2004

STUDIES OF ERGOT ALKALOID BIOSYNTHESIS GENES IN CLAVICIPITACEOUS FUNGI

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ABSTRACT OF DISSERTATION

Caroline Machado

The Graduate School
University of Kentucky
2004
STUDIES OF ERGOT ALKALOID BIOSYNTHESIS GENES IN CLAVICIPITACEOUS FUNGI

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By

Caroline Machado

Lexington, Kentucky

Director: Dr. Christopher L. Schardl, Professor of Plant Pathology

Lexington, Kentucky

2004

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ABSTRACT OF DISSERTATION

STUDIES OF ERGOT ALKALOID BIOSYNTHESIS GENES IN CLAVICIPITACEOUS FUNGI

*Neotyphodium* species, endophytic fungi associated with cool-season grasses, enhance host fitness and stress tolerance, but also produce biologically active alkaloids including ergot alkaloids associated with fescue toxicosis in grazing animals. One approach to reduce fescue toxicosis is to manipulate genes in the ergot alkaloid pathway. The gene, *dmaW*, encoding the first pathway-specific step in ergot alkaloid biosynthesis, was cloned previously from *Claviceps* spp. and its function was demonstrated by expression in yeast. Putative homologs have been cloned from *Neotyphodium coenophialum* (from tall fescue) and *Neotyphodium* sp. Lp1 (from perennial ryegrass).

In order to confirm the function of *dmaW* in ergot alkaloid production, *dmaW* in *Neotyphodium* sp. isolate Lp1 was knocked out by gene replacement. The *dmaW* knockout mutant produced no detectable ergovaline or simpler ergot alkaloids. Complementation with *Claviceps fusiformis* *dmaW* restored ergovaline production. These results confirmed that the cloned endophyte gene was *dmaW*, and represented the first genetic experiments to show the requirement of *dmaW* for ergot alkaloid biosynthesis.
*Neotyphodium coenophialum*, endophyte of the grass tall fescue (*Lolium arundinaceum*) has two homologs of *dmaW*. Considering the possible field applications in future, the Cre/lox site-specific recombination system was chosen because of the potential to sequentially knock out both homologs and obtain marker-free *dmaW* mutants of *N. coenophialum*. One homolog, *dmaW*-2, was disrupted by marker exchange, and the marker was eliminated by Cre, thus demonstrating the application of Cre/lox system in *N. coenophialum* to eliminate a marker gene. The *dmaW*-2 knockout did not eliminate ergovaline production, indicating that the *dmaW*-1 was probably also active in *N. coenophialum*.

A putative ergot alkaloid biosynthesis gene cluster was identified in *Claviceps purpurea* and *C. fusiformis*. *C. purpurea* and *C. fusiformis* produce different subsets of ergot alkaloids. Identification of nine common genes between them suggests the possible role of these genes in the early part of the ergot alkaloid biosynthetic pathway.

**KEYWORDS**: Neotyphodium endophytes, Ergot alkaloid biosynthesis, Cre/lox, *dmaW*, marker exchange

Caroline Machado

Date: 5/06/04
STUDIES OF ERGOT ALKALOID BIOSYNTHESIS GENES IN
CLAVICIPITACEOUS FUNGI

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Ergot alkaloids and their biosynthesis

Ergotism

Ergotism is the disease of humans or animals caused by consuming ergot-infected grains or feed. Ergot is the sclerotium body of the fungus *Claviceps*, and symptoms of the disease are caused by the alkaloids produced by the fungus and accumulated in the ergot. The toxic effects of ergot alkaloids have caused many poisoning epidemics on animals and humans throughout history. Ergot poisoning was reported in the middle ages in Central and Western Europe. Ergot was identified as a fungus in 1711 and the life cycle of *Claviceps purpurea* was described in 1853 by Tulasne. The first ergot alkaloid chemically purified was ergotamine, isolated in 1918 (Groger and Floss 1998).

Ergot alkaloid producing species

The fungi that produce ergot alkaloids are *Claviceps*, *Epichloë*, *Neotyphodium*, and *Balansia* spp. in the family Clavicipitaceae, order Hypocreales. Other fungi that have been reported to produce ergot alkaloids are *Aspergillus* and *Penicillium* spp. Ergot alkaloids have also been reported in green plants of the family Convolvulaceae, including species of *Ipomoea*, *Calistigia*, *Rivea* (Floss and Anderson 1980).

Ergot alkaloid biosynthesis

Ergot alkaloid biosynthesis has been studied well (Floss 1976) and most steps have been explained (Tudzynski et al. 2001) (Fig 1.1). The ergot alkaloids can be divided into different types: the clavine alkaloids, lysergic acid, simple lysergic acid amides and the more complex ergopeptines (Fig 1.1 and Fig 1.2).

The first experiments on the ergot alkaloid biosynthesis pathway were carried out with ergot growing parasitically on rye plants. Mothes and Weygand in 1958 (Mothes et al 1958) proposed a hypothesis for the origin of ergoline ring system by a condensation of tryptophan with an isoprenoid unit. They injected radioactively labeled tryptophan (\(^{14}\text{C}\)) into the plants and found that tryptophan was incorporated into ergonovine and the lysergic acid moiety. They
further confirmed their experimental results by conducting radioactive feeding experiment with a fermentation culture of *Claviceps* and obtained incorporation of radioactive tryptophan into elymoclavine (Gröger et al 1959). This was the first time the culture system was used to study ergot alkaloid biosynthesis, and since then this has become an important tool for elucidation of the biosynthetic pathway (Floss 1976).

Evidence for the involvement of an isoprene unit (from mevalonic acid) as the second precursor molecule was demonstrated with incorporation of mevalonate into the ergoline ring (Gröger et al 1960 and Birch et al 1960). Heinstein et al (1971) then demonstrated synthesis of dimethylallyl tryptophan (DMAT) from dimethylallylpyrophosphate and L-tryptophan, and also partially purified from a *Claviceps* sp the enzyme catalyzing this reaction, dimethylallylpyrophosphate: tryptophan dimethylallyl transferase [dimethylallyltryptophan (DMAT) synthase]. This was proposed to be the first pathway-specific reaction in ergot biosynthesis (Lee et al. 1976). DMAT N-methyl transferase activity was detected in cell free extracts of *Claviceps* spp., providing evidence that that N-methylation is the second step in ergot alkaloid biosynthesis. The subsequent closure of ring C in the ergoline structure was demonstrated by feeding experiments with labeled N-methyl DMAT (Otsuka et al., 1979; Otsuka et al., 1980).

Formation of chanoclavine-I from DMAT involves various reactions including the involvement of a cytochrome P-450 (Kozikowski et al.1988). Chanoclavine I is then converted to other clavine alkaloids, by chanoclavine cyclase, which generates a chanoclavine-I-aldehyde intermediate and closes the ring D and forms agroclavine. Agroclavine then may be oxidized to elymoclavine. Elymoclavine has been shown to be the precursor of lysergic acid derivatives (Floss and Anderson 1980).

Feeding experiments with lysergyl-amino acid peptides did not show incorporation into ergopeptine alkaloids. This suggested that lysergyl amino acids are incorporated after hydrolysis to lysergic acid, and that lysergyl amino acids are not free intermediates in the formation of peptide ergot alkaloids (Floss and Anderson 1980). Since no lysergyl-amide proposed as intermediates have been incorporated specifically into peptide alkaloids, it is unlikely that free intermediates occur in peptide chain formation during biosynthesis of ergopeptines. Therefore, it was suggested that the assembly of the peptide chain takes place on a multienzyme complex similar to the enzyme systems that catalyse the synthesis of peptide antibiotics (Floss 1976). These enzymes are nonribosomal peptide synthetases (NRPS), which have modular
configuration associated with amino acids. Each module contains specific domains for recognition, activation, thiolation and condensation of each amino acid (Marahiel et al. 1997). Ergopeptines are assembled from D-lysergic acid and three amino acids by NRPS designated lysergyl peptide synthetases (LPS), and different combination of three amino acids yields different ergopeptines (Riederer et al. 1996). LPS has two subunits, one to activate D-lysergic acid (LPS2) and another to activate the three amino acids (LPS1). Ergotamine, one of the predominant ergopeptines in *C. purpurea*, is derived from lysergic acid, L-alanine, L-phenylalanine and L-proline. Ergovaline, the ergopeptine synthesized by *Neotyphodium* endophytes is derived from lysergic acid, L-alanine, L-valine and L-proline (Fig 1.2). A list of various ergopeptines that differ in amino acid position I and II are listed in the table 1.1. (Panaccione and Schardl 2003).

**Regulation of the pathway**

Tryptophan has an important role in ergot alkaloid biosynthesis; in addition to being a precursor, tryptophan acts as an inducer of the alkaloid synthesizing enzymes in *Claviceps* spp. Though ergot-alkaloid biosynthesis is repressed at higher levels (22 mM) of inorganic phosphate; tryptophan can overcome the block of alkaloid synthesis by inorganic phosphate (Krupinski et al 1976). L-tryptophan triggers increased levels of DMAT synthase and possibly other enzymes in the pathway (Krupinski et al 1976, Floss et al 1974). Similar to other primary and secondary metabolic pathways, the ergot-alkaloid pathway is subject to feedback regulation. The enzyme DMAT synthase is inhibited by elymoclavine. Anthranilate synthetase, an enzyme in the tryptophan biosynthesis pathway, is inhibited by elymoclavine (Floss and Anderson 1980). Lysergic acid can feedback regulate the enzymes controlling key intermediates (clavines) in the pathway (Panaccione et al. 2003).

**Genetics of ergot alkaloid production**

Although the biochemistry of ergot-alkaloid biosynthesis was studied extensively in the 1960s to 1970s, the study of genes and enzymes involved in the biosynthesis has taken place only in recent years. The gene encoding DMAT synthase, *dmaW*, was the first ergot alkaloid pathway gene to be identified, cloned and sequenced (Tsai et al. 1995). The sequences of the peptide fragments from the protein purified from *C. fusiformis* isolate ATCC 26245 (Gebler and
Poulter 1992) were used design oligonucleotide primers and a PCR amplification product was cloned and sequenced. The function of the gene has been demonstrated by expressing the coding region in yeast (Saccharomyces cerevisiae). The dnaW gene encodes a 52 kDa protein (Tsai et al. 1995). Based on the dnaW sequence from C. fusiformis, the dnaW homolog (designated as cpd1) was cloned from C. purpurea strain P1 (Tudzynski et al. 1999) and its parent strain ATCC 20102 (Wang 2000). Using degenerate PCR, Wang and coworkers (Wang 2000)(Wang et al. 2004) identified and cloned dnaW-homologous sequences from Neotyphodium endophytes, Neotyphodium sp. Lp1, Neotyphodium coenophialum and Balansia obtecta.

Using chromosome walking, Tudzynski and coworkers (Tudzynski et al. 1999) identified more genes present in a cluster associated with the dnaW homolog in C. purpurea P1. Relationships of the cluster-associated genes suggested biosynthetic functions. Identification of a lysergyl peptide synthetase gene (cpps1) in the cluster suggested strongly that this was an ergot-alkaloid-biosynthesis gene cluster. Two other genes in the cluster were predicted to encode monomodular peptide synthetases, and one of these (cpps2) was characterized by targeted gene disruption to encode the protein subunit (LPS2) responsible for activation of D-lysergic acid (Correia et al. 2003).

A homolog of ccpps1, designated lpsA, was identified and cloned in a grass endophyte, Neotyphodium sp. Lp1. Analysis of lpsA by targeted gene knockout provided confirmation that the enzyme encoded by lpsA is involved in the ergot alkaloid biosynthesis (Panaccione et al. 2001). The lpsA knockout mutant did not produce any detectable ergovaline. Ergine (lysergic acid amide) and lysergyl-alanine were also missing in the mutant, providing evidence that simple lysergic acid amides arise from complex ergopeptides (Panaccione et al. 2003).

**Grass endophyte symbiosis**

**Endophyte biology**

The epichloë fungal endophytes belonging to the family Clavicipitaceae are symbionts of many cool-season C3 grasses of subfamily Poöideae (Scharld 1996). The sexual endophytes are classified as Epichloë species and the their asexual counterparts are Neotyphodium species. The family Clavicipitaceae comprises fungi that range from being mutualistic to pathogenic to plants, parasites of insects, nematodes and fungi.
In the tribe Poeae, *Lolium* and *Festuca* grass species are best known for harboring endophytes. The endophytes occupy intercellular spaces of the plant host, form systemic infections and live by feeding on extracellular substrates (Sbardl and Phillips 1997). The interactions of grasses and endophytes vary from mutualism to antagonism. In a mutualistic association the endophyte lives asymptomatically and grows systemically within the host plant, completes the lifecycle by infecting the seed and is transmitted vertically through seeds to the next generation of seedlings (Clay and Schardl 2002). *Neotyphodium* spp. and several *Epichloë* spp. endophytes exhibit this kind of association. In a pathogenic association, the endophytes suppress formation and maturation of grass inflorescences by forming the fungal sexual structure, namely a stroma (choke), around the flag leaf sheath of the emerging inflorescence. Thus, seed production of the host grass may be reduced or eliminated. The endophyte then spreads horizontally by ejecting ascospores; the spores that land on another grass plant can initiate the infection during flowering (Chung and Schardl 1997). Some of the *Epichloë* spp. exhibit only horizontal transmission, but several *Epichloë* spp. can exhibit dual transmission, i.e. in a single plant some tillers can produce choke and other tillers can produce infected seeds (mixed horizontal and vertical transmission) These mixed-transmission endophytes are also called pleiotropic symbionts (Schardl et al. 1997).

**Alkaloids produced by the endophytes**

The endophytes can be important agents of biological plant protection for forage and turf grasses against insects, nematodes, and grazing vertebrates (Sbardl and Phillips 1997). One of the important aspects of the grass-endophyte symbiosis is that endophytic fungi are capable of producing biologically active alkaloids. The four major classes of alkaloids produced by endophytes are the ergot alkaloids, lolines (saturated 1-aminopyrrolizidines), peramine (a pyrrolopyrazine) and lolitrems (indole-diterpenes) (Bush et al. 1997). The main role of alkaloids in the grass endophyte symbioses appears to be antiherbivore activity. Ergot alkaloids have antimammalian activity, lolitrems are tremorogenic neurotoxins (ryegrass staggers), lolines have anti-insect activity and peramine is an insect feeding deterrent. Different endophytes in association with their grass hosts can produce various combinations of alkaloids. *Neotyphodium coenophialum*, endophyte of tall fescue, can produce ergot alkaloids, lolines and peramine. *Neotyphodium* sp. Lp1, an endophyte of perennial ryegrass, can accumulate ergot alkaloids,
lolitrems and peramine (Bush et al. 1997). So far, no fungal endophyte that can produce all four classes of alkaloids has been identified.

**Benefits of grass-endophyte symbiosis**

The mutualistic endophytes that are transmitted vertically provide numerous benefits to their grass hosts with enhanced fitness and protection from biotic and abiotic stresses (Schardl and Phillips 1997). The plant host in turn provides nutrition, protects the symbiont from predators, and provides a means of dispersal for generations.

Many important endophyte benefits to the grasses are well studied in tall fescue with *N. coenophialum* and perennial ryegrass with *N. lolii* symbioses. These include enhanced competitiveness, improved root growth, increased drought tolerance, drought avoidance, mineral stress tolerance (Arechavaleta et al. 1989; Malinowski and Belesky 2000), resistance to certain nematodes (Kimmons et al. 1990; Elmi et al. 1999) and fungal pathogens like *Rhizoctonia* (Gwinn and Gavin 1992), and increased insect resistance (Rowan and Latch 1994)

**Livestock toxicosis**

The abnormalities that cattle and other animals suffer by grazing *N. coenophialum*-infected tall fescue are called fescue toxicosis (Schardl and Phillips 1997). Toxicosis problems suffered by animals grazing on tall fescue were noted in the 1930s, but the endophyte now called *N. coenophialum* was identified only in 1977 (Bacon 1977; 1995). Hoveland (Hoveland 1993) estimated the economic loss due to fescue toxicosis in 1990 as more than $600 million/year in the beef cattle industry in the U.S. The livestock that suffer usually exhibit loss of appetite and reduced weight gain, fat necrosis, loss of body temperature control, and rough hair coats (Schmidt and Osborne 1993). Cows that graze on endophyte-infected tall fescue show decreased levels of serum prolactin and milk production and show reduction in conception rates, whereas horses suffer from total agalactia (Porter and Thompson 1992; Cross 2003). Another symptom that livestock exhibit is fescue foot, usually seen in winter months where affected animals suffer from dry gangrene of the feet.

Whether ergot alkaloids, particularly ergopeptines, are involved in fescue toxicosis has not been demonstrated. It is possible that other ergot alkaloids like clavines and simple lysergic acid amides may also play a role in fescue toxicosis. *N. coenophialum* is also known to produce
other types of alkaloids, namely lolines and peramine (Bush et al. 1997). Whether these alkaloids (although not known to have anti-mammalian activity) act synergistically with ergot alkaloids or augment the effect is also unknown.

Prevention of livestock toxicosis

Fescue toxicosis is particularly problematic if the fields are overgrazed or if the animals are allowed to graze on grass that is flowering. The fungus is concentrated near the meristematic region and seed heads. Therefore, proper management of pasture and establishing clover and other forage mixes with endophytic fescue may be helpful for cattle (Cross 2003). Treatment of cattle with thiamin supplements has been reported to help alleviate the symptoms of toxicosis (Dougherty et al. 1991). Use of endophyte free tall fescue is the obvious solution, but due to the profound effect of endophyte on host fitness, competitiveness and biological protection against herbivores, the possibility of using endophyte free tall fescue is not always feasible (Bacon 1995). Identification of endophytes that are less toxic to livestock could be a solution. The strategy of removing *N. coenophialum* from tall fescue breeding stock, and reinfection with less toxic endophytes has been adopted to develop ‘MaxQ’ tall fescue (Bouton et al. 2000; Bouton 2003). Use of biotechnology to develop less to toxic endophytes by genetic modification of the ergot alkaloid biosynthesis genes is another promising approach (Schardl 1994). Such an approach was used in modification of a lysergyl peptide synthetase gene from *Neotyphodium* sp. Lp1, and resulted in a modified mutant that failed to produce ergovaline (Panaccione et al. 2001).

The advantage of a genetic approach is that the modification can be done in the native endophyte. Modification of endophyte to not produce ergot alkaloids will facilitate definitive tests on the role of ergot alkaloids in livestock toxicosis and also the roles of ergot alkaloids in other fitness enhancement traits and protection of grasses against biotic factors, especially nematode resistance and anti insect activity.

Identification and elimination of factors responsible for fescue toxicosis from the endophytes of forage grasses has been a long-term research goal for the project. My research project involves disruption of *dmaW*, which encodes DMAT synthase, the first step in the biosynthesis of ergot alkaloids. I show that the *dmaW*-knockout in *Neotyphodium* sp. Lp1 did not produce ergovaline or any other ergot alkaloids thus supporting the hypothesis that *dmaW* is required for ergot alkaloid biosynthesis. Because *N. coenophialum*, the endophyte of tall fescue,
harbors two homologs of \textit{dmaW}, and because the ultimate goal of this program is to have non-toxic endophyte that can be used in tall fescue cultivars, a different approach was chosen to knock out both \textit{dmaW} homologs in \textit{N. coenophialum}. Genetic modification usually involves the use of a genetic marker to select the modified fungus; but presence of a genetic marker in a cultivar may not be acceptable to society. Therefore, I chose to test a Cre/lox system to eliminate the marker used to disrupt one of the \textit{dmaW} homologs in \textit{N. coenophialum}.

My other major effort was to gain more information about other genes in the ergot alkaloid biosynthesis pathway. Based on the observation that genes involved in secondary metabolism usually occur in clusters in fungi (Keller and Hohn 1997; Young et al. 2001), genome regions around \textit{dmaW} were sequenced from \textit{Claviceps purpurea} and \textit{C. fusiformis} and a putative ergot alkaloid biosynthesis gene cluster was identified in both \textit{Claviceps} species.

Identification of genes involved in various steps in the pathway and disrupting them will help us to develop mutants that produce different subset of ergot alkaloids, which can be tested for their roles in fescue toxicosis. With this long-term goal and with the identification of a putative biosynthesis cluster from \textit{Claviceps} spp, the future goal can be to identify other genes involved ergot alkaloid biosynthesis in \textit{Neotyphodium} endophytes.
Table 1.1: Different ergopeptines that differ at amino acid position I and II. Amino acid position III is usually L-proline.

<table>
<thead>
<tr>
<th>amino acid position I</th>
<th>amino acid</th>
<th>position II</th>
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</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>ergovaline</td>
<td>ergotamine</td>
</tr>
<tr>
<td></td>
<td>L-leucine</td>
<td>L-isoleucine</td>
</tr>
<tr>
<td>L-valine</td>
<td>ergocornine</td>
<td>ergocristine</td>
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<td>L-ergokryptine</td>
</tr>
<tr>
<td>L-2-amiobutyric acid</td>
<td>ergonine</td>
<td>ergostine</td>
</tr>
<tr>
<td></td>
<td>ergoptine</td>
<td>L-ergoptine</td>
</tr>
</tbody>
</table>
Figure 1.1: Ergopeptide biosynthesis pathway
Figure 1.2: Simple and complex ergot alkaloids
Chapter 2

Gene disruption by marker exchange of dmaW in Neotyphodium sp. Lp1

Introduction

The endophytic fungi belonging to the genus Neotyphodium form symbioses with some cool-season grasses in the subfamily Poöideae, including several important forage and turf grasses (Scharl and Phillips 1997). The Neotyphodium spp. are seed-transmitted and provide numerous benefits to their grass hosts, such as protection from insect herbivores and mammalian herbivores, protection from abiotic stresses, and enhanced resistance to certain nematodes and fungal pathogens (Malinowski and Belesky 2000; Clay and Scharl 2002). One of the important aspects of the grass-endophyte symbiosis is the production of biologically active alkaloids by the endophytic fungi. The four major classes of alkaloids produced by endophytes are ergot alkaloids, loline alkaloids, peramine and lolitrems. Ergot alkaloids and lolitrems have anti-mammalian activity, lolines have anti-insect activity and peramine is an insect deterrent.

Endophytes associated with cool-season grasses can significantly enhance long-term persistence of forage grasses, but on the other hand some endophytes can cause toxicoses to animals that graze grasses with the endophytes. Ergot alkaloids produced by the endophytes have been thought to cause fescue toxicosis in livestock grazed on tall fescue. Ergovaline, the ergopeptine produced by Neotyphodium endophytes is thought to be an important factor in toxicosis on animals that graze on endophyte infected grasses (Lyons et al. 1986; Cross 2003). Ergot alkaloid biosynthesis has been well studied in Claviceps species (Floss 1976; Tudzynski et al. 1999), which are plant pathogens related to Neotyphodium species. The enzyme for the first pathway specific step, dimethylallyltryptophan synthase (DMAT synthase) was purified to homogeneity by Gebler and Poulter (1992), using a Claviceps fusiformis strain as source. Peptide fragments generated by CNBr cleavage were end-sequenced, and the partial peptide sequences were used to design PCR primers for cloning the corresponding dmaW gene from C. fusiformis (Tsai et al. 1995). Tudzynski et al (1999) identified a homologue of dmaW (designated cpd1) in C. purpurea strain P1. Subsequently, dmaW homologues were cloned from Neotyphodium sp. Lp1, an endophyte of perennial ryegrass (Lolium perenne L.), and from the tall fescue endophyte Neotyphodium coenophialum (Wang, 2000). Wang (2000) also provided evidence for the
association of $dmaW$ with ergot alkaloid producing fungi, hypothesizing the involvement of $dmaW$ in ergot alkaloid biosynthesis.

In order to confirm the role of $dmaW$ in ergot alkaloid biosynthesis, $dmaW$ of Neotyphodium sp. Lp1, a natural hybrid of Neotyphodium lolii and Epichloë typhina (Schartl et al. 1994) was disrupted. This chapter describes the knockout of $dmaW$ in Neotyphodium sp. Lp1 and complementation of $dmaW$ knockout with $C.\ fusiformis\ dmaW$. This was work conducted in collaboration with Dr. Jinghong Wang (Wang et al 2004).

Materials and Methods

Biological and molecular materials

Neotyphodium sp. Lp1 was isolated and maintained as described in Wang et al. (2004). For fungal DNA extraction, fungal culture was grown on cellophane layers on potato dextrose agar (PDA) for 5-6 days, mycelium was harvested and freeze dried and stored at -20°C. Fungal genomic DNA was isolated from 60 mg freeze-dried mycelium by the method described by Taha (Al-Samarrai and Schmid 2000). DNA concentration was measured by fluorometry using the DNA-binding fluorochrome Hoechst 33258 in a Fluorometer (Hoefer DyNA Quant 200, Hoefer Scientific Instruments, Amersham Pharmacia Biotech, San Fransisco, CA).

The oligonucleotide primers were from GIBCO custom primer (Life Technologies) and Integrated DNA Technologies (IDT Inc, Coralville, IA).

Generation of plasmid vector constructs for transformation

Plasmid constructs pKAES170 and pKAES157 were generated by Jinghong Wang as described in Wang et al. (2004).

Screening of fungal transformants by PCR and Southern blot

Transformation of Neotyphodium sp. Lp1 was conducted by Jinghong Wang as described in Wang et al. (2004). Once the transformants were grown, I transferred the transformants to PDA containing hygromycin B for sporulation, and single-spore isolation and screening. I initially screened transformants by PCR using primers dmaWe19.01d (5’-CACAGCACGGCGCCAATGTT-3’) and dmaWe19.02u (5’-CGCTGGCAACGAGACCATTC-
3’). Each 25 µl reaction mixture contained 1µl (approx 2-4 ng) DNA template, 1.25 mM each dNTP, 5 µM each of two primers, 1X PCR buffer, 1.25 units of TaqGold DNA polymerase (Applied Biosystems, Foster City, CA). The reaction mixture was held at 95°C for 9 min, then subjected to 35 cycles of the following program: 94°C for 35 sec, 55 °C for 40 sec and 72°C for 2 min in a Perkin Elmer Applied Biosystems thermal cycler (GeneAmp PCR system 2400). Clones that did not yield amplification products were further analyzed by Southern-blot hybridization.

Jinghong Wang and I conducted Southern blot analysis as follows: Hybridization probes were prepared by PCR with digoxigenin-dUTP (Roche-Boehringer, Indianapolis, IN) (Gebeyehu et al. 1987). A mixture of two fragments from the Neotyphodium sp. Lp1 dmaW region was used as probe. These fragments were amplified and labeled with digoxigenin by PCR with the following primers: dmaW e19copy2.3u (5’-GCAGTTTGGAGTATCTTTTG-3’) and 144dmaW.20d (5’-TCTATGTCAGAATCGTACCG-3’) for the probe just upstream of the dmaW coding region; and dmaWe19.07d (5’-CAACACCTTACACTTGACCC-3’) and dmaW19copy2.5u (5’-CTCGCCGGCATGCGTCAAAA-3’) for the coding sequence. Genomic DNA was digested with EcoRI and electrophoresed (Ausubel et al. 1999). DNA was transferred onto Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL) using the alkaline transfer method (Ausubel et al. 1999). The membranes were prehybridized and then hybridized at 42°C overnight with denatured probe, in prehybridization solution (Wang et al. 2004). Membranes were washed twice in 300 ml of 1X SSC, 0.1%SDS at 55-60°C for 30 min. Then the membrane was subjected to immunological detection using anti-digoxigenin antibody-conjugated alkaline phosphatase and CSPD ready to use substrate (Roche-Boehringer Indianapolis, IN).

Cotransformation

Protoplasts from the Lp1 dmaW knockout isolate were prepared as described by Murray et al (1992) except that mycelium was treated with 10 mg/ml Novozym 234 (Novo Biolabs, Wilton, CT). The protoplasts were cotransformed with linearized pKAES157 (containing C. fusiformis dmaW under the control of the tub2 promoter) in a 1:1 molar ratio with pAN8-1 (Mattern et al. 1988) carrying a phleomycin resistance marker by electroporation as described by Tsai et al (Tsai et al. 1992). After electroporation, protoplasts were mixed with 7 ml top complete regeneration medium (Panaccione et al 2001), then poured onto 20 ml complete
regeneration medium plate containing 75 µg/ml phleomycin (Sigma, St. Louis, MO). The plates were incubated at 21°C for 2-3 weeks and viable fungal colonies were transferred to PDA with phleomycin (75 µg/ml) for sporulation and single-spore isolation. The transformants were screened for integration of pKAES157 by using PCR with primers Ety-tub2-Cpu-dmaW (7801) (5’-CAAGTAACCGAGAAAATGCACAAAAAGCTCCA-3’) and Cpu-dmaW(-)-SalI (11052) (5’-CGGTGCACGCAAGACCCCTTGACATGA-3’).

A 6.3 kb fragment containing wt Lp1 wild type dmaW and the flanking sequences was amplified from Lp1 genomic DNA using primers dmaWe144(SacII).nest12d (5’-GCCCGCGGTAGTATAATTAACTGACTAAGCT-3’) and dmaWe144(SalI).11u (5’-CGTGCACGCTAAGTAGTTATCTTATTATAAGATACC-3’). I also cotransformed Lp1 dmaW ko with this 6.3 kb fragment containing Lp1 wild type dmaW in a molar ratio of 3:1 with pAN8-1 phleomycin resistant marker, by using the electroporation method described above.

Inoculation of ‘Rosalin’ seedlings

Perennial ryegrass cultivar Rosalin seedlings were inoculated as described by Latch and Christensen (1985). ‘Rosalin’ endophyte-minus seeds were surface sterilized and germinated on water agar for 5 to 6 days. The fungal mycelium was grown on PDA cellophane plates. The seedling meristems were slit and fungal mycelium was placed onto the wound. After inoculation, the seedlings were incubated at 21°C for 10 days in the dark and then in the light for 4-6 days. Then the seedlings were planted in flat trays containing soil and placed in the greenhouse. When the seedlings reached the 3 tiller stage, one tiller each was analyzed by the tissue-print immunoblot assay (TPIB) Gwinn et al., (1991) to determine the presence of endophyte. The preparation of N. coenophialum antigen and production of polyclonal rabbit antibodies for TPIB are described by An et al. (1993).

Generation of seeds from grass-endophyte symbiota

Perennial ryegrass ‘Rosalin’ plants inoculated with Neotyphodium sp. Lp1, dmaW ko and endophyte-minus plants were vernalized to induce flowering in the vernalization chamber. The vernalization chamber was set at the following parameters: step 1) For 14 days plants will have 11 hrs of light at 18.3°C and night 7.2°C, step 2) Seven days, 10 hrs of light at 12.8°C and night 1.6°C, step 3) Twelve days, 9.5 hrs of light at 7.2°C and night 1°C. step 4) Twenty one days, 9
hrs of light at 3°C and night -1°C, step 5) Seven days, 9.5 hrs of light at 7.2°C and night 1°C, step 6) Seven days, 10 hrs of light at 12.8°C and night 1.6°C, step 7) Seven days, 12 hrs of light at 18.3°C and night 7.2°C. The vernalization of the plants was conducted under Dr. Tim Phillips supervision (Dept. of Agronomy, University of Kentucky).

After the vernalization, plants were moved to the greenhouse, where the day temperature was set at 24°C and the night temperature was 21°C. The plants were given 15 hrs of day length period. The plants were transferred to bigger pots (6 1/2") and were randomly distributed so that no plant with same genotype (plant number) will be next to each other. Perennial ryegrass is an obligate outcrossing plant, therefore requires pollen from the different genotype for fertilization and successful seed production. To facilitate pollination, the flowering stalks were lightly brushed each morning to ensure the dispersal of pollen. In addition the greenhouse is equipped with good internal air movement system, which ensures the movement of pollen. After the seed had set and ripened, the seed heads were collected gently by cutting the inflorescences 2-3 inches below the lowest seed head and placed in a bag to dry. Once dry, the seeds were collected from the seed heads by manually separating them from the panicle.

**Ergot alkaloid analysis**

Pseudostems of perennial ryegrass ‘Rosalin’ with or without the endophyte were analyzed. Ergopeptines were extracted as described by Hill et al. (1993) with modifications as described by Panaccione et al. (2001). Dried leaf material (250 mg combined pseudostems and leaf blades) was ground to a fine powder in liquid nitrogen and extracted with chloroform : 0.01 M sodium hydroxide (9:1), filtered, and applied to silica gel-organic binder clean-up columns (Hill et al. 1993). Pigments were eluted with acetone:chloroform, and ergopeptines were eluted with 1.5 ml methanol and methanol elutes were concentrated to 50 µl with a Cenrivap concentrator (Panaccione et al. 2001), and diluted 1:1 with water to give 100 µl. To provide an internal standard for quantification, 100 ng of ergotamine-tartrate (Sigma, St. Louis, MO) was added to the plant material before extraction. Ergopeptines were analyzed by high-pressure liquid chromatography (HPLC) on a Microsorb C18, 5 µm particle, 4.6 X 250 mm column (Varian), as described by Annis and Panaccione (1998), except that fluorescence was detected on the basis of excitation at 310 nm and emission at 420 nm (Panaccione et al. 2001). I used this
method to conduct ergopeptine analysis at Dr. Dan Panaccione’s laboratory at West Virginia University.

Dr. Dan Panaccione also conducted ergopeptine analysis of some of the samples in this study during his sabbatical research at AgResearch Grasslands, Palmeston North, in New Zealand. These analyses were by an improved method modified from Spiering et al. (2002) and detailed in Wang et al. (2004).

**Results**

**dmaW knockout (ko) by homologous recombination in Neotyphodium sp. Lp1**

The plasmid construct pKAES170 contained a mutated *dmaW*, whereby 341 bp of the *dmaW* coding region from the start codon was replaced with *hph*. This plasmid was linearized by digestion with *Sal*I, then introduced into *Neotyphodium* sp. Lp1 by electroporation (Wang et al. 2004). The transformants were screened for homologous recombination by a negative PCR test employing two specific primers for screening, one of which was from the region of *dmaW* that was deleted in pKAES170. So, when the construct was integrated ectopically a 1.5 kb PCR product was amplified from *dmaW*, whereas the absence of this amplification product suggested gene replacement. Out of 125 transformants screened, one failed to yield this amplification product, but did yield amplified products from *hph* and the 3’- and 5’-flanking regions. Thus, this transformant was putatively was considered a *dmaW* knockout (ko) mutant.

The putative *dmaW* ko was analyzed further by Southern blot. Genomic DNA from two single-spore isolates of the ko, wild type (wt) Lp1 and a transformant with ectopic integration of the construct were digested with *Eco*RI. The probe was *dmaW* plus a portion of 5’-flanking sequence (Fig 2.1 panel A), as shown in the Figure 2.2. A 4.1-kb *Eco*RI fragment of wild-type *dmaW* had been replaced by 1.8-kb and 4.7-kb fragments in the ko mutant. The Southern result indicated that the 3’-flanking region of *dmaW* remained intact. In order to check if the 5’-flanking region of *dmaW* was also intact in the mutant, I conducted a PCR- walk from *hph* to 5’ flanking region (Fig 2.1 panel B and Fig 2.3). The PCR and the Southern blot analysis indicated that *dmaW* has been replaced with mutant *dmaW* in *Neotyphodium* sp. Lp1 by double-homologous cross-over, and that both 3’ and 5’ flanking regions were intact in the mutant. This mutant is referred as *dmaW* ko.
Complementation of *dmaW* ko with *Claviceps fusiformis dmaW*

To complement the *dmaW* ko mutant, a construct (pKAES157) consisting of *C. fusiformis dmaW* under the control of the *E. typhina* tub2 promoter (Byrd et al. 1990; Tsai et al. 1992; Wang et al. 2004) was introduced into *dmaW* ko by electroporation. The resulting transformants were screened by PCR for the integration of the construct. Out of 20 independent transformants screened, six of them had pKAES157 integrated into the genome. These transformants are designated as *dmaW* ct.

An attempt to use Lp1 wt *dmaW* to complement *dmaW* ko was unsuccessful as it resulted in no stable transformants.

Establishment of grass-endophyte symbiota

Because the ergot alkaloids are produced in grass-endophyte symbiota, but not reliably in *Neotyphodium* spp. cultures, *Neotyphodium* sp. Lp1 wild type (wt), *dmaW* ko and *dmaW* ct were introduced into perennial ryegrass cv. Rosalin to establish grass-endophyte symbiota. The frequency of endophyte infection after inoculation into perennial ryegrass were 17%, 14% and 18% for Lp1 wt, *dmaW* ko and *dmaW* ct respectively, suggesting that compatibility of these modified endophytes with their host had not been affected by the genetic manipulation.

Seed transmissibility of the modified endophyte

Seeds obtained from plants infected with Lp1 wt, endophyte-minus and *dmaW* ko transformants were germinated, and the seedlings were tested by TPIB for the presence of endophyte. All of the seedlings generated from the seeds of Lp1 wt and *dmaW* ko showed the presence of endophyte, and all the seedlings obtained from seeds of endophyte-minus were negative for endophyte.

Ergot alkaloid analysis

Five plants were analyzed for each treatment. Plants infected with the wild type *Neotyphodium* sp. Lp1 accumulated high levels of ergovaline and its stereoisomer ergovalinine (amount varied from 4.6 to 7.8 µg/ g dry weight of infected plant material), corresponding to HPLC peaks with retention times of 42.3 min and 51.4 min (Fig 2.4), respectively. Plants with *dmaW* ko as well as endophyte-minus plants had no detectable ergovaline or ergovalinine. Plants
infected with five independent \textit{dmaW} ct transformants accumulated a small but identifiable level of ergovaline and ergovalinine (mean of 0.15 g/g dry weight of infected plant material) (Fig 2.4). This result indicates that \textit{dmaW} ko eliminates the production of ergovaline in \textit{Neotyphodium} sp. Lp1.

**Discussion**

The results presented in this chapter demonstrate the first genetic test for the role in ergot alkaloid biosynthesis of \textit{dmaW}, which encodes DMAT synthase. \textit{dmaW} in \textit{Neotyphodium} sp. Lp1 was replaced with a construct containing partially deleted \textit{dmaW} by marker-exchange gene replacement, and when this \textit{dmaW} ko mutant was introduced into perennial ryegrass seedlings to form symbiotic associations, they established infection at a normal frequency as compared to that of wild type \textit{Neotyphodium} sp. Lp1. Furthermore, the modified endophyte was one hundred percent seed transmitted. Thus, the genetic modification of the fungus had not affected its host compatibility. The plants symbiotic with \textit{dmaW} ko lacked detectable ergovaline, showed that \textit{dmaW} ko did not produce ergovaline (Fig 2.4), the main product of the pathway, ergine, an alternate product of the pathway and chanoclavine, a key intermediate of the pathway (Wang et al. 2004). Thus, \textit{dmaW} was required for biosynthesis of ergovaline and other metabolites of the ergot alkaloid biosynthesis pathway, supporting the proposed role of DMAT synthase as a pathway specific step in the biosynthesis. Lee et al (1976) purified DMAT synthase from \textit{Claviceps} sp. and predicted that it would play a role as the determinant step in biosynthesis pathway. Cloned \textit{dmaW} from \textit{C. fusiformis} was demonstrated to express DMAT synthase in yeast (Tsai et al 1995). Therefore, the tests described in this chapter represent the first genetic evidence that DMAT synthase is essential for the biosynthesis of ergot alkaloids.

The complementation of \textit{dmaW} ko with \textit{C. fusiformis dmaW} (\textit{dmaW} ct) provided confirmation that the DMAT synthase was responsible for ergovaline production. Plants infected with \textit{dmaW} ct transformants indicated small but definitive levels of ergovaline and ergovalinine, whereas endophyte-minus plants and \textit{dmaW} ko symbiota completely lacked these peaks (Fig 2.4). An attempt to complement \textit{dmaW} ko with wild type Lp1 \textit{dmaW} was unsuccessful because no stable transformants were obtained. Complementation by \textit{C. fusiformis dmaW}, which is a well characterized \textit{dmaW} as it demonstrated to have DMAT synthase activity and authentic enzyme
product (Tsai et al 1995), was a confirmation that DMAT synthase was necessary for ergot alkaloid production.

The level of ergovaline synthesized by \textit{dmaW} ct symbiota was very low. It is possible that the low ergot alkaloid levels were due to poor expression of the \textit{C. fusiformis} protein in the \textit{dmaW} ct transformants. \textit{C. fusiformis dmaW} was expressed under \textit{tub2} promoter which is known to express constitutively, so it is a possibility that there was selection against high expression in the transformants if the effect was depletion of cellular pools of essential metabolites (tryptophan and DMAPP), or if DMAT generated by constitutive DMAT synthase activity was toxic to the fungus. Perhaps the only \textit{dmaW} ct transformants obtained were the ones that produced only low levels of the enzyme.

Identification and elimination of factors responsible for fescue toxicosis from the endophytes of forage grasses has been a long-term research goal. Modification of endophyte to not produce ergot alkaloids will allow us to conduct definitive tests on the role of ergot alkaloids in livestock toxicosis and also on the role of ergot alkaloids in other fitness enhancement traits and protection of grasses against biotic and abiotic factors.
Figure 2.1: (A) Maps of the wild type *dmaW* locus, targeting vector and expected map of locus after disruption. Vertical lines indicate *Eco*RI sites and double headed arrows indicate expected size of fragments in kb. Probe is indicated with horizontal line above. Southern blot result in Fig 2.2 (B) Map of the disrupted locus showing primer sites as pointed arrows used for PCR analysis (Fig 2.3)
Figure 2.2: Southern-blot analysis of Lp1 *dmaW* ko. Lane 1 contained total genomic DNA from the wild type (wt) Lp1; lanes 2 and 3 contained DNA from two single spore isolates of *dmaW* ko; and lane 4 contained DNA from an Lp1 transformant with ectopic integration of the vector. DNA was digested with *Eco*RI. The probe was *dmaW* plus a portion of the 5’-flanking region. Replacement of the 4.1-kb *Eco*RI fragment with 1.8-kb and 4.7-kb fragments indicated disruption of the gene. Fragment sizes and probes are indicated in Fig 2.1 panel A.
Figure 2.3: PCR analysis of the 5’ flanking region of Lp1 *dmaW* ko. One PCR primer targeted *hph*, and the other primer targeted a site upstream of the 5’-flanking region used in the disruption vector (Fig 2.1 panel B). The PCR product of 3 kb in the ko (lane 1 and 2) was expected if homologous crossover occurred in the 5’-flanking region during marker exchange mutagenesis. PCR from DNA of an ectopic transformant (lane 3) lacked the 3-kb fragment (the faint band is likely due to spillover from the adjacent well), as did wt Lp1 (lane 4) and the water control (lane 5). M is *Hind*III-cut bacteriophage lambda DNA as a size marker ladder.
Figure 2.4: Ergovaline analysis of Lp1 *dmaW* ko. HPLC analysis of ergovaline (42.3 min) and its stereoisomer ergovalinine in perennial ryegrass without endophyte (e-), or symbiotic with wild type Lp1 (wt), the *dmaW*-knockout mutant (ko) or the mutant complemented with *C. fusiformis* *dmaW* (ct). Detection was by fluorescence with excitation at 310 nm and emission at 410 nm. Asterisks mark the positions or expected positions of ergovaline and its stereoisomer ergovalinine.
Chapter 3
Knockout of dmaW in Neotyphodium coenophialum using a Cre/lox system

Introduction:

Neotyphodium coenophialum is a common endophyte of tall fescue, one of the most widely grown pasture and forage grasses in the U.S. The endophyte produces ergot alkaloids, which have been associated with fescue toxicosis, a complex of symptoms that cattle or other animals may suffer when grazed on N. coenophialum-infected tall fescue, and a major problem for livestock in Kentucky and much of the United States. The symptoms of tall fescue toxicosis include reduced weight gain, loss of body temperature control, reduced fertility and lactation and, in extreme cases, gangrene and death (Cross 2003). One of the strategies to ameliorate fescue toxicosis is to modify the fungus genetically to render it unable to produce ergot alkaloids. Genetic modification of the fungus requires a system to select the modified fungus, which usually involves introduction of an antibiotic resistance gene as an indicator (marker) of transformation. A genetically modified non-toxic endophyte with an antibiotic resistance gene may not be acceptable to society as a cultivar released in the environment. Therefore, I have embarked on the development of a marker-free strategy for gene knockout in N. coenophialum. I first tested a two-step disruption strategy published for Aspergillus nidulans, but found it to be infeasible (see Appendix 1). Therefore, I have chosen the Cre/lox system as a strategy to obtain marker free transformants.

Cre/lox is a site-specific recombination system derived from Bacteriophage P1 (Sternberg and Hamilton, 1981); Cre (cyclization recombination protein) is a recombinase that mediates site-specific recombination between a pair of specific target sequences designated loxP (locus of crossing-over in P1). The loxP site is a 34 bp region consisting of two 13 bp inverted repeats separated by a spacer region (Fig. 3.1A) that provides directionality of Cre-catalyzed recombination. Depending on the orientation of loxP sites relative to each other, Cre can either excise (Fig. 3.1B) or reverse (invert) the orientation of the region between them (Vergunst and Hooykaas, 1999). The Cre/lox system has been extensively used in mammalian systems, such as transgenic mice (Lakso et al. 1992) as well as in plant systems (Corneille et al. 2001; Hohn et al. 2001) to study the functions of genes and to remove selection markers in order to generate marker free transgenic organisms.
The tall fescue endophyte, *N. coenophialum*, has two homologs of *dmaW*, encoding dimethylallyl tryptophan synthase (DMAT synthase), the first pathway specific step in ergot alkaloid biosynthesis (Tsai et al. 1995). Here I demonstrate the use of the Cre/lox system to knock out a *dmaW* homolog in *N. coenophialum* by marker exchange, followed by removal of the selectable marker by Cre.

**Materials and Methods**

**Biological and molecular materials**

**Strains and culture conditions**

*Neotyphodium coenophialum* ATCC 62374 was grown and maintained on potato dextrose agar (PDA) at 21°C. For fungal transformation, cultures were grown in potato dextrose broth (PDB), shaking at 200 rpm for 8-9 days. For fungal DNA extraction, fungal cultures were grown on cellophane layers on PDA plates, harvested, freeze-dried and stored at –20°C. Fungal genomic DNA was isolated from 60 mg freeze-dried mycelium by the method of Taha (Al-Samarrai and Schmid 2000). DNA concentrations were measured by fluorometry using the DNA-binding fluorochrome Hoechst 33258 and a Hoefer DyNA Quant 200 fluorometer (Hoefer Scientific Instruments, Amersham Pharmacia Biotech, San Franciscoco, CA).

**Plasmids, primers and cloning supplies**

Plasmids pBCKS (+/-) and pBluescriptKS (+/-) were obtained from Stratagene Cloning Systems (La Jolla, CA). Gel purification employed the QIAquick gel extraction kit (Qiagen, Valencia, CA); plasmid DNA extraction employed the Qiagen miniprep kit (Qiagen, Valencia, CA). All the oligonucleotide primers were from Integrated DNA Technologies (IDT Inc, Coralville, IA). Ligations employed the Fastlink DNA ligation kit from Epicentre (Montgomeryville, PA). The bacterial transformations employed XL1Blue electroporation competent cells (Stratagene), and the cells were electroporated as per supplier’s instructions. Plasmid pQL123, containing *cre*, was generously provided by Dr. Peter Mirabito (University of Kentucky).
Construction of plasmid constructs

The *loxP-hph-loxP* cassette

pBCKS+ vector was digested with *BamHI* and half filled with dA and dG using Klenow fragment (DNA polymerase I large fragment). Two pairs of oligonucleotides [loxP1SalI(u) (5’-TCGAGATAACTTCGTATAGCATACATTATACGAAGTTATG-3’) with loxP1BHI(d) 5’-Phos TCCATAACTTCGTATAATGTATGCTATACGAAGTTATC-3’) and loxP2BHIfull(d) (5’-GATCGATAACTTCGTATAATGTATGCTATACGAAGTTATG-3’) with loxP2BHIhalf(u) (5’-Phos-TCCATAACTTCGTATAGCATACATTATACGAAGTTATC-3’)] were ordered. Each primer pair was annealed to form adapters as follows. The oligonucleotides were dissolved in H$_2$O to a concentration of 10 µM, mixed in a 1:1 ratio and heated at 70°C for 5 min, then cooled gradually to room temperature to anneal the oligonucleotides to form adapters consisting of *loxP* sites with *BamHI* and *SalI* sticky-end overhangs. As a source of the fungal-active hygromycin phosphotransferase gene, *hph*, pKAES080 (Tsai et al. 1992) was digested with *BamHI* and *SalI* and gel purified.

Ligation of four fragments: The two *loxP* adaptors, the *hph* fragment and linearized, half-filled pBCKS+ vector were mixed in a molar ratio of 45:45:1:1 (*loxP1; loxP2: hph: vector*) and ligated overnight at 16°C. The ligation mixture was mixed with XL1Blue electroporation competent cells, and the cells were electroporated (as per supplier’s instructions), then plated on LB plates containing chloramphenicol (25 µg/mL), plus 5-bromo-4-chloro 3-indolyl-D-galactoside (X-gal, 100 µl of 2% solution per plate) and Isopropylthiogalactoside (IPTG, 100 µl of 10mM solution per plate). White colonies were picked, then plasmid DNA was isolated, and analyzed by *BamHI* digestion and agarose-gel electrophoresis. A plasmid was identified that gave the expected fragments — a 1.3-kb insert with *loxP-hph-loxP*, and 3.4-kb vector — and was designated pKAES173.

Construction of mutant *dmaW-2*

A 2.9-kb fragment containing the 5’-flanking region of *dmaW-2* was amplified by PCR using primers dmaWe19copy2 SacII.13d (5’-GCCCGCGGCCCTTAGAATATAGTAGTATAATTAACCTTAC-3’) and *dmaW* e19copy2.cos BamHI.16u (5’-AAACGGATCCTGTGAAGAAGAGGACGAGCGAATAGC-3’), cut with *SacII* and *BamHI*, and cloned into pBSKS+ that had also been cut with *SacII* and *BamHI*.
Another 2.7 kb fragment containing the 3’ region of dmaW-2 starting 342 bp downstream of the translational start codon was amplified using primers dmaWe19copy2.cos BamHI.15d (5’-CTTCTTACAGGATCCGTTAACAACCTGCTATCTG-3’) and dmaWe19copy2 SalI.14u (5’-GCGTCGACAGTGATCAGGGATACCTTTGATTACA-3’). The 2.7 kb-fragment was then digested with BamHI and SalI and cloned in the BamHI and SalI sites of pBSKS+ to give pKAES148.

Plasmid pKAES148 was linearized with BamHI and treated with calf intestine alkaline phosphatase (Stratagene) to dephosphorylate the 5’-termini. Plasmid pKAES173 was digested with BamHI and the 1.3 kb fragment containing loxP-hph-loxP was gel purified. The fragment and linearized pKAES148 were mixed 1:2 (vector:insert), and ligated. After E. coli transformation, plasmid isolation and analysis, as above, a plasmid construct was identified with the 5’ and 3’ regions of the dmaW-2 locus flanking the loxP-hph-loxP cassette. This plasmid was designated pKAES174.

**Generation of a Pro\textsubscript{tub2}cre fusion**

First, the tub2 promoter region from pKAES080 (Tsai et al. 1992) was amplified by PCR using primers T3 (5’-ATTAACCCTCACTAAAGGGA-3’) and Cre5’tub2-3’(u) (5’-GTACGGTCAGTAAATTGGACATTTTCTCGGTTAC-3’). Primer Cre5’tub2-3’(u) had a partially overlapping sequence of 22 bp from the start codon of cre. PCR amplification of the cre coding region from pQL123 employed the primers Cre(d) (5’-ATGTCCAATTTACTGACCGTACAC-3’) and CreSacII(u) (5’-GCCCGCGGCTAATCGCCATCTTCCAGCAGGCG-3’). Products of these two amplification reactions were mixed and subjected to PCR with primers T3 and CreSacII(u) and generated and amplified a fusion product with the tub2 promoter and cre coding sequence. The PCR product was gel-purified, cut with SalI and SacII, and cloned into the SalI and SacII sites of pBSKS+. The resulting plasmid was designated pKAES175 (Fig 3.2).

**Generation of a Pro\textsubscript{dmaW}cre fusion**

First, the dmaW-2 promoter region of about 1120 bp flanking the start codon of dmaW-2 was amplified from pKAES148 by PCR using primers dmaWPrSalI(d) (5’-GCGTCGACCTCACTATCACGACTAAGGAGATAC-3’)}
and Cre5’dmaWPr3’(u) (5’-CGGTCAGTAAATTGGACATTGTGAAGAAGAGGAC-3’). Primer Cre5’dmaWPr 3’(u) included a 19-bp of overlapping sequence from the start codon of cre. The cre coding region from pQL123 was amplified by PCR using primers Cre(d) and CreSacII(u). Products of these two amplification reactions were mixed and subjected to PCR with primers dmaWPrSalI(d) and CreSacII(u) to generate and amplify a fusion product with the dmaW-2 promoter region and cre coding sequence. The PCR product was gel-purified, cut with SalI and SacII, and cloned into the SalI and SacII sites of pBSKS+. The resulting plasmid was designated pKAES176 (Fig 3.2).

**Fungal transformation**
Protoplasts were prepared as described by Murray et al (1992) except that mycelium was treated with 7 mg/ml of Novozym 234 (Novo Biolabs, Wilton, CT) and 3 mg/ml of Glucanex (Novo Nordisk Biochemicals, Franklinton, NC). Protoplasts were transformed with pKAES174 (4-7 µg for electroporation and 1-2 µg for PEG in 1 x10^6 to 10^7 protoplasts) that had been linearized by digestion with SalI. Transformation was by the electroporation (Tsai et al 1992) or PEG method (Panaccione et al. 2001). The protoplasts were then mixed with 7 ml of top complete regeneration medium (CRM, described below), then poured onto 20 ml CRM plates containing 48 µg/mL hygromycin B (Calbiochem, San Diego, CA). CRM contained, per liter: 304 g of sucrose, 1g NH₄NO₃, 1g KH₂PO₄, 1g NaCl, 0.25g anhydrous MgSO₄, 0.13g CaCl₂.2H₂O, 1 g yeast extract, 12 g potato dextrose broth powder, 1 g peptone, 1 g casein acid hydrolysate, and 7 g agarose. The transformation plates were incubated at 21°C for 3-4 weeks. The fungal colonies were transferred onto PDA with hygromycin B (48 µg/ml) for sporulation and single-spore isolation. On an average protoplast yield per mL was 2 x 10^8, and about 5 to 15 % of these were viable. Transformation efficiency was from 1.7 to 4.4 (Number of transformants per microgram of DNA).

**Screening of transformants by PCR**
DNA from single-spore-isolated transformants was isolated by a CTAB method (Doyle and Doyle 1990). PCR amplification was performed using primers dmaWe19copy2.1d (5’-AGAAACAGACAGGGCTATTTC-3’) and dmaWe19copy2.5u (5’-CTCGCCGGCATCGCTAAAA-3’), both specific for dmaW-2. Each 25-µl reaction mixture
contained 1 µl of DNA template, 1.25 mM each dNTP, 5 µM each of the two primers, 1X PCR buffer, and 1.25 units of TaqGold (Applied Biosystems, Foster City, CA) DNA polymerase. Reaction mixtures were held at 95°C for 9 min, then subjected to 35 cycles of the following program: 94°C for 30 sec, 61°C for 35 sec, 72°C for 3 min 10 sec in a Perkin-Elmer Applied Biosystems thermocycler (Gene Amp PCR system 2400).

**Southern blot analysis**

Restriction-endonuclease digestion of the genomic DNA, electrophoreses, and Southern blot were by standard protocols (Sambrook et al. 1989; Ausubel et al. 1999). The DNA was blotted onto positively-charged nylon membrane (catalog # 1 209 299, Roche-Boehringer, Indianapolis, IN) by the alkaline transfer method (Ausubel et al. 1999). Two hybridization probes were labeled with digoxigenin-dUTP (Roche-Boehringer, Indianapolis, IN) by PCR (Gebeyehu et al. 1987). A 500-bp-fragment probe for the upstream region of *dmaW*-2 was amplified by PCR with primers dmaW19copy2.9u (5’-ACCCGGGGAAATACACCAGGGGCTG-3’) and dmaWPrSalId (5’-GCGTCGACCTCATATCACGACTAAGGAGATAC-3’); the 1-kb probe for the region of *dmaW* downstream of the deleted portion was generated by PCR with primers dmaW19.07d (5’-CAACACCTTACACTTGACCC-3’) and dmaW19copy2.5u (CTCGCCGGCATGCCTAACCCCTG-3’). The membranes were prehybridized for 4-6 hours at 42°C in prehybridization solution [50% deionized formamide, 6X sodium chloride/sodium citrate (SSC) (Ausubel et al. 1999), 5X Denhardt's reagent, 0.1% sodium lauryl sulfate (SDS), 0.1% Na₂HPO₄·7H₂O, 50 mM Tris-HCl pH 7.5, 50 µg/ml herring sperm DNA]. The probe was denatured by incubation at 95°C for 12 min, and added to the prehybridization solution, then hybridization was performed at 42°C overnight. The membranes were washed once in 2 X SSC, 0.1% SDS for 5 min at room temperature, then twice at 65°C in 0.5 X SSC, 0.1% SDS for 25 min each. Immunological detection employed anti-digoxigenin antibody conjugated to alkaline phosphatase fragments and CSPD ready to use substrate according to the supplier’s instructions (Roche-Boehringer, Indianapolis, IN). The filters were exposed to double emulsion film (BioMax Light-2 Sigma, St.Louis, MO) to record the chemiluminescent signal.
Cotransformation

Each of plasmids pKAES175 and pKAES176, — containing \( \text{Pro}_{\text{tub2cre}} \) and \( \text{Pro}_{\text{dmaWcre}} \), respectively — was introduced into the \( \text{dnaW}-2 \) knockout mutant by cotransformation with pAN8-1, carrying a phleomycin-resistance gene (Mattern et al., 1988).

Protoplasts from the \( \text{dnaW}-2 \) knockout mutant were isolated as described earlier. The protoplasts were cotransformed by electroporation (Tsai et al., 1995) with pKAES175 or pKAES176: pAN8-1 in a molar ratio of 3:1. Transformants were selected on CRM plates containing 75 µg/ml phleomycin (Cayala, France) and screened by PCR using primers dmaWe19copy 2.1d and dmaWe19copy2.5u for pKAES175 and dmaW Pr SalI(d) and Cre SacII(u) for pKAES176.

Inoculation of seedlings

Tall fescue seedlings were inoculated as described by Latch and Christensen (1985). Kentucky 31 endophyte-free seeds were surface sterilized and germinated on wateragar for 5-6 days. The fungal mycelium was grown on PDA cellophane plates. The seedling meristems were slit and fungal mycelium was placed onto the wound. After inoculation, the seedlings were incubated at 21°C for 10 days in the dark and then in the light for 4-6 days. Then the seedlings were planted in flat trays containing soil and placed in the greenhouse. When the seedlings reached the 3 tiller stage, one tiller each was analyzed by tissue-print-immunoblot (TPIB) as described by Gwinn et al., (1991), to detect the presence of endophyte infection. The preparation of \( N. \text{coenophialum} \) antigen and production of polyclonal rabbit antibodies against it are described by An et al. (1993).

Ergovaline Analysis

Ergovaline was detected as described in Chapter 2 of this dissertation

Results

Homologous recombination in \( N. \text{coenophialum} \) ATCC 62374

The plasmid pKAES174, in was constructed with 341 bp of the \( \text{dnaW}-2 \) coding region (starting from the ATG initiation codon) deleted and replaced by a fungal-active \( \text{hph} \) gene flanked by \( \text{loxP} \) sites. This plasmid was introduced into \( N. \text{coenophialum} \) ATCC 62374 by
electroporation and polyethylene glycol (PEG) transformation. The transformants were screened by PCR analysis for homologous recombination. *N. coenophialum* has two homologs of *dmaW*. Therefore, in screening for knockouts of *dmaW*-2, I used the *dmaW*-2 homolog specific primers to PCR-amplify two fragments; one from wild-type 1.5 kb and the other from recombinant 2.5 kb if the construct was integrated into the genome. The recombinant 2.5 kb would replace wild-type 1.5 kb fragment if the homologous recombination had taken place (Fig 3.3). As controls, the wild-type *N. coenophialum* DNA was used as positive control template for amplification of the 1.5-kb fragment, and DNA from *N. uncinatum* (an endophyte of meadow fescue) was used as a negative control because it lacks *dmaW*. Out of 185 transformants screened, one did not amplify the wild-type 1.5-kb fragment but did amplify the 2.5-kb recombinant product (Fig 3.4). This transformant was considered a putative *dmaW*-2 knockout and screened further by Southern analysis to test the integrity of the 5’-flanking and 3’-flanking regions.

**Southern blot analysis**

Genomic DNAs of two single-spore isolates of a putative *dmaW*-2 knockout, an ectopic transformant where the mutant *dmaW* construct was integrated outside the targeted region, and *N. coenophialum* ATCC 62374 as a wild-type control, were digested with EcoRI, *Xba*I or *Hind*III, electrophoresis and blotted onto membranes. Two different probes labeled with digoxygenin were hybridized to the Southern blots, one to detect the upstream promoter region of *dmaW* and the other to detect the *dmaW* coding sequence downstream of the deleted region (Fig 3.5). When *Hind*III-digested DNA was hybridized with the upstream region probe (Fig 3.6), a 4.3 kb fragment was identified from wild type *dmaW*-1 *N. coenophialum* detected in all the lanes, indicating that the *dmaW*-1 was intact. Additionally, wild-type *dmaW*-2 (lane 3) had a 2.6-kb hybridizing fragment. The *dmaW*-2 knockout strain (lanes 1 and 2) lacked this fragment, but had the expected 3.4-kb fragment, confirming replacement of *dmaW*-2 with *loxP-hph-loxP* mutant *dmaW*-2. The ectopic transformant (lane 4) showed both the wild-type bands and the recombinant band indicating integration outside the targeted region.

Another Southern-blot analysis was conducted by digesting DNA with *Xba*I and probing the region 3’ of the *dmaW* coding region to check for any rearrangement in that region. As shown in Fig 3.7, the 5-kb hybridizing fragments (lane 3) was replaced by the expected 6-kb fragment (lanes 1 and 2), indicating homologous integration without rearrangement of the 3’-end
of the target site. DNA from all the lanes also had a 3.5-kb hybridizing fragment from \textit{dmaW-1}, further confirming that \textit{dmaW-1} was intact. These results indicated that \textit{loxP-hph-loxP} mutant \textit{dmaW-2} had replaced wild-type \textit{dmaW-2}, and the 5’ and 3’ flanking regions of \textit{dmaW-2}, as well as \textit{dmaW-1}, were intact in the disruptant. I will refer to this mutant as \textit{dmaW-2 ko} in the remainder of the chapter.

**Elimination of \textit{hph} in \textit{dmaW-2 ko}**

The \textit{dmaW-2} ko has \textit{loxP} sites flanking \textit{hph}, a substrate for the activity of Cre recombinase. To test if Cre is functional in \textit{N. coenophialum}, the \textit{dmaW-2} ko was cotransformed by electroporation with \textit{cre} expression construct pKAES175, and a phleomycin resistant vector pAN8-1. The transformants were screened by PCR for the integration of \textit{Pro\textsubscript{tm}}\textit{cre}. Six out of 22 transformants had the \textit{Pro\textsubscript{tm}}\textit{cre} integration into their genome. These transformants were screened further by PCR using the same set of the primers as used for \textit{dmaW-2} ko screening to check for the elimination of \textit{hph} (Fig 3.3). Elimination of \textit{hph} by Cre would result in a 1.2-kb fragment, compared to the 1.5-kb fragment in the wild type. The 6 transformants positive for \textit{Pro\textsubscript{tm}}\textit{cre} showed elimination of \textit{hph} (Fig 3.8). These transformants were plated on hygromycin B-containing medium, on which all failed to grow, further indicating the loss of \textit{hph}. Sequencing of these transformants to check for the junction of elimination of \textit{hph} showed retention of a single \textit{loxP} site as expected. These results demonstrated the function of Cre in specifically removing \textit{hph} that was located between \textit{loxP} sites.

**Cotransformation of \textit{dmaW-2 ko} with \textit{Pro\textsubscript{dmaw}cre}**

The construct pKAES176 has \textit{cre} under the control of the \textit{dmaW-2} promoter region, including 1120 bp region flanking the start codon of \textit{dmaW-2}. \textit{dmaW} is expressed in the \textit{N. coenophialum}-infected tall fescue and not in non-induced culture or in uninfected tall fescue (Wang 2000). Therefore, I undertook to check if \textit{cre} under the control of \textit{dmaW} promoter region would be expressed specifically when the fungus harboring the construct was introduced into the plants. The \textit{dmaW-2} ko was cotransformed with pKAES176 and pAN8-1 by electroporation. The transformants were screened by PCR for the integration of \textit{Pro\textsubscript{dmaw}cre} into the genome. Out of 30 transformants screened, four had \textit{Pro\textsubscript{dmaw}cre}. These four transformants were tested by PCR for the elimination of \textit{hph} as well as for the growth on hygromycin B-containing medium. All had
hph and were resistant to hygromycin B, indicating the presence of hph and suggesting that the dmaW promoter was not active in culture. These transformants were used to inoculate tall fescue ‘KY31,’ and the plants remain to be tested. Once the successful symbiota are established, these transformants will be checked for loss of hph, as expected if the dmaW promoter can drive the expression of cre in the symbiotum.

**Introduction of transformants into tall fescue cultivar KY31**

Ergot alkaloids are produced by *N. coenophialum* in grass-endophyte symbiota but not reliably in culture. Therefore, the *N. coenophialum* dmaW-2 ko transformant, dmaW-2 ko transformed with Pro_{dmaW}cre and Pro_{tub2}cre, and wild-type *N. coenophialum* ATCC 62374, were introduced into ‘KY31’ seedlings. Seedling infection frequencies for all transformants, as well as the wild-type *N. coenophialum* ATCC 62374, were extremely low compared to the normal frequency (15-25%), which is obtained with routine inoculations of tall fescue with other *N. coenophialum* isolates. TPIB results indicated that four out of 129 (3%) inoculated seedlings were positive for dmaW-2 ko, two out of 61 (3%) for Pro_{dmaW}cre, three out of 73 (4%) for Pro_{tub2}cre, and one out of 55 (2%) for wild-type. When the fungi were grown out of the seedlings and analyzed by PCR to check if they are the transformants that were introduced, only one seedling showed infection by dmaW-2 ko and the rest were wild-type *N. coenophialum*. These results indicated that a small percentage of KY31 seeds from the mainly endophyte-minus seed lot contained the wild-type endophyte, giving most of the TPIB-positive seedlings. Curiously, *N. coenophialum* ATCC 62374 had been inoculated into ‘KY31’ seedlings once previously, with a much more typical success rate of 20%. However, this isolate readily shows collapse of aerial hyphae in culture, a characteristic often associated with loss of host compatibility (M.J. Christensen, AgResearch, Palmerston North, New Zealand, pers. comm.). Thus, normal subculturing in the laboratory might have made affected compatibility with the plant. This will be tested by more inoculations with wild-type and dmaW-2 ko strains grown directly from infected plants, and inoculations with other isolates.

**Ergovaline analysis:**

Ergovaline analysis conducted on the plant e4393, the only plant that had established a successful symbiotum with dmaW-2 ko. This plant accumulated ergovaline (7 to 8 μg per g dry
weight of infected plant material) at a level similar to that of plants with wild type *N. coenophialum*.

**Discussion**

The results presented in this chapter demonstrate the use of the Cre/lox system in *N. coenophialum* to knock out *dmaW*-2 by homologous recombination, and the subsequent removal of the transformation marker by the action of Cre recombinase. Fescue toxicosis is a major problem in livestock agriculture and ergot alkaloids produced by Neotyphodium endophytes have been associated with fescue toxicosis. In chapter 2 we demonstrated the role of *dmaW* in encoding DMAT synthase, the first pathway specific step in ergot alkaloid biosynthesis in *Neotyphodium* sp. Lp1. *N. coenophialum* has two *dmaW* homologs, so I chose to use a Cre/lox system that potentially allows sequential use of the same marker gene in multiple gene knock-out strategies. A second reason for taking this approach is that the long term goal of this research is to have a modified strain of *N. coenophialum* that does not produce the toxic ergot alkaloids, and which can be used in the field application. The presence of a marker gene in the modified *N. coenophialum* will be a public concern, and use of Cre/lox system might help us to address this concern. For this reason I tested the feasibility of using a Cre/lox system in *N. coenophialum*.

The *dmaW*-2 homolog of *N. coenophialum* was targeted first because it encodes a predicted protein identical to that encoded by Lp1 *dmaW* (which is known to be functional as demonstrated in Chapter 2), and is transcribed in grass-endophyte symbiota (Wang. 2000). The frequency of marker exchange in the *N. coenophialum* *dmaW*-2 locus was less than 1%, which was similar to what was observed with *Neotyphodium* sp. Lp1 in Chapter 2. The *dmaW*-1 and *dmaW*-2 homologs share 96% identity at nucleotide level but are sufficiently divergent at the 5’ and 3’ flanking region to ensure that one or the other will be targeted depending on which flanking sequences are incorporated in the transforming plasmid. This information was useful to generate a homolog-specific construct to target one of *dmaW* homolog first. The results of PCR and Southern blot analyses indicated that *dmaW*-2 was replaced with the mutant *dmaW*-2 containing *loxP-hph-loxP*. Information on how far or on which chromosomal locations of *dmaW*-1 and *dmaW*-2 are located is unknown, but clearly *dmaW*-1 was not affected by marker exchange at *dmaW*-2.
A Pro<sub>dmaw</sub>cre construct was introduced into <i>dmaW-2</i> ko to facilitate elimination of <i>hph</i>. Inability of the transformants to grow on hygromycin B-containing medium, and the sequence of the region indicating the presence of only one loxP site, clearly demonstrated the function of Cre in specifically excising the <i>hph</i> gene that was between the loxP sites. To the best of my knowledge this is the first demonstration of the use of a Cre/lox system in a filamentous fungus. The <i>dmaW-2</i> ko without <i>hph</i> is not completely marker free because it has a phleomycin-resistance gene, which was introduced with <i>cre</i> for the reason that when <i>cre</i> was introduced without selection, there was no indication of transient expression of <i>cre</i> to facilitate loss of the <i>hph</i> marker (see Appendix 2). At this point it was not known if <i>cre</i> was not functional in <i>N. coenophialum</i> or that there was not enough transcript accumulated to translate Cre and drive the recombination. Introduction of <i>cre</i> with pAN8-1 demonstrated that <i>cre</i> is functionally expressed in <i>N. coenophialum</i> from a <i>tub2</i> promoter. The next attempt to produce a marker-free <i>dmaW-2</i> ko will involve introduction of Cre protein to <i>dmaW-2</i> ko.

<i>cre</i>, when expressed under the control of <i>tub2</i> promoter from <i>E. typhina</i>, was able to drive the expression of <i>cre</i> and eliminated the <i>hph</i> located in between loxP sites. A transformation protocol reported for <i>N. coenophialum</i>, where <i>hph</i> was expressed under the control of <i>tub2</i> promoter, produced hygromycin resistant transformants (Tsai et al 1992). Therefore the function of the 230 bp fragment used as <i>tub2</i> promoter was known. The <i>dmaW-2</i> promoter region of <i>N. coenophialum</i> has not been characterized, so I chose a large segment (1120 bp) of the upstream <i>dmaW-2</i> region in hopes of including the promoter. When <i>cre</i> was placed under the control of this region, Cre did not eliminate <i>hph</i> in culture. This was expected; given evidence from a study conducted by Wang (Wang 2000) that <i>dmaW</i> was expressed only in planta and not in non-induced culture. Expression in planta is expected based on reports that ergot alkaloid production is reliably detectable in tall fescue infected with <i>N. coenophialum</i> (Lyons et al 1986; Siegel et al 1990). The results obtained here compare the two promoter regions in culture and further support that <i>dmaW</i> promoter region is not functional in culture.

One of the difficulties in this study was the introduction of the <i>N. coenophialum</i> ATCC 62374; <i>dmaW-2</i> ko transformant as well as <i>dmaW-2</i> ko <i>Pro<sub>dmaw</sub>cre</i> and <i>Pro<sub>tub2</sub>cre</i> transformants into tall fescue ‘KY31.’ The difficulty introducing wild-type ATCC 62374 along with the transformants indicated that the genetic modification of <i>N. coenophialum</i> was not responsible for the reduced host compatibility, and the fact that <i>dmaW-2</i> ko was able to successfully colonize a
single plant (similar frequency as wild type) further supports that genetic manipulation did not affect host compatibility. Repeated subculturing of the ATCC 62374 culture in the laboratory might have allowed the fungus to lose host compatibility. This has been seen before with other endophyte species (C.L. Schardl personal observations, and M.J. Christensen, personal communications). \textit{N. coenophialum} ATCC 62374 strain was chosen because it is easier to transform in the laboratory (D.G. Panaccione personal communication). A critical test for \textit{dmaW-2} ko transformed with \textit{Pro_{dmaW}cre} was to introduce the transformant into plant to test the function of Cre in planta, but due to the host compatibility problem this test could not be completed.

Ergovaline, the primary ergot alkaloid produced by \textit{N. coenophialum} is synthesized in infected grass and probably is the only reliable indicator for expression of \textit{dmaW} or the ergot alkaloids. The only grass-plant that had infection with \textit{dmaW-2} ko transformant synthesized similar levels of ergovaline as compared to the plants with \textit{N. coenophialum} wild-type, suggesting that \textit{dmaW-1} also encoded DMAT synthase and was active in \textit{N. coenophialum}. This is an important finding and first genetic study of the role of both \textit{dmaW-1} and \textit{dmaW-2} in ergot alkaloid biosynthesis in \textit{N. coenophialum}. The \textit{dmaW} ko in Lp1 (Chapter 2) eliminated ergovaline and all other ergot alkaloids, and the predicted protein product of \textit{dmaW-2} of \textit{N. coenophialum} is identical to that of \textit{dmaW} from Lp1 and is transcribed in planta (Wang 2000). Thus, it appears that \textit{dmaW-2} should encode active DMAT synthase, and that the ko \textit{dmaW-2} was complemented with \textit{dmaW-1} in \textit{N. coenophialum}. The next step obviously will involve targeted disruption of \textit{dmaW-1} in a \textit{N. coenophialum} \textit{dmaW-2} ko strain.
Figure 3.1: Schematic diagram of the Cre/lox system. (A) Sequence of loxP site. The arrow above the spacer region indicates the direction of the loxP site. (B). Diagram explaining the excision of the region located between loxP sites by the action of Cre recombinase.
Figure 3.2: Electrophoretic analysis of PCR products generated for construction of *ProdmaW*-Cre and *Protub2*-Cre by SOE-PCR. (A) Products of first-round PCR-amplified DNAs containing the *dmaW* promoter region (lane 1), *cre* (lane 2) and *Protub2* (lane 3). (B) Second-round PCR-amplified *ProdmaW*-Cre (2.2 kb fragment in lane 4) and *Protub2*-Cre (1.3 kb fragment in lane 5). M1=125 bp marker, M2= 1 kb marker
Figure 3.3: Map of the \textit{dmaW-2} ko locus after Cre-mediated loss of \textit{hph}: A primer set, indicated as two arrows, is used for PCR-amplification of a 2.5 kb fragment from \textit{dmaW-2} ko with \textit{loxP-hph-loxP}, a 1.2 kb fragment from \textit{Δ-dmaW-2} after elimination of \textit{hph}, and a 1.5 kb fragment from \textit{wt dmaW-2} of \textit{N. coenophialum}. 

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\begin{figure}
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\includegraphics[width=\textwidth]{figure3.3.png}
\caption{Map of the \textit{dmaW-2} ko locus after Cre-mediated loss of \textit{hph}. A primer set, indicated as two arrows, is used for PCR-amplification of a 2.5 kb fragment from \textit{dmaW-2} ko with \textit{loxP-hph-loxP}, a 1.2 kb fragment from \textit{Δ-dmaW-2} after elimination of \textit{hph}, and a 1.5 kb fragment from \textit{wt dmaW-2} of \textit{N. coenophialum}.}
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Figure 3.4: PCR screening of *N. coenophialum* transformants for homologous recombinants in which mutated *dmaW*-2 (*dmaW*-2 ko) is substituted with the wild type (wt) gene. PCR with primer sets indicated in Fig 3.3 amplified a 1.5 kb fragment from wt *dmaW*-2 or a 2.5 kb fragment from *dmaW* ko with *loxP-hph-loxP* (lane 4). Nc= *N. coenophialum* as +control, Nu= *N. uncinatum* as -control. M1= lambda *HindIII* marker, M2=1 kb marker. Blank lanes indicate failed reactions.
Figure 3.5: Maps of the wild type $dmaW-2$ locus, targeting vector and expected map of the locus after marker-exchange mutagenesis. (A) Map showing HindIII sites (vertical lines). (B) Map of XbaI sites (vertical lines). Double headed arrows indicate expected fragments with sizes indicated in kb. The segment used as probe for the Southern blot in Fig 3.6 (A) and Fig 3.7 (B) are indicated.
Figure 3.6: Southern-blot analysis indicating *N. coenophialum dmaW*-2 ko.

Genomic DNAs were digested with *Hin*dIII. Lanes 1 and 2 contained DNA from two single-spore isolates of *dmaW*-2 ko, lane 3 contained wt *N. coenophialum* DNA, and lane 4 contained DNA from an ectopic transformant. The 4.3-kb band in each lane was from *dmaW*-1. Other fragment sizes were as indicated in Fig 3. 5 panel A. M = *Hin*dIII-cut bacteriophage lambda DNA as a size marker ladder.
Figure 3.7: Southern-blot analysis of *N. coenophialum* *dmaW*-2 ko. Genomic DNA was digested with *Xba*I. Lanes 1 and 2 contained DNA from two single-spore isolates of *dmaW*-2 ko, lane 3 contained wt *N. coenophialum*, and lane 4 contained DNA from an ectopic transformant. M=marker as in Fig 3.6. The 3.5-kb band in each lane was from *dmaW*-1. The other fragments were of expected sizes as indicated in Fig 3.5 panel B.
Figure 3.8: PCR analysis indicating elimination of hph by Cre recombinase: A PCR test for N. coenophialum dmaW-2 ko transformed with Protub2Cre. Transformants no. 1, 3, 11, 12, 14 and 17 have lost hph, as indicated by the 1.2-kb PCR product, PCR from N. coenophialum wt (Ncw) DNA amplified a 1.5-kb fragment, and PCR from dmaW-2 ko (Nck) amplified a 2.5-kb fragment as expected (See Fig 3.3). M is 1 kb marker.
Chapter 4

Comparison of *Claviceps purpurea* and *Claviceps fusiformis* genome regions near *dmaW* homologs

Introduction

*Claviceps purpurea*, *Claviceps fusiformis* and *Neotyphodium* species all belong to the family Clavicipitaceae, and many strains in both genera produce ergot alkaloids and have homologs of *dmaW*. The presence of *dmaW* in ergot alkaloid-producing fungi and absence in non-producing fungi has been shown by Wang (Wang, 2000). The ergot fungi (*Claviceps* spp.) cause disease on a broad range of cereal and grass species and form sclerotia on seed heads. Sclerotia — also known as “ergots” — contain diverse alkaloids, especially the derivatives of 4,4-dimethylallyltryptophan that are collectively known as ergot alkaloids. In medieval times ergot poisoning in humans was caused by consuming rye contaminated by ergots of *C. purpurea*. Ergot alkaloids are toxic to livestock that graze on grass or consume feed infested with ergot sclerotia. Different types of ergot alkaloids include clavine alkaloids, lysergic acid, simple amides of lysergic acid, and more complex amides with polycyclic peptides. *C. purpurea* produces mainly the peptide type (ergopeptines) of ergot alkaloids, and *C. fusiformis* produces the clavine type of ergot alkaloids.

Biosynthesis of ergot alkaloids involves multiple steps, which have been well studied (Floss 1976). The first determinant step is catalyzed by a prenyltransferase, dimethylallyltryptophan synthase (DMAT synthase), which catalyses the formation of dimethylallyltryptophan from dimethylallyl pyrophosphate (DMAPP) and tryptophan. The gene encoding DMAT synthase, *dmaW*, has been cloned from *C. fusiformis* (ATCC 26245) by Tsai et al. (1995) and then from *C. purpurea* ATCC 20102 by Wang (2000). Tudzynski et al (1999) cloned and analyzed the ergot alkaloid gene cluster from *C. purpurea* isolate P1. *C. purpurea* ATCC 20102 (the parent strain of P1) has two copies of *dmaW*, one is functional (demonstrated by expression in yeast; Wang, 2000) and the other one is a pseudogene with 7 bp insertion in the coding region.

In fungi, genes involved in secondary metabolism often occur in clusters (Keller and Hohn 1997). *dmaW* was first cloned from *C. fusiformis* by Tsai et al (1995), who first demonstrated activity of *dmaW* to encode the authentic dimethylallyltryptophan synthase enzyme.
(Tsai et al. 1995). The presence of any other ergot alkaloid biosynthesis genes in *C. fusiformis* has not been investigated previously. *C. fusiformis* produces clavine alkaloids, completing the pathway (Fig 1.1) at chanoclavine or agroclavine or elymoclavines.

The objectives of this study are to look for genes that are clustered with *dmaW* in *C. purpurea* and *C. fusiformis* and that may be involved in ergot alkaloid biosynthesis, and to compare the genes and their arrangements in two *Claviceps* species that produce different subsets of ergot alkaloids. This chapter describes the sequencing and identification by homology of genes/ORFs (open reading frames) putatively involved in ergot alkaloid biosynthesis, and the finding that at least nine such genes are shared by *C. purpurea* and *C. fusiformis*.

**Materials and Methods**

**Source of cosmid clones**

Genomic cosmid libraries of *C. purpurea* ATCC 20102 and *C. fusiformis* ATCC 26245 were prepared by J. Wang and H.F. Tsai, respectively (Wang 2000; Tsai et al. 1995). Three cosmid clones from the *C. purpurea* ATCC 20102 library — two (Cp26A07 and Cp27A01) containing a functional *dmaW* and one containing a non-functional *YdmaW* (Cp39F02) were chosen for sequence determination. These three cosmid clones were identified previously by Wang (2000) and demonstrated to contain functional and non-functional *dmaW* homologs. One cosmid clone from *C. fusiformis* ATCC 26245 (Cf26E11), positive for the presence of *dmaW*, was also chosen for sequencing. This cosmid clone was identified previously by Tsai (Tsai et al. 1995).

**Transposon-insertion libraries of cosmids**

The Genome Priming System (GPS –1, NewEngland Biolabs Inc.) is a transposon based system to generate a population of DNA sequencing templates by randomly inserting the transprimer transposon into target DNA. The transprimer transposon has two unique primer binding sites on both the ends, which allows sequencing DNA from both the strands of the target DNA. The transprimer encodes kanamycin resistance for selection of product DNA template. Transposition was carried out as per the manufacturer’s instructions. Each sample contained 2 µl of 10X GPS buffer (Reagent 1), 1 µl containing 0.02 µg of pGPS1.1 donor DNA (Reagent 2a),...
100 ng (in 1 µl) of target cosmid DNA, and 14 µl of H₂O to which 1 ul of TnsABC Transposase (Reagent 3) was then added. Reactions were incubated 10 min at 37°C and 1 µl of start solution (Reagent 4) was added and incubated for 1 hr at 37°C. The reaction was heat-inactivated at 75°C for 10 min. The DNA was introduced by electroporation into XL1-Blue electrocompetent cells (Stratagene), as per the manufacturer’s instruction. Colonies grown were selected on LB medium containing ampicillin (50 µg/ml) and kanamycin (20 µg/ml). For each of cosmids Cp26A07, Cp27A01 and Cf2611E, approximately 800 colonies were picked for storage and sequencing.

The colonies were picked into 96 well plates containing 900 ml of LB and 100 ml of 10X freezer medium [440 ml of 99% glycerol, 0.36M K₂HPO₄, 0.132 M KH₂PO₄, 0.017M Na-Citrate, 0.004M MgSO₄ and 0.068M (NH₄)₂SO₄], and backed up into 384 well plates as GPS libraries stored at –80°C. Each colony harbored an independent transposition, which was sequenced using two primers annealing to opposite ends of the transprimer transposon: primer S (5’-ATAATCCTTTAAAAACTCCATTTCCACCCCT-3’) and primer N (5’-ACTTTATTGTCA TAGTTTAGATCTATTTTG-3’).

**Cosmid Subcloning**

Cosmid DNA from Cp39F02 was prepared using Qiagen maxi prep kit (Qiagen), then treated overnight at 37°C with Plasmid-safe DNase (Epicentre), as per instructions, to remove *E. coli* DNA. DNase was inactivated by incubating at 70°C for 30 min. The DNA was ethanol-precipitated and the pellet was dissolved in 300 µl TE buffer, and filter sterilized using 0.22 µm Costar filters. Cosmid DNA was sheared into 2 kb fragments with a HydroShear (GeneMachines). The DNA was precipitated with 0.1 vol 3 M sodium acetate and 2 vol 95% ethanol, pelleted, and dissolved in 34 µl 10 mM Tris-HCl pH 8.0. The ends of the sheared DNA were repaired using the End-it DNA end-repair kit from Epicentre as per instructions. The reaction was stopped by incubating at 70°C for 10 min. The sheared end repaired DNA was size-separated by electrophoresis in Sea Plaque low-melting-temperature agarose gel, and fragments of 1.8 kb – 2.3 kb were extracted from the gel using QIAquick gel extraction kit (Qiagen). The eluted DNA was again ethanol-precipitated, and the pellet was dissolved in 6 µl of 10 mM Tris-HCl pH 8.0.

Vector pBCKS+ (Stratagene) was digested with *Sma*I and *Eco*RV, and 5’-terminal phosphates were removed by using calf intestine alkaline phosphatase (NewEnglad Biolabs).
DNA was extracted using phenol: chloroform: IAA (25: 24: 1) and precipitated with ethanol, DNA was purified again using Qiagen protocol (Special application: purification of plasmid DNA by other methods) (as per the instructions) and electrophoresed in a 2% SeaPlaque low–melting-temperature agarose gel. DNA was extracted from the gel by using QIAquick gel extraction kit (Qiagen) and eluted in 50 ul EB buffer (Qiagen). Ligation of the sheared DNA and purified vector employed the Fast-link DNA ligation kit (Epicentre). The ligated DNA was introduced into *E. coli* XL1-Blue electroporation competent cells (catalog# 200228 from Stratagene) by electroporation. Cells were plated on LB agar (with X-gal and IPTG) with chloramphenicol (25 µg/mL). Approx. 1000 -1200 white colonies were picked and arrayed, and each colony was sequenced using the two primers, KS (5’-TCGAGGTGCAGCTATC) and SK (5’-CGCTCTAGAACTAGTGGATC).

**Preparation of cosmid and plasmid DNA**

*Escherichia coli* colonies with cosmid clones and plasmid clones arrayed in 96 well plates were replicated into Whatman 2-ml-well plates (Whatman # 7701-5205) containing 2X YT medium (16 g of Tryptone, 10 g of yeast extract, 5 g of NaCl per liter, adjust to pH 7). The colonies were grown at 37°C for 15 – 17 hr for cosmid and 12-14 for plasmid clones in a HiGro (GeneMachines) shaking incubator at 425 rpm for cosmid and 525 rpm for plasmid, oxygenation 2 hr delay and gas flow cycle set at pulse timer 0.5 sec. The protocol for DNA extraction was as follows: The plates were spun at 1690 g for 1 min, and the supernatant was discarded. DNA was extracted from cells by using solution P1, P2 and P3 from Qiagen. RnaseA was added to solution P1 and 100 µl of P1 was added to each well, and the plates covered with capmats (Whatman # 7704-0104) were vortexed until pellets were completely suspended. Then 200 µl of P2 was added, gently mixed and incubated at room temperature for 5 min, 150 µl of P3 was added, mixed well and incubated at room temperature for 10 min. The lysate was then transferred to a lysate clarification filter plate on a uniplate collection plate (Whatman #s 7720-2830 and 7701-5750). The filter apparatus was spun for 15 min at 1690 g, and the filter plate was discarded. To each well of elute was added 200 µl isopropanol, and the plates were sealed with capmats and mixed by inverting 5-6 times. The plates were incubated 20 min at room temperature, and the DNA was pelleted by centrifugation for 30 min at 1690 g. Pellets were washed with 500 µl of 70% ethanol and the plates were spun for 10 min and the ethanol was discarded. Pellets were
dried for 30 min at room temperature, then the DNA was redissolved in 20 \mu l of water. DNA was quantified by Hoechst dye staining, measured with DyNA Quant 200 fluorometer (Hoefer Scientific Instruments, Amersham Pharmacia biotech. San Francisco, CA)) and fluoroimager (FluoroImager 595 Amersham Biosc. Corp, NJ).

**DNA Sequencing**

CEQ 2000 Dye terminator cycle sequencing with quick start kit (Beckman-Coulter) was used for sequencing as per manufacturer’s instructions. Each reaction contained, 500 ng cosmid DNA template, or 250 ng of plasmid template, 6.4 \mu M of sequencing primer, 2 \mu l of DTCS quick start master mix, 1.5 \mu l of sequencing buffer and H\textsubscript{2}O to 20 \mu l. DNA templates were heated at 96°C for 3 min before adding the rest of the components, then the sequencing reaction was run for 60 cycles for cosmid or 35 cycles for plasmid with the following profile in an Applied Biosystems model 9700 thermocycler: 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 min.

Sequencing reactions were stopped by adding of 2 \mu l of 3M NaOAc, pH 5.2, 2 \mu l of 100 mM Na\textsubscript{2}EDTA, pH 8.0, and 1 \mu l of 20 mg ml\textsuperscript{-1} glycogen to each well. Then 60 \mu l of 95% ethanol was added to each well, the plate was sealed with tape (Qiagen tape pads #19570) and vortexed for 5-10 sec. The plate was centrifuged for 5 min at 6102 g at 4°C. The supernatant was discarded; the plate was inverted in the centrifuge and spun at 8 g for 20 sec. The pellets were washed two times with 70% ethanol, each time centrifuging at 6102 g for 3 min. After the final wash the plate was inverted and spun at 8 g for 20 sec to remove residual ethanol. The samples were vacuum dried for 10 min and redissolved in 40 ul of sample loading solution (SLS; Beckman-Coulter). The samples were overlaid with a drop of mineral oil and subjected to sequence determination by capillary electrophoresis in the Beckman-Coulter CEQ2000 system.

As an alternative, sequencing reactions were cleaned with CleanSEQ (Agencourt Bioscience Corp.). To each sample was added 10 \mu l of CleanSEQ magnetic particle suspension, followed by 62 \mu l of 85% ethanol (for 20 \mu l vol reaction). The plate was sealed with tape and vortexed at medium speed for 10 sec. The sequencing plate was placed onto the magnetic plate (SPRI plate96-R ring magnetic plate, Agencourt) for 3 min to separate the beads from the solution. The cleared solution was removed by pipetting. The samples were washed with 100 \mu l of 85% ethanol at room temperature on the magnetic plate for 30 sec. The ethanol was removed...
by pipetting, and the sequencing plate was air-dried for 10 min. Each sample was redissolved in 40 µl of SLS and overlaid with a drop of mineral oil before subject to sequencing.

The contigs (see below, Sequence analysis) were stitched together by designing primers to walk out of each contig, and the distances between the contigs were analyzed by PCR amplification. The distance between the two contigs as detected by PCR amplification was sequenced as per the manufacturer’s instruction (Beckman-Coulter) to connect two contigs.

Sequence analysis

Sequences were assembled using Phrap/Consed package on the Linux platform. Phrap is a fragment assembly program for assembling shotgun DNA sequence data, and Consed is a program for viewing and editing assemblies assembled with Phrap assembly program. (David Gordon; gordon@genome.washington.edu).

Contig sequences were used as queries in BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) against the predicted protein database (blastx). In addition, contigs were submitted to FGENESH (http://www.softberry.com/berry.phtml) to predict the potential genes based on parameters for Aspergillus spp. and Neurospora crassa genomes. The predicted open reading frames (ORFs) were then further submitted to blastx and rpsblast.

Results

Analysis of sequences from Claviceps purpurea locus 1

Sequences assembled from the overlapping cosmids, Cp26A07 and Cp27A01 were designated locus 1. A 59.1 kb contig when subjected to BLAST and FGENESH analysis revealed nine ORFs upstream and eight ORFs downstream of dmaW (Fig 4.1; Table 4.1 and 4.2). The two ORFs, hypA and hypB just upstream of dmaW, were predicted to encode proteins significantly similar to hypothetical proteins from N. crassa and A. nidulans. Of these, hypA revealed similarity to nucleoside diphosphate sugar epimerase (conserved protein domain search) and hypB showed significant similarity to an uncharacterized conserved protein. Upstream of hypB were predicted genes whose inferred products were significantly similar to: (oxF) FAD/FMN-containing dehydrogenases, and (oxA) a short-chain-alcohol dehydrogenase from C.
purpurea (Accession # CAB39328 and CAB39316 respectively). Downstream of oxA were ORFs similar to (catA) a catalase from Emericella nidulans (AAG45152), and (oxP) a cytochrome P450 from Fusarium sporotrichoides (AAK33073). The three ORFs at the 5’ end of the contig included lpsB (cpps2), encoding a mono modular peptide synthetase, oxO (cppx3) (Tudzynski et al. 1999), whose predicted product showed similarity to NADH: flavin oxidoreductases (old yellow enzyme), and npsA (cpps3; Tudzynski et al. 1999), another gene which is predicted to encode a monomodular peptide synthetase. Sequence information of ORF npsA, oxO and part of lpsB were provided by P. Tudzynski (Universität Münster, Germany).

The 33 kb sequence downstream of dmaW revealed eight ORFs (Fig 4.1; Tables 4.1 and 4.2). This included two closely related tri-modular peptide synthetase genes, the first of which (lpsA-1) corresponded to the gene previously identified by Tudzynski et al. (1999), which they tentatively designated (cpps1). I have designated the two predicted tri-modular peptide synthetase genes lpsA-1 and lpsA-2 (Fig 4.1). Gene 1psA-2 had 89% identity at the nucleotide level to lpsA-1 (as detected by BLAST seq 2 analysis). lpsA-1 contains an intron in the terminal part of second module (Tudzynski et al. 1999) and lpsA-2 when compared with lpsA-1 for the intron region, also showed the presence of an intron at a similar position as lpsA-1 but was 11 bp longer than the intron of lpsA-1. Sequence analysis of the region between dmaW and lpsA-1 revealed an ORF designated oxH. The predicted product of oxH was significantly similar to that of Gibberella moniliformis fum3, which encodes a hydroxylase involved in fumonisin biosynthesis (Proctor et al. 1999). Between lpsA-1 and lpsA-2 was another ORF, hypC, which showed a match to a hypothetical protein from A. nidulans (EAA66313) in the database and for which no putative conserved domain was identified. In the 5 kb region downstream of lpsA-2, four ORFs (designated orf, Fig 4.1) with no significant matches by BLAST and conserved domain searches (data not shown in the table) were detected. These ORFs did not have any significant matches to genes or conserved protein domain in the database.

Analysis of sequences from Claviceps purpurea locus 2

Sequences assembled from Cp39F02 are designated C. purpurea locus 2. Cp39F02 cosmid clone has a non-functional dmaW, designated □ dmaW, with a 7 bp insertion in the coding region. Ten ORFs were detected in 26.5 kb of locus 2, three upstream and six downstream of □ dmaW (Fig 4.1; Tables 4.3 and 4.4). Three ORFs upstream of □ dmaW, □ lpsB,
oxF and hypB2, were related to genes from C. purpurea locus 1, not in the same order but in similar orientation (Fig 4.1). lpsB had similarity to lpsB, a monomodular peptide synthetase from C. purpurea. Only a small 3’-terminal part (125 bp) of lpsB seemed to be represented in the C. purpurea locus 2, so it is unlikely that this is a functional lpsB gene. lpsB did not predict any conserved protein domain by database search (Table 4.4). oxF had similarity to oxF, predicted to encode an oxidoreductase, FAD/FMN-containing dehydrogenase (Table 4.3) from C. purpurea locus 1, this is also most likely a non-functional gene because a small part of the oxF sequence is represented in C. purpurea locus 2 and also oxF did not predict any conserved protein domain by rpsblast search (Table 4.4). The predicted product of hypB2 showed significant similarity to a hypothetical protein gene from N. crassa (XP323696), and was predicted to encode an uncharacterized conserved protein.

There were six ORFs downstream of dmaW, hypD showed similarity to a hypothetical protein gene from M. grisea (EAA56058) and the protein predicted from hypD had a conserved domain similar to isoamyl acetate-hydrolyzing esterase and related enzymes (Table 4.4). The deduced protein product from vapA showed significant similarity to a vacuolar protein sorting-associated gene from Schizosaccharomyces pombe. The ORF, hypE showed similarity to a hypothetical protein gene from M. grisea and its deduced protein product showed similarity to a signalosome subunit. The deduced product of capB showed significant similarity to a capsule associated protein from Ustilago maydis and was predicted to have conserved domain similar to a lipopolysaccharide-modifying enzyme. hypF showed similarity to a hypothetical protein gene form M. grisea and its deduced protein product had similarity to a hydroxylase. The predicted product of ptpC had highly significant similarity to peptide transporter from Schizophyllum commune. Unlike the dmaW region in C. purpurea locus 1, where a tri-modular peptide gene was identified within 3 kb downstream of dmaW, no similar gene was present within 17 kb downstream of dmaW in C. purpurea locus 2.

Analysis of sequences from Claviceps fusiformis identify nine genes similar to those in the C. purpurea locus 1

In order to find out if C. fusiformis contains other genes involved in ergot alkaloids biosynthesis in a cluster as seen in C. purpurea ATCC 20102 and to determine the genes and their arrangement relative to those in C. purpurea, cosmid clone Cf26E11 containing dmaW was
sequenced. A total of 31.8 kb sequenced region revealed that \textit{dmaW} was located at one end of the cosmid clone and 13 other ORFs were upstream of \textit{dmaW} (Fig 4.1; Table 4.5 and 4.6). The ORFs \textit{hypG}, \textit{hypH} and \textit{hypI} showed significant similarities to hypothetical protein genes from \textit{A. nidulans} with matches to uncharacterized conserved proteins in the database. The predicted \textit{hypJ} product was significantly similar to predicted protein from \textit{A. nidulans} (EAA65743). The ORF \textit{apD} encoded a putative protein with significant similarity to methionine aminopeptidase from \textit{Leptospira interrogans}.

The contig in \textit{C. fusiformis} had nine genes/ORFs with similarity to \textit{C. purpurea} genes in the ergot alkaloid biosynthesis cluster 1 (Table 4.7). The seven genes from \textit{oxP} to \textit{dmaW} were arranged in the same order and orientation in \textit{C. fusiformis} and \textit{C. purpurea} (Fig 4.1). Upstream of \textit{oxP} were two ORFs, \textit{oxO} and \textit{lpsB} whose similarity to \textit{oxO} and \textit{lpsB} from \textit{C. purpurea} locus 1 with respect to deduced protein description, and conserved domains was similar; however, their order and orientation in \textit{C. fusiformis} was inverted relative to \textit{C. purpurea} locus 1 (Fig 4.1). The \textit{lpsB} of \textit{C. purpurea} contains an intron and \textit{lpsB} of \textit{C. fusiformis} when compared with \textit{lpsB} of \textit{C. purpurea} for intron region showed a different pattern and may not contain an intron in that position (preliminary observation). The description of the nine common genes and their deduced proteins shared between \textit{C. fusiformis} and \textit{C. purpurea} is shown in Table 4.7.

**Discussion**

The results presented in this chapter show the presence of ergot alkaloid gene cluster in \textit{C. purpurea} ATCC 20102 and \textit{C. fusiformis} ATCC 26245. \textit{Claviceps purpurea} and \textit{C. fusiformis} are closely related ergot alkaloid producing fungi. The end product of ergot alkaloid biosynthesis in \textit{C. purpurea} is ergopeptines, ergotamine is the major type of ergopeptines synthesized, other types are ergocrystine, ergocryptine, ergosine. In \textit{C. fusiformis} the ergot alkaloid biosynthesis ends with the production of clavine alkaloids, some strains of \textit{C. fusiformis} synthesize chanoclavine-I as the major compound and other strains produce agroclavine or elymoclavine.

Comparison of \textit{C. purpurea} and \textit{C. fusiformis} ergot alkaloid gene clusters is informative because these two \textit{Claviceps} species produce two different types of ergot alkaloids. \textit{C. purpurea} completes the pathway with production of more complex ergopeptines and \textit{C. fusiformis} completes the pathway early on with production of different sets of clavines. My results indicate the presence of nine common genes between \textit{C. purpurea} and \textit{C. fusiformis}, indicating these
might be involved in the early part of pathway up till the production of clavines in both Claviceps spp. Two genes encoding putative oxidoreductases oxA, a short-chain-alcohol dehydrogenase and oxF, FAD/FMN containing dehydrogenase might have functions in the early steps in the pathway of forming chanoclavine-I. Formation of chanoclavine-I from N-methyl-DMAT involves several steps requiring several monooxygenases/ oxidoreductases (Kozikowski et al. 1988). The role of a catalase (catA) is difficult to explain in the ergot alkaloid biosynthesis, although a catalase might be involved in the formation of chanoclavine-1-aldehyde from chanoclavine-I. The cytochrome P450 monooxygenase (oxP) and NADH dehydrogenase (oxO) might be good candidates for formation of clavines, agroclavine or elymoclavine, key intermediates in C. purpurea or end products of biosynthesis in C. fusiformis. Formation of agroclavine from Chanoclavine-1-aldehyde and elymoclavine from agroclavine require monooxygenases (Fig 1.1). A known enzyme in the pathway, agroclavine-17-monooxygenase, converts agroclavine to elymoclavine (Kim et al 1981) and this oxygenase is thought to be dependent on NADPH and molecular oxygen (Tudzynski et al 1999) and hence may be a cytochrome P450 monooxygenase. Possible roles of hypA and hypB products are obscure.

The presence of a lpsB homolog in C. fusiformis is intriguing. In C. purpurea lpsB encodes LPS2, the D-lysergic acid-activating module and lpsA-I encodes LPS1, which has the three amino acid modules involved in ergopeptide lactam synthesis (Tudzynski et al. 2001)(Correia et al 2003). The D-lysergyltripeptide lactams are assembled from D-lysergic acid and the three amino acids by non-ribsomal peptide synthetases, and ergopeptines are derived from D-lysergyltripeptide lactam (Tudzynski et al. 2001). C. fusiformis does not synthesize D-lysergic acid or lysergic acid derivatives, yet appears to harbor a gene (lpsB) whose product is known to be involved in the activation of D-lysergic acid, which has been demonstrated by gene knockout of lpsB in C. purpurea (Correia et al 2003). At this point the role of lpsB in C. fusiformis is completely not known but it will be interesting to find out if lpsB knockout of C. purpurea can be complemented with lpsB of C. fusiformis.

Tudzynski and coworkers sequenced ergot alkaloid biosynthesis cluster from C. purpurea strain P1 (Tudzynski et al. 1999), which is a laboratory-derived strain of C. purpurea ATCC 20102. They obtained sequences up to the gene for one tri-modular peptide synthetase, lpsA-I, and I confirmed the presence of the genes they had identified and extended my search further downstream of lpsA-I, identifying another homologue of the tri-modular peptide synthetase gene.
(lpsA-2). lpsA-1 and lpsA-2 are separated by ~ 5 kb region and share 89% identity. C. purpurea produces more than one type of ergopeptines, having more than one peptide synthetase genes might help the fungus to synthesize different kinds of ergopeptines. Peptide synthetases contain a separate module for each amino acid and each module harbors three domains, for recognizing and activating the substrate amino acid, for thiolation and for condensation (Marahiel et al. 1997). Ergopeptines are synthesized from trimodular peptide synthetase and different ergopeptines can be synthesized by substitution of amino acids in the tripeptide molecule region (Table 1.1).

There is a large variation in the genome regions around dmaW and [?]dmaW in C. purpurea locus 1 and locus 2. Two out of three ORFs that were similar between locus 1 and locus 2 seem to be highly truncated and non functional in locus 2. The region downstream of [?]dmaW has different genomic context compared to dmaW in locus 2. The role of [?]dmaW is not clear but non-functionality of [?]dmaW indicates that it is not required for ergot alkaloid biosynthesis in C. purpurea ATCC 20102.

So far three of the genes in ergot alkaloid biosynthesis have been characterized by gene knockout studies in Neotyphodium sp., Lp1 and Claviceps purpurea strain P1. The dmaW knockout in Neotyphodium sp. Lp1 (Wang et al. 2004) apparently blocked the entire pathway and eliminated production of all clavine and ergot alkaloids. The lpsA knockout (homolog of lpsA-1 in C. purpurea) in Neotyphodium sp. Lp1 did not produce any ergovaline (ergopeptine), ergine and lysergyl-alanine (simple lysergic acid amides), but did produce clavines. This provided evidence that formation of simple lysergic acid amides requires complex ergopeptides. There was an increase in the accumulation of lysergic acid and a novel clavine in lpsA knockout, but other clavines were not altered, suggesting that lysergic acid feeds back and regulates the pathway (Panaccione et al. 2001; Panaccione et al. 2003). This study by Panaccione et al. (2001) indicated that the lpsA operates downstream in the pathway compared to dmaW. dmaW is the first pathway specific step in the ergot alkaloid biosynthesis, blocking this step did eliminate production of all ergot alkaloids. The lpsB (cpps2) knockout in C. purpurea strain P1 accumulated D-lysergic acid and did not form ergopeptines (Correia et al 2003).

Now that a putative ergot alkaloid biosynthesis cluster in C. fusiformis and C. purpurea has been identified, studies might help in dissecting the pathway by individually knocking out other genes and determining in which other steps they operate. It will also be interesting to see if
ergot alkaloid biosynthesis genes in *Neotyphodium* sp. will be clustered. As *Claviceps* and *Neotyphodium* belong to the same family Clavicipitaceae, this might provide information about the evolution of the ergot alkaloid biosynthesis pathway in Clavicipitaceae. Preliminary sequencing of the region around *dmaW1* in *N. coenophialum* does not indicate clustering of genes (see Appendix 3); however, *N. coenophialum* has second homolog, *dmaW-2*. Obtaining sequences around *dmaW-2* would be critical to do a comparison between *Claviceps* and *Neotyphodium* species.
Table 4.1: Predicted open reading frames from sequences near *dmaW* in *Claviceps purpurea* locus 1.

<table>
<thead>
<tr>
<th>Orf description</th>
<th>Organism</th>
<th>Deduced protein description by blastx</th>
<th>Accession no.</th>
<th>Scores</th>
<th>% Identity/E value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>npsA</em></td>
<td><em>Claviceps purpurea</em></td>
<td>d-lysergyl peptide synthetase</td>
<td>CAB39315</td>
<td>876</td>
<td>41 / 0.0</td>
</tr>
<tr>
<td></td>
<td><em>C. purpurea</em></td>
<td>Peptide synthetase</td>
<td>CAD28788</td>
<td>551</td>
<td>30 / e-155</td>
</tr>
<tr>
<td><em>oxO</em></td>
<td><em>Neurospora crassa</em></td>
<td>NADPH dehydrogenase</td>
<td>XP_323805</td>
<td>335</td>
<td>52 / 2e-95</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Old yellow enzyme</td>
<td>Q02899</td>
<td>219</td>
<td>37 / 3e-60</td>
</tr>
<tr>
<td><em>lpsB</em></td>
<td><em>Claviceps purpurea</em></td>
<td>Peptide synthetase</td>
<td>CAD28788</td>
<td>1991</td>
<td>93 / 0.0</td>
</tr>
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<td><em>A. nidulans</em></td>
<td>Hypothetical protein</td>
<td>EAA58507</td>
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<td>29 / 3e-43</td>
</tr>
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<td></td>
<td><em>Fusarium sporotrichoides</em></td>
<td>Cytochrome P450</td>
<td>AAK33073</td>
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<td>Cochliobolus carbonum</td>
<td>HC-toxin synthetase</td>
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Table 4.2: Conserved protein domains deduced by BLAST analysis (rpsblast) from sequences near *dmaW* in *Claviceps purpurea* locus 1.

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<th>ORF designation</th>
<th>Conserved protein domain</th>
<th>Scores</th>
<th>E value</th>
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</thead>
<tbody>
<tr>
<td><em>npsA</em></td>
<td>AMP binding domain&lt;br&gt;Non ribosomal peptide synthetase modules and related proteins</td>
<td>284</td>
<td>7e-66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>237</td>
<td>2e-62</td>
</tr>
<tr>
<td><em>oxO</em></td>
<td>NADH: flavin oxidoreductase&lt;br&gt;NADH: flavin oxidoreductase, old yellow enzyme family</td>
<td>252</td>
<td>7e-68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>244</td>
<td>2e-65</td>
</tr>
<tr>
<td><em>lpsB</em></td>
<td>AMP binding domain&lt;br&gt;Non ribosomal peptide synthetase modules and related proteins</td>
<td>236</td>
<td>2e-62</td>
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<td></td>
<td></td>
<td>203</td>
<td>2e-52</td>
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<td>Cytochrome P450</td>
<td>89</td>
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<td>Catalase</td>
<td>433</td>
<td>6e-122</td>
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<td><em>oxA</em></td>
<td>Short chain dehydrogenase&lt;br&gt;FabG, dehydrogenases with different specificities, related to short-chain-alcohol dehydrogenases</td>
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<td>6e-27</td>
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<td>113</td>
<td>6e-26</td>
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<td>FAD binding domain&lt;br&gt;FAD/FMN containing dehydrogenases</td>
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<td></td>
<td>73</td>
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<tr>
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<td>Phytanoyl-CoA dioxygenase&lt;br&gt;Protein involved in biosynthesis of mitomycin antibiotics/polyketide fumonisin</td>
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<td><em>lpsA-1</em></td>
<td>Non ribosomal peptide synthetase modules and related proteins&lt;br&gt;Condensation domain</td>
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Table 4.3: Predicted open reading frames from sequences near \( \text{dmaW} \) in *Claviceps purpurea* locus 2.

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<th>Orf description</th>
<th>Organism</th>
<th>Deduced protein description by blastx</th>
<th>Accession no.</th>
<th>Scores</th>
<th>% Identity/E value</th>
</tr>
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<tbody>
<tr>
<td>( \text{lpsB} )</td>
<td><em>Claviceps purpurea</em></td>
<td>Peptide synthetase, monomodular</td>
<td>CAD28788</td>
<td>56</td>
<td>69 / 6e-06</td>
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<tr>
<td>( \text{oxF} )</td>
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<td>Oxidoreductase</td>
<td>CAB39328</td>
<td>76</td>
<td>43 / 6e-15</td>
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<tr>
<td>( \text{hypB2} )</td>
<td><em>Neurospora. crassa</em></td>
<td>Hypothetical protein</td>
<td>XP_323696</td>
<td>134</td>
<td>29 / 4e-30</td>
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<tr>
<td></td>
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<td>Hypothetical protein</td>
<td>EAA61806</td>
<td>149</td>
<td>30 / 1e-34</td>
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<tr>
<td>( \text{dmaW} )</td>
<td><em>Claviceps purpurea</em></td>
<td>Putative dimethylallyltryptophan synthase</td>
<td>CAC37396</td>
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<tr>
<td>( \text{vapA} )</td>
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<td>Hypothetical protein</td>
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<th>Scores</th>
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Table 4.4: Conserved protein domains deduced by BLAST analysis (rpsblast) from sequences near \( \square \) \( dmaW \) in \textit{Claviceps purpurea} locus 2.

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<td>( \square ) dmaW</td>
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<td>hypD</td>
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<td>7e-32</td>
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<td>vapA</td>
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<td>Putative lipopolysaccharide modifying enzyme</td>
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Table 4.5: Predicted open reading frames from sequences near *dnaW* in *Claviceps fusiformis*.

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<th>Accession no.</th>
<th>Scores</th>
<th>% Identity/E value</th>
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<td>EAA59168</td>
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<td>54 / 2e-62</td>
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<td><em>Leptospira interrogans</em></td>
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<td><em>Neurospora crassa</em></td>
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<td>47 / 9e-53</td>
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<td><em>Lycopersicon esulentum</em></td>
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<td>CAB43506</td>
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<td>41 / 1e-37</td>
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<td><em>Saccharomyces cerevisiae</em></td>
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<td><em>Emericella nidulans</em></td>
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<td>Accession no.</td>
<td>Scores</td>
<td>% Identity/ E value</td>
</tr>
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<td>Ajellomyces capsulatus Glomerella cingulata</td>
<td>Catalase isozyme P Catalase</td>
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<td>239</td>
<td>36 / 5e-62 236</td>
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<td>oxA</td>
<td>Claviceps. pupurea</td>
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<td>Oxidoreductase</td>
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<td>A. nidulans N. crassa</td>
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<td>31 / 3e-39 150</td>
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<td>hypA</td>
<td>Photorhabdus luminescens N. crassa Nostoc punctiformae Pseudomonas aeruginosa Streptomyces nogalater Shigella flexneri</td>
<td>Hypothetical protein Hypothetical protein Nucleoside-diphosphate sugar epimerase Nucleoside-diphosphate sugar epimerase Putative hydroxylase Putative oxidoreductase</td>
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<td>40 / 2e-29 101</td>
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<td>dmaW</td>
<td>Claviceps fusiformis C. purpurea</td>
<td>Dimethylallyltryptophan synthase Dimethylallyltryptophan synthase</td>
<td>JC4338 CAB39314</td>
<td>753</td>
<td>98 / 0.0 520</td>
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Table 4.6: Conserved protein domains deduced by BLAST analysis (rpsblastp) of putative genes near *dmaW* in *Claviceps fusiformis*

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<th>ORF designation</th>
<th>Conserved protein domain</th>
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<td><em>hypG</em></td>
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<td>7e-38</td>
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<tr>
<td></td>
<td>Uncharacterized conserved protein</td>
<td>150</td>
<td>5e-37</td>
</tr>
<tr>
<td><em>hypH</em></td>
<td>No putative conserved domain detected</td>
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<td></td>
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<td><em>hypI</em></td>
<td>Protein of unknown function</td>
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<td>7e-36</td>
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<td></td>
<td>Uncharacterized conserved protein</td>
<td>138</td>
<td>7e-34</td>
</tr>
<tr>
<td><em>hypJ</em></td>
<td>Predicted Kinase</td>
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<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Predicted aminoglycoside phosphotransferase</td>
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<td>0.002</td>
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<td><em>apD</em></td>
<td>Xaa-Pro aminopeptidase (amino acid transport and metabolism)</td>
<td>64</td>
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<tr>
<td></td>
<td>Methionine aminopeptidase (translation, ribosomal structure and biogenesis)</td>
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<tr>
<td><em>lpsB</em></td>
<td>Non ribosomal peptide synthetase modules and related proteins</td>
<td>74</td>
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<td><em>oxO</em></td>
<td>NADH: flavin oxidoreductase, old yellow enzyme</td>
<td>163</td>
<td>7e-41</td>
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<td></td>
<td>NADH: flavin oxidoreductase / 12-oxophytodienoate reductase</td>
<td>154</td>
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<td>Catalase</td>
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<td><em>oxA</em></td>
<td>FabG, dehydrogenases with different specificities, related to short-chain-alcohol dehydrogenases</td>
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<td></td>
<td>Short chain dehydrogenase</td>
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<td><em>oxF</em></td>
<td>FAD binding domain</td>
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<td>FAD/FMN containing dehydrogenases</td>
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<td><em>hypB</em></td>
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<td><em>hypA</em></td>
<td>Predicted nucleoside-diphosphate sugar epimerase</td>
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<td></td>
<td>Predicted dehydrogenase</td>
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<tr>
<td><em>dmaW</em></td>
<td>No putative conserved domain detected</td>
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Table 4.7: Description of the common genes shared between *Claviceps purpurea* locus 1 and *Claviceps fusiformis*

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Deduced protein description</th>
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<tr>
<td>lpsB</td>
<td>peptide synthetase, monomodular</td>
</tr>
<tr>
<td>oxO</td>
<td>NADPH dehydrogenase, old yellow enzyme</td>
</tr>
<tr>
<td>oxP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>catA</td>
<td>catalase</td>
</tr>
<tr>
<td>oxA</td>
<td>oxidoreductase, short-chain-alcohol dehydrogenase</td>
</tr>
<tr>
<td>oxF</td>
<td>oxidoreductase, FAD/FMN containing dehydrogenase</td>
</tr>
<tr>
<td>hypB</td>
<td>uncharacterized conserved protein</td>
</tr>
<tr>
<td>hypA</td>
<td>nucleoside-diphosphate sugar epimerase</td>
</tr>
<tr>
<td>dnaW</td>
<td>dimethylallyltryptophan synthase</td>
</tr>
</tbody>
</table>
Fig 4.1: Schematic map of genomic regions from dnaW-containing gene clusters from *C. fusiformis*, *C. purpurea* locus 1 and *C. purpurea* locus 2. Genes that are common are colored with black boxes. The orientation of genes is indicated by arrow heads. Vertical lines within each ORF indicate intron position.
Chapter 5
Conclusions and Discussion

Ergot alkaloids, the compounds synthesized by Clavicipitaceous fungi and several other organisms, have been an important part of history due to their toxic effect on humans and livestock. *Claviceps purpurea*, the ergot fungus, has been known to be cause for human poisoning due to consumption of bread contaminated with ergot (Floss and Anderson 1980). The *Neotyphodium* species, endophytes that are close relatives of *Claviceps* species, are thought to be involved in fescue toxicosis, a complex of symptoms that animals exhibit after grazing some *Neotyphodium*-infected grasses in which ergopeptine alkaloids are present (Schardl and Phillips 1997). Ergot alkaloid biosynthesis involves multiple steps, most of which have been explained (Tudzynski et al 2001). The enzyme for the first pathway specific step, dimethylallyltryptophan synthase (DMAT synthase) has been described (Shibuya et al 1990) and was reported to be a regulatory step in the biosynthesis (Krupinski et al 1976). The gene encoding DMAT synthase, *dmaW*, was identified and cloned from *Claviceps fusiformis* (Tsai et al. 1995) and *Neotyphodium* spp. (Wang 2000).

In this dissertation *dmaW* from *Neotyphodium* sp. Lp1 was disrupted by marker exchange and the *dmaW* knockout failed to produce ergovaline or any other ergot alkaloids. This is the first genetic evidence for the involvement of *dmaW*, encoding DMAT synthase, in ergot alkaloid biosynthesis. The *dmaW* knockout when complemented with *C. fusiformis dmaW*, the best characterized *dmaW*, was able to synthesize ergovaline, thus confirming that DMAT synthase is required for ergot alkaloid biosynthesis.

The *Neotyphodium* species form mutualistic associations with cool season grasses and provide numerous benefits by protecting the host grasses from biotic and abiotic factors and enhancing grass fitness and competitiveness (Malinowski and Beleski 2000). On the other hand, toxicosis on animals that graze on *Neotyphodium coenophialum*-infected tall fescue has been a major concern in agriculture. Elimination of factors responsible for fescue toxicosis from the endophytes has been the ultimate goal of this research project. The strategy is to use genetic approaches to modify the endophyte, and introduce the modified endophyte into tall fescue.
Molecular genetic modification usually involves the use of a genetic marker for transformation, the presence of which may not be acceptable in a released cultivar. In this dissertation I demonstrated the use of a Cre/lox system, a site-specific recombination system to eliminate the marker used in transformation. Application of Cre/lox system to *N. coenophialum* is particularly attractive as *N. coenophialum* possess two homologs of *dmaW* and Cre/lox can be used to knockout both the homologs, followed by removal of the selectable marker by Cre. The Cre/lox system developed for *N. coenophialum* in this thesis demonstrated the disruption of *dmaW*-2 and subsequent removal of marker by Cre. The application of Cre/lox system needs modification as Cre was introduced as an expression construct and this requires integration of cre into the genome to be functional. Introduction of Cre as protein would be one approach to avoid integration of cre into the genome.

Disruption of *dmaW*-2 in *N. coenophialum* did not eliminate the synthesis of ergovaline when introduced into tall fescue seedlings. The *dmaW*-2 homolog is predicted to encode identical protein as that encoded by *Neotyphodium* sp. Lp1. This provided new information that *dmaW*-1 also encoded DMAT synthase in *N. coenophialum*. The Cre/lox system can be used to knockout *dmaW*-1 and subsequently eliminate the marker by Cre. The possibility of using the same marker for sequential knockout makes the Cre/lox system more applicable to *N. coenophialum*.

Identification of a putative ergot-alkaloid biosynthesis gene cluster in *Claviceps purpurea* and *C. fusiformis* provided new information on arrangement and order of genes that are common in these two *Claviceps* spp. which produce different subsets of ergot alkaloids. *C. fusiformis* produces clavines and *C. purpurea* synthesizes more complex ergopeptides. The nine genes that are common between the two species are most likely to have roles in early steps of the pathway, which are shared between these two *Claviceps* spp. The presence of additional genes in *C. purpurea* gene cluster suggests their possible role in later steps in the pathway. One approach to know the role of these additional genes in *C. purpurea* would be to introduce these genes into *C. fusiformis* and check if *C. fusiformis* can synthesize the additional ergot alkaloids which are otherwise lacking.
Neotyphodium sp. Lp1 and N. coenophialum are proposed to have a similar ergot-alkaloid biosynthesis pathway as that of C. purpurea. C. purpurea produces several ergopeptines including ergotamine, whereas N. coenophialum and Neotyphodium sp. Lp1 produce ergovaline as their major type of ergopeptines. The enzymes involved in synthesis of ergopeptines are lysergyl peptide synthetases, which are non-ribosomally synthesized peptide synthetases. In Neotyphodium sp. Lp1 the knockout of part of lpsA, the gene encoding lysergyl peptide synthetase, failed to produce ergovaline and simple lysergic acid amides but did accumulate clavines and lysergic acid (Panaccione et al 2003). And, the dmaW ko from Neotyphodium sp. Lp1 eliminated the production of ergovaline and all clavines. These results confirmed that dmaW acts early on in the pathway such that its elimination blocks the entire pathway, whereas lpsA acts at a later step in the pathway such that knockout of lpsA does not affect the accumulation of clavines.

The putative ergot alkaloid biosynthesis gene cluster identified in Claviceps spp. might be helpful in identification of genes from Neotyphodium species. Identification of other genes involved in ergot alkaloid biosynthesis in Neotyphodium spp. would be important for two reasons: 1) to compare the genes between Claviceps and Neotyphodium spp. to provide information on evolution of the ergot alkaloid biosynthesis pathway, and 2) to molecularly dissect the pathway at various steps (similar to what was done by Panaccione et al. 2001 and this dissertation) to find out what genes/enzymes are involved in synthesizing different subsets of ergot alkaloids.

Ergot alkaloids, especially ergovaline are thought to be involved in fescue toxicosis of animals that graze on N. coenophialum infected tall fescue but this has not been demonstrated definitively. It is also possible that clavines and simple lysergic acid amides play a role in fescue toxicosis. Obtaining mutants that block the pathway at different steps might help us understand the ergot alkaloid pathway and the role of ergot alkaloids specifically in fescue toxicosis. Considering that Neotyphodium spp. provide profound fitness benefits and biological protection to their grass hosts, genetic modification of the endophyte will also allow us to test the role of ergot alkaloids in grass fitness and protection.

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Appendices

Appendix 1:
Two step knockout experiment to knockout $dmaW$ in $N. coenophialum$

Materials and Methods

Generation of plasmid constructs
Plasmids pBluescript KS+/- and pBSKS+ were from stratagene, pCB1004 from Dr. Mark Farman.

pKAES146

$N. coenophialum$ $dmaW-1$ knockout construct was constructed by cloning $BamHI$ and $Sall$ cut 4.8 kb fragment containing 341bp deletion in $dmaW-1$ coding region from the first ATG to the 3’ flanking region of $dmaW-1$ (total of 4.8kb fragment) which was amplified by PCR using primers dmaWe19copy2.cos BamHI.15d (5’-CTTCTTCACAGGATCCGTTTAACACTTTCCGCTATCTG) and dmaWe19cos Sall.37u (5’-GCGTCGACGGAAGTAGTCCTTTTACCTTAGTAAAGGC) into pBSKS+ that was cut with $BamHI$ and $Sall$. Another 4.1 fragment from 5’ flanking region that was amplified by PCR using primers dmaWe19cos SacII. 36d (5’-GCCCGCGGTAATGGAGCATTGGAGGTGACGTAAG) and dmaW e19copy2.cos BamHI.16u (AAACGGATCCTGTGAAGAAGAGGACGAGCGIATAGC) was digested with SacII and $BamHI$. The 4.1 kb fragment was cloned adjacent to SacII and $BamHI$ sites of the above pBSKS+ construct to give pKAES146.

pKAES147

pKAES146 was digested with SacII and $Sall$ and the 8.9 kb fragment containing both 5’ and 3’ flanking region of $dmaW-1$ was cloned into pCB1004 vector, which was digested with SacII and $Sall$. The resulting construct was pKAES147.
pKAES148

*N. coenophialum* *dmaW*-2 knockout construct. For details see the materials and methods in Chapter 3.

pKAES149

pKAES148 was digested with *Sac*II and *Sal*I and the 5.6 kb fragment containing both 5’ and 3’ flanking region of *dmaW*-2 was cloned into pCB1004 vector, which was digested with *Sac*II and *Sal*I. The resulting construct was pKAES149.

**Fungal culture and fungal transformation**

See chapter 3 of this dissertation for details

The experiment was done to target *dmaW*-1 and *dmaW*-2 simultaneously. Protoplasts of *N. coenophialum* ATCC62374 were transformed with linearized pKAES147 containing mutant *dmaW*-1 digested with *Sac*II enzyme and with pKAES149 containing mutant *dmaW*-2 seperately by electroporation (Tsai et al. 1992) and by PEG method (Panaccione et al. 2001). See Fig A 1.1 for outline of two step knockout process.

**Screening of transformants for the 1st step integration**

*N. coenophialum* transformants obtained after transforming with mutant *dmaW*-1 and mutant *dmaW*-2 construct were screened by PCR for the integration of the construct (step 1) at 3’ end and 5’ end with specific primers for *dmaW*-1 and *dmaW*-2 separately. Transformants were also checked for the presence of *hph* by PCR. The screen by PCR was a negative screen, the construct of *dmaW*-1 or *dmaW*-2 if integrated at the right locus would be too large to amplify by conventional PCR. Negative PCR result was further supported by amplifying *hph* and association of *hph* with *dmaW*. The putative transformants that did not amplify 3’ or 5’ end were further analyzed by Southern blot.

**Southern blot**

See chapter 3 of this dissertation for details.
Screening for two step knockout transformant

The putative transformants were subject to 2 to 3 rounds of single spore isolation on non-selective (non hygromycin) medium (Fig A 1.1). Spore suspension was prepared from the culture by adding a 3-5 mL of sterile water onto the plate and plating out the spore suspension on PDA plate. After 2-3 days when the conidia on PDA just began to germinate, each colony was picked individually under the dissecting microscope and placed on a grid plate (PDA without hygromycin) that accommodated 50 colonies per plate. After 2-3 weeks the colonies from the grid plate were replica plated onto PDA with hygromycin (hyg) plates using sterile toothpicks. The PDA with hygromycin grid plates were checked under the microscope for the presence of fungal inoculum. After 2-3 weeks, colonies from hyg – were compared with hyg+ for the growth. The colonies that failed to grow on hyg + were considered putative transformants and were single spore isolated on both hyg+ and hyg – for comparison of growth.

Results

One step integration

N. coenophialum dmaW-2 transformants

Out of 48 transformants screened for the integration of mutant dmaW-2 construct on 3’ and 5’ side of wt dmaW-2 in the N. coenophialum genome, two of the transformants showed putative integration on 3’ end and one of the transformant showed integration on 5’ end of wt dmaW-2 locus by PCR analysis. These 3 transformants were analyzed further by Southern blot and unfortunately two of them had multiple integration of the construct into the genome and the third one was complex to analyze and did not have the expected band size.

N. coenophialum dmaW-1 transformants

Total of 222 transformants were obtained by several rounds of electroporation and PEG transformation. Two (# 27 and # 30) out of 46 screened transformants showed putative integration of mutant dmaW-1 onto 5’ end of wt dmaW-1 locus by PCR. These two transformants were analyzed further by Southern blot analysis and one of them (# 27) showed expected band size for one step integration into the genome (Fig A 1.2). Transformant no. 27 was tested for the second step knockout process.
Two step knockout

Screening for 2\textsuperscript{nd} step knockout in putative \textit{dmaW-1} transformant

The transformant no. 27 was subjected to 3 rounds of single spore isolation on non hygromycin containing medium. Several rounds of spore suspension were used to pick colonies to replica pick on hyg\textsuperscript{+} and hyg\textsuperscript{−}. 4850 colonies were picked onto hyg\textsuperscript{−} medium and then replica picked onto hyg\textsuperscript{+} medium. Out of 4850 colonies, 56 of them failed to grow on hyg\textsuperscript{+} medium. (The spot on the grid showed the presence of hyphal bits and possibly spores but did not exhibit any growth). These 56 colonies were streaked again on hyg\textsuperscript{+} and hyg\textsuperscript{−} containing PDA plate and all of them grew on both hyg\textsuperscript{+} and hyg\textsuperscript{−} plates. DNA was extracted from several of these 56 colonies and analyzed for the presence of \textit{hph} and wt \textit{dmaW-1} by PCR and all of them showed the amplification for both \textit{hph} and wt \textit{dmaW-1} indicating that second step of knockout has not taken place.
Figure A1.1: Outline of two-step knockout process in *N. coenophialum*
Figure A1.2 Southern blot analysis indicating first step knockout in *N. coenophialum* transformed with *dmaW-I* construct. The genomic DNA was digested with *PstI*, *BamHI* and *EcoRI*. Lanes with no. 27, 30 and 29 indicate three different transformants and Nc is *N. coenophialum* wt. Tranformant no. 27 indicated the one step integration of *dmaW-I* construct into the genome.
Appendix 2:

Experiment to facilitate transient expression of Cre in *N. coenophialum dmaW-2* ko.

**Materials and Methods**

For construction of pKAES175, fungal transformation and PCR screening: see chapter 3 of this dissertation.

Fungal culture: *N. coenophialum dmaW-2* ko transformant was generated as explained in Chapter 3 and was grown and maintained on PDA similar to that of *N. coenophialum* ATCC 62374 (see Chapter 3 for details).

**Results**

**Screening of transformants for the function of Cre in *N. coenophialum dmaW-2* ko**

The construct pKAES175 has *cre* under the control of *tub2* promoter was introduced into *N. coenophialum dmaW-2* ko transformant by electroporation. Linear (with SacII) and circular form of pKAES175 was used for transformation. The transformants were selected on CRM medium (Chapter 3) containing no antibiotics. The transformants were transferred to PDA for sporulation and single spore isolation. Transformants were replica plated (as explained in Appendix 1) onto hygromycin plus (72 μg of hygromycin B per ml) and hygromycin minus containing plates. Out of 333 transformants screened, 40 of them did not grow on hygromycin plus medium. These transformants were analyzed by PCR as described in chapter 3 (Fig 3.3). All the transformants showed no indication of loss of *hph* by PCR analysis and all of them grew on hygromycin containing medium indicating Cre has not eliminated *hph* in *N. coenophialum dmaW-2* ko.
Appendix 3:

Sequence analysis of *N. coenophialum* cosmid Nc28B11 containing *dmaW-1* homolog.

Materials and Methods

Source of cosmid clone

Genomic cosmid library of *N. coenophialum* ATCC 90664 was constructed by H.-F. Tsai (unpublished data). A cosmid clone Nc28B11, containing *dmaW-1* was identified by J. Wang (2000). The cosmid clone Nc28B11 was chosen for sequence determination.

Preparation of transposon insertion library of cosmid Nc28B11, DNA sequencing and sequence analysis

See materials and methods in Chapter 4 of this dissertation.

Results

Analysis of sequences from *N. coenophialum* *dmaW-1* locus

Sequences assembled from the cosmid Nc28B11 revealed the presence of *dmaW-1* in the center of a 13.2 kb contig (Fig A 3.1). The gene *oxH* was located about 5 kb upstream of *dmaW-1*. The predicted product of *oxH* was significantly similar to that of *fum3* from *Gibberella moniliformis*, which is involved in fumonisin biosynthesis (Proctor et al. 1999) (Table A 3.1). Downstream of *dmaW-1* was an orf (*hypR*) whose predicted protein product showed similarity to a retrotransposon Tad1-1 from *N. crassa* (Fig A3.1 and Table A3.1). No other open reading frames were predicted upstream or downstream of *dmaW-1*.

Unlike *C. purpurea* and *C. fusiformis* genome regions near *dmaW* (Chapter 4) no genes having similarity to oxidoreductases and lysergyl peptide synthetase were present near *dmaW-1* in *N. coenophialum*. Only one gene from *N. coenophialum* had similarity to a gene from *C. purpurea* locus 1 however the location was quite different. In *C. purpurea*, *oxH* is located downstream of *dmaW* whereas in *N. coenophialum* *oxH* is located upstream of *dmaW*. *oxH* showed similarity to *fum3*, which encodes hydroxylase involved in fumonisin biosynthesis.
Table A3.1: Predicted open reading frames from sequences near *dmaW-1* in *Neotyphodium coenophialum*

<table>
<thead>
<tr>
<th>Orf designation</th>
<th>Organism</th>
<th>Deduced protein description by blastx</th>
<th>Accession no.</th>
<th>Scores</th>
<th>% Identity/E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxH</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Hypothetical protein</td>
<td>AAK01519</td>
<td>153</td>
<td>35 / 8e-35</td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em></td>
<td>Conserved hypothetical protein</td>
<td>NP_744958</td>
<td>148</td>
<td>33 / 3e-33</td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus nidulans</em></td>
<td>Hypothetical protein</td>
<td>EAA61518</td>
<td>137</td>
<td>53 / 5e-36</td>
</tr>
<tr>
<td></td>
<td><em>Gibberella moniliformis</em></td>
<td>Hypothetical protein</td>
<td>AAG27131</td>
<td>111</td>
<td>30 / 4e-22</td>
</tr>
<tr>
<td>dmaW-1</td>
<td><em>Neotyphodium coenophialum</em></td>
<td>dimethylallyltryptophan synthase</td>
<td>AAP81207</td>
<td>888</td>
<td>100 / 0.0</td>
</tr>
<tr>
<td></td>
<td><em>Epichloë typhina X N. lolii</em></td>
<td>dimethylallyltryptophan synthase</td>
<td>AAP81206</td>
<td>851</td>
<td>95 / 0.0</td>
</tr>
<tr>
<td></td>
<td><em>Claviceps purpurea</em></td>
<td>dimethylallyltryptophan synthase</td>
<td>CAB39314</td>
<td>596</td>
<td>67 / e-169</td>
</tr>
<tr>
<td></td>
<td><em>Balansia obtecta</em></td>
<td>dimethylallyltryptophan synthase</td>
<td>AAP92451</td>
<td>585</td>
<td>66 / e-166</td>
</tr>
<tr>
<td>hypR</td>
<td><em>Neurospora crassa</em></td>
<td>Hypothetical protein 2-retrotransposon Tad1-1</td>
<td>S43275</td>
<td>65</td>
<td>28 / 4e-08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Contains reverse transcriptase and cys finger domains</td>
<td>AAA21792</td>
<td>66</td>
<td>27 / 2e-08</td>
</tr>
</tbody>
</table>
Figure A 3.1: Schematic map of genomic regions near \textit{dmaW-1} from \textit{N. coenophialum}.

\textit{dmaW-1} is indicated with black box and vertical lines indicate introm position. The orientation of genes is indicated by arrow heads.
References


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Honors

Publication


Jinghong Wang, Caroline Machado, Daniel Panaccione and Christopher Schardl (1999) Ergot alkaloid biosynthesis genes cloned from Claviceps species and grass endophytes. The 20th Fungal Genetics Conference. March 23 - 28, 1999 Asilomar Conference Center, California, USA

