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Light-Induced Expression of a Blue Coral Protein in an Industrial Fungus

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Abstract

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The ultimate goal of this research project is to improve the growth and structural characteristics of an Ecovative Design LLC (Green Island, NY) production strain to produce commercial biomaterials for packaging. These biomaterials are produced from renewable resources and can be easily broken down after they fulfill their purpose, unlike the commonly used materials today (such as Styrofoam). In an effort to quantify the light-reactivity of the fungus, a codon-optimized DNA sequence coding a blue chromoprotein was introduced and utilized as a visual reporter gene. Transcriptional controlling sequences were identified from orthologs to specific light-regulated genes and were combined with a codon-optimized version of the coral Amil-CP Blue chromoprotein gene. This resulted in recombinant DNA constructs suitable for *Agrobacterium*-mediated transformation of the fungus. PCR and restriction enzyme digestion was used to verify the correct organization of the fragments making up each recombinant DNA molecule. Unfortunately, no evidence of the chromoprotein gene product could be detected by SDS-Polyacrylamide gel electrophoresis of total cell lysates from PCR-verified transformants. This research was supported by a grant to Ecovative Design from DARPA (BAA-16-50).

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Introduction

Ecovative and Mycelium Background

Ecovative Design, LLC is a leading biomaterials company located in Green Island, NY. The company's goal is to ultimately reduce or eliminate the use of toxic and unsustainable materials, such as Styrofoam, commonly found in packaging materials today. As an alternative, Ecovative has engineered products that are high performing, cost competitive, compostable, and fire-resistant by using agricultural waste and biological material. The agricultural waste is inoculated with live fungal mycelia, which in turn creates an innovative "Mushroom material" (1). Unlike plants, mushrooms do not use photosynthesis as a means to produce energy or use seeds to reproduce. Instead, they reproduce by spores (sexual and asexual), which can germinate under appropriate conditions to produce a mass of "interwoven, single-cell wide structures known as hyphae" (2). Masses of hyphae are known as mycelium (2). In other words, as this agro-fungal mixture continues to incubate, the mycelium is the essential hydrophobic glue that holds the material together. Throughout the growth process, the fungus secretes a variety of enzymes that break down the organic material present within the substrate acting as the host, to use as nutrition. Genetically engineering the fungal component of the bio-polymer is a priority for Ecovative: my task was to utilize a DNA transformation method which would potentially allow for the expression of a coral chromoprotein for use as a reporter for future gene expression studies of the fungus.

Ecovative's main production strain for the manufacture of engineered wood will be referred to in this thesis as simply the production strain used by Ecovative, for proprietary reasons. Ecovative's exact processes and technology are patentable trade secrets. They have a large library of fungal strains and the specific industrial uses of the strain of fungus being studied (and the identity of the strain itself) are patent-protected. While certain details will be omitted from this thesis, the overall goals and results will be discussed at length.

Ecovative Design Process

After Ecovative purchases agricultural waste material at a low cost from farmers in the local area, it goes through a pasteurization process. This pasteurization greatly reduces, but does not eliminate the bioburden of the material, which is then inoculated with the mycelium. This mixture is then placed in large plastic bags and sealed. The mycelium uses the agricultural waste as a substrate and consumes some of it. After a certain period of time (about 5 days usually), a matrix of white fibers is formed, which coats the dead plant material. This new material gets reground into loose particles (called the "Regrind" stage). These particles are then put into plastic molds (growth trays), which allow the mycelium to grow through and around the particles once again. This allows for more points of inoculation for the established fungus, and eventually forms a solid structure that fills the specific shape or mold of the growth tray. Once the material is completely solidified, it is removed from the mold and actively dried out to prevent more fungal growth. The biomaterial can be formed and dried out in structures of various shapes, depending on the needs of the customers (1). The research presented in this thesis will focus on helping Ecovative by establishing protocols needed to perform molecular genetic manipulations of Ecovative's fungal strain, which will potentially allow for new and novel growth characteristics of the fungus.

Benefits of Creating a Light-Responsive Reporter Gene System

The main goal of this project is to transform the fungal strain used by Ecovative in order to help optimize their process of making biodegradable materials. One way to achieve this is to genetically reprogram the fungus in order to enhance or modify its natural biological properties. One natural property of fungi is that they are able to respond to different stimuli in the environment, such as light. Similar to most life on the planet, fungi contain photoreceptors, which are proteins that have the ability to harvest light in order to generate a signal that stimulates a cellular response (3). This cellular response could be a variety of things, such as causing the reorientation of the direction in which the fungus is growing or altering the spectrum of gene transcription, which can cause new structures to form (3). By isolating the promoter and terminator of characterized light-reactive genes found in fungi, the expression of potentially any protein-coding sequence inserted in between can be made to be light-responsive. My specific project is to insert a gene for a blue chromoprotein from coral in between the promoter and terminator of these light-responsive genes to act as a visual reporter. By altering the DNA of their production strain, Ecovative will potentially be able to use this new genetically transformed fungus, hopefully improving their overall process.

Overview of the Molecular Biology of Light Perception and Response

The molecular analysis of the transcriptional response to light has been best studied in the ascomycete fungus, *Neurospora crassa*. White Collar-1 (WC-1), both a transcriptional activator and a blue light receptor, and White Collar-2 (WC-2), a transcriptional coactivator, form a complex, called the White Collar Complex (WCC). Once light reaches this complex, it transcriptionally activates a wide number of light-responsive genes by binding as a

heterodimer to specific elements within their promoters. This light response is eventually turned off by the action of one of these target genes for the WCC. When transcribed and translated, Vivid (*vvd*) interacts directly with the WCC in order to negatively regulate the light response that WCC initiated (4). This process is depicted in Figure 1 with the comparison of the gene present in the dark to the gene present in the light. When the fungus is placed in the dark, there is no transcription of the genes *vvd* or *gene x*, however, once the fungus is brought into the light, the WCC complex activates transcription of the gene which can then produce proteins.

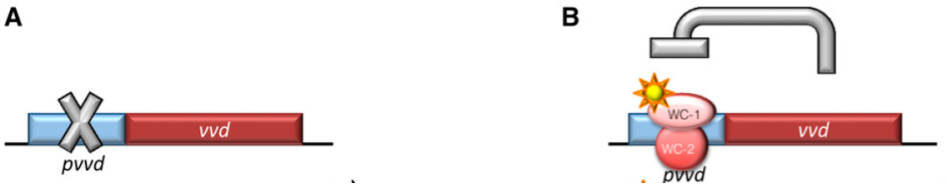

Figure 1. Figure taken from Hurley *et al.,* (2012). Outcomes of expression conditions from *vvd* promoter in *Neurospora*. A) In dark conditions all genes driven by *vvd* promoter are not induced. B) In light conditions in the wild type strain, the light activated WCC turns on expression at the *vvd* promoter. This activation leads to the production of WD that inhibits the activation at the *vvd* promoter by acting back on the WCC.

Light Regulated Genes in Neurospora crassa

In order to transform the Ecovative production strain, we utilized the promoter and terminator sequences of light-regulated genes from the well-studied model organism *Neurospora crassa* and inserted a codon-optimized coding sequence for a blue coral chromoprotein. The study done by Wu et al. (2014) gave some insight as to the specific light regulated genes identified in the bread mold, *Neurospora crassa*. We selected three light-regulated genes, *con-6* (conidiation-6), *bli-4* (bli-4 protein), and *bli-3* (blue light-induced-3). All of these genes are both light-responsive and expressed relatively strongly after exposed to

light for a period of time (5). The conidiation (con)-related genes especially, have been shown to be light responsive with rapid, high-level, sustained light responses (5). Although originally identified by Wu et al. (2014) in *Neurospora crassa* but the Ecovative production strain also has what appear to be likely orthologues. Figure 2 depicts (indicated by green arrows) these three light-responsive genes that I decided to focus on. The red arrows indicate some other genes in *Neurospora* that were highly expressed in the light but have no obvious orthologue in the Ecovative production strain. By isolating the promoter and terminator of these genes from our fungus and combining them with a visual reporter, the blue chromoprotein, I could then transform the Ecovative production strain with this new construct. The genes I focused on were *bli-4* and *con-6,* the Fall 2017 Bio 384 class worked with the genes, *bli-3* and *bli-4*.

Table 1 Transcripts with 16-fold or more induction by light

Locus	Symbol	Name	L15/D	L60/D	L120/D	L240/D
NCU00552	*al-1*	albino-1	8.3	6.9	4.1	2.4
NCU08699	*bli-4*	bli-4 protein	7.5	7.8	4.8	3.5
NCU02190		oxysterol binding protein	7.3	5.6	2.5	1.5
NCU03967	*vvd*	Vivid	6.6	5.1	4.0	3.1
NCU08770		hypothetical protein	6.4	7.6	2.3	0.7
NCU00582	*cry*	cryptochrome DASH	6.3	4.9	4.4	4.1
NCU10063		sugar isomerase	6.2	4.3	2.8	2.1
NCU08769	*con-6*	conidiation-6	6.0	8.5	7.5	2.4
NCU08626	*phr*	photoreactivation-deficient	5.3	4.0	1.9	1.2
NCU00585	*al-2*	albino-2	5.2	5.3	3.9	2.5
NCU07325	*con-10*	conidiation-10	4.8	7.1	4.3	1.5
NCU11424	*cao-2*	carotenoid oxygenase-2	4.8	4.1	2.8	2.1
NCU07434		short-chain dehydrogenase/reductase SDR	4.6	3.8	1.5	1.7
NCU07267	*bli-3*	blue light-induced-3	4.6	6.8	4.6	2.4
NCU11395		S-(hydroxymethyl)glutathione dehydrogenase	4.0	6.1	4.4	2.7
NCU06420		hypothetical protein	3.8	4.1	2.7	1.3
NCU01060		hypothetical protein	3.5	4.9	3.9	2.8
NCU09049		hypothetical protein	3.4	6.1	4.3	1.3
NCU03506		hypothetical protein	3.1	4.3	3.0	1.5
NCU05844		hypothetical protein	2.7	4.5	2.6	−0.5
NCU01861		short chain dehydrogenase/reductase family	2.2	5.6	4.5	1.1
NCU06597		hypothetical protein	2.0	5.4	3.3	0.6
NCU04823		NADP-dependent alcohol dehydrogenase C	1.6	4.5	3.5	1.0
NCU05652		hypothetical protein	1.4	4.6	3.5	0.7
NCU07345		hypothetical protein	1.2	4.3	3.2	0.4
NCU07337		hypothetical protein	1.2	4.2	4.1	2.1
NCU00716	*ncw-5*	non-anchored cell wall protein-5	−0.3	2.8	4.1	4.3

Figure 2. Information taken from the study done by Wu *et al.,* (2014). The genes listed are all light regulated and found in the bread mold, *Neurospora crassa.* The values represent the $\log_2$ change in expression compared to the dark at that specific time point. The green arrows point to the selected genes with high expression and are also present in the Ecovative production strain. The red arrows point to other genes that are highly expressed but are not found in the production strain.

Amil-CP Blue Chromoprotein as Visual Reporter

In order to quantify the light reactivity of any transformants, a codon-optimized chromoprotein originally identified in the coral *Acropora millepora* can be introduced as a visual reporter. In a published study of coral fluorescent proteins, it was found that the non-fluorescent chromoproteins, of a purple-blue color, have an intense absorption and are bright enough to be used as reporter proteins under visible light (6). One particular chromoprotein of interest that was mentioned in the study was Amil-CP, which is blue in color. This specific chromoprotein is bright enough to appear blue to the naked eye and does not fluoresce, so a specialized light microscope is not required to view it's expression (6). Amil-CP Blue has been expressed in *E. coli* and produced a bright blue color that was visible to the human eye. One example of this is seen in Figure 3. Since it has been demonstrated to be expressed in *E.coli*, it seemed like a good candidate to serve as a good visual reporter gene for inclusion into our new light-reactive constructs. Since coral and fungi are very far apart evolutionarily, the coding sequence for Amil-CP had to be codon-optimized for our fungus. Every organism has preferred codons that are used to encode particular amino acids, so the native coral gene would be unlikely expressed efficiently if it were inserted into the plasmid construct in it's original state. Figure 4 shows a comparison in the DNA sequences between the original coral sequence and the fungal codon-optimized sequence for Amil-CP Blue. Ideally, transformants of the fungus with an integrated construct should express this version of Amil-CP properly and appear blue in color when exposed to light.

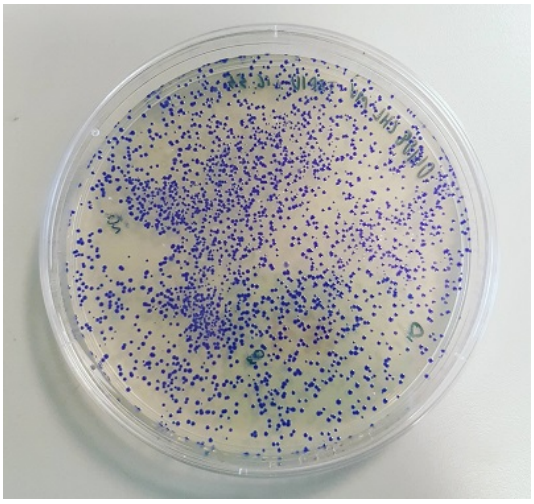

Figure 3. Photo of Amil-CP expressed in *E. coli*, visibly blue to the naked eye in *E. coli* colonies. (7)

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Optimized      1   ATGTCGGTCATCGCCAAGCAGATGACGTACAAGGTCTACATGAGCGGCACGGTCAACGGC
Original       1   ATGAGTGTGATCGCTAAACAAATGACCTACAAGGTTTATATGTCAGGCACGGTCAATGGA

Optimized     61   CACTACTTCGAGGTCGAGGGCGACGGCAAGGGCAAGCCCTACGAGGGCGAGCAGACCGTC
Original      61   CACTACTTTGAGGTCGAAGGCGATGGAAAAGGAAAGCCTTACGAGGGGGAGCAGACGGTA

Optimized    121   AAGCTCACCGTCACCAAGGGCGGCCCTCTCCCTTTCGCCTGGGACATCCTCTCGCCCCAG
Original     121   AAGCTCACTGTCACCAAGGGCGGACCTCTGCCATTTGCTTGGGATATTTTATCACCACAG

Optimized    181   TGCCAGTACGGCTCGATCCCCTTCACCAAGTACCCCGAGGACATCCCCGACTACGTCAAG
Original     181   TGTCAGTACGGAAGCATACCATTCACCAAGTACCCTGAAGACATCCCTGACTATGTAAAG

Optimized    241   CAGTCCTTCCCCGAGGGCTACACCTGGGAGCGCATCATGAACTTCGAGGACGGCGCCGTC
Original     241   CAGTCATTCCCGGAGGGCTATACATGGGAGAGGATCATGAACTTTGAAGATGGTGCAGTG

Optimized    301   TGCACCGTCTCCAACGACTCCTCGATCCAGGGCAACTGCTTCATCTACCACGTCAAGTTC
Original     301   TGTACTGTCAGCAATGATTCCAGCATCCAAGGCAACTGTTTCATCTACCATGTCAAGTTC

Optimized    361   TCCGGCCTCAACTTCCCTCCCAACGGCCCTGTCATGCAGAAGAAGACCCAGGGCTGGGAG
Original     361   TCTGGTTTGAACTTTCCTCCCAATGGACCTGTCATGCAGAAGAAGACACAGGGCTGGGAA

Optimized    421   CCTAACACCGAGCGCCTCTTCGCCCGCGACGGCATGCTCCTCGGCAACAACTTCATGGCC
Original     421   CCCAACACTGAGCGTCTCTTTGCACGAGATGGAATGCTGCTAGGAAACAACTTTATGGCT

Optimized    481   CTCAAGCTGGAGGGCGGCGGCCACTACCTCTGCGAGTTCAAGACCACCTACAAGGCCAAG
Original     481   CTGAAGTTAGAAGGAGGCGGTCACTATTTGTGTGAATTCAAAACTACTTACAAGGCAAAG

Optimized    541   AAGCCCGTCAAGATGCCCGGCTACCACTACGTGGACCGCAAGCTCGACGTCACCAACCAC
Original     541   AAGCCTGTGAAGATGCCAGGGTATCACTATGTTGACCGCAAACTGGATGTAACCAATCAC

Optimized    601   AACAAGGACTACACCAGCGTCGAGCAGTGCGAGATCAGCATCGCCCGCAAGCCTGTCGTC
Original     601   AACAAGGATTACACTTCGGTTGAGCAGTGTGAAATTTCCATTGCACGCAAACCTGTGGTC

Optimized    661   GCGTGA
Original     661   GCCTAA
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Figure 4. DNA sequence of the coding region for the Amil-CP Blue chromoprotein from the coral, *Acropora millepora* (bottom sequence "Original") aligned using BLAST to the fungal codon-optimized version (top sequence "Optimized"). Differences in the DNA sequences appear in red in the top optimized sequence.

Methods and Materials

Gibson Assembly of Light-Regulated Recombinant DNA Constructs

In order to transform the fungus, I had to create and use a plasmid, which is a strand of circular bacterial DNA that we use as a cloning vector. Once I insert the promoter, terminator and gene of our choosing into the backbone plasmid (pOSCAR), it is transformed into *E. coli* where multiple copies are made by the cells' replication machinery (8). These plasmids can then be used to transform the fungal cells by inserting our desired gene constructs. The vector that will be used in this case is pOSCAR, a plasmid developed for use in fungal transformation by ATMT (8). This vector, with the addition of the promoter, Amil-CP Blue gene and terminator will form our plasmid. In order to form the new constructs I used a technique called Gibson Assembly. This technique was developed by Dr. Daniel Gibson in 2009 in order to easily assemble multiple linear DNA fragments (9). This process is relatively simple and quick, requiring DNA fragments (either preexisting or created by PCR) to be incubated with a mix of enzymes containing an exonuclease, a polymerase, and a DNA ligase (9). Before assembling the plasmid, I first amplified the individual components of each construct that would be inserted into the pOSCAR plasmid vector.

Once the plasmid construct pieces have been confirmed by PCR to be of the correct size, a larger PCR reaction of 30µl instead of the usual 15µl is done and then separated by size via agarose gel electrophoresis. After the gels have finished running and are stained by ethidium bromide, the bands corresponding to each of the plasmid construct fragments are visualized, using a handheld UV light and then cut out from the gel using a sterile razor blade. DNA fragments were then purified and eluted from the gel slices via the use of the Illustra GFX DNA purification kit (GE Healthcare). This gel purification step helps to

separate the DNA that is to be used in Gibson Assembly from other DNA that may be present such as DNA that was accidentally amplified in the PCR reaction, or 'primer dimer' fragments that are often a result of PCR (11). Qubit Assays (ThermoFisher Scientific) were then used to quantify these gel-purified PCR samples in order to determine the concentration of each sample prior to Gibson Assembly.

For the Gibson Assembly reactions, four fragments of DNA need to be inserted together for each of the desired gene constructs. For my two constructs utilizing *bli-4* and *con-6* gene controlling sequences, the fragments consisted of the promoter, the Amil-CP Blue gene, the terminator (all produced by PCR) and the pOSCAR vector backbone, digested with the restriction enzymes, Hind III and Kpn I. These fragments are then incubated at 50ºC with the Gibson Assembly enzyme mix (New England Biolabs) for 120 to 180 minutes.

Table 1. PCR Primers Used in this Study.

Primer Name	Primer Use	PCR Product Length (Base-Pairs)	DNA Sequence (5' to 3')
ABCON6_U19 ABCON6_D20	*con-6* Promoter	1358	TCTGATCCAAGCTCAAGCTACTCGACAGCC TAGTGTGTTTC TGACCGACATAGTCGATAGAGTATTGGCG
ABCON6_U21 ABCON6_D22	*con-6* Amil-CP Blue	666	TCTATCGACTATGTCGGTCATCGCCAAGC GGACTACGATTCACGCGACGACAGGCTTG
ABCON6_U23 ABCON6_D24	*con-6* Terminator	1418	CGTCGCGTGAATCGTAGTCCGTCGGTTATT TG GTCGCGAGCGATCGCGGTACAGTTTGCTTT TGTTGCTTTG
ABBL41_U1 ABBL41_D2	*bli-4* Promoter	1392	TCTGATCCAAGCTCAAGCTAAAGTAC ATGTATCAGGGAAGG TGACCGACATTGTTGAGTGAGGAGGG TAG
ABBL41_U3 ABBL41_D4	*bli-4* Amil-CP Blue	666	TCACTCAACAATGTCGGTCATCGCCA AGC ACCAAGGACACATCACGCGACGACAG GCTTG
ABBL41_U5 ABBL41_D6	*bli-4* Terminator	1406	CGTCGCGTGATGTCCTTGGTCGGGCAC TG GTCGCGAGCGATCGCGGTACCGGCGG CACTGAGATTCCT
EV 615 EV 616	GPD "Control"	630	CGTTGGCGCTGAGTACATCG TCATACGTCGCCCCCTTCTC
EV 535 EV 223	Carboxin-Resistance	1773	TTAAACTGAAGGCGGGAAACGA GTGCATGAAGGCACGTAAGA
EV 589 EV 590	Amil-CP Blue	107	TGGGAGCGCATCATGAACTT TTGAGGCCGGAGAACTTGAC

Transformation of Recombinant DNAs into Bacteria, and Verification of Constructs

After the Gibson Assembly was completed, the resulting plasmid constructs are then transformed into *E. coli* by electroporation. Transformants were selected by plating on the antibiotic, spectinomycin. Plasmid DNA from *E. coli* was isolated by the alkaline lysis miniprep method using a Wizard EV kit (Promega). Correct conformation of plasmid DNA constructs were determined by both PCR by performing restriction endonuclease digestions. I used the online program, NEBcutter, http://nc2.neb.com/NEBcutter2 , to determine the appropriate restriction enzymes to use for diagnostic digests of the plasmid DNA. The enzymes chosen would cut the sequence 2 or 3 times, depending on the enzyme, and have resulting fragments of different sizes that could easily be distinguished on an agarose gel. The three enzymes used for the *con-6* construct were Eco RI (HF), Nde I (HF) and Xba I. For the *bli-4* construct, the enzymes Xba I, Xho I and Hind III, and Sph I and Eco RI were used. Each reaction consisted of a mix, containing water, 10X CutSmart Buffer and the desired enzyme or pair of enzymes, with the plasmid DNA. The reactions run overnight in a water bath heated to 37ºC, and then analyzed by agarose gel electrophoresis.

Agrobacterium tumefaciens Mediated Transformation (ATMT) of Basidiomycete Fungi

The ATMT (*Agrobacterium tumefaciens*- Mediated Transformation) transformation process is a multi-week procedure. *Agrobacterium* strain AGL-1 was transformed with the recombinant DNA previously transformed into *E. coli* by standard procedures. Transformants were selected on LB + Ampicillin (100 µg/ml) + Spectinomycin (100 µg/ml) plates and grown for 3 days at 27-28ºC (8). Selected antibiotic-resistant colonies of transformed *Agrobacterium* were independently isolated, reselected, and then verified via PCR for the presence of the recombinant DNA plasmid. For use in ATMT, fresh colonies were

innoculated into 10 ml of low-salt LB medium + 100 µg/ml Spectinomycin. Cultures were grown at 27ºC, shaking at 225 RPM until their optical density (O.D. 600) reached the range of 0.4 to 0.9 (usually 15-17 hours). Cells were harvested in sterile 15 ml conical tubes, and centrifuged at 3,500 X g in an Eppendorf Clinical Centrifuge (Model 5702 R) for 7 minutes. The supernatant was removed and the cells resuspended in 1 ml of Induction Medium and transferred to a sterile microfuge tube. The tubes were centrifuged (using an Eppendorf Microfuge) at 7000 rpm for 5 minutes. The supernatant was removed, and the pellet resuspended in 500 µl of Induction Medium. The centrifuge process was repeated one more time until the cells were resuspended in 150 µl of Induction Medium. The concentrated *Agrobacterium* culture was diluted to an O.D. 600 of 0.15 in a volume of 10 ml Induction Medium + 200µM Acetosyringone in a 50 ml sterile conical tube. This is called the pre-induction step. The entire tube was covered with foil (Acetosyringone is light-sensitive) and grown at 27ºC shaking at 250 RPM until cells reach an optical density (O.D. 600) of 0.3 (anywhere from 10 to 17 hours). Next, *Agrobacterium* cells were concentrated to an optical density (O.D. 600) of 0.8 by centrifugation, achieved by the removal of the appropriate volume of supernatant.

The next step was preparing fungal mycelium. Ecovative's production strain was grown on fresh Malt Extract agar plates (pH 7.5) for 3 days at 28ºC. Using sterile forceps, sterile Hybond-N filters were placed onto each ME agar plate. Prior to autoclaving, each of the membrane filters was dyed to a dark purple color with Nigrosin in order to be able to see the fungal growth better. The process was repeated for each plate used in a transformation experiment. The inoculated (fungal) plates were grown upside down in a 27ºC incubator for 12 - 24 hours until the fungal mycelia were visibly growing on the filter. Harvested

Agrobacterium tumefaciens from the previous Induction step was incubated with the fungal recipient colonies that had been transferred with their filters onto Induction Media agar plates containing 200µM Acetosyringone. 200µl of resuspended *Agrobacterium* cells transformed with a selectable marker plasmid (conferring resistance to the antibiotic Carboxin) was mixed with 150 µl of Agrobacterium transformed with our light-responsive Amil-CP Blue construct. This co-transformation mixture was pipetted onto the membrane and a sterile glass L spreader was used to gently disperse the cell suspension evenly over the entire membrane. The plates were briefly dried in a sterile hood and incubated upside down in an incubator set at 24ºC. This infection temperature was previously determined to be optimal for ATMT infection. The plates incubated for 4 days. Finally, after infection I selected against Agrobacterium by transferring the fungal membranes onto Malt Extract agar plates containing 200µM Cefotaxime + 5µg/ml Carboxin. These plates were typically incubated for a week at 28ºC, and then subcultured from the membranes (4-5 subcultures per colony) onto new plates with Cefotaxime + Carboxin for a second round of selection.

Fungal DNA Isolation

To confirm the integration of the plasmid into Ecovative's production strain, genomic DNA was isolated and PCR was performed using plasmid-specific primer sequences. Using a sterile toothpick, a roughly 1cm x 1cm square of fungal mycelia was scraped from the ME selection plates. The harvested cells were transferred into labeled microfuge tubes containing 400 µl of DNA extraction buffer. The extraction buffer contains 200mM Tris-HCl (pH 8.5), 250mM NaCl, 25mM EDTA and 0.5% SDS. The tubes containing the mycelia and buffer were vortexed for 2 minutes in order to fully mix the mycelial material with the extraction buffer, which improves the DNA yield. 200µl of 3M Sodium Acetate (pH 5.2) was then

added to each tube containing the mycelia and extraction buffer. Each tube was then vortexed again for 1 minute. The tubes were placed in a -20ºC freezer for a minimum of 15 minutes. The tubes were then centrifuged in a microfuge at room temperature at maximum speed for 15 minutes. After the spin, 300-400µl of the supernatant was pipetted into new, labeled microfuge tubes (avoiding the loose pellet). An equal amount of ice-cold isopropanol was added to the supernatant and the tubes were mixed by inversion 20 times. The tubes were then incubated in the freezer overnight to precipitate the genomic DNA (gDNA). gDNA was recovered the next day by centrifuging the tubes at top speed for 20 minutes in a cold room (4ºC), yielding a small white pellet. The supernatant was removed, and 300µl of cold 70% ethanol was added to each tube to wash the pellet. Once the tubes were mixed by inversion to dislodge the pellet, the tubes were centrifuged at top speed in the cold room for 15 minutes again. The supernatant was once again removed and one final spin of 15 seconds at room temperature allowed for collection of residual supernatant, which was removed by careful pipetting. The DNA pellets were dried in the fume hood for about 3 minutes to evaporate any remaining ethanol from the tubes. Dried DNA pellets were resuspended into 30µl of TE buffer (pH 8.0). gDNA samples were diluted between 1:2 and 1:4 in 10mM Tris (pH 8.0) prior for use as a template in PCR.

Fungal Protein Isolation

In order to check for the proper expression of the gene product of Amil-CP Blue in our transformed fungi, total cell lysates containing fungal proteins were isolated and analyzed using SDS-PAGE gel (13). Cellular protein lysates were isolated in the following manner. Once the fungal transformants are fully grown on selection plates (after 4 days), one entire colony was scraped using a sterile toothpick and inserted into a microfuge tube

containing 200µl Urea sample buffer. The Urea sample buffer contains 9M Urea, 1% SDS, 25mM Tris-HCl, 1mM EDTA, and 0.7M Mercaptoethanol. The Mercaptoethanol needs to be added freshly to the Urea buffer before cells are introduced so 10µl of concentrated Beta-Mercaptoethanol was added to 190µl of Urea sample buffer. The tube containing the cells and buffer was then vortexed for 45 seconds before being boiled (with a clamp) at 100ºC for 4 minutes. The tubes are then vortexed for 1 minute and re-boiled for 2 minutes. In order to pellet the lysate, the tubes are centrifuged in a microfuge at maximum speed for 5 minutes. 50µl of the lysate supernatant is removed and added to a newly labeled microfuge tube. 10µl of SDS sample buffer is then added to this new tube but the Beta-Mercaptoethanol has to be freshly added, therefore 0.5µl Beta-Mercaptoethanol is added to 9.5µl of sample buffer and added to the new tube. A pre-cast 4-15% TGX SDS-PAGE gel was used and 25µl of each sample was loaded into the wells of a pre-cast 4-15% TGX SDS-PAGE gel (Bio-Rad). For the protein marker, 15µl of NEB Prestained Broad Range Marker was added to 5µl of SDS sample buffer and loaded into the gel. The gel ran at 110 Volts for about 70 minutes before being stained overnight in Aqua-Stain (Bulldog Bio). Gels were imaged on a G-Box (SyGene).

Results/Discussion

Recombinant DNA Constructs

By using various online programs such as BLAST, NCBI and ORF finder, I was able to create recombinant DNA constructs consisting of the promoter and terminator sequences for the genes, *bli-4* and *con-6* with the Amil-CP Blue gene. Figure 5 shows the construct created for *bli-4* and Figure 6 shows the construct for *con-6.* These DNA fragments, once

inserted into the plasmid by Gibson Assembly, are then inserted into *E. coli* cells where it makes multiple copies of itself. While the construct is in the *E. coli* we can run tests such as PCR and restriction digests to see whether the plasmid has been correctly inserted into the DNA of the *E. coli* and if the plasmid contains all of our desired fragments. Once it is confirmed that all of the pieces are present in the plasmid, it can be used to infect the fungal cells and transform the DNA of the fungus.

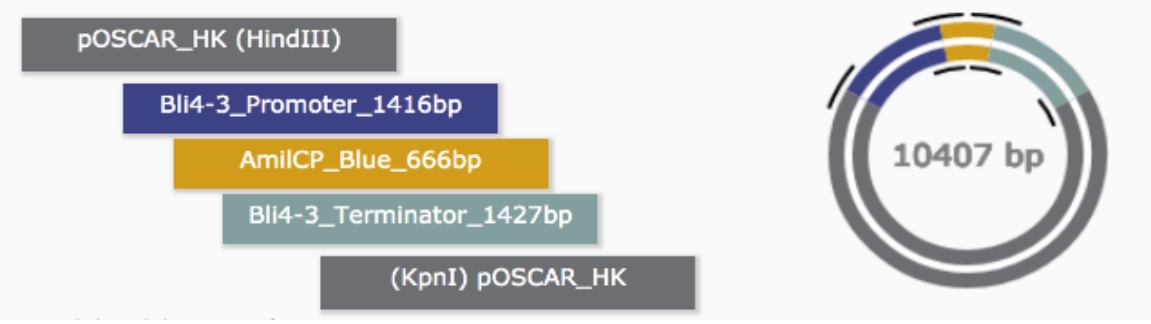

Figure 5. pGABLI4-3 plasmid construct including the plasmid in gray, *bli-4-3* promoter in dark blue, terminator in blue-gray and the Amil-CP Blue gene in yellow. Shows the sizes of each of the plasmid pieces in base pairs as well as the size of the whole plasmid.

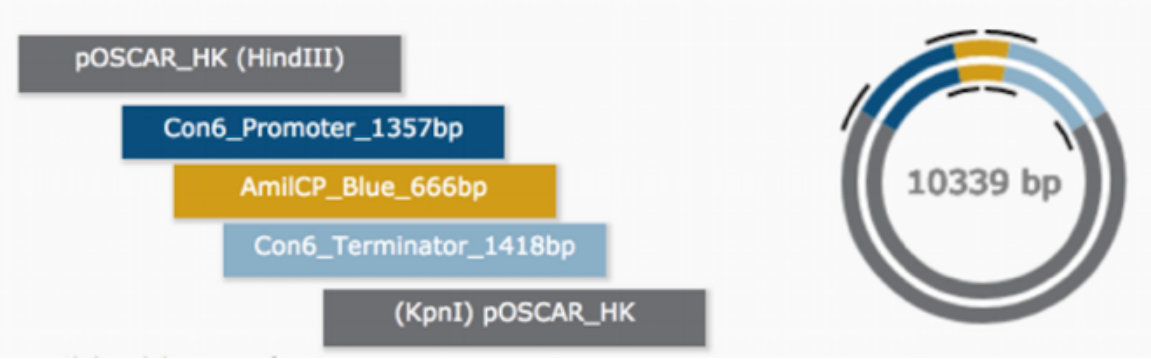

Figure 6. pGACON6 plasmid construct including the plasmid in gray, *con-6* promoter in dark blue, terminator in blue-gray and the Amil-CP blue gene in yellow. Shows the sizes of each of the plasmid pieces in base pairs as well as the size of the whole plasmid.

Construction and Verification of Light-Responsive Recombinant DNA Plasmids

PCR reactions utilizing the appropriate primers for the promoters and terminators of *bli-3, bli-4* and *con-6* yielded the desired products for the latter two constructs (Figure 7), but not for all of the necessary products for *bli-3*. I therefore decided to continue with the Gibson Assembly for the *bli-4* gene (done by the Fall 2017 Bio 384 class) and the *con-6* gene. The *bli-3* construct was missing the promoter fragments so I chose not to continue with the Gibson Assembly for that construct. After Gibson Assembly, the resultant recombinant DNA plasmids containing the codon-optimized Amil-CP Blue gene were transformed into *E. coli*. Verification of the correct plasmid constructs was done by a combination of PCR and diagnostic restriction enzyme digests.

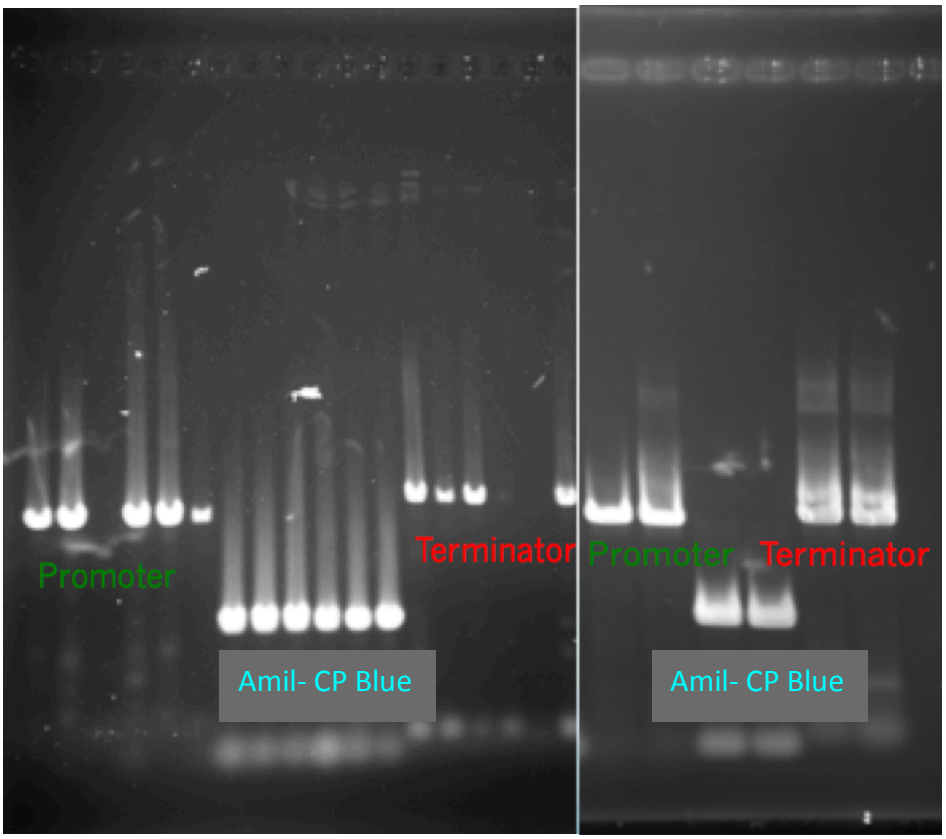

Figure 7. Individual plasmid constructs pieces amplified by PCR. Left gel picture shows the PCR products for pGABLI4-1 and right gel picture shows the PCR products for pGACON6. All bands that are present are of the expected sizes, green labels correspond to promoter sequences, blue labels correspond to the Amil-CP Blue gene and red labels correspond to terminator sequences.

As an example, for the *con-6* construct, I used, in separate digests, Eco RI (HF), Nde I, and Xba I. Eco RI and Xba I were predicted to cut the plasmid twice and Nde I cut it 3 times. Figure 8 shows the gel electrophoresis results of restriction digests of eight different plasmid DNA samples isolated from transformed *E. coli.* For Eco RI digests, two cuts were expected, resulting in expected plasmid fragments of 2.75kb and 7.58kb. The three Nde I sites cut should have resulted in fragments of 2.36kb, 2.49kb and 5.47kb. Xba I digests were expected to yield fragments of 3.0kb and 7.3kb. Not one plasmid DNA sample yielded the predicted sized fragments in every circumstance. Instead, two main 'sub-categories' of samples occurred where the sizes were as predicted for one or two enzymes but not for all three. One category was that the samples 1, 2, and 7 had clear bands for Eco RI at lengths ~7.5kb and ~2.5kb, which was expected, but faint or nonexistent bands for Nde I and only 1 single band at ~10.0kb for Xba I.

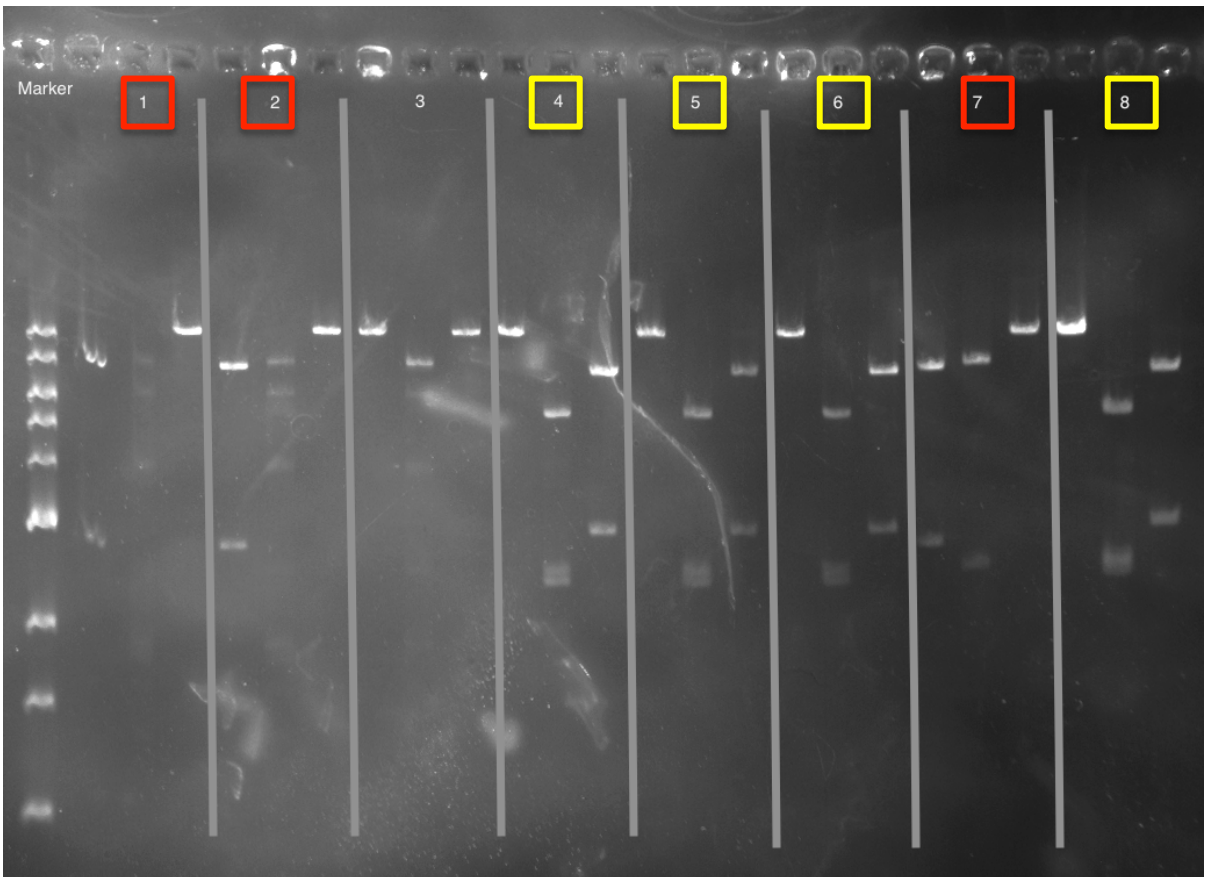

Figure 8. Gel for restrictive enzyme digests of the *con-6* plasmid. Three different enzyme digests (Eco RI, Nde I and Xba I) were used to cut plasmid DNA isolated from eight different transformed *E. coli* colonies. Each grouping of three lanes represents the different digested patterns for one sample, separated by vertical grey division lines. Lane 1 of each group corresponds to Eco RI digests, lane 2 corresponds to Nde I cuts and lane 3 corresponds to Xba I cuts. The numbers at the top of each group of three lanes represents the sample number. Samples circled in red followed category 1 and samples circled in yellow followed category 2. Sample 3 is the outlier that only had 1 fragment generated for each enzyme digest. Molecular weight marker (1kb ladder) containing the sizes 10.0kb, 8.0kb, 6.0kb, 5.0kb, 4.0kb, 3.0kb, 2.0kb, 1.5kb, 1.0kb and 0.5kb is in lane 1.

The second 'sub-category' was seen in samples 4, 5, 6 and 8 where only one band was present for Eco RI at ~9.0kb but clear bands at ~5.5kb and two at ~2.5kb for Nde I and at ~7.0kb and at ~3.0kb for Xba I. Either Eco RI made clear cuts or Nde I and Xba I made clear cuts, there were no samples that had all of them. This difference could be due to nucleotide polymorphisms between the sequenced species, different from Ecovative's production strain. Sample 3 had 1 strong band for each enzyme and is therefore an outlier.

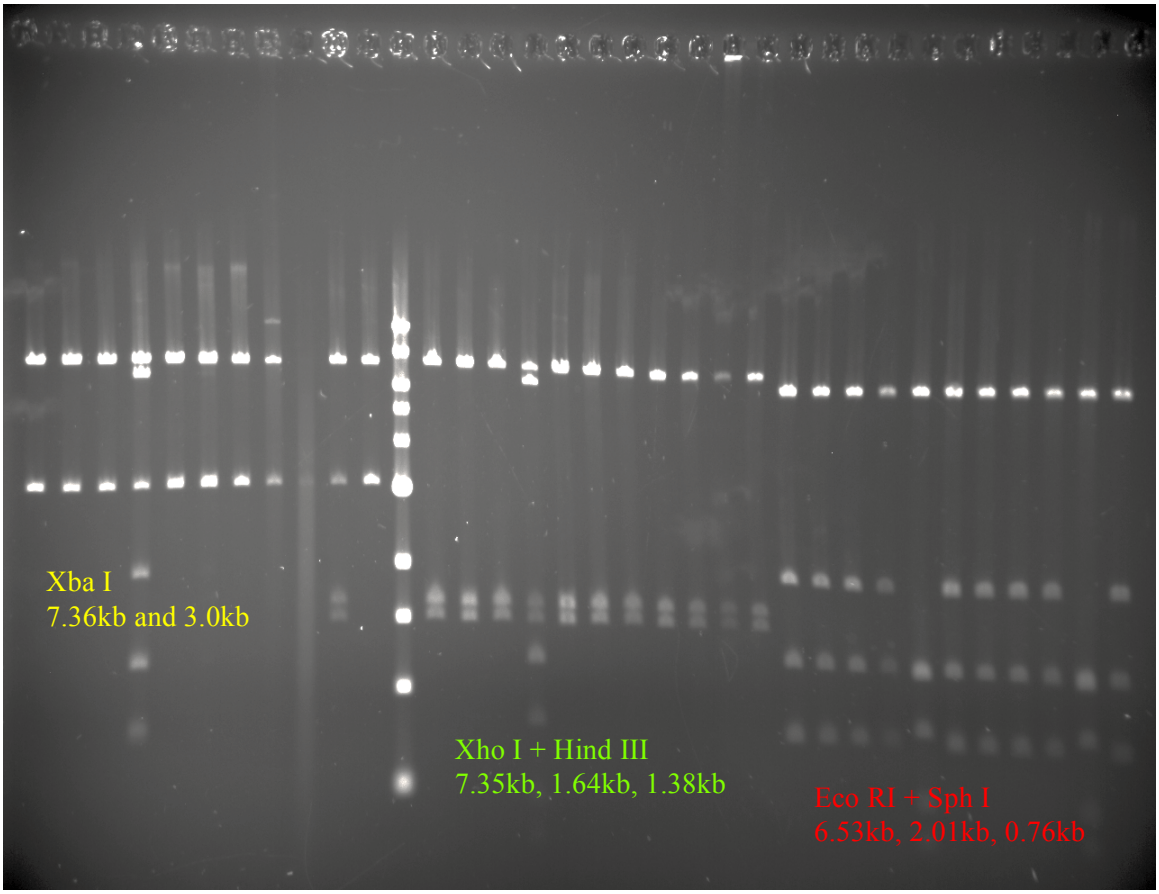

Figure 9. Gel illustrating restrictive enzyme digests of the *bli-4* plasmid. Three regimes of enzyme digests (Xba I, Xho I + Hind III, and Eco RI + Sph I) were used to cut plasmid DNA isolated from eleven different transformed *E. coli* colonies. Molecular weight marker (1kb ladder) containing the sizes 10.0kb, 8.0kb, 6.0kb, 5.0kb, 4.0kb, 3.0kb, 2.0kb, 1.5kb, 1.0kb and 0.5kb is in lane 12. Xba I cut samples are in the first section, Xho I + Hind III in the second section, after the marker and Eco RI + Sph I are in the third section.

The Fall 2017 Bio 384 class created the plasmid with the *bli-4* promoter and terminator controlling the expression of the Amil-CP Blue gene. They also performed restrictive enzyme digests on plasmid DNA isolated from their transformed *E. coli* colonies. after isolating DNA. The enzymes they decided to use were Xba I, Xho I + Hind III, and Eco RI + Sph I. Xba I was expected to cut twice and have two plasmid pieces at 7.36kb and 3.0kb. Xho I and Hind III in a double digestion were expected to make three cuts and

generate fragments of lengths 7.35kb, 1.64kb, and 1.38kb. Eco RI and Sph I used in another double digestion were expected to cut three times and generate three plasmid fragments of lengths 6.53kb, 2.01kb, and 0.76kb. The results of this restrictive enzyme digest (Figure 9) show that of the eleven isolated DNA samples digested with these enzymes, only one sample did not have all of the correct lengths for the pieces of plasmid after being cut by the enzymes. For the samples cut by Xba I, almost all of the samples, apart from three samples, had the correct plasmid restriction fragment lengths at ~7.0kb and ~3.0kb which was expected. For the double digestions using the enzymes Xho I and Hind III, a majority of the samples had three bands, which was expected, but none of these fragments matched the expected size. There was a band at ~6.0kb for each sample and a band at ~1.6kb both of which were expected but the third band was ~1.5kb instead of the 1.3kb band we were expecting. For the second double digest using Eco RI and Sph I, we were expecting three bands but instead we observed four bands. For most of the samples there were bands observed at ~6.5kb, ~2.0kb and ~0.7kb, all of which were expected. The fourth band of ~1.3kb and was not what we were expecting, so we were unsure as to why it appeared, and in a majority of the samples as well.

Diagnostic PCR Analysis of Selected Recombinant DNA Clones

After the restriction enzyme digests were performed for the *con-6* plasmid constructs, two samples from each of the two sub-categories of clones were subjected to PCR with the primers used to create the original fragments used in Gibson Assembly. This is another way to confirm that all of the correct fragments of our recombinant DNA plasmid were in fact inserted into the plasmid and cloned in *E. coli*. Two samples with the highest yield (brightest bands) from each of the two subcategories were selected, the latter determined from the

results of the restrictive enzyme digests for the *con-6* plasmid. A total of twelve PCR reactions were run. The results of these diagnostic PCR reactions are pictured in Figure 10.

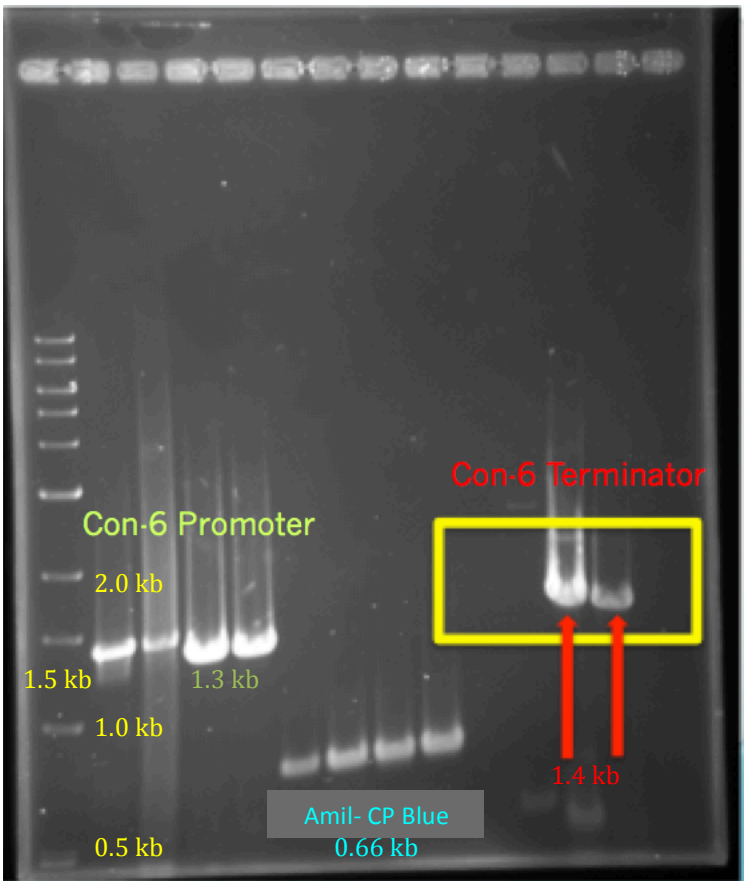

Figure 10. Gel electrophoresis of PCR products generated from transformed *E. coli* for the *con-6* recombinant DNA construct. Four samples of plasmid DNA, isolated from the transformed *E. coli* clones and confirmed to have the correct plasmid through restriction enzyme digests, were amplified through PCR with the primers used to construct the pieces of the plasmid. Arrows point to the two samples that had all three non-vector components, the *con-6* promoter, Amil-CP Blue and the *con-6* terminator. Green corresponds to the *con-6* promoter, blue corresponds to the Amil-CP Blue gene and red corresponds to the *con-6* terminator. The Amil-CP Blue bands on the bottom are actually all the same length, they appear to be slanted due to the gel being heated slightly during electrophoresis. Molecular weight marker (1kb ladder) containing the sizes 10.0kb, 8.0kb, 6.0kb, 5.0kb, 4.0kb, 3.0kb, 2.0kb, 1.5kb, 1.0kb and 0.5kb is in lane 1.

The gel depicted in Figure 10 shows the results of this PCR reaction to confirm the presence of the *con-6* recombinant DNA plasmid component pieces. The first four lanes after the marker correspond to the different plasmid clone templates that were amplified with the

upstream and downstream Gibson primers for the *con-6* promoter. All four of these clones have the *con-6* promoter part of the construct since they all yielded a PCR product of the expected size of ~1.4kb. The next four lanes (in the same order of clones) were PCR products amplified with the Amil-CP Blue Gibson primers and corresponded to the expected size of ~0.6kb. The last set of four lanes corresponded to PCR products amplified with the *con-6* terminator Gibson primers: only the plasmid templates in the last two lanes yielded the correct terminator sequence at an expected size of ~1.4kb. Since those two clones were the only ones of the four tested to have all of the correct plasmid pieces, I decided to continue with the transformation of *Agrobacterium* with those two *con-6* constructs, as well as Amil-CP Blue constructs with controlling sequences derived from *bli-4* (Bio 384, Fall 2017) and GPD (Ecovative Design).

Basidiomycete ATMT Co-Transformation with Amil-CP Blue and Carboxin-Resistance Constructs

Given that two of my *con-6* Amil-CP Blue have been confirmed by both restriction digests and PCR, these clones were deemed suitable for *Agrobacterium tumefaciens*-Mediated Transformation (ATMT) of the Ecovative production strain. *Agrobacterium tumefaciens* strain AGL-1 was transformed with my two *con-6* /Amil-CP Blue clones in order to be able to utilize *Agrobacterium*'s ability to infect filamentous fungi (8). This culmination of the ATMT process is pictured in Figure 11, showing both the fungus and *Agrobacterium* growing together on the dyed selection membrane.

Figure 11. Selection plate containing transformation of Ecovative's production strain of fungus by *Agrobacterium*.

In our case, we decided to perform a co-transformation, where two different sequences would be inserted into the fungus at the same time. We decided to insert a plasmid containing a Carboxin-resistance cassette along with our *bli-4* and *con-6* constructs in order to successfully transform the fungus. Carboxin is commonly used as a fungicide, this way, when the colonies were plated onto plates containing Carboxin, only the colonies that have been transformed with Carboxin-resistance would be able to grow (14). If these colonies were transformed successfully with the Carboxin-resistance, then they may have also been successfully transformed by the recombinant DNA as well. The antibiotic Cefotaxime selects against the *Agrobacterium*, whereas Carboxin-resistant fungal colonies have likely integrated the plasmid containing the Carboxin-resistance cassette. Since this was a co-transformation, a smaller subset of the Carboxin-resistant colonies should also have an integrated Amil-CP Blue cassette (usually no more than 50%, usually less). Colonies of mycelia were subcultured from the selective membrane and grown up on secondary selection plates containing Carboxin and Cefotaxime to further select for transformants (and against *Agrobacterium*). Genomic DNA was isolated from colonies that grew on these new plates and were tested

using PCR with primers specific for (1) a control DNA sequence already present in the fungus (GPD); (2) the Carboxin-resistance DNA sequence, and (3) our Amil-CP Blue recombinant DNA sequences.

The gel depicted in Figure 12 shows the PCR products amplified from Carboxin-resistant transformants using the control GPD primers and the Carboxin-resistance primers. The control primers were for a gene native to the production strain of fungus, GPD, used to make sure that the isolated DNA samples used are good DNA templates. As seen from the gel, each of the genomic DNAs yielded a GPD PCR product, indicated by a strong band at ~0.6kb. As a result, I can conclude that the genomic DNA samples are suitable templates for use in other PCR reactions. The results of this gel also indicate that all of the genomic DNAs, apart from one, yielded a PCR product using the Carboxin-resistance specific primers. Most of the PCR products were of the expected size of ~1.7kb but three gDNAs produced bands that were slightly smaller, at ~1.6kb. Unless this is an artifact, this difference in size may be due to a deletion somewhere between the two primers used. Overall, I can conclude that a majority of the samples did have the full-length Carboxin-resistance gene, showing that it was successfully integrated into the genomic DNA of the fungus.

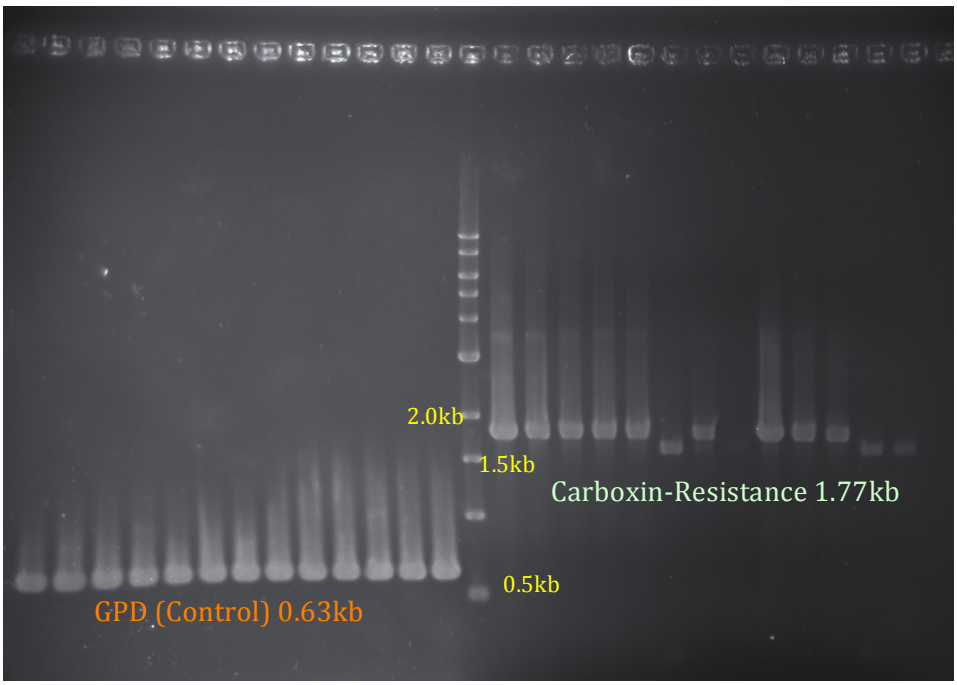

Figure 12. Gel electrophoresis of PCR products from genomic DNA isolated from Carboxin-resistant ATMT fungal transformants. Ecovative's production strain was co-transformed with both Carboxin-resistance and light-reactive Amil-CP Blue constructs. In the first set of reactions shown on the left of the gel, all twelve genomic DNAs had a PCR product of length of ~0.6kb, confirming that they all had the "control" GPD gene. Using Carboxin-resistance cassette primers, most of the same genomic DNAs yielded the expected product of ~1.7kb. Three genomic DNAs produced a product that was slightly smaller, at ~1.6kb.

Since all but one of the Carboxin-resistant colonies had the integrated Carboxin-resistance gene, this provided and adequate sample size for screening to see if our Amil-CP Blue construct has also been co-transformed into these individuals. Gel electrophoresis of the PCR reactions using the Amil-CP Blue-specific primers is shown in Figure 13. Of the eleven Carboxin PCR positives, only three of these yielded the expected Amil-CP Blue PCR product of ~0.107kb. One of these corresponded to the *bli-4* plasmid construct and two transformants contained an integrated copy of the *con-6* plasmid construct. These results confirm successful co-transformation of both Carboxin-resistance and Amil-CP Blue gene cassettes. However, despite the presence of the latter DNA sequences in the genome, there was no blue color of the co-transformed fungal mycelial colonies.

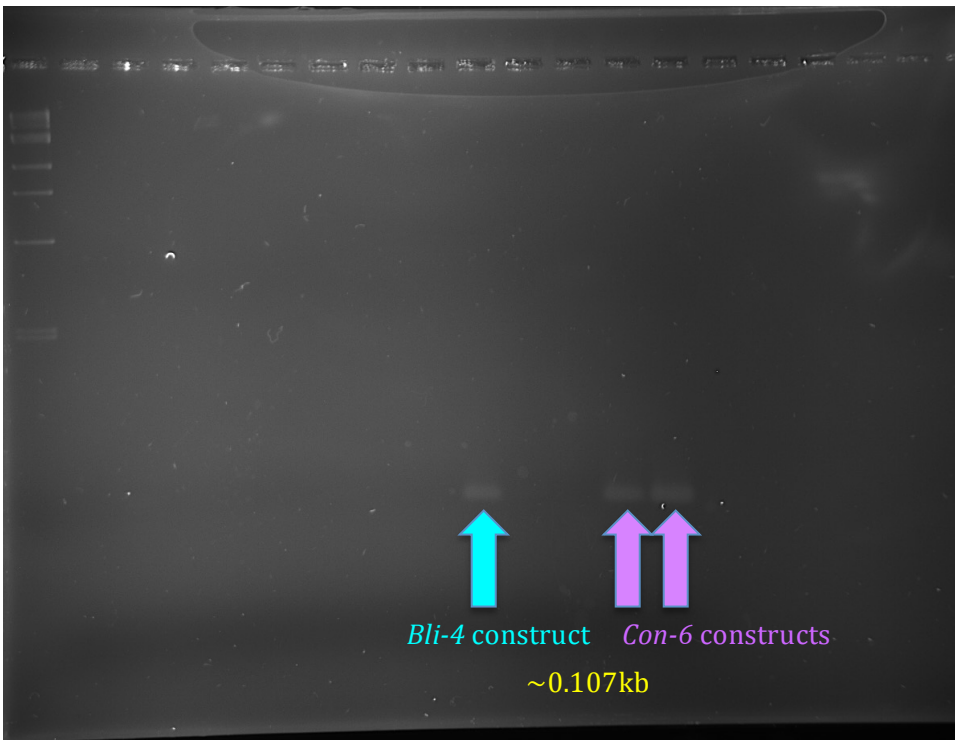

Figure 13. Gel electrophoresis of Amil-CP Blue PCR products from ATMT Carboxin-resistant transformants. Primers specific to an internal portion of the Amil-CP Blue gene were used in the PCR. Arrows point to Amil-CP Blue PCR products of length 0.107kb, one of which was derived from the *bli-4* construct and the others from the *con-6* construct.

Protein Isolation and SDS-PAGE Results

Given the lack of blue color in the three verified Amil-CP Blue transformants, I decided to check for expression of the protein product. Cellular proteins were isolated from the native Ecovative production strain and Amil-CP Blue transformants were analyzed by SDS-PAGE. If the expected Amil-CP Blue translation product of 25kD is expressed in the transformants, we would expect to see a band of this size superimposed on the spectrum of cellular proteins found in the production strain. Figure 14 shows the results of this protein extraction and comparison. Lanes 3 and 4 have the NEB protein marker and its overflow. The expected area surrounding the 25kDa region is highlighted in the Figure. Lanes 1 and 2 contain only proteins from Carboxin-only transformants. Lane 5 contains proteins from the transformant with the integrated *bli-4*/Amil-CP Blue construct. Lanes 6 and 7 correspond to

protein samples from the two verified *con-6*/Amil-CP Blue transformants. Lane 8 contains the cellular proteins from the untransformed Ecovative production strain, as a control for comparison.

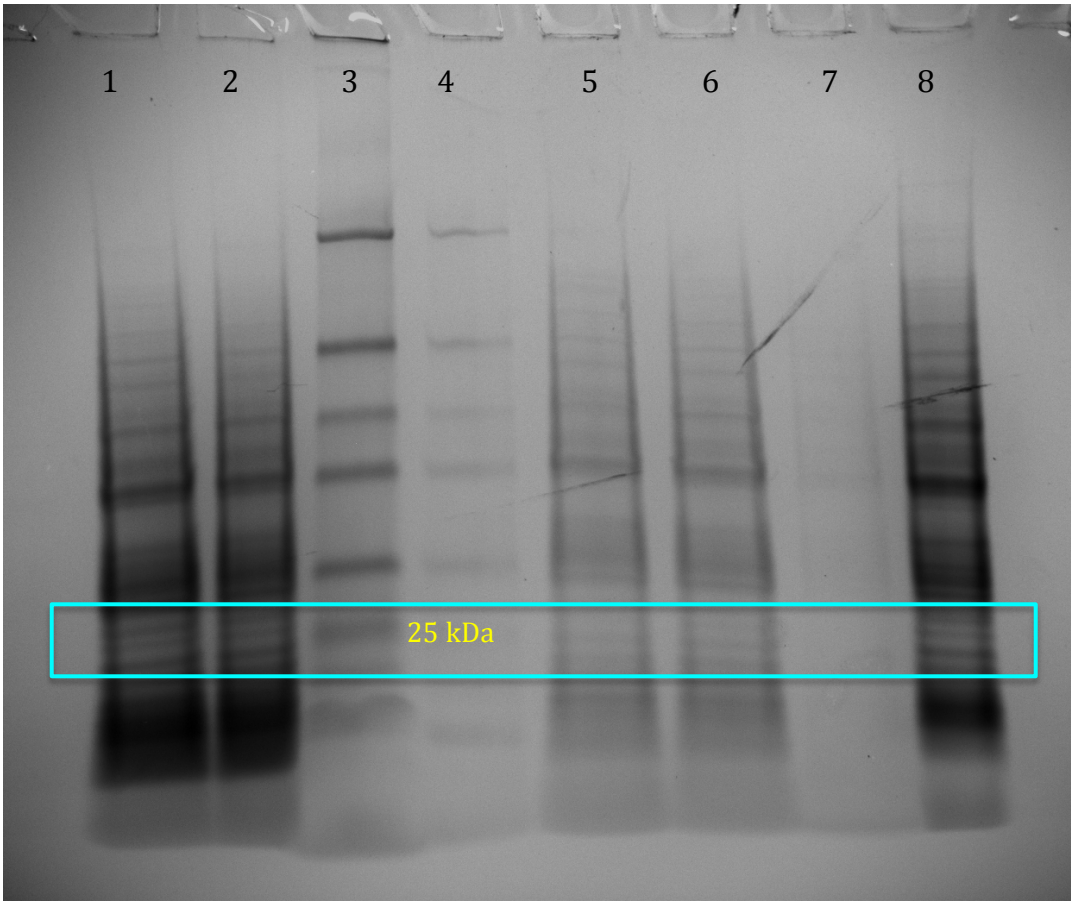

Figure 14. SDS-PAGE protein gel of total cellular lysates from control and Amil-CP Blue transformed fungal mycelia colonies. Lanes 1 and 2 correspond to lysates from Carboxin-only transformants. Lane 5 corresponds to a verified *bli-4*/Amil-CP Blue transformant. , Lanes 3 and 4 is the Broad Range protein marker (NEB) containing the sizes 80kDa, 46kDa, 30kDa, 25kDa, 17kDa, and 7kDa. Lanes 6 and 7 correspond to lysates from verified *con-6*/Amil-CP Blue transformants. Lane 8 corresponds to a lysate from the native, untransformed Ecovative production strain. We were expecting a significant Amil-CP Blue protein product at ~25kDa (blue box), but such product was seen.

The blue box in Figure 14 highlights the area surrounding the band at 25kDa where I expected to see a difference between the control and transformant lanes. There is no clear

difference in the protein spectrum between the Amil-CP Blue transformant and the controls, despite my finding that the gene is indeed present in the transformants.

Final Thoughts

Despite the PCR analysis demonstrating the presence of the codon-optimized Amil-CP Blue gene in three independent transformants, there was no clear change in the phenotype of the fungus. The lack of any distinct translation product corresponding to the expected size of 25 kDa suggests that the "block" may be at either the transcriptional and/or translational level. In order to test the former possibility, one could isolate RNA from both control and Amil-CP Blue transformed fungus samples and perform semi-quantitative PCR to verify (or not verify) the presence of the appropriately sized cDNAs. Another approach to integrate the Amil-CP Blue construct would be to utilize CRISPR technology, perhaps generating a larger sample size. The Genetics team at Ecovative has also attempted experiment similar to those described here, but they too were not able to see any phenotypic change in their transformants. However, they have demonstrated the presence of significant amounts of Amil-CP Blue mRNA, so it appears the block on expression is post-transcriptional. More work still needs to be done in order to see the change in the phenotype, but this project has been a good start. I hope my work during this thesis project will help future students and Ecovative to optimize their process in the future. I am excited to pass this project down to future thesis students and for the relationship between Ecovative and Union College to continue.

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