CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

Functional Analysis of Salt Stress Induced Genes from *Paulownia elongata*

A graduate project submitted in partial fulfillment of the requirements
For the degree of Master of Science in Biology

By Michel Chaires

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Thesis of Michel Chaires is approved by:

_____	_____
Stan Metzenberg, PhD	Date
_____	_____
Kerry Cooper, PhD	Date
_____	_____
Chhandak Basu, PhD, Chair	Date

California State University, Northridge

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Dedication

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Table of Contents

List of Figures

List of Tables

Abstract

Functional Analysis of Salt Stress Induced Genes from *Paulownia elongata*
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Bioethanol production has the potential to produce higher quantities of environment-friendly biofuel compared to other modern platforms. Currently, bioethanol is the main platform produced from sugar-based crops. These crops, however, compete with food supplies unlike the potential crops that could produce bioethanol from lignocellulosic biomass. Lignocellulosic biomass can generate higher energy output than sugar-based bioethanol, yet no efficient pretreatment methods currently exist that make biomass-produced bioethanol cost effective. Improving pretreatments as well as selecting an optimal bio-feedstock could play an important role for using biomass-produced bioethanol in the future.

An excellent candidate for biomass fuel production is a species of *Paulownia* native to China known as *Paulownia elongata*. The plant has beneficial characteristics like rapid growth rate, good quality biomass, and is resistant to climate changes. *Paulownia* resistance to abiotic stresses make it an ideal focus of research, studying the plant could improve crop yields by studying novel genes expressed in *P. elongata* undergoing different abiotic stresses.

Salinity, one of many abiotic stresses, is becoming increasingly hazardous to plants worldwide, but has not been studied in depth in plants at a molecular level. Salt affects the plant's overall physiology, particularly cellular mechanisms and numerous pathways. Understanding genes contributing to tolerance is crucial if biomass-produced bioethanol is to be mass produced in the future. This study involved a 10-day 125 mM NaCl treatment of *P. elongata* investigating changes in gene expression; RNA was extracted from salt stress plants; then barcoded cDNA libraries were prepared using the Illumina Truseq kit. The cDNA libraries of salt stressed and non-stressed control *P. elongata* were sequenced using an Illumina Miseq sequencing system with a 75 bp paired end run. The sequencing run produced an average of 9,809,716 reads per *P. elongata* sample. The Trinity software generated a *de novo* transcriptome from all reads pooled together. Finally, the Tuxedo suite programs analyzed the reads and compared them to the *de novo* transcriptome in order to quantify and calculate differential gene expression among control and salt stressed *P. elongata* plants. The analysis generated 216 upregulated, downregulated, turned on or turned off salt stress genes. Real time-quantitative PCR validated results from RNA-seq data. Genes were identified by BLASTing, and further investigation demonstrated that these genes potentially play different roles in the survival of *P. elongata* during salt stress. Function of novels genes can be used for downstream studies and hypothesized pathways can be tested in the future.

Chapter-I

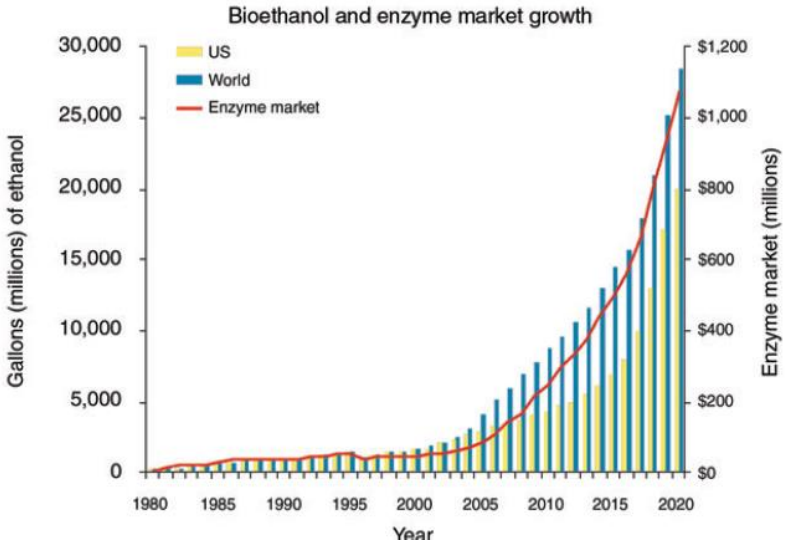
Introduction

Biofuel Technology

The decline in accessibility of petroleum-based fuel has sparked an innovative necessity within the scientific world to develop alternative forms of sustainable fuel. The concept of "biofuel" refers to fuel produced from various biological materials such as sucrose, starch, lignocellulosic biomass, gases and lipids processed through biological treatments producing alternatives forms of energy (Yuan et al., 2008). Biofuel is produced in the form of bioethanol, biodiesel and biogases; these are meant to replace or reduce the demand of diminishing fossil fuels which are still the main fuel source of energy today. The high demand for oil has led to a depletion in oil reserves estimated to exhaust completely in 35 to 40 years; other fossil fuel derived energy sources, like natural gases, are estimated to last for 70 more years (Shafiee & Topal, 2009). The dependency on combustible geologic deposits from organic materials have led to a huge scarcity of resources and an increase in demand for new forms of energy. In the US, bioethanol production tripled from 2.8 to 9 billion gallons between 2003 to 2008 and the production number continues to grow vastly each year (Tomes et al., 2010). Biofuel provides approximately 10% of the world's human energy use, while contributing to as much as 80% to 90% of energy in in the poorest regions of Latin America, Africa and Asia (Somerville, 2007). Due to all existing circumstances, including the environmental damage caused by emissions of gases from the burning of fossil fuels, there is an overall necessity for the world to find a "greener" solution. Biofuel production holds great promise in providing fuel less harmful to the environment, ultimately replacing or diminishing oil dependency. Leading countries, including the US, have invested money

in developing biofuel during the past few decades. The demand of ethanol for transportation purposes has contributed to a rapidly growing market of bioethanol production, and use of processing enzyme treatments, which facilitate biofuel production, have increased exponentially (Figure 1) (Weiland, 2010). The world of biotechnology opens up a greater depth of alternative resources and makes advancements in plant research; the biotechnology field has been expanding dramatically in the last 20 years. Understanding a plant's physiology and molecular mechanisms are key for improving treatments of biofuel production, advancing developments in genetically modified plants consequently improving biofuel processing, and clarifying many unknown aspects of gene expression related to environmental stresses (i.e. salinity) that are vital for plant survival.

Figure 1. Bioethanol Growth Worldwide, in the US and Enzyme Market Growth in Gallons between 1980 to 2020



Yellow bars represent the market growth of the US while blue represent the world's growth of market bioethanol. Red represents the enzyme market worldwide. Ethanol production is measured in gallons and enzyme market is calculated in market value (Weiland, 2010)

Biofuel Platforms

Biodiesel, bioethanol and biogas are the current major platforms of bioenergy products used as means for biofuel production (Figure 2). Biodiesel requires less processing than bioethanol or biogas and is derived from bioenergy feedstock lipids like vegetable oils and animal fats. These lipids are trans-esterified resulting in methyl or ethyl esters used as a biofuel source. Efficiency of transesterification is determined essentially by the catalysts used, temperature, and purity of the lipids (Meher et al., 2006). The base-catalyzed technique, commonly used for virgin oil in plants, is highly economical and produces 98% conversion yield (Ataya et al., 2007). Currently, there is a low energy production of biodiesel from sources including soybean, sunflower, canola, rapseed and palm. Although biodiesel production is simpler and has high conversion yield, some of the disadvantages include the issues in quality fuel and also a low amount of energy output.

Figure 2. Platforms Comparison of Biofuel and Bioenergy Crops

Platforms[a]	Feedstock[a]	NEB[b] GJ/ha/yr	NER[b]	CO_2 balance	Annual feedstock	Estab-lishment	Germ-plasm	Agricul. practice[d]	Ecological benefits
Ethanol from starch or sucrose	Maize	10–80	1.5–3.0	Positive	Yes	+++[c]	+++	+++	+
	Sugarcane	55–80	3.0–4.0	Positive	No	+++	+++	+++	+
	Sugar beet	40–100	2.5–3.5	Positive	Yes	+++	++	+++	+
	Sweet sorghum	85–300	5–10	Positive	Yes	+++	++	++	++
Ethanol from lignocellulosic feedstocks	Miscanthus	250–550	15–70	Possibly negative	Yes/No	+	+	+	+++
	Switchgrass	150–500	10–50	Possibly negative	No	+	+	+	+++
	Poplar	150–250	10–20	Possibly negative	No	+	++	++	+++
Biodiesel	Soybean	−20–10	0.2–0.6	Positive	Yes	++	+++	+++	+
	Canola	−5–2	0.7–1.0	Positive	Yes	+++	+++	+++	+
	Sunflower	−10–0	0.3–0.9	Positive	Yes	+++	++	+++	+

[a]Multiple platforms and crops are compared in a synthesis integrating information from multiple studies.
[b]Abbreviations: NEB, energy balance; NER, net energy ratio, which is the ratio of output to input energy needed to produce a fuel from a feedstock.
[c]Favorable features are indicated by + symbols, with +++ being the most favorable.
[d]Agricul. Practice, agricultural practice: how advanced is the current status of farming, harvesting, and processing.

Different platforms of biofuel describing types of feedstock, net energy balance (NEB), net energy ratio, carbon dioxide balance, annual feedstock capability, and favorable characteristics (Yuan et al., 2008)

The energy output of lipids from grains such as sunflower, canola, and sunflower was estimated to be approximately 10 to 40 GJ/ha (Figure 2), which is 5 times lower than other plant bio-feedstock such as lignocellulosic biomass (Yuan et al., 2008). Biodiesel does not meet the current energy demand of fuel; thus it cannot be currently meet the demands as an alternative sustainable form of biofuel.

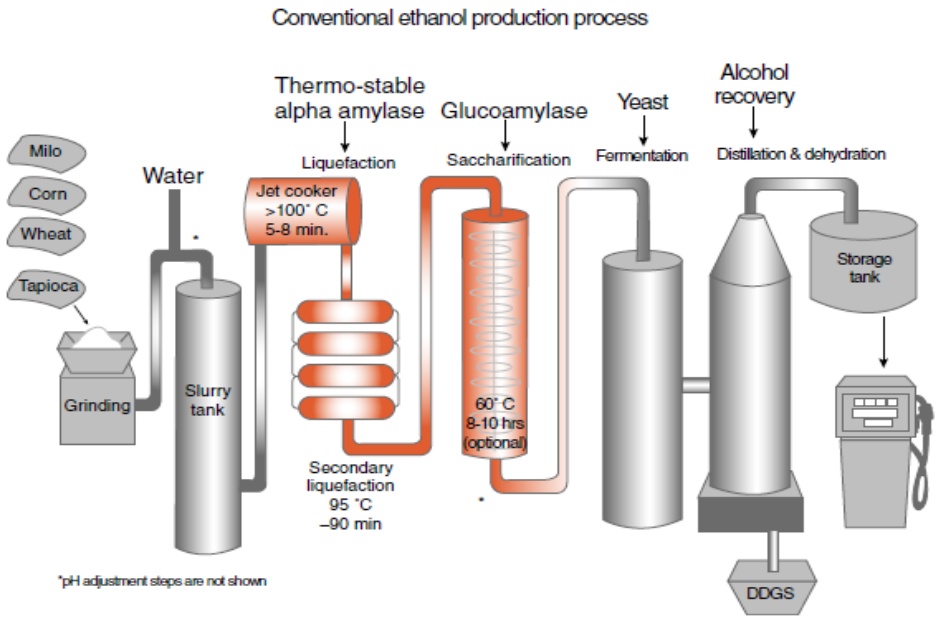
Biogas, one of the main platforms, is produced by biomass and other organic wastes through anaerobic gasification (Figure 5). Anaerobic digestion offers some benefits over other platforms; for example, the digestate can be used as a fertilizer for crops. Overall, these biogases would reduce greenhouse gases that would be otherwise emitted using fossil fuels. In 2008, European production of biogas was approximately 6 million tons of oil equivalent. Germany, one of the European leading biogas producers, has over 4,000 agricultural biogas production units operating in farms (Weiland, 2010). Currently, there is research underway, using algae as a source of hydrogen production, this involves a biological water-splitting reaction in which hydrogenase reduces protons in photosynthetic transport chains (Yuan et al., 2008). Biogas is found in the form of methane, hydrogen, and carbon monoxide, but unfortunately result in low energy balance (Figure 2) (Yuan et al., 2008). Research and development in areas of hydrogen production could accelerate advancements of plant-derived biogas. Microalgae could potentially produce about 15 to 300 times more biodiesel than traditional crops, yet current technology cannot realistically produce such quantities (Alam et al., 2015). Plant research might also unlock better methods resulting in higher net energy yields from biogas.

Bioethanol production is more complex process, deriving from different bio-sources such as starch, sucrose or lignocellulosic biomass. Sugars are either broken down through hydrolysis or obtained as monosaccharides which then undergo fermentation resulting in the production of bioethanol (Figure 4). Some of the most common feedstock used for starch and sugar-based ethanol are corn, sugarcane, sugar beet, wheat and sweet sorghum. Sugar-based fuel provides higher energy output than lipid-based fuel like biodiesel (Yuan et al., 2008). Brazil produces its bioethanol mainly from sugarcane while the US uses corn; combined, both countries make up more than half of the world's total bioethanol production (Sarkar et al., 2012). According to the Renewable Fuels Association, the US produced 14,806 million gallons (Figure 3) of bioethanol in 2015 (RFA, 2015). Unfortunately, using sugar-based fuel results in direct competition with food supplies, a factor argued by many opponents of biofuel. Bioethanol is also produced from lignocellulosic biomass, which goes through an additional pretreatment phase before hydrolysis and fermentation (Figure 5). Pretreatment of lignocellulosic biomass allows access of fermentable sugars, hydrolysis then breaks up the disrupted biomass into simpler sugars by cellulase activity, and finally fermentation produces bioethanol (Chen & Fu, 2016). The main approach today for producing bioethanol is using lignocellulosic biomass for syngas production (composed of carbon monoxide and hydrogen) also known as the Fischer-Tropsch process. An alternative approach relies on using enzymes and fermentation pretreatments to produce cellulosic ethanol (Figure 5).

These three platforms of biofuels come with their own set of advantages and disadvantages that play a big role in their ability to replace oil-based fuel. The

disadvantages serve as incentives for research and development in the area of biofuel technology.

Figure 3. Process of Conventional Bioethanol Production



Ethanol conversion starting from bioenergy sources leading to production. Sources are processed through liquefaction and saccharification. Final processing of fermentation, dehydration and distillation converts ethanol (Source: Genencor)

Lignocellulosic Biomass

Lignocellulosic biomass transcends other platforms of biofuel energy, not only because of potentially producing more biofuel, but due to the convenience of biomass crops not interfering with food supplies like traditional sugar crop-based fuels. Lignocellulosic biomass is considered second generation biofuel, traditional biofuel is

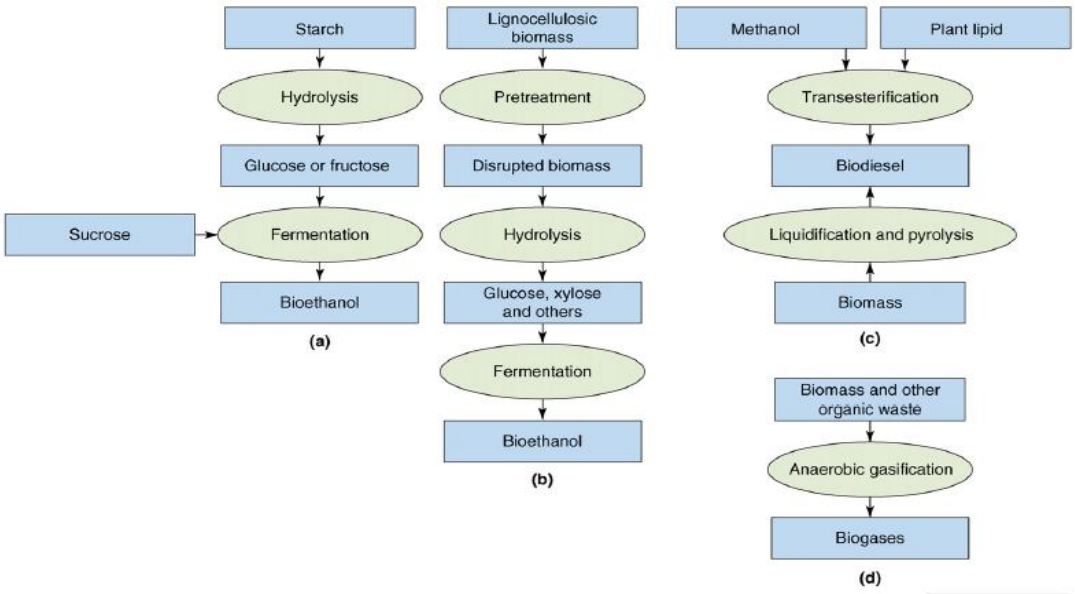
Figure 4. Bioethanol Production of Top Producing Countries Worldwide in 2015

Country	Millions of Gallons
United States	14,806
Brazil	7,093
European Union	1,387
China	813
Canada	436
Thailand	334
Argentina	211
India	211
Rest of World	391

Leading countries of bioethanol production in millions of gallons according to the Renewable Fuels Association (RFA, 2015).

obtained from sugar and starch-based crops is referred to as first generation bioethanol. Lignocellulosic biomass consists of three main components: cellulose, hemicellulose and lignin (Naik et al., 2010). Cellulose and hemicellulose contain polysaccharides while lignin is the organic compound responsible for binding cells and stabilizing cell wall structure. Lignocellulose is essentially “the woody part” and can include any type of grasses, sawdust or crop residues derived from plants (Sarkar et al., 2012). Utilizing biomass widens the options of numerous plants to be used as bio-feedstock compared to limited crop plants currently available for first generation fuel. The biggest hindrance of lignocellulosic biomass is breaking down the cell wall through pretreatments and saccharification, what is known as the recalcitrance issue (Figure 4). The cell wall itself contains approximately 75% polysaccharides, which could be potentially used for fuel, however, accessing these sugars is not optimal with current existing technologies (Naik et al., 2010).

Figure 5. Platform Processing of Different Biofuel Pathways from Various Feedstocks

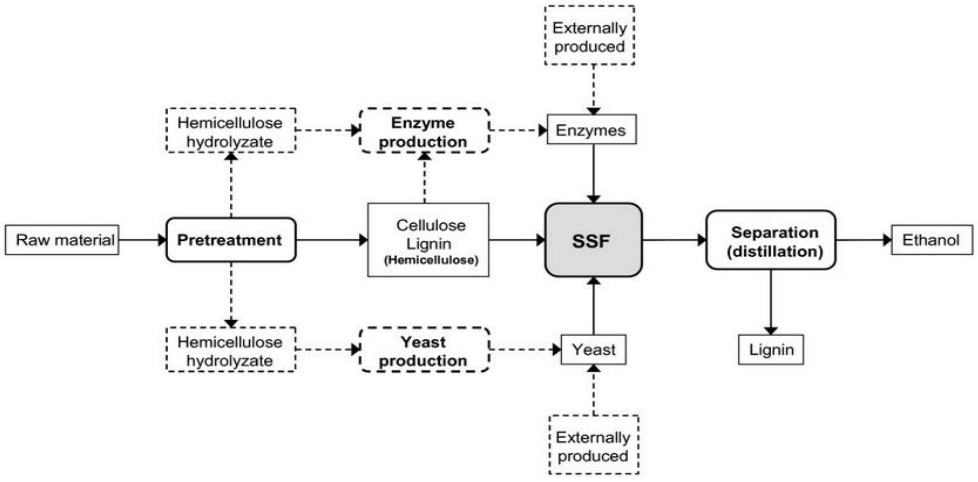


Different pathways leading to bioethanol production. (a) Starch and sucrose processing by hydrolysis and fermentation into monosaccharides then bioethanol. (b) Lignocellulosic is broken down by pretreatment, hydrolysis and fermentation into bioethanol. (c) Methanol and plant lipids go through transesterification to produce biodiesel while biomass is processed through liquidification and pyrolysis to produce biodiesel. (d) Biomass and other organic waste go through anaerobic gasification to produce biogases (Yuan et al., 2008).

Lignin locks in the structure of the cell wall membrane, finding methods of breaking down lignin could potentially disrupt the crystalline structure facilitating biomass-produced bioethanol; this disruption would improve enzymatic break down of polysaccharides (Mosier et al., 2005). One of the two main routes for bioethanol production used today are thermal processing, thermal decay and chemical reformation fall under this processing to produce a range of products such as syngas. Secondly, there is biochemical processing which breaks down polysaccharides into simple sugars through use of pretreatment enzymes (Naik et al., 2010). Sugar breakdown is responsible by hemicellulases or chemically through sulfuric acid (Mosier et al., 2005).

There are different techniques available for bioethanol production, each process has its own advantages and disadvantages, overall none of the existing processes has offered an efficient solution to the recalcitrant issue. Pyrolysis and/or gasification are two processes that produce synthetic liquids under the thermochemical pathway, these commercialized treatments are the more cost efficient as of now. These processes are less costly than the enzymatic pretreatment transformation, responsible for breaks down of cellulose and hemi-cellulose into simpler sugars (Damartzis & Zabaniotou, 2011). Enzymatic hydrolysis breaks down sugars and fermentation transforms the sugars into ethanol; when these two steps are done separately it is referred as SHF (separate hydrolysis and fermentation). Simultaneous saccharification and fermentation (SSF) and co-fermentation use microorganisms to carry out these two processes at the same time. New innovative research is underway to establish methods of developing genetically engineered microbes that can optimize SSF and SSCF (Figure 6) (Mosier et al., 2005).

Figure 6. Schematic Representation of Lignocellulosic Process through SSF



Outline of pretreatment, SSF processing and distillation of lignocellulosic biomass. Raw biomass materials go through enzymatic pretreatment breaking down sugars and then are processed through separate saccharification and fermentation (SFF) to produce ethanol (Olofsson et al., 2008).

Countries worldwide are on the race to develop improved treatment technologies focusing on developing biochemical processes for producing biomass; the US government proposed investing $150 million for biofuels as part of the 2007 Advanced Energy Initiative (Schubert, 2006). Other alternative approaches such as genetic modification and engineered biochemical pathways can facilitate the access of cellulose within the cell wall in the pretreatment stage of lignocellulosic biomass production. Different modernizing approaches in key processes of bioethanol production from biomass (pretreatment, saccharification, and fermentation) can reduce the associated biofuel production costs and efficiency (Yuan et al., 2008). For example, the efficiency of fermentation today is around between 40 to 48 percent while the maximum efficiency theoretically is 51 percent (Naik et al., 2010). Abiotic and biotic stress play important roles in improving biomass yield and offer knowledge ameliorating the saccharification of biomass. There are many challenges within biofuel, but as fossil fuel quantities diminish, biomass research increases the likelihood of having alternative forms of energy that are more cost efficient and less harmful to the environment.

Challenges of Biofuel Technology

Besides being a renewable resource, one of the greatest advantages of biofuel is its ability to be a competitive and cleaner source of fuel compared to petroleum-based energy. While ethanol and biodiesel are currently produced, they produce a positive carbon balance, meaning more carbon dioxide is emitted than is being used up in the environment, which is not favorable (Figure 2) (Yuan et al., 2008). Bioethanol sugar-based fuel obtained from crops, like corn or sugarcane, also establishes economic competition with food agriculture. The increase in demand leads to increase prices of

edible crops; therefore, biomass obtained from lignocellulosic sugars is a more appealing feedstock source. Obtaining lignocellulosic energy has some setback and challenges such as improving pretreatment, reducing the costs associated with pretreatment of cellulose, and finding more efficient methods of enzymatic hydrolysis and fermentation (Chen & Fu, 2016).

Setbacks of key processes of lignocellulosic biomass processing offer several industrial difficulties (Figure 7). Research in developing industrial fermenting yeast strains or synergistic enzymatic hydrolysis systems are plausible future improvements underway (Chen & Fu, 2016). Aside from the limitations of biogas, biodiesel and bioethanol production; biofuel technology also faces additional burdens that the agricultural industry has to combat such as dealing with abiotic and biotic stresses harmful to crop yield production. These challenges put research in biotechnology at the forefront of science, being beneficial for different industries aside from the biotechnology industry.

Future of Biofuel

First generation biofuel currently makes most of the world's bioethanol produced today, but at the same time using crop plants could be detrimental to the food market and to third world countries that heavily rely on these crops as a food source. Second generation biofuel biomass can potentially solve the problem and eliminate the existing food competition. A potential benefit of biomass is using renewable crops that yield large quantities of bioethanol and could also benefit the environment when grown. Third generation fuel is based on microalgae and microbes, this type of fuel might be capable of producing photo-biological hydrogen production, but the technology is currently being

developed. Producing microalgae biomass is typically more expensive and difficult than growing crops (Alam et al., 2015). Second generation fuel is the platform most likely to produce profitable amounts of bioethanol once pretreatment processes exist for easier access of lignocellulose in biomass. Selecting a plant that can grow quickly, resist biotic/abiotic stresses and which contains copious quantities of biomass is important for efficient yields of bioethanol production. One possible bioenergy feedstock that could be a great choice for lignocellulosic bioethanol production is *Paulownia elongata.*

Paulownia elongata

Selecting an optimal crop for biofuel processing is essential to improve efficiency and yield of production. Lignocellulosic biomass holds the greatest potential of the bioenergy sources, and thus opens the door for plant research of crops suitable for biomass biofuel. One plant demonstrating great features and is a promising source of biofuel is *Paulownia elongata* commonly known as Royal Empress Tree (name given to all plants under the genus *Paulownia*) (Stefanov et al., 2016). *P. elongata*, unlike other first generation biofuel plants, is not used as a food source and has more biomass available than most sugar crops such as corn or sugarcane. The plant, native to China, is adaptive to different types of climates ranging from -20°C to 47°C and has an estimated growth rate of 1 m^3 in 7-8 years (Donald, 1990). *Paulownia*, among other plants, can survive in diverse types of soil, including those that are contaminated. Studies have shown that *Paulownia* can improve the quality of soil and plantations can be planted over land used previously for mining. Soil that contain heavy metals and other contaminants can be reduced or removed through hyper-accumulation of metals in plants; this process is known as phytoremediation, another reason why *P. elongata* is a great biofuel plant

choice and a low cost tool that improves damaged soil (Doumett et al., 2008). Damaged lands, due to mining or other contamination factors, are perfect places for developing plantations of *P. elongata* where most standard agricultural crops would no longer be able to thrive in harsh soil environments. Food crops grown in these lands would most likely generate toxic produce that is not safe enough for human consumption.

Grown in plantations, *P. elongata* can be harvested in 4 to 7 years, unlike other hardwood trees that can take almost 80 years to mature (Ayrilmis & Kaymakci, 2013). The fast growth of the *Paulownia* species makes it a profitable option for biofuel production. *P. elongata* is completely renewable from root to leaves; its leaves and flowers have also been used for medicinal purposes (Ipekci & Gozukirmizi, 2003). A 2000-tree per hectare planting of *Paulownia* can yield up to 150 to 300 tons of wood within 5 years (Tisserat et al., 2013). *Paulownia* leaves can even be used for livestock fodder, highlighting the versatility of the plant being used for non-biofuel purposes. *Paulownia*'s different advantageous characteristics and usability have been the reason why different species of have been studied since it benefits more than on type of industry.

More than 65% of *Paulownia* is composed of cellulose or hemicellulose (Yadav et al., 2013). Current studies in genetic modification attempt to find better ways of accessing the sugars within cellulose, optimizing the treatment could make *P. elongata* an important source of bioethanol. The timber can be harvested in about 7 to 15 years and is light, soft, lightweight, and ring porous (Ates et al., 2008). The trees are renewable, once they are cut down they will grow back without having to replant them. Plant regeneration is a great advantage of *P. elongata*, over other plants. *Paulownia* can propagate from stem or root cuttings, saving money and labor in replanting (Ipekci & Gozukirmizi,

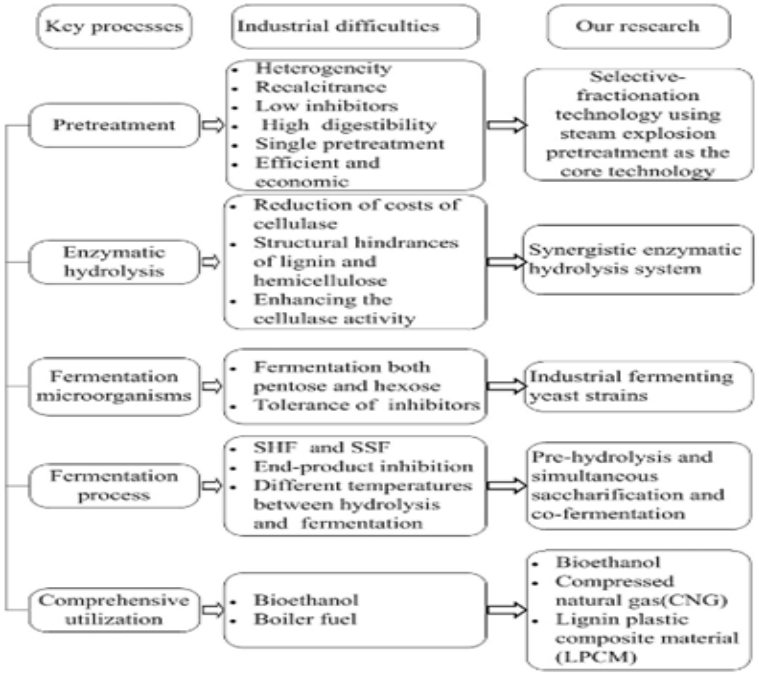
2003). Self-regeneration cuts down costs associated with initial planting of most crops. Self- regeneration makes *Paulownia* a recyclable bio-feedstock, appealing not only for the fuel industry, but for others such as the wood industry. In the U.S, *Paulownia* plantations are estimated to produce 250-275 m3/ha with 40-year-old *Paulownia* plants (Ian Nicholas, 2007). Plantations can be grown in different types of areas and regions. *P. elongata* is resistant to different types of soil and has been shown to be the second most tolerant plant to abiotic stresses among other *Paulownia* species (Ian Nicholas, 2007).

Different growth plantations exist in countries like Brazil and New Zealand, having nurseries and research areas of a variety of *Paulownia* species (Figure 6). Even though it could be a potential front runner as a source of biofuel production, *P. elongata* research is limited, there is not a vast amount of research on *P. elongata* or other *Paulownia* species. The *Paulownia* family is of great interest to other industries such as the wood industry due to its wood capabilities. The *Paulownia* wood, for example, has been studied for its use as a possible wood filler, its wood capabilities are excellent for wood composite production (Ayrilmis & Kaymakci, 2013). Production of *P. elongata* is highly dependent on the plant's ability to grow and its tolerance towards abiotic stresses affects its production.

Understanding *P. elongata* response to various biotic and abiotic stresses can play an important role in learning about the plant's resistance. These advancements could lead to possible improvements through genetic modification. *Paulownia* is known to be resistant to abiotic stresses such as temperature or abnormal pH in soil, but it is sensitive to salinity (García-Morote et al., 2014). Studying gene expression, in response to salinity

in *P. elongata*, could reveal important mechanisms and pathways of novel genes that have yet to be uncovered.

Figure 7. Summaries of Challenges and Countermeasures in Industrial Technologies of Lignocellulosic Ethanol



Outline of major key processes: pretreatment enzymatic hydrolysis, fermentation microoorganisms, and comprehensive utilization. Major industrial difficulties and current research of key processes (Chen & Fu, 2016).

Learning more about different biotic/abiotic stresses and their effect on gene expression can open the door for improvements in biotechnology and consequently improve bioethanol yields that could produce more economic biofuel. As of now, the transcriptome of most *Paulownia* has not been sequenced and there is limited genomic data in public databases (Li et al., 2014). Salt is one of the abiotic stresses that *P. elongata* is the least resistant to; genes involved in salt tolerance along with the complex integrated system cellular response to salinity are not completely understood. Studies of *P. elongata* and *P. tormentosa* have shown that salt stress can affect the plant's growth, photosynthesis and water use efficiency. Learning more about salt related gene

expression might be a useful biogenetic tool for the future (Gonçalves et al., 2008). *P. elongata* gene expression research can identify important information about genes responsible for plant tolerance of diverse harsh environments revealing more about the physiological responses due to different types of stresses; these would ultimately be profitable, not only for crops used for biofuel, but for those crops used for agriculture. Novel genomic studies could contribute in improving food supplies and agricultural resources. New research could save millions of dollars by saving crops lost each year due to abiotic stresses.

Figure 8. *Paulownia elongata* Plantation

12-year-old- *P. elongata* plantation growing near Opotiki, Bay of Plenty (Ian Nicholas, 2007)

Chapter-II

Salt Stress and Pathways

Worldwide Salinity Effects on Agriculture

In order to make biofuel technology a sustainable and profitable platform of fuel production, different methods of improving plants abiotic stress tolerance are pivotal. Salinity research, as a detrimental agricultural abiotic stress, is expanding becoming more threatening to agriculture and the environment. Today, salinity is turning into a serious environmental factor that is increasingly limiting crop production, germination and yields worldwide (Carillo et al., 2011). According to the UN, in 2013 salt induced crop loss was estimated at $441 per hectare corresponding to a global economic loss of $27.3 billion per year (UNU-INWEH, 2013). Salinity in soil is caused either through natural occurrences or secondary factors such as salinization of soil, surface water or as a result of agriculture and urbanization (Carillo et al., 2011). Since most plants are glycophytes, meaning they cannot withstand salinity, learning about the mechanisms and pathways of stress resistant plants can relay how plants resist salinity. The pathways are crucial for future of biofuel and agricultural productivity. An approximate 20% of cultivated land is currently affected by salinity (Gupta & Huang, 2014). The effects of salinity are destructive and reduce the amount of crops harvested, irrigation of land has been known to increase salinity leading to over-salinization of soil (Nasim et al., 2007).

Salt Tolerance Mechanisms and Physiological Effects of Salinity

Glycophytes can tolerate high levels of salt until salinity reaches 100-200 mM NaCl while their counter parts halophytes, plants resistant to salinity, can tolerate up to 300-400 mM NaCl (Carillo et al., 2011). At a molecular level salinity affects plants by

inhibiting photosynthesis, inducing several osmotic effects, and causing ionic stress. The two major players in plant death are decrease in water potential caused by high salinity and eventually a high Na^+ concentration level that becomes toxic at a cellular level (Sairam & Tyagi, 2004). Some examples of popular glycophytes are switchgrass or smooth cordgrass, thriving in high salt level soils. The majority of plants are glycophytes such as beans or rice, and most crop plants fall under this category. Salt sensitivity of the majority of crop plants emphasizes the threat brought forth by salinity. In a broad sense, salinity affects plants in a two-step fashion: salt compromises the capacity of the roots to intake water and high concentrations of salts become toxic, inhibiting physiological and biochemical processes such as nutrient uptake (Zhu, 2000).

High salt concentrations affect the ability of roots to uptake water causing a stunt in the shoot leading to an osmotic imbalance, inhibition of root and leaf growth and a decrease in intake of minerals and nutrients (Gupta & Huang, 2014). The plant reacts to an osmotic imbalance caused by the influx of Na^+ by accumulating osmoprotectants and osmolytes; these are compounds responsible for maintaining cell volume and fluid that vary between plant species (Yokoi et al., 2002). A plant's ability to make adjustments varies from hours to days contributing to the overall tolerance of the plant (Horie et al., 2012). Osmotic potential is an important factor that plays a big role across different types of abiotic stresses making salt stress studies beneficial for understanding ways of optimizing crops for biofuel production. Knowledge of the processes and pathways of water intake by plants roots is also vital for growing and producing plants.

Although soil salinity comes in the form of Cl^-, most research focuses on Na^+ due to its more profound effects on the overall plant metabolic processes, both are initially

compartmentalized into vacuoles by plants. Accumulation of ions mostly Na^+, in the transpiration stream reaches toxic levels that negatively affect photosynthetic components such as enzymes, chlorophylls and carotenoids (Petronia Carillo).

Figure 9. Summary of Stress Effects and Consequences as a Result of High Salinity

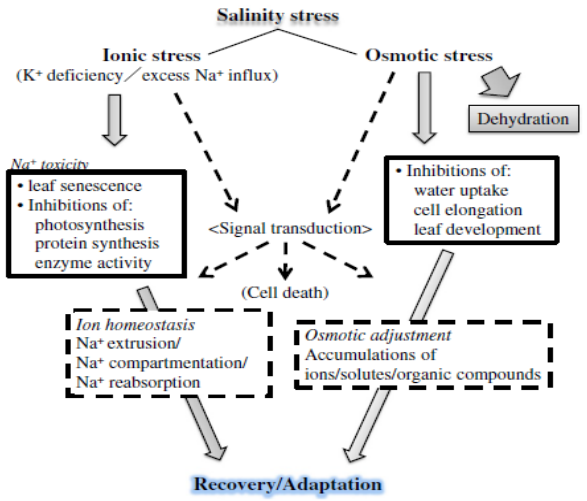


Diagram of salinity stress affecting plants through ionic and osmotic Stress. Ionic stress leads to Na^+ toxicity. Osmotic stress causes dehydration and inhibition of major processes. Plant attempts recovery through ion homeostasis and osmotic adjustments (Horie et al., 2012)

Excessive Na^+ accumulation leads to halting of pathways necessary for homeostasis, premature senescence and reduced growth (Horie et al., 2012). Excess Na^+ directly competes with ion intake of K^+ and Ca^{2+} ions, which contributes to its toxicity, as K^+ is involved in pivotal signaling mechanisms within the cell. Plant response to salt stress through three main mechanisms: osmotic tolerance, Na^+ exclusion, and tissue tolerance. Learning more about these mechanisms could reveal fundamental physiological and metabolic changes of the plant.

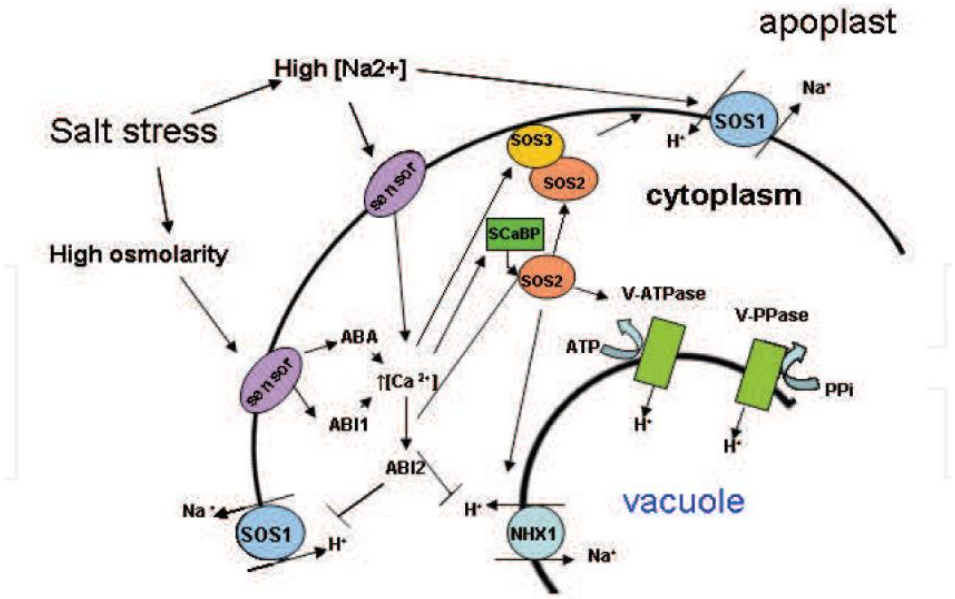
Biochemical mechanisms and pathways

Salt is involved in various principle mechanisms including, but not limited to, ion homeostasis, activation of antioxidant enzyme and compounds, generation of nitric oxide and hormone modulation, etc (Gupta & Huang, 2014). Antiporters, channels and transporters play an important role in mobilizing ions such as Na^+ or H^+ ions. The Salt Overly Sensitive (SOS) signaling pathway consists of proteins activated through gene expression induced by high salt levels. SOS genes regulate salt tolerance as well as pH homeostasis, membrane vesicle trafficking and vacuole functions (Gupta & Huang, 2014). The SOS1 Na^+/H^+ antiporter is an example of SOS signaling, the antiporter is responsible for regulating export and import of Na^+ and H^+ ions across the vacuole membrane (Carillo et al., 2011). Increase salinity can also disrupt electron transport chains in chloroplasts and mitochondria giving rise to oxidizing compounds known as reactivated oxygen species also known as ROS (Gupta & Huang, 2014). Superoxide and hydrogen peroxide are two examples of ROS induced by salinity. ROS removal is impaired under salt stress, therefore accumulation of ROS serves as a toxic by-product of stress metabolism and as a signal transduction molecule mediated by complex networks of stress response pathways (Carillo et al., 2011).

Nitric oxide (NO) regulates plant growth and development such as respiration, stomata closure and acts as a signaling molecule (Gupta & Huang, 2014). NO has been aided plant survival by contributing to an increase in production of antioxidant enzymes such as nitrate reductase and nitrite reductase (Manai et al., 2014). ROS accumulates as a result of salinity, acting as signal inducing molecule for NO synthesis. NO activates antioxidant enzymes. Once ROS is produced at a higher rate, it can no longer adapt metabolically (Groß et al., 2013). ABA, abscisic acid, is a well-studied hormone that

helps the plant improve the effects of stress conditions in plants, it works as a cellular signal that modulates salt tolerance genes (Gupta & Huang, 2014).

Figure 10. Pathways of Antiporter, SOS, and ABA Signaling during Salt Stress



Salt stress causes high osmolarity and excess Na^+ induce sensors that activate ABA mediated pathways. Signal induced NHX1 and V-ATPase/ATP antiporters to maintain ionic homeostasis within the vacuole. Different SOS proteins mitigate ionic balance within the cytoplasm and across the cell membrane (Carillo et al., 2011).

ABA is weakly acidic and initiates stomatal closure to prevent water loss by reducing turgor pressure through an efflux of K^+ signal cascades (Song & Matsuoka, 2009). ABA is induced by osmotic stress and water deficit in shoots and roots (Gupta & Huang, 2014). Salicyclic acid (SA) is another example of a hormone regulator of salt stress that improves tolerance by restoring membrane potential preventing K^+ loss (Gupta & Huang, 2014). SA is responsible for regulation of different genes in plants.

RNA sequencing and Salt Gene Expression

Salt stress induced genes which initiate pathways and mechanisms responsible for plant survival and tolerance have been studied indefinitely. New information about pathways and mechanisms can be used as tools in biogenetics. Gene expression techniques have changed in the past few decades; RNA-sequencing is a recent advancement in transcriptomics revolutionizing the way gene expression is studied. RNA-sequencing (RNA-seq) is a high-throughput method used for mapping and quantifying gene expression by fragmenting RNA, isolating mRNA then creating a cDNA coded library analyzed by bioinformatics software. The technique has been used to sequence *Arabidopsis thaliana* and higher eukaryotic transcriptomes like human cells (Wang et al., 2009). Some of the main advantages of RNA-sequencing over traditional methods such as microarrays include: detection of novel transcripts without need of species specific probes, increased specificity/sensitivity to gene expression, and digital sequencing read counts providing broader dynamic range. RNA-sequencing is a next generation sequencing technique that facilitates the process of understanding gene expression of organisms with un-sequenced genomes, and the specificity it offers can potentially uncover important genes that hold an important role in biotic and abiotic stresses in plants.

Abiotic stresses are complex interconnected networks; these mechanisms and pathways behind salinity play a crucial role in the ability of the plant to tolerate high salt concentrations and survive harsh conditions. Understanding and identifying key novel genes could be beneficial for genetic engineering and RNA-seq has been an instrumental tool in abiotic stress research of plants for the past few years. For example, RNA-seq-based research in *Mesembryanthemum crystallinum*, a halophyte ice plant, identified

important tolerance mechanisms and ABA responsive genes during salt stress (Tsukagoshi et al., 2015). RNA-seq analysis of Bermudagrass, identified genes regulating lignin synthesis and phytohormone signaling affecting cell wall loosening and cell growth (Hu et al., 2015). Considering cell wall loosening and lignin synthesis are connected to saccharification, these genes could potentially be used for developing bioengineered pathways facilitating pretreatment and enzymatic breakdown of polysaccharides. Studies in other abiotic stresses related to salt stress such as drought have presented ways in which imbalances of osmotic potential can hinder the reproduction stage of plants by affecting plant metabolism (Kakumanu et al., 2012; Tsukagoshi et al., 2015). *P. elongata* RNA-seq analysis could identify key regulatory salt genes linking important pathways and might be favorable for future biofuel-related studies.

Poverty and limited food availability affect more than 800,000,000 people, salinization in soil worsens the food-limited regions of the world. It is important to find ways of improving soil conditions for agricultural purposes as well as biofuel production (Himabindu et al., 2016). *P. elongata* is resistant to most abiotic stresses, understanding how gene expression unfolds under salt stress might offer a better insight on novel genes involved in preventing salinity from reaching toxic levels leading to plant senescence. These studies might serve as important alternatives to remediate worldwide issues with salinization and agriculture. *P. elongata* is a suitable option for second generation biofuel production.

Chapter-III

Functional Analysis of Salt Stress Induced Genes from *Paulownia elongata*

Background:

Paulownia elongata is one of the world's fastest maturing plants, and is capable of being an efficient and profitable feedstock for biofuel production. As the world leading countries are on a race to produce suitable environmentally-friendly forms of fuel, the world is straying away from the dependence of petroleum-based fuels. Bioethanol is one of the main biofuel platforms and generates the most energy output (Figure 2). Currently, bioethanol is produced as a result of hydrolysis and fermentation of starch and sucrose (Yuan et al., 2008). In 2005, the US produced more than 3.9 billion gallons of ethanol using about 14% of national corn crop (Somerville, 2007). Ethanol produced from lignocellulosic can produce higher amounts of bioethanol. In the US, the approximate yield of a 40-year old *Paulownia* plantation is about 250-275 m^3/ha (Ian Nicholas, 2007). *Paulownia elongata* is a front runner due to its high biomass content alongside other positive characteristics such as a fast growth rate, ability to regrow without re-planting, and resistance to numerous abiotic stresses. The wood of *Paulownia* is high quality and also usable for a variety of alternative purposes, the wood itself has great qualities such as light weight, insect resistance, heat resistant, etc (Tisserat et al., 2013).

Salinity is a detrimental abiotic stress that affects a plant's metabolic/cellular pathways indirectly and directly. Approximately 20% of the world's cultivated plants are affected by salinity, and thus a major potential abiotic stress impacting future biofuel production (Stefanov et al., 2016). At the molecular level, salinity activates a stress response through a complex network of signaling pathway and biochemical mechanisms.

P. elongata is known to be resistant to a wide variety of biological stresses, yet there is minimal knowledge of the plant's tolerance towards salinity (Ian Nicholas, 2007).

This study investigates gene expression levels during salt stress in *P. elongata.* Currently the genome of *P. elongata* has yet to be sequenced, thus raising challenges in investigating the transcriptome. However, exploring significant changes in gene expression, as a result of salt stress, could be fundamental in identifying genes that play an important role in the plant's survival and in other species as well. After being salt stressed for 10 days, *P. elongata* RNA was extracted, RNA-seq libraries prepared, sequenced and analyzed. Trinity software was used to generate a *de novo* generated transcriptome of *P. elongata,* and analysis with the Tuxedo software suite produced a list of genes that were statistically significant upregulated, downregulated, turned off or turned on during salt stress. Gene expression results from RNA-seq were validated by RT-qPCR. Genes were identified by BLASTing the sequences against NCBI's non-redundant (nr) database, and previous literature was examined for possible roles of these novel genes in principle mechanisms and pathways were hypothesized. These novel genes could play an important role in understanding plant tolerance mechanisms and in developing genetically engineered pathways. *P. elongata* novel genes can be used for downstream studies and functions of these novel genes can further knowledge of gene salt tolerance.

Materials and Methods

Salt Treatment Conditions

P. elongata were obtained from a nursery and approximately 3-years of age, grown inside the CSUN greenhouse. *P. elongata* plants were kept under a control temperature of 25°C,

75% relative humidity and under natural lighting from the sun. Salt stressed plant pots were irrigated with 1.5 L of 125 mM NaCl, while the controls did not receive NaCl treatment. Plants were salt treated daily for 10 days. This experiment used two biological replicate controls and two replicates for salt stress.

Total RNA extraction

Quick-RNA MiniPrep kit, per the manufacturer's instruction and included materials, yielded purified high-quality RNA. RNA degrades easily and safe practices had to be implemented: using RNase/DNase free tubes, RNase AWAY sprayed on counters/tools, and RNase/DNase free water, etc. Liquid nitrogen is used to disrupt the cell wall and cell membrane of plant tissue cells. *P. elongata* is placed in a mortar and crushed with a pestle, all wiped with RNase AWAY beforehand. Approximately 0.05 grams of *P. elongata* tissue is placed in the mortar, then liquid nitrogen is added, grains of sand can be added to facilitate grinding, but is not required. Different mortars should be used for control and the salt stress samples to avoid possibility of cross-RNA contamination. Frozen plant tissue is placed in 1ml RNase/DNase free tubes containing 600 µl ThermoFisher Scientific TRIzol Reagant; isolating the RNA, DNA and proteins from the frozen plant tissue, an exact volume of 100% ethanol is added to the mixture. The mixture is transferred to a Zymo Research spin column inside a collection tube and centrifuged at 12,000 x g for 1 min. using a Spectrafuge 24D centrifuge, the flow in the collection tube is discarded.

RNA is purified of DNA contamination by adding 40 µl of DNase I reaction mix and incubating the spin column at room temperature (≈25ºC) for 15 minutes. DNase I reaction mix is composed of 5 µl of DNase I and 35 µl of DNA digestion buffer. The

spin column is centrifuged 12,000 x g for 1 min and flow is discarded. Following centrifugation, 400 µl RNA Prep Buffer is added to the spin column and centrifuged for 30 sec., flow in the collection tube is discarded. Thereafter, 400 µl of RNA Wash buffer is added to the spin column and the mixture is centrifuged for 1 min., the wash is repeated. Final elution is performed by adding 50µl of RNase free water. Heating up RNase/DNase free water to 60°C for 10 min. improves yields and elution of RNA. Two samples of RNA were obtained from each of the two salt stress plants and two control plants labelled as follows: SA1, SA2, SB1, SB2, CA1, CA2, CB1, CB2.

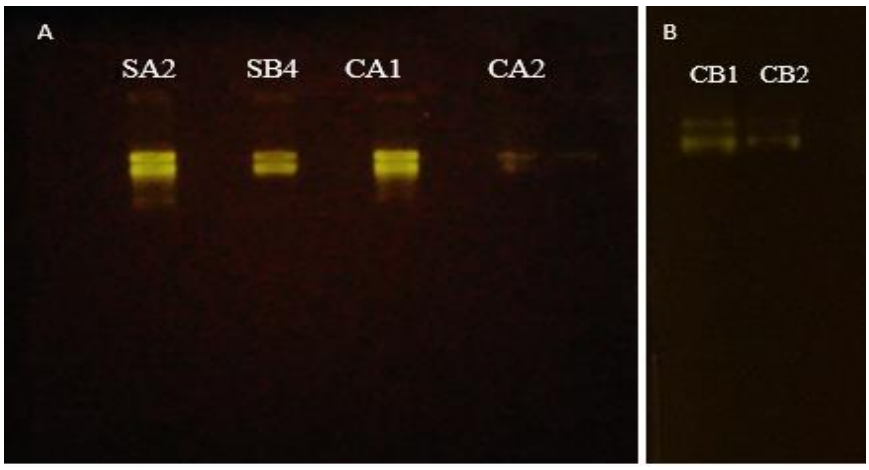
RNA samples are analyzed using the ThermoFisher Scientific Nanodrop 2000c spectrophotometer, which displays the absorption of RNA across different wavelengths. The Nanodrop serves as resourceful tool, as RNA purity is assessed by standard peaks of nucleic acids (Appendix 1). The Nanodrop is used a qualitative tool while the Invitrogen Qubit 3.0 fluorometer serves as a quantitative tool to measure RNA quantities within the samples (Table1). For both the Nanodrop and Qubit machines, only 1 µl of RNA is required to run the analysis per sample. Genomic DNA contamination is checked visually using gel electrophoresis (1% agarose) using an Invitrogen 1kb DNA Ladder to confirm there was no DNA contamination. A RNA staining gel, using a AMRESCO formaldehyde-free RNA gel kit, visually confirming the presence of RNA by displaying 28S and 18S rRNA bands. The RNA was quantified using an Alpha Innotech Fluorometer (Figure 11).

Table 1. Concentration of *P. elongata* RNA in ng/ml from .05 g of *P. elongata* Tissue Treated with 125 mM of NaCl for 10 Days using Qubit. Controls excluded NaCl Treatment

Biological Samples	ng/ml
SA1 (Salt Stress Plant A Sample 1)	5.8×10^4
SA2(Salt Stress Plant A Sample 2)	1.16×10^5
SB1 (Salt Stress Plant B Sample 1)	1.28×10^5
SB2 (Salt Stress Plant B sample 2)	1.34×10^5
CA1 (Control Plant A Sample 1)	1.36×10^5
CA2 (Control Plant A Sample 2)	1.3×10^4
CB1 (Control Plant B Sample 1)	3.44×10^4
CB2 (Control Plant A Sample 2)	1.1×10^4

*Bolded samples were chosen as best candidates for RNA sequencing

Figure 11. Gel of RNA Salt Stress and Control *P. elongata* Samples.



Gel A and Gel B shows 28S and 18S RNA bands of *P. elongata* salt stress and control replicates (SA2 and SB4 are salt replicates). CA2, CB1 and CB2 are RNA control replicates).

RNA-seq

Purification and Binding of RNA

Illumina TruSeq RNA preparation kit v2, including reagants, was used according to the manufacturer's instructions in order to generate cDNA barcoded libraries. The Low Sample Protocol of the kit was followed and due to the length of the experiment (Figure 12), the experiment can be divided into three parts across three days. To begin, 1 µg of RNA is diluted in RNase/DNase free water for a final volume of 50 µl into a .3ml 96-well PCR plate. Afterwards, 50 µl oligo-dT attached magnetic beads are added to the

mixture. The mixture is put in a thermocycler at 65ºC for 5 mins to facilitate binding. Mixture is washed with 200 µl of Bead Washing Buffer, consequently the well plate is placed on top of a magnetic stand for 5 mins. Subsequently, 50.0 µl Elution Buffer is added to the well plate. The plate is incubated for 2 mins at 80 ºC to isolate mRNA. The mRNA is isolated by adding 50µl of Bead Binding Buffer to each well plate containing the RNA and the plate is incubated at room temperature for 5 mins. The plate is placed on a magnetic stand, binding mRNA-attached beads, and the supernatant is discarded. Purified mRNA is prepared to be eluted and fragment, 19.5 µl of Elute, Prime, Fragment Mix is added and then the plate is placed in a thermocycler programmed for 94º C for 8 mins. The .3ml 96-well PCR plate is sealed with a micro-adhesive seal.

The Illumina TruSeq Low Sample Protocol then proceeds to first strand cDNA synthesis, 17 µl of supernatant, containing fragmented and primed RNA. The mixture is transferred to new 96-well .3ml PCR plate after being placed on a magnetic stand for 5 mins. Afterwards, 50µl of Superscript II, is added to the wells along with 8µl of First Strand Master Mix. First strand cDNA synthesis is completed by placing the plate in the thermocycler with the following parameters: 25ºC for 10 mins, 42ºC for 50 mins, 7ºC for 15 mins and hold at 4ºC. Second strand synthesis is initiated by adding 25µl of Second Strand Master Mix to each well and incubation in a thermocycler for one hour at 16ºC. AMPure XP Beads, 90µl, are added to separate the ds cDNA from the second strand reaction mix and the plate is incubated at room temperature for 15 mins. Bounded cDNA-AMPure XP Beads are isolated once the plate is put on top of the magnet stand, the supernatant is discarded. The wells are washed with 200 µl of 80% EtOH twice, discarding the supernatant each time while being place on the magnetic stand. The double

stranded cDNA is re-suspended by adding 52.5 µl of Resuspension Buffer. The plate is incubated for 2 mins, placed on the magnetic stand which isolates the cDNA in supernatant. The supernatant, 50 µl, is transferred unto another .3ml 96-well plate.
End Repair, adenylation of 3' ends, ligation of adaptors and PCR enrichment end repair fills in the 5' overhangs resulting from previous fragmentation, 10 µl of Resuspension Buffer is added to the *Paulownia* ds cDNA, then 40 µl of End Repair Mix is also added. The plate is sealed and incubated in a thermocycler for at 30ºC for 30 mins. AMPure XP Beads, 160 µl, are added to the wells and the plate is incubated at room temp. for 15 mins and then for 5 mins on the magnetic stand. A total of 200 µl of supernatant is discarded from the plate while on the magnetic stand; the plate is allowed to be dried for 15 mins. Plate is washed with 80% EtOH, adding 200µl, incubating the plate for 30 secs and the supernatant is discarded, this step is repeated one more time. The plate is removed from the magnetic stand and 17.5 µl Resuspension Buffer is added, then the plate is incubated for 2 mins. The supernatant of each well is transferred a new 96-well .3ml PCR plate completing end repair.

Adenylation of 3' end prevents self-ligation during adapter reaction. The process begins by adding 12. 5 µl A-Tailing Mix to each well of the plate, sealed with a micro-adhesive seal, the plate is placed in a thermocycler programmed: 37ºC for 30 mins, 70ºC for 5 min., hold at 4ºC. Adapters are ligated to ds cDNA for subsequent hybridization onto a flow cell. The well plate is heated at 30ºC for 10 mins in a thermocycler after being sealed with a micro-adhesive seal. Stop Ligation Buffer, 5µl, is added to the plate after being removed from thermocycler and 42µl of mixed AMPure XP Beads is added to each well. The place is incubated at room temperature for 15 mins and then placed on a

magnetic stand for 5 mins. The supernatant, 79.5 µl, is discarded and the well plate is washed twice with 200 µl of 80% EtOH, incubated for 30 secs and then supernatant is discarded. After the EtOH washes, the plate is dried for 15 mins at room temp., the plate is then removed from the magnetic stand. Resuspension Buffer, 52.5 µl is added, to each plate and is incubated for 2 mins. The well plate is placed on the magnetic stand once again at room temperature for 5 mins and then 50 µl of the supernatant from each well is transferred to a new 96-well .3ml PCR plate. Once transfer of supernatant is complete, 50 µl of mixed AMPure XP Beads is added and incubated at room temperature for 15 mins and then placed on a magnetic stand for 5 mins. Subsequently 95µl of the supernatant is removed and the plate is washed with 200 µl of 80% EtOH twice while on the magnetic stand, it is then incubated for 30 secs followed by complete removal of supernatant. After the plate is dried for about 15 mins, 22.5 µl Resuspension Buffer is added following a 2-minute incubation, the well plate is placed on a magnetic stand at room temperature for 5 mins. The supernatant, 20µl, is transferred to a new 96-well .3ml PCR plate.

The final part of the Low Sample Protocol involves using PCR to amplify adapter ligated DNA molecules to enrich the amount of *P. elongata* cDNA libraries. PCR primer cocktail, 5µl, is added to each well along with 25µl of Master Mix. The thermocycler is programmed under the following parameters: 98ºC for 10 secs; 15 cycles of 60ºC for 30 secs, 72ºC for 5 mins and hold at 10ºC. After running the PCR, 50 µl mixed AMPure XP Beads is added to each well containing 50µl of amplified cDNA. The plate is incubated for 15 mins, placed on a magnetic stand. Supernatant, 95µl, is removed and while on the magnetic stand, the well plate is washed with 200 µl of 80% EtOH twice, incubated for

30 secs followed by complete removal of the supernatant. After EtOH washes, the plate is dried for 15 mins at room temperature, the plate is then removed from the magnetic stand. Resuspension buffer, 32.5 µl is added, to each plate and incubated for 2 mins. The plate is placed on the magnetic stand for 5 mins at room temperature and then 30 µl of clear supernatant is transferred to a new 96-well .3ml PCR plate. The Invitrogen Qubit 3.0 Fluorometer quantified cDNA libraries of two salt stress and two control *P. elongata* samples (Appendix 2). Presence of cDNA within the libraries was validated using 1% agarose gel using Invitrogen 1kb DNA ladder (Figure 13).

Figure 12. Flow Chart of the Illumina RNA-seq

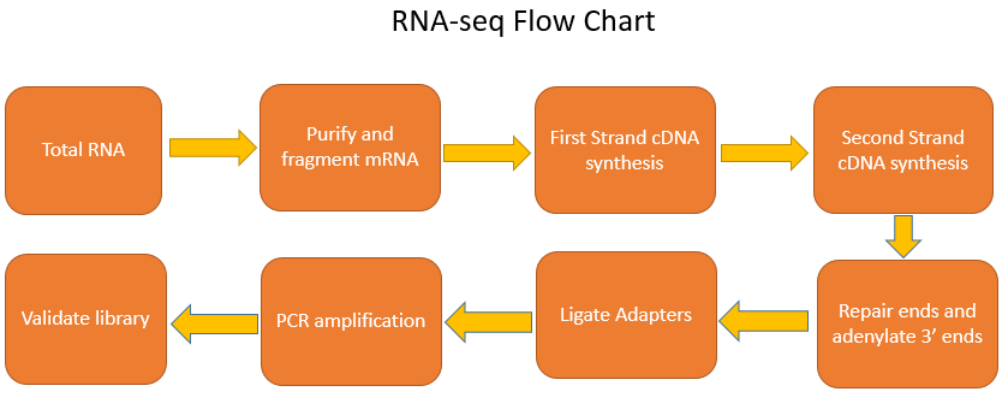
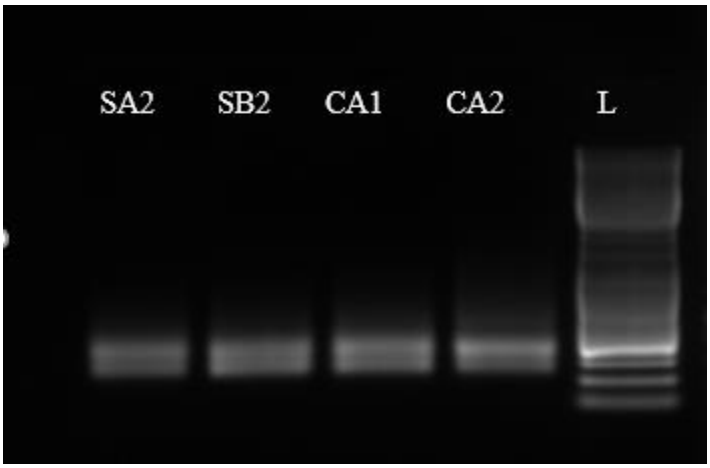


Figure 13. 1% agarose Gel of cDNA Libraries of *P. elongata* Salt stress and Control samples



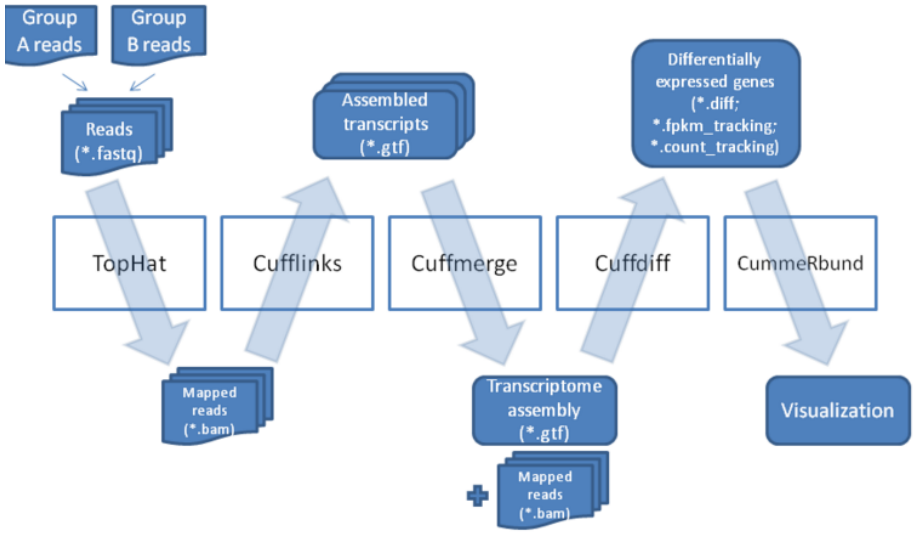
First two lanes from the left, SA2 and SB2, are cDNA libraries coming from salt stress plant 1 and 2 respectively. CA1 and CA2 are cDNA libraries from the control samples the final right is a 1kb DNA ladder.

RNA-seq gene expression analysis

The Illumina MiSeq Sequencing system at CSUN was used to sequence the barcoded cDNA libraries using the MiSeq Reagent Kit v3 (150-cycle) producing total number of reads for each cDNA sample (Table 2). *P. elongata* has no annotated genome available currently, therefore the bioinformatics Trinity software (v2.0.6) was used to generate a *de novo* transcriptome from all the pooled reads. The Tuxedo Software suite (v2.0.13) including TopHat, Cufflink, Cuffmerge and Cuffdiff were used to sort, map and quantify and determine expression differences between conditions from the RNA-seq reads. TopHat software mapped and aligned RNA-seq-reads to the *de novo* transcriptome using Bowtie software, a high-throughput short read aligner. Cufflinks software was used to quantify gene expression levels for each of the different samples, and Cuffmerge software was used to merge output data from Cufflinks. Cuffdiff compared the gene expression levels of the merged transcripts between the different samples and determined the differentially expressed genes during salt stress. Cuffdiff generated a list of genes that

were upregulated, downregulated, turned on or turned off by comparing abundance of transcripts between samples with statistically significant P-values.

Figure 14. Outline of Tuxedo Suite RNA-seq Analysis



Reads obtained from the de novo generated transcriptome of *P. elongata* were used in the Tuxedo suite for analysis. TopHat maps reads which are assembled into transcripts by Cufflinks and Cuffmerge assembles the different transcripts of each sample. Cuffdiff quantifies and sorts differentially expressed genes. CummerRbund can be used for visualization. (David C Corney, 2016)

Table 2. Total Reads of Sequenced Genomic cDNA of Salt Stressed *P. elongata* Samples and Controls

Biological Samples	Reads
SA2	9,545,616
SB2	10,196,304
CA1	10,586,624
CB2	8,910,322

NCBI Blast and Gene Selection

The Tuxedo software suite produced a list of statistically significant genes affected by salt treatment in *P. elongata* (Appendix 3). Genes that were downregulated, upregulated, turned off and turned on were selected and then BLASTed to find possible

matches. Novel genes were selected and confirmed by quantitative real-time PCR. Further research investigated possible functions in regard to their role in salt stress. Selection was based on genes that showed highest fold change in expression according to the RNA-seq data and the identity percentage from the NCBI BLAST results. Genes turned off and turned on were selected based on possible importance and high identity from NCBI BLAST results.

Table 3. Selected Salt Affected Genes in *P. elongata* Identified by NCBI Gene BLAST

P. elongata genes			
Down Regulated	Upregulated	Turned off	Turned on
Nitrate reductase	Extracellular Ribonuclease LE like	NADH Dehydrogenase Cytochrome C	Ethylene-responsive transcription factor ABR1-like
Pyruvate, phosphate dikinase	GDSL esterase/lipase At5g55050-like	Mitochondrial uncoupling protein 5-like	Aquaporin PIP2-4-like
Protein Gradient Regulation 5	21 kDa protein	Inactive leucine-rich repeat receptor-like protein kinase At5g06940	7-deoxyloganetic acid UDP-glucosyltransferase-like protein mRNA, complete cds
Fructose-bisphosphate aldolase 1	Glyceraldehyde-3-phosphate dehydrogenase GAPCP2, chloroplastic-like	CSC1-like protein	Tricyclene synthase Oc15
Magnesium-chelatase subunit ChlH	Glucan-1,3-beta-glucosidase 14-like	WRKY transcription factor 70	Extensin-2-like

Selected salt stressed *P. elongata* genes included a high identity of over 85% according to NCBI Blast. Each column is separated by category: upregulated, downregulated, turned off and turned on.

Quantitative real-time PCR

Primer Design and Optimization

Tuxedo software suite and Trinity software data was compared and validated using quantitative real-time PCR. Selected *P. elongata* salt stress genes were selected after

gene BLASTing. Primers were designed and ordered from IDT-website (Appendix 4). Primer Quest Tool Primer Assay design software, from the IDT-website, generated primers for each of the *P. elongata* genes using sequences obtained from the Tuxedo Suite analysis. Primers were optimized through a series of PCR experiments using *Paulownia elongata* DNA. The reaction conditions used for PCR were: 30s of initial denaturation at 95°C, 39 cycles of denaturation at 95°C for 5 seconds. Different annealing temperatures ranging from 49ºC-55ºC were selected, 1% electrophoresis confirmed which annealing temperatures worked optimally for each of the selected *P. elongata* genes.

Setup of *P. elongata* salt stress genes gene expression through RT-qPCR

HACTIN, an actin gene, was the housekeeping gene chosen for gene expression normalization also known as the reference gene. This study used iTaq Universal SYBR Green Supermix and Bio-Rad CFX96 real-time PCR detection machine. Each reaction mixture contained 1µl of cDNA, 5µl of iTaq Universal SYBR Green Supermix (2x), and 2µl of the corresponding forward and reverse primers (2 µmol/L). Three biological replicates of *P. elongata* gene reactions were used, one replicate of HACTIN reaction was used per gene, and one replicate of NRT negative control to ensure accuracy of gene expression. The temperature settings for the template was: 30s of initial denaturation at 95°C, 39 cycles of denaturation at 95°C for 5 secs. The annealing at temperature varied between 49-51°C (depending on optimized temperature of each gene) for 30 secs. The Bio-Rad CFX96 real-time PCR detection thermocycler ran the RT-qPCR and Bio-Rad software analyzed gene expression displaying gene quantification and melt curve analysis regarding primer specificity.

Results and Discussion

RNA Extraction/RNA-seq

Different RNA extraction protocols were performed to determine which would produce a viable yield of RNA needed for subsequent RNA sequencing. Extracting RNA from plants was hindered due to easy sample degradability and levels of contaminants in the samples. Frozen *P. elongata* tissue, after adding liquid nitrogen, needed thorough crushing as the cell wall impeded plant cell lysis resulting in low RNA concentrations. Zymo Research has an integrated DNase treatment as part of its protocol, which was key in producing high quality uncontaminated RNA. Alternative RNA protocols produce high yields of low quality RNA. Using most RNA extractions following a separate DNase treatment would end in very low yields of RNA not sufficient for RNA-seq. Zymo Research DNase treatment occurs in the same spin column used throughout the whole experiment minimizing any loss of RNA. RNA impurities also gave false reads and was responsible for high reduction in RNA concentrations after purification. Different RNA concentration reads by the Nanodrop machine would display inconsistent concentrations when the Nanodrop was used to analyze the samples. Although the Nanodrop spectrophotometer visualized early signs of contamination and confirmed presence of RNA by checking the absorbance peaks of the samples, it was highly unreliable as a quantitative tool and would produce erroneous reads. Ultimately, a more suitable tool was used for quantification purposes. The Qubit 3.0 Fluorometer provided more accurate quantification of RNA and was used as the final quantitative tool for quantifying DNA/RNA for all experiments. The Qubit machine uses fluorescent dyes to specifically

bind to nucleic acids, making its concentration readings specific and more accurate than concentration readings from the Nanodrop.

RNA-seq served as a useful tool to generate barcoded cDNA libraries, the technique holds various advantages over the traditional use of microarrays analysis of transcriptomes. The protocol requires extensive hours; it is recommended for the experiment to be split into multiple days to reduce chances of error. The use of magnetic beads to bind RNA/DNA and the magnetic stand in the Illumina TruSeq kit facilitated purification and isolation of RNA/DNA. The concentration of the *P. elongata* cDNA libraries in nanomolarity were as follows: SA2: 138.7, SB2:131.7, CA1:166.7, and CB2: 132.8. Sequencing produced a high amount of reads analyzed using Trinity Software. The reads generated from the MiSeq sequencer were as follows per *P. elongata* cDNA library: SA1: 9,545,616; SB2: 10,196,304; CA1: 10,586,624; and CB2: 8,910,322. The *P. elongata* genome, as is typically the case with most genomes, is not fully sequenced. Full genomic sequencing is required to perform microarrays; the results from microarrays can have low specificity and issues with hybridization. The Trinity Software can create a *de novo* transcriptome from reads, another advantage of RNA sequencing and its bioinformatics applications.

Salt Stress Gene Expression Analysis of Tuxedo Suite Software

The Tuxedo Suite software served as a useful tool to generate an assembled quantified transcriptome, sorting out gene expression levels between salt stress *P. elongata* samples. The Trinity Software played an important role in utilizing the reads as best as possible, particularly in the absence of a complete genome sequence. At first, the Poplar genome was used as a reference genome, but using the genome produced inaccurate results and

filtered out the majority of the reads from sequencing. We were able to use the majority of the reads for the *de novo* transcriptome using the Trinity Software, in comparison to using a known genome as reference such as Poplar. Future genome sequencing of *P. elongata* could improve efficiency of mapping reads. Each cDNA barcoded sample produced approximately 10,000,000 reads (Table 2). A total of 216 genes were calculated to have significantly changed in expression in *P. elongata* after a 10-day salt treatment. The expression of the genes was categorized as follows: 67 downregulated, 79 upregulated, 17 turned off, and 53 turned on. Gene expression of upregulated and downregulated reached up to 200-fold change (Appendix 3). Using FASTA sequences from the data analyzed, the identity of selected genes was determined through the NCBI Blast Tool (Table 3). Genes that produced significant alignments, had identity scores higher than 80% percent and low E-values were selected, thus identifying each of the genes. Identified salt stress genes were subsequently used to study possible novel functions in salt stress and their role based on examination of previous literature.

Table 4. Fold Change Generated from Tuxedo Suite algorithms

Upregulated	Fold Change	Down Regulated	Fold Change
Extracellular Ribonuclease LE-like	197.2	Nitrate reductase	117.3
GDSL esterase/lipase-like	32.6	PYDK	62.6
21 kDa protein	19.8	Magche	33.3
G3PD	17.2	PPGR5 protein	23.9
Glucan-1,3-beta-glucosidase 14-like	19.8	FBA1	22.6

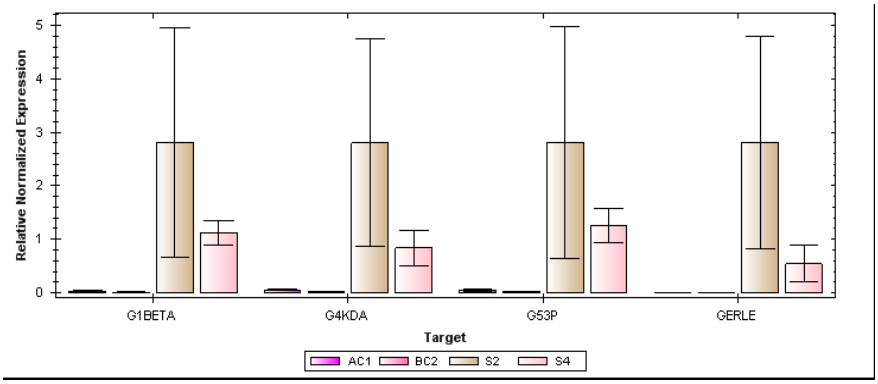
Fold change was calculated by PFKM values comparing salt stress and control expression generated by the Cuffdiff software within the Tuxedo Suite (fragments per base of exon).

Quantitative real time PCR Analysis

Performing RT-qPCR validated the results obtained from the RNA-seq as gene expression correlated accordingly across most of the genes that were upregulated, downregulated, turned off and turned on. CFX96 Bio-Rad Detection System calculated gene expression compared expression against the HACTIN reference gene (Figure 14). Normalized salt expression is also referred to as ΔΔCq or ΔΔCt, these values were used to calculate bar graphs by the software (Figure 15,16). Optimizing the primers of the selected salt stress genes was important to prevent possible unspecific hybridization and to ensure which annealing temperatures were optimal for each of the 20 genes. Optimization was achieved by performing PCR of primers with control *P. elongata* DNA using temperatures ranging from 49ºC up to 55ºC and checking PCR products by 1% agarose gel electrophoresis. The top five upregulated and downregulated were chosen while the most promising turned off and turned on genes from BLASTing were selected for RT-qPCR expression validation and further research. Upregulated and downregulated gene results (Figure 15, Appendix) showed a normal relative expression correlating to RNA-seq analysis. Turned on gene expression also correlated to that of the results of the RNA-seq analysis. The UDP gene did show an increase in expression across the control, which according to the RNA-seq analysis, should have shown no expression at all since this gene turned on after salt treatment. Turned off genes showed a variation of expression, while CSIL and SPROT expression correlated with the Tuxedo Suite analysis, WKR70, NADCYT and PKAL showed expression in controls and salt treatment inconsistent with the RNA-seq analysis. Possible error in primer design or non-specificity might account for the variation, future sequencing could be performed to determine if the

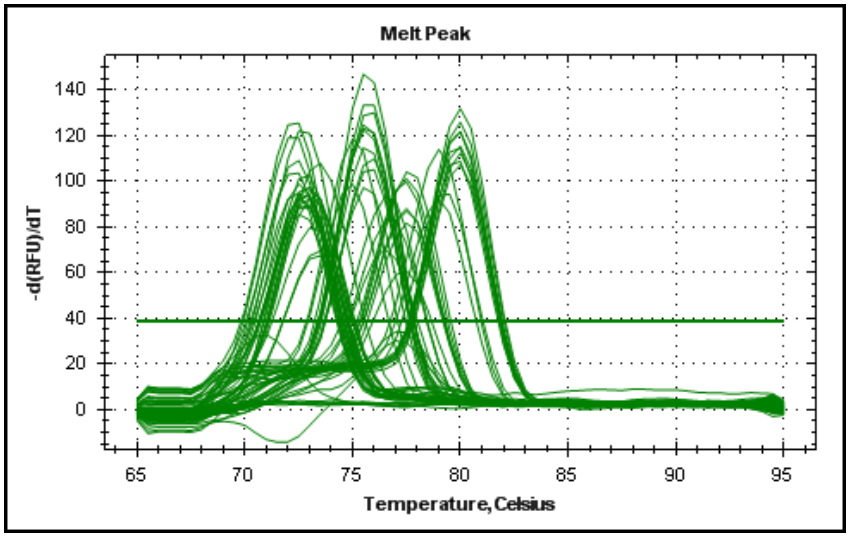
correct product was amplified by the RT-qPCR. The expression of the housekeeping gene might have also not been irregular between the salt replicates and the control, therefore normalization might not have been accurate. High standard deviation error bars are attributed to only using 3 replications per gene, more replicates would reduce the large margins of deviation. There was insufficient cDNA obtained from RNA-seq to be able to use more than 3 replicates, considering 20 genes were selected for RT-qPCR validation. Melting curve analysis was use as a diagnostic tool that ensured primers were producing single, specific DNA products. Melt curves plot graphs demonstrated that primers were binding to the specific DNA and creating a one DNA product. Dimers and non-specificity are factors that contributing to multiple peaks; dimer interaction might have been caused by less than optimal annealing conditions for certain genes or due to incorrect design of primer sequences. The negative change in fluorescence units across different temperatures determines shows the dissociation of DNA strands, multiple peaks represents more than one PCR product in melting curve analysis. Performing the experiment with more replicates and concentrating on fewer genes would have given better results. The quantity of cDNA used for the study was very small, more replicates and higher amounts of cDNA might be used in the future to improve RT-qPCR results, using more than on reference gene would normalize quantitative results as well.

Figure 15. Bar Plot of Gene Expression of Upregulated Genes through RT-qPCR



AC1 and BC2 are control plants while S2 and S4 are salt stressed *P. elongata* samples. G1BETA, G4KDA, G53, and GERLE were upregulated genes selected. Relative gene expression calculated by Bio-Rad CFX96 machine.

Figure 16. Melt Curve of Upregulated Salt Stress *P. elongata* by RT-qPCR



Peaks of 4 different primers of G1BETA, G4KDA, G53, and GERLE showing one peak per one set of gene primers. Each gene has 12 reactions: 3 SA2, 3 SA1. The negative change in relative fluorescence is measured across various temperatures were calculated by the Bio-Rad CXF96 system.

Table 5. Relative normalized expression, ΔΔCT, of *P. elongata* salt stressed genes

Genes	ΔΔ*CT*
Downregulated	
SPDCC	-2.2
SNADH	-3.1
PROTEIN5	-5.85
SFBAC	-12.99
SMCH	-53.3
Upregulated	
GERLE	523.48
GDSL	50.55
G4KDA	31.66

G53P	43.64
G1BETA	65.33
Turned off	
NADCYTO	1.54
SPROT	5.43
PKAL	-11.51
CSCIL	-9.0
WRKY70	-0.96
Turn on	
ABR1	2486.3
AQUAPIP	38.68
Exten2l	0.00
StriSynthase	7194.01
UDP	5.3

Generated values of downregulated, upregulated, turned on, turned off of salt stress. *P. elongata* generated by Bio-Rad CFX96 Real Time Detection machine. Values are average of two salt stress replicates, SA1 and SA2

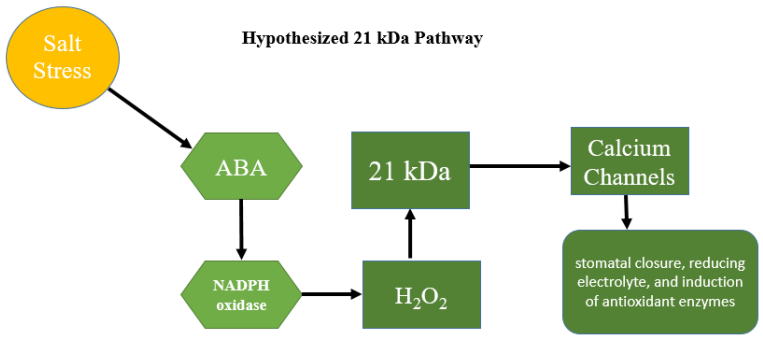
Hypothesized Gene Pathways and Mechanisms

Upregulated Novel Genes

The purpose of this study was to find novel genes that might play an important role in the survival of *P. elongata* or genes involved in response to major salt stress mechanisms and pathways. The 21kDa protein was a highly upregulated gene from the RNA-seq analysis, its function in salinity has not been studied in-depth. In a previous study, the 21kDa acted as a calmodulin-binding protein that was responsible for activating calcium channels (Jun et al., 1996). Studies have suggested that salt stress induces an ABA-mediated response responsible for activation of NADPH leading to hydrogen peroxide induction. The inductions consequently activates calcium channels, resulting in stomatal closure (Song & Matsuoka, 2009). It is possible that the 21kDa protein may serve as a signaling molecule of ABA, the protein activates as part of the salt stress response mechanisms associated with ABA. The 21kDA protein and ABA have previously been linked in a study which analyzes the accumulation of proteins in response to different abiotic stresses in rice. The results showed 21kDa protein accumulating in large quantities in response, to

not only salt stress, but to drought stress as well (Pareek et al., 1998). ABA is a phyto-hormone that plays a key role in stomatal closure induction, reducing electrolytes, and induction of antioxidant enzymes (Li et al., 2010) The 21kDa protein may act as a signal transduction molecule between hydrogen peroxide and the calcium channels (Figure 17). The relationship between hydrogen peroxide, the 21kDA and calcium channels during salt stress might reveal the role of 21 kDA in salt stress.

Figure 17. Hypothesized Pathway of 21 kDa Protein Upregulated by Salt Stress in *P. elongata*

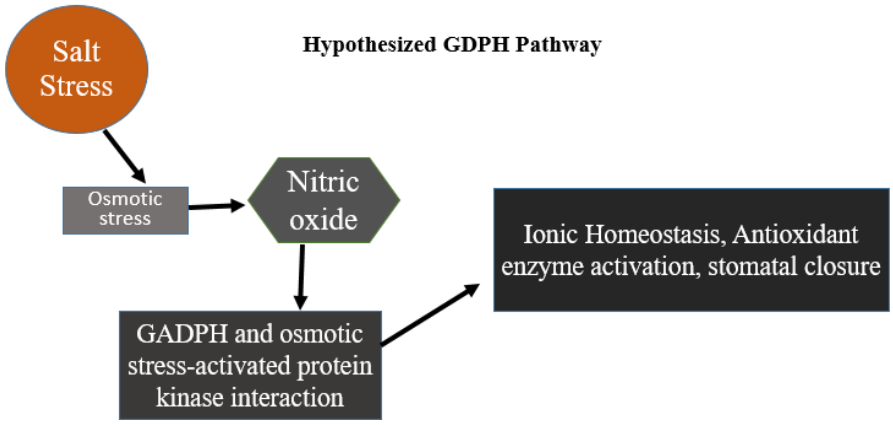


Salt stress induces ABA mediated pathways by activating NADPH oxidase inducing hydrogen peroxide across the plasma membrane. The 21 kDa may play act as a signal transduction signal between calcium channels and hydrogen peroxide involved in stomatal closure, reduction in electrolytes and induction of antioxidant enzymes.

Glyceraldehyde-3-phosphate dehydrogenase GAPCP2, chloroplastic-like also known as GADPH has been found to be associated nitric oxide (Wawer et al., 2010). Nitric oxide is known to help trigger the activation of redox-regulated pathways in order to help reduce ROS within the cell and has also been linked to stomatal closure and is a regulator of ionic imbalances (Gupta & Huang, 2014). GADPH has been suggested to interact with osmotic stress-activated protein kinase, regulated through NO activity (Wawer et al., 2010). Understanding more of this interaction might be beneficial since there is little known of osmotic stress-activated protein activation (Burza et al., 2006). Osmotic protein kinases have been known to play a role in regulation of stomatal closure.

GADPH and NO might serve important regulatory roles even though osmotic kinases have been traditionally associated with the ABA pathway (Wang et al., 2015).

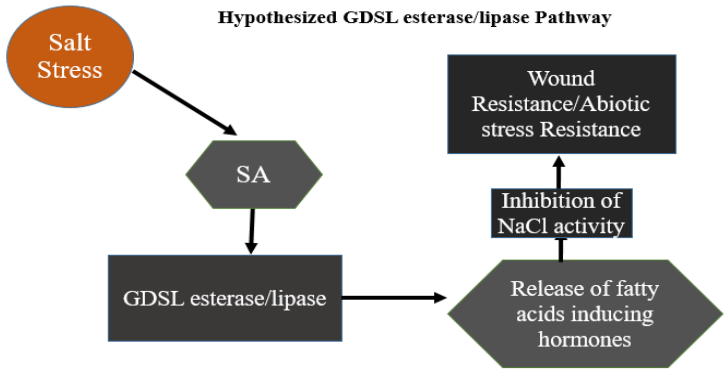
Figure 18 Hypothesized Pathway of GADPH Upregulated by Salt Stress in *P. elongata*



Salt stress increase osmotic stress in the plant activating a nitric oxide response that mediates different mechanisms. Nitric oxide indirectly or directly activates a suggested GADPH osmotic stress-activated kinase interaction that might play a role in ionic homoeostasis, enzyme activation, stomatal closure.

A third upregulated gene, GDSL esterase/lipase, has revealed to play a role in plant resistance to wound stress and pathogenic stress. A previous study, demonstrated that SA treatment induced activated overexpression of a GDSL esterase/lipase resulting in increased salt tolerance, therefore GDSL is suggested to be induced by SA as a response to salt stress (Jiang et al., 2012). Overexpression of GDSL esterase/lipase in *Arabidopsis* also showed inhibition of NaCl activity, possibly as a result of fatty acids release by GDLS esterase/lipase serving as hormone signal transduction molecules (Naranjo et al., 2006). A hypothesized pathway involves salt stress inducing SA activating GDSL esterase/lipase releasing fatty acids that inhibit NaCl activity providing overall salt tolerance.

Figure 19. Hypothesized Pathway of G2DSL Upregulated by Salt Stress in *P. elongata*

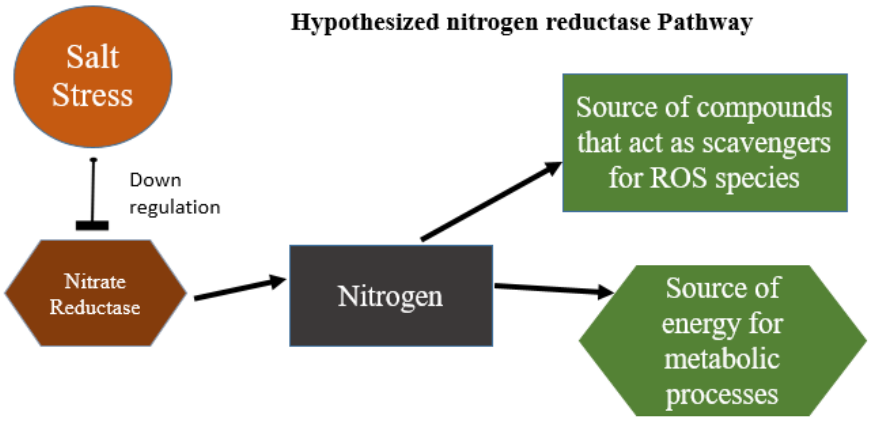


Salt stress induced salicyclic acid activates GDSL esterase/lipase. GDSL esterase/lipase has shown to increase wound resistance and abiotic stress resistance possibly by release of fatty acids initiated by hormones. (Naranjo et al., 2006).

Downregulated genes

Salinity affects gene expression by causing downregulation of genes, several downregulated novel genes are associated with inhibited mechanisms as a result of osmotic imbalance and Na^+ accumulation within plants. Nitrate reductase was one of the top five most downregulated genes, according to the RNA-seq analysis (Table 3). Nitrogen is a key source of energy for the plant and is derived from nitrate assimilation. Nitrogen reductase plays an essential role in nitrogen fixation, therefore the downregulation of nitrogen reductase, as a response to salt stress affects, the ability of the plant to obtain nitrogen (Baki et al., 2000). Salt stress studies have demonstrated that salinity inhibits nitrate reductase limiting the nitrogen source availability for the plant (Campbell, 1988). Several nitrogen containing compounds have been proposed as possible ROS scavengers freeing the cell of oxidative species caused by salt stress (Mansour, 2000). Downregulation of nitrate reductase could possibly halt major processes including photosynthesis and cellular respiration in plants.

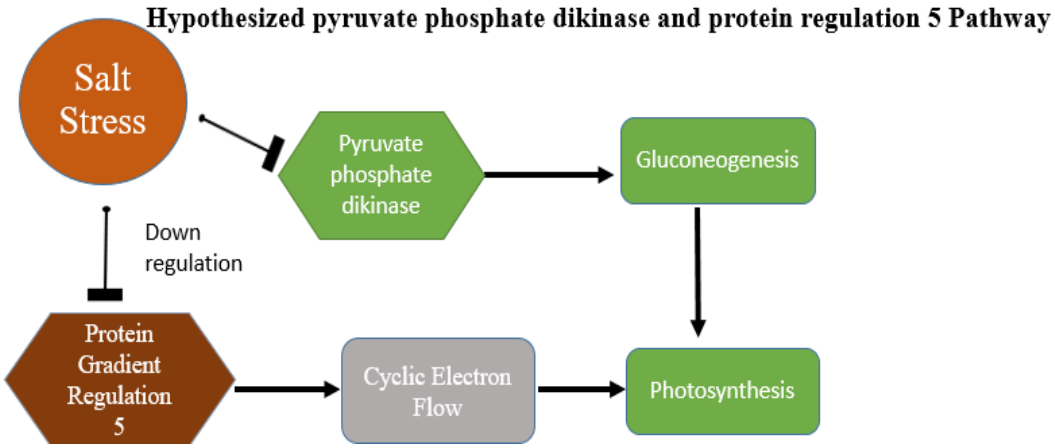
Figure 20. Hypothesized Downregulation of Nitrate Reductase Pathway by Salt Stress in *P. elongata*



Salt stress downregulates nitrate reductase, which is responsible for nitrogen fixation. Decrease in nitrogen fixation affects the plant's ability to produce energy and decreases salt tolerance.

Another downregulated gene, pyruvate phosphate dikinase, is a key enzyme in gluconeogesis (Lüttge, 1993). Pyruvate phosphate dikinase commonly referred to as PPDK is required for CAM to occur during the light dependent reactions of photosynthesis (Fißlthaler et al., 1995). In a similar fashion, protein gradient 5 gene, mediates cyclic electron flow during photosynthesis, which recycles electron, hence controlling the rate of photosynthesis (Johnson et al., 2014). Salt stress is known to deplete plant growth and these two downregulated genes play essential roles in plant growth and development. A decrease in photosynthetic rate is an early sign of the plant death and inability to produce enough energy necessary for metabolic survival. A summarized pathway (Figure 21), displaying the role of protein gradient 5 and pyruvate phosphate dikinase resulting in inhibition of processes leading to photosynthesis. Over expression of these two genes might increase the plant's ability to cope with toxic Na^+ levels that interfere with photosynthetic processes.

Figure 21. Hypothesized Downregulation of Pyruvate Phosphate Dikinase and Protein Gradient Regulation 5 as a Result of Salt Stress in *P. elongata*

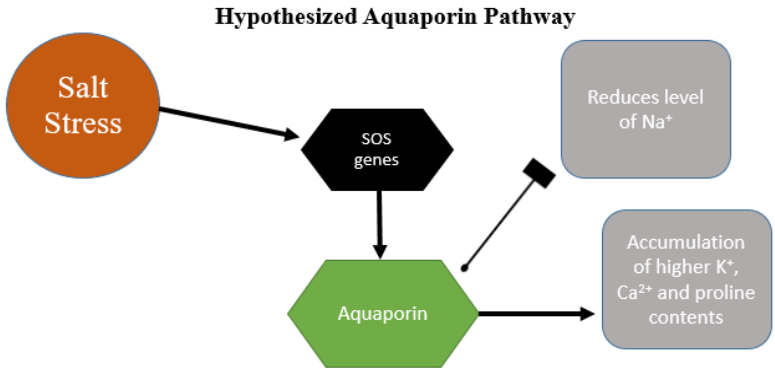


Salt down-regulates protein gradient 5 and pyruvate phosphate dikinase. Pyruvate phosphate dikinase is responsible for gluconeogenesis, necessary for photosynthesis. Protein gradient 5 plays a role in the cyclic electron flow in the light dependent reactions required for photosynthesis.

Turned on genes

Turned on genes found in this study could potentially be used to improve salt tolerance and prevent cell death due to salinity. Aquaporins, for example, are vital for uptake of soil water and other environmental stresses (Boursiac et al., 2005). Aquaporins are water channels, increasing permeability of the cell membrane allowing more water to enter the plant during a water deficit like the one that exist during salt stress. The SOS pathways and aquaporins have been linked to each other, different SOS genes activate aquaporins as a result of abiotic and biotic stresses (Gao et al., 2010) A hypothesized pathway shows how salt stress induces the SOS pathway to activate aquaporins, as a means of mediating high levels of salinity and initiating a response for ionic balancing.

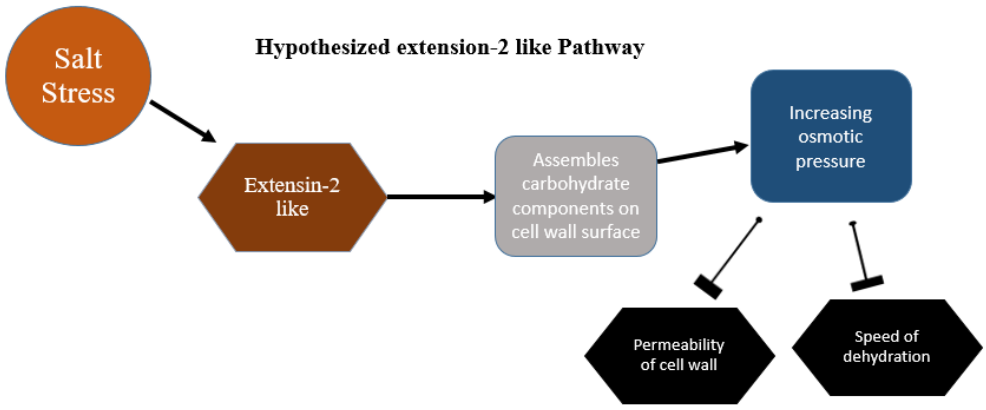
Figure 22. Hypothesized Downregulation of Aquaporin Pathway by Salt Stress in *P. elongata*



Salt stress induces SOS genes activating aquaporin channels in the cell membrane of the plant. Aquaporins are responsible for reducing levels of Na^+ and accumulation of higher K^+, Ca^{2+} and proline contents.

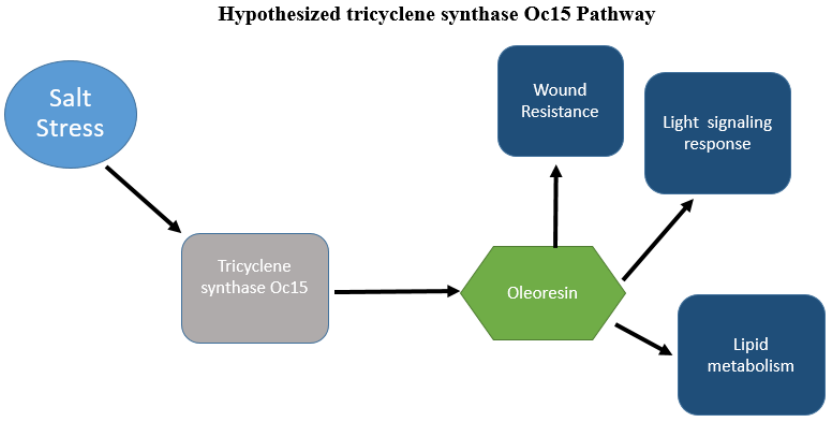
The gene coding for extensin, was another turned on gene from the RNA-seq serving as a possible mediator of osmotic pressure. Previous literature has associated extension with carbohydrate assembly components on the cell wall leading to increasing osmotic pressure. Maintaining osmotic pressure is important for the plant, as salinity decreases water intake and maintaining metabolic processes within the plant. Tricyclene synthase has been linked to high synthesis of lipids, such as oleoresin (Liu et al., 2015). Oleoresin also contributes regulating wound resistance and signaling a photosynthetic response (Lorio & Sommers, 1986). Salt induced activation of these genes might reveal the interactions of these genes in other major salt stress pathways.

Figure 23. Hypothesized Downregulation of Extension-2 like Pathway by Salt Stress in *P. elongata*



Salt induces extension-2 like is associated with assembling carbohydrate components on the cell wall surface which increases osmotic pressure.

Figure 24. Hypothesized Downregulation of Tricylene Synthase Oct15 Pathway by Salt Stress in *P. elongata*



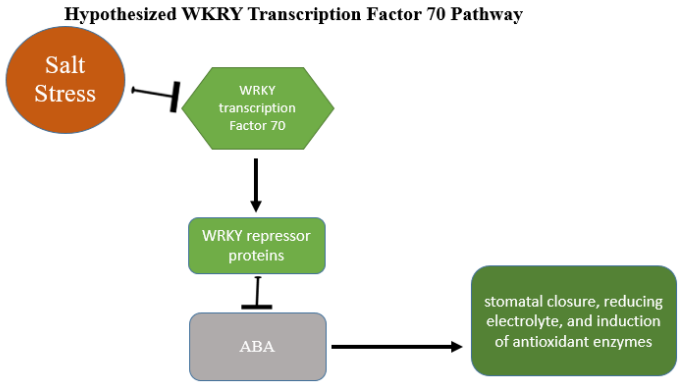
Salt stress induces tricyclene synthase Oc15 leading to oleoresin. Oleoresin associated with wound resistance. Light signaling response, lipid metabolism.

Turned off genes

The RNA-seq analysis listed a number of genes turned off due to the salt treatment in *P. elongata*. WRKY transcription factor 70 was a turned off gene belonging to a big family of WRKY transcription factors known to be involved in abiotic stresses. WRKY transcription factors can act as positive or negative regulators (Rushton et al., 2012). For

example, WRKY transcription factors act as a negative regulator to ABA during seed germination but positive regulators of ABA-mediated stomatal closure (Chen et al., 2012). WRKY transcription 70 could be a repressor protein, and turning off the gene would therefore induce an ABA response leading to salt tolerance by stomatal closure.

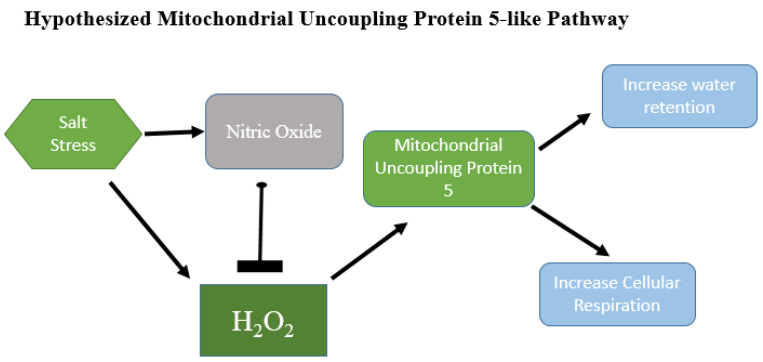
Figure 25. Hypothesized WRKY Transcription Factor 70 Pathway by Salt Stress in *P. elongata*



Salt stress inhibits WRKY transcript Factor 70 leading to downregulation of WRKY transcription Factor 70. ABA induction leads to stomatal closure, reducing electrolyte and induction of antioxidant enzymes.

Turned off genes from the RNA-seq analysis could reveal inhibition of genes as a result of high toxic Na^+ levels. Another turned off gene, mitochondrial uncoupling protein 5 is a mitochondrial protein regulating the proton gradient of respiratory chains (Santandreu et al., 2009). Overexpression of mitochondrial uncoupling protein 5 increases water retention and increases cellular respiration induced by an overproduction of ROS such as hydrogen peroxide (Begcy et al., 2011). Nitric oxide, under initial salt stress, reduces ROS activity, this might play a role in the cell turning off mitochondrial uncoupling protein 5 as a result of the cell's failure to adjust to salt tolerance and nitric oxide indirectly inhibiting expression of the protein (Figure 26).

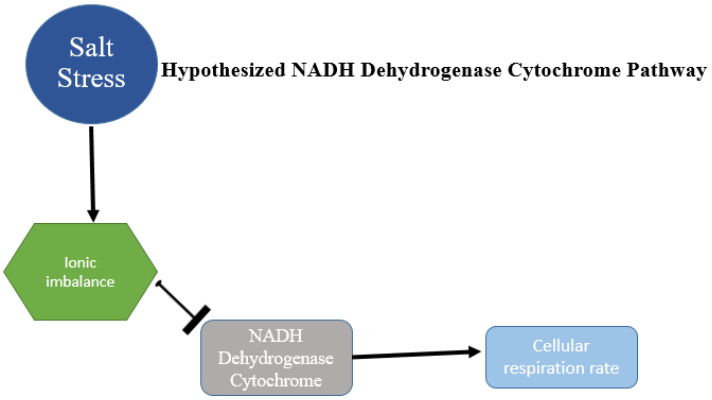
Figure 26. Hypothesized Mitochondrial Uncoupling Protein 5-like pathway of Salt Stress in *P. elongata*



Salt stress induces ROS species (hydrogen peroxide) which are inhibited by NO activity. Mitochondrial uncoupling protein 5 is turned off after salinity after NO indirectly turns off gene.

The results suggest that salinity initiates a reduction in energy expenditure by reducing cellular respiration while attempting to maintain photosynthetic energy production. NADH dehydrogenase cytochrome was another gene identified from the RNA-seq analysis. NADH dehydrogenase is responsible for uncoupled oxidations and influences the reduction level of NAD^+ in the plant (Eprintsev et al., 2011). NAD^+ plays an important role in the electron transport chain pathway of cellular respiration. Salt stress can impair the activities of NADH dehydrogenase cytochrome due to high ionic imbalance exertion on the mitochondrial transport chain (Begcy et al., 2011). Turning off the gene decreases cellular respiration, affecting energy expenditure of the plant. Turned off genes are notably genes that are inhibited by high concentrations of salt, further overexpression studies might improve plant salt tolerance.

Figure 27. Hypothesized NADH Dehydrogenase Cytochrome Pathway by Salt Stress in *P. elongata*



Salt stress causes an ionic imbalance caused primarily by excess Na^+ and inhibits NADH dehydrogenase cytochrome activity. Inhibition slows down the cellular respiration rate.

This study has shown an abundance of genes affected by salinity and the possible these genes have been linked to important pathways in previous literature. Salinity pathways and mechanisms are complex, but further research can increment knowledge and understanding of these complex networks. Novel genes are beneficial in understanding salt tolerance, not only for *P. elongata* but amongst other species. RNA sequencing proves to be a beneficial tool in analyzing transcriptomes that have yet to be completely sequenced. The Tuxedo Suite and Trinity software compared differential gene expression in *P. elongata* between salt stress and control biological samples, demonstrating the resourcefulness of RNA sequencing for future biofuel technology research. The 216 genes identified in this study can be used in the future for a variety of purposes. For example, comparison of these genes in other species under salt stress can identify differences and similarities in function. Performing a salt treatment of *P. elongata* over a longer period of time might shed important transgression in gene expression under salt stress.

The *Paulownia* plant is highly resistant to abiotic stresses; overexpression of novel genes might make the plant a more suitable candidate for bioethanol production.

Genetic modification of *P. elongata* could lead to improvements in lignocellulosic pretreatment methods as well as in biomass yield production. Possible hypothesized pathways in study can be used as a foundation to test cellular pathways that are not completely known. Further studies of these pathways might reveal important information about a plant's physiological response at a cellular level to salt stress. *P. elongata* shows to be a great candidate for biofuel production and for studying abiotic stresses considering the plants high resistance to environmental stresses.

Based on past and current studies, *P. elongata* is tolerant to a wide variety of biotic and abiotic stresses. It is an ideal bioenergy feedstock of bioethanol produced from lignocellulosic biomass. *P. elongata* can serve as a template plant for research in biofuel and as a tool for improving agricultural production in the future. This study revealed important novel genes that play different roles in the plant's ability to respond to salt stress. Understanding salinity is beneficial, for not only the future of biofuel, but for industries that used plants as their main source material. *P. elongata* is currently used for a wide variety of purposes and novel genes found in this study can help understand the response of *Paulownia* to salinity. Future studies, involving genetically modified *P. elongata*, can improve the tolerance of the plant to a variety of abiotic stresses. *Paulownia* holds great potential in producing bioethanol making it an ideal bioenergy feedstock for second generation biofuel.

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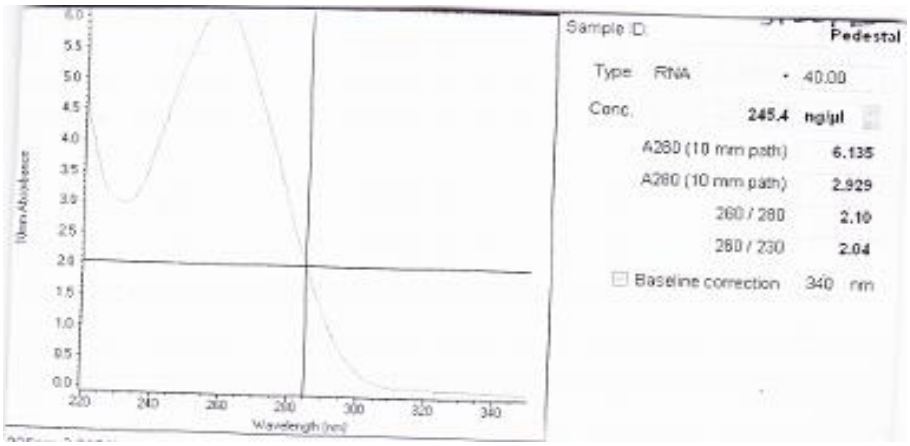
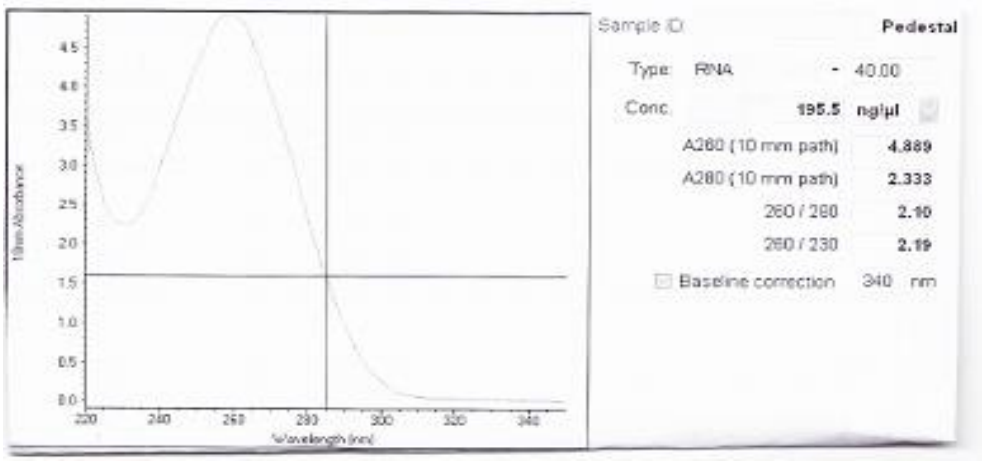
Appendix

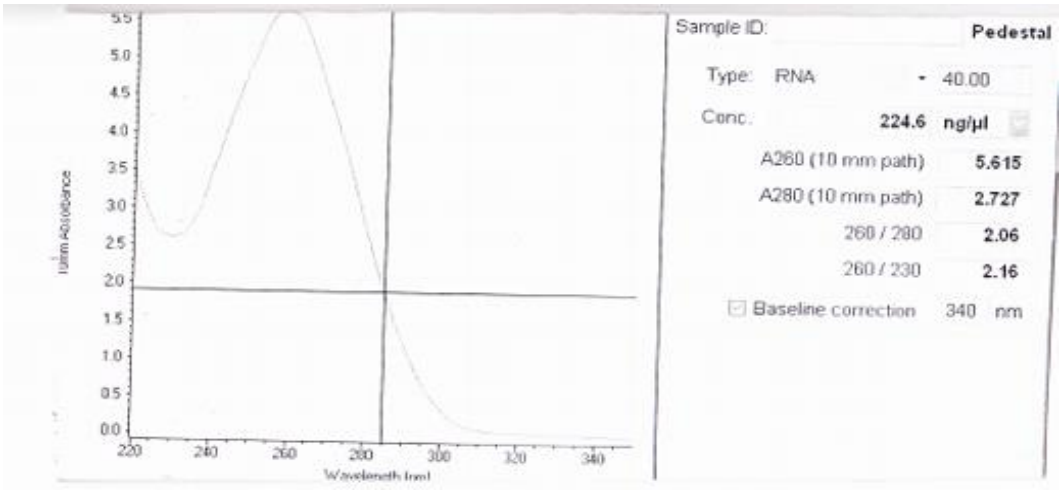
1.



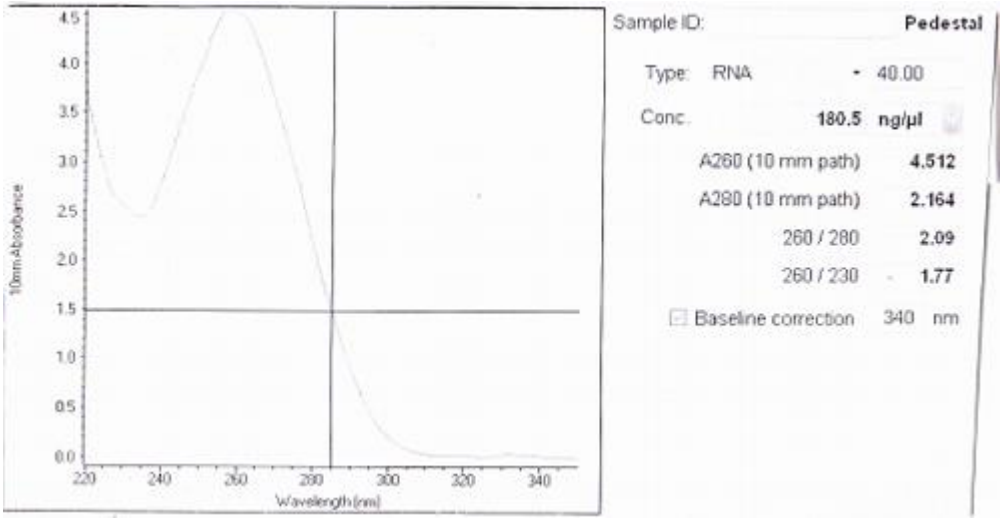
A. S1 (salt stress sample 1) Nanodrop Spectrometer results showing absorbance peaks across wavelengths and absorbance ratios



B. S2 (salt stress sample 2) Nanodrop Spectrometer results showing absorbance peaks across wavelengths and absorbance ratios



C. C1 (control sample 1) Nanodrop Spectrometer results showing absorbance peaks across wavelengths and absorbance ratios



D. C2 (control sample 2) Nanodrop Spectrometer results showing absorbance peaks across wavelengths and absorbance ratios

2. Nano-molarity of salt stress and control *P. elongata* cDNA samples generated from RNA-seq using Qubit fluorometer.

Biological Samples	nM
SA2	138.7
SB2	131.7
CA1	166.7
CB2	132.8

3. Total number of salt stress genes generated by Tuxedo Software suite gene expression analysis

P. elongata salt stressed genes	
Down Regulated	67
Upregulated	79
Turned off	17
Turned on	53
Total Genes	216

4. List of primers of selected Salt stress genes of *P. elongata* utilized for RT-qPCR

a) Primers of downregulated salt stress genes

Genes	Abbreviations	Primers
Downregulated		
Nitrate reductase	SNADH	F: 5'- GGCGGAAGAATGGTGAAATG-3' R: 5'- CGTCAACGTGAGAAGGTAGAA -3'
Pyruvate, phosphate dikinase	SPPDC	F: 5'- GAGGGTCTAGGAGTCTGATTGT-3' R: 5'- GGCAGCTCTGGACTTGTTATT-3'
Protein Protein Gradient Regulation 5	Protein5	F: 5'-AAGGAAACCAAGCCACTCTC-3' R: 5'- GCCATTGCTCTACACTCACA-3'
Fructose-bisphosphate aldolase 1-	SFBAC	F: 5'-GCCTGAACTTCGCTTCTTATTTG-3' R: 5'-GGTGATGACTTGAGGAGTGAT G-3'
Magnesium-chelatase subunit ChlH-	SMCH	F: 5'-CCCAGCTGCGACATACTAAA-3' R: 5'-AGTGGAGAAAGAGAGGGAGAG-3'

b) Primers of downregulated salt stress genes

Genes upregulated	Abbreviations	Primers
Extracellular Ribonuclease LE like	GERLE	F: 5'-CAATGCTGGAGGCACATTTC-3' R: 5'AGATGGGAACAGCCAGTTATAC-3'
GDSL esterase/lipase At5g55050-like	GDSL	F: 5'- GGGATCTGACCTACCTGAGAAA-3' R: 5'- CGCGCTCCTAGACCATAAATAC-3'
21 kDa protein	G4KDA	F: 5'-GGCGGAGATGAACCAGATAAA-3' R: 5'- CAGGTGTCCTCATCTGTCAAG-3'
Glyceraldehyde-3-phosphate dehydrogenase chloroplastic-like	G53P	F: 5'-CAGCAGAGAGAGAACCACATT-3' R: 5'- GATGGAAGCCACGGTAGAAATA-3'
Glucan-1,3-beta-glucosidase 14-like	G1BETA	F: 5'- CCGGTGTCTGAGAGGAATTATG -3' R: 5'- CGTGGAAGATAACCCTGTAACC-3'

c) Primers of turned off salt stressed genes

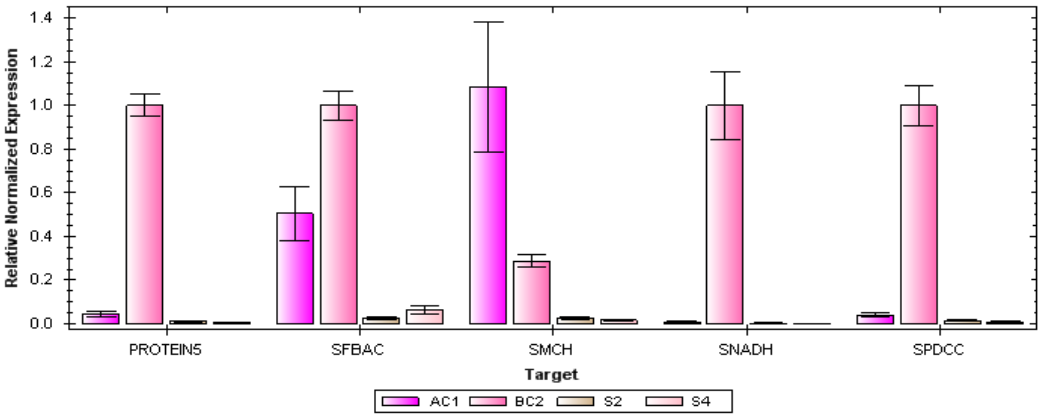
Turned off genes	Abbreviations	Primers
NADH Dehydrogenase Cytochrome C	NADCYTO	F: 5'- GATGATGCCACTGTTCCTCTTA-3' R: 5'- GGTGCTTTGACCAATGGATATG-3'
mitochondrial uncoupling protein 5-like	SPROT5	F: 5'- AGTGTGACAAAGAGCACGATAG-3' R: 5'- GCTGGACTGTGCCATGAA-3'
inactive leucine-rich repeat receptor-like protein kinase At5g06940	PKAL	F: 5'-CAG ATT GAG TTG TTT CTC CCA AAT-3' R: 5'- CAAGACCTTTAGCTTCACAAACC-3'
CSC1-like protein -F	CSC1L	F: 5'- CTCTCTTGACTTCTGCTGGTATC-3' F: 5'- CTTCTGTCCGAAGTACACACTC-3'
WRKY transcription factor 70	WRK70	F: 5'- CGGAGAGGTTGTTACAAG AGAA-3' R: 5'- CCTTCTGTCCATATT TCCTCCA -3'

d) Primers of turned on salt stressed genes

Turned on genes	Abbreviations	Primers
ethylene-responsive transcription factor ABR1-like	ARB1	F: 5'-CCTCTTCTGCTTCTTCCTCTTC-3' R: 5'-TCCTTGTGGCGTGAAATGTA-3'
aquaporin PIP2-4-like	AQUAPIP	F: 5'-GCAACCAAGAAACACTCAAACA-3' R: 5'-GGTGGCTCCAACCTCAATATC-3'
7-deoxyloganetic acid UDP-glucosyltransferase-like protein mRNA, complete cds	UDP	F: 5'-GCTCCACTGGGATTTGATTCT R: 5'
tricyclene synthase Oc15	Strisynthase	F: 5'-ACCCTTACCACAAGAGCATAAC-3' R: 5'-CATTTCCACCAGCTCTCCTAA T-3'
extensin-2-like	Exten2l	F: 5'-CCACGGTTTACGTGGATTTATTG -3' R: 5'-CTGAGTGCTTCATGGGAAGTTA-3'

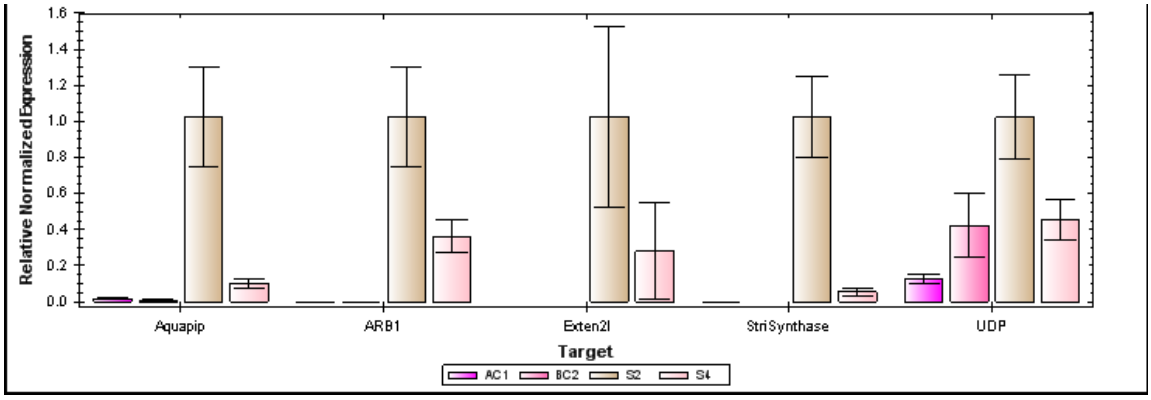
5. Bar Plots of RT-qPCR Gene Expression by CFX-96 BioRad

a) Bar plot of gene expression of downregulated genes through RT-qPCR



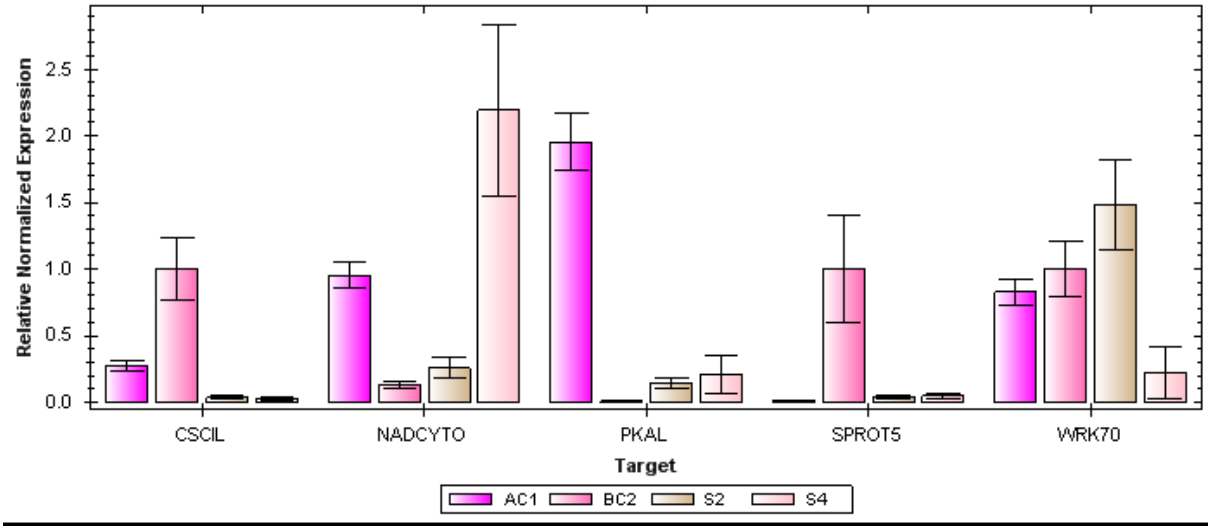
AC1 and BC2 are control plants while S2 and S4 are salt stressed P. *elongata* samples. Protein5, SFBAC, SMCH, SNADH, SPDCC were downregulated genes selected. Plot graph was generated by the CFX96 BioRad machine.

b) Bar plot of gene expression of turned on genes through RT-qPCR



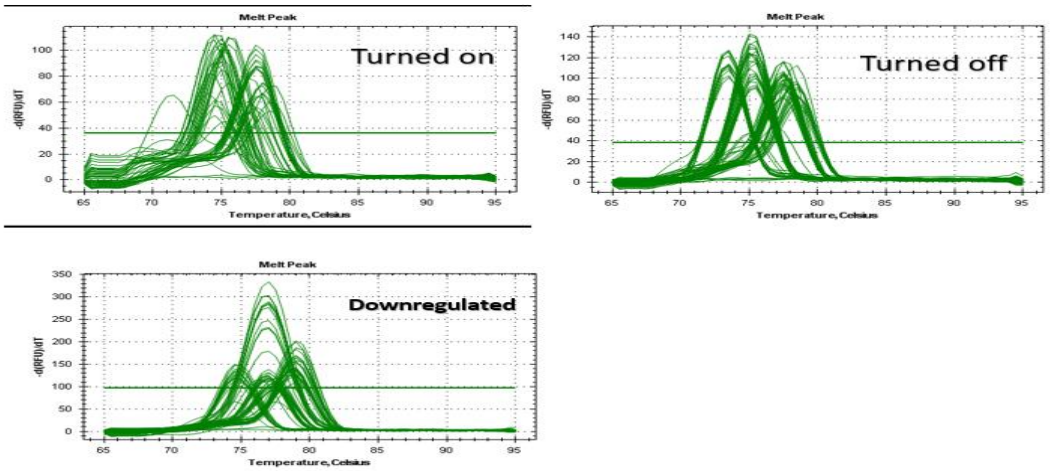
AC1 and BC2 are control plants while S2 and S4 are salt stressed P. *elongata* samples. Aquapip, ARB1, Exten2l, Strisynthase and UDP are turned on genes selected. Plot graph was generated by the CFX96 Bio-Rad machine.

c) Bar plot of gene expression of turned off genes through RT-qPCR



AC1 and BC2 are control plants while S2 and S4 are salt stressed *P. elongata* samples. CSCIL, NADCYTO, PKAL, SPROT5, WRK70 are turned off genes selected. Plot graph was generated by the Bio-Rad CFX96 machine.

a) Melt Curve of turned on, turned off and down regulated salt stress *P. elongata* genes by RT-qPCR



Peaks of plot show 4 different primers peaks belong to each set of primers analyzed. Turned on genes include Aquapip, ARB1, Exten2l, Strisynthase and UDP. Turned off genes CSIL, NADCYTO, PKAL, SPROT5, WRK70 are turned off genes selected. Downregulated genes are Protein 5, SFBAC, SMCH, SNADH, SPDCC. Melting curve was generated by the Bio-Rad CFX96 machine.