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A Thesis Submitted to

the Biotechnology Master's Program

in partial fulfillment of the requirements for

the degree in Master of Science

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Thesis Committee Supervisor/Chair

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Abstract

Plants are sessile organisms that are constantly exposed to a variety of abiotic and biotic environmental stresses. Some plants are known to be more tolerant to those environmental stressors than others; those are the extremophilic plants. Studying the stress response pathways in such plants is extremely important in developing transgenic crop plants with enhanced tolerance to environmental stresses. *Eutrema salsugineum* is an extremophilic plant that is known to be resistant to many abiotic stress factors such as drought, cold, salt, and nitrogen deficiency.

Experiments were carried out in KAUST by exposing the extremophilic plant to heat stress and exogenous ABA stress. RNA sequencing was done in order to get the transcriptome profile of the plant in response to the stresses. Trinity *De novo* transcriptome assembly was done followed by transcript abundance quantification and normalization using Kallisto. Differential expression analaysis was done to identify the differentially expressed transcripts in response to the different treatments in the shoot and root using the R bioconductor package EdgeR. The transcripts were annotated using EggNOG. The protein coding transcripts were identified by aligning them to the nr protein database using tblastx. Functional analysis of the DE transcripts to get the enriched terms was carried out using DAVID.

Trinity de novo assembly produced 49857 genes and 134493 transcripts. Out of the 134493 transcripts, 114692 (85.28%) transcripts had tblastx hits (protein coding). Thus, 19801 potentially non coding or novel transcripts have been identified. A large variety of genes were found to be differentially expressed depending on the pair-wise comparison. The genes were mainly involved in plant heat and ABA stress, ROS signaling pathway, ROS scavenging, secondary metabolite production, and lipid transfer.

Further investigation of the role of secondary metabolites such as flavonoids, and nitrogen and sulfur containing compounds in the abiotic stress response of *E. salsugineum* is needed since it appears to be a major mechanism used by the plant. The results of this research offer a wide variety of stress related genes in *E.salsugineum.* Investigation of the over-expression of some of these genes in stress sensitive plants will help in further understanding their functions and mechanisms of action.

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Chapter 1: Introduction

1.1 Effect of Stress Factors on Plants

Plants are sessile organisms that face are exposed to different biotic and abiotic environmental stresses throughout their life cycle. The abiotic stress factors include drought, extreme temperatures, and soil contamination by high salt concentration. The biotic stress factors include the variety of pathogens and herbivores that cause either mechanical or chemical damage to the plant. The adverse conditions can delay growth and development, reduce productivity, and eventually can cause death. Thus, these stress conditions can limit the agricultural yield worldwide and cause major economic losses (Krasensky and Jonak 2015).

The exposure of plants to environmental stress can cause damage to the structure of the cells and can disrupt many physiological functions. For instance, salinity, cold, freezing, and drought can all cause loss of turgidity in plant cells due to the disruption of the water potential. As a result, the plasma membrane structure is disrupted, the proteins are denatured and lose their function, and the reactive oxygen species (ROS) accumulate causing oxidative damage. This will eventually lead to photosynthesis inhibition, and metabolic impairment causing impaired fertility and growth, and early senescence (Fig 1 & 2) (Larcher 2003).

Plants vary with respect to their ability to withstand and respond to the harsh environmental conditions. Some plants are more stress sensitive than others. For example, extremophilic plants, such as *Eutrema salsugineum*, plants are those that are more able to tolerate stress than others. Two strategies exist for dealing with environmental stress: stress avoidance, and stress tolerance. Stress avoidance involves having inherited and stable morphological, physiological, and metabolic properties that enable the plant to withstand harsh environmental conditions. For example, cacti are plants that have different characteristics that enable them to withstand the harsh desert environment; their leaves are reduced to spines to minimize water loss by transpiration, and their stems are thick and fleshy to be able to store water. Stress tolerance involves the ability of a plant to acclimate to environmental stress. This is done by a variety physiological modifications such as changing gene expression or producing certain metabolites. These modifications are usually lost when the stress condition no longer exists (Levitt 1980).

Plants are known to have tolerance to specific single stresses, but recent research has also shown that plants have developed the ability to respond to stress combinations. Ten *A. thaliana e*cotypes were exposed to single stresses (salt, cold, heat, high-light, and flagellin) and 6 different double combinations of the mentioned stresses. Results revealed that 61% of the transcriptome changes in response to double stresses. It was also seen that plants prioritized between antagonistic responses for approximately 5-10% of the differentially expressed transcripts (Rasmussen et al. 2013). A functional clustering analysis was done for the overlapping target genes (529) between the different combined stress conditions and the resulting 4 clusters can be seen in fig.3 (Barah et al. 2016).

1.2 The Effect of Global Warming on Plants

Heat is an abiotic factor that can pose serious threats to the plant's growth and development, circadian rhythm, and immunity. For the model plant *Arabidopsis thaliana*, heat can be classified as warm ambient temperature (22-27°C), high temperature (27-30°C), and heat stress (37-42°C) (Liu et al. 2015). Due to global warming, the annual mean maximum temperature has increased by 0.35°C, and the annual mean minimum temperature has increased by 1.13°C during the period 1979-2003 (Peng et al. 2004). The rise in the mean annual temperature is expected to increase over the next years. This rise in temperature is causing significant losses of agricultural crops. For instance, the global maize and wheat yield has decreased by 3.8% and 5.5% respectively over the period of 1980-2008 (Lobell et al. 2011). It has been estimated that the global yields for the six most widely grown crops (wheat, rice, maize, barley, soy-beans, and sorghum) will decrease by 0.6~8.9% for every 1°C raise in temperature (Lobell and Field 2007).

Not only does global warming decrease the crop yield, but it also affects other aspects of the plant life cycle. For instance, a study in 2002 has shown that the average first flowering date of 385 British plant species has advanced by 4.5 days over the last decade (Fitter and Fitter 2002). Thus, understanding the plant's heat stress response and the different signal transduction

pathways that modulate such response specifically in more heat tolerant plants is of critical importance.

1.3 The Use of *Arabiodopis thaliana* in Studying Plant Stress Responses

Arabidopsis thaliana (thale cress) is a small angiosperm that is a member of the family Brassicacea (Amtmann 2009) It has been widely used as a model organism in botany since it was sequenced in the year 2000. The use of *A.thaliana* as a model organism is due to 1) A very small diploid genome of 125 mega base pairs spanning 5 chromosomes with fewer than 30,000 protein coding genes (Weigel and Mott 2009). 2) The presence of detailed physical and genetic map of all of its chromosomes. 3) A very short life cycle (6 weeks). 4) Easy and efficient transformation using *Agrobacterium tumefaciens.* 5) A large number of mutant strains.

As a model organism, *A.thaliana* has been used extensively over the years to study the plant's genotypic, phenotypic, and metabolic variations. It has also been used to study plant responses to biotic and abiotic stress. However, A.*thaliana* is a stress sensitive plant; thus, it has not been productive in such investigations (Kazachkova et al. 2013).

1.4 *Eutrema salsugineum*

Eutrema salsugineum (salt cress) is halophytic plants species that has been shown to be tolerant to a variety of abiotic stresses including salt, drought, and cold (Lee et al. 2013). It belongs to the family *Brassicaceae* to which *A. thaliana* belongs to. The genome of *E. salsugineum* was sequenced in 2013. The size of the genome is 241 Mb spanning 7 chromosomes with 26,531 protein coding genes. Repetitive sequences that are found mainly in pericentromeric regions constitute 51.4% of the halophote's genome (Yang et al. 2013).

1.5 Evidence for the Extremophilic Properties of *Eutrema salsugineum*

E. salsugineum was found to be able to distribute and store Na^+ under salinity stress by a highly coordinated control of ion movement across both the tonoplast (TP) and the plasma membrane (PM). For instance, an increased expression of the PM Na^+/H^+ exchanger SOS1 was observed in salt stressed roots and leaves. However, expression levels of the PM H^+- ATPase

isoform *AHA3*, and the Na^+ transporter *HKT1* did not change in salt stressed roots and leaves. The Na^+/H^+ exchanger *NHX1* was only detected in PM fractions of roots (Vera-Estrella et al. 2005).

One study aimed at investigating the transcript profile of *E. salsugineum* using a cDNA microarray containing 3628 unique sequences in response to salinity, cold, stimulated drought and re-watering after stimulated drought. The study revealed 154 differentially expressed transcripts under the investigated stresses with only 6 genes that were common between them. This is evidence that *E. salsugineum* exhibits a stimulus specific response (Wong et al. 2006).

An experiment investigated the biological function of a tonoplast AQP gene (*TsTIP1;2*) that has been found to be upregulated under a lot of stress conditions in *E. salsugineum.* Results revealed that the ectopic over-expression of the gene in A. *thaliana* increased the plant's tolerance to salt, drought, and oxidative stress. The gene was also found to transport H_2O_2 under oxidative stress into yeast cells and have water channel activity when expressed in *Xenopus* oocytes. The data collectively indicates that *TsTIP1;2* might have a role in the high stress tolerance of *E. salsugineum* (Wang et al. 2014b).

At an optimal temperature of 21°C, the metabolite levels of *E. salsugineum* and *A.thaliana* were found to be intrinisically different. *A. thaliana* had high levels of putrescine and fumarate while *E. salsugineum* had higher levels of amino acids such as alanine, asparagines, histidine, serine, phenylalanine, and leucine. At 4°C both species accumulated sucrose, but this accumulation was temporary in *A. thaliana* after the return to the optimal temperature. However, it was sustained in *E. salsugineum* (Benina et al. 2013).

A microarray study of cold acclimated *E. salsugineum* at a temperature of 4°C revealed the functional gene groups that are involved in cold tolerance. When considering down-regulated genes, the significantly affected functional groups included cell wall, photosynthesis, and hormones. When considering up-regulated genes, the significantly affected functional groups included glycolysis and biodegradation of xenobiotics (Lee et al. 2013).

A comparative study of *E. salsugineum* and *A. thaliana* under salinity stress focused on chloroplastic metabolism. *E. salsugineum* was found to have an intrinsic increased chlorophyll a/b ratio accompanied by higher Y_{11} value, lower non-photochemical quenching value, and a more active photosystem 1 (PS1). *E. salsugineum* was also found to have an intrinsic increase in

the production of H_2O_2 from plastoquinone pool. Under salt stress, the above mentioned variables were found to increase in *A. thaliana*. However, they remain unchanged in *E. salsugineum* which signals that the plant is metabolically primed to stress (Wiciarz et al. 2015).

Abiotic stresses such as salinity, drought, and low temperatures are known to cause the production of reactive oxygen species (ROS) which eventually lead to the accumulation of toxic aldehydes in plant cells. Aldehyde dehyrogenases (ALDHs) have a critical role in eliminating the toxic aldehydes (Singh et al. 2013). Seventeen members of ten plant *ALDH* families have been identified in *E. salsugineum*. In a gene expression analysis of *A. thaliana* and *E.salsugineum* under salt stress, most of the *ALDH* genes had the same expression levels in both plants except for *ALDH7B4* and *ALDH10A8*. This indicates that both genes might have a role in the extremophilic charachteristics of *E. salsugineum* (Hou and Bartels 2015).

Hydrogen peroxide (H_2O_2) is another ROS that modulates many biological and physiological roles in plants in low levels. However, high levels of H_2O_2can cause damage to cellular structures leading to severe effects (Ozyigit et al. 2016). Glutathione peroxidases (GPX) catalyze the reduction of H_2O_2 into water or alcohols to prevent any potential cellular damage. When studying the expression levels of *E. salsugineum GPX* genes, it was found that the gene family members were coordinately regulated under specific abiotic stress conditions and in different tissues (roots and leaves). For instance, three *GPX* genes (*GPX5, GPX7, GPX8*) were up-regulated in leaves under salt stress; While six *GPX* genes (*GPX1, GPX2, GPX3, GPX5, GPX7, GPX8*) were up-regulated in roots under salt stress. On the other hand, four *GPX* genes (*GPX1, GPX3, GPX4, GPX7*) in leaves under osmotic stress; While almost all *GPX* genes were up-regulated in roots under osmotic stress (Gao et al. 2014).

Based on the reviewed literature, it can be seen that the focus of stress tolerance studies using *E. salsugineum* has been salinity, drought, and cold responses. Thus, studying heat tolerance mechanisms in *E. salsugineum* is a necessary step in understanding the effect of global warming on the world's agricultural crops.

1.6 Comparing the Genomes of *A. thaliana* and *E. salsugineum*

It can be seen from sections 1.3 and 1.4, that the size of *E. salsugineum*'s genome is twice the size of *A. thaliana's* genome. The halophyte's genes have a high sequence identity (90%-95% at cDNA level) to *A. thaliana* (Taji et al. 2004). Due to the high genetic similarity between *E. salsugineum* and the model plant *A. thaliana* and the limitation of using *A. thaliana* to study stress responses, *E. salsugineum* has been extensively used over the past years as a model plant to study the signal transduction pathways that are involved in stress tolerance (Wong et al. 2006).

1.7 Thermotolerance

Plants have an inherent ability to survive exposure to temperatures above the optimal for growth (basal thermo-tolerance) and an ability to survive severe heat stress preceded by exposure to elevated but non-lethal temperatures or other types of stress (acquired thermo-tolerance) (Larkindale et al. 2005). Gene expression data under heat stress from different plant species, different tissue types, growth conditions and developmental stages revealed that high temperatures affect approximately 2% of the plant genome (Qu et al. 2013).

1.7.1 Controlling Thermo-tolerance

It has been reported that salicylic acid (SA)(Clarke 2004), jasmonic acid (JA) (Clarke et al. 2009), and ethylene signaling pathways are required for basal thermo-tolerance. ROS scavenging proteins (Miller et al. 2008), the transcriptional co-activator Multi-protein Bridging Factor 1C (*MBF1c*) (Suzuki et al. 2008), and Respiratory Burst Oxidase Homologue (*Rboh*) are also required for basal thermo-tolerance (Miller et al. 2008). On the other hand, acquired thermo-tolerance has been found to be controlled by Heat Stress Transcription Factors (*Hsfs*) and Heat Stress Proteins (*Hsps*) (Liu et al. 2015). Some signaling molecules such as ethylene, phospholipids, calcium, ABA, H_2O_2, and SA are also involved in acquired thermo-tolerance (Song et al. 2012).

1.7.2 Basal Thermotolerance

The major hormones produced by plants are gibberellins (GA), auxins, cytokinins, abscisic acid (ABA), salisylic acid (SA), ethylene (ET), jasmonates (JA), strigolactones, and brassinosteroids (BR). ABA, SA, ET, and JA are known to have critical roles in plant biotic and abiotic stress responses (Verma et al. 2016). Even though the four hormones have been shown to have roles in both types of stresses, ABA is more involved in plant abiotic stresses such as salinity, drought, cold, and heat stress (Lata and Prasad 2011; Zhang et al. 2006); SA, ET, and JA are more involved in plant responses to biotic stresses (Bari and Jones 2009). In the following section the separate role of the previously mentioned hormones will be explored with a focus on their role in basal thermotolerance. Evidence for crosstalk between the different hormones to mediate stress responses has been recently increasing; thus, this will also be explored in the next section.

1.7.2.1 Salicylic Acid (SA)

1.7.2.1.1 SA biosynthesis

Salicylic acid is a seven carbon phenolic compound found in nature. It is an endogenously synthesized phyto-hormone signaling molecule in plants. Three pathways are involved in the biosynthesis of SA in plants, the shikimic acid pathway, the phenylalanine pathway, and the isochorismate pathway (Khan et al. 2015).

1.7.2.1.2 The role of SA in stress tolerance

SA is involved in many plant physiological processes such as proline metabolism, photosynthesis nitrogen metabolism, glycinebetaine production, and antioxidant defense(Khan et al. 2015). It has also been shown to be involved in the induction of stress related genes and

responses to both biotic (Kumar 2014) and abiotic stresses such as salinity (Khan et al. 2014), drought (Fayez and Bazaid 2014), and heat in plants (Khan et al. 2013).

Many abiotic stress factors have been shown to induce enzymes involved in SA-biosynthesis such as isochromate synthase and phenylalanine ammonialyase (ICS & PAL) (Wildermuth et al. 2001). Exogenous application of SA to *A. thaliana* was found to enhance drought tolerance by up-regulating *ICS1 (Hunter et al. 2013).* Exposure of *A. thaliana* to ozone stress caused synthesis of SA with the involvement of ICS (Ogawa et al. 2005). SA accumulation occurred in barley roots and leaves due to the increased activity of PAL and benzoic acid hydroxylase under UV-B radiation and water deficit stress (Bandurska and Cieślak 2013).

1.7.2.1.3 The role of SA in thermo-tolerance

The role of SA in plant heat tolerance has been reported in many experiments (Larkindale et al. 2005; Wang et al. 2010; Khan et al. 2013).Transgenic *A. thaliana* seedlings that had an SA-decomopsing salicylate hydroxylase showed a decreased tolerance to heat (Larkindale and Knight 2002). SA was shown to decrease oxidative stress and electrolyte leakage, and improve maximum yield of PSII, Fv/Fm, and quantum yield of the PSII electron transport in heat stressed *Cucumis sativa* seedlings (Shi et al. 2006). Treatment of *T. aestivum* with SA increases stress tolerance by increasing proline production and restricting the formation of ethylene under HS (Khan et al. 2013).

1.7.2.1.4 The mechanism of SA to modulate abiotic stress

The signaling pathway and mechanism by which SA regulated response to abiotic stress is not yet understood. However, the SA-mediated plant stress tolerance mechanism has been shown to involve ROS signaling and the interaction of SA with mineral nutrients (Mn, Ca, Cu, Fe, P, Zn), osmolytes (GB & Pro), secondary metabolites (glucosinolates, alkaloids, anthocyanins, vanillin), and other phytohormones (ethylene & ABA) (Khan et al. 2015).

Salinity stress in *Vigna radiata* was shown to be alleviated by SA treatment which enhances the activities of ROS scavenging enzymes such as SOD, GPX, APX, CAT, and GR(Khan et al. 2014). Antioxidant enzymes such as CAT, APX, POD, and SOD had increased activities modulated by SA also in plants under drought (Saruhan et al. 2011)and cold stress (Mutlu et al. 2013). The uptake of plant beneficial minerals such as Mn, Ca, Cu, Fe, P, and Zn is regulated by SA minimizing oxidative stress under Pb stress (Wang et al. 2011a). SA increased photosynthesis in salt stressed *Brassica juncea* by decreasing cellular Na+ and Cl- ions and increasing the nutrient content (Syeed et al. 2010).

Osmolytes such as glycinebetaine (GB) and proline (Pro) contribute to maintenance of turgor pressure in stressed plants without interfering with any other metabolic processes (Misra and Saxena 2009). SA was shown to increase GB accumulation in plants under salinity, drought, and cold stress (Jagendorf and Takabe 2001). Photosynthesis and growth processes in salinity stressed *V. radiate* were restored by SA caused GB accumulation (Khan et al. 2014). SA was also shown to activate Pro biosynthesis enzymes and increase the Pro content under salt stress; this was accompanied by an increased salinity tolerance in *Lens esculenta (Misra and Saxena 2009).* Nitrogen assimilation and the heat stress impact on photosynthesis were alleviated by increased Pro production due to SA- mediated increase in the activity of γ-glutamyl kinase, and decrease in the activity of Pro oxidase (Khan et al. 2013).

Secondary metabolites are substances that are derived from primary metabolites; they are produced by plants as defense chemicals and are not involved in any metabolic activity (Irchhaiya et al. 2014). Exogenously applied SA to UV-B stressed *T. aestivum* increased the accumulation of anthocyanin and tocopherol (Hovarth et al. 2007). Production of sterols, xanthoproteins, saponins, and coumarins was induced in water stressed *Simarouba glauca* by the foliar application of SA (Awate and Gaikwad 2014).

1.7.2.2 Jasmonates (JAs)

1.7.2.2.1 JA biosynthesis

Jasmonates (JAs) are plant oxylipins that are involved in several developmental processes in plants such as vegetative growth, germination, and senescence. JAs also activate plant stress responses to biotic factors such as insect driven wounding or pathogen attack (Thaler et al. 2004), and abiotic factors such as salinity, cold, and heavy metal toxicity (Pauwels and Goossens 2011). Generally, oxylipins can be produced in plants either enzymetically, by α-DIOXYGENASES (α-DOXsas) or LIPOXYGENASES (LOXs), or non-enzymetically, by autooxidation of polyunsaturated fatty acids (Sharma and Laxmi 2015). There is a variety of active jasmonate compounds in plants including jasmonic acid (JA), cis jasmone (CJ), methyl jasmonate (MeJA), and jasmonoyl-isoleucine (JA-Ile) (Fonseca et al. 2009).

1.7.2.2.2 JA signal transduction pathway

The JA signal transduction pathway involves many repressors, transcription factors, and members of the ubiquitin proteasomal pathway. In the absence of a stimulus, jasmonic acid is not produced. This caused the jasmonate zim domain (JAZ) repressors to bind to the transcription activator MYC2 which leads to the recruitment of TPL and adaptor protein NINJA. The JAZ-NINJA-TPL complex causes the recruitment of HDA6 and HDA19 inhibiting the JA mediated gene expression (Fig. 4) (Sharma and Laxmi 2015).

In the presence of a stimulus, JA is produced and epimerized to JA-Ile which binds to Col1-JAZ-InsP5 co-receptor complex causing JAZ to be ubiquitinated and degraded by the proteosome. MYC2 and its homologs are then released causing them to bind to the G-box element that is found downstream of the JA responsive genes leading to the JA responses (Sharma and Laxmi 2015).

1.7.2.2.3 The role of JA in thermotolerance

Clarke et al. were the first to establish the role of the JA signaling cascade in basal thermotolerance of *A. thaliana*. Heat stressed plants accumulated a variety of JAs including JA, JAIle, OPDA, and MeJA. Cell viability of the heat stressed plants was maintained due to exogenous application of MeJA. Moreover, JA and SA signaling mutants were found to be significantly affected by exposure to heat (Clarke et al. 2009).

WRKY is a protein family found in *A. thaliana* whose members are known to be involved in a variety of stress responses. Exogenous application of JA induced the expression of a member of the WRKY family involved in heat stress, CaWRKY40. In addition, *NtLOX1* gene responsible for JA biosynthesis was found to be downregulated in plants with over-expressed *CaWRKY40* (Dang et al. 2013).

1.7.2.3 Ethylene (ET)

1.7.2.3.1 ET Biosynthesis

Ethylene is a gaseous phytohormone that regulates many metabolic and developmental processes in plants including senescence, growth, and fruit ripening; it also has a role in many abiotic and biotic environmental stress responses (Bleecker 2000). Ethylene is produced from methionine by converting S-adenosyl-L-methionine (AdoMet) to the non-protein aminoacid 1-aminocyclopropane-1-carboxylic acid (ACC), and then converting ACC to ethylene (Adams and Yang 1979). This 2 steps reaction is catalyzed by the enzymes ACC synthase and ACC oxidase respectively (Kende 1993).

1.7.2.3.2 Ethylene signal transduction pathway

There are five identified ethylene receptors located in the ER membrane ETR1, ETR2, ERS1, ERS2, EIN4. In the absence of ethylene, the ER receptors activate CTR1, a Ser/Thr kinase, which phosphorylates EIN2, an ER-bound protein, inactivating it. This prevents the transcription of the transcription factors EIN3, EIL1, and EIL2 inhibiting the ethylene responses.

In the presence of ethylene binding, The ER receptors inactivate CTR1 so that it cannot phosphorylate EIN2. Thus, EIN2 will be activated causing the transcription of the transcription factors and leading to the ethylene response (Fig. 5) (Shakeel et al. 2013).

1.7.2.3.3 The role of Ethylene in thermotolerance

Ethylene responsive element binding factors (ERFs) are a TF family found in *A.thaliana* that are involved in a variety of developmental processes and stress responses. ERF1 is an upstream molecule in both the ET and JA signal transduction pathways. Cheng et al. reported that salinity and drought stress treatments caused the upregulation of *ERF1*; moreover, overexpression of *ERF1* in *A. thaliana* caused increased tolerance to heat, salinity and drought stresses. Heat stressed *A. thaliana* over-expressing *ERF1* showed up-regulation of most heat tolerance genes (Cheng et al. 2013).

Heat stressed *Triticum aestivum* L., a heat sensitive cultivar, during early kernel development showed a significantly increased ethylene production in developing kernels, embryos, and flag leaves. The accumulation of ethylene caused kernel abortion and increased maturation. Treatment of the plant with an ethylene inhibitor prior to heat treatment inhibited kernel abortion and reduction in its weight (Hays et al. 2007).

Ethylene was also found to play a significant role in the ability of tomato pollen to withstand heat stress. Heat stressed tomato plants that were treated with an ethylene releaser had a 5-fold increase in the number of germinating pollen per flower. Inhibition of ethylene

biosynthesis in heat stressed tomato plants caused a 5-fold decrease in the number of germinating pollen per flower (Firon et al. 2012).

1.7.2.4 Abscisic acid (ABA)

ABA is a 15-C weak acid (Finkelstein 2013) that is formed by the cleavage of carotenoids which are derived from isopentenyl diphosphate. The genes that encode the enzymes involved in the ABA biosynthesis pathway include *ABA1, ABA2, ABA3, NCED,* and *AAO3* (Wasilewska et al. 2008).

A variety of genetic, biochemical, and molecular studies have been able to identify around 200 loci regulating the ABA response and thousands of genes regulated by ABA (Finkelstein 2013). The main ABA signaling components are a family of receptor proteins PYR/PYL/RCAR that act as negative regulators for the PP2C protein family (Ng et al. 2014). Inhibition of PP2C causes the activation of the kinase SnRK2 which targets the downstream components of the ABA network including bZIP transcription factors (ABF/ABRE/ABI5), ion channels, and NADPH oxidases (Fig. 6) (Hauser et al. 2011).

ABA is considered to be a master regulator for plant abiotic stress responses such as salt, drought, heat, and high light intensity (Bari and Jones 2009).The main abiotic factor that causes the accumulation of ABA in *A. thaliana* is reduced water availability for the cells. High ABA levels in plants is known to cause maintained seed dormancy, reduced water loss by transpiration through the stomata, and inhibited germination and lateral root formation (Hauser et al. 2011).

When *A. thaliana* was exposed to a combination of salt and heat stress, 699 transcripts were found to be upregulated as a result of the stress combination. Many of these transcripts were found to be associated with the hormone abscisic acid. Abscisic acid metabolism and signaling *A. thaliana* mutants exposed to the same combination of stress were found to be more stress sensitive than wild type plants (Suzuki et al. 2016). In another study, exogenous application of ABA to heat stressed *Cier arietinum* L. was shown to enhance the plant's thermotolerance indicated by the increased seedling growth compared to the ABA untreated

plants (Kumar et al. 2012b). Also in maize and *Phragmites communis*, exogenous application of ABA was found to enhance thermotolerance (Gong et al. 1998; Ding et al. 2010).

The mechanism by which ABA modulates the heat stress response is not yet fully understood. However, it has been shown that ABA can enhance thermo-tolerance by inducing HSPs or HSFs (Pareek et al. 1998; Rojas et al. 1999). Gong et al. reported that the action of ABA in modulating thermo-tolerance might involve calcium (Gong et al. 1998), while Song et al. reported that it might involve an increase in the levels of nitric oxide (Song et al. 2008).

1.7.2.5 Hormonal crosstalk

The network of crosstalk between the different plant hormones and its role in thermo-tolerance is not yet understood. However, evidence of the strong interaction between the hormones under other types of stress, mainly biotic, indicates that similar interactions might exist for the heat response as well (Verma et al. 2016).

Heat stressed *Pinus radiate* was tested for the levels of the different hormones. Results showed that the immediate heat stress response involved the production of both ABA and SA; this could be explained by the plant's need to regulate stomatal closure and oxidative membrane damage (Escandon et al. 2016).

1.7.2.5.1 Hormonal Crosstalk to regulate non-heat stress responses

SA and JA are known to regulate biotic stress responses in an antagonistic manner(Bari and Jones 2009); In tomato, the JA wound response was found to be inhibited by the exogenous application of SA (Doherty et al. 1988). NPR1 is thought to have a critical role in the anatagonistic relationship between both hormones. *npr1* mutant plants showed an inhibited suppression of JA-response genes such as vegetative storage protein (*VSP*), lipoxygenase 2 (*LOX2*), and *PDF1.2* by SA (Spoel 2003).

JA and ET were found to work synergistically to regulate stress genes after pathogen infection (Zhu et al. 2011). In pathogen infected tomato, positive interaction of JA and ET caused the transcription of genes encoding proteinase inhibitors (Verma et al. 2016). Also expression of *ERF1* and activation of pathogen related genes (*PR*) was found to require both JA and ET (Lorenzo 2002). ET and ABA are also known to crosstalk for controlling abiotic stress. Ethylene was shown to induce the transcription of DREBs, members of the ERF transcription factor family. ET is also know to oppose the effect of ABA in seeds and thus induces the release of dormancy and the start of germination (Arc et al. 2013).

Also an antagonistic interaction between ABA and JA and ET to regulate response to pathogens is suspected. Exogenous application of ABA was found to inhibit the basal and induced transcription of JA-ethylene- activated defense genes. On the other hand, inhibition of ABA resulted in the induction of basal and induced transcription of JA-ethylene defense genes (Anderson et al. 2004).

SA was shown to induce the accumulation of ABA under normal and salinity conditions improving photosynthesis and growth processes, and Osmotic adaptation in *S. lycopersicum.* In freezing stressed *Z. mays,* exogenous ABA treatment caused changes in the cellular SA and the oHCA (Pál et al. 2011).

1.7.2.6 Other molecules involved in basal thermo-tolerance

The wheat *Lipid Transfer Protein 3 (TaLTP3)* was found to be up-regualted in response to salt, water shortage, ABA, and heat treatments. Overexpression of *TaLTP3* in *A. thaliana* was reported to enhance basal thermo-tolerance by decreasing H_2O_2 accumulation and membrane damage in response to HS. Thus, it could be concluded that TaLTP3 is ROS scavenging protein (Wang et al. 2014a).

Plants that are stress tolerant protect themselves from the accumulation of ROS such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals by having strong antioxidant systems. Plants have evolved enzymatic and non enzymatic antioxidant systems. The enzymatic pathway includes a variety of ROS scavenging enzymes such as superoxide dismutase (SOD),

catalase (CAT), ascorbate peroxidase (APX), glutathione-S-transferase (GST), glutathione reductase (GR), and Glutathione peroxidase (GPX)(You and Chan 2015). For instance, APX and GPX are two ROS scavenging proteins that catalyze H_2O_2 in order to prevent its accumulation from causing damage to the plant cells (Ozyigit et al. 2016). The non enzymatic pathway includes carotenoids, flavonoids, GSH, and AsA (Gill and Tuteja 2010). In addition, soluble sugars such as fructans, and disaccharides are involved in regulating the rate of production of ROS (Couee et al. 2006).

Multiprotein bridging factor 1 (MBF1) is a highly conserved transcriptional coactivator that regulates a variety of processes such as hormone regulated lipid metabolism and endothelial cell differentiation (Takemaru et al. 1997; Brendel et al. 2002). *A. thaliana* has 3 genes that encode MBF1: *MBF1a*, *MBF1b*, *MBF1c* (Tsuda et al. 2004). In *A. thaliana*, exposure to heat, drought, salinity, ABA, SA, and pathogens was found to enhance the transcription of *MBF1c* (Rizhsky et al. 2004; Tsuda and Yamazaki 2004). Transgenic *A. thaliana* that constitutively express MBF1c were found to have enhanced tolerance to bacterial infection, heat, salinity, osmotic stress, and a combination of heat and osmotic stress. Results also revealed that the enhanced tolerance to heat and osmotic stress was caused by inducing the ethylene signal transduction pathway (Suzuki et al. 2005).

The mechanism of action of MBF1c in enhancing thermo-tolerance remains largely unknown. However, recent studies have revealed that MBF1c binds to DNA and controls a regulon of 36 different transcripts upon exposure to HS. The regulon was found to include DREB2A, 2HSFs, and a number of zinc finger proteins (Suzuki et al. 2011).

Reactive short-chain leaf volatiles (RSLV) have an α, β-unsaturated carbonyl bond in their structure and include (E)-2-hexanal and (E)-2-butenal . These molecules have been implicated in HS response; They are signaling molecules that cause the transcription of HS related transcription factors such as HSFA2, MBF1c, ZATs and DREB2A. In HSFA1s knockout plants, the expression of *HSFA2* and *MBF1c* induced by RSLV was inhibited but the expression of *DREB2A* and *ZATs* was not. This suggests that the RSLV signaling pathway comprises an HSFA1- dependant and –independent pathways (Yamauchi et al. 2015).

Studies revealed that fatty acids have a role in the responses to biotic and abiotic stresses through the remodeling of membrane lipid composition (Upchurch 2008). Linolenic acid was

found to be involved in abiotic and oxidative stress responses by inducing antioxidant systems such as Met sulfoxide reductase and galactinol synthase enzymes (Mata-Perez et al. 2015). Nitro-fatty acids (NO_2-FAs) are produced by the reaction of reactive nitrogen species with unsaturated fatty acids. Nitro-linolenic acid (NO_2-Ln) is involved in thermo-tolerance by inducing HSPs; it was also found to be involved in the response to oxidative stress caused by H_2O_2 accumulation and ROS by inducing the expression of ascorbate peroxidase (Mata-Perez et al. 2016).

Phospholipases are components of the phospholipid signaling network that is known to regulate plant growth and development. *A. thaliana* has 6 members of NPC phospholipase family, NPC1-6. Recently, studies have shown that NPC1 has a role in basal plant thermotolerance. In *npc1 A. thaliana* knockouts, basal thermotolerance was impaired; this was indicated by the lower survival rate and chlorophyll content seven days after exposure to HS. On the other hand, *A. thaliana* overexpressing NPC1 were found to have enhanced basal thermo-tolerance (Krckova et al. 2015).

Another group of phospholipases, PI-PLC, have also been implicated in the HS response. *A. thaliana plc* mutants had impaired basal and acquired thermo-tolerance. Complementation of the mutants with *PLC9* was able to rescue both types of thermo-tolerance. Moreover, overexpressing *PLC9* was found to further enhance thermo-tolerance (Zheng et al. 2012).

1.7.3 Acquired thermo-tolerance

Groups of genes that are either up-regulated or down-regulated in acquiring thermo-tolerance have been identified from different microarray and cluster analyses. Down-regulated genes include those encoding cytochrome P450 s, auxin-regulated genes, and genes involved in cell detoxification (glutathione S-tranferases) and disease resistance (*PR1, PR5*)(Larkindale and Vierling 2008). Up-regulated genes include those encoding HSPs (Hsp101, Hsp20s, organelle Hsp100/CIpB, and small Hsps), regulatory proteins (protein kinase, protein phosphatase, and transcription factors HsfA3, HsfA7a, NF-X1 DREB2A, DREB2B, DREB2C, DREB2H), stress related proteins (cold-regulated protein COR6.6 and late embryogenesis abundant proteins), and genes involved in photosynthesis, oxidative stress, and programmed cell death (Epple et al. 2003; Song et al. 2012; Lim et al. 2006). Phenotypic analysis of T-DNA insertion mutants in *A. thaliana* identified 8 up-regulated genes involved in acquired thermotolerance. The genes encode

cystolic ascorbate peroxidase, HsfA7a, NF-X1, ProOx, SGT1a, Hsp110, choline kinase, and thaumatin (Larkindale and Vierling 2008).

1.7.3.1 Heat Shock Proteins (HSPs)

HSPs are molecular chaperones required for maintenance and restoration of protein homeostasis. They prevent the aggregation of unfolded proteins produced due to stress and also restore the folding of the proteins. HSPs are divided into 5 classes based on their molecular masses (small Hsps, Hsp60, Hsp70, Hsp90, Hsp100) (Wang et al. 2004). sHSPs are divided into six classes based on their localization in the cytoplasm, nucleus, endoplasmic reticulum, mitochondria, or plastids (Siddique et al. 2008). They are involved in plant response to various stresses including salinity, cold, heat, and drought (Burke and O'Mahony 2001; Dafny-Yelin et al. 2008). For instance, *hsp18.1, hsp17.4,* and *hsp17.6A* were found to be involved in acquiring thermo-tolerance in *A. thaliana* (Dafny-Yelin et al. 2008).

1.7.3.1.1 Hsp70

Hsp70 family is divided into 4 groups localized to the cytosol, endoplasmic reticulum, plastids, and mitochondria (Lee and Schoffl 1996). *A. thaliana* has 18 Hsp70 family members, of which 14 belong to the DnaK subfamily and 4 to the Hsp11/SSE subfamily. Some of the genes were found to be up-regulated in *A. thaliana* under salt stress (Lin et al. 2001). Hsp70 has been reported to repress the DNA binding activities of HsfA1, HsfB1, and HsfA2 by direct interaction (Li et al. 2014a).

1.7.3.1.2 Hsp90

The function of Hsp90 protein family is controversial, but it is known to be the most constitutively abundant Hsp family in *A. thaliana* (Ludwig-Muller et al. 2000). Decreased level of Hsp90 in *A. thaliana* TU8 mutants was found to cause a decrease in acquiring thermo-tolerance(Ludwig-Muller et al. 2000). On the other hand when Hsp90 was inhibited in *A. thaliana* seedlings, it was found to cause the increased expression of *Hsp101* and *Hsp70* and

tolerance of the plant to heat (McLellan et al. 2007). Hsp90 has also been reported to enhance the DNA binding activities HsfA1, HsfB1, and HsfA2 (Li et al. 2014a).

1.7.3.1.3 Hsp100

Hsp100 protein family, CIp – caseinolytic protease proteins, are divided into two classes. They are associated with a great diversity of functions including acquired thermo-tolerance. Hsp60 and Hsp70 were found to be unable to resolubilize aggregates of unfolded proteins once they have formed. However, Hsp100 are able to resolubilize formed aggregates (Schrimer et al. 1996). Constitutive over-expression *Hsp101* in *A.thaliana* was found to enhance thermo-tolerance on transgenic plants. However, when the heat caused over-expression of *Hsp101* was reduced, *A. thaliana* showed decreased thermo-tolerance (Agarwal et al. 2002).

1.7.3.2 Heat Shock Factors (HSFs)

The transcription of *Hsps* is controlled by regulatory proteins called HSFs (Qu et al. 2013). The genome of *A. thaliana* was found to have 21 HSF members that can be categorized into 3 classes (A, B, and C) and 14 groups (A1-A9, B1-B4, and C1)(von Koskull-Doring et al. 2007). There are four genes in the *HsfA1* group, *HSFA1a, HSFA1B,HSFA1d,* and *HSFA1e.* All of those genes except *HSFA1e* have a role in triggering the HS response and acquiring thermo-tolerance (Yoshida et al. 2011). In fact, tolerance to heat, salt, oxidative, and osmotic stress were all compromised in the triple (*HSFA1a, HSFA1B,HSFA1d)* and quadruple knockout mutants of *A. thaliana:* This suggests that the HSFA1 group is involved in the response to a variety of environmental stresses (Liu et al. 2011; Liu and Charng 2012).

A variety of experiments have shown that under heat stress, HSFA1a induce the expression of many thermotolerance involved transcriptional regulators including other HSFs, MBF1C, bZIP2, DREB2A, and DREB2B (Yoshida et al. 2011; Liu et al. 2011).

Other studies show that HsfA1a is a master regulator that triggers the heat response by inducing HsfA1b and HsfA2. HsfA2 induces the expression of HSPs under heat stress. HsfB1 is

a co-regulator that improves the activity of both HsfA1a and HsfA2 (Baniwal et al. 2004). The function of HSFA2 in heat response irrelevant of HSFA1 has been explored in transgenic KO *A. thaliana*. Results showed that constitutive expression of HSFA2 in the absence of HSFA1 could still rescue the plant under heat stress but not under salt and osmotic stress (Fig.7) (Liu and Charng 2013).

1.7.3.3 DREB2A

The dehydration responsive element binding proteins (*DREB*) are a transcription factor gene family that has a critical role in plant stress responses. The ethylene responsive element binding factors regulate the expression of many stress induced genes by binding to a DRE/CRT cis-element that is present in the promoter region of these genes in an ABA independent manner (Lata and Prasad 2011). *DREB2* are known to be important in the heat and dehydration stress pathways (Li et al. 2014b).

The constitutive expression of *DREB2A* in *A. thaliana* resulted in enhanced target gene induction during heat and dehydration stress; however, the accumulation of DREB2A due to proteosome inhibitors did not induce target gene expression. Thus, DREB2A only is not sufficient to induce target gene expression (Morimoto et al. 2013).

1.7.3.4 bZIP

An ER membrane-tethered basic domain/leucine zipper (bZIP) transcription factor, bZIP28, was recently shown to regulate three heat stress genes, *BiP1, BiP2* and *UTR3(Gao et al. 2008). BiP1/BiP2* are ER localized molecular chaperones while *UTR3* is an ER and Golgi localized UDP-galactose transporter. In bZIP28 knockout muatant *A. thaliana,* expression of the three HS genes was not achieved; The mutation also caused decreased thermotolerance (Gao et al. 2008). In a study that investigated 45 *A. thaliana mutants* for basal and acquired tolerance, ABA signaling mutants (*abi1* and *abi2*) showed the most significant defect in acquired thermo-tolerance of root growth and seedling survival. Ethylene signaling mutants (*ein1* and *etr1*) and reactive oxygen metabolism mutants (*vtc1, vtc2, npq1,* and *cad2*) were defective in both basal and acquired thermo-tolerance, but the basal thermo-tolerance defect was more significant (Larkindale et al. 2005).

1.7.3.5 Other molecules involved in acquired thermotolerance

Exposure to heat stress is known to raise the cytoplasmic levels of Ca^{+2}. Calmodulin (CaM) is a Ca^{+2} sensing protein that is known to be involved in heat stress signal transduction pathways (Virdi et al. 2015). Experiments have shown that the cytoplasmic Ca^{+2} levels in wheat and rice increase within 4 and 7 min of heat stress respectively followed by induction of *CaM* genes. Ca^{+2} is proposed to be involved in increasing the DNA binding activity of HSFs through direct interaction. A model to explain how Ca^{+2} and *CaM* interact together to induce HS response proposes that the levels of NO increase due to HS causing the up-regulation of *AtCaM3*. The kinase AtCBK3 then binds to AtCaM3 in the presence of Ca^{+2} which causes the phosphorylation of HSFs (Liu et al. 2008). HSFs interact with heat shock elements (HSEs) causing the transcription of HSPs leading to the acquisition of thermo-tolerance (Queitsch et al. 2000). In sorghum, HSP90 was found to bind to CaM in a Ca^{+2}dependant manner (Virdi et al. 2009). In the presence of Ca^{+2} channel blockers and CaM antagonists, the heat stress induced increase in HSP90 was not observed which indicates that the level of HSP90 is regulated by the Ca^{+2}/CaM pathway (Virdi et al. 2011).

Salicylic acid was also found to have a role in acquired thermo-tolerance. SA induction deficient plants (sid2) were exposed to HS and their ability to acquire thermo-tolerance was investigated. Results showed that sid2 plants had a lower survival rate compared to wildtype plants. More interestingly, pretreatment of the mutant plants with H_2O_2successfully saved the plant from HS. SA was found to induce the expression of many HSFs, but HsfA2 was the most expressed. exogenous application of AsA, an H_2O_2scavenger, was reported to decrease the expression of HsfA2 induced by SA. These results show that SA regulates the expression of HsfA2 in response to HS and requires H_2O_2 to do so (Nie et al. 2015).

Systemic acquired acclimation (SAA) is defined as "the activation of acclimation mechanisms in systemic nonchallenged tissue". It was reported that SAA requires at least two steps: the spread of ROS from the initial site that was exposed to stress to the rest of the plant and a stress specific signal that causes the acquisition of stress tolerance. In *A. thaliana,* SAA to

HS was found to be regulated by a temporal spatial interaction between ABA and ROS. The ROS wave was involved in the propagation of electric signals (Suzuki et al. 2013).

1.8 RNA sequencing

The development of high-throughput next generation DNA sequencing (NGS) is considered a revolution in the field of transcriptomics because it allowed for RNA analysis through cDNA sequencing at massive scale. The main RNA-sequencing commercially available platforms are Illumina, Roche 454, Helico BioSciences, and Life Technologies (Ozsolak and Milos 2011). The technique involved converting the extracted RNA into a cDNA fragments library. Adaptors are attached to one or both ends of the fragments. Each fragment, with or without amplification, is sequenced in a high throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). The length of the reads ranges from 30-400 bp depending on the sequencing platform used. The sequenced reads are either aligned to a reference genome or transcripts, or assembled de novo (without the use of a reference genome) (Wang et al. 2009).

1.9 RNA sequencing Vs Microarray

From all the studies formerly discussed it can be seen that no comparative studies between *T.salsuginea* and *A.thaliana* have been performed using RNAseq yet. The only RNAseq studies that have been performed in this field have concerned only *A.thaliana,* and even those are very few.

For instance one research group carried out a genome wide RNA seq approach in order to study the transcription profile of *A.thaliana* in response to dehydration stress encountered for the first time and subsequent times. The results showed that there are 4 distinct types of dehydration stress memory genes that contribute to the enhanced dehydration stress response (Ding et al. 2013).

In a similar experiment to study the effect of mild stress on *A.thaliana* using genome wide RNAseq, 354 genes were found to be differentially expressed in 6 accessions under mild stress. Some of those genes were found to be involved in proline metabolism, abscisic acid signaling, and cell wall adjustments. In addition 87 genes were found to be specific for the leaf response to mild drought stress (Clauw et al. 2015).

The fact that researchers are still not adopting RNA seq analysis instead of microarray is hardly due to microarrays being more efficient. Instead this might be due to the RNA seq analysis being new to researchers and also because it is more expensive than microarrays and is more challenging with respect to data storage and analysis. However the shift to RNA seq analysis is expected due to the many advantages that it has over microarrays. RNA seq analysis does not require species or transcript specific probes like microarrays which mean that it offers unbiased detection of novel transcripts (Ozsolak and Milos 2011). It also is not limited by background and signal saturation like microarrays since it quantifies discrete, digital sequencing read counts and thus it offers a broader dynamic range. In addition RNA seq technology offers higher specificity and sensitivity than microarrays and an easier detection of rare low abundance transcripts(Wilhelm and Landry 2009; Guida et al. 2011; Wilhelm et al. 2010; Malone and Oliver 2011; t Hoen et al. 2008).

Many studies have been carried out to compare microarray and RNA seq technologies and all support the same conclusions previously mentioned: RNA seq offers many advantages not present with microarray. One study used both technologies on RNA samples from a human T cell activation experiment. There was a high correlation between the gene expression profiles generated by both technologies. However, the datasets showed that RNAs seq was more efficient in identifying genetic variants and detecting differentiating biological critical isoforms and low abundance transcripts. RNA seq was also able to detect more differentially expressed genes with greater fold change. Also RNA seq technology allowed the researchers to avoid the technical issues inherent to microarrays such as non specific hybridization, cross hybridization, limited detection range, and issues related to probe redundancy and annotation (Zhao et al. 2014).

In a similar study to compare gene expression profiles of colon cancer cells treated with 5-azadeoxy-cytidine (5-Aza) on both platforms. Results showed that the Spearman and Pearson correlation coefficients of both datasets were above 0.80 with 66-68% overlap. More interestingly, 33 IPA canonical pathways were identified by both technologies out of which 152 pathways were identified by RNA seq only and none were identified by microarray only (Xu et al. 2013).

Based on most of the studies carried out to compare the performance of microarray and RNA seq technologies it can be concluded that even though the RNA seq technology offers many advantages previously discussed, the microarray technology remains an accurate way of

measuring gene expression. Thus both techniques should be considered complementary to each other and not competitive (Nookaew et al. 2012).

1.10 RNA sequencing challenges

There are a few manipulation steps that need to be performed during the preparation of the cDNA library. This complicates the need to profile all types of transcripts. Small RNA sequences such as siRNAs, miRNAs, and piRNAs are easily sequenced after adaptor ligation. Large RNA molecules have to be divided into smaller fragments that range in size from 200-500 bp. Different fragmentation methods used include RNA fragmentation or cDNA fragmentation, which are known to produce specific biases in the produced fragments (Mortazavi et al. 2008; Nagalakshmi et al. 2008).

If the cDNA library is amplified, a number of identical short reads is produced which could be due to the abundance of those reads or PCR artifacts. Using biological replicates would help eliminate this problem (Wang et al. 2009). Another challenge with preparing the cDNA libraries is deciding whether to produce strand specific libraries. Those are very informative for transcript annotations (Wilhem et al. 2008; David et al. 2006; Dutrow et al. 2008), but are extremely tedious to produce (Cloonan et al. 2008). Also the experimenter needs to ensure that the antisense transcripts are not an artifact of reverse transcription (Wu et al. 2008).

The cost of RNA-seq is another research limiting step. The higher the sequence coverage, the more costly the sequencing is. Higher coverage requires more sequencing depth. In simple transcriptomes such as *Sacheromyces pombe* or *Sacheromyces cerevisiae,* sequencing depth is not a problem since there is no evidence for alternative splicing. However, organisms with larger genomes have more complex transcriptomes and thus require more sequencing depth. Once the RNA-seq reads are acquired, the challenge becomes data storage, retrieval, and analysis(Wang et al. 2009).

1.11 Study Objectives and Design

As previously discussed, the effect of global warming on the world crop yield cannot be overlooked. Understanding the mechanisms of heat stress response in thermo-tolerant plants is

critical to identify the target stress related genes that can be used to produce stress tolerant transgenic crops.

The main aim of this project is to assess the transcriptomic changes in the root and shoot of the extremophile *E. salsugineum* in response to heat stress and exogenous ABA application. This will be done through the following steps: 1) reporting the results of the plant transcriptome assembly. 2) inferring the statistically and biologically differentially expressed genes due to the different treatments and the different plant organs. 3) inferring the most enriched functional categories to which the DE transcripts belong to.

Figure 8 shows the workflow of the study. The RNA seq read were de novo assembled using Trinity assembler. The assembled reads were quantified and normalized using Kallisto. The transcripts were annotated using EggNOG. The R bioconductor EdgeR package was used for the differential expression analysis. The DE transcripts were aligned to Arabidopsis thaliana using blastx to identify the protein coding transcripts. Functional analysis was performed for the DE transcripts using DAVID.

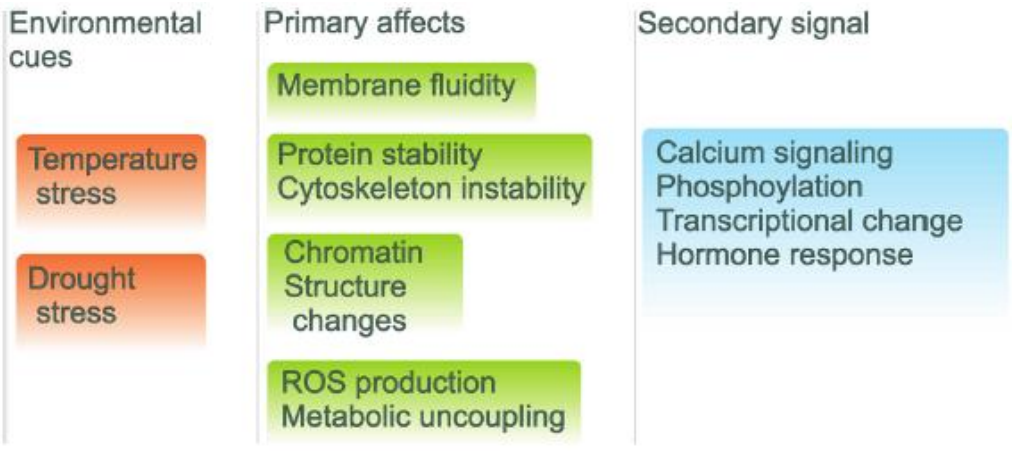

Figure 1: An overview of plant response to temperature and drought stress: The immediate effect includes disruption of the cell membrane fluidity, protein stability, and chromatin structure in addition to an increase in the production of reactive oxygen species. The

secondary signal includes an increase in the cytosolic calcium levels, phosphorylation, transcriptional change and the production of a variety of hormones including ABA, SA, ET, and JA (Bita and Gerats 2013).

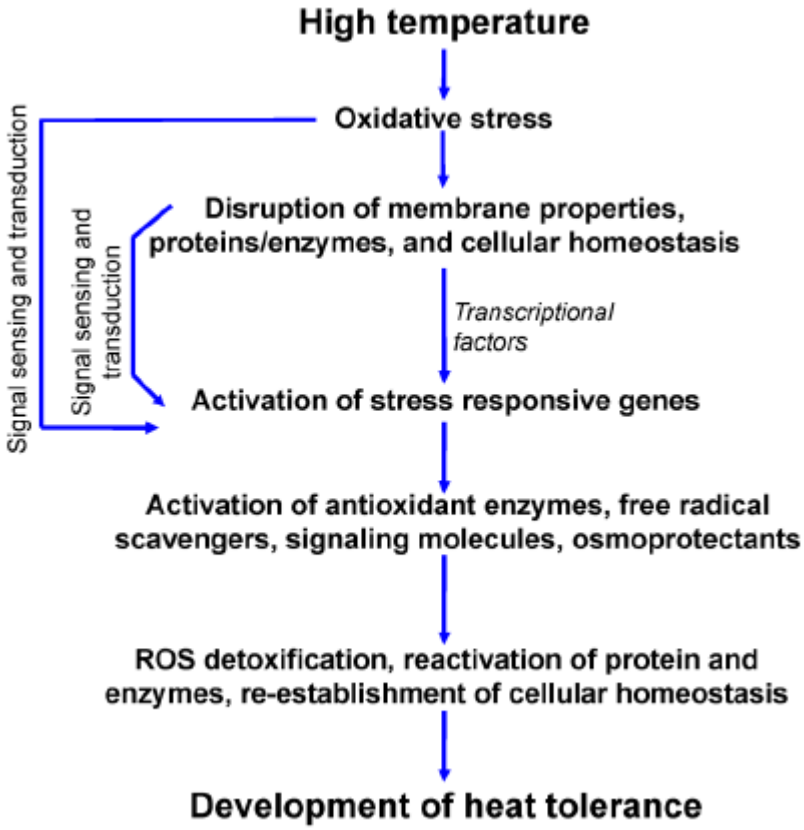

Figure 2: The plant molecular response to heat stress. High temperature causes the accumulation of ROS which leads to oxidative damage and disruption of cellular homeostasis. This ROS accumulation and the changes it causes act as a signal for activating the great variety of stress response genes which code for protein components of the plant antioxidant system, signaling molecules, and secondary metabolites. Recovery of cellular homeostasis is achieved and the plant develops heat tolerance (Hasanuzzaman et al. 2013).

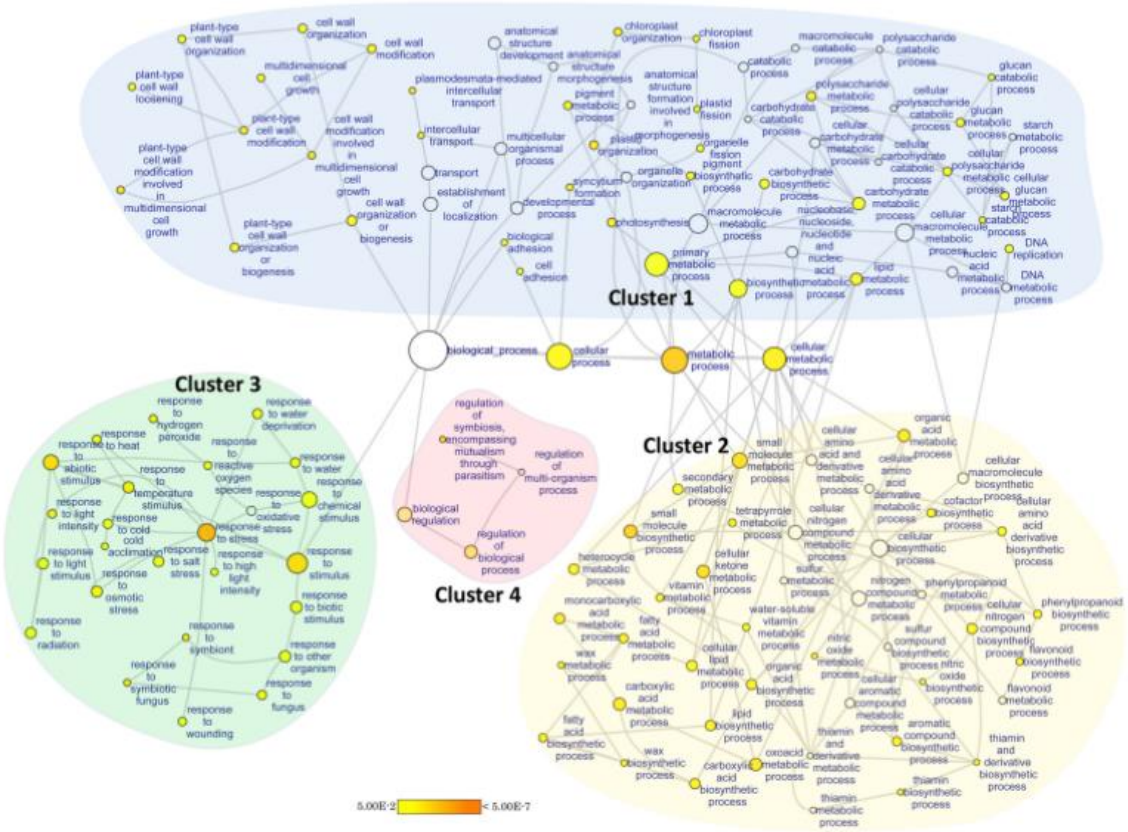

Figure 3: Gene Set Enrichment Analysis (GSEA) for the differentially expressed genes in plants subjected to single stresses (heat, high light, salt, cold, flagellin) and combined stresses (heat+flagellin+silwet, salt+heat, cold+high light, salt+high light, cold+flagellin+silwet, cold+silwet, heat+high light, cold+flagellin+silwet). Four clusters can be seen: Cluster 1 represents primary processes such as primary metabolism, photosynthesis, and reproduction. Cluster 2 represents secondary processes. Cluster 3 represents the different stress responses processes. Cluster 4 represents the processes that regulate the different stress responses. Nodes were colored according to their corrected P-values. The node size represents the total number of genes in each category (Barah et al. 2016).

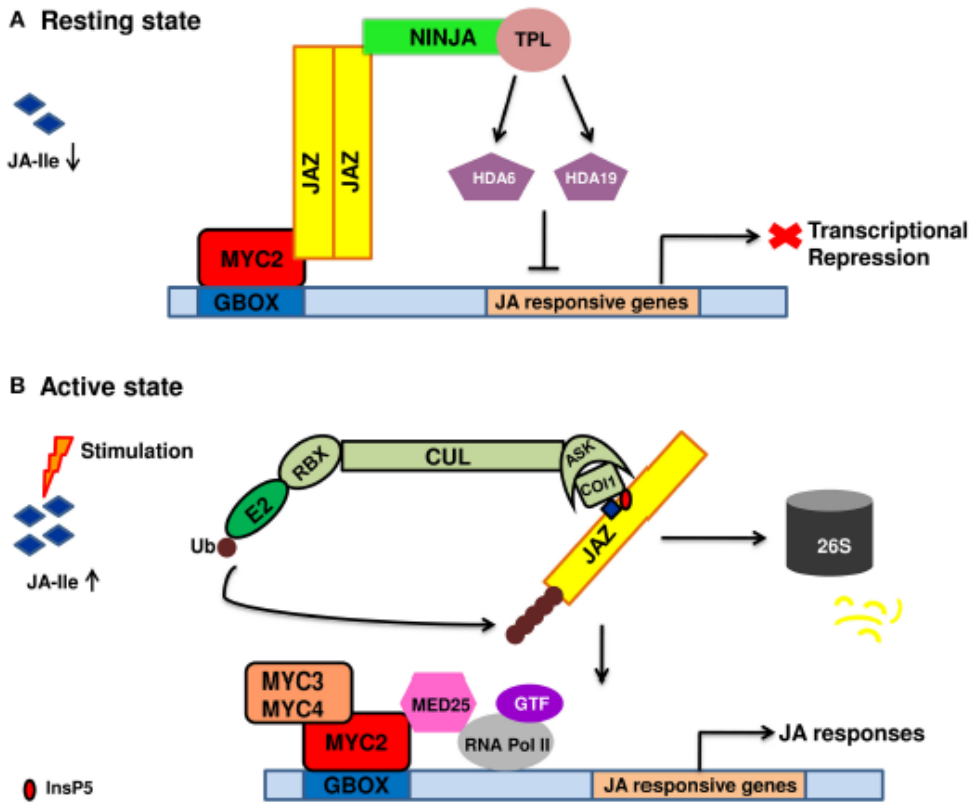

Figure 4: The JA signaling pathway. In the absence of a stimulus, jasmonic acid is not produced. This caused the jasmonate zim domain (JAZ) repressors to bind to the transcription activator MYC2 which leads to the recruitment of TPL and adaptor protein NINJA. The JAZ-NINJA-TPL complex causes the recruitment of HDA6 and HDA19 inhibiting the JA mediated gene expression. In the presence of a stimulus, JA is produced and epimerized to JA-Ile which binds to Col1-JAZ-InsP5 co-receptor complex causing JAZ to be ubiquitinated and degraded by the proteosome. MYC2 and its homologs are then released causing them to bind to the G-box element that is found downstream of the JA responsive genes leading to the JA responses (Sharma and Laxmi 2015).

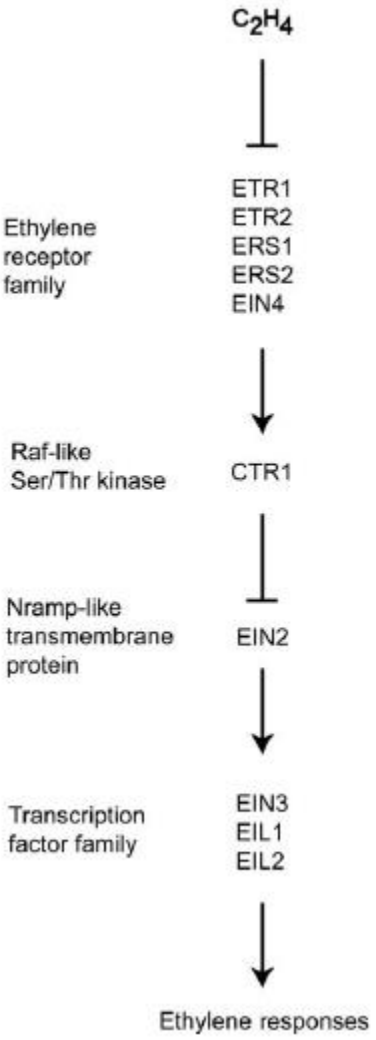

Figure 5: The ET signaling pathway. There are five identified ethylene receptors located in the ER membrane ETR1, ETR2, ERS1, ERS2, EIN4. In the absence of ethylene, the ER receptors activate CTR1, a Ser/Thr kinase, which phosphorylates EIN2, an ER-bound protein, inactivating it. This prevents the transcription of the transcription factors EIN3, EIL1, and EIL2 inhibiting the ethylene responses. In the presence of ethylene binding, The ER receptors inactivate CTR1 so that it cannot phosphorylate EIN2. Thus, EIN2 will be activated causing the transcription of the transcription factors and leading to the ethylene response (Shakeel et al. 2013).

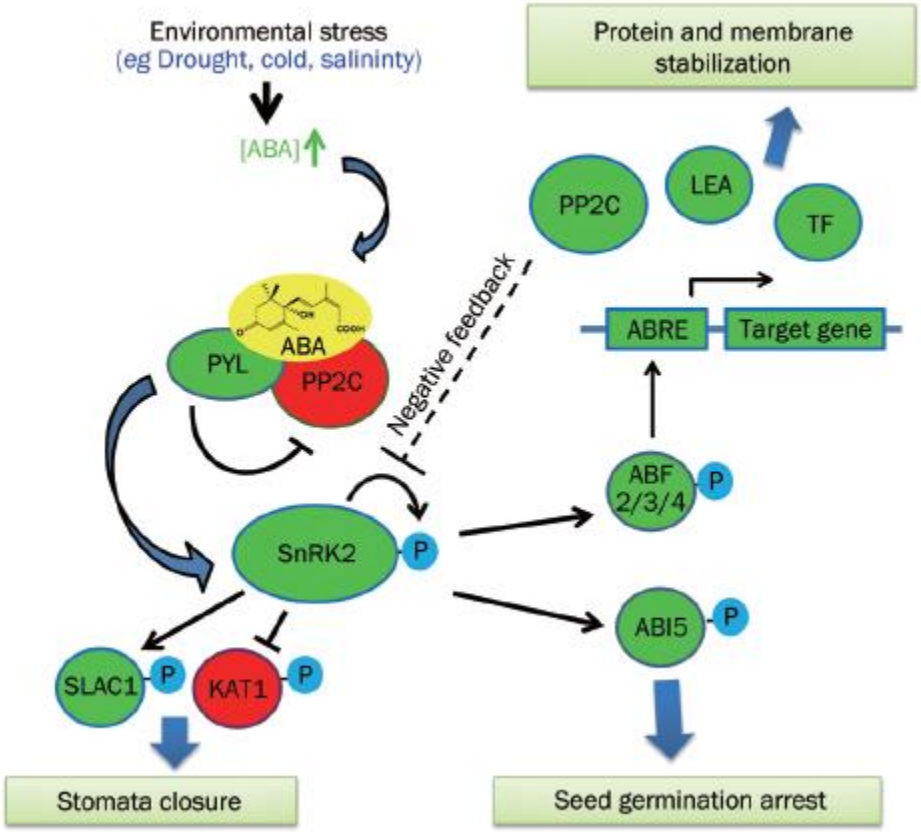

Figure 6: ABA signaling pathway. The main ABA signaling components are a family of receptor proteins PYR/PYL/RCAR that act as negative regulators for the PP2C protein family. Inhibition of PP2C causes the activation of the kinase SnRK2 which targets the downstream components of the ABA network including bZIP transcription factors (ABF/ABRE/ABI5), ion channels, and NADPH oxidases (Ng et al. 2014)

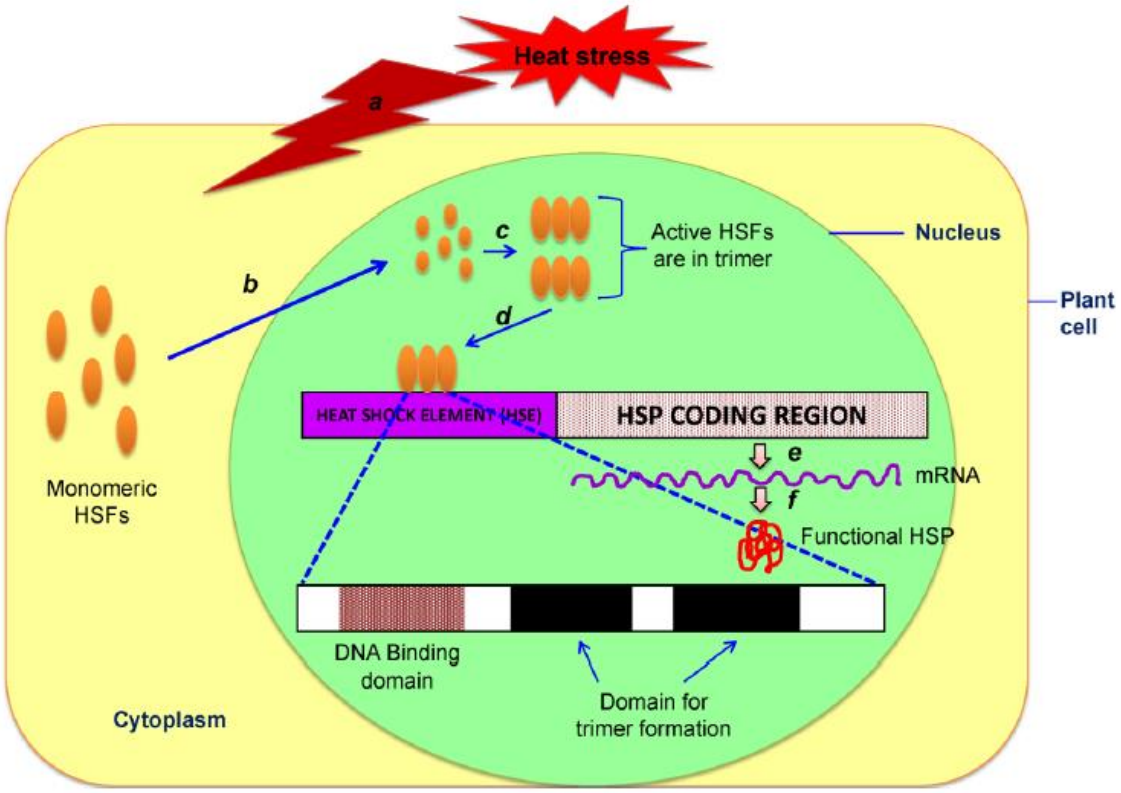

Figure 7: The molecular regulatory mechanism of heat shock proteins. In the presence of heat stress, the monomeric HSFs enter from the cytoplasm to the nucleus where they form trimers. The trimers will bind to the promoter (heat shock element) of the target gene. The HSE is composed of two domains for trimerization and one domain for DNA binding. Transcription produces the corresponding HSP that is responsible for thermotolerance (Hasanuzzaman et al. 2013).

Chapter 2: Methods

2.1 Experimental Setup

The experiments were carried out at King Abdulla University of Science and Technology (KAUST) in Dr. Magdy Mahfouz's lab. *E. salsugineum* seeds were sterilized in 10% (v/v) bleach for 10 min. They were then rinsed in sterile de-ionized water four times and stratified mixed with water at 4°C in the dark for 2 days. Seeds were grown on vertical square plates (1/2MS,1% Suc, pH 5.8, and 1.2% agar) in 22°C for 5 days. Seedlings were separately transferred into MS plates treated with 10uM ABA in 22℃, 16h light/8h dark for 7 days. The seedlings to be heat stressed were transferred into MS plates and grown in (37°C) for 5 days. Shoots and roots were collected separately from plates (0.1g) in eppendorf tube containing beads, immediately frozen in liquid nitrogen, and stored at −80°C.

Six *E. salsugineum* plants were used in this experiment. Two plants were treated with ABA (replicates), while two plants were treated with heat (replicates); the remaining two plants were used as control. Samples were taken from the root and the shoot. The resulting samples were root ABA1, root ABA2, root heat, root control1, root control2, shoot ABA1, shoot ABA2, shoot heat1, shoot heat2, shoot control1, and shoot control2. The root heat sample had no biological replicate. Total RNA was extracted from the samples and the cDNA library was constructed. Total RNA was isolated from shoot and root tissue using Trizol reagent. To eliminate any residual genomic DNA, total RNA was treated with ribonuclease-free DNase (Qiagen). . Single-end total RNA sequencing was performed.

2.2 Computational Analysis

Trinity *de novo* transcriptome assembler (v2.1.1), ran on the AUC server with the default parameters, was used for RNA-Seq reads assembly (Haas et al. 2013). Quantification and normalization of transcripts abundance was carried out using Kallisto (v0.42.4). Annotating the transcripts was performed using eggNOG (v4.5). To identify the protein coding transcripts, the transcripts were aligned to the nr database using tblastx; The TAIR10 IDs for the *A. thaliana* hits were extracted.

Differential expression analysis was performed using the R/Bioconductor package EdgeR (3.12.0). The R version used is the Wooden Christmas-Tree (3.2.3). Trimmed mean of *M*-values (TMM)-normalized transcript abundances generated by Kallisto were used for the differential expression analysis. The common BCV was manually set to 0.01 since one of the samples (root heat) has no biological replicate. Eight pair-wise comparisons were carried out (1. root ABA vs. root control, 2. root heat vs. root control, 3. shoot ABA vs. shoot control, 4. shoot heat vs. shoot control, 5. root ABA vs. shoot ABA, 6. root heat vs. shoot heat, 7. root ABA vs. root heat, 8. shoot ABA vs. shoot heat) using the e*xactTest()* function. The statistically significant differentially expressed (DE) transcripts were extracted at an FDR<0.01 and a logFC cutoff of 4.

The tblastx extracted TAIR10 IDs for the DEGs were uploaded on the Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.7). DAVID's functional annotation tool was used in order to investigate the most enriched annotation categories. The nine DAVID selected default annotation categories were used. The significantly enriched biological processes were visualized using bar-plots generated by R ggplot2 package (2.1.0).

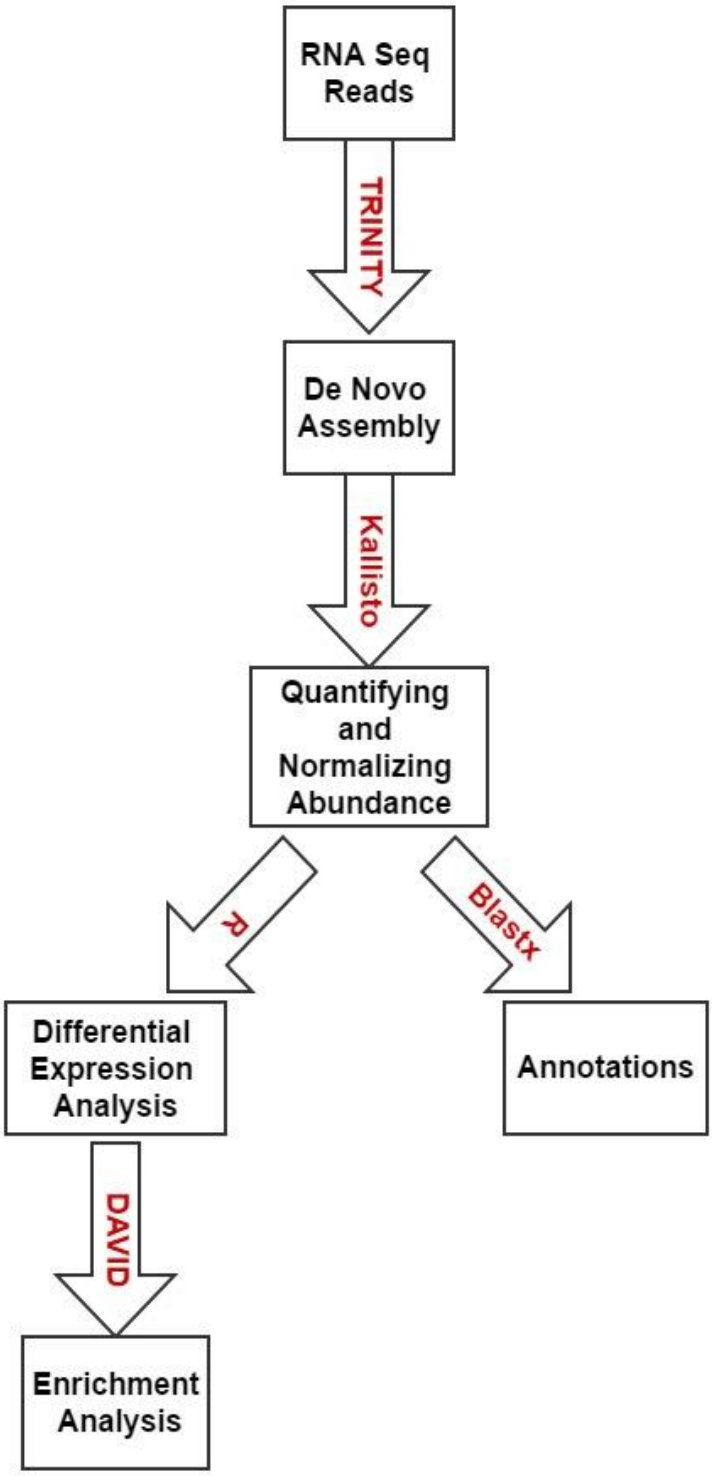

Figure 8: Overview of the work flow.

Chapter 3: Results and Discussion

Trinity de novo assembly produced 49857 genes and 134493 transcripts. The number of differentially expressed transcripts in each comparison at FDR<0.01 and a logFC cut-off of 4 can be seen in Table 1.

Out of the 134493 transcripts, 114692 (85.28%) transcripts had tblastx hits (protein coding). Thus, 19801 potentially non coding or novel transcripts have been identified. 110175 transcripts (81.92%) successfully aligned to *A. thaliana*.

3.1 The 50 most differentially expressed transcripts

Heat maps for the 8 comparisons were generated for the 50 most significantly differentially expressed transcripts. The annotations were used for the row clusters. Only the 20 most DE transcripts in each comparison will be discussed in details in the following section.

3.1.1 Root ABA vs Root Control

3.1.1.1 Up-regulated Transcripts

Late embryogenesis abundant group 1 domain-containing protein (FDR=3.14E-29). LEA proteins are a family of 7 hydrophilic protein groups that have been found to be induced by a variety of abiotic stresses that all lead to water deficit such as drought, salinity, heat, cold, and freezing temperatures. The proteins are known to be associated with water deficit in genereral, whether caused by external environmental stressors or by plant development under optimal growth (Battaglia and Covarrubias 2013). LEA2 proteins, dehydrins (FDR=3.25E-15), were found to be associated to anionic phopholipid vesicles and so are suggested to have a role in membrane stabilization (Koag et al. 2003).

Plant non specific lipid transfer proteins (FDR=3.47E-12) have the main function of transferring phospholipids between membranes. Plant LTPs were found to be involved to biotic and abiotic responses. The proteins were found to be either up-regulated or down-regulated in response to pathogen infection (Julke and Ludwig-Muller 2016). Overexpression of ns-LTP was shown to induce pathogen resistance in plants (Patkar and Chattoo 2006). Also LTPs are involved in the plant abiotic stress response to salt, drought, and cold stress (Julke and Ludwig-Muller 2016). LTPs are speculated to have antimicrobial activity (Cammue et al. 1995). They

were also shown to cause permeabilization of the pathogen's cell membrane (Regente et al. 2005). Finally some LTPs were found to bind to the calcium sensor calmodulin, or be phosphorylated which indicates that they might act as signaling molecules (Martin et al. 2007).

Phosphotases (FDR=5.62E-10) are a large protein family that catalyze the dephosphorylation reactions that may modulate the functions of proteins in signaling transduction pathways (Wang et al. 2013). The plant's ability to adapt to environmental stressors involves the removal of a variety of molecules from organelles, specifically membranes, and replacing them with new ones. This process is carried out by an internal vesicle trafficking system that is controlled by phosphatidylinositol (PtdIns) kinases and phosphatases (Kaye et al. 2011). The enzymes phosphorylate or dephosphorylate the hydroxyl group of the inositol ring in PtdIns (van Leeuwen et al. 2004). PP2C (a type 2C protein phophatase) is a core component of the ABA signal transuction pathway. It regulates the protein kinase (SnRK2) (Fan et al. 2016). Abiotic environmental stressors such as salt and osmotic stress were found to induce the production of PtIns (4,5)P2 and InsP3 in *A. thaliana.* PtdIns 5-phosphatases (5PTases) were found to be involved in the regulation of plant stress responses. Inositol polyphosphate 5-photphatase 7 was found to regulate the production of ROS and the response to salt stress in *A. thaliana* (Kaye et al. 2011).

3.1.1.2 Down-regulated Transcripts

Chitinases (FDR=1.54E-20) are proteins involved in the plant response to biotic stress. Their function is to break down chitin in the cell wall of fungi and inhibit the fungal growth. There are seven classes of chitinase proteins that are involved in a variety of functions including pathogen defense, temperature change, high salinity, metal and wounding stress, and hormone application such as SA, ET, and met-JA(Liu et al. 2010; Su et al. 2015).

Phenylalanine is an aromatic amino acid that is required for synthesis of proteins and serves as the precursor for phenylpropanoid plant secondary metabolites. Phe is synthesized from arogenate in plants. Aspartate aminotransferase (FDR=1.10E-11) were found to catalyze the first step of the aregonate pathway (Dornfeld et al. 2014).

Proline rich protein (FDR=3.25E-15) was also highly differentially expressed. Proline has been extensively shown to have a protective role against a variety of biotic and abiotic stresses in plants. It acts as a molecular chaperone that maintains protein homeostasis during stress. Several studies have also shown that it acts as an ROS scavenging osmolyte. Moreover, proline was found to have a role in stress signaling and protein translation (Szabados and Savoure 2010).

The most differentially expressed transcript in this comparison belongs to the potato inhibitor I family (FDR=3.14E-29). potato protease inhibitors are known to play major roles in the plant defense against pathogens and herbivores (Hartl et al. 2011). One type of potato protease inhibitors is the potato inhibitor I family which is found in the leaves, flowers, stems, and tuber sprouts (Fischer et al. 2015). PIN I and II proteins were found to inhibit digestives enzymes in the guts of microbes which interferes with their absorption of some essential amino acids that are needed for their growth and development (Chen 2008).

Inositol (FDR=2.91E-05) was down-regulated in this comparison. It was found that myo-inositl acts the main substrate for the production of PtdIns and phosphatidylinositides which are both essential for the structure and trafficking function of the endomembrane system and thus for auxin regulated embryogenesis (Luo et al. 2011).

3.1.2 Shoot ABA vs. Shoot Control

3.1.2.1 Up-regulated Transcripts

The plant non specific lipid transfer proteins were also up-regulated in the ABA treated shoot (FDR= 2.25E-19). The glucose 6 phosphate phosphate translocator (FDR= 3.38E-13), GPT2, controls the transport of glucose 6-phosphate across plastid membranes in exchange for inorganic phosphate (Niewiadomski et al. 2005). It was also found to modulate seedling development and to have a main role in the developing seedlings response to exogenous sugar.

GPT2 expression has been associated with senescence and impaired carbon metabolism (Dyson et al. 2014).

Heavy metal associated domain (FDR= 1.02E-10) was up-regulated in this comparison. It was shown that early signs of abiotic metal stress in barley were similar to signs of water deficiency stress. Thus, over-expression of dehydration related genes was cause in barley after the exposure to the metals Cd and Hg (Tamas et al. 2010). Moreover, seed germination in wheat was found to be inhibited due to exposure to the heavy metal As, which indicates the involvement of ABA in the heavy metal response of plants (Zhang et al. 2002).

3.1.2.2 Down-regulated Transcripts

The proline rich protein was also down-regulated in the shoot (FDR= 1.11E-11). Pathogenesis related protein 5 (FDR= 2.95E-19) was down-regulated in the ABA treated shoot. Plant reactions to abiotic and biotic stressors involve the physical strengthening of the cell wall through lignifications, callose deposition, and suberization. This is done through the production of PR proteins, phenolic compounds, and phytoalexins. PR proteins are a group of toxic proteins such as chitinases, glucanases, and lysosyme active proteins. There are 17 families of PR proteins including non specific lipid transfer proteins, ribosome inactivating proteins, peroxidases, chitinases, oxalate oxidases, and glucanases (Ebrahim et al.). PR proteins are known to be associated with systemic acquired resistance (SAR)(Van Loon and Van Strein 1999).

The amino acid asparagine is one of the main secondary metabolites produced in senescing leaves in plants since it has a high ratio of N:C and thus serves as a nitrogen transport molecule in the process of senescence. As is known as a key senescence associated gene and is speculated to have a role in the plant response to pathogens (Seifi et al. 2014). Asparagine synthetase (FDR= 1.43E-12) uses glutamine or ammonia as a nitrogen source to convert aspartate into asparagines in an ATP - dependant manner (Loureiro et al. 2013). During the process of senescence in barley, two asparagines synthetase categories are involved. One gene category is induced, while the other is repressed (Avila-Ospina et al. 2015).

The kinase family proteins (FDR= 1.10E-11) are involved in the ABA signal transduction pathway. As previously discussed, ABA interacts with the ABA receptors leading to the activation of the kinases SnRK2s which cause the phophorylation of many downstream substrates involved in the induction of the ABA response (Minkoff et al. 2015). Glucoronokinase (FDR= 2.69E-08) was recently characterized as the last missing enzyme of themyo-inositol oxygenase pathway to nucleotide sugars. The plant cell walls contain carbohydrate sugars that are made from nucleotide sugars. UDP-glucuronic acid is the main precursor of cell walls. One of the pathways that produce UDP-glucuronic acid involves the production of glucuronic acid from myo-inositol using the enzymes glucurokinase and phophorylase (Pieslinger et al. 2010).

Calcium binding protein (FDR= 2.75E-10) was down-regulated in ABA treated shoot. In the presence of ABA, the guard cells are hypersensitive to the changes in their internal concentration of calcium ions which cause the calcium ions to activate the protein SLAC1. This reaction eventually leads to the closure of the stomata. This is not the only pathway by which ABA can mediate stomatal closure (Brandt et al. 2015). The *A. thaliana* genome is known to encode for 9 salt sensitive 3 (SOS3)-like calcium binding proteins and 24 SOS2-like protein kinases. Two regulatory mechanisms for these two protein groups exist. The first is that the calcium binding proteins activate the kinases. The second is that the kinases phosphorylate the calcium binding proteins (Du et al. 2011).

2OG-Fe(II) oxygenase superfamily (FDR= 7.65E-09) are a class of enzymes that are widespread in bacteria and eukaryotes and that use a dioxygen molecule to catalyze the oxidation of an organic substrate. In plants, these enzymes are involved in the formation of ET and other plant hormones and secondary metabolites such as gibberellins, flavones, and anthocyanidins (Araving and Koonin 2001).

Cupins (FDR= 5.92E-08) are a family of proteins that are involved in a variety of biological processes in plants including acting as enzymes such as hydrolases, dioxygenases, dicarboxylases, isomerases, and epimerases. The protein family also has non enzymatic functions such as acting as transcription factors, binding to auxins, and seed storage (Stipanuk et al. 2011).

3.1.3 Root Heat vs. Root Control

3.1.3.1 Up-regulated Transcripts

The zinc finger domain (FDR=0) and leucine rich repeat (FDR=0) were both up-regulated in the root in response to heat. The basic region/leucine zipper (bZIP) family is one of the largest groups of transcription factors in plants. Members of this family are known to regulate a variety of developmental processes and responses to environmental stress (Llorca et al. 2015). bZIP28, was recently shown to regulate three heat stress genes, *BiP1, BiP2* and *UTR3*(Gao et al. 2008). A gene encoding a zinc finger protein in the grass species *Festuca arundinacea* (*FaZnF)* was found to be upregulated in response to salt, drought, heat, and wounding stresses.

As expected in this comparison, most of the significantly differentially expressed transcripts code for heat shock proteins and heat shock factors as seen in the heat map for RHXRC. The FDR values are 0 for the first 20 most DE heat shock transcripts. The major role of HSPs and HSFs in acquired plant thermo-tolerance was previously discussed in chapter 1. Exposure of the plant to heat stress causes the activation of heat shock factors which bind to heat shock elements on heat shock genes causing the transcriptions of heat shock proteins. *A. thaliana'*s genome is known to code for 21 HSFs, 13 Hsp20s, 18 Hsp70s, 7 HSP90s, and 8 HSP100s (Swindell et al. 2007). The CS domain (FDR= 0) has a compact anti-parallel beta-sandwich fold consisting of seven beta strands (Garcia-Ranea et al. 2002). The domain is known to be present in HSP20 and HSP90 (Singh et al. 2009).

Fructose biphosphate aldolase (FDR=0) was found to be highly phophorylated under heat stress in the root of the 2 plants *Agrostis scabra* and *Agrostis stolonifera*. Moreover, the phophorylation was higher in the thermo-tolerant *A. scabra* than in the heat sensitive *A. stolonifera* (Xu and Huang 2008). Ascorbate peroxidase (FDR= 1.12E-45) are a group of haem proteins that act as H2O2 scavengers in the chloroplasts and cytosol of plant cells. Seven types of ascorbate peroxidase exist in the genome of *A. thaliana* (Jespresen et al. 1997).

Cathepsin protein (FDR=0), a cysteine protease, was upregulated in the heated root. The cathepsin B protein in *A.thaliana* was found to be required for basal pathogen resistance. *Atcathb* triple mutants were also found to have delayed senescence with the accumulation of senescence marker protein SAG12. This indicates that cathepsin B might also have a role in the development of senescence in plants (McLellan et al. 2009).

3.1.3.2 Down-regulated Transcripts

Glutathione S transferase (FDR=0) is speculated to be a potential downstream target for the zinc finger protein (Martin et al. 2012). Glutathione S transferases are a group of enzymes that catalyse the addition of tripeptide glutathione to a number of substrates. Plant GSTs have been found to function in herbicide tolerance and a variety of stress responses such as the response to pathogens, heavy metal toxicity, and oxidative stress (Marrs 1996).

Pathogenesis-related protein Bet v I family (FDR=0) was down-regulated in heat treated root. Bet v 1 is the major birch pollen allergen and is a member of a large pathogenesis related protein family (Hoffmann-Sommergruber et al. 1997).

Serine threonine protein kinase (FDR=0) is a sucrose non fermenting 1-related protein kinase 2 (SnRK2). SnRK2 is a major component of the ABA abiotic stress response as previously explained in chapter 1. Overexpression of wheat *SnRK2.4* in *A. thaliana* was found to enhance the plant's tolerance to a variety of environmental stresses including drought, salt, and freezing (Mao et al. 2010).

A scorpion toxin like domain (FDR=0) was found to be down-regulated in the heat treated root. The auxin repressed 12.5 kDa protein (FDR=0) is expected to be differentially expressed since heat stress causes the accumulation of many plant hormones such as ABA, ET, and JA as previously discussed in chapter 1.

Another down-regulated protein in the heated root is able to Methylate 5-hydroxyferuloyl-CoA to sinapoyl-CoA and caffeoyl-CoA to feruloyl-CoA. It plays a role in the

synthesis of feruloylated polysaccharides and is involved in the reinforcement of the plant cell wall. It is increases the formation of cell wall- bound ferulic acid polymers in response to wounding and pathogen attack (EggNOG). One study showed that the drought stressed root of water melon had an up-regulated caffeoyl-CoA 3-O-methyl-transferase (*CCOAOMT*) gene. The protein is able to convert caffeoyl-CoA to feruloyl-CoA and thus has a major role in the synthesis of monolignols necessary for the strengthening of the cell wall (Yoshimura et al. 2008).

Peroxidases (FDR=0) are a family of haem containing enzymes that are responsible for oxidizing ROS and cell wall lignification and defense against pathogens (Novo-Uzal et al. 2014). In the water deficient root seedling of rice, peroxidases were accumulated and their activity was correlated with the strengthening of the cell wall (Le Gall et al. 2015).

3.1.4 Shoot Heat vs. Shoot Control

3.1.4.1 Up-regulated Transcripts

Like the RHXRC comparison the different heat shock proteins, CS domain, ascorbate peroxidase were up-regulated in the heat treated shoot. Galactinol synthase (FDR=2.77E-46) is a member of the glycosyltransferase 8 family. Members of this family catalyze the first step in the production of RFO (raffinose family oligosaccharides). A variety of abiotic stressors were found to cause the accumulation of RFO, which indicates that they have a role in the plant response to abiotic stress (Zhou et al. 2014).

CoM is a coenzyme known to act as a ROS scavenger like glutathionine. ComA (FDR=2.13E-45) is an enzyme that is required for the biosynthesis of ComA (Graham et al. 2002).

3.1.4.2 Down-regulated Transcripts

Peptidyl-prolyl cis-trans isomerase (FDR= 5.93E-65) (PPIases) are a family of ubiquitous proteins. The PPIases are responsible for isomeration of peptide bonds to accelerate the folding of some proteins. The wheat FKBP77 PPIase was found to be induced by heat stress (Kurek et al. 1999). A proline rich protein (FDR=4.47E-50) was also down-regulated in response to heat.

The glycine cleavage system protein H (FDR= 4.50E-62) belongs to the glycine cleavage system that catalyzes the reaction Glycine + H(4)folate + NAD(+) <==> 5,10-methylene-H(4)folate + CO(2) + NH(3) + NADH + H(+). The cleavage system includes 4 protein groups, one of which is the H protein group. The H protein group is responsible for converting P-protein to an active enzyme. The produced 5,10-methylene-H(4)folate is involved in the synthesis of a variety of secondary metabolites such as methionine, purine, and thymidylate (Kikuchi et al. 2008).

A 60S ribosomal protein (FDR=1.69E-55) was found to be down-regulated in heat treated shoot. Ribosomal protein L10 (RPL10) is a ubiquitous protein that has a role in the formation of the 80S ribosome by joining the 40S and the 60S subunits. RPL10 was found to have non-ribosomal related functions in plants. The genome of *A. thaliana* encodes 3 genes that encode RPL10. The three *RPL10* genes were found to be differentially regulated by the exposure to UV-light, suggesting their role in the plant response to this abiotic stress (Ferreyra et al. 2010).

ADP-ribosylation factors (Arf) (FDR=7.41E-47) are a family of small GTP binding proteins that are involved in intracellular trafficking. *A. thaliana* has two homologs for Arf, Arf1 and Arf3. Arf1 was strongly suggested to have a role in the transfer of proteins intracellularly in Arabidopsis (Lee et al. 2002).

3.1.5 Root ABA vs. Shoot ABA

3.1.5.1 Shoot Up-regulated Transcripts

A 14 kDa proline rich protein (FDR= 1.15E-46) and glucan endo-1-3beta-glucosidase (FDR=4.15E-36) were up-regulated in the shoot. A gibberellin regulated protein (FDR= 1.30E-41) was up-regulating in the shoot indicating the presence of GA and its downstream proteins. GAs are known to control photosynthesis in plants. Recently a gene network composed of 47 genes regulating photosynthesis and responsive to Gas was identifies including genes *RGA*, *MYBGa*, and *GID1* (Xie et al. 2016).

MLP-like protein (FDR= 9.09E-34), major latex protein-like proteins, belong to the pathogenesis related 10 protein-like protein family (Bet V 1). A recent study revealed that MLP43 acts as a positive regulator in *A. thaliana* exposed to ABA and drought stress. The protein was found to act upstream of the SnRK2s in the ABA signal transduction pathway (Wang et al. 2016).

Aminotransferases (FDR= 4.19E-32) or transaminases are a large group of enzymes that catalyze the conversion of one amino acid to another. One example of an aminotransferase in *A. thaliana* is class 1 glutamine aminotransferase (GAT1). A member of the GAT1 protein family was found to be highly up-regulated in the plant exposed to nitrogen stress causing the repression of shoot branching (Zhu and Kranz 2012).

Glycinebetaine (GB) is a glycine (FDR= 1.71E-31) derived quaternary amine whose accumulation has been involved in a variety of responses to abiotic stress. The nitrogenous compound is thought to be involved in ROS scavenging and osmotic adjustments in abiotic stressed plants (Giri 2011).

3.1.5.2 Shoot Down-regulated Transcripts

Previously discussed chitinase (FDR=8.06E-49), peroxidase (FDR=5.49E-36), and potato inhibitor family (FDR=9.09E-34) were found to be down-regulated in the shoot compared to the root.

Beta-glucosidase/fucosidase hydrolase, (FDR=8.06E-49) is a family 1 glycosyl hydrolase glycoprotein that can hydrolyse both beta glucosides and beta fucosides and is specific for isoflovanoids (Ketudat et al.). The GH1 family is known to be involved in a variety of important processes in plants such as biotic and abiotic stress response, cell wall remodeling and lignifications, and phytohormone activation (Opassiri et al. 2006).

Over-expression of lipid binding protein (FDR= 1.01E-37) FAR-1 (fatty acid and retinol binding protein) in tomato root cells was found to enhance the plant response to pathogen infection. The DE genes in root cells were involved in cell wall regulation and modification, biosynthesis of fatty acids associated compounds, and the phenylpropanoid pathway (Iberkleid et al. 2015).

Glucan endo-1-3-beta-glucosidase (FDR= 4.15E-36) is able to hydrolyse the beta D glycosidic bond in beta D glycans. Another name for this enzyme is callase which is able to break down the polymer callase. Callose is a major polymer in the cell wall of plants along with hemicelluloses and pectin (Mollet et al. 2013).

Plant defensins (gamma thionin family FDR=3.23E-34) are small cystein rich highly stable proteins that have a major role in the plant response to pathogenic attack. Some defensins are also involved in plant growth and development. They are known to act as antimicrobial compounds, proteinase inhibitors, and insect amylase inhibitors (Stotz et al. 2009).

Disease resistance protein 206 (DRR206) (FDR=4.82E-31) was found to be up-regulated in pea plant grown in a naturally infested soil with the pathogen *Fusarium oxysporum f. sp. pisi.* However, the biochemical function of the protein remains unknown (Hadwiger et al. 1992).

3.1.6 Root Heat vs. Shoot Heat

3.1.6.1 Shoot Up-regulated Transcripts

Only one out of the 50 most DE transcripts was up-regulated in the shoot compared to the root.

3.1.6.2 Shoot Down-regulated Transcripts

The previously described peroxidase (FDR=6.30E-18), proline rich protein (FDR=6.30E-18), cathepsin (FDR=1.28E-17), pathogenesis related protein Bet v I family (FDR= 1.49E-16), lipid transfer protein (FDR= 2.93E-12), and potato inhibitor family (FDR=2.00E-14) were all down-regulated in the shoot compared to the root.

Germin-like proteins (FDR= 1.28E-17) are auxin regulated ubiquitous proteins found in plants. Members of the protein family are involved in the plant defense response against pathogen attack (Lu et al. 2010). The protein family has various enzymatic properties including superoxide dismutase, polyphenol oxidase, AGPPase, and oxalate oxidase which allow them to function in plant responses against various and abiotic factors (Barman and Banerjee 2015).

3-hydroxyisobutyryl-CoA hydrolase-like protein (FDR=3.92E-15) is an enzyme that catalyzes the production of CoA and 3-hydroxy-2-methylpropanoate. The enzyme is involved in

a number of metabolic pathways including beta-alanine and propanoate metabolism and leucine, isoleucine, and valine degradation (Rendina and Coon 1957).

Pollen proteins Ole e I (FDR= 4.18E-14) are a group of pollen allergens. The function of these pollen allergens in the plant are not clearly understood but they are speculated to be involved in many pollen physiology related processes such as pollen germination, hydration, and pollen tube growth (Jimenez-Lopez et al. 2011).

Strictosidine synthase (SSL) (FDR= 2.99E-12) is a key plant enzyme in the biosynthesis of alkaloids. *A. thaliana* plants exposed to were found to have 3 SSL genes up-regulated in response to various phyto-hormones such as SA, ET, and met-JA and pathogens. This indicates the involvement of the protein in plant defense mechanisms (Sohani et al. 2009).

Phenylalanine ammonialyase (FDR=8.78E-12) (PAL) is a well studies enzyme that catalyzes the deamination of L-Phenylalanine to produce cinnamic acid and ammonia. The levels of this enzyme are known to fluctuate greatly over short periods of time in plant responses to many environmental stressors. Cinnamic acid, the product of PAL, is the precursor for the biosynthesis of many secondary metabolites including lignins (Camm and Neil 1973).

3.1.7 Root ABA vs. Root Heat

3.1.7.1 ABA Up-regulated Transcripts

The up-regulated transcripts in ABA treated root compared to heat treated one include serine threonine protein kinase, stress induced protein, H2O2 removal protein, peroxidase, glutathione S transferase, and pathogenesis related protein (FDR=0).

Thaumatin-like protein (FDR=0) belongs to the pathogenesis related protein family. ObTLP1 is a thaumatin like protein found in *Ocimum basilicumm* in response to Me-JA. The expression of the gene was found to be organ specific in unstressed conditions and is involved in the response to biotic and abiotic stresses and multiple phyto-hormone applications. The ectopic expression of the gene in *A. thaliana* was found to enhance the plant tolerance to pathogen infection and dehydration and drought stress (Misra et al. 2016).

3.1.7.2 ABA Down-regulated Transcripts

The down-regulated transcripts in ABA treated root include zinc finger protein, cathepsin, CS domain, fructose biphosphate aldolase, and heat shock proteins (FDR=0). Translationally controlled tumor protein (TCTP) is considered a stress related protein due to its up-regulation in stress related conditions. In bacteria, TCTP was suggested to belong to the family of heat shock proteins since it was found to protect the bacterial cell against heat shock induced death (Gnanasekar et al. 2009). *A. thaliana*'s genome has two TCTP genes that act as mitotic regulators (Toscano-Morales et al. 2015).

3.1.8 Shoot ABA vs. Shoot Heat

3.1.8.1 ABA Up-regulated Transcripts

60S ribosomal protein (FDR= 1.29E-51), ADP ribosylation factor (FDR= 3.47E-48), and proline rich protein (FDR= 5.63E-46) were up-regulated in the ABA treated shoot compared to the heat treated one.

Protein inhibitors such as trypsin inhibitor (FDR= 1.31E-53) are known to accumulate in plants in response to pathogen attack. Exogenous ABA application to barley was found to induce chymotrypsin, while exogenous ABA application was found to induce trypsin inhibitor (Casaretto et al. 2004).

Metallothioneins (FDR= 1.93E-47) are cysteine rich metal binding proteins. The rice genome was found to contain an MT gene *OsMT1e-P* that belongs to a family of 13 genes and 15 proteins which are all found in the rice genome. Over-expression of the gene was found to enhance stress tolerance. Under salinity stress, the gene is regulated in an organ specific manner. The encoded protein is thought to act as an ROS scavenger (Kumar et al. 2012a).

Dehydroascorbate reductase (FDR= 1.60E-45) is an enzyme that is necessary for the regeneration of ascorbate, an antioxidant, and the maintenance of the ROS scavenging ability (Shin et al. 2013).

3.1.8.2 ABA Down-regulated Transcripts

Heat shock proteins (FDR= 1.77E-73- 3.14E-49), peptidyl-prolyl cis-trans isomerase (FDR= 6.92E-60), CS domain (FDR= 1.87E-51), and galactinol synthase (FDR= 5.39E-46) were down-regulated in the ABA treated shoot compared to the heat treated one.

3.2 The Top 10 Most Enriched Biological Processes

3.2.1 Root ABA vs. Root Control

The two most enriched terms are "response to water" and "response to water deprivation". The main abiotic stress that has been found to cause ABA accumulation in plants is water deprivation(Hauser et al. 2011). ABA mediated processes, such as the control of stomatal opening, are known to be involved in the plant's response to water deprivation (Rossdeutsch et al. 2016). The ability of ABA to control stomatal opening allows for the reduction of water loss by transpiration when needed (Hauser et al. 2011). In a study to monitor the amount of accumulated ABA in a plant under varied water availability and relative humidity, the lowest amount of ABA was seen in well watered plants grown at high relative humidity. When the plants were exposed to a water deficit, ABA levels increased and were accompanied by a change in stomatal size (Giday et al. 2014).

As expected, the terms "response to abscisic acid stimulus" and "response to abiotic stress stimulus" were enriched. ABA is considered to be a master regulator for plant abiotic stress responses such as salt, drought, heat, and high light intensity (Bari and Jones 2009). The response to ABA stimulus is known to involve a family of receptor proteins (PYR/PYL/RCAR) as seen in figure (Ng et al. 2014). When comparing the transcript abundance of the ABA receptors in response to ABA in maize roots and leaves, the monomeric ABA receptors were involved in ABA signal transmission in the root while the dimeric forms of the same receptors were responsible for this function in the leaves (Fan et al. 2016).

The terms "lipid transport" and "lipid localization" indicate the ABA abiotic stress signaling pathway involves the storage of lipids. This is due to the fact that ABA accumulates

after exposure of the plant to abiotic stress causing the plant to enter a state of dormancy(Hauser et al. 2011). Lipid reserves would be needed for the dormant plant after recovery from the stress induced dormant state. Exogenous application of ABA to developing castor bean plant seeds was similarly found to enhance the accumulation of soluble sugar content by 6.3% followed by deposition of total lipid content by 4.9% (Chandrasekaran et al. 2014).

The terms “embryonic development ending in seed dormancy”, “seed development”, “fruit development”, and “reproductive developmental processes” stress further the role of ABA in inducing a plant dormant state under abiotic stress conditions; entering a state of dormancy involves ceasing reproductive processes including seed and fruit formation. In soft white spring wheat, ABA was found to induce seed dormancy during embryo maturation and to inhibit the germination of mature grains (Schramm et al. 2013). In *A. thaliana* seeds, four AtABCG transporters were found to transport ABA from the endosperm to the embryo to prevent germination (Kang et al. 2015). Commercial production of grapes requires the induction of dormancy release by hydrogen cyanamide. ABA was found to inhibit the dormancy release caused by HC (Zheng et al. 2015).

3.2.2 Shoot ABA vs. Shoot Control

Like the RAXRC pair-wise comparison, the two most enriched terms are “response to water deprivation” and “response to water”. However, the exposure of the shoot to ABA appears to cause the differential expression of genes associated with carbohydrates metabolism; this was not observed in the root samples. The most enriched terms include “glucosinolate metabolic process”, “glycosinolate metabolic process”, “glycoside metabolic process”. The mentioned carbohydrates appear to be broken down in response to ABA exposure, which shows in the enriched terms “glycoside catabolic process”, “glucosinolate catabolic process”, and “glycosinolate catabolic process”. Glucosinolates are organic compounds derived from glucose. They are simply sugars with a high content of nitrogen and sulfer. Glucosinolates represent a large store for nitrogen and sulfur in the plant cell (30% of cellular sulfur) (Falk et al. 2007). The catabolism of glucosinolates in response to exogenous ABA application might be explained by the increased need for nitrogen and sulfur under stress conditions so that they are available for primary metabolism (Janowitz et al. 2009). Glucosinolates are known to have a pungent smell

and are produced by plants as a defense mechanism against biotic stress (Janowitz et al. 2009). Due to the increased exposure of the shoot system to biotic stress, glucosinolates are present differentially in the shoot. In a study to analyse the proteome of *A. thaliana* exposed to ABA, nitrilase 1 and 2 (NIT1-NIT2) proteins were found to be up-regulated. These proteins have been hypothesized to control glucosinolates catabolism (Bohmer and Schroeder 2011).

The term "response to zinc ion" was also enriched in this comparison. bZIP transcription factors are known to be involved in regulating acquired thermotolerance (Gao et al. 2008). Two members of the *A. thaliana bZIP* families, *bZIP19* and *bZIP23,* were found to regulate the adaptation of plant to zinc deficiency. *bZIP 19* and 2*3* double mutants were found to be hypersensitive to zinc deficiency (Assuncao et al. 2010). bZIP10 is also a zinc deficiency transcription factor that was found to have a role in plant resistance to oxidative stress. Overexpressing *Bzip10* in *Brachypodium distachyon* was found to protect the plant and callus tissue from oxidative stress insults (Glover-Cutter et al. 2014). Thus, it is speculated that the application of ABA caused the upregulation of members of the bZIP transcription factors family to regulate the abiotic stress response; Since bZIP proteins are also involved in the plant's response to zinc deficiency, it is logical to have the response to zinc BP enriched.

3.2.3 Root Heat vs. Root Control

The most enriched biological process is "response to heat". As seen in figure 1, the plant's primary response to heat involves changes in the membrane fluidity, protein and cytoskeletal stability, and chromatin structure, accompanied by ROS production (Bita and Gerats 2013). The signal transduction mechanism induced by heat in plants can be summarized as seen in figure 2. The heat shock causes the accumulation of reactive oxygen species such as hydrogen peroxide. This oxidative stress leads to the disruption of the cell membrane properties, proteins, and cellular homeostasis. The homeostatic disruption causes the activation of a variety of transcription factors including the HSFs, DREB2A, bZIP, and the hormone signaling involved transcription factors. Transcription factors cause the activation of stress responsive genes which lead to the recovery of the cellular homeostasis by producing antioxidant enzymes, ROS scavengers, and signaling molecules. Restoring homeostasis eventually leads to heat tolerance in

plants (Hasanuzzaman et al. 2013). Other related highly enriched biological processes include "response to temperature stimulus" and "response to abiotic stimulus".

Evidence for the ROS accumulation in response to heat stress and the activation of plant antioxidant mechanisms is observed in the highly significant enrichment of the terms "response to inorganic substance", "response to hydrogen peroxide", "response to reactive oxygen species", "response to oxidative stress", and "hydrogen peroxide catabolic process". Reactive oxygen species include H_2O_2, $O_2^{\bullet-}$, $OH\bullet$, and $^1O_2$ (Gill and Tuteja 2010). ROS are produced in the cell organelles, plasma membrane and apoplast in response to abiotic stress. ROS accumulation is extremely reactive and toxic to lipids, nucleic acids, and proteins inside the plant cell (You and Chan 2015). The production of ROS acts as a signal for the activation of stress response pathways (Baxter et al. 2014). As previously mentioned, *E. salsugineum* genome was found to have 17 members of the aldehyde dehydrogenase family responsible for breaking down hydrogen peroxide (Hou and Bartels 2015). It was also found that different members of the GPX protein family, which are responsible for the break down of hydrogen peroxide into water and oxygen, were differentially abundant in different tissues such as roots and leaves in *E. salsugineum* in response to abiotic stress (Gao et al. 2014).

The plant responses to heat and high light intensity are expected have overlapping signal transduction pathways since high sunlight intensity is always accompanied by a higher temperature; thus, the enrichment of the term "response to high light intensity" is not unforeseen. Excess light conditions are known to induce the production of ROS in the chloroplast, peroxisome, vacuoles, and cytosol. Accumulation of ascorbate and glutathione in these compartments plays a critical role in the plant response to short term high light stress (Heyneke et al. 2013).

3.2.4 Shoot Heat vs. Shoot Control

The same terms that were highly enriched in the RHXRC comparison were highly enriched here. One term that was newly enriched in this comparison is "nitrogen compounds biosynthetic processes". It was observed that in the SAXSC comparison, genes that are involved in the breakdown of nitrogen rich glucosinolates were enriched. It is speculated that the heat

induced ABA accumulation causes the catabolism of glucosinolates releasing nitrogen that can be used to synthesize amino acids such as proline which have a central role in the plant heat response. Proline levels have been found to increase by more than a 100 fold during stress (Verbruggen and Hermans 2008). Proline has been extensively shown to have a protective role against a variety of biotic and abiotic stresses in plants. It acts as a molecular chaperone that maintains protein homeostasis during stress. Several studies have also shown that it acts as an ROS scavenging osmolyte. Moreover, proline was found to have a role in stress signaling and protein translation (Szabados and Savoure 2010). The biosynthesis of proline can occur using two precursors, glutamate and ornithine. However, the glutamate pathway appears to be the most prominent under stress conditions; the ornithine pathway is mainly involved in nitrogen recycling from arginine to glutamate (Liang et al. 2013).

3.2.5 Root ABA vs. Shoot ABA

The most enriched biological processes are those involved in scavenging ROS, specifically hydrogen peroxide as shown in the enriched terms "cellular response to hydrogen peroxide", "hydrogen peroxide catabolic processes", "cellular response to reactive oxygen species", and " cellular response to oxidative stress. It is expected to have more accumulation of ROS in the plant shoot than in the root since shoot cells have more chloroplasts and a large amount of the stress induced ROS is produced by the chloroplasts. It is also highly likely that the variety of genes that are involved in ROS scavenging are organ specific. For instance, Gao et al. have shown that 3 members of the GPX hydrogen peroxide scavenging protein family (GPX5, GPX7, GPX8) were up-regulated in the leaves under salt stress; six different members of the same protein family (GPX1, GPX2, GPX3, GPX5, GPX7, GPX8) were up-regulated in the roots under salt stress (Gao et al. 2014).

"Secondary metabolic process" is highly enriched in this comparison. Secondary metabolic processes are those that do not involve growth or maintenance of the cell. These processes usually occur in specific cells; thus, it is logical to have genes belonging to this annotation term expressed in a tissue specific way (GO:0019748). Genes belonging to this term include cytochrome P450, Nitrilase 3, and sulfotransferase 18. Cytochrome P450 is a

superfamily of haem containing enzymes that are involved in a variety of primary and secondary metabolic processes (Lamb and Waterman 2013). Members of the family are involved in detoxifying the cells from the ROS induced by stress. Nitrilases as discussed before are involved in the glucosinolate metabolism which was enriched in the shoot. *A. thaliana* contains 21 members of the sulfotransferase family (SOT). Members of this enzymatic protein family act on a variety of substrates such as glucosinolates, jasmonates, salycilic acid, and flavonoids, all of which are involved the plant thermotolerance. Sulfotransferases transfer a sulfuryl group from 3'-phosphoadenosine 5'-phophosulfate to the hydroxyl group of the substrate (Hirschmann et al. 2014).

"Lipid transport" and "localization" annotation terms were also enriched. Belonging to this term is the Lipid transfer protein 3. In wheat, LTP3 was up-regulated in response to heat and ABA treatments. Moreover, it was found to enhance basal thermo-tolerance in *A. thaliana* by acting as a ROS scavenging protein (Wang et al. 2014a). The reason lipid transport and localization genes are differentially expressed in the root and shoot is not yet fully understood.

3.2.6 Root Heat vs. Shoot Heat

The most enriched terms are "response to salt stress", "response to osmotic stress", and "response abiotic stimulus". The plants responses to heat, salt, and osmotic stress were found to overlapping in a variety of aspects. For instance, the ABA induced up-regulation of the small heat shock proteins (*sHSP*) in *Physocomitrella patens* was found to be caused under heat, salt, and osmotic stress (Ruibal et al. 2013). The term "response

Similar to the RAXSA comparison, terms related to the synthesis of secondary metabolites were enriched such as "phenylpropanoid biosynthetic process" and "flavonoid biosynthetic process", and "aromatic compound biosynthetic pathways". The phenylpropanoid pathway produces a variety of secondary metabolites in plants such as flavonoids, coumarins, and lignans. The pathway is also required for the biosynthesis of lignin (Fraser and Chapple 2011).

Flavonoids are a group of plant polyphenolic secondary metabolite, and include different classes such as anthocyanins, flavanols, flavonols, tannins, and proanthocyanidins. They are

known to be present in different amounts based on the plant organs (Debeaujon et al. 2001); In *A. thaliana*, 54 flavonoids were found to accumulate in an organ specific manner (Nakabayashi et al. 2014). Flavonoids are known to function as antioxidants; thus, they have a role in plant abiotic stress response. They are also involved in UV light protection and defense against plant pathogens (Kitamura 2006). Members of the cytochrome P450 family, which were also enriched in the RAXSA comparison, are involved in the biosynthesis of flavonoids (Winkel 2004). Other enzymes involved in flavonoid biosynthesis include chalcone synthase (CHS) and leucoanthocyanidin oxidase (LDOX) (Tian et al. 2008; Wang et al. 2011b).

3.2.7 Root ABA vs. Root Heat

Since it has become obvious from the existing literature that the plants mechanisms to respond to stress are extremely varied and overlapping, it was predicted to have the enriched GO terms in this comparison related to plant abiotic responses and ROS pathways. The enriched GO terms include “response to heat”, “response to reactive oxygen species”, and “response to light intensity”.

“Response to cadmium” was newly highly enriched in this experiment. Kulik et al. found that the SNF1-related protein kinase2 (SnRK2s) were transiently activated during the exposure to cadmium. The research concluded that SnRK2 has a role in the regulation of the plant response to cadmium stress through alleviating the ROS accumulation that results from the cadmium exposure (Kulik et al. 2012). SnRK2 is also known to control the downstream components of the ABA abiotic stress response network as seen in figure 6 (Ng et al. 2014). This explains why the response to cadmium was enriched due to the exposure of heat more than due to the exposure of ABA. It is speculated that the exposure of the plant to heat accumulates more ABA than the exogenous exposure of the plant to ABA, which causes the response to cadmium genes to be highly induced.

3.2.8 Shoot ABA vs. Shoot Heat

The only newly enriched term in this comparison compared to the RAXRH comparison is "defense response, incompatible interaction". This is not unpredicted since the plant responses to biotic and abiotic stress are known to overlap in many aspects. For instance, the main players in the basal thermotolerance pathways which are ABA, SA, ET, and JA are known to be involved in both biotic and biotic stresses. However, ABA is more involved in plant abiotic stresses such as salinity, drought, cold, and heat stress (Lata and Prasad 2011; Zhang et al. 2006); SA, ET, and JA are more involved in plant responses to biotic stresses (Bari and Jones 2009).

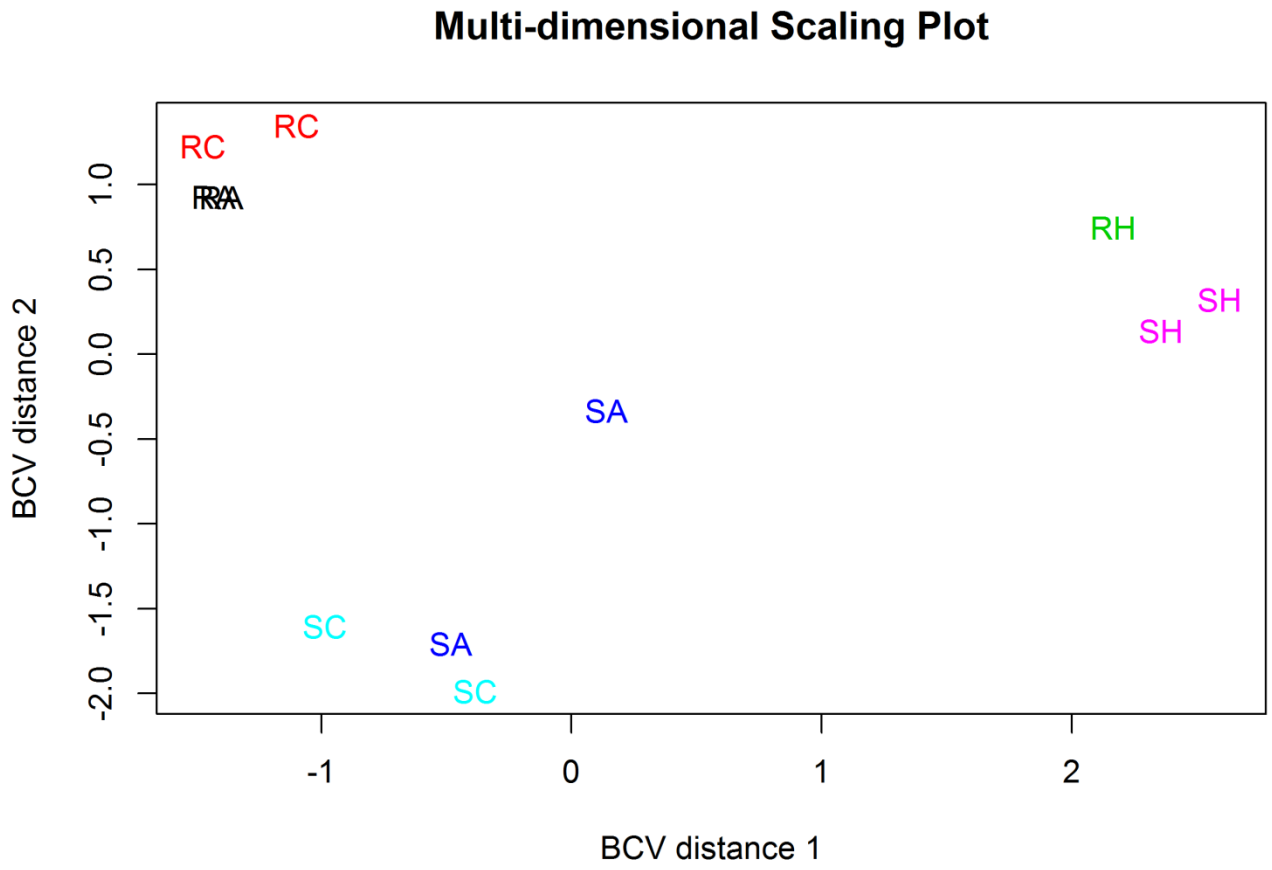

Figure 9: A multi-dimensional scaling plot using the biological coefficient of variation method shows how the samples cluster. The replicates cluster as expected. It can be predicted from the distance that the heat samples will have higher differential expression compared to the control samples than the ABA samples.

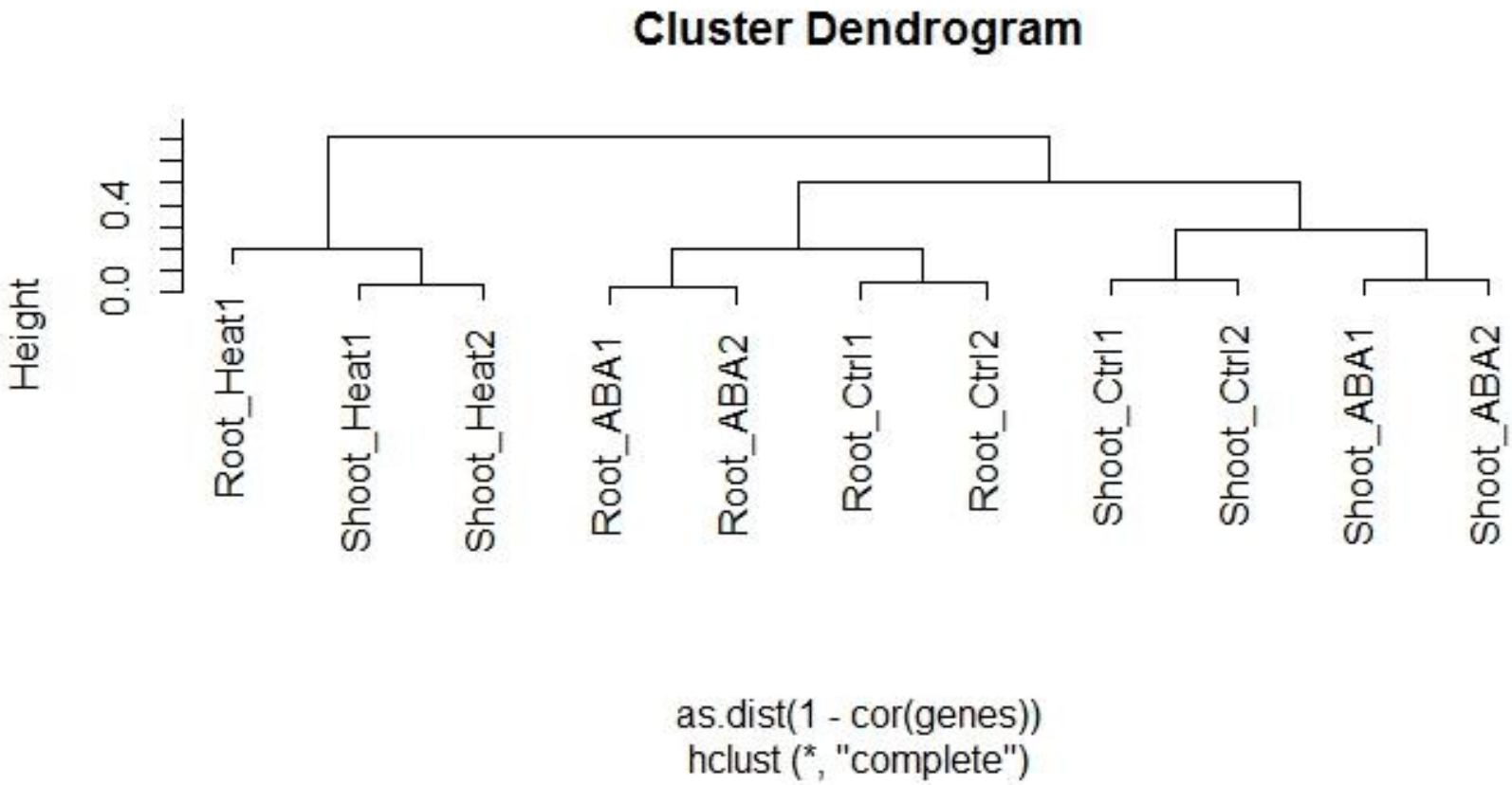

Figure 10: Hierarchical clustering of TMM normalized counts across replicates.

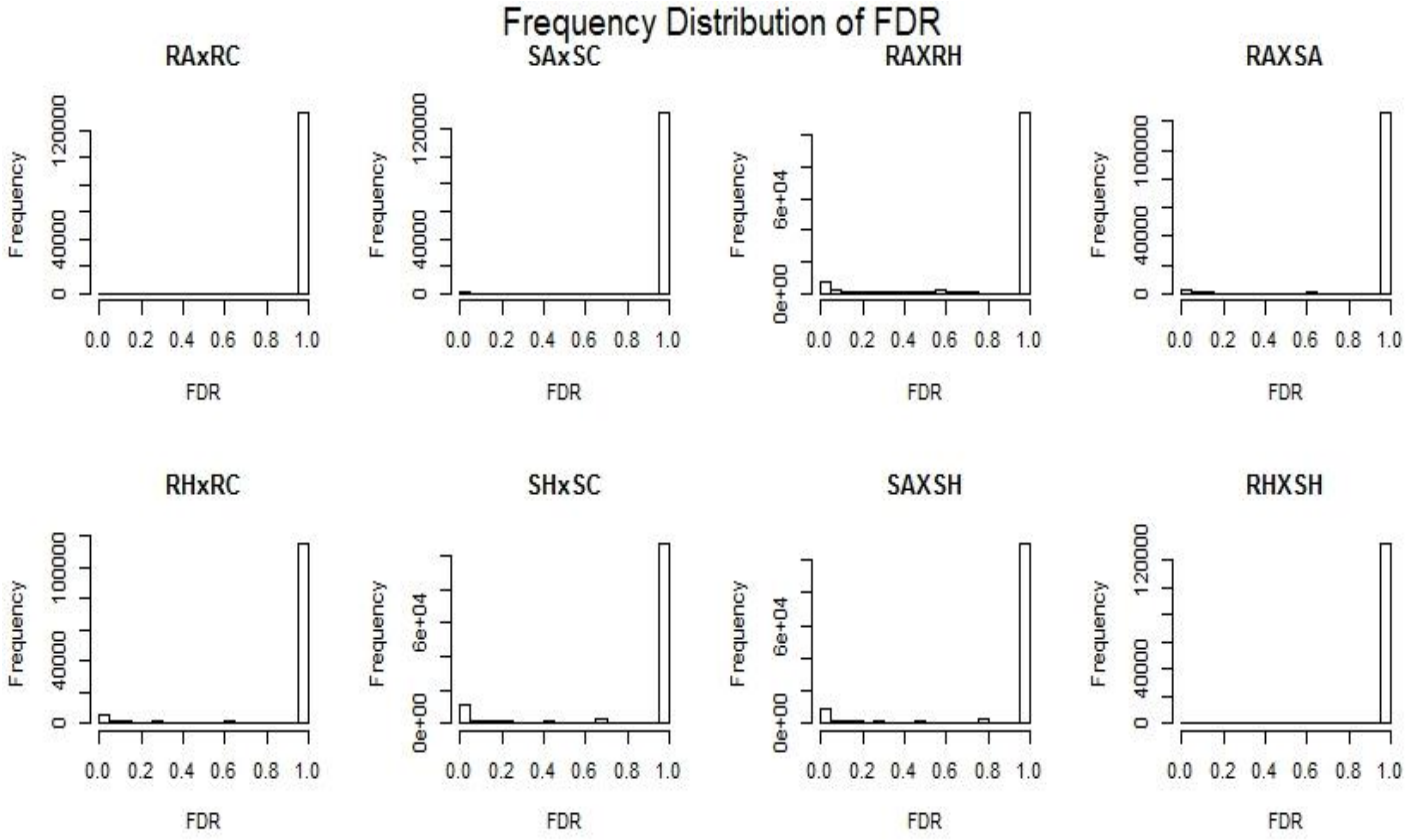

Figure 11: The histograms of the FDR frequency distribution for the 8 pair-wise comparisons show the statistically significant transcripts. It can be concluded that most of the transcripts in all the comparison have and FDR of 1; thus, most of the transcripts are not differentially expressed.

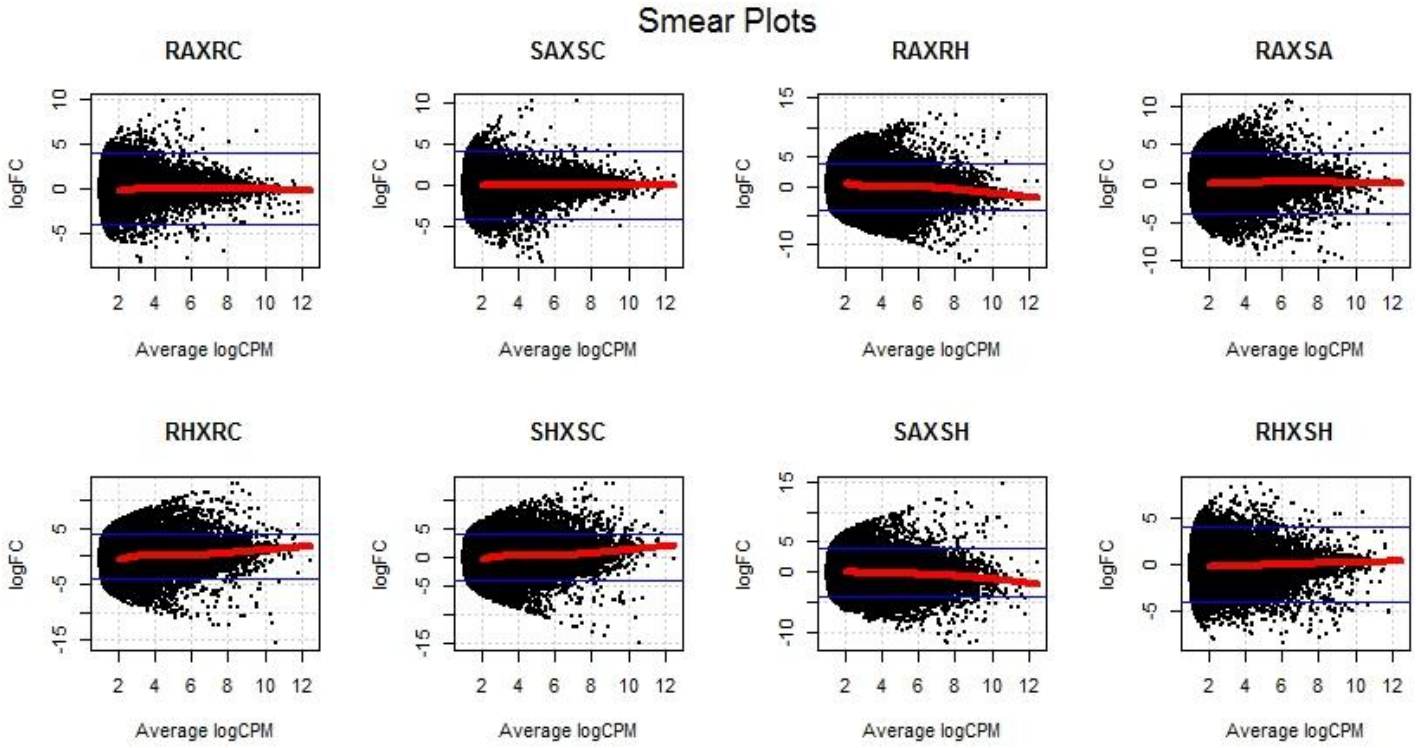

Figure 12: Smear plots of log fold-change vs. average log counts per million. A trend line is drawn in red. The blue ablines are drawn at a log fold-change cutoff = 4 (FDR>4, FDR<-4). The biologically significant transcripts can be seen above and below the ablines.

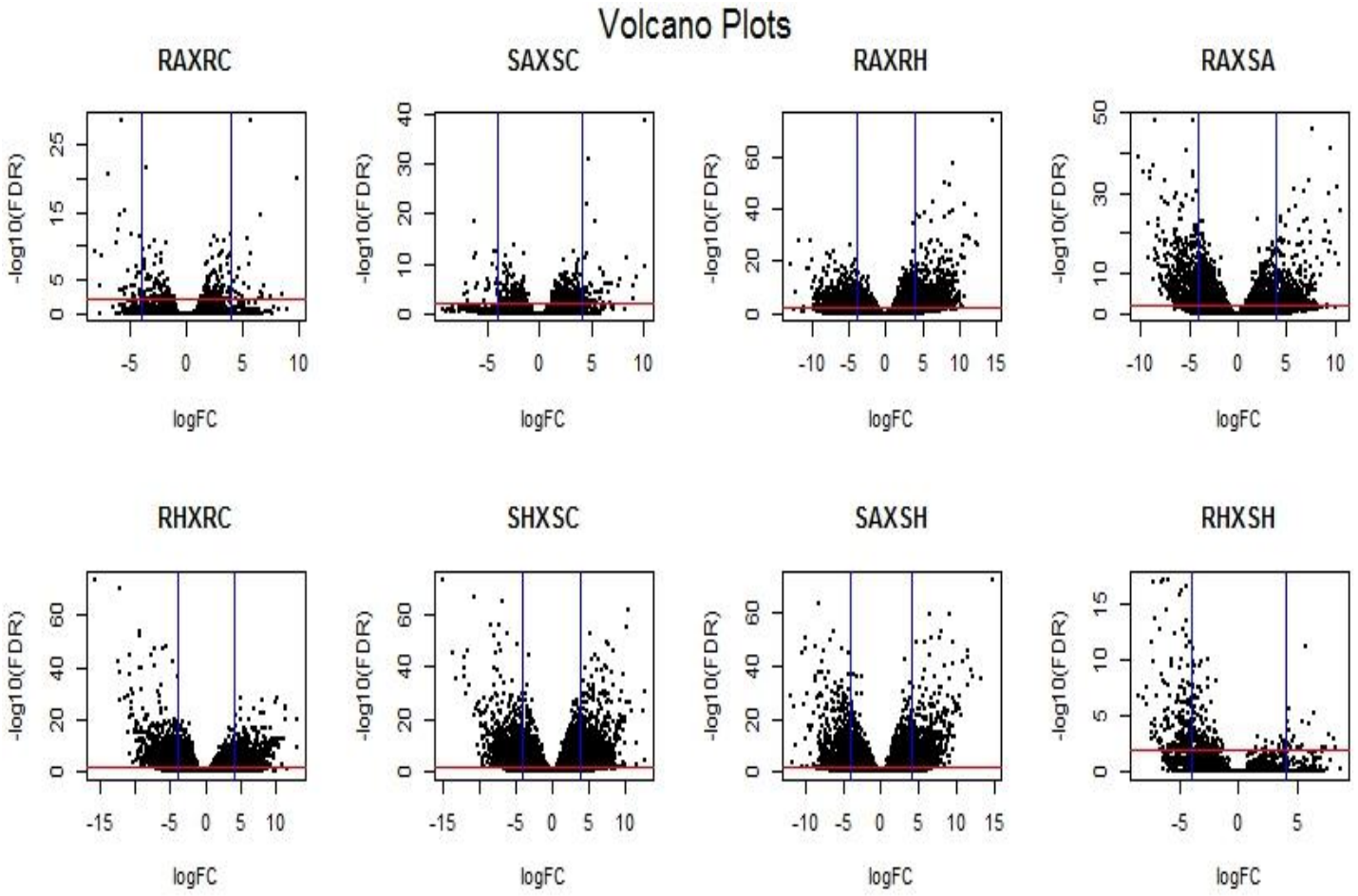

Figure 13: Volcano plots of –log10 (FDR) vs. log fold-change. The plots combine the statistical and biological significance. The blue vertical ablines are drawn at the log fold-change cutoff of 4 (FDR>4, FDR<-4). The horizontal red line is drawn at the FDR<0.01 (-LOG10 FDR>2). The biologically and statistically differentially expressed transcripts can be seen on the upper left and right sections of the plots

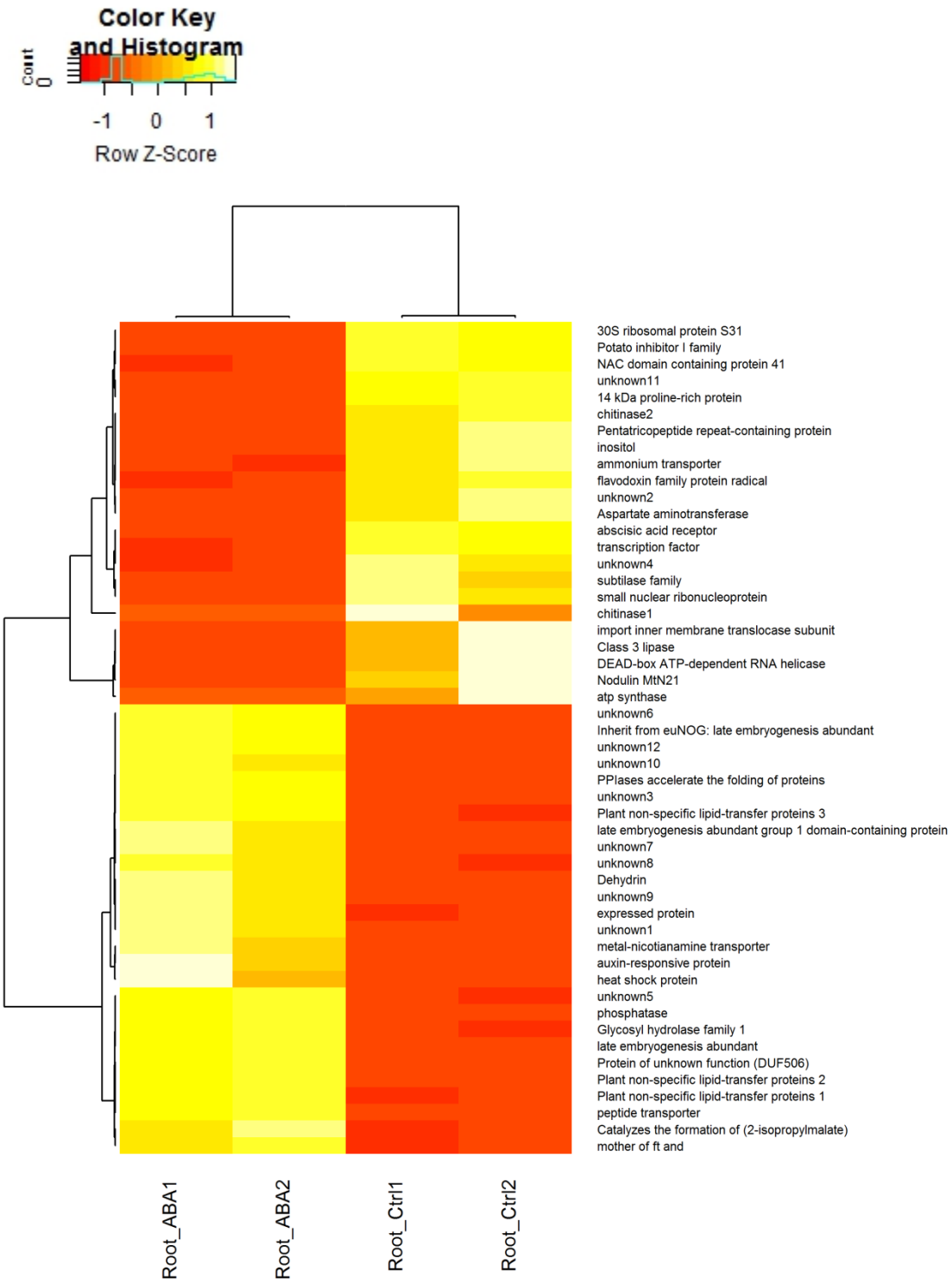

Figure 14: Heatmap of the 50 most DE transcripts in RAXRC.

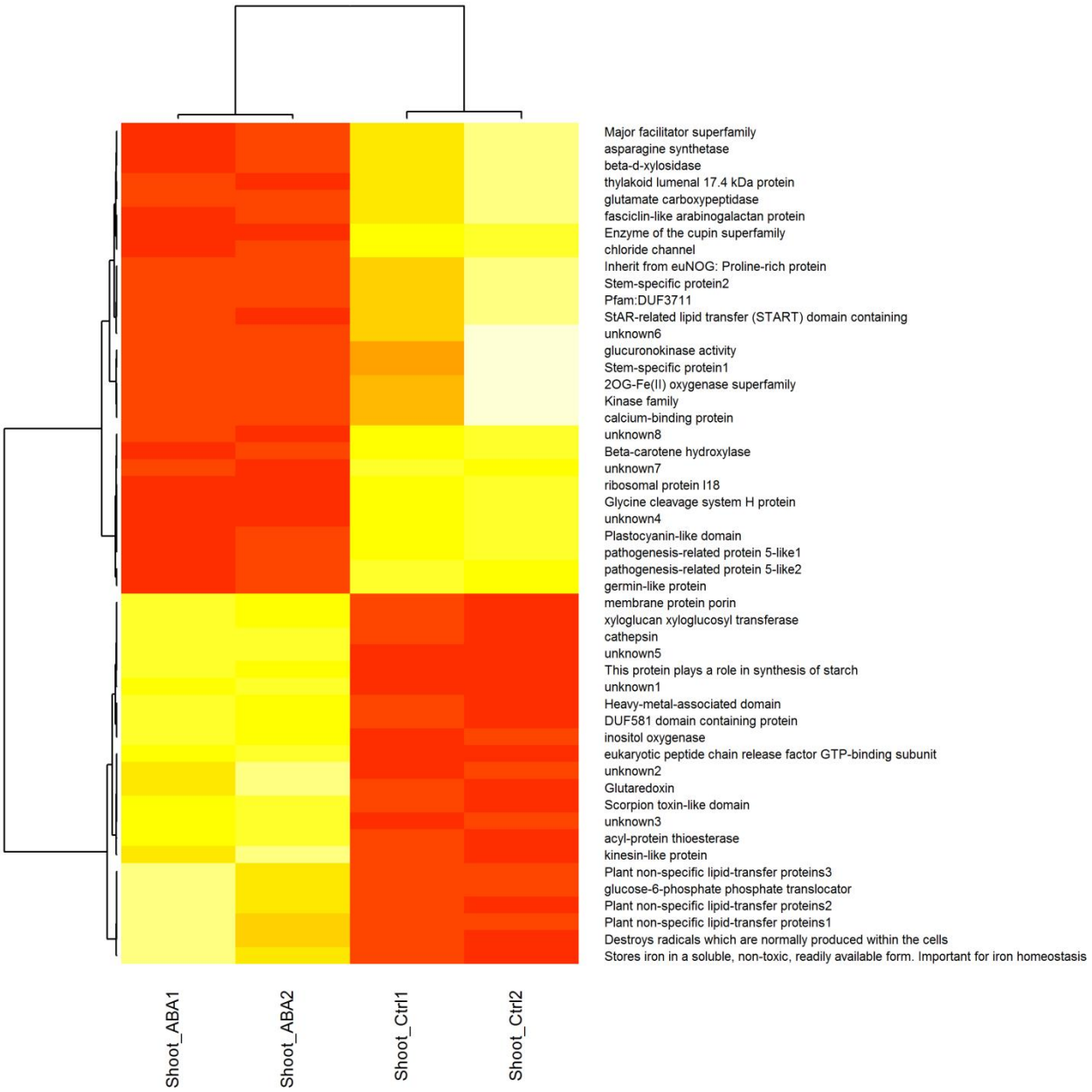

Figure 15: Heatmap of the 50 most DE transcripts in SAXSC.

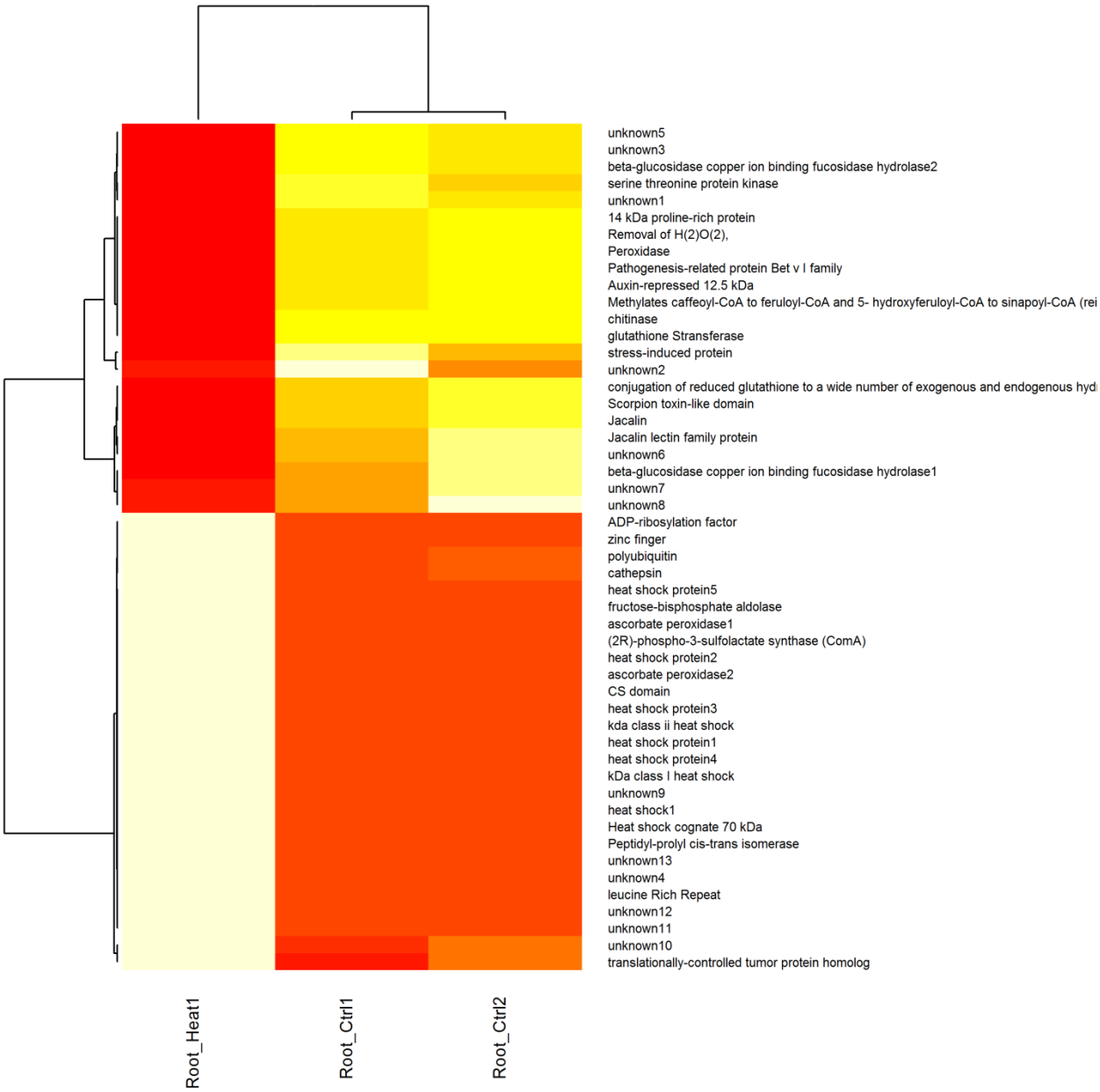

Figure 16: Heatmap of 50 most DE transcripts in RHXRC.

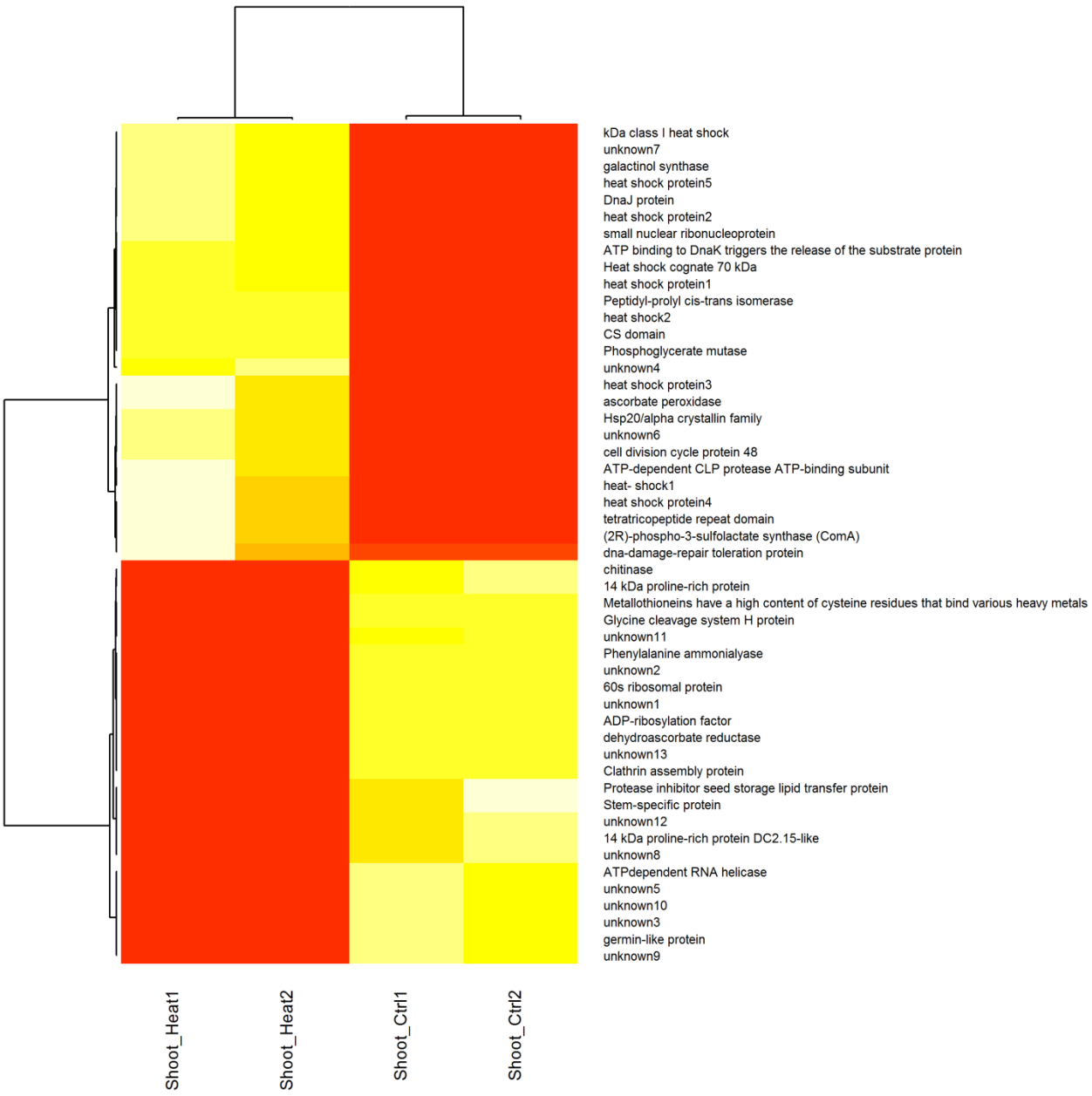

Figure 17: Heatmap of the 50 most DE transcripts in SHXSC.

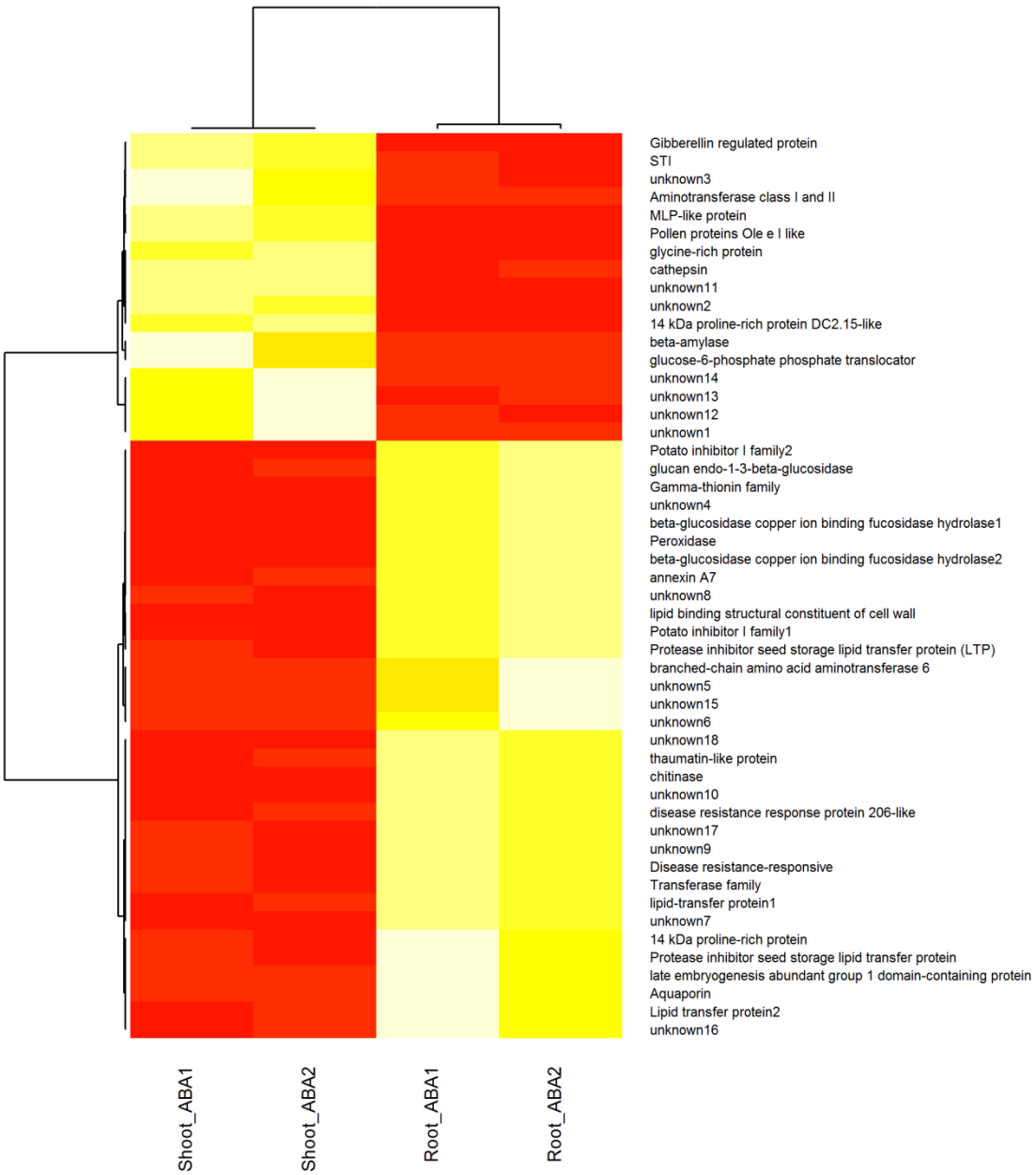

Figure 18: Heatmap of the 50 most DE transcripts in SAXRA.

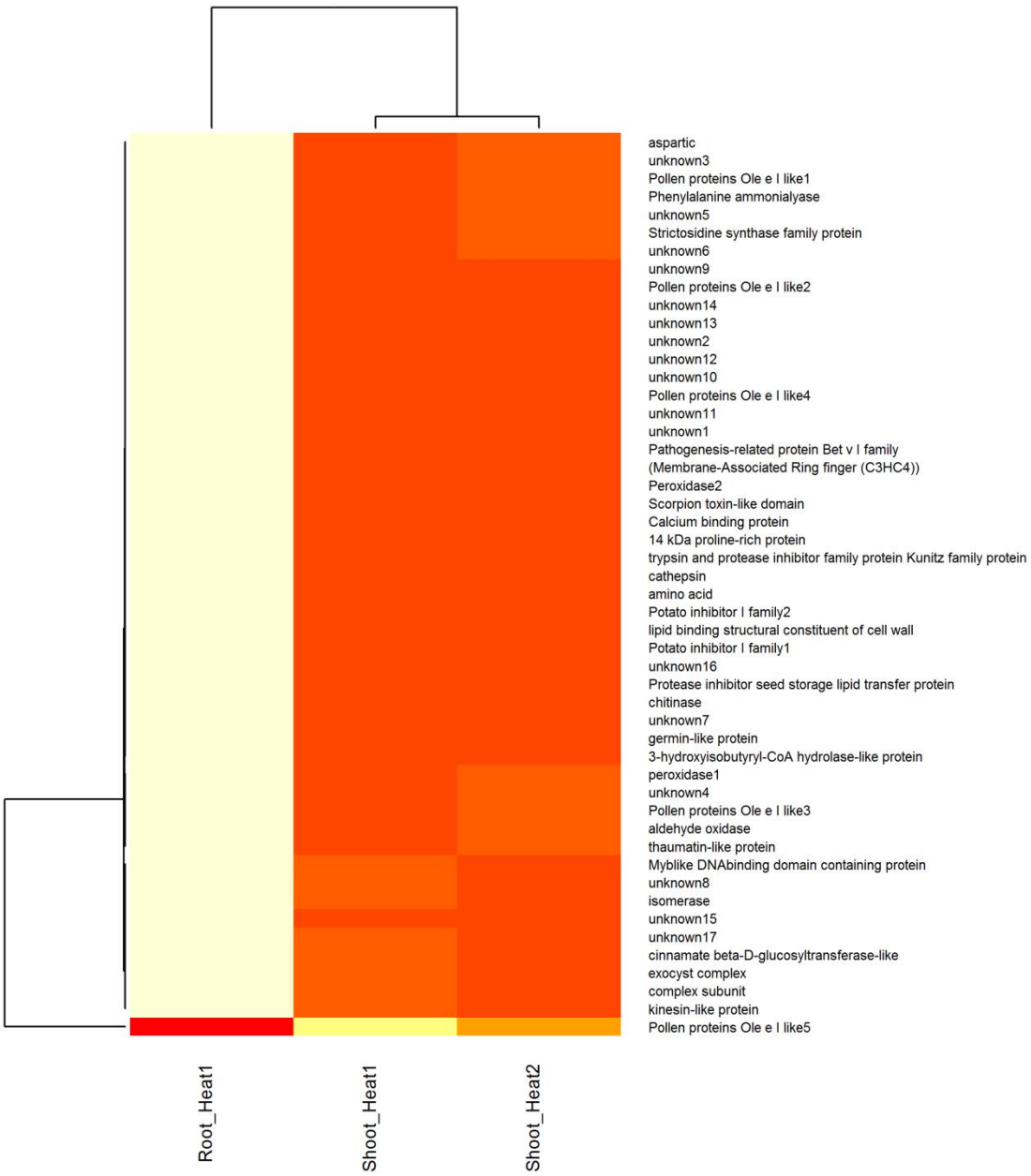

Figure 19: Heatmap of the 50 most DE transcripts in RHXSH.

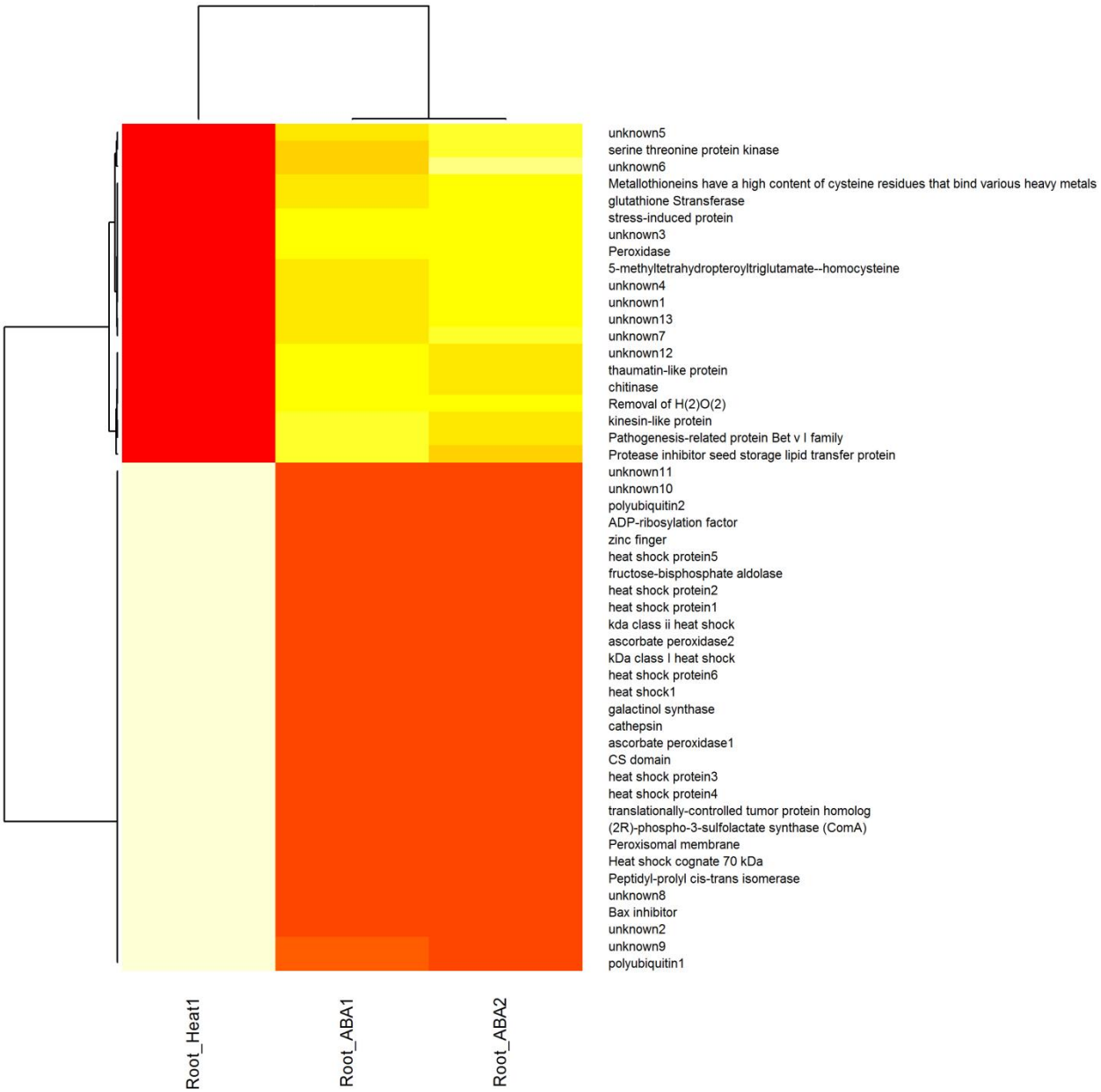

Figure 20: Heatmap of the 50 most DE transcripts in RAXRH.

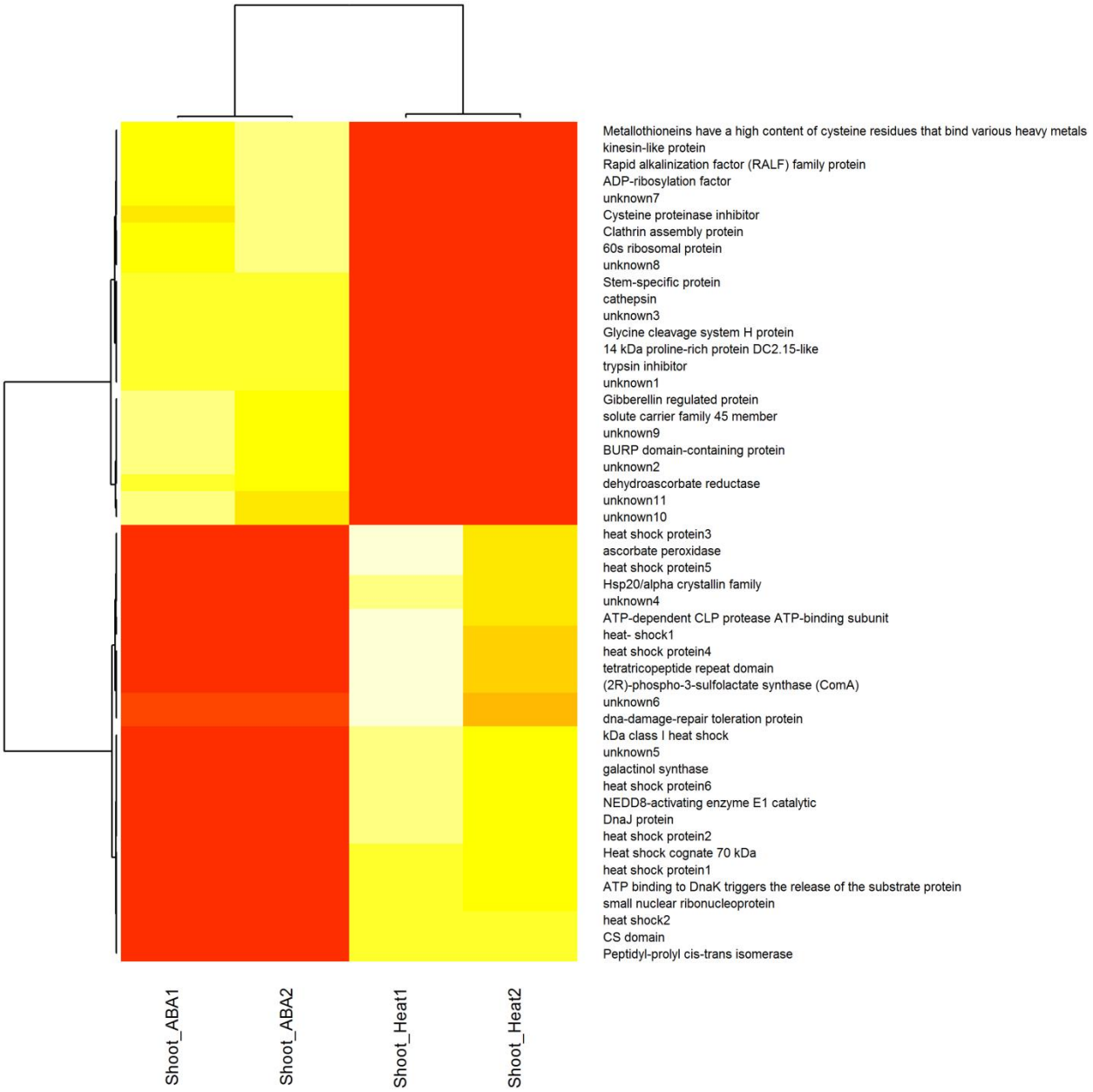

Figure 21: Heatmap of the 50 most DE transcripts in SAXSC.

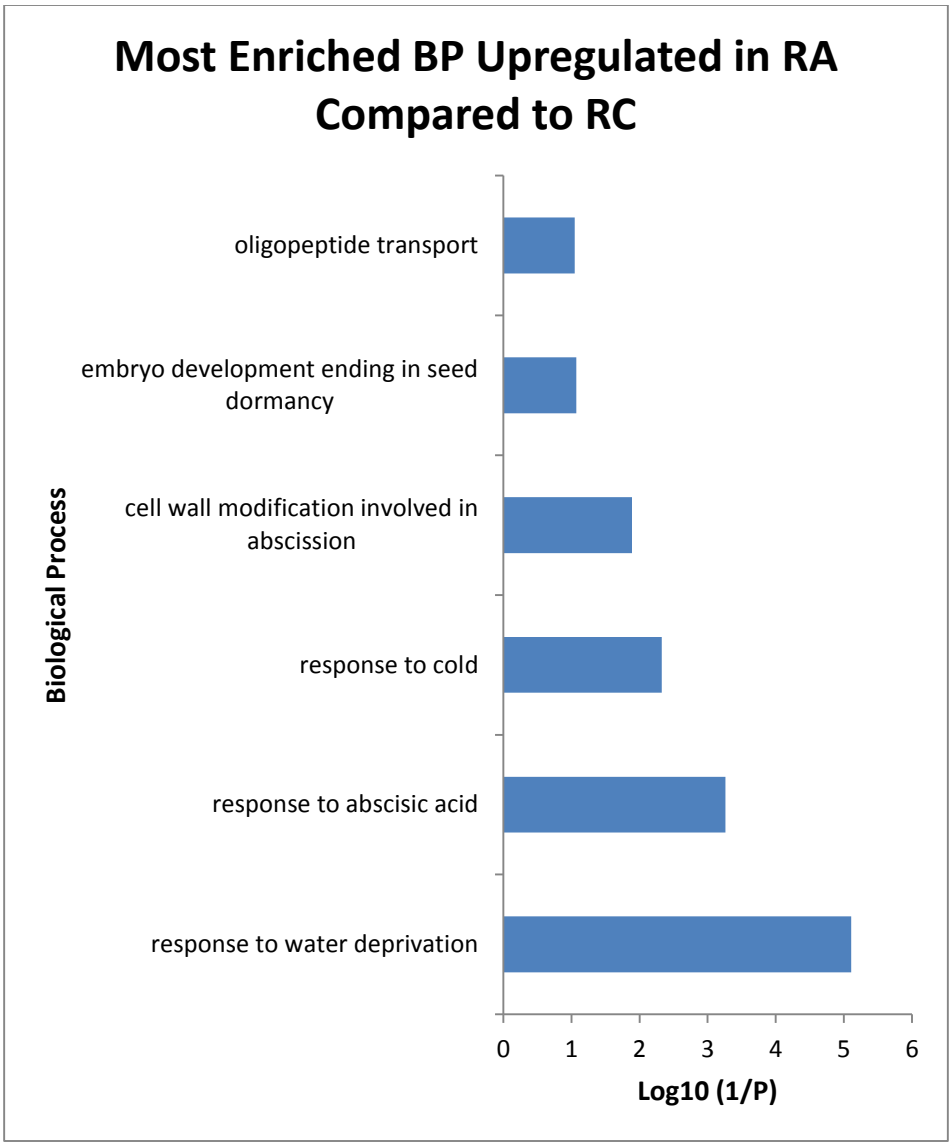

Figure 22: Most Enriched BP Upregulated in RA Compared to RC.

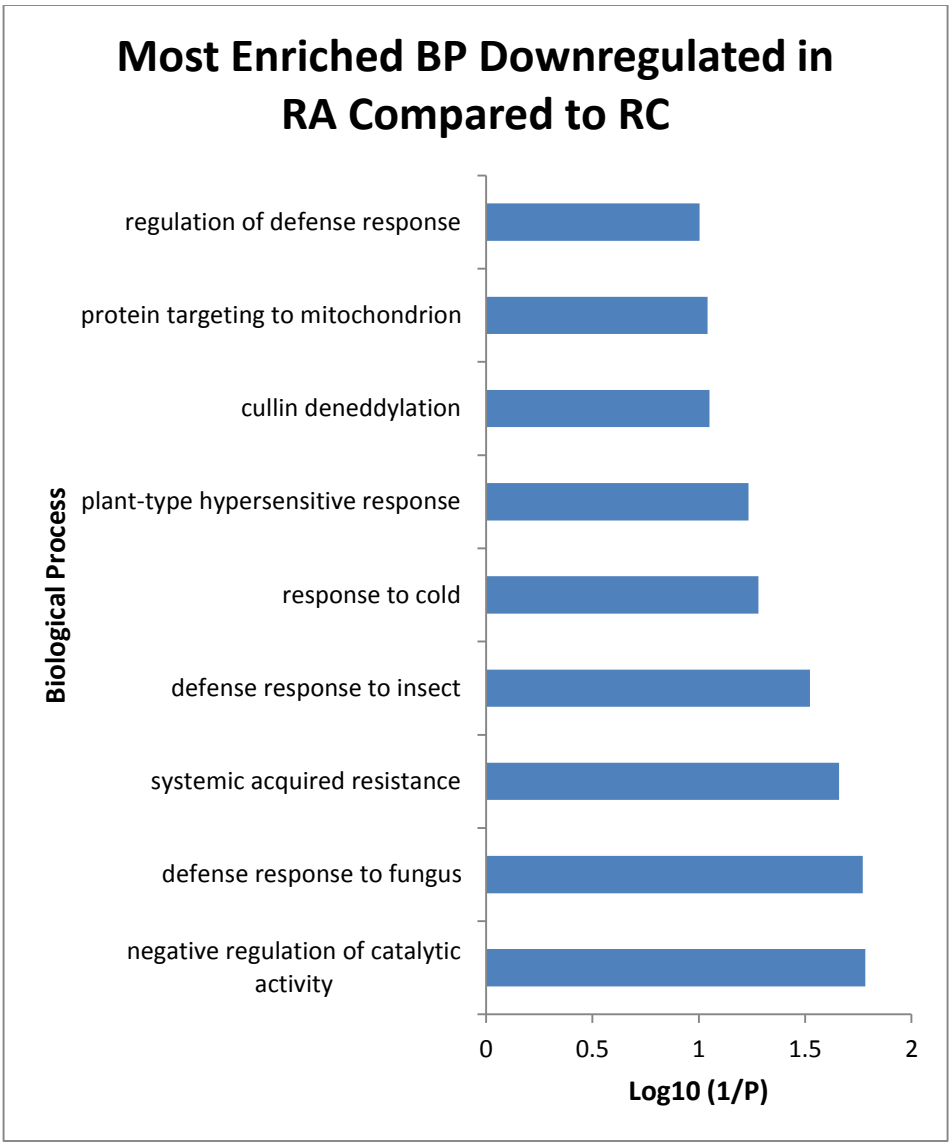

Figure 23: Most Enriched BP Downregulated in RA Compared to RC.

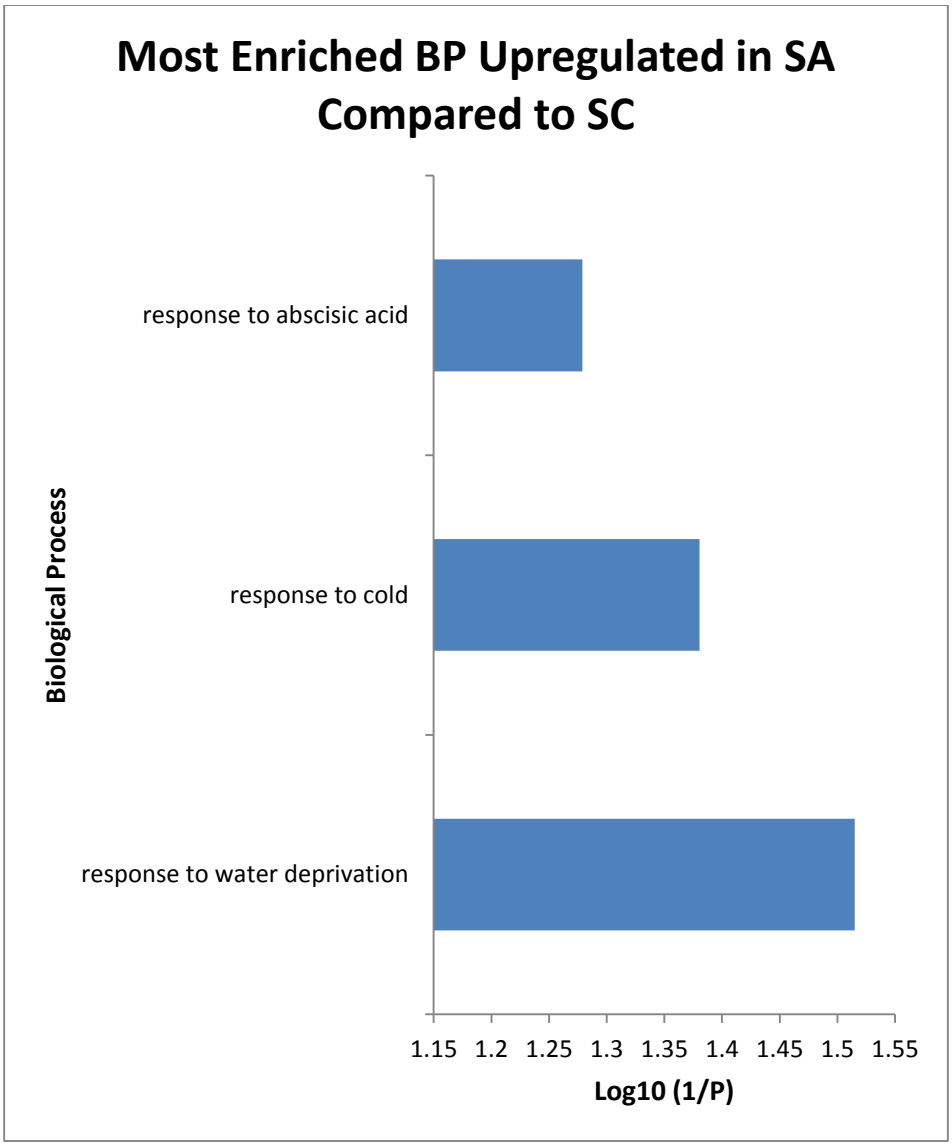

Figure 24: Most Enriched BP Upregulated in SA Compared to SC.

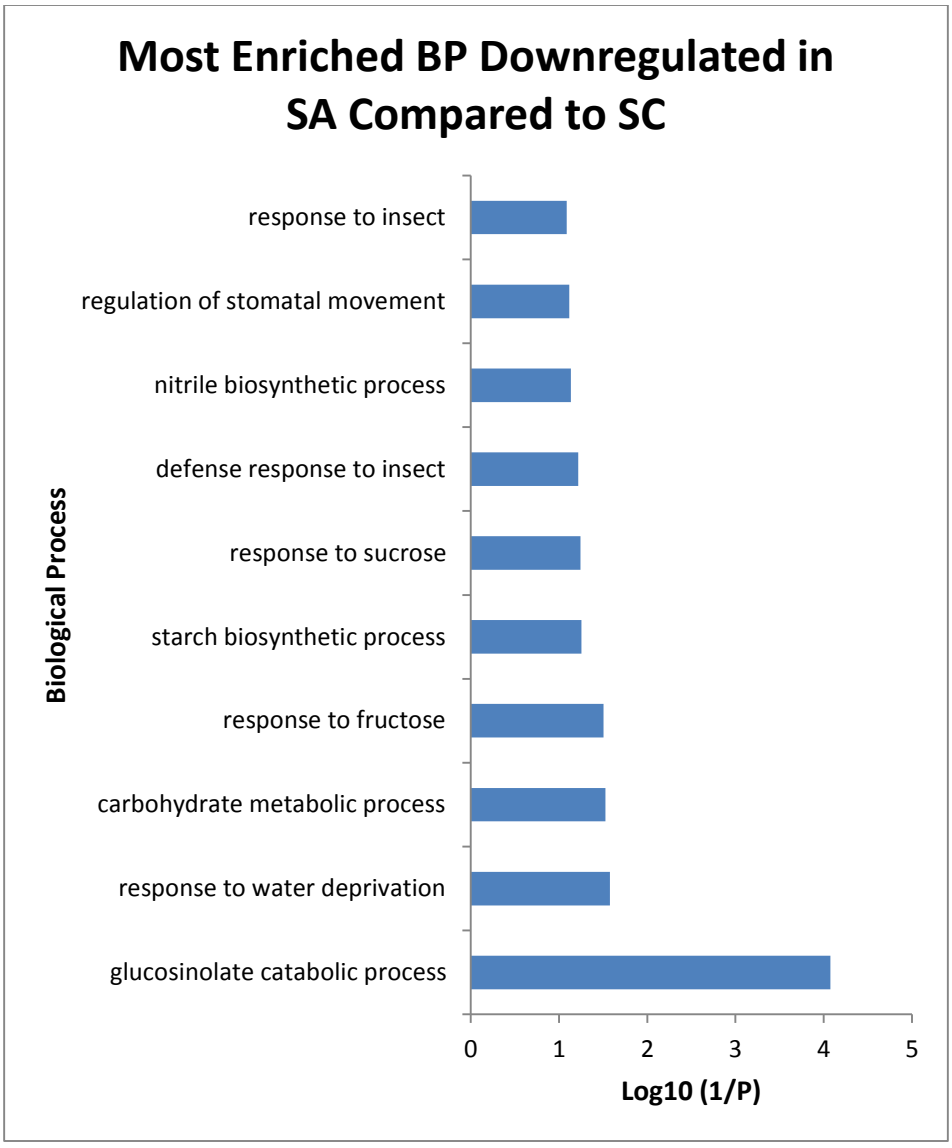

Figure 25: Most Enriched BP Downregulated in SA Compared to SC.

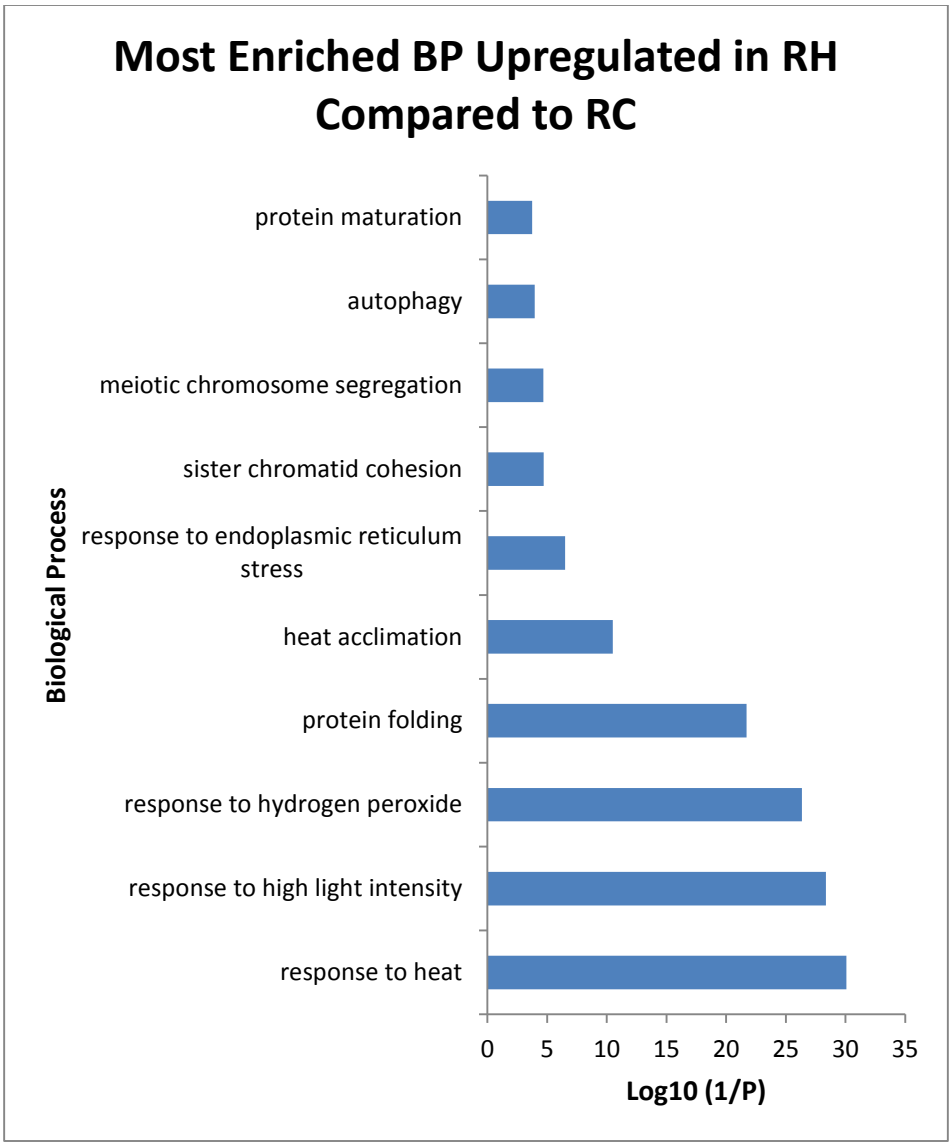

Figure 26: Most Enriched BP Upregulated in RH Compared to RC.

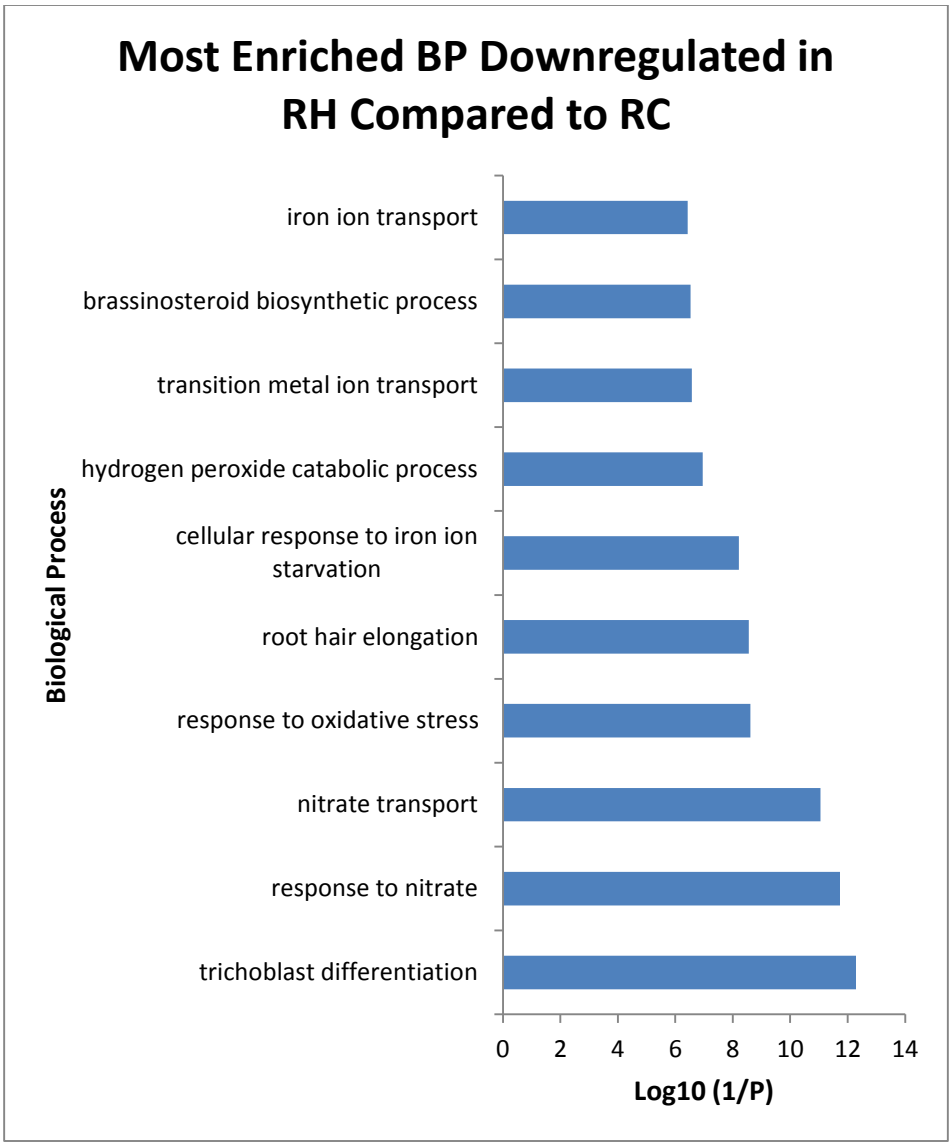

Figure 27: Most Enriched BP Downregulated in RH Compared to RC.

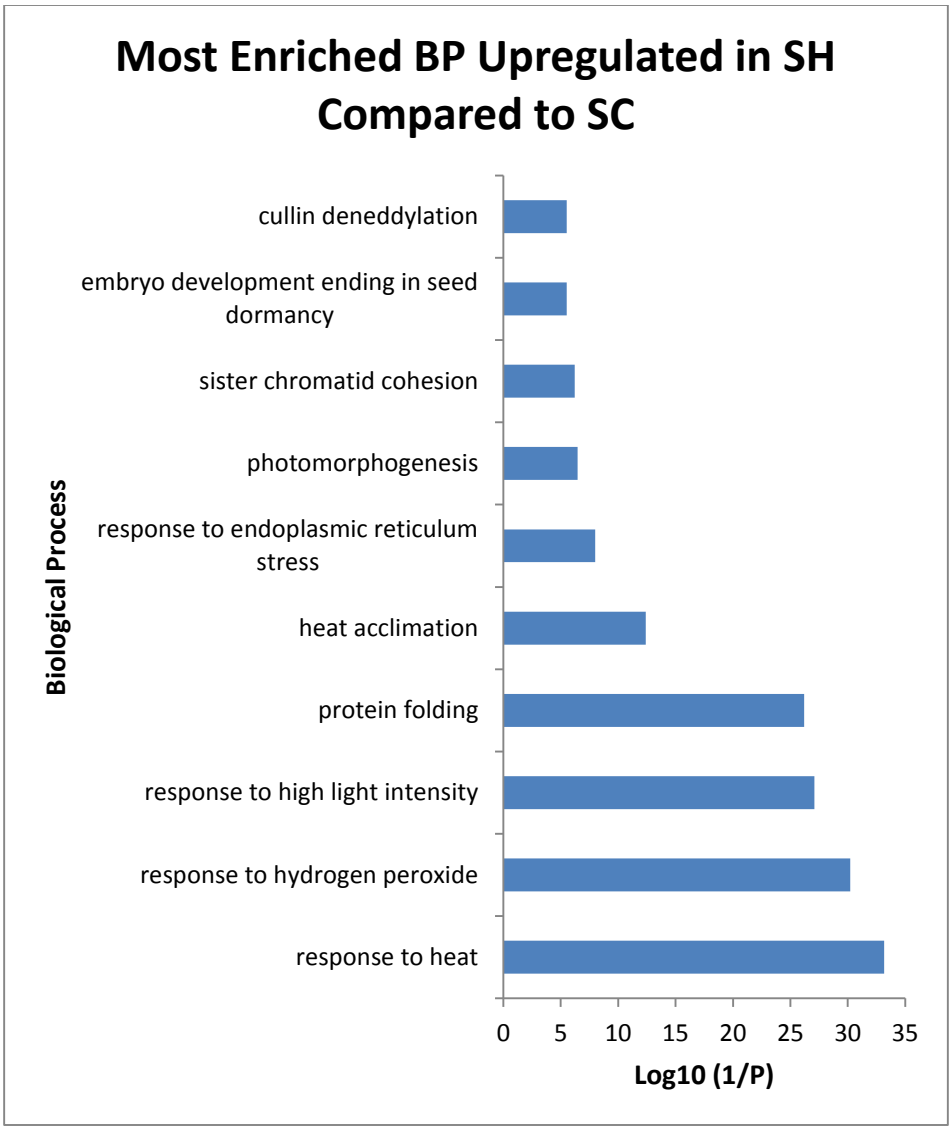

Figure 28: Most Enriched BP Upregulated in SH Compared to SC.

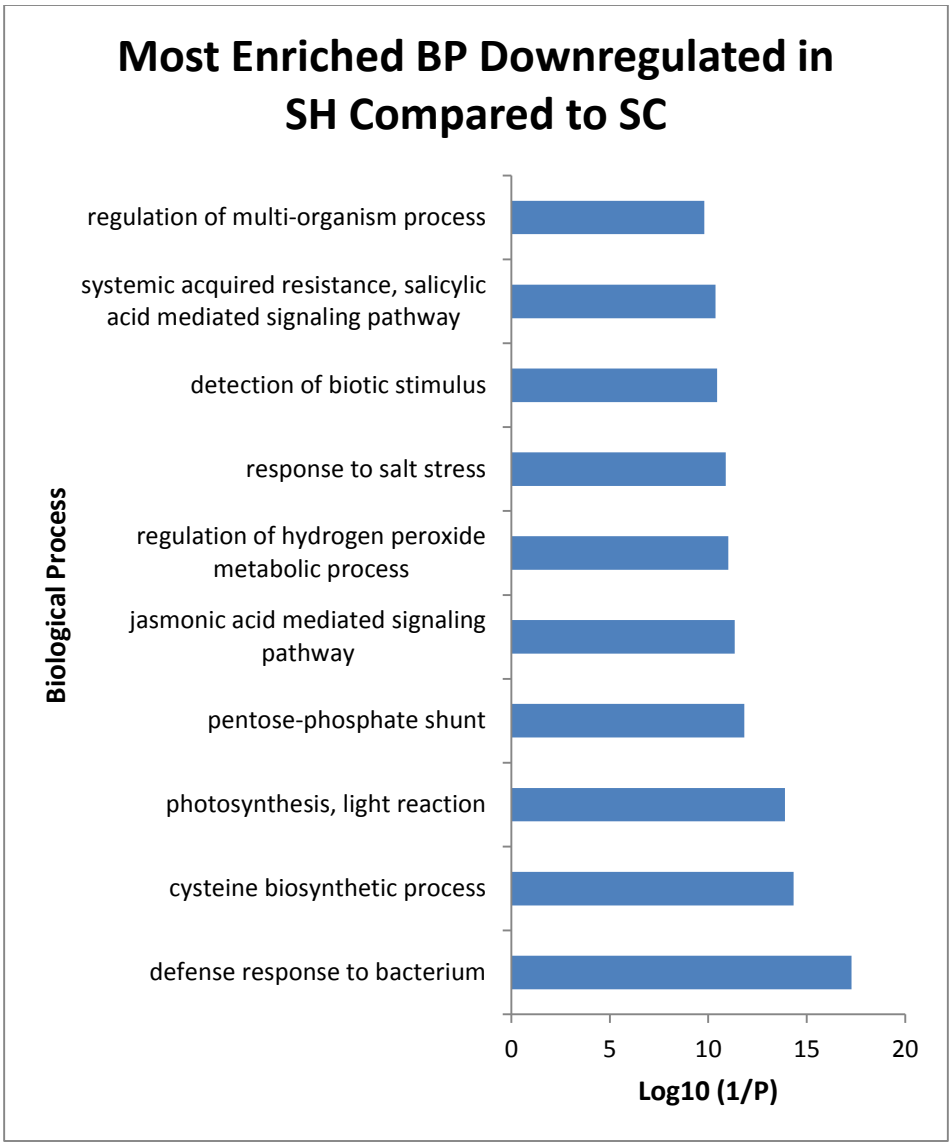

Figure 29: Most Enriched BP Downregulated in SH Compared to SC.

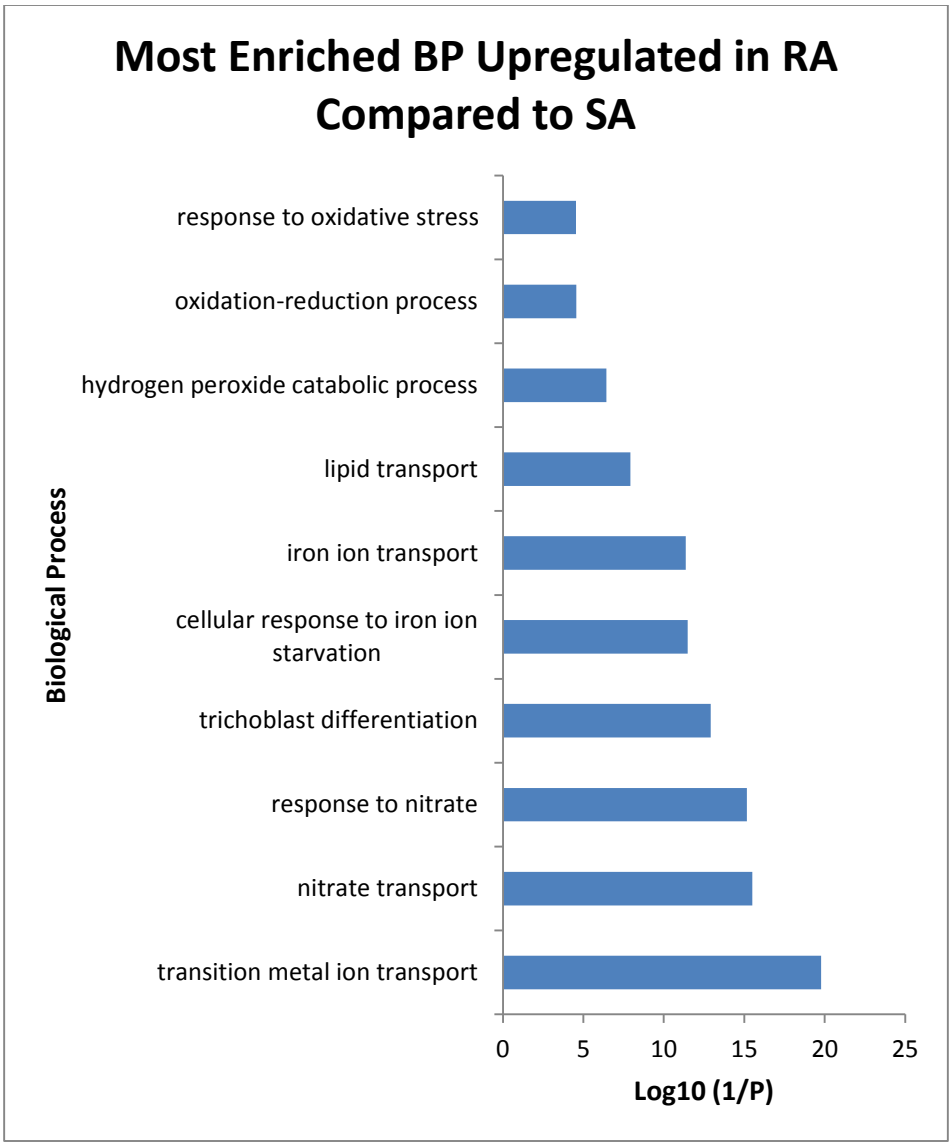

Figure 30: Most Enriched BP Upregulated in RA Compared to SA.

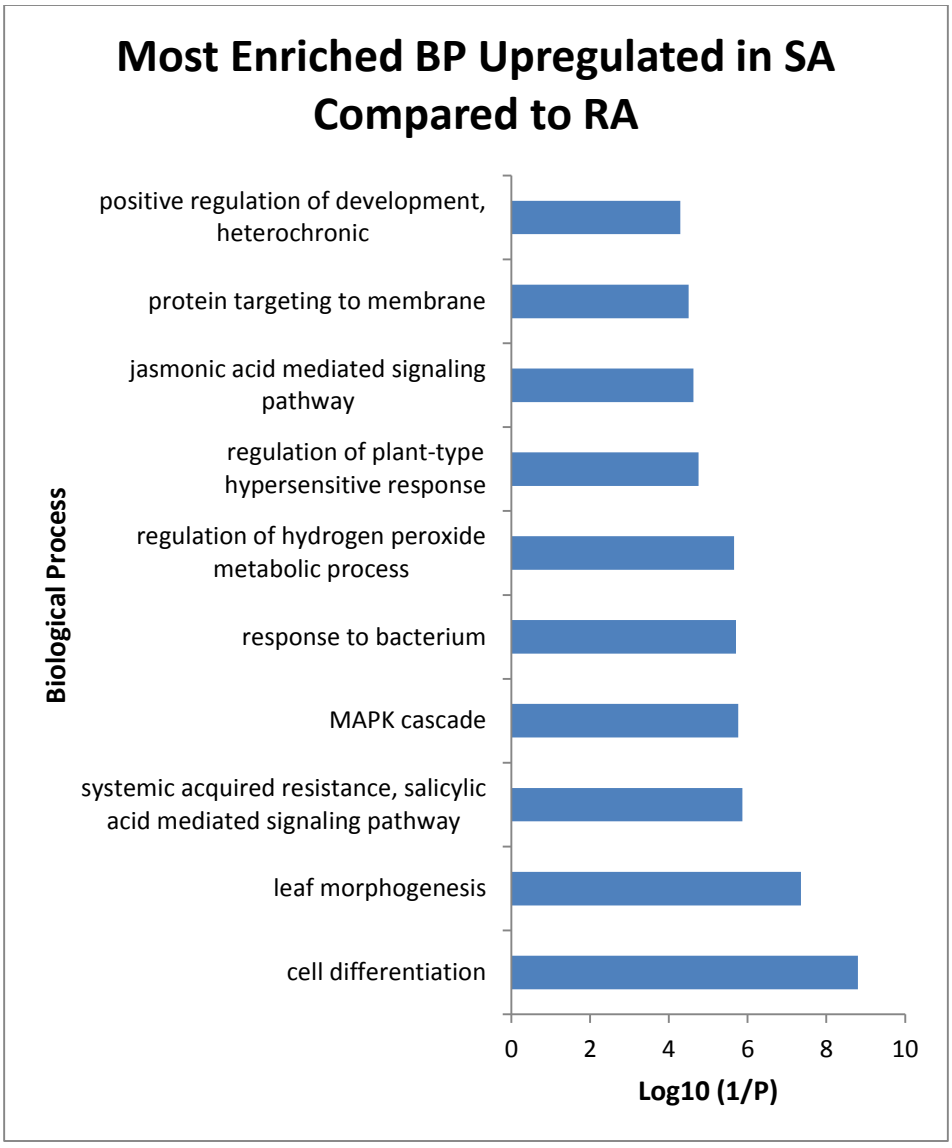

Figure 31: Most Enriched BP Upregulated in SA Compared to RA.

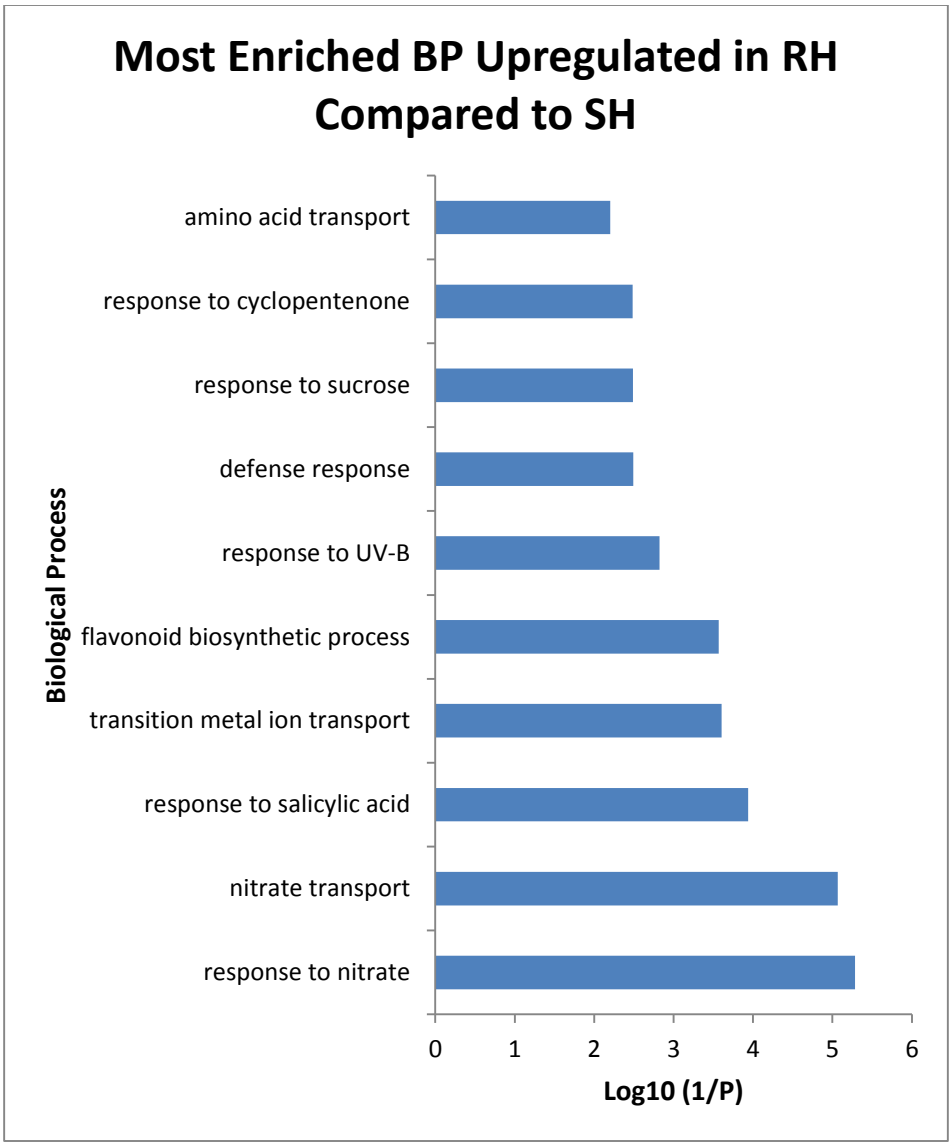

Figure 32: Most Enriched BP Upregulated in RH Compared to SH.

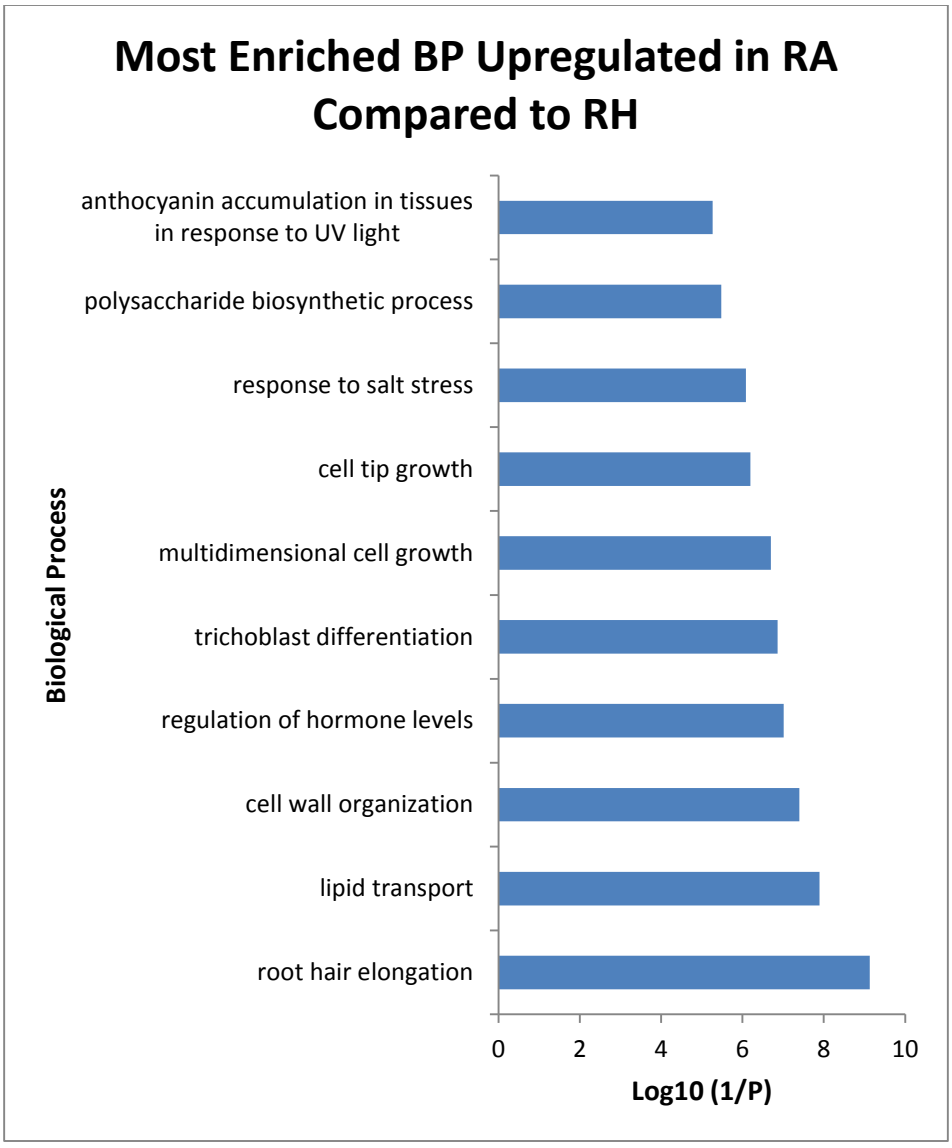

Figure 33: Most Enriched BP Upregulated in RA Compared to RH.

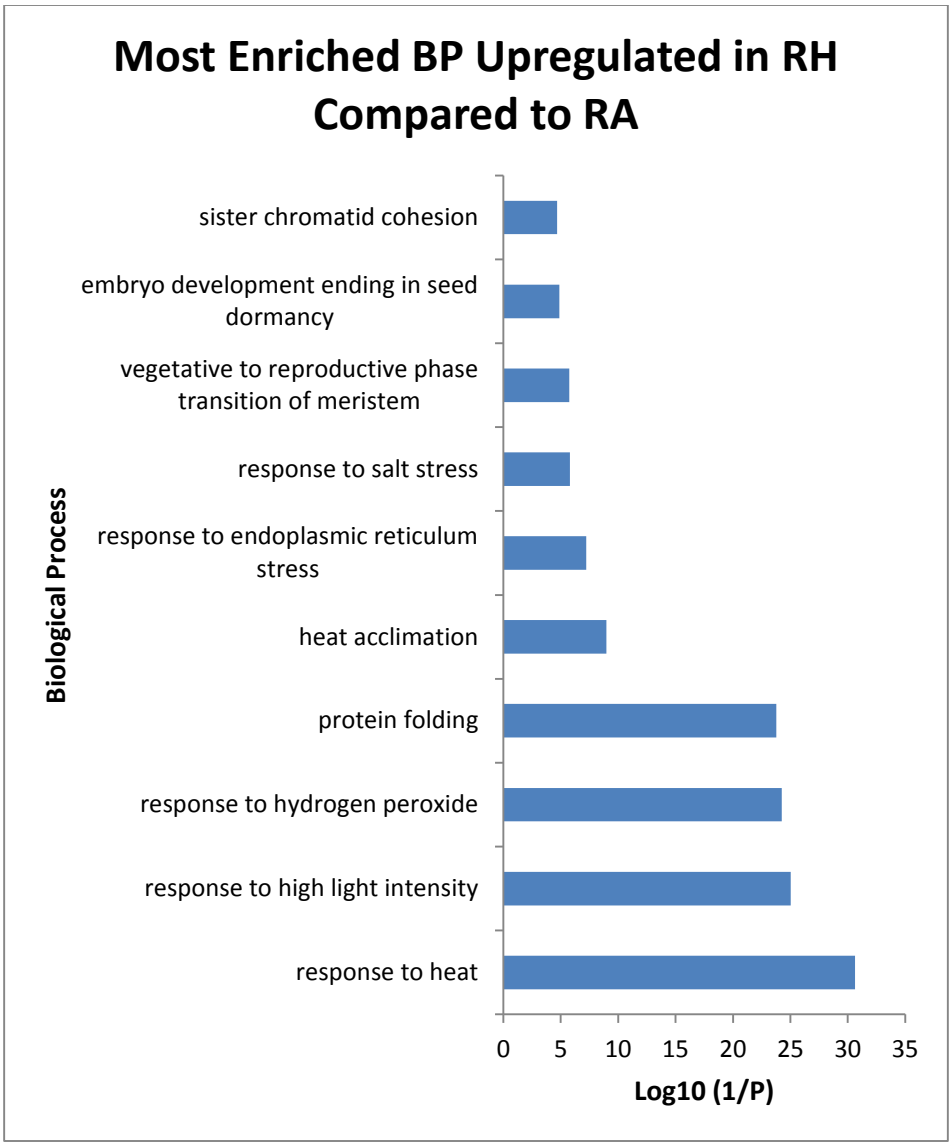

Figure 34: Most Enriched BP Upregulated in RH Compared to RA.

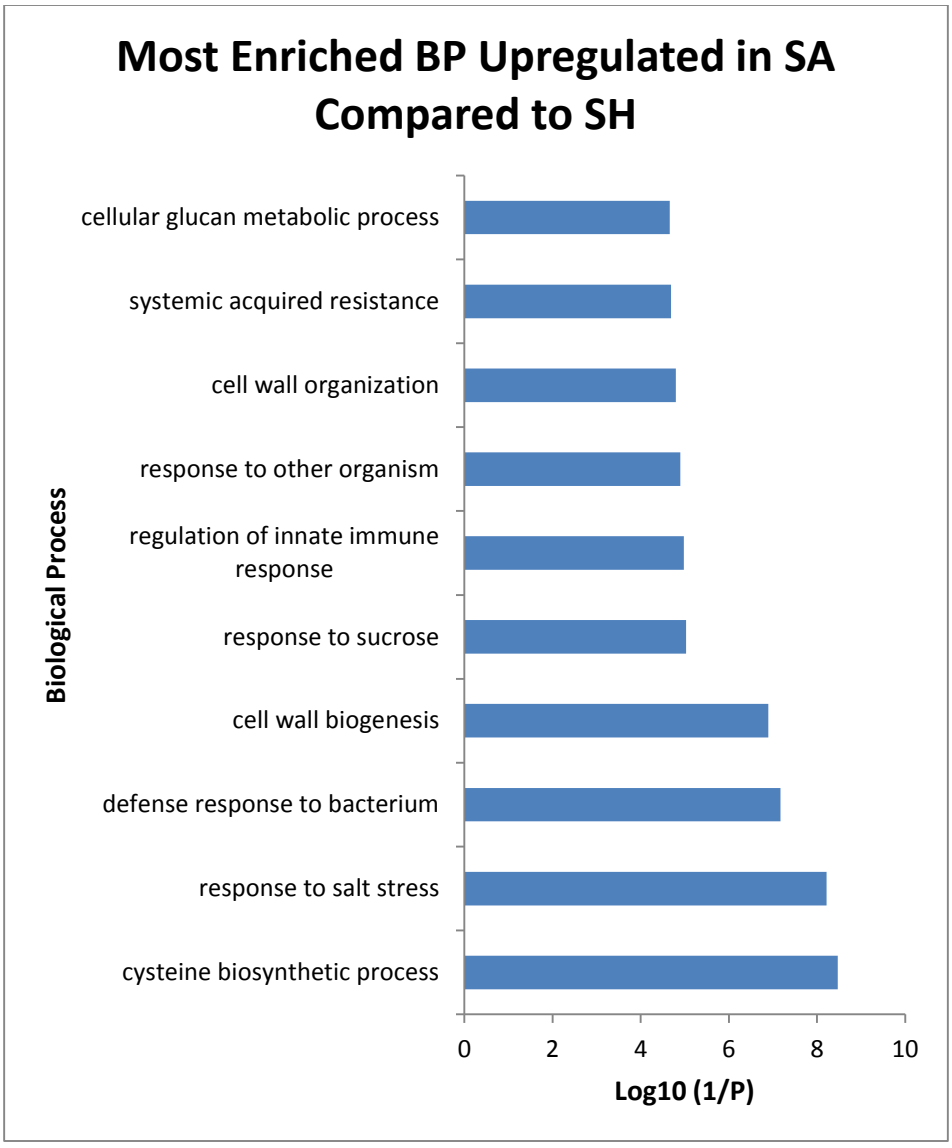

Figure 35: Most Enriched BP Upregulated in SA Compared to SH.

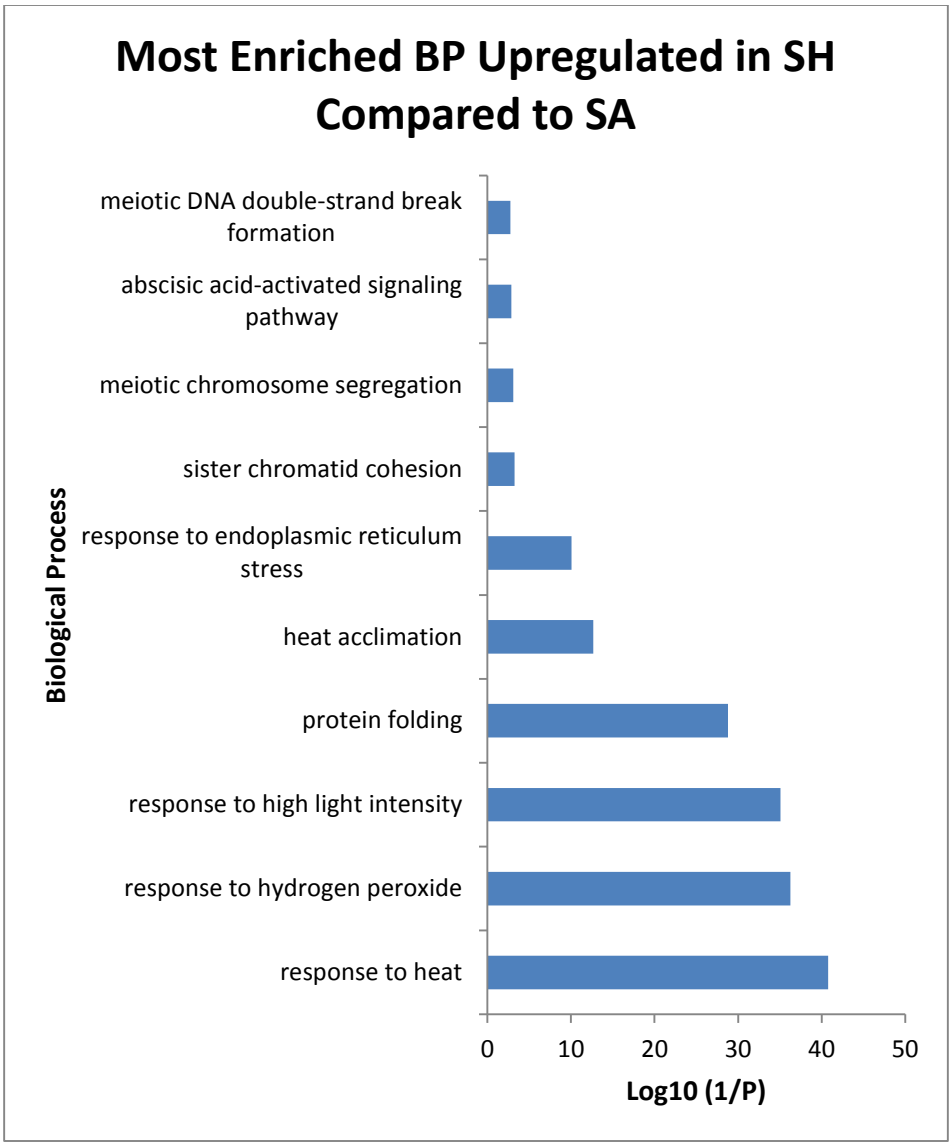

Figure 36: Most Enriched BP Upregulated in SH Compared to SA.

Table 1: The DE transcripts for the 8 pair-wise comparisons.

Pair-wise Comparison	DE transcripts	Up-regulated Transcripts	Down-regulated Transcripts	# of Enriched Clusters
Root ABA vs. Root Control (RAXRC)	50	23	27	8
Root ABA vs. Root Heat (RAXRH)	2236	1548	688	232
Root ABA vs. Shoot ABA (RAXSA)	830	307	523	90
Root Heat vs. Root Control (RHXRC)	1957	669	1288	198
Root Heat vs. Shoot Heat (RHXSH)	185	21	164	24
Shoot ABA vs. Shoot Control (SAXSC)	72	52	20	13
Shoot ABA vs. Shoot Heat (SAXSH)	2197	1292	905	202
Shoot Heat vs. Shoot Control (SHXSC)	2972	1195	1777	256

Chapter 4: Conclusions and Future Directions

Understanding the signal transduction pathways that control the stress response in the extremophile *Eutrema salsugineum* is a crucial step that will facilitate the development of stress resistant crops using genetic engineering technique. The KAUST produced RNA seq profile of the plant exposed to heat stress and exogenous ABA was analyzed to investigate the up-regulated and down-regulated genes and more importantly biological processes in response to the environmental stressors. *De novo* assembly of the RNA seq produced transcripts was performed. Differential expression analysis of the normalized transcript counts was carried out using 8 pair-wise comparisons. The differentially expressed genes were functionally annotated and clustered to find the enriched terms.

The response of the plant root to ABA was found to highly regulate genes involved in the response to water deprivation, ABA signaling pathway, lipid transport, and plant dormancy. The response of the plant shoot to ABA also involves water deprivation response, response to zinc and secondary metabolites related genes. The response of the plant root to heat mainly involved genes encoding heat shock proteins and ROS scavengers. The same genes were involved in the plant shoot response to heat in addition to nitrogenous compounds. When comparing the response of the root and the shoot to ABA, genes related to the production of secondary metabolites, ROS scavengers, and lipid transport proteins were differentially expressed. The heat response in the root and shoot was found to be differentially regulated by genes involved in salt stress response and the phenylpropanoid pathway. The genes that were differentially expressed between the two treatments, ABA and heat, in both organs were related to the response to heavy metals, the response to heat, and the response to oxidative stress. In general, it was concluded that heat induces a much higher differential expression in the plant compared to ABA treatment. The effect of the heat stress is also more widespread and involves a higher variety of genes. The shoot system appears to accumulate a wider variety of secondary metabolite in response to environmental stress.

Further investigation of the role of secondary metabolites such as flavonoids, and nitrogen and sulfur containing compounds in the abiotic stress response of *E. salsugineum* is needed since it appears to be a major mechanism used by the plant. The results of this research

offer a wide variety of stress related protein in *E.salsugineum.* Investigation of the over-expression of some of these genes in stress sensitive plants will help in further understanding their functions and mechanisms of action. The unknown functions of the proteins Ole eI and scorpion like proteins in stress tolerance are to be further investigated as well since they were shown to be differentially expressed in response to heat stress.

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