *HSFA2*, *HSP101*, and *HSP18.1* are different. *A. thaliana*, *B. arcuata*, and *B. depauperata* have different HSR to 35°C and 38°C heat stress, and transcription levels differ between the species.
• Hypothesis 3C (null): APX2, HSFA2, HSP101 and HSP18.1 transcription is not induced by moderately high temperature heat stress. APX2, HSFA2, HSP101 and HSP18.1 transcription are not upregulated by 35°C and 38°C heat stress.
MATERIAL AND METHODS

GROWTH OF PLANTS

Arabidopsis thaliana Columbia, Boechera depauperata, and Boechera arcuata seeds were stored in 4°C for long term storage. Seeds were washed with 95% ethanol for 1 minute, and then it was washed with Wash Buffer (69.8% water, 30% bleach, 0.2% Triton X-100) for fifteen minutes. The seeds were then washed five times with deionized autoclaved water. The seeds were then put on media plates consisting of 0.8% molecular biology grade agar with Murashige and Skoog salts and 2.5% sucrose. The plates were then wrapped with aluminum tin foil and put into the 4°C cold chamber to vernalize. Arabidopsis thaliana and Boechera arcuata seeds were vernalized for 3 days, and Boechera depauperata seeds were vernalized for 15 days. After vernalization the plates were un-foiled and moved into 22°C E-36L Percival growth chamber. Plants were grown in the 22°C growth chamber for 10 days with a light intensity set to 150μE.

HEAT SHOCK EXPERIMENTS

Ten day old plants were put into three identical Percival E-36 growth chambers, with light intensity set to 150 μE. The chambers were each set at a different temperature. One chamber was set to 22°C as a control, one chamber was set to 35°C, and the last chamber was set to 38°C. Samples were taken every 24 hours for up to 96 hours. Figure 6 shows the sample points in the heat stress experiment we performed for the CF and ion leakage experiments.

PHOTOSYNTHETIC MEASUREMENTS

To measure the effects of heat stress on photosynthesis, a JUNIOR-PAM by WALZ Chlorophyll Fluorometer was used to measure CF. Heat stressed samples were measured for CF immediately as they came out of the heat chamber.

ION LEAKAGE

To measure ion leakage, plants were stressed and samples were taken every 24 hours. The plants from each sample were cut at their stem and put into 15ml conical tubes. A
Figure 6. Moderately high temperature heat stress. Plants were grown for 7-10 days at 22°C then heat stressed at 35°C or 38°C for up to four days. Control and stress samples were taken at 0 hours, 24 hours, 48 hours, 72 hours, and 96 hours.

Thermol Scientific Orion was used with an ion meter to measure the concentration of ions in each conical tube per sample. Each sample was measured at zero hours, at one hour, two hours, three hours and four hours. After the fourth hour measurement, the tubes were then put into a -20°C freezer overnight. Samples were then thawed out and brought back to room temperature, and a final reading was taken as the total ion concentration for each sample.

**RNA ISOLATIONS**

RNA was extracted using BioPioneer kit BP-10 Spin Column Total RNA Mini-preps Super Kit. Samples were ground up into powder under liquid nitrogen condition. 100mg of grounded up tissue was used of each sample. 450ul of RLT solution was added to the sample, and then it was vortexed and incubated at 50°C for 1 minute. 250ul of 100% ethanol was added to the sample, then mixed and finally spun down. The aqueous layer was moved into BP-10 spin column, centrifuged at 8K rpm for 1 minute, and flow through was discarded. 500ul of RW solution was added to the column to wash and spun a second time at 8K rpm for two minutes and flow throw was discarded. 500ul of RPE solution was added to the column, centrifuged at 8K rpm for two minutes and flow through was discarded. Then the column was spun at 10K rpm for one minute and the column was transferred into a new 1.5ml eppendorf tube. 50ul of RNase-free H2O was added to the middle of the column; it
was incubated at 50°C for two minutes and then centrifuged at 10K rpm for two minutes. The RNA was kept at -80°C.

**CDNA SYNTHESIS**

BioPioneer first stand cDNA synthesis kit was used to make cDNA from our RNA samples. About 800ng of RNA per 20ul reaction was used. RNA was incubated at 70°C for five minutes, and then 13.5ul of RT SuperMix with 0.50l of M-MuLV RT was added. The samples were gently mixed and briefly spun down, and then kept on ice for five minutes. The samples were then put in a thermocycler at 42°C for one hour for elongation and followed by 94°C for five minutes to deactivate the Reverse Transcriptase.

**QPCR PRIMER DESIGN AND GENES OF STUDY**

QPCR primers were designed on the Integrated DNA Technologies website using the PrimerQuest™ PCR Design Tool. Parameters were set to give a melting temperature of 60°C and an amplicon size of 100-150 bases in length. The primers were designed off of *Arabidopsis thaliana* CDS. The primers were blasted against the genome using the BLAST tool on Arabidopsis Information Resource (TAIR) webpage to check the specificity for the proper gene.

**REFERENCE GENES**

Reference genes were used to normalize the data to a “control” baseline of expression. Reference gene’s expression does not change during the experimental conditions (Vandesompele et al., 2002). Reference genes were selected from a pool based on literature reviews and GENEVESTIGATOR’s RefGenes option (Hruz et al., 2011). The first reference gene used is actin 2, *ACT2* (AT3G18780), which is involved in many cellular housekeeping functions (Chandna et al., 2012). The second reference gene is the *SAND* family protein (At2g28390), which is found in the chloroplast and found across many developmental stages in *Arabidopsis* (Lamesch et al., 2012). *SAND* is continuously expressed, even under abiotic stress conditions (Zhu et al., 2013). *ACT2* and *SAND* are two genes that previously have been used as reference genes in other studies. The primers used can be found in Table 1.
Table 1. RT-qPCR Primers

<table>
<thead>
<tr>
<th>Genes of Interest</th>
<th>Forward Primers</th>
<th>Reverse Primer</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>APX2</td>
<td>CATCCCTGGTAGACAGGACAAG</td>
<td>CCATCCGACCAACACATCT</td>
<td>100 pb</td>
</tr>
<tr>
<td>HSFA2</td>
<td>CAAGTTTTCATTCGCAGCTCAATAC</td>
<td>GAGATGCTTCTGTTCCTGCTAAA</td>
<td>101 pb</td>
</tr>
<tr>
<td>HSP101 Arabidopsis</td>
<td>ATAGATGCACCGGCTGGTGATCTT</td>
<td>TCACCTCTCTTTGGGCCCCTTAGCA</td>
<td>124</td>
</tr>
<tr>
<td>HSP101 Boechera</td>
<td>GAGAGTGGAGTTGCTTTTGATGAT</td>
<td>AATAGGAATGATCCAGTTGGTTGT</td>
<td>140</td>
</tr>
<tr>
<td>HSP18.1</td>
<td>TTCTTCACGCCCATCTTCGCTTTG</td>
<td>CTTGCAAAGTCCGCTTTGACCAT</td>
<td>124</td>
</tr>
<tr>
<td>SAND</td>
<td>GATGACTGGCTCTACTCTCAA</td>
<td>GTTGTATCTTGTTAGGACAGATT</td>
<td>93</td>
</tr>
<tr>
<td>ACT2 Arabidopsis</td>
<td>CCTTACAGAGAGGTACATGTT</td>
<td>CTTGCTCAGTAGTCAACAGCAACAA</td>
<td>101</td>
</tr>
<tr>
<td>ACT2 Boechera</td>
<td>CCTTACCGAGAGGTACATGTT</td>
<td>CCTGCTCAGTAGTCAACAGCAACAA</td>
<td>101</td>
</tr>
</tbody>
</table>

**GENES OF INTEREST**

Genes of interest were chosen using a literature review, previous lab data and up-regulated genes from RNS-seq results. Primers for APX2, HSFA2, HSP101, and HSP18.1 were designed off of *A. thaliana* and tested using PCR on cDNA of *A. thaliana* and *Boechera* species, the product was run out on a gel. For the genes of interest, primers were designed using *A. thaliana* genome, and previously sequenced *Boechera* transcripts. The primers used can be found in Table 1.

**QPCR EXPERIMENTAL DESIGN**

Samples were collected and kept in the -80°C. The tissues were ground up using mortar and pestle. BioPioneer BP-10 Total RNA Miniprep Kit was used to isolate total RNA. BioPioneer RT-005 RT Master Mix was used to synthesis cDNA, 800ng of total RNA per 20µL reactions were used to synthesis cDNA. RT-qPCR: To find the efficiency of the primers, samples were diluted 1:10, 1:20, 1:40, and 1:80 for the standard curve. All samples were diluted 1:20 to find transcription levels. Primers were diluted to 7.5µM concentration. 20µL reactions were made in each well, consisting of, 6µL of (1:20) cDNA, 1µL of forward primers, 1µL of reverse primers, and 12µL of Sybr Grn master mix. The 96 well plates were loaded onto a Bio-Rad CFX96 Touch Real-Time PCR Detection System and Sybr Grn 96 well plate protocol was run followed by at melt curve, the elongation temperature was set to 60°C.
RNA Sequencing

Samples were collected and kept in the -80°C. Tissues were ground up into fine powder under liquid nitrogen condition using mortar and pestle. RNA was extracted and samples were sent off for library prep and RNA-sequencing by Scripps. Figure 7 shows the steps for library prep for each sample. TopHat was used in Ubuntu Linux GNU to analyze the data (Bergonzi et al., 2013; Trapnell et al., 2013). The steps were followed from Trapnell et al., 2013. The reads were aligned using TopHat and Arabidopsis thaliana TAIR10 as a reference genome. Transcript was assembled using Cufflinks, and differential analysis was done with Cuffdiff. CummeRbund was used to visualize the data in R.

Figure 7. RNA-seq library prep. RNA is isolated from the sample and then selected for using Poly-A tail. Random primers are used to generate cDNA, which then is cut to specific size, then an indexing adapter is bound to multiplex, and finally sequenced.

Single Seed Decent Production

One founder plant was chosen from the ‘mixed’ population to generate the SSD. Plants were grown on 0.6% MS agar plates, after two weeks of growth in a light chamber; they were transplanted onto soil in 3 inch pots and moved into a propagation room. After 1
month of growth in 3 inch pots, they were transplanted into 6 inch pots for maturation. After 2 months in the 6 inch pots, plants were cold treated to induce flowering. The cold treatment consisted of four weeks of diurnal treatment. Daytime was from 8AM-5PM, and nighttime was from 5PM-8AM. Daytime temperatures were: week one at 10°C, week two at 8°C, weeks three and four at 6°C. Nighttime temperatures were: week one at 8°C, week two at 6°C, and weeks three and four at 4°C. After the cold treatment, the plants were moved back into the propagation room at 22°C for seed production. *B. depauperata* and *B. drummondii* did not need cold treatment for them to produce flowers. Some plants that were cold treated (*B. arcuata, B. perennans, and B. hoffmannii*) needed their flowering stalks to be trimmed back. They produced too many flowering stalks, and the seeds did not mature. By cutting back the stalks, the plants were able to produce better quality seeds.

From each plant species, two offspring were moved forward to the next generation. Four plants were chosen to be collected for each line, but only two moved forward to the next generation. A total of four generations of self-fertilization was done to generate the final SSD.

**FLOWERING**

Plants were grown on 0.6% agar plates for 10-15 days in a growth chamber. The seedlings were then transplanted into three inch pots for four weeks and grown in our propagation room at 22°C for four weeks. After the four weeks they were transplanted into six inch pots. After eight weeks in six inch pots, the plants were put into the cold chamber for vernalization for four weeks following Table 2. After vernalization, the plants were returned to propagation room at 22°C. During the growth period, plants were fertilized every two weeks using high nitrogen (24% Nitrogen, 8% Phosphorus, and 12% Potassium). Post vernalization, plants were fertilized using high Phosphorus fertilizer (15% Nitrogen, 30% Phosphorus, and 15% Potassium). Plants were grown until seed pods matured and then the plants were dried and seeds collected.
Table 2. Temperature and Light Intensity for Vernalization

<table>
<thead>
<tr>
<th></th>
<th>Day Time (8AM-5PM)</th>
<th>Night Time (5PM-8AM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>10°C/150 uE Light</td>
<td>8°C/0 uE Light</td>
</tr>
<tr>
<td>Week 2</td>
<td>8°C/150 uE Light</td>
<td>6°C/0 uE Light</td>
</tr>
<tr>
<td>Week 3</td>
<td>6°C/150 uE Light</td>
<td>4°C/0 uE Light</td>
</tr>
<tr>
<td>Week 4</td>
<td>6°C/150 uE Light</td>
<td>4°C/0 uE Light</td>
</tr>
</tbody>
</table>
RESULTS

CF EFFICIENCY UNDER HEAT STRESS

During long-term heat stress *B. depauperata* protects PSII while *A. thaliana* and *B. arcuata* do not protect the PSII.

When a plant is under heat stress, light harvesting and conversion to photo-chemical energy efficiency is reduced. As a plant experiences stress, the efficiency of light conversion to photo-chemical energy drops. To determine if the impact of heat stress on photosynthesis I measured CF for all three species after 24, 48, 72, and 96 hours of heat stress. *B. depauperata* does not display a reduction of CF during heat stress at 35°C or at 38°C even the longest duration of stress i.e., after 96 hours of heat stress (Figure 8C). The CF of *B. arcuata* is reduced after heat stresses at 38°C, but is not significantly impacted after a stress 35°C (Figure 8B). The CF of *A. thaliana* is reduced after stresses at both 35°C and 38°C. In *A. thaliana* the low CF levels indicates that after the heat stresses imposed here the ability to convert light energy to photo-chemical efficiency is lost (Figure 8A).

Figure 8. PSII by species. Impact of stress on CF. CF was taken immediately following 35°C stress, 38°C stress, and 22°C no stress (CTL). The stress lengths were 24 hours, 48 hours, 72 hours and 96 hours. CF efficiencies for (A) *A. thaliana*, (B) *B. arcuata*, (C) *B. depauperata*. 
When looking at the effect of heat stress in more detail, we can see that all three species have very similar efficiency (CF values) under control conditions of 22°C (Figure 9A). But at 35°C, A. thaliana has a large reduction in efficiency after 48 hours of continuous stress (Figure 9B). At this temperature (35°C) B. arcuata is not significantly affected by heat stress. However, at 38°C both A. thaliana’s and B. arcuata’s CF is significantly reduced. It is notable that at this temperature (38°C) B. depauperata still has CF values that are similar to that at control conditions (Figure 9C). We can conclude from this data then that B. depauperata is able to protect PSII under both 35°C and 38°C heat stress conditions. B. arcuata can protect PSII from heat-induced damage at 35°C but not at 38°C, and A. thaliana is not able protect PSII at either 35°C or 38°C.

![Figure 9](image)

**Figure 9.** PSII by temperature. Impact of stress on CF. CF was taken immediately following 35°C stress, 38°C stress, and 22°C no stress (CTL). The stress lengths were 24 hours, 48 hours, 72 hours and 96 hours. CF efficiencies for (A) 22°C, (B) 35°C, and (C) 38°C.

**CELL DEATH**

B. depauperata displays membrane integrity at higher temperatures during long-term heat stress while A. thaliana and B. arcuate do not.

To measure cell death, we tested for membrane integrity. When a cell dies, plasma membrane integrity is lost, and cell content is expelled. In order to quantitatively examine cellular integrity and cell death I measured the level of ion-leakage intact leaves.
Plants were heat stressed for up to 96 hours, and ion leakage was measured after 4 hours submergence in de-ionized water. After a treatment at 35°C, *B. arcuata* had the greatest percentage of ion leakage or cell death (Figure 10B). After a treatment of 38°C *B. arcuata* and *A. thaliana* were both displayed ion leakage, while *B. depauperata* had low ion leakage indicating little cell death (Figure 10C). The pattern of ion leakage was different for *B. arcuata*. *B. arcuata* appears to have lost membrane integrity (i.e. ion leakage is high) at 35°C and 38°C (Figure 11B). Similar to the CF efficiency, there is little ion leakage seen in *B. depauperata* at both 35°C and 38°C (Figure 11C). This suggests that at both of these stress temperatures *B. depauperata* has intact cellular membranes.

![Figure 10](image)

**Figure 10.** Ion leakage by temperature. Impact of heat stress on membrane integrity. Ion leakage was measured following 35°C stress, 38°C stress, and 22°C no stress (CTL). The stress lengths were 24 hours, 48 hours, 72 hours and 96 hours. Ion leakage for (A) 22°C, (B) 35°C and (C) 38°C.

**RNA Sequencing**

Differential transcription analysis shows thermotolerance related genes up-regulated during basal and acquired heat stress.

RNA sequencing was done on tissue samples from *A. thaliana* root and shoot separately at 38°C basal heat stress, shoot tissue only from 43°C aquired heat stress and control samples. TopHat, Cufflinks and R were used to analyse the RNA-seq data. A heat map of the top 100 genes was made, and some genes that stand out in the heat map are *HSP101*, *ROF1*, *ROF2*, *APX2*, *HSP70*, *HSP70b*, *HSP17.6II*, *HSP17.4*, *HSP18.2*, *HSP90.1,*
Ion leakage was measured following 35°C stress, 38°C stress, and 22°C no stress (CTL). The stress lengths were 24 hours, 48 hours, 72 hours and 96 hours. Ion leakage for (A) *A. thaliana*, (B) *B. arcuata*, and (C) *B. depauperata*.

HSP21, HSFA7A, HSFA3, HSFA2, DREB2A, DREB2C, HSP23.6 (Figure 12). There are many unknown genes that show heat stress association when searched on TAIR. These genes can be looked into for future studies. A cold map of the top 100 genes down-regulated was also made and many of the genes that are down regulated are genes involved in cell metabolism (Figure 13). During heat stress, heat shock response genes are up-regulated and metabolism genes are down-regulated.

**RT-qPCR Gene Expression**

Boechera species have smaller fold change in HSR genes. In order to examine gene expression pattern in the three species during the long-term stress experiments (72 hours), plants were heat stressed at 35°C and 38°C and leaf samples were flash frozen with liquid nitrogen at the following times. Samples were processed and RNA was extracted. The RNA was converted to cDNA. RT-qPCR was performed using the cDNA to measure the expression levels of *APX2, HSFA2, HSP101* and *HSP18.1*. *ACT2* and *SAND* were used as reference genes. To calculate the fold change for each sample, primer efficiencies were calculated out (Table 3).
Figure 12. RNA-Seq heat map: Top 100 up-regulated genes in *A. thaliana* at 22°C control, 38°C basal heat stress shoot tissue, 43°C acquired heat stress shoot tissue, and 38°C basal heat stress root tissue.

After one hour at 35°C *A. thaliana* has an initial spike in transcription of *APX2*, *HSFA2*, *HSP101*, and *HSP18.1*. AT 38°C, *APX2* transcription (Figure 14A, Table 4) is up-regulated in all samples, suggesting continuous ROS production. However, *A. thaliana HSFA2* and *HSP101* are only up-regulated at hour 1, and from hours 6 to 72 transcription decays to control levels (Figure 15A, Table 5, Figure 16A, Table 6). *B. arcuata* at 35°C and 38°C has a spike in transcription for *APX2*, *HSFA2*, at hours 1 and 6, but transcription decays to control levels at 12 hours to 72 hours (Figures 14B and 15B). *B. arcuata* *HSP101*
Figure 13. RNA Seq cool map: Top 100 down-regulated genes in *A. thaliana* at 22°C control, 38°C basal heat stress shoot tissue, 43°C acquired heat stress shoot tissue, and 38°C basal heat stress root tissue.

Table 3. Primer Efficiency Values and R2 Values for the Trendline of Best Fit

<table>
<thead>
<tr>
<th></th>
<th>SAND</th>
<th>ACT2</th>
<th>APX2</th>
<th>HSFA2</th>
<th>HSP101</th>
<th>HSP18.1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R²=0.8033</td>
<td>R²=0.8645</td>
<td>R²=0.7642</td>
<td>R²=0.5477</td>
<td>R²=0.8033</td>
<td>R²=0.8814</td>
</tr>
<tr>
<td><em>B. arcuata</em></td>
<td>E= 2.576</td>
<td>E= 2.146</td>
<td>E= 2.549</td>
<td>E= 2.226</td>
<td>E= 2.451</td>
<td>E= 2.207</td>
</tr>
<tr>
<td></td>
<td>R²=0.7897</td>
<td>R²=0.8466</td>
<td>R²=0.7313</td>
<td>R²=0.8497</td>
<td>R²=0.8626</td>
<td>R²=0.8520</td>
</tr>
<tr>
<td><em>B. depauperata</em></td>
<td>E= 2.268</td>
<td>E= 2.165</td>
<td>E= 2.178</td>
<td>E= 2.110</td>
<td>E= 2.379</td>
<td>E= 1.909</td>
</tr>
<tr>
<td></td>
<td>R²=0.7828</td>
<td>R²=0.8767</td>
<td>R²=0.8489</td>
<td>R²=0.8726</td>
<td>R²=0.8940</td>
<td>R²=0.8018</td>
</tr>
</tbody>
</table>
Figure 14. **APX2** transcription fold change over control for (A) *A. thaliana*, (B) *B. arcuata*, (C) *B. depauperata*, ACT2 and SAND used as reference genes.

Table 4. Fold Change in **APX2** Transcription Per Species by Sample Point

<table>
<thead>
<tr>
<th></th>
<th>1 Hour</th>
<th>6 Hour</th>
<th>12 Hour</th>
<th>24 Hour</th>
<th>48 Hour</th>
<th>72 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. thaliana</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35°C</td>
<td>402,679.93</td>
<td>± 90,445</td>
<td>33,835.73</td>
<td>± 624.7</td>
<td>17,665.76</td>
<td>± 2,443.2</td>
</tr>
<tr>
<td></td>
<td>12,001.24</td>
<td>± 2,103.5</td>
<td>12,001.24</td>
<td>± 2,103.5</td>
<td>12,001.24</td>
<td>± 2,103.5</td>
</tr>
<tr>
<td>38°C</td>
<td>414,306.28</td>
<td>± 187,874</td>
<td>375,167.34</td>
<td>± 93,524</td>
<td>413,945.34</td>
<td>± 127,376</td>
</tr>
<tr>
<td></td>
<td>174,967.94</td>
<td>± 56,300</td>
<td>174,967.94</td>
<td>± 56,300</td>
<td>174,967.94</td>
<td>± 56,300</td>
</tr>
<tr>
<td><strong>B. arcuata</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35°C</td>
<td>656.26</td>
<td>± 396.73</td>
<td>325.24</td>
<td>± 143.47</td>
<td>58.81</td>
<td>± 33.77</td>
</tr>
<tr>
<td></td>
<td>38.17</td>
<td>± 19.28</td>
<td>38.17</td>
<td>± 19.28</td>
<td>38.17</td>
<td>± 19.28</td>
</tr>
<tr>
<td>38°C</td>
<td>862.88</td>
<td>± 503.01</td>
<td>156.11</td>
<td>± 79.09</td>
<td>18.16</td>
<td>± 9.65</td>
</tr>
<tr>
<td></td>
<td>5.77</td>
<td>± 2.06</td>
<td>5.77</td>
<td>± 2.06</td>
<td>5.77</td>
<td>± 2.06</td>
</tr>
<tr>
<td><strong>B. depauperata</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35°C</td>
<td>471.48</td>
<td>± 229.11</td>
<td>46.52</td>
<td>± 14.25</td>
<td>24.62</td>
<td>± 0.36</td>
</tr>
<tr>
<td></td>
<td>31.22</td>
<td>± 9.89</td>
<td>31.22</td>
<td>± 9.89</td>
<td>31.22</td>
<td>± 9.89</td>
</tr>
<tr>
<td>38°C</td>
<td>695.09</td>
<td>± 398.34</td>
<td>220.41</td>
<td>± 64.217</td>
<td>108.56</td>
<td>± 64.153</td>
</tr>
<tr>
<td></td>
<td>38.93</td>
<td>± 7.1664</td>
<td>38.93</td>
<td>± 7.1664</td>
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<td>± 7.1664</td>
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<tr>
<td></td>
<td>51.64</td>
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<td>± 15.224</td>
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<tr>
<td></td>
<td>55849.98</td>
<td>± 31839</td>
<td>55849.98</td>
<td>± 31839</td>
<td>55849.98</td>
<td>± 31839</td>
</tr>
</tbody>
</table>
Figure 15. HSFA2 transcription fold change over control for (A) A. thaliana, (B) B. arcuata, (C) B. depauperata, ACT2 and SAND used as reference genes.

Table 5. Fold Change in HSFA2 Transcription Per Species by Sample Point

<table>
<thead>
<tr>
<th></th>
<th>HSFA2</th>
<th>1 Hour</th>
<th>6 Hour</th>
<th>12 Hour</th>
<th>24 Hour</th>
<th>48 Hour</th>
<th>72 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. thaliana 35°C</td>
<td>97519625.46 ± 68453578</td>
<td>6800.74 ± 4654.54</td>
<td>32165.72 ± 21601.92</td>
<td>10524.12 ± 6711.778</td>
<td>26784.51 ± 18557.57</td>
<td>8141.29 ± 4588.086</td>
<td></td>
</tr>
<tr>
<td>A. thaliana 38°C</td>
<td>127230997.12 ± 85222277</td>
<td>11391335.15 ± 7780102.9</td>
<td>1752141.46 ± 1219511.1</td>
<td>114066.36 ± 33139.52</td>
<td>1177365.79 ± 830419.49</td>
<td>425416.71 ± 300426.24</td>
<td></td>
</tr>
<tr>
<td>B. arcuata 35°C</td>
<td>128.68 ± 73.50</td>
<td>78.59 ± 31.80</td>
<td>8.56 ± 3.66</td>
<td>11.50 ± 4.79</td>
<td>3.83 ± 1.18</td>
<td>8.79 ± 3.90</td>
<td></td>
</tr>
<tr>
<td>B. arcuata 38°C</td>
<td>168.71 ± 64.87</td>
<td>115.42 ± 55.94</td>
<td>19.72 ± 8.05</td>
<td>8.12 ± 1.29</td>
<td>16.50 ± 6.19</td>
<td>9.08 ± 5.98</td>
<td></td>
</tr>
<tr>
<td>B. depauperata 35°C</td>
<td>1006.93 ± 439.82</td>
<td>50.67 ± 12.16</td>
<td>33.94 ± 10.32</td>
<td>20.50 ± 0.38</td>
<td>18.06 ± 2.47</td>
<td>22.59 ± 6.48</td>
<td></td>
</tr>
<tr>
<td>B. depauperata 38°C</td>
<td>2305.85 ± 321.46</td>
<td>449.22 ± 47.09</td>
<td>278.18 ± 74.06</td>
<td>145.32 ± 21.08</td>
<td>134.68 ± 25.68</td>
<td>53712.42 ± 10551.20</td>
<td></td>
</tr>
</tbody>
</table>
Figure 16. *HSP101* transcription fold change over control for (A) *A. thaliana*, (B) *B. arcuata*, (C) *B. depauperata*, ACT2 and SAND used as reference genes.

Table 6. Fold Change for *HSP101*

<table>
<thead>
<tr>
<th></th>
<th>1 Hour</th>
<th>6 Hour</th>
<th>12 Hour</th>
<th>24 Hour</th>
<th>48 Hour</th>
<th>72 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. thaliana 35°C</em></td>
<td>8732.89 ± 2559.93</td>
<td>166.76 ± 91.09</td>
<td>615.71 ± 232.06</td>
<td>228.99 ± 128.57</td>
<td>684.07 ± 235.16</td>
<td>219.78 ± 47.03</td>
</tr>
<tr>
<td><em>A. thaliana 38°C</em></td>
<td>18980.86 ± 7057.71</td>
<td>1495.40 ± 399.33</td>
<td>2458.08 ± 603.88</td>
<td>1368.63 ± 742.63</td>
<td>1758.75 ± 427.85</td>
<td>1054.97 ± 216.03</td>
</tr>
<tr>
<td><em>B. arcuata 35°C</em></td>
<td>287.45 ± 69.33</td>
<td>3.00 ± 1.27</td>
<td>18.53 ± 10.36</td>
<td>26.55 ± 0.53</td>
<td>21.86 ± 0.68</td>
<td>5.33 ± 1.61</td>
</tr>
<tr>
<td><em>B. arcuata 38°C</em></td>
<td>131.37 ± 66.60</td>
<td>14.79 ± 7.23</td>
<td>4.63 ± 1.87</td>
<td>3.01 ± 1.53</td>
<td>4.10 ± 2.19</td>
<td>22.89 ± 14.52</td>
</tr>
<tr>
<td><em>B. depauperata 35°C</em></td>
<td>2510.59 ± 552.34</td>
<td>33.16 ± 15.94</td>
<td>125.58 ± 72.61</td>
<td>239.01 ± 93.93</td>
<td>130.02 ± 24.98</td>
<td>79.01 ± 17.49</td>
</tr>
<tr>
<td><em>B. depauperata 38°C</em></td>
<td>197.00 ± 116.00</td>
<td>261.69 ± 172.93</td>
<td>48.71 ± 32.63</td>
<td>20.14 ± 13.38</td>
<td>9.22 ± 5.89</td>
<td>2324.03 ± 1259.04</td>
</tr>
</tbody>
</table>
transcription increased only at hour 1 for both 35°C and 38°C, and transcription goes back to control levels for hours 6 through 72 (Figures 16B). *B. arcuata* *HSP18.1* has an increase in transcription at 48 hours and 72 hours at 38°C, but at 35°C for hours 1 through 72 transcription levels are the same as control (Figure 17B, Table 7). *B. depauperata* at 35°C only *HSP101* increases in transcription and only at hour 1; for all other genes and time points at 35°C transcription levels are the same as control (Figures 14C, 15C, 16C, and 17C). *B. depauperata* *APX2, HSFA2, HSP101,* and *HSP18.1* have an increase in transcription only at hour 72 at 38°C (Figures 14C, 15C, 16C, and 17C). It is important to note that *B. arcuata* and *B. depauperata* exhibit fold changes in transcription that are significantly lower than that of *A. thaliana* for all four genes.

Figure 17. *HSP18.1* transcription fold change over control for (A) *A. thaliana*, (B) *B. arcuata*, (C) *B. depauperata*, *ACT2* and *SAND* used as reference genes.
Table 7. Fold Change for HSP18.1

<table>
<thead>
<tr>
<th></th>
<th>1 Hour</th>
<th>6 Hour</th>
<th>12 Hour</th>
<th>24 Hour</th>
<th>48 Hour</th>
<th>72 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP18.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. thaliana 35°C</td>
<td>11756.65 ± 6741.60</td>
<td>11322.30 ± 6322.06</td>
<td>10024.81 ± 4232.12</td>
<td>1366.38 ± 729.49</td>
<td>1909.91 ± 666.37</td>
<td>1613.76 ± 910.04</td>
</tr>
<tr>
<td>A. thaliana 38°C</td>
<td>22790.42 ± 5868.57</td>
<td>20664.55 ± 263.01</td>
<td>130113.66 ± 9102.23</td>
<td>30868.23 ± 13031.67</td>
<td>47265.44 ± 373.61</td>
<td>22922.09 ± 10790.91</td>
</tr>
<tr>
<td>B. arcuate 35°C</td>
<td>0.03 ± 0.01</td>
<td>0.31 ± 0.16</td>
<td>0.00 ± 0.00</td>
<td>1.08 ± 0.62</td>
<td>1.57 ± 0.93</td>
<td>3.95 ± 2.38</td>
</tr>
<tr>
<td>B. arcuate 38°C</td>
<td>0.36 ± 0.21</td>
<td>1.89 ± 0.60</td>
<td>0.07 ± 0.04</td>
<td>2.14 ± 0.98</td>
<td>38.86 ± 26.76</td>
<td>65.12 ± 40.06</td>
</tr>
<tr>
<td>B. depauperata 35°C</td>
<td>2.62 ± 0.69</td>
<td>2.15 ± 1.48</td>
<td>2.38 ± 1.48</td>
<td>1.03 ± 0.57</td>
<td>1.92 ± 1.31</td>
<td>0.72 ± 0.42</td>
</tr>
<tr>
<td>B. depauperata 38°C</td>
<td>9.48 ± 5.98</td>
<td>7.20 ± 4.43</td>
<td>5.88 ± 4.07</td>
<td>0.27 ± 0.01</td>
<td>0.26 ± 0.15</td>
<td>105.91 ± 26.88</td>
</tr>
</tbody>
</table>

**SINGLE SEED DESCENT**

In a pooled wild population of seeds, there is genetic diversity. To separate genetic difference from a pooled population, we needed to produce single seed descent lines that have been self-fertilized for 4 generations or more. We took seeds from a single plant and grew four offspring, which made up the F1 generation. From the four offspring, we choose two plants to move forward to the F2 generation by growing up 4 plants. From the F2 generation, only one plant was chosen to move forward to the F3 generation. From the F3 generation, four plants were grown and one was chosen to make up the F4 generation and we will label this the Single Seed Descent, which should represent one genotype from the pooled population. Figure 18 shows the schematic setup for single seed descent for *B. depauperata*. Currently we are in the F3 generation for *B. depauperata*, and in the F2 generation for all the other species we work with in our laboratory.

**FLOWERING**

Some of the plants we were working with did not produce flowers at all or would take over one year to produce just a few inflorescences. In order to produce seeds for the single seed descent and other experiments I needed to optimize our growing conditions and determine what conditions would be necessary to induce flowering in the *Boechera* species. To induce flowering, we vernalized the plants for four weeks following the temperatures,
light intensity and day length described in Table 2. Plants that were vernalized produced many more flowering stalks compared to non-vernalyzed plants (Figure 19A). After vernalization, plants produced many flowering stalks, but many of the pods did not mature. We trimmed and reduced the number of flowering stalks and checked for mature pods, by trimming the number of flowering stalks to only 3 or 4, from ten or more, we were able to increase the number of mature seed pods (Figure 19B).
Figure 19. (A) Flowering rate for vernalization. (B) Seed pod production from trimming stalks.
DISCUSSION

SUMMARY

I have found that the three species in our study, *A. thaliana*, *B. arcuata* and *B. depauperata*, behave very differently under moderately high temperature long-term heat stress. *A. thaliana* CF loses efficiency both at 35°C and 38°C, while *B. arcuata* loses efficiency at 38°C only, and *B. depauperata* does not lose efficiency in either temperature. *B. arcuata* has a very leaky membrane while under heat stress and the most cell death, *A. thaliana* has a leaky membrane too, but not as leaky as *B. arcuata*. *B. depauperata* is able to withstand both 35°C and 38°C heat for up to 96 hours without a lot of damage and cell death from the heat stress. The gene expression profiles for all three plants are very distinct, where *A. thaliana* will turn on *APX2*, *HSFA2*, *HSP101*, and *HSP18.1* at both temperatures immediately, *B. depauperata* doesn’t increase transcription dramatically until 72 hours at 38°C. *B. arcuata* turns on transcription early in heat stress, but transcription decreases over time. Combining all the data together for *B. depauperata* of PSII efficiency, ion leakage, and differential transcription, it seems like it is not stressed at all. All three species behave in a unique manner to moderately high temperature long-term heat stress.

CF EFFICIENCY AND HEAT STRESS

PSII absorbs light and the energy from it can go down three paths: the energy can be used to free an electron to drive CO2 fixation, or it can be re-emitted as light, or it can be dissipated as heat (Maxwell and Johnson, 2000). Loss in PSII efficiency is one of the first signs of leaf stress (Maxwell and Johnson, 2000). High temperatures reduce CO2 fixation and consequently increase ROS production (Hewezi et al., 2008; Pinto-Marijuan and Munne-Bosch, 2014). *A. thaliana* does not lose PSII efficiency when stressed at 35°C until after 72 hours of continuous heat stress. But at 38°C, PSII efficiency is lost in *A. thaliana* as early as 24 hours into the heat stress. *B. arcuata* PSII starts to lose efficiency at 48 hours when heat stressed at 35°C, but PSII efficiency does not drop as much as *A. thaliana* by the 96th hour. *B. depauperata* seems to not be affected by 35°C or 38°C very much. We see a small decrease in efficiency at 72 hours at 38°C, but it isn’t as dramatic as *A. thaliana* or *B. arcuata*. *A. thaliana* is able to handle 35°C for some time, but 38°C is stressful for the
specie. Heat stressed at 38°C, *B. arcuata* loses efficiency more rapidly than *A. thaliana*. *B. depauperata* is least stressed by 35°C or 38°C heat stress. At 35°C *A. thaliana* is more stressed than *B. arcuata*, but at 38°C it is the other way around, with *B. arcuata* being more stressed. We had the three follow hypothesis:

1A: The three plant species have identical ability in protecting PSII from heat stress.
1B: The three plant species use distinct ability to protect PSII.
1C (null): PSII efficiency is not affected by moderately high temperature heat stress in these species.

We can reject the null hypothesis (1C) because heat has an effect on PSII efficiency. We can reject hypothesis 1A because *A. thaliana*, *B. arcuata*, and *B. depauperata* do not have identical PSII efficiencies for 35°C and 38°C heat stress. *A. thaliana* and *B. arcuata* do not protect PSII, and most importantly, *B. depauperata* is able to protect PSII under heat stress, making hypothesis 2 correct.

**ION LEAKAGE AND CELL DEATH**

When a plant is under heat stress, there is an increase production of ROS and an increase in ROS will trigger programmed cell death (Apel and Hirt, 2004; Pinto-Maarijuan and Munne-Bosch, 2014). ROS by itself does not activate programmed cell death, but when ROS levels increase and ROS scavenging is decreased, programmed cell death is induced (Apel and Hirt, 2004). ROS also plays a role as a signaling molecule that turns on the heat shock response during biotic and abiotic stress (Baxter et al., 2014). Oxidative stress and production of ROS has been shown to trigger APX2 transcription, a gene involved in ROS scavenging, which will protect tissues from heat-related damage (Baxter et al., 2014; Miller et al., 2008; Suzuki et al., 2013). It is also known that as the temperature of the cell increases, membrane fluidity increases and this can lead to loss of membrane integrity (Hemantaranjan et al., 2014).

It is interesting that *A. thaliana* starts to lose membrane integrity at 35°C after two days of continuous heat stress. I have also demonstrated that APX2 transcription increases dramatically at hour 1 at 35°C, then there is a drop, and at 48 hours and 72 hours there is a second small spike in transcription. At 38°C, membrane integrity is lost starting at day one, and APX2 transcription is continually on from hour one until hours 72. *A. thaliana* membrane integrity is not lost completely at 35°C, but at 38°C ROS levels are continually high, APX2
transcription is also continually high, and we see increased ion leakage in each consecutive sample until a zenith is reached at day 4. This suggests that ROS levels are high at the same time that ion leakage is high.

*B. arcuata*’s membrane is leaky at 35°C, losing about 40% of its total ions by day four. *APX2* transcription follows similar pattern as *A. thaliana* at 35°C, there is an initial spike in transcription, but it starts to decay over time, with a slight increase at 72 hours. At 38°C, within 48 hours the plant reaches greater than 80% ion leakage, the plant looks like it is dead. At 38°C, there is an increase in *APX2* transcription at hour 1, but it decays rapidly, and by the 12th hour transcription is non-existent. The difference in *APX* transcription levels at 35°C and 38°C between *A. thaliana* and *B. arcuata* is 1000 times greater in *A. thaliana*. *APX2* transcription is increased, but not maintained over time, and *B. arcuata* begins to die.

*B. depauperata* behaves very differently than *A. thaliana* and *B. arcuata*. At 35°C and 38°C, the plant ion leakage is very low, and membrane integrity is maintained. At 35°C there is almost zero *APX2* transcription at all sample points. The only increase in transcription we see is at 38°C after 72 hours of continuous heat stress. It is as if *B. depauperata* is not stressed at all at 35°C or 38°C. We hypothesized the follow:

2A: The three plant species have identical ability in protecting cells while under heat stress.
2B: The three plant species use distinct ability to protect cells from heat stress.
2C (null): Membrane integrity is not affected by moderately high temperature heat stress in these species.

The null hypothesis (2C) can be rejected because membrane integrity is affected by moderately high temperature heat stress. Hypothesis 2A can also be rejected because the three species do not have identical ion leakage. *A. thaliana* and *B. arcuata* are not able to protect cells from irreversible damage at 35°C and 38°C heat, while *B. depauperata* is able to protect membrane integrity and minimize cell death, thus hypothesis 2 holds true.

**HSFA2 TRANSCRIPTION EXPRESSION PROFILE**

*HSFA2* is a transcription factor regulating HSR, expression levels of *HSFA2* is highly upregulated by heat stress and it is essential in extending the duration of thermotolerance (Amano et al., 2012; Charng et al., 2007; Liu et al., 2011; Yoshida et al., 2011). Acquired thermotolerance decays faster with *HSFA2* knockout, and overexpression of *HSFA2* leads to
higher heat tolerance in many plant species (Charng et al., 2007; Ikeda et al., 2011; Larkindale and Vierling, 2008; Pillet et al., 2012). HSFA2 has been shown to interact with other heat shock proteins to extend heat shock response, and is only expressed in stressed plants (Meiri and Breiman, 2009; Scharf et al., 2012). HSFA1 and HSFA2 forms a superactivator complex for HSP encoding genes and are upregulated in long term heat stress (Scharf et al., 2012; Schramm et al., 2008; Yoshida et al., 2011). Our experiments are long last heat stress, so we are expecting HSFA2 expression in all three species to be very high and maintained throughout the heat stress regimen.

A. thaliana HSFA2 initial transcription is extremely high for both 35°C and 38°C; over millions of fold higher than the control sample at 1 hour. After one hour, the transcription level is still high, but not nearly as high as the initial spike in transcription. This shows that A. thaliana is under heat stress and HSFA2 transcription is induce to turn on HSR.

B. arcuata HSFA2 transcription is strong at both 1 hour and 6 hours at 35°C and 38°C. The fold change is not as incredible as A. thaliana, but there is increased transcription. After 12 hours of heat stress, transcription drops almost near control levels for both 35°C and 38°C, and HSR is not maintained.

For B. depauperata there is an initial increase in transcription of HSFA2 at hour 1 for both 35°C and 38°C. For 35°C after the first hour, transcription decays and is not increased. At 38°C there is a second and very large increased in transcription at 72 hours of continuous heat stress. B. depauperata only starts to experience heat stress after 72 hours of continuous heat exposer.

**HSP101 TRANSCRIPTION EXPRESSION PROFILE**

HSP101 is a molecular chaperone protein which has been shown to resolubilize heat-denatured proteins from insoluble aggregates, and it is required for high temperature survival and thermotolerance (Tonsor et al., 2008; Wu et al., 2013). HSP101 function is prolonged with HSA32, which prevents HSP101 decay (Lin et al., 2014; Wu et al., 2013). Plants with HSP101 mutations have lower acquired and basal thermotolerance (Hong and Vierling, 2000). HSP101 is activated by HSFA2 (Schramm et al., 2006). We are expecting HSP101 to follow a similar pattern to HSFA2, because it is activated by it.
A. thaliana has a large increase in transcription of HSP101 at 1 hour for both 35°C and 38°C. The expression decays after that, but transcription is maintained throughout the heat stress regimen for both temperature stresses. B. arcuata has very similar expression pattern as A. thaliana, there is an initial increase in expression, then it decays and maintained at low levels for both temperatures. B. depauperata also has an initial increase in transcription of HSP101, and then it decays afterwards, with one difference. At 38°C after 72 hours of heat stress, there is a second large increase in transcription. It takes B. depauperata to experience heat stress after a lot more heat exposure than A. thaliana and B. arcuata.

**HSP18.1 TRANSCRIPTION EXPRESSION PROFILE**

Small heat shock proteins are chaperone proteins that prevent the irreversible aggregation of proteins and can assist in refolding denatured proteins to maintain protein homeostasis (Haslbeck et al., 2005; Waters et al., 2008b). Small heat shock proteins range in size from ~15kD to 42kD; they play a role in normal growth conditions, and are an essential part of HSR (Neta-Sharir et al., 2005; Waters et al., 2008a). HSP18.1 is a small heat shock protein turned on by heat stress and under different temperatures, it binds to other HSP18.1 to form complex molecule (Eyles and Gierasch, 2010). Expression of HSP18.1 will tells us how many cellular proteins are being denatured from the moderately high heat stress.

In A. thaliana HSP18.1 is expressed initially at low levels at 35°C and then transcription decays after 12 hours, suggesting that 35°C does not denature many proteins inside the cell. At 38°C we see a gradual increase in transcription, peaking at 12 hours, and then decaying afterwards to lower levels. 38°C denatures more proteins than 35°C. B. arcuata HSP18.1 transcription is almost non-existent at 35°C. We see increased transcription at 38°C, after 48 hours of continuous heat stress. HSP18.1 may not be playing a major role in B. arcuata in protecting proteins from denaturing. Maybe there is another small heat shock protein that is protecting the plant, but the PSII data and ion leakage data suggests that B. arcuata may not be protecting proteins from denaturing, leading to cell death. B. depauperata has little to no transcription increase at both temperatures except at 38°C after 72 hours of continuous heat stress. B. depauperata proteins must not be denaturing at 35°C or 38°C.
The transcription profile of \textit{APX2}, \textit{HSFA2}, \textit{HSP101}, and \textit{HSP18.1} is different between the three species. \textit{A. thaliana} turns on heat shock response greatly, with fold change thousands of folds greater than control. \textit{B. arcuata} increase transcription, but not to the same level as \textit{A. thaliana}, which leads to the plant not being protected from heat stress. \textit{B. depauperata} only increases transcription at 38°C after 72 hours of continuous stress, 35 and 38 seems to not stress it out. We hypothesized that:

3A: The three plant species have identical transcription regulation for \textit{APX2}, \textit{HSFA2}, \textit{HSP101}, and \textit{HSP18.1}.

3B: The three plant species use distinct HSR and transcription levels for \textit{APX2}, \textit{HSFA2}, \textit{HSP101}, and \textit{HSP18.1} are different.

3C (null): \textit{APX2}, \textit{HSFA2}, \textit{HSP101} and \textit{HSP18.1} transcription are not induced by moderately high temperature heat stress.

The null hypothesis (3C) is rejected because moderately high temperature heat stress does induce transcription of \textit{APX2}, \textit{HSFA2}, \textit{HSP101}, and \textit{HSP18.1}. \textit{A. thaliana} has the greatest fold change in transcription for \textit{APX2}, \textit{HSFA2}, \textit{HSP101}, and \textit{HSP18.1}, while \textit{B. arcuata} transcription fold change occurs at hour 1 at both 35°C and 38°C and then decays back to control levels by hour 12, nullifying hypothesis 3C. \textit{B. depauperata} doesn’t seem to be heat stressed at 35°C at all, and only increases transcription of \textit{APX2}, \textit{HSFA2}, \textit{HSP101}, and \textit{HSP18.1} at 38°C after 72 hours of continuous heat stress. All three species have different transcription for change for the four genes of interest, so hypothesis 3B holds true.
CONCLUSION

It has been proposed that plants sense heat via at least five pathways: membrane fluidity, denatured proteins, Ca2+ fluxes, toxic byproduct buildup, and RNA unfolding (Horvath et al., 2012; Miller et al., 2008; Mittler et al., 2012). It has also been shown that heat stress increases ROS levels (Apel and Hirt, 2004; Miller et al., 2008; Suzuki et al., 2013). It is known that photosynthesis can be sensitive to heat stress (Maxwell and Johnson, 2000; Pinto-Marijuan and Munne-Bosch, 2014). In this thesis I have examined the impact of long-term stress on photosynthetic capacity, membrane integrity (cell death), and gene expression. My study has shown that B. depauperata is the most thermotolerant of the three species to long-term heat stress based on CF, ion leakage, and APX2, HSFA2, HSP101, and HSP18.1 transcription fold change. These findings are consistent with previous studies in the lab. It is clear from the gene expression patterns that while there are upregulation of HSFA2 and HSPs in Boechera, these expression levels are much lower than A. thaliana. It has been suggested that the HSFs and the HSPs are induced by the presence of damaged proteins. The lower levels of HSP expression in the Boechera species might be an indication that there are lower levels of denatured proteins in Boechera compared to A. thaliana. One of the other proposed temperature sensors in plants is a change in membrane fluidity. Based on the ion leakage data, it is clear that membrane integrity varies greatly by treatment and by species. Again the relatively low levels of gene expression for HSPs and low ion leakage in B. depauperata suggest that for most of the treatments studied here that B. depauperata does not “feel” the heat in the same manner as A. thaliana does. The little stress that we did observe in B. depauperata suggests that more research needs to be done to find the tipping point from homeostasis to heat stress.

A change in 3°C, from 35°C to 38°C, had a large effect on PSII efficiency, gene transcription, and cell death in our experiments. There are many species of plants that inhabit niche climates. A change in 3°C can lead to plant death, which may results in total population loss and decrease plant diversity. Loss of plant species can also lead to loss of habit for native wildlife such as birds and insects. It is important to understand what effects small changes in temperature have on native species. World temperatures are going up, plant
and animal species are going instinct, and we must stop it before all wildlife is lost. This study helps us understand the effects of 3°C change on two native California species.
FUTURE DIRECTIONS

The next step for us is to choose sample points to do RNA-seq and look at total
differential expression patterns and gene ontology. From the RNA-seq, we can look at
polymorphism in transcripts between *A. thaliana* and the *Boechera* species. RNA-sequencing
may lead to discovering novel pathways of HSR in the *Boechera* species.

*B. depauperata* was just starting to experience heat stress at 38°C after 72 hours of
continuous heat. In the next step the same experiments could be run on all three species at
40°C and compare it to the current data. We could also add more species of *Boechera* to do
these studies on.
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REFERENCES


