2009

TOWARDS ELIMINATION AND GENETIC MANIPULATION OF ERGOT ALKALOID PRODUCTION IN FUNGAL ENDOPHYTES

Simona Florea
University of Kentucky, sflor2@uky.edu

ABSTRACT OF DISSERTATION

SIMONA FLOREA

THE GRADUATE SCHOOL

UNIVERSITY OF KENTUCKY

2009

TOWARDS ELIMINATION AND GENETIC MANIPULATION OF ERGOT ALKALOID PRODUCTION IN FUNGAL ENDOPHYTES

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

Simona Florea
Lexington, Kentucky

Director: Dr. Dr Christopher L. Schardl, Professor
Department of Plant Pathology
Lexington, Kentucky
2009

ABSTRACT OF DISSERTATION

TOWARDS ELIMINATION AND GENETIC MANIPULATION OF ERGOT ALKALOID PRODUCTION IN FUNGAL ENDOPHYTES

Clavicipitaceous fungal endophytes provide several ecological benefits to their hosts. Besides improving host's growth characteristics, *Neotyphodium coenophialum*, the endophyte of tall fescue (*Lolium arundinaceum*), produces ergot alkaloids that have been proposed to be involved in fescue toxicosis. One approach to address the toxicosis problem is to genetically manipulate and modify *N. coenophialum* by knocking out a pair of homologous genes, (*dmaW*1 and *dmaW*2), encoding dimethylallyltryptophan synthase, the enzyme for the first and determinant step in ergot-alkaloid biosynthesis. In this study, disruption of *dmaW*2 was attempted using several disruption methods. Out of 1522 transformants screened, three putative knockouts were identified. Southern blot analysis of digested genomic DNA indicated that homologous gene replacement at *dmaW*2 locus took place while *dmaW*1 was still present. Chromosome separation followed by Southern-blot hybridization showed that the *dmaW* genes in *N. coenophialum* are located on different chromosomes.

The aim of this study was to obtain a nontoxic endophyte free of marker genes that could be used to inoculate popular tall fescue cultivars. Therefore the Cre/loxP system developed in this study allows reusing the marker gene for sequential transformations. Protoplasts from *Neotyphodium coenophialum*, *Neotyphodium uncinatum*, or *Epichloë festucae* isolates, containing a floxed hygromycin phosphotransferase (*hph*) gene (loxP::*hph*::loxP), were transfected with a Cre-recombinase expression plasmid and then cultured without selection. The marker was excised in 0.5-2% of the colonies, leaving a single loxP sequence. This strategy will help to reduce the concerns related to field release or commercialization of economically important grasses associated with manipulated fungal strains. It is expected that the technology will likely be adapted and applied in other fungal species.

Manipulation of the ergot alkaloid (EA) gene cluster from *C. purpurea* and *C. fusiformis* by introducing and expressing its genes in different fungal-grass symbionts was also investigated.

Heterologous expression of the ergot alkaloid cluster could result either in the synthesis of compounds similar to the ones produced by the host or in synthesis of novel compounds with new modes of action. Even though the results indicated that several EA genes were expressed in the new symbiota, none of the ergot alkaloids intermediates were detected.

Key words: endophyte, *dmaW*, gene disruption, Cre/loxP, transient transfection

Simona Florea
Student's Signature

Date 12-18-2009

TOWARDS ELIMINATION AND GENETIC MANIPULATION OF ERGOT ALKALOID PRODUCTION IN FUNGAL ENDOPHYTES

By

Simona Florea

Dr. Christopher L. Schardl
Director of Dissertation

Dr. Lisa J. Vaillancourt
Director of Graduate Studies

Date December, 2009

RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor's 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.

A library that borrows this dissertation for use by its patrons is expected to secure the signature of each user.

Name	Date

DISSERTATION

Simona Florea

The Graduate School

University of Kentucky

2009

TOWARDS ELIMINATION AND GENETIC MANIPULATION OF ERGOT ALKALOID PRODUCTION IN FUNGAL ENDOPHYTES

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

Simona Florea
Lexington, Kentucky

Director: Dr. Dr Christopher L. Schardl, Professor
Department of Plant Pathology
Lexington, Kentucky 2009

ACKNOWLEDGEMENTS

First and foremost, I would like to express my heartfelt appreciation to my advisor Dr. Christopher L. Schardl for his encouragement, motivation, constructive criticism, and support throughout my doctoral program years. Without his patient guidance and original ideas, this project would not have been possible. I would also like to thank my committee members Dr. Peter Nagy, Dr. Timothy Phillips, and Dr. Lisa Vaillancourt for their extremely valuable feedback and suggestions throughout the work on this project and to Dr. Charles Issel for accepting to serve on my committee as the outside examiner. I also would like to thank Dr. Daniel Panaccione of West Virginia University for performing the ergot alkaloid analysis in his laboratory, and for his invaluable assistance and helpful recommendations.

I would like to use this opportunity to acknowledge my colleagues, former postdoctoral fellows in Dr. Schardl's laboratory, who have made a huge difference in my career, in my life and will always be close to my heart. Dr. Caroline Machado, thank you for your friendship, guidance, for the immense amount of time you spent to help me make the first steps in the doctoral program, for your suggestions that have always improved my work on the projects. My many thanks also go to Dr. Jimmy Blankenship; thank you for everything you taught me in the laboratory, but most of all, for teaching me what resilience truly means, about how important it is to go on with your life no matter how hard it gets; thank you, for all the joy you brought with you at work, every day. At last, but not the least, I would like to thank Dr. Kalina Andreeva for her true friendship, for the never-ending support, for always giving priority to help me and others over her own needs, and for the long discussions that inspired me in my research. Kalina provided me

with extremely valuable assistance in the Southern-blot and chromosome separation experiments. I also would like to thank Walter Hollin and Al Byrd for all their laboratory support, to all members of Dr. Schardl's laboratory, and to all faculty and staff members of the Department of Plant Pathology for creating such a great working environment over the years.

I would like to express my deepest gratitude to my family and friends for their unconditional love and support throughout my life, especially to my sister Ramona, for believing in me more than I sometimes believed in myself. Without her, reaching these heights would have not been possible.

Thank you all!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FILES

Simona Florea-Dissertation.pdf .....2.3 MB

Chapter 1

Introduction

Ergot alkaloids: pharmacology and therapeutic use

Since ancient times the ergot alkaloids have impacted human lives in many ways. These complex compounds have harmful effects on the central nervous system (Pertz and Eich, 1999); however controlled dosages of ergot alkaloids have been used in traditional medicine against acute attack of migraines, as an aid during childbirth (Barceloux, 2008; Van Dongen and De Groot, 1995). Due to the structural resemblance to neurotransmitters such as dopamine and serotonin, the pharmacological activities of ergot alkaloids are complex (Schardl et al., 2006) . Their roles as agonists or antagonists vary depending on which receptors they bind (Stadler and Giger, 1984). For instance, lysergic acid derivatives, such as lysergic acid diethylamide (LSD), have psychedelic effects acting as partial agonists of 5-HT2A receptors (Barceloux, 2008). Ergotamine acts as a partial agonist of the 5-hydroxytryptamine (5-HT) and adrenolytic receptors, and due to its constrictive effects on the cranial blood vessels has been used in treatment of migraines (Wadworth and Crisp, 1992).

Ergonovine has partial antagonistic and agonistic activities on dopamine and serotonin receptors (Gilman et al., 1985). The vasoconstrictive effect of ergonovine on the coronary arteries leads to the arrest of the blood flow to the uterus. For this reason, ergonovine has been used during labor to reduce postpartum hemorrhages (Gilman et al., 1985; Kimball et al., 1989). Bromocriptine has affinity towards dopamine receptors, affecting the prolactin levels, being used in treatment of parkinsonism and other neurological diseases (Goetz, 1990). Fungi in the family Clavicipitaceae are known to

produce ergot alkaloids. Among these are the pathogenic *Claviceps* spp. known to cause ergotism in humans and animals due to the ergot alkaloids produced (Pazoutova et al., 2000).

Human poisoning and fescue toxicosis

Human intoxications with drastic social implications have been described over the ages. Examples of events associated with poisonings by ergots produced by *Claviceps purpurea* include the Salem Witch Trials in colonial Massachusetts (Aronson, 2003) and the Crusades (Billings, 1996). *Claviceps purpurea* infects the ears of rye and forage plants replacing the individual seeds with a dark fungal mass called ergot. These fruiting bodies are often rich in ergot alkaloids (Schardl et al., 2006). Humans consuming contaminated rye flour frequently developed symptoms of convulsive and gangrenous ergotism, such as fever, sweating, hallucinations, headaches, severe burning and muscle pain, and swollen and numb limbs (Eadie, 2003).

Ergot alkaloids are also produced by the symbiotic endophytes *Epichloë*, *Neotyphodium*, and *Balansia* spp., and have been proposed to be involved in toxicosis to animals grazing on infected grasses (Hemken et al., 1984; Poter, 1994; Schardl et al., 2006; Tor-Agbidye et al., 2001). In the recent decades, livestock toxicosis due to ergot alkaloids has had a serious impact on agriculture (Hoveland, 1993). Poisoning can be due to the presence of ergots produced by any of several *Claviceps* species, endophytic fungi such as *Epichloë* (anamorph *Neotyphodium)* spp. that complete their life cycles in association with cool-season grasses, or *Balansia* spp., which are warm-season grass symbionts (Schardl, 1996; Schardl et al., 2009). In the United States, cattle and horses grazed on tall fescue infected with the endophyte, *Neotyphodium coenophialum*, display

several toxicosis symptoms known as fescue toxicosis (Thompson and Stuedemann, 1993). The toxicosis problems are more frequent when the fields are heavily grazed (Cross, 2003) or when the grass is in flower and the animals are allowed to graze these fields (Schardl and Phillips, 1997). Ergot alkaloids display uneven distribution throughout the plants, with higher concentrations in the crown and pseudostems (Spiering et al., 2002) and in the seed heads of the plants (Rottinghaus et al., 1991). Symptoms of toxicosis are similar to those caused by *C. purpurea*, and are temperature dependent (Spiers et al., 1995).

The "summer slump" syndrome appears during hot periods and is manifested as convulsions, hyperthermia, poor weight gain, agalactia, reduced reproductive capability, and retention of winter coats. In contrast, "fescue foot" syndrome is observed in cold conditions when animals display dry gangrene potentially with loss of hooves, caused by the vasoconstrictive activity and lack of adequate oxygen supply to the extremities (Raisbeck et al., 1991; Spiers et al., 1995).

Endophyte-grass associations and ecological role of ergot alkaloids

Clavicipitaceous fungal endophytes colonize the aboveground parts of the host plant, living in the intercellular spaces and feeding on the plant's extracellular substrates (Clay and Schardl, 2002; Schardl and Phillips, 1997). The colonization of the plants by these endophytes can lead to mutualistic or antagonistic associations (Clay and Schardl, 2002). In mutualistic associations with *Neotyphodium* spp. (asexual derivatives of sexual *Epichloë* spp) the endophyte lives inside the plant without causing any damage, spreading systemically throughout the plant, and infecting the seeds. For these asexual endophytes, the only means of transmission is vertically through the infected seeds (Clay and Schardl,

2002; Craven et al., 2001; Malinowski and Belesky, 2006). However, in the pathogenic associations the endophytes grow asymptomatically until the plants reach the boot stage. At this stage, the fungal sexual structure, called a stroma (choke), forms around the flag leaf sheath, hampering the formation and maturation of grass inflorescences (Clay and Schardl, 2002). In this type of association (e.g. *Epichloë typhina* in *Dactylis glomerata, Phleum pratense* or *Lolium perenne*), the endophyte can only spread horizontally via the ejected ascospores formed in the stromata (Chung and Schardl, 1997). In other associations on the same plants some of the epichloae such as *E. festucae* on all of its known hosts, and *E. typhina* on *Poa nemoralis* and *Puccinellia distans* (Olejniczak and Lembicz, 2007; Schardl and Leuchtmann, 2005), can also exhibit vertical transmission. On these plants, while some tillers are prevented from forming inflorescences (due to the choke formation), others develop infected seeds.

Fungal endophytes provide several ecological benefits to their hosts, such as enhanced tillering and root growth, improved mineral uptake and resistance to drought (Arechavaleta et al., 1989; Malinowski and Belesky, 2000; Malinowski and Belesky, 2006), but may cause episodes of livestock toxicosis if they produce ergot alkaloids or indolediterpenes (Tor-Agbidye et al., 2001). The involvement of the ergopeptines in fescue toxicosis was demonstrated through feeding purified or synthesized ergovaline to animals (Cross, 2003; Spiers et al., 1995; Thompson and Stuedemann, 1993; Tor-Agbidye et al., 2001). However, the roles of the lysergic acid derivatives have not been evaluated due to the technical difficulties involved in alkaloid purification. Moreover, Hill et al. (2001) suggested that lysergic acid intermediates might play a role in toxicosis, observing that the transport of these compounds throughout the ruminant gastric

membranes is faster than ergopeptines. Aside from the animal toxicosis, several reports have indicated that some ergot alkaloids have effect against neonates of the insect *Agrotis ipsilon* (Potter et al., 2008), and clavine derivatives were found to have bacteriotoxic activity against *Escherichia coli* and *Salmonella typhimurium* (Eich and Pertz, 1999).

Ergot alkaloid biosynthesis and structure

Ergot alkaloids are complex compounds, and due to their pharmaceutical uses and involvement in historical toxic outbreaks, their synthesis has been studied intensively in *Claviceps* spp., So, many of steps in the ergot alkaloid biosynthetic pathway have been elucidated (Gröger and Floss, 1998). Furthermore, the genes believed to be involved in these steps have been identified in *Claviceps* spp. (Haarmann et al., 2005; Lorenz et al., 2007; Tsai et al., 1995). Due to the phylogenetic relationship with *Claviceps* spp. (Craven et al., 2001; Schardl et al., 2008), and to the presence of related genes (Fleetwood et al., 2007; Panaccione and Schardl, 2003; Wang et al., 2004), a comparable pathway should be present in *Epichloë, Neotyphodium* and *Balansia* spp.

Ergot alkaloids vary in complexity from the tetracyclic ergoline ring system present in ergopeptines to the less complex tricyclic related compounds such as chanoclavine **(Figure 1.1)**, and can be divided into different types: clavine alkaloids, lysergic acid, simple lysergic acid amides and ergopeptines (Correia et al., 2003; Schardl et al., 2006). Following their experiments with injected radiolabeled (^{14}C) tryptophan into rye plants ears infected with *C. purpurea*, and incorporation of the label into ergonovine and the lysergic acid, Mothes et al. (1958) proposed that formation of the ergoline ring system involves merging of tryptophan with an isoprenoid unit. Two years later, Birch et

al. (1960) showed that labeled (^{14}C) mavelonate incorporates into the ergoline ring, demonstrating the involvement of an isoprene unit in the aromatic prenylation.

The synthesis of dimethylallyl tryptophan (DMAT) from dimethylallyl diphosphate (DMAPP) and L-tryptophan **(Figure1.1)** was confirmed by Heinstein et al. (1971) who proposed that this reaction, catalyzed by the enzyme dimethylallyltryptophan (DMAT) synthase, is the first determinant step in the pathway (Heinstein et al., 1971; Tsai et al., 1995). In additional studies with *Claviceps* spp. the activity of DMAT *N*-methyl transferase was identified in cell-free extracts, indicating that the second step in ergot alkaloid biosynthesis is the *N*-methylation of DMAT (Otsuka et al., 1980). For the configuration of ring C and formation of chanoclavine I, two subsequent oxidations and a decarboxylation of *N*-methyl-DMAT take place. Subsequently, "chanoclavine cyclase", which likely represents two or three enzymes, catalyzes the closure of ring D and formation of tetracyclic ergolene cyanoclavine-I-aldehyde and agroclavine (Floss and Anderson, 1980; Kozikowski and Wu, 1988; Schardl et al., 2006). Oxidation of agroclavine to elymoclavine and elymoclavine to paspalic acid are NADPH-dependent, and might be both catalyzed by cytochromes P450 (Maier et al., 1988; Schardl et al., 2006), whereas the paspalic acid isomerizes to lysergic acid spontaneously (Schardl et al., 2006; Tudzynski et al., 2001).

In the later steps of the pathway, ergopeptide lactams (Komarova et al., 2002) are formed by the action of two nonribosomal peptide synthase (NRPS) subunits making up lysergyl peptide synthetase (Correia et al., 2003; Panaccione et al., 2001). A modular configuration characterizes the NRPS subunits, with each module implicated in the addition of an amino acid or other substituent. Each module is organized with an

adenylation domain, which recognizes the specific amino acid or carboxylic acid substrate, a thiolation domain also known as peptidyl carrier protein domain; and the condensation domain that links the substituent to the next substituent in the chain (Schardl et al., 2006; Schardl et al., 2009). The enzyme is atypical for fungal NRPS's in consisting of multiple subunits, one (LPS2) that activates lysergic acid and the other one (LPS1) that activates the three amino acids (Tudzynski et al., 2001). A final oxygenation results in the cyclol ring of the ergopeptine.

Various ergopeptines have been identified that have similar structure but differ at the amino acid positions in the tripeptide moiety (Schardl et al., 2006; Schardl et al., 2009; Tudzynski et al., 2001). Among the fungal endophytes belonging to family Clavicipitaceae, *Balansia obtecta* produces ergobalasine, whereas *Neotyphodium* and *Epichloë* spp. produce ergovaline. Most of the ergopeptines are formed from lysergic acid, amino acid -1, amino acid -2, and amino acid 3, which is most often L-proline, but is L-alanine in ergobalasine (Poter, 1994; Schardl et al., 2006) **(Table 1.1)**.

Genes in the ergot alkaloid producing fungi

Genes for complex secondary metabolic pathways are often clustered (Gueldener et al., 2002; Yu et al., 2004) and the pathways are spatially and temporally controlled at multiple steps (Floss and Anderson, 1980). For instance, tryptophan is a precursor of ergot alkaloids and in the same time plays a role in the induction of the ergot alkaloid biosynthesis enzymes (Rebek et al., 1983). The ergot alkaloid pathway is liable to feedback regulation, with lysergic acid regulating synthesis of key intermediates in the pathway (clavines) (Panaccione and Schardl, 2003; Schardl et al., 2006). Furthermore,

Krupinski et al. (1976) showed that high levels of inorganic phosphate inhibit the biosynthesis of ergot alkaloid, and this repression can be controlled by L- tryptophan, which induces increased activity of DMAT synthase enzyme.

Intensive studies of the biochemistry of ergot alkaloids using labeled compounds have taken place since the 1960's, whereas the molecular studies of the genes and enzymes required for their biosynthesis are more recent (Fleetwood et al., 2007; Haarmann et al., 2006; Panaccione et al., 2001; Tsai et al., 1995; Wang, 2000). Due to variation in names given to the orthologous genes identified in different species, Schardl et al., (2006) adopted a systematic set of names for the genes present in the *EAS* (ergot alkaloid synthesis) gene cluster. The genes already described through gene disruption were named according to the enzyme activities of the proteins they encode, while the genes with putative functions in the process to be characterized were named from *easA* to *easH* (Schardl et al., 2006; Tudzynski et al., 1999).

The gene designated *dmaW* encoding DMAT synthase, the first committed and rate limiting step of the pathway, was identified using peptide fragment sequences of the purified protein from *Claviceps fusiformis* (Gebler and Poulter, 1992), then cloned and sequenced (Tsai et al., 1995). Based on *dmaW* sequence from *C. fusiformis*(Tsai et al., 1995), several homologs of the gene were identified in *C. fusiformis* and *C. purpurea* (Wang, 2000), *Neotyphodium coenophialum, Neotyphodium lolii* x *E. typhina* Lp1 and *Balansia obtecta* (Wang, 2000). The role of *dmaW* as a first step in the pathway was demonstrated by gene disruption in *Neotyphodium* sp. Lp1 (Wang et al., 2004). The resulting mutant failed to produce ergovaline and chanoclavine I, providing further evidence for the role of DMAT synthase in the pathway.

The genes required for the biosynthesis of secondary metabolites are clustered, and identification of the genes linked with *dmaW* in the *C. purpurea EAS* cluster was completed by chromosome walking (Tudzynski et al., 1999). Using this approach, the *lpsA* gene, encoding lysergyl peptide synthetase subunit 1 (LPS1), was identified in close proximity to *dmaW* (Tudzynski et al., 1999). Furthermore, *lpsA* was identified and cloned from the grass endophyte isolate Lp1, and its function was confirmed through gene inactivation (Panaccione et al., 2001). In perennial ryegrass symbiota with the *lpsA* mutant, was observed an increased accumulation of the shunt product, 6,7-secolysergine, while ergovaline was absent (Panaccione et al., 2001).

Furthermore, *lpsB* and *lpsC,* predicted to encode monomodular peptide synthetases were localized in the cluster (Haarmann et al., 2005). Conducting gene disruption in *C. purpurea,* Correia et al. (2003) established that *lpsB* gene product is accountable for the activation of D-lysergic acid, the (LPS2) subunit of lysergyl peptide synthetase. The analysis of the alkaloid spectrum revealed that the *lpsB* mutant failed to produce ergopeptines but accumulated D-lysergic acid (Correia et al., 2003). Further chromosome walking experiments detected another gene, *cloA,* which was found to have a heme-binding motif similar to cytochrome P450 (Haarmann et al., 2006).

Using a gene disruption technique in *C. purpurea*, Haarmann et al. (2006) provided solid support that *cloA* encodes the enzyme that converts elymoclavine to D-lysergic acid **(Figure 1.1)**. Further analyses of the cluster identified another eight *EAS* genes (*easA* and *easC* to *easH*), and their putative functions were postulated based on bioinformatics (Haarmann et al., 2005; Schardl et al., 2006; Schardl et al., 2009) (Table 1.2). It was suggested that *easF* is the gene encoding *N*-methyltransferase (dependent on

AdoMet) responsible for methylation of DMAT (Otsuka et al., 1980), and the *easA* gene product was proposed to be similar to old yellow enzymes (with $FMNH_2$ as a cofactor) and participate in the epimerization of chanoclavine aldehyde. Two other genes-*easC* predicted to encode a catalase and *easE* thought to encode an FAD-containing monooxygenase or dehydrogenase-are believed either to account for the conversion of the *N*-methyl-DMAT to chanoclavine or for the formation of chanoclavine aldehyde (Coyle and Panaccione, 2005; Schardl et al., 2006).

A mutated version of *N. lolii lpsB* gene was used to disrupt its homolog in *E. festucae* which is the sexual ancestor of *N. lolii* (Fleetwood et al., 2007). The gene knockout analysis of *lpsB* confirmed its involvement in ergovaline biosynthesis, as previously shown in *C. purpurea* (Correia et al., 2003). Further, chromosome walking and sequence comparisons were carried out to identify other genes linked to *lpsB* in *N. lolii*. Using this approach, five more putative *eas* genes with homologues in *Claviceps* spp., - namely *easA*, *easE*, *easH*, *easF* and *easG* - were identified (Fleetwood et al., 2007). The presence of an *EAS* cluster in epichloë endophytes was expected, due to the phylogenetic associations between members of the family Clavicipitaceae.

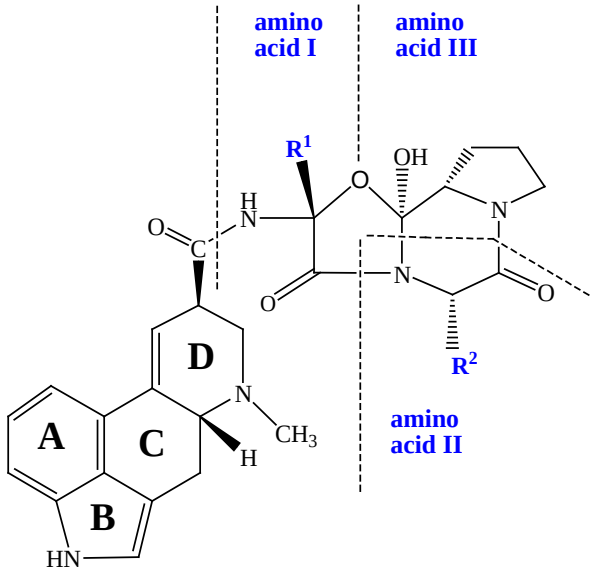
Agricultural impact

Fungi from family Clavicipitaceae produce ergot alkaloids with diverse profiles, and their accumulation is highly regulated throughout the pathway. Experiments have shown that different ergot alkaloids have different biological activities, and their accumulation at certain levels in the producing fungi might have important roles for their ecology (Tudzynski et al., 2001). To exemplify, a clavine alkaloid (agroclavine) has been

shown to have antimicrobial activity (Eich and Pertz, 1999), and the complex ergopeptines (ergovaline and ergotamine) have been shown to be effective against insects and mammals (Raisbeck et al., 1991; Thompson and Stuedemann, 1993). Animal performance is associated with the palatability of the plants (Marten et al., 1976), and the presence of ergot alkaloids in the grass-endophyte symbiota may be sensed by the grassing animals due to their bitter taste (Clay, 1990). Toxicosis to animals that graze grasses harboring ergot alkaloid-producing fungi, especially tall fescue infected with *N. coenophialum*, has been a major problem for agriculture. One approach to address this problem is to genetically manipulate the endophytes by knocking out genes involved in the key steps of the ergot alkaloid pathway, and inoculating the mutated strains into popular grass cultivars (Panaccione et al., 2001; Wang et al., 2004). Elimination of *N. coenophialum* from the popular tall fescue cultivars is not a feasible solution because, besides causing toxicosis episodes in livestock, the endophyte improves growth characteristics of the plants (e.g. tillering and root growth) and resistance to different stresses such as drought (Arechavaleta et al., 1989; Malinowski and Belesky, 2006).

The main goal of my research was to genetically manipulate and modify *N. coenophialum* endophytes to eliminate the factors implicated in fescue toxicosis while developing a system for marker elimination.

Table 1.1. Ergopeptines variations depending on the first two amino acid positions of the tripeptide moiety*



	Amino acid 1	R1	Amino acid 2	R2
Ergovaline	L-alanine	CH_3	L-valine	*i*-Pr
Ergosine	L-alanine	CH_3	L-leucine	*i*-Bu
Ergotamine	L-alanine	CH_3	L-phenylalanine	CH_2Ph
Ergocornine	L-valine	*i*-Pr	L-valine	*i*-Pr
Ergocryptine	L-valine	*i*-Pr	L-leucine	*i*-Bu
Ergocristine	L-valine	*i*-Pr	L-phenylalanine	CH_2Ph
Ergonine	L-2-aminobutyric acid	Et	L-valine	*i*-Pr
Ergoptine	L-2-aminobutyric acid	Et	L-leucine	*i*-Bu
Ergostine	L-2-aminobutyric acid	Et	L-phenylalanine	CH_2Ph

*In each case the third amino acid is L-proline

Table 1.2. Ergot alkaloid synthesis (*EAS*) cluster genes of *Claviceps purpurea* strain P1, with known and predicted functions

Gene	Putative function	Likely cofactors
dmaW	DMATrp synthase	
lpsB	LPS subunit 2	4'-phosphopanttheine, ATP
cloA	Elymoclavine oxygenase	Heme-Fe
lpsA1	LPS 1	4'-phosphopanttheine, ATP,Fe^{II}
lpsA2	LPS 1	4'-phosphopanttheine, ATP
easA	Reductase/Dehydrogenase (OYE)	$FMNH_2$
easC	Catalase	Heme-M
easD	Reductase/Dehydrogenase	NAD^+ or NADH
easE	Dehydrogenase or Monooxygenase	FAD
easF	Methyltransferase	AdoMet
easG	Reductase/Dehydrogenase	NAD^+
easH$_1$	Oxygenase/Hydroxylase	Fe^{II}
easH$_2$†	Oxygenase/Hydroxylase	Fe^{II}

† Likely pseudogene.

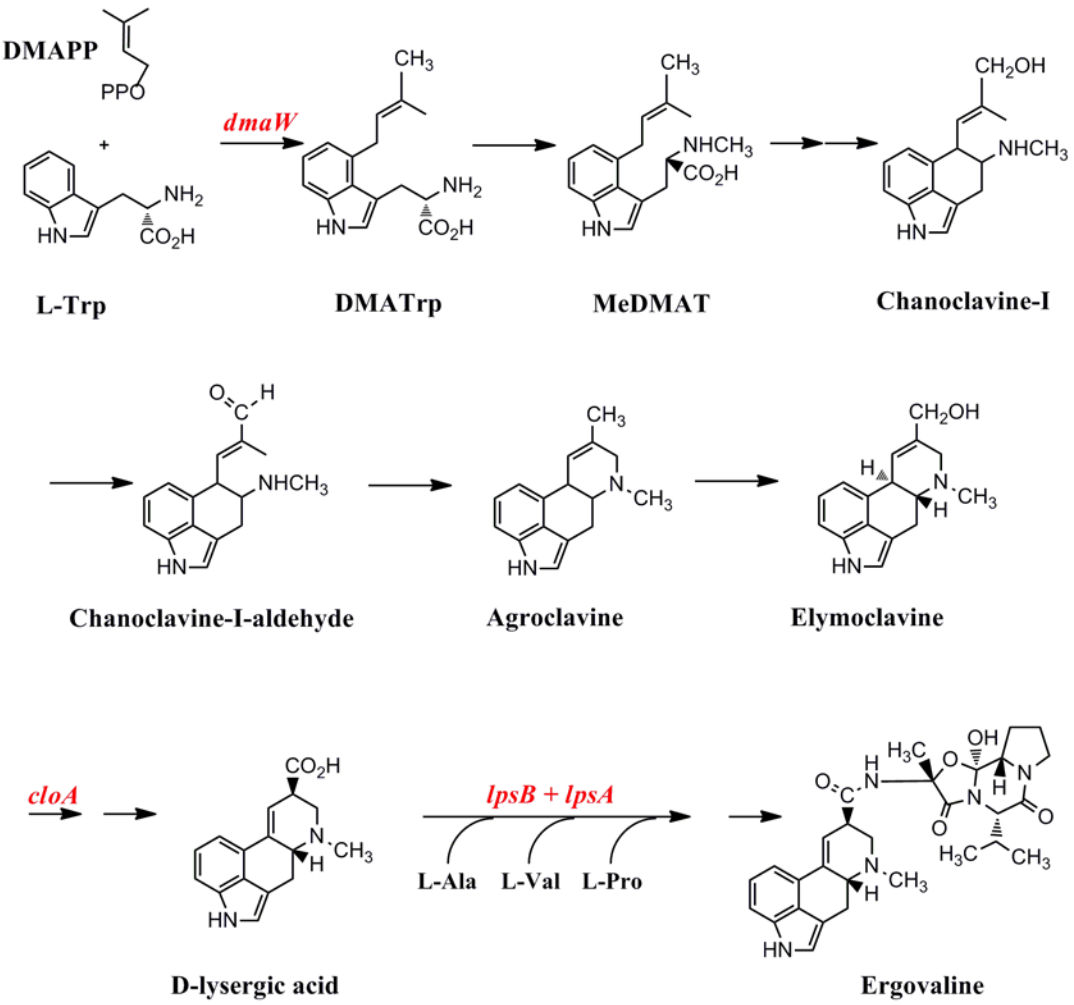


Figure 1.1 Ergot alkaloid biosynthetic pathway. The genes described through gene disruption are named according to the enzyme activities of the proteins they encode, and are represented in red.

Chapter 2

Disruption of the *dmaW2* gene encoding dimethylallyltryptophan synthase in *Neotyphodium coenophialum* strain e19

Introduction

Neotyphodium coenophialum is a mutualistic symbiont of tall fescue, one of the most extensively used grasses in the United States. The presence of the endophyte inside the grass does not produce harm to the host in any way. Moreover, this mutualistic association has been hypothesized to benefit both the endophyte and the plant; the endophyte providing several advantages such as increased drought tolerance, increased tillering and seed germination, as well as increased resistance to insects (Schardl, 1996; Schardl et al., 2004), while the plant provides nutrition, shelter and dispersal (Schardl and Phillips, 1997). Therefore, infected tall fescue is commonly planted in the U.S. for forage and conservation purposes (Buckner et al., 1979). However, animal performance on tall fescue is impaired by the endophyte toxicity which can lead to weight loss, decreased feed intake, agalactia, decreased fertility, gangrene of the limbs (Raisbeck et al., 1991; Tor-Agbidye et al., 2001), and increased respiration rate and body temperature (Hemken et al., 1984), symptoms commonly known as fescue toxicosis. The main factor believed to be involved in the toxicosis is ergovaline (Panaccione and Schardl, 2003), an ergot alkaloid produced by the endophyte.

Over time, efforts have been made to find solutions to this problem. The search for novel, less toxic endophytes and their introduction into tall fescue cultivars might reduce or eliminate livestock poisoning. However, studies have shown that tall fescue cultivar Jesup harboring the novel endophyte AR542 offered an improved animal intake

but the agronomic performance of the cultivar was lower than the performance of the same cultivar infected with the wild type endophyte (Bouton et al., 2002). In the past decade, efforts have been made to genetically manipulate the endophyte to make it unable to produce compounds involved in livestock poisoning. Nevertheless the attempt to genetically eliminate the toxicosis is impaired by the hybrid origin of *N. coenophialum*, which has resulted in an heteroploid endophyte with multiple genomes and several gene copies from the ancestral fungi (Clay and Schardl, 2002).

The *dmaW* genes encode dimethylallyl tryptophan synthase **(**DMAT synthase), and the role of the enzyme in the ergot alkaloid pathway has been demonstrated by gene disruption in *Neotyphodium lolii* x *Epichloë typhina* isolate Lp1, an endophyte of perennial ryegrass (Wang et al., 2004). One of the approaches being pursued to ameliorate fescue toxicosis is to knock out a pair of homolog genes (*dmaW*1 and *dmaW*2) in *N. coenophialum* expected to eliminate its ability to produce ergot alkaloids. The percent similarity of the two *dmaW* homologs in this endophyte is 96% at nucleotide level, but the flanking regions are different enough to target homologous recombination to disrupt the individual genes (Wang et al., 2004).

In this chapter, I describe several methods used in my attempt to disrupt the *dmaW*2 homolog gene in the endophytic fungus, *N. coenophialum*. The *dmaW*2 homolog was targeted first because it encodes a predicted protein identical with the protein encoded by the *dmaW* from isolate Lp1 (Wang, 2000).

Materials and methods

Biological materials

DNA from the fungal endophytes was isolated from fresh mycelium, following the method described by Al-Samarrai et al. (2000), using a Gen**o/**Grinder 2000 **(**SPEX CertiPrep, Metuchen, NJ, USA) and the DNeasy 96 Plant Kit **(**Qiagen, Valencia, CA, USA**)**. The DNA concentration was estimated using a Qubit fluorometer and Quant-iT™ DNA BR Assay Kit (Invitrogen, Eugene, OR). Bacterial cultures were grown in 50 ml LB medium at 37 °C for 12-14 h on a rotary shaker at 200 rpm, and plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen) or following the method of Ahn et al. (2000). The oligonucleotide primers used in this chapter were from Integrated DNA Technologies (Coralville, IA) and are listed in **Table 2.1**.

Plasmid used in *dmaW* 2 disruption method

The *dmaW*2-replacement vector pKAES174 (**Figure 2.1)** was designed by C. Machado (Florea et al., 2009) as follows: a 2.9-kb 5'-flanking region of *N. coenophialum dmaW*2 was amplified by PCR using primers dmaWe19copy2*Sac*II.13d and dmaWe19copy2.cos*Bam*HI.16u. The primers contained restriction sites (underlined) which were used for subsequent digestion of the PCR product and directional cloning into pBluescript KS+ (Stratagene Cloning Systems, La Jolla, CA). Similarly, the 3'flanking region of *dmaW*2 starting 342 bp downstream of the translational start codon was amplified using primers dmaWe19copy2.cos*Bam*HI.15d and dmaWe19copy2*Sal*I.14u. The PCR product was digested using the restriction sites (underlined) and directionally cloned into pBCKS+ (Stratagene Cloning Systems)

yielding pKAES148. Further, the plasmid pKAES148 was linearized with *Bam*HI, dephosphorylated by the action of calf intestine phosphatase (Stratagene Cloning Systems), and ligated with a fragment containing *hph* (encoding hygromycin phosphotransferase) flanked by loxP sites ("floxed"), which has been precut from pKAES173(Spiering et al., 2008) using *Bam*HI.

Split marker DNA preparation for transformation

A 2.6 kb PCR fragment was amplified using pKAES174 as template and a pair of primers, one (HYG_d1) designed to anneal to the upstream of the *dmaW*2 ORF, and the other (HYG_YG1) annealing just downstream of the *hph* gene. Similarly, a 3.5 kb fragment was generated using HYG_HY1 primer annealing upstream of the *hph* gene and HYG_u1 primer annealing downstream of the *dmaW2* gene. Primers HYG_YG1 and HYG_HY1 were designed to amplify a 534-bp overlapping region in the *hph* gene **(Figure 2.2).** The reactions were prepared in 25 µl volume with 20 ng template, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 200 nM of each primer, 2.5 units AmpliTaq Gold, and AmpliTaq Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied Biosystems, Foster City, CA, USA). The two PCR products were purified using QIAquick PCR purification kit (Qiagen Inc., Valencia, CA), and then used in protoplast transformation by electroporation. PCR reactions were performed in a model 2720 Thermal Cycler (Applied Biosystems) using the following program: 95 °C as the initial denaturation step for 9 min, a 30 s annealing step at 57 °C, a 72 °C extension step at 1 min/kb of sequence to be amplified, and then 34 cycles of 94 °C

for 30 s, 57 °C for 30 s and 72 °C for 1 min/kb of sequence to be amplified. After 35 cycles, a last 5 min extension at 72 °C was performed.

Single-stranded DNA preparation for fungal transformation

The *Sal*I-digested pKAES174 was used as a template for the amplification of single stranded fragments using individual primer dmaWe19copy2SacII.13d or dmaWe19copy2SalI.14u. LA Taq polymerase (Takara Bio Inc., Osaka, Japan) was used for the large (approximately 8 kb) DNA fragment amplification **(Figure 2.3)**. For the linear amplification reactions, the 50 µl reaction mixture contained 100 ng digested plasmid, 1x Ex Taq buffer, 2.5U of Ex Taq polymerase, 200 µM of each dNTP, and 200 nM primers (each primer in separate reaction). The temperatures were as follows: 95 °C for 1 min, followed by 15 cycles at 95 °C for 25 s, 72 °C for 9 min in a model 2720 Thermal Cycler (Applied Biosystems). The linear amplifications products were separately purified using the QIAquick PCR purification kit. Each purified product was ethanol precipitated using 0.3 M NaOAc and 2.5 volumes of 95 % ethanol and then dissolved in 10 µl H_2O. The two single stranded DNAs were separately mixed with 5 µg of RecA protein (Roche Molecular Diagnostics, Pleasanton, CA), then incubated for 30 min at 37 °C. The mixtures were kept in ice until used to transform *N. coenophialum*.

Transformation of *N. coenophialum* e19

Mycelium of *N. coenophialum* was grown in PDB medium with rotary shaking at 200 rpm for 7-10 days. Then protoplasts were prepared with slight adjustments from the protocol described by Murray et al., (1992). The enzyme mixture used for cell wall

removal contained 5 mg/ml β-glucanase (InterSpex Products, San Mateo, CA) or 5 mg/ml lysing enzymes (Sigma), 5 mg/ml driselase (InterSpex), 0.5 mg/ml zymicase I (InterSpex Products), 4 mg/ml glucanex (Novo Industri AS, Bagsvaerd, Denmark), and 3 mg/ml bovine serum albumin (Sigma, St Louis, MO) in osmotic solution (1.2 M $MgSO_4$, 50 mM sodium citrate, pH 5.8). Mycelium was then incubated at 30 °C for 3-4 h. Protoplasts were transformed either with 4-8 μg of *Sal*I-linearized pKAES174 DNA, 3-4 μg of the split marker DNA, or 1-2 μg of linear amplification DNA products. Transformation was completed by the electroporation (Tsai et al., 1992) or the PEG (polyethylene glycol) method (Panaccione et al., 2001) applied to 10^6-10^8 protoplasts.

The protoplasts were then suspended in 7 ml CRM-low (complete regeneration medium containing low melting temperature agarose from Seakem LE, FMC Bioproduct, Rockland, ME), and poured over 20 ml CRM plates containing hygromycin B (Calbiochem, San Diego, CA) at 48 μg/ml. The transformation plates were incubated at 21 °C for 4-5 weeks; then the fungal colonies were transferred onto PDA with 50 μg/ml hygromycin B for sporulation and single-spore isolation.

PCR screening to identify recombinants

PCR assays for the integration of the Δ*dmaW*2 mutant allele into the wild type locus, and replacement of the wild type *dmaW*2 was done using primer pair dmaWe19copy2.1d and dmaWe19copy2.5u. PCR reactions were carried out in 25 μl reaction mixture with 5-10 ng DNA template, 200 μM of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.2 μM of each primer, and 2.5 units AmpliTaq Gold, AmpliTaq Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied

Biosystems), in a model 2720 Thermal Cycler (Applied Biosystems). The temperature program was as follows: 9 min at 95 °C, 35 cycles of 94 °C for 30 sec, 61 °C for 35 sec, 72 °C for 3 min 10 sec.

Inoculation of the transformants into tall fescue 'Kentucky-31'

Surface disinfected 'Kentucky-31' endophyte-free seeds (Latch et al., 1985) were germinated at 21 °C on water agar in the dark until the shoot apical meristems were visible (7 days). At this stage, the *dmaW*2 knockout (Δ*dmaW*2) isolates, wild type (WT) *N. coenophialum* or ectopic transformant was inoculated into the seedlings by placing the mycelium inside a wound created in the meristem region as described by Latch and Christensen (1985). The plates with the inoculated seedlings were kept in vertical position (on edge of the plate) and incubated at 21 °C in dark conditions for 6 days followed by 5 days of light. The surviving seedlings were planted into soil and grown in the greenhouse. Tissue-print immunoblot (TPIB) (Gwinn et al., 1991) was performed when the seedlings reached at least 3 tillers to test for endophyte infection.

The *N. coenophialum*-specific antiserum and the development of TPIB were performed as described by An et al. (1993). Fungal mycelia were isolated from pseudostems of the infected plants and grown on PDA plates (Blankenship et al., 2001).

Ergot alkaloid analysis

For ergot alkaloid analysis, pseudostems from five plants inoculated with *dmaW*2 knockout isolates, two plants inoculated with an isolate possessing an ectopic integration of *dmaW2* and one WT infected plant were lyophilized and then ground to a fine powder.

The ergot alkaloid analyses were performed by Dr. Dan Panaccione at West Virginia University following a modification of the method described by Spiering et al. (2002) (Panaccione et al., 2003) as follows: the ground material was mixed with 1 mL of 2-propanol-water-lactic acid at (50:50:1 v/v/v) containing 1.111 µg/mL ergotamine tartrate as internal standard. The tubes containing the samples were agitated on a rotary shaker for 1 h, followed by 10 min centrifugation at 6000 x g. For each sample, 20 µl of the supernatant was analyzed by high-pressure liquid chromatography HPLC with wavelength fluorescence detection of 310 nm (excitation) and 410 nm (emission).

Chromosome separation

Mycelia of *N. coenophialum* e19 and *N. coenophialum* knockout strain (e7133) isolates were grown in PDB medium for 10 days. The protoplasts were prepared as described above and then suspended in GMB [0.9 M sorbitol, 125 mM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), pH 7.5]. Thereafter, the protoplasts (ca. $1x10^7$ protoplasts/ 90 µl) were mixed with 1.4% low melting temperature agarose (BioRad Laboratories, Hercules, CA, USA) at 1:1 volume, and poured into a mold to form the plugs. Then the plugs were incubated in SE buffer (2% SDS, 250 mM Na_2EDTA, pH 8.0) at 55 °C overnight. The plugs were transferred to 10 mL of 10x ET buffer (10 mM Tris, 500 mM Na_2EDTA, pH 8.0) with 1 mg/mL proteinase K (Sigma) and 100 mg of sodium lauroylsarcosine (Sigma), and incubated for 24 h at 50 °C followed by several washes in 10 mM Tris-HCl (pH 8.0).

The plugs for separation of chromosomes loaded into 0.6% chromosomal grade agarose gel (BioRad,). The gel ran in 0.5xTBE buffer at 12 °C in a CHEF DRII

Apparatus (BioRad). The contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Chu et al., 1986) conditions were set as follows: 6 V/cm for 370 h with pulsed intervals between 1100-6100 s and rotation angle 120°. The 0.5x TBE buffer was replaced every 48 h during the run.

Southern-blot analysis

Southern blot analysis was performed to check the replacement of the wild type *dmaW*2 with its mutated allele. The genomic DNAs were digested using *Xba*I restriction enzyme and electrophoresed in 0.8% agarose gel in 1x TBE electrophoresis buffer [89 mM Tris base, 89 mM boric acid, 2 mM ethylenediaminetetraacetic acid (EDTA) pH 8.2], and transferred from the gels to Hybond N^+-nylon membrane (GE Healthcare, Piscataway, NJ USA) following the manufacturer's instructions. The 3.6-kb PCR fragment used as a probe for the Southern blot was amplified from pKAES174 using primers 144.15d and dmaWe19copy2.5u. The fragment spanning the loxP::*hph*::loxP::Δ*dmaW*2 region stretched circa 800 bp upstream and 400 bp downstream *dmaW*2 flanking regions. The separated chromosomes were transferred from the gel to Hybond N^+-nylon membrane (GE Healthcare, Piscataway, NJ USA) following the manufacturer's instructions. The probe (used to identify on which of the chromosomes are located the *dmaW*1 and *dmaW*2 genes), was amplified using primer pair dmaW-delF and dmaW-delR. The probe specific for both genes was designed in the region that was deleted from e7133 strain. PCR reactions were carried out in 25 µl reaction mixture with 5-10 ng DNA template, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 200 nM of each primer, and 2.5 units AmpliTaq Gold, AmpliTaq

Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied Biosystems), in a model 2720 Thermal Cycler (Applied Biosystems).

The temperature profile was 94 °C for 5 min; 35 cycles at 94 °C for 20 s, with the annealing temperature of 58 °C for 20 s, and a final incubation at 72 °C for 7 min. The PCR products were purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA). The DNA probe was labeled with ^{32}P-dCTP using the Prime-a-Gene labeling kit (Promega, Madison, WI USA). The labeled probes were purified from unincorporated [^{32}P]dCTP by centrifugation through MicroSpin G-50 Columns (GE Healthcare, Piscataway, New Jersey, USA). The hybridization solution, conditions and the washing steps were done as described by Sambrook and Russell (2001).

Results

Selection of site-specific recombinants in *N. coenophialum*

Hygromycin B-resistant *N. coenophialum* isolates transformed with pKAES174 plasmid, split marker or single stranded DNA, were screened by PCR for homologous replacement of *dmaW*2. In the event of ectopic integration, the PCR screen was predicted to yield a 1.5-kb fragment from the wild-type locus and another fragment of 2.5 kb from the integrated pKAES174 sequence. In the case of homologous recombination the 2.5-kb amplification product was expected, but not the 1.5-kb product (**Figure 2.4 Panel A**). DNA from untransformed *N. coenophialum* e19 (wild-type) was used as PCR template for a positive control, and DNA from *N. uncinatum* CBS 102646 was used as negative control because it lacks *dmaW* (Wang et al., 2004). Out of 1522 transformants analyzed, 1376 were obtained using pKAES174 plasmid, 62 using the split marker method and 84

obtained using the RecA single-stranded DNA technique. Only three of all analyzed transformants were putative *dmaW*2 knockout, which did not give rise to the wild-type 1.5-kb fragment, but gave the 2.5-kb product. All of these were obtained by standard replacement strategy of transforming the pKAES174 plasmid into fungal protoplasts using the PEG or electroporation method. One putative *dmaW*2 knockout was designated e7133 and used in further experiments **(Figure 2.4 Panel B lane 12).**

Southern-blot analysis

Southern-blot analysis was performed with DNA isolated from *N. coenophialum* WT, an isolate possessing an ectopic integration, and e7133. In all isolates the presence of the *dmaW*1 gene was indicated by the 3.4 kb band **(Figure 2.5)**. The presence of WT *dmaW*2 was indicated by the 4.7 kb band in the *N. coenophialum* e19 as well as in the isolate possessing an ectopic integration, whereas the 5.2 kb and 6.8 kb bands from isolate e7133 were the sizes expected to be generated by the replacement of *dmaW*2 gene with loxP::*hph*::loxP::Δ*dmaW*2 fragment from pKAES174**.** These results indicated the occurrence of homologous recombination at the *dmaW*2 locus in the e7133 isolate.

Compatibility with tall fescue

To test the compatibility and the persistence of the genetically modified endophytes inside their host, *dmaW*2 knockouts, WT and ectopic transformants of *N. coenophialum* were introduced into tall fescue 'Kentucky-31' plants. Seedling infection frequencies were high at 67% for *dmaW*2 mutants, 27% for ectopic transformants and 54% for the wild-type *N. coenophialum* e19 strain **(Figure 2.6)**. Moreover, to confirm

that the inoculated fungus was present in the infected plants and exclude the possibility that such plants arose from seeds initially infected with WT endophyte, the fungus was grown out from 15 plants of each isolate. The DNA from the fungal isolates was checked by PCR using the primer set dmaWe19copy2.1d and dmaWe19copy2.5u, confirming that indeed the plants were infected with the inoculated isolates and not with the wild type (data not shown).

Ergot alkaloid analysis

The analyses of infected plant material, conducted by Dr. Dan Panaccione at West Virginia University, revealed that the *dmaW2* knockouts produced the same types and similar amounts of ergot alkaloids as the wild-type *N. coenophialum* e19, indicating that the unaltered *dmaW*1 gene fully complemented the function of *dmaW*2 in ergot alkaloid production.

Chromosome separation and gene mapping

Southern-blot analysis of separated chromosomes revealed that the two *dmaW* genes in *N. coenophialum* are located on different chromosomes. To further test if the *dmaW*1 was lost during the *dmaW*2 gene replacement, the probe for Southern-blot hybridization was designed in the 342 bp region of *dmaW*2 that is missing in the *dmaW*2 mutant strain. The results showed that in the wild type strain the probe hybridized to both *dmaW* genes, whereas in the mutant the band corresponding to *dmaW*2 was missing and *dmaW*1 was still present (**Figure 2.7**).

Discussion

In this Chapter, I presented gene replacement strategies used to knock out *dmaW*2 in *N. coenophialum*. The homologous recombination method is based on a linear recombinogenic fragment with long flanking regions surrounding a mutant gene, which facilitates the integration and replacement of the target gene with its mutant allele. To improve the transformation efficiency, several attempts involving split marker and RecA single-stranded DNA transformation methods were carried out. The first method is based upon the principle that only the transformants in which the overlapping resistance marker fragments recombine will recover and survive on medium with antibiotic (Fu et al., 2006). Occurrence of one recombination to repair the function of the resistance marker might be assisted by homologous recombination at the target locus. The second method is based on the idea that the single stranded DNA, along with RecA binding protein, are targeted to the homologous region of the double stranded DNA, facilitating the recombination (Spiering et al., 2008). However, in all attempts with these methods, homologous recombination at the *dmaW*2 locus did not occur. Therefore, knockout of *dmaW*2 was accomplished by the typical marker replacement method, which required laborious screening of 1376 transformants to identify the three putative *dmaW*2 gene replacements. Southern-blot analysis of digested genomic DNA indicated that homologous gene replacement at *dmaW*2 locus took place in e7133 isolate, while *dmaW*1 was still present. Further, chromosome separation followed by Southern-blot hybridization of the separated chromosomes indicated that *dmaW* genes in *N. coenophialum* are located on different chromosomes.

A previous attempt to knock out *dmaW*2, employed a different *N. coenophialum* strain, ATCC 62374, which proved very easy to transform and gave one homologous gene replacement among 185 transformants (Machado, 2004), compared to this study with *N. coenophialum* e19 for which 1522 transformants were screened to identify three transformants with the desired replacement. However, the pitfall of using ATCC 62374 was its propensity to lose compatibility with the host plant. The most likely explanation for this difference was that the strain ATCC 62374 may have been repeatedly subcultured (by the original collector or ATCC) without passage through the host plant. Maintenance of the endophyte compatibility with its host is important because the intent of this study is to obtain a non-toxic endophyte for use in forage tall fescue. Moreover, investigations of the ecological roles, phenotypes relevant to symbiosis and interactions between the genetically modified epichloae and their grass hosts, requires reintroduction of the mutated strains into the plants (Scott, 2001). Despite the fact that *N. coenophialum* strain e19 is not very controllable in the laboratory, the wild type along with the mutant strains were easily introduced into tall fescue 'Kentucky 31'. Ergot alkaloid analysis of the inoculated plants with the knockout *dmaW*2 strain confirmed the presence of the ergot alkaloids in this symbiotum indicating that other *dmaW* gene (or genes) complements the ergot alkaloid biosynthesis.

Table 2.1. Oligonucleotide primers used in Chapter 2

Primer name	Sequence
dmaWe19copy2SacII.13d	gcccgcggccccttagaatatagtagtataattaacttac
dmaWe19copy2.cosBamHI.16u	aaacggatcctgtgaagaagaggacgagcgiaatagc
dmaWe19copy2.cosBamHI.15d	cttcttcacaggatccgtttaacactttcgctatctg
dmaWe19copy2SalI.14u	gcgtcgacagtgatcagggatacctttgattaca
HYG_d1	gcgtgcgtatatctccttagactcg
HYG_YG1	ccattgtccgtcaggacattgttg
HYG_HY1	cgaagaatctcgtgcttcagcttcgatgtag
HYG_u1	ggaatgaatctgggttcagtaaatcttccagccg
dmaWe19copy2.1d	agaaacagacagggctattc
dmaWe19copy2.5u	ctcgccggcatgcgtcaaaa
dmaW-delF	gtaatggcaaagacactccac
dmaW-delR	actgccagttgcggcgttaat
144.15d	cgaatgtagattacaatggg

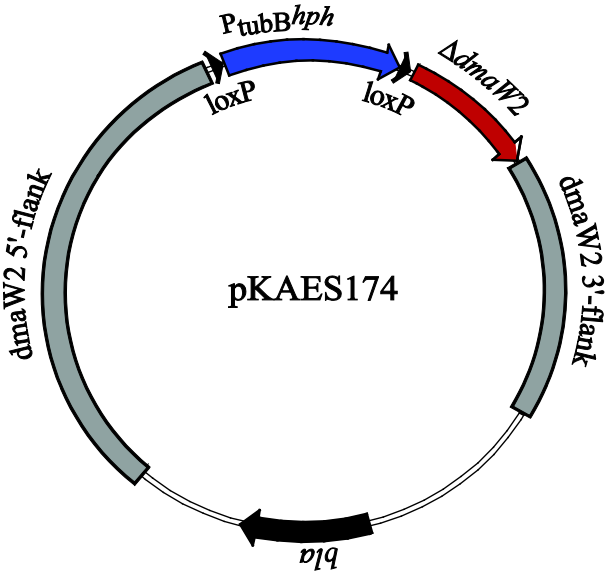


Figure 2.1. Plasmid pKAES174 constructed by substituting floxed *hph* into the *dmaW*2 gene sequence. The plasmid was used to knock out the *dmaW*2 gene by marker replacement. The loxP::*hph::*loxP cassette contains the marker gene *hph* (encoding hygromycin B phosphotransferase) under control of the *E. typhina tubB* promoter (P_{tubB}), and flanked by loxP sites. The plasmid contains the *bla* (beta-lactamase) gene conferring resistance to ampicillin.

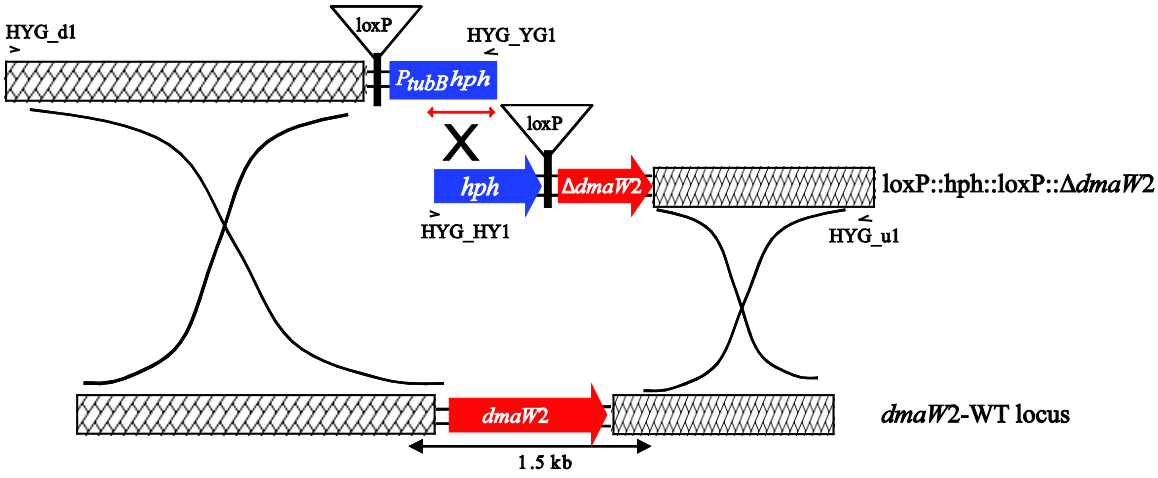


Figure 2.2. Map of the split marker disruption strategy**.** The pKAES174 was used as a template to amplify two fragments: a 2.6-kb PCR fragment containing the 5' flank of *dmaW*2 to the 3' end of *hph* was amplified using HYG_d1 and HYG_YG1 primers. A 3.5-kb fragment was generated using HYG_HY1 primer annealing upstream of *hph* and HYG_u1 primer annealing downstream of the 3'end of the right flank of *dmaW2*. Additionally, the HYG_YG1 and HYG_HY1 primers were designed to amplify a 534-bp overlapping region in the *hph* gene indicated by the red double headed arrow.

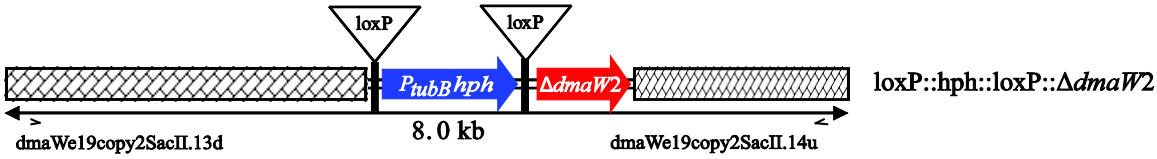


Figure 2.3. Single-stranded DNA gene disruption strategy. The pKAES174 was used as a template for amplification of single stranded fragments using one of the individual primers, dmaWe19copy2SacII.13d or dmaWe19copy2SalI.14u.

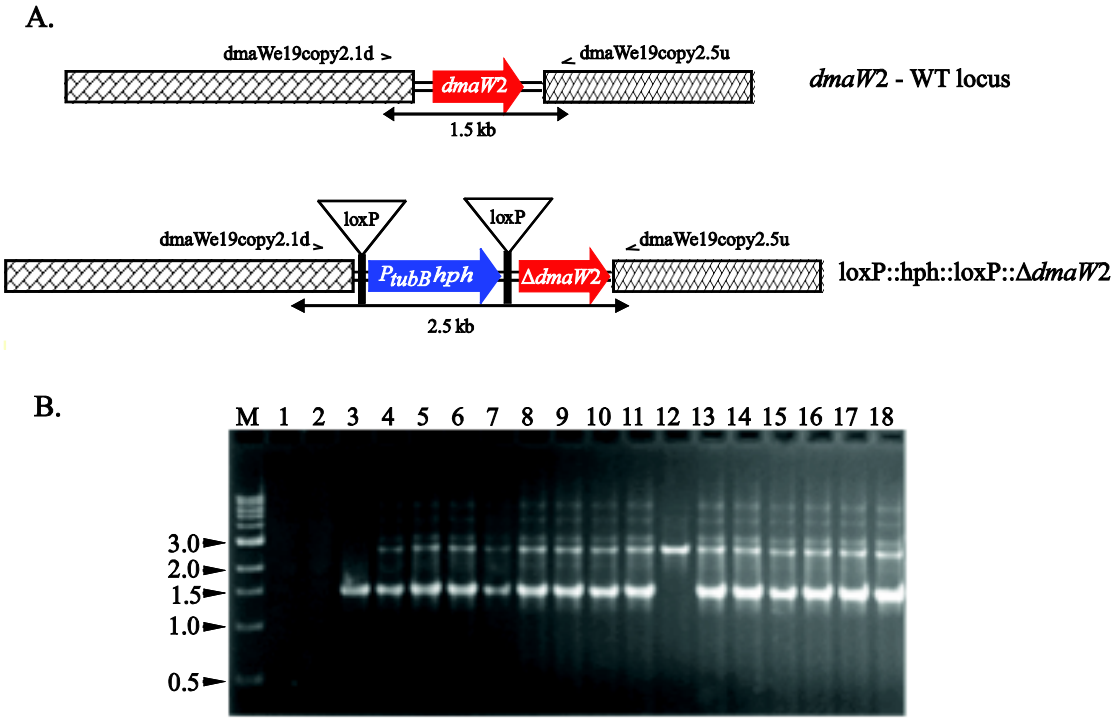


Figure 2.4. Gene disruption in *N. coenophialum*. **Panel A** Maps of the wild-type *dmaW*2 locus (*dmaW*2-WT), and the targeting vector and locus after marker exchange mutagenesis (loxP::*hph*::loxPΔ::*dmaW*2). **Panel B** PCR screen for *dmaW*2 gene replacement in *N. coenophialum*. Lanes contain size markers (lane M), and PCR products generated from the following templates: H_2O used as negative control (lane 1), *N. uncinatum* DNA as negative control (lane 2), wild-type *N. coenophialum* DNA as positive control (lane 3), and template DNA from 15 independent transformants (lanes 4-18). The transformants indicated in lanes 4-11 and 13-18 had ectopically integrated pKAES174. Transformant e7133 (lane 12) gave a 2.5-kb fragment from the construct but not the 1.5-kb fragment from the WT locus, indicating that it arose by homologous gene replacement.

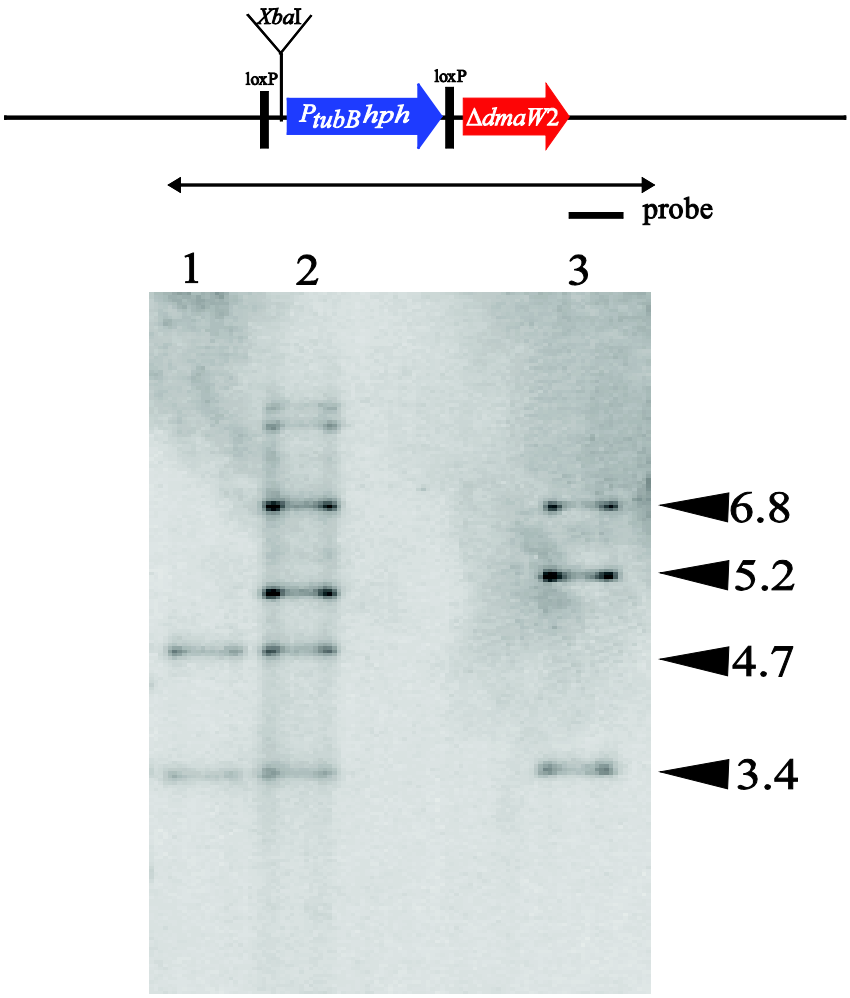


Figure 2.5. Southern-blot analyses indicating the *dmaW*2 gene knock out in *N. coenophialum*. The genomic DNA of the following isolates was digested with *Xba*I: *N. coenophialum* e19 (lane1); isolate possessing an ectopic integration pKAES174 (lane 2); and d*maW*2 knockout isolate e7133 (lane3). In all isolates the presence of the *dmaW*1 gene is indicated by the 3.4 kb band. The presence of the WT *dmaW*2 gene is indicated by the 4.7 kb band in the *N. coenophialum* e19 (lane1), as well as in the isolate possessing an ectopic integration (lane 2), whereas the 5.2 kb and 6.8 kb bands are generated by *dmaW*2 gene replacement (lane3) and ectopic integration (lane2).

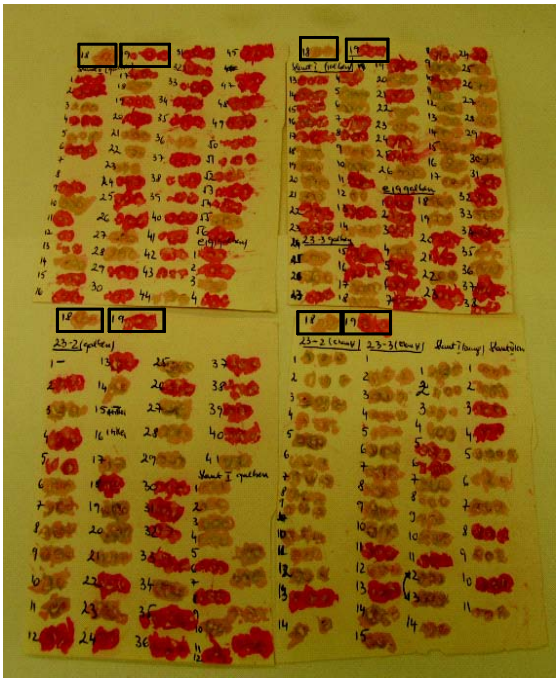


Figure 2.6. Tissue-print immunoblot (TPIB) performed to test for the endophyte infection of tall fescue seedlings inoculated with e7133. The blots were developed when the seedlings had at least 3 tillers. The negative (18) and positive (19) controls are represented in the black boxes.

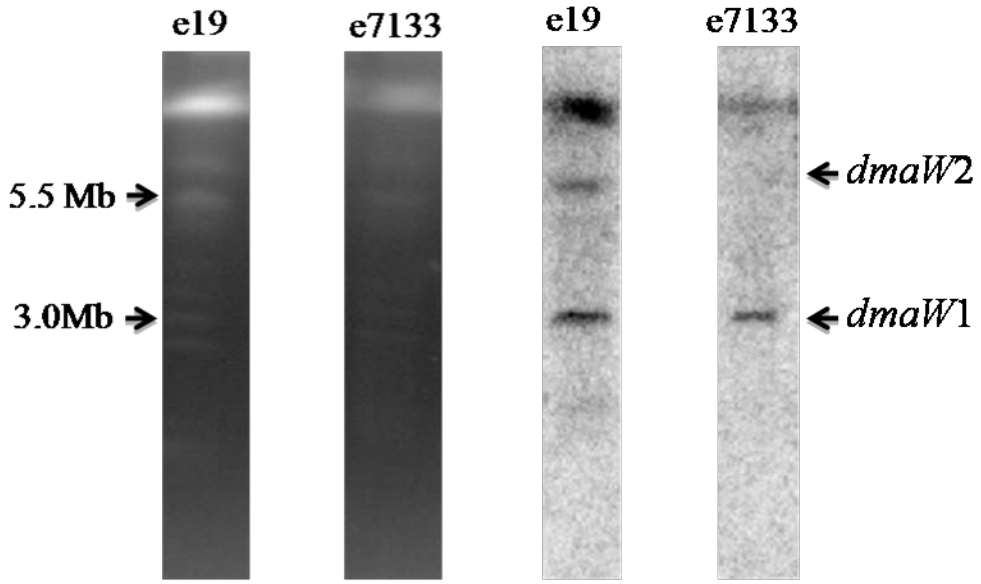


Figure 2.7. Chromosome separation and Southern-blot hybridization**.**

Panel A. Chromosomes of *N. coenophialum* e19 and the *dmaW*2 knockout mutant (e7133) separated by CHEF gel electrophoresis using CHEF DRII Apparatus (Biorad). Sizes of the two chromosomes containing *dmaW* genes are indicated in Mb. **Panel B**. Southern-blot analysis verifying that *dmaW*1 was not lost by the *dmaW*2 gene replacement and that the two *dmaW* gene copies are located on different chromosomes. A labeled 342 bp fragment identical in both genes but deleted from *dmaW*2 in knockout mutant e7133 was used as a probe. In the WT strain the probe hybridized to both *dmaW* genes, whereas in the mutant the band corresponding to *dmaW*2 was missing due to the gene replacement.

Chapter 3

Elimination of resistance markers from endophytic fungi

Introduction

Despite the fact that homologous recombination is not a frequent event in many filamentous fungi, gene replacement is routinely performed for elucidation of gene function in these organisms (Steiner et al., 1995). To facilitate such studies, mutant alleles are created by altering in vitro the target gene by using an antibiotic resistance marker gene to disrupt or replace the gene sequence (Chaveroche et al., 2000). The resistance marker allows selection of stable fungal transformants, and in a portion of these transformants the engineered mutant allele replaces the wild-type allele (Steiner et al., 1995). However, after identification of the desired gene replacement, the task of the selectable marker is completed and its presence in the fungal genome may become inconvenient for further experiments. In many filamentous ascomycetes, only two antibiotic resistance genes have proven useful, the hygromycin B-resistance gene, *hph*, (Gritz and Davies, 1983; Tsai et al., 1992), and the phleomycin resistance gene, *ble* (Mattern et al., 1994). The latter, however, requires a very expensive antibiotic, which acts by damaging DNA (Kross et al., 1982). Therefore, repeated use of *hph* would be the preferred approach for sequential gene deletions.

The limited availability of resistance markers is one of the reasons for developing a marker removal method suitable for a broader range of filamentous fungi that would allow the reuse of the same selectable marker for further manipulation. Moreover, molecular genetic studies of the epichloae illustrate why it is advantageous to have a

method for convenient removal of selectable markers in fungi. Such a method will be useful in cases of functional genetic redundancy, a common situation for the many epichloae that have arisen by interspecific hybridization, and thereby accumulated several gene copies from the parental strains (Moon et al., 2004). Knocking out multiple alleles requires either multiple antibiotic resistance markers, or repeated use of the same marker. A marker excision strategy can also yield genetically modified fungal strains lacking any foreign gene. In addition, field and greenhouse experiments are obligatory to test the fitness of plants inhabited by endophytes, as well as the persistence of the genetically modified endophytes inside their hosts (Scott, 2001), and genetically modified endophytes, such as *dmaW*-knockout strains of *N. coenophialum*, could be used commercially. Removal of the selectable gene would reduce the concerns and regulatory requirements regarding the presence of foreign genes in a genetically modified organism slated for environmental release.

Heterologous expression of the bacteriophage P1 recombinase enzyme, Cre, has been used extensively to remove selectable marker genes inserted between a pair of directly repeated loxP recombination sites (Sauer and Henderson, 1989; Sternberg and Hamilton, 1981). The excision events are specific, leaving a 34 bp loxP site in place. Numerous studies have reported the use of this Cre/lox system to study gene function and to remove markers from transgenic organisms including mammals such as mice (Schwenk et al., 1997; Xu et al., 2007); plants such as soybean (Li et al., 2007) and *Arabidopsis thaliana* (Shigaki et al., 2005), plant plastids (Corneille et al., 2001; Lutz et al., 2006), and fungi such as *Saccharomyces cerevisiae* (Sauer, 1987) and *Aspergillus* spp. (Forment et al., 2006; Krappmann et al., 2005).

In this chapter, I describe my efforts to develop a method for marker removal from fungal genomes with an adaptation of the Cre/lox system for rapid and convenient reuse of marker genes. The method does not require use of any additional selectable or counter-selectable marker gene. I tested the effectiveness of the technique in eliminating *hph* gene flanked by the loxP sites (floxed) in *N. coenophialum* e7133 and transformants of two other endophytic fungi *Epichloë festucae* and *Neotyphodium uncinatum*: Dr. Peter Mirabito (University of Kentucky) verified that the system also works well in *Aspergillus nidulans* (Florea et al., 2009).

Materials and methods

Biological materials

Fungal DNA was isolated from fresh mycelium as described in Chapter 2. The DNA concentration was estimated using a Qubit fluorometer and Quant-iT™ DNA BR Assay Kit (Invitrogen, Eugene, OR). Plasmid DNA was isolated from bacterial cultures using the Qiaprep Spin Miniprep Kit (Qiagen). The oligonucleotide primers used in this study were from Integrated DNA Technologies and are listed in **Table 3.1.**

Plasmids

The pKAES186 plasmid **(Figure 3.1 Panel A)** constructed by D.-X. Zhang (2008) carries a cassette containing *ble* and *cre* genes modified to function in filamentous ascomycetes and flanked by the loxP sites. The plasmid was designed with the *cre* gene following a putatively inducible promoter (from *lolC* of *N. uncinatum*) to induce *cre* expression in plant.

The pKAES175 plasmid **(Figure 3.1 Panel B)** containing a P_{tubB}*cre* cassette, was constructed by C. Machado (2004). The plasmid contains the *Epichloë typhina tubB* promoter region from pKAES080 (Tsai et al., 1992) linked to the Cre coding region from pQL123 (Liu et al., 1998), ligated into the *Sal*I and *Sac*II sites of pBluescript KS+.

Fungal strains

Experiments were performed with *Neotyphodium coenophialum* e7133 strain and *N. coenophialum* strain e19 isolated from tall fescue cultivar Kentucky 31 (Tsai et al., 1992), e7236, *Epichloë festucae* strain e7209 containing loxP::*hph*::loxP constructed for a virus-induced gene silencing (VIGS) experiment (K. Andreeva and C.L. Schardl, unpublished data), and *Neotyphodium uncinatum lolP*1-knockout mutant designated e7074 (Spiering et al., 2008). These epichloae were cultured and maintained as previously described (Blankenship et al., 2001; Wilkinson et al., 2000). All strains used in this chapter are described in **Table 3.2**.

Transformation of *N. coenophialum* with pKAES186 and screening for transformants

The mycelium of *N. coenophialum* e7133 was maintained on PDA plates containing 50 µg/ml hygromycin B. For protoplast isolation the fungus was ground and grown in flasks containing 50 ml PDB medium with rotary shaking at 21°C for 7-10 days. The protoplasts were isolated as described in Chapter 2 and then transformed with 7-10 µg pKAES186 DNA by the PEG method (Panaccione et al., 2001). The transformants were selected on CRM plates containing 25 µg/ml phleomycin and

screened by PCR for the integration of the plasmid using two sets of primers, one set [ble-tub(f) and ble(r)] specific for the *ble* gene and the other set [cre(f) and cre(r)] specific for the *cre* gene. The reactions were prepared in 25 µl volume reaction with 20 ng template, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 200 nM of each primer, 2.5 units AmpliTaq Gold, and AmpliTaq Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied Biosystems). The thermocycler program was set as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 58 °C for 35 sec, and 72 °C for 2 min, then a final incubation at 72 °C for 7 min. The screen for the presence of *hph* in the fungal genome was done with the primer set dmaWe19copy2.1d and dmaWe19copy2.5u, using the conditions described in Chapter2.

Protoplast fusion: screening for fusion and *hph* elimination

The fungal strain e7236 was obtained by co-transforming *N. coenophialum* e19 with pKAES175 and pAN8-1 (Mullaney et al., 1985) a plasmid carrying the *ble* gene, from transposon Tn5, for phleomycin resistance. For protoplast fusion, the protoplasts were isolated from e7133 and e7236 using the lysing enzymes described in Chapter 2. The protoplasts of the two strains were mixed in a 1:1 ratio of $1x10^6$ protoplasts each and then combined with three separate PEG solutions of different molecular weight (MW). The PEG solutions contained 40% PEG of 3500, 4000 or 6000 Dal mixed with Ca^{2+} amendments (1.8 M KCl, 150mM $CaCl_2$, 150 mM Tris pH 7.4). The protoplasts were recovered on CRM (described in Chapter 2) containing 25 µg/ml phleomycin or without antibiotic. The PCR screen for protoplast fusion and for *hph* elimination was carried out

using dmaWe19copy2.1d and dmaWe19copy2.5u primers, using the conditions described in Chapter 2.

Transient transfection of *Neotyphodium coenophialum, N. uncinatum* and *E. festucae*

Mycelia from the *Neotyphodium* spp. or *E. festucae* were grown in PDB medium. Then protoplasts were prepared as described in Chapter 2, except that during cell wall digestion the mycelium was incubated at 30 °C for 3-4 h for *N. coenophialum*, or 2 h for *N. uncinatum* and *E. festucae*. Using the PEG method (Panaccione et al., 2001) the fungal strains *N. coenophialum* e7133, *E. festucae* e7209 and *N. uncinatum* e7074, all containing a loxP*::hph::*loxP cassette integrated into the genome, were mixed with 5-7 µg pKAES175 DNA in a 100 µl suspension containing $1x10^7$ protoplasts. The treated protoplasts were recovered on CRM-low as described in Chapter 2, except that a 1-5 µl of the mixed suspension (containing 900-4000 protoplasts) was plated on medium without antibiotics. Due to slow recovery of *N. uncinatum* e7074 protoplasts on CRM, the following three-layer plate method was implemented. An 18 ml bottom PDA layer was overlain with 5 ml CRM-low. Then the protoplasts were suspended in 5 ml CRM-low at 45 °C and layered on top. The plates were incubated at 21 °C until colonies were visible by eye. Then, 50-400 colonies were transferred to PDA plates without antibiotics, grown until they sporulated, and single-spore isolated three times on PDA.

Screening of transformants following transient transfection with pKAES175

PCR reactions were carried out in 25 µl reaction mixture with 5-10 ng DNA templates, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 200 nM of each

primer, and 2.5 units AmpliTaq Gold, AmpliTaq Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied Biosystems), in a model 2720 Thermal Cycler (Applied Biosystems). PCR screening for marker elimination in *N. coenophialum* e7133 was done using primer pair dmaWe19copy2.1d and dmaWe19copy2.5u. The PCR program was as follows: 9 min at 95 °C, 35 cycles of 94 °C for 30 sec, 61 °C for 35 sec, 72 °C for 3 min 10 sec. The PCR screen for excision of *hph* from *N. uncinatum* e7074 employed primer set lolP15-3check loop2 and lolP13-5check loop2. PCR conditions were 95 °C for 9 min, then 35 cycles of 95 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 2 min. The PCR screens for *cre* in the fungal genomes were performed using primer set cre(f) and cre(r). The thermocycler program was 94 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 58 °C for 35 sec, and 72 °C for 2 min, then a final incubation at 72 °C for 7 min.

Sequencing reactions

PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced using the BigDye version 3 Terminator cycle sequence Kit (Applied Biosystems), according to manufacturer's instructions, with each of the PCR primers used as a sequencing primer. Sequencing reaction products were analyzed by capillary electrophoresis in an Applied Biosystems model 3730xl DNA analyzer at the University of Kentucky Advanced Genetic Technologies Center.

Southern-blot analysis

Genomic DNA of the fungal isolates was extracted by the method of Al-Samarrai and Schmid (2000). The quality of DNA was checked by electrophoresis and the DNA

was quantified using a Qubit fluorometer (Invitrogen). To test for the presence of *cre* or *hph*, genomic DNA was digested with *Apa*I and 2 µg DNA was electrophoresed in 0.8% agarose gel in 1xTBE electrophoresis buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.2). The DNA was transferred from the gels to Hybond N^+-nylon membrane (GE Healthcare, Piscataway, NJ USA) following the manufacturer's instructions. Labeled pKAES175 was used as the probe for integrated plasmid DNA (including *cre*). A 500 bp PCR product used as a probe for *hph* was amplified using primers HYG-HY1 and HYG-YG1 and pKAES174 as a template. The PCR reaction was performed in 25 µl with 5-10 ng DNA templates, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 200 µM of each primer, 2.5 units AmpliTaq Gold and AmpliTaq Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied Biosystems), in a model 2720 Thermal Cycler (Applied Biosystems). The PCR thermocycling profile was 94 °C for 5 min; 35 cycles at 94 °C for 20 s, annealing temperature 56 °C for 20 s, and 72 °C for 30 s; and a final incubation at 72 °C for 7 min. The PCR products were gel-purified using QIAquick Gel Extraction kit (Qiagen). Labeling was carried out using a Prime-a-Gene labeling kit (Promega). The labeled probes were purified from unincorporated [^{32}P]dCTP by centrifugation through MicroSpin G-50 Columns (GE Healthcare, Piscataway, NJ USA). The hybridization solution, conditions and the washing steps were done as described by Sambrook and Russell (2001).

Antibiotic sensitivity test

Isolates identified by PCR screen as having the *hph* gene looped out were replica-plated in Falcon 6-well plates (Becton Dickinson and Co, Franklin Lakes, NJ) on PDA

containing or lacking hygromycin B. Fungal mycelia were ground in 500 µl sterile water and aliquots of the suspensions were spread on the Falcon plates. Concentration of hygromycin B was 50 µg/ml for *N. coenophialum,* 80 µg/ml for *N. uncinatum,* and 650 µg/ml for *E. festucae*. The plates were incubated at 21°C for 3 weeks.

Inoculation of the transformants into tall fescue

Tall fescue (*Lolium arundinaceum*) seed sterilization and inoculation procedures were performed following the method described by Latch and Christensen (1985), whereas tissue-print immunoblot (TPIB) was performed when the plants had 3 tillers following the procedure described by Gwinn and Gavin (1991).

Results

Elimination of floxed *hph* by transformation with pKAES186 and compatibility with tall fescue

The e7133 isolate was transformed with pKAES186 carrying a cassette containing floxed *ble* and *cre* genes. Expression of *cre* driven by the *lol*C promoter would determine the excision of sequences located between loxP sites, namely, *hph*, *cre* and *ble*. More than 100 transformants were obtained from which 96 were screened by PCR for plasmid integration and presence of *cre* and *ble* genes in the fungal genome. Additional PCR screening was done to verify if *hph* was still present or eliminated from the fungal genome due to the activity of Cre recombinase. If the *hph* gene was eliminated by Cre, a PCR test would result in a 1.2 kb PCR fragment, compared to the 2.5 kb fragment from e7133 and a 1.5 kb fragment from the wild-type *dmaW*2 **(Figure 3.2. Panel A)**.

Unexpectedly, even though *cre* activity was driven by an in-planta inducible promoter, the *cre* gene was expressed in culture and, as a result *hph* was eliminated from the fungal genome. Using this method, the rate of elimination for *hph* among the phleomycin-resistant transformants was greater than 21%. One of these isolates was designed e7135 **(Figure 3.2. Panel B).** The results indicated that *cre* was expressed in culture and excised the *hph* gene with the retention of a loxP. However subsequent elimination of the *cre* and *ble* genes did not take place (Florea et al., 2007).

To learn if subsequent elimination of the *cre-ble* cassette would take place in planta, several transformants having *hph* eliminated from the fungal genome were inoculated into tall fescue seedlings. The fungus from the successfully inoculated plants was grown out from the plants and further screened by PCR for the presence of *cre* and *ble* genes. The results revealed that in all the transformants, the *cre-ble* cassette was retained in the fungal genome.

Screening for *hph* elimination by protoplast fusion

If fusion occurred between e7236, a transformant caring P_{tubB}*cre*, and e7133 harboring loxP::*hph*::loxP cassette, I expected Cre protein to be expressed and act on the loxP sites flanking the *hph* gene in e7133, eliminating it from the fungal genome. After the attempted protoplast fusion, several rounds of single-spore isolation were applied to 769 putative fused protoplasts. The PCR screen showed that none of the isolates obtained in the procedure gave the 1.2 kb band expected in case of *hph* gene excision.

Elimination of floxed *hph* from the *N. coenophialum* e7133 genome

The e7133 strain was subjected to transient transfection with pKAES175, and then the colonies were randomly picked and grown on PDA without antibiotic **(Figure 3.3)**. Genomic DNA was extracted from a portion of the colony of each isolate and screened by PCR for loss of *hph* from the mutated *dmaW*2 locus. Of these, 18 gave a 1.2-kb fragment size as expected if *hph* was excised due to the action of Cre recombinase on the flanking loxP sites. The remaining colony from each of these 18 isolates, was subjected to purification through three rounds of single-spore isolation, and then a second PCR test was conducted, which confirmed excision of *hph* in two of the isolates, designated e7202 and e7204 (**Figure 3.4 Panel A**). The sequences of the 1.2-kb PCR products from these two isolates showed the expected retention of a single loxP site. To learn if Cre expression was due to a transient presence of pKAES175 DNA, or due to stable integration of the plasmid into the fungal genome, a PCR screen was performed with *cre*-specific primers, cre(f) and cre(r). The strain e7236, which contains stably integrated *cre* in its genome, was used as positive control. The *cre* gene was detected in the positive control templates pKAES175 DNA and e7236 genomic DNA, but not in the genomic DNA of isolate e7202 or e7204 (**Figure 3.4 Panel B**).

To test for sensitivity to hygromycin B, the aforementioned 18 isolates and wild-type controls were grown in parallel in 6-well plates on medium containing or lacking the antibiotic. Isolates e7202 and e7204 failed to grow in the wells containing hygromycin B, but grew and sporulated normally in the wells lacking antibiotic. The other isolates grew both in antibiotic-containing and antibiotic-free wells, confirming that they still possessed *hph* **(Figure 3.5**).

Elimination of floxed *hph* in *N. uncinatum* and *E. festucae*

Protoplasts of *N. uncinatum* e7074, a gene replacement mutant with loxP::*hph*::loxP::Δ*lolP*1 (Spiering et al., 2008), were also subjected to transient transfection with pKAES175, and plated on medium without antibiotic, and individual colony isolates were checked by PCR for *hph* excision. Five of the isolates yielded the 610-bp PCR product predicted for the Cre-mediated loop-out of *hph*, and four of these failed to yield the 1945-bp *hph*-containing PCR product (**Figure 3.6 Panel A**). These isolates were also screened by PCR for *cre* in the fungal genome. The results showed that *cre* was present in the control strain but could not be detected in the strains having the *hph* looped out (**Figure 3.6 Panel B**). The five isolates were further single-spore isolated, and mycelium was inoculated on plates with hygromycin B (80 μg/ml) and without antibiotic. Two isolates failed to grow on the antibiotic plates, confirming the loss of *hph* (**Figure 3.7**).

Protoplasts of *E. festucae* e7209, an isolate transformed with the plasmid pKAES196 containing a loxP::*hph*::loxP cassette, were subjected to transient transfection with pKAES175, and then plated on antibiotic-free medium. The isolation of the putative transformants and the sensitivity tests were performed in collaboration with Dr. K. Andreeva. Sensitivity to hygromycin B was then assessed for 50 picked colonies. Mycelium from each isolate was ground and spread in wells with 650 μg/ml hygromycin B and wells without antibiotics. After three weeks of incubation, three isolates showed slower growth on antibiotic plates, but only one had clear indication of sensitivity to hygromycin B, indicating that *hph* had been lost (**Figure 3.8**).

Southern-blot test for the presence of *hph* and *cre* in fungal genomes

All isolates of *Neotyphodium* spp. or *E. festucae* identified by PCR as having lost *hph* were additionally tested by Southern-blot analysis. The results confirmed that these strains had the expected *hph* excision (**Figure 3.9**). These strains were further tested by Southern-blot hybridization to pKAES175 for *cre* or other plasmid segments in the fungal genome. The *cre* gene was absent from all strains except the positive control strain, e7236, which has stably integrated *cre*. However, pKAES175 plasmid backbone hybridized to fragments in the *E. festucae* e7213 DNA but not to the DNA of e7202, e7203, e7204, and e7205 isolates derived from *N. uncinatum* and *N. coenophialum* knockout strains. This was expected because e7213 derives from e7209, which was obtained by co-transforming two plasmids harboring a similar backbone as pKAES175 **(Figure 3.10).**

Discussion

In this chapter I describe a fast, simple and efficient strategy to generate marker free fungal mutants, based on transient expression of Cre recombinase following introduction of a fungal-active *cre* gene into protoplasts by standard transformation procedures. The strategy was applied to several endophytic fungi of grasses. In my efforts to eliminate the foreign gene from *N. coenophialum* e7133 and reuse the *hph* marker, I attempted two other strategies for Cre expression. One strategy was to attempt to fuse protoplasts of e7133 with a strain that had been stably transformed with the P_{tubB}*cre* construct, but *hph* deletion was not observed. Another strategy was to generate

transformants with pKAES186, a plasmid containing a potentially inducible *cre*, which together with the selectable *ble* gene, was also flanked by loxP sites. This approach eliminated the *hph* gene, but subsequent deletion of the *cre* and *ble* genes was not achieved, so the procedure left these two foreign genes in the fungal genome. Even if one of these approaches had succeeded, both were far more labor intensive than the successful approach of unselected transfection with pKAES175 to transiently express Cre.

In initial PCR screens following such treatment of *N. coenophialum* e7133 and *N. uncinatum* e7074, approximately 5% of colonies gave product sizes expected from the Cre-mediated elimination of *hph*. However, after single-spore isolation, required for purification of the nuclear genotypes, a second PCR screen confirmed the loss of *hph* for only two out of 384 *N. coenophialum* isolates, and two out of 96 *N. uncinatum* isolates. The difference in the number of fungal isolates with apparent Cre-mediated marker elimination before and after single-spore isolation may be explained by the fact that fungal protoplasts may be multinucleate, whereas single spores of *Epichloë* and *Neotyphodium* species are uninucleate (Kuldau et al., 1999). Therefore, it is possible that in protoplasts that took up the *cre* gene, marker excision occurred in some but not all of the nuclei. This resulted in a mixed colony, with the marker present in some parts of the colony and absent in others. Such a mixed colony was indicated for one of the isolates analyzed after transient transfection of *N. uncinatum* e7074 (see Figure 3.6, lane 5). In my screen, first I analyzed DNA of a portion of each colony by PCR, followed by single-spore isolation of the remainder of the colony for subsequent PCR confirmation. This technique apparently resulted in these conflicting results for many of the isolates.

Possibly, a higher percentage of confirmed marker gene loop-outs could be obtained by first single-spore isolating prior to the initial PCR analysis, but this would be a much more laborious approach.

Frequencies of stable transformants in the epichloae used in this study varied between 1.6 and 3.0 $x10^{-6}$. In contrast, the frequencies of confirmed marker deletion, based on the proportion of randomly picked colonies that had excised the *hph* gene, ranged from 5.2 to $21x10^{-3}$. These results allow an estimation of the frequency of DNA integration in transformation of *Neotyphodium* and *Epichloë* species, implying that one in approximately 10^3-10^4 protoplasts that take up exogenous DNA into their nuclei ultimately integrate that DNA into the genome. With the observed 0.5-2.1% frequency of marker deletion upon transient transfection, this is a very manageable approach for eliminating selectable markers from genetically modified fungal strains. The method was also tested on *Aspergillus nidulans* by Dr Mirabito (from the Biology department at the University of Kentucky). The protoplasts of a strain (TPM363-4) having foxed *yA* gene (responsible for green conidia color) were transiently transfected with pKAES175 or no DNA (mock transfection). The results indicated that for transiently transfected protoplasts with pKAES175, 2% of the colonies were yellow as expected in case of *yA* gene elimination. However, no yellow colonies were detected for the mock transfected protoplasts, suggesting that spontaneous elimination of floxed marked did not take place (Florea et al., 2009).

Over the years, the Cre/lox system has been used effectively for elimination of marker genes in different systems (Schwenk et al., 1997). New modifications to extend this approach have been developed (Forment et al., 2006; Krappmann et al., 2005;

Lambert et al., 2007). For instance, in the area of filamentous fungi, Forment et al., (2006) used a counter-selectable marker gene (*Neurospora crassa pyr*-4) flanked by loxP sequences to sequentially delete two target genes in the same strain of *A. nidulans*. With this system the authors used the same marker to sequentially inactivate two genes (*yA* and *wA*) responsible for conidium color. In their study, the plasmid with a regulatable *cre* gene was stably integrated into the fungal genome. The method described in this chapter differs from the previous Cre/lox approaches for marker gene removal by not requiring selection for the Cre plasmid or counter-selection for loss of the marker gene. Instead, I demonstrated that expression of Cre from plasmid pKAES175 during transient transfection is sufficient for marker gene removal with sufficient frequency for PCR screening.

In conclusion, I have presented a method for marker excision that offers advantages over the existing methods in being capable to generate fungal mutants entirely free of foreign genes. Furthermore, the strategy will obviate many concerns related to field release or commercialization of economically important grasses associated with manipulated fungal strains, allowing maintenance of the benefits provided by the endophytes. It is expected that the technology described in this chapter provides a reliable strategy for effective marker gene removal and will likely be adapted and applied in distantly related fungal species.

A version of this chapter has been published (Florea et al., 2009)

Table 3.1. Oligonucleotide primers used in Chapter 3

Primer name	Sequence
dmaWe19copy2.1d	agaaacagacagggctattc
dmaWe19copy2.5u	ctcgccggcatgcgtcaaaa
cre(f)	cctgtttcactatccaggttacgg
cre(r)	ccaatttactgaccgtacaccaa
lolP15-3check loop2	tcagagcacacttgcctcatcgtg
lolP13-5check loop2	acgtggtcatggacgttcgtag
Ble-tub(f)	tgagtcatgcacagaccacgat
ble(r)	cctgcaagcaattcgttctgtatc
HYG_HY1	cgaagaatctcgtgcttcagcttcgatgta
HYG_YG1	ccattgtccgtcaggacattgttg

Table3.2. Biological fungal strains used in third chapter

Species	Strain designation	Description
N. coenophialum	**e19**	The wild type strain
N. coenophialum	**e7133**	loxP::*hph*::loxP::Δ*dmaW*2 derived from the wild type e19 strain transformed with pKAES174.
N. coenophialum	**e7202**	loxP::Δ*dmaW*2 derived from e7133 by transient transfection with pKAES175.
N. coenophialum	**e7204**	loxP::Δ*dmaW*2 derived from e7133 by transient transfection with pKAES175.
N. coenophialum	**e7236**	Strain derived by co-transforming e19 strain with pKAES175 caring *cre* gene and pAN8-1 (Mattern et al.1988)
N. coenophialum	**e7135**	P_{tubB}*cre* P_{tubB}*ble* loxP::Δ*dmaW*2; derived from e7133 by transforming with pKAES186 harboring *cre* and *ble* genes floxed
N. uncinatum	**e7074**	Isolate harboring the disrupted *lolP*1 gene and the floxed *hph* gene(Spiering et al., 2008).
N. uncinatum	**e7203**	Isolate derived from e7074 by transforming this strain with pKAES175 plasmid harboring *cre* gene.
N. uncinatum	**e7205**	Isolate derived from e7074 by transforming this strain with pKAES175 plasmid harboring *cre* gene.
E. festucae	**e2368**	*E. festucae* wild type strain.
E. festucae	**e7209**	Isolate derived from e2368 by from co::transformation with pKAES196 and pKAES198; contains floxed *hph* from pKAES196 (K. Andreeva and C.L. Schardl, unpublished data)
E. festucae	**e7213**	Isolate derived from e7209 by transforming this strain with pKAES175 plasmid harboring *cre* gene. The isolate was identified as having excised *hph* gene due to transient expression of Cre recombinase.

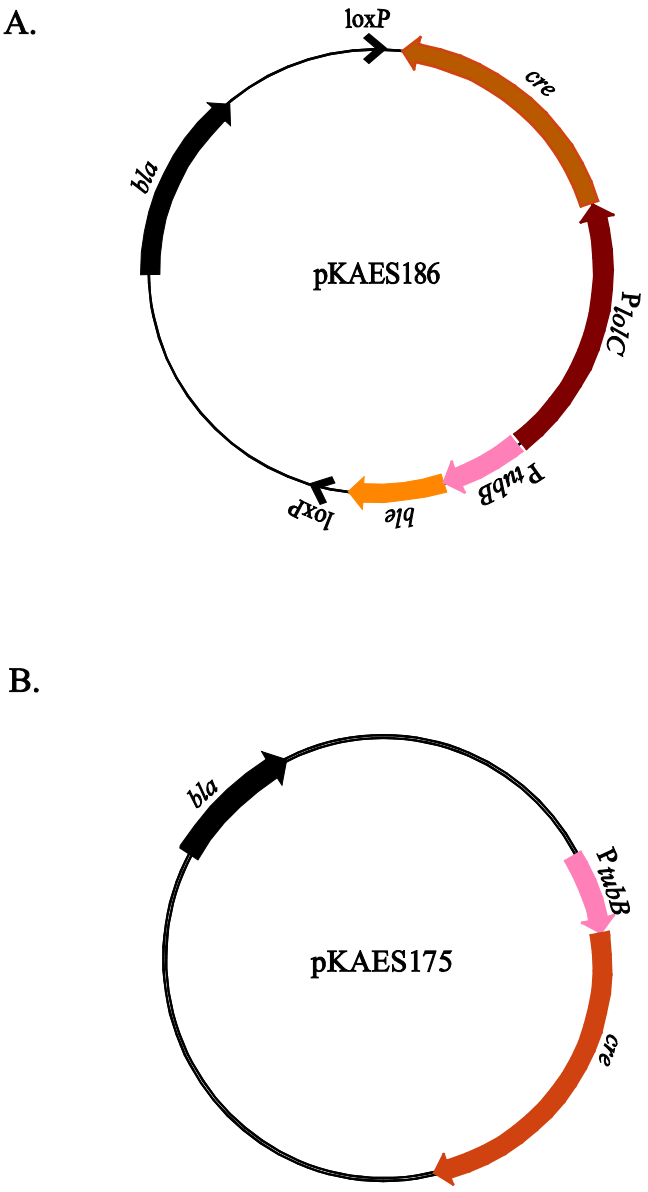


Figure 3.1. Maps of plasmids containing the *cre* gene. **Panel A.** pKAES186 is a plasmid containing *cre* linked to an inducible promoter, P_{lolC}, which together with the *ble* gene, was flanked by loxP sites. **Panel B.** Plasmid pKAES175 was designed for expression of the *cre* gene under control of P_{tubB}.

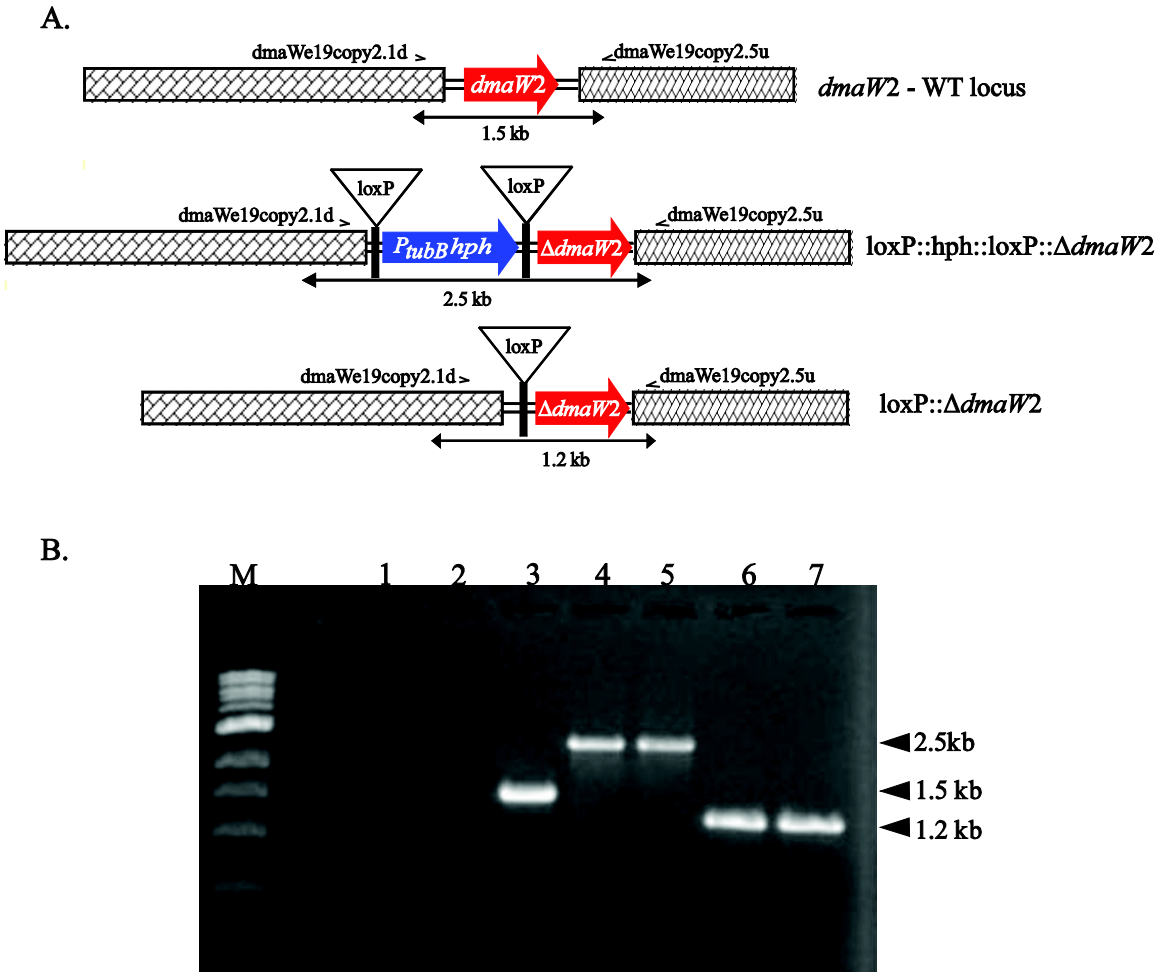


Figure 3.2 Elimination of cre gene using pKAES186 plasmid**. Panel A.** Maps of the wild-type *dmaW*2 locus (*dmaW*2-WT), the targeting vector and locus after marker exchange mutagenesis loxP::*hph*::loxP::Δ*dmaW*2, and the locus after excision of *hph* apparently by the activity of Cre recombinase (loxP::Δ*dmaW*2). The *dmaW*2-specific primers (arrows) used for PCR to identify homologous integration were dmaWe19copy2.1d and dmaWe19copy2.5u. **Panel B.** PCR screen to identify *hph* loop-outs after transforming pKAES186 into e7133. The transformants were screened using primer pair dmaWe19copy2.1d and dmaWe19copy2.5u to amplify a 2.5 kb region in e7133, a 1.2 kb in e7135 and 1.5 kb from the wild-type *dmaW* locus. Lanes contain size markers (lane M), and PCR products with the following templates: H_2O (lane 1) and *N. uncinatum* DNA (lane 2) used as negative controls, *N. coenophialum* e19 DNA as positive (lane3), e7133 DNA as positive control for the knockout (lanes 4 and 5), and e7135 isolate with excised *hph* resistance gene (lanes 6 and 7).

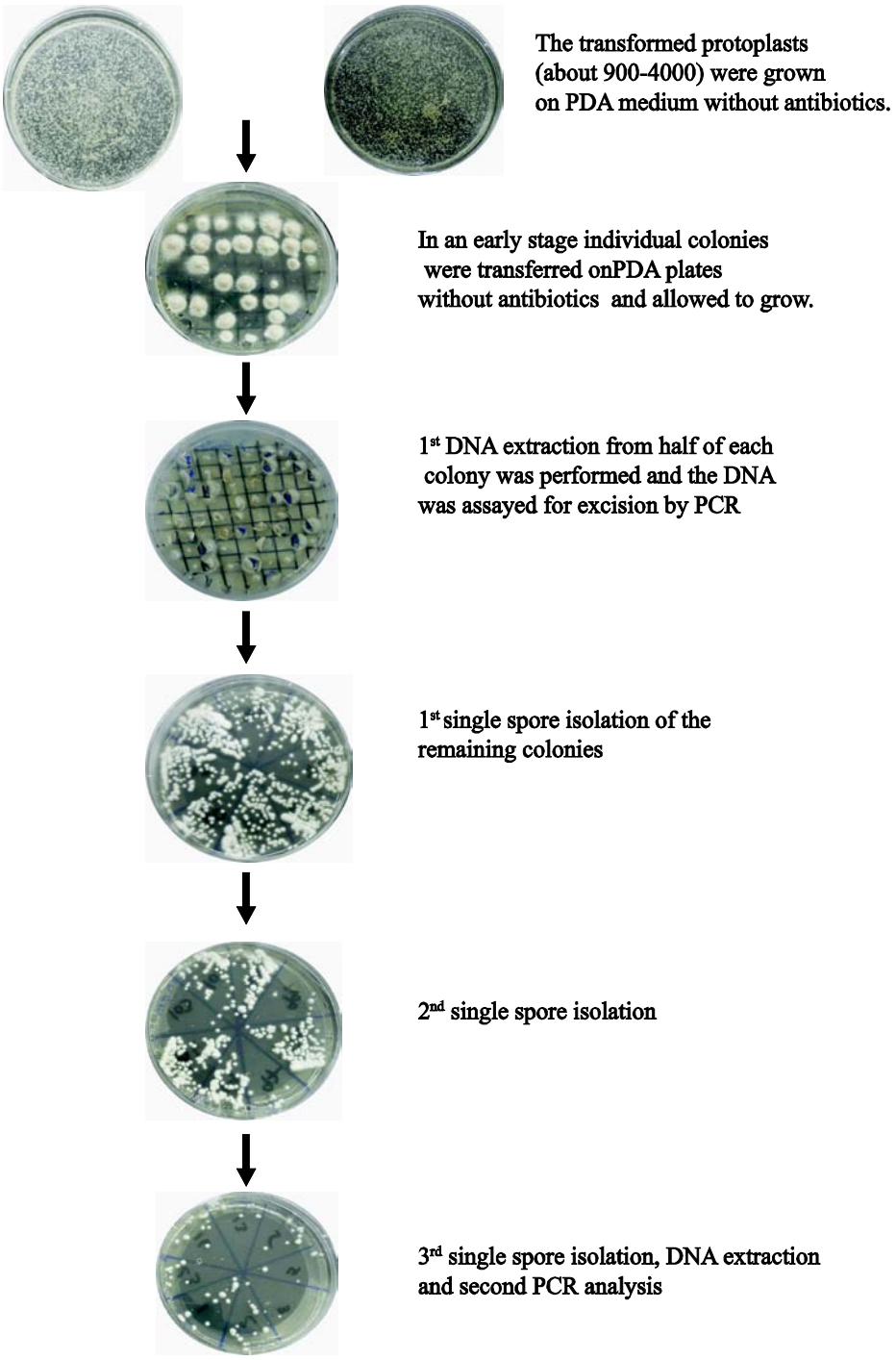


Figure 3.3. Strategy (steps) towards the identification of the fungal isolates having undergone marker excision after transient transfection with pKAES175.

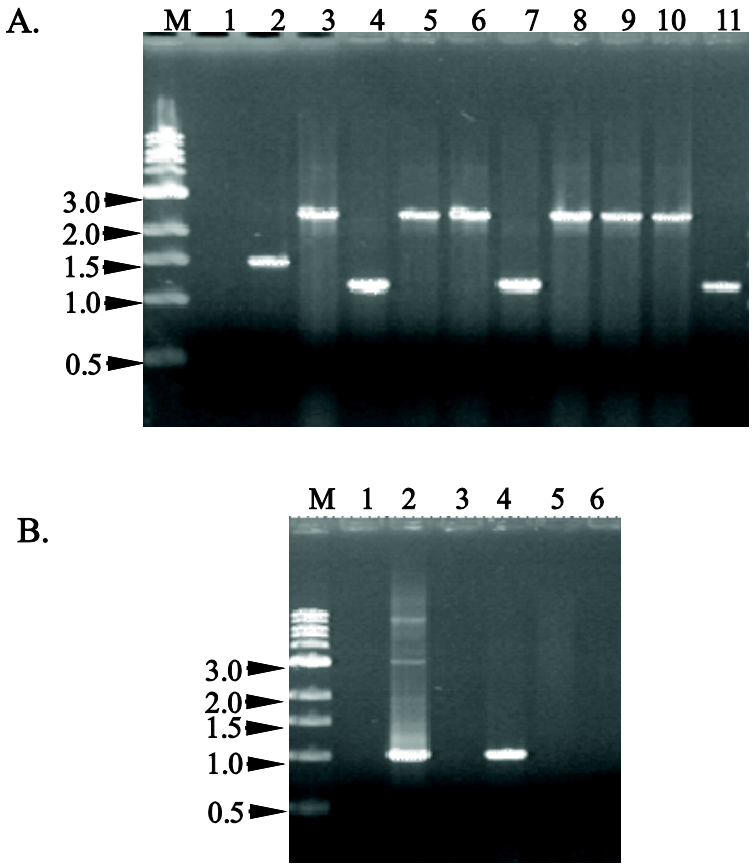


Figure 3.4. PCR screen for *hph* elimination and presence of *cre* in *N. coenophialum* genome. **Panel A.** PCR screen for excision of *hph*, resulting from site specific recombination between loxP sites. Lanes contain sizes markers indicated in kb (lane M), and PCR products with the following templates: H_2O used as negative control (lane 1), wild-type *N. coenophialum* DNA as positive control (lane 2), DNA from the loxP::*hph*::loxP::Δ*dmaW*2 transformant e7133 (lane 3), e7236 containing a *cre* gene integrated into the genome (lane 4), and derivatives of e7133 treated with plasmid pKAES175 (lanes 5-11). Isolates represented by lanes 5, 6, and 8-10 yielded the 2.5-kb fragment, expected if *hph* remained in the Δ*dma*W2 locus. Isolates represented by lanes 7 and 11 (e7202 and e7204, respectively) yielded a 1.2-kb fragment expected if the *hph* had been excised. **Panel B.** PCR screen for presence of *cre* in the fungal genome. Lanes contain size markers in kb (lane M), and PCR products with the following templates: H_2O as negative control (lane 1), pKAES175 DNA as positive control for indication of *cre* (lane 2), e7133 as negative control (lane 3), e7236 which previously has been transformed with *cre*, as positive control (lane 4), and isolates e7202 and e7204 (lanes 5 and 6), in which the floxed *hph* gene had been excised. Absence of a product in lanes 5 and 6 indicates that *cre* was not stably maintained in e7202 and e7204.

Figure 3.5 Response to hygromycin B of isolates derived from *N. coenophialum* e7133, which contained floxed *hph*, after transient transfection with pKAES175. Alternating rows contained (+) or lacked (–) hygromycin B. Red rectangles indicate the two hygromycin-B-sensitive isolates (e7202 and e7204) derived from the transiently transfection.

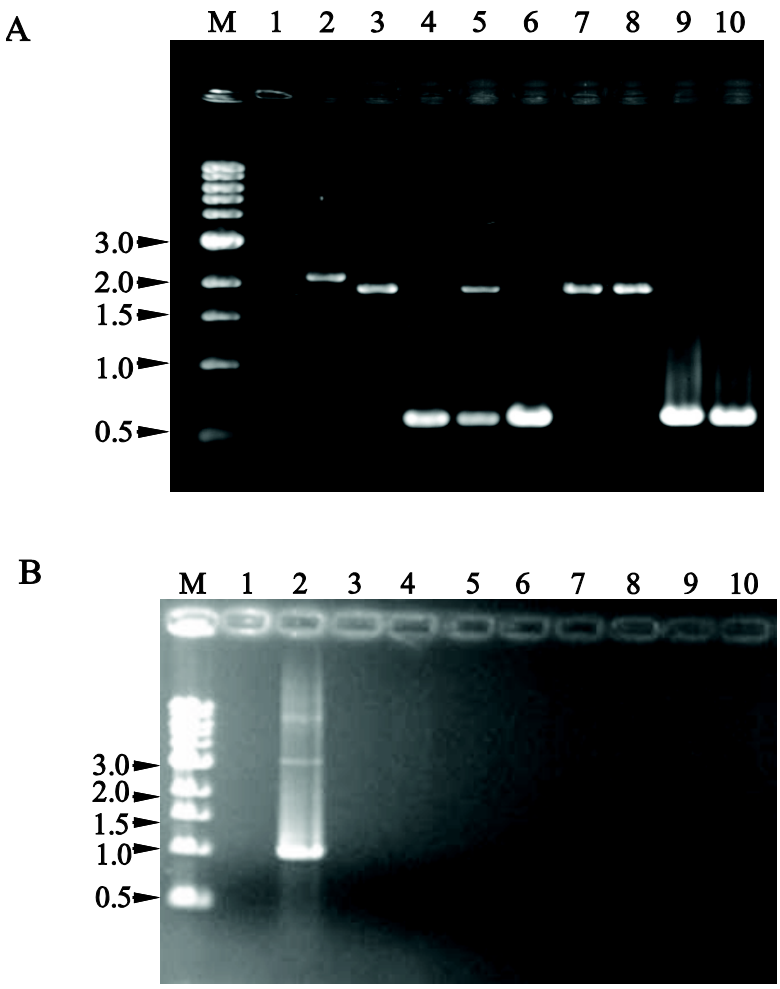


Figure 3.6. PCR screen for *hph* elimination and presence of *cre* in the fungal genome in *N. uncinatum***. Panel A** PCR screen for the excision of the *hph* gene from *N. uncinatum* Δ*lolP*1 strain e7074. Lanes contain molecular size markers (lane M; sizes indicated in kb), and products of PCR with the following templates: H_2O as negative control (lane 1), DNA of *N. uncinatum* CBS 102646 yielding the fragment from *lolP*1 (lane 2), DNA of isolate e7074 yielding the PCR product from Δ*lolP*1 (lane3), DNA from isolates from e7074 following transient transfection with pKAES175 (lanes 4-10). Product sizes in lanes 4, 5, 6, 9 and 10 are as expected in the case of *hph* gene excision, whereas product sizes in lanes 7 and 8 indicate that *hph* was not excised in those isolates. The presence of two PCR products in lane 5 indicates a mixture of nuclei with and without excised *hph*. **Panel B.** PCR screen for presence of *cre* in the fungal genomes. Lanes contain molecular size markers in kb (lane M), and products of PCR with the following templates: H_2O used as negative control (lane 1), pKAES175 DNA as positive control for indication of *cre* (lane 2), DNA of strain e7074 (lane 3), and DNA from isolates obtained after transient transfection of e7074 with pKAES175 (lanes 4-10). The same genomic DNAs were used as PCR templates for the respective lanes 3-10 of panels A and B.

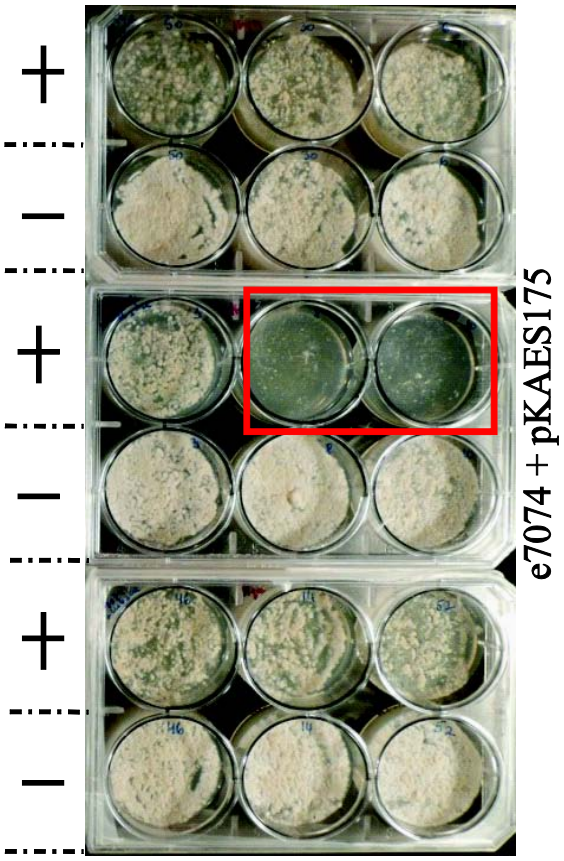


Figure 3.7. Response to hygromycin B of isolates derived from *N. uncinatum* e7074, which contained floxed *hph*, after transient transfection with pKAES175. Alternating rows contained (+) or lacked (–) hygromycin B. Red rectangles indicate the two hygromycin-B-sensitive isolates (e7201 and e7203) derived from the transiently transfection.

Figure 3.8. Response to hygromycin B of isolates derived from *E. festucae* e7209, which contained floxed *hph*, after transient transfection with pKAES175. Alternating rows contained (+) or lacked (–) hygromycin B. Red rectangles indicate one hygromycin-B-sensitive isolate (e7213) derived from the transiently transfection.

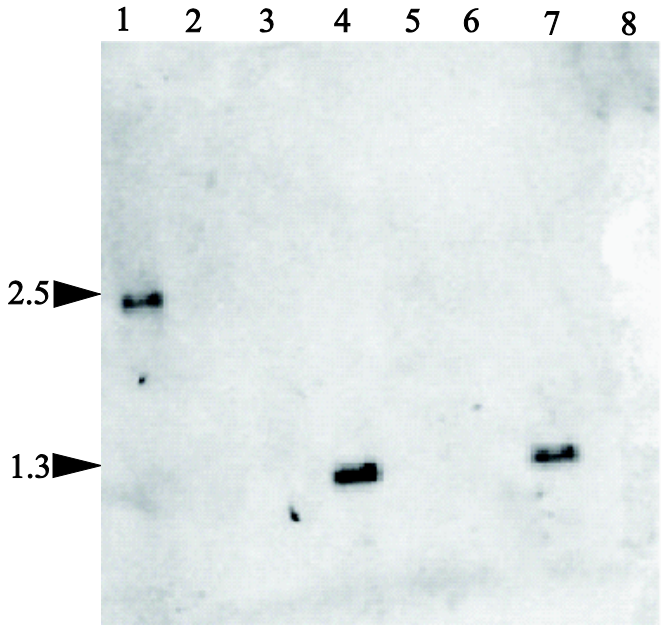


Figure 3.9 Southern-blot analysis of the floxed *hph* marker, indicating its loss from the transformants after transiently transfection with pKAES175 containing P_{tubB}*cre*. Lanes contained *Apa*I-digested genomic DNA from *N. uncinatum* transformant e7074 (control, lane 1), *N. uncinatum* e7201 (lane 2), *N. uncinatum* e7203 (lane 3), *N. coenophialum* e7133 (control, lane 4), *N. coenophialum* e7202 (lane 5), *N. coenophialum* e7204 (lane 6), *E. festucae* e7209 (control, lane 7), and *E. festucae* e7213 (lane 8). The Southern blot was hybridized with the *hph* probe.

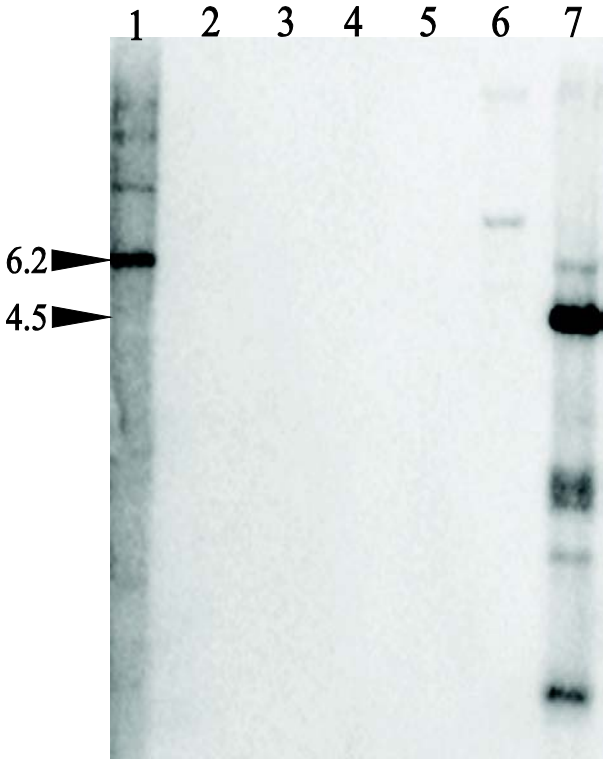


Figure 3.10. Southern-blot analysis of DNA from epichloë strains probed with the *cre* clone pKAES175. Lanes contained *Apa*I-digested genomic DNA from e7236 stably transformed with *cre* (control, lane 1), *N. uncinatum* e7201 (lane 2), *N. uncinatum* e7203 (lane 3), *N. coenophialum* e7202 (lane 4), *N. coenophialum* e7204 (lane 5), *E. festucae* e7213 (lane 6), and plasmid pKAES175 (control, lane 7). No hybridizing fragments were detected in lanes 2-5. However, hybridization of labeled pKAES175 to fragments in the e7213 DNA (lane 6) was expected because this strain derives from e7209 obtained by co-transforming two plasmids with a backbone similar to that of pKAES175. Calculated sizes of the major hybridizing fragments in lanes 1 and 7 are indicated in kb.

Chapter 4

Manipulation of ergot alkaloid (*EAS*) gene clusters in epichloae

Introduction

Fungal secondary metabolites, such as ergot alkaloids (EA) are not essential for the survival of the producing organisms. It is unclear what ecological roles these natural compounds play in the life of the producing fungi. A hypothesis is that they might provide competitive advantages against other microorganisms or play a defense role against organisms that feed on these fungi (Mylonakis et al., 2007).

Epichloë and *Neotyphodium* species produce not only EA but also other classes of alkaloids identified as indolediterpenes (lolitrem B), saturated aminopyrrolizidines (lolines), and pyrrolopyrazines (peramine) (Bush et al., 1997). The sets of the alkaloids produced by the endophytes vary among different species and more interestingly among different strains of the same species (Schardl and Phillips, 1997). For instance, *Neotyphodium uncinatum*, an endophyte of meadow fescue (*Lolium pratense*), produces lolines, whereas *N. coenophialum* produces lolines, peramine and EA, and different strains of *E. festucae* produce various combinations of the four classes of epichloë alkaloids (Schardl et al., 2006; Schardl and Phillips, 1997). Nevertheless, up to now, no single grass-endophyte association is known to produce more than three of these alkaloids types. For *Epichloë* and *Neotyphodium* spp., expression of these alkaloids occurs only *in symbio* (Panaccione et al., 2001; Wang et al., 2004). However, only *N. uncinatum* was found to produce loline alkaloids in defined media (Blankenship et al., 2001). The ability to producing these alkaloids in culture medium is an important and desired feature, since the outcome of functional genomic studies (such as knock-out of

gene function) could be identified and analyzed much faster in culture where the fungal growth is achieved in less than 10 days, in comparison to the more than three months required for inoculated plants to grow and be analyzed.

Over the ages, fungal EA have been intensively studied for their pharmaceutical capabilities (Eadie, 2003), and more recently molecular studies have helped us to learn more about the genes encoding enzymes that catalyze key steps in EA pathways. As in many filamentous fungi, in epichloae the genes regulating the production of EA are clustered and only few a steps in their biosynthetic pathway have been characterized through gene disruption (Correia et al., 2003; Fleetwood et al., 2007; Haarmann et al., 2006; Panaccione et al., 2001; Wang et al., 2004). However, the minimal set of genes that are required for the production of EA has not been identified. Heterologous expression of the ergot alkaloid synthesis (*EAS*) gene cluster could help in the identification of genes involved in the intermediate steps of the biosynthetic pathway, and may also result in detection of bioactive compounds with novel pharmaceutical capabilities which could lead to the development of new EA-based drugs.

In this chapter I describe my efforts to manipulate the *EAS* gene clusters from the pathogenic *C. purpurea* and *C. fusiformis* fungi by transforming *N. uncinatum* and *E. festucae* e2368 isolates with cosmid clones harboring clusters of *EAS* genes. Neither *N. uncinatum* nor *E. festucae* e2368 produces ergot alkaloids. Although, *E. festucae* harbors *EAS* genes that are homologous to the *C. purpurea* and *C. fusiformis* clusters, the expression of these genes could not be detected. The aim of my project had been to 1) identify the minimal set of genes required for EA biosynthesis 2) to determine if these

alkaloids can be produced in minimum medium and 3) to identify what profile of alkaloids would be produced.

Materials and methods

Biological materials

Neotyphodium uncinatum CBS 102646 was cultured from meadow fescue (*Lolium pratense*) stock plant number 167, and *Epichloë festucae* e2368 was isolated from stock plant number 2368 as previously described (Blankenship et al., 2001). The DNA from *N. uncinatum* and *E. festucae*, was isolated from fresh mycelium, using the method described by Al-Samarrai and Schmid (2000) and DNeasy 96 Plant Kit **(**Qiagen, Valencia, CA, USA**)**. The DNA concentration was estimated using a Qubit fluorometer and Quant-iT™ DNA BR Assay Kit (Invitrogen, Eugene, OR). Cosmid DNA was isolated from 50 ml bacterial cultures grown in LB medium at 37 °C for 12-14 h on a rotary shaker at 200 rpm following the method of Ahn et al., (2000). The oligonucleotide primers used in this chapter were from Integrated DNA Technologies, and are listed in **Table 4.1**.

Cosmid clones

The cosmid clones Cp7A26 **(Figure 4.1 Panel A)**, containing a cluster of nine *EAS* genes (*cloA*, *easC*, *easD*, *easE*, *easF*, *easG*, *dmaW*, *easH1*, *lpsA1*), Cp1A27 **(Figure 4.1 Panel B)**, containing a cluster of seven *EAS* genes (*easF*, *easG*, *dmaW*, *easH1*, *lpsA1*, *easH2* and, *lpsA2*), and Cp5A29 **(Figure 4.1 Panel C)**, containing a cluster of five *EAS* genes (*dmaW*, *easH1*, *lpsA1*, *easH2* and, *lpsA2*), derived from the same locus. These

were identified by Wang (2000) from a *C. purpurea* ATCC 20102 genomic library (Wang et al., 2004). Cosmid clone Cf11E26 **(Figure 4.1 Panel D)** contains a cluster of nine *EAS* genes (*lpsB*, *easA*, *cloA*, *easC*, *easD easE*, *easF*, *easG*, *dmaW*) and was identified by Tsai et al. (1995) from the *C. fusiformis* ATCC26245 cosmid library. All of these cosmid clones were in vector pMOcosX (Orbach, 1994) and harbor a *hph* gene modified for fungal resistance to hygromycin B (Calbiochem, San Diego, CA).

Transformation of *N. uncinatum* CBS 102646

Mycelium of *N. uncinatum* CBS 102646 was grown in PDB medium at 21 °C with rotary shaking at 200 rpm for 5-6 days. Then protoplasts were prepared with slight adjustments from the protocol described by Murray et al., (1992). The enzyme mixture used for cell wall removal contained 5 mg/ml β-glucanase (InterSpex Products, San Mateo, CA) or 5 mg/ml lysing enzymes (Sigma), 5 mg/ml driselase (InterSpex), 0.5 mg/ml zymicase I (InterSpex Products), 4 mg/ml glucanex (Novo Industri AS, Bagsvaerd, Denmark), and 3 mg/ml bovine serum albumin (Sigma, St Louis, MO) in osmotic solution (1.2 M $MgSO_4$ and 10 mM $NaHPO_4$, pH 6.0). Mycelium was then incubated at 30 °C for 2 h. The protoplasts were transformed with 2-4 µg DNA of the Cp7A26, Cp7A26, Cp5A29 and Cf11E26 cosmid. Each transformation with a single cosmid clone was performed using PEG (polyethylene glycol) method (Panaccione et al., 2001) applied to 10^7–10^8 protoplasts. Due to slow recovery of *N. uncinatum* protoplasts on CRM, the following three-layer plate method was implemented. The protoplasts were suspended in 5 ml CRM-low at 45 °C and layered on top of two layers (18 ml bottom PDA layer containing 80 µg/ml hygromycin B that was overlaid with 5 ml CRM-low).

The transformation plates were incubated at 21 °C for 4-5 weeks; thereafter the fungal colonies were transferred onto PDA with 90 µg/ml hygromycin B for sporulation and single-spore isolation.

Transformation of *E. festucae* e2368

Mycelium of *E. festucae* e2368 was grown in PDB medium with rotary shaking at 200 rpm for four days. Then protoplasts were prepared as described above and then transformed with 2-4 µg DNA obtained from cosmid clone Cf11E26. The transformation was completed by the PEG method (Panaccione et al., 2001) applied to 10^7–10^8 protoplasts. The protoplasts were then suspended in 7 ml CRM-low and poured over 20 ml CRM plates containing 600 µg/ml hygromycin B. The transformation plates were incubated at 21 °C for 4 weeks; then the fungal colonies were transferred onto PDA with 650 µg/ml hygromycin B for sporulation and single-spore isolation.

PCR screening for *N. uncinatum* transformants

All positive samples from transformation were assayed by PCR for the integrity of the cosmid clones inside the fungal genome. The reactions contained 25 µl reaction mixture with 5-10 ng DNA template, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.2 µM of each primer, 2.5 units AmpliTaq Gold and AmpliTaq Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied Biosystems). The cycling profile was set for 5 min at 94 °C, 40 cycles of 30 sec at 94 °C, 20 sec at 55-57 °C (depending on the primers set used) and 2-4 min at 72 °C, (depending on the

primers set used) followed by a final extension step of 10 min at 72 °C. PCR reactions were carried out in a model 2720 Thermal Cycler (Applied Biosystems).

For the isolates transformed with Cp7A26, Cp1A27, and Cp5A29 the PCR reactions were performed with several sets of primer pair specific for genes and regions between the genes of the *C. purpurea EAS* gene cluster. The following primer pairs **(Table 4.1)** were used: Cp-cloA(f) and Cp-cloA(r) specific for the *cloA* gene, Cp_easC(f) and Cp_easC(r) specific for the *easC* gene, Cp_easD(f) and Cp_easD(r) specific for the *easD* gene, Cp_easE(f) and Cp_easE(r) specific for the *easE* gene, Cp_easF(f) and Cp_easF(r) specific for the *easF* gene, Cp_easG(f) and Cp_easG(r) specific for the *easG* gene, Cp_dmaW(f) and Cp_dmaW(r), specific for the *dmaW* gene, lpsA1-7788d and lpsA1-9069u specific for the *lpsA*1 gene, lpsA2-9122d and lpsA2-10175u specific for the *lpsA*2, Cp_11 and Cp_12 for the region between *easD* and *easE*, Cp_19 and Cp_20 for the region between *dmaW* and *easH*1, Cp_21 and Cp_22 for the region between *easH*1 and *lpsA*1, Cp_25 and Cp_26 for the region between *easH2* and *lpsA2* genes, Cp_27 and Cp_28 for the region downstream of the *lpsA*2 gene. To check the integrity of the Cf11E26 clone inside the fungal genome, the following sets of primers for genes of *C. fusiformis EAS* gene cluster were used: Cf lpsB_f and Cf lpsB_r specific for *lpsB*, CfeasA_f and CfeasA_r specific for *easA*, CfcloA_f and CfcloA_r specific for *cloA*, CfeasC_f and CfeasC_r specific for *easC*, CfeasD_f and CfeasD_r specific for *easD*, CfeasE_f and CfeasE_r specific for *easE*, CfeasF_f and CfeasF_r specific for *easF*, CfeasG_f and CfeasG_r specific for *easG*, and the primers specific for *dmaW* gene CfdmaW_f and CfdmaW_r. The primers were designed to span the entire gene or region located between the genes.

PCR screening of *E. festucae* transformants

The isolates transformed with Cf11E26 were verified for the integrity of the clone inside the fungal genome, using the following sets of primers for genes present in the *C. fusiformis* ergot alkaloid gene cluster: Cf lpsB_f and Cf lpsB_r specific for *lpsB*, CfeasA_f and CfeasA_r specific for *easA*, CfcloA_f and CfcloA_r specific for *cloA*, CfeasC_f and CfeasC_r specific for *easC* gene, CfeasD_f and CfeasD_r specific for *easD*, CfeasE_f and CfeasE_r specific for *easE*, CfeasF_f and CfeasF_r specific for *easF*, CfeasG_f and CfeasG_r specific for *easG*, and the primers specific for *dmaW* gene CfdmaW_f and CfdmaW_r. The PCR reactions were performed in 25 µl final volume containing 5-20 ng of genomic DNA 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.2 µM of each primer, and 2.5 units AmpliTaq Gold, AmpliTaq Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied Biosystems, Foster City, CA, USA). The PCR conditions were set as follows: after an initial denaturation of 5 min at 94 °C, 40 cycles of 30 sec at 94 °C, 20 sec at 55-57 °C (depending on the primer set used) and 2-4 min at 72 °C followed a final extension step of 10 min at 72 °C and 30 min carried out in a model 2720 Thermal Cycler (Applied Biosystems).

Defined culture medium

N. uncinatum isolates carrying either one of the cosmid clones were grown in defined minimal medium and analyzed for EA production. Equal amounts of mycelia from each isolate were ground and used to inoculate plates containing 29.5 ml minimal medium. The medium contained 30 mM potassium phosphate, 30 mM 2(N-morpholino)

ethanesulfonic acid (MES), 15 mM urea as nitrogen source and 20 mM sucrose as carbon source, 2 mM $MgSO_4$, 0.6 µM thiamine, and a mixture of trace elements to give 3.6 µM H_3BO_3, 1 µM $CuSO_4$, 0.7 µM KI, 0.8 µM FeNa-ethylenediaminetetraacetic acid, 1 µM $MnSO_4$, 0.5 µM $NaMoO_4$, and 0.4 µM $ZnSO_4$ as previously described (Blankenship et al., 2001). The inoculated plates were incubated at 21 °C on a rotary shaker at 100 rpm for 20-24 days.

RNA extraction from plant material

Fresh pseudostem tissues from the inoculated plants were collected and flash-frozen in liquid nitrogen, and stored at -80 °C. The total RNA was extracted from 100 mg of each sample using the RNeasy Plant Minikit (Qiagen, Valencia, CA, USA). The RNA integrity was verified by 1% agarose gel electrophoresis and ethidium bromide staining, and then the RNA was stored at -80 °C until used for the reverse transcription reaction.

DNase treatment and reverse transcription

The total RNA extracts were treated with DNase using TURBO DNA-free kit (Ambion, Austin, TX). Treated RNA (1 µg) served as template for cDNA synthesis using the Monster Script 1^{st} strand synthesis kit (Epicentre Biotechnologies). The reactions prepared according to the manufacturer's recommendations were incubated at 42°C for 5 min and then at 60 ^{o}C for 40 min. Heating at 90°C for 5 min terminated the reaction. The cDNA samples were stored at -20 °C.

Inoculation of the transformants into perennial ryegrass (*Lolium perenne*) and meadow fescue (*Lolium pratense*) seedlings

Surface-sterilized endophyte-free seeds (Latch et al., 1985) were germinated in the dark for 5-7 days on plates containing water agar until the meristems of the seedlings were exposed. At this stage, the *N. uncinatum* isolates transformed with Cp7A26 , Cp1A27, Cp5A29 or Cf11E26 were inoculated into the etiolated seedlings of meadow fescue by placing the mycelia inside a wound created in the meristem region as described by Latch and Christensen (1985). The isolates of *E. festucae* transformed with Cf11E26 were similarly inoculated into seedlings of perennial ryegrass and meadow fescue. The plates with the inoculated seedlings were kept on the edge of the plate (in vertical position), and incubated at 21 °C in dark conditions for 6 days followed by 5 days of light environment. The surviving seedlings were planted into soil in the greenhouse. When the seedlings reached at least 3-5 tillers of each plant, two different tillers were blotted on a nitrocellulose membrane. For TPIB was performed with antiserum raised against *Neotyphodium coenophialum* crude protein extract (An et al., 1993).

Ergot alkaloid analysis

For EA extraction and analysis, the isolates grown in minimal medium were centrifuged to separate the fungal mass from supernatant, and the two phases were freeze dried. The successfully inoculated plants were grown in the greenhouse for five to six months. At this age, four pseudostems derived from each plant were collected, lyophilized and then ground to a fine powder. The ergot alkaloid extraction and analysis

was conducted by Dr. Dan Panaccione at West Virginia University as described by Panaccione et al. (2003).

Results

Identification of transformed isolates of *N. uncinatum*

Hygromycin B-resistant isolates transformed with the Cp7A26, Cp1A27 and Cp5A29 cosmid clones were checked by PCR for the presence of the *C. purpurea EAS* genes as well as for some regions located between the genes. The results indicated that in most of the isolates integration of the cosmid clone was incomplete; however, in two isolates designed e7385 and e7386 transformed with Cp7A26, one isolate transformed with Cp1A27 designed e7387, and two isolates transformed with Cp5A29 designed e7383 and e7384, PCR screening identified integration of the entire *EAS* cluster of genes. For the isolates harboring the Cp7A26 cosmid clone, PCR amplification revealed the presence of *cloA*, *easC*, *easD*, *easE*, *easF*, *easG*, *dmaW*, and *lpsA1* genes indicating a complete integration of the gene cluster present on the clone.

Similarly, for the strain containing Cp1A27 cosmid clone, PCR screen revealed the presence of *easF*, *easG*, *dmaW*, *easH*1, *lpsA*1, *easH*2 and, *lpsA*2 genes in the fungal genome, while *dmaW, easH*1, *lpsA*1, *easH*2 *and, lpsA*2 genes were identified in the two isolates bearing the Cp5A29 clone. DNA from *C. purpurea* ATCC 20102 was used as positive control for the assay **(Figure 4.2**). Furthermore, the seven hygromycin B-resistant isolates transformed with Cf11E26 were screened by PCR for the presence of *C. fusiformis EAS* genes. Among these transformants, only two isolates designed e7388 and e7389, had the complete cluster integrated into the genome. PCR screen identified the

nine genes — *lpsB*, *easA*, *cloA*, *easC*, *easD easE*, *easF*, *easG*, and *dmaW* — from the *C. fusiformis EAS* cluster **(Figure 4.3)**.

Identification of transformed isolates of *E. festucae*

The 11 hygromycin B-resistant isolates obtained by transforming *E. festucae* with cosmid Cf11E26 were screened for the presence of *C. fusiformis EAS* cluster genes. The results indicated that in several isolates the integration of the cosmid clone was incomplete; however, four of the isolates designed e7219, e7211, e7212, and e7382, had a complete integration of the nine genes (*lpsB*, *easA*, *cloA*, *easC*, *easD easE*, *easF*, *easG*, and *dmaW*) carried by the Cf11E26 cosmid. To make sure that the primers are specific only for *C. fusiformis* and do not amplify the *EAS* genes present in the wild-type *E. festucae* genome **t**he DNA of untransformed *E. festucae* e2368 was used as control **(Figure 4.4)**.

Compatibility analysis between the transformed strains and grass plants

Establishment of the symbiosis between endophytic fungi and their host plants is a requirement for ergot alkaloid production and analysis. Therefore, more than 300 meadow fescue plants were inoculated with *N. uncinatum* isolates harboring Cp7A26, Cp1A27, Cp5A29 or Cf11E26. Seedling infection frequencies were surprisingly low, between 0.3-0.6 percent. To further confirm that the inoculated isolates were present in the infected plants and exclude the possibility of initial infection with WT endophyte, the fungus was grown out from all TPIB-positive plants. DNA from the fungal isolates was extracted and checked by PCR for the presence of the genes from the cosmid clones. The

results indicated that one plant contained isolate e7385 isolate, and two plants each had isolate e7387, e7383 or e7384. Similarly, approximately 200 perennial ryegrass plants as well as 100 meadow fescue plants were inoculated with *E. festucae* isolates transformed with cosmid Cf11E26. TPIB assay indicated that the frequency of successfully inoculated plants was 3% for meadow fescue and 3.5% for perennial ryegrass. The fungi were isolated from infected plant pseudostems and grown on PDA plates. Their DNA was extracted and analyzed by PCR for the *EAS* genes. The results indicated that three meadow fescue plants and seven perennial ryegrass plants contained the *E. festucae* transformants.

EAS gene expression in planta

Gene expression was analyzed for fungal transformants in their host plants. Total RNA was isolated from infected plant samples and treated with DNase. To insure that there was no DNA contamination in the total RNA-DNase treated samples, a PCR screen was performed with primers specific for the ergot alkaloid genes. The primers were designed to span over the intron positions. The PCR screen resulted in no amplification demonstrating that no detectable DNA contamination was present in the RNA samples. The total RNA was then reverse transcribed into cDNA. The synthesized cDNA was then used as templates for PCR-detection of the *EAS* genes mRNAs using gene specific primers. No expression of any of the *EAS* genes was detected in the plants inoculated with *N. uncinatum* isolates harboring the different *C. purpurea* cosmid clones. However, expression of three *EAS* genes (*easD*, *easF* and *easG*) was detected from the samples

inoculated with the *N. uncinatum* isolate harboring the *C. fusiformis EAS* gene cluster **(Figure 4.5)**.

Similarly, cDNA from all positive *E. festucae* samples was examined for expression of the *C. fusiformis EAS* genes using gene specific primers. RT-PCR indicated expression of six of the genes (*easA*, *easD*, *easE*, *easF*, *easG*, and *dmaW*), whereas expression of *lpsB*, *cloA* and *easC* could not be detected **(Figure 4.6)**.

Analysis of ergot alkaloid composition

Ergot alkaloid analyses were performed by D.G. Panaccione (West Virginia University) on all plant samples that contain the modified fungal strains using detection standards for chanoclavine, agroclavine, setoclavine, ergine, and ergotamine. However, no production of any of the analyzed EAs could be detected in the examined plant-fungal symbionta.

Discussion

Heterologous expression of the *EAS* gene cluster in EA non-producing hosts is a potentially powerful tool for understanding the biosynthetic pathway and may result either in synthesis of compounds similar to the ones produced by the host or in synthesis of novel compounds with completely new modes of action. The biosynthesis of these complex compounds is possibly influenced by physiological and environmental signals and involves the activity of enzymes encoded by genes that are usually clustered in fungal genomes (Gardiner et al., 2004; Yu et al., 2004). Several functional analyses of the genes encoding enzymes involved in the key steps of the EA pathway has provided clear evidence for their roles in the biosynthesis of these metabolites (Correia et al., 2003;

Fleetwood et al., 2007; Haarmann et al., 2006; Panaccione et al., 2001; Wang et al., 2004). Due to the large size of the cluster and the complex genetic makeup of the host, isolation of the entire gene cluster is difficult. However, several cosmid clones harboring different groups of genes belonging to the *EAS* cluster have been identified in the genomic libraries of *C. purpurea* and *C. fusiformi*s (Tsai et al., 1995; Wang et al., 2004).

I explored the possibility that these cosmid clones could be used to manipulate the gene cluster by introducing and expressing its genes in different fungal-grass symbionts. Moreover, heterologous expression of the cosmid clones harboring different groups of genes could lead to the identification of the minimal gene set required for EA biosynthesis. The *C. fusiformis EAS* gene cluster shares eight homologous genes and one pseudogene (*lpsB*) with the *C. purpurea EAS* cluster (Lorenz et al., 2007), whereas the latter has at least four additional genes (*lpsA*1, *easH*1, *lpsA*2, *easH*2), of which products of the *lpsA* genes and perhaps the *easH* genes are involved in later steps of ergopeptine biosynthesis (Haarmann et al., 2008; Schardl et al., 2006). The common genes are believed to be involved in the early steps of the pathway. Although the gene function, order and direction of transcription for seven of these genes are conserved between the two clusters, the product encoded by *C. fusiformis* (*cloA*) is nonfunctional (Lorenz et al., 2007). Sequence analysis of the genome of another *Claviceps* sp., *Claviceps paspali*, indicated that this isolate carries at least nine *EAS* genes (Jinge Liu and C.L. Schardl, unpublished data). Analysis of the recently sequenced genome of *E. festucae* e2368 showed that, similar to *Claviceps* spp, the genes involved in EA biosynthesis are clustered, yet the gene order differs. However, gene expression analyses (U. Hesse and

C.L. Schardl, unpublished data), has revealed that the genes from the *E. festucae* ergot cluster are not expressed in isolate e2368 when symbiotic with meadow fescue.

Questions to be answered in this study was if the genes of the *EAS* cluster will be expressed in a heterologous host; and, if their expression could lead to the identification of the minimal set of genes required for biosynthesis of EA intermediate products. Such findings could facilitate experiments to assign enzymes to defined steps in the EA biosynthesis pathway. In similar studies, heterologous expression of a cosmid clone containing penicillin pathway genes *pcbAB*, *pcbC* and *penDE*, from *Pencilium chrysogenum* has been demonstrated in *Neurospora crassa* and *Aspergillus niger*. Neither *N. crassa* nor *A. niger* wild-type isolates possess a penicillin pathway, whereas protoplast transformation with the cosmid clone has resulted in isolates capable of producing penicillin V (Smith et al., 1992).

For the transformed epichloae isolates, depending on which of the *EAS* genes are present and expressed, the symbiota between the endophyte and grass host may accumulate different alkaloids such as chanoclavine-I, chanoclavine aldehyde, agroclavine, elymoclavine or more complex ergopeptines. So far it is known that expression of *dmaW*, encoding dimethylallyltryptophan synthase (Tsai et al., 1995), leads to the formation of DMATrp, which is further *N*-methylated (MeDMAT) by the action of an *N*-methyltransferase (Otsuka et al., 1980) assumed to be encoded by *easF* gene (Haarmann et al., 2005). Further, synthesis of chanoclavine-I, chanoclavine aldehyde, agroclavine, and elymoclavine involves at least five redox enzymes, believed to be encoded by *easA, easC, easD, easE* and *easG* (Haarmann et al., 2005; Schardl et al., 2006).

As the results presented in this chapter indicated, the *C. fusiformis* and *C. purpurea EAS* cluster of genes were present in the modified strains. Therefore, synthesis of different EA profiles was expected. However, no expression of the *C. purpurea EAS* genes was detected in transformed *N. uncinatum in symbio*, and expression of only three of the genes (*easD, easF*, and *easG*) was detected in symbiotic *N. uncinatum* carrying the *C. fusiformis* cluster. Furthermore, in symbiotic *E. festucae* transformants, expression of six of the genes (*easA*, *easD*, *easE*, *easF*, *easG*, and *dmaW*) was detected, whereas expression of one of the genes, *easC*, could not be detected in any of the samples. The *easC* gene is predicted to encode a catalase or related enzyme and is thought to be involved in one of the oxygenation steps leading to chanoclavine-I from MeDMAT, or in one of the two steps that lead to the synthesis of agroclavine from chanoclavine-I (Schardl et al., 2006; Tudzynski et al., 2001). Depending on which of the steps is catalyzed by EasC, expression of these six genes should result in synthesis of at least DMATrp, MeDMAT and chanoclavine-I. Nevertheless, none of these intermediates were detected by HPLC analyses.

The failure to detect any of the expected EA intermediates in the transformed epichloae may be explained by the possibility that *easC* and *easE* genes work together to produce chanoclavine-I from MeDMAT. If so, then the EasC could act to detoxify the peroxide that might be produced as a result of the EasE (flavin oxidoreduction) reaction. If *easC* is not expressed, it is possible that the peroxide would poison the FAD in EasE preventing further rounds of catalysis (Schardl et al., 2006). Evidence from recent studies indicated that the knockout of *easA*, *easC*, *easE* and *easF* changed the ergot alkaloid profile in *Aspergillus fumigates* (Coyle et al., 2008). In these studies, the *easF* knockout

isolates accumulated DMATrp; *easE* disrupted strains accumulated MeDMAT whereas *easA* knockout isolates accumulated chanoclavine aldehyde. Interestingly, the *easC* knockout isolates also failed to produce any EA intermediates (Coyle et al., 2008).

Whereas the genes involved in primary metabolic pathways are spread throughout the genome, most of genes required for the biosynthesis of secondary metabolism products are clustered and often located close to telomeres, and their regulation is controlled by global transcription factors (Shwab and Keller, 2008). These global regulators are encoded by genes located outside the gene cluster. Examples of such regulators are the LaeA nuclear protein which is thought to regulate the expression of the sterigmatocystin gene cluster in *Aspergillus nidulans*, and AreA, PacC and CreA zinc finger proteins which regulate the biosynthesis of aflatoxins and gibberellins in *A. flavus* and *Gibberella fujikuroi* respectively (Yu and Keller, 2005).

However, in epichloae, there is no indication of a global regulator for secondary metabolism, therefore regulation of the genes involved in EA biosynthesis is believed to be triggered by plant signals (Young et al., 2006). The endophyte growth being confined to the intercellular spaces of the grass plants, the nutritional condition of the plant (Faeth and Bultman, 2002), and the host and endophyte genotypes, may also influence the production of these alkaloids.

Table 4.1. Oligonucleotide primers used in Chapter 4

Primer name	Sequence	Primer name	Sequence
Cf lpsB_f	ggatgtatcttttgtagcagacg	Cp_11	gggaggtgaaattggtgctactgacg
Cf lpsB_r	caagtggaggagctttctg	Cp_12	caccaccttgcagaaagcctcctac
CfeasA_f	tgtcctcgtccaacctgttca	Cp_19	gccaactatacactgcaccacaac
CfeasA_r	ttggtccacgtcttgctggt	Cp_20	cctaattctcaacagcggataaag
CfcloA_f	tagtacctcgctaaactccatcg	Cp_21	atcctatctgctctcctgcctctcg
CfcloA_r	gctactctggctataccaagctc	Cp_22	tctccgaatacaaatcgtcgtctaacag
CfeasC_f	acaatctgggtcgctgttctg	Cp_25	gacgcaccaacctcgctatgaatgt
CfeasC_r	gcttcccaggtctctctcacag	Cp_26	gccgccggccttcataacg
CfeasD_f	gttgatgttggctccgtacact	Cp_27	ctgcggcgcgactggaagat
CfeasD_r	gtcatctgtgtccgccaaaatcttc	Cp_28	gaggcgcttcacggggagac
CfeasE_f	gtatcgtcttttaggtcctctcg	Cp_dmaW(f)	ctgtatcacgcttgcactcagcaga
CfeasE_r	gtatcgtcttttaggtcctctcg	Cp_dmaW(r)	aggcgtcgaaagaaattgggctgtc
CfeasF_f	tcgatctcacgttgttgaggactc	Cp_easC(f)	gacgttgaacggggatgtgcaaac
CfeasF_r	cagctcgtaaatgacaacatcgc	Cp_easC(r)	gtgcagagaggggaaacctcgtaatag
CfeasG_f	cttgttgtccgagacaaaatcgg	Cp_easD(f)	aggccacgggtccgagaaggtaata
CfeasG_r	gaccatattattgacgggaggca	Cp_easD(r)	gatattggccccgtacactggaatt
CfdmaW_f	gacaaaagctccagcaacg	Cp_easE(f)	cattcacgagttacaccattggc
CfdmaW_r	tcagggacaagtcacagcgt	Cp_easE(r)	cctctatggacggttcgactactc
lpsA2-9122d	cttcaagcaaccaatgcagc	Cp_easF(f)	cagaccaatgttccttttttggtcgc
lpsA2-10175u	ggtaggaaagtggcagtatg	Cp_easF(r)	cgctcaatgcgaaattgatgaagcc
lpsA1-7788d	ggttcccttcgaaaagc	Cp_easG(f)	gagattggtgaggaacgatgatctg
lpsA1-9069u	aaatggaatgaagaggcctc	Cp_easG(r)	tggaaagagtcttctacgcccattc
Cp-cloA(f)	tcgatcgacccaacgcagctgatct	Cp-cloA(r)	cggcacgaactttcttggacttgg

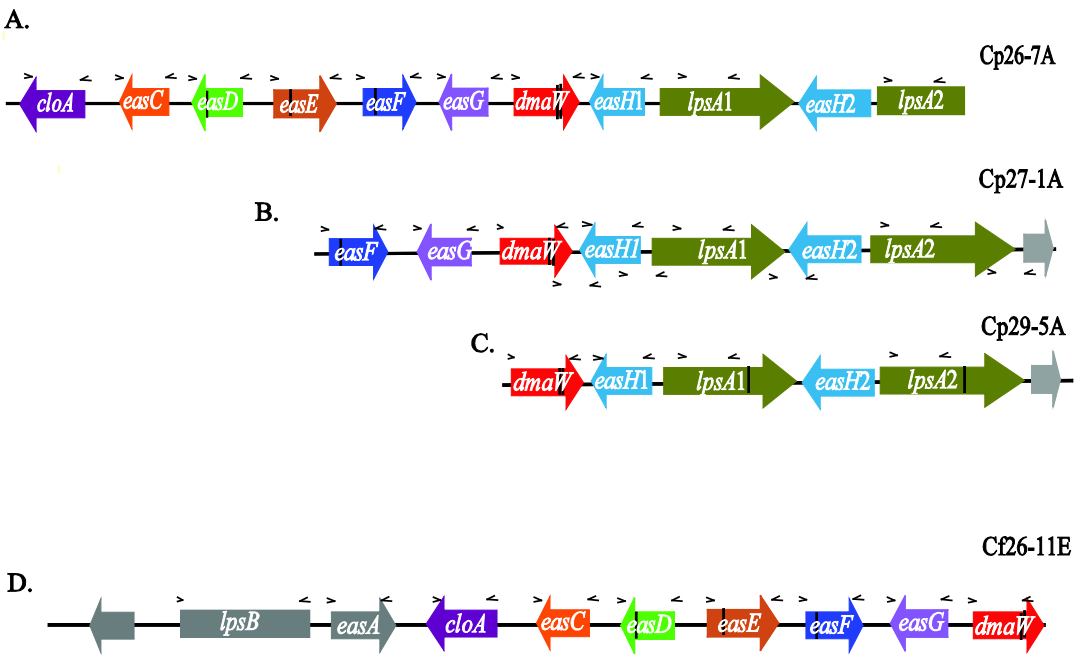


Figure 4.1. Maps of the cosmid clones used to transform *N. uncinatum* and *E. festucae*. The maps give a comparison and a graphic representation of the order and orientation of the genes comprised by the cosmid clones identified from genomic libraries of *C. purpurea* ATCC 20102 (Panels A, B, and C), and *C. fusiformis* ATCC 26245 (Panel D). Intron positions are indicated by black bars. Primer locations are indicated by small arrows. The maps were elucidated by Jinghong Wang, Caroline Machado and Ella V. Wilson.

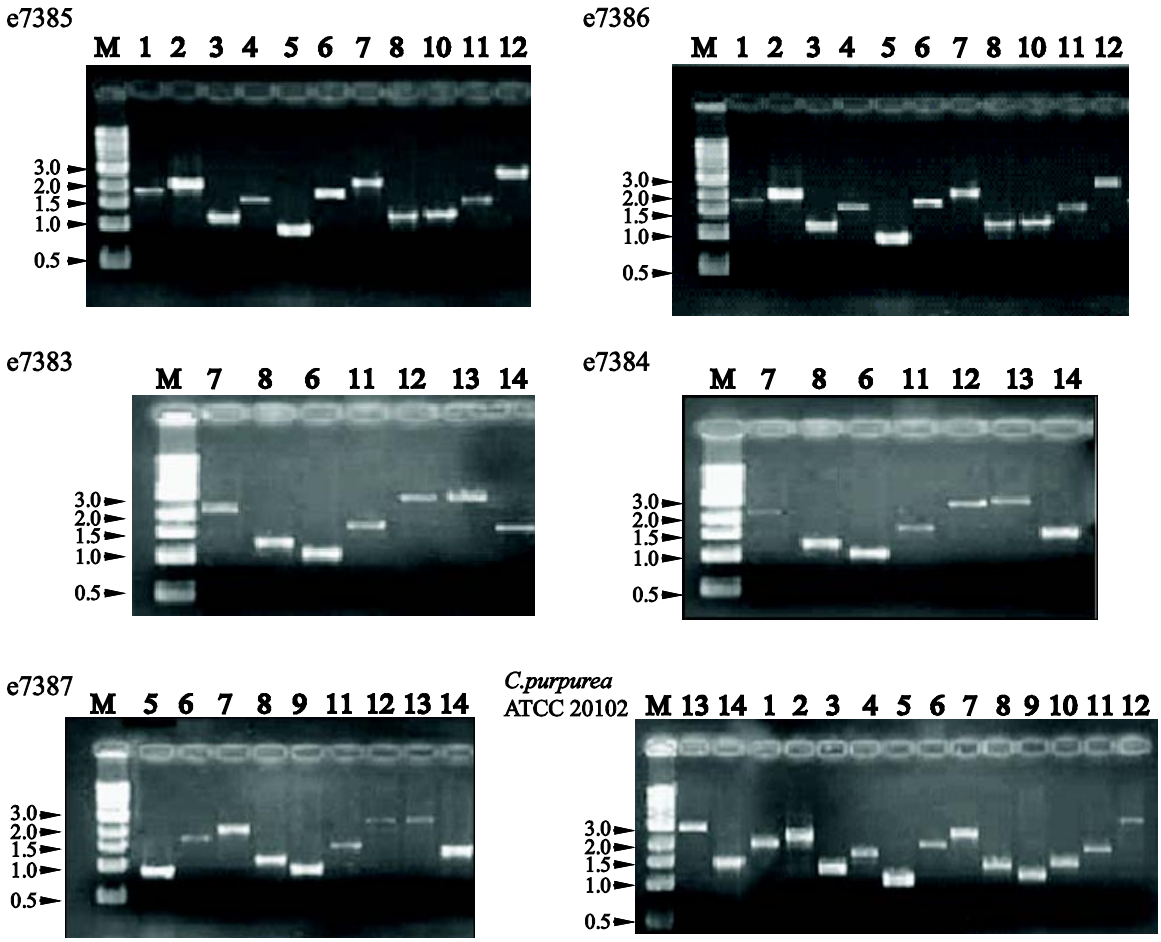


Figure 4.2. PCR test for the introduction of *C. purpurea EAS* genes into the *N. uncinatum* genome. **T**he lanes contain molecular size markers in kb (lanes M) and products of PCR using as template, DNA from *N. uncinatum* transformed with Cp7A26 (isolates e7385 and e7386), Cp1A27 (isolate e7387), and Cp5A29 (isolates e7383 and e7384). The DNA from *C. purpurea* ATCC 20102 was used as positive control. The primers specific for *cloA* (lanes 1), *easC* (lanes 2), *easD* (lanes 3), *easE* (lanes 4), *easF* (lanes 5), *easG* (lanes 6), *dmaW* (lanes 7), *lpsA1* (lanes 8), *lpsA*2 (lane 9), the regions located between *easD* and *easE* (lanes 10), between *dmaW* and *easH1* (lanes 11), between *easH1* and *lpsA1* (lanes 12), and between *easH*2 and *lpsA*2 (lanes 13), and the region downstream of *lpsA*2 (lanes 14).

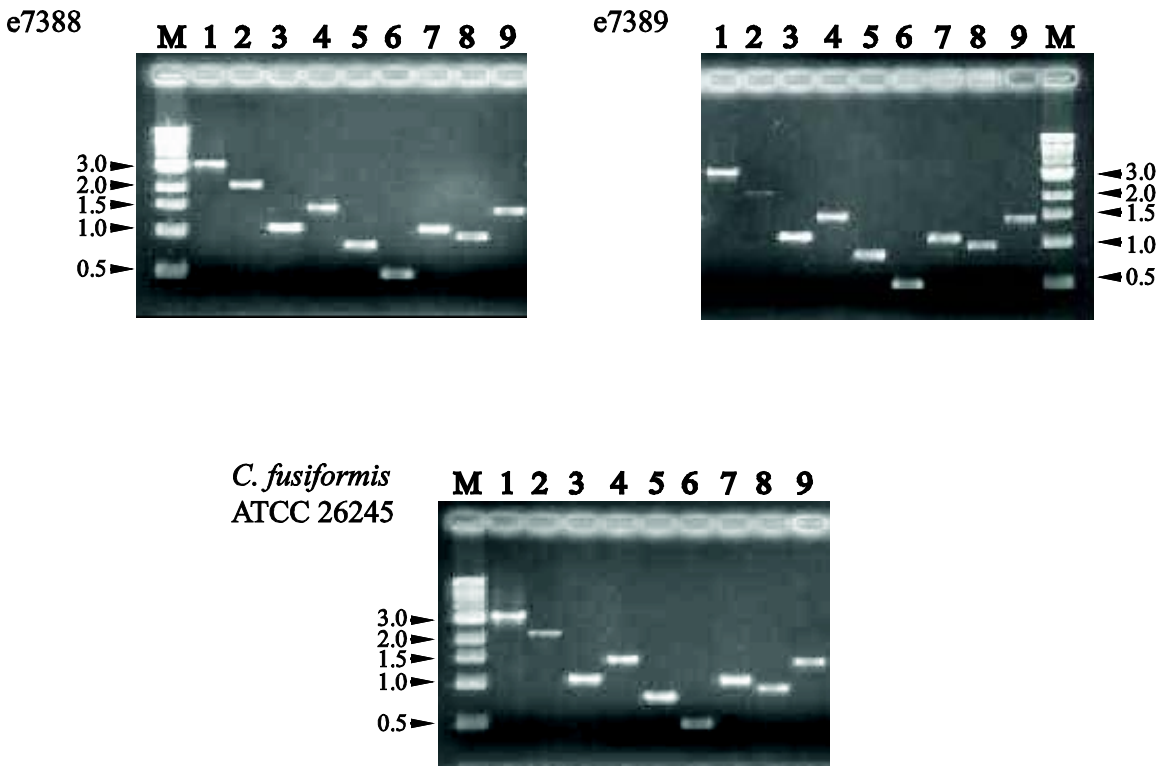


Figure 4.3. PCR test for introduction of the *C. fusiformis EAS* genes into the *N. uncinatum* genome. The *N. uncinatum* isolates e7388 and e7389, transformed with the cosmid clone Cf11E26, as well as *C. fusiformis* ATCC 26245 (used as a positive control), were tested for the presence of the *EAS* genes: *lpsB* (lanes 1), *easA* (lanes 2), *cloA* (lanes 3), *easC* (lanes 4), *easD* (lanes 5), *easE* (lanes 6), *easF* (lanes 7), *easG* (lanes 8), and *dmaW* (lanes 9). Size markers (lanes M) are indicated with sizes in kb.

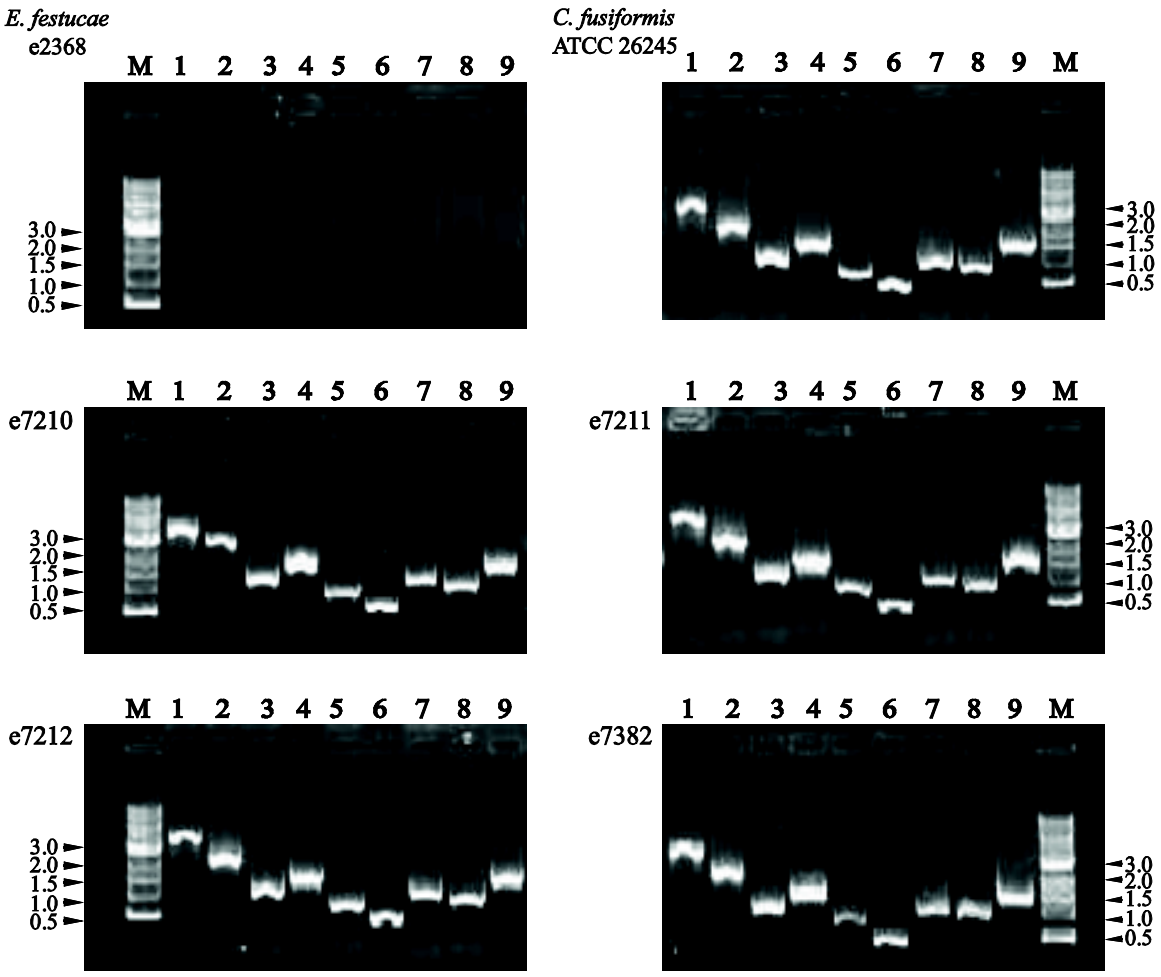


Figure 4.4. PCR test for the introduction of the *C. fusiformis* EA genes into the *E. festucae* genome. The isolates e7210, e7211, e7212, and e7382 carrying cosmid clone Cf11E26, along with *E. festucae* e2368 (as negative control) and *C. fusiformis* ATCC 26245 (positive control), were tested for the presence of the *EAS* genes *lpsB* (lanes 1), *easA* (lanes 2), *cloA* (lanes 3), *easC* (lanes 4), *easD* (lanes 5), *easE* (lanes 6), *easF* (lanes 7), *easG* (lanes 8), and *dmaW* (lanes 9). Size markers (lanes M) are indicated with sizes in kb.

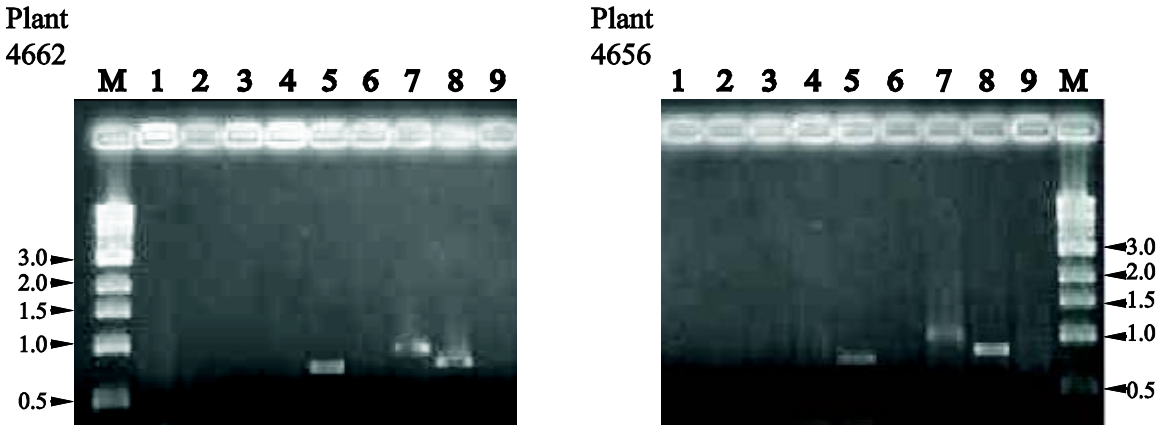


Figure 4.5. Expression of *C. fusiformis EAS* genes in *N. uncinatum* transformants in planta**.** RT-PCR was carried out with cDNA derived from samples from two plants (numbers 4662 and 4656) with *N. uncinatum* transformant infected with e7388 isolate. PCR was conducted with primers specific for each of the *C. fusiformis EAS* genes *lpsB* (lanes 1), *easA* (lanes 2), *cloA* (lanes 3), *easC* (lanes 4), *easD* (lanes 5), *easE* (lanes 6), *easF* (lanes 7), *easG* (lanes 8), *and dmaW* (lanes 9). The sizes of markers (lanes M) are indicated in kb.

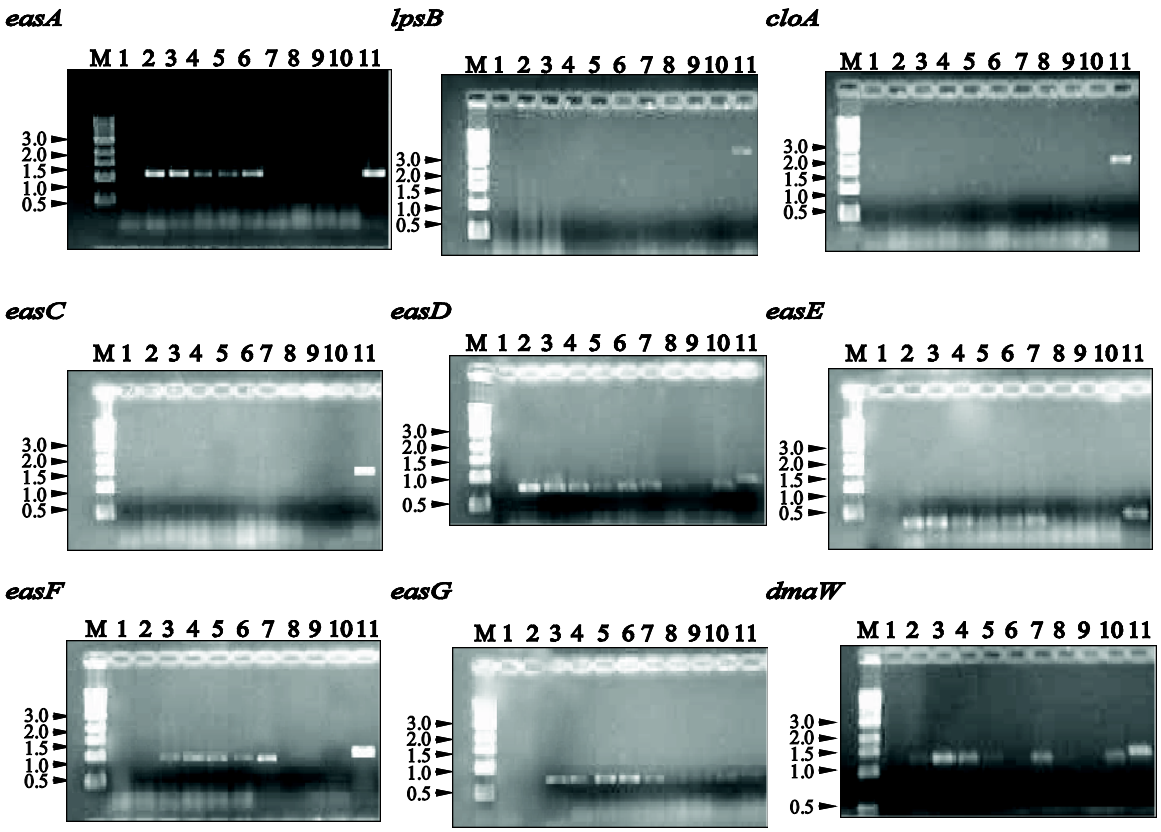


Figure 4.6 Expression of *C. fusiformis EAS* genes in *E. festucae* transformants in planta. RT-PCR was carried out with primers specific for each of the *C. fusiformis* EA genes: *easA, lpsB cloA, easC, easD, easE, easF, easG, and dmaW*. The samples are represented as follows: H_2O controls (lanes 1); meadow fescue plants 4711-1 (lanes 2), 4711-2 (lanes 3), and 4711-3 (lanes 4) harboring e7210 isolate; perennial ryegrass plant 4709 (lanes 5) harboring e7211 isolate; and the perennial ryegrass plants 4710-1 (lanes 6), 4710-2 (lanes 7), 4710-3 (lanes 8), 4710-4 (lanes 9), and 4710-5 (lanes 10) harboring e7210 isolate; DNA of *C. fusiformis* ATCC 26245 positive control (lanes 11). The size markers (lanes M) are indicated in kb.

Chapter 5

Conclusions and Discussion

Ergot alkaloids have been the subject of intense studies not only due to their pharmaceutical proprieties but also due to the toxicity to humans and livestock. Toxicosis to animals that graze grasses harboring ergot alkaloid-producing fungi, especially tall fescue infected with *N. coenophialum*, has been a major problem for agriculture (Paterson et al., 1995). One approach to eliminate the factors implicated in tall fescue toxicity is to genetically manipulate the endophytes by knocking out genes involved in the key steps of the ergot alkaloid pathway and inoculation of the mutated strains into popular grass plants.

In my doctoral dissertation, the main focus of the research was the disruption of *dmaW* genes in *N. coenophialum* e19, while developing a method for marker removal. My first attempt was to disrupt the *dmaW*2 gene using several disruption methods, such as split marker and RecA single-stranded DNA transformations. However, in all attempts with these methods, homologous recombination did not occur. Therefore, *dmaW*2 knockout was attained through a gene replacement strategy based on a linear recombinogenic fragment. This fragment had long (ca.3kb) regions from *dmaW*2 flanks surrounding the *dmaW*2 mutant gene, which had its 5'-portion replaced with an *hph* marker flanked by loxP sites. The long flanking regions facilitated the integration and replacement of the *dmaW*2 wild type gene with this mutant allele. Laborious screening of 1376 transformants identified three putative *dmaW*2 knockouts. Evidence from Southern-blot analyses of the digested genomic DNA, indicated that *dmaW2* was replaced in isolate e7133 while the homologous *dmaW*1 gene was apparently unaltered during the

transformation procedure. Moreover, chromosome separation and Southern-blot hybridization of the separated chromosomes indicated that the two *dmaW* genes in *N. coenophialum* are located on different chromosomes.

Further, the etiolated seedlings of tall fescue were easily inoculated with the *dmaW*2-disrupted isolates, suggesting that the mutated strains maintained compatibility with the host plant. The HPLC analysis of the ergot alkaloid content of the inoculated plants led to detection of ergovaline, demonstrating that the *dmaW*1 gene is functional and fully complements the ergot alkaloid biosynthesis.

In addition to causing the toxicosis episodes in livestock, the endophyte of tall fescue provides several ecological benefits to its host such as increased resistance to drought, improved mineral uptake, enhanced tillering and root growth (Arechavaleta et al., 1989; Malinowski and Belesky, 2000; Malinowski and Belesky, 2006). For this reasons, genetic modification of *N. coenophialum* to eliminate ergot alkaloids responsible for the toxicosis in tall fescue-endophyte symbiota could be a feasible solution to obtain popular non-toxic endophyte-infected tall fescue cultivars. However, commercialization of such cultivars could raise public concerns if they retain the selectable marker genes inside the fungal genome.

To address this problem, I developed a fast and efficient method for marker removal using the Cre/loxP system (Sauer and Henderson, 1989; Sternberg and Hamilton, 1981). The method is based on unselected transient transfection of fungal protoplasts with pKAES175 to transiently express Cre. I applied the method to several epichloae while Dr Peter Mirabito (University of Kentucky) tested the system in *Aspergillus nidulans* (Florea et al., 2009). The frequencies of confirmed marker elimination, based on the proportion of

randomly picked colonies that had excised the marker gene, varied between 5.2 to 21×10^{-3} demonstrating that this is a very manageable approach for eliminating selectable markers from genetically modified fungal strains. This method allowed me not only to remove the marker gene from the genome of isolate e7133, but also to reuse the same marker in further experiments that would eventually lead to the disruption of the *dmaW*1 gene. Moreover, marker removal would reduce the concerns and regulatory requirements regarding the presence of foreign genes in genetically modified organisms.

My research goal was the elimination of the ergot alkaloids from *N. coenophialum*-tall fescue symbiota. However, due to the heteroploid nature of *N. coenophialum* multiple gene knockouts are required to achieve this objective. Moreover, the slow growth of *N. coenophialum* makes this project very time consuming. Experiments are in progress towards the disruption of *dmaW*1 gene, which will be followed by the elimination of the marker gene and finally the inoculation of the double knockout into tall fescue cultivars. Maintenance of the endophyte compatibility with its host is important because the intent of this study is to obtain a non-toxic endophyte for use in forage tall fescue. Nevertheless, the plant genotype may influence the functionality of the modified endophyte as regards the fitness and protection of tall fescue cultivars (Hill, 2002). Therefore, studies are required to find the best tall fescue *N. coenophialum* combinations for maximum forage production and animal performance in different environments.

With the identification of an ergot alkaloid biosynthesis *EAS* gene cluster in *Claviceps* spp. and epichloë endophytes, studies will be concentrated to dissect the pathway by disrupting the genes to determine which steps they control. In such studies in

progress so far, four of the genes have been characterized by gene replacement studies (Correia et al., 2003; Fleetwood et al., 2007; Haarmann et al., 2006; Panaccione et al., 2001; Wang et al., 2004). Several cosmid clones harboring group of genes belonging to *EAS* cluster were identified in the genomic libraries of *C. purpurea* and *C. fusiformi*s (Tsai et al., 1995; Wang et al., 2004) and heterologous expression of the EA gene cluster in non-producing hosts could be used as a tool to understand the EA biosynthetic pathway.

I explored the possibility of manipulating the gene cluster from *C. purpurea* and *C. fusiformis* by introducing EA genes in different fungal-grass symbionts to determine if these genes will be expressed in a heterologous host. Their expression could lead to the identification of the minimal set of genes required for biosynthesis of EA and to the synthesis of compounds similar to the ones produced by the host or in synthesis of novel compounds. Even though the results indicated that several EA genes were expressed in the new symbiota, detection of the expected intermediates was not achieved. One speculation for these results could be that some of the genes such as *easC* and *easE* work in concert; with *easC* involved in the detoxification of the peroxide produce during the flavin oxidoreduction reaction catalyzed by EasE (Schardl et al., 2006). If the *easC* gene is not expressed, it is likely that the peroxide would poison the FAD in EasE preventing further rounds of catalysis. Moreover, ergot alkaloid pathway is subject to feedback regulation, with lysergic acid regulating synthesis of key intermediates in the pathway (clavines) (Panaccione and Schardl, 2003; Schardl et al., 2006). Therefore, it is possible that feedback inhibition might be involved at different steps in the EA pathway.

Appendix

Method used to disrupt *dmaW1* homolog gene encoding dimethylallyltryptophan synthase in *Neotyphodium coenophialum* strain e19

Materials and methods

Plasmids used for *dmaW* 1 disruption method

The pKAES146 (**Figure A.1 A**) constructed by C. Machado (2004) contains a 4.1 kb *Bam*HI-*Sal*I right flanking region of the *dmaW*1 gene and a 4.8 kb *Bam*HI-*Sac*II left flanking region including the disrupted *dmaW*1, cloned into pBSKS+ vector. The vector carries the beta-lactamase (*bla*) gene for bacterial resistance to ampicillin. The pKAES267 (**Figure A.1 B**) was constructed by inserting a 1.3-kb fragment containing the *hph* gene for hygromycin B-resistance flanked by loxP sites into the pKAES146 vector. The loxP::*hph*::loxP insert was cut out from pKAES173 (Spiering et al., 2008) using *Bam*HI restriction enzyme and ligated into the *Bam*HI site of pKAES146. The ligation reaction was performed using Fast-link DNA Ligation kit (Epicentre, Madison, WI, USA) with100 ng pKAES146 vector and 210 ng loxP::*hph*::loxP insert in a 15 µl ligation reaction. For bacterial transformation, 1 µl of the 1:10 diluted ligation reaction was mixed with 50 µl XL-1 blue *Escherichia coli* strain electro-competent cells (Stratagene, La Jolla, CA, USA), and electroporating at 1.7 kV, 200 Ω, and 25 µF, then mixed with SOC (Sambrook and Russell, 2001) medium and incubated with shaking for 1 h at 37 °C. The bacterial suspension was spread on LB agar plates containing 50 µg/ml of ampicillin. The pKAES268 (**Figure A.1 C**) was obtained by inserting the 10.2 kb *Sac*II-*Sal*I fragment cut from pKAES267 into the *Bam*HI site of the pBCKS+ vector (Stratagene Cloning Systems). Both the vector and the 10.2 kb *Sac*II-*Sal*I fragment were end-repaired using

DNA End-Repair Kit (Epicentre Biotechnologies, Madison, WI, USA). After dephosphorylation of the blunt-ended vector, the ligation was done using the DNA ligation kit (Epicentre Biotechnologies, Madison, WI, USA). The bacterial transformation was performed as described above. Thereafter, the bacterial mixture was spread on LB agar plates containing 30 of μg/ml chloramphenicol and incubated at 37 ^{o}C overnight. Single colonies were isolated and grown overnight in 5 ml LB broth with 30 μg/ml of chloramphenicol on a rotary shaker. Plasmid DNA was extracted by Qiaprep Spin Miniprep Kit (Qiagen, Valencia, California).

Transformation of *N. coenophialum* e7202 and e7204

Mycelium of either e7202 or e7204 *N. coenophialum* strains was grown in PDB medium with rotary shaking at 200 rpm for 7-10 days. Then protoplasts were prepared as described in Chapter 2 and the transformations were performed with 5-7 μg of *Sna*BI linearized DNA of pKAES267 and pKAES268. Transformation was completed by the PEG (polyethylene glycol) method applied to 10^6–10^8 protoplasts. The protoplasts were mixed with 7 ml CRM-low (complete regeneration medium containing low melting agarose from Seakem LE, FMC Bioproduct, Rockland, ME), and poured over 20 ml CRM plates containing 48 μg/ml hygromycin B (Calbiochem, San Diego, CA). The transformation plates were incubated at 21 °C for 4-5 weeks; then the fungal colonies were transferred onto PDA with 50 μg/ml hygromycin B for sporulation and single-spore isolation.

PCR screening for *dmaW*1 homologous identification

PCR screen for homologous integration of the *dmaW*1 mutant allele into the wild type locus, and replacement of the wild type *dmaW*1 was done using the forward primer dmaW(f) (TTATTGGATGAAACCTTAGCTAGTTGGC) and the reverse primer dmaWe19(-10) (CTCGCCGGCATGCGTCAAAT). The PCR program was as follows: 9 min at 95 °C, 35 cycles of 94 °C for 30 sec, 59 °C for 35 sec, 72 °C for 3 min 10 sec. All the PCR reactions were carried out in 25 µl reaction mixture with 5-10 ng DNA template, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.2 µM of each primer, and 2.5 units AmpliTaq Gold, AmpliTaq Gold PCR buffer with $MgCl_2$ (1.5 mM final conc.) provided by the manufacturer (Applied Biosystems, Foster City, CA, USA), in a model 2720 Thermal Cycler (Applied Biosystems).

Results

Selection of site-specific recombinants in *N. coenophialum* e7202 and e7204

Hygromycin B-resistant isolates transformed with pKAES267 and pKAES268 were analyzed by PCR for homologous replacement of *dmaW*1. In the event of ectopic integration, the PCR screen was predicted to yield a 1.7-kb fragment from the wild-type locus and another fragment of 2.7 kb from the integration of either pKAES267 or pKAES268 DNA. In case of homologous recombination the 2.7-kb amplification product was expected, but not the 1.7-kb product. Due to the low level of replication of the pKAES267 in the *E. coli* XL1-Blue electroporation competent cells (Stratagene, La Jolla, USA) the concentration of DNA isolated from this plasmid was extremely low. Following several transformations, only 37 transformants were obtained using DNA from

pKAES267, however, 570 transformants were obtained using pKAES268. The results and analysis of all of these transformants showed that the integration of the vector carrying the disrupted *dmaW*1 gene took place in ectopic location of the genome without replacing the wild type allele of the gene.

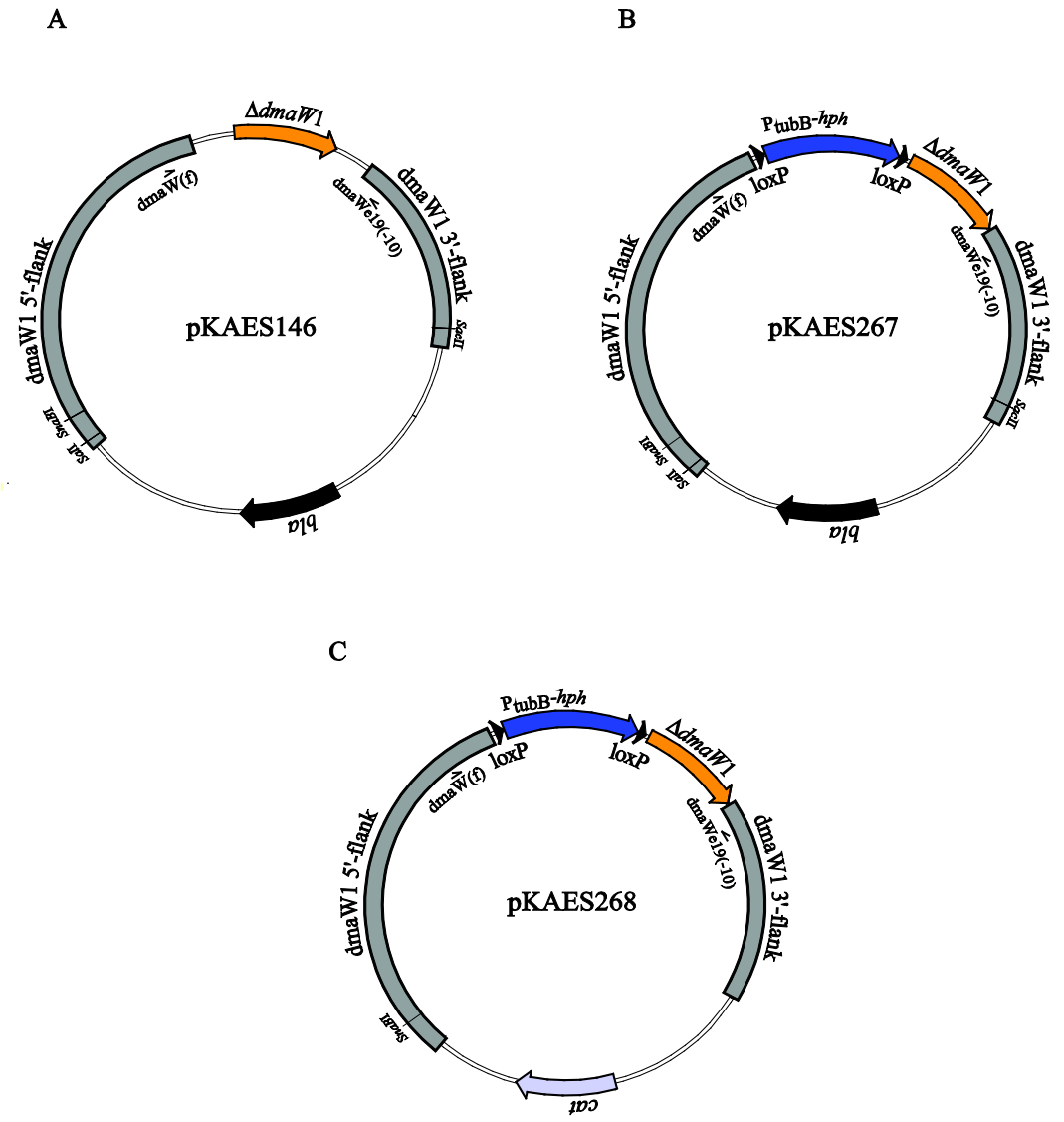


Figure A.1 Plasmids used in *dmaW*1 gene disruption experiments. **Panel A.** pKAES146 is a plasmid containing the disrupted *dmaW*1. **Panel B.** pKAES267 containing the *hph* gene flanked by the loxP sites cloned into the *Bam*HI site of pKAES146. **Panel C.** pKAES268 constructed by cloning the *Sal*I-*Sac*II fragment from pKAES267 into pBCKS+ vector

References

Ahn, S. C., Baek, B. S., Oh, T., Song, C. S. and Chatterje, B., 2000. Rapid mini-scale plasmid isolation for DNA sequencing and restriction mapping. Biotechniques 29**,** 266–368.

Al-Samarrai, T. H. and Schmid, J., 2000. A simple method for extraction of fungal genomic DNA. Letters in Applied Microbiollogy. 30**,** 53-56.

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

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

Aronson, M., Witch-hunt: Mysteries of the Salem witch trials. Atheneum, New York, 2003.

Barceloux, D. G. (Ed.) 2008. Medical Toxicology of Natural Substances: foods, fungi, medicinal herbs plants and venomous animals. John Wiley & Sons, Inc.

Billings, M., The Crusades: Five Centuries of Holy Wars. New York: Sterling, 1996.

Birch, A. J., McLoughlin, B. J. and Smith, H., 1960. The biosynthesis of the ergot alkaloids. Tetrahedron Lett. 7.

Blankenship, J. D., Spiering, M. J., Wilkinsona, H. H., Fanninb, F. F., Bush, L. P. and Schardl, C. L., 2001. Production of loline alkaloids by the grass endophyte *Neotyphodium uncinatum*, in defined media. Phytochemistry 58**,** 395-401.

Bouton, J. H., Latch, G. C. M., Hilla, N. S., Hovelanda, C. S., McCannc, M. A., Watsonc, R. H., Parishc, J. A., Hawkinsd, L. L. and Thompsond, F. N., 2002. Reinfection of Tall Fescue Cultivars with Non-Ergot Alkaloid–Producing Endophytes Agronomy Journal 94**,** 567-574.

Buckner, R. C., Powell, J. B. and Frakes, R. V., Historical development. In: R. C. Buckner, L. P. Bush, Eds.), Tall fescue. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Inc, Madison, Wisconsin, 1979, pp. 1-8.

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

Chaveroche, M. K., Ghigo, J. M. and d'Enfert, C., 2000. A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. Nucleic Acids Research. 28.

Chu, G., Vollrath, D. and Davis, R. W., 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science. 234**,** 1582-1585.

Chung, K. R.and Schardl, C. L., 1997. Sexual cycle and horizontal transmission of the grass symbiont, *Epichloë typhina*. Mycological Research 101**,** 295-301.

Clay, K., 1990. Fungal endophytes of grasses. Annual. Rev. Ecol. Syst. 21**,** 275-297.

Clay, K. and Schardl, C. L., 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. The American Naturalist 160**,** S99-S127.

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

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

Coyle, C. M., Goetz, C. G. and Panaccione, D. G., 2008. Clustered genes common to both *Aspergillus fumigatus* and ergot fungi control early steps within ergot alkaloid pathway. Phytopathology. 98**,** S214.

Coyle, C. M. and Panaccione, D. G., 2005. An ergot alkaloid biosynthesis gene and clustered hypothetical genes from *Aspergillus fumigatus*. Applied and Environmental Microbiology. 71**,** 3112–3118.

Craven, K. D., Blankenship, J. D., Leuchtmann, A., Hignight, K. and Schardl, C. L., 2001. Hybrid fungal endophytes symbiotic with the grass *Lolium pratense*. Sydowia 53**,** 44–73.

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

Eadie, M., 2003. Convulsive ergotism: epidemics of the serotonin syndrome? . The Lancet Neurology. 2**,** 429-434.

Eich, E. and Pertz, H., Antimicrobial and antitumor effects of ergot alkaloids and their derivatives In: V. Kren, L. Cvak, Eds.), Ergot: The Genus Claviceps. Harwood Academic Publishers, Amsterdam, The Netherlands, 1999, pp. 441–449.

Faeth, S. H. and Bultman, T. L., Endophytic fungi and interactions among host plants herbivores and natural enemies. In: T. Tscharntke, B. A. Hawkins, Eds.), Multitrophic Level Interactions. Cambridge University Press 2002, pp. 89-123.

Fleetwood, D. J., Scott, B., Lane, A. G., Tanaka, A. and Johnson, R. J., 2007. A complex Ergovaline Gene Cluster In *Epicloe* Endophytes of Grasses. Applied and Enviromental Microbiology. 2571-2579.

Florea, S., Andreeva, K., Machado, C., Mirabito, P. M. and Schardl, C. L., 2009. Elimination of marker genes from transformed filamentous fungi by unselected transient transfection with a Cre-expressing plasmid. Fungal Genetics and Biology. 46**,** 721-730.

Florea, S., Machado, C., Zhang, D., Panaccione, D. G. and Schardl, C. L., Towards the elimination of ergot alkaloid biosynthesis genes in *Neotyphodium coenophialum*. In: N. Z. G. Association, (Ed.), 6th International Symposium on Fungal Endophytes of Grasses, Christchurch, New Zealand, 2007, pp. 477-479.

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

Forment, V. J., Ramón, D. and MacCabe, A. P., 2006. Consecutive gene deletions in *Aspergillus nidulans*: application of the Cre/loxP system. Current Genetics. 50**,** 217–224.

Fu, J., Hettler, E. and Wickes, B. L., 2006. Split marker transformation increases homologous integration frequency in *Cryptococcus neoformans*. Fungal Genetics and Biology 43**,** 200-212.

Gardiner, D. M., Cozijnsen, A. J., Wilson, M. M., Pedras, C. and Howlett, B. J., 2004. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. Molecular Microbiology. 53**,** 1307-1318.

Gebler, J. C. and Poulter, C. D., 1992. Purification and characterization of dimethylallyl tryptophan synthase from *Claviceps purpurea*. Archives of Biochemistry and Biophysics 296**,** 308-313.

Gilman, A. G., Goodman, L. S. and Rall, T. W., The Pharmacological Basis Of Therapeutics. Macmillan Publishing Company, New York, 1985, pp. 931-45.

Goetz, C. G., 1990. Dopaminergic agonists in the treatment of Parkinson's desease. Neurology. 40**,** 50-54.

Gritz, L. and Davies, J., 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. . Gene 25**,** 179-188.

Gröger, D. and Floss, H. G., 1998. Biochemistry of ergot alkaloids– achievements and challenges. Alkaloids Chem Biol. 50**,** 171–218.

Gueldener, U., Heinisch, J., Koehler, G. J., Voss, D.and Hegemann, J. H., 2002. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Research. 30.

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

Haarmann, T., Lorenz, N. and Tudzynski, P., 2008. Use of a nonhomologous end joining deficient strain (Δku70) of the ergot fungus *Claviceps purpurea* for identification of a nonribosomal peptide synthetase gene involved in ergotamine biosynthesis. Fungal Genetics and Biology. 45**,** 35-44.

Haarmann, T., Machado, C., Lubbe, Y., Correia, T., Schardl, C. L., Panaccione, D. G. and Tudzynski, P., 2005. The ergot alkaloid gene cluster in *Claviceps purpurea:* extension of the cluster sequence and intra species evolution. Phytochemistry 66**,** 1312–1320.

Haarmann, T., Ortel, I., Tudzynski, P. and Keller, U., 2006. Identification of the cytochrome P450 monooxygenase that bridges the clavine and ergoline alkaloid pathways ChemBioChem 7**,** 645–652.

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

Hemken, R. W., Jackson, J. A. J. and Boling, J. A., 1984. Toxic factors in tall fescue. Journal of Animal Science 58**,** 1011-1016.

Hill, N. S., 2002. Performance of tall fescue germplasms bred for high- and low-ergot alkaloids. Crop Sci. 42**,** 518-523.

Hill, N. S., Thompson, F. N., Stuedemann, J. A., Rottinghaus, G. W., Ju, H. J., Dawe, D. L. and Hiatt, E. E., 2001. Ergot alkaloid transport across ruminant gastric tissues. J. Anim. Sci. 79**,** 542–549.

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

Kimball, B. P., Lipreti, V. L. and Aldridge, H. E., Quantitative Arteriographic Responses To Ergonovine Provocation. Subjects With Atypical Chest Pain, Vol. 64. American Journal Cardiology, 1989, pp. 778-82

Komarova, E. L., Shain, S. S. and Sheichenko, V. I., 2002. Isolation of the ergot strain Claviceps purpurea (Fr.) Tul. VKM-F-3662D producing the lactamic alkaloid ergocornam. Applied Biochemistry and Microbiology. 38**,** 567-571.

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

Krappmann, S., Bayram, O. and Braus, G., 2005. Deletion and allelic exchange of the *Aspergillus fumigatus veA* locus via a novel recyclable marker module. Eukaryot Cell. 4**,** 1298-307.

Kross, J., Henner, W. D., Hecht, S. M. and Haseltine, W. A., 1982. Specificity of deoxyribonucleic acid cleavage by bleomycin, phleomycin and tallysomycin. Biochemistry 21**,** 4310-4318.

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

Kuldau, G. A., Tsai, H. F. and Schardl, C. L., 1999. Genome sizes of *Epichloë* species and anamorphic hybrids. Mycologia. 91**,** 776-782.

Lambert, J. M., Bongers, R. S. and Kleerebezem, M., 2007. Cre-lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Applied and Environmental Microbiology. 73**,** 1126–1135.

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

Li, Z., Xing, A., Moon, B., Burgoyne, S., Guida, A., Liang, H., Lee, C., Caster, C., Barton, J., Klein, T. and Falco, S., 2007. A Cre/loxP-mediated self-activating gene excision system to produce marker gene free transgenic soybean plants. Plant Molecular Biology. 65**,** 329-341.

Liu, Q., Li, M. Z., Leibham, D., Cortez, D. and Elledge, S. J., 1998. The univector plasmid-fusion system, a method for construction of recombinant DNA without restriction enzymes. Current Biology. 8**,** 1300-1309.

Lorenz, N., Wilson, E. V., Machado, C., Schardl, C. L. and Tudzynski, P., 2007. Comparison of ergot alkaloid biosynthesis gene clusters in *Claviceps* species indicate loss of late pathway steps in evolution of *C. fusiformis*. Appl Environ Microbiol 73**,** 7185-7191.

Lutz, K. A., Svab, Z. and Maliga, P., 2006. Construction of marker-free transplastomic tobacco using the Cre-loxP site-specific recombination system. Nature Protocols. 1**,** 900-910.

Machado, C., Studies of eregot alkaloid biosynthesis genes in clavicipitaceous fungi. Plant Pathology, Vol. PhD. University of Kentucky, Lexington, 2004.

Maier, W., Schumann, B. and Gröger, D., 1988. Microsomal oxygenases involved in ergoline alakloid biosynthesis of various *Claviceps* strains. Journal of Basic Microbiology. 28**,** 83–93.

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

Malinowski, D. P. and Belesky, P. D., 2006. Ecological importance of *Neotyphodium spp*. grass endophytes in agroecosystems. Grassland Science 52 1-14.

Marten, G. C., Jordan, R. M. and Hovin, A. W., 1976. Biological Significance of Reed Canarygrass Alkaloids and Associated Palatability Variation to Grazing Sheep and Cattle. Agron J. 68**,** 909-914.

Mattern, I. E., Punt, P. J. and Van den Hondel, C. A. M. J. J., 1994. A vector of *Aspergillus t*ransformation conferring phleomycin resistance. Nutrition and Food Research Institute, Medical Biological Laboratory, The Netherlands.

Moon, C. D., Craven, K. D., Leuchtmann, A., Clement, S. L. and Schardl, C. L., 2004. Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. Molecular Ecology 13**,** 1455-1467.

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

Mullaney, E. J., Hamer, J. E., Roberti, K. A., Yelton, M. M. and Timberlake, W. E., 1985. Primary structure of the trpC gene from Aspergillus nidulans. Mol Gen Genet. 199**,** 37-45.

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

Mylonakis, E., Casadevall, A. and Ausubel, F. M., 2007. Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathogens 3:e10.

Olejniczak, P. and Lembicz, M., 2007. Age-specific response of the grass *Puccinellia distans* to the presence of a fungal endophyte. Oecologia 152**,** 485-494.

Orbach, M. J., 1994. A cosmid with a HyR marker for fungal library construction and screening. Gene. 150**,** 159-162.

Otsuka, H., Quigley, F. R., Groger, D., Anderson, J. D. and Floss, H. G., 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., Johnson, R. D., Wang, J. H., Young, C. A., Damrongkool, A., Scott, B. and Schardl, C., 2001. Elimination of ergovaline from a grass-Neotyphodium endophyte symbiosis by genetic modification of the endophyte. Proceedings of

the National Academy of Sciences of the United States of America 98**,** 12820-12825.

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

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

Paterson, J., Forcherio, C., Larsonts, B., Samfordt, M. and Kerleyf, M., 1995. The effects of fescue toxicosis on beef cattle productivity. Journal of animal science. 73**,** 889-898.

Pazoutova, S., Olsovska, J., Linka, M., Kolinska, R. and Flieger, M., 2000. Chemoraces and Habitat Specialization of Claviceps purpurea Populations. Appl. Environ. Microbiol. 66**,** 5419-5425.

Pertz, H. and Eich, E., Ergot alkaloids and their derivatives as ligands for serotoninergic, dopaminergic, and adrenergic receptors. In: V. Kren, L. Cvak, Eds.), Ergot: The Genus Claviceps. Harwood Academic Publishers, Amsterdam, The Netherlands, 1999, pp. 411- 440.

Poter, J. K., Biotechnology of Endophytic Fungi of Grasses. In: C. W. Bacon, J. F. j. White, Eds.). CRC Press, Boca Rathon, Florida 1994, pp. 103-123.

Potter, D. A., Stokes, T., Redmond, C. T., Schardl, C. L. and Panaccione, D. G., 2008. Contribution of ergot alkaloids to suppression of a grass-feeding caterpillar assessed with geneknockout endophytes in perennial ryegrass. Entomol Exp Appl.

Raisbeck, M. E., Rottinghaus, G. E. and Kendall, J. D., Effects of naturally occurring mycotoxins on ruminants. In: J. E. Smith, R. S. Henderson, Eds.), Mycotoxins and Animal Foods. CRC Press, Boca Raton, 1991.

Rebek, J. J., Tai, D. F. and Shue, Y. K., 1983. Synthesis of ergot alkaloids from tryptophan. J. Am. Chem. SOC. 106**,** 1813-1819

Rottinghaus, G. E., Garner, G. B., Cornel, C. N.and Ellis, J. L., 1991. HPLC method for quantitating ergovaline in endophyteinfested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. J Agric Food Chem 39.

Sambrook, J. and Russell, D. W., 2001. Molecular cloning: a laboratory manual 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sauer, B., 1987. Functional expression of the Cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. Molecular and Cellular Biology. 7**,** 2087–2096.

Sauer, B. and Henderson, N., 1989. Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome. Nucleic Acids Research. 17**,** 147-161.

Schardl, C. L., 1996. *Epichloë* species: fungal symbionts of grasses. Annual Review of Phytopathology 34**,** 109-130.

Schardl, C. L., A, L. and Spiering, M. J., 2004. Symbioses of grasses with seedborne fungal endophytes. Annual Review of Plant Biology. 55.

Schardl, C. L., Craven, K. D., Speakman, S., A, S., Lindstrom, A. and Yoshida, R., 2008. A novel test for host-symbiont codivergence indicates ancient origin of fungal endophytes in grasses. Systematic Biology. 57**,** 483-498.

Schardl, C. L. and Leuchtmann, A., The epichloë endophytes of grasses and the symbiotic continuum. In: J. Dighton, J. F. White, P. Oudemans, Eds.), The Fungal Community: its organization and role in the ecosystem. CRC Press, Boca Raton, Florida, 2005, pp. 475-503.

Schardl, C. L., Panaccione, D. G. and Tudzynski, P., 2006. Ergot alkaloids- Biology and Molecular Biology Chemistry and Biology 63**,** 45–86.

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

Schardl, C. L., Scott, B., Florea, S. and Zhang, D.-X., Epichloë Endophytes: Clavicipitaceous Symbionts of Grasses. In: H. Deising, (Ed.), The Mycota Plant Relationships, 2nd Edition. Springer-Verlag Berlin Heidelber, 2009, pp. 275-305.

Schwenk, F., Sauer, B., Kukoc, N., Hoess, R., Muller, W., Kocks, C., Kuhn, R. and Rajewsky, K., 1997. Generation of Cre recombinase-specific monoclonal antibodies, able to characterize the pattern of Cre expression in cre-transgenic mouse strains. Journal of Immunological Methods 207**,** 203–212.

Scott, B., 2001. *Epichloë* endophytes: fungal symbionts of grasses. Current Opinion in Microbiology 4**,** 393–398.

Shigaki, T., Vyzasatya, R. R., Sivitz, A. B., Ward, J. M., Sze, H. and Hirschi, K. D., 2005. The Cre-loxP recombination-based reporter system for plant transcriptional expression studies. Plant Molecular Biology. 58**,** 65–73.

Shwab, E. K. and Keller, N. P., 2008. Regulation of secondary metabolite production in filamentous ascomycetes. Mycological Research. 112**,** 225-230.

Smith, M. W., Feng, D. and Doolittle, R. F., 1992. Evolution by acquisition: The case for horizontal gene transfers. Trends in Biochemical Sciences 17**,** 489-493.

Spiering, M. J., Davies, E., Tapper, B. A., Schmid, J. and Lane, G. A., 2002. Simplified extraction of ergovaline and peramine for analysis of tissue distribution in endophyte-infected grass tillers. J Agric Food Chme 50**,** 5856–5862.

Spiering, M. J., R, F. J., Zhang, D., Machado, C., Grossman, R. B. and Schardl, C. L., 2008. Role of the *LolP* cytochrome P450 monooxygenase in loline alkaloid biosynthesis. Fungal Genetics and Biology. 45**,** 1307–1314.

Spiers, D. E., Zhang, Q., Eichen, P., Rottinghaus, G. E., Garner, G. B. and Ellersieck, M. R., 1995. Temperature-dependent responses of rats to ergovaline derived from endophytes infected tall fescue. Journal of Animal Science. 73**,** 1954-1961.

Stadler, P. A. and Giger, R., Ergot alkaloids and their derivatives in medical chemistry and therapy. In: L. P. Krosgard, C. H. Christensen, H. Kofod, Eds.), Natural products and drug development. Munksgaard, Copenhagen, 1984, pp. 463-485.

Steiner, S., Wendland, J., Wright, M. C. and PhiLippsen, P., 1995. Homologous recombination as the main mechanism for DNA integration and cause of rearrangements in the filamentous ascomycete *Ashbya gossypii*. Genetics 140**,** 973-987.

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

Thompson, F. N. and Stuedemann, J. A., 1993. Pathophysiology of fescue toxicosis. Agriculture, Ecosystems & Environment. 44 263–281.

Tor-Agbidye, J., Blythe, L. L. and Craig, A. M., 2001. Correlation of endophyte toxins (ergovaline and lolitrem B) with clinical disease:fescue foot and perennial ryegrass staggers. Veterinary and human toxicology. 43**,** 140–146.

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

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

Tudzynski, P., Correia, T. and Keller, U., 2001. Biotechnology and genetics of ergot alkaloids. Applied Microbiology and Biotechnology 57 593-605.

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

Van Dongen, P. W. and De Groot, A. N., 1995. History of ergot alkaloids from ergotism to ergometrine. European Journal of Obstetrics&Gynecology and Reproductive Biology 60 109-16.

Wadworth, A. N. and Crisp, P., 1992. Co-dergocrine mesylate. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in age-related cognitive decline. Drugs Aging. 2**,** 153-17.

Wang, J., *dmaW* encoding tryptophan dimethylallyltransferase in ergot alkaloid biosynthesis from Clavicipitaceous fungi. Plant Pathology, Vol. PhD. University of Kentucky, Lexington, 2000.

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

Wilkinson, H. H., Siegel, M. R., Blankenship, J. D., Mallory, A. C. and Bush, L. P., 2000. Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. Molecular Plant-Microbe Interactions. 13**,** 1027–1033.

Xu, Y., Xu, G., Liu, B. and Gu, G., 2007. Cre reconstitution allows for DNA recombination selectively in dual-marker-expressing cells in transgenic mice. Nucleic Acids Research. 35**,** 19 e126.

Young, C. A., Felitti, S., Shields, K., Spangenberg, G., Johnson, R. D., Bryan, G. T., Saikia, S. and Scott, B., 2006. A complex gene cluster for indolediterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. Fungal Genet Biol. 43**,** 679-693.

Yu, J.-H. and Keller, N. P., 2005. Regulation of secondary metabolism in filamentous fungi. Annu. Rev. Phytopathol. 43**,** 437-58.

Yu, J., Chang, P. K., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Payne, D. A., Linz, J. E., Woloshuk, C. P. and Bennett, J. W., 2004. Clustered pathway genes in aflatoxin biosynthesis. Appl Environ Microbiol. 70**,** 1253-62.

Zhang, D., Loline Alkaloid Biosynthesis Gene Expression In Epichloë Endophytes Of Grasses. Plant Pathology, Vol. PhD. University of Kentucky, Lexington, 2008.

VITA

NAME Simona Florea

PLACE OF BIRTH Cluj-Napoca, Romania

EDUCATION

08/2003-12/2009	University of Kentucky, College of Agriculture, Department of Plant Pathology, Lexington, KY
Degree:	PhD in Plant Pathology (Defense on December 2, 2009) - pending
10/2001-02/2003	University of Agricultural Sciences and Veterinary Medicine Cluj, Romania
Degree:	Master of Sciences in Plant Breeding and Quality Control of Seeds
10/1996-07/2001	University of Agricultural Sciences and Veterinary Medicine, Cluj, Romania
Degree:	Bachelor of Science in Agricultural Sciences

HONORS

2003-2009	University of Kentucky, Target of Opportunity Research Assistantship (TORA)

PROFESSIONAL EXPERIENCE

05/2002-07/2003	Research Specialist at the University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania

PUBLICATIONS

Florea, S., Andreeva, K., Machado, C., Mirabito, P. M., and Schardl, C. L.(2009). Elimination of marker genes from transformed filamentous fungi by unselected transient transfection with a Cre-expressing plasmid. Fungal Genetics and Biology. 46**,** 721-730

Schardl, C. L., Scott, B., **Florea, S**., and Zhang, D.-X. (2009). Epichloë endophytes: clavicipitaceous symbionts of grasses. In The Mycota, vol. V, 2nd ed. Springer, Heidelberg, Germany

Florea, S., Andreeva, K., Machado, C., Mirabito, P. M., and Schardl, C. L. (2009). Elimination of selectable marker from various filamentous fungi by transient expression of Cre recombinase. Poster at 25th Fungal Genetics Conference at Asilomar, California, USA

Andreeva, K., **Florea, S**., Young,C., An,Z., Voisey, C.R., Panaccione, D.G., Farman, M.L., and Schardl, C.L. (2009). Analysis of secondary metabolite gene clusters in the genome of *Epichloë festucae***.** Poster at 25th Fungal Genetics Conference, Asilomar, California, USA

Florea, S., Machado, C., Panaccione, D.G., and Schardl, C.L. (2008) Towards the elimination of ergot alkaloid biosynthesis genes in *Neotyphodium coenophialum* Flash-and-Dash Poster Presentation at the American Phytopathological Society, Centennial Meeting in Minneapolis, Minnesota, USA

Schardl, C.L., Panaccione, D.G., Potter, D.A., **Florea, S**., Hesse, U., Konnova, E., Liu, M., Machado,C., and Stokes, J.T. (2007). Genetic tests of ecological roles of ergot alkaloids produced by an epichloë endophyte of ryegrass. Grassland Association: Endophyte Symposium. p. 172 New Zealand

Florea, S., Machado, C., Panaccione, D.G., and Schardl, C.L. (2007). Towards the elimination of ergot alkaloid biosynthesis genes in *Neotyphodium coenophialum*. New Zealand Grassland Association: Endophyte Symposium. pp. 477-479, New Zealand

Florea, S., Machado, C., Panaccione, D.G., and Schardl, C.L (2006) Towards the elimination of ergot alkaloid biosynthesis genes in *Neotyphodium coenophialum* Poster at the American Phytopathological Society, Canadian Phytopathological Society, and Mycological Society of America Joint Meeting Quebec City, Canada

Florea, S. (2004). Roles of ergot alkaloids produced by plant-symbionts and pathogens Oral presentation at Center for Ecology, Evolution and Behavior Spring Research Symposium at University of Kentucky, USA

Machado, C., Wang, J., **Florea, S**., Panaccione, D.G., and Schardl C.L. (2004). Elimination of ergot alkaloid biosynthesis genes in *Neotyphodium* endophytes of forage grasses. Poster Abstract Proc. 5th Int. Symp. on *Neotyphodium*/Grass Interactions, Fayetteville, Arkansas, USA