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Expression Analysis of a Glucose Oxidase Transgene in Tobacco

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SENIOR THESIS APPROVAL

This Honors thesis entitled

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Date

Expression Analysis of a Glucose Oxidase Transgene in Tobacco

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Abstract

Glucose oxidase (GOX) is an enzyme in plants that catalyzes the oxidation of glucose to hydrogen peroxide and Glucono delta-lactone[5]. We have expressed GOX under the control of an estrogen inducible system, XVE, to analyze the gene's expression under this system compared to a system using the 35s system. The 35s system contains a promoter that constitutively turns on the GOX gene in the *Nicotiana tabacum* plant which causes the gene to always be turned on. Leaf disc assays were performed with discs from 35s, XVE, and also wild type plants (not containing the GOX gene) in order to extract protein to show GOX expression. With 35s and wild type acting as controls, the XVE discs were subjected to both water and 100 uM estradiol applications for differing amounts of time (24 and 48 hrs). Western blots displayed results exhibiting different expression of protein in XVE:GOX discs when subjected to the estradiol versus water solution. *Nicotiana tabacum* therefore shows considerable induction within as little as 48 hrs. The XVE plants were subjected to other assays to confirm the inducibility seen in the leaf disc assays. Discs taken from both water and estradiol applications were taken and placed in glucose for 8 days causing stress on the plants which could be viewed by the loss of the green color. The discs that were effectively induced by the estradiol showed greater effects due to the glucose. Along with being placed in glucose, discs were also placed in a starch media containing glucose for 7 days. Discs demonstrated blackening in the media within several hrs. These results suggest that the XVE system used in the *Nicotiana tabacum* shows an ability to be induced with 100 uM estradiol and alters the GOX expression in the plant.

Introduction

In this experiment, two transgenic lines of tobacco were used to study the plant's responses to oxidative stress and the defense mechanisms produced in response to that stress. These lines were

modified to produce the protein Glucose Oxidase (GOX) from a gene found in the insect *Helicoverpa zea*, commonly known as the corn earworm. Fungi, as well as insects, naturally produce this enzyme which acts as an anti-fungal and anti-bacterial agent, but the constant production of this enzyme leads to the buildup of oxidative stress in the organism[5]. In the first transgenic line, the gene that was transformed contained the 35s promoter, which is able to express the gene constitutively[2]. In addition to this line, another transgenic line was created with a construct that contained the XVE system, which acts by an estrogen-inducible promoter. This gives us the ability to control expression of the gene[1]. During expression, the GOX protein is able to break cellular glucose down into hydrogen peroxide (H2O2) and Glucono delta-lactone. The H2O2 in the reaction then acts as a reactive oxygen species (ROS) that breaks down into H2O and O-. This O- ion causes oxidative stress by oxidizing cellular structures and proteins within the cell[4]. When the protein is present throughout the life of the tobacco plant, the plant must have ways to adapt to this reactive oxygen species (ROS)[3].

In our experiment, we are interested in looking at how exactly the plant is able to adapt to this presence of reactive oxygen species. By studying this, we are hoping to learn more about the defense mechanisms that the plant uses. This is not only important research in plant biology, but due to the homology between plants and animals, this research could help identify defense mechanisms that are used in humans to fight stress-related diseases[6,7].

Materials and Methods

Throughout all of the following experiments, the 35s:GOX and XVE:GOX transgenic lines used were produced previously from the combination of a transgene GOX, which codes for a protein called glucose oxidase, which was isolated from the insect *Helicoverpa zea,* with the promoters 35s and XVE promoter system. These constructs were then inserted into the *Nicotiana tabacum* plants. We took seeds from these previously created transgenic lines and used them for our plants. Specific lines that we used for our experiment were determined by previous research that showed a more consistent expression of the glucose oxidase gene. We grew our lines in a basement under fluorescent lights that

could be adjusted in intensity by modifying how close they were to the plants and could also be placed on timers that would have specific light and dark cycles. Not only was this helpful in maintaining a constant amount of light and temperature for our plants, but their isolation was important for the plants not to experience cross pollination. The plants were sufficiently spread apart to allow light to contact each plant.

Polymerase Chain Reaction

The first step before proceeding with further testing of the plants is to run PCR. PCR determines whether or not the plant contains the gene of interest. In order to extract the plant DNA for use in PCR, we used the InstaGene Matrix DNA Extraction Kit from Bio-Rad and followed their protocol. 3 uL of this DNA was then combined with a master mix solution containing 0.5 uL of a complimentary primer, 5 uL of buffer, 0.125 uL of Taq Polymerase, 0.5 uL of dNTPs, and 15.875 uL of water. This procedure was reproduced for each transgenic plant that was tested using separate PCR tubes for each one. Once all of the samples were primed for PCR, they were placed in the thermocycler and allowed to amplify 20-30 times. The samples were then able to be run through Agarose gel electrophroesis to confirm the DNA.

DNA Extraction

Leaf discs were cut from whole leaves that were taken from the growing plant for the extraction of protein. The discs were initially flash frozen with liquid nitrogen, then placed into a mortar to be ground. Extraction buffer (1mL/g of tissue) containing, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 6 mM β-Mercapto-ethanol, 0.5 mM PMSF, and 5 μg/mL aprotinin was added to the plant tissue. This mixture was place in a microcentrifuge tube, then centrifuged for 15 min. The supernatant was collected and centrifuged for an additional 5 min. 5 μL was then used in a protein concentration assay with BioRad reagent to determine the concentration of protein in each sample.

Western Blot

Samples prepared from DNA extraction were then used in a western blot to show the expression

of the protein. The amount (in μL) of each sample of protein varied depending on the concentration of the protein in that sample. 25 μg of protein was added along with 5μL of 4X LDS sample buffer, 2 μL of 10X reducing reagent, and the appropriate amount of water in order to reach a total of 20 μL for each sample. The samples were then heated at 70°C for 10 min, quick spun (~10 seconds) in the centrifuge, then loaded into a gel. We then made 1X-SDS running buffer by diluting a 20X MES-SDS stock solution. A final volume of 1L (per Gel-rig used) of running buffer was needed. We prepared the gel by taking the tape off, placing the gel in the chamber, filling the middle with running buffer, checking for leaks, filling the outer container ~3/4 with running buffer, then removing the comb. The next step was to load the marker. The samples containing the protein were then loaded and were run at 150 V till the blue color was at the bottom. The gel was then transferred onto Nitrocellulose paper by using a blot module to create a "sandwich" which was filled with transfer buffer and run at 200 mA for 1.5 hrs. The Nitrocellulose paper was then placed in a solution of blocking buffer for at least an hr (can be left overnight). The Nitrocellulose paper was then incubated with the primary antibody on a rotating plate, to allow it to attach to our protein on the Nitrocellulose paper. The solution contained a certain dilution of our antibody to blocking buffer, depending on which antibody was used. After at least 1 hr of incubation with the primary antibody (often times overnight), the Nitrocellulose paper was then washed with PBS Tween. The Nitrocellulose paper was then added to a solution containing the secondary antibody at a certain dilution of antibody to blocking buffer. This was placed on the rotating plate for at least 1 hr. The Nitrocellulose paper was then washed again with a solution of PBS tween. Finally, a 10 mL solution containing 10 mL of deionized water and a sigma FAST tablet was added to the Nitrocellulose paper and allowed to incubate for around 30 min or until a purple color appeared. *Note that it is important to keep the Nitrocellulose paper wet throughout the process of the western blot.

Starch Iodide Assay

The starch Iodide solution was created by adding 2 g of starch (2%), 0.5 g of Agarose Gel

(0.5 %), and 0.83 g of Potassium Iodide (KI) (20mM) per 100 mL of solution made. Leaf discs or a whole leaf was placed on a plate and autoclaved, then shortly mixed with a magnetic stirrer and autoclaved for an additional 5 min. After being autoclaved, the solution was kept mixed with the stirring bar and let cool to 50 °C. (Do not pour until then: If it is still hot the solution will kill the plant and denature proteins) Then the starch Iodide solution was pipetted onto a plate containing the leaf discs. The discs will have a tendency to float but can be pushed down and will stay when the solution begins solidifying. Do this until the gel is solid and the discs are on the bottom of the gel. Flip over and put under light. Results should be seen within 24 to 48 hrs.

Glucose Necrosis Assay

This assay is a way of producing a large amount of reactive oxygen species by placing leaves in a 20 mM solution of glucose. Glucose acts as the substrate for the GOX produced by the transgene that we have placed in our tobacco plants. The GOX causes the glucose to be converted to hydrogen peroxide. This hydrogen peroxide is then at high levels in the cell. The plant's defense system must find a way to get rid of the hydrogen peroxide before it begins taking electrons from other places in the cell and causing serious damage[4]. We placed the leaves in the solution and examined the effects seen on the outside of the leaf. The effects were examined at day 1 and day 7.

XVE Induction Method

Plants that were transgenically modified with the XVE:GOX construct are considered to be inducible for the glucose oxidase gene. This means that the glucose oxidase in these plants are not normally expressed, but can be turned on when subject to a chemical or signal that affects the promoter. While there is more than one possible chemical that has been found to be effective in inducing the XVE promoter system, the one that we found to have the most evidence of success was estradiol. In this procedure, we soaked leaf discs in a 100 μm solution of estradiol (with 01.% Tween20) and in a separate plate, soaked discs from the same plant in a water control. The leaf discs were subjected to the estradiol solution for differing amounts of time in order to see how quickly we could induce the gene

and see GOX expression. We confirmed the induction through western blotting.

XVE Glucose and Starch Iodide assay

The tobacco plants with the XVE promoter system were also subjected to the Glucose Necrosis and Starch Iodide assays in tandem to see the cellular effects of the reactive oxygen species on this system as well.

Results

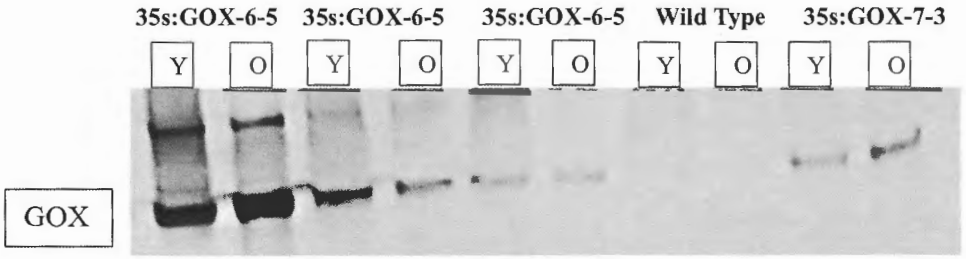

Figure 1. The Y and O in each sample represents Young Leaves' and Old Leaves' Proteins. This western blot shows GOX presence in each leaf and in the same concentration for each individual plant.

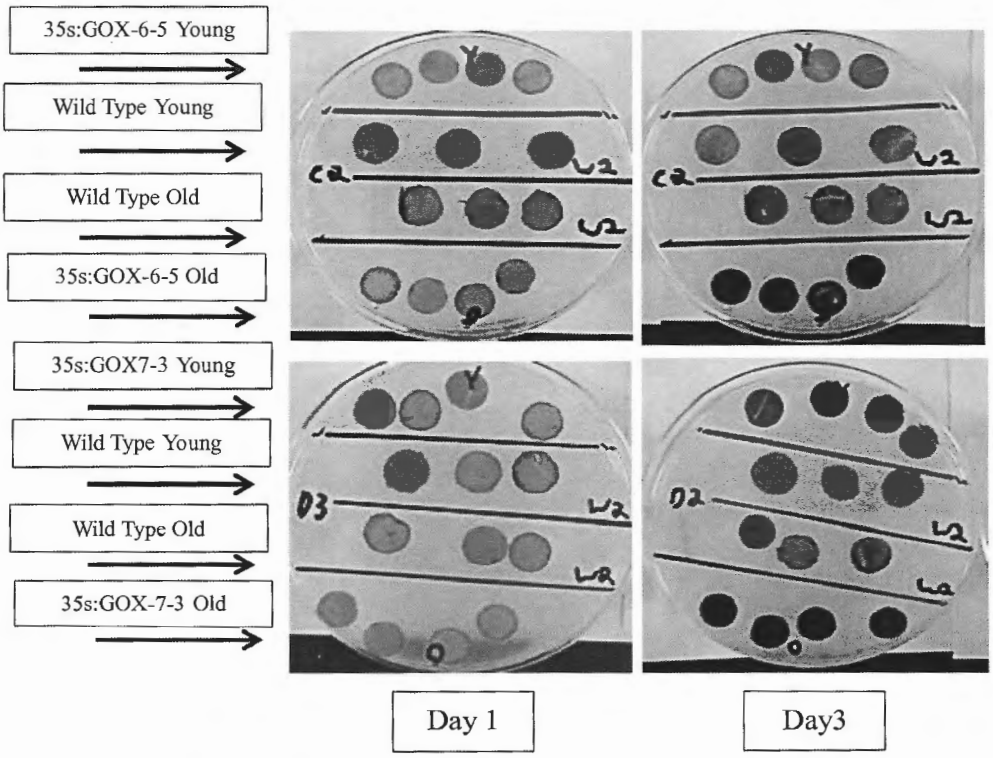

Figure 2. H_2O_2 production assay. Two different 35s:GOX lines and non-transgenic control (WT) were used. Additionally discs were taken from developing young (Y) leaves and mature older (O) leaves. Black presence indicated the production of H_2O_2.

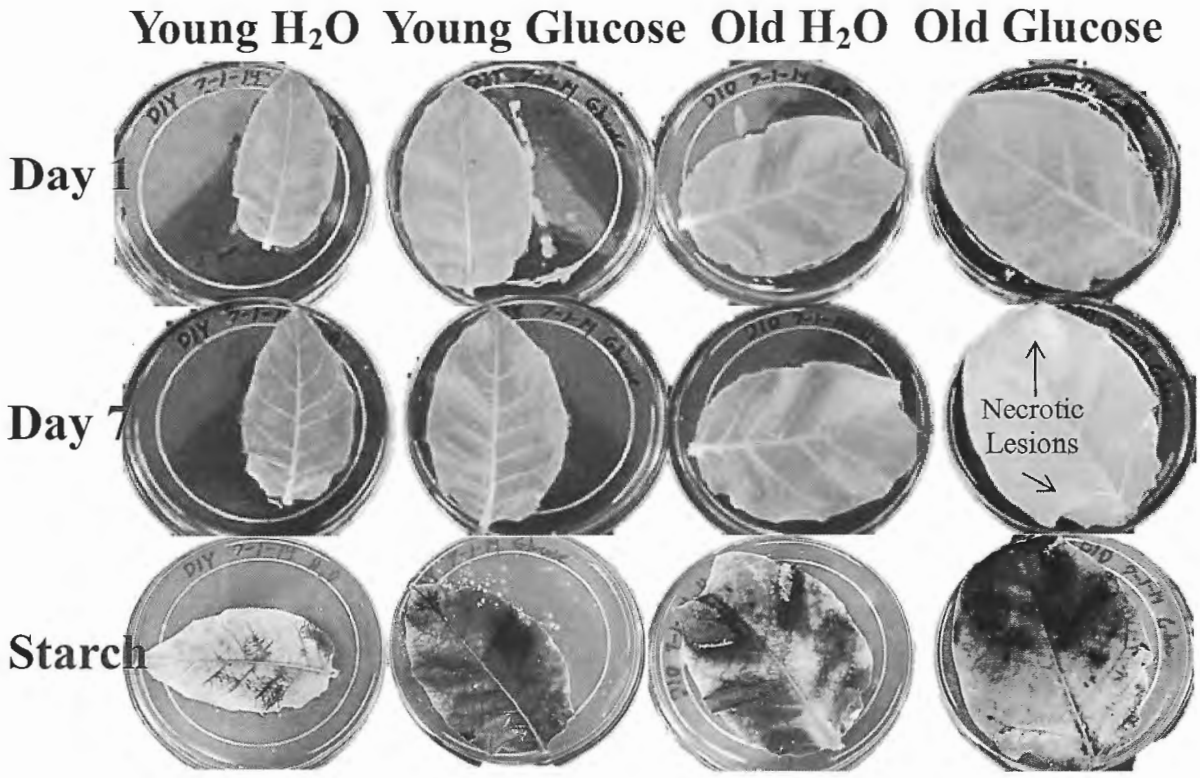

Figure 3. Glucose assay show includes young and old leaves in both a standard water solution and a 20mM Glucose solution.

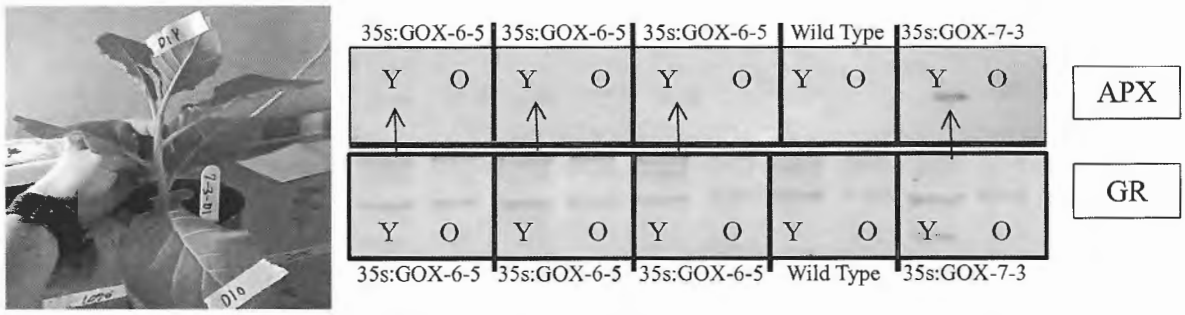

Figure 4. Western blot showing positive and negative results of Y (young leaves) and O (old leaves).

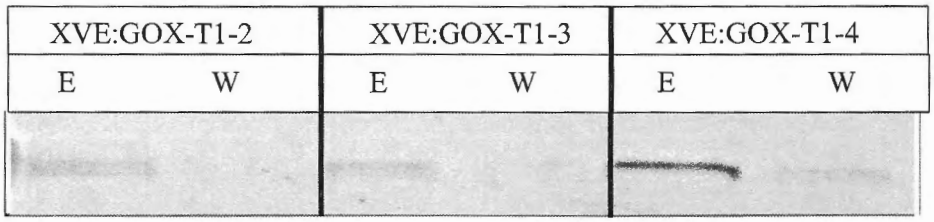

Figure 5. Western blot analysis showing induction of the GOX protein under the control of the XVE promoter. Leaf disc were soaked in 100um estradiol (E) or water (W) for two days prior to protein extraction.

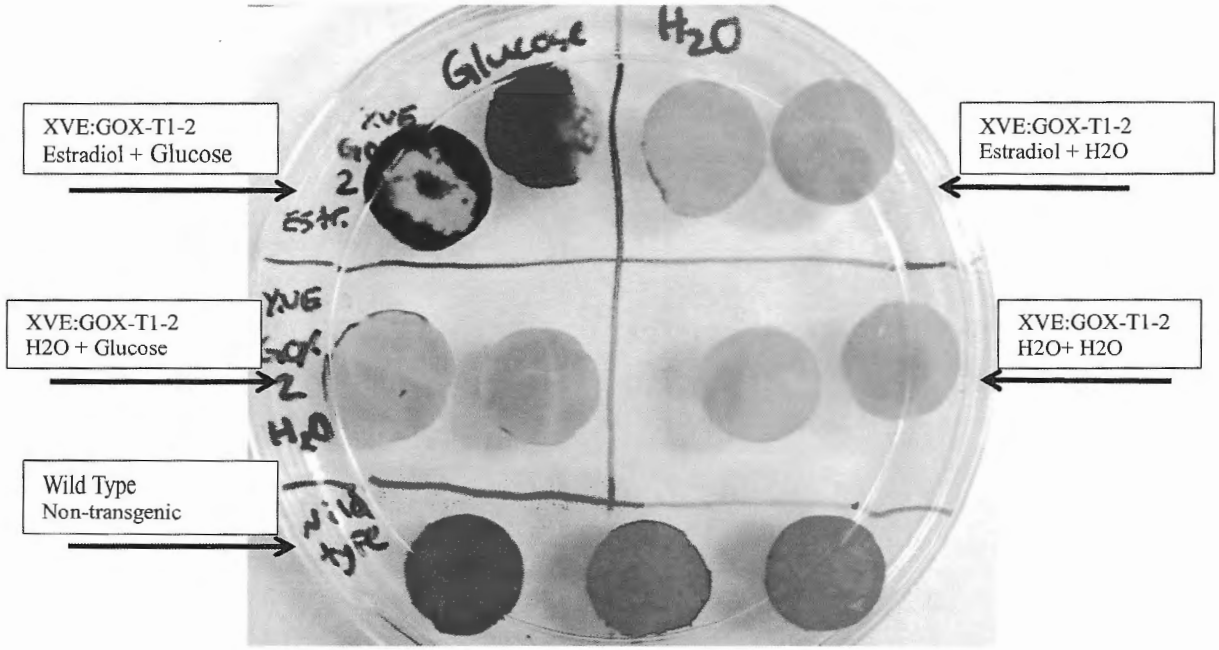

Figure 6. Starch-Iodide assay of an XVE:GOX transgenic line and non-transgenic control (WT) showing oxidative stress. The black leaf discs show successful induction of the GOX gene by estradiol treatment.

Discussion

After ensuring that the plants that were being tested actually had the GOX gene present, the 35s:GOX constitutive line was tested by using different aged leaves to see if there was any differences in the expression of the GOX gene between them. As the western blot in Figure 1 shows, in each respective line of plants, the young and old leaves show the same levels of GOX expression. What this shows is that throughout the life of the plant, it is not only able to maintain its expression, but also the expression to the same level.

The fact that the young and old leaves show the same expression of GOX does not necessarily mean that they have the same effect to the addition of reactive oxygen species. In Figure 2 leaf discs from old and young leaves were subjected to a starch iodide assay that shows the production and presence of hydrogen peroxide. There are wild type discs that show what the lack of reaction with the starch iodide should look like. As you can see, essentially all of the leaf discs looked the same by day three other than the old leaf discs from the GOX plant line. The test shows the presence of hydrogen peroxide able to interact with the iodide and produce the black color; we see that all except the old leaves stayed a brown color. We would expect this in the wild types, because they do not contain the gene that is producing the hydrogen peroxide. We see though that in the young leaves that even have the GOX gene being expressed (which was confirmed by the western blot in Figure 1) they do not have the same hydrogen peroxide levels as the old leaves. In Figure 3, we also see that when the old leaf is subjected to the same harsh conditions as the young leaf, that the old leaf has more adverse effects.

The fact that the old leaves were less capable of handling the stress than the young leaves is not surprising. What we were interested in learning was what exactly caused the younger plant to be able to better handle the stress than the old leaf. The answer to this question was ultimately a question about the leave's defense mechanism against the oxidative stress. In this experiment, we used several well known proteins that have been connected with defending against oxidative stress. Through western blot analysis, we were able to see that Ascorbate Peroxidase (APX) was expressed at higher levels in the

younger leaves which would agree with the increased ability of younger leaves to handle stress. Glutathione Reductase (GR) is another known anti-oxidant protein that we checked the expression levels of between the two aged leaves[4]. This, on the other hand, did not show differing levels of expression, so it may not make as much of a direct impact against the oxidative stress created in this way. Future studies would utilize more anti-oxidant proteins to see how they were expressed to try and gain a better understanding of the overall defense mechanism that the plant is using when it is stressed.

Along with the 35s promoter system, we also worked with a tobacco plant with the XVE promoter system. As shown in Figure 5, there was an expression GOX in the estradiol treated leaves in three separate lines, while leaves from the same plant placed in water solutions did not show the expression of GOX. This confirmed that the estradiol solution was able to induce the GOX gene with two days of exposure. Once the XVE plant induction was confirmed, we used the same assays used with the 35s promoter system plants to see if we saw similar results. As shown in Figure 6, we saw positive results on the leaf discs that were induced and subjected to a glucose solution as we would have expected to see due to GOX being turned on and being overloaded with substrate. This result was seen by the necrosis in the leaf discs in the estradiol treated solution. More tests are certainly required, but these results suggest that induced as well constitutively promoted GOX is capable of breaking down glucose and cause significant damage to the plants.

Conclusion

Using this oxidative stress model in the *Nicotiana tabacum* plant could lead to a better overall understanding of the defense pathways in not only plants, but in humans due to the numerous similarities seen between plant and animal systems. This is of high importance as oxidative stress is a problem seen in humans that interrupts other cellular process and is known to cause mutations in the DNA which lead to cancer.

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Thesis Reflection

For my honors thesis project, I got the opportunity to work in a lab at Ouachita Baptist University under Dr. Nathan Reyna in conjunction with several other undergraduate students for approximately ten weeks during the summer of 2014. While I did gain invaluable experience in the lab during this time, I might argue that I learned more in other areas of my life from this experience.

One of the particular areas that I feel I grew from this experience was in responsibility. The fact that I was responsible for my own work and that the work that I produced was something that was actually contributing to the knowledge of others, instilled in me a sense of responsibility that I have not previously had. While I have worked before, and even had a leadership role in that work, I feel that this opportunity has been a unique experience that has required me to really grow out of the mindset that what I am doing does not actually mean that much and doesn't really affect those around me significantly. Having up to this point in my education only learned about scientific principles and acquiring knowledge based on the work of others, I feel that it is easy for me to naturally think subconsciously that science is only expanded by those who have spent many years in the lab and have many more years of scientific and educational experience than me. Therefore at the beginning of this experience, I would say that I had very little belief that I could really contribute to the knowledge in the scientific community. For the most part, previous to this experience, the work that I had done in science labs had essentially just been experiments that have had a known result by the professor, that I was repeating only for the purpose of learning the protocol and repeating work done previously by others. Very few times have I had to think about why I was performing the procedures that I was, in those experiments. In this experience, Dr. Reyna certainly had to guide me through the whole process constantly, but one of the things that I feel he did a great job of was consistently making me think about what I was actually doing and why. One of the areas that I, as a student, have been able to get away with up to this point is trusting that what other people are telling me as fact and not having a mind that questions what is going on. This is specifically dangerous as a scientist, as people are constantly

learning that others were not correct in their theories and other assumptions, but also just in general as a learner. While it may be okay to accept that others, more experienced than you in any given area, are telling you something, the problem comes when you rely on just what they are telling you without a thought about why that is actually the case. If practiced constantly, this practice will lead to a lack of depth in knowledge and a less than effective understanding of whatever you are learning. I can say that I have been able to see a large change in my mindset since having this experience in the way that I study and learn for my classes now. I feel that this lesson that I learned was very necessary in gaining responsibility for my own learning which I feel will be very beneficial in my upcoming educational endeavors as well as just life. As a senior in college, the fact that I am about to be required to be mature and take complete responsibility for my life is becoming more and more clear every single day. This research project has been ultimately the best experience that I have had in preparing me for taking on that responsibility thus far.

While there was importance placed on actually producing these effective results, the Patterson Summer Research program and specifically Dr. Reyna in my personal experience, created an atmosphere of learning as well. I would say that this was very important in my ability to have confidence to work independently. Understanding that I was expected to produce effective work, but also knowing that I was not completely expected to be flawless in the lab was essential in this experience. If there was no room at all to make a mistake, I would have been fearful to work without asking about every single little thing that I did. This effectively would have been just like every other lab that I had previously experienced. During this research project, I certainly made a fair number of mistakes. While I certainly never want to make mistakes, especially when it pertains to something of this level of importance, I feel that these mistakes may have been the thing that caused me the most personal growth. Like I have stated previously, most of the educational experiences that I have had have essentially been memorization and required very little critical thinking. In most reproduced labs for the biology and chemistry classes I have taken up to this point, it was important to do them correctly,

but often times if something did not work the way that you were planning on it working, you did not have the time to go back and redo it until it worked. The next lab, you had a completely different lab to complete, so you could not really go back and keep trying to get it right or you would get behind. Also, because there was a new lab assignment every week, the overall importance of that one lab was lessened due to the fact that you plenty more that could make up for that one. This, I feel, produced an atmosphere that decreased the importance of accuracy. While I understand the importance and some of the benefits of getting a wide array of lab experience, I feel that depth is certainly another aspect which is important. This summer I had a significant amount of time working with the same set of plants and on approximately the same experiment, mainly with just different steps and slight different variations. This allowed me to and required me to learn the ins and outs of the protocols and not just brush off the experiment if there was something that went wrong. If there was something that did not go the way we were expecting, or was just did not work at all, there was always a time in the lab where we would try to get to the root of the problem. There may have been an error made by me or one of the other workers in the lab, something that was wrong with the materials that we were using, or maybe a malfunction in one of the machines that we were using. Our job at that point was to use the results that we saw as clues to what may have gone wrong. Initially, most of the protocols I was relatively unfamiliar with, and Dr. Reyna, who was much more experienced in analyzing the results of these protocols, was able to use his previous experience to effectively figure out the step that was most likely the result of the ill effect. As I began to gain experience in running these experiments, I began to understand why each step was performed and what the solutions added were effectively doing at the molecular level which helped me to begin understanding what was going on when we saw certain results. Learning more in depth the protocols of this specific lab experiment was important for our work, but I can say that I will be able to take the sense for a need of accuracy and depth of knowledge away from this specific part of the process. This process really allowed me to see that the sense of asking questions and truly understanding what I was performing was an extreme weakness of mine as a learner.

Another aspect of growth that was the result of my research over the summer was learning how to work with others. Over the summer there were three different kinds of relationships that I was able to experience. There was the relationship that I had with Dr. Reyna as the boss, I worked in close tandem with a guy name Jesse, and there was a guy named Colby who was working in the same lab, but was working on a completely different project than both Jesse and I. First off, in my relationship with Dr. Reyna, I got a good sense of what kind of things that you seek to accomplish as you are working for someone else. As he was the one that graciously gave me the opportunity to work in his lab, I wanted to not only produce good work for the sake of good work, but as a way to show appreciation to him. The research that I was conducting was an extension of something that he had already started and worked on for about ten years. To do less than my best, I would have seen as a waste of his time not only now, but to the work that he has already put in. Along with that, I felt a desire to not disappoint him. Unfortunately at times I feel that my work did not show that desire to do my best work and desire not to disappoint him. While in many jobs if you have errors, you are likely to either be given a short a leash or will get fired, I was fortunate that Dr. Reyna had an understanding that mistakes were going to happen and he was more interested in seeing that mistakes were not made because of a lack of effort. As I mentioned earlier, failure is one of the things that I would say caused me to grow the most. When I did make mistakes, I felt like I was letting Dr. Reyna down. This feeling that would stay with me for quite a while was something that I certainly do not want in the future in anything that I do. I would say this has given me even more drive than I initially had, to put in the work it requires to perform well and not make mistakes. While we were conducting slightly different experiments, Jesse and I were working on the same species of plant and were often times either working on the same protocols or were helping the other while we had dead time in our own project. This was a great experience. There were times when I had already done something that he was doing for the first time and vice versa. The similarity of our projects allowed us to help one another constantly. I feel that this willingness and ability to help one another is an effective way of working and getting things done. One of the great things that I got

out of having that relationship is that with being so involved in the work that he was doing, I was essentially able to experience two different projects at once. I not only had to know what was going on in my experiment, but had to keep up with his so that I could teach him what to do next if I had previously done that protocol or just be able to help him as an extra pair of hands. We were able to spend time together relating our projects in order to get a greater understanding of the way our plants worked as a whole. We also used previously collected data to compare ours with in order to make more connections. I also got to get a taste of what another undergraduate student, Colby, was working on. Colby was working a project that was new to the lab. Most often when I would help him, it was a matter of being there as an extra set of hands, but through that I was able to learn more about what exactly it was that he was doing. Like other things that I have talked about, this was beneficial to me in more than just learning specifically about the biology at hand. I was able to see how you can learn from others just by being willing to help them even though you might not know what you are doing. This has been evident to me since this experience. By getting involved in things that you are not already good or knowledgeable at, is so beneficial in growing your knowledge as an individual.

Mentioning all of the ways that I have grown personally from this experience without mentioning the significance that it played on my idea of science and understanding of what real research is would be missing a big part of what I learned through this experience. One of the immediate things that I learned is how long things take to actually obtain results. In my personal experience, I had worked close to seven weeks before what I was trying to accomplish actually happened. That is not relatively that much amount of time, but that step was one of the initial steps that were to happen before any real data could be extracted from the plant system that I was working with. Not to say this is completely insignificant, but if you take a look at the bigger picture this is a very small step on the road to the finish line. One of the things that I was able to see from a better perspective was how the scientific community is constantly gathering data about biological systems, but that gathering the data does not mean anything until you are able to analyze the data that you collect. Once you acquire this for

your own experiment, you must then see how you can take that and apply it in other situations and see if you get the same results. Ultimately it takes someone to piece together the puzzle of parts acquired from years of extensive research from many places to make breakthroughs happen in science. I certainly know that I may not work as quickly as people that are much more experienced, but I have gained an understanding of the amount of time and work required to make a significant advance in science. Along with seeing the conceptual picture of science through a clearer lens, I was also able to gain practical lab skills through this experience. From using casual lab instruments such as a mortar and pestle, to using thermocyclers and many other advanced pieces of lab equipment, I was able to better my lab techniques while simultaneously gaining knowledge of equipment that I had previously not been exposed to.

The specific ways that I have mentioned that I have grown from this opportunity are something that has a concrete cause and effect. There were moments that I can look at and say, because of going through that specific task, I grew in this specific way. I can sit and playback a specific moment and recall how exactly it affected me. There has also been a difference, though, that I can see which does not have a clear cut reason behind it. This difference is the fact that I have developed a mindset that will allow me to become successful in the future. I can't tell you why I have the mindset now and did not have it before, but I can tell you that I was so far away from realizing that mindset before this opportunity and that it was absolutely necessary for me to be where I am now.