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Genetic Analysis of the Biosynthesis of Pharmaceutically Important Dihydroergot Alkaloids by Heterologous Expression of *Claviceps gigantea* Genes in the Fungus *Neosartorya fumigata*

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Genetic analysis of the biosynthesis of pharmaceutically important dihydroergot alkaloids by heterologous expression of *Claviceps gigantea* genes in the fungus *Neosartorya fumigata*

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Thesis submitted to the Davis College of Agriculture, Natural Resources and Design at West Virginia University

In partial fulfillment of the requirements for the degree of

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Abstract

Genetic analysis of the biosynthesis of pharmaceutically important dihydroergot alkaloids by heterologous expression of *Claviceps gigantea* genes in the fungus *Neosartorya fumigata*

Ergot alkaloids are a family of secondary metabolites produced by several species of fungi. The structural similarities of ergot alkaloids to neurotransmitters give them the ability to help treat several neurological and cognitive diseases, such as dementia, Alzheimer's and Parkinson's disease. These pharmaceuticals are currently being synthesized from more complex ergot alkaloids, however they would be more efficiently synthesized from dihydroergot alkaloids. Understanding the genes involved in the synthesis of dihydroergot alkaloids, such as dihydrolysergol and dihydrolysergic acid, and elucidating their pathways will be beneficial. The Mexican maize ergot pathogen *Claviceps gigantea* has been reported to produce dihydrolysergol, which is an oxidized product of the precursor ergot alkaloid festuclavine. Due to the discovery of an ergot alkaloid synthesis gene, *cloA*, catalyzing multiple oxidations on a similar substrate, it was hypothesized that expression of *C. gigantea cloA* in a festuclavine-accumulating mutant of the model fungus *Neosartorya fumigata* would yield dihydrolysergol. HPLC and LC-MS analyses of the *cloA* transformed strains, demonstrated that the modified strain produced dihydrolysergic acid, the fully oxidized product of dihydrolysergol. Since dihydrolysergol was expected to be the pathway end product of *C. gigantea*, the genomic sequence data was analyzed and extracts of field-grown *C. gigantea*. The natural alkaloid analysis results supported the finding that dihydrolysergic acid is the end product of the *C. gigantea* pathway, rather than dihydrolysergol. *C. gigantea* is the first organism demonstrated to produce the pharmaceutically important compound dihydrolysergic acid as its pathway end product and heterologous expression of its *cloA* gene may serve as the basis of a more efficient approach to production of DHLA for pharmaceutical purposes. Additional attempts to increase the yield of dihydrolysergic acid in our modified strain, such as removal of introns, expression from an alternative promoter, and coexpression with an additional ergot alkaloid synthesis gene, *easA*, were conducted. The removal of introns and the alternative promoter had no significant effect on the yield of dihydrolysergic acid compared to the original *cloA* transformants in ANOVA ($p = 0.58$ and $p = 0.76$). However, the mutants expressing *cloA-easA* had conversion rates two-fold higher than the mutants expressing *cloA* ($p = 0.01$). The data illustrate that CloA of *C. gigantea* catalyzes a six-electron oxidation of festuclavine to dihydrolysergic acid.

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Literature Review/Introduction

Ergot Alkaloids

Ergot is a fungal infection caused by *Claviceps* species that affect rye, wheat, corn, and several other monocot plants. Ergot alkaloids are agriculturally and pharmaceutically important, though many *Claviceps* species produce ergot alkaloids, which have caused mass poisonings, called ergotism, as a result of accumulations of these alkaloids in grain crops. Ergot alkaloids also have caused reduced weight gain and fitness in grazing animals as a result of their accumulation in related endophytic fungi (*Epichloë* species) that colonize forage grasses (Robinson and Panaccione, 2014). However, in 1918, Sandoz marketed ergot alkaloids as bulk pharmaceutical chemicals, when it was discovered they had clinical uses (Schiff, 2006). Many fungi in the genus *Claviceps* (in the family Clavicipitaceae) produce a variety of ergot alkaloids, including clavines and lysergic acid derivatives. A different set of ergot alkaloids are produced by the distantly related fungus *Neosartorya fumigata*, from the family Trichocomaceae. Over 80 distinct types of ergot alkaloids have been isolated, mainly from the genus *Claviceps* (Schiff, 2006).

Ergot alkaloids are a family of secondary metabolites and represent the largest group of nitrogenous fungal metabolites in nature (Schiff, 2006). Ergot alkaloids are limited to a narrow set of species, such as those found in the families Clavicipitaceae and Trichocomaceae, so they have a limited distribution in nature. Ergot alkaloids play a role in plant defense, because they have harmful effects on vertebrate and invertebrate herbivores (Panaccione et al., 2006; Potter et al., 2008).

Ergot alkaloids are small molecules that are heavily derivatized from the amino acid tryptophan. Tryptophan is an indole derivative and is a precursor to the neurotransmitter serotonin. Ergot alkaloids also have structural similarities to the neurotransmitters dopamine, adrenaline, and

noradrenaline, making them useful pharmaceuticals. Ergot alkaloids' pharmaceutical effects are the result of their agonism or antagonism at serotonergic, dopaminergic, and adrenergic receptors in the central nervous system, as well as at adrenergic receptors in blood vessels (Schiff, 2006; Gerhards et al., 2014). This allows ergot alkaloids to be used clinically to treat several neurological and cognitive diseases, such as migraines, dementia, Alzheimer's, and Hyperprolactinemia (Morren and Galvez-Jimenez, 2010; Baskys and Hou, 2007; Winblad et al., 2007; Kerr et al., 2010; Table 1). Several important pharmaceuticals are semisynthetic derivatives of dihydrolysergic acid or its intermediate dihydroelymoclavine (i.e. dihydrolysergol), which are ergot alkaloids rarely encountered in nature; therefore elucidating their biosynthetic route would be beneficial (Bilovol and Panaccione, 2016; Fig. 1; Table 1).

Figure 1: Structures of the dihydroergot alkaloids dihydrolysergol and dihydrolysergic acid compared to their potential pharmaceutical products. Green arrows indicate the pharmaceuticals dihydrolysergol is

capable of producing and blue arrows indicate the pharmaceuticals dihydrolysergic acid is capable of producing.

Table 1: Ergot Alkaloids and their Pharmaceutical Uses

Ergot Alkaloid	Brand Name	Clinical use(s)	Current semisynthetic derivation	Efficient semisynthetic derivation
Nicergoline	Sermion	Senile dementia, Alzheimer's, cerebral thrombosis	From LA via other ergot alkaloids	From DHlysergol, lysergol, or DHLA
Cabergoline	Caberlin, Dostinex	Hyperprolactinemia, pituitary prolactinomas	From LA via other ergot alkaloids	From DHLA
Pergolide	Permax	Parkinson's (elsewhere, withdrawn in USA, 2007)	From LA via other ergot alkaloids	From DHlysergol
Ergoloid mesylates	Hydergine	Senile dementia	From ergopeptines or LA via other ergot alkaloids	From DHergopeptines or DHLA
DHergotamine	DHE 45, Migranal	Migraines	From ergotamine or LA via other ergot alkaloids	From DHergotamine or DHLA

Abbreviation: LA- lysergic acid, DH – dihydro, DHLA – dihydrolysergic acid

Ergot Alkaloid Pathway

Individual species in two major fungal families, Clavicipitaceae and Trichocomaceae, are known to produce ergot alkaloids. All fungi that produce ergot alkaloids share the same early biosynthetic steps that lead to the formation of the ergoline ring structure, before diverging into specific ergot alkaloid lineages (Coyle et al., 2014). For the production of all ergot alkaloids, the ergot alkaloid synthesis (*eas*) genes that are involved are all found in clusters in each genome of the producing organism. No gene that is critical to the production of ergot alkaloids is located outside of an *eas* gene cluster (Bilovol and Panaccione, 2016). Modifications to the ergoline rings by enzymes in the *eas* gene cluster results in chemical products that are used as the basis for pharmaceutical drugs. Gene clusters from several fungi have been elucidated through genome mining, knock-out experiments, and overexpression experiments (Gerhards et al., 2014).

The ergot alkaloid pathway in both *Claviceps* species and *N. fumigata* is initiated by the prenylation of tryptophan, which is catalyzed by dimethylallyltryptophan synthase encoded by the gene *dmaW* (Fig. 2). Coyle et al. (2010) demonstrated that chanoclavine-I-aldehyde is the last shared intermediate in the ergot alkaloid pathway for *Claviceps* and *N. fumigata* (Fig. 2). The branch point between these pathways occurs during the D ring closure, as a result of the products of different alleles of *easA* have functional differences. EasA is an oxidoreductase of the ‘old yellow enzyme’ class, which reduces double bonds that are conjugated to either aldehydes or ketones. In the ergot alkaloid pathway, EasA reduces the double bond conjugated to the aldehyde group of chanoclavine-I-aldehyde, which allows for ring closure by Schiff base formation (Coyle et al., 2010). In *N. fumigata*, the 8,9 double bond in chanoclavine-I-aldehyde is reduced by the reductase form of EasA (Fig. 2). The derived iminium ion is further reduced by EasG to form festuclavine, which can be acetylated at carbon 2 and/or prenylated at carbon 9 to produce fumigaclavine derivatives (Fig. 2) (Wallwey et al., 2010; Cheng et al., 2010). In most *Claviceps* species, an isomerase form of EasA transiently reduces chanoclavine-I-aldehyde before re-oxidizing it to produce the 8,9 unsaturated agroclavine (Fig. 2) (Coyle et al., 2010). In *C. purpurea* and *Epichloë* species agroclavine is oxidized to produce elymoclavine, elymoclavine is further oxidized and isomerized to produce lysergic acid, and lysergic acid is assimilated into ergopeptines and/or lysergic acid amides (Fig. 2) (Robinson and Panaccione, 2014). As a result of this major difference, fungi in the family Clavicipitaceae synthesize lysergic acid derivatives, in which carbons 9 and 10 in the D ring of the ergoline ring structure are unsaturated and carbon 17 is oxidized (Fig. 2) (Panaccione et al., 2014; Schardl et al., 2006; Lorenz et al., 2009). Fungi in the family Trichocomaceae synthesize clavine derivatives, in which carbons 9 and 10 in the D ring are fully saturated and carbon 17 remains as a methyl group (Fig. 2) (Panaccione et al., 2012; Wallwey and Li, 2011). Lysergic acid derivatives are produced exclusively by the family Clavicipitaceae

and not by model organisms, like *N. fumigata*, in which their modification might be facilitated (Robinson and Panaccione 2014).

Coyle et al. (2010) also created two mutant strains of *N. fumigata*. The first was an *easA* knockout strain, which was created by integrating pAFOYE1, along with the hygromycin resistance plasmid pMOcosX, into *easA* (reductase) in order to disrupt its function. The disruption construct, pAFOYE1, contained a 595 bp fragment from the 1,131 bp coding sequence of *easA* from *N. fumigata* ligated into the T/A overlap vector pCR2.1. This mutant strains pathway ended at chanoclavine-I-aldehyde, which is the last common intermediate between the fumigaclavine C and lysergic acid pathways. This strain was also beneficial, because it allowed Coyle et al. (2010) to investigate the functions of *easA* isomerase compared to *easA* reductase. By cotransforming *easA* isomerase from *C. purpurea* along with pBC-phleo, a phleomycin resistance plasmid, into the *easA* KO strain, they were able to modify the ergot alkaloid profile. Instead of detecting the usual alkaloids such as festuclavine and fumigaclavines A,B, and C, they detected setoclavine, isosetoclavine, and large amounts of agroclavine (Coyle et al. 2010).

Robinson and Panaccione (2014) engineered a strain of *N. fumigata* to transform agroclavine into lysergic acid (Fig. 2). This was done by using an *N. fumigata easA* knockout strain as host fungus, so that the pathway stopped at chanoclavine-I-aldehyde. A construct containing *easA* (isomerase) and a second gene, *cloA*, both from an *Epichloë* species, was transformed into the *N. fumigata easA* knockout strain, so that the EasA isomerase converted chanoclavine-I-aldehyde into agroclavine and CloA converted agroclavine into lysergic acid. The gene *cloA*, which stands for clavine oxidase, was required for complete oxidation of carbon 17 of both agroclavine and elymoclavine during the synthesis of lysergic acid (Fig. 2). The gene encodes a P450 monooxygenase and is only found in fungi that produce lysergic-acid derivatives (Robinson

and Panaccione, 2014). It was concluded that CloA catalyzes a six-electron oxidation of agroclavine to lysergic acid and that either CloA or EasA isomerizes the 8,9 double bond in the D ring to the 9,10 position. CloA had multiple catalytic activities, including a two-electron oxidation of agroclavine to elymoclavine and an additional two sets of two-electron oxidations to convert elymoclavine to lysergic acid. The lack of detectable elymoclavine in the extracts of the developed strain indicated that CloA could execute successive oxidations and an isomerization without releasing the substrate (Robinson and Panaccione, 2014).

Two species from Clavicipitaceae, *Claviceps gigantea* and *Claviceps africana*, have been reported to produce dihydroergot alkaloids, however the genes involved in their biosynthetic pathways have not been investigated. Dihydroergot alkaloids differ from lysergic acid-derived alkaloids due to carbons 9 and 10 being saturated and carbon 17 being oxidized (Bilovol and Panaccione, 2016), giving them different, unique pharmaceutical properties. These species are similar to *N. fumigata*, due to chanoclavine-I-aldehyde being reduced by EasA reductase to produce festuclavine. Barrow et al. (1974) investigated the dihydroergot alkaloid pathway through an approach based on feeding labeled precursors to the fungus *Claviceps africana*. They showed that the dihydroergot alkaloid pathway in *C. africana* and by extension *C. gigantea* proceeded from festuclavine to dihydroelymoclavine to dihydrolysergic acid to dihydroergosine (Fig. 2). It is predicted that festuclavine could be transformed into dihydroelymoclavine then dihydrolysergic acid by oxidizing carbon 17, analogous to the agroclavine to lysergic acid pathway. Dihydrolysergic acid derivatives are also solely produced by the family Clavicipitaceae and not by model organisms, such as *N. fumigata*, in which their modification may be facilitated (Robinson and Panaccione, 2014). Many of the genes that are necessary in the biosynthesis of the lysergic acid-derived ergot alkaloids have been characterized, but the genes that are necessary in the

synthesis of dihydroergot alkaloids have yet to be studied (Bilovol and Panaccione, 2016). Among the genes in the *eas* cluster, *cloA* is the reasonable candidate to conduct the first oxidation of carbon 17 of festuclavine to produce dihydroelymoclavine, due to the discovery of its catalytic functions on a similar substrate, agroclavine (Robinson and Panaccione 2014).

Figure 2: Lysergic acid pathway (left; Robinson and Panaccione, 2014), predicted dihydroergot alkaloid pathway (middle; Barrow et al., 1974), and fumigaclavine pathway (right; Bilovol and Panaccione, 2016).

Both intermediates and products are shown along with the enzymes responsible (indicated next to arrows). Double arrows express the omission of one or more intermediates. The ergoline ring structure is shown in the top right for reference to descriptions in text.

In *N. fumigata*, festuclavine is transformed into fumigaclavines through the hydroxylation and acetylation of carbon 2 and/or the prenylation of carbon 9 (Fig. 2). The gene *easM* encodes for the festuclavine-hydroxylating enzyme, which hydroxylates festuclavine into fumigaclavine B (Fig. 2). Bilovol and Panaccione (2016) prepared an *easM* knockout strain of *N. fumigata*, so the ergot alkaloid pathway stops at and accumulates large quantities of festuclavine (30.9 amol/conidium). The strain was derived by replacing part of the *easM* coding sequence with a hygromycin resistance gene, in order to disrupt the gene's function. This development is beneficial, because festuclavine is the last common intermediate in both the fumigaclavine branch in *N. fumigata* and in the dihydroergot alkaloid pathway in certain *Claviceps* species. This mutant strain will be useful for studying genes important in the synthesis of dihydroergot alkaloids, since festuclavine is the precursor to dihydroergot alkaloids (Bilovol and Panaccione 2016). The candidate gene, *cloA*, was tested through expression in an *N. fumigata easM* knockout (*easM* KO) strain and an *N. fumigata easA* knockout (*easA* KO) strain.

Claviceps gigantea

A new species of *Claviceps* was described in the 1960s (Fuentes et al. 1964). The fungus grew on maize and was reported due to the toxic effects it caused when ingested. The fungus is recognized by the large, soft sclerotia that replace the kernels on maize. Large numbers of microconidia occur on the sclerotia, and they were isolated to prove they were the etiological agent (Ullstrup, 1973). The sclerotia produced the typical stipe and stroma of other *Claviceps* species, however, they were much larger than any other described species, so the new species *Claviceps*

gigantea was described. *C. gigantea* has a very limited geographical distribution and is only found at high altitudes in Mexico, where the climate is cool and humid (Ullstrup, 1973).

C. gigantea was studied for ergot alkaloids in Agurell and Ramstad's work in 1965. Festuclavine and dihydroelymoclavine (synonym dihydrolysergol) were two alkaloids isolated from the sclerotia of the fungus. This was the first time dihydrolysergol was isolated from a natural source, and it is relevant because dihydrolysergol is closely related to dihydrolysergic acid. For example, it had previously been chemically cleaved from dihydrolysergic acid methyl ester (Agurell and Ramstad, 1965). Overall, most species of *Claviceps* produce derivatives of lysergic acid. However, it is believed that *C. gigantea* is capable of producing other dihydroergot alkaloids, based on the isolation of dihydrolysergol from the fungus. This fungus may be an appropriate genetic resource for studies on dihydroergot alkaloid biosynthesis.

Rationale and Hypothesis

Though many steps in the ergot alkaloid pathways have been clarified, the dihydroergot alkaloid pathway remains to be elucidated. As a result, dihydroelymoclavine and dihydrolysergic acid used for pharmaceutical purposes must be chemically modified from more complex, naturally occurring ergot alkaloids. The *easM* knockout strain of *N. fumigata* created by Bilovol and Panaccione (2016) is the ideal host for my planned experiment, since it accumulates high amounts of festuclavine, which is the precursor to dihydroergot alkaloids (Barrow et al., 1974). Additionally, the discovery of the multiple catalytic functions of the enzyme CloA make it a reasonable candidate to oxidize carbon 17 of festuclavine to transform it into dihydroelymoclavine or dihydrolysergic acid. I intend to test the gene *cloA* from the fungus *C. gigantea*, due to this fungus being reported to produce dihydroelymoclavine. Therefore, it is hypothesized that *cloA* from *C. gigantea* introduced into the *easM* knockout strain of *N. fumigata* will result in the

oxidation of festuclavine into dihydroelymoclavine or perhaps dihydrolysergic acid (Fig. 2). The objective of this project is to modify a strain of *N. fumigata* to produce dihydroelymoclavine or dihydrolysergic acid as its final product. This was done by transforming the gene *cloA* from *C. gigantea* into the festuclavine-accumulating *easM* knockout strain of *N. fumigata*.

Materials and Methods

***C. gigantea* gene cluster**

The *C. gigantea eas* gene cluster (Figure 9B) was identified by querying the genome's sequence with individual *eas* genes from other *Claviceps* species. Additional *eas* genes were identified by blastx comparisons to the nonredundant nucleotide database at NCBI.

***C. gigantea* natural alkaloid analysis**

C. gigantea sclerotia samples were obtained from Montecillo, Mexico (Fig. 3). The head of the sclerotia was pulverized with 10 glass beads (3-mm diameter) in 300 μ L of HPLC-grade methanol by a Fastprep 120 (Bio101, Carlsbad, CA) for 30s at 6 m/s. Alkaloids were extracted for 1 hour, then the sample was pelleted by centrifugation and 20 μ L of supernatant was analyzed by HPLC with fluorescence detection.

Figure 3: Image of three *C. gigantea* sclerotia samples analyzed for natural alkaloid production. The top row are the sclerotia heads, which were analyzed for alkaloid production, and the bottom row are the corresponding sclerotia tails.

Resequencing of pseudogene *lpsA* from *C. gigantea*

Part of the pseudogene *lpsA* was amplified from *C. gigantea* genomic DNA using primer combination 14 (Table 2) using Phusion high-fidelity polymerase (Thermo Fisher, Waltham, MA). The fragment contained 743 bp corresponding to nucleotides 3997 to 4739 in the *lpsA* coding sequence and contained the suspected frameshift mutation (GenBank: KY906251). Phusion polymerase creates products with blunt ends, so the amplified product was purified (Qiagen) and ligated into *Sma*I-digested pUC18. This construct was then transformed into *Escherichia coli*, plasmid prepped (Qiagen), and sequenced to confirm frameshift mutation found during the characterization of *C. gigantea*'s *eas* gene cluster (Eurofins, Louisville, KY).

Preparation of transformation constructs

Four different constructs were prepared in attempts to accumulate and increase the yield of dihydrolysergic acid (Fig. 5). The first construct contained *cloA* from *C. gigantea* and a bidirectional *easA-easG* promoter from *N. fumigata* (Fig. 4, Fig. 5A). The second construct

contained an intron free version of *cloA* from *C. gigantea* and the bidirectional *easA-easG* promoter from *N. fumigata* (Fig. 4, Fig. 5B). The third construct contained an intron free version of *cloA* from *C. gigantea* and a strong *gpdA* promoter from *N. fumigata* (Fig. 4, Fig. 5C). The fourth construct contained an intron free version of *cloA* from *C. gigantea*, the bidirectional *easA-easG* promoter from *N. fumigata*, and *easA* from *C. gigantea* (Fig. 4, Fig. 5D). All of the PCR amplifications used Phusion high-fidelity polymerase (Thermo Fisher, Waltham, MA) and followed the same protocol, with an initial denaturation at 98°C for 30s, followed by 35 cycles of 98°C (15 s), AT°C (15 s), and 72°C (ET s), and a final extension at 72°C for 60s (AT=annealing temperature, ET=extension time, Table 2). The 25 µL PCR mixture contained 11 µL distilled/deionized water, 5 µL 5x buffer (100 mM KCl, 20 mM Tris-HCl pH 7.4, 1.5 mM MgCl₂), 4 µL dNTPs (final concentration 200 µM each), 1.25 µL forward primer (final concentration 0.5 µM), 1.25 µL reverse primer (final concentration 0.5 µM), 2 µL DNA template, and 0.5 units of Phusion high-fidelity polymerase. All PCR products were run on agarose gels (0.8% wt/vol) in 0.5 x TBE (45 mM Tris-Borate, 1 mM EDTA) with ethidium bromide (500 ng/mL).

***C. gigantea cloA* construct**

To test whether CloA from *C. gigantea* would catalyze oxidation of festuclavine, an *easA/easG* promoter-*cloA* construct was prepared (Fig. 5A), for expression of *cloA*, in a festuclavine-accumulating *easM* knockout strain of *N. fumigata*. Primer combination 1 was used for amplification of *cloA* from *C. gigantea* genomic DNA (Tooley et al., 2006) by PCR (Fig. 4, Table 2). The reverse primer in the combination was assembled with an oligonucleotide that contained a *HindIII* restriction site (AAGCTT) near its 5' end to facilitate cloning of the product (Fig. 5A). Primer combination 2 was used for PCR amplification of the *easA-easG* promoter from *N. fumigata* wild type strain Af293 (Fig. 4, Table 2). The forward primer in the combination was assembled with an oligonucleotide that contained an *XbaI* restriction site (TCTAGA) near its 5'

end (Fig. 5A). The two fragments were joined by adding equimolar amounts of the two initial PCR products into a fusion PCR, using primer combination 3 (Fig. 4, Table 2) to form a construct for expressing *C. gigantea cloA* in *N. fumigata* (Fig. 5A). The *easA/easG* promoter-*cloA* construct was double-digested with *Hind*III and *Xba*I, then ligated into *Hind*III/*Xba*I-digested pBC-phleo (Fungal Genetics Stock Center, Manhattan, KS) to allow for selection for phleomycin resistance after fungal transformation (Fig. 5A). The plasmid was transformed into *E. coli*, plasmid prepped (Qiagen, Gaithersburg, MD), and the construct was confirmed by enzymatic digests and DNA sequencing (Eurofins, Louisville, KY). The confirmed plasmid was then transformed by established methods (Robinson and Panaccione, 2014) into the festuclavine-accumulating *easM* knockout strain of *N. fumigata*.

***C. gigantea* intron free *cloA* construct**

To test whether an intron free version CloA from *C. gigantea* would better convert festuclavine to dihydrolysergic acid, an *easA/easG* promoter- intron free *cloA* construct was prepared (Fig. 5B) for expression in the same *N. fumigata easM* knockout strain. Primer combination 4 was used to amplify an intron version of *cloA* with *easA-easG* promoter and 3'UTR extensions from the 1,524 bp cDNA (see mRNA analysis) (Fig. 4, Table 2). Due to the cDNA not having the *cloA* 3' UTR, a *cloA* 3'UTR segment with *cloA* extension was also individually amplified from *C. gigantea* DNA using primer combination 5 (Fig. 4, Table 2). The reverse primer in the combination was assembled with an oligonucleotide that contained an *Eco*RI restriction site (GAATTC) near its 5' end to facilitate cloning of the product (Fig. 5B). The 5' UTR was included in the bidirectional *easA/easG* promoter, so it did not need individually amplified. Primer combination 2 was used for PCR amplification of the *easA-easG* promoter with *cloA* extension from *N. fumigata* wild type strain Af293 (Fig. 4, Table 2). The forward primer in the combination was assembled with an oligonucleotide that contained an *Xba*I restriction site (TCTAGA) near its

5' end (Fig. 5B). The three fragments were joined by adding equimolar amounts into a fusion PCR, using primer combination 6 (Fig. 4, Table 2) to form a construct for expressing a fully processed version of *cloA* from *C. gigantea* in *N. fumigata* (Fig. 5B). The *easA/easG* promoter-intron free *cloA* construct was double-digested with *EcoRI* and *XbaI*, then ligated into *EcoRI/XbaI*-digested pBC-phleo (Fungal Genetics Stock Center, Manhattan, KS) to allow for selection for phleomycin resistance after fungal transformation (Fig. 5B). The plasmid was transformed into *E. coli*, plasmid prepped (Qiagen, Gaithersburg, MD), and the construct was confirmed by enzymatic digests and DNA sequencing (Eurofins, Louisville, KY). The confirmed plasmid was then transformed with the same methods, into the festuclavine-accumulating *easM* KO.

***C. gigantea* *gpdA* promoter construct**

To test whether a stronger promoter from *N. fumigata* would increase expression of *cloA* in attempts to increase conversion of festuclavine to dihydrolysergic acid, a *gpdA* promoter- intron free *cloA* construct was prepared (Fig. 5C) for expression in the *N. fumigata* *easM* knockout strain. Primer combination 7 was used to amplify an intron-free version of *cloA* with *gpdA* promoter and 3'UTR extensions from the 1,524 bp cDNA (see mRNA analysis) (Fig. 4, Table 2). Primer combination 8 was used for PCR amplification of the *gpdA* promoter with *cloA* extension from *N. fumigata* wild type strain Af293 (Fig. 4, Table 2). No restriction site needed to be assembled to the primer, because the sequence already contained an *XbaI* restriction site (TCTAGA) near its 5' end (Fig. 5C). Equimolar amounts intron-free *cloA*, *gpdA* promoter, and 3'UTR (from the previous section) were combined in a fusion PCR, using primer combination 9 (Fig. 4, Table 2) to form a construct for analyzing the effect of a new, stronger promoter (Fig. 5C). The *gpdA* promoter-intron free *cloA* construct was double-digested with *EcoRI* and *XbaI*, then ligated into *EcoRI/XbaI*-digested pBC-phleo (Fungal Genetics Stock Center, Manhattan, KS) to allow for selection for phleomycin resistance after fungal transformation (Fig. 5C). The plasmid was transformed into *E.*

coli, plasmid prepped (Qiagen, Gaithersburg, MD), and the construct was confirmed by enzymatic digests and DNA sequencing (Eurofins, Louisville, KY). The confirmed plasmid was then transformed with the same methods, into the festuclavine-accumulating *easM* KO strain.

***C. gigantea easA-cloA* construct**

To test whether CloA required a species specific EasA to function properly, an *easA-easA/easG* promoter- intron free *cloA* construct was prepared (Fig. 5D) for expression in an *N. fumigata easA* knockout strain. The bidirectional promoter had to be reamplified using primer combination 10, in order to add on *easA* and *cloA* extensions (Fig. 4, Table 2). The *easA* segment with bidirectional promoter extension was amplified from *C. gigantea* DNA using primer combination 11 (Fig. 4, Table 2). The forward primer in the combination was assembled with an oligonucleotide that contained an *Xba*I restriction site (TCTAGA) near its 5' end to facilitate cloning of the product (Fig. 5D). The construct was assembled by adding equimolar amounts *easA*, bidirectional promoter, *cloA*, and 3'UTR into a fusion PCR, using primer combination 12 (Fig. 4, Table 2). The reverse primer for the fusion product was assembled with an oligonucleotide that contained a *Hind*III restriction site (AAGCTT), due to *easA* from *C. gigantea* already having an *Eco*RI site in its sequence (Fig. 5D). The *easA-cloA* construct was double-digested with *Hind*III and *Xba*I, then ligated into *Hind*III/*Xba*I-digested pBC-phleo (Fungal Genetics Stock Center, Manhattan, KS) to allow for selection for phleomycin resistance after fungal transformation (Fig. 5D). The plasmid was transformed into *E. coli*, plasmid prepped (Qiagen, Gaithersburg, MD), and the construct was confirmed by enzymatic digests and DNA sequencing (Eurofins, Louisville, KY). The confirmed plasmid was then transformed with the same methods, into an *easA* KO *N. fumigata* strain.

Figure 4: PCR images resulting from the primer combinations in Table 1. Arrows indicate fragments of different lengths. Sizes of relevant fragments of BstEII-digested bacteriophage λ DNA is indicated to the left of the image. If = intron free.

Figure 5: A) pBC-phleo vector containing the bidirectional *easA/easG* promoter-*cloA* construct (Construct 1). B) pBC-phleo vector containing the bidirectional *easA/easG* promoter-intron free *cloA*-3'UTR construct (Construct 2). C) pBC-phleo vector containing the *gpd* promoter-intron free *cloA*-3'UTR construct. D) pBC-phleo vector containing the *easA*-bidirectional *easA/easG* promoter-intron free *cloA*-3'UTR construct.

Table 2: Primers and PCR Protocol Information

Primer Combination	Primer Sequences	Product	Length (bp)	Annealing	
				Temperature (°C)	Extension Time (s)
1	GAGTAGGCACTCCGACCATGTCACTAACATCGTTTTACGCC + CTACAAGCTTCGACTAGGCCACCCACACC	<i>cloA</i> with bidirectional promoter extension	2491	63	75
2	GACCTCTAGACATTGCTTCTAATCCACCAAGTACTTG + GGCGTAAACGATGTTAGTGACATGGTGCGGAGTGCCTACTC	Bidirectional promoter with <i>cloA</i> extension	814	64	30
3	GACCTCTAGACATTGCTTCTAATCCACCAAGTACTTG + CTACAAGCTTCGACTAGGCCACCCACACC	Bidirectional promoter- <i>cloA</i> fusion construct	3253	63	100
4	GAGTAGGCACTCCGACCATGTCACTAACATCGTTTTACGCC + CCGCCGATGTCATACTCAACTAGTATGTGACTTCAGTCCATC	IF <i>cloA</i> with bidirectional promoter and 3' UTR extensions	1562	63	40
5	GACTGAAGTCACATACTAGTTGAAGTATGACATCGGCG + CACAGAATTCGAACCTCTGTGACGTGCGCAGATTG	3' UTR + <i>cloA</i> extension	422	63	20
6	GACCTCTAGACATTGCTTCTAATCCACCAAGTACTTG + CACAGAATTCGAACCTCTGTGACGTGCGCAGATTG	Bidirectional promoter- <i>cloA</i> -3' UTR fusion construct	2713	64	90
7	GTACCAGACGAATCTACACAATGTCACTAACATCGTTTTACGCC + CCGCCGATGTCATACTCAACTAGTATGTGACTTCAGTCCATC	IF <i>cloA</i> with <i>gpd</i> promoter and 3' UTR extensions	1564	61	40
8	GATTCTAGAAGTCCTGAATAGTAG + GGCGTAAACGATGTTAGTGACATTGTGTAGATTCGTCTGGTAC	<i>gpd</i> promoter with <i>cloA</i> extension	990	64	40
9	GATTCTAGAAGTCCTGAATAGTAG + CACAGAATTCGAACCTCTGTGACGTGCGCAGATTG	<i>gpd</i> promoter- <i>cloA</i> -3'UTR fusion construct	2889	60	90
10	GAGTTTGGACGTGGACATTGCTTCTAATCCACCAAGTAC + GGCGTAAACGATGTTAGTGACATGGTGCGGAGTGCCTACTC	Bidirectional promoter with <i>cloA</i> and <i>easA</i> extensions	835	64	30
11	GACATCTAGACTGGCGACGTCACAGAAGTTC + GGTGATTAGAAGCAATGTCCACGTCCAACTCTTCAAC	<i>easA</i> with bidirectional promoter extension	1322	63	60
12	GACATCTAGACTGGCGACGTCACAGAAGTTC + CACAAAGCTTGAACCTCTGTGACGTGCGCAGATTG	<i>easA</i> -bidirectional promoter- <i>cloA</i> -3'UTR fusion construct	4054	63	180
13	CTATAGAGTAGGCACTCCGCAC + CTACGCCGACTACTGTTGTCC	<i>cloA</i> internal sequences (cDNA)	1524 -1621	58	45
14	CAGTTCGAGCACGTCTTCTCG + GTGCTCGACAATCATGAGGTC	<i>lpsA</i> internal sequences	743	60	30

Fungal Transformation

The *easA/easG* promoter-*cloA* construct, the *easA/easG* promoter- intron free *cloA* construct, and the *gpdA* promoter- intron free *cloA* construct were transformed into an *N. fumigata easM* KO strain, and the *easA*- *easA/easG* promoter- intron free *cloA* construct was transformed into an *N. fumigata easA* KO strain. All constructs were transformed with a phleomycin resistance vector containing the gene, *ble*, from *Aspergillus niger*.

Protoplasts were prepared by growing fungal cultures in 50 mL of malt broth at 37 °C with shaking at 80 rpm overnight. The broth was discarded and replaced with 15 mL 0.7 M NaCl containing 40 mg lysing enzyme (Sigma-Aldrich, St. Louis, MO) and 1 g VinoTaste Pro (Gusmer Enterprises Inc., Mountainside, NJ), then shaken for an additional 2 hours at 37 °C and 80 rpm. Protoplasts were separated from hyphae by filtering through miracloth and then were pelleted by

centrifugation (3 min at $3,000 \times \text{rpm}$). The protoplasts were washed with 10 mL 0.7 M NaCl and 5 mL STC (1 M Sorbitol, 1 M Tris (pH = 7.4), 1 M CaCl_2), and then resuspended in a final volume of STC, so that a 100 μL would have 5×10^6 protoplasts. A polyethylene glycol (PEG) solution was prepared by adding 2 volumes of 37.5% PEG 8000 to 1 volume of PEG amendments (1.8 M KCl, 150 mM CaCl_2 , 150 mM Tris (pH = 7.47)) and was added to the protoplast solution in the amount of $\frac{1}{4}$ the final volume that the protoplasts are in. Once the final protoplast solution was prepared, 125 μL was added to 10 μL of the desired plasmid and incubated on ice for 30 minutes. An additional 1 mL of PEG solution was added to the solution and incubated at room temperature for 20 minutes. The solution was then split across 3 petri dishes (about 365 μL each) and mixed with 15 mL molten TM102 media (Rykard et al., 1982). Once solidified, an additional layer of molten TM102 mixed with 800 $\mu\text{g/mL}$ phleomycin (InvivoGen, San Diego, CA) was added. The plates were incubated for 3 days at 37 °C and the blue fungal colonies were replated on malt extract agar plates and analyzed by HPLC with fluorescent detection.

mRNA analysis

Transformed fungal cultures were grown in 50 mL of malt extract broth in a 250-mL Erlenmeyer flask for 1 day at 37°C with shaking at 80 rpm. The produced fungal mat was placed in a petri dish and incubated at 37°C for an additional day. The RNeasy Plant Mini kit (Qiagen, Gaithersburg, MD) was used to extract RNA from approximately 80 mg of fungal mat. RNA was treated with DNaseI (RNeasy Mini kit) in place of step 6 of the protocol and was reverse transcribed with Superscript IV (bypassing the optional RNase H step). Resulting cDNA was diluted 1:10 and amplified by PCR using Phusion high-fidelity DNA polymerase and primer combination 13 (Table 2) to test for the presence of *cloA*. The resulting gel showed two bands, so the PCR product was reloaded onto a 0.8% agarose gel and run at 25 V for 4 hours to effectively separate these bands, for easier individual purification with Qiaquick columns. Both purified

versions of cDNA were ligated into *Sma*I-digested pUC18 and transformed into *E. coli* (Fig. 10B), in order to have higher concentrations for sequencing, and the resulting plasmids were sent for sequencing (Eurofins, Louisville KY).

Agroclavine feeding study

The ability of *cloA* from *C. gigantea* to utilize both festuclavine and agroclavine as precursors was tested by feeding agroclavine to a *C. gigantea cloA*-bidirectional *easA/easG* promoter transformed *N. fumigata* strain. Agroclavine is oxidized to lysergic acid by *cloA* from *Epichloë* sp. Lp1. Twelve cultures of the transformed strain and *N. fumigata easM* KO strain were grown from 60,000 conidia in 200 μ L of malt broth. Six cultures of each were supplemented with 2 μ L of agroclavine (10 nmol/ μ L in 2 μ L methanol), whereas the other six cultures were supplemented with 2 μ L of methanol as a control. Twelve malt extract broth controls were also treated with the same supplements. The cultures grew for 1 week in a 37°C incubator. After incubation, the cultures were extracted by pulverization with 300 μ L HPLC-grade methanol and 10 glass beads (3-mm-diameter) in a Fastprep 120 (Bio101, Carlsbad, CA) for 30s at 6 m/s. The extracted alkaloids were analyzed by HPLC with fluorescence detection for the presence of lysergic acid.

Compartmentalization study

In order to test whether the substrate and enzymes were being localized in different compartments, a compartmentalization study was conducted. Transformed *N. fumigata* cultures were grown in 50 mL of malt extract broth in a 250-mL Erlenmeyer flask for 2 days at 37 °C with shaking at 80 rpm for sufficient sporulation. The resulting fungal mat was divided into equal thirds. Two of the thirds were homogenized with 200 μ L 0.1 M phosphate buffer (pH = 7.6; Kim et al., 1981), and 0.1 M phosphate buffer supplemented with NADPH (7.5 mg/mL), respectively. The

homogenized solutions were transferred to 2-mL microcentrifuge tubes, supplemented with 150 μ L 0.06% Tween 20, and incubated for 2 hours at 23°C. After incubation, equal parts of HPLC-grade methanol were added to each solution, pelleted by centrifugation, and 90 μ L were analyzed by HPLC with fluorescence detection. The remaining third was analyzed for ergot alkaloids as the control.

HPLC and LC-MS analyses of the transformants

Colonies from each transformant were grown on malt agar for 3-5 days. Samples were taken with the wide end of a 1,000 μ L pipette tip and alkaloids were extracted with 300 μ L HPLC-grade methanol at 23°C for 1 hour. The extracts were pelleted by centrifugation and 20 μ L were analyzed by reverse-phase high-performance liquid chromatography (HPLC) with fluorescent detection. All samples were separated with a multilinear binary gradient of 5% acetonitrile in 50mM ammonium acetate to 75% acetonitrile in 50 mM ammonium acetate on a 150-mm by 4.6-mm C18 column (Phenomenex Prodigy ODS3, 5 μ m particle size; Torrance, CA; Panaccione et al., 2012). A dihydrolysergic acid standard was prepared by dissolving 1 mg ergoloid mesylate (Sigma) in 100 μ L of 1.2 M NaOH at 75°C for 6 hours, succeeded by the addition of 1.2 M HCl to neutralize the standard.

Festoclavine and dihydrolysergic acid were quantified from all three transformed strains derived from *easM* KO. Festoclavine, dihydrolysergic acid, and fumigaclavine C were quantified from the transformed strain derived from *easA* KO. Agroclavine, lysergic acid, and isolysergic acid were quantified from agroclavine fed cultures. All alkaloids were quantified by comparing their peak areas to standard curves prepared from dihydroergocristine (for alkaloids fluorescing at 272 and 372 nm) and ergotamine (for alkaloids fluorescing at 310 and 410 nm). The percent of festoclavine converted into dihydrolysergic acid and the percent of agroclavine converted to

lysergic acid were calculated and compared by analysis of variance (ANOVA). Statistical analyses were conducted with JMP (SAS, Cary, NC).

For liquid chromatography-mass spectrometry (LC-MS) analysis, week old cultures grown on malt extract agar were washed with 4 mL of HPLC-grade methanol. The samples were pelleted by centrifugation, concentrated for 4-5 hours in a vacuum centrifuge to a volume between 50-100 μ L, and 10 μ L were injected into the LC-MS. The concentrated samples were analyzed by previous methods (Ryan et al., 2013) on a Thermo Fisher LCQ DecaXP LC-MS. Dihydrolysergic acid samples were analyzed on a Thermo Fisher Q Exactive ultrahigh-performance liquid chromatograph mass spectrometer (UHPLC-MS). Analytes were ionized by electrospray ionization in positive mode, a spray voltage of 3.5 kV, a capillary temperature of 300°C, and were detected by scanning for ions with m/z 200-400. Samples were separated on a 50-mm by 2.1-mm Hypersil Gold column (Thermo Fisher, 1.9 μ m particle size) in a linear gradient of 5% acetonitrile with 0.1% formic acid to 75% acetonitrile with 0.1% formic acid over a 10 minute period with a flow rate of 300 μ L/min (Ryan et al., 2013). Dihydrolysergic acid has an m/z = 271.2 and was eluted at about 7.8 minutes. After an initial full scan of m/z = 200-400, ions of m/z = 271.2 were fragmented and analyzed.

Results

Function of *C. gigantea cloA*. A construct for expressing *C. gigantea cloA* was successfully transformed into the *N. fumigata easM* KO strain. A total of 52 transformants were obtained from several transformation experiments, and 33 produced a novel peak that co-eluted with dihydrolysergic acid as evidenced by HPLC with fluorescence detection (Fig. 6). No other novel peaks of significance were observed in the transformants, but relatively large quantities of festuclavine were still observed. The recipient strain, *N. fumigata easM* KO, accumulated large

quantities of festuclavine (data not shown) and nothing that eluted near dihydrolysergic acid (Fig. 6) The accumulation of dihydrolysergic acid in the transformants was supported by LC-MS analyses in which the *cloA*-transformed strains produced a parent ion ($m/z = 271.2$) consistent with the $[M+H]^+$ of dihydrolysergic acid (data not shown). The data indicate that CloA catalyzes a six-electron oxidation of festuclavine to dihydrolysergic acid as opposed to stopping at dihydroelymoclavine (Fig. 2) as expected based on the reported ergot alkaloid profile of *C. gigantea* (Agurell and Ramstad, 1965). The accumulation of dihydrolysergic acid, however, was low in comparison to the abundance of festuclavine in the transformed strains. Potential reasons for this low accumulation, as well as attempts to increase the yield of dihydrolysergic acid are discussed below.

Figure 6: HPLC analysis of transformant strain, dihydrolysergic acid (DHLA) standard, and *easM* ko host strain.

Analysis of the natural ergot alkaloid profile of *C. gigantea*. Due to *C. gigantea* catalyzing the production of dihydrolysergic acid instead of dihydroelymoclavine, three samples of *C. gigantea* sclerotia (Fig. 3) were obtained from Dr. Carlos De Leon (Monticello, Mexico) and analyzed by HPLC (Fig. 7) and LC-MS (Fig. 8) for natural alkaloid production to further investigate its ergot alkaloid biosynthetic capacity. Each of the three sclerotia samples accumulated chanoclavine,

festuclavine, and dihydrolysergic acid (Fig. 7). This alkaloid profile differed from that reported by Agurell and Ramstad (1965), which included chanoclavine, festuclavine, and dihydroelymoclavine. Even though a compound with the mass of dihydroelymoclavine was not detected it is possible the concentration was below the limit of detection. Another possibility is that ergot alkaloid profile varies among isolates of *C. gigantea*.

Figure 7: Natural ergot alkaloid HPLC chromatogram for *C. gigantea* sclerotia. DHLA = dihydrolysergic acid, C = chanoclavine, F = festuclavine.

Figure 8: LC-MS mass spectra for *C. gigantea* sclerotia compared to DHLA standard. Top left is the parent ion ($m/z = 271.2$) for DHLA in *C. gigantea* and bottom left is fragment ions ($m/z = 253.1, 210.1, 228.1,$

and 194.2) for DHLA in *C. gigantea*. This data can be compared to the DHLA standard mass spectra, with the top right being the parent ion and the bottom right being the fragment ions.

Characterization of *C. gigantea* eas gene cluster. The *eas* gene cluster in the *C. gigantea* genome was characterized to obtain evidence for the type of ergot alkaloids the fungus was theoretically capable of producing. The *eas* cluster (GenBank: KY906251) was studied by identifying contigs that contain *eas* genes by querying the genome's sequence (Panaccione and Schardl, unpublished) with individual *eas* genes from other *Claviceps* species. Additional *eas* genes were identified in the originally identified contigs by blastx comparisons of upstream and downstream portions of the contigs to the nonredundant nucleotide database at NCBI. The *eas* cluster contained homologs of the following *eas* genes: *dmaW*, *easF*, *easC*, *easE*, *easD*, *easA*, *easG*, *cloA*, *lpsA*, *lpsB*, and *easH* (Fig. 9B). The gene cluster indicates the fungus was once capable of producing a dihydroergopeptine derivative of dihydrolysergic acid. This hypothesis was supported by the observation that the product of the gene *cloA* from *C. gigantea* oxidized carbon 17 of festuclavine to produce dihydrolysergic acid.

Figure 9: A) Ergot alkaloid synthesis (*eas*) cluster from wild-type *Neosartorya fumigata*. B) Predicted *eas* cluster from *Claviceps gigantea*. *Eas* genes found in both clusters are grey, whereas genes exclusive to *N. fumigata* are white and genes exclusive to *C. gigantea* are black. Ψ : pseudogene.

The gene *lpsA* encodes lysergyl peptide synthetase subunit 1 and acts downstream of *CloA* in the pathway to convert dihydrolysergic acid into ergopeptines. Sequencing of *lpsA* from *C. gigantea* along with comparison to other sequenced *lpsA* alleles revealed a frameshift mutation, which would almost certainly render the gene nonfunctional. The frameshift is believed to occur

between 4345 bp and 4352 bp, since the regions upstream and downstream this frameshift match other sequenced *lpsA* alleles. This frameshift has a span of 8 nucleotides and there should only be 6 nucleotides in this area to have the correct reading frame. This frameshift mutation results in a premature termination codon appearing after amino acid 1458 (4374 bp). The original LpsA product should have been 3581 amino acids in length (encoded by 10743 bp), meaning that more than half of the coding sequence would not have been translated in the correct reading frame. When comparing the *C. gigantea lpsA* sequence data (GenBank: KY906251) to the *Periglandula ipomoeae lpsA* sequence data (GenBank: AEV21218.1) the altered amino acids are an alanine and a valine. The removal of an adenine in the frameshift (located at 4350 bp) in *C. gigantea* would reinstate a valine in the correct position. It does not appear any nucleotide removals in the frameshift will reinstate an alanine. However between 4346 bp and 4348 bp there are three adenines, making it plausible that one of these nucleotides is the second single nucleotide insertion causing the frame shift. The removal of one of these adenines would code for glutamic acid and reinstate the correct reading frame. Such a mutation is consistent with the accumulation of dihydrolysergic acid as the pathway end product in the *C. gigantea* sclerotia and it is predicted that a functional *lpsA* homolog would accumulate dihydroergopeptines. It should be noted that the *C. gigantea* genome was obtained from an isolate independent of those analyzed by LC-MS. This observation indicates that the dihydrolysergic acid profile is typical among several independent isolates of *C. gigantea* from Mexico.

Approaches to increase production of dihydrolysergic acid. In order to check for *cloA*'s expression and to see if the introns in *cloA* were being spliced out correctly, mRNA was extracted from the transformants, reverse-transcribed into cDNA, and were sequenced. PCR analyses of cDNA prepared from mRNA from the *cloA*-transformed strain indicated that approximately half

of the mRNA was fully processed, whereas the other half was larger than expected (Fig. 10A). Sequencing results identified two versions of mRNA, the larger being 1,621 and the smaller being 1,524 bp. The larger fragment retained 1 out of the 7 introns, specifically intron 4, and the smaller fragment was free of introns and likely responsible for the small conversion of dihydrolysergic acid (Fig. 10 C). However, the retention of intron 4 results in a frame shift that leads to a stretch of 6 incorrect amino acids, followed by a premature termination codon, causing the loss of 288 amino acids, which presumably renders the resulting protein nonfunctional (Fig. 10D). It was plausible that *cloA* mRNA containing intron 4 could be an issue limiting the production of dihydrolysergic acid in the transformed strain.

D

Amino Acid Sequence

MSLTSFYAPNPWLLLTASVCVAFPWVVKSIYNLYFHPLRNVPGPRLAALTNFYAFYWNWIREGGYSKQFS
 QLHQHYNPVRIGPNSVHTNQIELYDVIFKGGSAWLKDWQFYQHFNVDAMIGSKHFRTYRDHLAPLYA
 QRTVDGLAPKVRENLELCAARISNAARTGKTVNLAKLLRLFSTSVILYNIFSVDISLYEDDGFHPLEAFEYIM
 AQSWL FVTYPMMSAWLSFIPGTIFSRLDSSWKTFFMKYCTVWNDEDVRLQLESDEQSIKNSHKSXYFAM
 KREGNEEKNNIIPNPVDDVFNFISSGSDTTAYTIAAAFFYILSSPPVCAKLVQELDDNRSIIRDDLNNYKIKGL
 PYLNAVIKETLRIAVPLPGCLPRVVPKGGIAVGSFHLPGTSLSLTHQVISFNEEIFPSSQSFLPQRWLGPDAV
 GLDKWNVAFSRGPRQCIGTTLAYLEVRICALAYFFSRFQMTLTGSCGDRLRWVDRFVAANVDEVEVRIVAD
 RWTEVTY*

Amino Acid Sequence with Intron 4

MSLTSFYAPNPWLLLTASVCVAFPWVVKSIYNLYFHPLRNVPGPRLAALTNFYAFYWNWIREGGYSKQFS
 QLHQHYNPVRIGPNSVHTNQIELYDVIFKGGSAWLKDWQFYQHFNVDAMIGSKHFRTYRDHLAPLY
 AQRTVDGLAPKVRENLELCAARISNAARTGKTVNLAKLLRLFSTSVILYNIFSVDISLYEDDGFHPLEAFEYI
 MAQSWL CECFGP*

Figure 10: A) *cloA* cDNA. Arrows indicate fragments of different lengths. Sizes of relevant fragments of *Bst*EII-digested bacteriophage λ DNA is indicated to the left of photograph. B) pUC18 containing *cloA* cDNA. C) Genomic *cloA* with introns and *cloA* as expressed in transformants with some mRNA containing intron 4 (larger band in Fig. 6A) and some with no introns (smaller band in Fig. 6B). D) Amino acid sequence for *cloA* and *cloA* with intron 4 retained. The amino acid sequence after the 5'-splice junction of intron 4 is highlighted yellow.

In an attempt to increase turnover of festuclavine to dihydrolysergic acid, new versions of the *cloA* expression construct were prepared using the fully processed cDNA as the template. This fully processed version of *cloA* was expressed by the original *N. fumigata easA-easG* promoter to test the effectiveness of removing the intron, and was also expressed by a *gpdA* promoter to analyze if a stronger promoter would increase the accumulation of dihydrolysergic acid. Both constructs were analyzed by HPLC and LC-MS for production of dihydrolysergic acid. HPLC data is shown for both transformed strains (Fig. 12), LC-MS data is shown for *gpdA* promoter-*cloA* transformed strain to confirm the product is dihydrolysergic acid (Fig. 11). LC-MS data for intron free *cloA* is not shown. The conversion yields of festuclavine to dihydrolysergic acid were similar, indicating the removal of introns ($p = 0.58$) and a stronger promoter ($p = 0.75$) did not have a significant impact on production of dihydrolysergic acid (Table 3). Since *gpdA* promoter-*cloA* transformed strains did not produce larger amounts of dihydrolysergic acid, it did not seem relevant to analyze the expression of *cloA* in these transformants.

Figure 11: LC-MS mass spectra for *gpd-if cloA* transformed strain compared to LC-MS data for DHLA standard. Top left shows the parent ion ($m/z = 271.2$) for transformed strain and bottom left shows fragment ions ($m/z = 253.1, 210.1, 228.1,$ and 194.2) for transformed strain. These values can be compared to the DHLA standard, with top right showing parent ion ($m/z = 271.2$) and bottom right showing fragment ions ($m/z = 253.1, 210.1, 228.1,$ and 194.2).

In previous studies conducted by Robinson and Panaccione (2014) *cloA* and *easA* from *Epichloë* sp. Lp1 were cotransformed into an *N. fumigata easA* knockout strain. The gene *easA* (isomerase) converted chanoclavine-I-aldehyde to agroclavine, the precursor for Lp1 *cloA* in their study. Since *cloA* was not transformed individually it was questioned whether CloA requires EasA, preferentially from the same source, to function optimally. The gene *easA* from *C. gigantea* was PCR fused to the free end of the bidirectional *N. fumigata easA-easG* promoter in the intron-free *cloA* construct. The construct was transformed into an *N. fumigata easA* KO strain and analyzed via HPLC (Fig. 12). The addition of *easA* (isomerase) from *C. gigantea* converted chanoclavine to our precursor, festuclavine, which was then oxidized to dihydrolysergic acid by *cloA* (Fig. 12). However, the *easA* KO strain still contained the gene, *easM*, the product of which converted

festuclavine into fumigaclavine B, which was ultimately converted to fumigaclavine C via the *N. fumigata* fumigaclavine pathway (Fig. 12). Since festuclavine was also being converted to fumigaclavine C, fumigaclavine C had to be taken into account when calculating the percent conversion of dihydrolysergic acid. Even with the diversion of some of the substrate to fumigaclavine C, the yield of dihydrolysergic acid increased with the addition of *C. gigantea easA* to the construct ($p = 0.01$, Table 3).

Figure 12: HPLC analysis of intron free-*cloA* transformed strain (top), *gpd-cloA* transformed strain (middle), and *easA-cloA* transformed strain (bottom). All three analyses also include dihydrolysergic acid

standard, *easM* ko host strain (top and middle), and *easA* ko host strain (bottom). DHLA = dihydrolysergic acid, F = festuclavine, C = chanoclavine, Fum C = fumigaclavine C.

Additionally, since it was speculated that CloA was being localized in different subcellular locations than the festuclavine substrate, the strain containing *cloA* expressed from the *gpd* promoter was homogenized and the resulting solutions were incubated with 0.06% Tween 20, and either 0.1 M phosphate buffer (pH = 7.6) or 0.1 M phosphate buffer supplemented with NADPH (7.5 mg/mL). The homogenized solution was analyzed via HPLC and the quantitative data was analyzed using ANOVA and Tukey-Kramer HSD (Table 4). The percent conversion to dihydrolysergic acid was only significantly different between the control and the homogenized solution incubated with phosphate buffer + NADPH. The other two comparisons were not significantly different.

Table 3: Amount of dihydrolysergic acid converted from festuclavine

Modified strain	Percent conversion to DHLA
<i>cloA</i>	1.6 ± 0.7 A
intron-free <i>cloA</i>	2.1 ± 0.5 A
<i>gpd promoter</i>	1.9 ± 0.5 A
<i>easA-cloA</i>	4.3 ± 1.5 B

Values labeled with different capital letters differ significantly in ANOVA (p = 0.01). DHLA = dihydrolysergic acid.

Table 4: Homogenization effect on the production of dihydrolysergic acid

Homogenization Method	Percent conversion to DHLA
Control	2.6 ± 0.4 A
Phosphate Buffer	3.7 ± 0.6 AB
Phosphate Buffer + NADPH	4.6 ± 1.2 B

Values labeled with different capital letters differ significantly in ANOVA and Tukey-Kramer HSD (p = 0.02). DHLA = dihydrolysergic acid.

Another possible issue that could have been limiting the production of the alkaloid is the temperature at which the transformants were grown, since *C. gigantea* is only found in two regions in Mexico, both having cool, humid climates. This made it plausible that the *C. gigantea* enzymes

had a different optimal temperature for activity than *N. fumigata*. Transformed strains were grown at 24 °C, 37 °C, and 50 °C and analyzed by HPLC with fluorescent detection. The optimal temperature for *C. gigantea* gene expression was 37°C, which is the same optimal temperature for *N. fumigata* (Table 5).

Table 5: Temperature Effect on the Production of Dihydrolysergic Acid

Temperature	Percent conversion to dihydrolysergic acid
24 °C	1.8 ± 0.2 A
37 °C	3.6 ± 0.4 B
50 °C	N/A

Values labeled with different capital letters differ significantly in ANOVA (p = 0.003)

Substrate specificity of *C. gigantea* CloA. In order to test substrate specificity of *C. gigantea* CloA, the *cloA*-transformed strains were fed agroclavine. HPLC analysis showed the agroclavine-fed transformants accumulated lysergic acid (15.1) and isolysergic acid (21.2) (Fig. 13, Table 6). This indicates that CloA from *C. gigantea* is capable of oxidizing both festuclavine and agroclavine as substrates.

Figure 13: HPLC analysis of transformed *cloA* strain fed agroclavine. Top image is chromatogram at 310 nm/410 nm wavelength and bottom image is chromatogram at 272 nm/372 nm wavelength. LA = lysergic acid, Iso-LA = isolysergic acid, Iso-S = isosetoclavine, S = setoclavine, Agro= agroclavine.

Table 6: Amount of lysergic acid converted from agroclavine

Modified strain	Percent conversion to LA
<i>cloA</i> + agroclavine	0.93 ± 0.13
<i>easM</i> KO + agroclavine	ND
malt broth + agroclavine	ND

The *cloA* mutant strain that was fed agroclavine was significantly different from the other trials in a Wilcoxon/Kruskal-Wallis test with $p = 0.003$. ND = none detected; LA = lysergic acid.

Discussion

The expression of CloA in an *N. fumigata easM* knockout strain altered the fungus to imitate the dihydrolysergic acid pathway. Bilovol and Panaccione (2016) synthesized the *easM* KO strain to demonstrate the role of *easM* in the *N. fumigata* fumigaclavine pathway. This transformant also serves as a transformation recipient to investigate the oxidation of festuclavine to dihydroergot alkaloids, based on Barrow et al.'s (1974) finding that festuclavine was a precursor to dihydroergot alkaloids in *Claviceps africana* and by extension *Claviceps gigantea*. Agurell and Ramstad (1965) isolated ergot alkaloids from natural *C. gigantea*, including festuclavine and dihydroelymoclavine. As a result, it was predicted a *C. gigantea cloA*-transformed strain of *N.*

fumigata would convert festuclavine into dihydroelymoclavine. The mutant strain, however, produced dihydrolysergic acid rather than dihydroelymoclavine. These data support the findings of Robinson and Panaccione (2014) of CloA catalyzing multiple oxidations. It appears the *C. gigantea* allele of CloA performs a two-electron oxidation of festuclavine to dihydroelymoclavine, followed by two sets of two-electron oxidations to convert dihydroelymoclavine to dihydrolysergic acid. Also consistent with data reported by Robinson and Panaccione (2014), there was an absence of dihydroelymoclavine and other intermediates in our transformants, further indicating that CloA binds festuclavine and completes successive oxidations, before releasing dihydrolysergic acid. It was also discovered that CloA from *C. gigantea* was able to utilize both festuclavine and agroclavine as substrates, meaning that it is capable of functioning in both dihydrolysergic acid and lysergic acid pathways.

Due to the unexpected production of dihydrolysergic acid instead of dihydroelymoclavine, alkaloids from samples of *C. gigantea* sclerotia were analyzed. Surprisingly, in contrast to the findings of Agurell and Ramstad (1965), data from three different sclerotia showed accumulation of dihydrolysergic acid, which was not thought to be produced in nature. The data however, supported our findings that the product of *cloA* was responsible for the production of dihydrolysergic acid. Additionally, having dihydrolysergic acid as the ergot alkaloid pathway end product in the fungus was supported by DNA sequence analysis revealing that the gene encoding lysergyl peptide synthetase subunit 1, *lpsA*, had a frame shift mutation, likely abolishing the genes function. It was predicted that the correction of the *lpsA* mutation would further the dihydrolysergic acid pathway to dihydroergosine, as observed by Barrow et al. (1974) for the related fungus *C. africana*. Nevertheless, *C. gigantea* is the first discovered ergot fungus to have dihydrolysergic acid as its pathway end product.

Furthermore, after *cloA* was sequenced from cDNA reverse-transcribed from mRNA from the mutant strains, it was discovered that about half of our mRNA was fully processed, containing no introns, whereas the other half of mRNA still contained one of the seven introns, specifically intron four. This discovery was not surprising, considering some of the *cloA* introns in *C. gigantea* had unusual 3' splice junctions, with polyA chains followed by a guanine, instead of the conventional YAG. These unusual 3' splice junctions make it plausible that the host strain, *N. fumigata*, might have problems recognizing the 3' splice and fail to splice out the intron. An intron-free version of *cloA* to see if the removal of the fourth intron would have an effect on the production of dihydrolysergic acid. The constructs were successfully transformed and still produced dihydrolysergic acid, however there was not a significant difference between the yields in ANOVA ($p = 0.58$). As a result, it could be concluded that the retention of intron four did not have an effect on the production of dihydrolysergic acid.

Additionally, it was questioned whether a stronger promoter would assist in increasing the expression of *cloA*, hopefully increasing the turnover of dihydrolysergic acid. The intron-free version of *cloA* was expressed from an *N. fumigata* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter, which was assumed to be stronger than the *N. fumigata easA-easG* bidirectional promoter, due to its original role for expressing a gene important for glycolysis. These mutant strains also produced dihydrolysergic acid, however there was not a significant difference between the yield produced by this strain compared yield produced by the original strain in ANOVA ($p = 0.75$). As a result, it could be concluded that a different promoter did not have a significant effect on the production of dihydrolysergic acid.

Moreover, since Robinson and Panaccione (2014) coexpressed CloA with the oxidoreductase, EasA from the same organism, it was questioned whether CloA functionally

required coexpression with a species specific EasA to more efficiently generate dihydrolysergic acid. The coexpression of *cloA* and *easA* both from *C. gigantea* in an *N. fumigata easA* KO produced two-fold more dihydrolysergic acid ($p = 0.006$), indicating that the two enzymes may interact in the pathway and that *cloA* from *C. gigantea* may function better with *easA* from *C. gigantea* rather than *easA* from *N. fumigata*. This yield was significantly different from the original yield in ANOVA, however it is important to note that the overall yield was still low.

Similarly, it was also predicted that the enzymes and substrates may be localized in separate compartments. To test this, the transformants were homogenized and incubated with tween, and either 0.1 M phosphate buffer (pH = 7.6) or 0.1 M phosphate buffer (pH = 7.6) supplemented with NADPH (7.5 mg/mL). These homogenized solution incubated with 0.1 M phosphate buffer supplemented with NADPH was significantly different in production of dihydrolysergic acid from the control in ANOVA ($p = 0.02$). However, it cannot be concluded if the enzymes may be localized in a separate compartment than the substrate or if the addition of NADPH helped in the production. It is also important to note that the overall yield from this experiment was also low. Another possibility that could be effecting the expression of *cloA* and/or the turnover of dihydrolysergic acid is the random integration of the constructs into the genome, as a result of our traditional transformation methods. Depending on where the construct is integrated it could be highly expressed, lowly expressed, or not even expressed at all. Multiple transformants were analyzed and their conversions of dihydrolysergic acid were averaged in attempts to minimize this issue.

Lastly, it was questioned whether *cloA* from *C. gigantea* could oxidize substrates other than festuclavine. Agroclavine was fed to the mutant strains and analyzed the ergot alkaloids secreted into the media by HPLC. As a result, it was discovered that *cloA* from *C. gigantea* can

oxidize agroclavine to lysergic acid, the same as *cloA* from *Epichloë* sp. Lp1 in Robinson and Panaccione's study (2014). This means that our allele of *cloA* is capable of oxidizing both festuclavine and agroclavine, making it functional in both the dihydrolysergic acid pathway and in the lysergic acid pathway.

Overall, the potential of the gene *cloA* from *C. gigantea* was unlocked to produce the pharmaceutically important ergot alkaloid dihydrolysergic acid by genetically engineering a strain of *N. fumigata*. Several significant pharmaceuticals, such as nicergoline (Sermion) and pergolide (Permax), are currently being derived from lysergic acid, however semisynthetic derivation from dihydroelymoclavine and/or dihydrolysergic acid would be more efficient. These drugs are pharmaceutically relevant due to their structural similarities to neurotransmitters, allowing them to treat cognitive and neurological disorders, such as migraines, dementia, Alzheimer's, and Parkinson's disease (Morren and Galvez-Jimenez 2010; Baskys and Hou 2007; Winblad et al. 2007; Perez-Lloret and Rascol 2010). The generation of dihydrolysergic acid in such a fast-growing organism would have a tremendous impact on drug modification and turnover for these pharmaceuticals, when an alternative starting material is created. Additionally, understanding the genes in the pathway could assist in producing libraries of ergot alkaloid derivatives with altered pharmacology.

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