THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMICAL ENGINEERING

DEVELOPMENT OF *AGARICUS BISPORUS* AS A PLATFORM FOR HETEROLOGOUS EXPRESSION OF BIOPHARMACEUTICALS

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ABSTRACT

Agaricus bisporus (the button mushroom) is examined as a viable platform for commercial-scale therapeutic protein expression in light of newly developed genetic manipulation techniques, coupled with established methods for rapid and economical biomass production. To this end, studies were undertaken to optimize protein expression using the β-glucuronidase (GUS) reporter system. In the course of these experiments, it was discovered that by manipulating the commercial bi-layered growth system, high levels of heterologous protein could be accumulated in the mushroom despite the absence of the cognate transgene. Further investigation into this unexpected result, including RT-PCR experiments and analysis of expression in basidiospores and compost mycelium, led to a hypothesis that protein expressed in the lower compost layer is shuttled upward during development of the fruiting body, which forms from the upper casing layer.

This work set the stage for investigation into the mechanism of this phenomenon, prompting studies with an epitope-tagged native glyceraldehyde-3-phosphate dehydrogenase (GPD-HA) gene to explore whether protein transport was due solely to the presence of a heterologous gene, or whether native proteins could also be translocated. In a separate line of experiments, designed to boost heterologous expression levels, genetic constructs were assembled with GUS flanked by the untranslated regions from RNA-2 of the Cowpea Mosaic Virus

(CPMV), sequences which have been shown to increase translation rate in transgenic plants and could potentially have the same effect in the mushroom.

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DEDICATION

This thesis is dedicated to the loving memory of my grandfathers: Michael Curson, whose tremendous patience and infallible work ethic are a continual inspiration, and Bill Woolston, whose positive attitude and determination will always be a source of strength.

ACKNOWLEDGEMENTS

I would like to give thanks to my advisors, Dr. Wayne Curtis and Dr. C Peter Romaine, whose mentorship and guidance on this project have been invaluable. Special thanks are also owed to Dr. Carl Schlagnhaufer for his patience in teaching me the experimental techniques that have been used in the generation of this thesis. I am also indebted to the team at the Penn State Mushroom Research Facility for their help growing and harvesting mushroom fruiting bodies. Finally, I would like to acknowledge Jeff Larsen for exceedingly helpful discussions on the finer aspects of molecular biological techniques.

CHAPTER I

INTRODUCTION AND BACKGROUND

The recent H1N1 pandemic identified a need for manufacturing systems capable of rapid therapeutic protein production in response to imminent virus threats. With the development of a robust *Agrobacterium*-mediated transformation protocol for *Agaricus bisporus*[1,2], the potential has arisen for the common button mushroom to meet this need, and work in the Romaine lab has aimed at investigating heterologous expression in this organism. With over 1.3 million tonnes of *Agaricus bisporus* produced annually, biomass scale-up is fast and economical, with the capability to produce 30 kg of tissue per square meter in 32 days[3] at a low cost of approximately $2.00 per kilogram[4]. Downstream processing technologies have been developed to take advantage of the localization of protein to the harvestable fruiting body to reduce the time and cost associated with protein purification. Furthermore, it has been recently reported that the first step in the glycosylation pathway in basidiomycetes is identical to that in humans, facilitating the production of proteins free of immunogenic sugar residues[5]. For these reasons the Romaine Lab began systematic efforts to maximize protein expression in *A. bisporus* in an effort to develop an economically feasible commercial process for rapid biopharmaceutical production.

CHAPTER II

DISCOVERY OF A LONG-RANGE PROTEIN TRANSPORT PHENOMENON IN *AGARICUS BISPORUS* LINES EXPRESSING β-GLUCURONIDASE

Initial studies in protein optimization were undertaken with GUS under control of a native *Agaricus* laccase promoter, designated L-GUS. Mushrooms were grown following commercial methodology (**Figure 1**): Supplemented compost is inoculated with *Agaricus* mycelium spawn maintained on sterilized rye grain. After two weeks, this compost layer is completely colonized. A second layer (casing layer) comprised of peat moss is spread over the compost and inoculated with casing inoculum (CI), a second culture of *Agaricus* maintained on vermiculite. This creates a bi-layered substrate system, in which the starvation conditions created by the nutrient-deficient peat moss induce the fruiting of the mushroom in approximately 17 days. This initial crop represents the majority of biomass generated in the cycle, with subsequent fruiting events (breaks) occurring at roughly one-week intervals with decreasing biomass yield and quality.

Figure 1 - Commercial Growth of Agaricus bisporus

Preliminary trials with L-GUS used to inoculate both the compost layer and casing layer yielded promising results. GUS activity was highest in the first break, and gradually diminished over the next two weeks (**Figure 2**). The percentage of the total GUS protein harvested in each break (55%, 25%, 20%) paralleled the typical trend in biomass production[6]. Because the latter two breaks account for a significant portion of the total protein extractable from a single crop, the total time from spawning to final harvest extends up to seven weeks. Even when coupled to an optimistic four-week period for genetic engineering, the total time between identifying the sequence of a target protein and producing a transgenic crop approaches three months. Although this is still faster than many other heterologous expression systems, this time must be reduced if therapeutics are to be produced in response to an immediate disease threat.

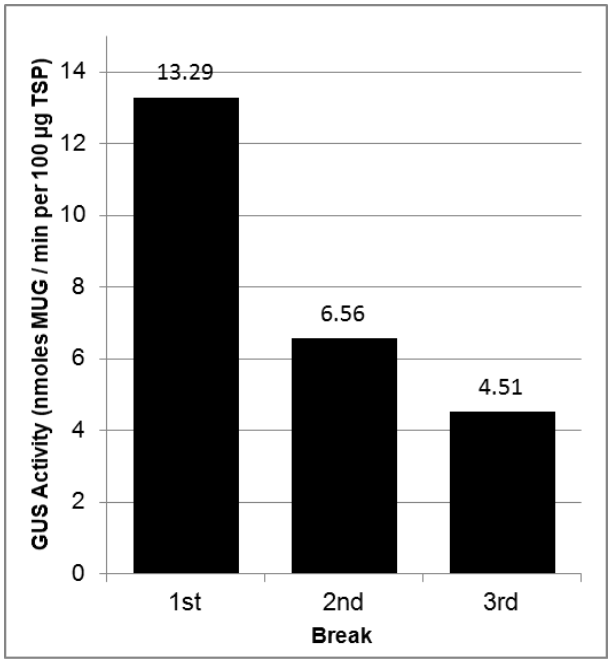

Figure 2 - GUS Protein Expression Level by Mushroom Break

Toward this goal, experiments were conducted using a wild-type (WT) lower spawn with a GUS-expressing CI. Theoretically, this could shorten the process by two weeks, as the wild-type culture could colonize the compost layer concurrently with transformation efforts. However, when this method was used with L-GUS CI and WT spawn, GUS activity diminished by 97% relative to L-GUS CI and spawn (**Figure 3**). q-PCR analysis of the fruiting body showed similar trans-gene copy numbers between the two treatments (**Figure 4**), thus gene dilution from the WT compost layer could not explain the down-regulation of GUS expression in the fruiting bodies spawned from the CI.

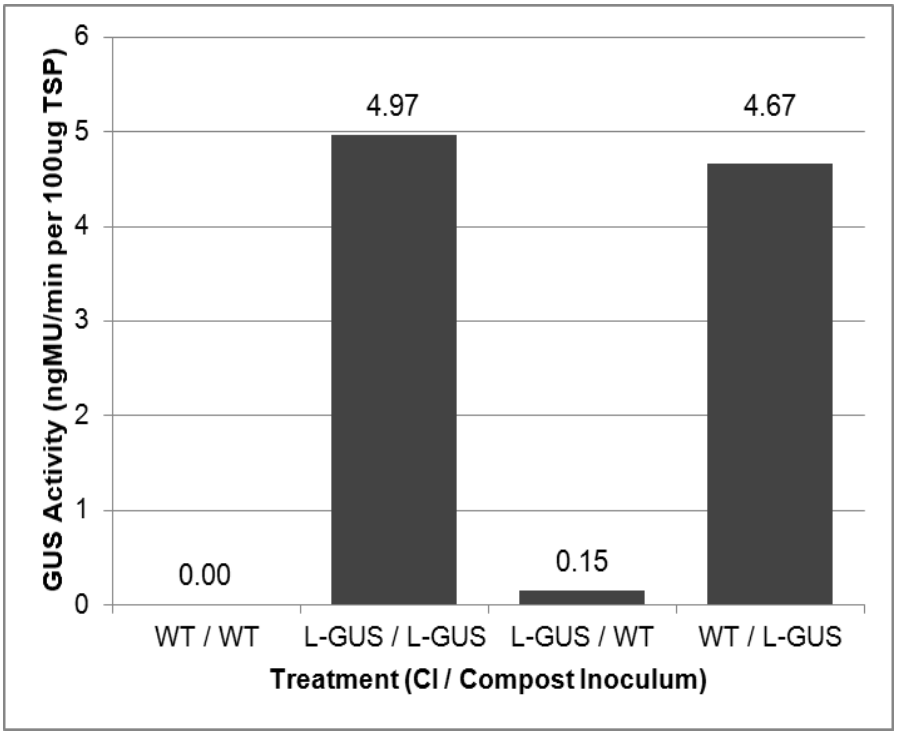

Figure 3 - GUS Activity within the Fruiting Body in Initial Mixed Inoculant Study where X/Y gives the genotype of the (lower inoculums)/(upper casing).

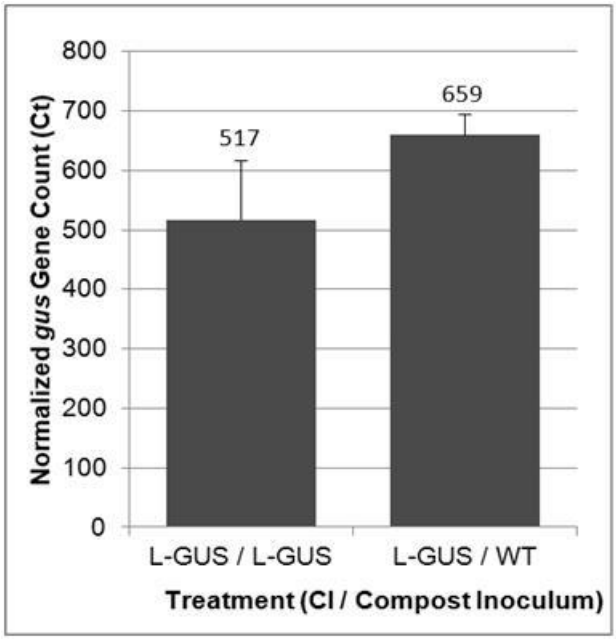

Figure 4 - qPCR for gus gene in the fruiting body from Initial Mixed Inoculant Study

In contrast, when the opposite treatment was tested, L-GUS spawn overlaid by wild-type CI (WT/L-GUS), the *gus* gene was not detected in the resulting mushrooms by PCR even though the WT/GUS tissue contained concentrations of GUS protein similar to the L-GUS/L-GUS treatment (**Figure 5**) Furthermore, large explants (relative to PCR samples) from five mushrooms in this treatment failed to spawn on malt extract plates supplemented with Hygromycin B, verifying the absence of the *hygR* selectable marker gene (**Figure 6**). When PCR analysis was extended to all treatments – WT/WT, L-GUS/L-GUS, L-GUS/WT and WT/L-GUS – the genotype of the fruiting body also matched that of the CI (**Figure 5**). Thus while the genotype of the mushroom was determined by the CI, the phenotype was be determined by the compost layer inoculum.

Most interestingly, GUS activity in the L-GUS/L-GUS treatment was only marginally higher than in the WT/L-GUS treatment (**Figure 5**), even though mushrooms in the latter case lacked the *gus* gene. Furthermore, RT-PCR failed to detect *gus* transcript in the fruiting body of WT/GUS (**Figure 5**), indicating that GUS accumulating in the fruiting body was *not* synthesized locally. Though it was initially difficult to believe that protein could be detected without the cognate gene or transcript, we hypothesized that protein expressed in the lower compost layer, the only transgenic portion of this treatment, was shuttled upward through the casing layer and into the mushroom during its development.

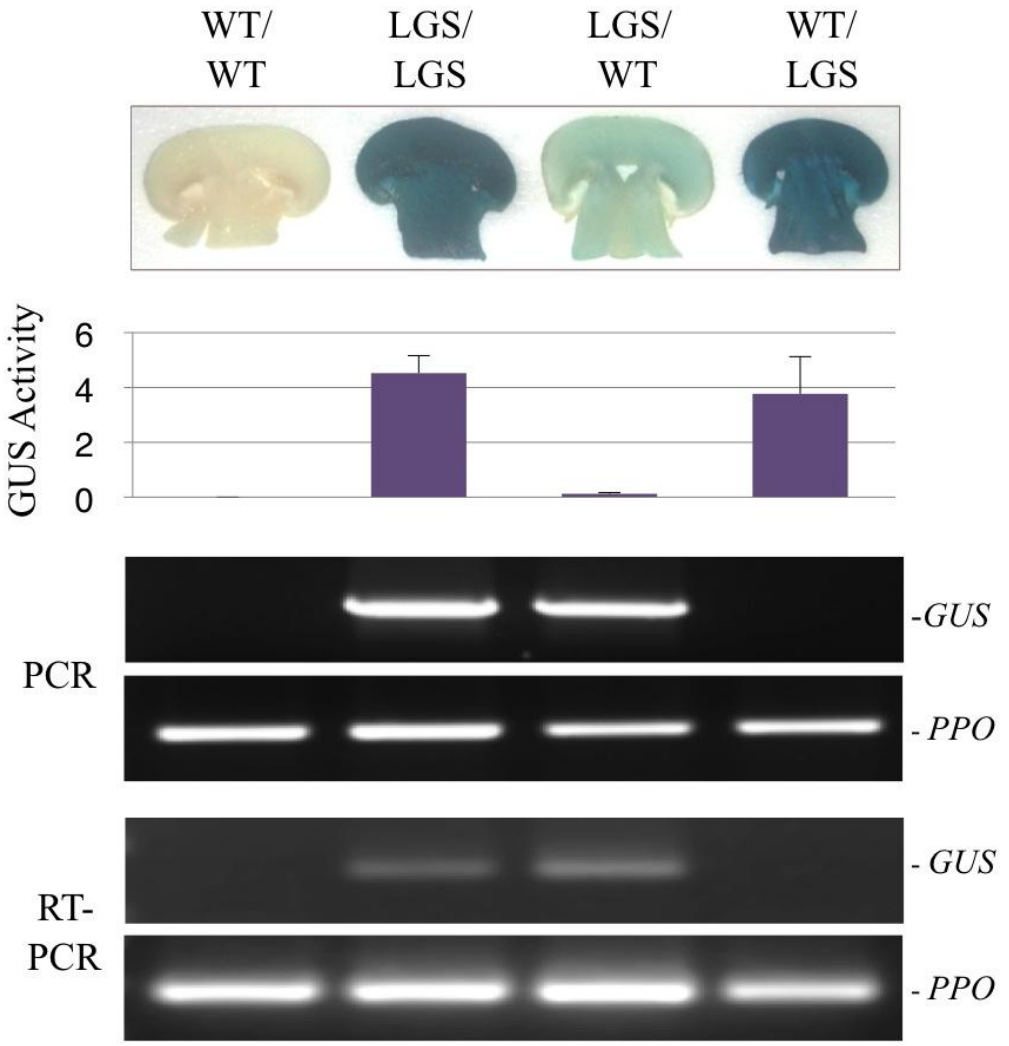

Figure 5 - GUS activity and molecular analyses of mushrooms grown using wild-type and transgenic inoculants under control of the laccase promoter.

Indicated is the upper layer inoculant/lower layer inoculant for the bi-layered cultivation substrate. WT: wild-type line; GUS lines carrying the *laccase* promoter (LGS) From top to bottom: **Histological GUS assay**, **Quantitative GUS assay -** Enzyme activity is expressed as nmol MUG hydrolyzed/min/100 µg total soluble protein, and represents the mean value of two independent experiments), **PCR analysis of the *GUS* gene** - The predicted 163-bp *GUS* amplicon (*GUS*) and 403-bp amplicon for the endogenous polyphenol oxidase 1 (*PPO*) gene, included as a PCR control, are indicated. **RT-PCR analysis of the *GUS* transcript**. Indicated are the predicted 163-bp and 403-bp amplicons for the *GUS* transcript and endogenous *PPO* transcript control, respectively.

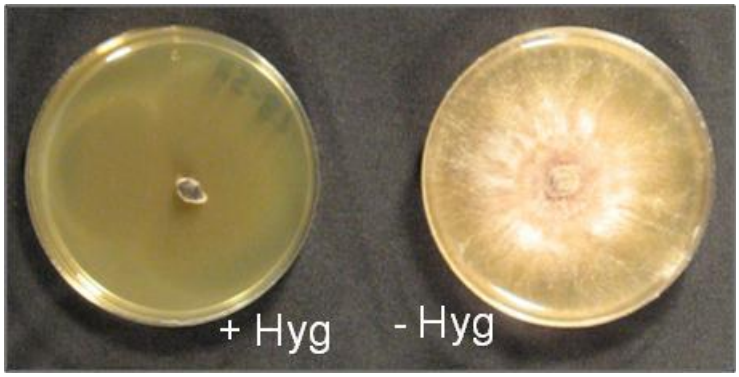

Figure 6 - Transient Antibiotic Resistance in Mushroom Tissue Explants

To our knowledge, such a mechanism has never been reported, but would account for the observed differences in GUS activity between the three treatments: From transcriptome analysis **(Figure 7)** and the primary literature, it is known that the *laccase* promoter is most active in the mycelium[7, 8.] In the transgenic system (L-GUS/L-GUS) therefore, we expect the majority of the GUS protein to be expressed in the lower compost, where the promoter is active. If compost-layer proteins are shuttled upwards into the fruiting body, then the low GUS mushroom protein levels of L-GUS/WT can be explained, as no GUS is synthesized in this wild-type compost layer. The similarly high levels detected in L-GUS/L-GUS and WT/L-GUS mushrooms are also explained, as the relative protein contribution of the CI and fruiting body are minimal compared to the lower compost layer. Thus for the *laccasse* promoter, the genotype of the CI (and hence the mushroom) had a minimal impact on the total GUS protein extracted from the mushroom.

Agaricus Line[1]	*Agaricus* Gene	Protein ID	Fruiting Body Rank[2]	Vegetative Mycelium Rank[2]	Fruiting Body: Mycelium Ratio[3]
HGS	Hydrophobin A (*HYPA*)	133693	1	2,488	108
DGS	Fruiting body-specific D (*FBSD*)	193061	5	1,180	27
LnGS	Lectin (*LCTN*)	194888	11	62	2.4
AGS	β-Actin (*ACTN*)	192120	86	79	0.975
LGS	Laccase 2 (*LCC2*)	135709	7,127	114	0.007

[1] Used in the present study and carrying the *GUS* transgene driven by the promoter of the indicated native gene.
[2] Represents the expression of each gene relative to the other 10,413 genes in the *Agaricus* genome. Data were developed using an Agilent array of four replicates for each of fruiting body tissue and vegetative mycelium grown in compost.
[3] The ratio is calculated from the raw expression data and not from the rank.

Figure 7 - Tissue-Specificity of Various Agaricus Promoters Assessed by Transcriptome Analysis

RT-PCR confirmed that at least some GUS was synthesized in the fruiting body in L-GUS/L-GUS, despite the relative inactivity of the promoter in this tissue. Thus we suspected that the protein accumulated in the mushroom was a combination of *de novo* synthesis in the fruiting body *and* protein transported from the mycelium of both the CI and compost layers. To investigate this hypothesis, previous experiments were expanded to include constructs with GUS under control of three other promoters: Glyceraldehyde-3-phosophate dehydrogenase (G-GUS), Actin (A-GUS), and Hydrophobin (H-GUS). With G-GUS, the results were identical to studies with L-GUS: Relative protein expression patterns and the presence or absence of the transgene and cognate transcript were the same with both promoters (**Figure 7**). The genotype of the mushroom matched that of the CI in all cases, and high GUS activity was found in the WT/G-GUS treatment despite the absence of the transgene.

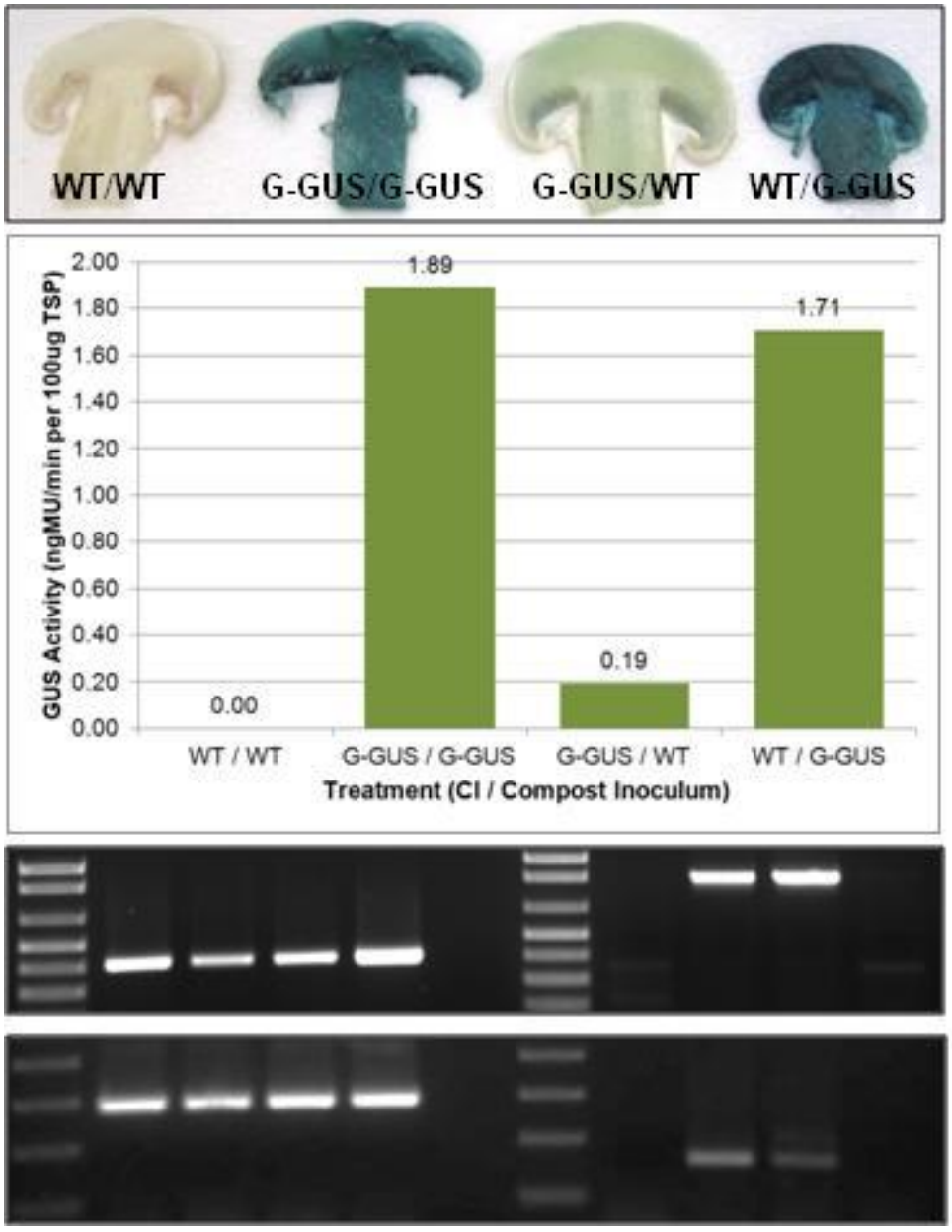

Figure 8 - GUS activity and molecular analyses of mushrooms grown using wild-type and transgenic inoculants under control of the GPD promoter.

From top to bottom: **Histological GUS assay**, **Quantitative GUS assay** - Enzyme activity is expressed as nmol MUG hydrolyzed/min/100 µg total soluble protein, **PCR analysis of the *GUS* gene**. From left to right: WT/WT, G-GUS/G-GUS, G-GUS/WT, WT/G-GUS. The first set of lanes are reactions with GUS primers, and the second set are with the control PPO primers. **RT-PCR analysis of the *GUS* transcript**. The same labeling system is used as for PCR.

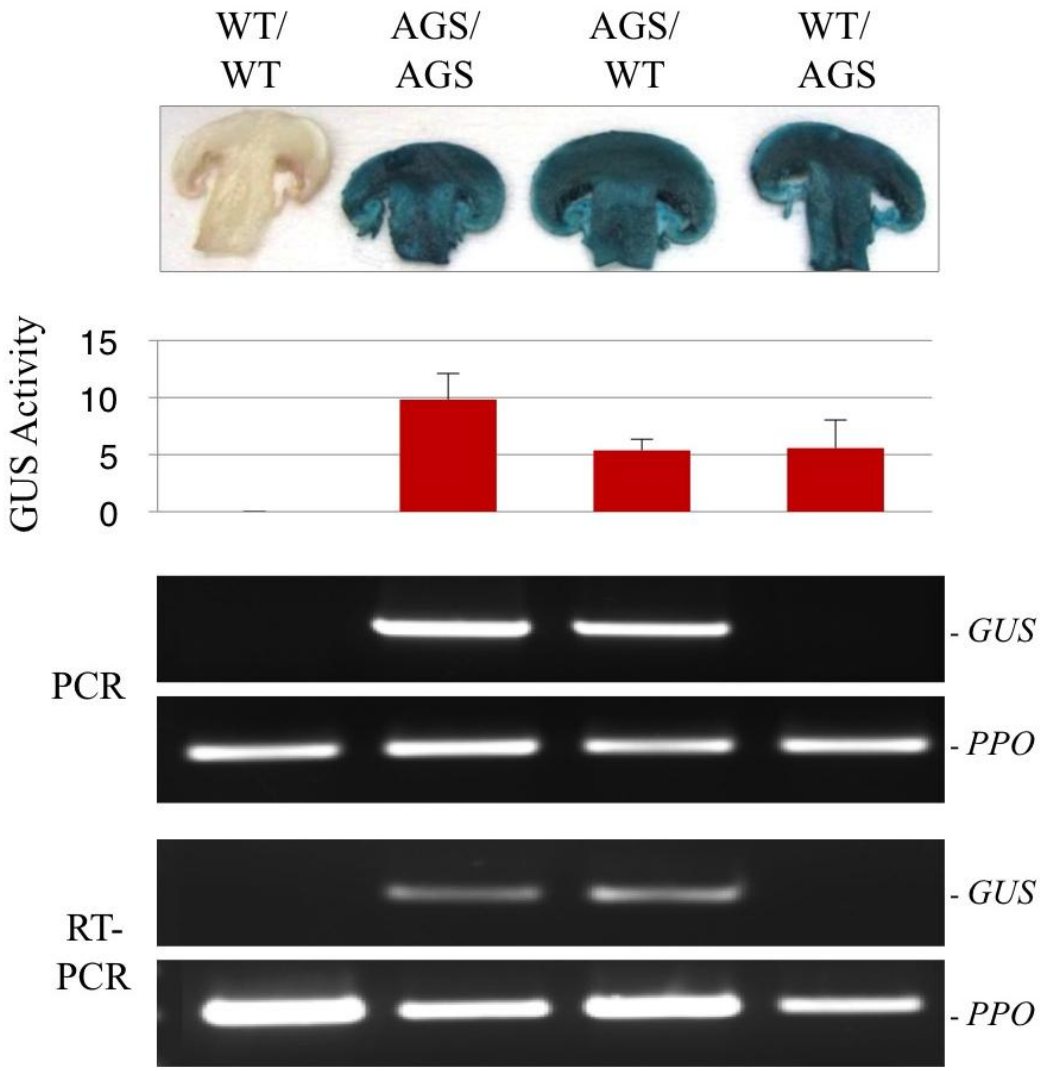

Figure 9 - GUS activity and molecular analyses of fruiting bodies grown using wild-type and transgenic inoculants under control of the Actin promoter.

Figure layout is the same as in Figure 5, except using A-GUS lines in place of L-GUS Lines

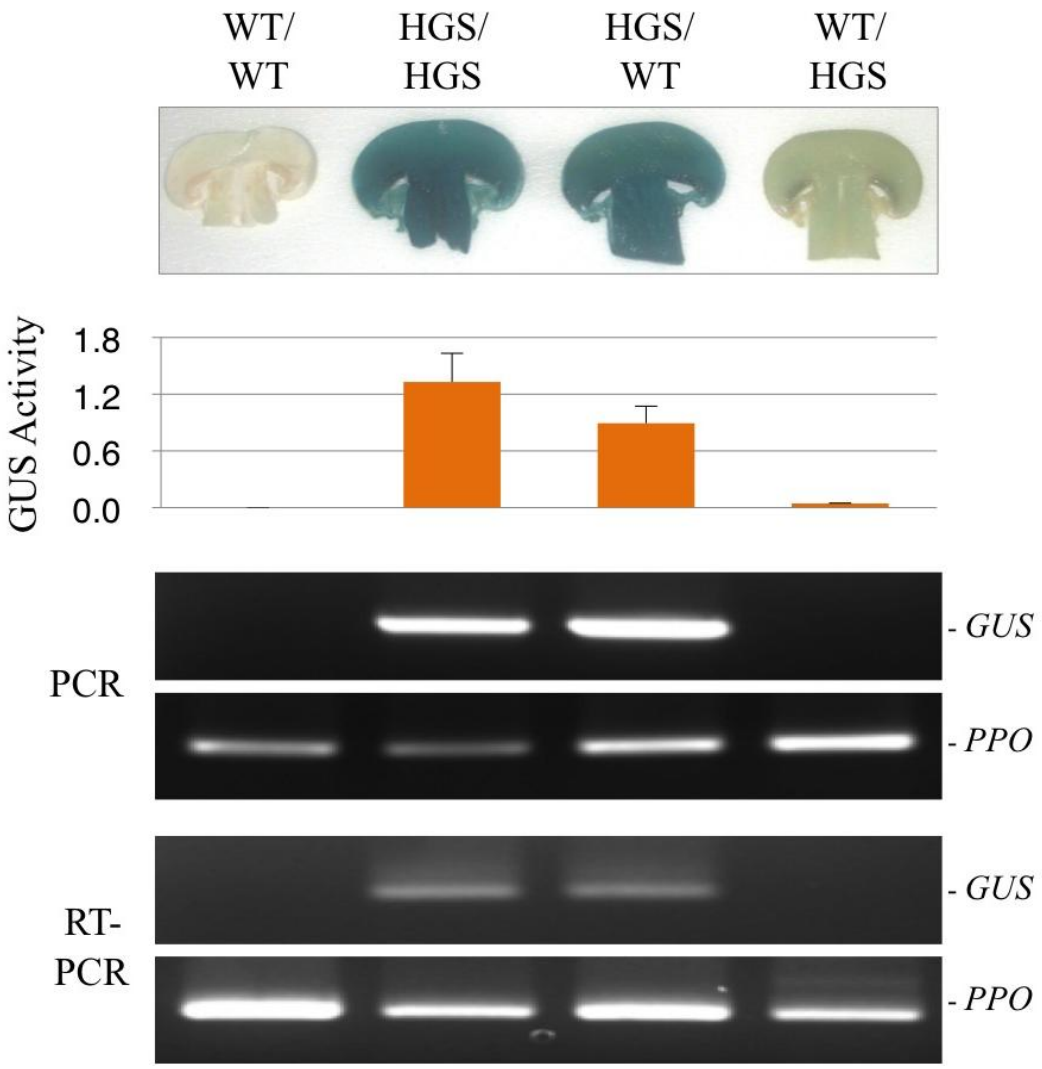

Figure 10 - GUS activity and molecular analyses of mushrooms grown with wild-type and transgenic inoculants with the hydrophobin promoter.

Figure layout is the same as in Figure 5, except using H-GUS lines in place of L-GUS Lines

GUS activity in WT/A-GUS and A-GUS/WT was roughly half of that in A-GUS/A-GUS, while RNA and DNA profiles were identical to those in other lines **(Figure 8).** Transcriptome analysis of the mushroom shows that the actin promoter is constitutively active **(Figure 7)**, in agreement with its behavior in other fungal systems[9, 10], so expression in the fruiting body accounts for a greater portion of the total protein than with the *laccase* promoter. Thus the contribution from *both* layers was significant, with loss of expression in either one resulting in a noticeable drop in total GUS accumulated in the fruiting body.

With the hydrophobin line, results were opposite to those obtained with the L-GUS and G-GUS lines: With H-GUS CI and wild-type spawn (H-GUS/WT), GUS activity in the fruiting body was roughly equivalent to that in H-GUS/H-GUS (**Figure 9**). RNA and DNA profiles were identical to those for the previous promoters. Hydrophobin is most active in the fruiting body[11], where its hydrophobic cysteine residues waterproof the mushroom[12]. Thus the majority of GUS expression is localized to the fruiting body, so the loss of this expression in WT/H-GUS significantly reduces the total GUS activity. H-GUS/WT differs from H-GUS/H-GUS only in the protein contribution from the lower compost, where the promoter is largely inactive and loss of this contribution barely impacts total GUS.

We next asked whether this protein translocation phenomenon could be harnessed to increase overall GUS activity. Previous optimization attempts involved only a single transgenic line under one promoter in both the compost and CI, based on the assumption that the compost layer contributed no protein to the

mushroom. By our hypothesis, GUS in the fruiting body is a result of protein synthesized *de novo* and protein shuttled upward from the lower compost and CI layers. Thus a combination of different lines in the two layers could allow the accumulation of high levels of protein if appropriate promoters were chosen for the respective layers. Specifically, we sought the combination of a highly active mycelial promoter for the compost inoculum and an active fruiting body promoter for the CI. Such a system could be advantageous over using a single promoter, making use of the differential tissue specificity of each promoter. We referred to this approach as 'spatial gene stacking' as a potential technology for enhanced protein expression levels.

Three further promoters were included in this study: Lectin (LE-GUS), a sugar-binding protein used in biological recognition; and Fruiting-Body-Specific Promoters B (FB-GUS) and D (FD-GUS), promoters upregulated in the fruiting body whose functions are not yet known. In each case, use of L-GUS in the compost layer with another promoter in the CI (?-GUS/L-GUS) was compared to use of this second promoter in both layers (?-GUS/?-GUS). Results are shown in **Figure 10**.

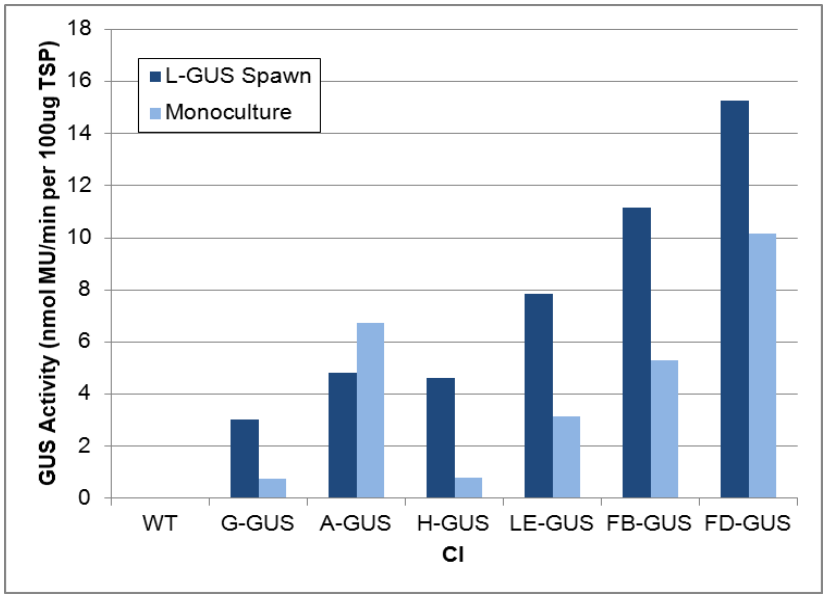

Figure 11 - Protein Results of the Spatial Gene Stacking Experiment

In five of the six treatments this method increased total GUS activity. The effect ranged from a 1.5-fold increase (FD-GUS/L-GUS vs. FD-GUS/FD-GUS) to almost 6-fold (H-GUS/L-GUS vs. H-GUS/H-GUS), with the highest total protein resulting from FD-GUS/L-GUS. In the case of the A-GUS, combination with L-GUS in the compost resulted in a decline in total protein. In this experiment, actin-controlled expression in the compost was higher than laccase-controlled expression. Future studies will repeat this experiment substituting A-GUS for L-GUS in the compost layer, with the highly active FB-GUS and FD-GUS lines as CI. Such a system will take advantage of the highest experimental expression levels currently available in both tissue types.

The experiments described above thus lead to three major findings: 1) The genotype of the mushroom was determined solely by the genotype of the casing inoculum. 2) The phenotype of the mushroom was a result of protein synthesized

in the fruiting body *and* protein expressed in the lower compost layer that is shuttled up to the fruiting body during its development through a previously unreported translocation mechanism. 3) This mechanism can be harnessed to increase GUS activity by using a mycelial-specific promoter in the compost and a fruiting body-specific promoter in the CI. This presents an interesting conundrum from a regulatory perspective, as we have demonstrated the ability to accumulate heterologous protein in a fruiting body with a wild-type genotype. Current regulations define a genetically modified product based on *genetic* composition, thus these mushrooms may not be considered a G.M. crop (although the lower compost would be transgenic). An important concern with using mushrooms for protein production is the potential for the release of transgenic spores. However, spores isolated from WT/L-GUS mushrooms were incapable of growth under selection pressure, indicating a wild-type genotype (**Figure 10**).

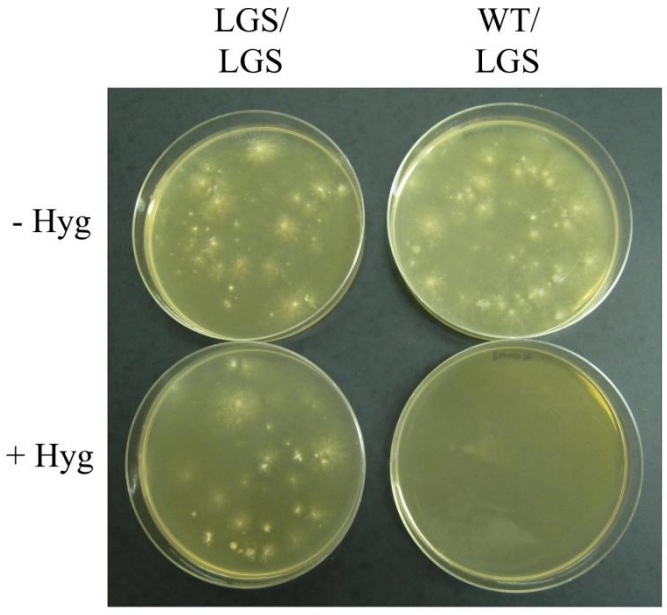

Figure 12 - Germination of Spores on Hygromycin Plates

It was also determined that this wild-type genotype could be driven by CI inoculation rates lower than used commercially (**Figure 12**), thus using WT/GUS provides high levels of biocontainment with minimal losses in heterologous protein expression levels. The addition of this feature to the arsenal of benefits provided by *Agaricus bisporus* further enhance its viability for the rapid manufacture of heterologous therapeutic proteins.

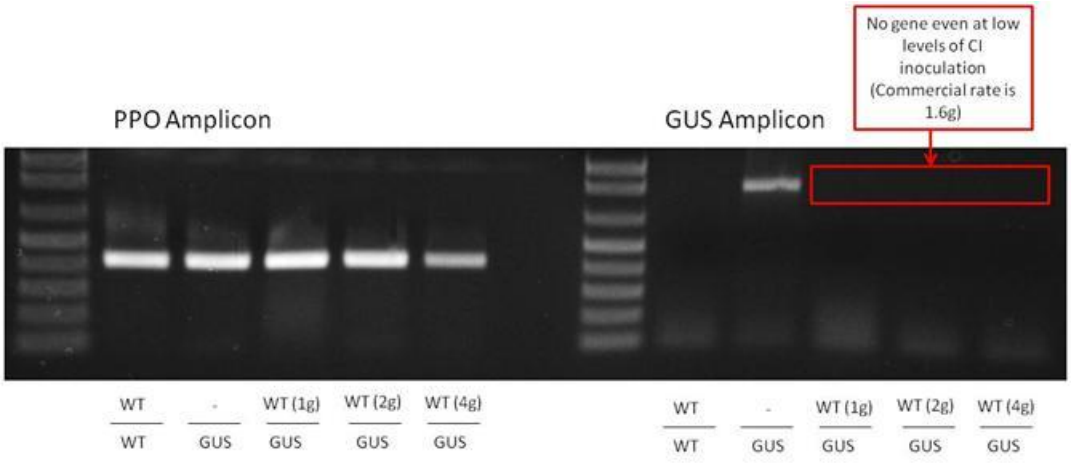

Figure 13 - PCR Analysis of Fruiting Bodies Derived from L-GUS Spawn and Varying Quantities of Wild-Type Casing Inoculum

CHAPTER III
ONGOING AND FUTURE WORK

Discovery of the protein transport phenomenon described in the previous chapter prompted further research into the mechanism of protein transport. Specifically, the fundamental question arose as to whether our observations were an artifact due to using the GUS protein, or whether *Agaricus bisporus* has a robust transport system for the shuttling of *multiple native* proteins from the mycelium to the fruiting body. Concurrently, further experiments were undertaken to increase heterologous expression levels by the use of novel binary vectors which have showed success recently in plant systems. At the time of writing this thesis, both of these projects are still in progress, and the details given in this chapter focus specifically on the rationale and theory behind the experiments, and the preliminary work that has been completed so far.

Investigation of the Specificity of the Protein Transport Phenomenon with an Epitope-Tagged Native Protein (GPD-HA)

In regard to whether the observed protein transport was an artifact caused by the presence of transgenic GUS protein, or a ubiquitous system also targeting native proteins, either possibility is plausible: Many organisms (including humans) demonstrate characteristic responses to foreign proteins, thus it is plausible that protein transport in *Agaricus* is simply an ‘immune-like’ response.

On the other hand, a global protein transport system can be rationalized from an energy conservation standpoint. The lower compost provides the developing mycelium with far more nutrients than the peat moss in the casing layer, therefore it may be energetically favorable to synthesize fruiting body proteins in the lower layer and shuttle them upwards, rather than expressing them *de novo* in the nutrient-deprived fruiting body.

To address the question experimentally, we sought a native protein whose accumulation in the fruiting body could not only be detected and quantified, but also identifed as synthesized either in the compost layer or the casing layer. To this end, a native mushroom gene encoding the key glycolysis protein glyceraldehyde-3-phosphate dehydrogenase[13] (GPD2) was cloned and appended with a 3' hemaglutinin (HA) epitope tag. The resulting modified gene was placed under the control of three tissue specific promoters (GPD, laccase, and hydrophobin) and then transformed into *Agaricus*. **(Figure 14)**

The presence of this essentially native protein (named GPD-HA) could be detected and quantified by Western blotting with antibodies to the HA tag, and would be distinguishable from the unaltered GPD protein, which, lacking the HA epitope tag, is undetectable by anti-HA antibodies. It was hypothesized that a repetition of the full-factorial overlay experiments in the previous chapter but with GPD-HA-expressing lines as the transgenic inoculum, would show whether native proteins were also subject to translocation by direct comparison to the results with GUS as the protein of interest. The genetic engineering and transformation components of this project were completed over the summer of

2010 (as described in the Materials and Methods section), and work is currently underway to identify the most highly expressing transformed lines for each promoter to use in a full factorial study.

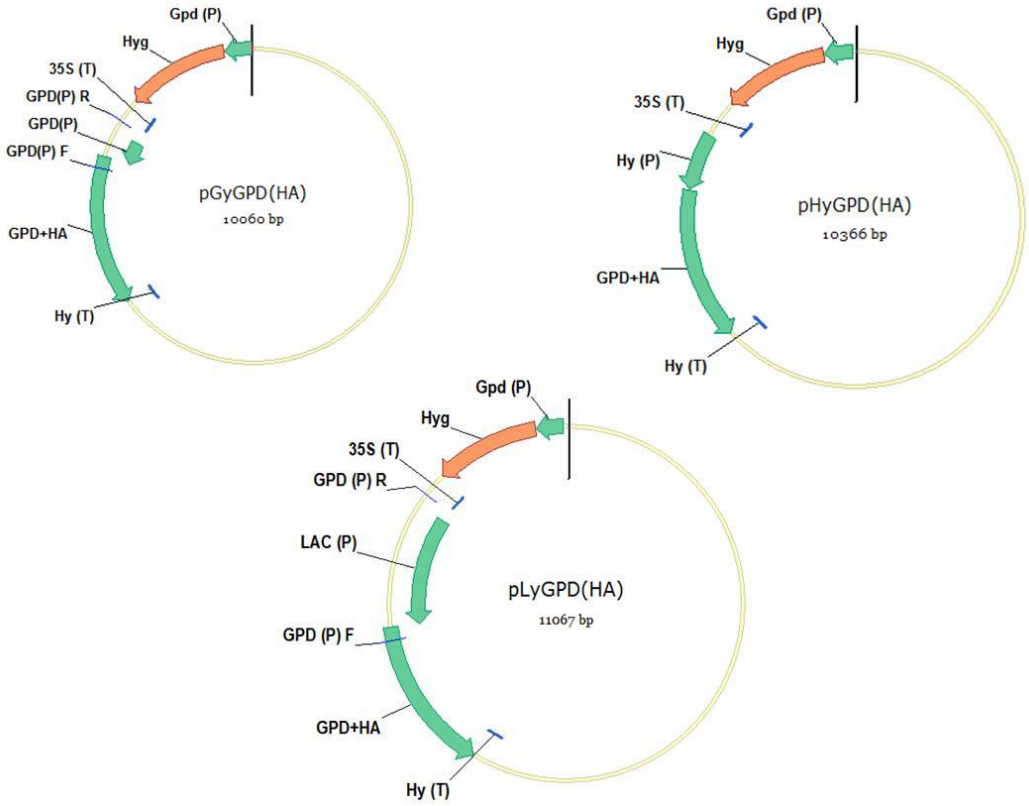

Figure 14 - HA-Tagged GPD Constructs

Evaluation of the Cow-Pea Mosaic Virus Leader Sequence for Improved Heterologous Expression Level

Recently, Lomonossoff et. al reported the high-level stable expression of a variety of foreign proteins in *N. benthamiana* by using the 5' and 3' untranslated regions (UTRs) of the RNA-2 gene from the Cowpea Mosaic Virus (CPMV)[14, 15]. These flanking sequences are thought to increase the translation rate of the mRNA

encoded by the gene, and provide the highest levels of heterologous expression in plants achieved to date without the use of viral replication. It was hypothesized that these UTRs might provide similar increases in expression level in *Agariucs bisporus*. The pEAQHT plasmid, which contains these viral leader sequences, as well as the pEAQHT-19 plasmid, which in addition to these sequences contains the P19 silencing suppressor gene, were made available to the Curtis Lab by MTA.

A cloning strategy was devised to transfer the GUS protein used in our preliminary studies into these vectors, to evaluate their effect on heterologous expression level. The pEAQHT vectors both use kanamycin resistance (NPTII) as their selectable marker, which is not compatible with *Agaricus*. Thus, the cloning process was made more complicated by the need to transfer the CPMV-GUS cassette from the pEAQHT vectors to a binary vector encoding Hygromycin B resistance, the marker used by our lab in transformation of the mushroom. A total of three constructs were generated: (1) pLy-EAQHT-GUS - The GUS gene under control of the CaMV promoter and flanked by the CPMV leader sequences. (2) pLy-EAQHT-GUS-19 - The same as the previous construct but with the addition of P19. (3) pLy-401-Nos - A control construct with GUS driven by the CaMV promoter but with no leader sequences. All constructs used the nopaline synthase terminator and hygromycin as a selectable marker (**Figure 15)**.

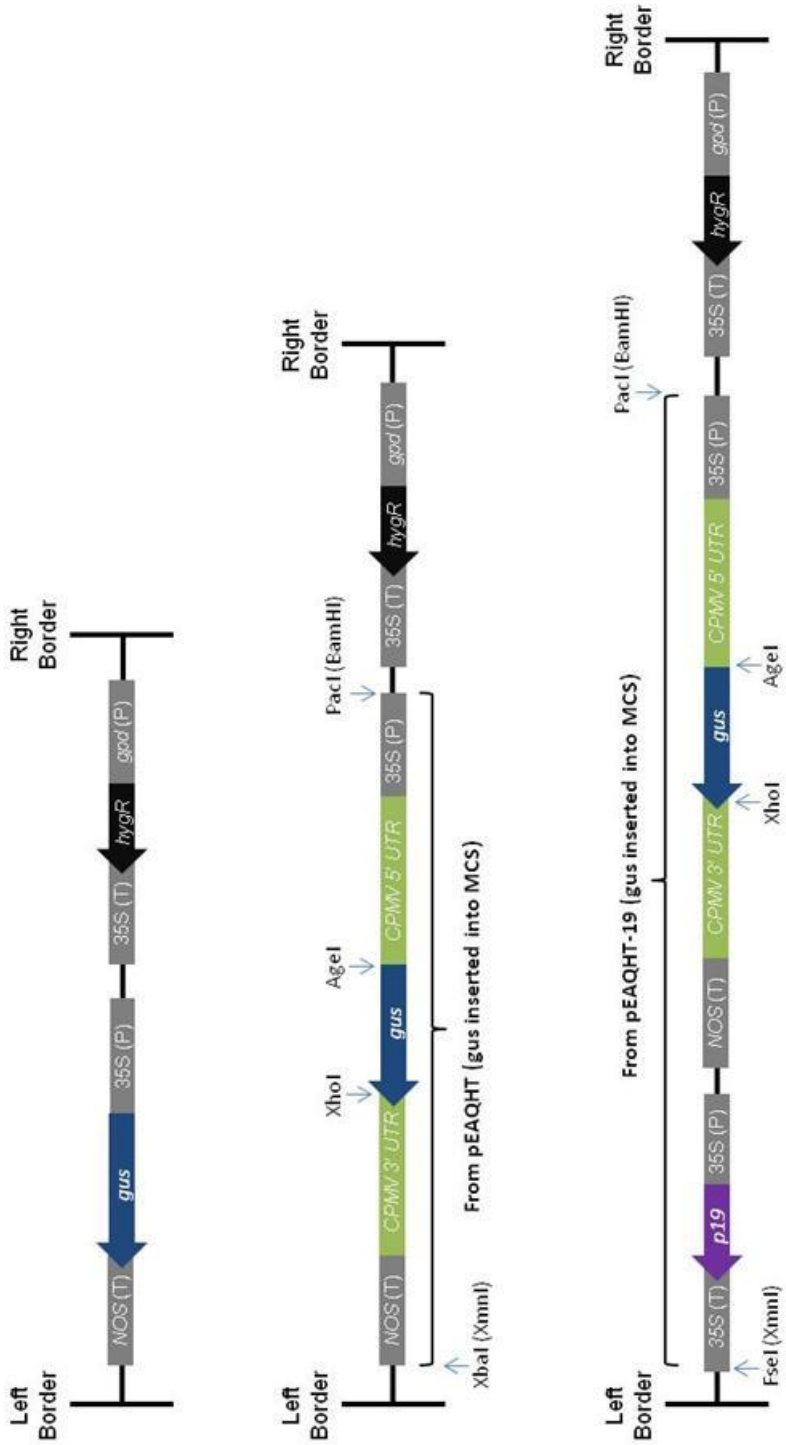

Figure 15 - Genetic Elements of Constructs to Evaluate Potential of CPMV For Increased Expression Level

From left to right: pLy401GUS, pLyCPMV-GUS, pLyCPMV-GUS-19, as described in the text

These constructs were completed and sequenced in early April 2011, and have been transformed into *Agrobacterium tumefaciens* for eventual transfer to *Agaricus bisporus.* Upon completion of the transformation process, expression will be quantified by growing mycelial cultures on plates containing X-Gluc, the synthetic GUS substrate which leaves a blue residue after cleavage by the enzyme, giving a qualitative indication of expression level (the same chemical process as the staining of mushroom tissue shown in Chapter II). Promising transformed lines will be used to generate fruiting bodies, from which protein will be extracted and quantified according to the MUG assay.

CHAPTER IV

CONCLUSION

The button mushroom (*Agaricus bisporus)* has been investigated herein for its potential use in the production of biopharmaceuticals. The key limitation in this effort has been the low protein expression levels achievable thus far. However, results from this work point to several methods to increase expression to economically favorable levels: A novel protein transport system was discovered, whereby protein from the mycelial spawn in the bottom layer of compost was shuttled upwards to the fruiting body during its development. Based on this system, a 'spatial gene stacking' strategy was developed to take advantage of the tissue specificity of various promoters and increase protein accumulation by up to six-fold. Furthermore, experiments are currently underway to utilize developments from plant technology to maximize protein production. Specifically, mushroom expression vectors have been constructed that use viral leader sequences from Cowpea Mosaic Virus, which have shown heightened translation rates in model plant systems. These will soon be evaluated for their potential to increase expression level in *Agaricus bisporus*. Such constructs have never been evaluated in fungi, but even if the button mushroom does not emerge as a platform for large-scale protein production, the work reported in this thesis has revealed an interesting protein transport phenomenon that has never before been known.

CHAPTER V

MATERIALS AND METHODS

This chapter describes the experimental procedures used in the above research. The results from Chapter II have recently been written up for submission to a peer-reviewed journal, and thus the text of some of the methods detailed below may be identical to an eventual publication.

Fungal Cultures

Commercial intermediate white hybrid strains of *A. bisporus* were used throughout this study. Wild-type cultures were maintained on malt extract agar (MEA; 20 g/L malt extract, 2.1 g/L MOPS pH 7.0, 15 g agar) and transgenic cultures on MEA containing 50 µg/mL hygromycin B.

GUS Constructs (Chapter II)

ß-glucuronidase (*GUS*; beta D-glucuronoside glucuronosohydrolase; EC 3.2.1.31) gene expression constructs were assembled in the *A. bisporus* transformation vector pBHg, which contains the hygromycin phosphotransferase (*HPT*; *EC* 2.7.1.119) gene conferring resistance to hygromycin B as a selection marker[1].

The figure below **(Figure 15)** depicts the general structure of the gene constructs and provides specifications on the gene elements.

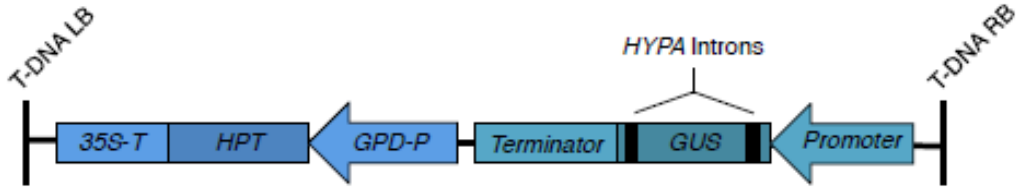

Figure 16 - Organization of the GUS Constructs used in Chapter II

GPD Constructs

The gpd2 gene was PCR-amplified with Phusion (New England Biolabs, Ipswich, MA) by the manufacturer's protocol with High GC Buffer. Genomic *Agaricus* DNA template for the reaction was extracted from wild-type fruiting body tissue as described above. The primer targeting the 5' end of the gene was TGACGACCCGGGATGGTTTGTCTCTCGCTTGCATAC, which contains an XmaI cutsite, and the 3' primer was TTTAAAGAGCTCTAAGCGTAATCAGGAACATCGTAAGGGTAGCCGATG CCAGCCTTGGCGTC, which contains a SacI site and adds the HA tag (GYPYDVPDYA) immediately upstream of the stop codon. Purified reaction product was incubated with Taq Polymerase (New England Biolabs, Ipswich, MA) to add noncoded adenosine residues by the standard protocol, then cloned into a pCR-TOPOII Vector (Invitrogen, Carlsbad, CA) and sequenced. The gene was excised using the aforementioned cut sites and ligated into either pLy or pHy, empty binary vectors with the SacI and XmaI cut sites between the hypA terminator and either the lcc2 or hypA promoter, respectively, to generate pLyGPD-HA and pHyGPD-HA. To generate pGyGPD-HA, the lcc2 promoter was excised from pLyGPD-HA with SmaI and BamHI, and replaced with the

GPD2 promoter. This sequence was amplified from plasmid pAGN30 with the forward primer GGATCCCAGAGGTCCGCAAGTAGATT and reverse primer CCCGGGGGCGATAAGCTTGTTGTGTG and cloned and sequenced in the TOPO vector, before being cut with SmaI and BamHI and ligated into the promoter-deficient vector. All ligation products were verified by colony PCR with primers that spanned the border between the vector and the insert.

CPMV Constructs

pLyEAQHT-GUS and pLyEAQHT-GUS-19 were each generated in two steps: First, the GUS gene was amplified by PCR from the plasmid pAGN237 with the AgeI and XhoI cutsites on the 5' and 3' end, respectively. The forward primer was ACCGGTATGGTCGATCTCCGCGTA, and the reverse primer was CTCGAGTCACACCTGAAGAGAGAA. After sequening in the TOPO vector, this gene was ligated into either pEAQHT or pEAQHT-19 to generate pEAQHT-GUS and pEAQHT-GUS-19. In the second step, the cassette containing the cauliflower mosaic virus (CaMV) promoter, the CPMV RNA-2 leader sequences, the GUS gene, and the nopaline synthase terminator (NosT) was excised from pEAQHT-GUS with PacI and XbaI, blunt-ended with Klenow (New England Biolabs, Ipswich, MA) and ligated into an empty binary vector containing only the hygromycin B resistance gene in the T-DNA region, to make pLyEAQHT-GUS. In the case of pEAQHT-19, the P19 gene was included in the excised cassette, which was cut with PacI and FseI, blunt-ended with Klenow, and ligated into the same empty vector to create pLyEAQHT-GUS-19.

To generate the control construct, pLy401Nos, the Nos terminator was amplified from pEAQHT by PCR with Taq, sequenced in the TOPO vector, then ligated into plasmid pAGN401, which contains GUS under the control of the CaMV promoter. The forward primer was CCTAGGGATCGTTCAAACATTTGGC and the reverse primer was GGATCCGATCTAGTAACATAGATGACACC, which contain the AvrII and BamHI cut sites, respectively. This cassette was then excised with PvuII and BamHI, blunt-ended with klenow, and ligated into the same empty vector as the other constructs. All ligation products were verified by colony PCR with primers that spanned the border between the vector and the insert.

Agaricus Transformation

Agrobacterium-mediated transformation of *A. bisporus* lamallae tissue was carried out using bacterial strain AGL-1 and 30 µg/mL hygromycin B for selection[1,2]

Preparation of Clonal Inoculants

A 250-mL flask containing either 50 mL rye grain, 0.8 g calcium carbonate, 0.8 g calcium sulfate, and 60 mL Milli-Q water (lower layer inoculant) or 50 g of a proprietary matrix (Lambert Spawn Co. Coatesville, PA and Sylvan Inc., Kittanning, PA) and 75 mL Milli-Q water (upper layer inoculant) was autoclaved for 30 min. Each flask of inoculant was seeded with three mycelial agar blocks of

A. bisporus, and kept at room temperature for 2-3 wks with occasional shaking to redistribute the inoculum.

Fruiting Body Production

Fruiting bodies were cultivated as described elsewhere[16] except 15 g of lower layer inoculant and 1.8 kg of compost were mixed and then packed in a 25-cm diameter plastic container, and 25 g of upper layer inoculant and 1.5 L of peat-based substrate were mixed and overlaid on the compost. Fruiting bodies were harvested, rinsed with water, diced, and stored as an aggregate sample by treatment at -20 °C for PCR analysis and GUS assay and -78 °C for RT-PCR analysis.

Histological GUS Assay

Longitudinal slices (2-3 mm thick) of freshly harvested fruiting bodies were incubated for 1.5-4 h in 10 mL X-Gluc substrate (100 mM potassium phosphate pH 7.0, 25 mM ascorbic acid, 0.02% Triton X-100, 0.08% ethanol, 0.5 mg/mL 5-bromo-4-chloro-indoxyl-beta-D glucuronic acid (Gold Biotechnology, St. Loius, MO). To analyze GUS enzyme activity in vegetative mycelium grown in compost, 1.75-cm holes were drilled into the sides of the 12-cm diameter plastic pots containing compost used for fruiting body production, and moistened 2.3-cm diameter Whatman 3MM filter paper discs (Whatman Inc., Clifton, NJ) were affixed with tape to cover the holes. After the 14-day compost *Agaricus* colonization phase, the discs were removed and placed in X-Gluc substrate for 2

hours. To assess GUS activity in vegetative mycelium grown in axenic culture, a mycelial colony growing on MEA contained in a 6-cm diameter Petri plate was flooded with 5 mL X-Gluc substrate and incubated overnight.

Quantitiative GUS Assay

Frozen fruiting body tissue (3 g) was homogenized in 9 mL of extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 10 mM mercaptoethanol, 0.1% Triton X-100, 0.1% sarkosyl) for 1 min with a PT 10-35 GT polytron (Kinematica, Inc., Lucerne, Switzerland). The extract was clarified at 11,000 *g* for 15 min and the protein concentration determined by the Bradford method[17]. GUS activity was quantified by a fluorometric assay with a 4-methylumbeliferyl B-D-glucuronide (MUG; Sigma Chemical Co., St Louis, MO) substrate[18]. The value was reported as the mean of the ratio of the molar rate of formation of 4-methyl-7-hexacoumarin (MU) to the total soluble protein.

HPT Activity Assay of Basidiospores and Fruiting Body Tissue Cultures

To screen basidiospores for the co-transformed *HPT* gene conferring hygromycin B-resistant selection, a fruiting body approaching full maturity was soaked in a 10% commercial bleach solution (final concentration 0.6% NaClO) for 1 min, and then rinsed exhaustively with sterile Milli-Q water. Using a scalpel, the stem and veil tissue were excised to expose the lamellae, and the cap was suspended from a hooked wire over a sterilized 9-cm diameter filter paper disc within a sterilized glass chamber. After an overnight incubation, the discharged basidiospores were

washed from the surface of the paper with sterile Milli-Q water. A 100-µL aliquot of a turbid basidiospore suspension (>100,000 basidiospores/mL as determined by hemocytometry) was spread onto each of a 10-cm diameter Petri plate of MEA and MEA containing 100 µg/mL hygromycin B, and the plates were incubated at room temperature for 3-4 wks. To assay fruiting body tissue for hygromycin B resistance, a 0.5-cm disc of internal cap tissue was transferred aseptically onto each of a 6-cm diameter Petri plate of MEA and MEA containing 50 µg/mL hygromycin B. Plates were incubated at room temperature for 2-3 wks.

Isolation of DNA

DNA was extracted from frozen fruiting body tissue (100 mg) using the LETS procedure (Chen et al., 1999) with a FastPrep FP-120 system (Thermo Fisher Scientific, Waltham, MA). DNA was stored in TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 8.0) at -20 °C.

Isolation of RNA

RNA was extracted from frozen fruiting body tissue (100 mg) using the RNAqueous Kit (Applied Biosystems, Foster City, CA) and FastPrep system. RNA was treated with 2 U DNAse in Tris-HCl pH 7.5, 2.5 mM MgCl2, 0.5 mM CaCl2 at 37 °C for 1 h, followed by standard phenol extraction and ethanol precipitation. RNA was stored in TE buffer at -78 °C.

PCR

Amplification was carried out in a final volume of 25 µL containing 0.75 U *Taq* DNA polymerase with Standard *Taq* Buffer (New England Biolabs, Ipswich, MA), 200 µM dNTPs, 0.2 µM each primer and 10-50 ng DNA template. Primer set: Fwd 5'CGTGACAAGAACCATCCAAGCG3' and Rev 5'GGGTAGCCATCACAAACAGCAC3' were used to amplify a 163-bp sequence in the *GUS* gene. As a DNA template control, a separate reaction was run using primer set: Fwd 5'CGACGGGTGTGAACGCAAAGG3' and Rev 5'CAATCAGTCGATCAACGTTCGC3', which defined a 403-bp sequence in the native polyphenol oxidase 1 (*PPO*) gene[19]. Thermocycling parameters were: 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min.

RT-PCR

RT-PCR was performed (25 µL final volume) with a MasterAmp RT-PCR Kit (Epicentre Biotechnologies, Madison, WI) using 30-100 ng RNA template and the PCR primer sets for the *GUS* and *PPO* genes in separate reactions.

Thermocycling parameters were: 60 °C for 20 min (RT); 94 °C for 2 min; 40 cycles of 94 °C for 1 min and 60 °C for 1 min; 72 °C for 7 min.

Restriction Digests and Purification

Digests were carried out with enzymes purchased from New England Biolabs in 50 uL reactions (1x NEB Buffer [1-4], 1x BSA, 1-5uL template DNA). Enzyme quantity was determined from the reported reaction rate from NEB, and was

adjusted to ensure the reaction would be completed after 1 hour, up to a maximum of 10% v/v to ensure minimal star activity. Reaction products were separated by electrophoresis and purified with a Qiagen Gel Extraction Kit (Qiagen Inc, Valencia, CA)

DNA Ligation

Ligations were performed with NEB T4 DNA Ligase (NEB, Ipswich, MA) with an insert:vector molar ratio of 3:1, as quantified either by Nanodrop or gel electrophoresis with an NEB 1-kb ladder. 20uL reactions were incubated overnight at 16°C before being heat-shocked into chemically competent *E. coli* cells.

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EMPLOYMENT:

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R&D Co-Op in Dow Coating Materials (Summer 2009 - 3rd Rotation) **2007 - 2009**

- Designed and executed experiments to investigate kinetics of a diisocyanate polymerization reaction
- Evaluated film properties of hybrid (polyurethane, polyolefin, acrylic latex) coatings
- Co-authored two internal research publications (in progress)

Manufacturing Co-Op in Environmental Operations (Spring 2008 - 2nd Rotation)

- Organized and led a week-long turnaround of plant for improvement and maintenance
- Successfully employed Six Sigma methodology to mitigate process nuisance alarms
- Lead training sessions for other staff in VBA macro programming and JMP

R&D / TS&D Co-Op in Fabric and Surface Care (Fall 2007 - 1st Rotation)

- Designed, built and evaluated successful new methodologies for scientific evaluation of cleaning agents
- Nominated by manager for "Co-Op of the Year Award"

Sanofi-Aventis Pharmaceuticals ……..Bridgewater, NJ
R&D Internship in Chemical Development **Summer 2007**

- Scaled up synthesis of major process impurities for analysis
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The Pennsylvania State University …….University Park, PA
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- Devised exam, homework and quiz problems, developed and taught lectures, and provided general assistance for *Advanced Organic Mechanisms* (CHEM 535), *Bioprocess Engineering* (CH E 438), and *Introduction to Biomolecular Engineering* (CH E 340)

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- Employed standard molecular biology and biotechnology techniques (ligation, transformation, etc.)

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- Examined technical / economic feasibility of integrated bioprocesses for environmentally useful organisms
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