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Transforming moss P. patens with lignin peroxidase through heterologous protein expression.

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University of Louisville

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TRANSFORMING MOSS P. *PATENS* WITH LIGNIN PEROXIDASE THROUGH
HETEROLOGOUS PROTEIN EXPRESSION

By

Jesse Lee Rozsa
B.S. University of Buffalo 2015

A Thesis
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville
In Partial Fulfillment of the Requirements
For the Degree of

Masters of Science in Biology

Department of Biology
University of Louisville
Louisville, Kentucky

August 2019

TRANSFORMING LIGNIN PEROXIDASE USING A NOVEL HETEROLOGOUS
PROTEIN EXPRESSION SYSTEM

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Jesse Lee Rozsa

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A Thesis Approved on

August 7, 2019

By the following Thesis Committee:

_____

Dr. Mark P. Running

_____

Dr. Nobuyuki Matoba

_____

Dr. Mike Perlin

_____

Dr. David Schultz

ABSTRACT

TRANSFORMING MOSS P. *PATENS* WITH LIGNIN PEROXIDASE THROUGH
HETEROLOGOUS PROTEIN EXPRESSION

Jesse Rozsa

August 7, 2019

Lignin peroxidase is an enzyme secreted by white-rot fungi

Phanerochaete chrysosporium. Our laboratory is attempting to produce several

lignin degrading enzymes in our protein expression system that utilizes the moss,

Physcomitrella patens. During our experiments we discovered that this particular

species of fungus produces altered splicing variants that are not represented in

the previously reported reference sequence. Despite several attempts to produce

a positive transformant containing a desired transgene encoding; lignin

peroxidase, production of the proper construct eluded our efforts, possibly due to

toxic effects associated with lignin peroxidase expression. Our laboratory has

created a stepping stone for other laboratories to integrate into future research

relating to the expression of fungal proteins in a moss heterologous protein

expression system.

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INTRODUCTION

Lignin peroxidase is an extracellular peroxidase that helps digest the lignin of a plant. The word lignin is derived from the Latin word "*lignum*", meaning wood. Among the organisms that produce the enzyme lignin peroxidase is the white-rot fungus *Phanerochaete chrysosporium*. *P. chrysosporium* does not use the digested lignin as a fuel source directly, but rather it digests the lignin in order to gain access to the cellulose beneath it. Lignin is the second most abundant naturally occurring compound in the world (Coconi-Linares et al. 2014). Lignin, a phenolic polymer derived from hydroxycinnamoyl alcohols, is known for being recalcitrant to degradation by most enzymes. An explanation for the increase of interest in lignin and lignin degrading enzymes recently, is due to the potential of biofuel applications which are otherwise impeded by the presence of lignin.

The function of lignin is usually associated with providing the structural support to allow the transportation of water and nutrients, structural support of the organism itself to stand upright, and protection from biological attacks. Lignin has a complex chemical structure but is constructed from three basic monomers, p-hydroxyphenyl (H) guaiacyl (G), and syringyl (S). These units are derived from p-coumaryl, coniferyl, and sinapyl alcoholic precursors, respectively. Biosynthesis of lignin is a process in which monomers undergo radical coupling reactions to form racemic (composed of equal amounts of left and right-handed enantiomers), cross-linked, and phenolic polymers giving lignin a large array of

complexity. Interestingly, the lignin of gymnosperms contains almost entirely G units whereas the lignin of dicotyledonous angiosperms are comprised mostly of G and S units. All of the different units of lignin can be found in monocotyledonous lignin (Poovaiah et al. 2014). Even further, the structure of lignin can vary among different tissues and ages of the same individual organism (Lu et al. 2017).

Lignin biosynthesis developed during the bridge from aquatic to terrestrial inhabiting plants. The precursor of lignin biosynthesis is believed to have developed in aquatic moss (Espiñeira et al. 2011). Lignin was one of the key factors that allowed plants to overcome the obstacles in terrestrial ecosystems. Lignin provided plants with structural rigidity to stand upright as well as withstand the pressure produced during transpiration (Weng and Chapple 2010).The development of a system to digest lignin did not mature until long after the takeover of land by plants. The organisms that developed these systems include but are not limited to fungi such as *P. chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, and some bacteria (Kantelinen et al. 1988, Hatakka 1994).

The biodegradation of lignin by white rot fungi is best described by Leonowicz and colleagues (Leonowicz et al. 1999) as well as Ten Have (Ten Have and Teunissen 2001). In short, *P. chrysosporium* produces hydrogen peroxide while also excreting a multitude of enzymes including, lignin peroxidase. The enzyme uses hydrogen peroxide for activity and is capable of cleaving and oxidizing lignin compounds. During these reactions it is noted that a pure oxygen atmosphere enhanced the production of hydrogen peroxide in fungal cultures

(Faison and Kirk 1983). Studies have shown that enzymes as large as peroxidases and laccases cannot directly make contact with lignin due to the pore size of the cell wall. Therefore, it has been proposed that low molecular weight cofactors may play a role. Manganese, veratryl alcohol, oxalate, and 2-chloro-1,4-dimethoxybenzene have all been found to be present during the degradation of lignin and are believed to play the role of cofactor during fungal decay (Ten Have and Teunissen 2001).

Lignin peroxidases are glycoproteins. Glycoproteins are proteins that comprise a carbohydrate group attached to its polypeptide chain. *N*-glycosylated proteins have the carbohydrate attached on the amide side chain of asparagine whereas *O*-glycosylated proteins have an attachment to the oxygen on a serine or threonine (Walsh 2006). There are many different functions of glycosylation of proteins. These include protein folding, cell to cell adhesion, a quality control mechanism to ensure correct orientation of folding of the protein, and much more (Trombetta 2003). Lignin peroxidase is a protein with a molecular mass from 38 to 43k Da. Approximately 2 to 3k Da of the molecular weight of lignin peroxidase can be attributed to N-glycosylation. It is likely that lignin peroxidase also contains O-linked carbohydrates because the enzyme has multiple potential sites for O-glycosylation One of the most important points of glycosylated proteins is that the mechanism for *N*-glycosylation is conserved across eukaryotes and archaea. (Gold et al. 1993).

Lignin peroxidase is transcriptionally regulated; thus, the mRNA will be processed like most secreted enzymes. The ribosome-RNA complex will be

transported to the rough endoplasmic reticulum to be translated. During translation is when *N*-glycosylation will occur. The glycosylated product will conform and fold properly making it functional. If the enzyme requires *O*-glycosylation, the process will proceed post-translationally in the Golgi apparatus. Lignin peroxidase has a folding motif that contains eight major alpha helices, eight minor helices, and three short antiparallel beta sheets. It is also segregated into two different domains by a heme group that is only accessible through two small channels. Once properly folded, the enzyme will proceed in a vesicle from the Golgi to be secreted from the cell (Ten Have and Teunissen 2001).

Our laboratory is interested in producing lignin degradation enzymes for use in biofuel production and industrial applications. We are trying to accomplish this with the help of our collaborative group of artificial membrane scientists and engineers to produce a bio-inspired membrane containing several enzymes for easy degradation of lignin in a scalable vat of complex lignin polymers. Our lab is accomplishing this using a specific protein expression system.

Our laboratory is trying to express lignin peroxidase in a moss heterologous protein expression system. The moss we are using is *Physcomitrella patens*. *P. patens* has been of interest for heterologous protein expression for some time (Reski et al. 2018). We are taking advantage of a mutant strain of *P. patens* in which the cells of the moss act more like a unicellular form of algae (Thole et al. 2014). The mutant moss can easily express foreign genes and does not develop into specific tissues found in wild type moss. Our strain of moss replicates as a single cell that does not undergo

differentiation, and thus the strain could be considered immortal. The lignin peroxidase gene we are using is derived from the white-rot fungus *P. chrysosporium*. Because moss is a eukaryote, the co and post-translational modification of the enzyme should not interfere with the production of the enzyme. This is because of the fact that *N*-glycosylation is conserved in all eukaryotes as stated above. *O*-glycosylation in plants is mostly conserved and should not be an issue during the transformation process. P. *patens* is expected to be able to fold the protein expressed in its proper motif. The protein should be processed through the endoplasmic reticulum similarly to how it would in its native organism.

Aside from *P. patens*, there are many other heterologous protein expression systems that have been extensively studied including tobacco, tomato, insect cells, and various yeast and bacteria strains. Our lab believes that the moss *P. patens* is the best option for heterologous protein expression because producing a lignin peroxidase in a lignin containing plant such as tomato or tobacco could potentially compromise the integrity of the plant. Fields and farming equipment are also needed to grow large amounts of tobacco which could potentially add unexpected maintenance costs to our budget. Moss is seen as a more favorable option because it can be grown in solid media or in suspension culture. It can be scaled to increase the output as well. It is a eukaryote; thus it can complete complex protein folding motifs and post translational modifications (Gomes et al. 2019). Bacteria do not have the capability to process this type of gene to become functional.

METHODS AND MATERIALS

Phanerochaete chrysosporium (RP 78) was grown in suspension culture as described by Tien and Tu (Tien and Tu 1987). Specifically, *P. chrysosporium* was grown on top of potato dextrose agar in 5 mL flasks for 7 days at 37 degrees Celsius. The culture was then inoculated into a complex liquid media containing trace elements and veratryl alcohol (Tien and Kirk 1988). Flask culture is also exposed to high oxygen concentration for 30 seconds once per day for 5-7 days. This is accomplished by opening the stopper and placing a sterile tube into the flask that is attached to an oxygen dispensary tank. The oxygen is allowed to flow slowly to not disturb the sample. The rubber stopper is then immediately placed back onto the flask. During this time, the flask is also being shaken at 150 RPM for all hours of growth.

The tissue was washed with distilled water through a cheesecloth. The tissue is then cut into several pieces (100 mg) and wrapped in foil to be stored in minus 80 degrees Celsius or processed further.

The 100 mg tissue sample is crushed in a liquid nitrogen bath using a mortar and pestle for 20 minutes. The cell powder was used for RNA extraction using the RNA extraction protocol described by Qiagen RNAeasy plant mini kit. RNA was sampled on a Nanodrop, to determine purity as well as concentration.

A reverse transcriptase reaction was performed on the RNA sample converting it to cDNA first by using a poly dT mix at 70 degrees for 5 minutes. The mix of RNA and poly dT was then added to a reverse transcriptase PCR reaction. The reaction was set to 25 degrees Celsius for 5 minutes, followed by 42 degrees for 60 minutes, followed by 72 degrees Celsius for 15 minutes, and finally 4 degrees to finish.

A PCR reaction was set up using proofreading KOD polymerase with lignin peroxidase gene specific primers (Table 2). The reaction was setup for 30 cycles to produce an amplicon of approximately 1300 bp in size with an initialization temperature of 94 degrees Celsius for 3 minutes. The 3-part cycle consisted of a denaturation temperature of 94 degrees Celsius for 30 seconds, an annealing temperature was 58 degrees Celsius for 30 seconds, and an elongation temperature of 72 degrees Celsius for 1 minute and 30 seconds. After 30 cycles there was a 10-minute extension phase of 72 degrees Celsius. Gel electrophoresis was used to confirm the success of the reaction and size of the amplified DNA.

The DNA was then purified using ethanol precipitation techniques. A Nanodrop was used to measure the concentration and purity of the DNA. A TOPO cloning reaction was performed using competent *E. coli* cells and the pENTR/D vector (Table 1). After incubation at room temperature for 30 minutes, the vector and DNA were added to 25 µL of chemically competent cells. The cells were then heat shocked at 42 degrees Celsius for 45 seconds then immediately transferred to an ice bucket for 5 minutes. 300 µL of LB media was added to the

competent cells. The tubes were then placed into a 37-degree Celsius incubator shaking at 200rpm for 2 hours. Then, 150µL of the media with competent cells was plated onto kanamycin containing agar plates. The plates were placed in a stationary 37-degree Celsius incubator for approximately 14 to 16 hours.

Colonies were screened using colony PCR with the lignin peroxidase specific primers. The PCR reaction was setup for 30 cycles to produce an amplicon of approximately 1300bp in size with an initialization temperature of 94 degrees Celsius for 3 minutes. The 3-part cycle consisted of a denaturation temperature of 94 degrees Celsius for 30 seconds, an annealing temperature was 58 degrees Celsius for 30 seconds, and an elongation temperature of 72 degrees Celsius for 1 minute and 30 seconds. After 30 cycles there was a 10-minute extension phase of 72 degrees Celsius. Positive colonies were then inoculated in 5 mL of liquid LB media overnight at 37 degrees shaking at 200 rpm. Plasmid extraction and purification was performed on the cells as well as rescreening for the lignin peroxidase gene.

The extracted plasmid with the lignin peroxidase insert was linearized using the NotI restriction enzyme, following the condition information by the manufacture (New England BioLabs). The plasmid and enzyme solution were placed in a 37-degree incubator for 2 hours. Proteinase K was used to disable the functionality of the restriction enzyme. The digests were lowered onto agarose and the DNA separated via electrophoresis to confirm linearity of the plasmid.

The plasmid of the fragment containing the lignin peroxidase gene was then cloned into the circular destination vector T1OG (Li et al. 2017) using an LR Clonase reaction. T10G was then linearized using EcoNI digestion enzyme in similar protocol as the pENTR vector. The linearized plasmid was then transformed in competent *E.* coli cells similarly to the TOPO cloning reaction. The cells were plated on ampicillin containing plates and incubated at 37 degrees Celsius for 14 to 16 hours. Colonies were screened using colony PCR for the lignin peroxidase specific gene. The PCR reaction was setup for 30 cycles to produce an amplicon of approximately 1300bp in size with an initialization temperature of 94 degrees Celsius for 3 minutes. The 3-part cycle consisted of a denaturation temperature of 94 degrees Celsius for 30 seconds, an annealing temperature was 58 degrees Celsius for 30 seconds, and an elongation temperature of 72 degrees Celsius for 1 minute and 30 seconds. After 30 cycles there was a 10-minute extension phase of 72 degrees Celsius. Positive colonies were inoculated into 5mL of LB media and incubated at 37 degrees while shaken at 200rpm overnight. Plasmid extraction was performed on the colonies.

Plasmids that were confirmed to have the inserted gene were then linearized using the EcoNI digestion enzyme similarly to the above reaction of linearization. The linearized plasmid was then transformed into the moss *Physcomitrella patens* in accordance to Hiwatashi and Hasebe (Hiwatashi & Hasebe). In summary, the moss protonema must be between 7-10 days into their development cycle before use. The process used was a PEG-mediated transformation. The plated moss was incubated for 7-10 days after

transformation. The cellophane holding the moss was transferred to a selection media containing zeocin after initial growth phase. The selection lasted for 7 days. The selection media contained zeocin. Positive transformants were then chosen to undergo another growth phase for 7-10 days. After the second growth phase, the transformants were placed for 7 days on a secondary selection media containing zeocin.

The moss individuals that survived were then screened using DNA extraction and PCR using lignin specific primers to confirm positive transformation.

RESULTS

The Invitrogen Gateway technology was employed to transform *P. patens.*
The vectors we used in this study are depicted in Figure 1. We then found our
reference sequence from a paper published in 1987 (Tien and Tu 1987) (Figure
2) and developed primers for amplification of the target gene lignin peroxidase
(Table 1) as well as 2 more primers to be used for DNA sequencing (not shown).

pENTR/SD/D-TOPO
2.6 kb

ApaLI(2281)

pBR322_origin

rrnB_T2_term

HpaI(501)
AflIII(554)
ApaI(567)

rrnB_T1_term(427, 470)
M13_pUC_fwd_pri(522, 544)
M13_forward20_pri(537, 553)
attL1(569, 668)

SacII(672)
NotI(672)
EagI(672)

T7_transl_en_RBS(684, 700)
attL2(726, 825)
T7_pro(843, 861)
M13_reverse_pri(862, 880)

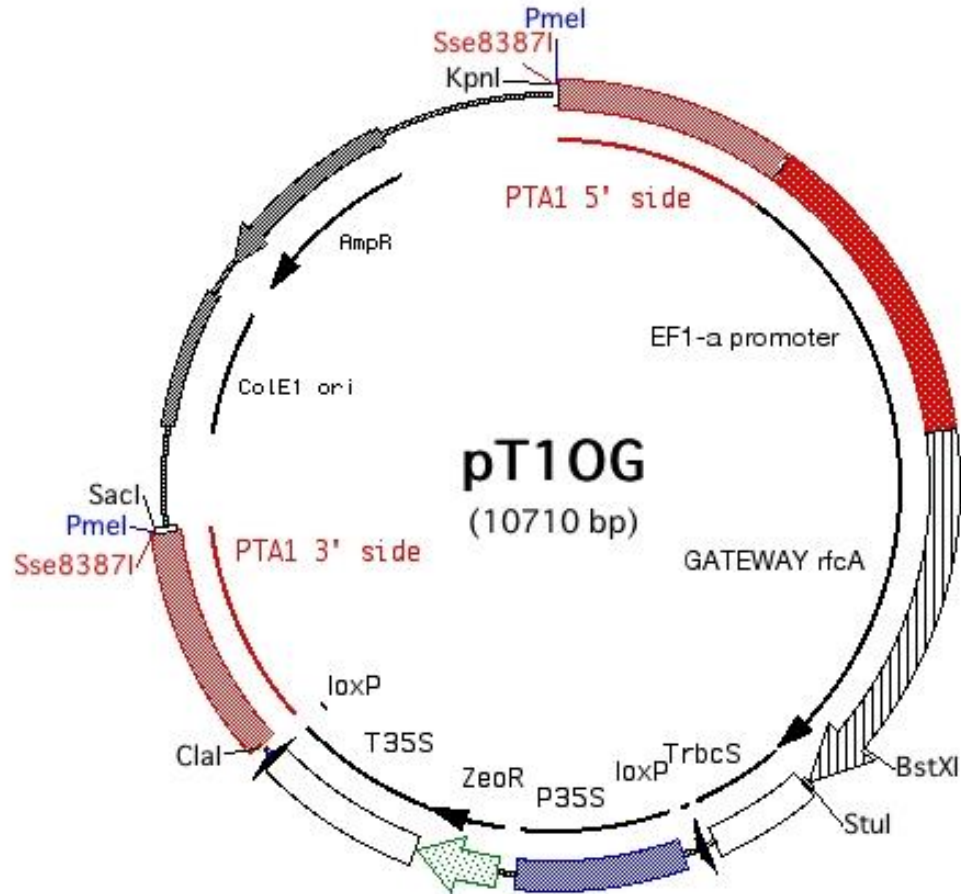EcoRV(838)

ORF 2

NruI(1077)

KanR2

12
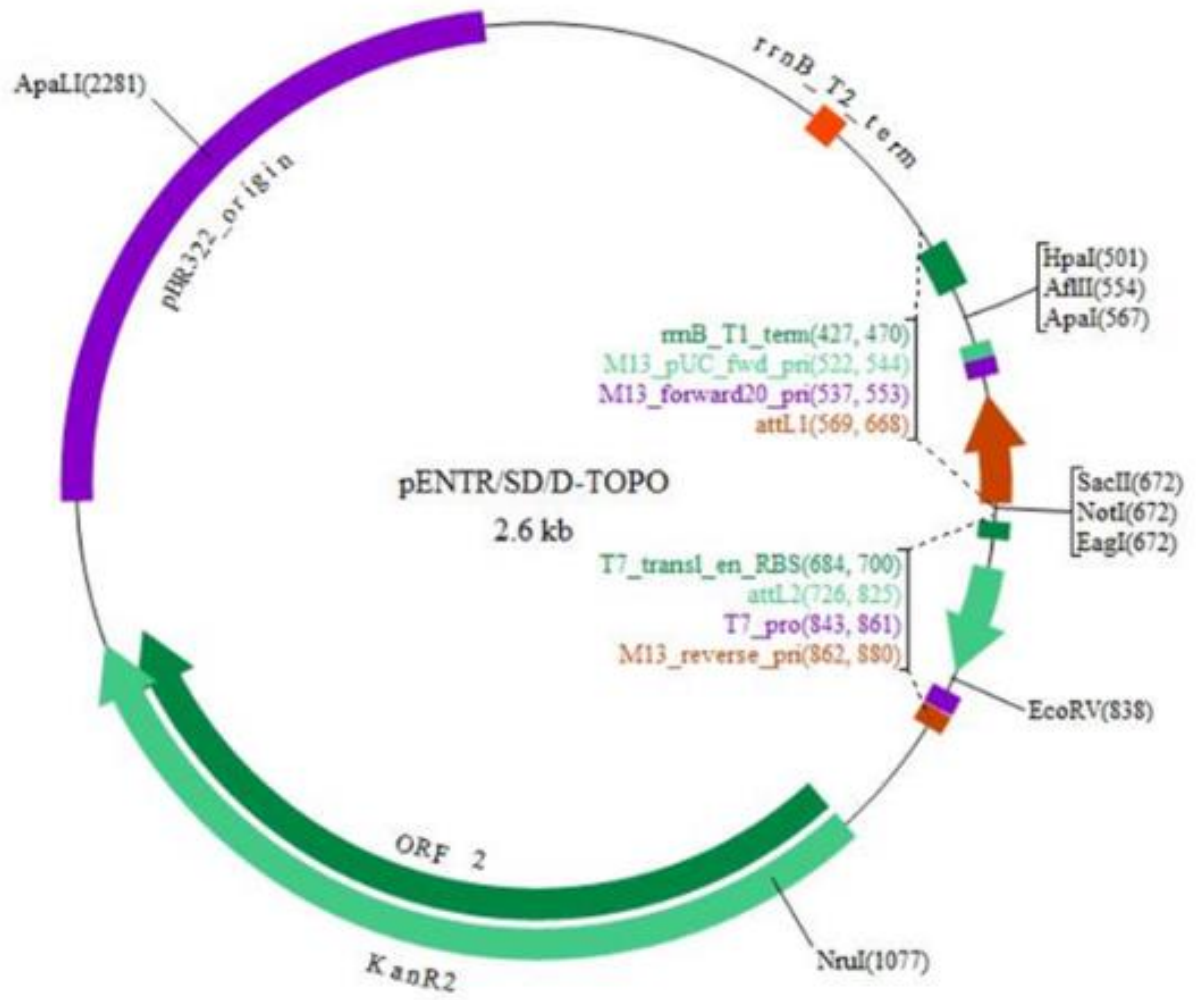
Figure 1. pENTR from Invitrogen Gateway Technologies and pT1OG vectors were used in this experiment (Li et al. 2017). This technology uses site-specific recombination properties of bacteriophage lambda. This technology also enables rapid and highly efficient transfer of a DNA insert, easy protein expression and functional analysis. It is easy to maintain the orientation and reading frame of the insert as well as convert any vector into a Gateway destination vector. The pENTR vector contains a kanamycin resistance gene for selection purposes in bacteria as well as an *E. coli* replication origin. The pT1OG vector contains the EF1-alpha promotor which is a constitutive promotor for moss, the Gateway reading frame cassette insertion site, the LoxP site which is the site of recombination for cre-dependent gene expression, the

Cauliflower mosaic virus 35S promotor, which is a constitutive promotor in plants, a

zeocin antibiotic resistance gene for selection in plants, a T35S transcriptional terminator,

sites homologous to the non-essential moss gene PTA1 for homologous recombination

gene replacement, ColE1, which is an origin of replication site in *E. coli*, and an ampicillin

antibiotic gene.

Target Gene	Forward (5') Primer	Reverse (3') Primer
LiP-H8	CAC CAT GGC CTT CAA GCA GCT CTT GCC	TTA GTG GTG GTG GTG GTG GTG AGC ACC CGG AGG CGG AGG

Table 1. Primers for directional cloning by adding four bases (CACC) to the 5'

primer. The overhang in the vector anneals with these four bases, stabilizing the insert in

correct orientation, which results in blunt end PCR product.

ATGGCCTTCAAGCAGCTCTTCGCAGCTATCTCTCTCGCTCTCTTGCTCTCGGCTGCGAACGCGGCTGCGGTGATC
GAGAAGCGCGCCACCTGTTCCAACGGCAAGACCGTCGGCGATGCGTCGTCGTGCGCTTGGTTCGACGTCCTGG
ATGATATCCAGCAGAACCTGTTCCACGGCGGCCAGTGCGGCGCTGAGGCGCACGAGTCGATTCGTCTCGTCTTC
CACGACTCCATCGCAATTTCGCCCGCCATGGAGGCACAGGGCAAGTTCGGCGGCGGTGGTGCTGACGGCTCCA
TCATGATCTTCGACGATATCGAGACTGCGTTCCACCCTAACATCGGTCTCGACGAGATCGTCAAGCTCCAGAAGC
CATTCGTTCAGAAGCACGGTGTCACCCCTGGTGACTTCATCGCCTTCGCTGGTCGTGTCGCGCTCAGCAACTGCC
CTGGTGCCCCGCAGATGAACTTCTTCACTGGTCGTGCACCTGCTACCCAGCCCGCTCCTGATGGCCTTGTCCCC
GAGCCCTTCCACACTGTCGACCAAATCATCAACCGTGTCAACGACGCAGGCGAGTTCGATGAGCTCGAGCTTGTC
TGGATGCTCTCCGCGCACTCCGTCGCAGCGGTGAACGACGTCGACCCGACCGTCCAGGGTCTGCCCTTTGACTC
GACCCCCGGAATCTTCGACTCCCAGTTCTTCGTCGAGACTCAGCTTCGTGGTACCGCCTTCCCCGGCTCTGGTG
GCAACCAAGGCGAGGTCGAGTCGCCGCTCCCTGGCGAAATTCGCATCCAGTCCGACCACACTATCGCCCGCGAC
TCGCGCACGGCGTGTGAATGGCAGTCCTTCGTCAACAACCAGTCCAAGCTCGTCGATGACTTCCAGTTCATCTTC
CTCGCCCTCACCCAGCTCGGCCAGGACCCGAACGCGATGACCGACTGCTCGGATGTTATCCCGCAGTCCAAGCC
CATCCCTGGCAACCTCCCATTCTCGTTCTTCCCCGCTGGCAAGACCATCAAGGACGTTGAGCAGGCGTGTGCGG
AGAGCCCCTTCCGACTCTCACCACTCTCCCGGGCCCCGAGACGTCCGTCCAGCGCATCCCTCCGCCTCCGGGTG
CTTAAATGATGCCATACAGAATACTCCTCAAACCGACTGTAACGGTGGCCGGCTAACTCGTGACGGAACTTCGGC
TTTACTAGATTTCATTCATCGTATCTCTGCACCTAACTACGAATCTCATTCGTCTACTTCCTTCTTACGATATTCCTT
GCGCGTGGGCTTATGAAATATCGGTGCACATCC

Once we produced several milligrams of *P. chrysosporium* tissue, we followed the steps above to harvest cells, isolate RNA, perform reverse transcription, and PCR to amplify the lignin peroxidase cDNA using gene specific primers (Table 1). We confirmed the lignin peroxidase gene was present in our samples by gel electrophoresis (Figure 3).
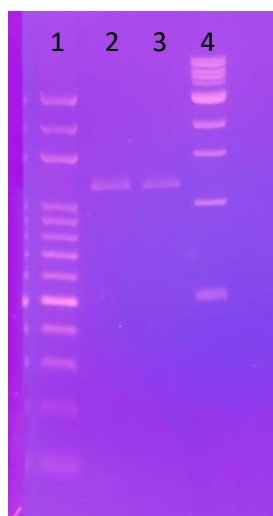

Figure 3. This gel depicts the Lignin peroxidase gene amplified through PCR using cDNA. KOD proofreading polymerase was used for PCR. Lane 2 shows 1:10 dilution of the lignin peroxidase gene amplified at around 1300 bp. Lane 3 shows 1:20 dilution of the lignin peroxidase gene amplified at around 1300 bp. The cDNA came from a reverse transcriptase reaction from the RNA of *P. chrysosporium*. Lane 1 is a 100 bp ladder and lane 4 is a 10 kb ladder.

Once the proper band was confirmed, we performed the TOPO cloning reaction as described by Invitrogen Gateway technologies. The plasmid was transformed into *E. coli*; colonies surviving selection on kanamycin were

screened using colony PCR, with several positive transformants identified as depicted in Figure 4.
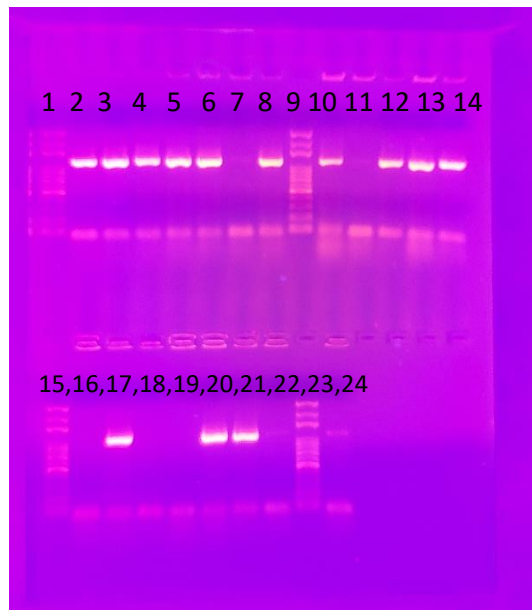

Figure 4. Results from a colony PCR using lignin peroxidase specific primers to identify colonies that were successfully transformed using the pENTR vector. Lanes 1, 9, 15, and 23 all contain a 100 bp ladder. Lanes 1-6, 8, 10, 11-12, 17, 20, and 21 contain positive lignin peroxidase inserts with the expected 1300 bp band. Lanes 7, 11, 16, 18, 19, 22, and 24 are negative for the lignin peroxidase gene.

Several colonies were chosen as candidates for amplification of the vector containing the lignin peroxidase insert. These colonies were inoculated into LB media and cultured overnight. The vectors were then extracted and purified from the colonies during our plasmid extraction protocol. The results of the plasmid extraction can be found on Figure 5 and Figure 6.
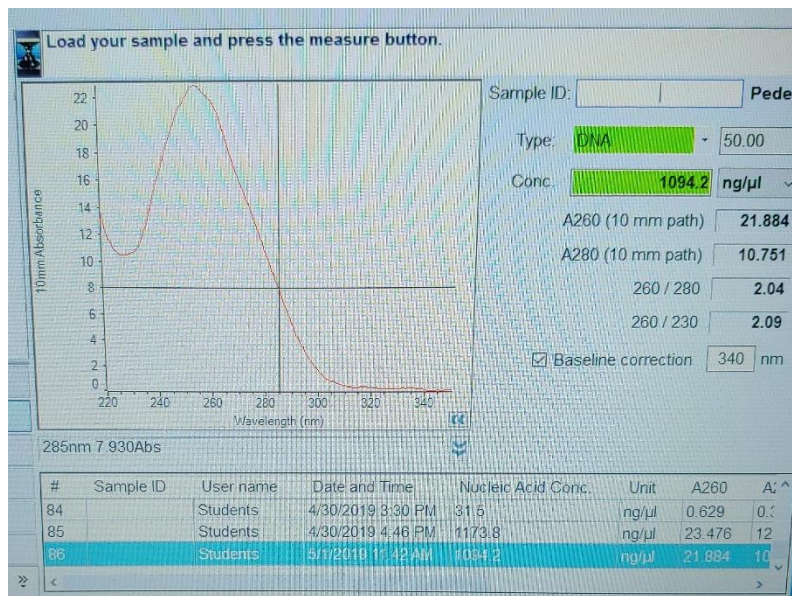
Figure 5. This shows the quality of DNA on a nanodrop after plasmid extraction as well as the concentration of the DNA for later processing.
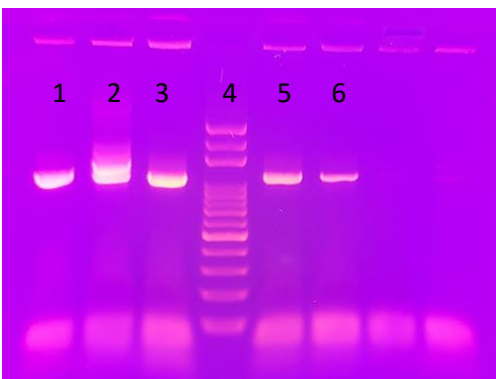
Figure 6. This gel depicts the expected 1300 bp insertion from the plasmid after plasmid extraction of the pENTR vector from the colonies grown. PCR was done using lignin peroxidase specific primers. Lane 4 contains a 100 bp ladder.

The samples were then sent to Eurofins Genomics to be sequenced. The forward primer, reverse primer (Table 1), 2 sequencing primers within the sequence (Not shown), as well as 2 backbone primers (Not shown) were used to

confirm the sequence. The combined results of this can be found on Figures 7

and Figure 8.
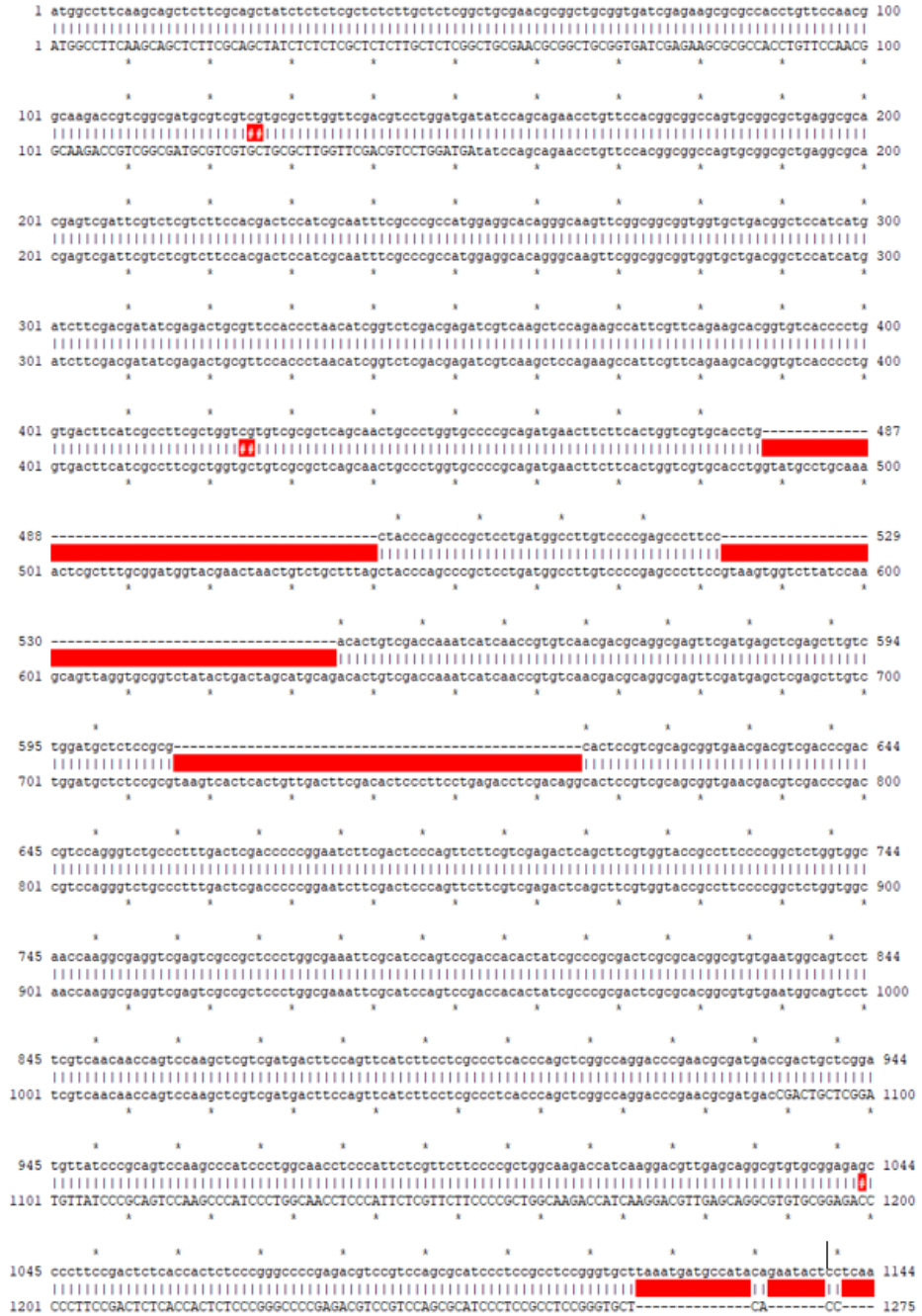
Figure 7. The sequence data of the lignin peroxidase reference (top) and the plasmid DNA extracted from colony 5 with the pENTR vector insertion (bottom). This figure shows 3 gaps approximately 50 base pairs in length. These 3 gaps in the sequence are a

complete match to 3 of lignin peroxidase's exons. There are also two silent mutations where a guanine and cysteine have been swapped around base-pairs 126 and 424.
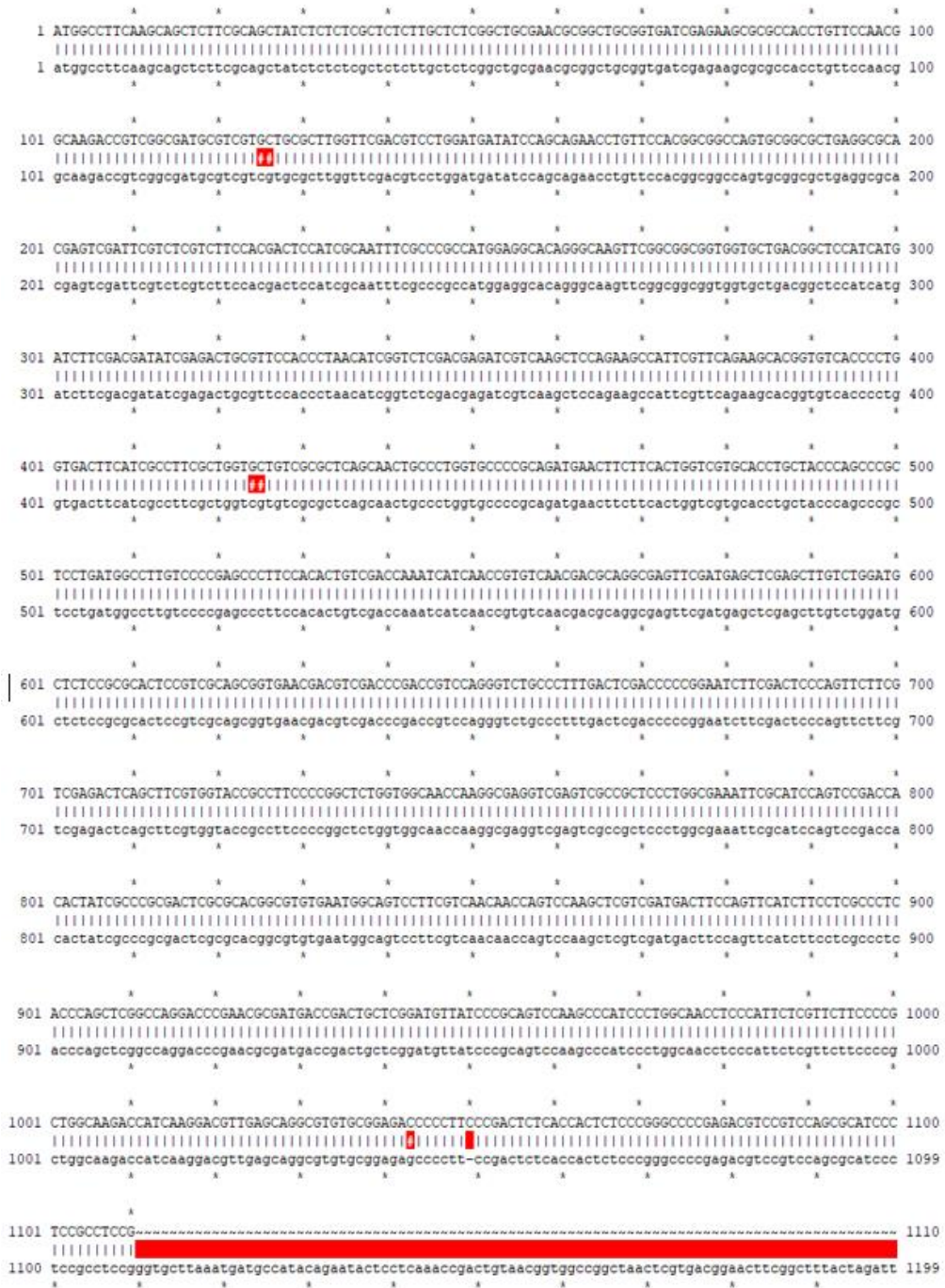

Figure 9. These data show the sequence of the reference lignin peroxidase (top) and the plasmid DNA extracted from colony 10 with the pENTR vector insertion (bottom). There are two mutations which change a cysteine to a serine as well as an arginine to an

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alanine. These can be found around base pairs 126 and 424 respectively. There is also a

deletion that causes a frame shift at 1049.

The purified plasmid was then digested to release the cloned insert from

the vector. The confirmed linearization of the pENTR vector can be seen in

Figure 9.
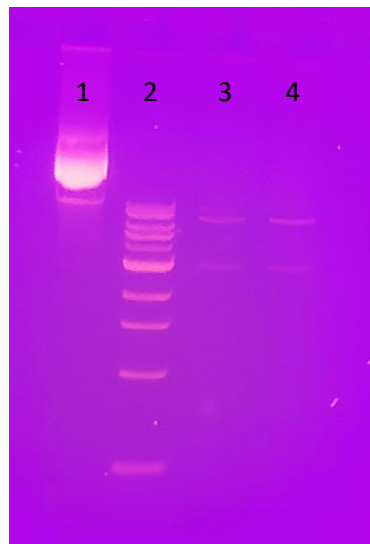
Figure 9. The linearization of the T10G vector using EcoRV digestion enzyme.
Lane 1 depicts the control used (cDNA). Lane 2 contains a 10 kb ladder. Lane 3 and 4
were two samples for digested plasmids.

Once confirmed to be linearized, the LR reaction provided by Invitrogen

was performed allowing the insertion from the entry vector to be collected by the

destination vector. The destination vector was then screened from colonies of *E.*

coli cells. The positive cells were inoculated in LB media and grown overnight.

The plasmids from the cells were then extracted and purified. The destination

vector was then linearized using digestion enzymes. Once confirmed through gel

electrophoresis, the linearized plasmid was transformed into *P. patens* (Figure

10). Unfortunately, none of the putative transformants per date have been demonstrated to contain the transformed lignin peroxidase gene. None of the cells survived transformation.

Figure 10. Protonema cells of P. *patens* that have undergone the transformation protocol.

DISCUSSION

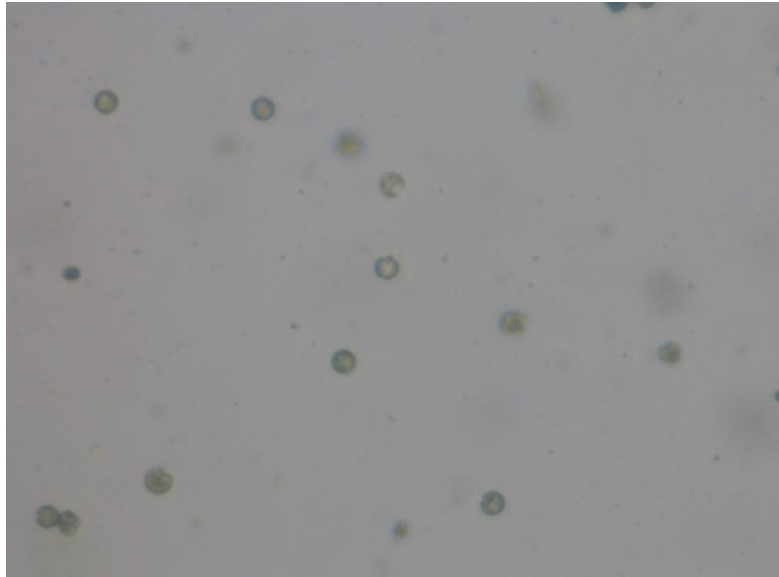In this work, we began with growing the lignin peroxidase gene source organism, *P. chrysosporium* under laboratory conditions. The trace elements are essential for the production of lignin peroxidase as well as exposure of cells to oxygen. It was described by Tien and Kirk that, in a higher concentration of oxygen environment, the fungal tissue will produce more enzyme (Tien and Kirk 1988). Thus, we used Erlenmeyer flasks with rubber stoppers. Every day, the stopper would be opened and pure oxygen from a gas tank would be flowed into the bottle for 30 seconds. It was described that lignin peroxidase expression builds to a peak at around the 5th day of growth and then slowly declines in expression (Hatakka 1994).

Once the tissue samples were grown sufficiently, an RNA extraction of the desired gene from *P. chrysosporium* was performed. Extraction of RNA from fungal tissue samples was particularly difficult. Our lab is accustomed to extracting RNA from plant tissue using liquid nitrogen and a mortar and pestle. Normally, plant tissue can be pulverized within 3 to 5 minutes of the first application of liquid nitrogen. Fungal tissue, however, would not give satisfactory results greater than 10 ng without first pulverizing for 20 minutes.

Once our cDNA was ready, we decided to perform a Taq polymerase PCR before our KOD polymerase PCR reaction. This was mainly because of the expense of using KOD polymerase in PCR reactions. Once the Taq polymerase

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confirmed lignin peroxidase was present in our tissue samples we continued to use KOD polymerase. Unlike Taq polymerase, KOD polymerase has proof reading capability to allow us to get nearly exact matches to our gene of interest for transformation (McInerney et al. 2014). KOD polymerase also showed a 3 fold increase in PCR product formation in comparison to Taq. (Benson et al. 2003).

When we used our gene specific primers on our cDNA, our first results showed many non-specific bands in the gel. This was not an issue because gel extraction and purification were always an option. However, we decided that a 2-step protocol could be better with an annealing/extension temperature of 67 degrees Celsius. We found that this allowed our primers to bind more efficiently to their respective sites.

We sought to convert the RNA into cDNA in hopes the introns were eliminated from the gene of interest. The reasoning behind this is during moss transformation using *P. patens*, we expected the gene of interest to be processed by *P. patens* through heterologous protein expression. In theory, *P. patens* would be able to splice the introns from our fungal gene of interest (Brown 1986). However, we did not want to rely heavily on the regulation aspects of our transformation system but rather express our functional gene without the need of post transcriptional regulation such as alternative splicing mechanisms that may vary among moss and fungi.

During the first round of this experiment, it was discovered that the DNA sequence contained putative introns not present in the reference sequence deposited in GenBank (Figure 7). At first, one could mistake this as genomic

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DNA contamination. After investigation, it was learned that the lignin peroxidase gene was reported to contain 8 introns (Tien and Tu 1987). Our experimental data show that the amplicon contained 3 introns, thus we reasoned that the likelihood of genomic contamination was minimal. Analysis of the sequences of lignin peroxidase splice variants showed that the exon and intron sequences exactly matched those of the genomic sequence DNA. It is possible that fungus may have an alternative splicing mechanism to produce other functional proteins from this transcript; however, none were identified when investigated on multiple databases. We decided to still continue with the experiment with the hopes that *P. patens* may be able to splice out the introns. These introns contain stop codons. If the introns are not cut from the amplicon, the theoretical translated protein would end prematurely and produce a non-functional protein. Moss transformation was performed 8 separate times with fresh moss samples. Controls were used in these transformations and no positive transformant with the lignin peroxidase gene was recovered whereas multiple controls succeeded. The controls were a combination of empty T1OG plasmids as well as the PGX8 plasmid we use in the labs that have been proven to work in moss before. This combination would tell us if the reagents had gone bad, or if the T1OG plasmid by itself was not compatible with our moss.

We found that this incomplete splicing had been deliberated on before. As described by Macarena and Fernando (Macarena et al. 2005), splice variants of different lignin peroxidase and manganese peroxidase transcripts were observed in multiple cultures. They explained that, since the presence of stop codons in

several of the introns would prevent the synthesis of an active enzyme, the transcripts arose as a result of incomplete processing and not alternative splicing. There are multiple reports of different genes where alternative splicing has been detected in *P. chrysosporium (Larrondo et al. 2004, Braesel et al. 2015, Yang et al. 2015)*. We believe that what we have found here is an alternative splicing variant that does not form a functional protein due to the presence of premature stop codons and whose purpose is unknown. Our data agree with Macarena and Fernando with the "altered" splicing model. (Larrondo et al. 2004, Macarena et al. 2005). In a paper that was published in 2015 (Yang et al. 2015), researchers found splicing alterations of *Lac1* gene that resulted in premature stop codons similarly to the altered splicing found in our laboratory. The group concluded that the altered spliced genes in *P. chrysosporium* were not correlated with the unconventional GT-TG splice site (Yang et al. 2015) Another group of researchers found that *invA1* genes in a fungus called *Paxillus involutus* also contained retained introns or "erroneously removed exonic sequences" (Braesel et al. 2015). All of these described cases of altered splicing resulted in a frameshift and premature stop codons.

We decided to continue using a different colony that did not have these introns. Due to time constraints, no transformation was able to be performed. We believe that our problems laid with our choice in digestive enzymes. At first our choice for linearization was the Not1 digestive enzyme. We believed that this would cleave the pENTR vector into two distinct pieces, which we confirmed. However, the enzyme may have cut too close to our insertion on the vector,

possibly rendering the LR reaction beyond repair. Thus, we attempted to use a different enzyme, EcoNI. This resulted in a linearized plasmid; however, the LR reaction still produced no *E. coli* colonies. The control during the LR reaction were just T1OG plasmids without our insert. There were several colonies growing on the control plate. More troubleshooting is needed before we can draw a conclusion from these data.

We sequenced pENTR vectors from many E. *coli* transformant colonies and found that the reference sequence did not match our experimental sequence 100% even without the altered splicing introns. This reference sequence is approximately 30 years old and we used the same strain (Tien and Tu 1987) however, there may have been some errors or individual variations that could explain difference with the sequencing results from 1988. Regardless, the sequencing differences were GC mutations and thus could affect the desired protein. These mutations changed a cysteine to a serine as well as an arginine to an alanine. There was also a consistent deletion at the end of the sequence near the reverse primer that may be due to degradation of sequencing quality in long sequencing runs.

After several attempts of transformation, we questioned if technique and experience could have been a factor. We decided to have an experienced colleague and myself perform transformation on the same day with the same reagents. The experienced colleague not only tried to transform the moss using my insertion but also had several other plasmids and insertions to transform that day. After the first selection phase, we found that all the moss that were

transformed with the lignin peroxidase gene were not viable. However, the other genes that were transformed that day had positive results. We determined the most likely explanation was that the use of the T1OG construct may have caused lethality due to constitutive expression of the lignin peroxidase gene. More troubleshooting is needed. In addition, the use of an inducible expression vector may be more successful.

A possible problem that was foreseen was expressing a lignin degrading enzyme in a plant that could biosynthesize lignin molecules required for its function. It is known that moss contains parts of the biosynthesis pathways required for making lignin (Ligrone et al. 2000). However, no evidence supporting the existence of lignin in mosses has yet been found (Espiñeira et al. 2011). Lignin peroxidase is not known to interfere with degrading our moss cells in its traditional function.

This work demonstrated the difficulty of working with *P. chrysosporium*, especially its apparently frequent use of alternative splicing to encode apparently non-functional proteins.

CONCLUSION

We have shown that performing a successful transformation of moss using fungal genes can be difficult; however, our laboratory has produced viable transformants with other genes from fungal tissue. It appears that the altered splicing model was our biggest hurdle. In future projects, we will screen our fungal tissue to ensure no unwanted altered splicing constructs are present.

A test that could be performed to see if lignin peroxidase would interfere with the moss cells would be to grow moss on a media and then present commercially available lignin peroxidase to the moss. It is unlikely moss will be lysed by the lignin peroxidase alone; however, it is still a possibility due to lignin peroxidases ability to oxidize high redox-potential aromatic compounds (Martínez and Technology 2002).

The future of biofuels looks promising. *P patens* as well as the mutant of *P. patens* we believe are viable hosts for a multitude of eukaryotic gene expression. Not only could this system favor biofuels but also pharmaceuticals. The applications for our expression system are numerous and need further research to develop into efficient and trustworthy laboratory tool.

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CURRICULUM VITA

NAME: Jesse Lee Rozsa

ADDRESS: Department of Biology
                Life Sciences Building
                University of Louisville
                Louisville, KY 40292

DOB: Buffalo, New York – November 5, 1992

EDUCATION
& TRAINING: B.S., Biological Sciences
                University of Buffalo
                2011-15

                Research Intern
                Roswell Cancer Institute Buffalo, NY
                Winter 2015

                Research Intern
                LifeCell Dx Buffalo, NY
                2014-15

                Environmental Technician
                Sienna Environmental Technologies Buffalo, NY
                Summer 2015

                Eurofins Genomics
                Laboratory Lead Louisville, KY
                2015-17