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Characterization of peroxidase and catalase genes in defense mechanisms of Physcomitrella patens

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Characterization of peroxidase and catalase genes in defense mechanisms of *Physcomitrella patens*

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Lauren Marie Ciulla

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Abstract

Systemic acquired resistance (SAR) and the hypersensitive response (HR) are two important induced defense mechanisms in plants. SAR is the development of an enhanced resistance to a pathogen due to a prior encounter. It results in faster and more effective defensive action within the plant upon a second-time pathogen attack. HR is a plant defense mechanism that utilizes reactive oxygen species (ROS) to attack pathogens at the site of an infection. ROS can be generated in many ways; however, it is specifically known that plants use the enzyme peroxidase to generate the ROS hydrogen peroxide during HR. Plants also use the enzyme catalase to generate water from hydrogen peroxide in order to contain and control the toxicity. Much is known about SAR and HR in economically important vascular plants such as rice and corn; however, they have only recently been identified in nonvascular plants such as moss. This study aims to identify and characterize the roles of peroxidase and catalase in HR and SAR in the model moss species *Physcomitrella patens*; specifically, how the expression of peroxidase and catalase genes in this species is affected by exposure to the fungal elicitor β-glucan. This will provide insight into the SAR and HR of moss and other nonvascular plants and into the evolution of plant defense mechanisms.

Introduction

Evolution and Survival Mechanisms

Some of the most common interactions that all organisms experience are predation, parasitism, and pathogen exposure. These interactions may put an individual in immediate danger. The organism can either die or change its behavior, surroundings, or molecular make-up in order to survive. Over time, as generations are exposed to these interactions, responses that help them to survive may become innate. The organisms that have the best response survive, while others die ultimately passing the "most fit" genetic make-up to future generations (Mayr, 2001). This process is "decent with modification" and is called evolution (Mariam Webster). In order to survive, evolution has led to a plethora of diverse and complex survival mechanisms across all life on earth such as predation, parasitism, and pathogen exposure. Although, predation, parasitism, and pathogen exposure function in different ways, they are similar in that they are driving forces of evolution of survival mechanisms in organisms (Hart, 1990).

All of these three relationships involve the attack, harm, or death of an organism. Predation is typically when one organism—the prey—gets eaten by another—the predator. An example of this is a cheetah attacking and eating a gazelle. In this scenario, the cheetah is the predator and the gazelle is the prey. Because of this, gazelles tend to live in herds and over time evolution has selected for fast gazelles, as the fastest can escape the cheetah. Predator-prey relationships have forced evolution of survival and eating habits and decisions between both the predator and the prey (Stevens, 2010).

Parasitism is an extremely common interaction that is experienced by many organisms. It is a type of symbiosis in which there is a parasite and a host. Often, the

parasite will steal and utilize the host's nutrients for itself, leaving the host weakened. A common example of this is an intestinal parasite, which dwells within the hosts intestines and lives off of the nutrients the host has acquired. The parasites could also dwell in a host because it provides the parasite safety. Parasites have forced the evolution of habits, such as when termites isolate other infected termites, and also immune systems (Stevens, 2010).

Pathogens have especially forced the evolution of intricate and specific immune systems. A pathogen is a bacteria, fungus, virus, or other type of microorganism that can lead to disease when within its host organism. In order to fight a pathogen that has entered the host, the host must change parts of itself in order to make itself less appealing and suitable for the pathogen (Alberts, 2002). These changes have developed into what is known as the immune system.

Humans and Pathogens

Parasites and pathogens have been important evolutionary drivers in the development of the human immune system. The human immune system is extremely intricate and can specifically target numerous types of parasites and pathogens. The first line of defense from foreigners in humans is called the innate defense system and it limits exposure to the inside of the body. This can be done by moving away from the foreign body, the use of hair, mucous, and skin (Figure 1) (Janeway, 2001). This line of defense alone is somewhat effective, however, if the foreign bodies cross the barrier into the human, there are two other lines of defense that protect the human from disease—nonspecific and adaptive.

The second line of defense is the nonspecific line of defense. This consists of immediate changes in the molecular makeup of the human that are not specific to the type of pathogen that is invading it. Typically, the molecular players in this line of defense are phagocytotic leukocytes such as, macrophages, natural killer cells, and granulocytes (Figure 1). These cells devour and degrade the pathogen, alert other immune system cells to travel to the site of infection (inflammation), and often invoke fever in an attempt to accelerate defense mechanisms and decrease microbial growth (Janeway, 2001); (Abbas, 2015). This line of defense is extremely important, allows for a very quick immune response, and is effective at killing many pathogens. The second line of defense, however, is not specific to pathogen type, is not diverse in its response, and does not provide immunological memory (Abbas, 2015). These functions are a result of the third line of defense.

The third line of defense is referred to as the adaptive immune system. This system is mostly limited to vertebrates and is very complex. Unique characteristics of an adaptive immune system are: specificity, diversity, and immunological memory. The organism acquires specificity to parasites through the use of antibodies that are created by plasma cells from B cells (Abbas, 2015). The number of possible distinctive antibodies created by the body through gene alterations is seemingly endless—reaching 10^{12} possibilities (Alberts, 2002). Because there are so many antibody possibilities, it is extremely likely that one will match specifically to the pathogen that is infecting the body-causing specificity of attack. The responses of the adaptive immune system are also diverse in that there are many different ways that the body can format this response. For example, it could kill infected cells directly using helper T cells, make the pathogen more

likely for phagocytosis through opsonization, or stimulate the upregulation of other helper cells. Finally, the adaptive response provides immunological memory through memory B cells which carry an antibody previously exposed to a specific pathogen and respond more quickly to the same pathogen upon second exposure (Figure 1) (Abbas, 2015).

All three of the lines of defense in the human immune response make this response efficient and robust. The first line prevents exposure and infection, the second response very quickly and abruptly reacts to infection, and the third, although slower, provides specific attack and memory for subsequent infections. Although the human immune system is so intricate and powerful, it is not the only immune system meriting study and research. Immune systems of other organisms hold many similarities to humans, but they also contain many interesting differences due to various habitats, lifestyles, and physiological characteristics. It is important to study all types of immune systems from many types of life in order to better understand this important response as a whole.

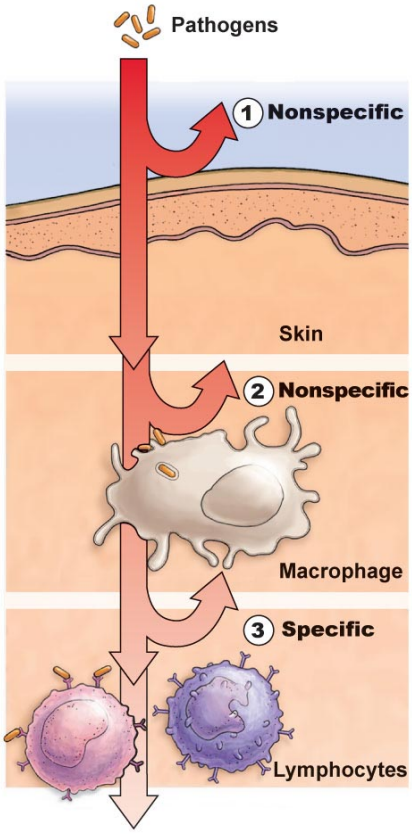

Figure 1. The three lines of the human immune system. The human immune system consists of three lines of defense. Step 1 shows an example of the first line of nonspecific defense—the skin. Step 2 shows an example of the second line of nonspecific defense—a macrophage. Step 3 shows an example of the third line of specific defense, also called the adaptive immune system. This step shows lymphocytes that often help to create specific attacks, like antibodies. (Pearson Education, 2010)

Plants and Pathogens

The plant immune system, although somewhat similar to the human immune system, is very unique to plant physiology and characteristics. Plants are often exposed to pathogens and many classes of microbes that can cause damage their growth or reproduction. Plants are capable of responding to these pathogens and protecting themselves (Jones, 2006). Because they are sessile, unlike humans, plants must utilize a wide array of defensive mechanisms to avoid death.

In plants, there are two major categories of pathogen response—systemic acquired resistance (SAR) and the hypersensitive response (HR) (Winter, 2014); (Mur, 2008). SAR is the development of an enhanced resistance to a pathogen due to a prior encounter. It results in faster and more effective defensive action within the plant upon a second-time pathogen attack (Conrath, 2006). The SAR defense response is initiated throughout the entire organism if a local response has been started and if it is the second time the plant has been exposed to the specific pathogen. The plant recognizes specific pathogens via pattern-recognition receptors—receptors that identify pathogen molecular markers. The initiation of SAR in response to pathogen identification vis a pattern-recognition receptor is dependent upon signaling by salicylic acid (SA)—a plant hormones synthesized by the plant (Qing-Ming, 2015). Specifically, accumulation of SA causes activation of the nonexpressor of pathogenesis and related genes1 (NPR1). Monomeric NPR1 goes into the nucleus of the plant cells and causes expression of antimicrobial genes, ultimately causes a systemic defense response (Mach, 2015). Although accumulation of SA results in the beginning of the SAR, this can also trigger a localized HR response (Hartman, 2016).

HR acts as an immediate defensive response often in the form of self-initiated cell death at the site of infection (Govrin, 2000). By killing cells that surround the pathogen, the plant can isolate the infection and prevent more of itself from becoming infected. It is very important that HR is properly regulated by the cell because errors in regulation could result in the killing of healthy, uninfected cells.

Vital to the HR in plants are reactive oxygen species (ROS). ROS are chemically reactive and toxic to cells and result in significant damage (Bailey-Serres, 2006). When

localized to an infected area, ROS are advantageous members of the HR, often resulting in the death and containment of the pathogen. This localization of ROS to a pathogen is often referred to as an oxidative burst (Wojtaszek, 1997). Often, ROS are stored and accumulated in vacuoles, so that they can be quickly and efficiently released when necessary. One enzyme that serves to generate ROS in the form of hydrogen peroxide and create oxidative bursts in plants is peroxidase (Zhang, 2004). Because of its toxic nature, plants also utilize the enzyme called catalase to convert hydrogen peroxide into water. This contains and controls the ROS and rids of the cell of ROS once the HR is over (Caverzan, 2012). It is expected that these two enzymes— peroxidase and catalase—work together and in somewhat of a cycle during HR.

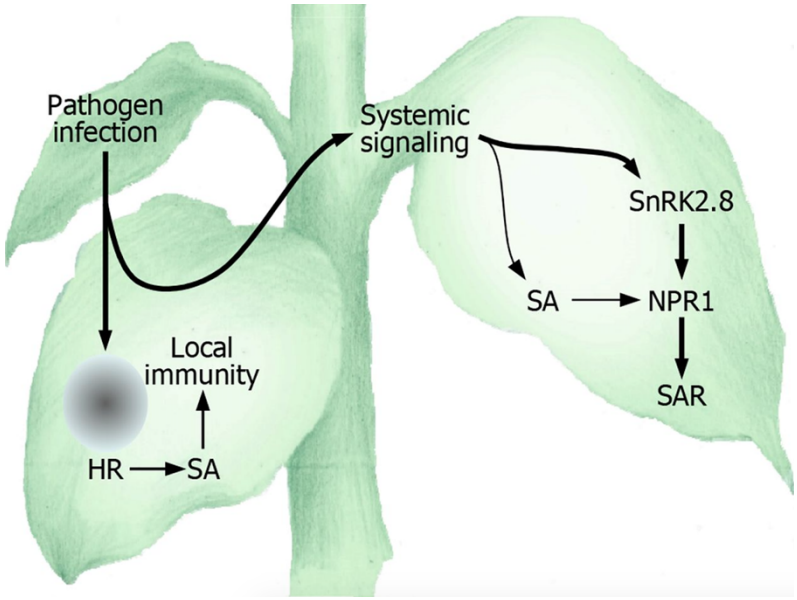

Figure 2. Systemic acquired resistance and the hypersensitive response in plants. Pathogen infection at a localized site on the plant leaf can stimulate a HR (local immune response). Accumulation of SA turns on NPR1 which causes expression of antimicrobial genes leading to a systemic response, SAR. (Lee, 2015)

Vascular and Nonvascular Plants

Plants are divided into two major types: vascular and non-vascular. This divide is based on the presence of a vascular system and ultimately is a major divide between the higher and lower plants (Figure 3). Vascular plants, or higher plants, have specialized tissues for transporting water and nutrients and often grow tall, while nonvascular, or lower plants do not have these specialized tissues and grow on the surface of trees or the ground (Panawala, 2017). Much is known about the immune system and genetics of vascular plants because these plants are often utilized as crops and are important for the economy. Extensive research has been conducted on these plants, like corn, in order to protect them from pathogens and strengthen their defense systems. Significantly less is known about the lower plants, however, recently there have been some major discoveries in these species' mechanisms.

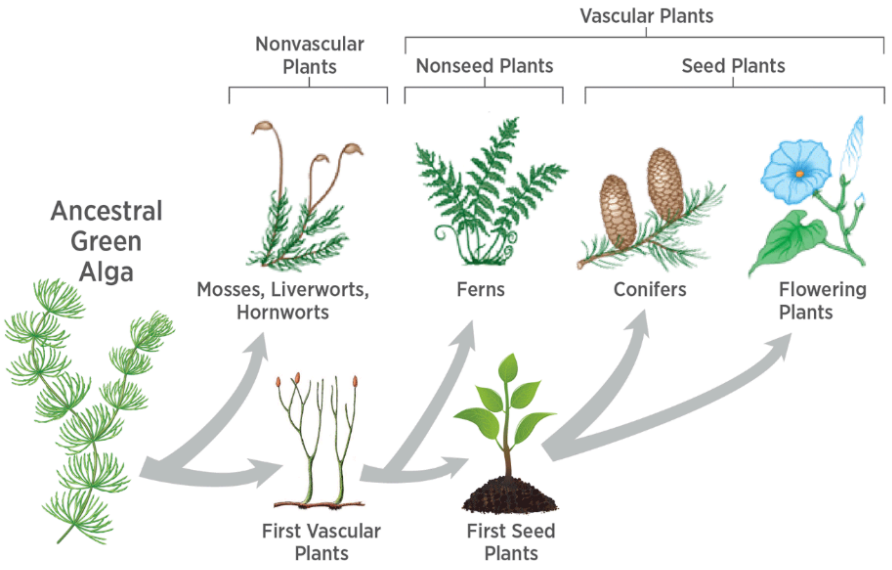

Figure 3. Vascular and Nonvascular plants. This figure highlights the divide between vascular and nonvascular plants. Ancestral green alga is thought to have had an evolutionary split between nonvascular plants (mosses, liverworts, and hornworts) and the first vascular plants. Vascular plants have since divided into those with seeds and those without (doTERRA International, 2019).

Physcomitrella patens

Although much is known about the immune system of vascular, higher plants, there is still much unknown about the immune systems of nonvascular lower plants, like moss. It is suspected, however, that there are many similarities between the higher and lower plant immune systems, as they are evolutionarily related. The moss species, *Physcomitrella patens* is often utilized as a model species for lower plant immune system research because it is easily cultured, and its entire genome has been sequenced (Figure 4) (Cove, 2009).

New research has found many similarities in the HR between vascular and nonvascular plants (Oliver, 2009). Peroxidase, the enzyme responsible for creating ROS, has been detected in response to fungal elicitors in the nonvascular moss *P. patens*, further bridging a knowledge gap between the vascular and nonvascular plants. Two identical peroxidase genes, *Prx34A* and *Prx34B*, have also been identified in *P. patens* (Lehtonen, 2009). Catalase, the enzyme responsible for the breakdown of ROS, is expected to be present in *P. patens* due to its similarities with vascular plants, however, less is known about this enzyme and no specific catalase gene has been identified in the moss.

Current research has identified peroxidase in other mosses and is also working to quantify the amount of and time of release of the peroxidase enzyme in response to fungal elicitors. A change in gene expression of peroxidase and catalase following immediate release of peroxidase would be expected, as the organism would need to replenish both enzyme supplies. From a genetic and evolutionary perspective, it would be

especially compelling to understand and characterize these changes in the gene expression of peroxidase and catalase in response to fungal elicitors in *P. patens*. Continued research of nonvascular plants, especially in regard to gene expression, may result in findings that are applicable to both nonvascular and vascular plants and therefore lead to a broader and more complete understanding of the evolution of plant defense mechanisms.

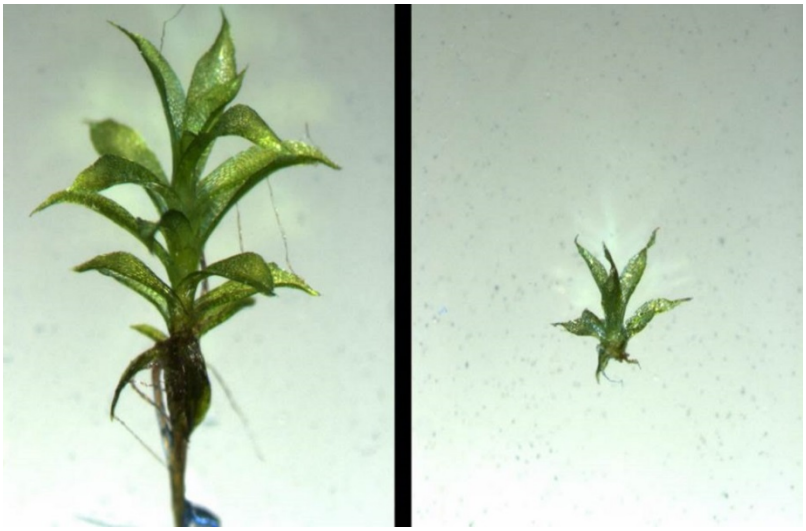

Figure 4. Photo of *Physcomitrella patens*. This is an example micrograph photo of the model moss species, *Physcomitrella patens* obtained from Wageningen University. (Wageningen University, 2017)

Experiment and Hypothesis

The central objective of this study is to identify and characterize the roles of peroxidase and catalase in HR. This will be accomplished by determining changes in gene expression of peroxidase and catalase in response to fungal pathogen elicitors over 48-hour periods post-treatment. The changes in gene expression are of importance because they ultimately control cellular levels of the enzymes and therefore the HR altogether. As genes become upregulated, production of their enzymes increases, therefore increasing the enzyme's functionality and response. For example, an upregulation of the peroxidase gene would correspond to increased peroxidase production, while an upregulation of the catalase gene should correspond to increased catalase production. By observing and analyzing these changes in gene expression, the timing of the cycling of peroxidase and catalase throughout the HR can be determined.

Found changes in gene expression of peroxidase and catalase will also serve as important genetic comparisons to vascular plants. Because this research has been conducted in vascular plants, vascular plants have evolved from nonvascular plants, and the HR has recently been discovered in nonvascular plants, it is suspected that the HR will be similar in the two plant groups. This analysis will allow for a genetic and evolutionary evaluation between vascular and nonvascular plants and ultimately it will provide a better understanding of the evolution of plant defense systems.

I hypothesize that changes in gene expression will be delayed compared to the immediate peroxide release and will be seen within an hour of pathogen exposure. Upon pathogen exposure, the peroxide is immediately used. When the plant's stores of peroxide

begin to run low, the plant will need to replenish its supply. The gene expression of peroxidase will increase at this time, which I suspected will be approximately 30 minutes after exposure to pathogen.

I also hypothesize that the peroxidase gene will be upregulated before the catalase gene and will also be turned off before the catalase gene in order to maintain appropriate levels of peroxide. This hypothesis supports the idea that the peroxidase and catalase levels cycle during the HR. After an increase in peroxidase, an increase in catalase should ensue in order to break down the peroxide and prevent its toxicity from traveling to healthy parts of the plants. The phenotypic cycling of peroxidase and catalase levels should be mimicked by the genetic regulation of the two enzymes, but with a slight time delay.

Methods

Maintenance of *P. patens* Cultures

The moss species *P. patens* was used in this experiment because it is the model moss organism and can be grown on culture easily and sterilely. *P. patens* was grown and cultured in on sterile BCD agar medium (Cove, 2009). BCD stock was made in 800mL increments with 8mL of stock B solution (25g $MgSO_4.7H_2O$ filled to 1L with diluted water), 8mL of stock C solution (25g KH_2PO_4 filled to 1L with diluted water), 8mL of stock D solution (101g KNO_3, 1.25g $FeSO_4.7H_2$ filled to 1L with diluted water), 3.84 g of agar gel, and 0.739 g of ammonium tartrate. The solution was then microwaved for 7-8 minutes and swirled until the agar dissolved. It was then autoclaved at 121° C and 15 psi for 25 minutes and 800ul of 1M autoclave sterilized $CaCl_2$ was added to the solution after autoclave. This final solution was poured into petri dishes and allowed to solidify before used for culture.

P. patens was placed onto BCD plates using utensils sterilized by 95% EtOH and heated by flame for approximately 30 seconds. About 7-8 *P. patens* colonies were placed onto each dish. The *P. patens* was stored under fluorescent lights that were timed to mimic typical light-dark phase (16 hours lights on, 8 hours lights off).

Identification of Gene and Primer Design

Previous studies have identified two identical peroxidase genes, *Prx34A* and *Prx34B*, in *P. patens* (Lehtonen, 2009). Using the primer design program associated with the National Center for Biotechnology Information (NCBI), forward and reverse primers

associated with the peroxidase gene were designed and ordered to Butler University (Table 1).

Using the Basic Local Alignment Search Tool (BLAST) associated with NCBI, the *P. patens* genome was analyzed and compared to other plants, allowing for putative catalase homolog identification. These homologs were used to design forward and reverse primers associated with the catalase gene and primers were ordered to Butler University (Table 1).

Rubisco forward and reverse primers were also ordered. This primer served as a control gene throughout the experiment because this gene is responsible for photosynthetic fixation of carbon in plant chloroplasts and therefore does not vary (Karcher, 1995)

Table 1. NCBI accession numbers and primer sequences for *P. patens* genes of interest.

GENE NAME	ACCESSION NUMBER	PRIMER SEQUENCE
Rubisco	AB1020708.1	F:CTGCATTGCCCTTGCGATTC R:GATGACGCCACAGTCACAGA
Peroxidase isozyme 1-like (Prx1)	XM_024508862.1	F:CAATACGCTACTCGCGACTCTGT R:CGTCTCTTCGACCGCCATA
Catalase isozyme 1-like (Cat1)	XM_024519324	F:AAGATGTACACGCGGGAAGG R:CTTGCACGTTCGATTTGGGG
Catalase isozyme 2-like (Cat2a)	XM_024505089.1	F:GGAGACCGCAGTCGATGAGT R:CGGAGAGGCCTCAATATGGG
Catalase isozyme 2-like (Cat2b)	XM_024546406.1	F:AGGCATTGTGCTCATTCAGGA R:ACCGGACCTCTAGGACCAAC

Fungal elicitor exposure

P. patens samples were treated with the fungal elicitor, beta-glucan. Beta-glucan is a fundamental component of fungal cell walls and elicits an immune reaction in *P. patens* (Fesel, 2016). 50mg of beta-glucan and 10mL of H_2O were combined to make a 5mg/mL beta-glucan stock solution. Plants samples were treated with 50uL of 0.5mg/mL beta-glucan by diluting 100uL of stock solution with 900uL of H_2O.

P. patens samples were transferred to new plates, four samples per plate, that were divided into four sections. Samples were treated with 50uL of 0.5mg/mL beta-glucan either 30 minutes, 1 hour, 2 hours, 6 hours, 24 hours, or 48 hours before gene extraction. These treatments were always conducted at the same time of day in order to avoid confounding circadian rhythm issues.

RNA extraction

After the beta-glucan treatment, *P. patens* tissue was frozen in liquid nitrogen and then RNA was extracted using a Qiagen RNeasy Plant Mini Kit. Extractions were quantified using a BioTek microplate reader and Gen 5 software. RNA was frozen at -76^{o} C until qRT-PCR was performed.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed on RNA extraction in order to analyze gene expression levels at the various beta-glucan exposure times. qRT-PCR was performed using an Applied Biosystems kit. Forward and reverse primers used were: *Rubisco* (control), *Prx1*, *Cat1*, *Cat2a*, and *Cat2b*. Each time interval (0hr, 30minutes, 1hour, 2hours, 6hours,

24hours) underwent qRT-PCR analysis of all five primers used. Three replicates of each trial were performed and averages of the three replicates were used in analysis. Expression data was normalized using the results of the *Rubisco* gene expression.

Analysis of gene expression

Relative amounts of mRNA per each of the genes (*Rubisco*, *Per1*, *Cat1*, *Cat2a*, *Cat2b*) were compared to themselves across the 24-hour time interval and their trends were observed. T-tests and ANOVAs were used to statically analyze differences in gene expression between peroxidase and catalase.

Results

Gene Identification

A catalase protein from Arabidopsis (NP_195235) was identified in NCBI. Protein BLAST was run using the Arabidopsis protein to identify three putative catalase homologs in the *P. patens* genome. All three catalase homologs had e-values of 0, indicating that the amino acid sequence is highly conserved between Arabidopsis and *P. patens*. Primers were designed for all three *catalase* genes, as well as for *peroxidase* and *Rubisco* genes (Table 2).

Table 2. Accession numbers and primer sequences for identified genes in *P. patens*.

GENE NAME	ACCESSION NUMBER	PRIMER SEQUENCE
Rubisco	AB1020708.1	F:CTGCATTGCCCTTGCGATTC R:GATGACGCCACAGTCACAGA
Peroxidase isozyme 1-like (Prx1)	XM_024508862.1	F:CAATACGCTACTCGCGACTCTGT R:CGTCTCTTCGACCGCCATA
Catalase isozyme 1-like (Cat1)	XM_024519324	F:AAGATGTACACGCGGGAAGG R:CTTGCACGTTCGATTTGGGG
Catalase isozyme 2-like (Cat2a)	XM_024505089.1	F:GGAGACCGCAGTCGATGAGT R:CGGAGAGGCCTCAATATGGG
Catalase isozyme 2-like (Cat2b)	XM_024546406.1	F:AGGCATTGTGCTCATTCAGGA R:ACCGGACCTCTAGGACCAAC

Melt Curve Analysis

Following 40 rounds of qRT-PCR**,** a melt curve analysis was done on the amplification products to determine if the primers are specific for their one intended gene of interest. One peak per gene indicates that the primers only amplified one product, thus

confirming that the primers are specific. Multiple peaks during the melt curve analysis would indicate that the primers were not specific for the gene of interest, but instead multiple targets were amplified. All primers used (*Rubisco*, *Per1*, *Cat1*, *Cat2a*, *Cat2b*) showed one peak and therefore all primers were specific to their gene of interest (Figure 5).

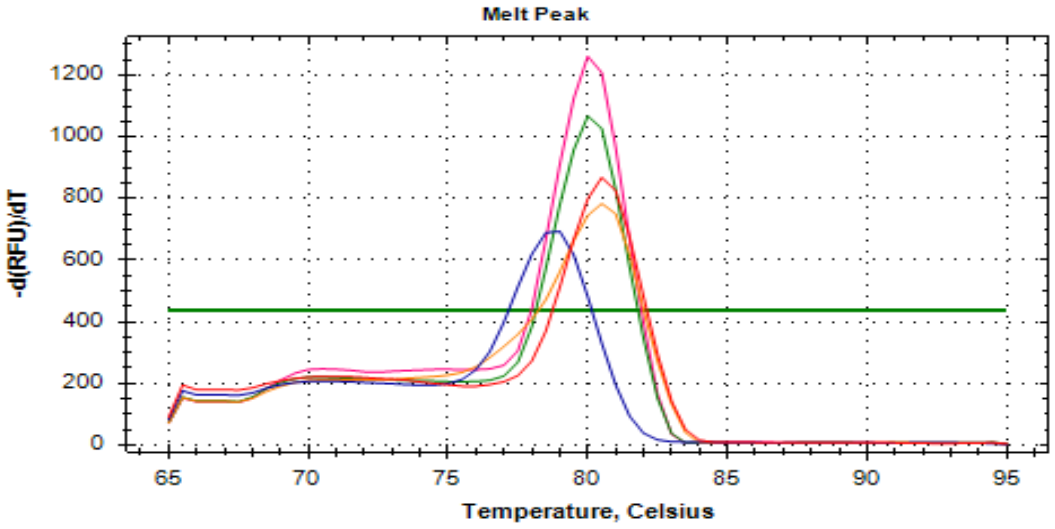

Figure 5. Melt curve analysis for primers designed for genes of interest in *P. patens*. The x axis represents change in temperature and the y axis represents change in fluorescence. Red=*Rubisco*, Blue=*Prx1*, Orange=*Cat1*, Green=*Cat2a*, Pink=*Cat2b*

Initial Gene Expression Studies

Initial gene expression studies were conducted in the summer of 2018. These studies attempted to identify changes in the expression of the peroxidase and catalase genes after 15 minutes, 30 minutes, 1 hour, 2 hours, 6 hours, 24 hours, and 48 hours of fungal pathogen exposure. Although numerous trials were conducted, no significant patterns or similarities were found (Figures 6-9). For example, in some trials, the *Prx1* gene decreased in expression over the first 24 hours after elicitor exposure (Figure 6a), whereas in other trials the expression of this gene increased over the first 24 hours after

exposure (Figure 6b). These inconsistencies occurred for the other three genes in question (Figure 7a,b – 9a,b). The average fold change in gene expression per time period for each gene was calculated and no significant patterns were found in any gene (Figures 6c-9c). The results of these initial gene expression studies were therefore inconsistent.

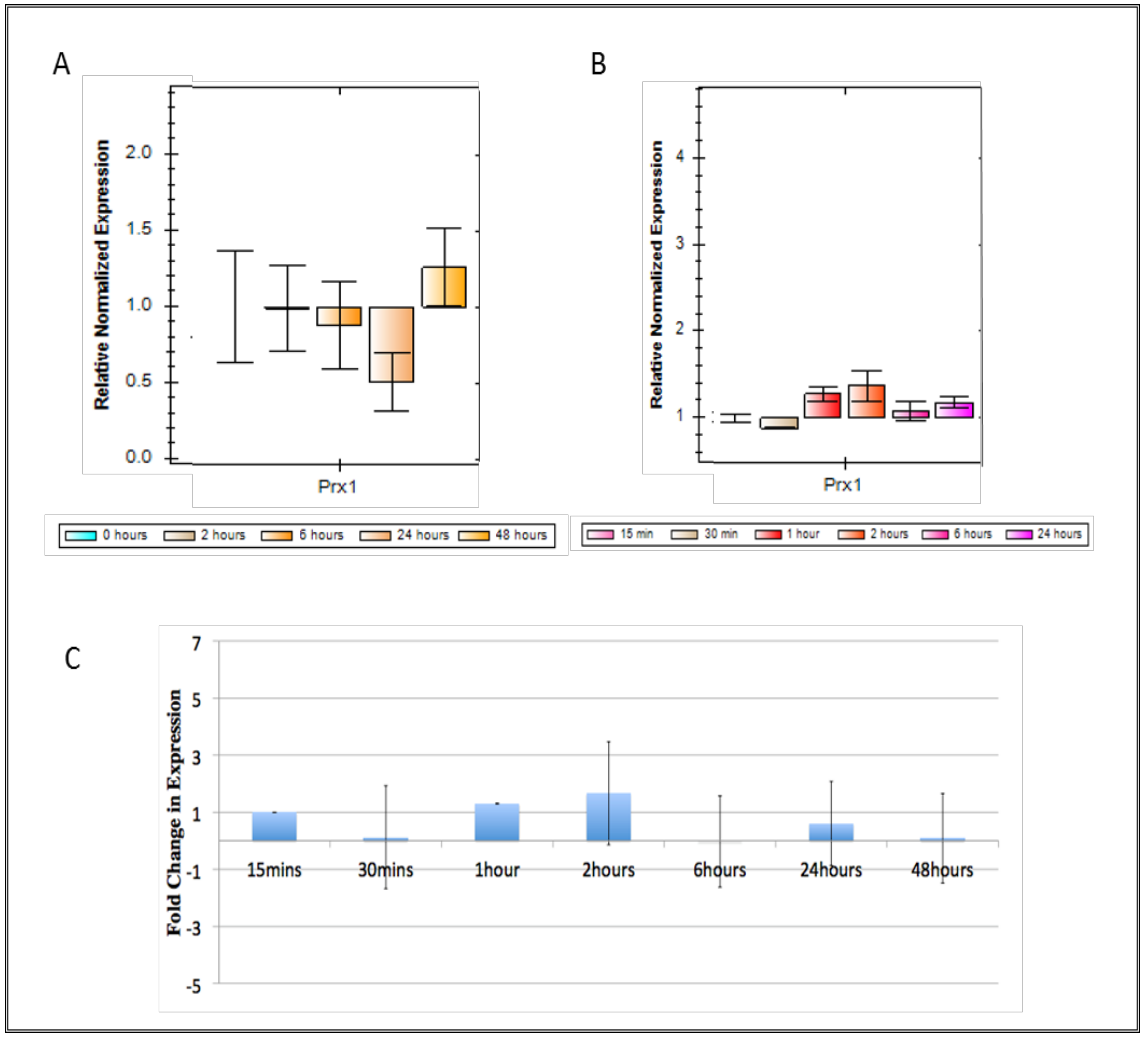

Figure 6. Gene expression patterns in *Prx1 peroxidase* gene of *P. patens*. Figures 6A and 6B show representative gene expression patterns of the *Prx1 peroxidase* gene from two separate trials. Figure 6A shows data after 0, 2, 6, 24, and 48 hours of pathogen exposure. *Prx1* shows a 0.5 decline in gene expression at 24 hours with an increase to approximately 1.3 increase at 48 hours, relative to the control (*Rubisco*). Figure 6B shows data after 15 min, 30, 1, 2, 6, and 24 hours of pathogen exposure. *Prx1* shows a 0.2 increase at 2 hours with a decrease back to 1.1 at 24 hours, relative to control. No comparable patterns are noticed between the *Prx1* gene in the two separate trials. Figure 6C shows the average fold change in *Prx1* gene expression of all trials conducted in the initial studies. No trends are noticed. Error bars represent standard deviation from the means.

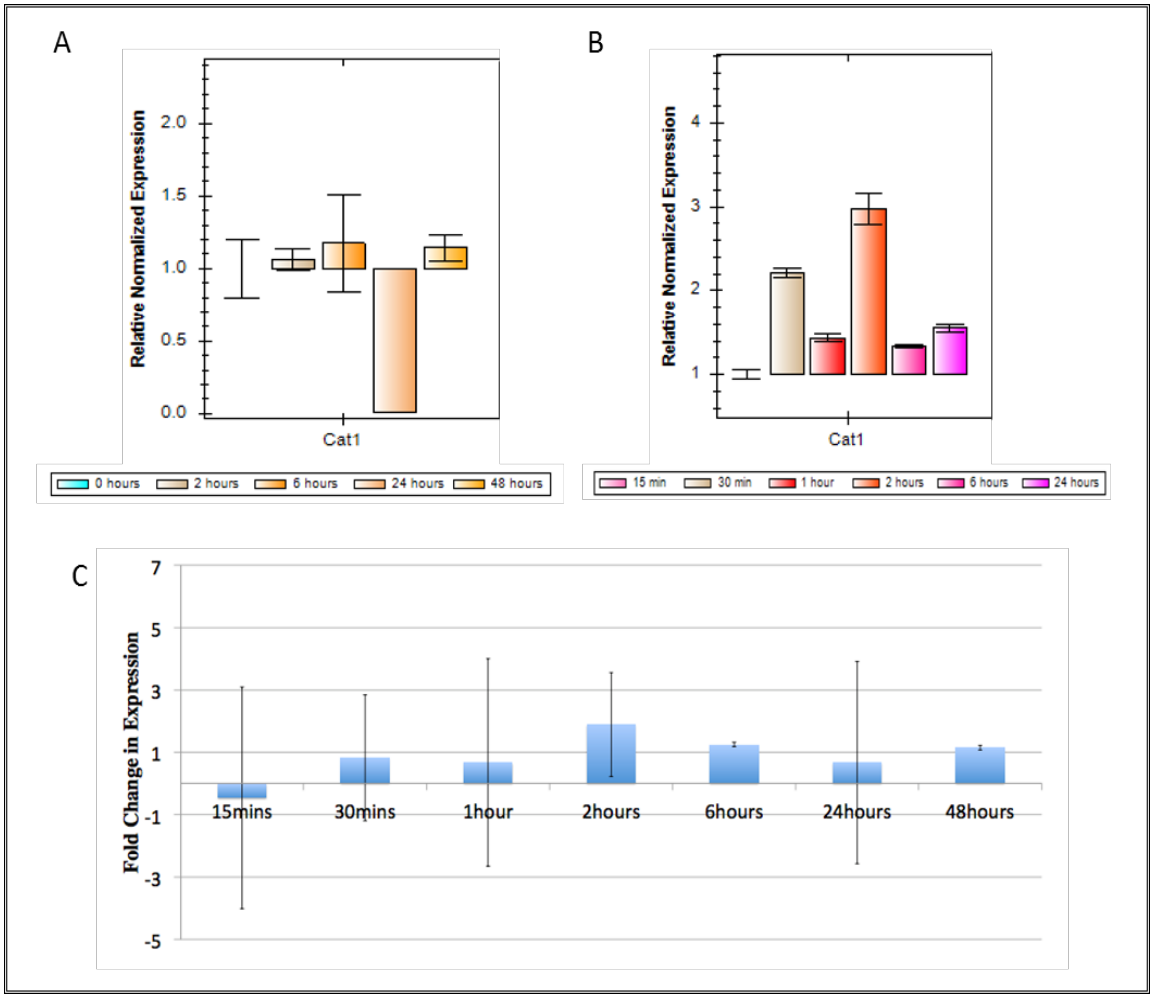

Figure 7. Gene expression patterns in *Cat1 catalase* gene of *P. patens*. Figures 7A and 7B show representative gene expression patterns of the *Cat1 catalase* gene from two separate trials. Figure 7A shows data after 0, 2, 6, 24, and 48 hours of pathogen exposure. *Cat1* shows a 1.0 decline in gene expression at 24 hours with an increase to approximately 1.3 increase at 48 hours, relative to the control (*Rubisco*). Figure 7B shows data after 15 min, 30, 1, 2, 6, and 24 hours of pathogen exposure. *Cat1* shows a 3.0 increase at 2 hours with a decrease back to 1.2 at 6 hours, relative to control. No comparable patterns are noticed between the *Cat1* gene in the two separate trials. Figure 7C shows the average fold change in *Cat1* gene expression of all trials conducted in the initial studies. No trends are noticed. Error bars represent standard deviation from the means.

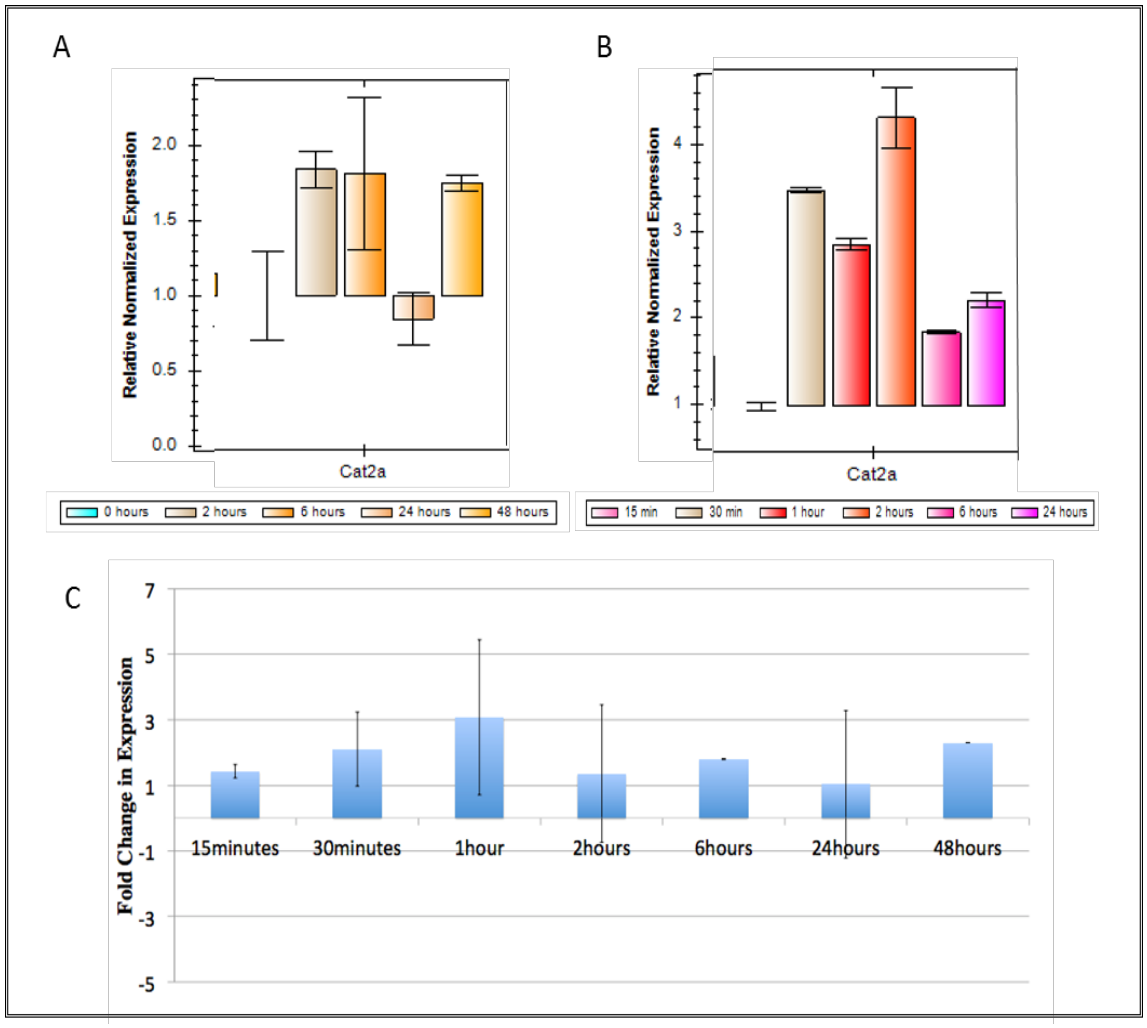

Figure 8. Gene expression patterns in *Cat2a catalase* gene of *P. patens*. Figures 8A and 8B show representative gene expression patterns of the *Cat2a catalase* gene from two separate trials. Figure 8A shows data after 0, 2, 6, 24, and 48 hours of pathogen exposure. *Cat2a* shows a 1.8 increase in gene expression at 2 and 6 hours with a decrease to approximately 0.8 at 24 hours, relative to the control (*Rubisco*). Figure 8B shows data after 15 min, 30, 1, 2, 6, and 24 hours of pathogen exposure. *Cat2a* shows a 3.3 increase at 30 mins with a decrease back to 1.8 at 6 hours, relative to control. No comparable patterns are noticed between the *Cat2a* gene in the two separate trials. Figure 8C shows the average fold change in *Cat2a* gene expression of all trials conducted in the initial studies. No trends are noticed. Error bars represent standard deviation from the means.

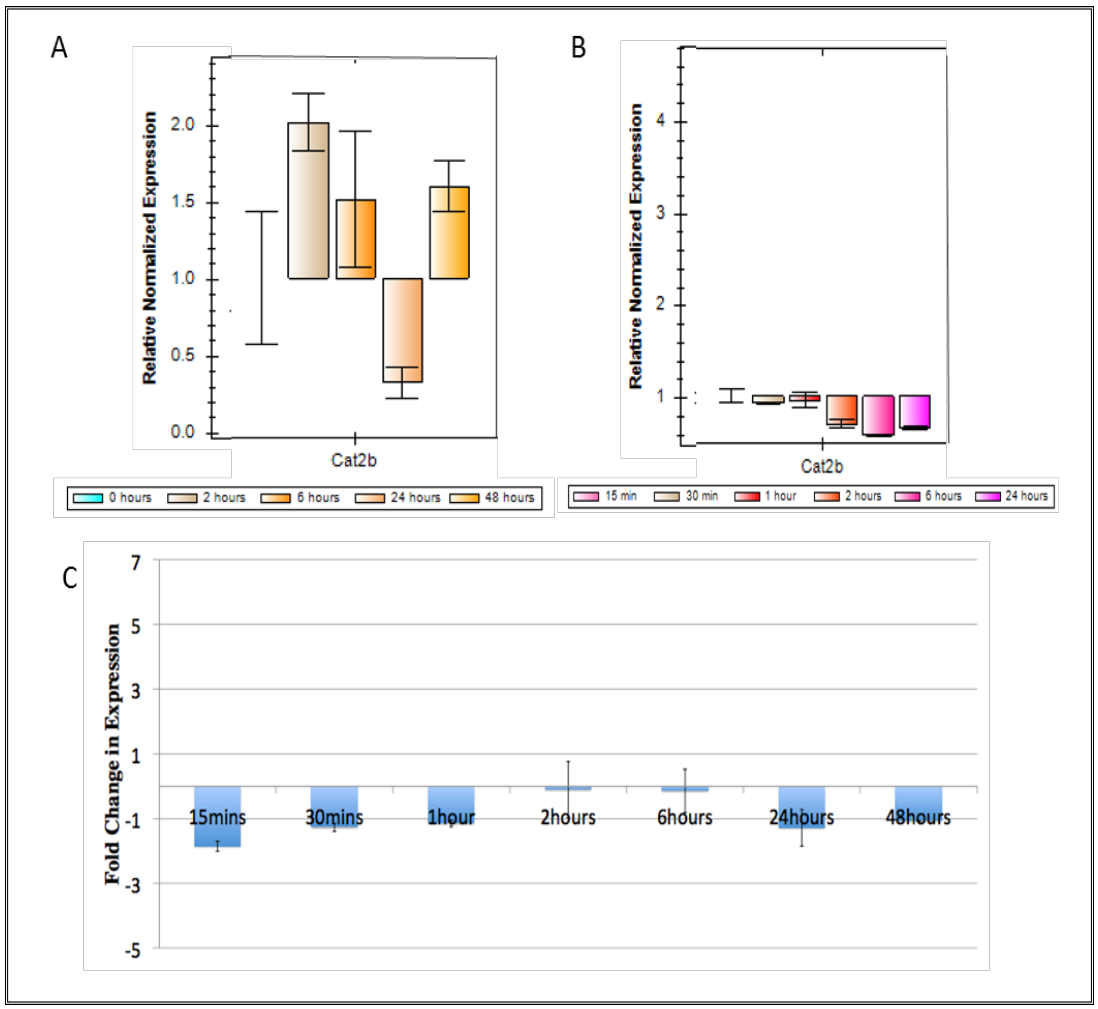

Figure 9. Gene expression patterns in *Cat2b catalase* gene of *P. patens*. Figures 9A and 9B show representative gene expression patterns of the *Cat2b catalase* gene from two separate trials. Figure 9A shows data after 0, 2, 6, 24, and 48 hours of pathogen exposure. *Cat2b* shows a 2.0 increase in gene expression at 2 with a decrease to approximately 0.3 at 24 hours, relative to the control (*Rubisco*). Figure 9B shows data after 15 min, 30, 1, 2, 6, and 24 hours of pathogen exposure. *Cat2b* shows almost no gene expression change throughout the 24 hours. No comparable patterns are noticed between the *Cat2b* gene in the two separate trials. Figure 9C shows the average fold change in *Cat2b* gene expression of all trials conducted in the initial studies. No trends are noticed. Error bars represent standard deviation from the means.

We hypothesize that circadian rhythm disturbances via overhead laboratory lighting served as the underlying cause of the gene expression inconsistencies. Throughout the initial gene expression studies, no standard procedure of overhead

laboratory room lighting was used. Moss received timed standard lighting directly above their storage shelves, however, the overhead laboratory lights were sometimes left on throughout both the day and night. We predict that the inconsistent lighting practices utilized within the laboratory had a stressful affect on the circadian rhythms of the moss. Because light acts as the circadian rhythm time cue, leaving the lights on or off for irregular amounts of time may disrupt entrainment rhythms and disturb or weaken many physiological systems, such as the immune system. Proper laboratory light would attempt to align moss circadian rhythms with the 24-hour sun and moon cycle using a typical 16-h light 8-h dark lighting schedule. The exact mechanism of the effect of the irregular light time cue on the circadian rhythms of *P. patens* is not fully understood and future experiments will seek to better understand this process.

Final Gene Expression Studies

Before beginning the final gene expression studies, *P. patens* was placed on a 16-h light 8-h dark overhead room lighting schedule in addition to their timed 16-h light 8-h dark shelf lighting. This was done for 2 months before any new data was taken in order to ensure that circadian rhythms were entrained to a normal 24-h cycle. These final gene expression studies once again attempted to detect any changes in expression of the peroxidase and catalase genes after 30 minutes, 1 hour, 2 hours, 6 hours, and 24 hours of fungal pathogen exposure. Data from the multiple trials was more consistent and showed noticeable trends in gene expression (Figure 10). Therefore, we predict that this new overhead lighting schedule was successful at entraining rhythms.

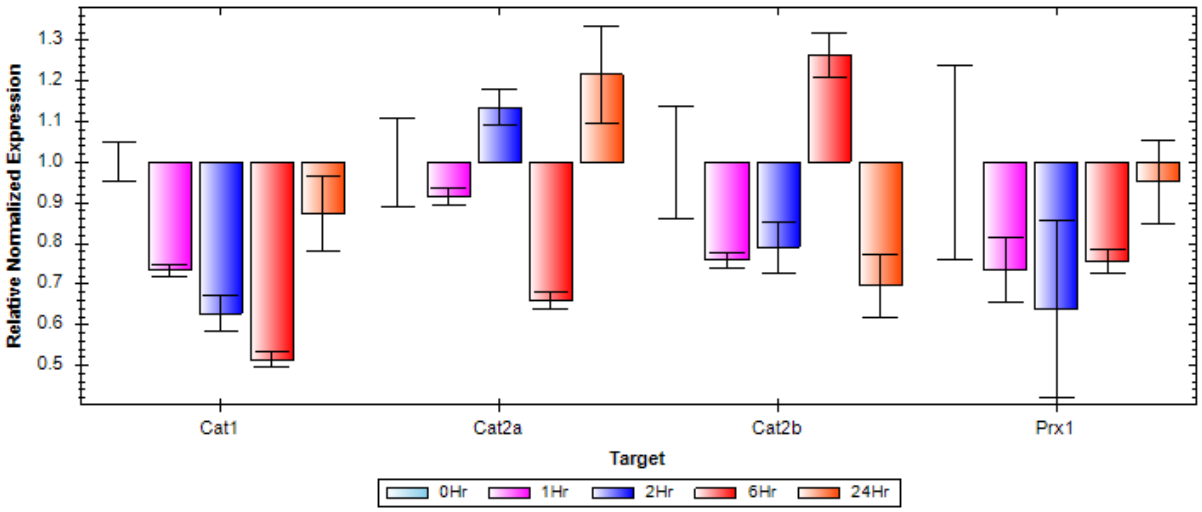

Figure 10. Gene expression trends over 24 hours in *peroxidase* and *catalase* genes in *P. patens*. This is representative gene expression data of *Cat1*, *Cat2a*, *Cat2b*, and *Prx1* taken from one trial during the final gene expression studies. Data was recorded at 0, 1, 2, 6, and 24 hours after pathogen exposure. *Cat1* shows a progressive decrease in expression from 0-6 hours. *Cat2a* shows a sharp drop in expression after 6 hours. *Cat2b* shows a sharp increase in expression after 6 hours. *Prx1* shows decrease in expression from 0-6 hours. Data was taken relative to the *Rubisco* control.

These patterns suggest that *catalase* genes have an overall decrease from 0-6 hours after pathogen exposure. Immediately after pathogen exposure (potentially from 0-6 hours), it is likely that moss will down regulate *catalase* gene to ensure that *catalase* does not remove peroxide from the system. *Cat2a* and *Cat2b* show large increases in expression at 24 and 6 hours respectively (Figure 10). At these times, the plant would benefit from upregulating *catalase* in an attempt to rid the plant of peroxide after the pathogen has been destroy.

The results of the *peroxidase* gene are more unclear than that of *catalase*. *Prx1* shows significant decreases in gene expression at 0-6 hours and then increases back to 1 at 24 hours (Figure 10). Immediately after pathogen exposure, it would be logical that the

plant would upregulate *Prx1* in an attempt to make more peroxide and destroy the pathogen. Therefore, no hypothesis can be made about the happenings of the *Prx1* gene at this time.

Conclusions

Although the results of the initial gene expression studies were inconsistent, some noticeable trends were observed in in the final gene expression studies. Gene expression of *peroxidase* and *catalase* in the final studies which controlled for circadian rhythm disturbances, showed patterns that likely correlate with the physiological happenings of the infected *P. patens*. Specifically, *catalase* genes showed large decreases in expression from 0-6 hours, which may indicate the moss's need for defensive peroxide at these times. More trials will seek to both further this hypothesis and delve further into physiological and mechanistic specifics. Additionally, this study demonstrated the affect of circadian rhythm disturbances on the moss immune system. It is likely that unregulated overhead lighting resulted in the numerous inconsistencies found in the initial gene expression studies. Future studies will attempt to regulate this disturbance and to understand the mechanisms of this disturbance.

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Figures

Figure 1: Pearson Education 2010.

Figure 2: Lee H, Park Y, Seo P, Kim J, Sim H, *et al*. 2015. Systemic immunity requires SnRK2.8-mediated nuclear import of NPR1 in *Arabidopsis*. *The Plant Cell* 27: 3425-3438.

Figure 3: Physcomitrella Photo Retrieved from Wageningen University, 2017: https://www.wur.nl/en/newsarticle/Better-understanding-of-plant-cell-biology-via-research-into-the-moss-Physcomitrella-patens.htm.

Figure 4: Vascular and nonvascular plants Photo Retreived from doTERRA International, 2019.
https://www.doterra.com/US/en/botany-living-organism-plant-evolution.