University of Kentucky

UKnowledge

2004

STUDIES OF ERGOT ALKALOID BIOSYNTHESIS GENES IN CLAVICIPITACEOUS FUNGI

Caroline Machado
University of Kentucky, cmach0@uky.edu

Right click to open a feedback form in a new tab to let us know how this document benefits you.

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

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.

Caroline Machado

Date: 5/06/04

STUDIES OF ERGOT ALKALOID BIOSYNTHESIS GENES IN
CLAVICIPITACEOUS FUNGI

By

Caroline Machado

Christopher L. Schardl

_____
Director of Dissertation

Lisa J. Vaillancourt

_____
Director of Graduate Studies

5/06/04

_____

RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctors degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with permission of the author, and with the usual scholarly acknowledgments.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

DISSERTATION

Caroline Machado

The Graduate School

University of Kentucky

2004

STUDIES OF ERGOT ALKALOID BIOSYNTHESIS GENES IN
CLAVICIPITACEOUS FUNGI

_____

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

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FILES

Chapter 1
Introduction

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}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 dimethylallylpyrophospate and L-tryptophan, and also partially purified from a *Claviceps* sp the enzyme catalyzing this reaction, dimethylallyl-pyrophosphate: 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 *dmaW* gene encodes a 52 kDa protein (Tsai et al. 1995). Based on the *dmaW* sequence from *C. fusiformis,* the *dmaW* 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 *dmaW*-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 *dmaW* 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 *cpps1*, 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 (Schardl 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 (Schardl 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 (Schardl 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 pyrrolpyrazine) 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 anti-mammalian 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 the1930s, 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 *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 *dmaW* homologs in *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 *dmaW* homologs in *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 *dmaW* were sequenced from *Claviceps purpurea* and *C. fusiformis* and a putative ergot alkaloid biosynthesis gene cluster was identified in both *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 *Claviceps* spp, the future goal can be to identify other genes involved ergot alkaloid biosynthesis in *Neotyphodium* endophytes.

Table 1.1: Different ergopeptines that differ at amino acid position I and II. Amino acid position III is usually L-proline.

amino acid position I	amino acid position II			
	L-valine	L-phenylalanine	L-leucine	L-isoleucine
L-alanine	ergovaline	ergotamine	ergosine	β-ergosine
L-valine	ergocornine	ergocristine	ergokryptine	β-ergokryptine
L-2-amiobutyric acid	ergonine	ergostine	ergoptine	β-ergoptine
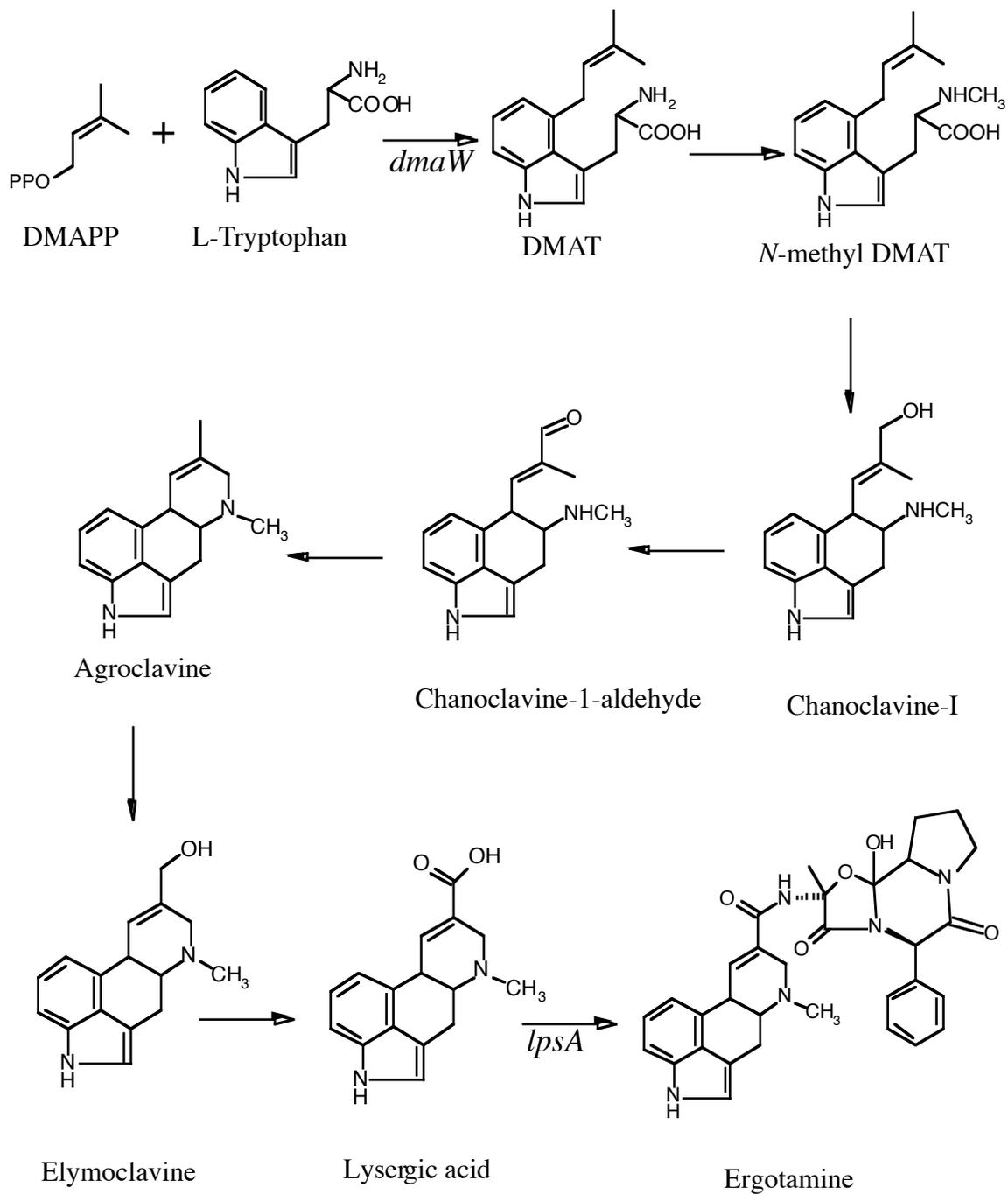
Figure 1.1: Ergopeptine biosynthesis pathway
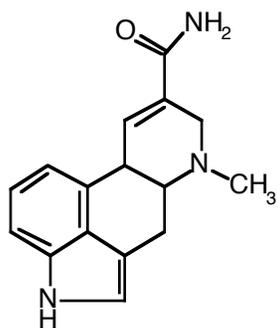
Lysergic acid amide
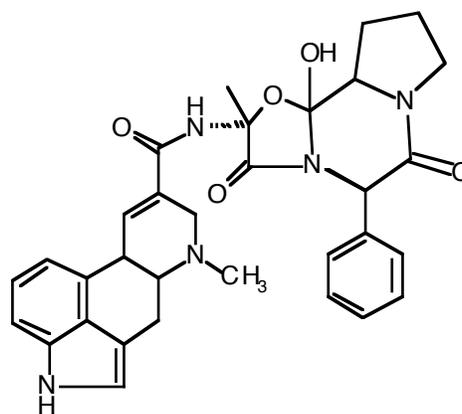
Ergotamine
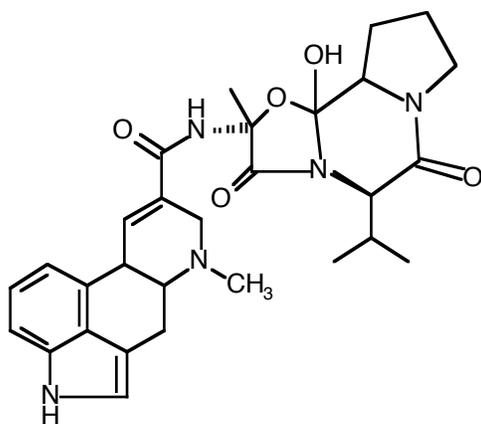
Ergovaline

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 (Schardl 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 Schardl 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* (Schardl 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 Francisco, 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 *Eco*RI 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'-CAAGTAACCGAGAAAATGACAAAAGCTCCA-3') and Cpu-dmaW(-)-SalI (11052) (5'-CGGTCGACGCAAAGACCCTTGACATGA-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'-GCGTCGACGCTAACTACTAGTTATCTTATTATAAAGATACC-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.

17

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 *dmaW* ct transformants accumulated a small but identifiable level of ergovaline and ergovalinine (mean of 0.15 µg/g dry weight of infected plant material) (Fig 2.4). This result indicates that *dmaW* ko eliminates the production of ergovaline in *Neotyphodium* sp. Lp1.

Discussion

The results presented in this chapter demonstrate the first genetic test for the role in ergot alkaloid biosynthesis of *dmaW*, which encodes DMAT synthase. *dmaW* in *Neotyphodium* sp. Lp1 was replaced with a construct containing partially deleted *dmaW* by marker-exchange gene replacement, and when this *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 *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 *dmaW* ko lacked detectable ergovaline, showed that *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, *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 *Claviceps* sp. and predicted that it would play a role as the determinant step in biosynthesis pathway. Cloned *dmaW* from *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 *dmaW* ko with *C. fusiformis dmaW* (*dmaW* ct) provided confirmation that the DMAT synthase was responsible for ergovaline production. Plants infected with *dmaW* ct transformants indicated small but definitive levels of ergovaline and ergovalinine, whereas endophyte-minus plants and *dmaW* ko symbiota completely lacked these peaks (Fig 2.4). An attempt to complement *dmaW* ko with wild type Lp1 *dmaW* was unsuccessful because no stable transformants were obtained. Complementation by *C. fusiformis dmaW,* which is a well characterized *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 *dmaW* ct symbiota was very low. It is possible that the low ergot alkaloid levels were due to poor expression of the *C. fusiformis* protein in the *dmaW* ct transformants. *C. fusiformis dmaW* was expressed under *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 *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.
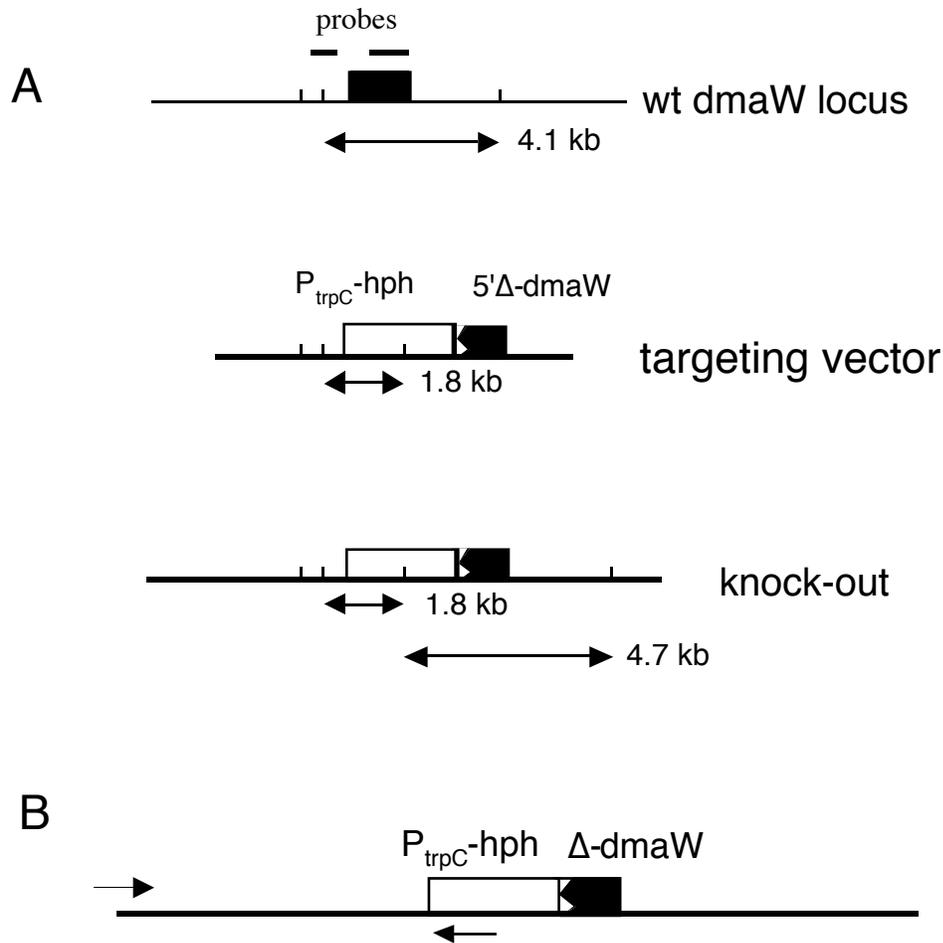
Figure 2.1: (A) Maps of the wild type *dmaW* locus, targeting vector and expected map of locus after disruption. Vertical lines indicate *Eco*RI sites and double headed arrows indicate expected size of fragments in kb. Probe is indicated with horizontal line above. Southern blot result in Fig 2.2 (B) Map of the disrupted locus showing primer sites as pointed arrows used for PCR analysis (Fig 2.3)
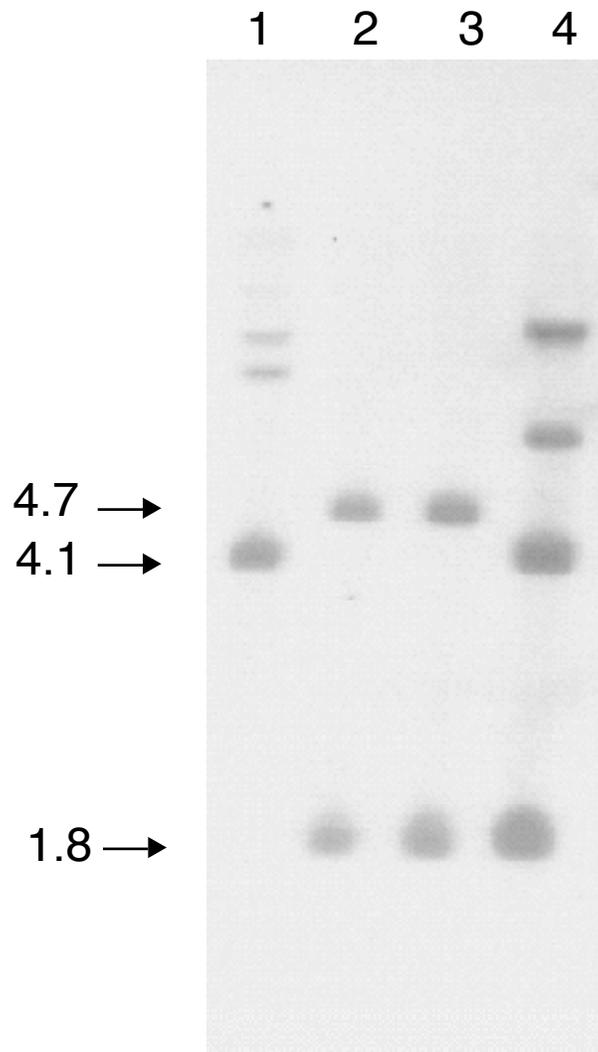
Figure 2.2: Southern-blot analysis of Lp1 *dmaW* ko. Lane 1 contained total genomic DNA from the wild type (wt) Lp1; lanes 2 and 3 contained DNA from two single spore isolates of *dmaW* ko; and lane 4 contained DNA from an Lp1 transformant with ectopic integration of the vector. DNA was digested with *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
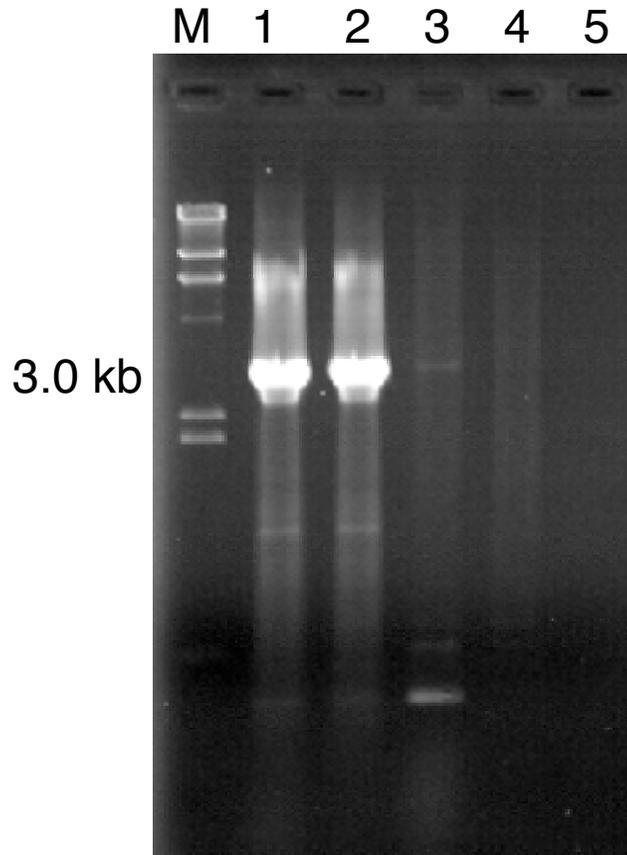
Figure 2.3: PCR analysis of the 5' flanking region of Lp1 *dmaW* ko. One PCR primer targeted *hph*, and the other primer targeted a site upstream of the 5'-flanking region used in the disruption vector (Fig 2.1 panel B). The PCR product of 3 kb in the ko (lane 1 and 2) was expected if homologous cross-over 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 *Hin*dIII-cut bacteriophage lambda DNA as a size marker ladder.
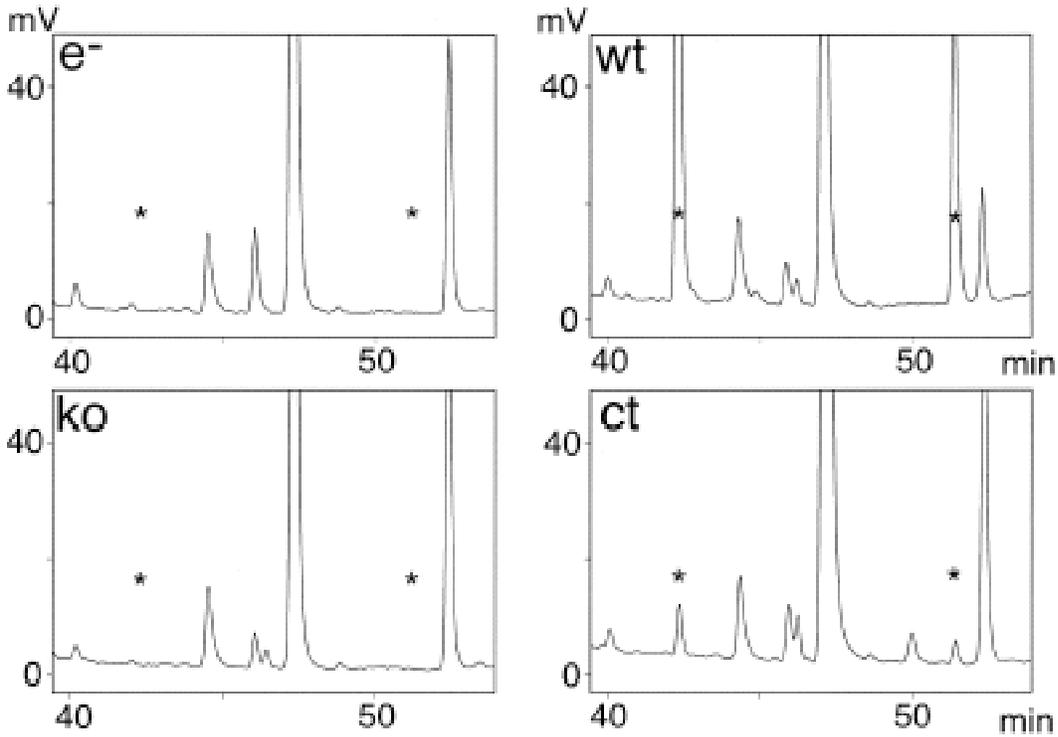
Figure 2.4: Ergovaline analysis of Lp1 *dmaW* ko. HPLC analysis of ergovaline (42.3 min) and its stereoisomer ergovalinine in perennial ryegrass without endophyte (e-), or symbiotic with wild type Lp1 (wt), the *dmaW*-knockout mutant (ko) or the mutant complemented with *C. fusiformis dmaW* (ct). Detection 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 Franscisco, 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 *Bam*HI 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_2O 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 *Bam*HI and *Sal*I sticky-end overhangs. As a source of the fungal-active hygromycin phosphotransferase gene, *hph*, pKAES080 (Tsai et al. 1992) was digested with *Bam*HI and *Sal*I 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 (*loxP*1; l*oxP*2: *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-β 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 *Bam*HI 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'-GCCCGCGGCCCCTTAGAATATAGTAGTATAATTAACTTAC-3') and *dmaW* e19copy2.cos BamHI.16u (5'-AAACGGATCCTGTGAAGAAGAGGACGAGCGIAATAGC-3'), cut with *Sac*II and *Bam*HI, and cloned into pBSKS+ that had also been cut with *Sac*II and *Bam*HI.

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'-CTTCTTCACAGGATCCGTTTAACACTTTCGCTATCTG-3') and dmaWe19copy2 SalI.14u (5'-GCGTCGACAGTGATCAGGGATACCTTTGATTACA-3'). The 2.7 kb-fragment was then digested with *Bam*HI and *Sal*I and cloned in the *Bam*HI and *Sal*I sites of pBSKS+ to give pKAES148.

Plasmid pKAES148 was linearized with *Bam*HI and treated with calf intestine alkaline phosphatase (Stratagene) to dephosphorylate the 5'-termini. Plasmid pKAES173 was digested with *Bam*HI 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~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 *Sal*I and *Sac*II, and cloned into the *Sal*I and *Sac*II sites of pBSKS+. The resulting plasmid was designated pKAES175 (Fig 3.2).

Generation of a *Pro~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'-GCGTCGACCTCATATCACGACTAAGGAGATAC-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

*Sal*I and *Sac*II, and cloned into the *Sal*I and *Sac*II 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, Franklington, 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 *Sal*I. 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 NH_4NO_3, 1g KH_2PO_4, 1g NaCl, 0.25g anhydrous $MgSO_4$, 0.13g $CaCl_2.2H_20$,

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'-

AGAAACAGACAGGGCTATTC-3') and dmaWe19copy2.5u (5'-

CTCGCCGGCATGCGTCAAAA-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 dmaWe19copy2.9u (5'-ACCCGGGGAATACAACCACT-3') and dmaWPrSalI(d) (5'-GCGTCGACCTCATATCACGACTAAGGAGATAC-3'); the 1-kb probe for the region of *dmaW* downstream of the deleted portion was generated by PCR with primers dmaWe19.07d (5'-CAACACCTTACACTTGACCC-3') and dmaWe19copy2.5u (CTCGCCGGCATGCGTCAAAA). 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% $Na_4HPO_4.7H_2O$, 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 $Pro_{tub2}cre$ and $Pro_{dmaW}cre$, respectively — was introduced into the *dmaW-2* knockout mutant by cotransformation with pAN8-1, carrying a phleomycin-resistance gene (Mattern et al., 1988).

Protoplasts from the *dmaW-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. 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. coenophialum* ATCC 62374

The plasmid pKAES174, in was constructed with 341 bp of the *dmaW-2* coding region (starting from the ATG initiation codon) deleted and replaced by a fungal-active *hph* gene flanked by *loxP* sites. This plasmid was introduced into *N. 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 *Eco*RI, *Xba*I or *Hin*dIII, 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 *Hin*dIII-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 *dmaW-1*, further confirming that *dmaW-1* was intact. These results indicated that *loxP-hph-loxP* mutant *dmaW-2* had replaced wild-type *dmaW-2*, and the 5' and 3' flanking regions of *dmaW-2*, as well as *dmaW-1*, were intact in the disruptant. I will refer to this mutant as *dmaW-2* ko in the remainder of the chapter.

Elimination of *hph* in *dmaW-2* ko

The *dmaW-2* ko has *loxP* sites flanking *hph*, a substrate for the activity of Cre recombinase. To test if Cre is functional in *N. coenophialum*, the *dmaW-2* ko was cotransformed by electroporation with *cre* expression construct pKAES175, and a phleomycin resistant vector pAN8-1. The transformants were screened by PCR for the integration of $Pro_{tub2}cre$. Six out of 22 transformants had the $Pro_{tub2}cre$ integration into their genome. These transformants were screened further by PCR using the same set of the primers as used for *dmaW-2* ko screening to check for the elimination of *hph* (Fig 3.3). Elimination of *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 $Pro_{tub2}cre$ showed elimination of *hph* (Fig 3.8). These transformants were plated on hygromycin B-containing medium, on which all failed to grow, further indicating the to loss of *hph*. Sequencing of these transformants to check for the junction of elimination of *hph* showed retention of a single *loxP* site as expected. These results demonstrated the function of Cre in specifically removing *hph* that was located between *loxP* sites.

Cotransformation of *dmaW-2* ko with $Pro_{dmaw}cre$

The construct pKAES176 has *cre* under the control of the *dmaW-2* promoter region, including 1120 bp region flanking the start codon of *dmaW-2*. *dmaW* is expressed in the *N. coenophialum*-infected tall fescue and not in non-induced culture or in uninfected tall fescue (Wang 2000). Therefore, I undertook to check if *cre* under the control of *dmaW* promoter region would be expressed specifically when the fungus harboring the construct was introduced into the plants. The *dmaW-2* ko was cotransformed with pKAES176 and pAN8-1 by electroporation. The transformants were screened by PCR for the integration of $Pro_{dmaw}cre$ into the genome. Out of 30 transformants screened, four had $Pro_{dmaw}cre$. These four transformants were tested by PCR for the elimination of *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_{tub2}cre* construct was introduced into *dmaW-2* ko to facilitate elimination of *hph*. 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 *hph* 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 *dmaW-2* ko without *hph* is not completely marker free because it has a phleomycin-resistance gene, which was introduced with *cre* for the reason that when *cre* was introduced without selection, there was no indication of transient expression of *cre* to facilitate loss of the *hph* marker (see Appendix 2). At this point it was not known if *cre* was not functional in *N. coenophialum* or that there was not enough transcript accumulated to translate Cre and drive the recombination. Introduction of *cre* with pAN8-1 demonstrated that *cre* is functionally expressed in *N. coenophialum* from a *tub2* promoter. The next attempt to produce a marker-free *dmaW-2* ko will involve introduction of Cre protein to *dmaW-2* ko.

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

        One of the difficulties in this study was the introduction of the *N. coenophialum* ATCC 62374; *dmaW-2* ko transformant as well as *dmaW-2* ko *Pro_{dmaw}cre* and *Pro_{tub2}cre* transformants into tall fescue 'KY31.' The difficulty introducing wild-type ATCC 62374 along with the transformants indicated that the genetic modification of *N. coenophialum* was not responsible for the reduced host compatibility, and the fact that *dmaW-2* 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 loose host compatibility. This has been seen before with other endophyte species (C.L. Schardl personal observations, and M.J. Christensen, personal communications). *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 *dmaW-2* ko transformed with $Pro_{dmaw}cre$ 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 *N. coenophialum* is synthesized in infected grass and probably is the only reliable indicator for expression of *dmaW* or the ergot alkaloids. The only grass-plant that had infection with *dmaW-2* ko transformant synthesized similar levels of ergovaline as compared to the plants with *N. coenophialum* wild-type, suggesting that *dmaW-1* also encoded DMAT synthase and was active in *N. coenophialum*. This is an important finding and first genetic study of the role of both *dmaW-1* and *dmaW-2* in ergot alkaloid biosynthesis in *N. coenophialum*. The *dmaW* ko in Lp1 (Chapter 2) eliminated ergovaline and all other ergot alkaloids, and the predicted protein product of *dmaW-2* of *N. coenophialum* is identical to that of *dmaW* from Lp1 and is transcribed in planta (Wang 2000). Thus, it appears that *dmaW-2* should encode active DMAT synthase, and that the ko *dmaW-2* was complemented with *dmaW-1* in *N. coenophialum*. The next step obviously will involve targeted disruption of *dmaW-1* in a *N. coenophialum dmaW-2* ko strain.

A
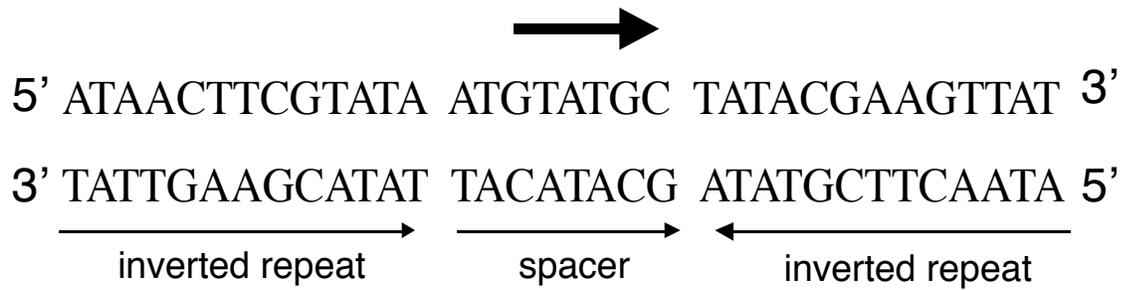
B
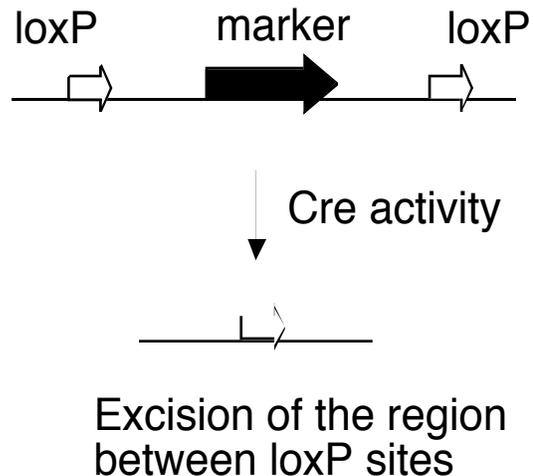
Figure 3.1: Schematic diagram of the Cre/*lox* system. (A) Sequence of *loxP* site. The arrow above the spacer region indicates the direction of the *loxP* site. (B). Diagram explaining the excision of the region located between *loxP* by the action of Cre recombinase.
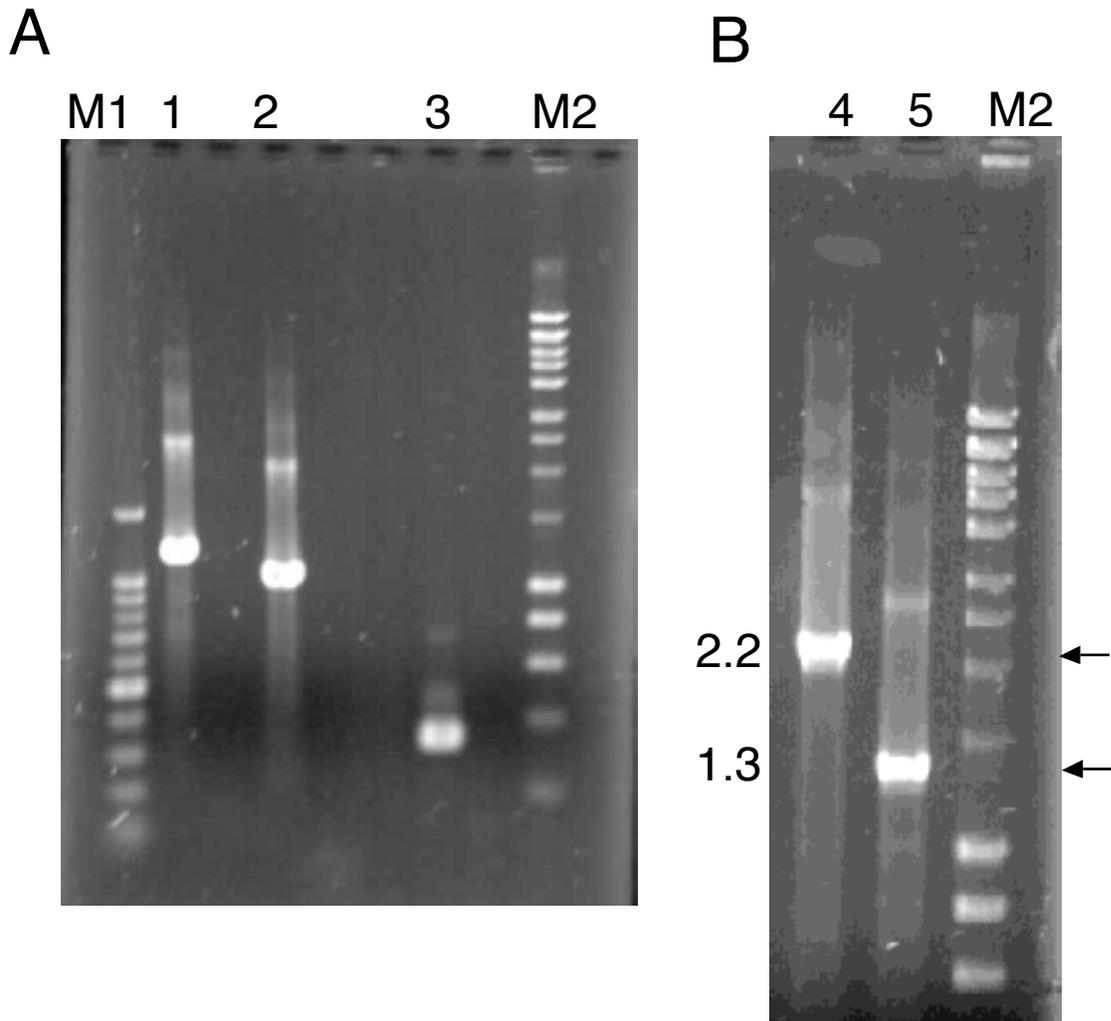
Figure 3.2: Electrophoretic analysis of PCR products generated for construction of *ProdmaW*-Cre and *Protub2*-Cre by SOE-PCR. (A) Products of first-round PCR-amplified DNAs containing the *dmaW* promoter region (lane1), *cre* (lane 2) and *Protub2* (lane 3). (B) Second-round PCR-amplified *ProdmaW*-Cre (2.2 kb fragment in lane 4) and *Protub2*-Cre (1.3 kb fragment in lane5). M1=125 bp marker, M2= 1 kb marker
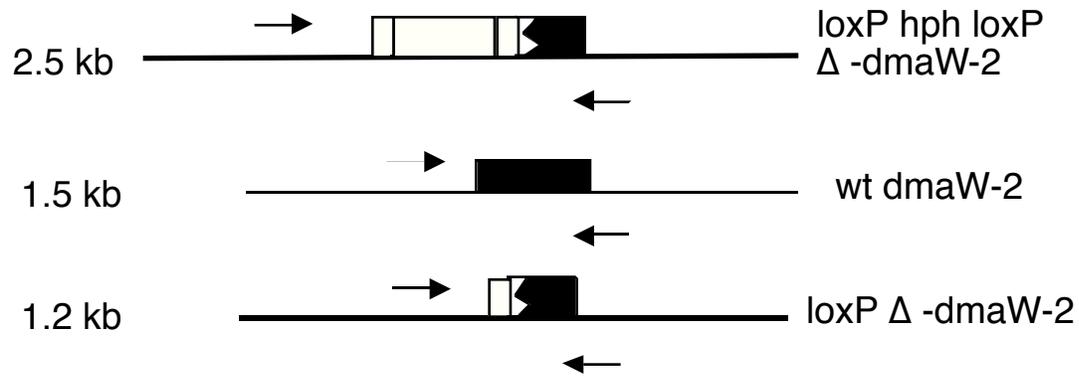
Figure 3.3: Map of the *dmaW*-2 ko locus after Cre-mediated loss of *hph*: A primer set, indicated as two arrows, is used for PCR-amplification of a 2.5 kb fragment from *dmaW*-2 ko with *loxP-hph-loxP*, a 1.2 kb fragment from Δ-*dmaW*-2 after elimination of *hph,* and a 1.5 kb fragment from wt *dmaW*-2 of *N. coenophialum*.
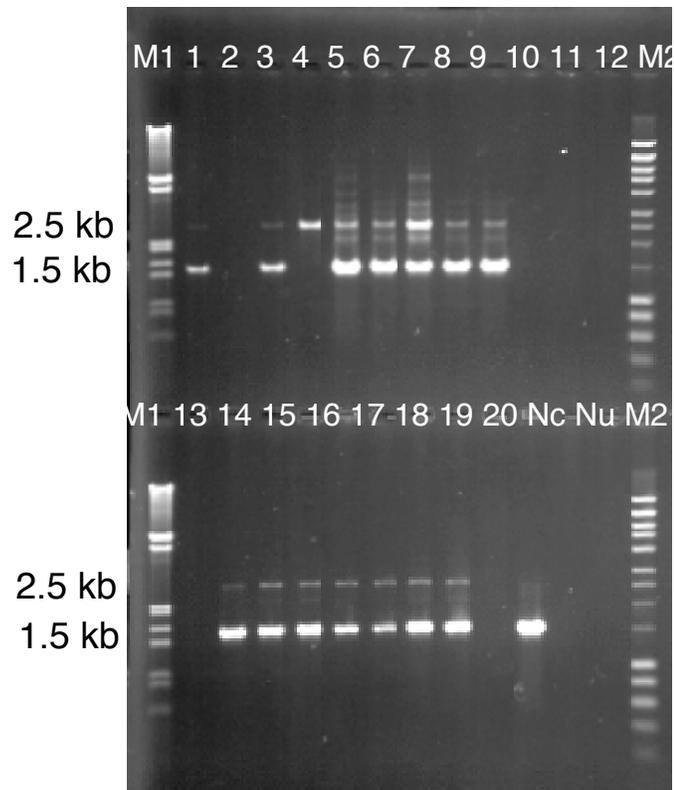
Figure 3.4: PCR screening of *N. coenophialum* transformants for homologous recombinants in which mutated *dmaW-2* (*dmaW-2* ko) is substituted with the wild type (wt) gene. PCR with primer sets indicated in Fig 3.3 amplified a 1.5 kb fragment from wt *dmaW-2* or a 2.5 kb fragment from *dmaW* ko with *loxP-hph-loxP* (lane 4). Nc=*N. coenophialum* as +control, Nu= *N. uncinatum* as -control. M1= lambda *Hind*III marker, M2=1 kb marker.Blank lanes indicate failed reactions.
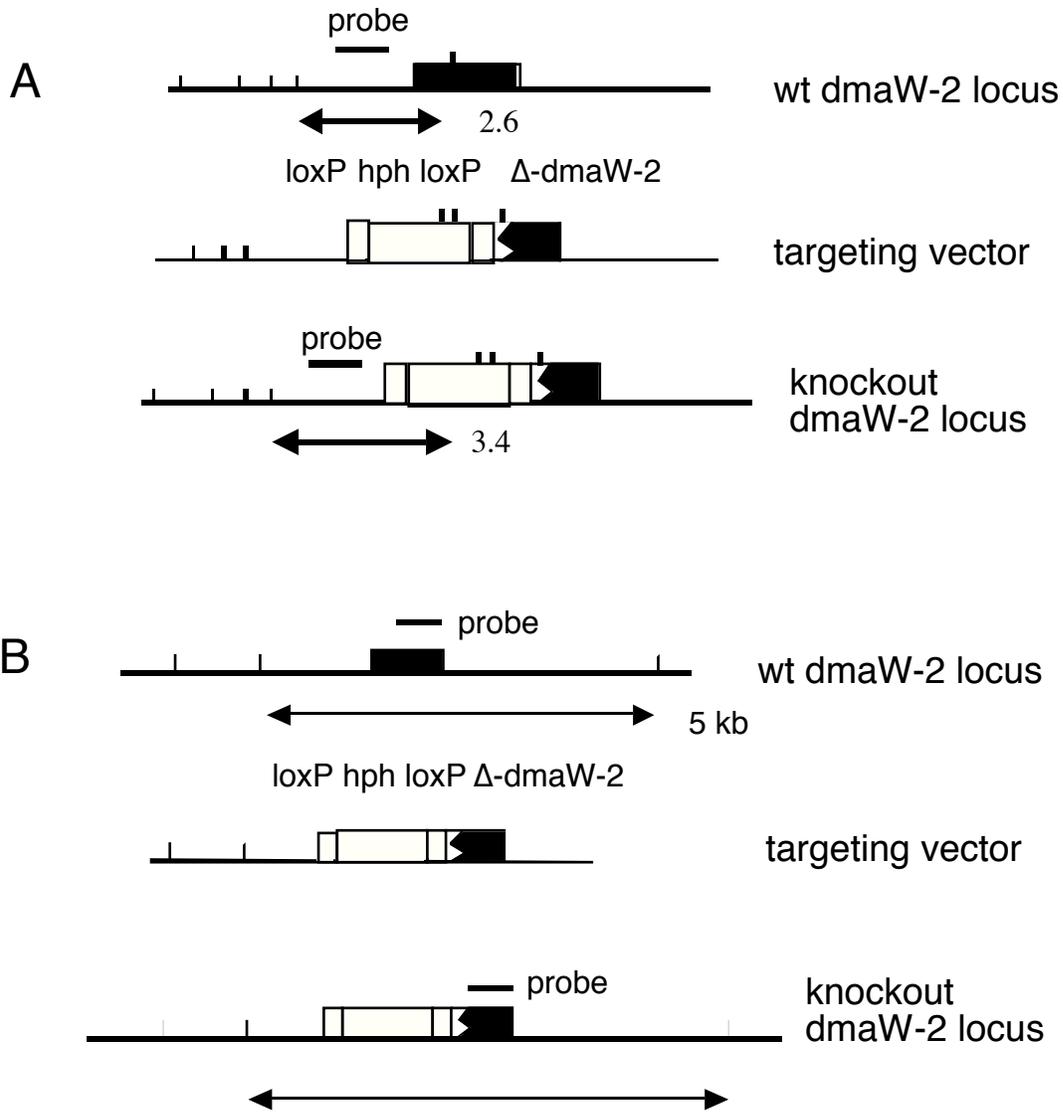
Figure 3.5: Maps of the wild type *dmaW*-2 locus, targeting vector and expected map of the locus after marker-exchange mutagenesis. (A) Map showing *Hind*III sites (vertical lines). (B) Map of *Xba*I sites (vertical lines). Double headed arrows indicate expected fragments with sizes indicated in kb. The segment used as probe for the Southern blot in Fig 3.6 (A) and Fig 3. 7 (B) are indicated.
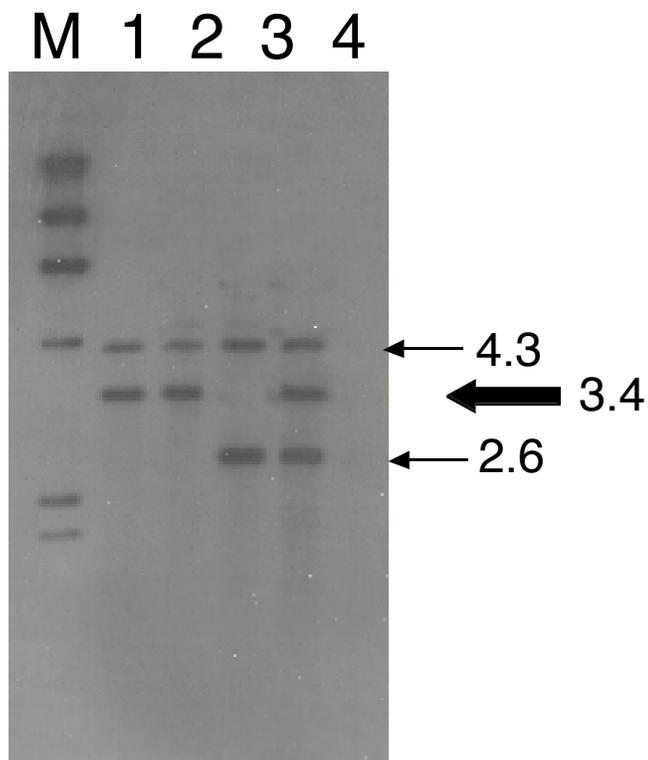
Figure 3.6: Southern-blot analysis indicating *N. coenophilalum dmaW-2* ko. Genomic DNAs were digested with *Hin*dIII. Lanes 1 and 2 contained DNA from two single-spore isolates of *dmaW-2* ko, lane 3 contained wt *N. coenophialum* DNA, and lane 4 contained DNA from an ectopic transformant. The 4.3-kb 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
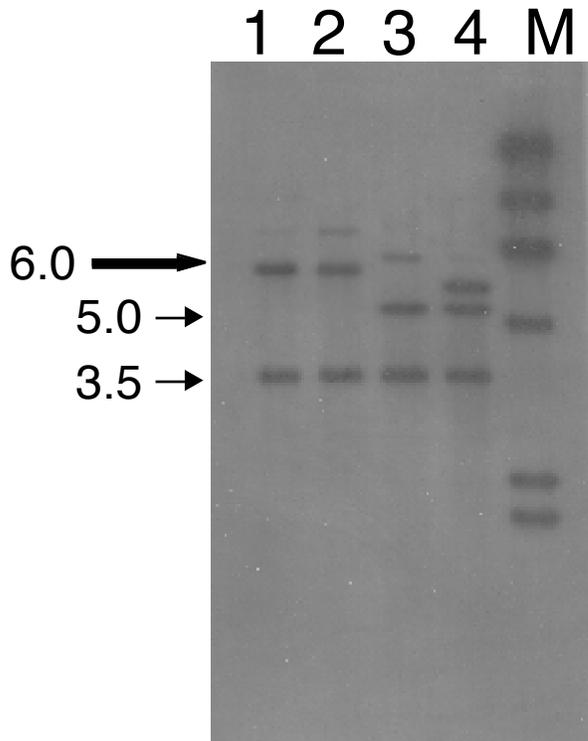
Figure 3.7: Southern-blot analysis of *N. coenophialum dmaW*-2 ko. Genomic
DNA was digested with *Xba*I. Lanes 1 and 2 contained DNA from two single-
spore isolates of *dmaW-2* ko, lane 3 contained wt *N. coenophialum* ,and lane
4 contained DNA from an ectopic transformant. M=marker as in Fig 3. 6.
The 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.
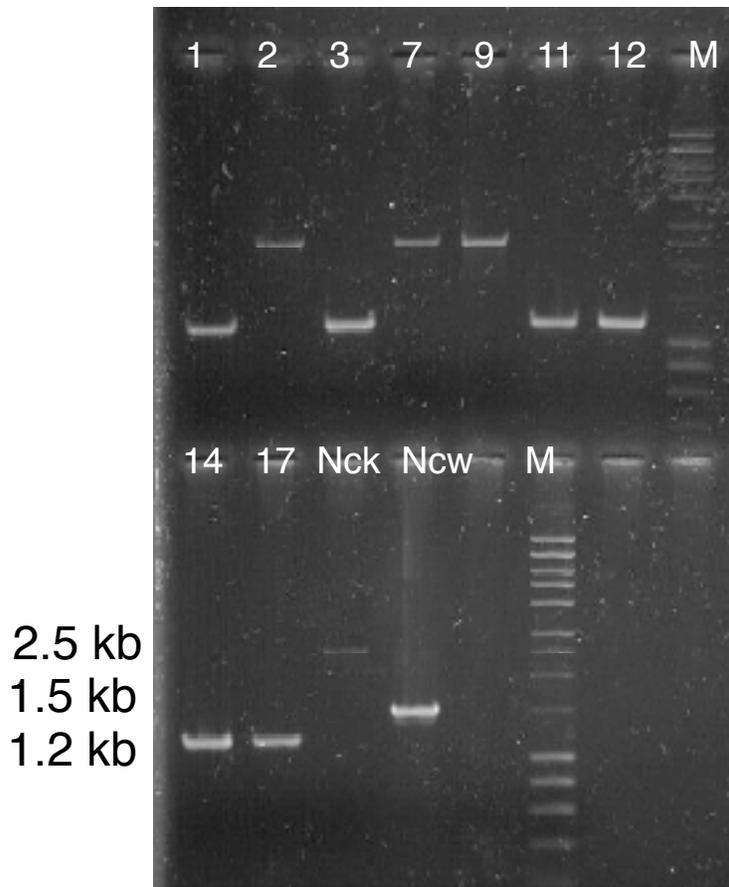
Figure 3 8: PCR analysis indicating elimination of *hph* by Cre recombinase:
A PCR test for *N. coenophialum dmaW-2* ko transformed with *Protub2*Cre.
Transformants no. 1, 3, 11, 12, 14 and 17 have lost *hph,* as indicated by the
1.2-kb PCR product, PCR from *N. coenophialum* wt (Ncw) DNA amplified
a 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-γ,γ-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*.

Materals 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 *ΨdmaW* (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 H$_2$O 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 K$_2$HPO$_4$, 0.132 M KH$_2$PO$_4$, 0.017M Na-Citrate, 0.004M MgSO$_4$ and 0.068M (NH$_4$)$_2$SO$_4$], 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'-ATAATCCTTAAAAACTCCATTTCCACCCCT-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 (NewEnglnad 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'-TCGAGGTCGACGGTATC) 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 μl of water. DNA was quantified by Hoechst dye staining, measured with DyNA Quant 200 fluorometer (Hoefer Scientific Instruments, Amersham Pharmacia biotech. San Franscisco, CA)) and fluoroimager (FluoroImager 595 Amersham Biosci.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 μM of sequencing primer, 2 μl of DTCS quick start master mix, 1.5 μl of sequencing buffer and H_2O to 20 μl. 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 μl of 3M NaOAc, pH 5.2, 2 μl of 100 mM Na_2EDTA, pH 8.0, and 1 μl of 20 mg ml^{-1} glycogen to each well. Then 60 μl 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 μl of CleanSEQ magnetic particle suspension, followed by 62 μl of 85% ethanol (for 20 μl 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 μl 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.*

51

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* (*cpox3*) (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 Ψ*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 Ψ*dmaW* (Fig 4.1; Tables 4.3 and 4.4). Three ORFs upstream of Ψ*dmaW*, Ψ*lpsB*,

ΨoxF and hypB2, were related to genes from *C. purpurea* locus 1, not in the same order but in similar orientation (Fig 4.1). Ψ*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. Ψ*lpsB* did not predict any conserved protein domain by database search (Table 4.4). Ψ*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 Ψ*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 Ψ*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 Ψ*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 *dmaW* was located at one end of the cosmid clone and 13 other ORFs were upstream of *dmaW* (Fig 4.1; Table 4.5 and 4.6). The ORFs *hypG*, *hypH* and *hypI* showed significant similarities to hypothetical protein genes from *A. nidulans* with matches to uncharacterized conserved proteins in the database. The predicted *hypJ* product was significantly similar to predicted protein from *A. nidulans* (EAA65743). The ORF *apD* encoded a putative protein with significant similarity to methionine aminopeptidase from *Leptospira interrogans*.

The contig in *C. fusiformis* had nine genes/ORFs with similarity to *C. purpurea* genes in the ergot alkaloid biosynthesis cluster 1 (Table 4.7). The seven genes from *oxP* to *dmaW* were arranged in the same order and orientation in *C. fusiformis* and *C. purpurea* (Fig 4.1). Upstream of *oxP* were two ORFs, *oxO* and *lpsB* whose similarity to *oxO* and *lpsB* from *C. purpurea* locus 1 with respect to deduced protein description, and conserved domains was similar; however, their order and orientation in *C. fusiformis* was inverted relative to *C. purpurea* locus 1 (Fig 4.1). The *lpsB* of *C. purpurea* contains an intron and *lpsB* of *C. fusiformis* when compared with *lpsB* of *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 *C. fusiformis* and *C. purpurea* is shown in Table 4. 7.

Discussion

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

Comparison of *C. purpurea* and *C. fusiformis* ergot alkaloid gene clusters is informative because these two *Claviceps* species produce two different types of ergot alkaloids. *C. purpurea* completes the pathway with production of more complex ergopeptines and *C. fusiformis* completes the pathway early on with production of different sets of clavines. My results indicate the presence of nine common genes between *C. purpurea* and *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-1* 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-1,* and I confirmed the presence of the genes they had identified and extended my search further downstream of *lpsA-1*, 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 Ψ*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 Ψ*dmaW* has different genomic context compared to *dmaW* in locus 2. The role of Ψ*dmaW* is not clear but non-functionality of Ψ*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

56

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.

Claviceps purpurea Orf description	Organism	Deduced protein description by blastx	Accession no.	Scores	% Identity/ E value
npsA	*Claviceps purpurea*	d-lysergyl peptide synthetase	CAB39315	876	41 / 0.0
	C. purpurea	Peptide synthetase	CAD28788	551	30 / e-155
oxO	*Neurospora crassa*	NADPH dehydrogenase	XP_323805	335	52 / 2e-95
	Saccharomyces cerevisiae	Old yellow enzyme	Q02899	219	37 / 3e-60
lpsB	*Claviceps purpurea*	Peptide synthetase	CAD28788	1991	93 / 0.0
oxP	*A. nidulans*	Hypothetical protein	EAA58507	177	29 / 3e-43
	Fusarium sporotrichoides	Cytochrome P450	AAK33073	117	22 / 5e-25
	Gibberella zeae	Cytochrome P450	AAM48836	117	23 / 6e-25
	Fusarium pseudograminearum	Cytochrome P450	AAM48852	115	23 / 1e-24
catA	*A. nidulans*	Hypothetical protein	EAA57781	378	43 / e-103
	Emericella nidulans	Catalase	AAG45152	375	43 / e-102
	Ajellomyces capsulatus	Catalase isozyme P	AAF01463	374	42 / e-102
	Glomerella cingulata	Catalase	CAC35154	374	43 / e-102
oxA	*Claviceps. pupurea*	Oxidoreductase	CAB39316	499	95 /e-140
oxF	*Claviceps. pupurea*	Oxidoreductase	CAB39328	879	93 / 0.0
hypB	*A. nidulans*	Hypothetical protein	EAA61806	155	30 / 1e-36
	N. crassa	Hypothetical protein	XP_323696	139	29 / 1e-31
hypA	*Photorhabdus luminescens*	Hypothetical protein	NP_929597	133	32 / 3e-30
	N. crassa	Hypothetical protein	XP_327760	98	30 / 2e-19

58

Table 4.1: Continued

Orf description	Organism	Deduced protein description by blastx	Accession no.	Scores	% Identity/ E value
hypA	*Nostoc punctiformae*	Nucleoside-diphosphate sugar epimerase	ZP_00112459	49	23 / 7e-05
dmaW	*Claviceps purpurea*	Dimethylallyltryptophan synthase long isoform	AAP81209	926	100 / 0.0
	C. purpurea	Dimethylallyltryptophan synthase short isoform	CAB39314	919	100 / 0.0
	C. fusiformis	Dimethylallyltryptophan synthase	JC4338	645	68 / 0.0
	Balansia obtecta	Dimethylallyltryptophan synthase	AAP92451	639	68 / 0.0
	Neotyphodium coenophialum	Dimethylallyltryptophan synthase	AAP81207	609	67 / e-173
oxH	*Aspergillus nidulans*	Hypothetical protein	EAA61518	139	33 / 9e-32
	Pseudomonas putida	Conserved hypothetical protein	NP_744958	135	37 / 9e-31
	P. aeruginosa	Hypothetical protein	AAK01519	133	37 / 5e-30
	Gibberella moniliformis	Fum 3p hydroxylase	AAG27131	107	31 / 2e-22
lpsA-1	*Claviceps purpurea*	d-lysergyl peptide synthetase	CAB39315	6206	96 / 0.0
	A. nidulans	Hypothetical protein	EAA59538	1414	29 / 0.0
	Neotyphodium lolii	Peptide synthetase	AAL26315	1408	54 / 0.0
	A. nidulans	Hypothetical protein	EAA64650	1383	29 / 0.0
	Gibberella zeae	Hypothetical protein	EAA69381	1287	28 / 0.0
	Hypocreae virens	Nonribosomal peptide synthetase: NRPS	AAM78457	1263	29 / 0.0
hypC	*A. nidulans*	Hypothetical protein	EAA66313	46	27 / 1e-04
lpsA-2	*C. purpurea*	d-lysergyl peptide synthetase	CAB39315	3759	85 / 0.0
	Hyporcrea virens	Nonribosomal peptide synthetase: NRPS	AAM78457	907	30 / 0.0
	N. lolii	Peptide synthetase	AAL26315	706	53 / 0.0
	Cochliobolus carbonum	HC-toxin synthetase	A45086	746	26 / 0.0
	A. nidulans	Hypothetical protein	EAA59538	978	30 / 0.0
	A. nidulans	Hypothetical protein	EAA64650	971	30 / 0.0

Table 4.2: Conserved protein domains deduced by BLAST analysis (rpsblast) from sequences near *dmaW* in *Claviceps purpurea* locus 1.

ORF designation	Conserved protein domain	Scores	E value
npsA	AMP binding domain	284	7e-66
	Non ribosomal peptide synthetase modules and related proteins	237	2e-62
oxO	NADH: flavin oxidoreductase	252	7e-68
	NADH: flavin oxidoreductase, old yellow enzyme family	244	2e-65
lpsB	AMP binding domain	236	2e-62
	Non ribosomal peptide synthetase modules and related proteins	203	2e-52
oxP	Cytochrome P450	89	2e-18
catA	Catalase	433	6e-122
oxA	Short chain dehydrogenase	116	6e-27
	FabG, dehydrogenases with different specificities, related to short-chain-alcohol dehydrogenases	113	6e-26
oxF	FAD binding domain	73	2e-13
	FAD/FMN containing dehydrogenases	44	9e-05
hypB	Uncharacterized conserved protein	108	2e-24
hypA	Predicted nucleoside-diphosphate sugar epimerase	65	2e-11
dmaW	No putative conserved domain detected		
oxH	Phytanoyl-CoA dioxygenase	79	2e-15
	Protein involved in biosynthesis of mitomycin antibiotics/ polyketide fumonisin	58	2e-09
lpsA-1	Non ribosomal peptide synthetase modules and related proteins	283	7e-76
	Condensation domain	154	3e-37
hypC	No putative conserved domain detected		
lpsA-2	Non ribosomal peptide synthetase modules and related proteins	284	3e-76
	Condensation domain	158	3e-38

Table 4.3: Predicted open reading frames from sequences near ΨdmaW in *Claviceps purpurea* locus 2.

Claviceps purpurea Orf description	Organism	Deduced protein description by blastx	Accession no.	Scores	% Identity/ E value
Ψ*lpsB*	*Claviceps purpurea*	Peptide synthetase, monomodular	CAD28788	56	69 / 6e-06
Ψ*oxF*	*Claviceps. pupurea*	Oxidoreductase	CAB39328	76	43 / 6e-15
hypB2	*Neurospora. crassa*	Hypothetical protein	XP_323696	134	29 / 4e-30
	Aspergillus. nidulans	Hypothetical protein	EAA61806	149	30 / 1e-34
Ψ*dmaW*	*Claviceps purpurea*	Putative dimethylallyltryptophan synthase	CAC37396	481	90 / e-135
hypD	*Magnaporthe grisea*	Hypothetical protein	EAA56058	360	62 / 2e-98
	Neurospora crassa	Hypothetical protein	XP_328432	296	52 / 4e-79
vapA	*Magnaporthe grisea*	Hypothetical protein	EAA55783	590	56 / e-167
	Neurospora crassa	Hypothetical protein	XP_327306	574	51 / e-162
	Aspergillus nidulans	Hypothetical protein	EAA63909	532	53 / e-150
	Schizosaccharomyces pombe	Putative vacuolar protein sorting-associated protein	CAB62421	280	33 / 8e-74
hypE	*M. grisea*	Hypothetical protein	EAA55781	253	39 / 8e-66
	N. crassa	Hypothetical protein	XP_327305	207	31 / 5e-52
	A. nidulans	Hypothetical protein	EAA63918	159	33 / 2e-37`
capB	*N. crassa*	Hypothetical protein	XP_326285	607	55 / e-172
	M. grisea	Hypothetical protein	EAA56304	584	52 / e-165
	A. nidulans	Hypothetical protein	EAA58972	542	50 / e-152
	Ustilago maydis	Capsule associated protein	AAL05058	178	25 / 4e-43
	Filobasidiella neoformans	Capsule associated protein	AAD44757	204	26 / 6e-51
hypF	*M. grisea*	Hypothetical protein	EAA53358	288	43 / 2e-76
	N. crassa	Hypothetical protein	XP_322703	286	42 / 9e-76
ptpC	*M. grisea*	Hypothetical protein	EAA51337	910	56 / 0.0

Table 4.3: Continued

Orf description	Organism	Deduced protein description by blastx	Accession no.	Scores	% Identity/ E value
ptpC	*M. grisea*	Hypothetical protein	EAA48646	808	50 / 0.0
	Schizophyllum commune	Peptide transporter MTD1	AAF26618	602	41 / e-170

Table 4.4: Conserved protein domains deduced by BLAST analysis (rpsblast) from sequences near Ψ*dmaW* in *Claviceps purpurea* locus 2.

ORF designation	Conserved protein domain	Scores	E value
Ψ*lpsB*	Not detected		
Ψ*oxF*	Not detected		
hypB2	Uncharacterized conserved protein	107	5e-24
Ψ*dmaW*	Not detected		
hypD	Isoamyl acetate-hydrolyzing esterase and related enzymes	132	7e-32
vapA	Membrane coat complex retromer, subunit VPS5/SNX1 (Intracellular trafficking, secretion and vesicular transport)	69	4e-12
hypE	Signalosome subunit (post translational modification, protein turnover, chaperones and signal transduction mechanism)	84	7e-17
capB	Putative lipopolysaccharide modifying enzyme	84	1e-16
hypF	Prolyl 4- hydroxylase alpha subunit homologues	44	6e-05
ptpC	Sexual differentiation process protein (signal transduction mechanisms)	287	5e-78
	Oligopeptide transporter protein	183	2e-46

Table 4.5: Predicted open reading frames from sequences near *dmaW* in *Claviceps fusiformis*.

Claviceps fusiformis Orf designation	Organism	Deduced protein description by blastx	Accession no.	Scores	% Identity/ E value
hypG	*Aspergillus nidulans*	Hypothetical protein	EAA59167	251	71 / 2e-65
	Agrobacterium tumefaciens	Uncharacterized conserved protein	NP_534111	168	57 / 1e-40
hypH	*A. nidulans*	Hypothetical protein	EAA59168	381	64 / e-104
hypI	*A. nidulans*	Hypothetical protein	EAA59167	167	55 / 1e-40
	Caulibacter erescentus	Conserved hypothetical protein	NP_419337	152	53 / 5e-36
hypJ	*A. nidulans*	Predicted protein	EAA65743	149	33 / 1e-34
	A. nidulans	Hypothetical protein	EAA62371	136	31 / 5e-31
apD	*Mycobacterium avium* subsp. *paratuberculosis*	Hypothetical protein MAP3935	NP_962869	239	54 / 2e-62
	Leptospira interrogans	Methionine aminopeptidase	NP_713137	165	40 / 6e-40
	Pseudomonas putida	Iron-sulfur cluster binding protein	NP_744785	97	33 / 2e-19
lpsB	*Claviceps purpurea*	Peptide synthetase, mono modular	CAD28788	270	60 / 0.0
oxO	*Neurospora crassa*	NADPH2 dehydrogenase	XP_323805	209	47 / 9e-53
	Lycopersicon esculentum	12-oxophytodienoate reductase	CAB43506	159	41 / 1e-37
	Trichodesmium erythraeum	NADH: flavin oxidoreductase, old yellow enzyme	ZP_00074744	159	40 / 1e-36
	Saccharomyces cerevisiae	Old yellow enzyme	NP_015154	152	39 / 7e-36
oxP	*A. nidulans*	Hypothetical protein	EAA58507	89	27 / 2e-16
	Fusarium pseudograminearum	Cytochrome P450	AAM48852	60	31 / 6e-08
	Gibberella zeae	Cytochrome P450	AAM48884	60	39 / 1e-07
catA	*A. nidulans*	Hypothetical protein	EAA57781	245	39 / 1e-63
	Emericella nidulans	Catalase	AAG45152	242	39 / 7e-63

Table 4.5: Continued

Orf designation	Organism	Deduced protein description by blastx	Accession no.	Scores	% Identity/ E value
catA	*Ajellomyces capsulatus*	Catalase isozyme P	AAF01463	239	36 / 5e-62
	Glomerella cingulata	Catalase	CAC35154	236	36 / 5e-61
oxA	*Claviceps. pupurea*	Oxidoreductase	CAB39316	332	72 / 3e-90
oxF	*Claviceps. pupurea*	Oxidoreductase	CAB39328	610	63 / e-173
hypB	*A. nidulans*	Hypothetical protein	EAA61806	164	31 / 3e-39
	N. crassa	Hypothetical protein	XP_323696	150	31 / 5e-35
hypA	*Photorhabdus luminescens*	Hypothetical protein	NP_929597	130	40 / 2e-29
	N. crassa	Hypothetical protein	XP_327760	101	42 / 8e-21
	Nostoc punctiformae	Nucleoside-diphosphate sugar epimerase	ZP_00112459	61	27 / 2e-08
	Pseudomonas aeruginosa	Nucleoside-diphosphate sugar epimerase	ZP_00136570	59	31 / 6e-08
	Streptomyces nogalater	Putative hydroxylase	AAF01810	50	25 / 2e-05
	Shigella flaxneri	Putative oxidoreductase	NP_709986	50	25 / 3e-05
dmaW	*Claviceps fusiformis*	Dimethylallyltryptophan synthase	JC4338	753	98 / 0.0
	C. purpurea	Dimethylallyltryptophan synthase	CAB39314	520	67 / e-146

Table 4.6: Conserved protein domains deduced by BLAST analysis (rpsblastp) of putative genes near *dmaW* in *Claviceps fusiformis*

ORF designation	Conserved protein domain	Scores	E value
hypG	Protein of unknown function	153	7e-38
	Uncharacterized conserved protein	150	5e-37
hypH	No putative conserved domain detected		
hypI	Protein of unknown function	145	7e-36
	Uncharacterized conserved protein	138	7e-34
hypJ	Predicted Kinase	39	0.001
	Predicted aminoglycoside phosphotransferase	39	0.002
apD	Xaa-Pro aminopeptidase (amino acid transport and metabolism)	64	2e-11
	Methionine aminopeptidase (translation, ribosomal structure and biogenesis)	45	2e-05
lpsB	Non ribosomal peptide synthetase modules and related proteins	74	1e-13
oxO	NADH: flavin oxidoreductase, old yellow enzyme	163	7e-41
	NADH: flavin oxidoreductase / 12- oxophytodienoate reductase	154	3e-38
oxP	Cytochrome P450	61	5e-10
catA	Catalase	269	6e-73
oxA	FabG, dehydrogenases with different specificities, related to short-chain-alcohol dehydrogenases	116	6e-27
	Short chain dehydrogenase	111	2e-25
oxF	FAD binding domain	81	8e-16
	FAD/FMN containing dehydrogenases	67	1e-11
hypB	Uncharacterized conserved protein	121	3e-28
hypA	Predicted nucleoside-diphosphate sugar epimerase	71	2e-13
	Predicted dehydrogenase	39	7e-04
dmaW	No putative conserved domain detected		

Table 4.7: Description of the common genes shared between *Claviceps purpurea* locus 1 and *Claviceps fusiformis*

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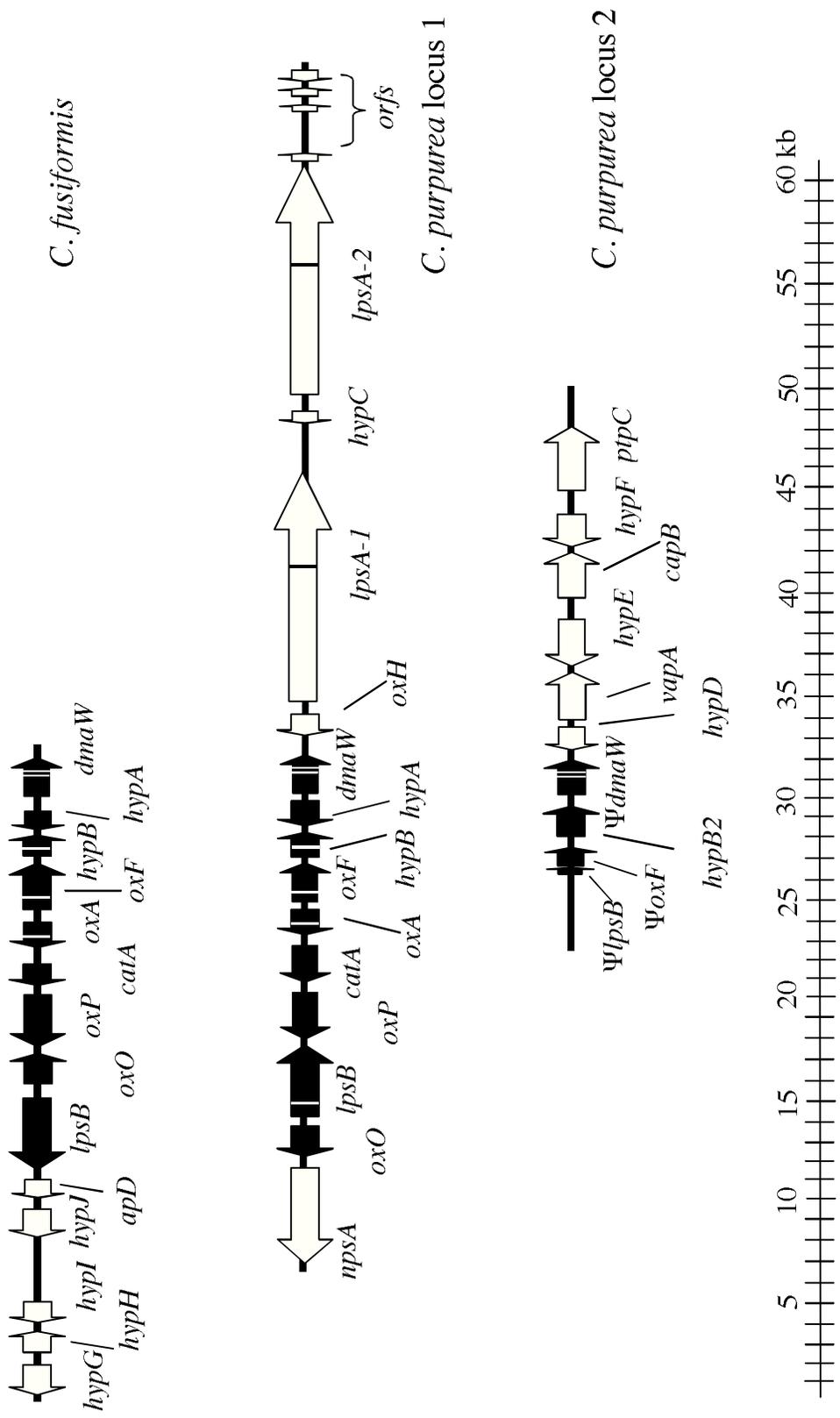
Fig 4.1: Schematic map of genomic regions from *dmaW*-containing gene clusters from *C. fusiformis*, *C. purpurea* locus 1 and *C. purpurea* locus 2. Genes that are common are colored with black boxes. The orientation of genes in indicated by arrow heads. Vertical lines within each ORF indicate intron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

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 *Bam*HI and
*Sal*I 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'-
CTTCTTCACAGGATCCGTTTAACACTTTCGCTATCTG) and dmaWe19cos SalI.37u (5'-
GCGTCGACGGAAGTAGTCCTTTTACCTTAGTAAAGGC) into pBSKS+ that was cut with
*Bam*HI and *Sal*I. 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
(AAACGGATCCTGTGAAGAAGAGGACGAGCGIAATAGC) was digested with *Sac*II and
*Bam*HI. The 4.1 kb fragment was cloned adjacent to *Sac*II and *Bam*HI sites of the above
pBSKS+ construct to give pKAES146.

pKAES147

      pKAES146 was digested with *Sac*II and *Sal*I 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
*Sac*II and *Sal*I. 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 2nd step knockout in putative *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+ and hyg –. 4850 colonies were picked onto hyg- medium and then replica picked onto hyg + medium. Out of 4850 colonies, 56 of them failed to grow on hyg + 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+ and hyg – containing PDA plate and all of them grew on both hyg+ and hyg – plates. DNA was extracted from several of these 56 colonies and analyzed for the presence of *hph* and wt *dmaW-1* by PCR and all of them showed the amplification for both *hph* and wt *dmaW-1* indicating that second step of knockout has not taken place.

Transformation

Transfer

Single spore isolation

Analysis - PCR, Southern blot

Step 1

Streak on hyg minus (non selection)
2 to 3 rounds

Conidial suspension

Pick individual colonies and place on
PDA grid plate without selection

hyg -

Replica plate onto hyg plate

hyg +

Identify colonies growing on hyg-
and not on hyg +

hyg -

hyg +
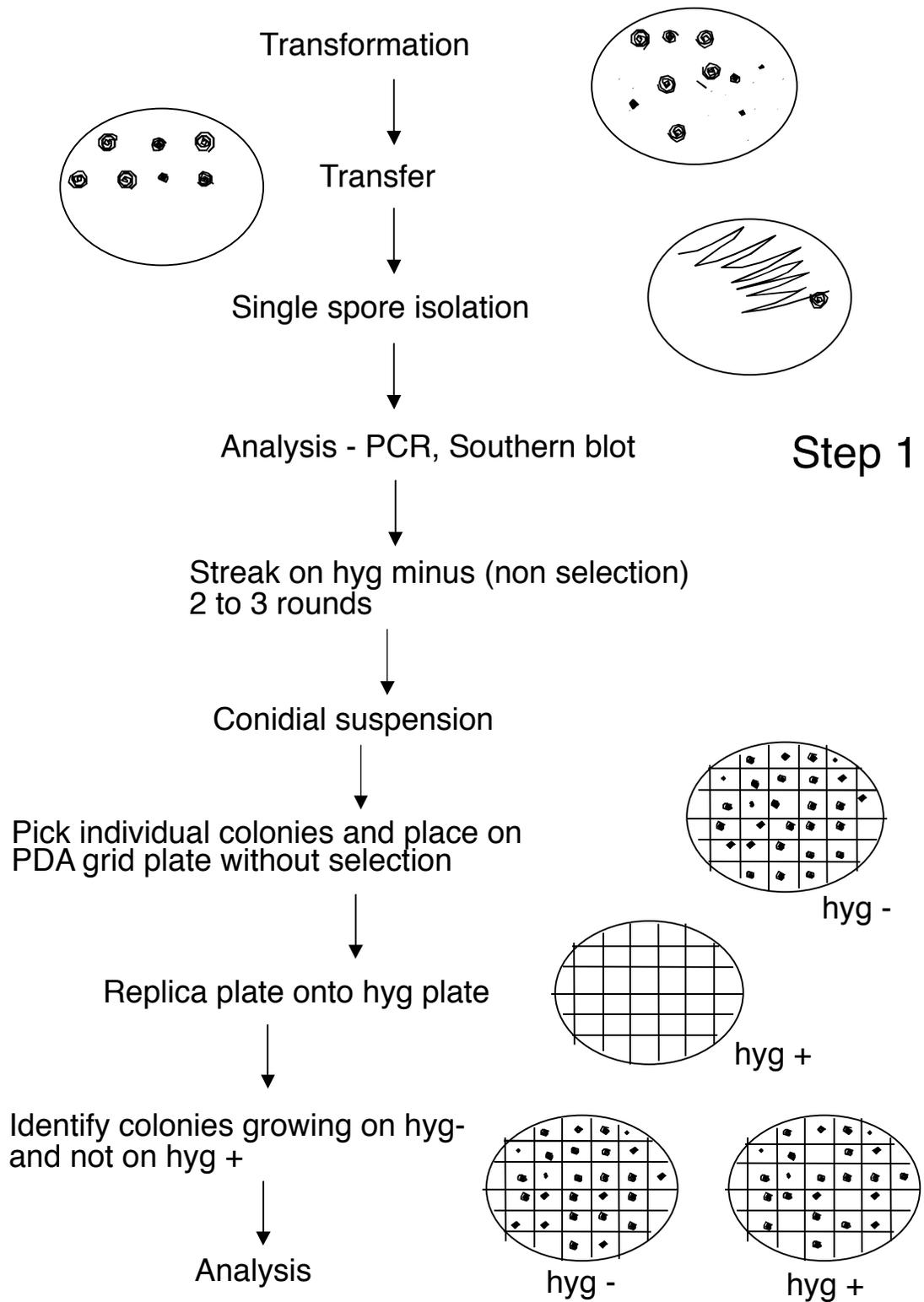
Analysis

Figure A1.1 : Outline of two step knockout process in *N. coenophialum*
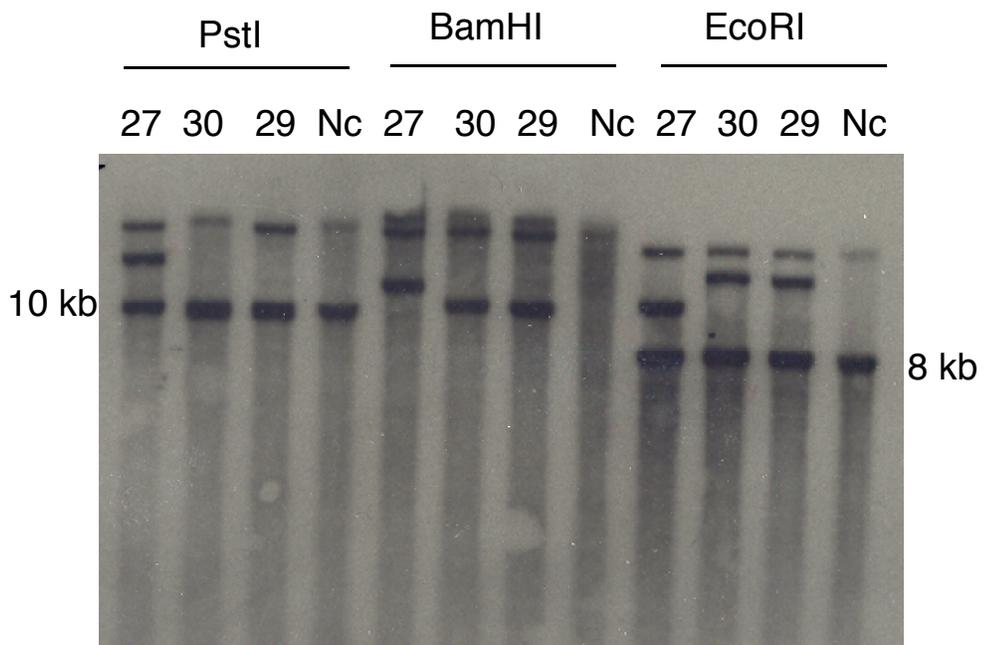
Figure A1.2 Southern blot analysis indicating first step knockout in *N. coenophialum* transformed with *dmaW-1* construct. The genomic DNA was digested with *Pst*I, *Bam*HI and *Eco*RI. Lanes wih no. 27, 30 and 29 indicate three different transformants and Nc is *N. coenophialum* wt. Tranformant no. 27 indicated the one step integration of *dmaW-1* 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 *Sac*II) 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*

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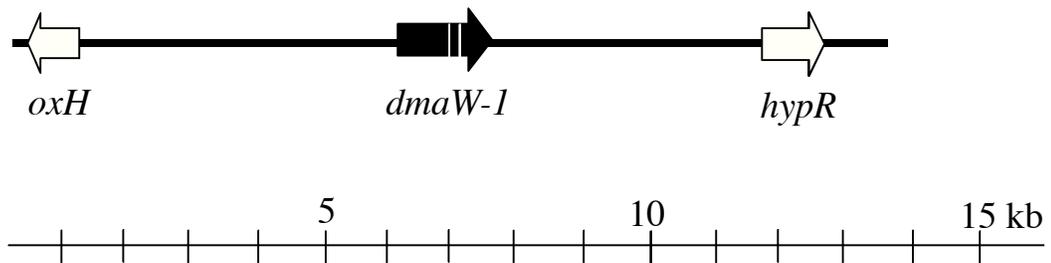
Figure A 3.1: Schematic map of genomic regions near *dmaW-1* from *N. coenophialum*.

dmaW-1 is indicated with black box and vertical lines indicate introm position. The

orientation of genes is indicated by arrow heads.

References

Al-Samarrai, T. H. and J. Schmid (2000). A simple method for extraction of fungal genomic DNA. Lett. Appl. Microbiol. **30**: 53-56.

An, Z.-Q., M. R. Siegel, W. Hollin, Tsai H-F, D. Schmidt and C. L. Schardl (1993). Relationships among non-*Acremonium* sp. fungal endophytes in five grass species. Applied and Environmental Microbiology **59**: 1540-1548.

Annis, S. L. and D. G. Panaccione (1998). Presence of peptide synthetase gene transcripts and accumulation of ergopeptines in *Claviceps purpurea* and *Neotyphodium coenophialum*. Canandian Journal of Microbiology **44**: 80-86.

Arechavaleta, M., C. W. Bacon, C. S. Hoveland and D. E. Radcliff (1989). Effects of the tall fescue endophyte on plant response to environmental stress. Agronomy Journal **81**: 83-90.

Ausubel, R. M., R. Bremt, R. E. Kingston, D. D. Moore and J. G. e. a. Seidman (1999). Current Protocols in Molecular biology, John Wiley & Sons. New York.

Bacon, C. W. (1995). Toxic endophyte-infected tall fescue and range grasses: historic perspectives. Journal of Animal Science **73**: 861-870.

Bacon, C. W., J. K. Porter, J. D. Robbins and E. S. Luttrell (1977). Epichloe typhina from toxic tall fescue grasses. Applied and Environmental Microbiology **34**: 576-581.

Birch, A. J., B. J. McLoughlin and H. Smith (1960). The biosynthesis of the ergot alkaloids. Tetrahedron Lett. **7**: 1-3.

Bouton, J. H. (2003). Commercial applications of endophytic fungi. In: white Jr., J.F., Bacon, C.W., Hywel-Jones, N.L., Spatafora, J.W (Eds.), Mycology. Clavicipitacean fungi: evolutionary biology, chemistry, biocontrol and cultural impacts. vol 19, Marcel-Dekker Inc, New York and Basel: 495-516.

Bouton, J. H., N. S. Hill, C. Hoveland, M. A. McCann, F. N. J. Thompson, L. Hawkins and G. C. M. Latch (2000). Performance of tall fescue cultivars infected with non-toxic endophytes. Proceedings of 4th International Neotyphodium/grass interactions symposium, Soest, Germany: 179-185.

Bush, L. P., H. H. Wilkinson and C. L. Schardl (1997). Bioprotective alkaloids of grass-fungal endophye symbioses. Plant Physiology **114**: 1-7.

Byrd, A. D., C. L. Schardl, P. J. Songlin, K. L. Mogen and M. R. Siegel (1990). The B-tubulin gene of *Epichloë typhina* from perennial ryegrass (*Lolium perenne*). Current Genetics **18**: 347-354.

Chung, K.-R. and C. L. Schardl (1997). Sexual cycle and horizontal transmission of the grass symbiont, *Epichloë typhina*. <u>Mycological Research</u> **101**: 295-301.

Clay, K. and C. L. Schardl (2002). Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. <u>The American Naturalist</u> **160**: S99-S127.

Corneille, S., K. Lutz, Z. Svab and P. Maliga (2001). Efficient elimination of selectable marker genes from the plastid genome by the CRE-*lox* site-specific recombination system. <u>The Plant Journal</u> **27**(2): 171-178.

Correia, T., N. Grammel, I. Ortel, U. Keller and P. Tudzynski (2003). Molecular cloning and analysis of the ergopeptine assembly system in the ergot fungus *Claviceps purpurea*. <u>Chemistry and Biology</u> **10**: 1281-1292.

Cross, D. L. (2003). Ergot alkaloid toxicity. <u>In: white Jr., J.F., Bacon, C.W., Hywel-Jones, N.L., Spatafora, J.W (Eds.), Mycology. Clavicipitacean fungi: evolutionary biology, chemistry, biocontrol and cultural impacts. vol 19, Marcel-Dekker Inc, New York and Basel</u>: 475-494.

Dougherty, C. T., L. M. Lauriault, N. W. Bradley, N. Gay and P. L. Cornelius (1991). Induction of tall fescue toxicosis in heat stressed cattle and its alleviation with thiamin. <u>Journal of Animal Science</u> **69**: 1008-1018.

Doyle, J. J. and J. L. Doyle (1990). Isolation of plant DNA from fresh tissue. <u>Focus</u> **12**: 13-15.

Elmi, A. A., C. P. West, R. T. Robbins and T. L. Kirkpatrick (1999). Endophyte effects on reproduction of a root-knot nematode (*Meloidogyne marylandi*) and osmotic adjustment in tall fescue. <u>Grass and Forage Science</u> **55**: 166-172.

Floss, H. G. (1976). Biosynthesis of ergot alkaloids and related compounds. <u>Tetrahedron</u> **32**: 873-912.

Floss, H. G. and J. A. Anderson (1980). Biosynthesis of ergot toxins. <u>In: A Study in secondary metabolism. Academic Press, NewYork</u>: 17-67.

Floss, H. G., J. E. Robbers and P. F. Heinstein (1974). Regulatory control mechanisms in alkaloid biosynthesis. <u>Recent Advances in Phytochemistry</u> **8**: 141-178.

Gebeyehu, G., P. Y. Rao, P. SooChan, D. A. Simms and L. Klevan (1987). Novel biotinylated nucleotide analogs for labeling and colorimetric detection of DNA. <u>Nucleic Acids Research</u> **15**: 4513-4534.

Gebler, J. C. and C. D. Poulter (1992). Purification and characterization of dimethylallyl tryptophan synthase from Claviceps purpurea. <u>Archives of Biochemistry and Biophysics</u> **296**: 308-313.

Groger, D. and H. G. Floss (1998). Biochemistry of ergot alkaloids-achievements and challenges. In: The alkaloids: Chemistry and biology, vol 50. Academic Press, London: 171-218.

Groger, D., K. V. Mothes, H. Simon, H. G. Floss and F. Weygand (1960). Uber den Einbau von melalonaure in das ergonlinsystem der clavin-alkaloide. Z. Naturforsch Teil **15b**: 141-143.

Groger, D., H. J. Wendt, K. V. Mothes and F. Weygand (1959). Untersuchungen zur Biosynthese der Mutterkornalkaloide. Z. Naturforsch Teil **14b**: 355-358.

Gwinn, K. D., M. H. Collins-Shepard and B. B. Reddick (1991). Tissue print immunoblot, an accurate method for the detection of *Acremonium coenophialum* in tall fescue. Phytopathology **81**: 747-748.

Gwinn, K. D. and A. M. Gavin (1992). Relationship between endophyte infestation level of tall fescue seed lots and Rhizoctonia zeae seedling disease. Plant Disease **76**: 911-914.

Heinstein, P. F., S.-L. Lee and H. G. Floss (1971). Isolation of dimethylallylpyrophosphate: tryptophan dimethyl transferase from the ergot fungus (Claviceps spce). Biochemical and biophysical research communications **44**: 1244-1251.

Hill, N. S., G. E. Rottinghaus, C. S. Agee and L. M. Schultz (1993). Simplified sample preparation for HPLC analysis of ergovaline in tall fescue. Crop Science **33**: 331-333.

Hohn, B., A. A. Levy and H. Puchta (2001). Elimination of selection markers from transgenic plants. current Opinion in Biotechnology **12**: 139-143.

Hoveland, C. (1993). Importance and economic significance of the *Acremonium* endophytes to performance of animals and grass plants. Agriculture Ecosystems and Environment **44**: 3-12.

Keller, N. P. and T. M. Hohn (1997). Metabolic pathway gene clusters in filamentous fungi. Fungal Genetics and Biology **21**(1): 17-29.

Kim, I.-S., S.-U. Kim and J. A. Anderson (1981). Microsomal agroclavine hydroxylase of Claviceps species. Phytochemistry **20**: 2311-2314.

Kimmons, C. A., K. D. Gwinn and E. C. Bernard (1990). Nematode reproduction on endophyte infected and endophyte free tall fescue. Plant Disease **74** (757-761).

Kozikowski, A. P. and J. P. Wu (1988). Probing ergot alkaloid biosynthesis: Identification of advanced intermediates along the biosynthetic pathway. Journal of American Chemical Society **110**: 1970-1971.

Krupinski, V. M., J. E. Robbers and H. G. Floss (1976). Physiological study of ergot: induction of alkaloid synthesis by tryptophan at the enzymatic level. Journal of Bacteriolgy **125**: 158-165.

Lakso, M., B. Sauer, J. Mosinger, B., E. J. Lee, R. W. Manning, S. Yu, K. L. Mulder and H. Westphal (1992). Targeted Oncogene Activation by Site-Specific Recombination in Transgenic Mice. PNAS **89**: 6232-6236.

Latch, G. C. M., M. J. Christensen and G. J. Samuels (1985). Artificial infection of grasses with endophytes. Annals of Applied Biology **107**: 17-24.

Lee, S.-L., H. G. Floss and P. F. Heinstein (1976). Purification and properties of dimethylallylpyrophosphate: tryptophan dimethyl transferase, the first enzyme of ergot alkaloid biosynthesis in *Claviceps*. sp. SD 58. Archives of Biochemistry and Biophysics **177**: 84-94.

Lyons, P. C., R. D. Plattner and C. W. Bacon (1986). Occurance of peptide and clavine ergot alkaloids in tall fescue grass. Science **232**: 487-489.

Malinowski, D. P. and D. P. Belesky (2000). Adaptation of endophyte-infected cool season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Science **40**: 923-940.

Marahiel, M. A., T. Stachelhaus and H. D. Mootz (1997). Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem. Rev. **97**: 2651-2673

Mattern, I. E., P. J. Punt and C. A. M. J. J. van den Hondel (1988). A vector of Aspergillus transformation conferring phleomycin resistance. Fungal genetics newsletter **35**: 25.

Mothes, K. V., F. Weygand, D. Groger and H. Grisebach (1958). Untersuchungen zur Biosynthese der Mutterkorn-Alkaloide. Z. Naturforsch Teil **14b**: 355-358.

Murray, F. R., G. C. M. Latch and D. B. Scott (1992). Surrogate transformation of perennial ryegrass, *Lolium perenne*, using genetically modified *Acremonium* endophyte. Molecular and General Genetics **233**: 1-9.

Otsuka, H., J. A. Anderson and H. G. Floss (1979). The stage of N-methylation in ergot alkaoid biosynthesis. Chem. Comm. **15**: 660-662.

Otsuka, H., F. R. Quigley, D. Groger, J. A. Anderson and H. G. Floss (1980). In vivo and in vitro evidence for N-methylation as the second pathway specific step in ergoline biosynthesis. Planta Medica **40**: 109-119.

Panaccione, D. G., R. D. Johnson, J. H. Wang, C. A. Young, P. Damrongkool, B. Scott and C. L. Schardl (2001). Elimination of ergovaline from a grass-Neotyphodium endophyte

symbiosis by genetic modification of the endophyte. <u>Proceedings of the National Academy of Sciences of the United States of America</u> **98**(22): 12820-12825.

Panaccione, D. G. and C. L. Schardl (2003). Molecular genetics of ergot alkaloid biosynthesis. <u>In: white Jr., J.F., Bacon, C.W., Hywel-Jones, N.L., Spatafora, J.W (Eds.), Mycology. Clavicipitacean fungi: evolutionary biology, chemistry, biocontrol and cultural impacts. vol 19, Marcel-Dekker Inc, New York and Basel</u>: 399-424.

Panaccione, D. G., B. A. Tapper, G. A. Lane, E. Davies and K. Fraser (2003). Biochemical outcome of blocking the ergot alkaloid pathway of a grass endophyte. <u>Journal of Agricultural Food Chemistry</u> **51**: 6429-6437.

Porter, J. K. and F. N. J. Thompson (1992). Effects of fescue toxicosis on reproduction in livestock. <u>Journal of Animal Science</u> **70**: 1594-1603.

Proctor, R. H., A. E. Desjardins, R. D. Plattner and T. M. Hohn (1999). A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in *Gibberella fujikoroi* mating population A. <u>Fungal Genetics and Biology</u> **27**: 100-112.

Riederer, B., M. han and U. Keller (1996). D-lysergyl peptide synthetase from the ergot fungus *Claviceps purpurea*. <u>Journal of Biological chemistry</u> **271**: 27524-27530.

Rowan, D. D. and G. C. M. Latch (1994). Utilization of endophyte-infected perennial ryegrass of increased insect resistance. <u>in: Biotechnology of endophytic fungi of grasses. CRC press, Boca Raton</u>: 169-183.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular cloning: a laboratory manual. <u>Cold Spring Harbor Laboratory, Cold Spring Harbor, New York</u>.

Schardl, C. L. (1994). Molecular and genetic methodologies and transformation of grass endophytes. <u>in: Biotechnology of endophytic fungi of grasses. CRC press, Boca Raton</u>: 151-165.

Schardl, C. L. (1996). Epichloe species: fungal symbionts of grasses. <u>Annual Review of Phytopathology</u> **34**: 109-130.

Schardl, C. L., A. Leuchtmann, K.-R. chung, D. Penny and M. R. Siegel (1997). Coevolution by common descent of fungal symbionts (Epichloë spp.) and grass hosts. <u>Molecular Biology and Evolution</u> **14**: 133-143.

Schardl, C. L., A. Leuchtmann, H. F. Tsai, M. A. Collett, D. M. Watt and D. B. Scott (1994). Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloe typhina*. <u>Genetics</u> **136**(4): 1307-1317.

Schardl, C. L. and T. D. Phillips (1997). Protective grass endophytes where are they from and where are they going? Plant Disease **81**(5): 430-438.

Schmidt, S. P. and T. G. Osborne (1993). Effects of endophyte-infected tall fescue on animal performance. Agriculture Ecosystems and Environment **44**: 233-262.

Shibuya, M., H.-M. Chou, M. Fountoulakis, S. Hassam, S.-U. Kim, K. Kobayashi, H. Otsuka, E. Rogalska, J. M. Cassady and H. G. Floss (1990). Stereochemistry of the isoprenylation of tryptophan catalyzed by 4-(γ,γ-dimethylallyl) tryptophan synthase from Claviceps, the first pathway-specific enzyme in ergot alkaloid biosynthesis. Journal of American Chemical Society **112**: 297-304.

Siegel, M. R., G. C. M. Latch, L. P. Bush, F. F. Fannin, D. D. Rowan, B. A. Tapper, C. W. Bacon and M. C. Johnson (1990). Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. Journal of Chemical Ecology **16**: 3301-3315.

Spiering, M. J., E. Davies, B. A. Tapper, J. Schmid and G. A. Lane (2002). Simplified extraction of ergovaline and peramine for analysis of tissue distribution in endophyte-infected grass tillers. Journal of Agricultural and Food Chemistry **50**(21): 5856-5862.

Sternberg, N. and D. Hamilton (1981). Bacteriophage P1 site-specific recombination. Journal of Molecular Biology **150**: 467-486.

Tsai, H.-F., H. Wang, J. C. Gebler, C. D. Poulter and C. L. Schardl (1995). The Claviceps purpurea gene encoding dimethylallyltryptophan synthase, the committed step for ergot alkaloid biosynthesis. Biochemical and biophysical research communications **216**(1): 119-125.

Tsai, H. F., M. R. Siegel and C. L. Schardl (1992). Transformation of *Acremonium coenophialum*, a protective fungal symbiont of the grass *Festuca arundinacea*. Current Genetics **22**(5): 399-406.

Tudzynski, P., T. Correia and U. Keller (2001). Biotechnology and genetics of ergot alkaloids. Applied Microbiology and Biotechnology **57**(5-6): 593-605.

Tudzynski, P., K. Holter, T. Correia, C. Arntz, N. Grammel and U. Keller (1999). Evidence for an ergot alkaloid gene cluster in *Claviceps purpurea*. Molecular and General Genetics **261**: 133-141.

Vergunst, A. C. and P. J. J. Hooykaas (1999). Recombination in the plant genome and its application in biotechnology. Critical Reviews in Plant Sciences **18**(1): 1–31.

Wang, J., C. Machado, D. G. Panaccione, H.-F. Tsai and C. L. Schardl (2004). The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. Fungal Genetics and Biology **41**(2): 189-198.

Wang, J. (2000). *dmaW* encoding tryptophan dimethylallyltransferase in ergot alkaloid biosynthesis from Clavicipitaceous fungi. <u>Ph.D Thesis. University of Kentucky.</u>

Young, C., L. McMillan, E. Telfer and B. Scott (2001). Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Penicillium paxilli*. <u>Molecular Microbiology</u> **39**: 754-764.

Vita

Name   Caroline MACHADO
Date of Birth   August 30, 1970
Place of Birth Karkala, India

Education

Master of Science, Agricultural microbiology (1995) Thesis: "Mycorrhization helper bacteria and their influence on growth of cowpea, *Vigna unguiculata* (L.) Walp". University of Agricultural Sciences, Bangalore, Karnataka, India.

Bachelor of Science, Agriculture (1992). University of Agricultural Sciences, Bangalore, Karnataka, India.

Honors
Dissertation year fellowship (2002-2003)
University of Kentucky, Research Challenge Trust Fund I Fellowship (1999 - 2000), (2000 - 2001) and (2001 - 2002)

Publication

Wang, J., C. Machado, D. G. Panaccione, H.-F. Tsai and C. L. Schardl (2004). The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. Fungal Genetics and Biology 41(2): 189-198.

Caroline Machado, J. Wang, D.G. Panaccione and C. L. Schardl (2003). Eliminating ergot alkaloid biosynthesis genes in the endophytes of forage grasses. The 4th International symbiosis society congress. August 17-23, 2003. Halfax, NovaScotia, Canada.

Caroline Machado, Jinghong Wang, D.G. Panaccione and C. L. Schardl (2003). Eliminating anti-mammalian toxin genes in the endophytes of forage grasses. Presented at the 4th I.E. Melhus graduate student symposium; APS annual meeting. August 9-13, 2003. Charlotte, North Carolina, USA.

Caroline Machado, Jinghong Wang and Christopher Schardl (2001). The gene for the determinant step in ergot alkaloid synthesis by *Neotyphodium coenophialum* and other grass endophytes. The Joint meeting of the American Phytopathological Society, Mycological Society of America and Society of Nematologists. August 25 - 29, 2001. Salt Lake City, Utah, USA

Christopher Schardl, Jinghong Wang, Caroline Machado (2001). Dimethylallyltryptophan synthase genes associated with endophyte ergot alkaloids. The 21st Fungal Genetics Conference. March 13 - 18, 2001 Asilomar Conference Center, California, USA

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

Bagyaraj, D.J. and Caroline Machado, (1996). Phosphorus concentration in soil solution optimum for VA Mycorrhizal symbiosis in *Leucaena leucocephala*. Annals of Forestry, 4 (2): 123-128.

Bagyaraj, D.J. and Caroline Machado, (1996). Effect of soil solution P levels on VAM symbiosis in Mulberry. First International Conference on Mycorrizae. August 4 - 9, 1996. University of California, Berkeley, California, USA.

Caroline Machado and Bagyaraj, D.J. (1995). Mycorrhization helper bacteria and its influence on growth of cowpea. In: Mycorrizae: Biofertilizers for the future. Proceedings of the Third National Conference. new Delhi, India. pp 192-196.