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Quantification of Salicylic Acid and Analysis of Defense-Related Genes in the Moss Physcomitrella patens

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In Partial Fulfillment
of the Requirements for Graduation Honors

Ryan Joseph Eller

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This thesis is dedicated to Harry and Billie Reinholt; two people who dedicated their lives to the plants they grew and their family.
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1.1 Abstract

The moss *Physcomitrella patens* is an invaluable non-vascular plant specimen to study genetic interactions because its entire genome is sequenced. Therefore, any gene studies conducted in more evolved vascular plants can be transferred to this specimen fairly easily by utilizing comparative genomic techniques made possible through the National Center for Biotechnology Information (NCBI). The questions that were asked in this thesis revolved around the Systemic Acquired Resistance, or the basic mechanism that allows plants to protect themselves against biotic and abiotic stressors. Two main approaches were used to analyze this complex system. First, an analytical chemistry technique was used to quantify the products formed from this system, mainly the plant hormone salicylic acid. Second, the defense-related genes that code for these hormones were also analyzed. The findings of this study are strictly preliminary, but it does appear that the defense-related PAL genes play a major role in plant defense when exposed to a pathogen-like elicitor.

1.2 Introduction

We experience pathogens every day from the bacteria sitting on our desk to the virus lying on the doorknob. In order to stay healthy we normally practice good hygiene and we may even avoid pathogen infested areas, such as hospital waiting rooms, when we can. If all else fails, we have our immune system that protects us even if the pathogens enter our bodies. However, it may seem as though plants have a harder time avoiding pathogens since they are stationary and cannot practice good hygiene. Despite these disadvantages plants are able to protect themselves from the pathogens they encounter due to their complex "immune system." One of the first barriers that plants employ to deter pathogens is their physical barriers. Similar to the epidermis of our skin, plants use their cell walls which are made of cellulose and their cuticle which is made of cutan and cutin to prevent pathogens from entering into their interior.
1.2.1 Hypersensitive Response

Similarly, if pathogens breach the plant’s exterior, the plant initiates a series of localized reactions in order to contain the pathogen. This localized response, otherwise known as the hypersensitive response (HR), is the second defense plants have against pathogens. Resistance (R) proteins bind to antigens found on the surface of pathogens, which in turn causes a signal cascade that results in the formation of local lesions that contain antimicrobial compounds (Orlandi et al., 1992; Koyanagi et al., 2000). In the second phase, the triggered cells begin to rapidly generate superoxide, peroxide, and hydroxyl radicals (Baker et al. 1993) which are highly toxic. Superoxide is produced in plants by the enzyme NADPH oxidase, which is functionally similar to activated neutrophils (Lamb and Dixon, 1997) – a type of white blood cells that helps fight off infection in mammals. These oxidizing agents, as well as salicylic acid, aid in the expression of the disease-fighting mechanism of the HR. The thickening of the plant’s cell wall followed by programmed cell death or apoptosis in the adjacent cells (Bowman, 2011), normally isolates and quarantines the pathogen to a certain area preserving the rest of the organism. In addition, various pathogen-related (PR) genes are activated during the HR. Some of these genes code for PR proteins which may be hydrolytic enzymes (e.g. \( \beta-1,3\)-glucanases and chitinases) or proteins that have antimicrobial properties (Durner et al., 1997).

1.2.2 Systemic Acquired Resistance

A more encompassing response is the systemic acquired resistance response (SAR) which prevents infection in a broader manner due to the expression of additional pathogenesis-related genes. Various studies such as Grüner et al., 2003 suggest that the HR activates genes that increase the production of pathogen-related proteins that are involved in the SAR. Some of the hormones that have been studied, and are proposed to be used in this response, are ethylene, jasmonic acid, and salicylic acid (Oliver et al., 2009). These hormones trigger various anatomical and physiological changes in the plant which include senescence, growth inhibition, and the release of phytoalexins.
(antimicrobials). However, in previous research certain hormones were triggered depending on the nature of the pathogen (Oliver et al., 2009).

### 1.2.3 Salicylic Acid

Salicylic acid (SA) has been found to play a prominent role in the SAR response in tobacco plants infected with the tobacco mosaic virus and in diseased *Arabidopsis thaliana* (Lennon et al., 1997). Salicylic acid appears to have a wide range of functions such as acting as a signaling molecule for the SAR, which induces the expression of other defense-related genes (Shulaev et al., 1995), increasing the production of H$_2$O$_2$, inducing apoptosis, or activating genes normally induced by fungal elicitors or wounds (Shirasu et al., 1997; Kauss et al., 2011; Mur et al., 2002). The biosynthesis and eventual metabolism of SA involves the expression and silencing of a multitude of genes that result in the formation of enzymes that act as the regulators of the biosynthetic pathway. From the evidence presented in previous papers (Dempsey et al., 2007), I believe that there are two primary paths (See Figure 1) involved in SA production from chorismate, the end-product of the shikimate pathway. From our current knowledge, the IC and PAL pathways, whose names are derived from their corresponding regulatory genes, are these two paths.

According to Dempsey et al., 2011 the isochorismate (IC) pathway is the primary route for SA production in *A. thaliana* and is regulated by the ICS gene. SA also undergoes numerous modifications, such as glucosylation, methylation, and amino-acid conjugation, after it is produced in the cells (See Figure 2). Most modifications render SA inactive. For example, methylation allows for the transport of SA since it inactivates SA while increasing its membrane permeability. Glucosylation also inactivates SA and allows for vacuolar storage of the modified molecule. Thus, the quantity of free SA in the cell at any time is regulated by the rate of production and the metabolism of free SA.
Figure 1: Potential pathways for the biosynthesis of SA. The IC pathway is shown on the left while the phenylalanine ammonia-lyase (PAL) pathway is shown on the right. Open arrows indicate flux in the pathways, while the arrows above the arrows (Dempsey et al., 2011) indicate the enzymes that convert the metabolites are shown.
Figure 2: Modifications of SA. Arabidopsis proteins that have the capacity to catalyze the reaction are shown. All modifications with the exception of SA-2-sulfonate have been detected in plants, including Arabidopsis (Dempsey et al., 2011).

1.2.4 Physcomitrella patens

One of the many problems with studying an intricate system such as the SAR is that it has numerous layers of complexity, which makes it significantly harder to study. If scientists try to study this process in complex organisms, then the problems may become exacerbated. Therefore, when studying a complex response such as the SAR, it may be beneficial to use a less complex model. While all plants are complex, bryophytes tend to be less complicated than some of their more evolved relatives who have developed
vascular tissue. Thus, by studying the complicated SAR response in this comparatively simple organism, the task of elucidating the mechanisms involved in SAR becomes much easier. The model that was used in this experiment was a bryophyte that has been studied intensively and, as a result, has had its entire genome sequenced. Ultimately, this allows for a more simplified analytical and genetic analysis.

_Physcomitrella patens_ is a bryophyte that belongs to the *Funariaceae* family and is found scattered through the temperate areas of the world with the exception of South America (Rensing et al., 2009) inhabiting wet areas surrounding rivers, lakes and are present in moist forests. It is characteristic of moss to transfer between a haploid and a diploid phase of the life cycle (Figure 3). A diploid sporophyte produces a sporangium that releases spores (n), which develop into either male or female gametophytes. Sperm produced by the male gametophyte’s antheridia is washed into a female gametophyte’s archegonia which contains an egg. Fertilization occurs and a 2n sporophyte is produced, which produces spores via meiosis and the process repeats. The moss is useful for scientific study since it is small, anatomically simple, and the dominant phase of its life cycle is the haploid gametophyte. The moss’s haploid nature allows for more simplified genetic analysis (Rensing et al., 2009).
Figure 3: General moss life cycle (Campbell and Reece, 2005)
Despite the research conducted on vascular plant "immune systems" such as the economically important plants such as *Zea mays* (Corn) and *Glycine max* (Soybeans), less is known about the response in the less derived non-vascular plants. Due to the complex nature of vascular plants, by studying the less intricate non-vascular organisms, it may be easier to determine the relationship between these genes and the complex defense pathways they trigger.

### 1.2.5 *Pythium irregulare*

In addition, to trigger the SAR response in *P. patens* in order to study SA and the genes that control its production, it is necessary to use either a pathogen or a chemical elicitor that mimics a pathogen. In this experiment, I used the pathogen *Pythium irregulare* to trigger an immune response and the chemical elicitors chitosan and β-Glucan. *P. irregulare* is a soil-borne, white, non-sporulating, filamentous fungus that belongs in the Oomycetes class. *P. irregulare* is found worldwide on hundreds of various plants. Generally, oomycete fungi can reproduce either sexually producing oospores or asexually via zoospores (Figure 5).
During infection *P. irregulare* uses hyphal germ tubes (Van West et al., 2003) to enter through the moss cell walls or the Oomycete may infect the plant indirectly by forming sporangia on the mycelium, which produce zoospores that infect the moss by forming a germ tube (Katawczik, 2008) from which the appressorium forms. Subsequently, the penetration peg, which penetrates the cell wall, grows from the appressorium (Figure 6). This process of infection followed by some form of reproduction is what allows the fungi to efficiently infect over 200 host species on all major continents excluding Antarctica (Farr et al., 2004). Conversely, *P. irregulare* may survive saprophytically on the dead plant tissue in the hyphal form by producing new strands of mycelium on the infected plant indefinitely until either the food source is depleted or other environmental conditions make living difficult. If either of these conditions is met, the Oomycete will produce a dormant oospore allowing the organism to wait for more favorable growing conditions. It is important to note that *P. irregulare* is a necrotrophic pathogen, which

**Figure 5: Pythium Root Rot Life Cycle** (OMAFRA Staff, 2012).
correlates to its affinity to infect and kill host tissue. Conversely, biotrophic pathogens colonize living plant tissue and receive nutrients from the plant without killing the host cells.

1.2.6 Necrotrophic vs. Biotrophic Pathogens

The difference between biotrophic and necrotrophic pathogens is imperative in determining the success of the plant’s defense mechanisms. For example, necrotrophic pathogens seemingly would benefit from plant defense mechanisms that trigger apoptosis while biotrophic pathogens would suffer since they receive nutrients from living cells. Fittingly, plants have adapted methods to respond to both types of pathogens. If the plant is attacked by biotrophic pathogens, the HR and, if needed, the SAR is triggered, which

**Figure 6: Process of fungal infection.** A) Germ tube formation from spore; B) Appressorium formation from germ tube; C) Penetration peg formation from appressorium (Wilson and Talbot, 2009).
attempts to quarantine the pathogen in cells and then destroys the cell with highly toxic oxidizing agents. If the pathogen is necrotrophic, the plant utilizes a different mechanism that employs the jasmonic acid (JA) signaling pathway. Research has suggested that JA pathways recruit rhizosphere microbial communities for their anti-herbivory and antimicrobial properties (Carvalhais et al., 2013). Even though both SA and JA pathways aid in particular responses to pathogens, several experiments have suggested that there is cross talk between the two pathways that inhibit the other when one is triggered (Zarate et al., 2007; Carvalhais et al., 2013). Since \textit{P. irregulare} is a necrotrophic pathogen a logical prediction would be that the amount of jasmonic acid should be elevated in comparison to salicylic acid upon infection with \textit{P. irregulare}.

1.2.7 Salicylic and Jasmonic Acid

Besides being one of the main active components of Aspirin (acetylsalicylic acid), salicylic acid is a phenolic phytohormone that is produced in plant tissue after a hypersensitive response to a pathogen or other forms of stress. Due to the research on Arabidopsis and other vascular plants (Oliver et al., 2009), salicylic acid has been found to be integral to the SAR response. As mentioned previously, jasmonic acid is also important in other defense related mechanisms that aid the plant in disease and herbivory prevention. Therefore, understanding these pathways genetically and chemically is an important aspect of plant research due to its connection with improving plant health by utilizing a protection mechanism that is already present in plants.

However, detecting and quantifying salicylic acid amid the copious quantities of other cellular components is a task that requires an efficient extraction procedure and finely calibrated instrumentation. Previous studies such as Segarra et al., 2006; and Forcat et al., 2008 have outlined various methods to isolate the hormone, both of which utilized High-Performance Liquid Chromatography – Mass Spectrometry (HPLC-MS). The methods involve various extraction steps that are performed under acidic conditions followed by running the purified samples through HPLC-MS under basic conditions (Segarra et al., 2006) and under acidic conditions (Forcat et al, 2008). Therefore, this study will utilize the methods outlined in these previous studies in order to quantify the levels of SA in the
bryophyte model. In particular, this experiment will rely mainly upon the separation and quantification abilities of the HPLC-MS.

1.2.8 High Pressure Liquid Chromatography (HPLC), UV-Vis Spectrophotometry, and Mass Spectrometry

HPLC is a chemical tool that separates compounds based on their physical differences. While chromatography, in general, can separate compounds based on polarity and size, Reverse Phase HPLC involves passing an analyte through a column that contains a modified silica stationary phase that has a polar alkyl group attached to the silica. In addition, a relatively non-polar mobile phase is passed through the column to elute the compounds. Therefore, depending on the polarity of the analytes in the matrix, the analytes will elute from the column at different times, which effectively separates the compounds. Once the compounds have eluted from the column they can be detected by using several instruments. The UV-Vis detector identifies the compound by measuring the difference between the light inputted into the sample and the light emitted from the sample at a particular wavelength (the wavelength of light inputted into the sample varies depending on the target compound). Unfortunately, UV-Vis is only sensitive enough to detect relatively large amounts of a compound (in the ppm range), but smaller amounts tend to not be easily detectable. Thus, when the concentration falls below 1-5ppm the mass spectrometer (MS) is used to determine the concentration since it has a greater sensitivity. In addition to having greater sensitivity, the MS is also able to isolate certain masses and determine the compound’s identity by analyzing the fragmentation patterns that are produced when the machine fragments the selected ion. By utilizing these three machines, it is possible to separate a targeted compound from a complex matrix, determine if there is a large or small quantity of the compound via UV-Vis and/or MS, and finally determine the exact identity of the compound by using fragmentation data collected by the MS.
1.3 Thesis Statement

I hypothesize that during the course of 24 hours, the salicylic acid level will be minimal upon exposure to P. irregulare. In addition, the PAL and ICS1 genes (see page 9) will be expressed at high levels within several hours of being exposed to chitosan and/or β-Glucan and then will normalize over the course of the next 12-24 hours.

2.1 Methods and Materials for the Quantification of Salicylic Acid

2.1.1 Plant Material and Growth Conditions

Physcomitrella patens was cultivated on solidified (1% [w/v] agar) BCD mineral media [250 mg L⁻¹ KH₂PO₄, 250 mg L⁻¹ MgSO₄·7H₂O, 1.01 g L⁻¹ KNO₃, 12.5 mg L⁻¹ FeSO₄·7H₂O, 8 g L⁻¹ agar, with 1 mL added of trace element solution, adjusted to a pH of 6.5 with KOH] on 100x15mm petri dishes at 22°C with a 16-h-light/8-h-dark regime under Sylvania F40/CWP 4100K white light (long-day conditions).

2.1.2 Plant Inoculations

Originally, the fungus, P. irregulare, was to be used in the experiment. However, after working with the fungus, it appeared as though chemical elicitors would provide more consistent results. Therefore, several immune system elicitors were used to activate the P. patens SAR response. β-glucan (Sigma Aldrich) and chitosan (Sigma Aldrich) were the two chemical elicitors used in the genetic experiments that focused on the activation of PAL and ICS1 genes, while Pythium irregulare was used primarily during the analytical chemistry experiments that attempted to design a method to quantify salicylic acid. Since the analytical experiments turned out to be largely qualitative, the less reliable inoculation of the moss to P. irregulare did not create any substantial deviations with the results. P. irregulare was grown on potato dextrose agar (PDA) [4.0 g L⁻¹ potato starch, 20.0 g L⁻¹ glucose, and 15.0 g L⁻¹ agar] and grown on 100x15 mm petri dishes at 22°C.
The inoculations of all moss samples treated with the fungus, or the chemical elicitors were conducted at zero, two, four, and twelve hours.

2.1.3 Preparation of Standards and Buffers for High-Powered Liquid Chromatography

Even though SA was the primary compound of interest, standards for both SA and JA were prepared in order to optimize the HPLC-ESI-MS system. If quantification of SA proved successful, JA was meant to undergo analysis for the purpose of another related study. SA and JA standards were prepared from Analytical Grade 99.5-100.5% Salicylic Acid (Sigma Aldrich) and BioReagent Grade Jasmonic Acid (Sigma Aldrich) in the 200ppb to 300ppm range. The linear range for the MS was found to be from 500ppb to 50ppm. Liquid chromatography buffers and extraction buffers were also made from ammonium acetate (Flinn Scientific Inc.), CHROMASOLV® Plus for HPLC Methanol (Sigma Aldrich), CHROMASOLV® >99.9% Acetonitrile (Sigma Aldrich), and ASC Reagent Grade Glacial Acetic Acid (Fisher Scientific).

2.1.4 Optimization of the Mass Spectrometer for Salicylic and Jasmonic Acid

Due to the structures of salicylic and jasmonic acid, deprotonation of both structures by subjecting the compounds to a basic solvent appeared to give the best MS signals. Salicylic acid (SA) and jasmonic acid (JA) were found to contain prominent fragment ions at 92.7 and 93.9m/z for SA, and 58.9 m/z for JA under basic conditions (pH is approximately 6.6). However, at low concentrations (200-500ppb) the signals of salicylic acid were diminished by post source decay presumably at the capillary. Increasingly negative capillary voltages appeared to hinder the [M-H]- signal at 137 m/z in favor of the 119.9 m/z signal, which is suggested to be salicylic acid with a deprotonated carboxyl group and a cleaved hydroxyl group at the ortho position by Dr. Kaz Surowiec at Texas Tech University (See Table 1 and Figure 7 for an outline of precursor and product ion observed). However, when the samples were run through the LC-MS at higher capillary voltages (which favor the jasmonic acid fragmentation) SA fragments from the 137 ==> 93.9 were quantifiable down to concentrations of 200ppb. The 93.9 m/z fragment from SA is presumably the phenol fragment (pKa of 9.95) while the 92.9 m/z fragment is the
more easily seen deprotonated phenol fragment (since the MS is operating in the negative mode). JA was also found to contain strong daughter ions of 59.9 m/z when the [M-H]⁻ ion at 209 m/z was fragmented via MS/MS. However, a potential pitfall was discovered when the 119.9m/z signal was also found to occur naturally in JA standards (which is also present in SA standards). The fragment at 119.9m/z was found to break down into 58.9m/z which is why fragmentation via MS/MS is ideal in positively identifying and quantifying SA and JA.

Table 1: Fragmentation data observed during SA and JA optimization.

<table>
<thead>
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<tr>
<td><strong>Precursor Ion</strong></td>
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<td>:---------------</td>
</tr>
<tr>
<td>Salicylic Acid (Fig.7A)</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Jasmonic Acid (Fig.7B)</td>
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Figure 7: Salicylic and Jasmonic Acid Fragments.

A) Salicylic Acid; B) Jasmonic Acid; C) Deprotonated Phenol; D) Phenol; E) Deprotonated Carboxyl with cleaved hydroxyl group; F) deprotonated acetic acid.

2.1.5 Optimization of High-Powered Liquid Chromatography for Salicylic and Jasmonic Acid

High-powered liquid chromatography was used in tandem with the mass spectrometer in order to separate the target compounds from the other cellular impurities in the sample. A Phenomenex Luna 3u C18(2) 100A column was used with a solvent gradient of 100%A to 100%B for 20 minutes. The system was then allowed 5 minutes to re-equilibrate to 100% A. Mobile phase A consisted of a 10mM ammonium acetate solution containing 95% H2O and 5% CH3CN while mobile phase B consisted of a 10mM ammonium acetate solution containing 5% H2O and 95% CH3CN. Both of these mobile phases were adjusted with NaOH to a pH of approximately 6.6. SA and JA standards showed excellent resolution with SA eluting at around 3 minutes and JA at around 6.5 minutes.

2.1.6 Quantification of Salicylic Acid in Moss Samples

Approximately 300mg of fresh moss tissue was collected and weighed. To eliminate inconsistencies with varying water content of moss tissue, the samples were freeze dried via a VirTis Benchtop lyophilizer. Prior to lyophilizing, the moss samples were boiled for three seconds in order to remove any residual agar attached to the plant. Moss samples were then immediately dried with a chemwipe and frozen with liquid nitrogen. The
frozen samples (roughly 300mg) were placed in a VirTis Benchtop lyophilizer for at least 3 hours. After the sample was freeze dried, the mass of the sample was calculated by differential weighing.

2.1.7 Extraction of Salicylic Acid and other Metabolites

Extraction of the targeted salicylic and jasmonic acid was performed by adding 750 μL of a MeOH based extraction buffer (90:9:1 MeOH-H_2O:HOA) as described by Segarra et al., 2006 to roughly 50mg of freeze dried tissue. After each addition of the extraction buffer, samples were vortexed and centrifuged for 6 minutes at 10,000 rpm in a Beckman Model J2-21M Induction Drive Centrifuge. After centrifuging, the green colored supernatant was collected and the process was repeated several times.

2.1.8 Concentration of Salicylic Acid samples

Due to the relatively small quantities of salicylic acid expected from the small amount of moss tissue harvested, the supernatant was concentrated by using gaseous nitrogen to evaporate the MeOH. Concentration by roto-evaporation and lyophilization was also attempted, but proved to be less efficient due to loss of sample in the collection vessel.

2.1.9 Re-suspension of Salicylic Acid and Clean Up

Re-suspension of the dried salicylic acid was accomplished by saturating the dried solute encrusted on the sides of the test tube with a water based extraction buffer (0.05% acetic acid in 85:15v/v of H_2O-MeCN) as described in Segarra et al., 2006. Due to the considerable amount of noise reported by the LC-MS, cleanup by using a combination of charcoal and Varian and Discovery C18 SPE columns were used to filter out organic compounds. Due to the poor performance of the Discovery C18 columns, Varian C18 SPE columns were used primarily for sample cleanup. After filtering, cleaned extracts were filtered through a 0.2 micrometer syringe filter and injected into the LC-MS.
2.1.10 Correction of Noise by Utilizing Standard Addition

Ideally, an internal standard such as benzoic acid (in the case of salicylic acid) would allow for any sample loss or background interference. However, since benzoic acid is a precursor for the formation of salicylic acid in the plant tissue, these internal standards would have failed to work. Instead, before injection, the samples were spiked with 200 ppb and 500 ppb salicylic acid standards.

2.2 Results and Discussion for Salicylic Acid Quantification

Despite numerous and varied approaches to elucidate an efficient method of SA quantification via LC-ESI-MS/MS three major roadblocks hindered the efforts proposed in this thesis. 1) The hindrance of the SA signal by other compounds in the plant matrix, 2) the inconsistent performance by the university’s Varian 310 LC-triple quad (possibly caused by the first factor), and 3) the volatility of SA prevented accurate and reproducible quantification of salicylic acid in the moss tissue. Each one of these roadblocks was discovered through a series of small related experiments, which are described in the sections below. More importantly, however, is the fact that many of these problems (SA signal hindrance and SA volatility) were somehow surmounted by Forcat et al., 2008 even though the samples were prepared in a similar fashion and my LC column was identical to theirs. Yet, despite all of the factors that hindered my efforts to quantify SA and JA, I was able to validate the potential of analyzing these two substances simultaneously as stated in previous studies (Forcat et al., 2008; Engelberth et al., 2003; and Segarra et al., 2006).

2.2.1 Potential of SA and JA Quantification via HPLC-MS/MS

One finding of this study is there is substantial evidence that supports the findings of previous studies, which state that SA and JA can be measured simultaneously by utilizing reverse phase liquid chromatography (RPLC) in tandem with mass spectrometry (Forcat et al., 2008; Engelberth et al., 2003; and Segarra et al., 2006). By running JA and SA standards through the HPLC system in conjunction with the Varian 310 LC-triple quad mass spectrometer, I was able to achieve a good separation between the two compounds.
of interest due to RPLC, and excellent identification of the compounds by using multiple reaction monitoring on the MS (See Figure 8).

2.2.2 Hindrance of SA Signal by Compounds in the Plant Matrix

One of the first issues that became apparent from the first mass spectrometer analysis of the plant extract (prepared by using the method outlined in Forcat et al., 2008) was the abundance of other cellular material that confounded the total ion chromatogram (TIC). Even after separation via high powered liquid chromatography (HPLC), the other contaminants in the sample were highly noticeable during the organic buffer wash at the end of the solvent gradient (See Figure 9). More troublesome was the fact that contaminants suppressed the salicylic acid signals. In order to validate this claim, plant extracts were spiked with SA standards and then run through the LC-MS. It is apparent from Figure 10 that the SA signal that was present when running the standard is not even observable in the extract spiked sample. Therefore, it is interesting that Forcat et al., 2008 was able to see a signal for SA since the sample matrix suppresses the signal. Additional, sample cleanup utilizing charcoal and Varian C18 SPE columns were utilized and the visible results can be clearly seen in Figure 11. However, it appears as though the matrix still suppresses the signal even with this extra purification step as seen from Figure 12.
Figure 8: Chromatograms that demonstrate the separation and identification power of LC/MS. A) Chromatogram in MRM mode of 137/92.7, which is characteristic of SA and B) 209/58.9, which is characteristic of JA. C) UV/Vis chromatogram is included to illustrate the ambiguity of UV/Vis chromatography at low concentrations.
Figure 9: MS and UV/Vis impurity chromatograms. MS and UV/Vis illustrate the abundance of residual impurities present in the unfiltered moss sample. A) The total ion chromatogram (TIC) and C) the UV/Vis chromatogram illustrates that additional compounds are eluting from the column as the mobile phase gradient becomes increasingly organic. B) The TIC and D) the UV/Vis chromatogram of the blanks even show additional compounds eluting from the column at the 10 minute mark, which is when the gradient is 50% A (aqueous) and 50% (organic) demonstrating residual matrix eluting from the column.
Figure 10: Suppression Chromatograms. The suppression of the SA signal can be clearly seen in A) the extracted ion chromatogram (XIC) for SA of a moss sample that has been spiked with 200ppm SA, since no definitive peak can be seen around the 3 minute mark. The XIC for JA B) and the UV/Vis chromatogram C) are included to illustrate that no traces of any compound of interest could be identified.
Figure 11: Salicylic Acid Cleanup. Salicylic Acid Matrix before (A) and after (B) sample cleanup.
Figure 12: **SA Cleanup Chromatograms.** Plant extract spiked with a standard solution of SA to yield a final concentration of 5ppm. A) The XIC of the 5ppm standard solution is given as a reference to compare to B) the SA spiked moss sample run through a Varian C18 SPE column, and C) another SA spiked moss sample run through a C18 SPE column and charcoal. Notice that the addition of charcoal allows for the expression of the SA signal.
2.2.3 Mass Spectrometer Inconsistencies

One of the principal components of good science is reproducibility. Reliable and reproducible data is necessary to compare data from multiple runs where a single variable is being changed. Thus, in well controlled experiments, the differences between samples are due to the alteration of the independent variable. While instrumentation and user-created variability is inevitable, these extraneous sources of variability should be minimized. Therefore, since the mass spectrometer’s performance varied over the course of time in which this experiment was being conducted, the confidence in the data collected decreases significantly. The reliability of the instrument was questioned after performing two calibration curves spanning the course of two and a half months. While the variation of each standard (since the standards were run in triplicate) was small, the peak intensities between identically made standards were significantly different (Figure 13). Even though the settings of the MS were nearly identical, the significantly smaller signal observed in the second calibration could be attributed to several factors: contamination of the LC column or front-end contamination of the MS. By performing the method as described in Forcat et al., 2008 one explanation could be that the LC column may have become contaminated with residual matrix material. By reducing the efficiency of the column, the resolution of the salicylic acid will decrease due to band broadening. Thus, when the salicylic acid runs through the mass spectrometer, the SA will arrive at the detector over a longer period of time (producing a smaller signal since the SA is more dilute), instead of arriving “in bulk” over a shorter period of time (resulting in a larger signal). However, after observing the chromatograms seen in Figure 13, which compares identically prepared standards, I believe that a lower resolution is probably not the cause of the difference since the width of the peak corresponding to SA does not appear to be significantly different. However, I do believe that the moss sample contaminated the column since the baseline of the second calibration was found to be significantly more unstable (this was determined by using the Varian’s MS analyzing software and is not easily recognizable from the chromatograms due to different scalings). The extra contamination that I saw was probably caused by the other components in the matrix, since, in the Forcat method, no sample cleanup (such as solid
phase extraction) was utilized. As mentioned in the previous section, the cellular contaminants seemed to suppress the SA signal. Thus, it is more likely that residual contamination may have decreased the signal of SA during subsequent trials, including the runs involved in the second MS calibration.

**Figure 13: SA Calibration Chromatograms.** A) TIC of a standard solution containing only 50ppm of SA run on 6-13-2013, and B) an identically prepared standard solution containing only 50ppm of SA run on 7-17-2013.
2.2.4 SA Volatility

Another important observation was that while running percent recoveries, salicylic acid seemed to readily disappear throughout the plant cleanup procedure. SA standards were quantified after almost every step in the sample preparation procedure in order to detect for contamination or sample loss. While SA was not analyzed after the lyophilization step, it appeared as though the majority of SA was lost after the concentration step in which the MeOH was evaporated from the extracted tissue. After consulting several organic chemists, our initial thought was that the salicylic acid was sublimating during the concentration step.

Further experiments and literature research appear to support this claim. Many papers suggest that in order to minimize SA sublimation, the pH of the solution containing SA should be increased in order to convert SA into sodium salicylate, which is less volatile. Articles such as Verberne et al., 2002 explain that efforts to extract SA from plant tissues have “frequently been found to be low and variable.” In their study, the researchers added $10\mu L$ of 0.2 M sodium hydroxide in order to lower the volatility of SA. Doing additional follow-up experiments that adjusted the pH before concentrating with N$_2$ appear to support these findings (See Figure 14). The researchers also speculate that the addition of an internal standard would also compensate for any loss of sample due to sublimation as long as the initial concentration of SA is large enough (so that the final concentration is quantifiable) and the internal standard used has almost an identical volatility as SA.

It’s important to note that these two speculations are directly related to the success of this experiment. First, the initial SA concentrations collected from the Arabidopsis plants in the Forcat et al., 2008 study was found to be approximately 13.5ppm. To put this value into perspective, the linear range for SA for the mass spectrometer was from 500ppb to 50ppm, so the 13ppm initial concentration is fairly high. In addition, Forcat et al. uses $^2$H$_4$SA and $^2$H$_2$JA as their internal standards. Thus, since they have a fairly large amount of initial SA and since their internal standard has the same volatility as SA, they are able to 1) retain enough SA to quantify, and 2) normalize for any SA that they lost by utilizing their radioactive internal standard.
Figure 14: Effect of pH Chromatograms. A quick experiment to determine the effects of altering pH during concentration of SA. A SA standard was dried with N₂ in a test tube under different two different pH's. The pH of the first standard solution was around a pH of 3 since the solution used was the extraction buffer containing a 90:9:1 mixture of MeOH, H₂O, and acetic acid. The second solution was a 1:1 mixture of the extraction buffer and mobile phase B. The final pH of this solution was approximately 5.6. Notice that the basic solution yielded a slightly higher signal than the more acidic solution.
2.3 Quantification of SA Conclusions and Future Directions

In conclusion, the attempt to quantify salicylic acid highlights several important findings. First, the amount of initial SA present in the sample is important to the analysis. If there is a small amount of SA extracted from small amounts of sample, then there may not be enough SA left at the end of the sample preparation in order to quantify. Second, the complex matrix of moss tissue appears to suppress SA signals. Even though JA standards were prepared, JA was not tested since the priority of this analysis was quantifying SA. However, the mass spectrometer was set to monitor multiple reactions (MRM mode) so that the product ions of both SA and JA would be identified if they were formed.

While the data is qualitative at best, the initial findings of this study found that JA was either non-existent in the samples or that the JA signals were being suppressed as well. Third, the use of appropriate internal standards is imperative to the success of quantifying this rather volatile compound. By designing an internal standard that has the same properties of the compound of interest, the researchers in Forcat et al. were able to compensate for any substantial loss of product. According to some sources such as Verberne et al., 2002, these losses can be on the magnitude of 20-50% reduction.

While it is not discussed in the sections above, one additional point should be made about the Forcat et al. method. In their study, the researchers use a set of mobile phases that have a pH of around 2.5. However, the researchers analyze SA with the MS scanning in the negative mode. Since SA has a pKa of 2.98 (PubChem), then this would suggest that in solution the SA would prefer to be positively charged over being negatively charged. However, the researchers then decided to use the MS to scan for all of the negatively charged SA species. While optimizing the MS system with SA standards I found dramatically reduced sensitivity when performing the Forcat et al. method, which is why I designed a basic mobile phase (pH of approximately 6.5) and ran the MS in the negative mode. However, it is important to note that only small initial concentrations of SA will be dramatically affected by the reduced sensitivity of running the positively charged SA ions through a system that is scanning for negatively charged SA ions.
In the future, studies should focus on two areas. First, it is evident that an internal standard with similar volatilization properties as SA be used to remedy the sample loss due to volatilization during sample prep. Finally, the sample should be cleaned prior to injection to reduce the suppression of the SA signals from the matrix and to prevent the LC column and the MS from accumulating residual moss matrix. While other factors listed in this thesis should be considered as well, it seems as though these two points were the major roadblocks that hindered my efforts.

3.1 Methods and Materials for the Analysis of Defense-Related Genes

3.1.1 Plant Material and Growth Conditions

Moss samples, growth conditions, and inoculations are as described in sections 2.1.1 and 2.1.2.

3.1.2 Plant Inoculation and RNA Extraction

*P. patens* specimens approximately 10mm in diameter were inoculated with either 30 μL of 1mg/ml Chitosan or 30 μL of 1mg/ml β-Glucan in a laminar flow hood to maintain sterility at 0, 2, 4, and 12 hours. Due to several findings in the preliminary studies, the moss samples were rinsed in weigh boats filled with DI water prior to mRNA extraction (See Figure 15). After being dried with blotting paper and chemwipes, the moss plug was flash frozen with liquid nitrogen and crushed with a pestle in a 500 μL plastic tube. mRNA was extracted with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturers’ protocol. RNA concentrations for individual samples were determined by using a BioTek Synergy H1 plate reader. Samples were discarded unless they met the minimum concentration of 15ng/μL.
3.1.3 Genomic Analyses and Primer Design

Sequences from defense-related genes PAL1, PAL2, PAL3, and PAL4 from *Arabidopsis* were collected from NCBI (See Supplementary Data Table 2) and their corresponding protein sequences were determined. Predicted *P. patens* protein sequences were derived from BLASTP searches which returned hits with E values less than $1.00 \times 10^{-8}$. The predicted proteins were then converted into their respective mRNA sequences. Oligonucleotide primers for these defense-related mRNA sequences were designed using the Primer3 program designed by the University of Massachusetts Medical School. Since the BLASTP search results for the Arabidopsis PAL1, PAL2, PAL3, and PAL4 proteins were identical to the corresponding *P. patens* proteins, the top 9 hits were used, and primers were designed for these 9 predicted mRNA sequences. In addition, other defense related genes such as isochorismate synthase 1 (ICS1), UDP-glycosyltransferase 74 F1 (UGT74F1), HXXD-type acyl-transferase-like protein (EPS1), auxin-responsive GH3 family protein (PBS3), enhanced disease susceptibility 5 protein (EDS5), enhanced susceptibility disease 1 protein (EDS1), EIN4, regulatory protein NPR1 (NPR1), ethylene response factor 1 (ERF1), PAD3, and 1-aminocyclopropane-1-carboxylate synthase-like protein 1 (ACS2) were studied, entered into the BLASTP search engine,
and primers were created for these genes in the same method as detailed with the PAL genes (See Figure 16).

3.1.4 Primer Screening and Analysis

To determine the relative efficiency of the primers, genomic P. patens DNA was combined separately with the individual primers and semi-quantitative PCR was performed. Following analysis of the PCR products, select primers were compared against the NCBI genomic profile of P. patens in order to identify whether the lack of DNA amplification could be attributed to primers spanning introns.

3.1.5 Running qRT-PCR and Subsequent Data Analysis

Since time and money were limited, genes that demonstrated high amplification on genomic DNA, and genes that were involved directly in the SA pathway were analyzed with qRT-PCR. These genes include PAL5, PAL6, PAL7, PAL8, PAL9, ICS1, and the control gene Actin. qRT-PCR was set up by using a Power SYBR® Green RNA to Cr™ 1-Step Kit (Applied Biosystems) according to the manufacturer’s protocol. Inoculated sample mRNA (0, 2, 4, and 12 hour) were added into separate vials which were homogenized and then added into seven separate tubes where primers were added. After an additional homogenization step, 20 μL of the mixture was added into three wells. By homogenizing frequently, I believe that I was able to minimize the standard deviation despite doing the pipetting by hand. Qualitative real-time PCR was carried out on an BioRad CFX Connect™ Real-Time PCR Detection System using the following conditions: 48.0°C for 30 min, followed by 95 °C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60 °C for one minute, followed by a five minute melt curve. The resulting data was analyzed with Biogazelle’s qbasePLUS software.
3.2 Results and Discussion for the Analysis of Defense-Related Genes

The analysis of the defense-related genes in *P. patens* illustrates several important findings. 1) The research presented in this thesis may allow future research to be conducted on other non-PAL or ICS related defense genes since many of the primers have already been created and tested against genomic DNA. 2) The PAL genes that are involved in the SAR response in the vascular plant, *Arabidopsis thaliana*, are also involved in defense mechanisms in the non-vascular plants (Harshavardhan et al., 2010). 3) The ICS1 gene constructed from the Primer3 program should be redesigned if the ICS pathway is to be studied. 4) The PAL primers constructed are all positively correlated with one another suggesting that these primers either bind to the same PAL gene or that there are multiple PAL genes that all appear to be expressed similarly. 5) The response time of the non-vascular plant to external stimuli (in this case, the elicitor reaction time) appears to be extremely fast.

3.2.1 Non-PAL or ICS Gene Applications

Since a number of genes related to the defense mechanism of the vascular plant, *A. thaliana*, were analyzed and had primers designed for them in the non-vascular *P. patens*, this allows for the opportunity to explore the role of these SAR genes in non-vascular plants. Ten of these genes were screened against genomic DNA, and at least one set of primers bound to and replicated the gene of interest in semi-quantitative PCR. These genes include: ACS2-2, PBS3A-2, UGT74F1-1, EDS5-2, EDS5-1, EIN4-3, EIN4-4, EIN4-5, EIN4-6, EIN4-7, NPR1-1, ERF1-1, PAD3-2, EPS1-1, and ACS2-2 (the efficiency of some of these genes can be seen in Figure 16). It is important to note that some of the primers were inconsistent between PCR runs. However, when analyzing some of the inconsistent primers, I found that many of the primers either spanned an intron or a fairly large intron was situated between the two primers, which may have decreased the efficiency of the replication.
Figure 16: Analysis of select primers on genomic Physcomitrella DNA. The well contents are described in the diagram below the image. Red boxes indicate the presence of a band.

3.2.2 Role of PAL and ICS in the SAR Response in Non-Vascular Plants

Probably one of the most substantial findings from this research project is the evidence that supports the findings of Ponce de León et al., 2013 and Wolf et al., 2010 – that the PAL genes observed in vascular plants (which mediate defense-related physiological changes) is not an exclusive feature to vascular plants. Therefore, the evidence suggests that the PAL pathway is an ancestral trait that is conserved in vascular and non-vascular plants. However, after running melt curves on the ICS gene (Figure 17) it is obvious that the ICS primer needs to be redesigned. Principally, the melt curve demonstrates the exclusivity of the primers. Every primer should only have one peak associated with it (which denotes an amplification target). Thus, if a primer has more than two peaks (which means the primer is amplifying two different DNA sections) or none (which means the primer did not amplify anything), then the primer should be redesigned to have
a higher specificity for the segment that is to be amplified. However, the melt curve also
demonstrates that all of the PAL gene primers are highly efficient.

![Melt Peak Analysis](image)

**Figure 17: Melt Peak Analysis.** Melt Peak Analysis of defense related PAL and ICS
genes and the reference gene Actin.

### 3.2.3 Implication of Spearman Correlation Analysis of PAL Genes

The fact that the same nine PAL primer sets were matches for four different Arabidopsis
PAL genes is troubling since this leads to a substantial amount of ambiguity. Since qRT-PCR
demonstrates that all of the PAL primers were amplified at different time points and
since the Spearman correlation test (Figure 18) demonstrates that there is a positive
correlation between PAL primers 8 and 9 (the other Spearman correlations between the
other PAL primers returned similar results), this demonstrates that the PAL primers are
all acting similarly to each other. In other words, whenever PAL 6 is up-regulated, so are
the other PAL genes. This suggests two or possibly more scenarios. The simplest explanation for these results is that all of the PAL primers bound to the same mRNA product that was encoded from one of the PAL genes. Another possibility is that different primers bound to different mRNA products encoded from different PAL genes, but these PAL genes all behave similarly. From the data collected here it is impossible to know the fine details of what was actually expressed, but it is possible to know that PAL is being expressed in one form or another when the plant is being stressed by pathogenic stressors.

![Figure 18: Spearman correlation plot of PAL 8 vs. PAL 9. It is worthwhile to note that all of the additional Spearman correlations that were plotted analyzing all PAL interactions returned perfect Spearman correlations of +1. This indicates that each of the PAL genes are perfect monotone functions of the other.](image)

### 3.2.4 Response Time of PAL

One extremely interesting implication suggested from the data is the fact that PAL was up-regulated within minutes of inoculating with β-glucan. As illustrated in Figure 19, the gene expression (measured in relative quantities due to the normalization with Actin performed by qBasePLUS by Biogazelle) in the 0 hour treatment is larger than the 4 and 12 hour treatments. It is important to note that all of the PAL genes demonstrated the same
trend as seen in Figure 13, except only two gene samples were able to quantify the 12 hour treatment. Yet, due to the perfect positive correlation seen in the Spearman Correlation analysis, I believe as though the two genes that had results for the 12 hour treatment (PAL 8 and PAL 9) are good representatives of the PAL genes as a whole, since this study is strictly preliminary. One potential explanation for the trends seen in Figure 19 is that PAL is expressed almost immediately after the plant recognizes a pathogenic attack. The plant may be able to react so quickly that it can up-regulate the PAL gene to significant levels within the initial 5-10 minutes of sample prep before freezing the moss in liquid nitrogen. While defense-related gene time studies appear to be lacking, one study conducted by Sharma et al., (1996) on the ozone induced response of salicylic acid, seems to corroborate my findings. Sharma et al., gives evidence that the vascular plant, Arabidopsis, produces increased levels of SA within an hour of being exposed to 300ppb ozone. Since ozone may trigger a similar defense-related response, the rapid activation of PAL genes to produce the increased levels of SA observed in Sharma et al. appears to be plausible. Since increased levels of SA were observed in under an hour, I would assume that the genes controlling the formation of SA would be activated at least under an hour since gene activation must occur before the hormone is produced. In addition, the idea that a non-vascular plant can react to external stimuli on a scale that is comparable to a vascular plant is a fascinating proposition. However, due to time restraints, additional replicates of this experiment were not performed, and thus statistical ANOVA and Post-Hoc tests determining differences between the treatment levels could not be performed.
Figure 19: Relative gene expression. Relative gene expression after being normalized by the reference gene Actin for PAL 8 (the models and algorithms implemented in the qBasePLUS analysis program are given in Hellemans et al., 2007). Notice that the 0 hour sample appears to be much larger than the 4 and 12 hour treatments. The standard error is denoted by error bars (black lines).

3.3 Analysis of Defense-Related Genes Conclusions and Future Directions

It is evident that I have only scratched the surface of gene interactions involved in non-vascular plants. However, the data presented in this thesis seems to lend a small amount of support to many papers already published. PAL does indeed appear to be active in non-vascular plants as already stated by Oliver et al., 2009 and Ponce de León, 2013. The response time of plants to external stimuli appears to be remarkably fast, but, this was already demonstrated in Sharma et al., 1996. However, the novel findings in this study
demonstrate the relationship between PAL activation and time. From the data collected it appears as though PAL is expressed in the first few hours of being inoculated with a pathogen (or an elicitor such as β-glucan). However, it has not escaped my notice that the data presented here is strictly preliminary. Due to the lack of biological replicates, it is impossible to conduct any statistical analysis on the significance of these findings, and, as such, any conclusions based on the findings listed here should be made with caution. In order to support these findings at least 3-6 additional replicates should be performed so that a one-way ANOVA with post hoc Tukey tests (if applicable) may be run to validate the findings. Furthermore, an additional moss sample should be “inoculated” with water in order have a better control. Since there is evidence that the zero hour control in this study resulted in an up-expression of PAL, a sample that is treated with water would allow for a more accurate baseline. In addition, the PAL pathway is not the only route that results in SA formation. Since the ICS1 gene did not bind correctly in this study, continued efforts to design a functional primer should be undertaken so that the complete pathway of SA formation can be understood since previous research suggests that the IC pathway is dominant to the PAL pathway.

4.1 Overall Conclusions and Final Remarks

The research conducted in this thesis has demonstrated the potential of taking a two-sided approach to analyzing a particular pathway involved in P. patens. The goal was to analyze the genetics of the PAL gene and correlate the expression levels to the amount of SA that was produced by utilizing powerful quantitative equipment. Despite the numerous papers published on each of these key aspects, I have yet to come across one that incorporates both of these approaches into a single paper. Therefore, from the experiences gained researching this biological system, I can say that looking at a problem from two different perspectives can give helpful insights that can assist with navigating potential pitfalls. Thus, while it may be possible to devote one’s time to understanding one perspective of a biological mechanism in depth, novel, emergent properties may begin to arise if a more holistic view is applied leading to a better understanding of the system of interest as a whole.
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Supplementary Data

Table 2: List of designed primers for defense-related genes in *P. patens*. Target genes in Arabidopsis are listed in green and their corresponding proteins are listed under the Protein Seq Ref ID column according to their NCBI reference numbers. These proteins were used in the BLASTP to search for similar proteins in *P. patens*. Top results were collected, transformed back into mRNA sequences, and listed under mRNA Ref ID. Primers were designed by using Primer3 program hosted by the University of Massachusetts Medical School.

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<td>TAGCTTCCCTGGCCATTACGA</td>
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</table>

### HXXXD-type acyl-transferase-like protein

<table>
<thead>
<tr>
<th>Gene Name ID</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_126135.1</td>
<td>EPS1</td>
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</tbody>
</table>

**Purpose:**
Pathogen-induced SA accumulation

<table>
<thead>
<tr>
<th>Protein Seq Ref ID</th>
<th>E Val</th>
<th>mRNA Ref ID</th>
<th>Primer ID (FWD</th>
<th>REV)</th>
<th>SEQ</th>
<th>Product Size</th>
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<tbody>
<tr>
<td>XP_001763897.1</td>
<td>8.00E-30</td>
<td>XM_001763845.1</td>
<td>RE_F-EPS1-1</td>
<td>RE_R-EPS1-1</td>
<td>GCAATCGACTACATCGAGCA</td>
<td>187</td>
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<td>GGATGATGAGCCCTTCAAAA</td>
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</table>

### Auxin-responsive GH3 family protein

<table>
<thead>
<tr>
<th>Gene Name ID</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_125118.1</td>
<td>PBS3 or GH3</td>
</tr>
</tbody>
</table>

**Purpose:**
Catalyzes the conjugation of specific amino acids (e.g. Glu) to its preferred acyl substrates

<table>
<thead>
<tr>
<th>Protein Seq Ref ID</th>
<th>E Val</th>
<th>mRNA Ref ID</th>
<th>Primer ID (FWD</th>
<th>REV)</th>
<th>SEQ</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP_001782182.1</td>
<td>1.00E-135</td>
<td>XM_001782130.1</td>
<td>RE_F-PBS3-1</td>
<td>RE_R-PBS3-1</td>
<td>TTTCCCCCTCCTTCCTTA</td>
<td>319</td>
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<td></td>
<td>AAATCTGGGAGGTCAAAAATC</td>
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<tr>
<td>XP_001764319.1</td>
<td>3.00E-129</td>
<td>XM_001764267.1</td>
<td>RE_F-PBS3-2</td>
<td>RE_R-PBS3-2</td>
<td>TTAATCGGAGCTAGTGTTGC</td>
<td>255</td>
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<td></td>
<td>CTGGAATGAGCGGAGGTGGA</td>
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</table>
### Enhanced disease susceptibility 5 protein

**Gene Name ID**
NM_120955.5

**Purpose:**
Component of salicylic acid–dependent signaling for disease resistance

<table>
<thead>
<tr>
<th>Protein Seq Ref ID</th>
<th>E Val</th>
<th>mRNA Ref ID</th>
<th>Primer ID (FWD</th>
<th>REV)</th>
<th>SEQ</th>
<th>Product Size</th>
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</thead>
<tbody>
<tr>
<td>XP 001771784.1</td>
<td>1.00E-159</td>
<td>XM_001771732.1</td>
<td>RE_F-EDS5-1, RE_R-EDS5-1</td>
<td>TTGCAAGAGGCAGGAGT</td>
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<td>XP 001762919.1</td>
<td>5.00E-157</td>
<td>XM_001762867.1</td>
<td>RE_F-EDS5-2, RE_R-EDS5-2</td>
<td>ACAAAAGAGGAAGCAGA</td>
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<tr>
<td>XP 001761217.1</td>
<td>2.00E-150</td>
<td>XM_001761165.1</td>
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<td>AGGTCTCTGCTTTGGGAATCT</td>
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### Enhanced disease susceptibility 1 protein

**Gene Name ID**
NM_114678.3

**Purpose:**
Component of salicylic acid–dependent signaling for disease resistance

<table>
<thead>
<tr>
<th>Protein Seq Ref ID</th>
<th>E Val</th>
<th>mRNA Ref ID</th>
<th>Primer ID (FWD</th>
<th>REV)</th>
<th>SEQ</th>
<th>Product Size</th>
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<tbody>
<tr>
<td>XP 001753768.1</td>
<td>7.00E-09</td>
<td>XM_001753716.1</td>
<td>RE_F-EDS1-1, RE_R-EDS1-1</td>
<td>GGTGGTGCTCTACGTCCATT</td>
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### EIN4

**Gene Name ID**
NM_202489.1

**Purpose:**
Ethylene Receptor

<table>
<thead>
<tr>
<th>Protein Seq Ref ID</th>
<th>E Val</th>
<th>mRNA Ref ID</th>
<th>Primer ID (FWD</th>
<th>REV)</th>
<th>SEQ</th>
<th>Product Size</th>
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<tr>
<td>XP 001769490.1</td>
<td>0.00E+00</td>
<td>XM_001769438.1</td>
<td>RE_F-EIN4-1, RE_R-EIN4-1</td>
<td>TTGCAGTGCCCTTTGTCTCAC</td>
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<tr>
<td>XP 001774409.1</td>
<td>3.00E-162</td>
<td>XM_001774357.1</td>
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<td>TCTTGCGCTACTGCACCTATT</td>
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<tr>
<td>XP 001762445.1</td>
<td>8.00E-162</td>
<td>XM_001762393.1</td>
<td>RE_F-EIN4-3, RE_R-EIN4-3</td>
<td>CGACTTTTCGCTGTGATGTA</td>
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<tr>
<td>XP 001751520.1</td>
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<td>XM_001751468.1</td>
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<td>XP 001754898.1</td>
<td>4.00E-155</td>
<td>XM_001754846.1</td>
<td>RE_F-EIN4-5, RE_R-EIN4-5</td>
<td>ATGCCTTAGCTTTACACAG</td>
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<td>XM_001756097.1</td>
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<td>TCACTGGCGGTCAACTCTTG</td>
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<tr>
<td>XP 001772050.1</td>
<td>1.00E-129</td>
<td>XM_001771998.1</td>
<td>RE_F-EIN4-7, RE_R-EIN4-7</td>
<td>ATCCCTTATGCGCCGAGA</td>
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### Regulatory protein NPR1

**Gene Name ID**: NPR1  
**Protein Seq Ref ID**: XP_001778211.1  
**mRNA Ref ID**: XM_001778159.1  
**Primer ID (FWD | REV)**:  
- **RE_F-NPR1-1**: CAAACGTGCACGTGAGAAAGA  
- **RE_R-NPR1-1**: TATTTGCGTGCAACTTCAGC  
**Product Size**: 296

**Purpose**: Regulation of SA dependent gene expression for SAR

### Ethylene response factor 1

**Gene Name ID**: ERF1  
**Protein Seq Ref ID**: XP_001779786.1  
**mRNA Ref ID**: XM_001779734.1  
**Primer ID (FWD | REV)**:  
- **RE_F-ERF1-1**: AGCTGAGGTCCGAGATTCAA  
- **RE_R-ERF1-1**: GAATCGGTTGTGATCCTCGT  
**Product Size**: 156

**Purpose**: Encodes a transcription factor that regulates the expression of pathogen response genes that prevent disease progression

### PAD3

**Gene Name ID**: PAD3 or BA2-H  
**Protein Seq Ref ID** and **mRNA Ref ID**:  
- **XP_001779518.1**: TGTGTGACCATCGCCACTAT  
- **XP_001751225.1**: GCAATTGGACACAAACAACG  
- **XP_001754538.1**: AGCTGCGATGGAGGTTCTTA  
**Primer ID (FWD | REV)**:  
- **RE_F-PAD3-1**: TGTGTGACCATCGCCACTAT  
- **RE_R-PAD3-1**: GCCCATTCCTTGTCTTGTGT  
- **RE_F-PAD3-2**: GCAATTGGACACAAACAACG  
- **RE_R-PAD3-2**: GTGGATACATGGGGGATGAG  
- **RE_F-PAD3-3**: AGCTGCGATGGAGGTTCTTA  
- **RE_R-PAD3-3**: GTCATACCTCAGGCTTGTG  
**Product Size**: 257, 273, 279

**Purpose**: Encodes for a cytochrome P450 monooxygenase that converts BA to SA

### 1-aminocyclopropane-1-carboxylate synthase-like protein 1

**Gene Name ID**: ACS2  
**Protein Seq Ref ID** and **mRNA Ref ID**:  
- **XP_001778276.1**: 8.00E-12  
- **XP_001783780.1**: 6.00E-60  
**Primer ID (FWD | REV)**:  
- **RE_F-ACS2-1**: GGCTGCATTATTCCACTGGT  
- **RE_R-ACS2-1**: TGCAAGCACTGATTTCAAGG  
- **RE_F-ACS2-2**: AACCTGTAATCCTGGCGATG  
- **RE_R-ACS2-2**: AGCCAGTACAGCCTCTTCCA  
- **RE_F-ACS2-3**: AGCTGCGATGGAGGTTCTTA  
- **RE_R-ACS2-3**: GTCATACCTCAGGCTTGTG  
**Product Size**: 387, 163

**Purpose**: Ethylene Synthesis