Identification of a Gene Involved in Production of Pharmaceutically Important Lysergic Acid Amides

Kelcie Britton, Chey Steen, Jessi Sampson, Daniel Panaccione

Hello, my name is Kelcie Britton and my research is entitled " Identification of a Gene Involved in Production of Pharmaceutically Important Lysergic Acid Amides." My research has been done in association with the plant and soil sciences department at West Virginia University and in tandem with my co authors Chey Steen, Jessi Sampson, and my mentor Dr. Daniel Panaccione.

Ergot alkaloids are best known for their role in human and animal toxicosis, which is broadly termed ergotism. Examples of events attributed to ergotism through history include the Salem Witch trials and St. Anthony's Fire. In contrast, alkaloids were also used in early homeopathic treatments, primarily targeting women during childbirth. More modernally, alkaloid derivatives are useful precursors in pharmaceuticals for neurological and circulatory diseases. Specifically, these therapeutics are useful in treating dementia, migraines, and hyperprolactinemia. A large quantity of these effective therapeutics are derived from lysergic acid amides, as pictured on the left.

Broadly, ergot alkaloids are classified into 3 branches based on their respective biosynthesis and subsequent biochemical activity. Ergopeptines and clavines have largely been explored genetically and biochemically. Some mechanisms involved in lysergic acid amides biosynthesis still remain illusive, this is largely due to the absence of a suitable species to study these amides.

M. brunneum has since been seen to produce lysergic acid amides, specifically LAH, into its growth medium and has a sequenced genome.

Pictured here is an excerpt of the ergot alkaloid pathway, specifically focused on the lysergic acid amide branch. This branch is unique in that there are two alternative terminal end products pictured at the tip of either arrow. Lysergic acid alpha hydroxyethylamide (hereafter referred to as LAH) is pictured at the tip of the yellow arrow and ergonovine pictured at the tip of the blue arrow. In the case of *M. brunneum* LAH is produced preferentially over ergonovine. Even though Ergine appears later in the pathway than LAH, it is a spontaneous byproduct of LAH that is only produced in low quantities. The two proteins highlighted in yellow represent those that will be explored in my presentation.

I began my research by asking what the role of the gene *easP* was. This gene was present in the *M. brunneum* ergot alkaloid gene cluster, (seen below) where all of the genes associated with a certain pathway are generally clumped together. EasP was present on the far left relative to the other genes known to be a part of the ergot alkaloid pathway.

Genetically, I interrupted the functionality of the *easP* gene in *M. brunneum.* This was done with CRISPR technology using a construct that introduced resistance to phosphinothricin. Antibiotic resistance was used to select transformation colonies for further biochemical and genetic screening. An example of a transformant can be seen below, where the yellow regions represent the original *easP* gene, and the blue region represents the phosphinothricin resistance that was conferred.

Genetically confirmed mutants were then analyzed using HPLC. Quantities of alkaloids were normalized using the fungal sterol ergosterol which was quantified using liquid chromatography-mass spectroscopy. Pictured in the bottom right are fungal killed larva that were used for alkaloid quantification.

When we initially began this project, we suspected that *easP* was the only gene left to convert the precursor intermediate to LAH. Without functional *easP* we expected that there would be no/or leaky production of LAH and an increased amount of ergonovine in mutants. Instead, there was still LAH and ergonovine in the *easP* knock-out fungus. This was specifically interesting to me and later led to further exploration. When comparing the normalized amount of alkaloids, it was seen that LAH was reduced by approximately 50% in the mutant. This can be visualized in the top graph, where the left bar represents the *easP* mutant. Consistent with this observation, the proportion of the total lysergic acid amide that was ergonovine significantly increased as pictured in the bottom graph, where the left bar represents the *easP* mutant. Based on the genetic analysis, we suspect EasP to function as a carboxylesterase in the conversion of the precursor to LAH.

Based on the data presented in the previous slide, we hypothesize that another gene encodes an enzyme similar in function to *easP* exists in the *M. brunneum* genome. I used the sequence of *easP* and compared it to all of the other genes present in the *M. brunneum* genome. From this comparison, I identified a candidate gene with a very high similarity to *easP*, which is now called *estA*. There was no other candidate gene with a comparison value less than 1, suggesting that *estA* was the best candidate.

The results of the first part of my work with *easP* lead to a second research question for me to work on. I asked what the role of *estA* is in LAH biosynthesis.

I used the same CRISPR technology to mutate the *estA* gene in the already prepared *easP*-lacking *M. brunneum* background. In order to select transformants for the double knock out strain, hygromycin resistance was used. From the selected colonies, transformants were screened similarly to before, using genetic analysis which can be seen in the figure to the right. The yellow portions of the sequence represent the wild type *estA* gene, and the interruption of blue represents hygromycin resistance that was conferred. Once transformation was confirmed, I then performed alkaloid analysis where samples of the wild type, *easP* knockout and double knockout strains were prepared simultaneously.

Following alkaloid analysis, it was seen that there was not a significant difference in alkaloid secretion between the *easP* knockout and the *easP*, *estA* double knock out fungus. LAH secretion and ergonovine secretion maintained significant differences between the mutant strains and wild type strains, supporting my previous data. Differences ini LAH secretion can be seen in the figure at the top of the slide, where the first two bars labeled “A” represent the mutant strains. Differences in ergonovine can be seen in the bottom figure, where the first two bars labeled “A” represent the mutant strains. These data suggest that *estA* does not have a role in LAH biosynthesis.

To further explore *easP* and its role in LAH biosynthesis, we decided to functionally analyze EasP. To functionally analyze my *easP* gene, I transformed the *easP* construct alongside ampicillin resistance into a competent strain of *E. coli*. Following genetic selection, I performed a protein gel analysis to show that the protein EasP was inducible in the *E. coli* transformants. This can be seen to the right. Where the brighter bands represent more protein present in the induced strains. This can be seen to the right where the brighter bands represent more protein present in the induced strains. I am currently using the mutant *E. coli* strains and proprietary substrates to explore the EasP protein function. Data will be collected using a combination of spectrophotometry and High performance liquid chromatography in line with previously established protein activity assays.

Based on all of the work that has been done thus far in relation to EasP, it can be concluded that EasP does have a significant role in the production of LAH. Based on genetic results, the protein encoded by *easP* has a role indicative of a carboxylesterase, which is being biochemically explored using my EasP expression protocol. Finally, due to the unique results from the first part of my presentation, it is predicted that there is a functional redundancy in *M. brunneum* that is allowing LAH to still be produced in *easP* mutants. But, data does not suggest that estA is the functional redundancy given that there was no significant difference in alkaloid production of double mutants.

Collectively, my work has provided light to the last unknown step in the lysergic acid amide pathway. Additionally, appears to be *easP* the first easterase identified in the whole ergot

alkaloid pathway. In reference to pharmaceutical development, my work provides background information and tools for modifying ergot alkaloids in the future.

Thank you for taking the time to listen to my presentation. I would like to thank my advisor, Dr. Daniel Panaccione, as well as the NIH and the Arnold and Mabel Beckman Foundation.