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A TRANSCRIPTOMICS APPROACH TO STUDY GENES EXPRESSED UNDER SALT STRESS IN *CAMELINA SATIVA* VAR. *SUNESON*

MARIO AMIN SOLIMAN
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Reviewed and approved* by the following:

Dr. Shobha D. Potlakayala
Assistant Professor of Biology
Thesis Supervisor

Dr. Sairam V. Rudrabhatla
Associate Program Chair of Biology
Faculty Reader

Dr. Ronald Walker
Director of the Honors Program
Honors Advisor

* Signatures are on file in the Schreyer Honors College

ABSTRACT

Agricultural production and quality are adversely affected by various abiotic stresses including cold, drought, salinity, radiation, and light intensities. The influence of stress has a direct impact on yield and productivity of crop plants particularly during vegetative plant growth. Biotechnological intervention that augments traditional/conventional breeding has yielded crops with improved input and output traits. In the present study, a transcriptomics approach has been taken to study the gene expression of selected genes during salt stress in *Camelina sativa*.

To study the transcriptome of the stress response, two-week old *Camelina sativa* seedlings were exposed to different time regimes of salt stress. Total ribonucleic acid was extracted from the treatments and complementary deoxyribonucleic acid was synthesized. Reverse transcription polymerase chain reaction was performed to check the presence of eight stress-related genes expressed in *Camelina* and a housekeeping gene. This was followed by a detailed transcriptomic analysis by looking at relative changes in gene expression of all the chosen genes using a quantitative reverse transcription polymerase chain reaction. Results showed various trends of gene regulation, indicating their significance in salt stress response in *Camelina*. Further analysis from this study will lead to a deeper understanding of molecular mechanisms involved in salt stress and also identify potential gene(s) for genetically engineering salt tolerance into this economically valued biofuel crop.

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Chapter 1: Introduction

1.1 General introduction

Agricultural production and yield are adversely affected by various environmental challenges such as drought, salinity, extreme temperatures, light intensities, and radiation (UV). Plant response to stress becomes most critical during two stages of the plant life cycle, the seed germination and reproductive phases. Plants have developed innate mechanisms through the course of their evolutionary history due to targeted selection response to abiotic and biotic stress at cellular and metabolic level (Tuzhikov et al 2011). Estimates of crop losses due to abiotic and biotic stresses can amount to billions of U.S. dollars worldwide. Since 2013, the agricultural industry in California alone, the top U.S. agricultural producer, lost $44.7 billion due to crops lost to drought (Campbell, 2014). Abiotic stress is the basic principle cause of crop yield loss worldwide, reducing crop yield by 50%, which inevitably will lead to an economic and financial loss.

Additionally, according to the United States Census Bureau, the working-age population (18 to 64) is expected to increase by 42 million between 2012 and 2060, from 197 million to 239 million. The alarming increases in population, especially in developing countries, poses an urgent need to develop complementary technologies to plant breeding, thus allowing for increasing world food output and reducing food insecurity by minimizing crop loss due to abiotic and biotic stress (Chrispeels, et al. 2000). Recent advances using the modern tools of recombinant DNA technology have allowed farmers to grow new plant varieties with improved traits to meet environmental challenges and consumer needs of 21st century.

The promising recombinant DNA research from public-private partnerships marked a significant turning point in agricultural biotechnology for crop improvement. Since the first introduction of *Bt* (*Bacillus thuringiensis*) in major food crops, such as soybean, cotton, and corn, there has been dramatic increases in the planting and production of transgenic crops worldwide. The successful outcomes through genetic engineering have resulted in isolating and manipulating genes from diverse and exotic source techniques and incorporating into crop species. Some of the examples of commercial products from biotechnology include crops with resistance to pests and diseases, abiotic and biotic stress, improving nutritional and post-harvest quality (Godliving Y.S. Mtui et al. 2011).

Besides population, the challenge of climate change over the next decade poses a serious threat to agriculture production worldwide (Hatfield et al 2011). Global warming, leading to changes in temperature and availability of water to plants, can have a significant impact on the overall plant growth and development. Introduction of traits for developing stress tolerant crops can be accomplished through conventional breeding, marker assisted breeding, and through genetic engineering. The conventional breeding approach has not been very successful largely due to breeding being a slow and long-term process and the stress resistance in plants being influenced by multigenes. The availability of whole genome sequences for model crops and some crop plants in the past decade has allowed for rapid testing of candidate genes and transcription factors for abiotic stress tolerance by up/down regulating them in homologous and heterologous systems. Research has shown a vast array of transcription factors showing promising response to various abiotic stresses. These include *bZIP* (Basic leucine Zipper),

DREB/ERF (Dehydration responsive element binding and ethylene responsive element binding protein), *MYB* (myleocytomatosis oncogene), *NAC* (N-terminus DNA-binding domain) and *WRKY*, *ZF* (Zinc finger) (Reugera et al. 2012).

As described earlier, the intrinsic response of plants upon exposure to various abiotic stress will lead to physiological changes, and changes at both the cellular and metabolic level. This will lead to plants synthesizing defense proteins such as *LEA* (late embryogenesis abundant proteins), osmolytes such as proline, glycine betaine, trehalose, polyamines and hormones such as abscisic acid and methyl jasmonate.

Chapter 2: Literature Review

2.1 What is abiotic stress?

2.1.1 Cold/ Frost

Low temperature can cause a significant impact on the growth and development of the plant. A cascade of proteins, metabolites, and genes have been shown to express under cold stress causing dehydration and membrane damage due to accumulation of ice crystals (Khan et al. 2015). Cold stress in some of the tropical fruit crops leads to the accumulation of proline and endogenous nitric oxide levels. The exposure of cold stress to plants will lead to detrimental phenotypes with symptoms such as reduced leaf expansion and wilting, chlorosis (yellowing of leaves), necrosis (death of tissue), poor germination, and stunted seedlings. The reproductive development of the plant is also hindered from its normal activities under exposure to cold/chilling stress (Jenks et al. 2005).

2.1.2 Drought

Among the various abiotic stresses that could potentially impact plants during its life cycle, drought stress is most complex and devastating on a global scale as a result of the climate changes. It is expected by 2025 there will be a 30% global crop production loss as result of shortages of water (Khan et al. 2015). Drought in crop plants leads to a wide phentotypic and genotypic variability with a major effect on the reduction of growth in the plant. As water deficit conditions progress, the leaves of the plant begins to change color (blue-green), indicating the presence of the stress leading to crop damage.

Tolerance to drought is a quantitative trait, with a complex phenotype, often confounded by plant phenology. In drought conditions, the roots of the plant exhibit a

decrease in water potential. Upon further exposure, the plant will accumulate abscisic acid and eventually stomatal closure occurs. The closure of the stomata reduces the plant's leaf relative water content. After drought exposure, the plant will exhibit drought stress after a certain amount of time that is dependent on the stage of growth, plant species, and the water-holding capacity of a plant. As a plant is subjected to water deficit conditions, osmotically active compounds such as amino acids and sugars start to accumulate and lead to a decrease in osmotic potential. This leads to production of reactive oxygen species (ROS) such as superoxide, singlet oxygen, hydrogen peroxide, hydroxyl radicals leading to plant death. Osmotically active amino acids that are up-regulated include proline and glycine betaine. The osmotic adjustment allows a plant to absorb water, maintain turgor, and survive longer. In addition to the osmotic adjustment, some plants, such as Sorghum, are coated with a thick epicuticular wax allow the plant to retain their leaf turgor for longer periods of time after the initial exposure of drought stress (Jenks et al. 2005).

2.1.3 Heat

Prolonged high temperatures or heat stress, a global threat to agricultural crop and yield, causes various morphological, anatomical, physiological, and biochemical changes in plants. (Jenks et al. 2005). Heat stress causes severe cellular injury causing cellular apoptosis, affecting the survival of plants. High temperatures induce primary effects including protein denaturation and aggregation, and increased fluidity of membrane lipids. Upon further exposure, secondary effects, include enzyme inactivation in chloroplast and mitochondria, protein degradation, inhibition of protein synthesis, and loss of membrane integrity. Some additional heat-stress effects include a decreased of

rate of growth, reduced ion flux, and production of reactive oxygen species (ROS), which are reactive compounds (Jenks et al. 2005).

After immediate exposure to heat, the plant begins the expression of heat stress-related genes as means of defense; some of the proteins include the heat shock proteins. Heat shock proteins (HSP) are a family proteins produced by cells in response to heat stress; the HSP family are 10 to 200 kDa (Jenks et al. 2005).

2.1.4 Salt stress effects on plant survival, growth, and development

Salinity is one of the most complex developmentally regulated traits limiting the productivity of agricultural crops in approximately 830 Mha worldwide (Rengasamy et al. 2006), with adverse effects on germination, plant vigor, and crop production. Salinity has been a threat to the agricultural crops and continues to effect 20% of all cultivated land (Jenks et al. 2005). The poor water quality of irrigation channels continues to jeopardize crop yield leading to a substantial financial loss. Increased salinity and sodium levels cause ionic imbalance and affects lipid and protein composition in the cell membrane and the cytosol of plant, thus leading to the overall impact on plant growth and development. Current research efforts are focused toward identifying salt tolerance genes, the role of nitric oxide as a signaling molecule triggered in response to salinity stress on plant growth and development. After cellular receptors recognize induced salt stress, stress responsive genes are expressed resulting in the generation of secondary messengers, ROS, and inositol phosphates (Khan et al. 2015).

2.1.4.1 NaCl causes both ionic and osmotic stress

The apoplast in plants is the free diffusional space outside the plasma membrane including the cell wall, intracellular spaces, and the space within dead structures such as xylem and phloem. Apoplasts with high salt concentrations result with primary and secondary effects that can negatively impact the plant's survival, growth, and development. The primary effects presented by highly concentrated apoplasts include ionic toxicity, disequilibrium, and hyperosmolality. At high concentrations, Na^+ and Cl^- can inhibit cytosolic and organellar activity, therefore proper concentrations are required for normal cellular activity (Jenks et al. 2005). As concentrations of Na^+ and Cl^- increase to 0.4M or greater, enzymatic activities are inhibited due to change in the protein structure. The structure of a protein is dependent on hydrophobic-electrostatic balance, which is disturbed at high concentrations of Na^+ and Cl^-, therefore appropriate Na^+ and Cl^- concentrations are essential. Additionally, at low concentrations of 0.1 M, Na^+ can be toxic to cells; this is an indication of its direct effect on physiological and biochemical activity (Jenks et al. 2005). In addition to ionic toxicity, high salt concentrations can lead to a decrease in water potential causing a low state of turgidity, also known as hyperosmotic shock. A reduction in turgor impedes cellular expansion. A significant decrease in apoplastic water potential leads to dehydration as water loss is increased with negative pressure.

2.1.4.2 Secondary effects of salt stress

Fluctuation of NaCl introduces a manifold of secondary effects that include but not limited to potassium (K^+) acquisition, membrane dysfunction, impairment of photosynthesis, generation of reactive oxygen species (ROS), and cell apoptosis (Jenks et

al. 2005). K^+ is an essential plant nutrient and is required for plant growth and reproduction. Potassium plays a vital role in activation of enzymes, ATP production, osmo-regulation, protein synthesis, and photosynthesis by regulating the stomata. Sodium uptake is mediated by voltage-dependent channels and independent cation channels. Na^+ competes with K^+ uptake through sodium-potassium co-transporters, particularly during unequal ionic concentrations. At the appropriate concentration of sodium, a plant is functional and healthy at a molecular level, however, high sodium concentrations can lead to cell toxicity, which can be alleviated Ca^{2+} via regulation of K^+/Na^+ selective accumulation (Jenks et al. 2005).

Additionally, hyperosmotic and ionic stresses can cause secondary metabolic effects that if left unaddressed, may impede plant growth and development. For example, salt stress causes oxidative stress, one of many consequences, and increases reactive oxygen species, such as superoxide radicals, hydrogen peroxide (H_2O_2), and hydroxyl radical, which are all produced in aerobic cellular processes during photorespiration (Jenks et al. 2005).

2.1.4.3 Plant adaptations to NaCl Stress

Halophytic, plants that are native saline environments, and glycophytic plants use various cellular processes to tolerate salt. Other plants that are not able to withstand salt stress develop adaptive morphological structures that allows them to avoid salt; some morphological structures include bladder on leaves that serve as salt sinks or reservoirs (Jenks et al. 2005). However, even plants that cannot tolerate salt proceed through conserved physiological processes upon exposure to salt conditions.

Researchers are focused to understanding the mechanisms by which halophytes

acquires in order to tolerate high saline environments. Through researching extreme halophytes such as *Salicornia* and *Sueada*, it was determined many salt-adaptive features within the genes responsible for stress tolerance (Jenks et al. 2005). Further understanding of salt stress genes was attained from studies on wheat and tomato and their halophytic relatives. Additionally, further research with glycophytes such as tobacco and Sorghum plants proved that glycophytes have the capabilities to adapt to saline environments. Collective research concluded that all plants, halophytes and glycophytes, are able to tolerate and adapt to saline environment to an extent.

2.2 Gene Regulation in Plants

Gene regulation in plants occurs in many different ways. Transcriptional regulation, histone modifications, epigenetics, and post-transcriptional regulation all contribute to genetic regulation in plants. *MYB, BZIP*, and *WRKY* transcription factors also contribute to genetic regulation in plants (Watson et al. 2014).

Gene regulation occurs through transcription. Transcriptional regulation can occur using cis-acting elements or trans-acting factors. Cis-acting elements are DNA sequences located closely to the gene that is being expressed. There are five important types of cis-acting elements: promoters, terminators, enhancers, silencers, and insulators. Promoters are binding sites on DNA where transcription factors attach. Promoters start the transcription of a gene and can increase expression levels. Examples of promoters include the CAAT box and the TATA box. Terminators are sequences that cause RNA polymerase to dissociate from the template DNA strand and release the newly synthesized RNA chain. Terminator sequences are usually poly-adenylation signals, with repeats of AAUAAA. Enhancers are sequences that can bind with activators to activate

the transcription of a gene or enhance the transcription of a gene. Silencers are sequences that reduce or turn off expression of a gene. Silencers cause genes to either have decreased expression or no expression at all. Insulators are sequences that "block activation of the promoter by activators bound at the enhancer" (Watson et al. 2014). Insulators can cause decreased expression of a gene or no expression of a gene. Trans-acting factors are proteins that bind to cis-acting elements in order to control expression. Trans-acting factors cause genetic expression at specific times and in specific tissues. Trans-acting factors have two domains, which are required to bind to DNA and activate transcription. There are four important motifs that trans-acting factors use: helix turn helix, zinc finger, leucine zipper and helix loop helix. The helix turn motif has two helices which press against the DNA. The recognition helix "fits into the major groove of DNA" while the other helix fits against the top groove of DNA (Watson et al. 2014). The zinc finger motif has an alpha helix and zinc fingers, which fit against the DNA. Each zinc finger "inserts an alpha helix into the major groove" of the DNA while the zinc ion "interacts with cysteine and histidine residues and serves a structural role" when binding with DNA (Watson et al. 2014). The leucine zipper motif consists of two helices that both insert into the major groove of DNA. These two helices also form a protein dimer. The helix loop helix has two helices connected by a loop, which form a dimer. Each of these motifs directly affect the expression levels of particular genes. If an improper motif is used on a gene, expression can be reduced or no expression may occur. If the proper motif is used on a gene, enhanced or regular expression of the gene may occur.

Moreover, gene regulation is a post-transcriptional regulation. The primary example of post-transcriptional regulation is RNA splicing. RNA splicing is a process in

which introns are removed and exons are linked together after transcription has occurred. Alternative splicing is the process in which pre-mRNAs can be spliced in multiple ways. Alternative splicing can occur in five ways. First, introns can be regularly excised and exons are ligated together. Second, exons can be skipped. Third, “exons can be extended by selecting an alternative downstream 5’ or upstream 3’ splice site” (Watson et al. 2014). Fourth, some introns can be retained and pasted to exons. Fifth, alternative exons can be derived from alternative Poly-A sites. Although the gene number remains the same, many different protein products can be produced by splicing the pre-mRNA in a number of different ways (Watson et al. 2014). Alternative splicing regulates gene expression by producing different protein products. Different protein products affect cellular function and development.

Additionally, gene regulation occurs through histone modification. Histones are proteins that DNA wraps around and subsequently control gene expression through chemical and/or physical changes to DNA. Histones can experience the addition and removal of acetyl, methyl, phosphoric, and ubiquitin groups, which can lead to changes in gene expression. Histone acetyltransferases (HATs) are enzymes that cause an acetyl group to be added to a histone. Acetylation of a histone generally increases the transcription of a gene. The acetylation of histone tails creates euchromatin, a less tightly packed form of chromatin. Acetyl groups also help bind transcription factors to DNA by creating specific binding sites, known as bromodomains. Histone deacetylases (HDACs) are enzymes that remove an acetyl group from a histone (Watson et al. 2014). Deacetylation of histone tails creates heterochromatin, a tightly packed form of chromatin. Deacetylation causes decreased expression of a gene and can lead to silencing

in some cases. “Methylation of DNA by enzymes called DNA methylases” can lead to transcriptional silencing (Watson et al. 2014). Methylation can inhibit protein binding which can lead to silencing. Histone phosphorylation can lead to transcriptional activation. Phosphorylation can activate gene expression by stimulating HATs to acetylate genes. Finally, ubiquitination can lead to increased gene expression for some genes and decreased gene expression for other genes. In some cases, ubiquitination can lead to the creation of euchromatin, or can signal for transcription factor recruitment, which leads to enhanced transcription of a gene. In other cases, ubiquitination can lead to the creation of heterochromatin or can lead to restriction of SR proteins, which can lead to transcriptional silencing.

Epigenetic regulation occurs when environmental factors influence genetic expression. Methylation can occur through epigenetic regulation. “Maintenance methylases modify hemimethylated DNA” and cause gene silencing after DNA replication has occurred (Watson et al. 2014).

The *MYB* transcription factors have three imperfect repeats of 53 amino acids and a helix turn helix binding domain. *MYB* transcription factors contribute to many different functions in plants, such as metabolism, development, stress signal transduction pathways and the regulation of cell morphogenesis. One specific example of a *MYB* transcription factor interaction involves cold stress (Watson et al. 2014). MYB transcription factors are up-regulated by cold stress and subsequently increase expression of *CBF* genes.

The *bZIP* transcription factors have a sixteen amino acid region with heptad repeats of leucines. These transcription factors regulate pathogen defense, light and stress signaling, seed maturation and flower development. One specific example of a *bZIP*

transcription factor, OsAB15 in rice, is caused by salt stress (Jenks et al. 2005).

2.3 Proteins

2.3.1 Kinases

Kinases, an enzyme that catalyzes the transfer of phosphate groups, is specific to the substrate in which it binds to; the process is known as phosphorylation. Conversely, the reverse process is called dephosphorylation (Watson et al. 2014). Kinases, a part of the phosphotranseferases, are an important class of enzyme-dependent metabolic pathways, such as glycolysis. In glycolysis, phosphorylation occurs four times. Additionally, kinases are essential in gene regulation. In regards to plants, much like animals, kinases allow the plant to increase or decrease the expression of particular genes that may responsible for stress response (Watson et al. 2014).

The phosphorylation of proteins by protein kinases can directly alter the function of proteins. Phosphorylation of proteins can increase or decrease protein activity, stabilize proteins, or initiate the cellular apoptosis (Watson et al. 2014). Kinases, accompanied by cyclin-dependent kinases, play a pivotal in gene regulation. Cyclin-dependent factors (CDKs) are essential in the regulation of the cell cycle. Cyclin, a protein, mediates the progression of cells through the cell cycle by binding CDKs (Watson et al. 2014).

2.3.2 Transcription Factors

Transcription factors are proteins that bind to specific DNA sequences, which allow it to control the transcription of genetic information from DNA to mRNA. A transcription factor is able to control the transcription by itself or with other proteins, by promoting or blocking the recruitment of RNA polymerase. RNA polymerase is an

enzyme that is required (along with a transcription factors) to form the transcription initiation complex, which is required for the commencement of transcription. In eukaryotes, general transcription factors (GTF) are required for the transcription of all genes. Most GTFs are designated as TFII, TFIIB, and most of them are multimeric proteins (Pahi et al. 2015). In prokaryotes, there is only one general transcription factor, known as sigma factor.

Transcription Factor IID (TFIID)

Transcription factor (TFIID) is composed of 14 subunits including the TATA box binding protein (TBP) and recognizes the promoter and recruits TFIIB. TATA box is making a pseudo-twofold sequence-specific interaction with two threonines and two asparagines of TBP. TFIID coordinates the activities of approximately 70 polypeptides required for the initiation of transcription. Primarily, TFIID serves a channel for regulatory signals and binds to the core promoter, a region that initiates transcription of a particular gene, to enable to the proper positioning of the polymerase (Pahi et al. 2015).

Transcription Factor IIB (TFIIB)

The transcription factor IIB (TFIIB) recruits polymerase II and TFIIF-PolII complex at the transcription start site. Some TFIIB mutants result in a shift of transcription start site. Some TFIIB mutants result in a shift of transcription start site. TFIIB consists of four domain, which are termed the C terminus, the B linker, the B reader, the zinc ribbon. Each one of the four domains interacts with various domains of the RNA Polymerase II, an enzyme that catalyzes the transcription of DNA synthesis (Pahi et al. 2015). The B terminal extends in the grove near the active site. Upon binding of the RNA polymerase II, the B reader and B linker domains undertake a sterio-chemical

change that enabled the binding of an essential second magnesium ion.

Transcription Factor IIF (TFIIF)

This transcription factor (TFIIF) recruits polymerase II to the existing DNA-TFIIB-B complex, positioning the polymerase II over the start site. Some TFIIF mutants result in a shift of transcription start site. TFIIF is encoded by three primary genes, which are general transcription factor IIF polypeptide I (GTF2F1), general transcription factor IIF polypeptide II (GTF2F2), and general transcription factor IIF polypeptide IIL (GTF2F2L) (Kim et al. 2015). TFIIT disables the RNA polymerase II from contacting DNA outside the promoter by binding to RNA polymerase II when the enzyme is already unbound to any other transcription factors (Barnes et al. 2015). Additionally, TFIIF helps facilitate the stabilization of RNA polymerase II while it is in contact with TFIIB.

Transcription Factor IIE (TFIIE)

Transcription factor IIE (TFIIE) is heterotetrameric protein and appears to create the docking site for the transcription factor II H (TFFIIH). TFIIE also modulates TFIIH enzymatic activities. TFIIE is encoded by two genes, which are general transcription factor IIE subunit 1 (GTF2E1) and general transcription factor IIE subunit 2 (GTF2E2). TFIIE has the ability to bind to single stranded DNA due its zinc ribbon motif (Barnes et al. 2015).

Transcription Factor IIA (TFIIA)

The TFIIA is another transcription factor that is required for transcription in vivo. The function of TFIIA is somewhat unclear but is believed to help the other factors to bind. TFIIA is not required for transcription in vitro. TFIIA is encoded by two genes, which are general transcription factor IIA, 1 (GTF2A1) and general transcription factor

IIA, 2 (GTF2A2) (Kim et al. 2015). Additionally, TFIIA interacts with the TATA-binding protein (TBP) to facilitate the formation of the transcription initiation complex. TFIIA also act as a coactivator for transcription factors.

Transcription Factor IIH (TFIIH)

Transcription factor IIH or TFIIH is a multimeric protein that is composed of 9 subunits and has a helicase activity, which unwinds the DNA duplex at the start site, allowing DNA Pol II to bind to the transcription factor template strand. TFIIH has a kinase activity that phosphorylates Pol II in the beginning of elongation. TFIIH consists of ten subunits that include XPD, XPB, p62, p52, p44, p34 and TTDA. The ten subunits of TFIIH forms the core complex of the transcriptions factor (Barnes et al. 2015). XPD is an essential protein that links core complex to the cyclin-activating kinase-sub complex, which is composed of *CDK7, MAT1*, and *cyclin H*). The transcription "bubble" is initiates using the *ERCC2/XPD* and *ERCC3/XBP* subunits, which use helicase and ATPase to facilitate the process (Barnes et al. 2015). TFIIH is part of the transcription initiation complex, thus upon binding, transcription begins.

2.4 Genes Studied

2.4.1 *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*)

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) is a gene that encodes a member of the *glyceraldehyde-3-phosphate dehydrogenase* protein family. The *GAPDH* enzyme, ~37kDa, breaks down glucoses by facilitating the catalysis of the sixth step of glycolysis, which converts glyceraldehyde 3-phosphotase to D-glycerate 1,3-biphosphate. The process occurs in the cytosol of eukaryotic cells via SN2 reactions. Additionally, *GAPDH* been found to partake in uracil DNA glycosylase activity in the nucleus of the

cell. *GAPDH* has been shown to be constitutively and uniformly expressed at high notable levels in most tissues and cells, therefore it is a housekeeping gene (Fang et al. 2015). Researchers use *GAPDH* as a loading control for western blots and control for qRT-PCR.

This housekeeping gene (*GAPDH),* has been shown to be associated with diseases such as degenerative diseases, plexiform neurofibroma, and osteochondritis dessecans. Additionally, *GAPDH* expression has shown a striking increase expression in thyroid and lung cancer tissue indicating its involvement in cancerous cells (Tokunaga, 1987). Using cells from patients with breast cancer, glycolaldehyde, a diose, was shown to inhibit cell growth by inactivating *glucose 6-phosphate dehydrogenase, GAPDH,* copper (Cu), and Zinc (Zn) and often prematurely induces cellular apoptosis (Fang et al. 2015). Patients with Alzheimer's and Huntington's diseases show decreased levels of *GAPDH* expression alluding the intertwining networks between *GAPDH* and the psychological diseases.

2.4.2 Dehydrin (DHN)

Dehydrin (DHN) is a pivotal protein that is expressed in plant response and adaptation to abiotic stresses. The presence of DHN has been characterized by the presence of polar amino acids, such as glycine (Hanin et al. 2015). DHN has been found to accumulate in maturing seeds or during vegetative tissues that have been exposed to salt, drought, and temperature stresses (Shi et al. 2015; Hanin et al. 2015). The DHN structure is comprised of three domains, which are Y, S, and K domains. The K domain is characterized by its amphiphilic a-helix motif and consists of 15 lysine-rich amino acids.

Dehydrin constitutes of late embryogenesis abundant (LEA) proteins, extremely

hydrophilic proteins. DHN proteins, much like LEA proteins, are found among many angiosperm and gymnosperm plant species. Additionally, DHN proteins have been found to have a pivotal role in dehydration-induced cells and help facilitate the recovery of drought-stressed plants (Shi et al. 2015; Hanin et al. 2015). Exposure to drought and salt stress, the production of abscisic acid (ABA) is exacerbated, indicating plant stress. The production of ABA via stress signal transduction pathways ABA increased the levels of DHN (Hanin et al. 2015).

2.4.3 *Heat shock factor A2A* (*HSFA2A)*

The *heat shock factor A2A* (*HSFA2A*) is a transcription factor of the HSP (heat shock protein) family. Although the functions *HSFA2A* has not been thoroughly studied, overexpression of *HSFA2A* was observed under salt, heat, and dehydration stress (Shukla et al. 2015); this indicates that *HSFA2A* is a stress-responsive gene.

2.4.4 *Metallothionen 3* (*MT3*)

Metallothionen 3 (*MT3*) is pivotal in detoxifying excess copper and metals to limit the oxidative damages in the cell. The *metallothionen* protein family, cys-rich, exploit their low molecular weight to bind to heavy metals (Tomas et al. 2014). Additionally, *metallothionen* is believed to play a role in metal homeostasis (Tomas et al. 2014).

2.4.5 *Phenylalanine ammonia-lyase-1* (*PAL1*)

Phenylalanine ammonia-lyase-1 (*PAL1)* is a gene that codes *phenylalanine ammonia-lyase-1*, an enzyme vital to the phenylpropanoid pathway. Phenylpropanoids, a family of organic compounds in plants that are composed of phenylalanine, provide protection to plants from ultraviolet light and pathogens. Tobacco plants over expressing

L-Phenylalanine ammonia-lyase-1 have shown increased levels of cholorogenic acid, which is associated with reduced susceptibility to infection and fungal pathogens such as Cerocospora nicotianae (Jia et al. 2015). Additionally, *PAL1* plays an important role in catabolic (nitrogen and carbon production) and metabolic pathways, including tyrosine metabolism, phenylalanine metabolism, nitrogen metabolism, phenylpropanoid biosynthesis, and finally alkaloid biosynthesis II.

This gene, *PAL1*, has been studied more in gymnosperms, seed-producing plants, for its role in defense responses of pine trees, especially *Pinus taeda.* The *PAL1* gene enables the *Pinus taeda* trees to produce low-weight phenolic compounds under certain abiotic and biotic stresses (Jia et al. 2015). The *PAL1* gene responds to external abiotic stresses by accumulating salicylic acid, and developing microscopically defined hyper-sensitive-response, which was proved to be light dependent.

2.4.6 *Pyridoxal kinase salt overly sensitive 4* (*PLSOS4*)

Pyridoxal kinase salt overly sensitive (*PLSOS4*) is involved in the salt stress signaling pathway (Soda et al 2013). Salt overly sensitive 4 (SOS4) codes for a pyridoxal kinase involved in the pyridoxal-5'-phosphate pathway. This phosphate is an essential component for ligands and enzymes related to particular ion transporters (Shi et. al. 2002). Additionally, it is evident that *PLSOS4* plays a key role in the development of root hairs (rhizoids) in plants (Shi et al. 2002).

2.4.7 *Pathogenesis related protein-1* (*PR1*)

Pathogenesis related protein-1 (*PR1*) plays a role in plant response due to abiotic stress. In particular, *PR1* is responsible for stress response due salicylic acid stress. The gene was observed to express in *Arabidopsis*, a small flowering plant in the Brassicaceae

family, with cauliflower mosaic virus (Jirage et al. 1999). This gene, *PR1*, has been observed to increase when a plant is stressed with salicylic acid. Additionally, *PR1* and *PDF1.2* (*putative defensing-like protein 1.2*) have been used in combination to enhance plant tolerance to jasmonic acid and salicylic acid (Kachroo et al. 2003).

2.4.8 *Salivaricin A modification enzyme* (*SalT*)

Salivaricin A modification enzyme (*SalT*) expression has been observed in rice (*Oryza sativa L.*) under salt stress. In-situ hybridization and gel blots showed increased expression of *SalT* in younger tissue of the rice plant (Gracia et al. 1998). Under abscisic acid (ABA), gibberellic acid, and salt induced-stress, an increased expression was observed indicating *SalT*'s involvement in plant response to salt stress.

2.4.9 *Salinity induced factor V* (*SIFV*)

Salinity induced factor V (*SIFV)* is a hypothetical protein, however, it has been predicted that it is required for the plant's viability (Soda et al. 2013).

2.4.10 *Salinity induced factor VG* (*SIFVG*)

Salinity induced factor VG (*SIFVG)* is a hypothetical protein, however, it is speculated that it is involved in vegetative growth (Soda et al. 2013).

2.4.11 *WRKY transcription factor 70 (WRKY70)*

WRKY transcription factor 70 (WRKY70) plays a key role in plant defense. The gene, *WRKY70*, regulates the plant's response to drought, cold, salinity, and other abiotic stresses including UV and heavy meatal toxicity (Can-fang et al. 2012). *WRKY70* has been used build plant tolerance towards biotic and abiotic stresses. When *WRKY70* is upregulated, enhances a plant's resistance against E. cichoracearum (Li et al. 2006). Conversely, when *WRKY70* is downregulated, the plant's resistance to E. cichoracearum

is inhibited.

2.5 Gene Expression Analysis

The expression of specific genes regulates the function and order of all living cells. Therefore, it is important to quantitatively and qualitatively study gene expression via various laboratory techniques. Throughout the last decade, molecular techniques have drastically advanced resulting in the identification and cure of many diseases. Some of the techniques include northern blotting (RNA analysis), western blot (protein analysis), fluorescent in situ hybridization (FISH), DNA microarray, RNA sequencing, polymerase chain reaction (PCR), reverse-transcription polymerase chain reaction (RT-PCR), and quantitative polymerase chain reaction (qRT-PCR).

2.5.1 Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a modified type of polymerase chain reaction (PCR). This molecular technique is used to qualitatively detect RNA expression. Reverse transcriptase, an enzyme, facilitates the synthesis of cDNA, which is later amplified. In addition to reverse transcriptase, a primer that is complementary to the targeted mRNA is used in the procedure. The targeted strand is amplified using the primers and Taq polymerase. The products of RT-PCR are separated on an agarose gel via gel electrophoresis. Using the RT-PCR technique, a researcher can check for the presence of a specific gene in a given sample. If the specific gene is present, a band will be present in the gel. Additionally, RT-PCR will show the level of gene expression.

2.5.2 Quantitative Polymerase Chain Reaction (qRT-PCR)

Quantitative polymerase chain reaction (qRT-PCR) is a molecular laboratory technique that stems from polymerase chain reaction (PCR). This reaction monitors the amplification of DNA in real time and allows researchers to view the increase in the amount of DNA as it is amplified in real time. The qRT-PCR procedure is carried out in a thermal cycler that illuminates each reaction mixture with a beam of light that is set at a particular wavelength. The reaction begins as a standard polymerase chain reaction (PCR) and a reporter probe is added the reaction. In the annealing phase of the procedure, the primer and probe anneal to the targeted DNA.

In the PCR procedure, the cycle is repeated approximately 25-50 times. The fluorescence is measured for few brief seconds during the last phase of each cycle and the process is repeated for all reactions. The increase in DNA products is quantified as the double stranded fluorescently tagged DNA is detected. SYBR Green is the reporter that is generally used in qRT-PCR. The results of qRT-PCR results in C_T values.

2.5.3 Cycle threshold (C_T)

A cycle threshold (C_T) value is the number of cycles required for the fluorescent signal to the cross the threshold or exceed the background noise. C_T levels are used to quantify the amount of copied DNA and is inversely proportional to the amount of target DNA. Low C_T values indicate increased amount of target DNA, while high C_T values indicate a decreased amount of target DNA.

2.6 Project Rationale

Camelina sativa, an oilseed crop of the *Brassicaceae* family, is an herbaceous annual dicot C3 crop that has been regaining popularity to cultivate as a low cost biofuel crop and a rich source of omega-3 fatty acids. A transcriptomics study following salt stress was analyzed to determine the changes in the gene expression to gain a better understanding of the molecular mechanisms involved in abiotic stress in *C. sativa*. Additionally, by identifying transcripts that regulate the magnitude of abiotic stress, it is possible to predict the metabolic pathways that are involved and ultimately further refine the composition of these pathways. This would be an important step toward elucidating the molecular networks of abiotic stress in plants and lead to the genetic engineering of the crop.

Two-week old *Camelina sativa* seedlings were exposed to different time regimes of salt (NaCl) stress at three different concentrations, 100 mM, 200 mM, and 500 mM. In response to the salt stress, plants displayed visual phenotypical changes. Molecular techniques including RT-PCR and qRT-PCR were used to study differential gene expression in *Camelina sativa* under salt stress conditions. The project was aimed to identify stress-related genes and draw conclusions that will be able to be used by other researchers studying the effects of abiotic stress on plants.

Outcome: Identification of genes responding to salt stress.

Chapter 3: Material and Methods

3.1 Experimental Design

The greenhouse of Penn State Harrisburg's Central Pennsylvania Laboratory for Biofuels was used to grow *Camelina sativa* var. *Suneson* seedlings and perform salt stress experiments. The greenhouse was maintained at 26°C with a 14-hour light photoperiod at 25-40 $\mu Em^{-2}s^{-1}$. Thirty-six four inch pots were set up using a high porosity soil, HP Mycorrhizae from Pro-Mix (Rivière-du-Loop, Québec, Canada). Ten seeds of *Camelina* were sown per individual pot and the pots were watered every day and seedlings were grown for a period of two weeks (Figure 1). Salt stress treatments were performed on two-week old *Camelina* seedlings, followed by the collection of leaf tissue, total RNA extractions, complementary DNA synthesis, and quantitative reverse transcription polymerase chain reaction using abiotic stress-related genes as markers.

Figure 1. Two-week old *Camelina sativa* seedlings grown in four-inch pots in the greenhouse to perform salt stress experiments

3.2 Solution preparation

Three different concentrations, 100 mM, 200 mM and 500 mM of NaCl solutions were prepared and autoclaved prior to use.

3.3 Plant Growth and Salt Stress Treatments

All the experimental pots containing *Camelina* seedlings were watered every day at the same time of the day (10 am) with 100 mL of water for a period of two weeks (14 days). The following day, i.e. on the 15th day of the experiment, *Camelina* seedlings were subjected to different salt stress treatments. Nine pots each of *Camelina* seedlings received 100 mL of either 100 mM, 200 mM or 500 mM of NaCl solution instead of water. The control group of plants received 100 mL of water.

3.4 Plant Material Collection and Tissue Storage

After the salt treatments were performed, *Camelina* leaf tissue was collected at 0, 3, 6, and 12 hours (four different time points) in three biological replicates, flash frozen in liquid nitrogen, and stored at -80°C prior to molecular analysis. In summary, the experiment had 13 biological samples collected in triplicates from a total of 4 different treatments: control (no salt) and 100 mM, 200 mM or 500 mM concentrations of NaCl and tissues collected after 0, 3, 6, and 12 hours of salt exposure.

3.5 Total RNA Extraction and cDNA Synthesis

Total RNA from all samples was extracted using 500 mg of leaf tissue using the Spectrum^{TM} Plant Total RNA Extractions Kit (Sigma, St. Louis, MO, USA) followed by the synthesis of complementary DNA using the High-Capacity cDNA Archive Kit (Life Technologies, Frederick, MD, USA). All procedures were followed according to the manufacturer's instructions. Optical densities of total RNA and cDNA were measured

using a Nanovue Plus. A 260/280 nm ratio of ~1.8 for DNA and ~2.0 for RNA was used as an indication of sample purity.

3.6 Primer Design and Quantitative RT-PCR

The GeneScript Primer Design Program (https://www.genscript.com/sslbin/app/primer) was used to design the primers (Sigma, St. Louis, MO, USA) for the following abiotic stress related genes and one housekeeping gene in order to perform qRT-PCRs (Table 1).

Gene name	Sequence of Forward (F) and Reverse (R) primers	Locus Number	Origin
GAPDH	F 5'-ATGATGTGTGCTGCTGAC-3'	GR880018	*Panicum virgatum*
	R 5'-GTAGGACGAGTTCTTGTTCTG-3'	SW_H39_7_O16_050_1	
HSFA2A	F 5'-CGAATGTTCTTCTTCTGGAATT-3'	NM_001057776.2	*Oryza sativa japonica*
	R 5'-GCTGAAATTACTCCGTAACTTT-3'		
MT3	F 5'-AATGGACACGAGAGGTTG-3'	NM_001202970.1	*Arabidopsis thaliana*
	R 5'-TTGTTGTTGTTGTTGTTGTTC-3'		
PLSOS4	F 5'-CTTGGCTTCTATGTTGACTCCTA-3'	AT5G37850.1	*Arabidopsis thaliana*
	R 5'-TTCTGATGGCTTCCGATAAGTAAT-3'		
PR1	F 5'-CACAACAATAACCATTATCAACT-3'	AY064023	*Arabidopsis thaliana*
	R 5'-GAAGAACAAGAGCACCTAC-3'		
SalT	F 5'-ATGACGCTGGTGAAGATT-3'	LOC_Os01g24710.2	*Oryza sativa japonica*
	R 5'-TGTAGTTGAAGGCAATGGA-3'		
SIFV	F 5'-ATGAGGACTTTAGGTTGGAACTC-3'	LOC_Os01g21200.1	*Oryza sativa japonica*
	R 5'-GGAATAGTGGCGGGCGAC-3'		
SIFVG	F 5'-ATACATCACCGCTGGATT-3'	LOC_Os01g21020.1	*Oryza sativa japonica*
	R 5'-TCAGTTGTGCCTCAAGAG-3'		
WRKY70	F 5'AGCACTTCTCTATCTTCAG-3'	NM_115498	*Arabidopsis thaliana*
	R 5'-GTACAGTACATACACTCATTAG-3'		

Table 1. List of forward and reverse primers used to perform qRT-PCR

Quantitative RT-PCR reactions were performed using 150 ng of cDNA in a 96 well plate using 10µl of 2X SYBR Select Master Mix (Life Technologies, Frederick, MD, USA), 5 µl of diluted cDNA reaction mixture, and 2.5 µl each of 5 µM

concentration of forward and reverse primers. The qRT-PCR reactions were performed using the following conditions: 2 minutes at 55°C, 5 minutes at 95°C, and 40 cycles of the one step cycling of 15 seconds at 95°C, 15 seconds at the standardized annealing temperature for each specific primer (temperatures ranging from 48.5-62.6°C), and 30 seconds at 72°C. The qRT-PCR reaction ended with a final cycle of 15 seconds at 95°C, 15 seconds at 60°C, and 15 seconds at 95°C. Each qRT-PCR reaction was performed in triplicate on individual biological samples.

Relative gene expression was calculated in terms of C_T values using the algorithm provided by Eppendorf Mastercycler® ep *realplex*. The final cycle threshold (C_T) value for each biological sample was calculated as the means of all C_T values obtained from the three technical replicates. The qRT-PCR reaction products were later used to run a 1% agarose gel to visually observe the amplified gene products.

Chapter 4: Results and Discussion

High salinity is a major abiotic stress that limits plant growth and development leading to losses in agricultural crop production. Plants sense these stresses and manifest their responses through phenotypic and molecular responses. The goal of this study was to look at the gene expression patterns at the onset of stress after exposure to different NaCl concentrations.

4.1 Plant Growth and Phenotypic Changes in Response to NaCl stress treatments

The experiment was setup in the greenhouse using thirteen different treatments (table 2). The growth conditions of the greenhouse were carefully monitored to ensure the consistency of the set physical parameters such as photoperiod, light intensity, humidity, and the amount of water the plants received. A high porosity soil, HP Mycorrhizae, was used for the growth of the seedlings to ensure good water absorption. Additionally, care was also taken to make sure that the watering was done at the same time of the day to rule out the possibility of additional stress due to water deficiency.

	Exposure time of NaCl stress to seedlings			
Salt Concentrations	**0 Hour**	**3 Hour**	**6 Hour**	**12 Hour**
Control (Water)	T1	T2	T6	T10
100 mM NaCl		T3	T7	T11
200 mM NaCl		T4	T8	T12
500 mM NaCl		T5	T9	T13

Table 2. Treatments used in the present study indicating different concentrations of NaCl stress on two-week old *Camelina sativa* seedlings. Also shown are the time points at which leaf tissue was collected.

Two-week old *Camelina* seedlings were subjected to three different concentrations of NaCl stress (100 mM, 200 mM, and 500 mM) over 3, 6, and 12 hour post treatment. Changes in leaf color was observed as a reflection of the phenotypical changes occurring in the seedlings. Accordingly, the first onset of phenotypical stress was noticed at 12 hours for 100 mM NaCl treatment, 6 hours for 200 mM NaCl treatment, and 3 hours for 500 mM NaCl treatment (Figure 2). These time points were therefore chosen for analyzing gene expression during the early stages of stress development. The idea is to study all the genes that are activated in *Camelina* irrespective of the NaCl concentrations used in this study.

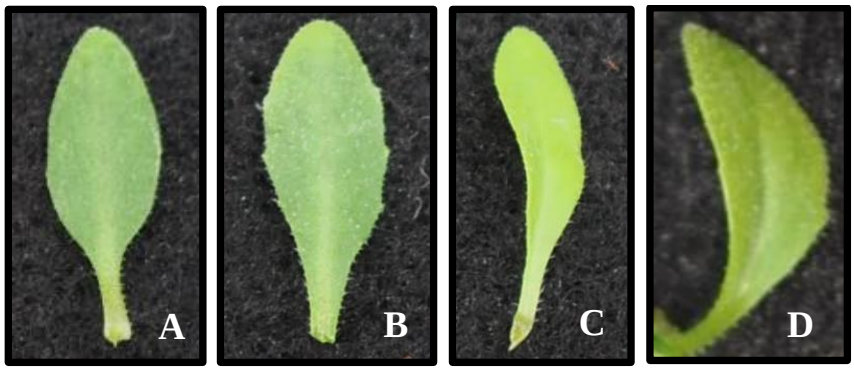

Figure 2. Phenotypic response of *Camelina* seedlings after exposure to NaCl treatment. A) Control, 0 hour B) 100 mM, 12 hour C) 200 mM, 6 hour D) 500 mM, 3 hour

During leaf sample collection, yellow or brown leaves was avoided to obtain good yield of RNA. Gloves were used during the collection process to prevent nuclease activity leading to the degradation of RNA. Following, samples were immediately flash frozen in liquid nitrogen to arrest metabolic activity and then stored at -80°C. The relative phenotypic effects of NaCl stress after 12 hours for three different concentrations is depicted in Figure 3. The seedlings in the control treatment did not show any changes in the leaves as expected. The seedlings exposed with 100 mM NaCl showed the least effect

of stress while the seedlings with 500 mM NaCl showed the most deleterious effects. The higher the concentration, the faster the color changes were observed as expected.

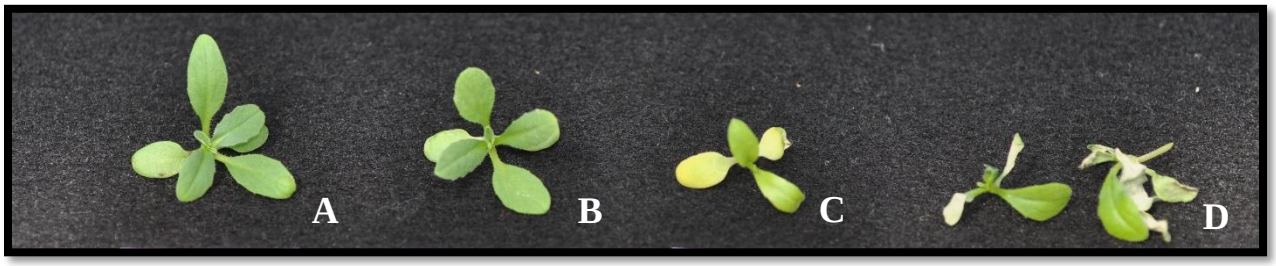

Figure 3. Phenotypical response of *Camelina* seedlings after 12 hours of exposure to varying concentrations of NaCl A) Control B) 100 mM C) 200 mM D) 500 mM

The seedlings stressed with 100 mM NaCl showed the least amount of yellowing after 12 hours of NaCl exposure compared to the other two concentrations of NaCl. Comparatively, the seedlings exposed to 200 mM and 500 mM showed more drastic phenotypic effects in terms of wilting, curling, and yellowing of leaves indicating the possible degradation of chlorophyll pigments. Particularly, the seedlings exposed to 500 mM NaCl were shrunken and wilted very quickly and eventually lead to fatal damage of the seedlings. This could be due to a significant reduction of photosynthetic activity in the seedlings.

4.2 Total RNA Extraction and cDNA synthesis

Camelina leaves were collected for total RNA extraction from the treatments indicated in Table 3. These treatments (T5, T8, and T11) represent the stages at which the initial stress response was observed after the seedlings were challenged with 100 mM, 200 mM, and 500 mM NaCl solutions respectively.

	Exposure time of NaCl stress to seedlings			
Salt Concentrations	**0 Hour**	**3 Hour**	**6 Hour**	**12 Hour**
Control (Water)	T1	T2	T6	T10
100 mM NaCl				T11
200 mM NaCl			T8	
500 mM NaCl		T5		

Table 3. Treatments used in the present study to extract RNA and synthesize cDNA

Total RNA from selected samples was extracted using the frozen leaf tissue. Following, a total of 0.2 µg of total RNA per individual sample was used to synthesize complementary DNA. Optical densities of the samples were measured at 260 nm to estimate the concentration of RNA and DNA. Readings were also taken at 280 nm to confirm the purity of the samples by looking at 260/280 ratios of ~2.0 for RNA and ~1.8 for cDNA.

4.3 RT-PCR and qRT-PCR

A total of eight well characterized salt stress related genes were chosen to investigate the transcript levels of those genes and their role in NaCl stress in *Camelina*. To confirm the presence and look at the primer specificity of these eight reference genes and the internal control, *GAPDH* gene, a reverse transcription PCR was performed using 60 ng of cDNA from the T1 (zero hour control) sample. The RT-PCR products were analyzed using a 0.8% agarose gel. The presence of positive bands for all the genes indicated that all the nine genes chosen for this study were present in *Camelina sativa*

var. *Suneson* (Figure 4).

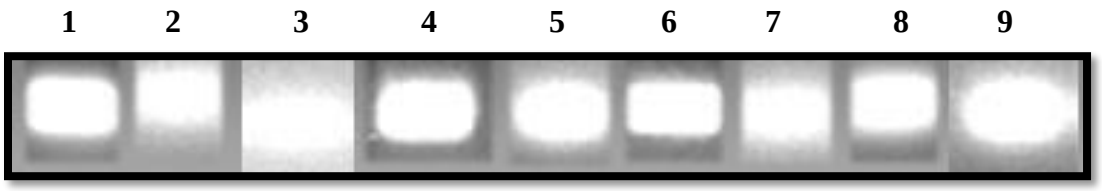

Figure 4. RT-PCR showing the presence of nine genes used in this study. (Lane 1: *GAPDH*; Lane 2: *HSFA2A*; Lane 3: *MT3*; Lane 4: *PLSOS4*; Lane 5: *PR1*; Lane 6: *SalT*; Lane 7: *SIFV*; Lane 8: *SIFVG*; Lane 9: *WRKY70*

4.3.1 Quantitative RT-PCR

Quantitative RT-PCR reactions for all the eight reference genes and one housekeeping gene was performed as described in section 3.6. Each qRT-PCR reaction was performed in triplicates on the individual samples. The melting curve analysis indicated that there was no primer-dimers and the designed primers and optimized primer annealing temperatures were specific to the genes amplified indicating primer specificity. The qRT-PCR products were also used to run a 1% agarose gel to visually observe the amplified gene products.

Housekeeping genes are required to maintain the basic cellular function. The *GAPDH* housekeeping gene was used to as an internal control in this study. In order to quantify the constitutive expression levels of this internal control, the untreated control samples from different time points (0, 3, 6, and 12 hours), a qRT-PCR was performed. The cycle threshold (C_T) values obtained for any time point of the control treatment showed uniform values indicating the same amount gene expression (Figure 5).

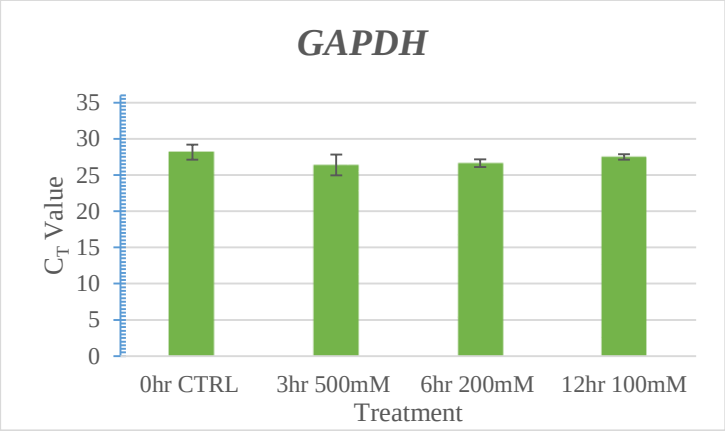

Figure 5. Cycle threshold (C_T) values of *GAPDH* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

This indicated that the housekeeping gene is expressed uniformly at all time points as expected. Therefore, it was decided to use only the 3 hour time point for the control treatment to estimate the relative gene expression for all the eight reference genes. The qRT-PCR products were then analyzed on a 1% agarose gel as indicated in Figure 6.

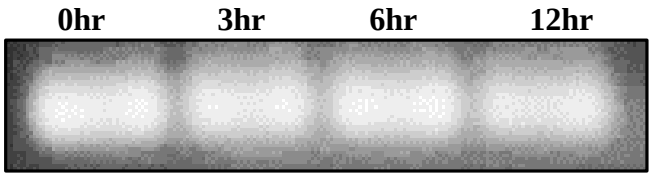

Figure 6. Expression of the housekeeping gene *GAPDH* in 0, 3, 6, and 12 hour control samples

Any expression changes that would be seen in the other eight stress related genes would then reflect only the changes due to salt stress alone and not due to other various factors. The band intensities in all the four lanes was uniform as expected. This also correlated very well with the uniform C_T values obtained as discussed above (Figure 5). In summary, it was noted that the expression level of the housekeeping gene, *GAPDH*, used

as an internal control was constant all time points (0, 3, 6, and 12) confirming that there was no change in the expression. Previous research showed that all the eight reference genes chosen in this study were upregulated during abiotic stress (Garcia et al. 1998; Machens et al. 2014; Tomas et al. 2014; Soda et al. 2015; Song et al. 2015). To evaluate the relative gene expression of the eight abiotic stress responsive genes, qRT-PCR reactions were performed on the chosen treatment samples using gene specific primers listed in Table 1. To quantify the overall differences in the transcript levels for all the genes, under each of the abiotic stress treatments, a C_T value was calculated based on the program, Mastercycler ep *realplex*. The average C_T values and standard deviations were calculated using data from the technical replicates. Most of the average C_T values were ranging from 12-28 indicating a moderately high levels of expression (Figures 8-12). All the qRT-PCR reaction mixes were also used to run an agarose gel (Figure 13).

The gene *SIFVG* had the highest level of expression among all the tested stress candidate genes (i.e., had the lowest median C_T value of 11.19), while the *MT3* gene had the lowest level of expression (highest median C_T values, 38.37). Typically low C_T values indicate high amount of gene expression (upregulation) and high C_T values represent low amount of gene expression (downregulation). All the C_T values obtained in this study correlated well with the DNA band intensities observed on the agarose gel. This reiterates the authenticity of the qRT-PCR analysis in this entire study. The remaining six genes indicated relative expression levels that ranged between the lowest and highest C_T values. Three of the eight genes (*SIFVG*, *WRKY70*, and *SalT*) showed a good correlation of gene upregulation in 3, 6, and 12 hour treatments when compared to the control. This trend was also seen in the bands as indicated in Figure 13. In fact, *SIFVG* and *WRKY70* showed

a consistent and gradual increase of gene expression starting between 3 and 6 hour and 6 and 12 hour treatments. These elevated levels of expression indicates that these two genes would be involved in providing stress tolerance to the plants.

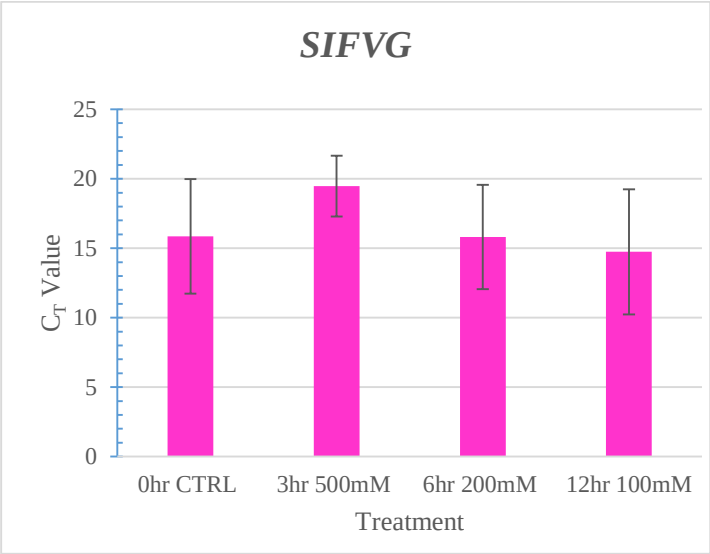

Figure 7. Cycle threshold (CT) values of *SIFVG* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

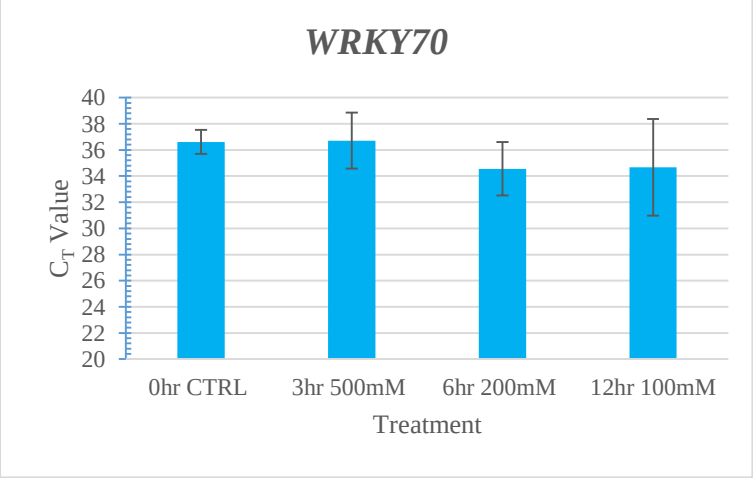

Figure 8. Cycle threshold (C_T) values of *WRKY70* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

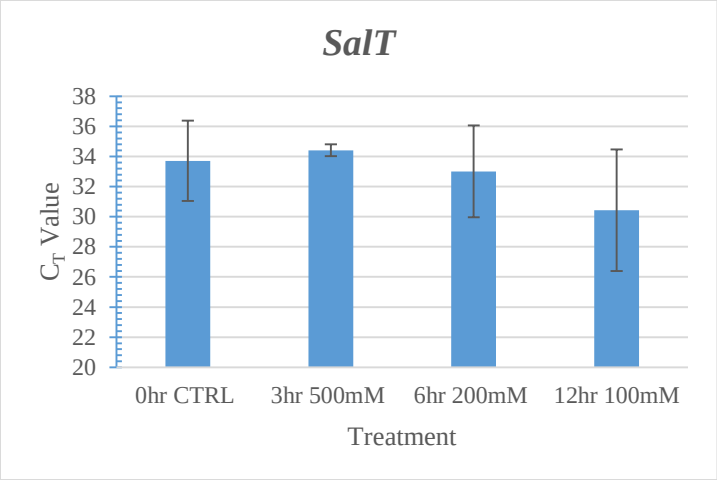

Figure 9. Cycle threshold (C_T) values of *SalT* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

The analysis of the gel electrophoresis indicated that the *PR1* and *SalT* genes were upregulated in all the time points (3, 6, and 12 hours) when compared to the zero hour control. Similar trend of upregulation was observed for the *SalT* gene as indicated by the gradual reduction of C_T values from zero hour to all the other treatments. However the C_T values and the intensity of the gene amplification products did not correlate very well for the *PR1* gene.

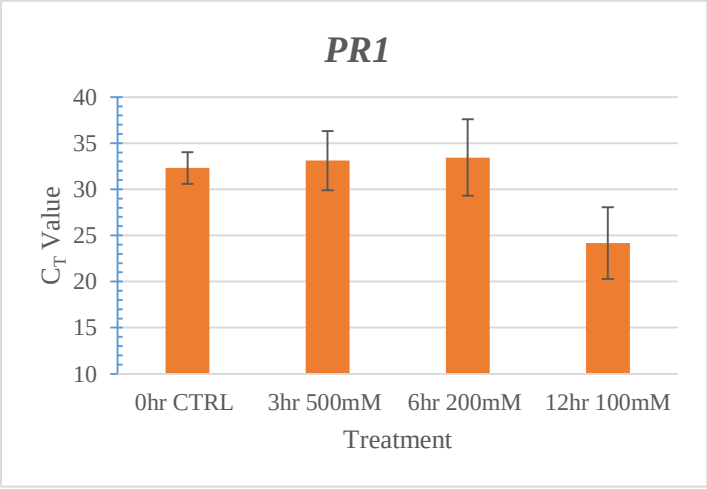

Figure 10. Cycle threshold (C_T) values of *PR1* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

For the *MT3* and *SIFV* gene, the C_T values decreased across all the three different salt treatments when compared to the control; this indicated an upregulation of the *MT3* and *SIFV* gene expression as discussed in Tomas et al. 2014 and Soda et al. 2013 respectively. However, the gel analysis did not correlate the same and this could be due to the loss of the qRT-PCR product while loading the agarose gel.

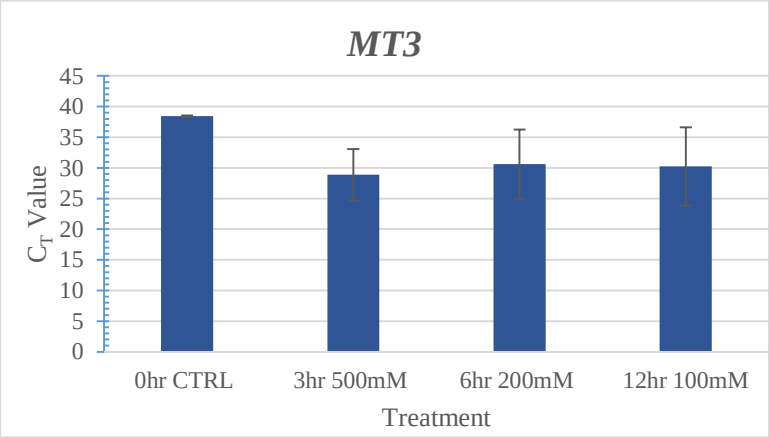

Figure 11. Cycle threshold (C_T) values of *MT3* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

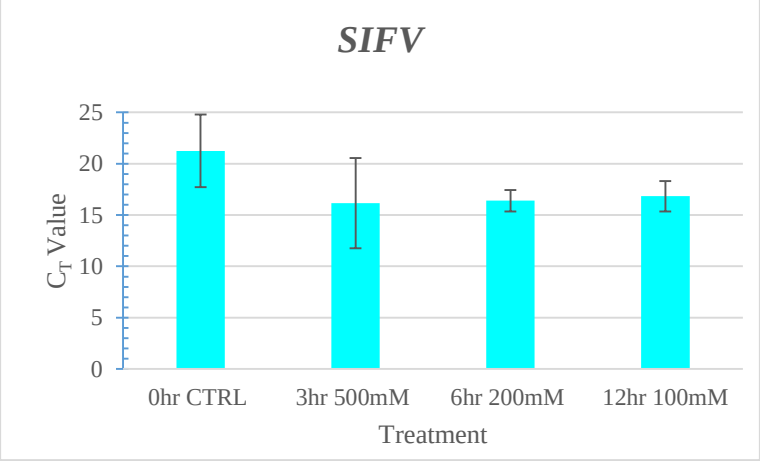

Figure 12. Cycle threshold (C_T) values of *SIFV* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

Of the eight reference genes studied, the C_T values for the *HSFA2A* and *PLSOS4* genes were not obtained for all the three technical replicates and therefore it was not possible to represent the variations in gene expression in the form of a graph. However, the qRT-PCR reaction products were run on an agarose gel to look for any visual differences in the band thickness in order to get a general idea of the changes in gene expression. Sometimes genes behave differently in different plant species. The *HSFA2A* gene expression agarose results indicated an unexpected trend of downregulation in *Camelina* during salt stress. Unexpectedly, the band intensities for 3, 6, and 12 hour treatments were lower compared to the zero hour control. This could either be a technical error of losing the samples while loading the gel or for some unknown reason. Therefore, further analysis is needed to confirm the *HSFA2A* gene expression patterns in *Camelina*. The *PLSOS4* gene did not result in C_T values and the agarose gel showed faint bands indicating no change in expression levels.

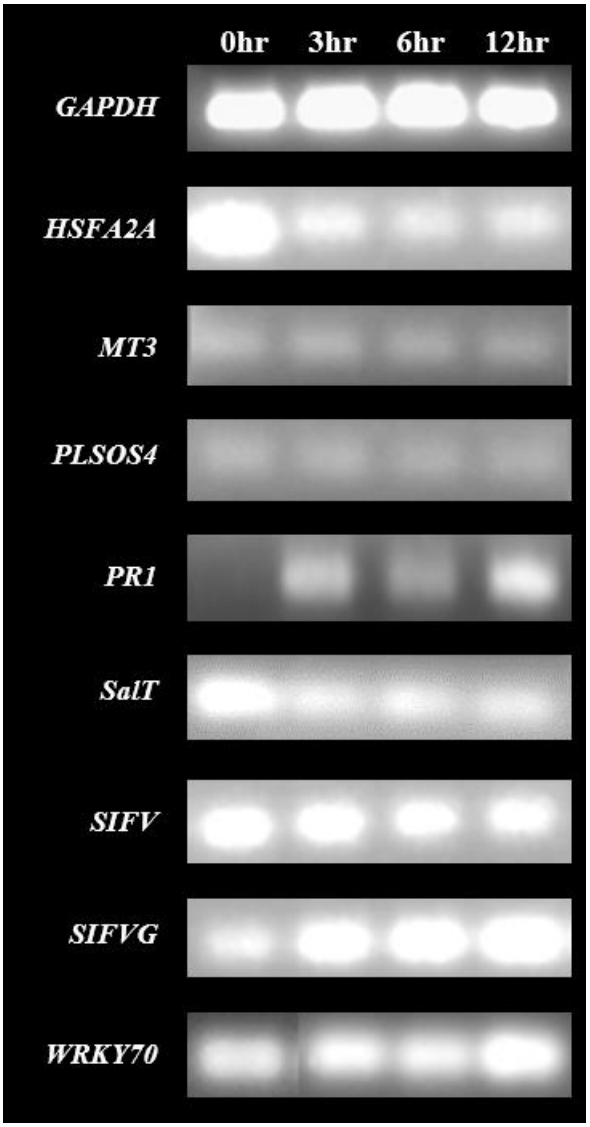

Figure 13. Analysis of qRT-PCR gene amplification products

In summary, varying levels of gene expression was observed for all stress related genes used in this study indicating that they are all responsive and involved during exposure to salt. All the six genes, expect for *HSFA2A* and *PLSOS4*, followed the expected trend of upregulation in gene expression. Quantitative RT-PCRs need to be repeated at least two more times using all the biological replicate samples to confirm the observed trends. This would eliminate any ambiguities that were observed in this

research. The limited time frame of this honors' project provided substantial preliminary data and thus further research would uncover more specific gene regulation patterns.

Chapter 5: Conclusion

In general, plants respond to biotic and abiotic stresses in various ways including changes in gene expression. Abiotic stress due to salt, cold, and/or drought is a widely researched phenomenon in many plants. However, there is a very limited amount of study on the biofuel crop plant, *Camelina sativa*. The present research was aimed at getting a fundamental knowledge of the expression patterns of eight selected genes that are important during salt stress in *Camelina*.

After the plants were challenged with 100 mM, 200 mM or 500 mM concentrations of NaCl, the plants clearly showed phenotypic changes in the leaf color and texture. Yellowing of leaves was a reflection of early response of plants with all salt concentrations and further leading to browning and wilting of the leaves particularly with 500 mM NaCl treatment.

Genes are regulated temporally and/or spatially in response to various external and internal stimuli. Quantitative RT-PCR is a very sensitive and accurate method for measuring the relative expression of genes. Using this technique, changes in gene expression of *HSFA2A, MT3, PLSOS4, PR1, SalT, SIFV, SIFVG* and *WRKY70* were analyzed. Initially, a RT-PCR was performed to confirm the presence of *GAPDH* (internal control, housekeeping gene) and the eight reference genes in *Camelina*. Consequently, expression analysis was performed using qRT-PCR followed by gel electrophoresis. The results showed that increased concentrations of salt affected gene expression indicated through the presence of different levels of gene transcripts. An expected correlation was observed between the C_T values and the visual representation of the DNA bands confirming the authenticity of the qRT-PCR results.

Expression of genes is also known to be regulated after transcription through post-transcriptional, translational, and post-translational modifications. Therefore, mRNA concentrations sometimes may not necessarily be an accurate measure of the amount of active functional protein. Hence it is possible that better correlation between abiotic stress and gene expression could be revealed by monitoring protein levels using western blot analysis. Also, it might be interesting to measure the complete metabolomic profile of the salt stressed plants to get a better understanding of the overall functioning of the plant.

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Figure 1: Two-week old *Camelina sativa* seedlings grown in the greenhouse

Figure 1. Two-week old *Camelina sativa* seedlings grown in the greenhouse

Figure 2: Phenotypic response of *Camelina* seedlings

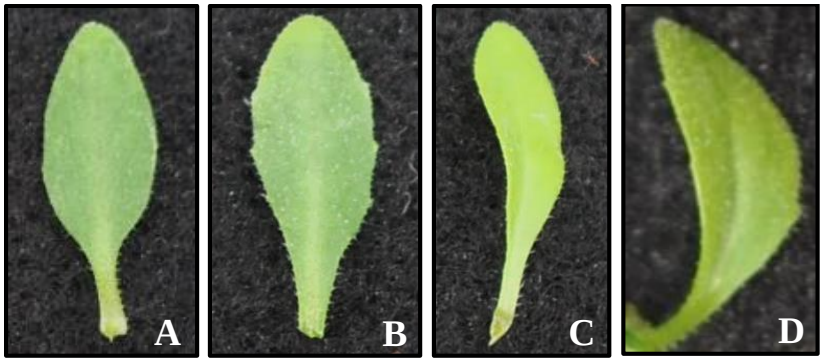

Figure 2. Phenotypic response of *Camelina* seedlings after exposure to NaCl treatment. A) Control, 0 hour B) 100 mM, 12 hour C) 200 mM, 6 hour D) 500 mM, 3 hour.

Figure 3: Phenotypical response of *Camelina* seedlings after 12 hours of exposure

Figure 3. Phenotypical response of *Camelina* seedlings after 12 hours of exposure to varying concentrations of NaCl A) Control B) 100 mM C) 200 mM D) 500 mM

Figure 4: RT-PCR showing the presence of nine genes used in this study

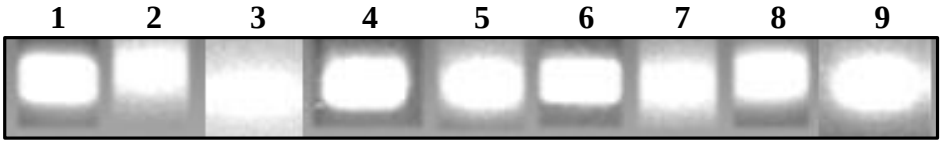

Figure 4. RT-PCR showing the presence of nine genes used in this study. (Lane 1: *GAPDH*; Lane 2: *HSFA2A*; Lane 3: *MT3*; Lane 4: *PLSOS4*; Lane 5: *PR1*; Lane 6: *SalT*; Lane 7: *SIFV*; Lane 8: *SIFVG;* Lane 9: *WRKY70*)

Figure 5: Cycle threshold (C_T) values of *GAPDH*

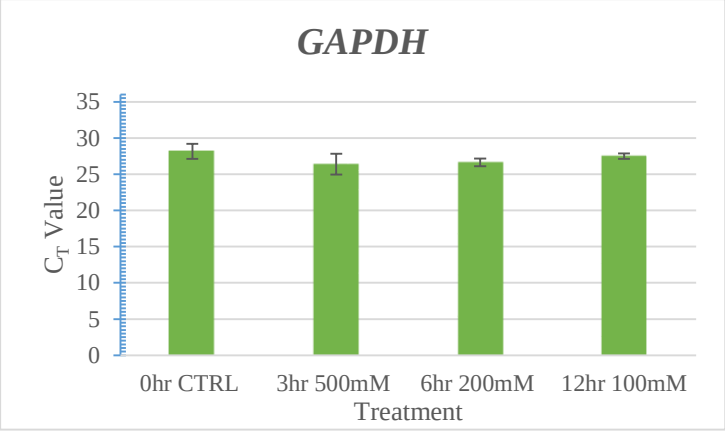

Figure 5. Cycle threshold (C_T) values of *GAPDH* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

Figure 6: Expression of the housekeeping gene *GAPDH*

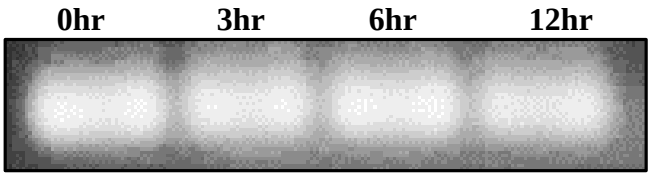

Figure 6. Expression of the housekeeping gene *GAPDH* in 0, 3, 6, and 12 hour control samples

Figure 7: Cycle threshold (C_T) values of *SIFVG* gene

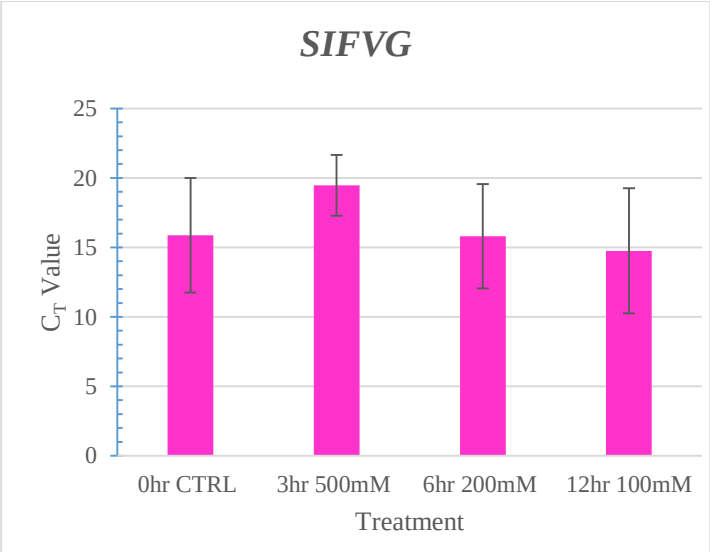

Figure 7. Cycle threshold (C_T) values of *SIFVG* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

Figure 8: Cycle threshold (C_T) values of *WRKY70* gene

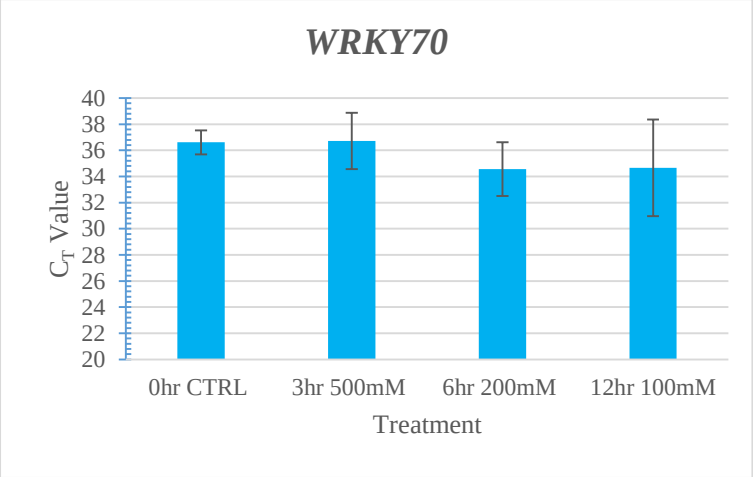

Figure 8. Cycle threshold (C_T) values of *WRKY70* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

Figure 9: Cycle threshold (C_T) values of *SalT* gene

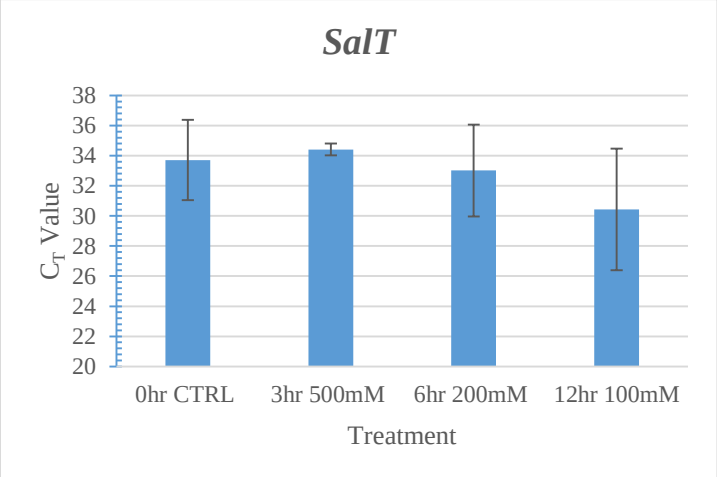

Figure 9. Cycle threshold (C_T) values of *SalT* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

Figure 10: Cycle threshold (C_T) values of *PR1* gene

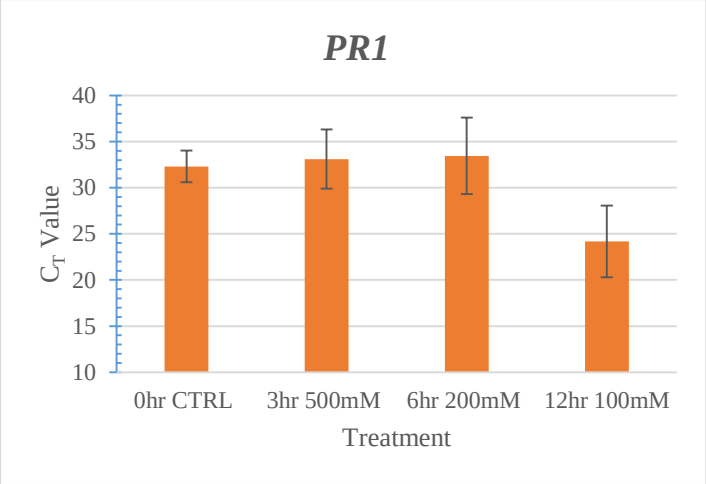

Figure 10. Cycle threshold (C_T) values of *PR1* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

Figure 11: Cycle threshold (C_T) values of *MT3* gene

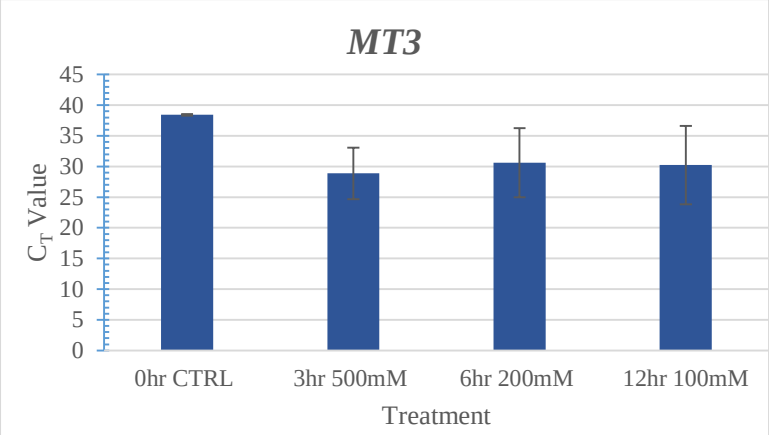

Figure 11. Cycle threshold (C_T) values of *MT3* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

Figure 12: Cycle threshold (C_T) values of *SIFV* gene

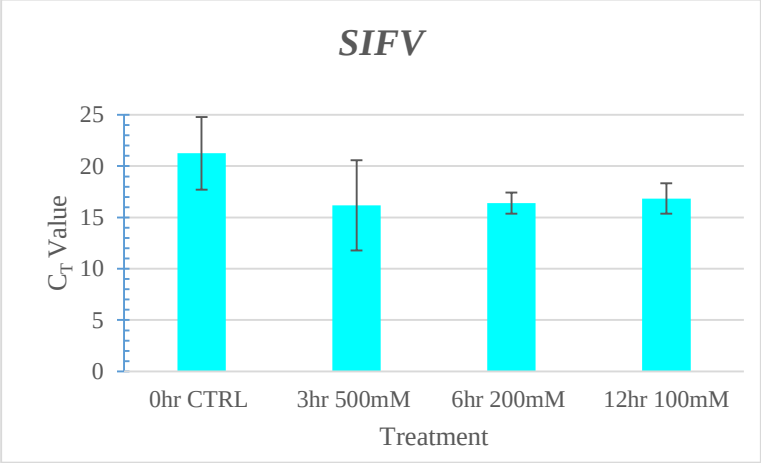

Figure 12. Cycle threshold (C_T) values of *SIFV* gene across the four treatments. Each data point (bar) represents the mean of ± standard error of the three technical replications.

Figure 13. Analysis of qRT-PCR gene amplification products

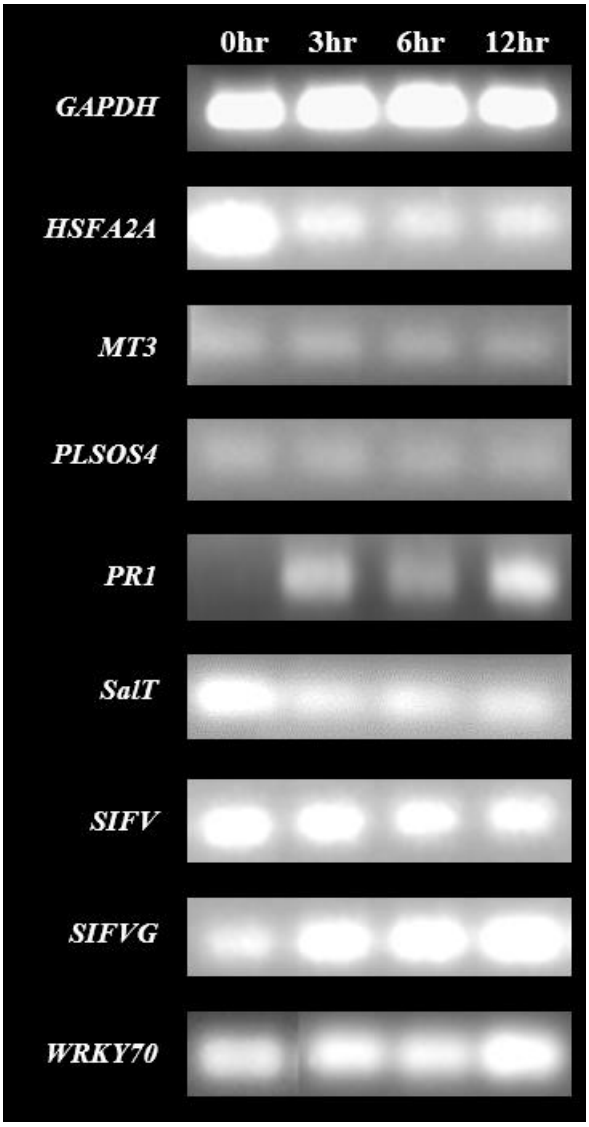

Figure 13. Analysis of qRT-PCR gene amplification products

Table 1: List of forward and reverse primers used to perform qRT-PCR

Gene name	Sequence of Forward (F) and reverse (R) primers	Locus Number	Origin
GAPDH	F 5'-ATGATGTGTGCTGCTGAC-3'	GR880018	*Panicum virgatum*
	R 5'-GTAGGACGAGTTCTTGTTCTG-3'	SW_H39_7_O16_050_1	
HSFA2A	F 5'-CGAATGTTCTTCTTCTGGAATT-3'	NM_001057776.2	*Oryza sativa japonica*
	R 5'-GCTGAAATTACTCCGTAACTTT-3'		
MT3	F 5'-AATGGACACGAGAGGTTG-3'	NM_001202970.1	*Arabidopsis thaliana*
	R 5'-TTGTTGTTGTTGTTGTTGTTC-3'		
PLSOS4	F 5'-CTTGGCTTCTATGTTGACTCCTA-3'	AT5G37850.1	*Arabidopsis thaliana*
	R 5'-TTCTGATGGCTTCCGATAAGTAAT-3'		
PR1	F 5'-CACAACAATAACCATTATCAACT-3'	AY064023	*Arabidopsis thaliana*
	R 5'-GAAGAACAAGAGCACCTAC-3'		
SalT	F 5'-ATGACGCTGGTGAAGATT-3'	LOC_Os01g24710.2	*Oryza sativa japonica*
	R 5'-TGTAGTTGAAGGCAATGGA-3'		
SIFV	F 5'-ATGAGGACTTTAGGTTGGAACTC-3'	LOC_Os01g21200.1	*Oryza sativa japonica*
	R 5'-GGAATAGTGGCGGGCGAC-3'		
SIFVG	F 5'-ATACATCACCGCTGGATT-3'	LOC_Os01g21020.1	*Oryza sativa japonica*
	R 5'-TCAGTTGTGCCTCAAGAG-3'		
WRKY70	F 5'AGCACTTCTCTATCTTCAG-3'	NM_115498	*Arabidopsis thaliana*
	R 5'-GTACAGTACATACACTCATTAG-3'		

Table 1. List of forward and reverse primers used to perform qRT-PCR

Table 2: Treatments used in the present study

	Exposure time of NaCl stress to seedlings			
Salt Concentrations	**0 Hour**	**3 Hour**	**6 Hour**	**12 Hour**
Control (Water)	T1	T2	T6	T10
100 mM NaCl		T3	T7	T11
200 mM NaCl		T4	T8	T12
500 mM NaCl		T5	T9	T13

Table 2: Treatments used in the present study indicating different concentrations of NaCl stress on two-week old *Camelina sativa* seedlings. Also shown are the time points at which leaf tissue was collected.

Table 3: Treatments used in the present study to extract RNA and synthesize cDNA

	Exposure time of NaCl stress to seedlings			
Salt Concentrations	**0 Hour**	**3 Hour**	**6 Hour**	**12 Hour**
Control (Water)	T1	T2	T6	T10
100 mM NaCl				T11
200 mM NaCl			T8	
500 mM NaCl		T5		

Table 3: Treatments used in the present study to extract RNA and synthesize cDNA

Academic Vita

Mario A. Soliman

1370 Armitage Way
Mechanicsburg, PA 17057

Phone: (717) 824-2405
Email: mas6552@psu.edu

Objective

I am pursuing a medical degree following my undergraduate studies. I am currently working toward my bachelors in life science and will be applying to medical school for the fall 2016 entering class.

Education

The Pennsylvania State University **0**8/12 **-** Present
Capitol College, Middletown, PA
Pursuing Bachelors in Life Science

Schreyer Honors College student
Thesis title: A Transcriptomics Approach to Study Genes Expressed Under Salt Stress in *Camelina Sativa* var. Suneson

Relevant Courses –

- Biology 230H – Molecules and Cells
- Biology 240W – Function and Development of Organisms
- Biology 141 – Physiology
- Biology 142 – Physiology Laboratory
- Chemistry 210, 212H, 213 – Organic Chemistry I, II, and laboratory
- Biology 322H – Genetics Analysis
- Biology 496 – Research in Biology (Thesis work)
- Biology 400 – Teaching Biology
- Biology 401 – General Biochemistry
- Biology 411– Medical Embryology
- BIOTC 459 – Plant Tissue Culture and Biotechnology
- BMB 401 – Biochemistry

Professional Experience

Research Intern June 2015 – August 2015
Harvard Medical School – Catalyst Program **Boston, MA**

- The Summer Clinical and Translational Research Program (SCTRP) is a ten-week mentored, summer research program designed to enrich the pipeline of my understanding and interest in pursuing clinical and translational research
- Participated in weekly seminars with Harvard faculty and graduate students focusing on topics such as research methodology, health disparities, and ethics
- Primarily focused my research on the cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling
- Identified an agonistic drug that triggers the STING pathway
- Completing a biostatistics course at Harvard Medical School
- Gaining valuable research techniques such as mammalian cell culture, immunofluorescence, isolation of primary immune cells, and protein purification

Research Intern June 2014 – August 2014
National Science Foundation – REU, Penn State Harrisburg Biofuels Laboratory **Middletown, PA**

- Worked with professional staff to design and utilize eight assays to better understand the biochemical, molecular, and physiological changes that are occurring within the biofuel crop *Camelina sativa*, while subjecting it to drought, salinity, or temperature stresses
- Gained laboratory experience in plant tissue culture, plant transformation, gene cloning, and gene expression
- Attended the 2014 Disappearing Boundaries Summer Research Meeting at Elizabethtown College discussing G protein-linked receptors
- Presented my project on Biochemical Analysis of *Camelina sativa* Under Abiotic Stress at Penn State Harrisburg NSF-REU Meeting

Chemistry and Biology Tutor August 2013 – Present
Pennsylvania State University Learning Center **Middletown, PA**

- Clarify academic content in all chemistry courses offered at Penn State Harrisburg, including inorganic and organic chemistry, laboratories, as well as several biology courses including Physiology, Function and Development of Organisms, and Molecules and Cells
- Work with students in a one-on-one learning and small groups environments

- Assess the students' study skills: note-taking, time management, problem-solving, and test-taking skills

Chemistry and Biology Teacher Assistant and Grader August 2013 – Present
Penn State Harrisburg Chemistry and Biology Department **Middletown, PA**

- A recitation instructor for a 200-level biology course
- Assist with the teaching of chemistry and biology courses including principle microbiology and biological sciences and their relevant laboratories
- Grade and tabulate student quizzes, assignments, lab reports, and exams
- Address, communicate, and resolve student questions and concerns

Lab Assistant/Volunteer January 2013 – Present
Penn State Harrisburg Biofuels Laboratory **Middletown, PA**

- Broadly crossed-trained in areas that include but not limited to PCR, RT-PCR, qRT-PCR, southern blot, media and buffer preparation, RNA and DNA extraction, cDNA synthesis, biolistic and agrobacterium mediated transformation with professional staff
- Assisted with several projects including the iTRAQ analysis of salt stress response in the biofuel crop *Camelina sativa*
- Trained and mentored students from Susquehanna, Harrisburg, Middletown, and Central Dauphin Schools who visit the lab
- Help create and coordinate activities for visiting high school students

Accomplishments and Affiliations

- Dean's List (every semester from Spring 2013 to Spring 2015)
- Volunteered for 10 weeks at The Good Shepherd Hospital in the Emergency Department in Cairo, Egypt, during revolution, in the summer of 2012 — 06/2012 – 08/2012
- Member and Sponsor Assistant of the Student Red Cross Club (SRS) — 08/2012 – 12/2012
- Physician Observer/ Volunteer at Holy Spirit Hospital (107 hours) — 01/2013 – 06/2015
- Two manuscript (Co-authorship) - iTRAQ analysis of salt stress response in the biofuel crop *Camelina sativa* — 01/2014 – 08/2014
- Assisted the PI and Co-PI of the Penn State Harrisburg Biofuels Lab with NSF, USDA, and foundation grant submissions — 01/2014 – Present
- Active member of the National Society of Leadership and Success — 08/2014 – Present
- Volunteer at the Penn State Hershey Medical Center Emergency Department (112 hours +) — 10/2014 – 05/2015
- Recipient of the Penn State Harrisburg Travel Award that was used for a research trip to Peru — 10/2014 – 03/2014
- Recipient of the Schreyer Honors College Scholarship — 01/2015
- Recipient of the Graham Kenneth Honors Scholarship — 01/2015
- Worked in ACEER Laboratory in Puerto Maldonado, Peru and explored the effects of the newly built Transoceanic Highway on the Tambopata River; research results were published in the National Geographic Journal — 03/06/2015 – 3/15/2015
    - Developed first aid kits in the Infierno villagers in Peru
- Recipient of the Eric A. and Josephine S. Walker Award for leadership, scholarship, and citizenship qualities — 4/17/2015
- Attended the 2015 American Society of Plant Biologists Conference and was invited as a guest speaker — 4/18/2015
- Recipient of the Sanders Schreyer Honors Scholarship — 08/2015
- Recipient of the Capitol College Honors College Scholarship — 08/2015
- University of Rochester School of Medicine URD Conference — 08/15/2015

Skills

- Strong organizational, time management, communication, analytical skills, and peer advising
- Trained in laboratory molecular techniques including PCR, RT-PCR, qRT-PCR, and SDS PAGE
- Trained in genetic transformation, plant and animal tissue culture, and protein isolation
- Proficient in Microsoft Office (Word, Excel, PowerPoint, and Publisher), EndNote, Google Sketch and Solid Works
- Fluent in English, Arabic, and French