2015

The Role of RNAi in Downregulation of Physcomitrella Patens Defense Genes and the Preliminary Steps of PEG Mediated Transformation of Mosses

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For Honors Program use:

Level of Honors conferred: University: Summa cum laude

Departmental: High honors in Biology

The Role of RNAi in Downregulation of *Physcomitrella patens* Defense Genes and the Preliminary Steps of PEG Mediated Transformation of Mosses

A Thesis

Presented to the Department of Biological Sciences

College of Liberal Arts and Science

And

The Honors Program

of

Butler University

In Partial Fulfilment of Requirement for

Graduation Honors

Adam Jeffrey Beswick

March 18, 2015

Dedication

This thesis is dedicated to all family and friends, especially my parents, Jeff and Tammy Beswick. I would also like to dedicate this to both Dr. Philip Villani and Dr. Nat Hauck for their instrumental guidance and support the last four years.

Acknowledgements

I would like to thank Dr. Philip Villani for the generous amounts of time, guidance, and resources he has given to me to make this thesis possible.

Dr. Nat Hauck for his irreplaceable knowledge and ideas.

The Department of Biological Sciences for their resources and support.

The Butler University Honors Program for their unwavering assistance.

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Abstract

When attacked by a pathogen, the moss *Physcomitrella patens* will undergo both a hypersensitive response (HR) as well as a systematic acquired resistance (SAR) response. The SAR response turns on genes that ready the plant for future pathogen attacks. The hormone jasmonic acid (JA) is one of the products of SAR response in *P. patens*. In this study interference RNA (RNAi) was used to decrease gene expression of the allene oxide cyclase (AOC) gene, a key gene in the production pathway of JA. Using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), RNAi was found to significantly lower AOC gene levels in mosses, both before and after infection with the fungal pathogen *Pythium irregulare*. Furthermore RNAi was tested to see if it created a phenotypic difference when infecting *P. patens*. Lastly, the preliminary steps in a polyethylene glycol (PEG) mediated transformation were started by attempting to isolate single celled protoplasts in the mosses *Mnium cuspidatum* and *Ceratadon purpureus*. This transformation would cause a permanent knockout of a gene, as opposed to the short term decrease in expression achieved by RNAi.

Abbreviations

MAMP: Microbe Associated Molecular Patterns

HR: Hypersensitive Response

PCD: Programmed Cell Death

ROS: Reactive Oxygen Species

SAR: Systematic Aquired Resistance

JA: Jasmonic Acid

LOX: 13-lipoxygenase

RNAi: Interference RNA

PEG: Polyethylene Glycol

AOC: Allene Oxide Cyclase

siRNA: Small Interfering RNA

Introduction

Defense Mechanisms

All organisms are susceptible to predation, parasitism and disease. Each unique organism has an evolutionary background in which its ancestors devoted exorbitant amounts of time and energy into maintaining good health. The goal of life, from an evolutionary standpoint, is to survive and reproduce; to pass genes on to the next generation. Without good standing health, reproduction is greatly hindered, and this is precisely why organisms devote so much to defense mechanisms.

Some of the most commonly recognized defense strategies are employed by animals. For example, the quills of a porcupine which are an extremely effective physical defense mechanism. Chemical defenses are also common in animals, one of the most widely recognized and feared being that of the Striped Skunk. However some animals have even more intriguing defense strategies. Such as the Blast Ant which can commit suicide by contracting its abdominal muscles, rupturing its gaster and mandibular glands, releasing an explosion of sticky, irritating liquid. This ultimate sacrifice is often enough to scare away predators and can save many other ants in the colony (Jones et al. 2004). While people may believe animals to have the more exciting defense systems, plants have equally effective strategies.

Plants harbor similar mechanical and chemical defenses seen in animals. Many tree and shrub species have thorns or prickles which work to deter herbivory. Similar, in herbivory defense, many plants harbor toxic chemicals that prevent animals from eating their foliage. While visible defense systems get major recognition for the work they do, it is the microscopic defenses of plants which allow them to survive just as much as the macroscopic ones. Defense against pathogens, diseases, and parasites are all vital to maintaining health and plants have adapted successful techniques in doing so.

Pathogen-host Relationships

To maintain good health organisms need to have successful defenses against microorganisms. Commonly known as pathogens, they can come in the form of bacteria, viruses, fungi or parasites. While many microbes cause no harm at all to their host, pathogens do. One extreme example is the parasite *Toxoplasma gondii*. It infiltrates the body of a rat or bird and makes its way to the brain where it alters brain function which causes the animals to no longer fear feline predators. This behavior change is to the parasites advantage because in order to continue its lifecycle it must be fully digested by a second animal, i.e. a cat. While not all pathogens are this brutal, many do seek to kill their host by altering their physiology (Dubey and Jones, 2008). Without proper defenses plants and animals would not survive.

However not all microorganisms are pathogens. In fact without microbes, humans and other living organisms could not exist. There are three distinct types of relationships that microbes have with their hosts: commensalism, mutualism, and parasitism. All microorganisms that live on or within other organisms with no effect on the hosts are commensalism relationships. The host simply provides a place for the microbe to survive and thrive, without receiving costs or benefits. Mutualistic microbe-host relationships are ones in which both parties are receiving benefits. The microorganism gets a place to live and it provides some type of service to the host; fends off harmful pathogens, breaks down food, etc. An example of this is the fungal mycorrhizae that have a symbiotic relationship with the root nodules in many plants. The fungi fix nitrogen to be used by the plants, and in turn the plants provide nutrients and a place to live for the fungi. The parasitic relationship, is not so friendly.

Pathogens are parasitic. This relationship benefits the parasite and comes at a cost for the host. Often times pathogens will only weaken its host, not kill it. This way the parasite can

be sustained for a longer period of time without looking for a new host. However in some cases the parasite will seek to kill, or the host will simply die after being in a weakened state for an extended period of time. Dodder is a parasitic plant that tightly wraps itself around a host plant and uses its haustoria adaptation to penetrate the host's stem, and siphon nutrients.

The potentially deadly impact of pathogens on their hosts is why organisms must have systems in place to prevent infection. Dodder, as mentioned above, is a quick spreading and deadly plant pathogen that can affect many key agricultural crops including: onions, tomatoes, potatoes, and cotton. Unless preventative measures are taken, 90% and even up to 100% of these crops can be lost (Tanase et al., 2012). We as humans are equally susceptible and have our own defense systems as well.

Human Pathogen Interaction

Some of the most dangerous diseases in human history, including malaria, smallpox and tuberculosis are all pathogenic outbreaks (Karlsson et al, 2014). Because of their prevalence in human society pathogens are an important part of public health, economics, and agriculture. Millions of dollars each year are spent in the medical fields to create vaccinations or medicines to help patients with diseases. Because of their direct relationship with humans it is important to continue to study and learn more about pathogen-host, particularly, pathogen-human relationships.

However, microbes have also affected humans indirectly. As far back as 700 B.C. wheat rust fungus plagued early wheat farmers and caused the creation of new Roman gods, "Robigo" and "Robigus" to whom they sacrificed plants and animals to in order to ensure healthy crops. A more commonly known historical impact of a pathogen would be the "late blight" in the 1840's that decimated the potato crop in Ireland year after year resulting in thousands of deaths due to

starvation ("Plant disease and their worldwide impact", 2011). Finally, Butler's campus has also felt the effects of a pathogen. The Emerald Ash Borer, a species of weevil, has plagued Butler's Ash tree population and has resulted in unhealthy trees with minimal foliage, and the removal of dead Ash trees.

Plant Pathogen Interaction

The biggest difference between plants and animals in defense is mobility. Animals have the ability to move away from threats, while plants to not have that luxury. The first line of defense for plants is structural. A waxy cuticle covers leaves and layers of bark form to prevent microbes from entering the organism. The nature of plant cells is also provides a structural defense. Plant cells have a primary and a secondary cell wall which are used to prevent microbial invasion. However, despite the structural defenses, pathogens will inevitably infiltrate plants, which is when plants need to turn to their chemical defenses.

The first step for plants in a successful defense is detection. Plants resistance genes are able to detect microbe-associated molecular patterns (MAMP's) commonly found in microbes. This detection triggers basal resistance, also known as innate immunity. This non-specific response simply provides and immediate defense and attempts to slow the infiltration of the pathogen. If the pathogen overcomes the innate immunity plants may counter with a hypersensitive response (HR). HR is a type of programmed cell death (PCD) which will occur at the site of the infection. The goal is to limit the water and nutrient supply the pathogen needs in order to survive. A HR response is usually triggered by the presence of effector molecules introduced by the pathogen (Freeman and Beattie, 2008). Although a fairly drastic response, sacrificing a few cells to save the rest of the plant can be worth it.

Another early response to infection is an oxidative burst of reactive oxygen species (ROS). Just like innate immunity, ROS production can be trigger by the presence of MAMP's. ROS have two main roles in plant defense. First they strengthen cell walls by linking glycoproteins, thereby fortifying the wall. Second, they act as signaling molecules to send messages to other parts of the plant that they are under attack (Torres et al., 2006). One signal they send is to defense genes in the plant. This signal turns these genes on, beginning transcription and translation.

Finally, SAR is a longer term solution for the plants to protect themselves against pathogens. Again, triggered by the presence of MAMP's, the plant produces a signal that is sent to all healthy tissue to induce the expression of defense genes (Shanks, 2014). This signal is able to prepare the rest of the plant to be attacked, allowing them time to get their defenses ready. Once SAR is instituted in the plant, quick responses to future attacks can be made. A number of plant hormones play an important role in the subsequent defense; jasmonic acid (JA), salicylic acid and ethylene. Each activates pathogenesis-related (PR) defense genes. PR defense genes play key roles in signaling pathways and encode for antimicrobial protein products (Winter et al., 2014). SAR has been well established in vascular plants but its role in defense for non-vascular plants is less studied.

JA has been associated with plant defense and SAR in moss, but has never been directly found to affect plant survival in the presence of a pathogen. JA is produced most commonly by the 13- lipoxygenase (LOX) pathway. Induced by the presence of a pathogen, the first step is transforming phospholipase D to 13-hydroperoxide, catalyzed by LOX. From there the molecule undergoes a series of reactions controlled by the AOS and AOC genes. These genes play a key role in the synthesis of JA by controlling the enzymes that are essential in the JA pathway (Avanci et al., 2010) (Figure 1).

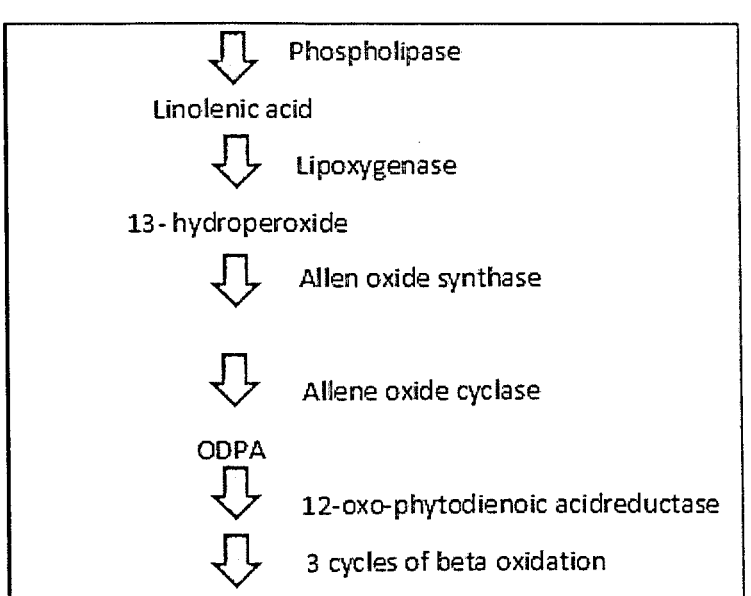

Figure 1. The production pathway of jasmonic acid.

Vascular Plants

Vascular plants are the most recent evolutionary branch of plants, they are characterized by their vascular tissues. These tissues are able to distribute key resources, water and nutrients, throughout the plant. Xylem is specialized to carry water, necessary for photosynthesis, to the photosynthetic parts of the plant. Phloem, a second transport tissue, then brings the sugar produced by photosynthesis to the heterotrophic parts of the organism. These tissues along with others are what allow vascular plants to grow much larger than non-vascular plants.

Non-vascular Plants

Non-vascular seedless plants, also known as Bryophytes, consist of mosses, liverworts and hornworts. In my research three different species of mosses were used.

Often considered "lower plants" because they were the earliest group of plants to evolve, non-vascular plants lack the specialized tissues that vascular plants have. For that reason they grow close to the ground where water and nutrients are readily available. They have no true leaves or roots, but instead have single sheet of cells where photosynthesis takes place and rhizoids, root like structures that do not absorb nutrients but help in anchoring the plant to a substrate. Because they don't rely on gaining nutrients from the soil, non-vascular plants often grow on rocks, trees, and other plants, and despite not going vertically, can cover vast areas.

Physcomitrella patens is a model organism when working with mosses. Like all bryophytes it occupies a unique evolution position for plants, between algae and vascular plants and therefore research done on it can have applications to both groups. *P. patens* in particular requires simple growth conditions and is easily manipulated in the lab (Schaefer and Zrÿd., 2001). However, its best attribute and the reason it is so commonly used for genetic studies is that its genome has been fully sequenced. This knowledge allows for specific genes to be targeted and up-regulated, down-regulated, transformed, or knocked-out. By altering specific genes you can then learn what their functions are in the organism.

Less information is known about the genome of *Mnium cuspidatum*, a second moss used in this study. Living all across North American, *M. cuspidatum* is a common moss but not as commonly used in genetic studies as *P. patens* (Streb, 2001).

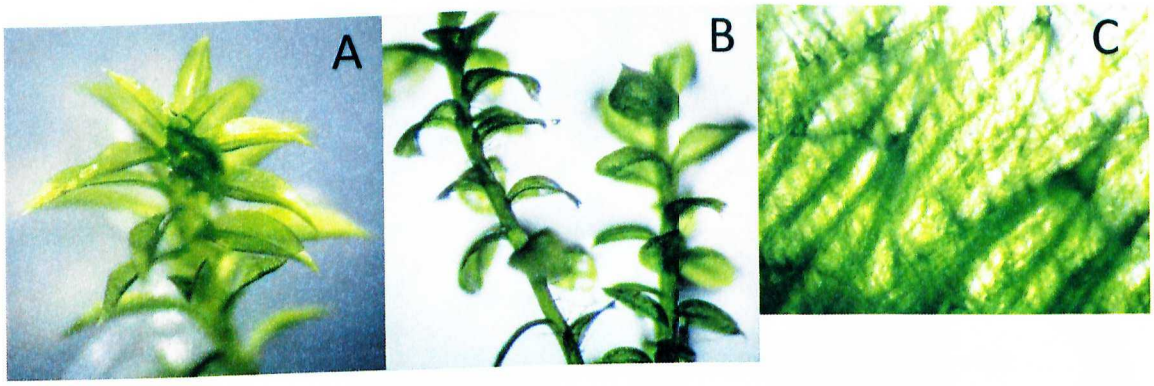

Figure 2. From left to right: (A) *Physcomitrella patens*, (B) *Mnium cuspidatum* and (C) *Ceratadon purpureus.*

Lastly, *Ceratadon purpureus*, commonly known as fire moss was also used in this study. It gets its name from the growth pattern of fluffy clumps that resemble a flame. Similar to *M. cuspidatum*, it is common across North America, and its genome is also not sequenced (Rook, 2004).

Mosses are unique in their life cycles, which are unlike vascular plants. Mosses begin as a haploid spore which first produces protonemata, a filamentous chain of cells, and continues to grow until it is a full grown gametophore. Gametophores are dioecious, the females have egg storing archegonium and the male's sperm filled antheridia. In this stage, the moss is still haploid in nature. When sperm and egg come together a diploid zygote is formed. This zygote will grow in the archegonium of the female gametophyte and eventually grow a tall stalk, which at the top of lies the sporangium. At this stage the moss is now diploid. Sporangium produce haploid spores which are eventually released from the top of the stalk, and the life cycle repeats (Campbell et al., 2008). The most identifiable and common stage of this cycle is the haploid gametophyte stage. This differs greatly from almost all other living organisms which are diploid for the vast majority of their life cycles (Figure 3).

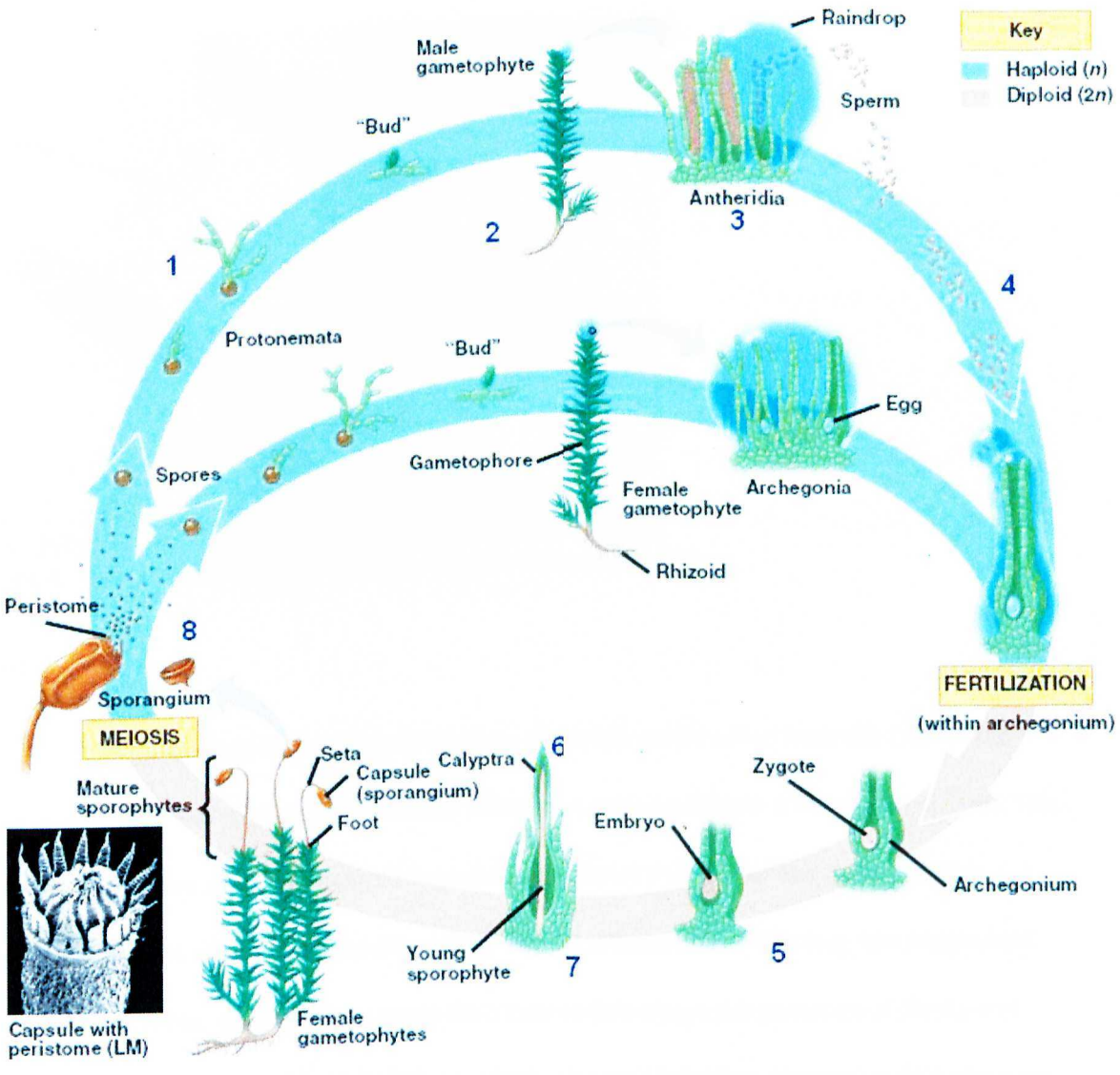

Figure 3. The life cycle of moss (Campbell et al., 2008)

Pythium irregulare

The pathogen used in this study is the soilborne pathogen, *Pythium irregulare*. Once a plant is infected by *P. irregulare*, it experiences blight and root rot, both severely stunting plant growth. It can affect many plants, including the majority of important crops in the United States (Katawczik, 2008). A member of the class Oomycete you can identify *P. irregulare* under microscope by its thin, filamentous hyphae (Figure 4).

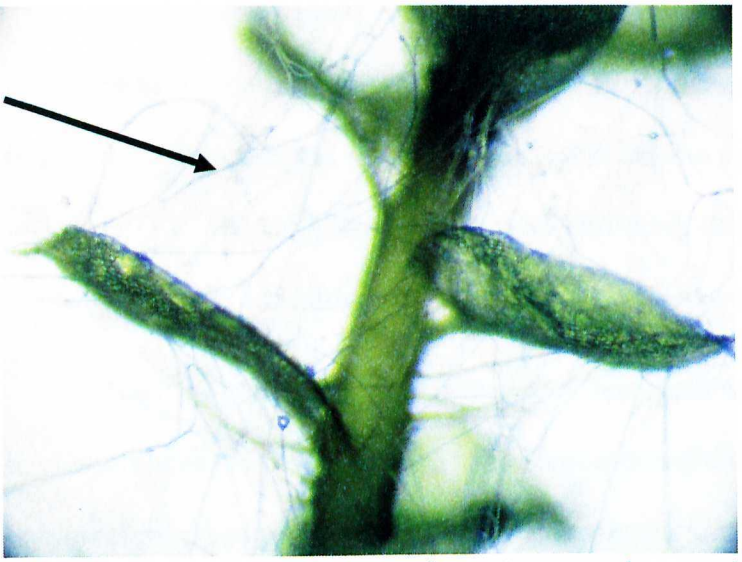

Figure 4. *Pythium irregulare* (arrow) growing on *Mnium cuspidatum*.

RNAi

The central dogma of biology is the flow of genetic information from the DNA in the nucleus of a cell, through transcription and translation and resulting in a functional protein. This process which takes place millions of times every second in the cells of living organisms is responsible for the abundance and diversity of life. As science and technology has progressed, particularly genetics, we now know more than ever before about the genomes of plants and animals. With this increased knowledge, new technologies have been discovered that allow the DNA and RNA within an organism to be manipulated and studied. This type of genetic work is extremely valuable because it can identify the specific roles that proteins have, as well as identify the genes responsible for making those proteins. The more we can learn about how the vastly complex genomes of organisms work, the better equipped scientists will be in finding ways to integrate that knowledge into modern medicine, helping to cure plant, animal and human diseases.

Interfering RNA (RNAi) is one of these new technologies that has quickly become a key tool in the field of genetics. RNAi is the use of small, 21 base pair, single-stranded RNA fragments to disrupt mRNA before it can be translated into a protein. Two types of RNAi can be utilized by cells, microRNA (miRNA) and small-interfering RNA (siRNA). The differences between the two are subtle, and both do the same job which is decreasing gene expression.

The RNAi pathway is found in many eukaryotes and begins with either the delivery of double stranded RNA to the cell or with the production of it in the nucleus. From there the enzyme Dicer chops up the long strand into approximately 21 base pair long segments. The double stranded RNA then unwinds and one piece, the guide strand, is incorporated into the RNA-induced silencing complex (RISC), while the other strand is degraded. This RNA and protein complex will then find a complementary sequence of mRNA and uses the catalytic argonaute component of RISC to cleave the mRNA strand (Zamore et al., 2008). Once the mRNA strand is cleaved, it can no longer be translated. In this way RNAi can be used to decrease gene expression (Figure 5).

The use of RNAi does not cause permanently decreased levels of gene expression. Because RNAi does not affect transcription it is only a short term control of expression.

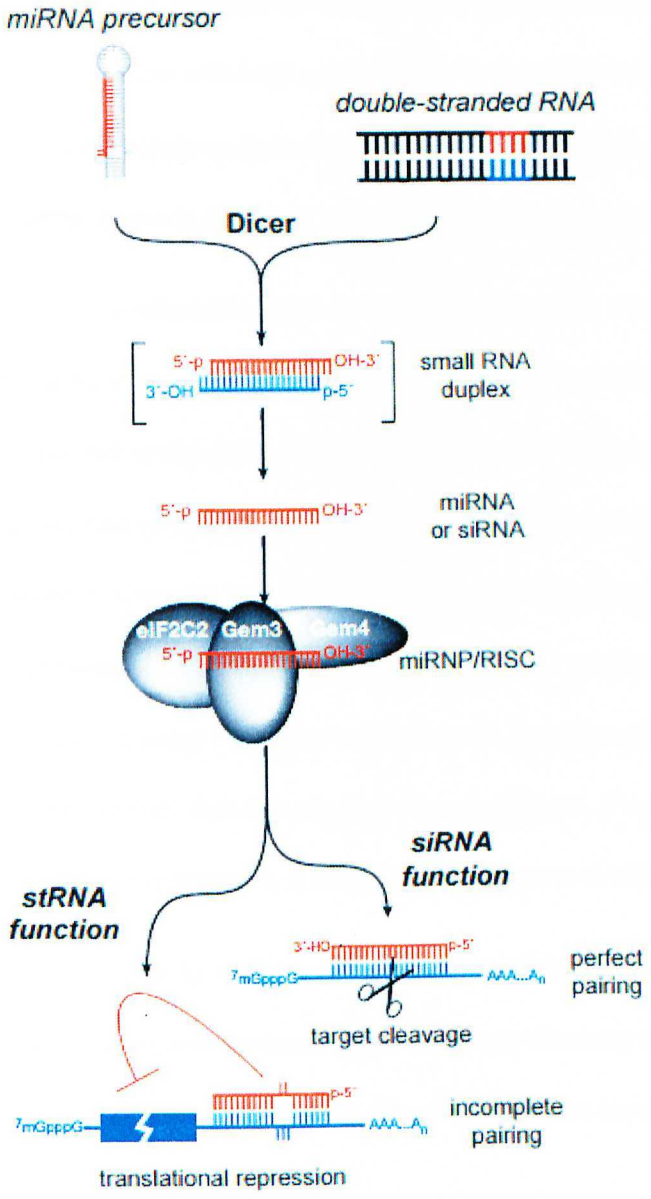

Figure 5. RNAi pathway, beginning with double stranded RNA and finishing with a silencing RNAi molecule (Hutvágner and Zamore, 2002).

Gene Knockout

Complete gene-knockouts are a long term method of decreasing gene expression. RNAi is limiting because in order to continue to down-regulate a gene, you must continually apply it. A genetic transformation is needed for a knockout. The most common method for this

transformation is polyethylene glycol (PEG) - mediated DNA transfer to protoplasts. Knockouts have been done with high success rates in *P. patens* due to its unique ability to integrate foreign DNA by homologous recombination (Hohe et al., 2004). Protocols exist for PEG mediated transformation in *P. patens* but are lacking in other mosses.

Transformation is the integrating of genetic material into an organism via a vector. PEG-mediated transformation requires an *E. coli* vector which harbors plasmid DNA, which is transferred to the plant. Protoplasts, plant cells with the cell wall digested away, have the highest rate of uptake for plasmids and therefore isolating protoplasts is the first step in the transformation process. The plasmid is transferred from the *E. coli* to the protoplasts where it gets integrated into the genome (Hiwatashi and Hasebe, 2011). The construct of the plasmid is designed to integrate with the target gene, therefore disrupting the sequence and causing the protein product that is made to be non-functional, if even transcribed and translated at all. Once the DNA has integrated, it stays in the genome and will permanently disrupt the gene function.

Similar to RNAi treatments, gene knockouts are an ideal way to study the function of specific genes and proteins. By permanently silencing a gene you can explicitly see what its function is within an organism. This targeted gene silencing has great potential to help cure many diseases.

Thesis Research and Hypothesis

JA production is an important part of the SAR response in vascular plants. Because vascular plants evolved from their non-vascular ancestors, it is safe to assume that they may contain similar defense responses. It has been previously shown that the introduction of *P. irregulare* to *P. patens* causes an increase in gene expression of genes in the JA pathway (Bryce

Fawcett, unpublished). However to further understand how integral the role of JA is in pathogen defense its production must be stopped and the plant infected.

I hypothesize that the introduction of RNAi would decrease the levels of mRNA created by the AOC gene. Due to the AOC gene product playing an integral role in the production of JA, this decreased gene expression would also decrease JA levels in the plant. I also hypothesize that *P. patens* which had been exposed to RNAi would be more easily killed by the pathogen compared to *P. patens* samples that had not been exposed.

Finally, as part of my thesis research I also wrote protocol for the isolation of protoplasts of *M. cuspidatum* and *C. purpureus*. Isolating single cells without cell walls is the first step in attempting a gene knockout in these moss species. Whereas RNAi only decreases gene expression for a period of time, a full gene-knockout would permanently decrease expression. This first step is crucial because stable protoplasts are the first step in the transformation process.

Materials and Methods

Plant and Pathogen Growth Conditions

P. patens was used in the RNAi experiment while *M. cuspidatum* and *C. purpureus* were used in the protoplast isolation protocol. All three mosses are ideal for use in the lab because they grow easily on media and can be transferred to new media quickly and sterilely. All mosses were grown in Dr. Villani's lab. *P. patens* was grown on sterilized BCD agar with alternating 12 hours of fluorescent lighting at 27.5° C and 12 hours of dark. BCD stock was made in 1 L increments with 10 mL of stock B containing 25 g $MgSO_4 \bullet 7H_2O$ filled to 1 L with H_2O, 10 mL of stock C containing 15 g KH_2PO_4 filled to 1 L with H_2O and adjusted to pH6.5 with 4M KOH, 10 mL stock D containing 101 g KNO_3 1.25 g $FeSO_4 \bullet 7 H_2O$ filled to 1 L with H_2O, and 920 mg di-ammonium tartrate, then brought to a volume of 1 L by H_2O and adjusted to pH6.5 before the addition of 4 g agargel. Solution was then microwaved for 7 minutes or until boiling, and swirled to dissolve agar, before being autoclaved at 121° C at 15 psi for 25 minutes. After autoclave sterilization 1 mL of 1 M $CaCl_2$ was added before the solution was poured into petri dishes and let sit to solidify before use. *M. cuspidatum* and *C. purpureus* were grown on BCDAT medium which was made in 1 L increments with 10 mL of stock B, 10 mL of stock C, 10 mL of stock D, 1 mL of alternative TES containing 55 mg $CuSO_4 \bullet 5H_2O$, 614 mg H_3BO_3, 55 mg $CoCl_2 \bullet 6 H_2O$, 25 mg $Na_2MoO_4 \bullet 2 H_2O$, 55 mg $ZnSO_4 \bullet 7 H_2O$, 389 mg $MnCl_2 \bullet 4 H_2O$, and 28 mg KI filled to 1 L with H_2O, and 920 mg di-ammonium tartrate, then brought to a volume of 1 L by H_2O before addition of 4 g agargel. The solution was microwaved, autoclaved, and 1 mL of 1 M $CaCl_2$ added in the same manner as above.

Mosses were propagated using utensils that were flamed sterilized with 95% EtOH. Fully grown mosses were removed and diced into small pieces before being transferred to a new

petri dish. Approximately 7 colonies were on each dish. Acetate membranes were placed on the top of the medium before mosses were added. *C. purpureus* did not require dicing due to its already filamentous structure.

Pythium irregulare, a common fungus known to infect mosses was the pathogen used in this study. *P. irregulare* was obtained from sterile samples kept in Dr. Villani's lab. It grew on PDA medium at 1/8th strength, allowing the pathogen to maintain its virulence by not supplying it satisfactory amounts of nutrients. For 500 mL of PDA, 2.438 grams of stock PDA containing 15 g/L agar, 20 g/L dextrose, and 4 g/L potato extract was combined with 6.563 g of BD Difco agar and brought to 500 mL with H_2O (Fawcett, 2014). Samples were grown at room temperature, 25°C, in the shade.

Protoplast Isolation

P. patens and *M. cuspidatum* were grown on BCD alternative medium in order to encourage chloronemata growth which is easier to isolate protoplasts from due to the tissue being largely undifferentiated (Hiwatashi and Hasebe, 2011). *C. purpureus* was grown on BCD medium because it was already in a chloronemata state.

Two hundred mL of an 8% mannitol (Sigma Aldrich) solution was made and autoclaved at 121° C at 15 psi for 25 minutes, let cool, and refrigerated. One-tenth of a gram of Driselase (Sigma Aldrich) was added to and dissolved in 5 ml of 8% mannitol in a sterile 15 mL conical tube. The solution was then centrifuged for 5 minutes at 6000 rpm. Supernant was syringe filtered using a 10 mL syringe and 0.22 µm pore size syringe filter, and transferred to a sterile 15 mL conical tube. Plant tissue was then added. When using *C. purpureus*, moss (0.2 g dry weight collected from one plate) was added directly to the tube. A tissue grinder was required for *P. patens* and *M. cuspidatum* before they could be added to the solution. Grinder was sterilized

using a 10 second 95% EtOH rinse, followed by a sterile H_2O rinse. Plant tissue (0.2 g dry weight collected from one plate) was combined with sterile H_2O in a 50 mL conical tube and pulsed on low power for 1 second increments until large plant clumps had been disassembled. Excess H_2O was then poured off and the remaining moss was added to the filtered solution. Next solution was incubated at 25°C for 30 minutes and mixed gently 5 minutes. *C. purpureus* only required 5-10 minutes in incubation whereas *P. patens* and *M. cuspidatum* required the full 30 minutes. Solution was then filtered through a 70 µm nylon cell strainer, allowing protoplasts to run through. This final solution was pipetted onto BCD plates and left to grow under the same lighting conditions listed above.

RNAi Treatments

RNAi was custom ordered from Gene Link using a cDNA sequence for the AOC gene in *P. patens*. The sequence found via the National Center for Biotechnology Information website by searching "*Physcomitrella patens* Aoc" (Table 1). This sequence was sent to Gene Link, and custom siRNAi's were made and sent to Butler. The Gene Link package included three separate RNAi's, aoc-1, aoc-2, and aoc-3. Each a different 21 base pair sequence coinciding with a part of the AOC gene. These sense strand segments are highlighted in the cDNA sequence.

cDNA Sequence	GCCCTTGGACACTGACATGGACTGAAGGAGTAGAAAACTCAGCGAGTAGCTTCGTGTGACCAGGTC TGGAAGTGGGTCTCAATTCAGTGCTTGTTGAAAGTGGGTGCATAGTAGCCGTGAGGTCGAGAGGGA GCTGGTGTGTGTGAGTTGCAGAGATGGCAGCGAGAGGCGCGTCCCCAGGGCATGTGCAGGAGCTGT TTGTGTACGAGATCAATGAGCGCGACCGTGGTAGTCCTGTGTTCTTGCCCTTCGGAGGAAAGAAAC AGCCGGGGACTGATGCGCATGTCAACAGCCTCGGGGACCTTGTGCCGTTCTCTAACAAGATATATG ACGGGTCATTGAAGACTAGGCTGGGAATCACGGCGGGTCTCTGCACGCTGATCTCTCACAGCGACC AGAAGAACGGTGATCGCTACGAAGCCTTGTACTCTTTCTACTTCGGCGACTACGGCCACATCTCCG TTCAGGGGCCATACATCACATACGAGGACTCGTACCTCGCGATCACGGGAGGCTCGGGGATCTTCG CGGGCTGCTACGGTCAGGCGAAGCTGCACCAGATCATCTTCCCCTTCAAGCTCTTCTACACGTTCT ACCTGCAAGGAATTAAAAAGCTCCCAGAAGCGTTGTGCGCCCCGTGCGTGCCGCCCTCGCCGTCCG TGGCTCCAGCTGATGAGGCTAAGCAGTGTCTTCCCAACCACGTCGCACCCAATTTCACAAAATAGA CACGATTTCTCCCCATCGAACCCGCCATCGGGTGCGTCGACTCCTCGATGTAAGCCAACGCTGCGC AGGACCCATAACGGCTGGCGCTGCAGCACCGGCTCTTCTAGCCAGCGCGCTAGTCGAGCAGCAGCG ACTCAAACCAGTCGATCAATGTGGATTCGAATACATAAAACTGTCAATATAATACAATATCTCTTC TTCTCAAGCTAAAAAAAAAAAAAAAAAAAAAAA
Aoc-1	UGAGAGAUCAGCGUGCAGATT
Aoc-2	UCGUAUGUGAUGUAUGGCCTT
Aoc-3	AUUCCUUGCAGGUAGAACGTT

Table 1. *Physcomitrella patens* AOC gene sequence (cDNA sequence) and AOC RNAi segments manufactured by gene link. Highlighted sections designate the regions of the cDNA sequence from which RNAi constructs were made.

Two nmols of each AOC RNAi were supplied and Gene Link guaranteed a minimum of 70% silencing with at least one of the RNAi's supplied. Upon arrival the RNAi's were stored in -20°C. Before first use the RNAi's were resuspended to a concentration of 20 µmolar using 1 mL H_2O in each tube. They were then incubated at 90°C for 1 minute, followed by 37°C for 60 minutes in water baths, per Gene Link instructions, and finally stored at -80°C.

RNAi was applied to *P. patens* and RNA extracted to see if it was successful in lowering levels of JA. A total of four treatments were done. Using 4-way divided BCD plates, one clump of *P. patens* was placed in opposite corners of two plates. All treatments were applied two hours prior to RNA extraction. The treatments were: a control with nothing added, elicitor only, RNAi only and RNAi plus elicitor. The elicitor used was 1 mg/mL β-glucan and is used to elicit a defense response in the plant by mimicking a pathogen. In each treatment 10 µL of β-glucan and 10 µL of RNAi was used. The RNAi used was a mix of 15 µL of all three provided RNAi's. This was

done for a higher chance of significantly decreased expression. Both the elicitor and RNAi were applied to the moss by releasing some liquid from the tip of the micropipette, sucking it back up, moving to another spot on the moss and repeating until all the liquid was expelled. This technique ensured the entire moss was exposed.

RNA Extractions

RNA extraction of *P. patens* was done with a Qiagen RNeasy Plant Mini Kit, using protocol for purification of RNA from plant cells and tissues and filamentous fungi. RNA extractions were quantified via BioTek microplate reader and corresponding Gen 5 (version 2.00) software.

qRT-PCR

Extracted RNA of treated samples was submitted to qRT-PCR for observing gene expression levels. RNA samples of 70 ng/µL were combined with Biosystem's Power SYBR Green RNA-to-CT 1-Step Kit and F/R AOC primer mix (2.5 µM). mRNA levels were measured by a BioRad CFX Connect Real Time PCR Detection System. Primers used were, a forward/reverse AOC, as well as actin (control). Three replicates were made for each treatment and final levels were an average of the replicates. Statistical data was obtained using the software on the Bio Rad CFX Manager.

Primer	Sequence
AOC PW1_AOC_F	GCATGTCAACAGCCTCGGGG
ACO PW2_AOC_R	GCTGGAGCCACGGACGG

Table 2. Forward and reverse primers used for qRT-PCR.

Phenotyping

To assess the ability of the RNAi to cause a phenotypic difference in *P. patens* capacity to defend itself from a pathogen (*P. irregulare*), a phenotyping experiment was conducted. Three *P. patens* clumps were transferred to separate petri dishes, and 24 hours prior to inoculation, three treatments were given: a negative control with nothing added, a positive control with 20 µL of β-glucan elicitor, and an experimental group with 20 µL of β-glucan elicitor and 20 µL of RNAi mix.

A second phenotyping trial was conducted with the same negative and positive control treatments but with a slight addition to the experimental group. The 20 µL of RNAi mix was added 24 hours prior to inoculation, and 10 µL of RNAi was also added every 24 hours to maintain a presence of RNAi for the moss. Ideally this will allow for continued exposure and for a consistent decrease in gene expression.

Treatments were then monitored and checked daily for phenotypic differences. Micrographs were taken, starting at 24 hours post inoculation to document differences.

Inoculation Procedure

Prior to inoculation, *P. irregulare* was transferred to a fresh 1/8th PDA plate by cutting out a small square of medium with pathogen grown on top. The piece was then placed on the edge of a new 1/8th PDA plate and allowed to grow under shaded conditions. For inoculation, a piece of medium containing the pathogen was cut out at the edge of the pathogen growth, to ensure it will continue to grow and infect the moss sample.

Results

Chloronemata Growth

The first step in the isolation of protoplasts was to grow chloronemata rich moss. By dicing up *P. patens* and *M. cuspidatum* and transferring it to the BCDAT medium, intermediate chloronemata growth was achieved (Figure 6). *P. patens* had more success than *M. cuspidatum*. The BCDAT medium initially caused undifferentiated, filamentous growth, but over time the gametophore stalks began to sprout up. Figure 6 shows a plate where high amounts of chloronemata growth was achieved. However, Figure 7 is a micrograph of the same plate showing more stalk growth than chloronemata. It is unknown why this inconsistent growth was observed, however the filamentous growth was encouraging. Less success was observed in *M. cuspidatum*. Figure 8 is a micrograph of *M. cuspidatum* on BCDAT medium. You can see small amounts of chloronemata growth underneath the leafy green gametophore that is dominant. Both *P. patens* and *M. cuspidatum* micrographs can be contrasted with Figure 9, *C. purpureus*, which is shown in a fully chloronemata state.

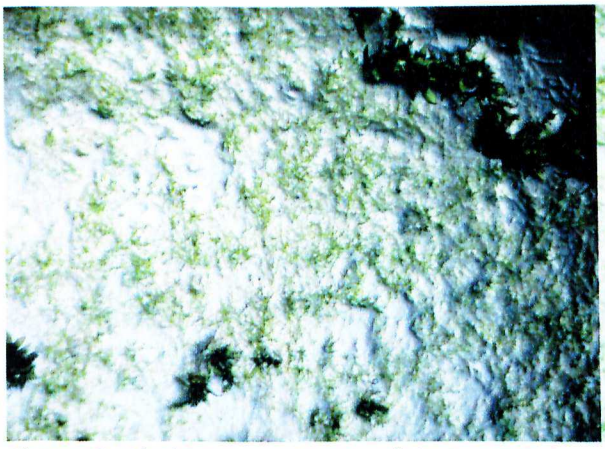

Figure 6. Choloronemata growth in *P. patens*.

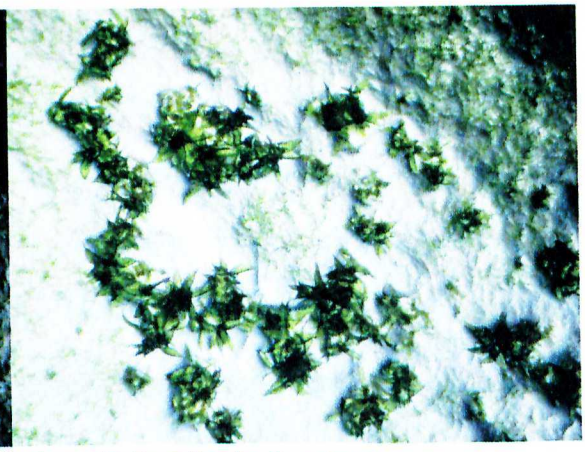

Figure 7. Budding in *P. patens*.

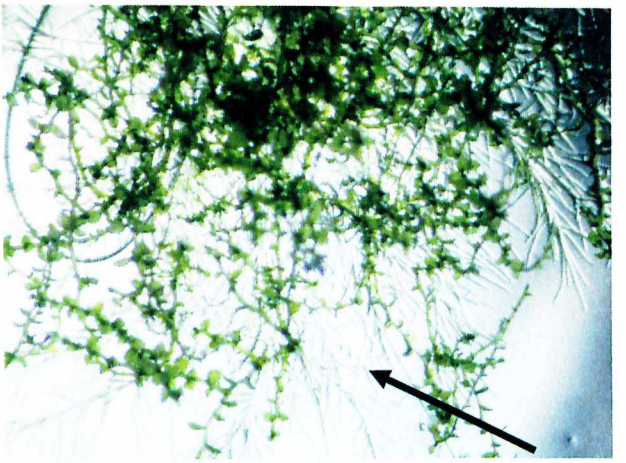

Figure 8. *M. cuspidatum* with minimal chloronemata growth (arrow).

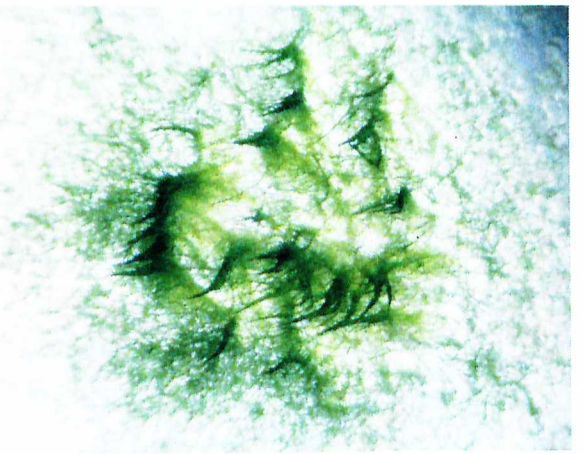

Figure 9. *C. purpureus* in a fully chloronemata state.

Protoplast Isolation

Attempts at isolating single cells was done for all three mosses. The protocol which was followed was designed for *P. patens* and was the first step in the process of transformation. Limited success in growing chloronemata rich moss affect the ability to isolate protoplasts from *P. patens* and *M. cuspidatum*. No significant amount of living protoplast cells were obtained for either moss. The breakdown of the cell wall depends on the surface area of each cell to the Driselase chemical. When the moss is not filamentous, less cells come in contact with the Driselase, therefore much lower yields are seen.

Protoplast isolation was achieved with great success in *C. purpureus*. Despite the protocol not being for this particular moss, minor changes led to impressive yields. Figure 10 is a micrograph of one drop of filtered protoplasts. The round and oval shaped cells are distinctive of protoplasts. Without cell walls to give them a uniform structure, they become round.

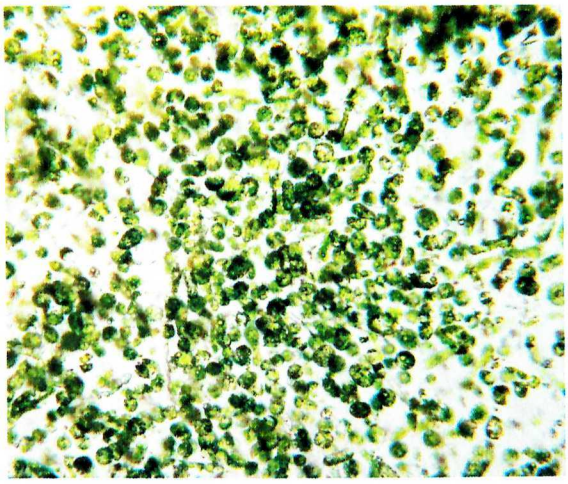

Figure 10. *C. purpureus* protoplasts (40x magnification)

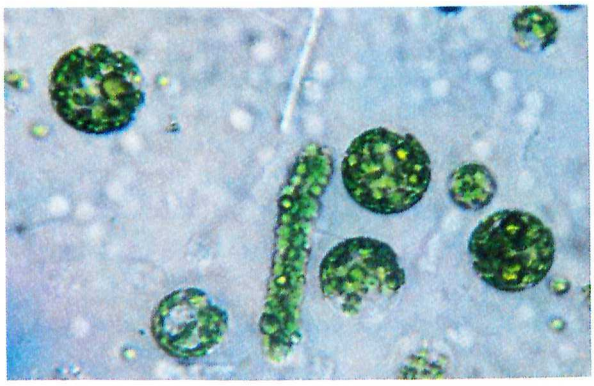

Figure 11. *C. purpureus* protoplasts (100x magnification)

AOC Gene Levels Determined by qRT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) allows for the quantification of RNA levels in an organism. This technique was used to determine whether or not the RNAi would effectively decrease levels of the AOC gene in *P. patens*. Four treatments were used: elicitor only, RNAi only, elicitor and RNAi, and a negative control with nothing. The

role of the elicitor is to mimic a pathogen, thus jumpstarting the defense mechanisms within the moss.

The negative control group had moderate levels of gene expression (Figure 12). In moss with the elicitor added, levels were at their highest due to the elicitor's ability to successfully mimic a pathogen. Finally, both the RNAi only treatment, as well as the elicitor and RNAi treatment showed the lowest levels of gene expression. Therefore showing the RNAi's ability to successfully lower gene levels both on its own, and in the presence of a pathogen.

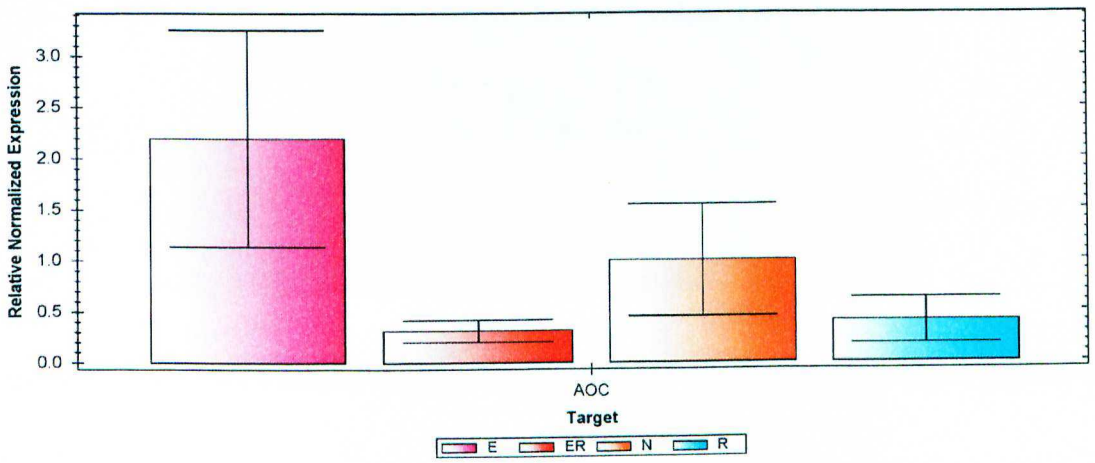

Figure 12. Allene Oxide Cyclase (AOC) expression in *P. patens* treated with 20 µL of 1 mg/mL β-glucan elicitor (E), 20 µL of 1 mg/mL β-glucan elicitor and 20 µL of RNAi (ER), no treatment (N), and 20 µL of RNAi (R).

Phenotyping

Phenotyping was done to determine whether RNAi application would reduce the mosses ability to survive a pathogen assault. After observing decreased levels of AOC gene products by qRT-PCR the goal was to test the ability of RNAi to cause a phenotypic difference. Continuous treatment with RNAi did not cause a phenotypic change in the mosses reaction to *P.*

irregulare compared to a positive and negative control. Figures 13-15 show the day by day progression of pathogen infection in each treatment, no phenotypic differences were observed.

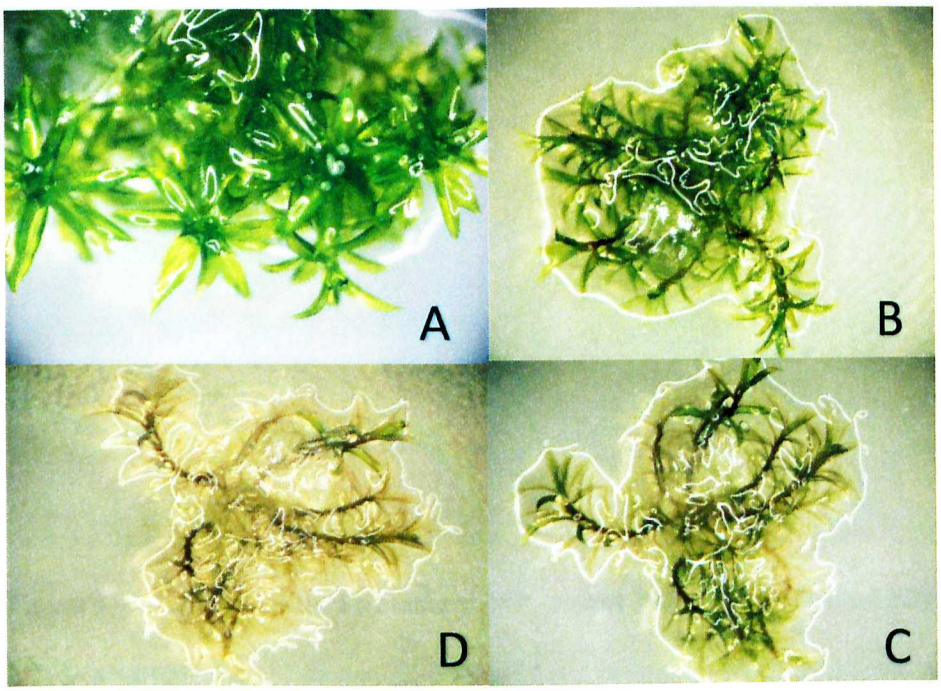

Figure 13. Daily RNAi treated *P. patens* infected with *P. irregulare* at 0 (A), 3 (B), 8 (C), and 11 (D) days.

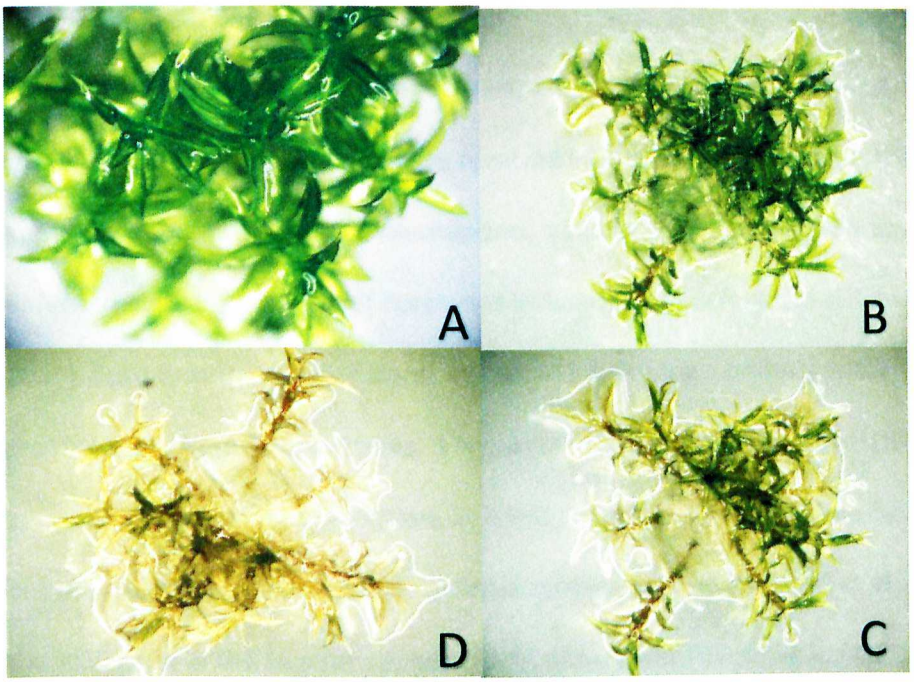

Figure 14. Negative control treatment of *P. patens* infected with *P. irregulare* at 0 (A), 3 (B), 8 (C), and 11 (D) days.

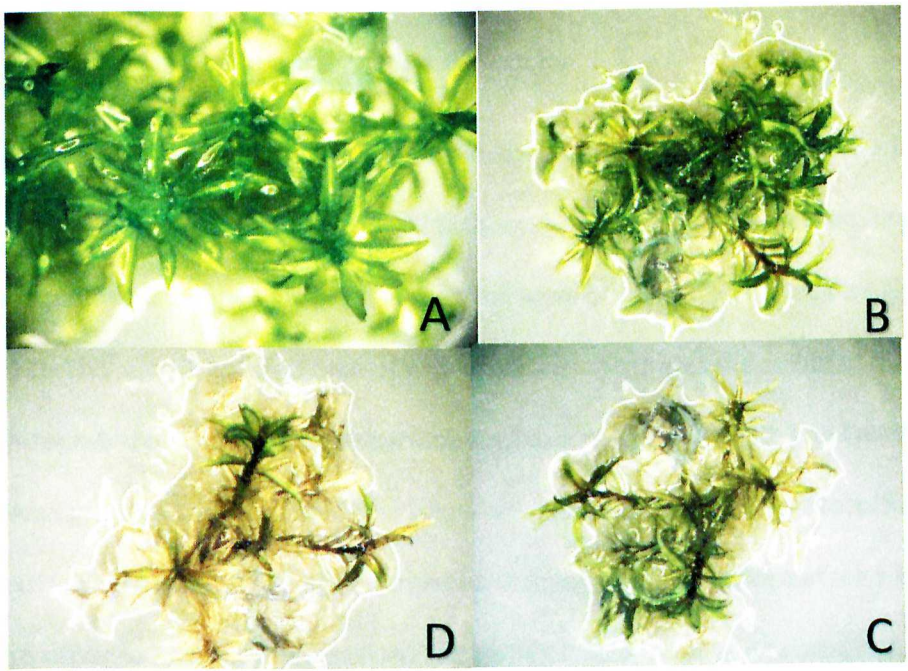

Figure 15. Positive control treatment of *P. patens* infected with *P. irregulare* at 0 (A), 3 (B), 8 (C), and 11(D) days.

Discussion

Mosses occupy a unique evolutionary position between aquatic algae and vascular plants. Given this position they have received little attention from researchers and much is still unknown about their defense mechanisms. Vascular plants utilize their transport tissues in defense and use a plethora of hormones including JA to defend against pathogens (Avanci et al., 2010). Furthermore vascular plants exhibit SAR following a primary pathogen attack can help protect them from future attacks. It is unknown how much of these vascular plant defense systems are also utilized in mosses. JA levels do increase with infection by a pathogen in mosses, but the exact role that JA plays in defense remains unknown (Avanci et al., 2010). By targeting the AOC gene in the JA pathway with RNAi, a temporary decrease in AOC gene products was observed. This decreased expression could have implications for moss survival post pathogen inoculation.

RNAi Successfully Decreases Gene Expression

RNAi is designed to decrease expression of targeted gene products, but before this study minimal application of RNAi technology on mosses had been explored. By using qRT-PCR, decreased levels of AOC gene expression were observed in mosses treated with RNAi (Figure 12). This significant decrease of expression demonstrates the ability of RNAi molecules to be integrated by the plant after surface application. Use of RNAi in previous studies with *Physcomitrella patens* called for more complex transformation protocol in order to integrate the RNAi (Schaefer et al., 1991). It remains unknown how effective surface application is compared to other methods of integration. The qRT-PCR took place two hours after application of 20 µL or RNAi mixture, this short amount of time was enough for the RNAi to decrease gene expression levels, but how long it is actively blocking expression is unclear. This remains to be determined.

To further examine the longevity of externally applied RNAi's ability to lower gene expression a more extensive qRT-PCR experiment is required. After applying RNAi, gene levels should be checked every hour until gene expression is back to normal levels. This experiment would produce a curve that plots RNAi's effectiveness over time. This information is valuable in future studies as it can be used to determine when additional application of RNAi is needed to maintained decreased levels of expression.

RNAi worked effectively for short term gene silencing. It significantly decreased AOC gene product levels by surface application. This finding has many implications for future studies. The ability to temporarily silence a gene product allows for identification of that genes role in certain processes. Other genes within the pathway for JA could be silenced, or other genes related to PR defense could be looked at to assess their role in plant defense systems.

RNAi Ineffective in Phenotyping Experiments

After seeing significantly decreased AOC gene product levels in qRT-PCR, RNAi was used to assess its ability to cause a phenotypic difference in mosses ability to defend itself against a pathogen. To understand how RNAi can affect the mosses phenotypically, RNAi was added to a small clump of *P. patens* which, 24 hours later was inoculated with *P. irregulare*. The RNAi treatment was compared to a positive control with β-glucan elicitor, also added 24 hours prior to inoculation, and a negative control with nothing added. A first trial saw no noticeable phenotypic differences between the three treatments over a two week period. All mosses were completely covered by the pathogen after one day and within a week were mostly brown. By the two week mark mosses were completely brown and dead. The RNAi treated moss did not fall victim to the pathogen any quicker than the other moss samples, and the elicitor treated moss did not survive any better.

After observing no significant phenotypic differences in the first trial, a second trial was conducted in which RNAi was added daily to the RNAi treated moss sample. The same 20 µL of RNAi was added 24 hours prior to inoculation, but 10 µL was also added every 24 hours post-inoculation for seven days. Despite the addition of more RNAi, no significant differences were observed between the controls and the RNAi treatment. Similar problems with the ability of RNAi to cause phenotypic differences were observed by Bezanilla et al. (2003) who concluded that if a key gene is being silenced it is possible to see no phenotypic changes due to redundant functions in related genes. This means that one possible explanation for the lack of differences is that despite the RNAi knocking out the AOC gene, another gene created a similar gene product to do the same job. Another possible explanation is that other plant hormones besides JA play more important roles in plant defense.

It was not a lack of RNAi that was a problem, by the third application of 10 µL the moss was sitting in a pool of RNAi containing liquid (Figure 13). It is possible that this pool of liquid affect the moss in its ability to uptake the RNAi. This problem of not knowing how long the RNAi that is applied is actually able to decrease gene levels, brings back the need for further qRT-PCR research to be done to determine the longevity of RNAi in plant systems.

Finally, the moss samples were infected by the pathogen by cutting out a small square of agarose gel containing growing ends of *P. irregulare*. However it is possible that the amount of pathogen compared to small amount of moss used resulted in the pathogen being able to completely overtake each moss treatment so thoroughly that no phenotypic differences could be observed. That is to say that the pathogen was so overpowering on the positive and negative controls that it was able to attack them just as quickly as the RNAi sample, so the rate of infection was almost identical. To combat this, either a smaller amount of pathogen should be

used to infect, or larger clumps of moss. This would allow for a more lengthy infection period and a higher possibility of seeing phenotypic differences.

Short-term to Long-term Gene Knockout

RNAi has been an instrumental tool in discovering the roles of many genes. The ability to temporarily knockout gene function gives insight into what, when, and why a gene does what it does. In this case it allowed further insight to the role of JA in pathogen defense. However, temporary knockouts can only give so much information, a long-term knockout allows for a deeper look into gene function. *Physcomitrella patens* is an ideal genetic model organism for full gene knockout studies and knockouts have been done before with success (Schaefer and Zrÿd, 1997). This type of knockout not only allows you to assess gene function over a short period of time, but gives insight to how the loss of a gene can affect the plants ability to survive and reproduce on at a longer time scale. This type of knockout is the next step in determining exactly how important the AOC gene, and other PR genes are in plant defense. PEG-mediated transformation has seen the highest rates of transformation in *P. patens* to date (Schaefer and Zrÿd, 2001).

Chloronemata Growth- The First Step in PEG-Mediated Transformation

There are multiple ways to create a gene knockout, but the most successful method has been PEG-mediated transformation. Protocol for this transformation by Hiwatashi and Hasebe (2011) calls for the moss to be in a chloronemata state, citing that chloronemata rich moss has higher transformation rates than caulonemata rich moss. Lower transformation rates of caulonemata could be linked to the fact that caulonemata rich moss has a tendency to sprout buds more quickly than chloronemata moss (Reski and Abel, 1985). Ideal cells for transformation are easier to obtain from filamentous, undifferentiated cells than from cells that

are part of the gametophyte, or gametophyte bud. To begin the exploration of PEG-mediated transformation, growth of chloronemata rich moss was attempted.

Chloronemata growth was achieved with inconsistent success in *Physocmitrella patens* and *Mnium cuspidatum*. A special medium was used to promote chloronemata growth from protonemata. Protonemata is simply undifferentiated tissue growth. Propagating protonemata from BCD plates to the special BCD alternative plates led to varying degrees of chloronemata growth rates. Figures 6 and 7 show the inconsistency of chloronemata growth patterns in *P. patens*. However, despite only obtaining relative amounts of success in chloronemata growth, the second step in PEG-mediated transformation was attempted, the isolation of protoplasts.

Protoplast Isolation

Protoplasts are individual cells which have been stripped of their cell wall. Once the cell wall has been dissolved away, cells no longer maintain a square shaped, but are round (Figures 11 and, 12). Isolation was attempted in all three moss types, with the most success coming from *C. purpureus*. This moss was being grown in a 100% filamentous state (Figure 9) and thus it was easier for the cell walls to be dissolved away because they had more contact with the Driselase enzyme. In isolation attempts with *P. patens* and *M. cuspidatum* less filamentous moss was available for the enzyme to attack, with a lower surface area, less digestion of the cell wall occurred.

Isolation was done following Hiwatashi and Hasebe's protocol (2011), however slight changes led to increased results. The protocol called for a 30 minute digestion period of the moss in the Driselase mixture, however when using *C. purpureus* this time was reduced to 7 minutes. Original trials at 30 minutes led to over digestion of cellular matter and killed cells. Because the moss had a high surface area to volume ratio not as much time was needed to

digest away the cell walls. By reducing the time to 7 minutes, the Driselase had enough time to dissolve away cell walls, but not damage the cells in any way (Figure 11).

Trials with *P. patens* and *M. cuspidatum* were less successful because the moss used did not have the same amount of surface area in contact with the Driselase, resulting in less cell wall digestion. In an attempt to create a greater surface area to volume ration two methods were used. The first was a fine dicing of the moss with a scalpel prior to addition of the moss to the Driselase solution. This method worked with minimal results. Few protoplasts were found, but not at the density of when using *C. purpureus*. A second method was to use a tissue grinder. The grinder macerated the tissue, but caused too much damage to the cells. After the 30 minutes with the Driselase solution, all plant cells were dead. No protoplasts were observed when the tissue grinder was used to macerate the moss.

Following isolation of *C. purpureus* protoplasts, approximately 250 µL of solution containing the cells was put on BCD plates and allowed to grow. However, in three attempts at growing full mosses from single cells, all plates were thoroughly contaminated. It remains unclear which step in the isolation process caused contamination. In future attempts, new solutions should be made for all steps, and extra precautions should be used to keep all elements sterile.

Conclusion

Using the moss *Physcomitrella patens* and RNAi constructs, this study looked at how successful RNAi can be in decreasing gene expression. Using qRT-PCR it was clear that surface application of RNAi significantly lowered AOC gene products in *P. patens*. This finding supported the hypothesis that RNAi would be able to affectively alter gene expression. However, further examination was required to assess RNAi's ability to cause a phenotypic difference. In a phenotyping experiment, mosses treated with RNAi did not die any more quickly than mosses that had not been treated with RNAi. This finding did not support the original hypothesis that RNAi would be able to cause a phenotypic difference.

Furthermore, the mosses *Mnium cuspidatum* and *Ceratadon purpureus* were used to explore the beginning steps of a PEG-mediated transformation process. Step one, the growth of chloronemata rich moss, was achieved with inconsistent results. A high efficiency of isolation of single celled protoplasts in *C. purpureus* was achieved, while efficiencies were much lower in *P. patens* and *M. cuspidatum*.

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