The Role of *CYP72A13* in *Arabidopsis thaliana* Stress Response

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ABSTRACT

Plants vary tremendously with respect to growth conditions and defense mechanisms. Cytochrome P450s (CYPs), a family of enzymes that play a major a role in affecting growth and defense responses, have been widely studied in plants and other organisms. In this project, a model organism, *Arabidopsis thaliana*, was used to study one CYP extensively through various procedures and stress conditions including heat stress, osmotic stress and biotic stress. Molecular genetics allowed us to isolate plants with altered expression of *CYP72A13*, whose function has been associated primarily with defense mechanisms. Previous studies have shown that this gene is up-regulated with respect to various defense mechanisms, particularly against certain predators, in addition to being involved with osmotic stress response. Here, we explore the role that *CYP72A13* does or does not play under heat, osmotic, and biotic stresses. We found that no significant conclusions could be drawn indicating the role played by *CYP72A13* in heat shock defense, but our results indicate that the gene may be involved in both defense against osmotic stress and against biotic stress. More research should be conducted on *CYP72A13* to better understand this role. The broader implications are that CYPs can be useful and perhaps critical enzymes that play many roles in a variety of organisms. Further exploration of this gene would contribute to the broader understanding of the CYP72As in plants.

INTRODUCTION:

Cytochrome P450s (CYPs) are one of the largest families of enzymes with over thousands genes and catalyze extremely diverse reactions throughout nature. The scientific world began its fascination with and study of CYPs in the late 1950s and to this day continues to look to CYPs for answers to many questions, such as how the evolution of biochemistry in many species came about. CYPs are important in biochemical functions of all organisms, from humans to bacteria to plants. In general, CYPs catalyze a multitude of reactions, especially those that lead to the precursors of crucial macromolecules and those that are involved in the biosynthesis of hormone signaling molecules, pigments, and defense compounds (Bak *et al.*, 2011). It has been suggested that their ability to be involved in such diverse mechanisms and reactions is based on their structural heterogeneity and their molecular dipoles, which may aid in protein recruitment for catalysis (Boddupalli *et al.*, 1995).

One specific example of the importance of CYPs in our world is that their ability to make sterols, a crucial component of the eukaryotic cellular membrane, depends directly on a particular CYP (*CYP51*). As a result, without CYPs, it would be hard for multicellular life on Earth to exist at all (Nelson, 2013). Another example that attests to the importance of CYPs on our planet is the fact that certain CYPs provide a waterproof coating for plants so that over time, as they began to leave the water and settle on

land, they did not dehydrate. This coating was created as a result of pathways that evolved over time for making cutins and suberins that were used in forming epicuticular waxes. This function of plants' coats was provided early on by *CYP86* and was and is still absolutely crucial for without such waterproof protection, the land would be "surrounded by an algal pond scum and little more" (Nelson, 2013).

Although CYPs are involved in all organisms, they play a very crucial role in plants, particularly with respect to their role in signaling and defense pathways and mechanisms. For starters, one percent of all plant genes are CYPs and these genes play important roles in agriculture, biochemistry, and medicine (Nelson, 2013). In order to best understand their role in plants in particular, we must begin with understanding the structural components of these enzymes. CYPs are hemethiolate enzymes that share a catalytic center (heme with iron). They work by activating and heterolytically cleaving oxygen and reducing one of its own atoms to form water; thus they are subsequently classified as monooxygenases. With respect to plants, CYPs are bound to membranes and are attached at the cytoplasmic surface of the endoplasmic reticulum. Most importantly, in order to be active, CYPs must be coupled to electron-donating proteins (or other electron donors) (Bak *et al.,* 2011).

Plant CYPs are more complex than animals because their evolution selected for diverse biochemistry. Plants, unlike nearly all animals, must stay in one place and defend themselves against a multitude of predators such as herbivorous animals, fungi, viruses, and bacteria (Nelson, 1999). As a result, plants depend on CYPs for nearly every aspect of their biology. Nelson and colleagues compared the entire genomes of rice, grape, and papaya to examine the differences in CYP families between plant species (2008). This experiment showed that the earliest and oldest CYP families were devoted to the essentials and basics regarding biochemistry of sterols to allow for eukaryotic cell evolution. On the other hand, the more recent CYP families seem to have driven the diversity of plants with respect to aiding in the synthesis of pigments, odorants and genus specific secondary metabolites (Nelson, 2008).

Secondary metabolism is a crucial aspect of plants that allow them to respond to environmental stresses. A very big part of secondary metabolism (products that aid plants but are not actually required for basic growth and development) are the defense mechanisms the plant utilizes. CYPs play a role in plants' various stress responses to biotic and abiotic factors and it is these responses that allow us to see the potential function of many CYPs. Jasmonates, for example, are involved in certain defense mechanisms against herbivorous predators. Jasmonate is a plant hormone that is produced as a result of a major signaling pathway involved in most plant defenses against insects called the octadecanoid pathway. It has been known that jasmonate levels rise steeply in response to insect damage and subsequently trigger the production of many other proteins involved in plant defense. Most importantly, jasmonate induces transcription of a host of certain genes that encode key enzymes in all the major pathways for secondary metabolite synthesis (Taiz and Zeiger, 2010).

Certain CYPs, such as *CYP72A13*, are up-regulated upon application of *cis*-jasmone (Bruce, 2008). *Cis*-jasmone is a defense volatile and experimentation utilizing this volatile mimics insect attack on plants. Further experimentation has shown that certain plant volatiles associated with the biotic stress of insect damage, which elicits *cis*-jasmone signaling, are known to switch on defense metabolism in *Arabidopsis* (Bleeker *et al.,* 2009). Specifically, research has shown that jasmonate/*cis*-jasmone is essential for insect defense in *Arabidopsis* (McConn *et al.,* 1997). Transcriptomic analyses of *cis*-jasmone-induced plants showed that certain CYPs (including *CYP81D11* and *CYP72A13*) were strongly up-regulated (Bruce, 2008). There have also been studies conducted that show when plants are under the biotic stress of insect predation, there is an upregulation of *CYP72A13,* once again attesting to the role of this gene somewhere in the defense pathways and mechanisms of plants. Experiments such as these indicate that up-regulation of certain CYPs under certain stresses in certain organisms attests to the immense diversity and broad functions of this family of enzymes.

In addition to CYPs in general playing a role in defense mechanisms, *CYP72A* genes in particular play specific roles in plant defense and in other areas of plant development and maintenance. For

example, *CYP72A1* has been suggested to be involved in the biosynthesis of indole alkaloids (Irmler, 2000), and this is very crucial for plants because indole alkaloids are involved in plant development and stress defense (Wang, 2004). In addition to playing defensive roles in plants, *CYP72A* proteins play an important role as triterpene-oxidizing agents that are involved in producing glycyrrhizin, the main sweet-tasting component of licorice (Seki *et al.,* 2011). Rice genomes have also been located in a region of a *CYP72A* gene cluster in a clone of a specific chromosome, attesting to the crucial roles *CYP72A* genes play in a global staple crop (Wang, 2004). And although the function of *CYP72A*s in *Arabidopsis thaliana* in general is unknown, studies have been done that have identified the genetic structures of CYP72As (large clusters of eight genes and one pseudogene) and that gene duplication probably caused the groups of genes to have similar functions (Bak *et al.,* 2011).

The goal of this study was to examine the function of *CYP72A13* in *Arabidopsis thaliana* stress response. Because *CYP72A13* has been implicated in plant defense, we hypothesize that plants with less *CYP72A13* expression should be less tolerant of stress.

We approached the question through utilizing mutant characterization. First, we isolated knockdowns of *CYP72A13* to compare them to the wildtype plants. We then determined which mutants were good potential single and double mutants. Finally, we placed these plants under various abiotic and biotic stresses including heat shock, osmotic stress, and caterpillar (*Trichoplusia ni*) predation stress.

MATERIALS AND METHODS

Plant Growth:

Seedlings were grown on BASTA plates, which contain one packet of Linsmaier and Skoog Basal Medium at half strength, 1.5% sucrose, 0.8% phytoblend agar, and BASTA (25 ug/mL). Separate plates without BASTA were also made for non-transgenic plants. Growth media was autoclaved to ensure proper sterilization. *pTP4 DNA construct contains a T-DNA encoding BASTA resistance and an artificial microRNA to reduce *CYP72A13* expression.

Seeds of the plants of interest were sterilized by rinsing the seeds with 70% ethanol/0.05% Triton X100 for 15 minutes and then aspirating off the solution. Then 95% ethanol/0.05% Triton X100 was used for 10 minutes and 95% ethanol was used for 5 minutes to transfer the seeds to drying filters. The dried seeds were then spread uniformly over the plate. The seeds were stratified with 3-5 days at 4˚C in the dark.

The plates were then transferred from the cold chamber to the growth chamber. The generic growth conditions for *Arabidopsis* seedlings include using a continuous light cycle at 75 µM of photons per square meter per second and a temperature of 22˚C. A continuous light cycle is utilized in one growth chamber and a day/night light cycle is used in the other. The samples were kept in the chamber and observed for two weeks.

Plant Transplantation and Growth Conditions:

After the transgenic plants were identified, they were placed into pots containing an equal mix of Super Fine Fafard Germination Mix, Redi-Earth, and Vermiculite. The contents were mixed thoroughly to allow for even spreading of materials. The same amount of mix was placed into each pot to allow for nearly identical growth conditions. Pots were watered until soaked.

The transgenic plants were then transplanted using forceps from the plates into the pots. The pots were then placed in the growth chamber at 25˚C and 50% humidity. The chamber utilized a 16 hours of light, 8 hours of dark day/night cycle.

Genomic TDNA Mutant Verification:

After about a week, leaves from the mature plants in the growth chamber were plucked and their tissue was crushed using a pestle. 500 mL of PEB (Plant DNA extraction buffer, containing 200 mM Tris,

25 mM EDTA, 200 mM NaCl, and 0.5% SDS) was then quickly added. The tubes were vortexed for about ten seconds and then centrifuged for ten minutes. The supernatant was collected and added to new tubes, where 400 µL of isopropanol was added. Precipitated DNA was washed with ethanol and resuspended in Tris/EDTA (TE) buffer (10 mM Tris-HCL, 1 mM EDTA). After PCR was conducted, genomic DNA was tested using a 1% agarose gel, 100 bp ladder, forward primer TDNA LBb1.3 and forward primer 72A9811LP.

The forward primers TDNA LBb1.3 and 72A9811LP (Table 1) and the reverse primer 72A9-811RP2 (Figure 3A) were used to help distinguish between the wildtype and the A9 mutants. The LBbl.3/RP2 set produces a product when the *CYP72A9* gene is disrupted within a TDNA (only produces a product if the insert is present) and forward primer 72A9811 LP was utilized to verify the Col-0 (WT) genotype. The LP/RP2 primer set yields a product only if there is no TDNA insert present.

Table 1. Primers used in this study.

Name	Sequence (5′ to 3′)	Use
TDNA LBb1.3	ATTTTGCCGATTTCGGAAC	Forward primer that is located on TDNA insert in mutants and will only produce a product if the TDNA insert is present
72A9811LP	GCATTCTCAATTCAAAACATGG	Forward primer that is used to indicate the presence of mutants. It will form a product with RP if there is no TDNA insert present
72A9-811RP2	TGATCTACAAGGCATTAACC	Reverse primer that will form a product with LBb1.3 if there is a TDNA insert present. Will also form a product with LP if there is no TDNA insert present.
Ubiquitin Forward Primer	GGTATTCCTCCGGACCAGCAGC	Forward primer used to help standardize the samples to get the same amount of cDNA in each sample to compare functionalities of samples in the A11 and A13 genes. Ubiquitin was used as a control for total cDNA.
Ubiquitin Reverse Primer	CGACTTGTCATTAGAAAGAAA GAGATAACAGGAACGG	Reverse primer used to help standardize the samples to get the same amount of cDNA in each sample to compare functionalities of samples in the A11 and A13 genes. Ubiquitin was used as a control for total cDNA.
72A11i4 Forward Primer	GTTAAGTCAACACCAAGATTGG	Forward primer used to compare samples (that have been standardized with ubiquitin primers) in the A11 background/functionality to aid in determining whether or not we have potential mutant samples.
72A11i4 Reverse Primer	AGAATCTCTGGAGTATCAATGC	Reverse primer used to compare samples (that have been standardized with ubiquitin primers) in the A11 background/functionality to aid in determining whether or not we have potential mutant samples.
72A13i4 Forward Primer	CGATTTGCTGGGTATACTTC	Forward primer used to compare samples (that have been standardized with ubiquitin primers) in the A13 background/functionality to aid in determining whether or not we have potential mutant samples.
72A13i4 Reverse Primer	AAGGAAAGAAGGAGACTTGG	Reverse primer used to compare samples (that have been standardized with ubiquitin primers) in the A13 background/functionality to aid in determining whether or not we have potential mutant samples.

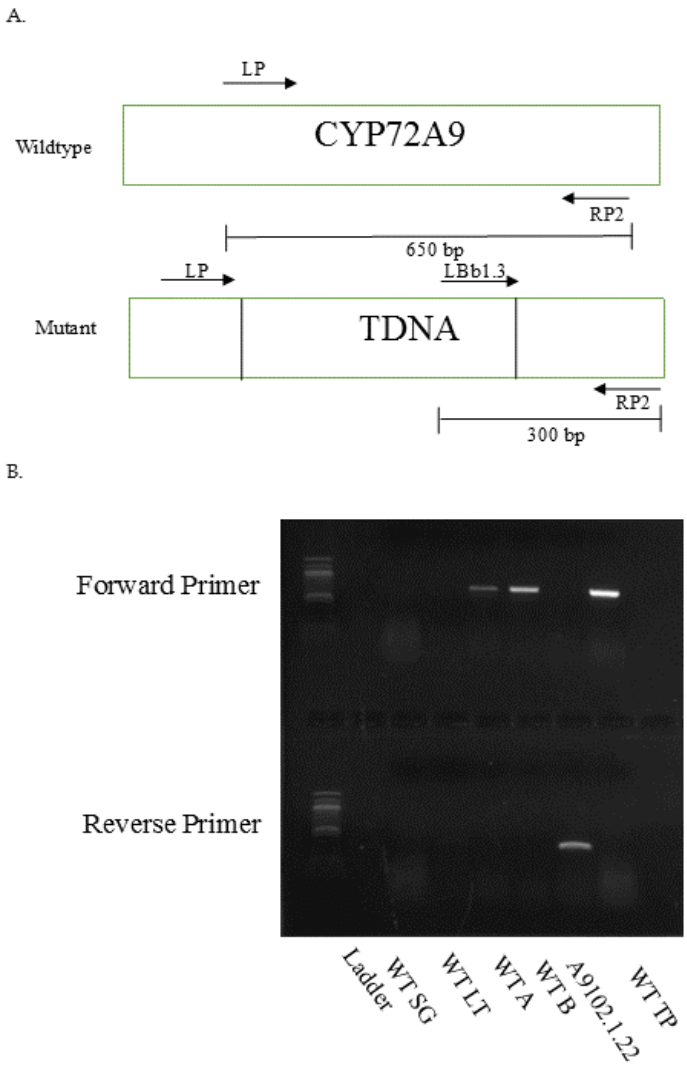

Figure 3. Primers in WT and Mutant with TDNA insert. A) Diagram illustrates how we distinguished A9 mutants from WT. Top diagram shows genomic DNA for normal A9 gene. Bottom illustrates how A9 was disrupted by TDNA insertion. Primers were selected that allowed us to get 650 bp product if normal and 300 bp if product is not normal. Not to scale (See Table 1). B) Gel showing products from both primer sets. The A9102.1.22 mutant is the only sample here with the T-DNA. (LP/RP2 and LBb1.3/RP2).

Next, Polymerase Chain Reaction (PCR) was used to test the *CYP72A9* gene locus (See Figure 3). PCR reaction was conducted using 12.5 µL GoTaq Green Master Mix (Promega, Madison, WI), 7.5 µL dH_2O, 2 µL DNA, 1 µL $MgCl_2$, 1 µL forward primer, and 1 µL reverse primer. Cycle conditions for the PCR were 95˚C for four minutes, 95˚C for 30 seconds, 58˚C for 30 seconds, 72˚C for two minutes, 72˚C for ten minutes, and 4˚C on infinite hold. The portion of the cycle with 95˚C for 30 seconds, 58˚C for 30 seconds, and 72˚C for two minutes was repeated 30 times.

Gel electrophoresis was used to test the presence or absence of the TDNA insertion. Gels contained 1/2x TBE, 1% agarose and ethidium bromide (EtBr). We expected bands of 250 bp for the LBb1.3/RP2 primer set and 650 bp for the LP/RP2 primer set.

RT-PCR for *CYP72A13* Gene Expression:

cDNA generated using RT-PCR was amplified using PCR and allowed us to see which parts of the DNA strand were used in coding different proteins and showed which genes were being expressed in the tissue that was used to create the cDNA. Three processes were utilized to acquire this expression data: RNA isolation, cDNA synthesis, and PCR.

RNA isolation was conducted to verify mutant expression to synthesize cDNA for further testing using the Qiagen RNeasy Plant Mini Kit and protocol. Frozen leaves from plant tissue samples were ground with a frozen pestle in a tube. Then, RLT buffer and mix buffer were added to the sample in the tube (sample size could be no greater than 100 mg) and the sample was grinded again. After vortexing, the lysate was transferred to a spin column placed in a 2 mL collection tube and was centrifuged for 2 minutes at full speed. The supernatant was then transferred to a new microcentrifuge tube. Ethanol was added to the cleaned lysate and was mixed by pipetting. The sample (about 650 µL) was transferred to an RNeasy spin column (pink colored) placed in a 2 µL collection tube. This was centrifuged for 15 seconds and the flow-through was then discarded. 700 µL buffer RW1 was added to the RNeasy spin column and the column was centrifuged for 15 seconds at over 10,000 rpm. Then, 500 µL buffer RPE was added to the spin column which was centrifuged for another 15 seconds at over 10,000 rpm. Then, 500 µL buffer RPE was added again and the column was centrifuged for 2 minutes at over 10,000 rpm. Finally, the RNeasy spin column was placed in a 1.5 µL collection tube, and 30 µL of RNase-free water was directly added to the spin column membrane twice, successively, to bring the total added RNase-free water to 60 µL. *An important step to follow: if not using DNAse, one must add the 700 µL RP1 buffer to the RNeasy spin column. If using DNAse, one must not add RP1 buffer.

cDNA synthesis was conducted using the Invitrogen SuperScript III First-Strand Synthesis System. First, RNA was mixed with oligodT and dNTPs and then denatured at 65˚C for 5 minutes. This mix was then placed on ice for about one minute. Next, the cDNA mix was created. 8 µL of RNA per sample was used, in addition to 2 µL 10x RT buffer, 4 µL 25 mM $MgCl_2$, 2 µL 0.1 M DTT, 1 µL RNaseOUT, and 1 µL SuperScriptIIIRT. After preparing this mix, 10 µL of this 1/5 diluted cDNA mix was added to each RNA/primer mixture and was collected by brief centrifugation. OligodT was incubated for 50 minutes at 50 ˚C. After reactions were terminated at 85˚C for 5 minutes and chilled on ice, the reactions were collected by brief centrifugation and 1 µL of RNase H was added to each tube. The tubes were incubated for 20 minutes at 37˚C, to 4˚C, then spun down. *An oligodT primer was used for cDNA synthesis, thus the total cDNA of all polyA-containing RNA molecules was generated.

PCR was then utilized to amplify the first-strand cDNA obtained in this synthesis reaction. 1/5 dilutions of each cDNA sample were used in PCR for testing. A super master mix, containing 9.5 µL dH_2O, 12.5 µL green mix, and 1 µL primer mix was synthesized and an even amount was placed in multiple tubes (primer mix contained ubiquitin forward and reverse primers, 72A11i4 forward and reverse primers, and 72A13i4 forward and reverse primers (Table 1). Each tube contained 23 µL of the mix (dH_2O, green mix, and primer mix) to which 2 µL of cDNA from the different plant samples was added, resulting in a total of 25 µL mix per tube. Cycle conditions for the PCR were 95˚C for two minutes, 95˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for one minute, 72˚C for five minutes, and 4˚C on infinite hold. 30 cycles were used for *A11/A13* while 24 cycles were used for ubiquitin.

Stress Conditions:

The plants were placed under three primary forms of stress:

Initially, the plants were placed under heat shock stress. Magenta boxes were made using a mixture of half-strength Linsmaier and Skoog Basal Medium, sucrose, and phytoblend. Samples were grown under normal growth conditions using around 90 µM of photons per square meter per second using a day/night cycle (16 hours of light, 8 hours of dark) and at 25˚C. After 3 weeks, plant samples were transferred to a chamber for 3 hours. The plants were placed in a chamber with a temperature of 38˚C

Celsius. They were then brought back into the chamber at 25˚C (Figure 6). A week later, at 4 weeks old, plant samples were observed and their pictures were taken.

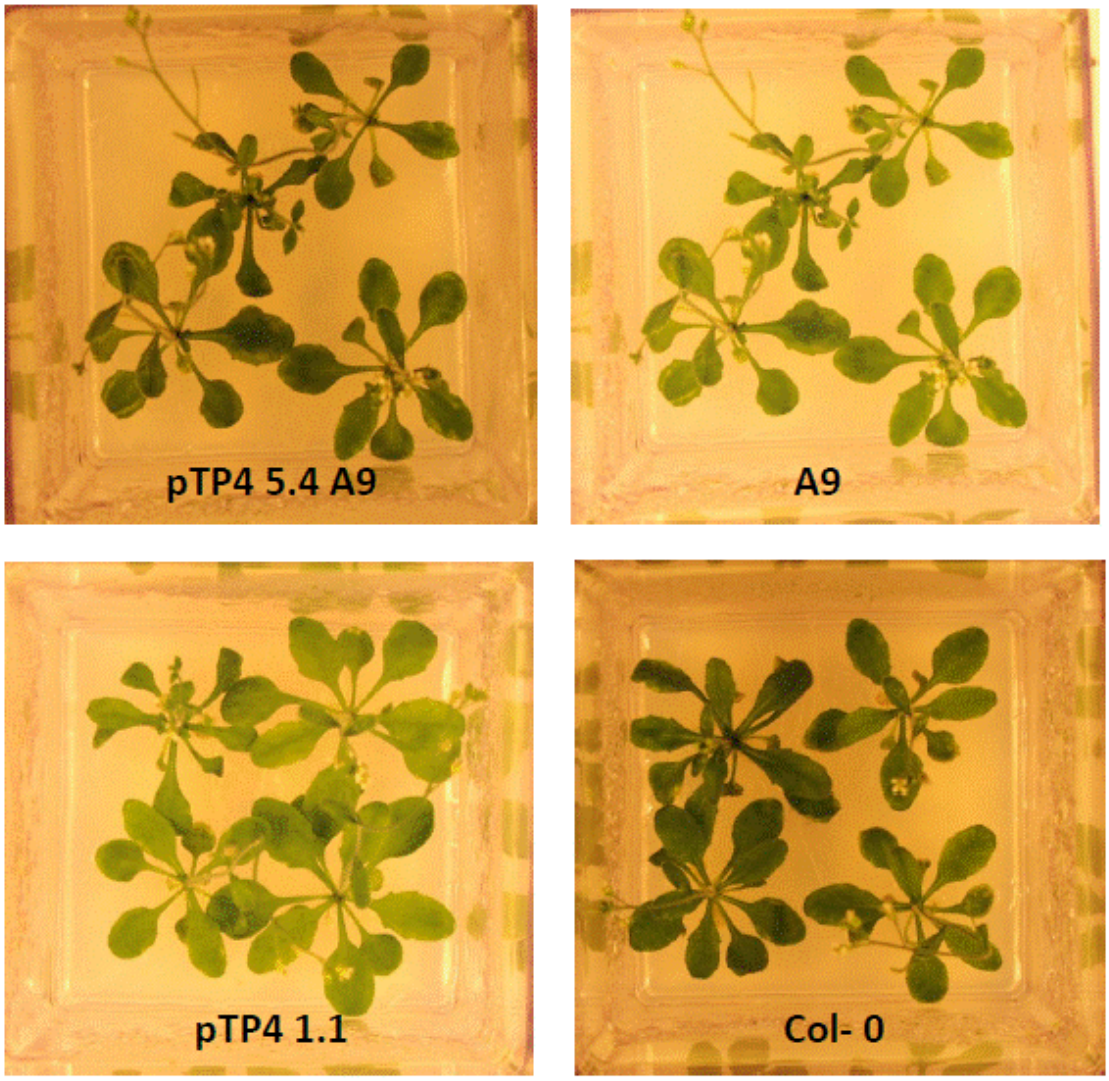

Figure 6. Heat shock. Samples were organized in the above fashion with the WT and A9 controls on the left side and the mutants in the pTP4 and pTP4 A9 backgrounds on the right side. The specific conditions include day/night light cycle (16 hours of light, 8 hours of dark) along with a temperature of 25˚C. For 3 hours, the plants were placed in a chamber with a temperature of 38˚C. They were then brought back into the chamber at 25˚C.

Next, the plants were placed under osmotic stress (via utilization of mannitol to simulate the drought-like effects of osmotic stress). Two experiments using mannitol were set-up; one involved growing plants on normal media and then transferring plants to mannitol-media plates, and the second experiment involved growing plants on mannitol-media plates from the beginning.

The plants were split up into different categories with mannitol media. Media was made using water, 1 pack of LS (Linsmaier and Skoog) salts (half-strength LS Basal Medium), phytoblend and 30g of sucrose. The components were mixed and split into four flasks each containing 250 mL of mix. Then, four sets of mannitol plates were made using different concentrations of mannitol: 0 mM, 100 mM, 300 mM and 500 mM. Various amounts of water were added to each flask to bring total to 500 mL. Then 4g of agar (phytoblend) was added to each flask. Flasks were autoclaved (with 20 minutes of sterilization). Mix was poured into plates in hood; 12 plates per flask were poured.

Plant samples were grown on regular media plates (see protocol for making regular plant media plates) and were grown for five days and then transferred to mannitol-media plates. These plates were labeled 0, 100, 300, and 500 mM for their varying mannitol concentrations (Figure 7).

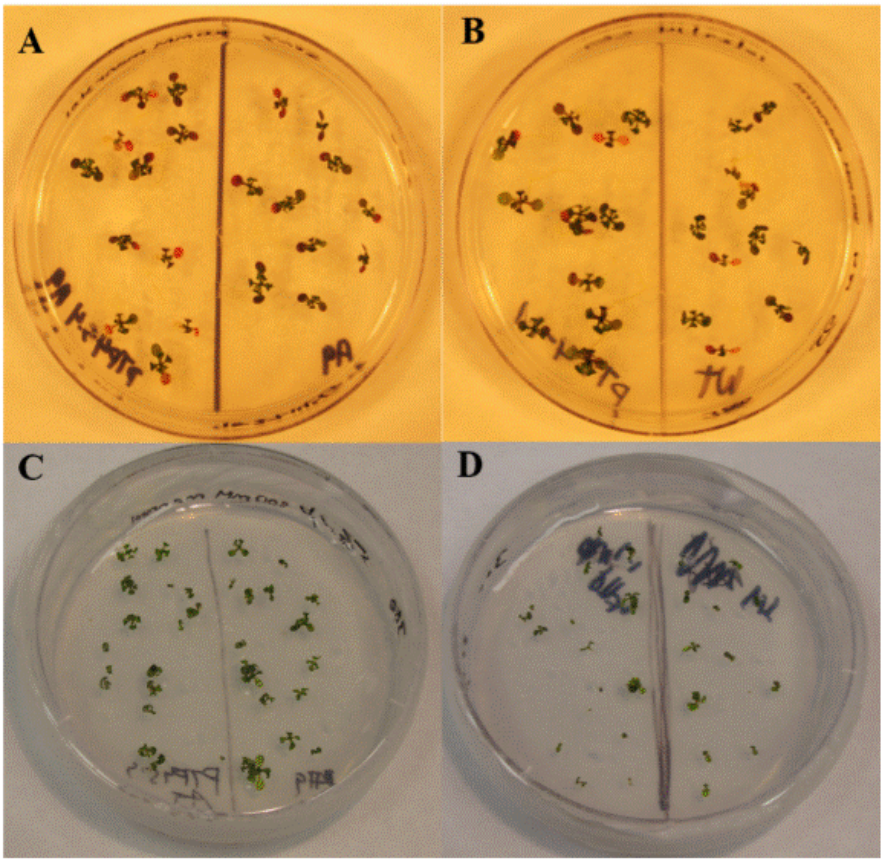

Figure 7. Mannitol media plate experiments to simulate drought-related effects of osmotic stress. Samples were organized in the above fashion in two separate experiments. A and B are from the first experiment, which utilized growing WT, A9, pTP4-1.1, and pTP4-5.4 A9 samples in regular growth media and then transferring plants into above plates with mannitol media. C and D are from the second experiment, which consisted of growing the same plant samples directly in the mannitol-media plates from the onset. A) pTP4 5.4 A9 samples were grown on the left and A9 samples were grown on the right. B) pTP4 1.1 samples were grown on the left and WT samples were grown on the right. C) pTP4 5.4 A9 samples were grown on the left and A9 samples were grown on the right. D) pTP4 1.1 samples were grown on the left and WT samples were grown on the right. Two of each of the plates above were grown in 0, 100, 300, and 500 mM/mL concentrations of mannitol.

Plant samples were grown on mannitol media plates directly from the onset (rather than being transferred). Same plates were used as in previous experiment. Plates were once again labeled 0, 100, 300, and 500 mM/mL (Figure 7).

Finally, the plants were placed under biotic stress utilizing caterpillars. *T. ni* (six days old) were placed on three week old plants. The caterpillars were allowed to eat the plant for seven days before being frozen and dried. Silk nets were placed over the pots the plants and caterpillars were in to allow air to come through while simultaneously preventing the caterpillars from escaping.

RESULTS

To verify whether lines of plants were transgenic, the first step was to identify which plants were able to survive in the BASTA plates, indicating that they have taken up the transgene and BASTA resistance gene (showing that they have BASTA antibiotic resistance). These plants were transgenic based on their big, dark green, and upright position in the plates (Figure 2). It was crucial to eliminate non-transgenic plants that are attached to the transgenic plants of interest to avoid falsely selecting a plant without the mutation.

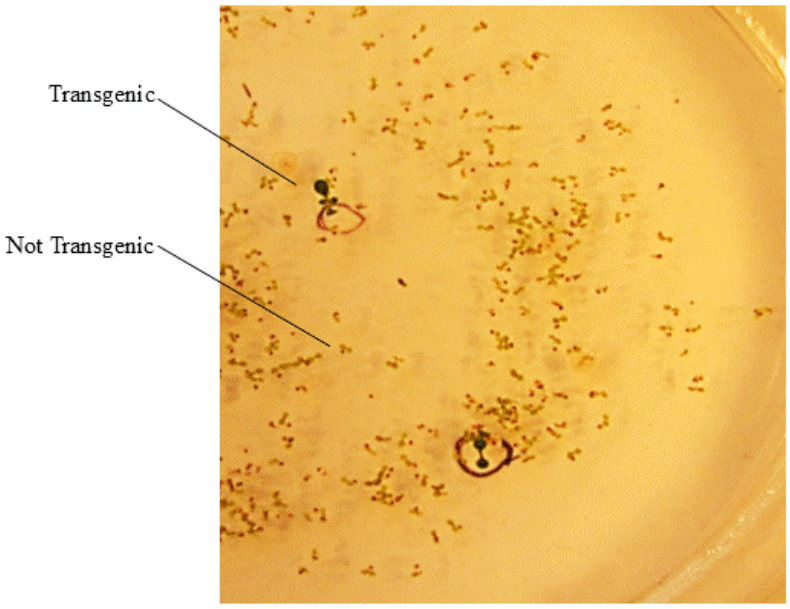

Figure 2. Transgenic plant identification. Plants were grown on growth media for 8 days at two stages (stage 1: 3 days at 4°C in dark, covered completely in aluminum foil). This was the stratification stage with no growth. They were then moved to growth chamber and kept there for 7 days at 25 C with 24 hours continuous light cycle at ~30 micromoles of photons per square meter per second.

Once these plants were chosen based on being transgenic, the plants were tested to see whether they still had the *CYP72A9* mutation. To do this, genomic DNA was isolated and tested by PCR (Figure 3). The results indicated that the transgenic plants we picked had the A9 mutation. Figure 3 shows how we distinguished A9 mutants from the WT samples. This is evidenced by gel electrophoresis because genomic DNA bands are present, thus indicating the successful disruption of the A9 gene and allowing us to say that the plants still had the A9 mutation.

Next, the plants were tested to see whether the pTP4 amiRNA construct knocked down expression of the *CYP72A13* gene. Figure 4 shows RNA isolation and because bands were present there was sufficient RNA for cDNA synthesis. Figure 5 shows that through RT-PCR and ubiquitin standardization, pTP4-1.1 is a good potential single mutant and pTP4-5.4A9 is a good potential double mutant. Ubiquitin forward and reverse primers were used to standardize the plant samples to try and get

the same amount of cDNA in each sample. This aids us because it is easier to compare our samples' functionalities in the *CYP72A11* and *CYP72A13* backgrounds when we have the same amount of cDNA in all of our samples.

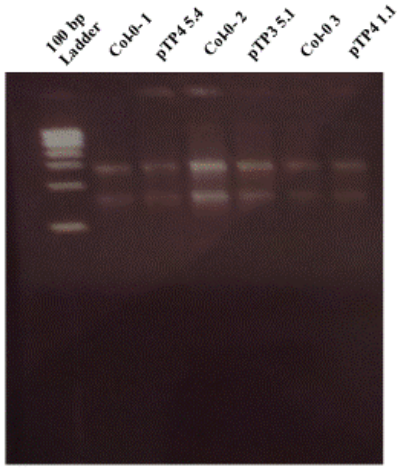

Figure 4. RNA isolation. Shows relatively uniform RNA from each plant sample.

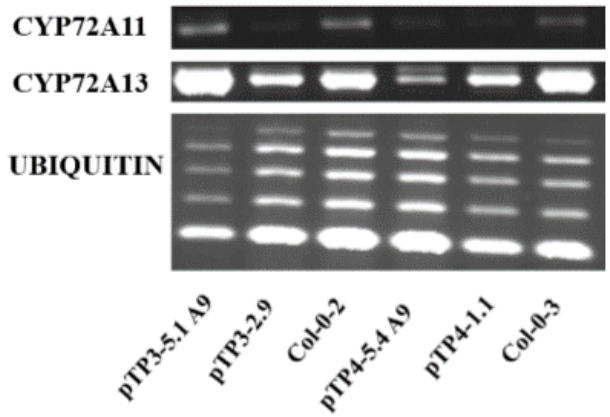

Figure 5. RT-PCR. Gel electrophoresis of PCR from cDNA samples indicated below with gene specific primers indicated on the left.

In addition to using the ubiquitin forward and reverse primers, the 72A11i4 forward and reverse primers and the 72A13i4 forward and reverse primers were also used to test our cDNA and compare our bands to determine if our samples, pTP4 5.4 A9 and pTP4 1.1, were good potential mutants by comparing the relative strengths of bands (for example, comparing the pTP4 1.1 band in the A11 background using the 72A11i4 forward/reverse primers to the WT band also in that background and then comparing the pTP4 1.1 band in the A13 background using the 72A13i4 forward/reverse primers to the Col-0-3 band also in that background) (Figure 5). We were able to determine that pTP4 1.1 was a good potential single mutant because it is mutated at one gene and in comparing the pTP4 1.1 band to the Col-0-3 band in both the A11 and A13 backgrounds, the pTP4 1.1 band was fainter in both cases. We also determined that pTP4 5.4 A9 was a good potential double mutant because it is mutated at 2 genes and when comparing

the pTP4 5.4 A9 band to the Col-0-3 band in both the A11 and A13 backgrounds, the pTP4 5.4 A9 band was fainter, indicating it was not as functional. This assumption could be made because the cDNA levels of the two bands were similar in strength when looking at the ubiquitin uniformity (Figure 5).

Most importantly, A13 expression is decreased for pTP4 5.4 A9 and pTP4 1.1, meaning both of these samples had decreased expression when compared to the Col-0-3 bands. Thus the primers used in this comparison allowed us to clearly see the differences in mutant and wildtype expression. However, it is also important to note that in the gel run using ubiquitin primers to standardize cDNA, pTP4 1.1 is very similar in band strength to Col-0-3, whereas the pTP4 5.4 A9 band is bigger than the Col-0-3 band. Thus it is easier to make comparisons between pTP4 1.1 to Col-0-3 in the A11 and A13 backgrounds than it is to compare pTP4 5.4 A9 to Col-0-3 in the A11 and A13 backgrounds, but because the pTP4 5.4 A9 band was somewhat similar in strength to the Col-0-3 band we were still able to compare pTP4 5.4 A9 bands to Col-0-3 bands in the A11 and A13 functionalities (Figure 5). The cDNA generated using RT-PCR was then amplified using PCR. *CYP72A11* was used for comparison's sake because it is the next closest homolog to *CYP72A13*, thus we needed to verify that *CYP72A11* expression is unchanged by the amiRNA targeting *CYP72A13*. Interestingly, the gel shows that there is some decrease in *CYP72A11* expression. This finding must be taken into account when analyzing the phenotypes.

After confirming that we had good single and double mutants based on decreased functionality at the A11 and A13 functionalities, we were able to design stress response tests to examine whether the *CYP72A13* gene was required for stress resistance. Heat shock, osmotic stress (using mannitol), and biotic stress (from caterpillars) were all set up through four separate experiments.

The heat shock test was conducted to see whether *CYP72A13* played a defensive role in response to drastic changes in heat conditions. Col-0 and A9 controls were compared to the pTP4 1.1 single mutant and the pTP4 5.4A9 double mutant. The samples that were used were "mid-range" samples, in that they were not too healthy nor were they too withered. The four selected samples were all a similar height and each sample had four leaves. In both the mutant samples and the wildtype samples, the leaves appeared a similar shade of green with withering at the tips. Thus when directly comparing samples from both controls to both mutants, no significant phenotypic differences were seen. (Figure 6). These results are consistent with the literature and with what we expected.

For the mannitol experiment, both a qualitative and quantitative analysis was performed where the Col-0 control was compared to the pTP4 1.1 single mutant and the A9 control was compared to the pTP4 5.4 A9 double mutant. According to Figure 7, for the initial mannitol experiment where the plants were grown first on regular media and then transferred to 300 mM/mL mannitol plates later on, the pigments of the Col-0 and pTP4 1.1 samples looked fairly similar; no significant differences were able to be discerned. Specifically, some samples have red and pink tips, while others are dark green, and most samples are withered except for a select few. In addition, the pigments of the A9 and pTP4 5.4 A9 samples looked fairly similar as well, and so no significant differences were able to be discerned. Specifically, both the pTP4 5.4 A9 and A9 control samples were short and about half of them had bright red tips. The A9 control samples had a little darker tone of red on the tips but no drastic differences were noted between the pTP4 5.4 A9 and A9 samples (Figure 7). Thus, an accurate conclusion could not be drawn strictly from qualitative analysis of the plant samples.

For the same experiment, where the plants were transferred from regular media plates to 300 mM plates, a quantitative analysis was also performed. Anthocyanins were extracted from the plants and measured based on absorbance at 530 nm and 657 nm. However, based on our analysis, anthocyanin

levels were not statistically different in the control samples, Col-0 and A9, from the mutant samples, pTP4 1.1 and pTP4 5.4 A9 (Figure 8).

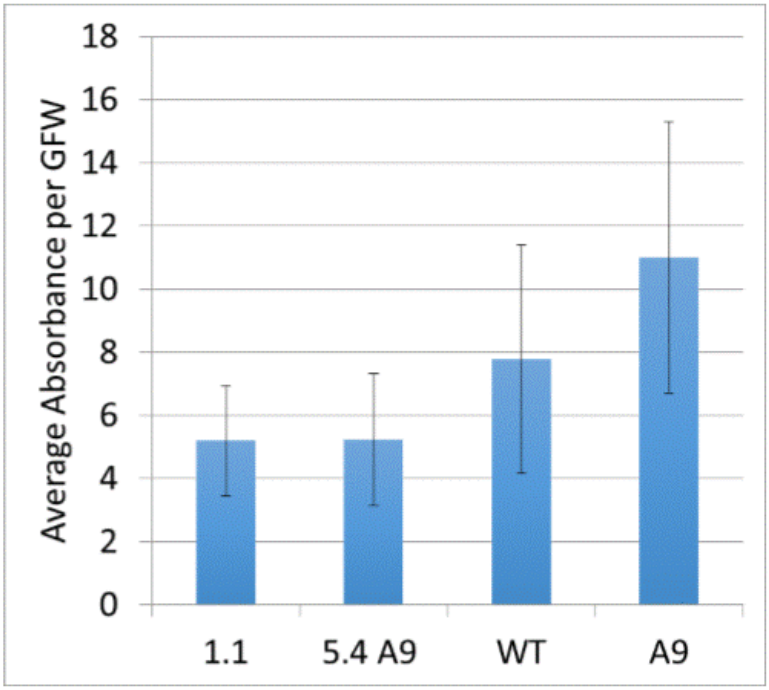

Figure 8. Average anthocyanin levels. Samples were placed under wavelengths of 530 nm and 657 nm in a spectrophotometer to determine average absorbance. Error bars indicate the uncertainty in the reported measurement of anthocyanin levels. Standard error of the mean was 1.06 for pTP4-1.1, 1.86 for pTP4-5.4 A9, 2.04 for WT and 2.82 for A9. Numbers are values ± standard error. Error bars show the data are not statistically significant. *N*= 3 to 8 for all samples.

The final mannitol experiment consisted of growing plants using 300 mM media plates from the start, rather than growing them first on regular growth media plates and later transferring them to mannitol plates. A qualitative analysis was performed, and the results indicated that, after two weeks of being placed in the growth chamber, the plants had no significant pigmentation or differences when compared to one another (Figure 7). The Col-0 control plants were compared directly to the pTP4 1.1 single mutant plants, and the A9 control plants were compared directly to the pTP4 5.4 A9 double mutant plants, and no significant differences were noted between these two sets of plants.

For the biotic stress experiment, caterpillars were placed on sample plants and were monitored for the amount of the plant they ate. It was noted that the caterpillars that ate the mutant plants, pTP4 1.1 and pTP4 5.4 A9, were not drastically different from the caterpillars that ate the control plants, Col-0 and A9. Quantitatively, the average weight of caterpillars feeding on the Col-0 control plants was 6.398 mg and the average weight of the caterpillars feeding on the pTP4 1.1 single mutant plants was 9.448. In addition, the average weight of caterpillars feeding on the A9 control was 7.627 mg and the average weight of the caterpillars feeding on the pTP4 5.4 A9 double mutant plants was 5.392 mg. The experiment consisted of weighing caterpillars who fed on one of the four types of plants, Col-0, A9, pTP4 1.1, and pTP4 5.4 A9 (Figure 9).

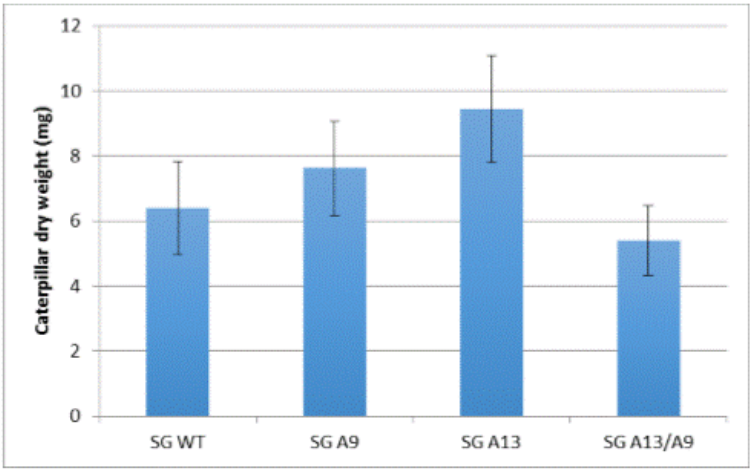

Figure 9. Caterpillar dry weights by plant sample. Pots of plant samples WT, A9, pTP4-1.1 and pTP4-5.4 A9 were set up with caterpillars of the species *Trichoplusia ni*. Caterpillar dry weights were calculated with Mettler Toledo sensitive balance from TCNJ Chemistry Department. Error bars indicate the uncertainty in the reported measurement of caterpillar dry weights. Numbers are values ± standard error. Error bars show the data are not statistically significant. *N*= 11 to 20 for all samples.

DISCUSSION:

Many plants are able to withstand somewhat drastic changes in temperature in their environments with the help of specific genes that are upregulated when the plant is put in such a stressful situation. The heat shock response allows cells in a plant to withstand a variety of stress conditions and is activated by increased synthesis of heat shock proteins (HSPs), which protect cellular proteins from stress-induced denaturation. One specific HSP, HSP90, has been shown to be crucial in regulating heat shock responses, and a mutation that eliminates CYP40, which is required for full HSP90 function, has been shown to prevent HSP90 from initiating the heat shock response in *Saccharomyces cerevisiae* (Duina *et al.*, 1998). Examples such as this show that CYPs do in fact play roles in heat shock response.

We tested the mutant plant samples under heat shock conditions. We tested *CYP72A13* because of the connection between HSPs and other CYPs, because the expression of the *CYP72A11* gene changes under heat shock stress and because *CYP72A11* is a homolog to *CYP72A13* (Winter *et al.*, 2007). However the results, acquired through phenotypic analysis, indicate that *CYP72A13* does not seem to defend the plant from heat shock, and thus our hypothesis was not supported. There were no noticeable differences between the *CYP72A13* mutant and control plants. This was expected because we did not see anything in the literature or in the data from previous experimentation that would indicate that *CYP72A13* specifically was responsible for mounting a defense response to heat shock. Thus the original hypothesis- that *CYP72A13* does not play a role in heat shock stress response in *Arabidopsis thaliana*- seems to be supported. What this means is that, although we saw a potential relationship between heat shock causing changes in *CYP72A13* expression due to HSPs and their relationship with CYPs, we did not see any differences between our mutants and controls, indicating that no defense response was initiated by *CYP72A13* against heat stress.

Next, with respect to osmotic stress, the literature suggests that *CYP72A13* is upregulated under this particular stress and thus we expected to see similar results (Winter *et al.*, 2007). Two separate mannitol experiments, one where plants were grown first on regular growth media and transferred later on to mannitol media and another where plants were grown on mannitol media from the start, were

conducted. The first setup was a shock to established plants and the second setup was a shock that impacted germination. The two setups were used to observe how the mutants responded to similar stresses at different stages in the plants' life cycles.

The first experiment indicated that, after transferring plants from regular growth media to 300 mM mannitol media, the samples exhibited pigmentation changes, becoming somewhat purple and red. I expected to see an increase in pigmentation, or anthocyanins, in the mutants. Anthocyanins are flavonoid pigments that can be induced by various abiotic stresses, including osmotic stress via mannitol. They serve as antioxidants that quench reactive oxidative species (ROS), which would allow plants facing abiotic stresses to increase their photosynthetic capabilities as ROS are known for limiting photosynthesis in plants under stress conditions (Kovinich *et al.,* 2014). More specifically, there is a relationship between anthocyanin production and osmotic stress. The literature in this field shows that increasing sucrose concentration or adding mannitol to a culture medium, resulting in an increase in osmotic potential, subsequently causes an increase in production of cyaniding 3-gluocoside, peonidin 3-glucoside and peonidin 3-p-coumaroylglucoside. These are three anthocyaninsthat are affected by osmotic stress and increasing osmotic potential as high osmotic potential appears to stimulate the methylation of anthocyanins (Do and Cormier, 1991). As a result, I expected to see an increase in anthocyanin in the mutants compared to the controls because the mutants have *CYP72A13* knocked down and, if we are to believe that *CYP72A13* is responsible for defense against osmotic stress, I expected to see more anthocyanin to protect the plant to make up for the fact that the defense gene is not present. However, there was no noticeable pigmentation difference between Col-0 samples and pTP4 1.1 samples and between A9 samples and pTP4 5.4 A9 samples. The plant may compensate for not having the supposed-defense gene, *CYP72A13*, by increasing anthocyanin synthesis. However, no major differences were obvious, so we tested further with spectroscopy.

The spectrophotometer was thus utilized to better observe differences in anthocyanin levels. This quantitative analysis allowed us to conclude that anthocyanin levels were greater in the controls (Col-0 and A9 samples) compared to the mutants (pTP4 1.1 single mutant and pTP4 5.4 A9 double mutant) (Figure 9). This went against what we expected as we thought the anthocyanin levels in the mutants would be higher than the anthocyanin levels in the controls because the mutants lack *CYP72A13* and without the gene, the plant should increase anthocyanin synthesis to combat the lack of defense. The mutant plants did not mount the typical stress response. This suggests that *CYP72A13* could be required for initiating the defense response. A follow up experiment would be to grow the plants longer to determine whether the mutants are more severely damaged by the stress.

The second experiment was where the plants were grown directly on 300 mM/mL mannitol media from the start. The observed pigmentation on these samples was only slightly visible, thus no significant comparisons between mutants and controls could be made (Figure 7). There was no difference between anthocyanin synthesis between Col-0 samples and pTP4 1.1 samples or between A9 samples and pTP4 5.4 A9 samples. Perhaps changes to the protocol of the experiment must be made, such as changing the mannitol concentration or keeping the plants on the growth media or the mannitol media for longer periods of time. As of now, we can only state that it seems that *CYP72A13* plays some role in osmotic stress response in *Arabidopsis thaliana*, based on the results from the plants grown on regular media and transferred to mannitol media. *CYP72A13* appears to be needed for the normal response to osmotic shock, but not the early growth under osmotic stress.

In addition to understanding abiotic stress responses for *CYP72A13*, it was also important to include biotic stresses as the literature suggests that this gene is involved in defense responses that are initiated under biotic stress. *Arabidopsis thaliana* has been known to be able to defend itself against abiotic stresses such as excessive heat and drought conditions, but it is also known, in addition to many other plants, for its ability to counteract biotic predation from a variety of organisms, including parasites (Bar-

Nun and Mayer, 2008). In addition, another important defense compound, *cis*-jasmone, has been shown to induce certain *Arabidopsis* genes that affect the plant's defense against aphids and their parasitoids. Most importantly, it has been shown that certain CYPs, such as *CYP72A13*, are upregulated upon application of *cis*-jasmone (Bruce, 2008), which artificially induces the insect defense responses.

We tested this upregulation of *CYP72A13* by placing our plant samples under caterpillar biotic stress. We expected to see a greater average weight for the caterpillars that fed on the mutants and a lower average weight for the caterpillars that fed on the controls. The data suggests that *CYP72A13* is most likely needed for defense. Because pTP4 1.1 and pTP4 5.4 A9 both have *CYP72A13* knocked down, the potential defense mechanisms initiated by the gene would not be able to occur. In the controls, because Col-0 and A9 both have *CYP72A13* (the gene is not knocked down), the potential defense mechanisms of the gene should occur as normal. As a result, the caterpillars feeding on the mutants should be bigger than the caterpillars feeding on the controls because the mutant plants are not able to mount a successful biotic stress defense response, whereas the controls, having *CYP72A13*, are able to utilize the gene's potential defense capabilities and prevent the caterpillar from eating the entire plant. However, a simple t-test was conducted, and the result was not less than 0.05, indicating that the mutant and wildtype data are not significantly different. In addition, although the data are variable because the caterpillars were not uniformly dry, there is a trend of more mass in the insects feeding on mutant plants, thus suggesting that *CYP72A13* is most likely needed for defense.

All of this information is crucial in understanding the bigger picture, which is that CYPs play important roles in plants in various areas such as growth and defense mechanisms. These secondary metabolites are important because they allow the plant to survive in adverse conditions (be it arid, dry climates or extremely cold, wet ones, or even areas where predation rates are high). In addition, plants respond differently to their environments and to various environmental stresses because of their specific genetic makeup and how certain genes are upregulated in response to certain environmental cues such as heat shock, osmotic stress, or biotic stress such as caterpillar predation. This study shows that the *CYP72A13* enzyme is more important in osmotic shock than in heat shock and most likely plays a role in insect defense.

A better understanding of plants and how they utilize CYPs and subsequently different molecular and biochemical pathways will allow us to induce productive change in the areas of crop yield and production (and in agricultural practices in general). As the world continues to face numerous challenges such as an immense population boom, food shortages, and environmental problems such as global warming, it is important to be able to efficiently grow crops in bulk that can withstand adverse conditions and stresses. Studying plant stress response in a model plant is the first step towards a better understanding of crop plants in general. Understanding how to best induce certain defense and growth mechanisms in key crops will allow us to more efficiently produce enough food to sustain our continuously expanding population. CYPs play a major role in this goal and thus understanding CYPs and how they work may just be the key to saving the world.

LITERATURE CITED:

Bak, S., Beisson, F., Bishop, G., Hamberger, B., Hofer, R., Paquette, S., Werck., Reichhart, D. (2011). Cytochromes P450. *Arabidopsis Book, 9.*

Bar-Nun, N., Mayer, A.M. (2008). Methyl jasmonate and methyl salicylate, but not *cis*-jasmone, evoke defenses against infection of *Arabidopsis thaliana* by *Orobanche aegyptiaca*. *Department of Plant and Environmental Sciences, The Hebrew University of Jerusalem. 8,* 91-96.

Bleeker, P.M., Haring, M.A, Kant, M.R., Schuurink, R.C., Van Wijk, M. (2009). Plant Volatiles in Defence. (2009). *Advances in Botanical Research. 51,* 613-616.

Boddupalli, S.S., Deisenhofer, J., Hasemann, C.A., Kurumbail, R.G., Peterson, J.A. (1995). Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure. 3,* 41-62.

Bruce, T. J., Matthes, M. C., Chamberlain, K., Woodcock, C. M., Mohib, A., Webster, B., Napier, J. A. (2008). *cis*-Jasmone induces Arabidopsis genes that affect the chemical ecology of multitrophic interactions with aphids and their parasitoids. *Proceedings of the National Academy of Sciences. 105*, 4553-4558.

Do, C.B., Cormier, F. (1991). Accumulation of peonidin 3-glucoside enhanced by osmotic stress in grape (*Vitis vinifera* L.) cell suspension. *Plant Cell, Tissue, and Organ Culture. 24, 49-54.*

Duina, A.A., Kalton, H.M., Gaber, R.F. (1998). Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of heat shock response. *The Journal of Biological Chemistry. 231,* 18974-18978.

Irmler, S., et al. (2008). Indole alkaloid biosynthesis in Catharanthus roseus; new enzyme activities and identification of cytochrome p450 CYP72A1 as secologanin synthase. *The Plant Journal, 24* (797).

Kovinich, N., Kayanja, G., Chanoca, A., Riedl, K., Otegui, M.S., Grotewold, E. (2014). Not all anthocyanins are born equal: distinct patterns induced by stress in *Arabidopsis*. *Planta. 240,* 931-940.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E., Browse, J. (1997). Jasmonate is essential for insect defense in Arabidopsis. *Proceedings of the National Academy of Sciences. 94,* 5473-5477.

Nelson, D. (2013). A world of cytochrome P450s. *Philosophical Transactions of the Royal Society, 368* (1612)

Nelson, D.R. (1999). Cytochrome P450 and the Individuality of Species. *Archives of Biochemistry and Biophysics. 369*, 1-10.

Seki, H., Sawai, S., Ohyama, K., Mizutani, M., Ohnishi, T., Sudo, H., Fukushima, EO., Akashi, T., Aoki, T., Saito, K., Muranaka, T. (2011). Triterpene functional genomics in licorice for identification of CYP72A154 involved in the biosynthesis

of glycyrrhizin. *Plant Cell. 23,* 4112-4123.

Taiz, Lincoln and Eduardo Zeiger. *Plant Physiology.* 3rd ed. Sunderland, Mass.: Sinauer Associates, 2010. Print.

Wang, Y., Li, Q., & He, Z. (2004). Blast fungus-induction and developmental and tissue-specific expression of a rice P450 CYP72A gene cluster. *Chinese Science Bulletin, 49*(2), 131-135.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., Provart, N.J. (2007). An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets. *PLOS One. 2,* e718.