Development of a Bioluminescent Sensor of Isothiocyanates through Genetic Modification of *Pseudomonas fluorescens* and Validation on Crude Plant Materials

by
Octavia Lydia Hogaboam

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented March 2, 2020
Commencement June 2020

AN ABSTRACT OF THE THESIS OF

Octavia Lydia Hogaboam for the degree of Honors Baccalaureate of Science in Microbiology presented on March 2, 2020. Title: Development of a Bioluminescent Sensor of Isothiocyanates through Genetic Modification of *Pseudomonas fluorescens* and Validation on Crude Plant Materials.

Abstract Approved: _____

Kristin Trippe

Isothiocyanates (ITCs) are produced by roots in a variety of agricultural plants, such as those within the *Brassicaceae* family. ITCs act as fumigants, and are used to control microbial plant pathogens by adding *brassica*-origin seed meal to soil prior to replanting. However, there is suggestion that the presence of residual ITCs in soil may inhibit seed germination in recently planted crops. These chemical compounds are difficult to detect, where a biological sensor may be a strategic indicator for when the soil is safe to replant in. Some microbes, including *Pseudomonas fluorescens,* exhibit resistance to ITCs mediated by an induced biochemical pathway. *P. fluorescens* is a root-colonizing non-pathogenic microbe, making it an ideal and safe biological sensor for agricultural use. To accomplish this, we linked an ITC-responsive promoter (*saxA* promoter) to the *ilux* operon, an enhanced version of the *lux* operon with an additional FMN reductase. The resulting biosensor was responsive in a dose-dependent manner to the aliphatic ITC, sulforaphane, in the concentration range of 1-100 μM. Further tests were conducted using extracted fluid from plant material of broccoli and daikon for the experimental group, while utilizing clover, alfalfa, and mung bean as a negative control group. The biosensor was responsive to the *Brassica*-related plants, and did not luminesce in response to the negative control extracts. The biosensor was also responsive to the seed meal of *Brassica juncea* and *Sinapis alba,* which are common seed meals used in biofumigation. Future experiments will determine the biosensor's responsiveness to other ITCs and their precursors, glucosinolates, and detection in seed meal amended soils.

Key Words: Pseudomonas, Isothiocyanates, Luminescence, Genetics, Detection

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Octavia Lydia Hogaboam, Author

Acknowledgements

I would like to express my appreciation to my supervisor and mentor, Dr. Kristin Trippe, for assigning this project to me, encouraging me throughout my undergraduate career, and providing helpful feedback during the writing process. This experience has taught me so much that I would not have gotten doing short summer internships. She not only gave me a place to conduct research, but another home where I was surrounded with positivity and motivation.

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This 3-year project is the result of the combined effort and support by the people in the lab and others involved in my life.

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Introduction

Isothiocyanates (ITCs) are volatile compounds that *Brassicaceae* plants use as a defense mechanism in response to physical damage to the plant. In this process, species containing glucosinolates (specifically β-D-thioglucoside-*N*-hydroxysulfates or GLS) enzymatically form ITCs by an interaction between the myrosinase and GLS that are held in separate compartments. Upon tissue damage, the glucosinolates (GLS) come into contact with myrosinase, which cleaves the β-D-glucose and spontaneously forms ITC. The products of this degradation process are volatile and toxic, capable of terminating microbes and nematodes residing in the soil [1]. Some species that are capable of producing ITCs, like *B. carinata* (Abyssinian mustard), *B. nigra* (Black mustard), *B. juncea* (Indian Mustard), *and B. rapa* (Field Mustard) have GLS in the roots, stems, leaves, and reproductive organs, but at varying quantities at different developmental stages [2].

Farmers have co-opted this defense mechanism by generating *Brassicaceae* plant-derived ITCs for biofumigation, which is a method of removing pests and pathogens from soil organically. One method is to grind up the seeds of *Brassica*-origin plants to amend the soil with seed meal before covering the land with a tarp for approximately a week. Another approach is to take fully grown *Brassicaceae* plants like mustard and till them to release the ITCs in the form of green manure. These processes take a different amount of time, but the overall mechanism of biofumigation remains the same [3]. Although the reduction of pathogens and pests is typically beneficial, there is significant evidence suggesting that ITCs inhibit seed germination. In addition to that, ITCs coming into direct contact with leaves led to chlorosis and cell death, as demonstrated by studies done on *Arabidopsis thaliana*, a model organism in plant biology. It was observed that concentrations of 10-100 μM of ITCs led to reversible root growth inhibition in *A. thaliana* under standardized conditions [4].

Since ITCs inhibit the germination of seeds post-fumigation, there is a critical need for fast, accurate, and simple methods that detect residual ITC concentrations in a soil matrix. Current methods of quantifying ITCs in soil involve spectroscopic analysis after a reaction of ITCs with dithiols like 1,2-benzenedithiol, to generate cyclic condensation products. For example, the product 1,3-benzodithiole-2-thione is stable and can be quantified at 365 nm using ultraviolet spectrometric detection. Another method involves quantification using high

performance liquid chromatography (HPLC) by extracting the ITCs with methanol [5]. Although there are multiple venues for chemical detection using spectroscopic analysis and liquid chromatography, there has not been development of a biological sensor capable of the same function. The biological sensor would require less specialized and expensive equipment, be easily applicable, and would be fast enough to detect ITCs within a day.

Current methods of ITC detection are not amenable to detecting remnant concentrations of ITCs that may inhibit germination. As such, the purpose of our study was to develop a biosensor that has the following properties: 1) is able to detect ITCs at μM concentrations, 2) is resistant to the anti-microbial action of ITCs, and 3) is amenable to genetic manipulation. *Pseudomonas fluorescens* SBW25 meets all of these criteria. It is a non-pathogenic root colonizer and is capable of stimulating plant growth. This microbe can also cause induced systemic resistance, which functions like a vaccination that protects the plants from other harmful pathogens [6]. Since ITCs are meant to terminate microbes that could be pathogenic to new crop growth, it is crucial that the microbe being used is capable of surviving exposure. There are a number of Pseudomonads that are capable of resisting aliphatic isothiocyanates like sulforaphane, using a gene complex called *sax*. The *saxCAB* genes are capable of resisting ITCs, which had been demonstrated in the pathogen-host relationship, *Arabidopsis-Pseudomonas* [7]. Through a search of the presence of the *sax* gene among *Pseudomonas fluorescens strains*, it was found that the strain SBW25 contained *sax* in its genome [8]. We concluded that the strain would be used in the development of the biosensor due to its root colonization ability and resistance to ITCs.

The luminescence aspect of the biosensor can be generated by the gene, *luxCDABE*, which originated from *Photorhabdus luminescens*. The *luxAB* portion of the gene encodes for the luciferase enzyme, which is the catalyst for the reaction that produced luminescence. The *luxCDE* encodes for an enzyme complex that produces the substrate for the luciferase to react to [9]. This reaction occurs as follows:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + hv$$

To keep this oxidization reaction continual, the fatty acid reductase, transferase, and synthetase would need to turn the FMN back into $FMNH_2$ to bypass a limiting factor. To

accomplish that, researchers added an additional FMN reductase in order to enhance luminescence in *Escherichia coli*, dubbing the gene *ilux* [10]. With the combination of the *saxA* promoter from the *sax* gene and the *ilux* operon, a *Pseudomonas fluorescens* bioluminescent sensor could be produced to detect ITCs without requiring methanol and liquid chromatography.

In the current study, we designed, constructed, and validated a *P. fluorescens* biosensor that is reactive to ITCs. We used variety of molecular microbiological techniques to ultimately transformation the microbe into the final strain. Once the biosensor was constructed, it was tested in bioluminescent assays with a controlled quantity of the known aliphatic ITC, sulforaphane. The last aspect of the current study was to use crude substances like extracted plant matter and seed meal as a form of validation of the biosensor's capabilities.

Part I: Development

Development of a Bioluminescent Isothiocyanate Sensor through Genetic Transformation of *Pseudomonas fluorescens*

Purpose

The objective of this phase of the project was to genetically modify a *P. fluorescens* strain to detect isothiocyanates in a dose-dependent manner without background expression. This was completed by combining the *ilux* operon with the *saxA* promoter, deleting the constitutive background promoter, *p1*, and selecting for gentamicin resistance during the transformation process. Confirmation of the biosensor's functionality was done using a concentration gradient of sulforaphane, an isothiocyanate produced by broccoli, in a kinetic luminescence assay.

Methods

Plasmid Preparation:

The plasmid vector, *pUC18T-Tn7T-mini-lux-Gm,* contained Tn7 sites for genome integration into SBW25, the *lux* gene responsible for bioluminescence, and the gentamicin resistance gene for selection during transformation. A custom multiple cloning site synthesized by Viola Manning using Genewiz (*see Appendix*) was inserted into the plasmid by digesting each fragment with XcmI/SacI restriction enzymes and ligating under standard conditions. The *p1* promoter, a constitutive promoter present upstream of the *lux* operon in the original vector, was simultaneously removed from the plasmid. The resulting plasmid, pHT1, was sequenced with VM-109/110 primers (*see Appendix)* and confirmed with EcoRI/HinDIII restriction enzyme digests and gel electrophoresis.

The *saxA* promoter was isolated from the genomic DNA of *Pseudomonas cannabina* ES4326 strain using PCR amplification with VM-093/094 primers (*see Appendix*). The PCR product was digested with EcoRI/BamHI restriction enzymes, purified with Zymo Research DNA Clean & Concentrator, and quantified using a Qubit fluorometer. By using the multiple cloning site that was previously inserted, pHT1 was linearized via restriction digestion and the promoter fragment was inserted upstream of the *lux* gene. After ligation, the insertion of the promoter was confirmed by sequencing the plasmid with VM-114 (*See Appendix).* The resulting plasmid, a combination of pHT1 and the *saxA* promoter, was renamed pHT3 (*See Figure 1.1).*

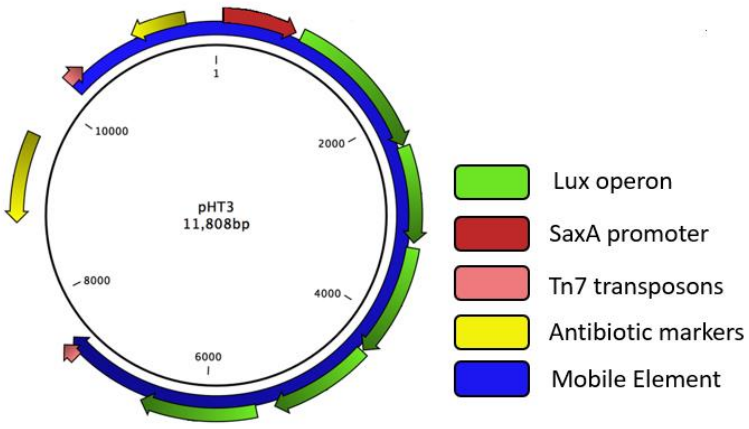

Figure 1.1. *Plasmid map of the first functioning plasmid, pHT3 that had an inducible luminescent response to sulforaphane without background expression when integrated into Pseudomonas fluorescens strain SBW25.*

To potentially increase the luminescent signal, an enhanced *lux* operon, *ilux* [10], replaced the original operon in pHT3, while still keeping the inserted *saxA* promoter. The difference between the original *lux* operon and *ilux* is the addition of an FMN reductase downstream, labeled, "ilux frp," as shown in Figure 1.2, and a series of mutations on the *luxAB, luxCD,* and *lux E* genes. In order to add the operon to generate our bioluminescent sensor, we requested the plasmid from the paper directly.

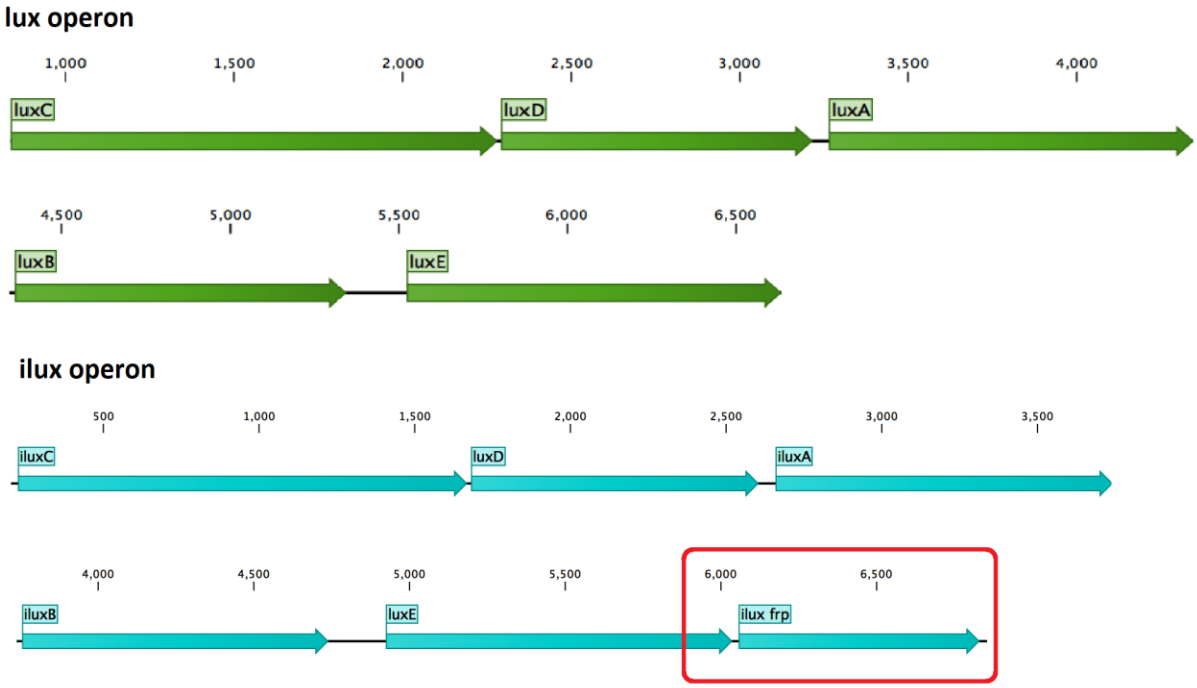

Figure 1.2. *Layout of lux operon and ilux operon indicating the difference between the two genes with the ilux frp, FMN reductase, gene downstream from the rest of the original operon.*

The addition of *ilux* was achieved through Gibson Assembly of two large fragments from pHT3 and the pGEX-*ilux* [10]. The pHT3 fragment was PCR amplified with VM-159/160 and the pGEX fragment with VM-161/162 (*See Appendix).* The amplified DNA was visualized on a 1% agarose gel, gel purified using QIAGEN Gel Extraction Kit, and quantified with Qubit fluorometry. Using the provided NEBuilder HiFi DNA Assembly 2x Master Mix, 75 ng of each fragment was assembled to create pHT9. An equivalent protocol was used to create pHT8, a control plasmid using pHT1 to ensure no background expression in the absence of the *saxA* promoter. The newly generated plasmids were digested using BglII/PstI to confirm that the fragments were in the correct orientation.

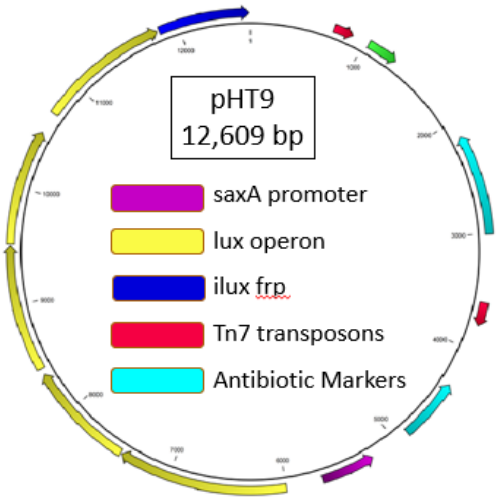

Figure 1.3. *Plasmid map of final pHT9 plasmid containing the crucial components of the isothiocyanate biosensor.*

Bacterial Transformation:

Each of the generated plasmids were used to transform *Escherichia coli NEB10-β by* heat-shocking at 42°C and selecting on Luria broth (LB) agar plates containing 50 µg/mL carbenicillin and 15 µg/mL gentamicin. The successful clones containing the desired plasmid were selected and grown overnight in equivalent liquid media. The plasmids were subsequently purified using a Zyppy Plasmid Miniprep Kit (Zymo Research, RPI) and quantified using a Qubit fluorometer. The plasmids were then electroporated into *Pseudomonas fluorescens SBW25*, with the addition of a helper plasmid, pTNS3. Transformed *P. fluorescens* SBW25 colonies expressing resistance to carbenicillin and gentamicin were selected for further study. Genomic DNA was extracted from selected colonies and integration of the plasmid was confirmed using PCR primer pairs VM-024/142 and VM-026/143 (*See Appendix*), which were compared directly to the genomic DNA of *SBW25.*

Luminescence Assays:

The first functional biosensor, pHT3-2, resulting from the integration of pHT3 into the genome of SBW25, encoded the *saxA* promoter driving expression of *luxCDABE* operon. To test its functionality, the clone pHT3-2 was added to a solution of Luria Broth with 50 µg/mL carbenicillin, 15 µg/mL gentamicin and a concentration gradient of 0-100 µM sulforaphane (in 25 µM intervals, diluted from a 100 mM stock suspended in DMSO). The bacterial density was measured by OD_{600} (at 0.5 cm), and diluted to 0.005 OD. These

solutions were distributed in 100 μL volumes in a 96-well white, opaque plate. Each concentration was measured in four replicates in order to average and evaluate consistency. Rather than using the plastic cover on the plate, the lid was removed and replaced with a Breath-Easy® sealing membrane. The plate reader was programmed to measure luminescence every 30 minutes for 20 hours. Between readings, the cultures were incubated at 31°C degrees agitated linearly at 500 cpm (3 mm).

The final biosensor, pHT9-3, encoded the enhanced *ilux* operon. We used a similar plate reader protocol as described above to compare the Relative Light Units (RLUs) at the varied sulforaphane concentrations over time. The parameters were adjusted to optimize expression using extensive experimentation. The final concentration gradient experiment conducted with pHT9-3 contained 0, 5, 10, 25, 50, 75, and 100 μM sulforaphane concentrations, an initial bacterial optical density of 0.05, an incubation temperature of 29°C, and an interval decreased to every 15 minutes. All results generated by the Gen5 software were exported and interpreted manually in Microsoft Excel.

Results and Discussion

The goal of this project was to develop a *P. fluorescens* strain that is bioluminescent in response to ITCs. The first sensor, pHT3-2, exhibited a peak at an approximate time of 8 hours regardless of the concentration of ITCs present in the solution. The sensor exhibited almost no background luminescence in relation to the other concentrations, drawing a flat line in *Figure 2.1*, indicated by the dark blue line containing 0 µM sulforaphane. The peak luminescence measured for 100 µM of sulforaphane was at approximately 700 RLUs for the pHT3-2 clone.

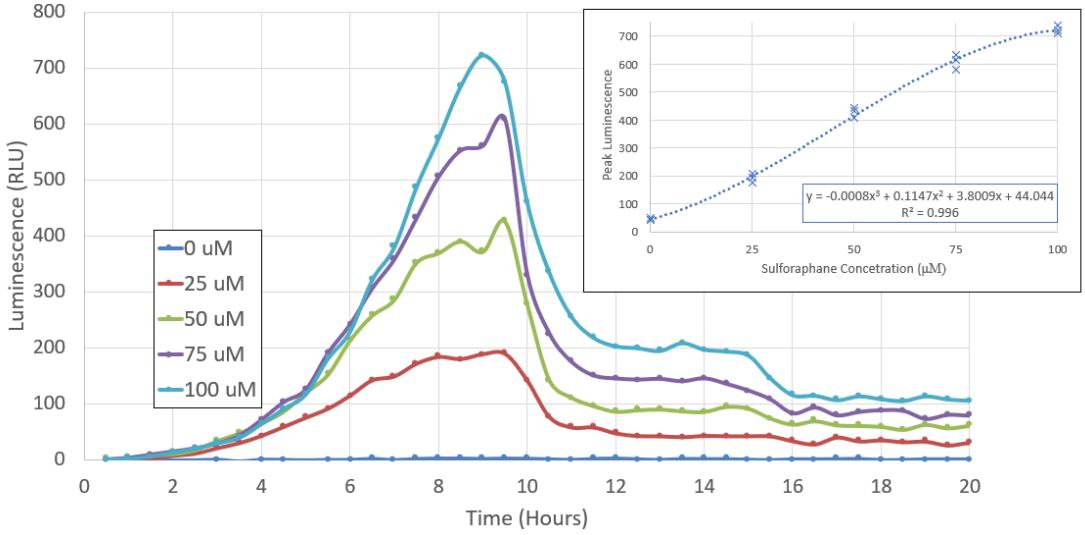

Figure 2.1. *Luminescence of pHT3-2 in response to varying sulforaphane concentrations (0, 25, 50, 75, 100 uM) under specified growing conditions.*

It was concluded that even though the background caused by the constitutive promoter was eliminated, the peak luminescence was too low to be adequate for detecting small amounts of ITC that would be present in soil. Clone pHT9-3, which encodes the *ilux* operon, under slightly different parameters, exhibited a peak luminescence at 100 µM of approximately 5700 RLUs, while the background remained constantly flat at 0 µM of ITC (*See Figure 2.2*). To obtain increased luminescence, we reduced the incubation temperature to accommodate the ideal growing conditions of *P. fluorescens (*29°C). The starting optical density was also adjusted, where it was found that a higher starting value, to a limit, lead to an earlier and more prominent peak luminescence prior to the spontaneous drop in

expression. The lowest concentration of sulforaphane, 5 µM, gave an average peak luminescence value at approximately 500 RLU, which is the equivalent of the 50-75 µM values in the pHT3-2 assay (*See Figure 2.1).* Thus, the results obtained with the *ilux* operon demonstrated a significant improvement over the ***luxCDABE*** operon when it was integrated into the *SBW25* genome.

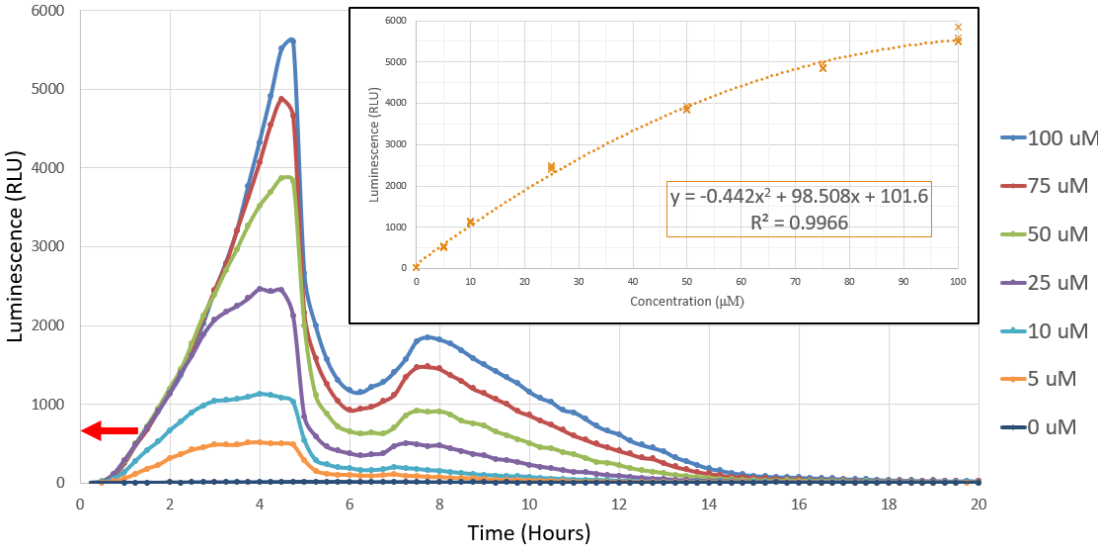

Figure 2.2. *The P. fluorescence strain SBW25, clone pHT9-3, responsive to a concentration gradient ranging from 0-100 µM of sulforaphane with adjusted parameters. The red arrow indicates where the peak 100 µM pHT3-2 was.*

We could detect relative light units generated by 5 µM of sulforaphane, so we tested the limitations of the *ilux*-based biosensor by measuring luminescence in a range of 0-20 µM sulforaphane in 1 µM intervals to determine if smaller intervals would be accurately detected. In the concentration gradient shown in *Figure 2.3,* there is visible separation between each quantity, although there were some overlapping data points with 12 µM and 13 µM. This issue prompted adopting a new technique for loading 96-well plates involving multi-channel pipetting to increase consistency among the replicates. However, despite the occasional overlapping, the *ilux*-based biosensor exceeded expectations in detecting sulforaphane concentrations.

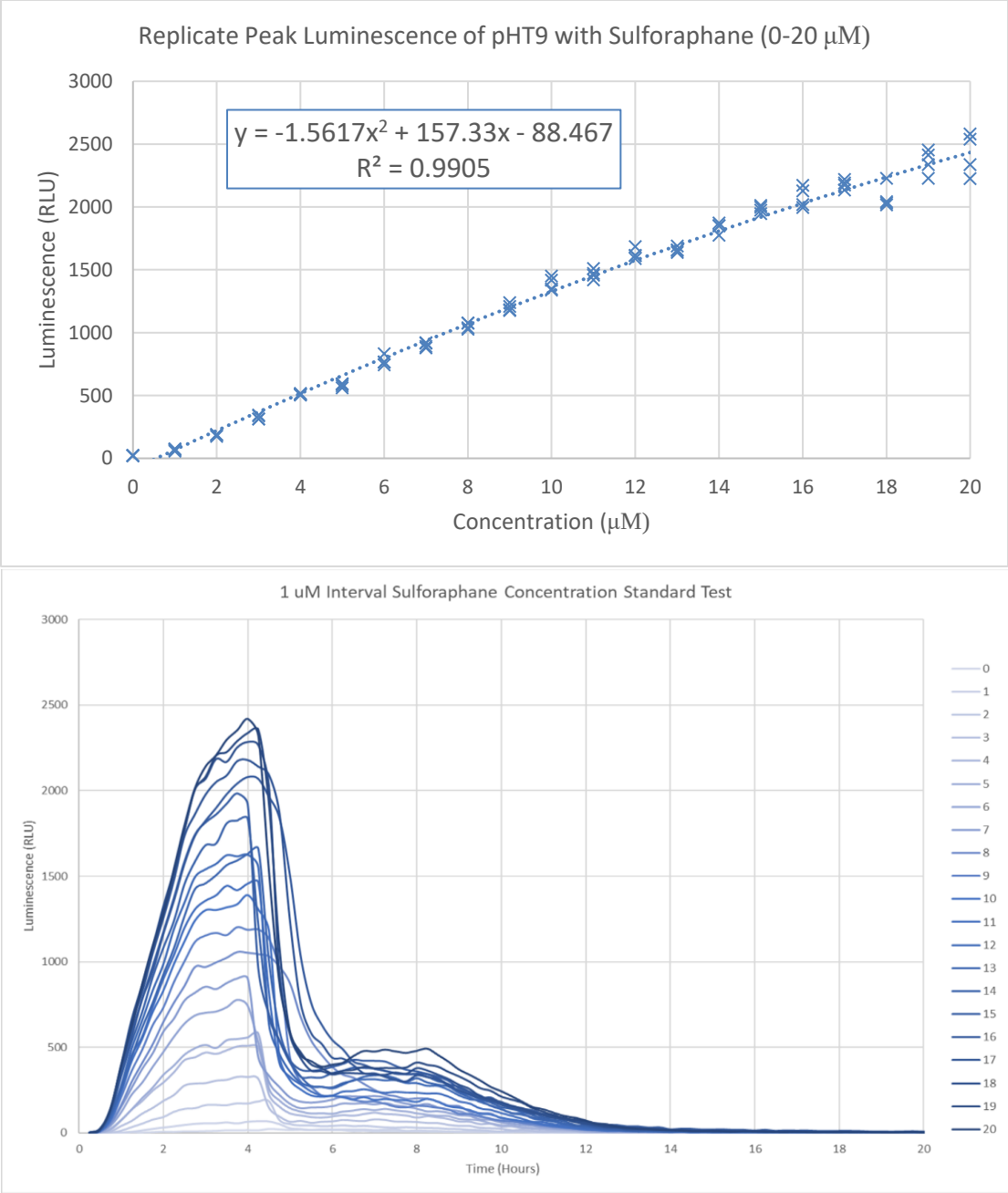

Figure 2.3. *Sulforaphane Concentration Gradient Test on pHT9-3 clone from 0-20 μM at 1 μM Intervals to Show Limitations and Specificity of the Biosensor.*

In all of our experiments, we observed a sharp drop in expression of the *ilux* gene after reaching peak luminescence. To determine if this peak correlated with growth of the bacteria, we tracked the optical density over time and compared that to the luminescence values. Although these tests had to be conducted separately due to microplate restriction, luminescence peaked consistently around 4 hours. It was found that as the bacteria

progressed from exponential growth to stationary phase (*See Figure 2.3*), the expression of *ilux* would drop around the approximate inflection point. This indicated that the level of luminescence was likely population and growth dependent.

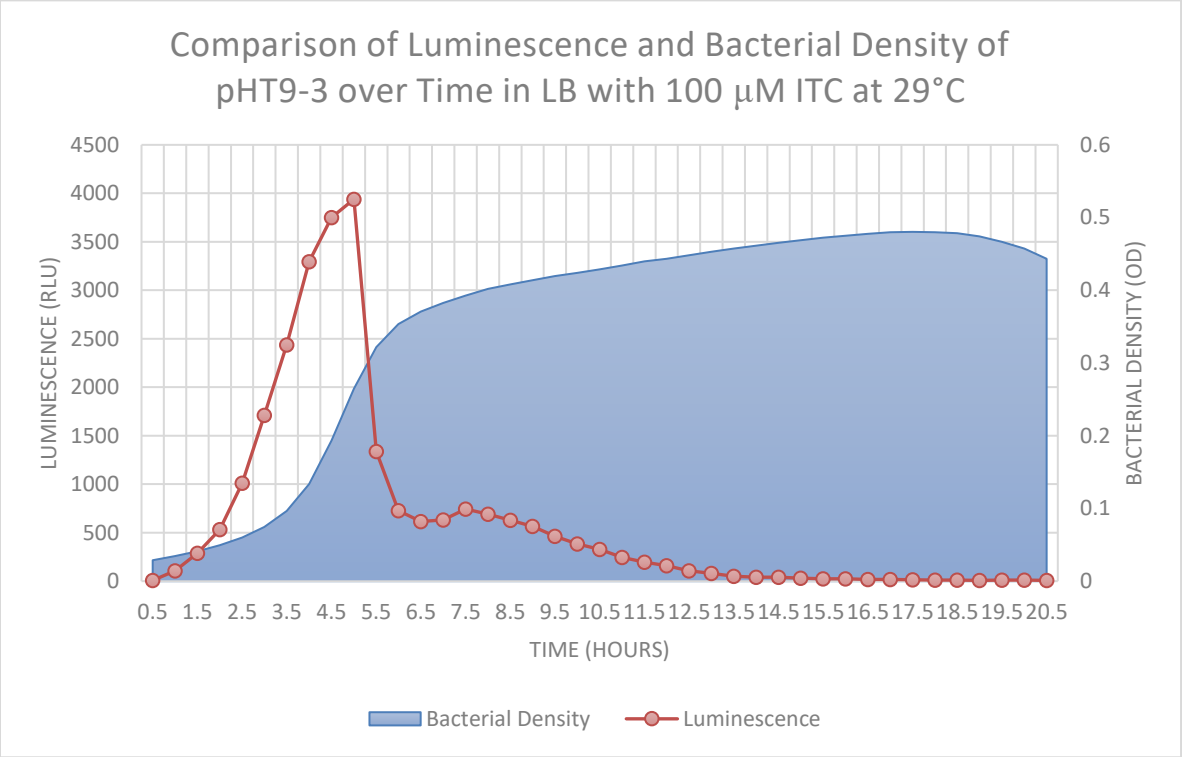

Figure 2.4. *Direct comparison of the luminescence timing with an OD_{600} growth curve under the same growing conditions as Figure 2.2. Note: The kinetic assays for each curve on this graph were each done separately due to plate incompatibility.*

In order to make sure that the sulforaphane was not inhibiting or affecting the growth of *SBW25-pHT9*, optical density curves were generated in the presence of different concentrations of sulforaphane. The results presented in *Figure 2.4* indicated that regardless of the sulforaphane concentration (0-100 µM), the growth pattern remained constant. This demonstrated that SBW25 is resistant to sulforaphane, confirming what had already been known about the organism's genome.

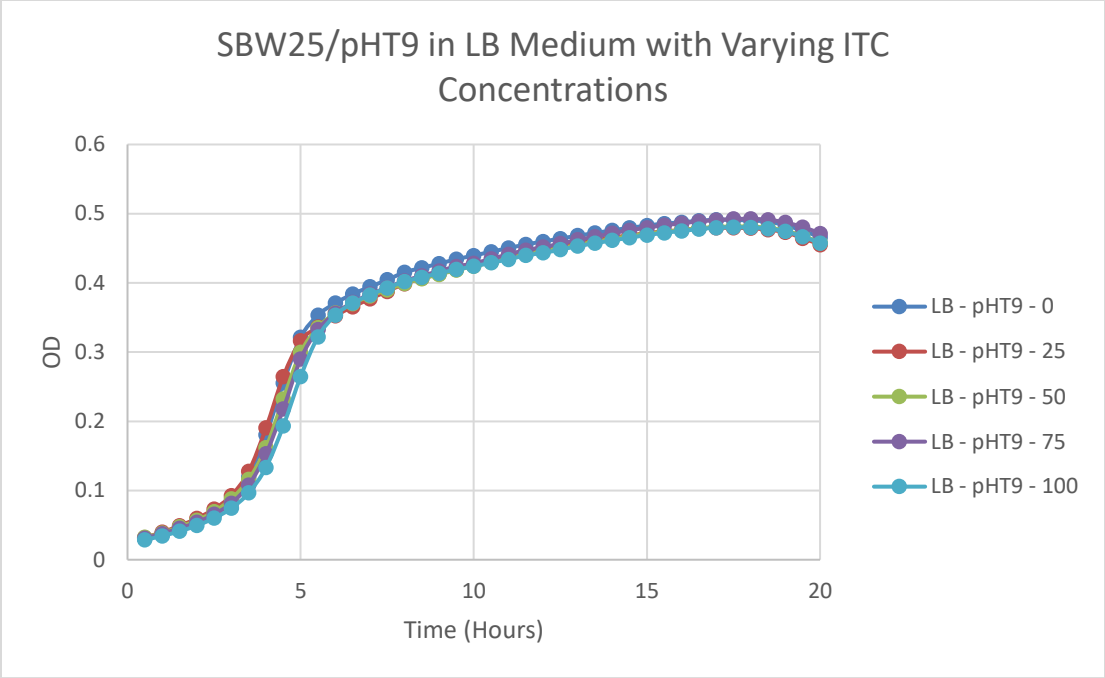

Figure 2.5. *OD_{600} growth curves of pHT9-3 clone in a kinetic assay with 0-100 μM sulforaphane concentrations under standardized parameters specified in Figure 2.2.*

The results of our experiments demonstrated that SBW25-pHT9 reports the presence of sulforaphane in a standardized amount, and could be used to quantify unknowns if compared directly to standard curve. Originally, it was meant to detect the presence or absence of ITCs in a given solution like a visible switch. The biosensor exceeded expectations and was capable of ITC detection in a consistently dose-dependent manner. However, these results were limited to sulforaphane. The ultimate goal is for this biosensor to detect ITCs in seed-meal amended soils in general, which may be more complex than the standardized experiments used to validate functionality.

Part II: Validation

Validation and Optimization of an Isothiocyanate Biosensor with *Brassica* Plant Extracts and Seed Meal Leeching Products

Purpose

The objective of this phase of the project was to confirm the *ilux*-based biosensor would interact with crude plant-based extracts in a comparable manner to the standard curve. To validate this, we extracted fluid of lysed *Brassicaceae* sprouts, as ITCs are produced in damaged tissue. We also determined the efficacy of the biosensor on pure seed meal leeched with water. With this information, the concentration of ITCs can be measured for future research in determining safe levels for crop replanting on recently fumigated soil.

Methods

Plant Extraction

To validate the efficacy of the biosensor, tests were conducted with extracts from roots and seed meal. Broccoli and daikon seeds were sterilized using a surface-sterilization protocol, where the seeds were first soaked in 70% ethanol for 3 minutes. This was followed by a 15 minutes soak in a 1.2% bleach solution amended with 0.1% Tween 20, an additional detergent that reduces the ability for bacteria and fungi to survive in the crevices of the seeds. After a thorough washing process, the seeds were left overnight in a sterile hood to dry completely prior to use. The broccoli and daikon seeds were germinated in small petri dishes containing 0.5x Murashige and Skoog Medium (MSB) for the cultivation of plant cultures. In addition to that, the media contained 0.3% agar in order to facilitate easy extraction of the rooted sprouts with minimal disturbance of the fragile root hairs. Seeds were incubated for a up to 4 days under artificial lighting (8-hour day, 16-hour night) at 23°C.

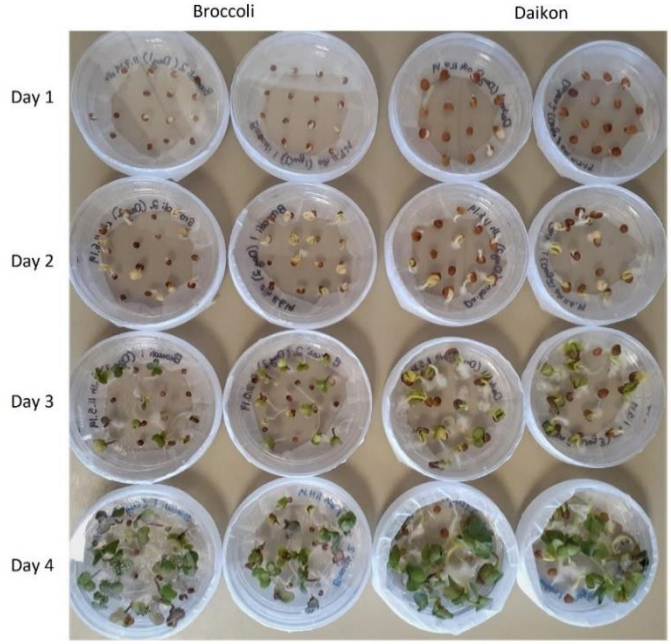

Figure 3.1. *Image of the morphologies of the germinated broccoli and daikon radish seeds from days 1-4 of incubation on 0.5x MSB and 0.3% agar. Each plate is wrapped with breathable cloth tape to allow for respiration and to maintain consistent moisture.*

The germinated seeds were ground in specialized tubes containing steel beads at 1500 RPM for 10 minutes. The samples were centrifuged at 15,000 RPM for 5 minutes, and the remaining supernatant was transferred and sterilized using a syringe filter. The assumption was that the supernatant contained the isothiocyanates and any other unspecified compounds that the bacteria may react with. Supernatant (100 μL) was added to 900 μL of Luria Broth along with the pHT9-3 at a final optical density of 0.05. Luminescence was measured on a

plate reader as described in Part I. Different germination times were assessed to determine when the plants exhibited the greatest ITC concentrations.

The same protocol was used on non-brassica plants to confirm that the biosensor would be unresponsive to plants that do not produce ITCs. The plants that were tested were mung bean, alfalfa, and clover due to their quick germination time and lack of evidence of exhibiting any isothiocyanate production. Therefore, they would serve as a negative control to ensure no background expression from reacting to other plant metabolites.

Seed Meal Leeching

The seed meal was incubated in a rotating shaker at room temperature with a 1:10 ratio (seed meal : water) by mass in 2.0 mL microcentrifuge tubes. The centrifuging and syringe filtering were conducted as described above with the plant extractions. The two seed meal types that were tested were *Sinapis alba* (*S.a.*), white mustard, and *Brassica juncea* (*B.j.*), Indian mustard, which are both Brassicas that are used in biofumigation.

Figure 3.2. *Image of Brassica-origin seed meal in powdered form indicated lack of requirement for the extraction protocol and need for standardized leeching process. This also shows comparison in the composition of the two different substances, where the B. juncea is clumpier and likely contains more moisture than S. alba.*

These 96-well plate tests were optimized to obtain high luminescence readings (*See Figure 2.2 for parameters)* while maintaining a controlled concentration gradient to account for slight variations in each assay. These variations could have been due to bacteria being in a different phase at the beginning of the test and other factors that cause a slightly different peak height each time.

Results and Discussion

The objective of the experiment was to validate the biosensor's ability to be applied to plant-based substances and to analyze any interferences that other unidentified compounds may cause.

The biosensor produced 3,000 RLUs in response to the extracted broccoli fluid, which was approximately 25 µM sulforaphane when directly compared to the standard curve of pure sulforaphane. The extract from daikon, which is another known plant producer of ITCs, resulted the detection of 6,000 RLUs. However, the peak from daikon extract was significantly delayed despite the pattern or the primary and secondary peak being similar. This may indicate that a compound in the daikon is inhibiting the bacterial growth and causing a delay in the exponential phase that drives the *ilux* expression in the bacterial population. This particular test merely demonstrated that the biosensor was capable of detecting isothiocyanates at varying intensities, however, there were limitations in the amount of information that can be interpreted from the daikon assay.

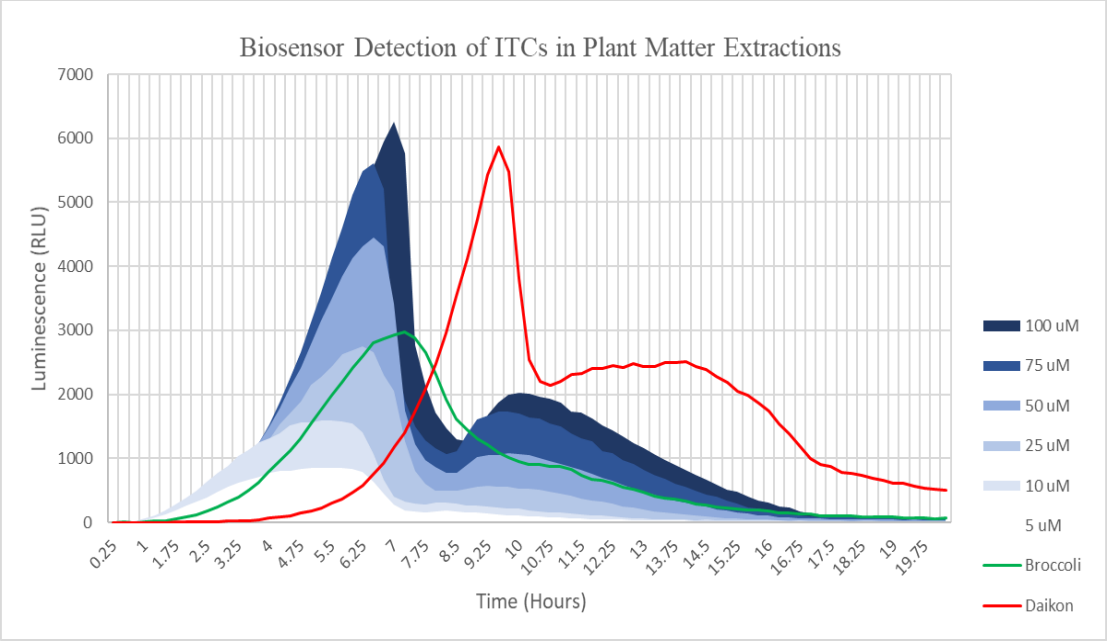

Figure 4.1. *Broccoli and daikon extractions in H_2O after Day 3 of germination compared to the 0-100 µM sulforaphane gradient over time.*

To determine if the biosensor reacted to non-ICT containing plant extract, we tested it with various non-brassica origin plants. Although it appears that there is a small amount of expression of approximately 20-25 RLUs (*Figure 4.2*), by placing the 0 µM background

luminescence obtained with pure sulforaphane, it is clear that the bacteria were expressing a small baseline luminescence regardless of the presence of the plant extract. This meant that the biosensor was not reacting to the non-brassica plants and therefore was only capable of reacting to the isothiocyanates. When compared to the maximum luminescence at 5,700 to 6,000 RLUs, the 20-25 RLUs are not significant enough to be a concern. Even in the concentration gradient test in *Figure 2.3*, 1 µM of sulforaphane generated an average luminescence of approximately 70 RLUs, which is three times as large as the non-brassica background.

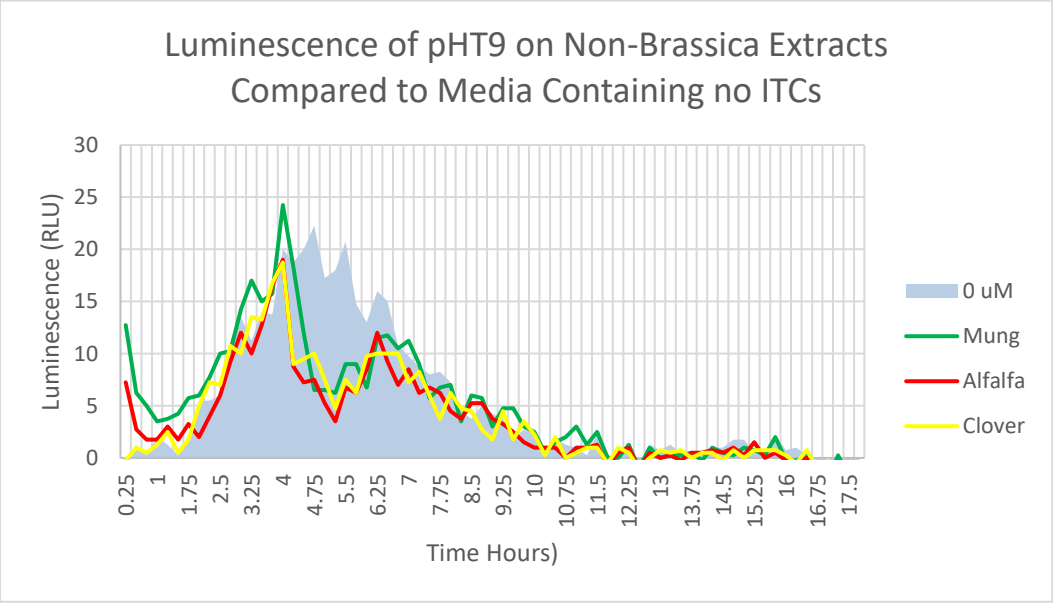

Figure 4.2. *Baseline luminescence of the biosensor in 0 µM sulforaphane compared with extracted fluid from mung, alfalfa and clover germinated for 3 days.*

In order to generate a possible explanation for the delayed peak of the daikon sample shown in *Figure 4.1.*, an optical density test compared the growth of the bacterium in 100 µM of sulforaphane to the growth in a solution of daikon extracted after two days of germination. The results of this experiment clearly showed that the exponential phase of pHT9 was delayed by approximately 4 hours, which would explain the frame shift while still exhibiting a similar dual-peak pattern. This suggests that the daikon may contain another defensive compound designed to inhibit microbial growth that *P. fluorescens* SBW25 is not naturally resistant to.

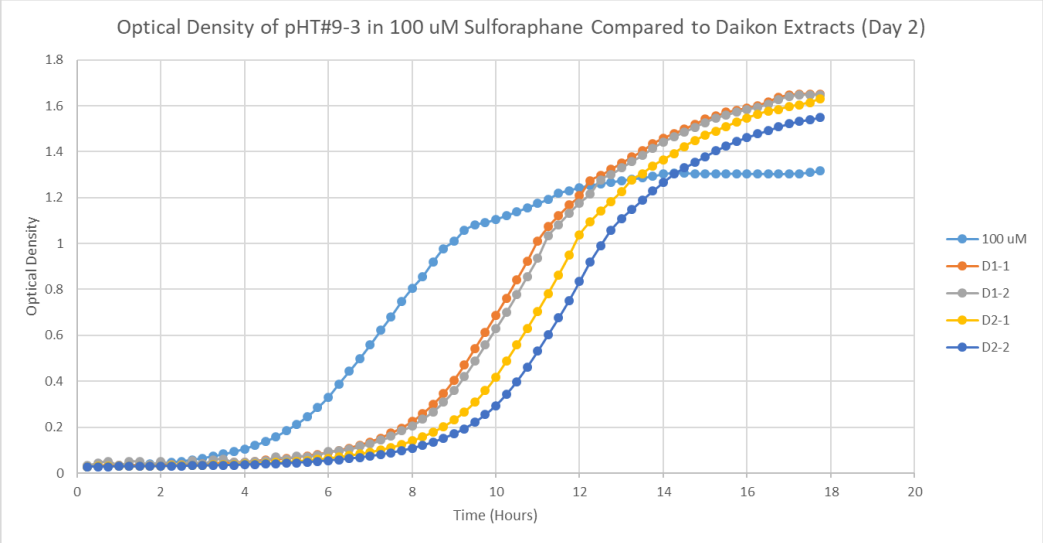

Figure 4.3. *Optical density graph comparing between daikon extract solutions (two extracts, two replicates of each extract to a solution of 100 μM sulforaphane to check for inhibition.*

Since the initial tests were done after a set day of germination, further experimentation was conducted to compare the varying incubation lengths and the corresponding ITC quantities detected by the biosensor. In this experiment, the broccoli was plated on different days, and simultaneously extracted (*See Figure 4.4*). In Day 1 of germination, the broccoli extract had a concentration of 5 μM according to the standard curve. In Day 2, there was a slight increase in the amount of ITC detected with some variability between the two replicate extracts between approximately 7.5 and 10 μM. In Day 3, the luminescence became more prominently peaked and at its highest halfway between sulforaphane's 10 μM and 25 μM luminescence values. Then, the detected concentration appeared to decrease back to approximately the concentration present in Day 2 of germination. The prediction is that this has to do with sprout morphology between the last two days (See *Figure 3.1.)*

The sprouts focus on root growth during the first three days, where on the third day they have visibly a significantly large amount of small root hairs. Then, on the fourth day of development, the focus changes to increasing leaf production and the roots gradually become longer and less juvenile. Previous literature evidence indicates that glucosinolates, the precursors to isothiocyanates, are present in all parts of brassica plants, but at varying

quantities in different growth stages [2]. This test provided more that the transition between days three and four are changes in the growth stage of the plant.

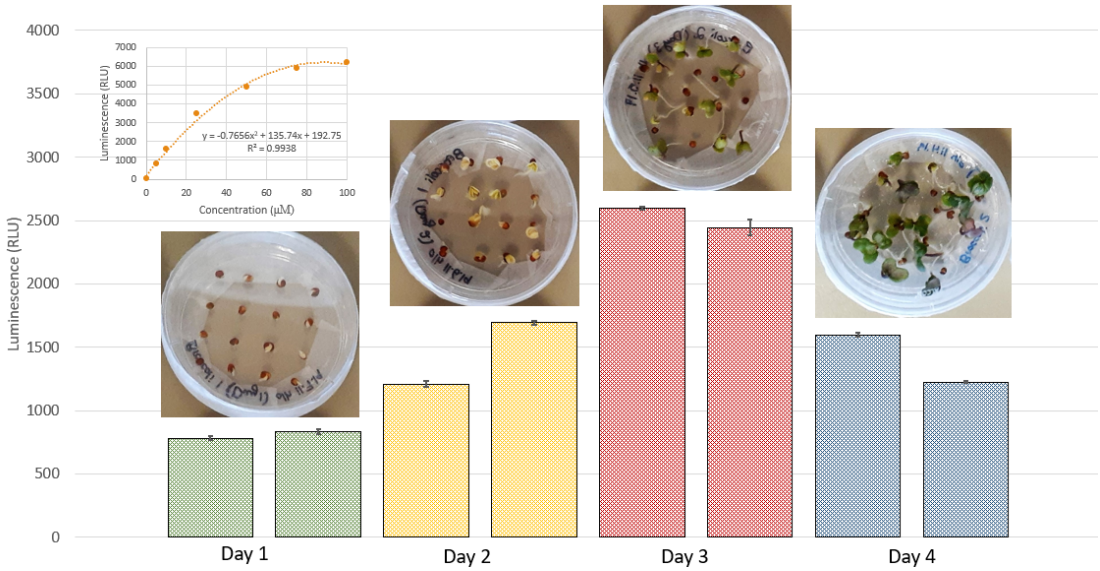

Figure 4.4. *Demonstration of the change in sulforaphane produced by broccoli sprouts on varied days of germination when compared to a 0-100 μM concentration gradient.*

The same experiment was repeated with daikon radish to further analyze how the peaks may change either position or height throughout the phases of growth. By looking at *Figure 4.5*, it is apparent that as the days progress, the peak retracts gradually closer to the peaks present in the concentration gradient of sulforaphane. Unfortunately, none of these peaks resembled the same intensity that had occurred in the initial test from *Figure 4.1*. However, this test is more reliable in quantification due to having two extracts exhibiting similar outcomes despite being independent of one another (*See Figure 4.5*). There appeared to be a strong delay occurring during the first few days, which may indicate some kind of inhibitory action on the growth of the bacterial cells. The samples also had varied peaks, which is why they were not processed into a bar graph similar to that of the broccoli. While the peaks for the concentration gradient occurred at the standard 4.25 hours, the peaks for Day 1 post daikon germination occurred between 9.5 and 10.5 hours of the kinetic assay. In Day 2, the peaks occurred between 6.25 and 7.25 hours without a change in the luminescence. Then, progressing into Day 3, the peaks increased in height slightly and occurred at 5.25 hours. Finally, on the last day of this test, the peaks reached 4.25 hours at

the same rate as the original concentration gradient and a similar pattern with the secondary peak. In addition to that, the concentration of detected ITCs increased to approximately 20-45 µM, which was significantly higher than earlier. Either this is indicating a change in the metabolism or enzymatic activity of the plant, or there are other isothiocyanates that the biosensor is detecting that have not been identified.

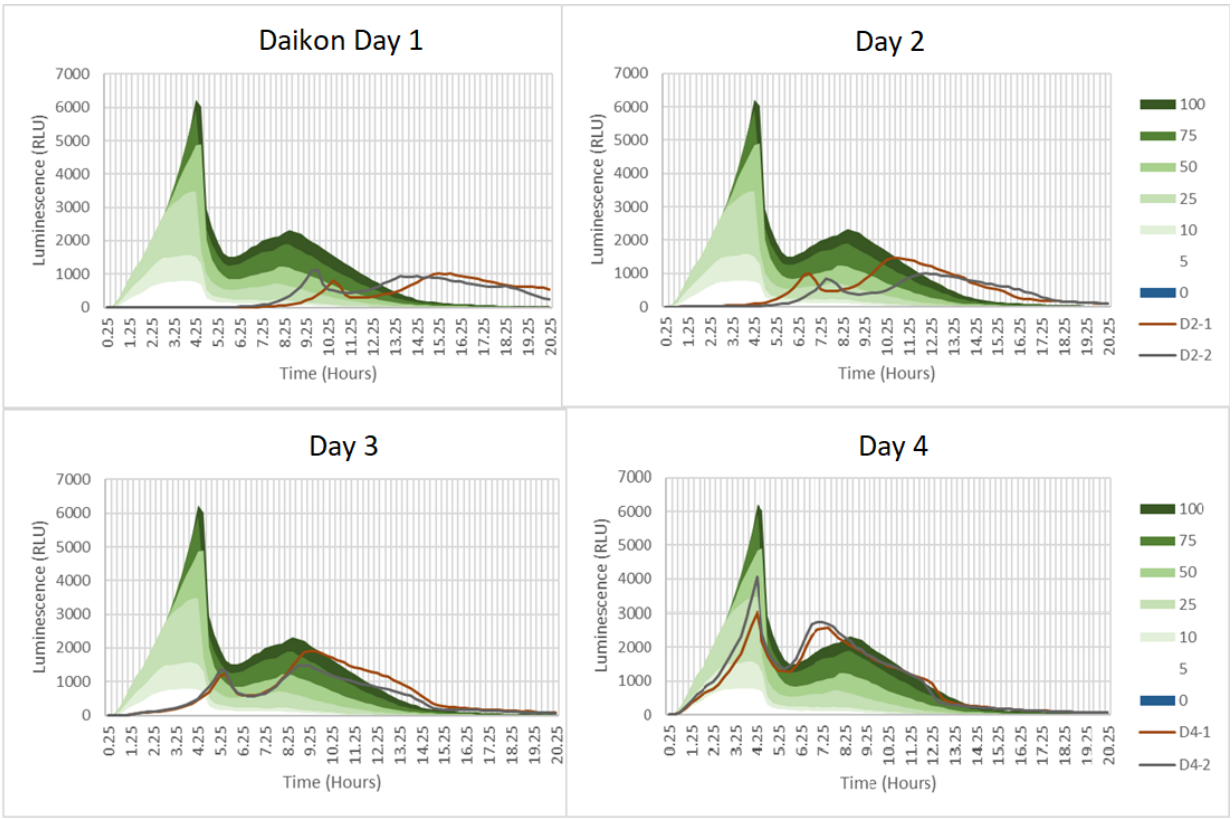

Figure 4.5. *Demonstration of the change in sulforaphane or other ITCs produced by daikon sprouts on varied days of germination when compared to a 0-100 µM concentration gradient.*

After conducting a variety of experiments on extracted plant matter, seed meal was introduced as a progressive step towards the biosensor's final objective. The plot in *Figure 4.6* shows the results from 24 hours of leeching *B. juncea* and *S. alba* in water at room temperature. The other extractions were done after 2, 5, and 48 hours of leeching, but 24 hours yielded the most prevalent results. Extracts for the seed meal were measured in the exact same manner as the plant extracts with the optimized parameters. The results of the experiment showed that these two varieties of seed meal contained approximately the same

quantity of detectable ITC by the biosensor at approximately 10 μM of sulforaphane equivalence.

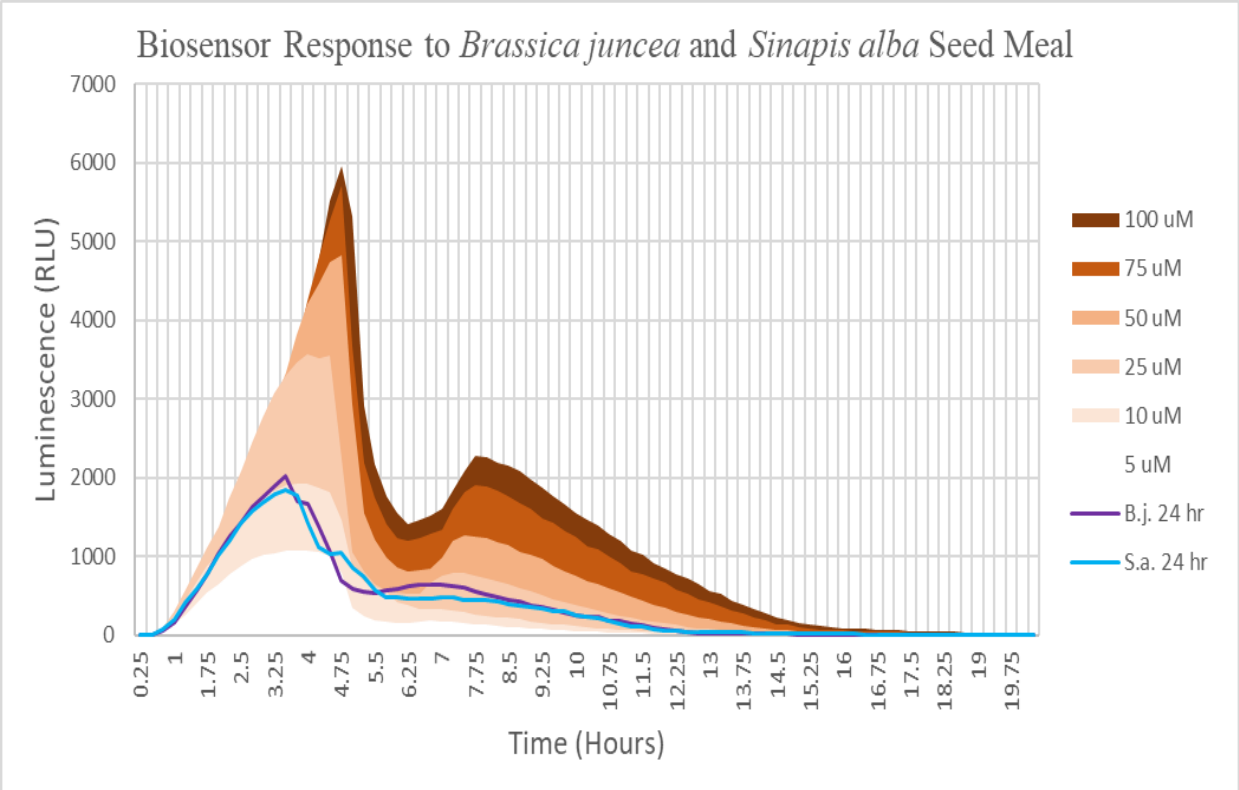

Figure 4.6. *Reported luminescence of biosensor in response to added B. juncea and S. alba in kinetic assay with 0-100 μM sulforaphane for comparison.*

Conclusion

This project demonstrated that the combination of the *saxA* promoter and the *ilux* operon integrated into the genome of *P. fluorescens* could generate a dose-dependent bioluminescent sensor of isothiocyanates (ITCs). The biosensor was capable of producing luminescence in response to sulforaphane, and further research is still being conducted. This mainly targeted the *brassica*-origin plants like mustard varieties and broccoli that are avid producers of sulforaphane, the main ITC of the project. Since we can detect low amounts of sulforaphane (1 μM), the next step involves acquiring seed-meal amended soils, or products of fumigation. With evidence that it the biosensor can detect ITCs in pure seed meal, there are good prospects of being able to detect ITCs at lower concentrations. If there is still not enough expression for the concentrations present, previous literature has demonstrated that generating more copies of the operon can increase overall expression without significantly increasing the background [8]. The gene would produce an abundance of the enzyme complex needed to initiate the biochemical pathway to luminescence, so theoretically, there could be an increase in overall expression.

Significant progress is being made to further understand what other compounds this gene interacts with and how those results can be interpreted in an applied setting. For example, we have acquired compounds like sulforaphene, an ITC present in radish, 4-hydroxybenzyl isothiocyanate, an ITC isolated from *S. alba*, and glucosinalbin, a precursor from *S. alba,* to name a few. More research needs to be done on the function of the *sax* gene itself to understand the population dependency observed earlier. Ultimately, this biosensor could be applied to agricultural use as a detector of trace ITCs rather than the expensive alternative. Research on the subject will continue for the pursuit of general molecular knowledge while developing an applicable device for farmers.

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Appendix

Table 1: *Primer specifications used during the genetic development of the biosensor for either synthesis or confirmation of sequence. Note: All primers that do not have a Tm listed were primers used for sequencing of the DNA. The Tm listed for the rest is the Tm used in the experimental PCR reactions.*

Primer	Target	% GC	Tm (°C)	Sequence (5'→3')
VM-093	saxA	48.4%	72°C	GCAGGATCCCATCACTGACATAAAGCACTTC
VM-094	saxA	43.8%	72°C	CTGATGAATTCTGGAGTTGATCCTGTCGATAC
VM-109	MCS	44%	-	ATACTTGAGCCACCTAAC
VM-110	MCS	44%	-	CATGCTCTTCTCTAATGC
VM-113	pHT	45%	-	AGTATAGGAACTTCAGAGCG
VM-114	pHT	44%	-	GGAAAGATTTCAACCTGG
VM-024	gDNA	44%	60°C	CACAGCATAACTGGACTGATTTC
VM-026	gDNA	50%	60°C	ATTAGCTTACGACGCTACACCC
VM-142	gDNA	55%	60°C	TGTCGTACTACGTTGCCGTG
VM-143	gDNA	55%	60°C	AGCAGTTCGATGGTATCCGC
VM-159	pHT-fw	40%	56°C	CCTAATTGTAAGTGGAATGC
VM-160	pHT-rv	50%	56°C	TTTAATGGTATGGCGGCC
VM-161	ilux-fw	36%	72°C	GCGGCCGCCATACCATTAAAGGATCCATGA CTAAAAAAATTTCATTCATTATTAC
VM-162	ilux-rv	49%	72°C	GCATTCCACTTACAATTAGGCCGCTTACCTT CTGGCAAG
Multiple	Cloning	Site	Sequence	GAGCTCTTCGGATCCACCGAATTCCACTGCA GCGATATCAACCCCGGGTGCAAGCTTCTTCA GCTGTAGCGGCCGCCATACCATTAAATGGAT GGCAAAT

Table 2: *Protocols for generation of fragments used to synthesize final plasmid products that became the biosensor of isothiocyanates.*

DNA/Primers	Reaction Solution	PCR Cycle
P. cannabina ES4326 VM-093/094	5 µL Q5 5x Buffer 0.5 µL dNTP (10 µM) 1.25 µL VM-093 (10 µM) 1.25 µL VM-094 (10 µM) 0.25 µL Q5 Polymerase 1 µL DNA (10 ng/µL) 15.75 µL dd H_2O	98°C (3 min) → 98°C (10s) → 72°C (30s) → 72°C (2 min) → cycle 34x → 4 °C (Hold)

pHT1/pHT3 Backbone for Gibson Assembly VM-159/160	5 µL Q5 5x Buffer 0.625 µL VM-159 (10 µM) 0.625 µL VM-160 (10 µM) 0.5 µL dNTPs (10 µM) 0.25 µL Q5 Polymerase 1 µL DNA (1 ng/µL) 17 µL dd H_2O	98°C (3 min) → 98°C (10s) → 56°C (20s) → 72°C (3 min) → 72°C (2 min) → cycle 34x → 4°C (Hold)
pGEX-*ilux* Backbone for Gibson Assembly VM-161/162	5 µL Q5 5x Buffer 0.625 µL VM-161 (10 µM) 0.625 µL VM-162 (10 µM) 0.5 µL dNTPs (10 µM) 0.25 µL Q5 Polymerase 1 µL DNA (1 ng/µL) 17 µL dd H_2O	98°C (3 min) → 98°C (10s) → 72°C (20s) → 72°C (3 min) → 72°C (2 min) → cycle 34x → 4°C (Hold)

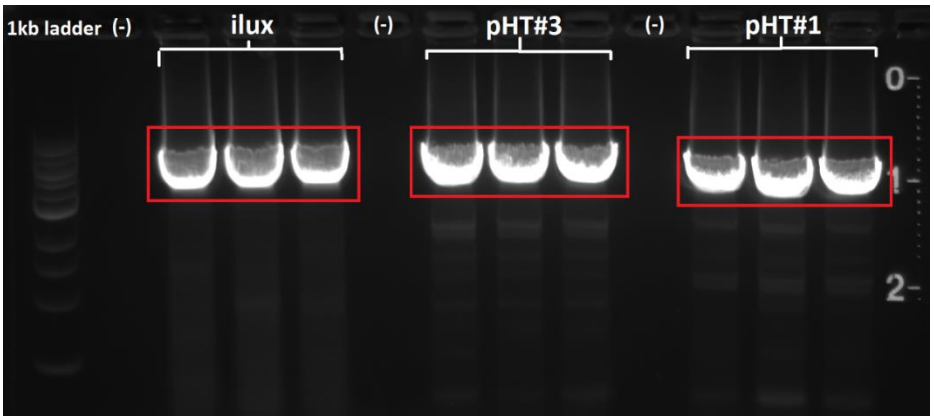

Figure 5.1: *Demonstration of fragment size after PCR reaction with VM-159/160 and VM-161/162 on pHT3, ilux, and pHT1 prior to gel purification and Gibson Assembly.*

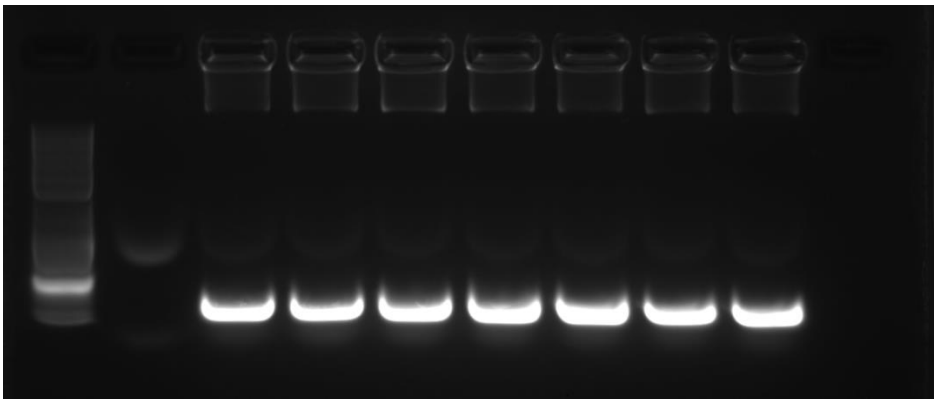

Figure 5.2: *Confirmation of pHT9 integrating into the SBW25 genome with primer pair VM-024/142 with a 1 kb size ladder and a genomic DNA prep of SBW25 for comparison.*

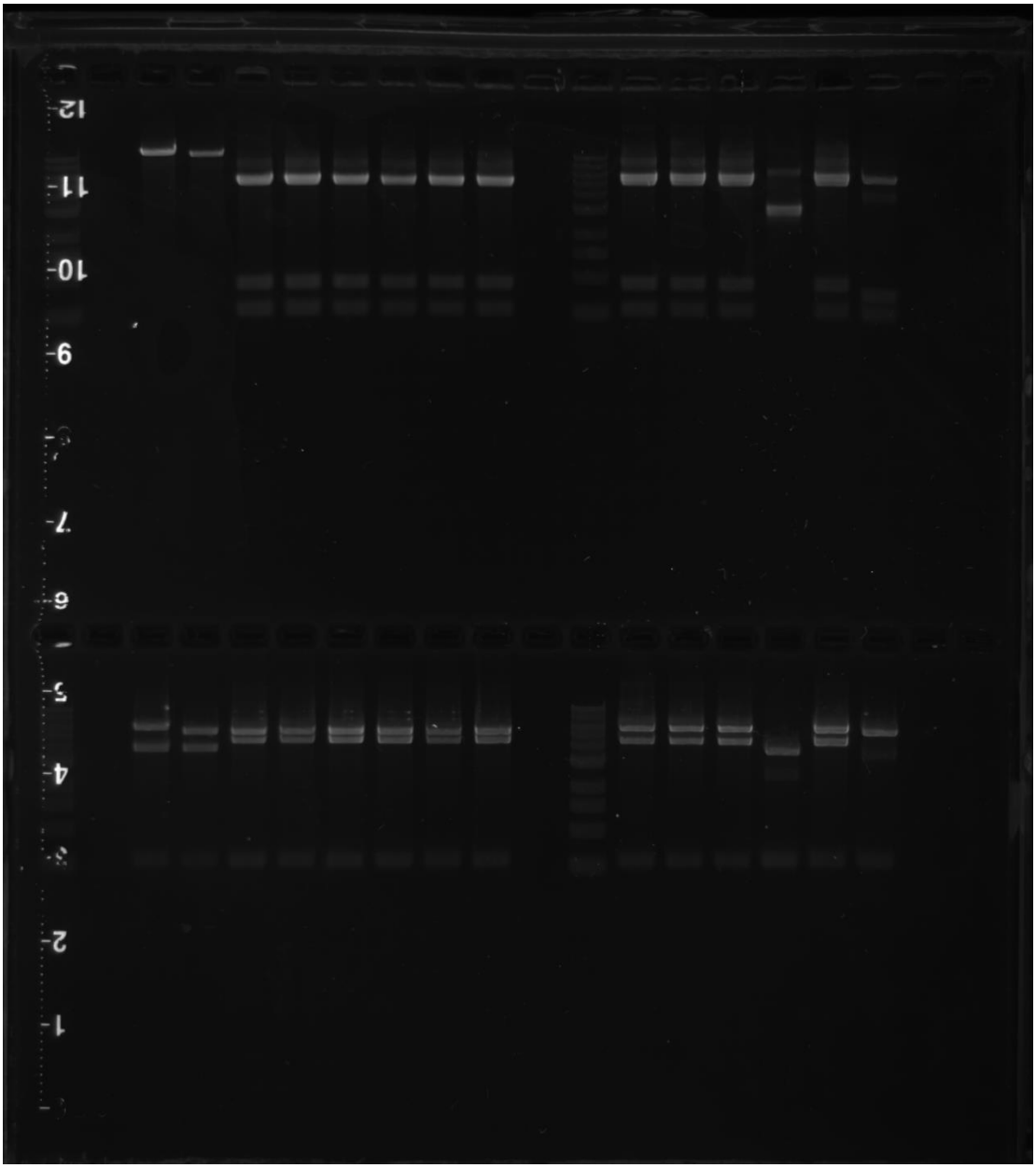

Figure 5.3: *Gel electrophoresis of enzyme digests of pHT9 and pHT8 confirming the correct configuration of the final plasmids. Those that exhibited the desired banding were to be integrated into SBW25 via electroporation.*