Heat Shock-Based Activation of Systemic Acquired Resistance in *Arabidopsis thaliana*

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**Abstract:** Crop wastage, especially that caused by pests and pathogens, is becoming an increasingly pressing problem. Genetic technology and its advances may be applied to agriculture and farming in order to combat the wastage. Previous research has attempted to use genetic techniques to make plants more resistant to drought, increased salt concentrations in the environment, decreased nutrients in the soil, and other detriments to crop growth. In this project, genetic technology was leveraged to attempt to create a genetically modified plant that had an increased ability to resist pathogenic and pest-based damage. In particular, the experimental plant *Arabidopsis thaliana* had its Systemic Acquired Resistance immune pathway modified to better resist the bacterium *Pseudomonas syringae*. A concern with using crops that have enhanced immune systems, however, is that pests and pathogens will evolve to attain resistance to the enhanced immune response. To prevent this, the enhanced immune system was further modified to only activate when it was exposed to an external stimulus. This way, the enhanced immune system was not always activated. Therefore, pests and pathogens had a decreased amount of time to acclimate to or experience the enhanced system, thus slowing down the evolution of resistance to the plant’s enhanced immune system. In this project, the crop wastage is addressed with the consideration of a potential evolution of resistance by pests and pathogens, a novel exploration. Although the edits to experimental plants’ genomes did not generate a statistically significant resistance to pests and pathogens when compared to unmodified plants, a likely source of error is identified and explained for future research to address. This work’s contribution towards the identification of a functional solution to crop wastage due to pests and pathogens has multiple potential positive implications including fighting food shortages and beneficially impacting the agricultural sector of the global economy.
Introduction

Global population growth is one of the most pressing challenges to agriculture in the twenty-first century [3-6, 29]. With the world population projected to reach ~9.5 billion by 2050 [3,5,6], farmers will need to increase agricultural outputs to meet global food needs. However, crop yields are significantly impacted by environmental stresses which can cause significant setbacks to improving agricultural productivity [3,8,9]. For instance, biotic and abiotic stresses, such as drought, frost, salinity, insects, pests, and viral or bacterial pathogens, cost about 220 billion US dollars of losses to agriculture each year [1,30]. Specifically, pests and pathogens account for 20%-40% of global losses in crops such as wheat, soybean, maize, and rice [3,5]. Faced with future challenges of producing food in these dynamic environmental conditions, farmers and scientists must work together to apply various technologies to expedite food production. An escalation in the amount of research being done to resolve these issues in the field of genetic engineering highlights its importance as a solution to agricultural concerns [7].

Genetic engineering is one of the most promising technologies used to combat these problems and has been used by researchers to improve plant resistance to various extreme and unfavorable environmental conditions, such as pests and pathogens [3,7-9]. Precise genomic modifications or insertions of transgenes may be used to help improve plant response to pathogenic diseases by enhancing defense mechanisms and immune responses [18,19,22,26,27].

To create engineered plants, scientists most commonly use Agrobacterium-mediated transformation or biolistic bombardment [24,27]. While slightly different, both processes introduce DNA into the host plant cell genome. In the context of improving plant resistance to pathogens, introduced DNA could produce enzymes whose products activate plant defense pathways and catalyze the onset of an immune response [22,25,26].

One example of a plant defense system is Systemic Acquired Resistance (SAR), a pathway that induces a global immune response throughout a plant after localized exposure to a pathogen [12-17,21]. At the site of the infection, resistance efforts consist of cell wall modifications, the production of phytoalexins and pathogenesis-related (PR) proteins, and the activation of hypersensitive response (HR) [13,14]. To prevent further spread of the pathogen, small molecules such as the PR proteins salicylic acid (SA) and piperolic acid (Pip) are produced at the site of infection and spread through the plant. Both molecules are important for establishing an immune response against pathogens; however, Pip is also involved in the activation of SAR [16,17,20,21]. Until recently, the specific role of Pip in initiating SAR was unknown. Through the use of untargeted metabolomics of mutant Arabidopsis lines, the Sattely Lab from Stanford University discovered that the protein flavin-dependent monooxygenase (FMO1) converts Pip to N-hydroxy-pipolic acid (NHP), a principal metabolite that activates SAR in A. thaliana [16].

As shown in (Fig. 1), the biosynthetic pathway for the production of NHP involves 1) the conversion of L-Lysine to dehydro-Pipolic Acid by PLP-dependent aminotransferase (encoded by ALD1), 2) the
conversion of dehydro-Pipecolic Acid to Pipecolic Acid by Reductase (encoded by SARD4), and 3) the conversion of Pipecolic acid to NHP by Flavin-dependent monooxygenase (encoded by FMO1) [12,15-17]. Through the use of gas chromatography-mass spectrometry, the Sattely Lab was able to determine that the expression of ALD1 and FMO1, two of the three genes found in the NHP-encoding pathway of A. thaliana, could create a functional biosynthetic pathway for the formation of NHP. The third gene, SARD4, does not need to be expressed to produce NHP. Transient expression assays of ALD1 and FMO1 in Nicotiana benthamiana and Solanum lycopersicum resulted in an increased resistance to the bacterial pathogen *Pseudomonas syringae*, one of the most common plant pathogens [35], in systemic tissues [16]. While constant expression of these genes could be a desirable feature in plants, one concern is the possibility of attacking pathogens developing resistance [3,19].

Due to this concern, a system was designed in which ALD1 and FMO1 were only expressed when the plant was subjected to an external stimulus. The stimulus caused increased expression of ALD1 and FMO1, which allowed for the manipulation of NHP expression, thereby extending control over the initiation of SAR. After engineering the edited gene construct using the Benchling software, a transient assay was performed to transiently express the gene constructs in the A. thaliana experimental organisms. Finally, the titers of *P. syringae* were compared between treated and untreated plants. To create engineered genes, the Golden Gate Cloning method was used to create a plasmid which contained a backbone with a heat-shock promoter, ALD1 and FMO1, and a terminator [28].

**Methods**

Using Benchling software, three artificial gene constructs were assembled. The pJC139 vector was the experimental group and had a Heat Shock Promoter and an ALD1-P2A-FMO1 sequence. The pJC140 vector was the positive control and had a 35S Promoter and an ALD1-P2A-FMO1 sequence. The pJC141 vector was the negative control and had a 35S promoter and a luciferase gene. The 35S Promoter was used to continually express the genes in the vector, while the Heat Shock Promoter was used to activate the genes on the vector after the cell
had been subjected to a heat shock. The experimental and positive control groups had the ALD1-P2A-FMO1 sequences since the roles of these groups were to produce NHP and active SAR. The negative control group’s plants were not modified with regards to their immune responses, but they expressed a luciferase gene to verify that the edited gene constructs had been successfully taken up by the plant. Replicability of the construct is ensured by the availability of the ALD1-P2A-FMO1 sequences in Appendix C.

The Arabidopsis Information Resource (TAIR) database for A. thaliana was used to find the coding DNA (cDNA) sequences for FMO1 and ALD1. These sequences were ordered from Twist Bioscience with the addition of Type II restriction enzyme-compatible cut and binding sites. Golden Gate Assembly was used to create a final T-DNA vector for each of the three groups, which was used during the Agrobacterium transformation into A. thaliana leaf tissue.

Figure 2. Experimental A. thaliana plants outside the growth chamber. The yellow labeling card indicates the gene construct transformed within the plant. For example, the number “141” suggests that the respective plant has transformed the pJC141 construct (139 represents the experimental group, 140 the positive control, and 141 the negative control).

The final T-DNA vectors were transformed into DH5-alpha E. coli cells, plated on agar plates containing appropriate antibiotics (streptomycin, kanamycin, and gentamycin), and incubated overnight for ~16 hours. For each Golden Gate assembly, Sanger sequencing was performed on plasmids extracted from a single colony to verify the integrity of the coding sequence. Upon verification, T-DNA constructs with the desired sequence were transformed into Agrobacterium strain GV3101 using the freeze-thaw method with liquid nitrogen and plated on Lysogenic Broth (LB) plates containing kanamycin and gentamicin, which select for the T-DNA vector and helper plasmid pMP90, respectively. The freeze-thaw method was chosen for its balance of cost-effectivity and high transformation rates [34].

In parallel, A. thaliana plants were grown under controlled conditions at 22°C under short-day conditions (8 hours of light and 16 hours of darkness) (Fig. 2) [32]. As the plants grew, leaf identification was performed and the petioles of leaves eight through ten and thirteen through fifteen were marked for future reference.

Five-week-old plants were infiltrated with prepared Agrobacterium cultures that harbored the engineered T-DNA (Fig. 3). For each construct, infiltration was carried out on three individual plants. Twenty-four hours after the infiltration, the plants underwent a 42°C heat shock treatment (Fig. 4). All plants underwent a heat shock treatment even though only the experimental plants were expected to undergo changes in gene expression due to the heat shock. Two days
after infiltration, two leaves from each plant were removed and analyzed for luciferase expression. ~48 hours are required before a heat shock’s effects are felt on the expression of edited genes with a HSP promoter [15]. This was to verify the successful transfer of Agrobacterium into leaf tissue. Four days later, infiltration with DC3000 P. syringae bacteria into upper leaves was carried out. The four-day gap allowed ALD1 and FMO1 expression, and thus NHP production, to be maximized within the plant [15]. Three days after the bacterial infiltration, leaves were harvested, and bacteria was plated onto solid media for analysis of growth counts using ImageJ. Previous research has suggested that three days is an appropriate amount of time for the bacterial infection to take effect and for the plant’s immune response to react [32].

**Artificial Gene Constructs**

Three gene constructs were created during the experiment. The purpose of two of the gene constructs was to induce an immune response in the plants (both in different ways), and the purpose of the third gene construct was to set a baseline for what a natural response by a plant to a bacterial infection would look like.

The first gene construct, pJC139, is controlled by a Heat Shock promoter (HSP) (Fig. 5). This promoter will only activate the expression of the other genes in the construct when the plant containing this gene construct is exposed to a heat shock. The second and third gene constructs, pJC140 and pJC141, respectively, contain a 35S promoter, which continually expresses the genes in the construct (Fig. 6). The 35S promoter was used because the second and third constructs were positive and negative controls; hence, they were to continually express their genes. pJC139 and pJC140 contained an ALD1-P2A-FM01 sequence because they are both meant to induce Systemic Acquired Resistance through the production of NHP in the experimental plants. The pJC140 construct, because of its 35S promoter, did so continuously, serving as a positive control. The pJC139 construct did so only when the plants were subjected to a heat shock because it was the experimental group. All three constructs contained antibiotics and the luciferase gene. All three contained a luciferase gene because the activation of this gene would emit a green color from the plant, signifying successful uptake of the gene constructs into the plant. Without this, it would have been difficult to ascertain whether the infiltration of the gene
constructs into the plants had occurred or not. All three plants were also grown in kanamycin and gentamicin so that only E. coli containing the gene constructs would be infiltrated into the experimental plants (the gene constructs transformed into E. coli contained kanamycin and gentamicin resistance markers). The pJC14 construct only contained a 35S promoter in combination with a luciferase gene because the plants taking up these vectors were to be the negative controls, and therefore, their SAR immune responses were not to be modified.

These three constructs were made using Golden Gate Assembly via the Benchling software. The components used in assembly were from the Dan Voytas Lab’s database of gene constructs. The three constructs were ordered for usage in the
experiments and their sequences were verified via Sanger sequencing.

Results

Primary Leaf Infiltration

*Figure 7A.* Six sample leaves transformed with the pJC141 construct under light conditions. The bright/white spots indicate the emission of light, a process caused by the expression of the luciferase gene in the transformed gene construct.

Primary leaf infiltration successfully occurred during the experiment. Primary inoculation of experimental plants involved the transformation of the three gene constructs into the plants. This transient expression was verified by the activation of the luciferase genes from within the experimental plants. If the plants emitted green light, it could be inferred that the luciferase gene—and thus the rest of each gene construct—had been transformed into the experimental plant successfully.

Light was emitted from the leaf tissue of the negative control plants, thus indicating expression of the transformed gene constructs in the plant (Fig. 7). Similarly, it can be concluded that the gene constructs had been transformed into the pJC139 and pJC140 plants because they underwent the same primary inoculation steps and were treated under the same conditions as the pJC141 plants. pJC141 plants were analyzed for luciferase expression because they were the negative control and were not modified with regards to their immune responses.

*Figure 7B.* Six sample leaves transformed with the pJC141 construct under dark conditions.

Thus, they were tested for the success of the primary inoculation steps.

Because light was emitted from these plants, it is possible to conclude that they had successfully taken up the gene constructs of interest and that transient expression of the gene construct had begun.

Secondary Leaf Infiltration

During the secondary leaf infiltration, *P. syringae* was inoculated into the experimental plants. All experimental plants were exposed to the bacteria and were inoculated under the same conditions. Evidence for the inoculation is present in the existence and growth of bacterial colonies on plated mixtures of the inoculated experimental plant leaves. Because plated mixtures of leaves from pJC139, pJC40, and pJC141 plants grew bacterial colonies, it is
possible to conclude that secondary inoculation occurred successfully in all the experimental plants.

*Bacterial Colony Growth*

Bacterial colonies numbered in the thousands on the analyzed plates (Fig. 8).

Using Gimp Photoshop Software, pictures of the agar plates containing the

**Heat Shock**

<table>
<thead>
<tr>
<th>Plant</th>
<th>No Shock</th>
<th>Shock 1</th>
<th>Shock 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJC139</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>pJC140</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>pJC141</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 8.* Bacterial colonies grown from a leaf mixture of experimental plant leaves. The number of bacterial colonies on each agar plate were counted using ImageJ, an image analysis software. pJC139 corresponds to the plant containing the pJC139 construct, and this data was collected from the leaves of experimental group plants. pJC140 corresponds to the plant containing the pJC140 construct, and this data was collected from the positive control units. pJC141 corresponds to the plant containing the pJC141 construct, and this data was collected from the negative control units.
bacterial colonies were separated from the surrounding countertop. These pictures were then uploaded to the ImageJ software, and the number of colonies on each plate was counted (Table 1).

To compare the number of bacterial colonies between the experimental, positive control, and negative control groups, an analysis of the means was performed (Table 2). The mean number of bacterial colonies that grew on the agar plates of the post-heat-shocked plants from each group then underwent statistical analysis. Post-heat-shocked plant data was used to eliminate the consideration of differences in bacterial colony growth in pre- and post-heat-shocked plants. Difference of means analysis between the experimental and positive control and experimental and negative control groups suggested no statistically significant differences in the number of bacterial colonies between the experimental and either positive or negative control groups (Fig. 9).

### Table 1.
The number of bacterial colonies on each plate.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Number of Bacterial Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJC139 No Shock</td>
<td>47</td>
</tr>
<tr>
<td>pJC139 Shock 1</td>
<td>2067</td>
</tr>
<tr>
<td>pJC139 Shock 2</td>
<td>3222</td>
</tr>
<tr>
<td>pJC140 No Shock</td>
<td>1138</td>
</tr>
<tr>
<td>pJC140 Shock 1</td>
<td>4642</td>
</tr>
<tr>
<td>pJC140 Shock 2</td>
<td>1592</td>
</tr>
<tr>
<td>pJC141 No Shock</td>
<td>2459</td>
</tr>
<tr>
<td>pJC141 Shock 1</td>
<td>2613</td>
</tr>
<tr>
<td>pJC141 Shock 2</td>
<td>847</td>
</tr>
<tr>
<td>Mock Trial</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Mean number of *P. syringae* colonies that grew on the agar plates in a 28°C incubator for 2 days from the post-heat-shocked experimental (heat-shock controlled expression of ALD1-P2A-FMO1), positive control (continuous expression of ALD1-P2A-FMO1), and negative control groups (no enhanced expression of SAR) plant leaf tissue mixture.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Number of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJC139 (Experimental Group)</td>
<td>2645</td>
</tr>
<tr>
<td>pJC140 (Positive Control Group)</td>
<td>3117</td>
</tr>
<tr>
<td>pJC141 (Negative Control Group)</td>
<td>1730</td>
</tr>
</tbody>
</table>

Discussion

The protocols used in this project were chosen based on their high success levels and/or because they have been suggested by previous research. For instance, the Golden Gate Assembly method was chosen because of its convenient and time-efficient character relative to the Digest and Ligate or Gibson Assembly methods. The *A. thaliana* plants were grown using conditions determined to be suitable for experimentation [32-34], and the primary
and secondary inoculation steps were chosen because they have been shown to work with a significantly high effectivity (i.e., inoculum was transformed into experimental plants with high rates of success) [12,15-17, 30-32].

A lack of statistically significant differences in the number of bacterial colonies that grew on the experimental, positive control, and negative control plates suggests a lack of effectiveness in the Heat Shock promoter’s ability to activate SAR in a plant (the experimental and positive control groups were expected to have fewer colonies due to their more effective immune systems). Specifically, the lack of statistically significant differences in the mean number of bacterial colonies between the experimental and negative control group implies that a heat shock-based activation of SAR is not viable and will not effectively trigger increased levels of NHP production in plants. However, the lack of statistically significant differences in bacterial colony growth between (1) the positive control and experimental group and (2) the positive control and negative control poses a concern. This result suggests that artificially enhanced SAR expression in plants is not effective and is not significantly different from leaving a plant’s immune responses unchanged. This conclusion contradicts previous research, which has determined that the continuous expression of SAR improves a plant’s immune response [15].

Because the positive control group does not appear to be expressing SAR, it may be inferred that an error occurred in either the construction of the gene or the inoculation of the gene construct. A review of the gene constructs and Sanger sequencing data suggests that the gene constructs did not contain errors. The gene constructs for all three vectors contained accurate copies of the genes of interest. For example, the gene constructs for pJC139 and pJC140 were searched for the ALD1-P2A-FMO1 DNA sequence. Each construct was also searched for the DNA sequence of its respective promoter, luciferase gene, and antibiotic resistance marker. This search yielded no
errors, and the Sanger sequencing showed no errors in the physical copies of the gene construct.

An analysis of the primary inoculation procedures revealed a possible source of error. The reagent preparation steps were performed accurately and were verified by comparing laboratory records with published protocols. The inoculation occurred successfully, confirmed by the activation of the luciferase gene in all the negative control plants. However, a potential source of error lies between the primary and secondary inoculations. During the experimental procedure, six days were left between primary inoculation and heat shock activation of the experimental control plants; in contrast, some protocol publications suggest leaving only four to five days between primary inoculation and heat shock activation. This is a possible source of error because transient expression in plants is not permanent. Rather, transient expression reaches its peak levels a certain number of days after it is initiated, and after the peak is reached, expression levels begin to decline. By waiting two more days between primary inoculation of the experimental plants and heat shock activation, it is likely that transient expression of the gene construct decreased, leading to a less effective SAR response—a response more comparable to a natural, unenhanced response from a plant [16]. This may explain the lack of statistically significant differences in bacterial colony growth between the positive and negative control plants.

In order to develop a plant whose SAR response is controlled by an external stimulus, future research is required. Although a viable gene construct was built, more testing is required to confirm that a solution to the question of controlling SAR activation has been found. One way to advance work on this issue is to make improvements to the testing methods.

In this experiment, only six experimental plant units were tested. In order to draw conclusions that can be generalized to larger populations, tests must be conducted on larger samples of plants and in more species of plants to create a more comprehensive idea of how SAR can be controlled.

Furthermore, another improvement to the protocol would be to change the duration of time between primary inoculation of experimental plants and heat shock/secondary inoculation. As mentioned previously, changing this duration could affect the levels of transient expression of gene constructs in a plant. Decreasing the duration from six to four days after primary inoculation may allow the heat shock and secondary inoculation to occur at a time when transient expression levels are increased, thereby allowing for a more practical solution to be developed.

This experiment created further opportunities for novel research in the field. Specifically, this experiment unveiled new gaps that encourage other research projects. More in-depth research on changes in transient expression levels under different circumstances is a possibility. Quantitative studies that map how transient expression varies over time in different plants would
help address a gap identified by this experiment. For instance, mapping levels of transient expression of a gene construct over two weeks in A. thaliana could help researchers understand how transient expression levels change in this model organism. Creating a database containing this information could be a possible outcome, and this information could be used in planning future research projects related to genetics and in creating a model of transient expression levels based on plant species.

Another consideration for a future project is to attempt to control the activation of SAR in A. thaliana, but rather than using transient expression, use floral dipping to create a stable line plant. A stable line plant will not only express a transformed gene construct, it will also pass on the genes to offspring plants. This “permanent” transformation of a gene construct into a plant’s genome can be done using floral dipping, a facile method in which stable line A. thaliana plants are frequently produced. In this method, transformation of female plant gametes is accomplished by submerging A. thaliana inflorescences in a solution of 5% sucrose, 0.05% nonionic organosilicone surfactant (often Silwet L-77), and Agrobacterium cells containing the gene constructs that will be transformed into the plant [34]. Then, the plant sets seeds, and the seeds are tasted for the presence of the gene constructs of interest. Although this method only has a minimum transformation frequency of 1%, its simple nature can allow researchers to produce hundreds of transgenic plants in three months using just 60 plants [34]. Floral dipping provides an opportunity for researchers to address the issue of controlling SAR activation without having to consider a possible decline in the expression of a transformed gene construct. Using floral dipping and stable line plants instead of transient expression in similar experiments would address another gap unveiled by this project—how controlling immune responses in plants is dependent upon transformed gene construct expression levels.

Furthermore, variations to this experiment can also be carried out as novel research projects. Because the way transient expression levels change over time is different among different plants, this research project opens a gap relating to how SAR activation can be controlled in other plant species through transient expression methods [19]. By setting up experiments similar to the one conducted in this project, controlling SAR can be researched in other plants as well. Between these experiments, many of the methods would vary, but a similar structure would be preserved. This would help address another gap opened by this research project.

In addition, attempting to control other immune responses and systems in plants is a possible endeavor for future research. Many other immune responses in plants exist, and the uniqueness of many of these systems makes them important targets for future research. For instance, learning how to control these immune responses would further enable scientists to exercise management over plant immunology, and this could help decrease crop or plant losses due to pests and pathogens.

Conclusion

This research project aimed to create a variant of A. thaliana whose SAR immune response could be controlled by external stimuli. This project has two primary stakeholders: researchers and companies engineering genetically modified crops (GMCs). Researchers could study the genes
regulating SAR in different plants, in combination with other genes, or in combination with other promoters. In addition to researchers, GMC-producing companies are likely stakeholders in this project. Global crop production is related to the interests of such businesses, and since the results of this project may impact the way global agriculture is conducted, GMC-producing businesses are likely to hold a stake in them. Specifically, they could help produce a more cost-effective, pathogen-resistant plant product.
Appendix A: Protocols and Detailed Methods

By supplementing model plants with NHP, Dr. Elizabeth Sattely’s lab was able to identify NHP as a metabolite inducer of SAR [16]. Furthermore, her lab characterized the pathway through which L-Lysine is converted to NHP. Intermediate steps involve the conversion of L-lysine into dehydro-pipecolic acid and the subsequent conversion of dehydro-pipecolic acid into pipecolic acid. Pipecolic acid is, in turn, converted to NHP. These conversions are catalyzed, respectively, by the PLP-dependent aminotransferase, reductase, and flavin-dependent monooxygenase enzymes. These enzymes are encoded by ALD1, SARD4, and FMO1, respectively. Dr. Sattely and her colleagues found, by overexpressing combinations of these genes and evaluating the results using liquid-chromatography/mass-spectrometry (LCMS) untargeted mutagenesis, that only ALD1 and FMO1 needed to be overexpressed to overproduce NHP [16]. Furthermore, Dr. Sattely showed, via in planta experiments, that the transient expression of edited genes overexpressing ALD1 and FMO1 enhanced the SAR response in tomato plants [16].

Preparation of LB Agar Plates

37 g of premixed LB-agar powder per liter of molten agar were used. The LB-agar powder was transferred into a bottle to be subjected to autoclaving. Some excess volume was necessary to prevent the molten agar from boiling in the autoclave. Sterile water was transferred (1:1 ratio of water to desired media volume) to the same bottle and swirled until a colloid was formed. The bottle was covered and taped down with an autoclave tape. The bottle was placed in the autoclave and subjected to 121°C and 20psi for 30 minutes [30]. A 1000x stock solution was created and spectinomycin was prepared. A 60°C water bath was also prepared. After the agar mix was subjected to the autoclave under the aforementioned conditions, about 75% of the bottle was submerged in the prepared water bath. The agar plating station was prepared by spraying the area with a 70% ethanol solution. Then, a flame was positioned adjacent to the area where plates were poured [30]. The flame was lit, and spectinomycin was diluted into the molten agar sample at a 50µg/mL concentration. Even distribution of spectinomycin throughout the bottle was ensured, and the solution was poured into each plate one at a time next to the flame. A pipette was used to measure the desired amount of agar per plate. The plates then solidified. The plates were tested to ensure that spectinomycin functioned as desired [30].

Golden Gate Assembly of pJC III (Level 0 ALD1-P2A-FMO1 J6-J9) cDNA Sequences

Golden Gate assembly of the fragments was carried out to clone the ALD1-P2A-FMO1 DNA fragment into a pJC221 MoClo vector backbone (to make J6-J9 fragments components of a B module). 11µx of H2O, 2µx of T4 DNA Ligase Buffer, 0.5µx of DTT, 0.5µx of BSA, 1µx of ALD1 Twist Fragment, 1µx of P2A annealed Oligo, 1µx of FMO1 Twist Fragment, 1µx of pJC221, 1µx of T4 DNA Ligase, and 1µx of BbsI were used as reactants. After performing the necessary reactions, the product was subjected to a thermocycler for five minutes at 37°C, 10 minutes at 16°C, 15 minutes at 37°C, eight minutes at 80°C, and held at 10°C. The first and second conditions were repeated between 10-40 times. The plasmid was then heat shock transformed into DH5 alpha E. coli
Golden Gate Assembly of pJC132 (HSP-ALD1-P2A-FMO1-35S in B Module)

Golden Gate assembly was used to create the HSP Promoter-ALD1-P2AA-FMO1-35S Term. 11.6µx of H2O, 2µx of Ligase Buffer, 0.5µx of BSA, 0.5µx DTT, 1µx of PJC199 (HSP Promoter), 0.5µx of pJC III (ALD1-P2A-FMO1), and 1.3µx of MC-9-35-II (35ST), 0.6µx of PJC225 (B Module), 1V of T4 DNA Ligase, and 1µx BsaI were used in the reactions. After performing the necessary reactions, the product was subjected to a thermocycler for five minutes at 37°C, 10 minutes at 16°C, 15 minutes at 37°C, eight minutes at 80°C, and held at 10°C. The first and second conditions were repeated between 10-40 times. The plasmid was then heat shock transformed into DH5 alpha E. coli cells and plated on LB plates with 50mg/L carbenicillin.

Golden Gate Assembly of the final T-DNA Vector

Golden Gate assembly was used to create the final T-DNA vector that was used in plant transformation. This pJC139 (HSP-ALD1-P2A-FMO1 T-DNA) vector was created using 11.3µx of H2O, 2µx of Ligase Buffer, 0.5µx of DTT, 0.5µx of BSA, 1µx of pMOD A-5801 (luciferase), 1.1µx of pJC132 (HSP-ALD1-P2A-FMO1-35T), 1µx of pMOD-C0000, 0.6µx of pTrans-220, 1µx of T4 DNA Ligase, and 1µx of AarI. After performing the necessary reactions, the product was subjected to a thermocycler for five minutes at 37°C, 10 minutes at 16°C, 15 minutes at 37°C, eight minutes at 80°C, and held at 10°C. The first and second conditions were repeated between 10-40 times. The plasmid was then heat shock transformed into DH5 alpha E. coli cells and plated on LB plates with 50mg/L kanamycin.

Creation of Liquid Culture

Two colonies of each E. coli sample—the sample containing the pJC III (Level 0 ALD1-P2A-FMO1 [6-9]) cDNA sequences, the sample containing the pJC132 (HSP-ALD1-P2A-FMO1-35S in B Module) term, and the sample containing the pJC139 (HSP-ALD1-P2A-FMO1 T-DNA) vector—were selected to create a 5mL overnight liquid culture containing 50mg/L spectinomycin, 50mg/L carbenicillin, and 50mg/L kanamycin, respectively [34]. Engineered vectors were extracted from the overnight cultures, and extracted DNA was sequenced with Sanger sequencing.

Transformation into Agrobacterium

The T-DNA with the desired sequence, pJC139, was transformed into Agrobacterium strain GV3101 using the freeze-thaw method with liquid nitrogen and plated on LB plates with 50mg/L kanamycin and 50mg/L gentamycin [34]. Preparing the competent cells started with the inoculation of 250mL of LB with 100µL of a fresh culture of the desired Agrobacterium strain (GV3101 in this experiment). Cells were grown overnight at 28°C and 300rpm shaking. Cells were pelleted at 4500xg for 10 minutes before supernatant was discarded. The pellet was resuspended in 25mL of fresh LB and 205µL cells were aliquoted in 1.5 mL tubes. Tubes were snap frozen in liquid nitrogen and stored at -80°C. To transform the Agrobacterium, the frozen competent cells were almost completely thawed then placed on ice. 1-5µL of standard miniprep binary plasmid was added and mixed gently. The tubes were frozen in liquid nitrogen for 10 seconds, then placed in a 37°C water bath for 5 minutes [34]. The tubes were shaken.
horizontally at 28°C and 300rpm for 20-60 minutes. Afterwards, they were plated on a LB plate with the appropriate antibiotic. Colonies were grown in a 28°C incubator for two days to yield between 20-500 colonies [34].

**Reagent Setup**

To prepare the Lysogenic Broth (LB), 10g of Tryptone, 5 g of Yeast Extract and 5 g of NaCl was resuspended into 0.800 L of distilled water. Distilled water was added so the solution has a volume of 1 L. The solution was autoclaved for 121°C for 20 minutes, cool it down to 50°C, and add the desired antibiotic. 20 mL of LB agar was poured for every 10 cm Petri dish. To prepare the antibiotics, for *P. syringae* strains containing plasmids, 15 µg/mL kanamycin and 2 µg/mL cycloheximide were used to prevent fungal contamination [31]. To prepare the 10mM magnesium chloride solution, a 1M stock solution was diluted by adding 1 mL to a 99 mL distilled water solution. To induce SAR in *Arabidopsis*, a wild type strain of the plant, such as Pto DC3000, or a derivative containing an avirulent effector—such as AbrRpt2, AvrRm1, or AvrRps4—is necessary. All steps were carried out in a sterile location to prevent contamination of any reactant. The DC3000 strain was used in conjunction with the DC3000 strain expressing AvrRpt2 from a plasmid [31].

**Plant Growth**

To plant the A. thaliana plants, seeds were stratified and sown in an appropriate solid mixture. They were covered with a transparent plastic lid and moved to a controlled environment room where they grew at 22°C under short-day conditions (8 hours of light and 16 hours of darkness) [32]. The plants were checked every two days. The plants were watered to ensure that the soil remained humid. Excess water was prevented from being poured into the tray. After two weeks, the *Arabidopsis* seedlings were transferred to individual pots. There were again covered using a transparent plastic lid for one week. Four- to five-week-old plants, if healthy, were deemed appropriate for bacterial inoculations via infiltration [32].

**Identification of Arabidopsis Leaves**

Accurate identification of the correct leaves on the A. thaliana plant was crucial for the inoculation process. Cotyledons and the first and second leaves were identified as the first opposing leaves. The third and future leaves sprouted in a roughly 130-degree angle from the previous leaf. Leaves 3 through 6 were increasingly larger and rounded. Half of *Arabidopsis* plants grew clockwise, and the others grew counterclockwise [32]. The eighth and beyond leaves had similar sizes and an elongated shape. The petiole of the eighth, ninth, and tenth leaves, were permanently labeled, as were the petioles of leaves 13, 14, and 15 in a different color [32].

**Preparation of Bacterial Inoculum**

Sterile conditions were used during the preparation of bacterial inoculum. *P. syringae* was streaked out from a -80°C stock culture onto a LB agar medium plate supplemented with spectinomycin (50µg/mL), kanamycin (50µg/mL), and gentamycin (10µg/mL). When working with plasmid-containing strains, the bacteria was cultured in the presence of its corresponding antibiotic to ensure plasmid maintenance. The plates were incubated at 28°C for two days. Afterwards, the bacterial biomass was scraped out from a fresh Petri dish and suspended in 10mM Magnesium Chloride. The OD600 was adjusted to 0.1 by adding
10mM Magnesium Chloride as needed. The OD600 was confirmed using a spectrophotometer [32]. Bacterial overgrowth was prevented, and a constant temperature was maintained to prevent OD600 alteration. An OD600 of 0.1 in bacterial suspension of *P. syringae* corresponded to approximately 5x10^7 colony forming units (cfu) per mL. Serial dilutions were performed by adding 100 microliters of the mixed inoculum to 900 microliters of 10mM Magnesium Chloride in a 1.5 mL tube and mixing by using the vortex. This step was repeated to obtain a bacterial suspension with an OD600 = 0.001 (5 x 105 cfu/mL inoculum) [32]. Next, an aliquot of the 5 x 105 cfu/mL inoculum was collected and two more serial dilutions were completed (1:10 and 1:100). Then, the dilutions were plated in LB agar plates and incubated at 28 °C. This colony was used as the inoculum control [32].

**Primary and Secondary Inoculations for SAR Activation**

Inoculations on the plant leaves were performed during the first hour of the light cycle for the plants. The number of *Arabidopsis* plants to be used for each treatment were then selected. The plants were randomly assigned to each treatment group. A needleless syringe was used to pressure infiltrate the 5 x 105 cfu/mL inoculum or mock solution into the abaxial face of the eighth, ninth, and tenth leaves [32]. Inoculum concentrations were maintained higher than those used for the secondary inoculation. Leaves that had been infiltrated properly did not look different from other leaves one to two hours after the infiltration. The eight ninth and tenth leaves were infiltrated by gently pressing the syringe onto the leaf until the area around the syringe grew darker. The plants were then returned to the controlled environment room. After one day, the secondary inoculation was performed. A Pto DC3000 suspension was prepared using the same steps as used for the primary inoculation [32]. This was a standard proliferation assay; thus, the inoculum concentration was low enough to allow multiple rounds of bacterial replication. This helped the development of differences between treatment types. Using a needleless syringe, the DC3000 5 x 104 cfu/mL suspension was infiltrated into the abaxial face of the thirteenth, fourteenth, and fifteenth leaves [32]. The plants were returned to the controlled environment.

**Bacterial Recovery from Plant Samples and Determination of CFU**

Four days after secondary inoculation, a 10-millimeter-in-diameter disc was taken from the center of each leaf infiltrated in the secondary inoculation with the aid of a cork-borer. The discs were placed into a 1.5 mL tube containing 500 microliters of 10mM magnesium chloride. The process was repeated for each inoculated plant. Then, plant samples were homogenized by using pestles. 500 microliters of 10mM magnesium chloride were added to the solution achieve a volume of 1 mL [32]. A vortex was used to mix the solution. 1:10 serial dilutions were made and plated on LB agar plates containing 2 µg/mL of cycloheximide. Multiple serial dilutions were plated. Each dilution was prepared by adding 100 microliters of a solution made by adding 100 microliters of bacterial suspension to 900 microliters of 10mM magnesium chloride to each Petri dish. Plates were incubated for two days at 28°C [32].

**Appendix B: Glossary of Field-Specific Terms**

Agrobacterium-Mediated Transformation: the process which uses the bacterial
pathogen Agrobacterium tumefaciens to transfer foreign genes into a host plant.

Biolistic Bombardment: a method for the delivery of DNA to cells by high-speed particle bombardment with a pressurized gene gun.

Metabolism: biochemical processes occurring within an organism to maintain life.

Metabolite: a molecule or substance required for metabolism in an organism.

Metabolome: the complete characterization of metabolites present in an organism or biological sample.

Metabolomics: the study of metabolites, their derivatives, and intermediates.

Transgene: a gene that is introduced into the genome of an organism but is not naturally found in the genome of the organism.

Untargeted Metabolomics: the comparison of metabolomes of control units and experimental units to identify differences between.

Appendix C: cDNA Nucleotide Sequences

Following are the cDNA sequences ordered from Twist Bioscience with the addition of Type II restriction enzyme-compatiable cut and binding sites.

BbsI cut sites are colored orange

BbsI binding sites are colored green

J6 – ALD1 – J7

TTTgaagacaaATATGGTCACTATGTTCCTTTTA GTTGGGCCTCACCCTTTATGCTCTTCTCCATCAA AAATCCCAAGGCTAGTTGGAACCTGAGATG GAAGAAACTTGGTGGGACGACAAAAACCTTTGGC GAACGTAATTGAGAAACTAAGAAATAATT ATTGTTTCTCAGAATCACAAGCAGTAACTT GAGCACAATTGAAAGCATCCCAAATGTAACATT GATAAGCCTTGGAACACTGTGATACAACAGAGC

J7 – P2A – J8

TTTgaagacaaAGGTTCTggatctggagctactaattttttcttttacttaagctgagatgcctaggtttggaaccc TCGttttcttTTT

J8 – FMO1 – J9
TTTgaagaacaATTGGCTTCTAACTATGA
TAAGCTTACTCTCTCGAGAGTACCCATTCAATCG
GTGCTGTTGTTAGCGGATTTACGAGCCGCTAAG
AACCTATGCGATTCGAGTTTGCAGGAGGATTGAGA
GCTGCATTTAGACGACAGCATAAACATACATCG
GCTGCGACTGCGATTTACGAGTTTCGGAGACTTTCC
ATGGCCCAATAATAGAGATGACACAAACTTTCC
CACCTTACCTGAGATATTAGATTACTTTGAA
TCTTATGCAAACATTTTGTATCTCTCTAAATT
CATGAAGTTTGGCTCTAAGTCCATCGAAGTAA
GTTCCATCGTGGTAGCCGAAACTCTCCAGATG
GGGCAATTTGATAGGCCTAACCAAAGCTTCCTTAGAACTC
TGATAGGGCCTAACCAGCATTCTTATTAGAACCCTC
TCTTGGGTCACTCTCTCTCTCTCTCTCTTCTAGGGCGG
TAGTTTCCAAGTTCATCCAGCCGAGATATGTTTATT
TGGAAGCTACTCTCTGGAAATATGGTCTAAA
ACCAACATTTCTCTCGAGGAAGACTATGCTT
TTGGTCACATGCAACAAGCTATTGTTTCCA
TGATGAGAACAGCGATACGGAGAATTTCCTTGTCAAATGGCGATCATACCGGAGAATTTC
TTTGAGGAAGCGGATAAAGGGATGATCCGGTT
TGATGGCAAGAAGAAGCTCAAAGCTATTGTTC
CTGAACCTTTGCTTACGGCAACTTGTGGCC
CCGAAAACCCTGTATGGGAAGTTGCTGTTCCGA
TCTGAGATTTCTGAGATATCTGAGCTCGATGCA
TTTGAAGTTGTTGTGTGTACCGGGAAATA
CGGAGGATTTCCAAAGATTAGGCCTTAGA
CACCAGAAGGGCCCGGAGATGTTCACAGGAAA
TTTAGGCTACAGTGAATAGCGCACAGTGAAGTGA
AAGAAGATTTGGATCAATTCTTAAAAGAAATG
GAAGTCCATTGGATTTCCAGTCCACGATCAAAC
AAAAATGAGCCGCTATTGGAAGAGGAGAACATCC
GCTGCATTTGCTTCTTGGCTTAGGTTCCGAGCTCAGGAGAAATCC
GAACATCTTTGCTATTTGGGACATGGAAGCACCA
CATTATGGGTGTTGGGGCTTACCATCTTTCTT
GTTCTACTCTCTCGAGAGTCTCTCAGTGGCTCCA
TGATAGGGCCTAACCAGCATTCTTATTAGAACCCTC
TCTTGGGTCACTCTCTCTCTCTCTCTCTTCTAGGGCGG
TAGTTTCCAAGTTCATCCAGCCGAGATATGTTTATT
TGGAAGCTACTCTCTGGAAATATGGTCTAAA
ACCAACATTTCTCTCGAGGAAGACTATGCTT
TTGGTCACATGCAACAAGCTATTGTTTCCA
TGATGAGAACAGCGATACGGAGAATTTCCTTGTCAAATGGCGATCATACCGGAGAATTTC
TTTGAGGAAGCGGATAAAGGGATGATCCGGTT
TGATGGCAAGAAGAAGCTCAAAGCTATTGTTC
CTGAACCTTTGCTTACGGCAACTTGTGGCC
CCGAAAACCCTGTATGGGAAGTTGCTGTTCCGA
TCTGAGATTTCTGAGATATCTGAGCTCGATGCA
TTTGAAGTTGTTGTGTGTACCGGGAAATA
CGGAGGATTTCCAAAGATTAGGCCTTAGA
CACCAGAAGGGCCCGGAGATGTTCACAGGAAA
TTTAGGCTACAGTGAATAGCGCACAGTGAAGTGA
AAGAAGATTTGGATCAATTCTTAAAAGAAATG
GAAGTCCATTGGATTTCCAGTCCACGATCAAAC
AAAAATGAGCCGCTATTGGAAGAGGAGAACATCC
GCTGCATTTGCTTCTTGGCTTAGGTTCCGAGCTCAGGAGAAATCC
GAACATCTTTGCTATTTGGGACATGGAAGCACCA
CATTATGGGTGTTGGGGCTTACCATCTTTCTT
GTTCTACTCTCTCGAGAGTCTCTCAGTGGCTCCA
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