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The Role of Ethylene in Systemic Acquired Resistance in Moss

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The Role of Ethylene in Systemic Acquired Resistance in Moss

A Thesis written by Keiffer Williams

Butler University Class of 2016, Biology

College of Liberal Arts and Sciences

Mentored by Dr. Nathanael Hauck

Dedication

This thesis is dedicated to all of those who have built me into the individual that I am today, whether through parenting, teaching and mentorship, or friendship.

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This thesis was not written through my own efforts alone, and so I would like to give credit to where it is due. I thank Dr. Nat Hauck for the use of his lab and knowledge of all things genetics and moss-related. I am deeply grateful for his willingness to work with and challenge me as a student, as well as teaching me when and how to take a coffee break.

I would also like to thank Dr. Philip Villani for his additional guidance and direction during the bulk of this research during BSI, and for reviewing the first draft of this thesis. I found his advice and insight to always be beneficial and worthwhile.

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Table of Contents

Abstract

The moss species *Physcomitrella patens* and *Amblystegium serpens* were used to categorize the effects of the phytohormone ethylene in the plant-pathogen response mechanism systemic acquired resistance (SAR) in non-vascular plants. The results outlined in this thesis found that individual exposure to the ethylene precursor molecules ACC and ethephon both up-regulated ethylene responsive genes in *P. patens*, demonstrating a positive response to ethylene exposure in the moss. Exposure to ACC and ethephon in *A. serpens* followed by inoculation with the fungal pathogen *Pythium irregulare* demonstrated an ambiguous resistance response induced by ethylene exposure in the moss. The growth rate of *P. irregulare* on BCD media when exposed to concentrations of ACC and ethephon was also measured, to determine any inhibitory effects of the ethylene precursor molecules on fungal pathogen growth.

Introduction

A Struggle for Existence

Since the dawn of the earliest known micro-organism on Earth some 3.5 billion years ago (Mojzsis *et. al* 1996), there has always been a struggle for existence. To be alive is to actively engage and respond to the natural environment, taking in compounds and converting them into energy and waste, ultimately to reproduce and pass on genes to the next generation to continue the cycle of life. In this imperfect world, resources and the space necessary to live and reproduce in are often randomly and unevenly distributed, and some organisms harbor traits that make them more efficient than others at acquiring these scant resources in their environments. Hence, the very existence of life brokers a harsh yet necessary counter-measure: competition, brought about by the crucial tenets of natural selection, and death, for those not naturally fit enough to survive and reproduce.

This inevitable struggle for existence was deemed by the renowned naturalist Charles Darwin as natural selection, and serves as the driving force behind the evolution and diversification of life (Darwin 1864). Darwin's concept of natural selection is both defined and driven by its four central tenets: more individuals in a generation are produced than can survive, variation in adaptations among individuals exists and is heritable, individuals that inherit adaptations better suited to their environment are more likely to survive, and speciation occurs when a sexual population is isolated for an extended period of time (Darwin 1864).

Since every organism on earth is driven by the mechanisms of natural selection, it has been deduced that species have survived through co-evolution with one another, each engaging in inter and intra-species competition for similar resources (Soulé 1985). Central to this concept

of co-evolution is that species are inter-dependent upon one another (Soulé 1985). Some of these inter-dependent relationships between species are mutualistic and are defined as symbiotic, or a relationship between two or more organisms living together in a prosperous manner (Mauseth 2014). However, most interactions between organisms are typically exploitative or detrimental to one organism, while greatly benefitting the other (i.e. predator/prey, parasitic and pathogenic relationships) (Mauseth 2014).

Mechanisms of Exploitation

In the case of exploitative interactions, organisms all throughout the natural world have developed intricate mechanisms of defense to counteract acts predation, parasitism, and pathogenic attack. Mechanisms that deter predation can be found across all kingdoms of life, from the venomous spines and intense coloration of lionfish (Albins & Hixon 2011) to the thorns present on a variety of woody plant species (Milewski et. al 1990). Parasitism is a distinct interaction from predation, one in which the parasite survives only by living in a host organism (Mauseth 2014), such as the notorious bot fly of Central America (Miton 1996).

Pathogens and their interactions are distinguishable from both predators and parasites, in that they are specific to viruses, bacteria, and fungi, and they incur the rapid spread of disease or death in their host (Mauseth 2014). Pathogenic relationships between pathogens and their hosts are common among both plant and animal kingdoms, and the complexities of these relationships are frequently brought about by the exquisite processes of co-evolution (Berbee 2001).

Pathogens and Civilization

The need for a thorough understanding of human-pathogen interactions is quite obvious, if one only briefly reflects upon the development of civilization across the past millennia.

Between the years 1347 and 1351, the infamous Black Death swept across Europe, killing an estimated 1/3rd of the population (Gottfried 1983). Although the precise culprit of the infamous plague is frequently debated (Prentice *et. al* 2004), it is generally accepted that it was caused by the bacteria *Yersinia pestis* brought to Europe by rats and fleas stowed away on trade-ships returning from the East (Gottfried 1983). The onset of modern medicine and sanitation procedures greatly reduces the probability of plague outbreaks in developed civilizations, though pathogenic outbreaks—such as the recent Ebola and Zika virus—are still a comment threat to the populations of developing nations (World Health Organization, www.WHO.int).

Plant-based pathogens have also drastically affected civilizations in modern times. The infamous Irish Potato Famine was caused by the fungal pathogen *Phytophthora infestans*, a tuber-rotting pathogen (Haas et. al 2009, Reader 2008). From 1845-1848, the famine was responsible for the starvation of an estimated 1 million, and the emigration from Ireland of approximately 2 million (Reader 2008). *P. infestans* destruction is still relevant, as it remains the leading cause of disease in potato—the fourth largest food crop—worldwide (Reader 2008). Clearly, a more holistic and thorough understanding of plant-pathogen interactions and plant mechanisms of defense is necessary to better combat the spread and onset of plant disease, particularly in agriculturally relevant plant species.

The Hypersensitive Response (HR)

Through the processes of co-evolution, plants as an entire kingdom have evolved specialized responses to pathogenic attack. There are two main methods of response, the first of which is known as the hypersensitive response. The hypersensitive response, or HR, entails the localized cell-death of plant cells in response to a direct pathogen invasion in an attempt to

prevent the spread of a pathogen (Goodman and Novacky 1994, Heath 2000). This response occurs as a defensive measure by a plant to isolate the invasion of a plant pathogen by inducing cell apoptosis (programmed cell-death), preventing the further spread of the pathogen throughout the plant (Heath 2000).

The process of HR is instigated by direct interaction of a plant cell with a pathogen, or indirectly through plant-recognition of pathogen avirulence proteins and plant-resistance gene activation (Heath 2000). Generally, plants have resistance genes specific to pathogen avirulence proteins that match one another in a "gene-for-gene relationship", although there are several exceptions to this rule (Heath 2000). The activation of plant-resistance genes in response to pathogen proteins typically occurs within dying and adjacent plant cells, although the spread of pathogens is typically not contained by cell-death alone (Heath 2000). Therefore, it is currently understood that the induction of cell-death in the HR acts less as a defensive mechanism, and more-so as a signal to induce a defensive response (Heath 2000).

Systemic Acquired Resistance (SAR)

Contrary to a localized response as seen in HR, systemic acquired resistance (SAR) is the process of inter-cellular signaling to activate defensive genes throughout a plant once a pathogenic presence has been detected (Ryals *et. al* 1996). This method of pathogen resistance in plants is extremely beneficial, as it allows a plant that is undergoing a pathogen attack in one part of its structure to signal other non-invaded parts, causing the up-regulation of defensive (or pathogenesis-related; PR) genes. An SAR response is typically recognized through phenotypic observations and descriptive methods, but can also be understood through the quantification of SAR-related genes (Ryals *et. al* 1996). These SAR-related genes are usually PR genes, and are

used as common markers for the presence of an SAR response (Ryals *et. al* 1996). Though a variety of PR genes exist across different species of plants and each can be differentially expressed in an SAR response (Ryals et. al 1996), these PR genes express proteins that are commonly believed to contribute directly to the induced resistance in plants (Durant and Dong 2004, Ryals *et. al* 1996).

Plant Hormones and Pathogen Resistance

The process of signaling to induce an SAR response throughout a plant body is carried out via plant hormones, although hormones are also crucial for several other signaling processes. Hormone function in plants is known to range from biotic and abiotic stress responses to signaling in the process of growth and development (Bari and Jones 2009). Plant hormones have many different groups of classification, including auxins, gibberellins (GA), abscisic acid, cytokinins, salicylic acid (SA), jasmonic acid (JA), brassinosteroids, peptide hormones, strigolactones, and ethylene (Bari and Jones 2009). Of the multiple and various plant hormones, JA, SA, and ethylene are known to play crucial roles in plant defense regulation and response in vascular plants (Bari and Jones 2009). SA has shown to act as a defensive response to biotrophic pathogens (pathogens that feed on living host tissues), while JA and ethylene are typically involved in defense responses to necrotrophic pathogens (pathogens that kill host tissue then feed) and herbivorous insects in vascular plants (Bari and Jones 2009, Glazebrook 2005).

Plant hormones are also known to interact with one another while mounting a PR response (Glazebrook 2005). These interactions can either be synergistic or antagonistic, depending on the set of genes that are being activated in response to a stressor (Bari and Jones 2009, Glazebrook 2005). Coordination has been shown between JA and ethylene in several

different types of genes, although expression of other types of genes only requires one of these hormones (Glazebrook 2005). A prime example of JA and ethylene coordination is found in the transcriptional activation of ethylene response factor 1 (ERF1) in the model vascular plant *Arabidopsis thaliana* (Lorenzo *et. al* 2003). The activation of ERF1 through coordination of JA and ethylene allows for a more efficient expression of the transcription factor rather than the expression of ERF1 being acted on either hormone alone (Lorenzo *et. al* 2003). ERF1 is a transcription factor that serves to prevent pathogen-induced disease progression through the regulation of pathogen response genes (Lorenzo *et. al* 2003).

In stark contrast, coordination of two hormones can also inhibit physiological responses such as growth or defense. As demonstrated in *Arabidopsis thaliana* and *Oryza sativa* (rice), signaling interactions between GA and JA inhibit the growth inducing capability of GA (Yang *et. al* 2012). SA and JA expressed together have been known to inhibit one another, and the coordinated signaling of JA and ethylene has also been known to have negative inhibitory effects in certain circumstances (Glazebrook 2005).

Ethylene and Ethylene Pre-cursors in Pathogen Resistance

Ethylene is perhaps the most diverse of the three main defense response hormones. Ethylene is a small, gaseous molecule consisting of two double bonded carbons and their attached respective hydrogen atoms (Figure 1). It functions as a signal molecule that can bind to various receptors to activate responses in vascular plants including senescence (aging), fruit ripening, seed germination, and defense (Bleecker and Kende 2000). Ethylene is synthesized by S-adenosyl-L-methionine (AdoMet) via 1-aminocyclopropane-1-carboxylic acid (ACC) in vascular plants (Figure 2). The two enzymes responsible for catalyzing the synthesis of ethylene

in vascular plants are ACC synthase and ACC oxidase (Bleecker and Kende 2000). The regulation of ethylene biosynthesis is largely controlled by the expression of ACC synthase genes, and ACC synthase presence is enhanced by ethylene promoting factors such as stress conditions or auxin (Yang and Hoffman 1984).

In addition to the biosynthesis of ethylene via ACC (Figure 2), ethylene can also be made available for use by plants through ethephon (Figure 1B). Ethephon is a widely used plant growth regulator in the agricultural industry, primarily for senescence and fruiting in crops (www.sigmaaldrich.com); however, there is evidence that ethephon exposure in plants is also capable of activating PR genes (Lawton *et. al* 1994). Plants exposed to ethephon are able to metabolize the chemical into ethylene for further use throughout the plant body (Foster *et. al* 1992, Audley *et. al* 1976).

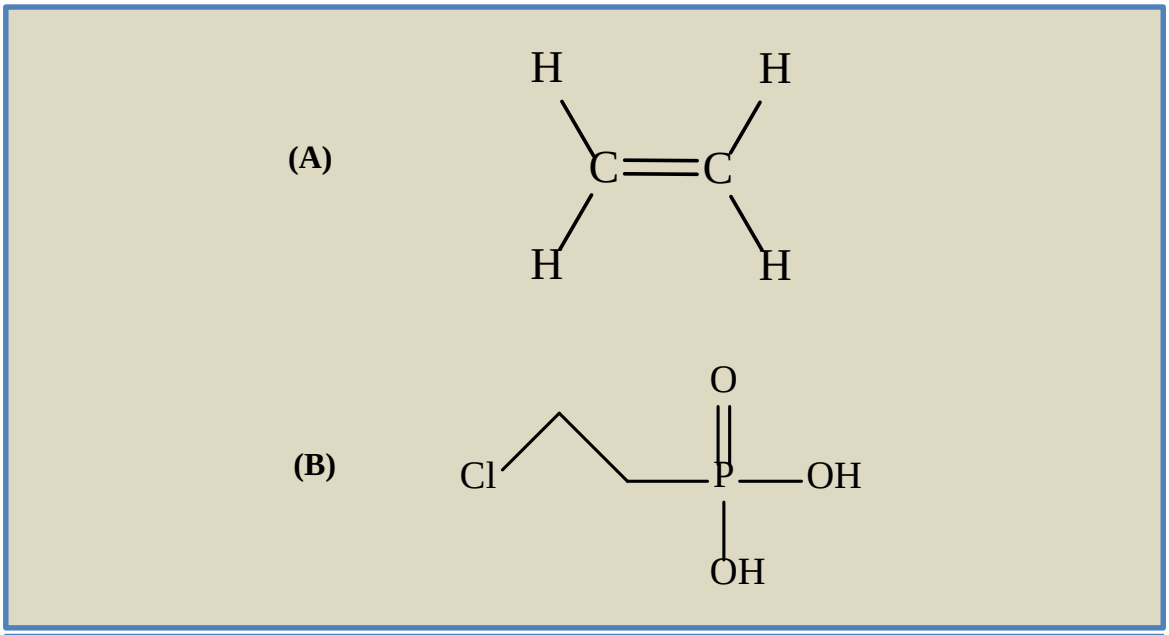

Figure 1. The molecular structure of ethylene (A), and the molecular structure of ethephon (B). Ethylene is a common plant hormone in vascular plants used for various processes ranging from senescence to pathogen resistance. Ethephon is a common plant growth regulator used in agriculture. It can be metabolized by plants into ethylene.

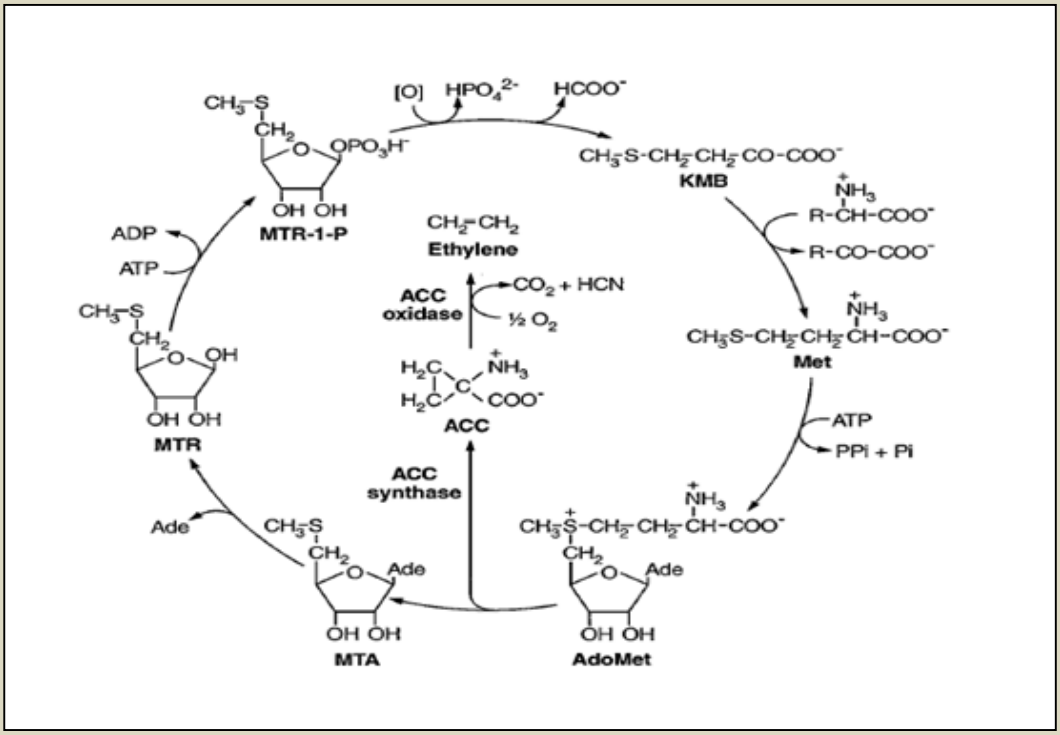

Figure 2. The Biosynthetic Pathway of Ethylene in vascular plants. (Bleecker and Kende 2000). From the methyl transferase AdoMet, ACC is synthesized by the enzyme ACC synthase. ACC is then subsequently oxidized by ACC oxidase into ethylene.

Ethylene Responsive Genes

Several classes of ethylene responsive genes and their role in pathogen resistance in vascular plants have been studied extensively. Some of these gene families include the ERF1 transcription factor (Lorenzo *et. al* 2003), the ETR1 ethylene receptor (Clark *et. al* 1997), and the EIN3 class of ethylene signaling molecules (Bleecker and Kende 2000). For synthesis of ACC, the genes that code for ACC synthase and ACC oxidase are also of high importance in regards to ethylene production in vascular plants (Bleecker and Kende 2000; Figure 2). These genes all function interchangeably to respond to ethylene and carry out the various functions of this

diverse signaling molecule. In many cases such as with the ERF1 transcription factor, these ethylene responsive genes work in conjunction with PR gene activation and the SAR response (Lorenzo *et. al* 2003).

Non-vascular Plant Pathogen Resistance

Currently, little is known with regard to hormonal signaling of PR gene activation and the SAR response in non-vascular plants (mosses), although there are efforts starting to take place to change this. The mosses are the simplest of land plants, evolving roughly 450 million years ago (Rensing *et. al* 2008). Being the first of the land plants to evolve, the mosses lack a vascular system. Similar to veins and arteries in humans, vascular tissue in plants consists of xylem and phloem used to transport water and nutrients, respectively, throughout the plant body (Mauseth 2014). In addition to transporting essential nutrients, phloem in vascularized plants is known to transport hormones for signaling responses (Mauseth 2014). Knowing that vascular tissue aids in the transport of plant hormones in the higher plants, this gives rise to the question of how lower plants carry out hormone signaling with their non-vascular tissue systems.

Knowledge of hormone signaling and defense responses in non-vascular plants is minimal, but steadily growing. Due to recent efforts, both HR and SAR have been shown to be present in non-vascular plants (Oliver *et. al* 2009; Winter *et. al* 2014). The Winter *et. al* 2014 study was conducted by the Hauck lab, and demonstrated the presence of SAR in non-vascular plants. Since then, subsequent work in the Hauck lab has followed to further elucidate the potential role of hormones in the SAR response of mosses. JA has been demonstrated to enact an SAR response in moss (Shanks BSI 2013), while SA does not seem to elicit an SAR response (Grabinski Honors Thesis, 2014). GA, a hormone known for growth and a wide range of

developmental processes in vascular plants (Richards *et. al* 2001) was recently shown to play very little role, if any, in the SAR response (Hebert BSI 2014). Herein, I examined the potential role of ethylene in the SAR response of mosses.

Significance

It is abundantly evident that plants play a vital role in the sustenance of human civilization. As with every other form of life, plants are susceptible to death, ranging from drought to pathogenic attack, to the common practice of human over-exploitation. The agricultural significance of the various crop plants such as corn, wheat, rice, and the aforementioned potato can never be overstated, as they provide nutrition and sustenance for, arguably, the entire human population. The obvious agricultural importance of these plants has led to countless efforts worldwide to understand and improve the ability of these plants to survive predation and pathogenic attacks, and these efforts have paid off considerably. However, in the abundant complexity of life and its concurrent struggle, there is always more to be understood and acted upon to curb survivability and sustainability to humanity's own favor.

In examining the role of SAR in non-vascular plants, the evolutionary ancestors of vascular plants, it is possible to provide further insight into the capabilities of pathogen response in agriculturally relevant plants. In this study, examining the role that ethylene has in non-vascular pathogen response can aid in teasing out the details of how early plants respond to an extremely diverse and essential plant hormone. Through these efforts, the origins of SAR and pathogen response in plants can be further elucidated, providing a more holistic view of the plant-pathogen interaction, and what can be done to further improve pathogen resistance in plants.

The mosses *Amblystegium serpens* and *Physcomitrella patens* were selected for use in this study (Figure 3A, B). *A. serpens* has a uniquely elongated morphology (Figure 3A), which made it ideal for phenotypic observations of a potential SAR response in the moss. *P. patens* is a novel moss species with its entire genome sequenced (Figure 3B, Rensing *et. al* 2008), and thus was used for the selective quantification of ethylene responsive and biosynthesis genes. The fungal pathogen *Pythium irregulare* was used for pathogen inoculation procedures, due to its virulence, and its ready availability in the Hauck lab.

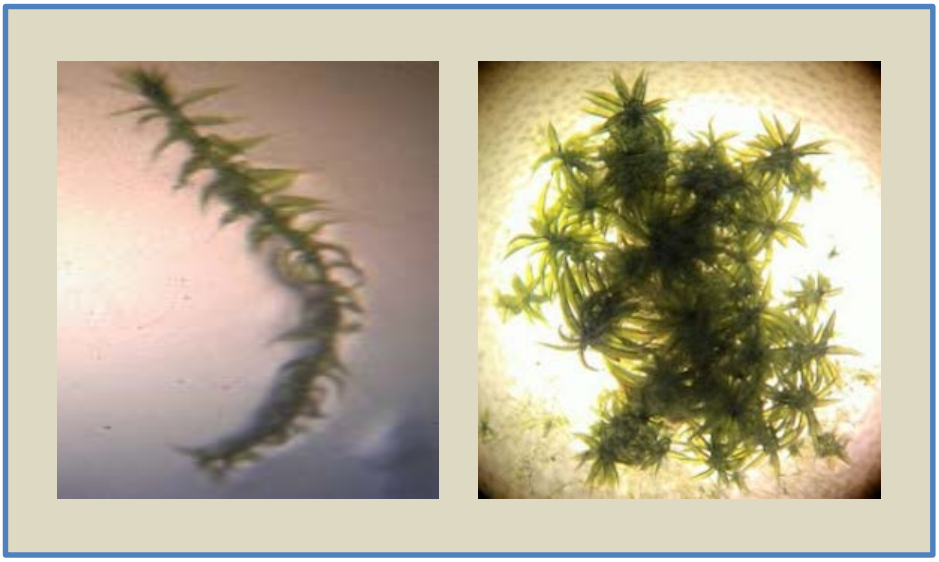

Figure 3. The creeping feathermoss *Amblysegium serpens* (A) and the spreading earthmoss *Physcomitrella patens* (B). *A. serpens* can be distinguished through its uniquely elongated morphology. *P. patens* is a novel moss species, due to its recently sequenced genome (Rensing *et. al* 2008).

Hypothesis

I hypothesize that exposure to the precursor molecules ACC and ethephon in the creeping feathermoss *A. serpens* prior to *P. irregulare* pathogen inoculation will elicit an SAR response in the moss. In addition, I hypothesize that exposure to ACC and ethephon in the spreading earthmoss *P. patens* will have increasingly higher levels of expression of defense genes 2, 24, and 48 hours post-exposure relative to non-treated control specimens. I also predict that exposure to ACC and ethephon will have no significant change in the growth pattern of *P. irregulare* on PDA media.

Materials and Methods

Culturing and Maintenance of moss specimens and fungal pathogen

The moss species *P. patens* was used in this study to determine the effect of ethylene treatment in moss at the genotype level while the species *A. serpens* was used to determine any relative levels of a mounted defense response at the phenotype level in response to ethylene treatment, followed by pathogen inoculation. The fungal pathogen *P. irregulare* was used to carry out pathogen inoculation procedures on *A. serpens* moss specimens.

P. patens is a novel moss species that has its entire genome sequenced (Rensing *et. al* 2008). Having its genome sequenced, it was an efficient model species to use to find homologous sequences of ethylene responsive genes using BLAST software. The unique morphology and radial-growth pattern of *P. patens* also made it efficient for sub-culturing and collection for RNA extraction (Figure 1A).

A. serpens served as an ideal specimen for the phenotypic pathogen response portion of this study, due to its elongated morphological shape (Figure 1B). This elongated morphology also makes *A. serpens* a valuable specimen to demonstrate the effects of an SAR response in moss, which was demonstrated by Winter *et. al* (2014).

Moss specimens were cultured and maintained at room temperature on BCD media at 21°C on a 12-hour light, 12-hour dark cycle using fluorescent overhead lights. BCD media was made in 400 mL increments, in accordance to BCD media protocol. Media was made by addition of sterile DI (de-ionized) water to a flask, filling it to half the desired media volume. Then, added to the flask was 4 milliliters each of solution B, solution C, and solution D, followed by 400 microliters of trace elements. This solution was then transferred to a graduated cylinder, where

the other half portion of sterile DI water was added. The final volume of the media solution was poured back into the flask, in which 1.92 grams of agar gel and 0.368 grams of ammonium tartrate was added to the media solution. The solution was then microwaved until the agar was fully dissolved. This was followed by autoclaving the aqueous media at 121°C at 15 psi for 30 minutes. After autoclaving, 400 microliters of sterilized 1 M calcium chloride was added to the solution. Final BCD media was then poured onto petri plates and allowed to cool overnight.

Individual specimens of *P. patens* were propagated from sterile, healthy adult specimens by dividing a selected adult specimen in half. Half of the specimen was then transported to a fresh BCD media plate, with 10-12 newly propagated specimens being placed on each fresh plate. This process was performed as needed when plates became over-abundant with specimens of *P. patens*.

A. serpens were also propagated onto fresh BCD media plates on an as needed basis, with 10-12 specimens being placed onto each fresh plate. Sterile *A. serpens* specimens were propagated by dividing a grown specimen in half and transporting one-half to a fresh plate.

P. irregulare was selected as an inoculation agent in this study due to its status as a universal fungal pathogen, and its accessibility through both the Hauck and Villani labs. *P. irregulare* was cultured at 21°C in a low-light environment on PDA media plates. PDA media was made in 400 milliliter increments at 1/8 strength, in order to enhance fungal growth. Sterile DI water was poured into a flask, filling it to approximately half the final 400 milliliter volume. 1.9 grams of Potato Dextrose Agar (PDA) powder was added to the flask, followed by 5.25 grams of Bacto agar. The solution was then transferred to a graduated cylinder, where the remaining volume of sterile DI water was added. The solution was transferred back to the flask

and then microwaved until all solutes were dissolved. The PDA media was then autoclaved at 121 C at 15 psi for 30 minutes. Following the autoclave, the final PDA media was poured onto petri plates and allowed to cool overnight.

Determination of an ACC or Ethephon Mediated SAR Response

Due to ethylene's state as a gaseous molecule, which makes it difficult and challenging to work with, the ethylene precursor molecules ACC and ethephon were used to examine an SAR response in *P. patens*. The methods for detecting an SAR response have been described previously (Winter *et. al* 2014).

ACC mediated experiments were conducted by applying 20 microliters of 20 micromolar concentrated ACC to individual *A. serpens* specimens at intervals of 2, 24, and 48 hours prior to *P. irregulare* pathogen inoculation. A 0-hour control was also used, in which no ACC was applied to *A. serpens*. Each treatment (control, 2, 24, and 48 hour) consisted of two specimens of *A. serpens*. *P. irregulare* inoculation was performed by applying a 3-mm^3 plug of PDA media with *P. irregulare* present directly to an individual moss specimen. Specimens were observed 7 days after *P. irregulare* inoculation to determine any relative levels of pathogen response.

Ethephon trials were performed in an identical manner as the aforementioned procedure; however, ethephon was applied at a higher concentration of 100 micromolar. This higher concentration was selected due to uncertainty of ethephon's effective ability of breaking down into ethylene upon exposure to specimens of *A. serpens*.

For each pathogen inoculation procedure, a 3 mm^3 plug of *P. irregulare* on PDA media was taken from a stock plate of *P. irregulare* and transported to a fresh PDA plate, in order to create a new *P. irregulare* growth front. *P. irregulare* growth fronts were given between 18-24

hours to develop, and were initiated approximately 24 hours prior to inoculation treatments of *A. serpens*.

Identification of ethylene response genes

The selection of *P. patens* ethylene response genes used in this study were acquired through prior knowledge of experiments in the Hauck lab and the use of the National Center for Biotechnology Information (NCBI) database and its Basic Local Alignment Search Tool (BLAST) program. A list of the *P. patens* genes selected for quantification, including their accession number and their putative function, can be viewed in Table 2 of the results section.

Putative *P. patens* ethylene response gene sequences were compared using BLAST to compare known homologs of ethylene response genes found in the genome of *Arabidopsis thaliana,* a model vascular plant species. Potential ethylene response homologs from the recently sequenced genome of *P. patens* (Rensing *et. al* 2008) were compared against known ethylene response genes in *A. thaliana*. This led to identification of the ethylene response homologs used in this study. The forward and reverse primers of these genes can be viewed below in Table 1.

Primers for each respective ethylene response gene were created using the NCBI Primer-BLAST program. Primer sequences were approximately 20 nucleotide bases in length, and included both the forward and reverse primers. Primers were synthesized and purchased from Sigma-Aldrich Corporation, and prepared for use as instructed by the company (Sigma-Aldrich, Saint Louis, MO). A list of the primers used during this study can be viewed below (Table 1).

Gene	Forward Primer	Reverse Primer	Product Length
ACC Synthase	AGCTCTGGCAGTGAATGCTT	CCAGGCATTTCTCCCTCGTT	199 base pairs
EIN3a	AATCGTCAAGTACCAGGCGG	GCACTCCTTTTTCCAACGGG	179 base pairs
EIN3b	AGGCCTGGAAAGTTGGTGTT	GCCATGTGGCACTTTCCTTG	132 base pairs
ERFa	CGGAGATTCGTGACAAGATTGG	ATTCAGCTGGAGGAGGCGACAAG	173 base pairs
ETR1	AGCTCTGGCAGTGAATGCTT	CCAGGCATTTCTCCCTCGTT	199 base pairs

Table 1. The forward and reverse primers of *P. patens* genes used for quantification in this study.

RNA Extraction and Gene Quantification

RNA was extracted from *P. patens* using RNeasy kits purchased from Qiagen, and the extraction was performed as according to the manufacturer (Qiagen, CA). Once extracted, the RNA was quantified using Nanodrop technology. RNA quantification was performed as according to the manufacturer (Thermo Fisher Scientific, MA). Gene quantification from the acquired RNA was performed using the common molecular technique qRT-PCR (quantitative Reverse Transcriptase-PCR). Quantification was performed as instructed by the manufacturer (Applied Biosystems, CA).

Each RNA extraction and subsequent quantification consisted of four treatment groups of 0, 2, 24, and 48 hours. Each treatment is relative to the amount of time prior to extraction that *P. patens* was exposed to either 20 micromolar ACC or 100 micromolar ethephon, with 0 hours serving as the control. For each extraction, six specimens of *P. patens* were used for each treatment group in order to harness enough RNA to be quantified prior to quantification.

Pythium irregulare growth experiments

P. irregulare growth was measured on 1/8 strength PDA media exposed to either ACC or ethephon to determine if either chemical hindered the growth of the pathogen. Each of the three growth experiments consisted of three separate treatments (control, ACC, and ethephon) with four samples per treatment. PDA media was created using the previously mentioned protocol. However, following the autoclave period of the protocol, ACC and ethephon were added to their respective treatment groups. Consistent with the SAR experiments, ACC-exposed PDA media consisted of a 20 micromolar ACC concentration, while ethephon-exposed PDA media consisted of a 100 micromolar ethephon concentration.

P. irregulare growth fronts from stock plates were initiated 18-24 hours prior to the start of each experiment, with three experiments total being conducted. At the start of each experiment, one $3mm^3$ plug of *P. irregulare* from the established growth front was placed on each of the sample plates, and allowed 24 hours to establish a front. After 24 hours, the growth of the pathogen was measured using circular transects in four different directions on the plate. Four individual measurements of the growth of each fungal front were taken over the course of an approximately 12-hour time period. Each treatment group consisted of four different sample plates, with each plate consisting of four transect measurements. The four transect measurements on each sample plate were measured at the respective time-interval, and then the four measurements for each sample plate were averaged. The average growth of each sample plate was then averaged with the growth average of the sample plates relative to that treatment, at each measurement time-interval. The averages of each treatment at each measurement time-interval were then graphed for comparison across three separate trials. In addition, the growth-rate slope of each treatment group across the three trials were compared using a 2-tailed T-test, in order to

determine any statistical differences between the growth rates of each of the three treatment groups.

Results

ACC mediated SAR Response in Amblystegium serpens

As aforementioned, an SAR response in moss has been previously demonstrated by Winter *et. al* 2014. In this current study, the potential role of ethylene in an SAR response mediated by ACC was examined in the moss species *A. serpens*. Specimens of *A. serpens* were exposed to 20 microliters of 20 micromolar ACC at various time points prior to inoculation by the fungal pathogen *P. irregulare.* Specimens of *A. serpens* were treated with 20 micromolar ACC at time points of 2, 24, and 48 hours prior to pathogen inoculation. Each time point represented a different treatment group, with an additional non-inoculate control and a 0 hour inoculation control treatment group included in the experiment. Each treatment group consisted of two individual specimens of *A. serpens*. The experiment was replicated five times. The results are shown in Figure 4A below.

All results from the 0-hour to 48-hour ACC treatments were examined with respect to the non-inoculate control. The 0-hour treatment specimen displayed signs of pathogen invasion and degradation throughout the body of the plant, including necrosis of the plant tissue and leafy structures (Fig. 4A). The 0-hour specimen seemingly failed to mount a response to pathogen invasion. The 2-hour ACC specimen also demonstrated signs of pathogen invasion and a failure to mount a defensive response (Fig. 4A). Severe tissue necrosis of tissue was present throughout the specimen, and no healthy or leafy tissue was visible after 7 days of pathogen exposure. The 24-hour treatment specimen also seemed to mount little to no response to pathogen invasion.

Tissue necrosis was present throughout the specimen, with no signs of healthy tissues present throughout the specimen (Fig. 4A). Finally, the 48-hour specimen exhibited signs of tissue necrosis on both proximal and distal ends of its body. However, some green and leafy tissue persisted in the mid-region of the specimen; though the onset of tissue necrosis in this region was still present (Fig. 4A).

Ethephon mediated SAR Response in Amblystegium serpens

The role of ethylene in an SAR response in the moss species *A. serpens* was also examined using the ethylene precursor chemical ethephon. Experimental parameters for ethephon mediated treatments were identical to ACC mediated treatments. However, 100 micromolar ethephon concentrations were used, rather than the 20 micromolar ACC concentrations. The experiment was replicated three times, and the results can be viewed in Figure 4B below.

Results from the 0-hour to 48-hour specimens were examined with respect to the non-inoculate specimen. The 0-hour treatment showed signs of necrosis throughout the specimen, with severe necrosis present on both ends of the moss specimen. However, some green leafy tissue persisted in the mid-region of the specimen (Fig. 4B). In the 2-hour treatment, tissue necrosis was again present throughout the specimen, although some green and leafy tissue still persisted in the central region of the specimen (Fig. 4B). The 24-hour treatment displayed a similar pattern as the aforementioned treatment specimens, with tissue necrosis present throughout, but some green, leafy tissue persisting in the middle region of the specimen (Fig. 4B). The 48-hour treatment exhibited mild tissue necrosis throughout, with only the lower region

of the moss exhibiting signs of severe necrosis. Contrary to the other treatment results, the 48-hour treatment seemed to retain green and leafy tissue on the upper portion of its body (Fig. 4B).

Figure 4. Survival of *A. serpens* 7 days after inoculation following ACC or ethephon pretreatement 0, 2, 24 or 48 hours prior to inoculation (A) *A. serpens* 7 days after *P. irregulare* pathogen inoculation. Specimens were either left untreated (0 hour) or treated with 20 microliters of 20 micromolar ACC 2, 24, or 48 hours prior to pathogen inoculation. Non-inoculate specimens were used for comparison purposes. **(B)** *A. serpens* specimens 7 days after pathogen inoculation. Specimens were left either untreated (0 hour) or treated with 20 microliters of 100 micromolar ethephon 2, 24, or 48 hours prior to pathogen inoculation, with non-inoculate specimens used for comparison purposes.

Ethylene Response and Biosynthetic Pathway Gene Identification

The ethylene response and ethylene biosynthetic pathway genes that were used in this study were identified through utilizing the NCBI's BLAST program. As mentioned previously, *P. patens* has a fully sequenced genome (Rensing et. al 2008). The *P. patens* genes used in this study have been identified as the known homolog of genes present in the model vascular plant *A. thaliana*. This was determined through comparison of the gene sequences using the NCBI BLAST program. All genes used in this study are listed in Table 2 below. Interestingly enough, after searching the literature and using BLAST to identify a potential homolog, there was no known homolog for the ACC biosynthetic pathway gene ACC Oxidase found in this study (Table 2).

Gene Name	Accession Number	Putative Gene Function
Acc Synthase	XP 001778276.1	Converts AdoMet to ACC
ACC Oxidase	No Known Homolog	No Known Homolog
EIN3a	XP 001766792.1	Ethylene Signaling Molecule
EIN3b	XP_001753917.1	Ethylene Signaling Molecule
ERFa	XP_001782760.1	Ethylene Response Factor
ETR1	XP 001751520.1	Ethylene Receptor

Table 2. *P. patens* ethylene response and biosynthetic pathway genes identified using NCBI's BLAST program.

Ethylene Response Gene Quantification with ACC and Ethephon

The selective gene response of *P. patens* to ACC and ethephon exposure was quantified using qRT-PCR, as mentioned previously in the Materials and Methods. Each trial consisted of four separate treatments of 20 micromolar ACC at 0, 2, 24, and 48 hour time periods prior to RNA extraction. Results were relative to the 0 hour control. The constitutively expressed RuBisCO gene was used as a loading control to ensure that differential expression of genes was due to the time of treatment, rather than irregular loading of the RNA. Trials for both ACC and ethephon exposure were replicated numerous times, with the results shown representing the overall trend of gene expression in *P. patens* when exposed to ACC or ethephon across various time points. These results are shown in Figures 5 and 6 below.

Quantified ACC Response

The results displayed in Figure 5 represent two independent ACC-treated quantifications acquired from the same *P. patens* RNA extraction. It is evident in both trials that there is a consistent pattern of gene expression present in the moss from 0-48 hours (Figure 5). As expected, there is no level of expression at 0 hours in either trial, as expression levels are all relative to the 0 hour control treatment (Figure 5). However, at 2 hours post-ACC exposure, all genes were up-regulated at substantially higher levels than the control level of expression (Figure 5). At 24 hours, the relative levels of expression drop slightly in both trials, but still remain at a substantial level of expression (Figure 5). By 48 hours, most genes had returned to their initial 0-hour expression levels, albeit the ACC synthase gene in trial 1, and both trials of the ETR1 gene (Figure 5). It is also evident that the ETR1 gene demonstrated a very strong response to ACC exposure, exhibiting a far greater change in expression than all other genes in both trials, as well as a slightly increased level of expression maintained relative to the control after 48 hours

(Figure 5). In addition, ACC synthase appeared to show a decrease in expression at 48 hours relative to the control in trial 2 (Figure 5).

Quantified Ethephon Response

The results in Figure 6 were taken from a single quantification trial of ethephon-treated *P. patens.* It should be noted that the 48 hour treatment data is absent in these results, due to an error that was made in the extraction of RNA in this trial (Figure 6). However, the trend of expression in subsequent trials demonstrated that 48-hour treatments across all genes in ethephon-treated *P. patens* showed expression levels similar or equivalent to 2-hour treatments. In addition, the results in Figure 4 were unable to be replicated, due to a lack of RNA available for a second quantification.

As seen previously in the ACC-mediated response (Figure 5), there is a common trend of expression across the treatment groups in the ethephon-mediated response (Figure 6). The 2-hour treatments across the ACC synthase, ERFa, and ETR1 genes all showed little to no change in expression, while both EIN3a and b appeared to show a decrease in expression at 2-hours (Figure 6). However, at 24-hours across all treatments, all genes showed an increase in gene expression relative to the 0-hour control (Figure 6). Interestingly, ACC synthase demonstrated a substantial fold-increase in expression of 120, while ETR1 also demonstrated a large fold-increase of 60 (Figure 6). ERFa and EIN3a both showed fold-increases of approximately 20, while EIN3b only showed an increase in expression by an approximate 2-fold change (Figure 6).

Trial A

Trial B

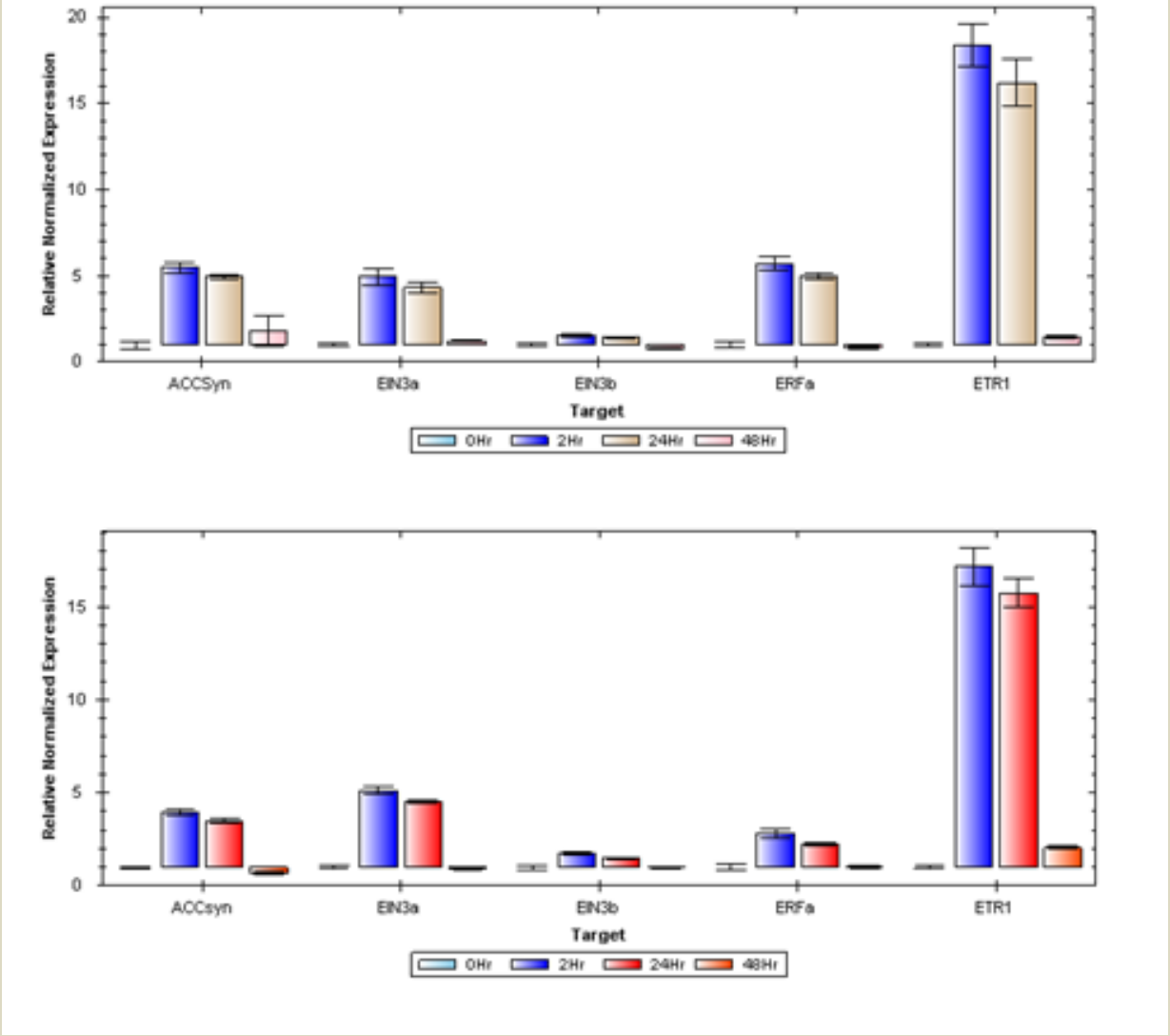

Figure 5. The quantitative gene expression of select ethylene response genes in *P. patens* following treatments of 20 micromolar ACC at 0, 2, 24, and 48 hour time points. Change in expression were measured in relation to the 0-hour control treatments of each gene, with the RuBisCO gene used as a loading control to ensure equal levels of mRNA were used in each sample. Each treatment consists of three samples averaged together to provide the average level of expression for that treatment. Results from both trials **(A)** and **(B)** were obtained from the same RNA extraction treatment.

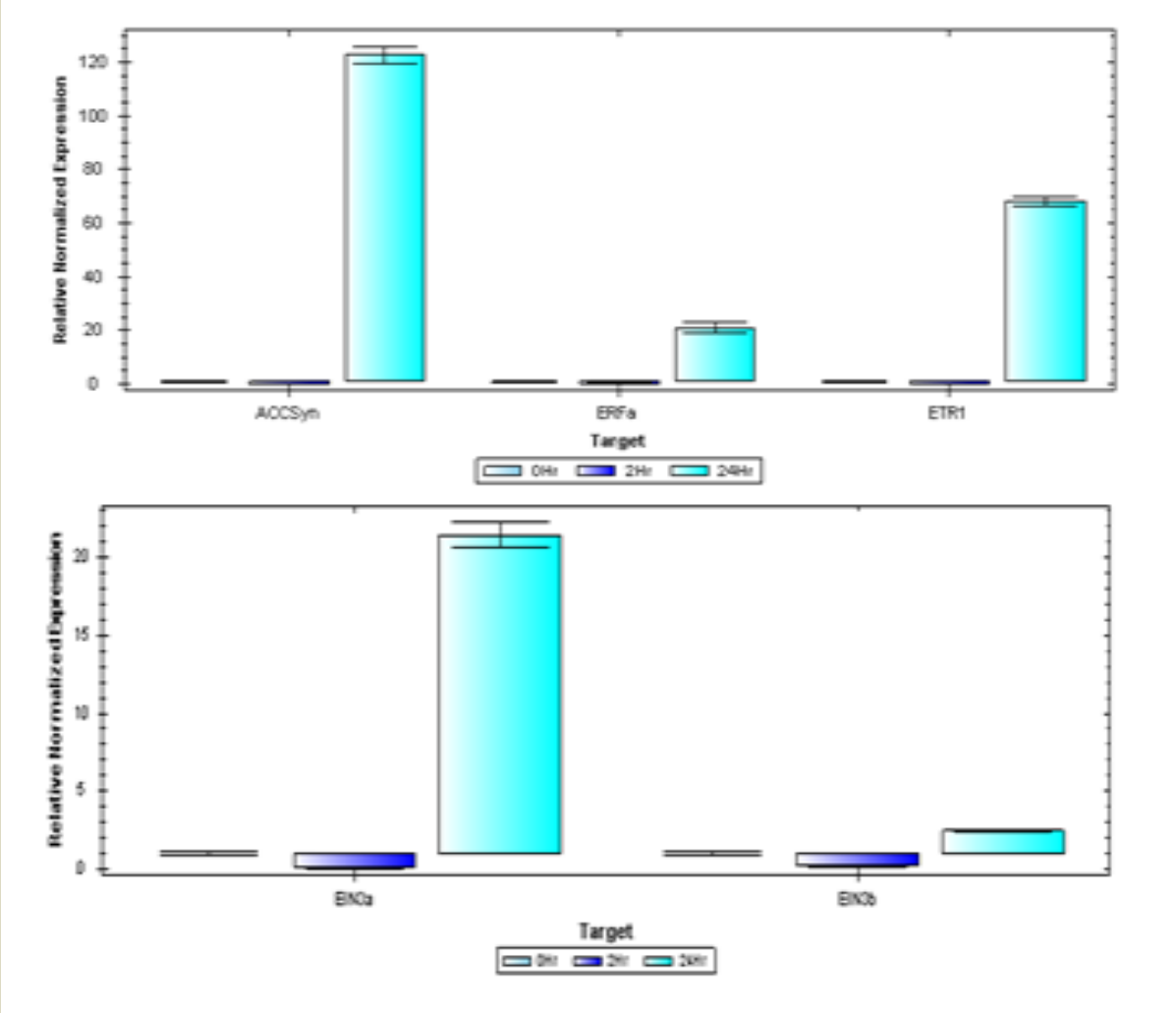

Figure 6. The quantitative gene expression of select ethylene response genes in *P. patens* following treatments of 100 micromolar ethephon at 0, 2, and 24 hour time points. Due to an error in extraction, 48-hour time point data was omitted from these results. It should also be noted that although on different respective graphs, these results were all obtained from the same batch of extracted RNA. All changes in expression are relative to the 0-hour control, with RuBisCO used as a loading control.

Pythium irregulare Growth Response to ACC and ethephon

The growth rate of *P. irregulare* was examined across three separate treatment groups of normal PDA media, ACC infused PDA media, and ethephon infused PDA media. PDA media was created under the same aforementioned protocol found in the Materials and Methods. However, following the autoclave period of PDA media, varying amounts of stock ACC and ethephon were added to each respective treatment flask. ACC flasks were brought to 20 micromolar concentrations, while ethephon flasks were brought to 100 micromolar concentrations, so as to keep concentrations consistent with the previous experiments. The experiment was replicated in three separate trials, with each treatment group consisting of four sample plates. A 3 mm^3 plug of *P. irregulare* was placed upon each sample plate 24 hours prior to the beginning of each trial in order to allow for a growth front to be initiated.

It should be noted that the first experimental trial consisted of irregular time points, with *P. irregulare* growth measurements recorded at 0, 3, 5, and 13 hours. Although the time points are irregular, the change in slope of each treatment sample were under examination in this study, and so irregularity of measurements would have a minimal effect on the rate of fungal growth over time. These experiments were conducted across three separate trials, and the results are shown in Figure 7 below.

In a combined comparison of all three trials, it was found that there was no significant difference between the growth rate of *P. irregulare* when exposed to ACC vs. control ($p>0.9$), ethephon vs. control ($p>0.2$), and ACC vs. ethephon ($p>0.3$) plates (Figure 7). However, it should be noted that in trials 1 and 3, the average initial growth front of *P. irregulare* in ethephon treatment plates was much less when compared to both control and ACC treatments (Figure 7).

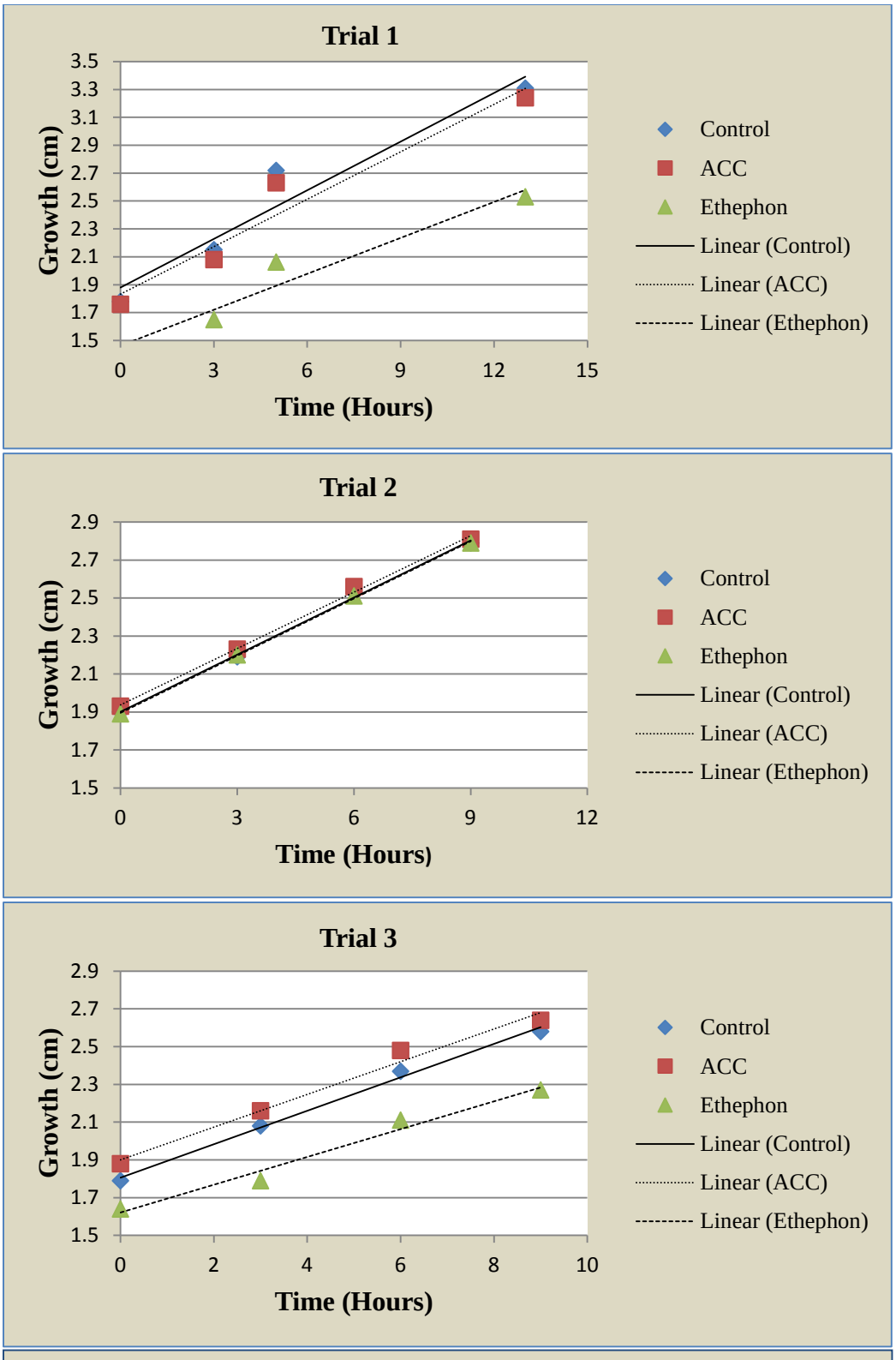

Figure 7. The average growth rate of *P. irregulare* exposed to ACC and ethephon infused PDA media across three separate trials. ACC media concentration was 20 micromolar; ethephon media was 100 micromolar. Control media consisted of unaltered PDA media. Each data point represents the average of four samples from a treatment group at a particular time point. It should be noted that in trial 1, time points are not evenly dispersed.

Discussion

Was an SAR response induced with ethylene exposed Amblystegium serpens?

In the ACC-exposed treatments of *A. serpens* prior to pathogen inoculation, it appears that little to no pathogen response was mounted in any of the four treatment groups (Figure 4A). This was expected in the 0-hour control group, but not in the other three ACC treatments. There was seemingly no differentiation among the 2, 24, and 48 hour time treatments in terms of a progressive pathogen response, although at the 48-hour time treatment there appears to be green and leafy regions still present near the center of the specimen (Figure 4A). These results did not support my initial hypothesis that ACC would elicit a clear SAR response in *A. serpens.*

Ethephon-exposed treatments of *A. serpens* yielded similar results to ACC treatments (Figure 4B). Again, a mounted pathogen response in *A. serpens* across various time-points was seemingly lacking. From the results given in Figure 4B, it is difficult to project a discernable difference among the four treatment groups ranging from the 0-hour treatment with no ethephon to the 48-hour ethephon treatment. These results also failed to support my hypothesis that, like ACC, ethephon exposure would evoke an SAR response in *A. serpens*.

Both of the results in Figure 4 were taken from single ACC and ethephon experiments, although prior and subsequent experiments yielded very similar results. The apparent lack of phenotypic pathogen response seems to imply that ethylene, at least when acting alone, has little to no effect on the pathogen response in mosses. As mentioned previously, ethylene typically works in conjunction with JA in vascular plants to respond to plant pathogens (Bari and Jones 2009 and Glazebrook). The same situation could very well be the case for mosses. Further

examination of hormone signaling coordination in the mosses is needed in order to further understand if ethylene plays a more decisive role in the pathogen response of mosses.

There are, however, several caveats in this portion of the study that must be addressed. It is plausible that the concentrations of both ACC (20 micromolar) and ethephon (100 micromolar) were not potent enough to induce a pathogen response in *A. serpens*, although this seems unlikely; changes in gene expression were detected in the gene quantification results for both ACC and ethephon treatments using the same respective concentrations (Figures 5 and 6). Also, the addition of a positive pathogen response control would have been beneficial for comparative purposes. This could be done through the use of β-glucans, an essential component to the fungal cell wall of *P. irregulare*. Future work would benefit by including this positive control to compare and contrast the differences between the known-pathogen response activation and the currently ambiguous ethylene-treated pathogen response. It is also plausible that the pathogenic inoculum size relative to the size of the moss itself was too large, and the moss was simply over-powered by the pathogen. Future work in using smaller inoculum plugs could potentially clear up whether or not this factor was playing a significant role in the moss's ability to mount a pathogen response.

Implications of Gene Identification

The genes identified for use in this study through the use of the NCBI's BLAST program (Table 2) provided further insight into the evolutionary history of the mosses and vascular plants. All genes searched for in this study yielded similar gene homologs when comparing the sequenced genomes of *P. patens* and *A. thaliana*, with the exception of the ACC oxidase gene (Table 2). Upon intensive searching of the available primary literature and thorough

investigation of the NCBI gene database, no known homolog for the ACC oxidase gene could be found for *P. patens.* However, a recently published study of the green algae *Spirogyra pratensis*—an ancestral species of land plants—found evidence for a conserved ethylene biosynthesis pathway (Chuanli *et. al* 2015). The study presents evidence that *S. pratensis* has at least one homolog of both ACC synthase and ACC oxidase (Chuanli *et. al* 2015). This also presents a convoluted case for the presence of ACC oxidase in *P. patens.* While this implies that the ACC oxidase gene is conserved and present in land plants, further searching through the primary literature and the NCBI database yields few reliable hits for a likely ACC oxidase homolog in *P. patens*. With the status of *P. patens* being a novel moss used for plant research, it is plausible that current lab-cultured stocks of *P. patens* have gradually evolved away from the conserved method of ethylene biosynthesis, though this seems unlikely. A second alternative could be that *P. patens* is truly unique in that it has its own novel process of ethylene synthesis. It is clear that further work must be done in characterizing the ethylene biosynthetic pathway in *P. patens* in order to properly determine whether ACC oxidase is present in the pathway, or if the moss in culture has recently lost the conserved ability to synthesize ethylene.

Consistent Gene Response Patterns to ACC exposure in P. patens

The gene response of *P. patens* when exposed to ACC provided a very consistent pattern of expression across two independent trials (Figure 5). These results demonstrate very clearly that *P. patens* was able to respond to and metabolize ACC through the up-regulation of ACC synthase, and that ACC was likely being synthesized into ethylene by the up-regulation of the ethylene responsive genes ERFa, EIN3a,b, and ETR1(Figure 5). The pattern of expression indicates that upon initial exposure of only 2-hours, *P. patens* has a substantially increased response to ACC. Across all genes across both trials, genes were up-regulated substantially

higher than the non-ACC exposed control moss (Figure 5). This up-regulation was especially noticeable in ETR1, which in both trials shows a greater than 15 fold-increase in expression (Figure 5). At 24 hours, hyper-expression of all genes was still occurring, with levels dropping only a few fold. At 48 hours, it is evident that most genes had returned to their normal levels of expression, save for ACC synthase and ETR1, which still showed slight increases in expression from normal levels (Figure 5).

As aforementioned, all genes selected in this study play a role in the ethylene response in vascular plants (Bleecker and Kende 2000, Clark *et. al* 1997, Lorenzo *et. al* 2003). The results shown in Figure 5 provide evidence that the response to ethylene is essentially the same in the mosses, as each gene in Figure 5 is a precise or likely homolog of the ethylene biosynthesis and response genes present in *A. thaliana*. As also previously noted, ERFa, protein in the family of the ERF1 proteins, plays an indirect role in pathogen response in vascular plants through aiding in the transcription of pathogen response genes (Lorenzo *et. al* 2003). This provides evidence that ethylene may play a role in the pathogen response of *P. patens*, although in vascular plants ERF1 is typically activated in conjunction with ethylene and JA (Lorenzo *et. al* 2003).

Additionally, the substantial increase in expression of ETR1—an ethylene receptor protein—could be due to the positive feedback effect that ethylene production is known to have in vascular plants (Kende 1993). As ethylene is detected in a plant, more is produced, and therefore a greater increase in production of an ethylene receptor protein such as ETR1 would make logical sense. It is also curious that EIN3a and b, both in the same class of ethylene signaling molecule, had starkly different levels of expression, with EIN3a showing a much greater expression than EIN3b (Figure 5). These two molecules could play slightly different roles

in ethylene response that requires the greater expression of EIN3a over EIN3b, but little is known regarding this matter.

Gene Response Patterns to Ethephon exposure in P. patens

Unlike ACC, obtaining a concise response to ethephon exposure in *P. patens* was a much more difficult task, as is evident in Figure 6. Although ethephon showed a relatively consistent response to ethephon exposure across all genes in Figure 6, these results proved difficult to replicate. The given results are lacking a 48-hour treatment, and they only consist of one trial. However, although the 48-hour response is missing from Figure 6, there was data collected during this study for the 48-hour response that showed a very similar pattern of expression levels to that of the 2-hour expression (data not shown).

At 2-hours of exposure to ethephon in *P. patens*, it is clear in Figure 6 that little to no change in expression occurred in any of the five selected genes. After 24 hours, all six genes showed a substantial increase in gene expression, especially ACC synthase, EIN3a, and ETR1 (Figure 6). It should also be noted that ACC synthase demonstrated a very substantial increase in expression of 120 fold (Figure 6). While this could likely have been due to an error in analysis during qRT-PCR, it is still of interest that ACC synthase was being up-regulated by ethephon, a chemical that is not involved directly in the ACC biosynthetic pathway. It could be that the eventual break-down of ethephon to ethylene could have triggered the expression of ACC synthase, since ACC synthase is typically active when ethylene is present.

The results obtained in Figure 6 demonstrating a pattern of delayed expression at 2 hours but greatly increased expression at 24 hours in ethephon is in stark contrast to the ACC results in Figure 5. Since ethephon is metabolized rather than synthesized by a biosynthetic pathway like

ACC, it is possible that upon exposure it took *P. patens* some time to break down ethephon into ethylene. Due to inconsistencies in results and the differing patterns of expression in comparison with ACC, It is clear that much more work with ethephon needs to be carried out in further studies in order to better understand its potential effect on the pathogen response in *P. patens*. A further exploration of its role in a moss species could provide a deeper understanding of the role that ethephon could play in pathogen resistance in vascular plants, as it is currently used as a plant growth regulator (www.sigmaaldrich.com).

No Apparent Change in Growth of P. irregulare on ACC or Ethephon Plates

Across all three trials of *P. irregulare* growth measurements, there was no significant difference found among the growth-rate of the fungus on control, ACC, and ethephon plates (Figure 7). The differences in the slopes compared directly between control and ACC, control and ethephon, and ACC and ethephon across all three trials showed essentially little to no significant differences. These results were expected; although there was some consideration that ethephon might affect fungal growth, given its seeming delayed effect of ethylene production in *P. patens* previously mentioned.

In both trials 1 and 3, *P. irregulare* initially grew slower in the presence of ethephon compared with ACC or the control plates (Figure 7). This could be due to a growth inhibiting effect of ethephon until it is broken down. However, it could also be due to inconsistencies in methodology of placing pathogen plugs onto the ethephon media, or the proper preparation of ethephon media (as might very well be the case in trial 2). Regardless, though ethephon might have a growth effect on *P. irregulare*, there is no strong evidence that either ACC or ethephon have a negative effect on the growth rate of *P. irregulare.*

Future Directions

The evidence accumulated through this study has provided the opportunity to take several different directions forward in order to further understand the role that ethylene has in an SAR response in moss. The results given from the phenotypic SAR study with *A. serpens* indicates that further experimentation needs to be done in order to better define if ethylene plays a definitive role in an SAR response. The addition of a positive defense response control through the use of β-chitosan would prove especially useful for comparison purposes. Utilizing different concentrations of ACC or ethephon might also be effective in activating an SAR response in *A. serpens*. Direct exposure of gaseous ethylene could also be a potential option in order to understand if ethylene plays a role in SAR, although this route was initially avoided in this study due to the difficulty and expense of working directly with ethylene gas. Lastly, working in conjunction with other hormones such as SA and JA would benefit this study, as it is highly plausible that ethylene is not capable of initiating an SAR response in moss alone, as is often the case in vascular plants (Lorenzo *et. al* 2003).

The effects of ACC on gene expression in *P. patens* yielded consistent results, although it is clear that more work needs to be done with ethephon. A potential next step would be to acquire known PR gene homologs in *P. patens* and test to determine with greater certainty that ethylene is capable of activating PR genes and potentially initiating an SAR response. ERFa is a transcription factor known to play an indirect role in pathogen response in vascular plants (Lorenzo *et. al* 2003), and so it is quite likely that PR genes could be activated through exposure to ethylene in *P. patens*. Another option is to treat *P. patens* with ethylene prior to pathogen inoculation, as was done with *A. serpens*, as well as expose *P. patens* directly to *P. irregulare* and then examine the changes in gene expression. Examining changes in gene expression at

smaller time intervals between 0 and 2 hours would also be insightful in better understanding just how quickly *P. patens* is capable of responding to ethylene/initiating a pathogen response. An additional option is to use RNAi technology to knock-out ethylene response genes in *P. patens*, and then quantify the effects of ethylene exposure on gene expression following the knock-out. This could aid in determining if the change in regulation of one gene can significantly affect the change in regulation of another if the gene is rendered inactive through knock-out.

The study of the growth-rate of *P. irregulare* on ACC and ethephon was performed in part to determine if either chemical was significantly affecting the virulence of the fungus. The evidence implies that a delaying effect might be present on the initial growth of the fungus, but there is no significant change in the growth-rate of *P. irregulare* when exposed to either ACC or ethephon. For more concise validation of data, further experiments should be performed in order better understand the potential delaying effect that ethephon has on fungal growth.

Conclusion

This study aimed to elucidate the role of the vital plant hormone ethylene in systemic acquired resistance in moss. In order to do this, the moss species *A. serpens* and *P. patens* were used as test subjects, while the known ethylene precursor molecules ACC and ethephon were used for ethylene exposure, and the fungal pathogen *P. irregulare* was used for inoculation purposes. In *A. serpens,* it was inconclusive whether ethylene played a direct role in an SAR response in both ACC and ethephon treatments. Further work in categorizing the SAR response in *A. serpens* is needed in order to provide more conclusive results. Exposure of *P. patens* to ACC induced a substantial increase in ethylene responsive genes at 2 and 24 hours, while exposure to ethephon caused a delayed increase in expression of ethylene responsive genes in *P. patens.* Continued work with ethephon is needed in order to provide more substantial evidence that it is able to consistently up-regulate ethylene responsive genes upon exposure to *P. patens.* Finally, it was found that neither ACC nor ethephon had a significant effect on the growth rate of *P. irregulare.*

The goal of this study was to provide further insight into the origins of pathogen resistance in plants, in an effort to better understand how plants have evolved their mechanisms of defense against pathogens. It is with hope that this study will someday aid in a more holistic understanding of plant-pathogen resistance, particularly in agriculturally relevant plants. In order to better shape the future, it is crucial to understand the past. By examining the capabilities of pathogen resistance in non-vascular plants, the evolutionary ancestors of all vascular and agricultural plants, that is what this study set out to do.

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