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Ether Bridge Formation and Chemical Diversification in Loline Alkaloid Biosynthesis

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ETHER BRIDGE FORMATION AND CHEMICAL DIVERSIFICATION IN LOLINE ALKALOID BIOSYNTHESIS

DISSERTATION

A dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food and Environment at the University of Kentucky

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2014

ABSTRACT OF DISSERTATION

ETHER BRIDGE FORMATION AND CHEMICAL DIVERSIFICATION IN LOLINE ALKALOID BIOSYNTHESIS

Loline alkaloids, found in many grass-*Epichloë* symbiota, are toxic or feeding deterrent to invertebrates. The loline alkaloids all share a saturated pyrrolizidine ring with a 1-amine group and an ether bridge linking C2 and C7. The steps in biosynthesis of loline alkaloids are catalyzed by enzymes encoded by a gene cluster, designated *LOL*, in the *Epichloë* genome. This dissertation addresses the enzymatic, genetic and evolutionary basis for diversification of these alkaloids, focusing on ether bridge formation and the subsequent modifications of the 1-amine to form different loline alkaloids.

Through gene complementation of a natural *lolO* mutant and comparison of *LOL* clusters in strains with different loline alkaloid profiles, I found that *lolO*, predicted to encode a 2-oxoglutarate-dependent nonheme iron (2OG/Fe) dioxygenase, is required in formation of the ether bridge. Through application of isotopically labeled compound to *Epichloë uncinata* culture, I established that *exo*-1-acetamidopyrrolizidine (AcAP) and *N*-acetylnorloline (NANL) are true pathway intermediates. Application of AcAP to yeast expressing *lolO* resulted in production of NANL, establishing that LolO is sufficient to catalyze this unusual oxygenation reaction.

After ether formation, modifications on the 1-amino group give loline, *N*-methylloline (NML), *N*-formylloline (NFL) and *N*-acetylloline (NAL). A double knockout of *lolN*, predicted to encode an acetamidase, and *lolM*, predicted to encode a methyltransferase, produced only NANL. Complementation of the double knockout with wild-type *lolN* and *lolM* restored the loline alkaloid profile. These results indicate that LolN is involved in deacetylating NANL to produce norloline, which is then modified to form the other lolines. Crude protein extract of a yeast transformant expressing LolM converted norloline to loline and NML, and loline to NML, supporting the hypothesis that LolM functions as a methyltransferase in the loline-alkaloid biosynthesis pathway. The alkaloid NAL was observed in some but not all plants symbiotic with *Epichloë siegelii*, and when provided with exogenous loline, asymbiotic meadow fescue (*Lolium pratense*) plants produced *N*-acetylloline (NAL), indicating that a plant acetyltransferase converts loline to NAL.

I further analyzed the basis for loline alkaloid diversity by comparing the *LOL* clusters in the *Epichloë* and *Atkinsonella* species with different loline alkaloid profiles, and found that *LOL* clusters changed position, orientation and gene content over their evolutionary history. Frequent, independent losses of some or all late pathway genes, *lolO, lolN, lolM* and *lolP*, resulted in diverse loline alkaloid profiles. In addition, phylogenetic analyses demonstrated transspecies polymorphism of the *LOL* clusters.

Based on my findings, I established that in *Epichloë* and *Atkinsonella* species the ether bridge is formed on acetamidopyrrolizidine. My study of the loline alkaloid profile of *Adenocarpus decorticans* (Fabaceae) suggests that these plants probably use a similar strategy at least with respect to ether-bridge formation. Further diversification steps of loline alkaloids in grass-Clavicipitaceae symbiota are carried out by enzymes of both *Epichloë* species and the host plant. Finally, I present evidence that *LOL* clusters have evolved by balancing selection for chemical diversity.

Keywords: loline alkaloids, *Epichloë*, ether bridge, biosynthesis, chemical diversification

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ETHER BRIDGE FORMATION AND CHEMICAL DIVERSIFICATION IN LOLINE ALKALOID BIOSYNTHESIS

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Chapter One

Introduction

1.1. Plant associated alkaloids

Alkaloids are basic, nitrogen-containing organic molecules that are produced in many plant species as chemical defense compounds against herbivores and pathogens (Freeman and Beattie, 2008)(Schardl & Chen, 2010supplementary, Woolley, 2001). Among them are several well-known chemicals like nicotine from tobacco (*Nicotiana tabacum*), caffeine from coffee (*Coffea arabica*) and tea (*Camellia sinensis*), morphine from opium poppy (*Papaver somniferum*), and so on. Alkaloids are typically produced from primary metabolites, usually amino acids (Woolley, 2001, Ziegler & Facchini, 2008), such as tryptophan (precursor of ergot alkaloids, vinblastine, and quinine), tyrosine (precursor of berberine, morphine, and dopamine), and arginine (precursor of nicotine). Caffeine, on the other hand, is an example of an alkaloid derived from a purine nucleotide instead of amino acids.

Although plant alkaloid biosynthesis pathways often begin with the limited source of precursors, the product structures are immensely diverse, with more than 10,000 structures of alkaloids described from vascular plants (Wink, 2001). The elucidation of a plant alkaloid biosynthetic pathway typically begins by tracking isotopically labeled metabolites to identify precursors and intermediates in the pathway. Further steps include reverse genetics to identify enzymes and genes involved in the biosynthesis pathway. These studies enable a reasonable hypothesis on the series of reactions in the biosynthesis. Isotopic feeding of hypothesized intermediates and further studies on identification and characterization of hypothesized genes through forward or reverse genetic methods are used in the next steps to establish the whole biosynthesis pathway. Recent advances in genomic and transcriptomic analyses of alkaloid-producing plants also facilitate the identification of genes in alkaloid biosynthesis (Leonard et al., 2009).

The alkaloids originate from nitrogen-rich compounds; therefore, they could be very costly for the plant to produce. Phylogenetic studies of alkaloid-producing plants have shown that the production of specific alkaloids is sporadic within plant families rather than consistent in taxonomic groups (Wink, 2003), reflecting the varied defense strategies other than chemical defense that are utilized by different plants. The *Acacia* plants serve as a good example of plants utilizing different defensive strategies. Neotropical species of the genus *Acacia* are divided into two groups depending on their association with symbiotic ants of the genus *Pseudomyrmex.* The *Acacia* species that do not form symbiosis with ants are found to produce various chemicals — such as alkaloids and cyanide — for defense against herbivores, whereas the species that are symbiotic with the ants tend to produce much less defensive chemicals, but instead invest in hosting these symbiotic ants and rely on the symbionts to defend against herbivores (Rehr et al., 1973).

The grasses (Poaceae) are well documented to be capable of synthesizing alkaloids such as 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in chemical defense against microbial pathogens and herbivores (Sicker et al., 2000, Macias et al., 2009, Niemeyer, 1988). These hydroxamic acids are stored as their β-D-glucoside forms and are cleaved off the glucoside to release the active form when needed (such as cell wounding by the herbivore). The genes involved in hydroxamic acid biosynthesis have been characterized in maize, and interestingly, they form a gene cluster (Sicker et al., 2000). Such clusters are also a common theme for fungal secondary metabolite genes (Keller et al., 2005). Aside from producing alkaloids themselves, the cool-season grasses (subfamily Pooideae) also take another route in chemical defense. Many of these grasses host endophytic fungi (*Epichloë* spp.) in their intercellular spaces, benefiting in return by various alkaloids produced by the fungus to defend against herbivores (Clay & Schardl, 2002).

1.2. Grass-*Epichloë* symbiosis

The *Epichloë* species (family Clavicipitaceae) are endophytic symbionts of many cool season grasses. The infection frequency varies within and between host species, but within populations it can be consistent. For example, it has been found in Illinois that two native grass species, *Festuca paradox* and *Festuca obtusa*, are nearly 100% infected (Spyreas et al., 2001). Populations of the grass species *Elymus canadensis*, a native prairie grass in North America, are also found to be highly infected (100%) (Vinton et al., 2001). The high frequency of infection in the wild populations suggests that the endophytes might contribute a selective advantage to the host grasses. The infection frequency is not stable, however. A survey of natural grasses in Europe found that infection frequency is closely related to drought conditions. Mediterranean regions that are subject to summer drought stress harbor the highest infection frequency (Lewis et al., 1997, Hesse et al., 2003, Hesse et al., 2005). Increased CO_2 also resulted in higher endophyte infection frequencies in greenhouse conditions (Brosi et al., 2011). These population dynamics of infection reflect the selective pressure imposed on the grass-*Epichloë* symbiosis.

The interactions of these endophytes and grasses vary dramatically depending on fungal and host genotypes and environmental conditions. The fungal endophyte typically colonizes the host systemically throughout the whole above ground tissues, and grows intercellularly by means of intercalary growth, such that hyphae lie roughly parallel to the longitudinal axes of plant cells (Christensen et al., 2008, Christensen et al., 2002). The symbiont obtains nutrients from the host, but unlike many pathogenic fungi, it does not form haustoria or similar intracellular structures (e.g., arbuscules). The outcome of the grass-*Epichloë* interactions is complex and interesting, spanning a spectrum from pathogenic to mutualistic depending on the fungal reproduction mode (Chung et al., 1997, Sampson, 1933, Schardl, 2010). In pathogenic interactions, the fungus can overgrow the plant tissue at the onset of the host inflorescence differentiation, and eventually replace the flowering structure to form the fungal reproductive structure (stroma), causing the disease that is referred to as grass choke disease (Chung et al.,

1997). After fertilization, ascospores develop in perithecia embedded in the stroma, are ejected, and are spread by air currents to adjacent plants to initiate new infections (horizontal transmission). In the mutualistic grass-*Epichloë* interaction, the fungus remains intercellularly localized in the host inflorescence and further into the ovule, thereby transmitting to the next generation through seeds (vertical transmission) (Schardl et al., 2004). Some of the endophyte species, such as *Epichloë typhina* in orchard grass (*Dactylis glomerata*), are completely horizontally transmitted, choking all of the infected inflorescences (Western & Cavett, 1959, Alderman et al., 1997). Asexual species transmit solely through the seeds, and some sexual species exhibit both modes, choking some tillers but also allowing some tillers to develop normally and transmit through the seeds.

In addition to chemical defense against herbivores, the endophyte can benefit grass hosts in various other ways by enhancing survival and growth (Clay et al., 2005, Clay et al., 1993, Bazely et al., 1997), such as increasing seed production (Clay, 1987, Madej & Clay, 1991) and tolerance of drought (Kannadan & Rudgers, 2008, Malinowski et al., 1997, Nagabhyru et al., 2013). Hence, endophyte infection of grasses could result in significant ecosystem effects. For example, tall fescue grass infected with *Epichloë coenophiala* are more competitive compared with uninfected tall fescue plants, and the symbiosis with the endophyte can result in major change of plant community compositions (Clay et al., 2005).

An increased resistance against herbivores associated with grass-*Epichloë* symbioses is conferred by the various alkaloids produced by the fungus. Four classes of alkaloids have been reported to be produced by *Epichloë* species in *symbio*: ergot alkaloids, lolitrems, loline alkaloids, and peramine (Panaccione, 2005, Schardl et al., 2009, Panaccione et al., 2000, Young et al., 2009, Scott et al., 2013, Schardl et al., 2013c). In fact, some of the alkaloids are so neurotoxic that animals heavily grazing on infected grasses with certain alkaloids can suffer severe symptoms and even die. Ergot alkaloids produced by *Epichloë coenophiala* causes the notorious fescue toxicosis, in which case sheep, horses, and cattle grazing on infected tall fescue may lose their extremities, or in less extreme cases have poor fertility or weight gain (Bacon et al., 1977, Bacon, 1995, Schardl et al., 2006, Porter & Thompson, 1992). Lolitrems produced by the perennial ryegrass symbiont *Epichloë festucae* var. *lolii* causes livestock to loose balance and muscular control, a syndrome known as ryegrass staggers (Latch et al., 1984, Gallagher et al., 1984). These two classes of alkaloids are toxic to both vertebrate and invertebrate herbivores; therefore, although they are very effective defensive compounds benefiting the plant, these endophyte alkaloids can cause huge economic losses in livestock agriculture (Allen & Segarra, 2001). The other two classes, loline alkaloids and peramine, are toxic or feeding deterrents specifically active in invertebrates (Schardl et al., 2013a), and hence are highly desirable traits selected in grass breeding. Understanding the biosynthesis of the alkaloids provides the basis for molecular manipulation of the endophytes for improved forage, pasture and turfgrass cultivars.

1.3. Biological effects of loline alkaloids

Loline alkaloids all share a core *exo*-1-aminopyrrolizidine structure with an

unusual ether bridge on C2 and C7. Different modifications on the 1-amine group define different loline alkaloids. Structures of common loline alkaloids and the related pyrrolizidine alkaloids are shown in Figure 1.1.

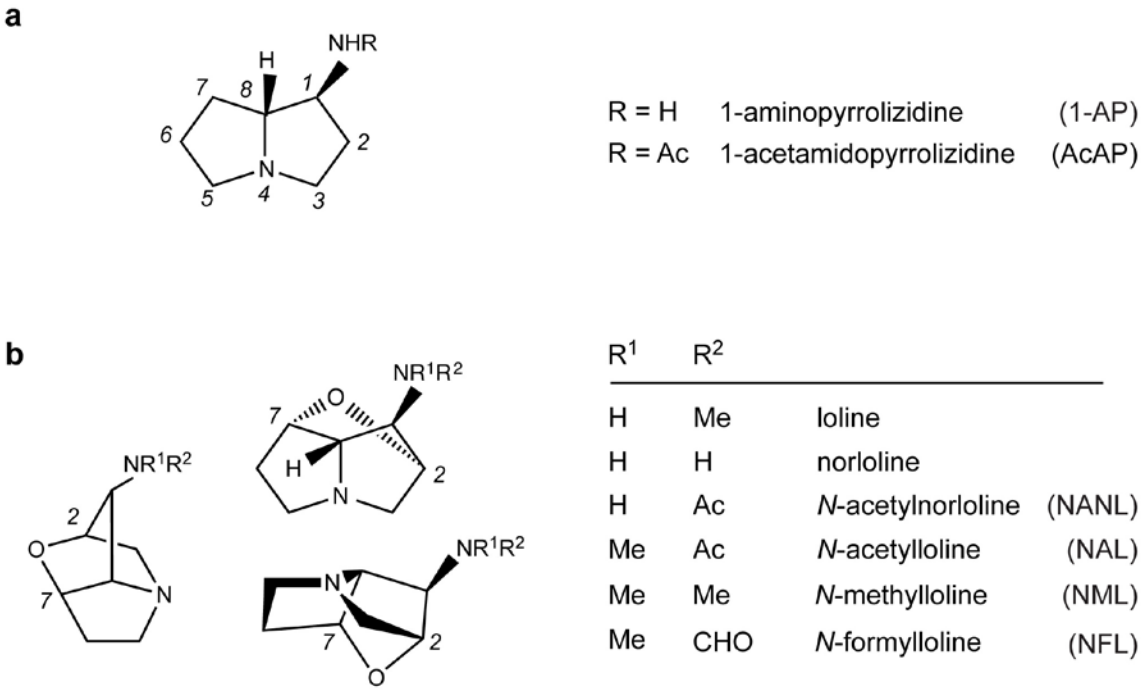


Figure 1.1. Perspective illustrations of loline alkaloids and related pyrrolizidines. (a) Loline alkaloids related pyrrolizidines, and (b) loline alkaloids found in grass-*Epichloë* symbiosis.

Loline alkaloids are most prominent in their broad-spectrum insecticidal effects and the ability to deter feeding. It has been reported that lolines are associated with anti-feeding effects against bird-cherry oat aphid (*Rhopalosiphum padi*; Homoptera) (Siegel et al., 1990). *In vitro* tests of *N*-formylloline (NFL), *N*-acetylloline (NAL), *N*-methylloline (NML), and semi-synthetic loline derivatives (Riedell et al., 1991) showed feeding deterrence against fall armyworm (*Spodoptera frugiperda*; Lepidoptera) larvae. NAL reduced weight gain in European corn borer (*Ostrinia nubilalis*; Lepidoptera), but no feeding behavior change was observed in the study, so the effect was probably due to metabolic toxicity. NFL, NAL, and NML are almost as potent as nicotine in insecticidal activity against green bug (*Schizaphis graminum* Rondani), and interestingly NML shows a similar regression curve to nicotine sulfate, indicating a similar pharmacological mechanism (Riedell et al., 1991). The rice pest, rice leaf bug (*Trigonotylus caelestialium;* Heteroptera), is also affected by loline alkaloids produced by meadow fescue (*Lolium pratense*) infected with *Epichloë uncinata* or Italian ryegrass (*Lolium multiflorum*) with *Epichloë occultans* (Shiba & Sugawara, 2009). Insects feeding on the former showed greater effect, probably due to higher loline alkaloid production. An *in vitro* test showed that the survival of the rice leaf bug larvae was adversely affected by artificial diets supplemented with NFL even at a low concentration of 50 μg/g (Shiba & Sugawara, 2009). Tests of NFL and *N*-acetylnorloline (NANL) on adult Argentine stem weevil (*Listronotus bonariensis*) (Jensen et al., 2009) showed marked reduction in oviposition. A further study of loline alkaloid effects on Argentine stem weevil larvae showed that NANL and NFL might affect the beetle in different ways. At high concentrations (800 and 1600 μg/g) toxicity of NFL reduced the growth, development and survival of the larvae, whereas NANL caused high mortality of the larvae but with little effect on the growth and development of the larvae (Popay et al., 2009). The broad-spectrum insecticidal and feeding deterrent effects of lolines make them attractive as an environmental-friendly alternative insect control agent in the field.

1.4. Loline alkaloid biosynthesis pathway and *LOL* gene cluster

Although loline alkaloids were known to be associated with endophytes (Siegel et al., 1990), the biosynthesis of loline alkaloids by the fungus was not established until loline alkaloids were produced in cultures of *Epichloë uncinata* strain e167 (Spiering et al., 2002). Suppression subtractive hybridization PCR showed that two genes, *lolA* and *lolC*, were up-regulated during loline alkaloid production in cultures of *E. uncinata* e167, and these genes were present only in endophytic fungi that produce loline alkaloids *in planta*, indicating a tight association with the alkaloid production (Spiering et al., 2002). Like other fungal specialized (secondary) metabolites, for which genes involved in the biosynthesis are often clustered (Keller & Hohn, 1997), two redundant gene clusters that included *lolA* and *lolC* were later found to be associated with loline alkaloid production (Spiering et al., 2005).

The endophytes that produce NFL, as well as the other loline alkaloids, have a cluster of 11 genes in the following order: *lolF*, *lolC*, *lolD*, *lolO*, *lolA*, *lolU*, *lolP*, *lolT*, *lolE, lolN, and lolM* (Spiering et al., 2005, Schardl et al., 2013b). Bioinformatic analysis of these genes has suggested that most or all encode biosynthetic enzymes or regulatory

proteins in loline alkaloid biosynthesis. Other strains that produce NANL or the bicyclic *exo*-1-acetamidopyrrolizidine (AcAP) as the end product have varied gene contents within the 11 aforementioned genes, and either lack or have inactivating mutations in late pathway genes (Schardl et al., 2013b), which will be discussed in detail in Chapters 2 and 3 of this dissertation. Based on the predicted functions of *LOL* genes and the already established steps (discussed later), I propose the biosynthetic pathway shown in Figure 1.2, which is similar to previous proposals (Schardl et al., 2013b) except that, as I show in Chapter Two, LolE is not required for ether bridge formation. Also, support for the proposed LolN and LolM steps is presented in Chapter Three.

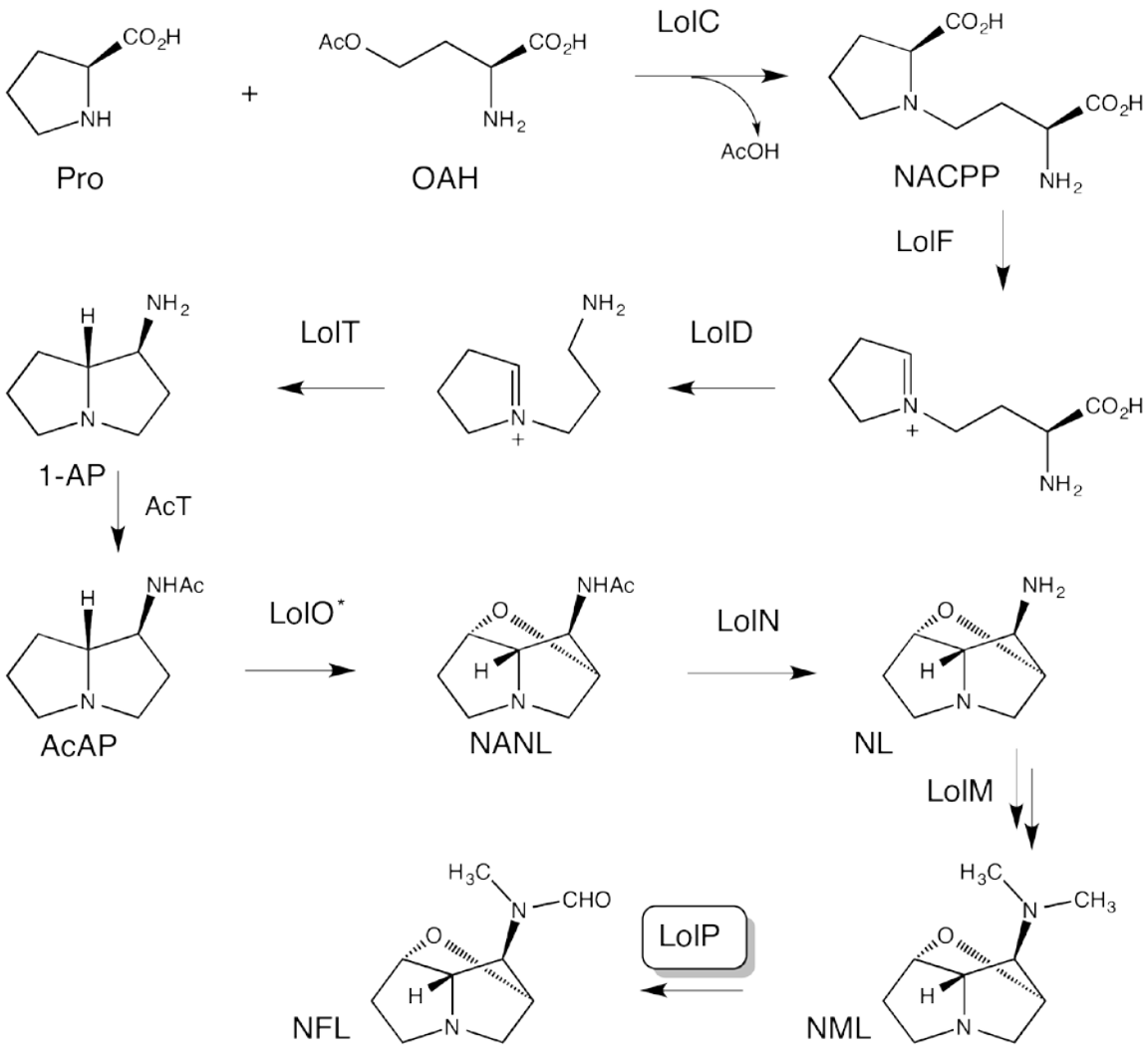


Figure 1.2. Proposed loline alkaloid biosynthesis pathway. Shown are known precursors, intermediates, and end product of the loline alkaloid biosynthesis pathway. The enzymes that are proposed to function in specific steps from bioinformatic analysis are shown above the arrows. The function of LolP (circled) has been established. AcT = acetyltransferase. The asterisk (*) indicates ether formation catalyzed solely by LolO, as I discuss in Chapter Two of this dissertation.

Isotopic enrichment experiments have established that L-proline (Pro) is the precursor that contributes N-4, C-5, C-6, C-7, and C-8 of the loline alkaloids (Figure 1.1), and that homoserine is the precursor that contributes C-1, C-2, and C-3 and the 1-amino group nitrogen, whereas S-adenosylmethionine provides the carbon of methyl and formyl groups on the 1-amine (Blankenship et al., 2005). Based on the precursors involved, it seems likely that the biosynthesis of lolines begins with an unusual γ-substitution that condenses proline and the homoserine moiety of *O*-acetylhomoserine. LolC, which is a homolog of *Aspergillus nidulans O*-acetylhomoserine (thiol) lyase (homocysteine synthase), is the most likely candidate to catalyze this unusual γ-substitution reaction to produce *N*-(3-amino-3-carboxypropyl) proline (NACPP) (Figure 1.2). Knocking down *lolC* by RNA-interference (RNAi) resulted in reduced loline alkaloid production in the culture, indicating its involvement in the biosynthesis pathway (Spiering et al., 2005). Furthermore, deuterium atoms on isotopically labeled NACPP incorporated into NFL (Faulkner et al., 2006), indicating that NACPP is a true intermediate in loline alkaloid biosynthesis. Subsequent decarboxylations are proposed to be catalyzed by an FAD-containing monooxygenase, LolF, and a pyridoxal-phosphate (PLP)-containing decarboxylase, LolD, in consecutive steps. The function of *lolD* in loline alkaloid biosynthesis was tested by gene knockout, as discussed in the Appendix C of this dissertation. Another PLP-containing enzyme, LolT, was proposed to close the ring to produce *exo*-1-aminopyrrolizidine (1-AP), which has been established by isotopic labeling as a pathway intermediate (Faulkner et al., 2006). The fact that 1-AP is an intermediate indicates that the pyrrolizidine ring forms before ether bridge formation in loline alkaloid biosynthesis.

The ether bridge of loline alkaloids is unusual in that it connects two inactivated bridgehead carbons, and it is probably functionally important in its pharmacological effects. It has been proposed that the ether bridge probably prevents loline alkaloids to be metabolized into a pyrrole byproduct as is typical for plant pyrrolizidine alkaloids, and which causes mammalian hepatotoxicity and carcinogenicity (Chen et al., 2010, Petroski & Stanley, 2009, Powell & Petroski, 1992). LolO and LolE, both of which are predicted 2-oxoglutarate-dependent non-heme iron dioxygenases, have been proposed to catalyze this reaction (Spiering et al., 2005). Functional characterization of these two genes and tests of their roles in ether bridge formation is described in Chapter Two.

After ether formation, different modifications on the 1-amine group give rise to the various loline alkaloids. *N*-formylloline, NANL, and NAL, are the most abundant lolines found in the symbiota. Small amounts of norloline, loline, and NML are also observed, indicating that they are also pathway intermediates. A predicted cytochrome P450 enzyme, LolP, has been characterized and is required for oxygenation of NML to produce NFL, the pathway end product, and is not required in earlier steps (Spiering et al., 2008). The biosynthetic origins of other loline alkaloids have not been established yet. An acetamidase and a methyltransferase, encoded by *lolN* and *lolM*, respectively, were proposed to catalyze formation of loline and NML from NANL. Functional characterization of these genes and biosynthesis of NAL are discussed in Chapter Three.

Other than grasses that are symbiotic with fungi of the *Epichloë* and *Atkinsonella* spp., loline alkaloids are also reported in *Adenocarpus* spp. (Fabaceae) (Ribas & Alonso De Lama 1953, Veen et al., 1992) and *Argyreia mollis* (Convolvulaceae) (Tofern et al., 1999). However, there is no knowledge on correlation of possible symbionts and loline alkaloids in the latter two systems. I propose that a possible fungal endophyte in *Adenocarpus* spp. is responsible for loline alkaloid production, and describe the effort to search for such a symbiont in Chapter Four, and also describe the series of pyrrolizidine-like alkaloids and loline-like alkaloids found in the *Adenocarpus* spp.

In Chapter Five of this dissertation, I present my work to investigate the origin of the *LOL* cluster and the evolutionary history of *LOL* clusters in *Epichloë* and *Atkinsonella* spp. through bioinformatic analysis. I found that the gene content, orientation, and location of the *LOL* clusters are dynamic, which reflect diversifying selection of the loline alkaloids. Phylogenetic analyses also demonstrated that the polymorphism of *LOL* clusters in *Epichloë* and *Atkinsonella* spp. is transspecies; specifically, polymorphisms in *Epichloë bromicola* have been maintained since before the divergence of *Epichloë* species. I also present evidence that recombination events have occurred between deeply diverged *LOL* loci during evolution of the *Epichloë* species.

Chapter Two

Investigation of ether bridge formation in loline alkaloid biosynthesis

A version of this chapter has been published:

Pan, J., Bhardwaj, M., Faulkner, J. R., Nagabhyru, P., Charlton, N. D., Higashi, R. M., Miller, A.-F., Young, C. A., Grossman, R. B., Schardl, C. L., 2014. Ether bridge formation in loline alkaloid biosynthesis. *Phytochemistry* 98, 60-68.

MB, under direction of RBG, accomplished the chemical synthesis of AcAP and D4-AcAP, and characterized AcAP with high-resolution MS and NMR. JRF constructed *lolE* and *lolO* RNAi plasmids pKAES225 and pKAES226, respectively, transformed *E. uncinata* e167 with these plasmids and inoculated the transformants to asymbiotic meadow fescue. JRF also checked loline alkaloid profiles in cultures of the two RNAi transformants of e167 and identified AcAP as a possible new pathway intermediate. JRF also constructed the *lolO* yeast expression plasmid pKAES205. RMH performed FTICR-MS of AcAP.

I conducted all other experiments described in this chapter, including but not limited to the following: I confirmed the *lolO* RNAi result, purified AcAP from natural sources to compare to the synthetic material, analysis and annotations of gene sequences, conducting all gene knockout and complementation experiments, and conducting all experiments involving application of isotopically labeled compounds to plants, fungal cultures and transformed yeast cells.

2.1. Introduction

The lolines (Figure 1.1) are saturated *exo*-1-aminopyrrolizidines with an oxygen bridge between carbons 2 (C2) and 7 (C7), causing the pyrrolizidine ring to be strained. Such an ether linkage between unactivated bridgehead carbons is a characteristic rarely found in natural metabolites. Isotopic enrichment experiments have previously identified L-proline (Pro) and L-homoserine as precursors in a loline-forming biosynthetic pathway that proceeds via *N*-(3-amino-3-carboxypropyl)proline (NACPP) and 1-AP (Figure 1.2) (Faulkner et al., 2006, Blankenship et al., 2005). These findings indicate that the ether bridge forms after the completion of the pyrrolizidine ring system, which in turn excludes many common routes of ether formation in natural products such as reduction of acetals or hemiacetals (Dominguez de Maria et al., 2010).

In several epichloae, a gene cluster, designated *LOL,* has been identified with up to 11 genes, in the order of *lolF, lolC, lolD, lolO, lolA*, *lolU, lolP, lolT*, *lolE*, *lolN*, and *lolM*, and is strictly associated with biosynthesis of lolines (Spiering et al., 2005, Kutil et al., 2007, Schardl et al., 2013b). The predicted products of *LOL* genes include three pyridoxal-phosphate (PLP)-containing enzymes (LolC, LolD, and LolT) and four enzymes involved in oxidation/oxygenation reactions (LolF, LolO, LolP, and LolE). Among the potential oxidizing enzymes, LolP has been functionally characterized to catalyze oxidation of NML to NFL (one of the most abundant loline alkaloids found in grasses) and is not required for earlier steps (Spiering et al., 2008). LolF is likely to be involved in pyrrolizidine formation (Schardl et al., 2007), and, as an FAD-containing monooxygenase, it probably would not provide the oxidative potential for steps in formation of the ether bridge. Hence LolO and LolE, predicted to be non-heme iron α-ketoacid-dependent dioxygenases, are the most likely candidate enzymes for catalyzing ether bridge formation. In this chapter, I demonstrate that LolO is solely responsible for the ether bridge formation, and that LolE is not involved in this process. A new pathway intermediate, *exo*-1-acetamidopyrrolizidine (AcAP) (Figure 1.1) was identified. I also present evidence that AcAP is the direct biosynthetic precursor of the loline alkaloids.

2.2. Results

2.2.1. Identification of exo-*1-acetamidopyrrolizidine*

Expression of the *lolO* RNAi construct in transformed *E. uncinata* e167 altered the loline alkaloid profile, giving a major peak of a previously unknown compound with a 12.0 min retention time in the GC (Figure 2.1). Although the same peak was also observed in the vector-only and wild-type controls, the area of this peak relative to NFL and NANL was much greater in extracts from the RNAi strain cultures compared to the controls. The mass spectrum of the compound had no match when searched against the organic spectral database at http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi. FTICR-MS determined the mass to be 169.1335, which is within 0.059 ppm of the theoretical mass of protonated 1-acetamidopyrrolizidine (AcAP), an aminopyrrolizidine alkaloid related to lolines but lacking the ether bridge (Figure 1.1).

The newly discovered compound (presumably AcAP), but none of the lolines, was also identified as the only loline-related metabolite produced in *Elymus canadensis* plant 4814, symbiotic with a strain (designated e4814) of the endophyte species *Epichloë canadensis*.

In order to determine the identity of the newly discovered compound and its relative configuration (*exo* or *endo*) with certainty, I purified presumed AcAP from tillers collected from plant 4814 and compared it to synthetic (±)-*exo*-1-acetamidopyrrolizidine ((±)-*exo*-AcAP). The synthesis of (±)-*exo*-1-acetamidopyrrolizidine was published in Pan et al. (Pan et al., 2014). NMR and GC-MS spectra of the purified AcAP and the synthetic (±)-*exo*-AcAP were similar, and spectra of an equimolar mixture of the two were consistent with their hypothesized identity (Pan et al., 2014 supplementary data). Thus, the compound newly isolated from *Epichloë*-symbiotic grass was determined to be *exo*-AcAP. For simplicity, from now on, *exo*-AcAP detected from the symbiosis is referred to as AcAP.

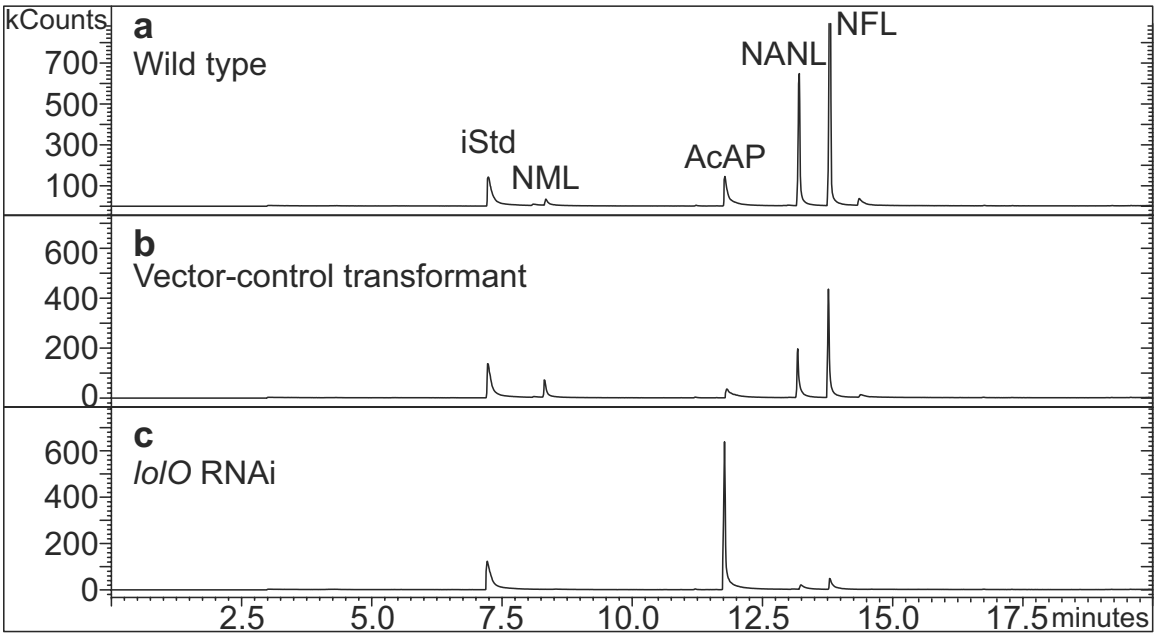


Figure 2.1. GC-MS total ion traces of *E. uncinata* RNAi transformant and controls showing loline-alkaloid profiles from 25 day-old cultures. The internal standard (iStd), quinoline, was used for quantification. (a, b) Chromatograms of products from wild-type *E. uncinata* e167 and the vector-only transformant, respectively; (c) chromatogram of products from a *lolO* RNAi transformant.

2.2.2. Meadow fescue infected with **lolO** *RNAi transformants accumulated relatively higher amounts of AcAP compared with controls*

The *lolO* RNAi and vector-only transformants were inoculated to endophyte-free (E-) meadow fescue (*Lolium pratense*), the natural host of the fungus. A substantially higher amount of AcAP over total loline alkaloids was observed in plants with the *lolO* RNAi transformants compared with controls (Figure 2.2), supporting the hypothesized role of *lolO* being involved in oxygenation of AcAP to form tricyclic loline alkaloids.

2.2.3. Accumulation of AcAP is associated with **lolO** *mutations*

Different loline alkaloid profiles were associated with grasses symbiotic with various strains and species of *Epichloë* and the related grass symbiont, *Atkinsonella hypoxylon* (Figure 2.3). Genomes of several of these symbionts were sequenced to identify and characterize all *LOL*-cluster genes in each. Strains from plants with AcAP, but without lolines, consistently had mutated *lolO* but apparently functional genes *lolF, lolC, lolD, lolA, lolU, lolT* and *lolE* in the *LOL* clusters (Figure 2.3). For example, the *lolO* gene of *Epichloë brachyelytri* E4804 had a frame-shift mutation in the first exon and a deletion that extended into the second exon (Figure 2.4). Likewise, in *A. hypoxylon* B4728, *lolO* had a large deletion extending through the first exon and part of the second, and a frame-shift mutation in the second exon (Figure 2.4). In contrast, the *lolO* genes appeared intact in *Epichloë festucae* E2368, which produced NFL and other loline alkaloids, and *Epichloë amarillans* E57, which produced NANL as the final product. These results suggested that *lolO* is required for conversion of AcAP to NANL.

The alkaloids were profiled for additional endophyte-symbiotic plants representing seven grass species in order to further test the correlation between *lolO* and production of fully cyclized lolines. Three distinct loline-alkaloid profiles were observed. Plants with *E. festucae* E2368, *E. uncinata* e167, and *E. coenophiala* e19 accumulated loline, NANL, NAL, NML, and NFL**.** Plants with *E. amarillans* E57 and *E. canadensis* e4815 had NANL, but no other fully cyclized lolines, and plants with *E. amarillans* strains E721, E722 and E862, *E. brachyelytri* E4804, *E. canadensis* e4814, and *A. hypoxylon* B4728 had AcAP, but none of the lolines. Genome sequences and sequences of PCR products revealed that each strain that produced several lolines or only NANL had intact *lolO* genes, whereas those producing only AcAP all had mutant *lolO* genes (Figures 2.3 and 2.4). In addition, the defective *lolO* genes differed in positions of insertions and deletions (Figure 2.4), implying independent origins of *lolO*-inactivating mutations, all four of which were associated with the accumulation of AcAP.

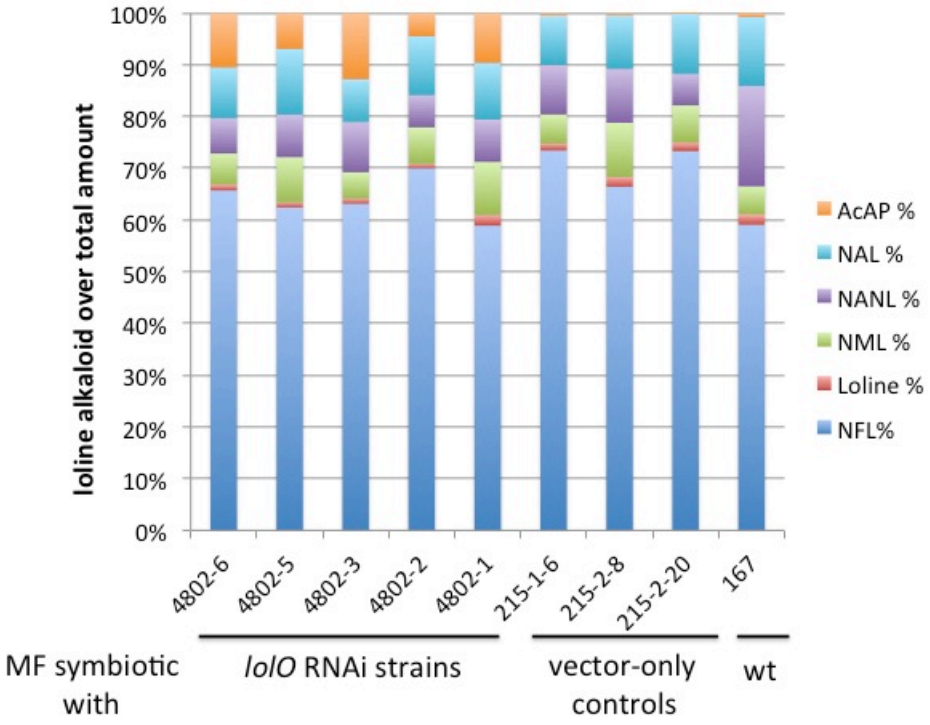


Figure 2.2. Loline alkaloid profiles of meadow fescue symbiotic with *E. uncinata* strains. Shown are meadow fescue plants symbiotic with *E. uncinata* transformed with *lolO* RNAi transformant e4802, plant control transformants 215-1 and 215-2, and the wild-type *E. uncinata* strain e167. Each alkaloid level is indicated as a percentage of total loline alkaloids detected in the symbiotum. Numbers after each plant designation indicate independently inoculated plants.

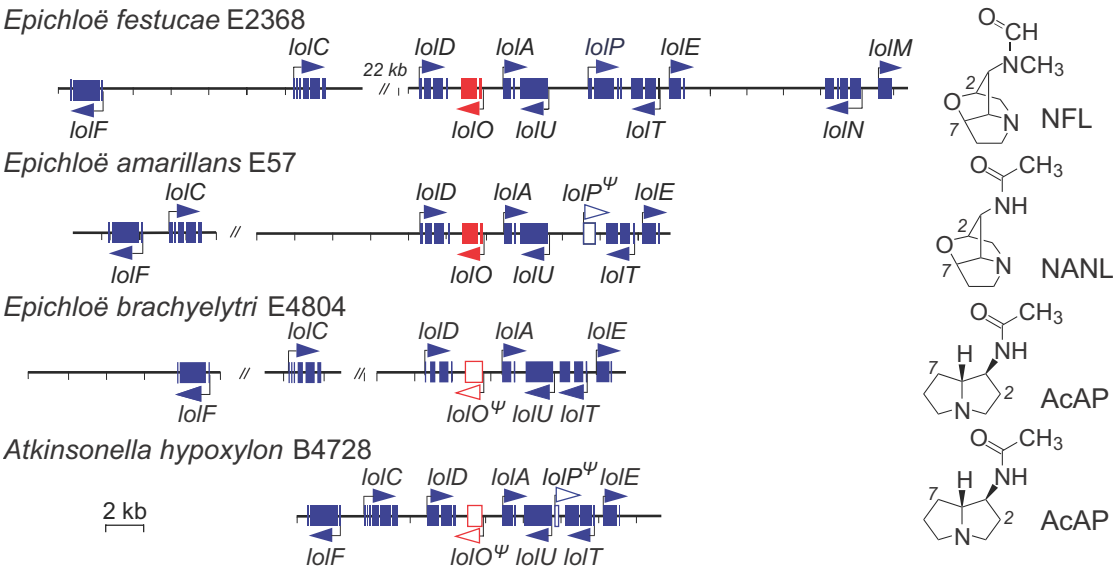


Figure 2.3. Comparison of *LOL* clusters in four fungal species with different loline alkaloid profiles. The *LOL* genes are drawn to scale, with filled boxes representing the exons, and gaps between boxes representing introns. Arrows indicate directions of transcription. Empty boxes indicate pseudogenes, and *lolO* is depicted in red. The chemical structure shown beside each cluster indicates the pathway end product found in each strain.

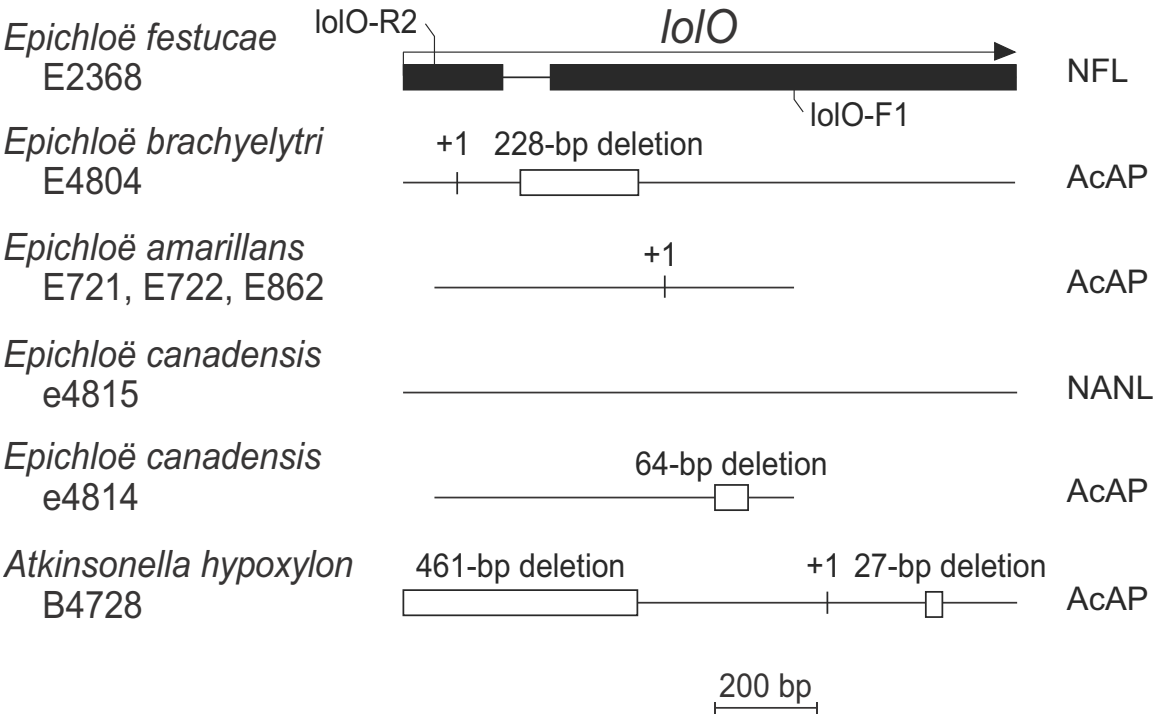


Figure 2.4. Schematic representation of *lolO* from species that differ in alkaloid profiles. The coding region of *lolO* from *E. festucae* E2368 is represented by filled boxes. Binding positions of the primers used for amplification of *lolO* fragments (for E721, E722, E862 and e4814) are indicated as lolO-F1 and lolO-R2. Sequence coverage of the *lolO* gene in each strain is indicated by a horizontal black bar. Sequence variations are shown as +1 to indicate a frameshift, and open boxes as deletions with the size indicated above each box. The compound listed to the right of each map indicates the pathway end-product detected in plants infected with the respective strain.

2.2.4. Heterologous expression of wild-type **lolO** *complemented a natural* **lolO** *mutant*

A genetic complementation experiment was conducted to test the hypothesis that *lolO* mutations caused the loline alkaloid pathway to terminate at AcAP. *Epichloë canadensis* e4814, possessing a mutated *lolO*, was transformed with pKAES309, which contains wild-type *lolO* with its own promoter, cloned from *E. festucae* E2368. Three independent transformants were obtained and introduced back into endophyte-free *El. canadensis* plants. Expression of *lolO in symbio* was checked by RT-PCR with primers targeting the region that included the deletion in e4814. Sequences of the RT-PCR products indicated that the pKAES309 transformants transcribed both the endogenous mutated *lolO* and the introduced wild-type *lolO*, whereas only the mutated *lolO* was transcribed in the wild-type and vector-only controls. Furthermore, plants inoculated with pKAES309 transformants showed accumulation of both AcAP and NANL (Figure 2.5), supporting the hypothesized role of LolO.

2.2.5. Labeling from L-[U-$^2H_7$]Pro showed retention of the deuterium atoms in lolines

In order to determine if exchange of hydrogen on the Pro-derived ring occurs during loline alkaloid biosynthesis, L-[U-$^2H_7$]Pro was applied to *El. canadensis* plants with symbiotic *E. canadensis* e4814. Loline alkaloids were extracted and analyzed by GC-MS. Heptadeuterated AcAP was observed at the front edge of the AcAP peak (Figure 2.6). A +7 *m/z* enrichment was observed in the parent ion (*m/z* = 169 to 176), and in the major fragment ion, which contains the Pro-derived ring (*m/z* = 83 to 90). Similarly, L-[U-$^2H_7$]Pro was applied to *E. uncinata* e167 cultures, and loline alkaloids were analyzed by GC-MS. The expected labeled forms of the alkaloids were all observed, including,the heptadeuterated AcAP (data not shown), and the hexadeuterated loline, NANL, and NFL (Figures 2.7, 2.8, and 2.9). Taking NFL labeling as an example, extract from the culture with L-[U-$^2H_7$]Pro, but not from culture with all-proton Pro (data not shown), gave a GC peak adjacent to the peak corresponding to NFL (Figure 2.9). This novel peak was determined by MS to contain mainly hexadeuterated NFL. A +6 *m/z* enrichment was observed in the parent ion (*m/z* = 183 to 189), its likely deformylated derivative (*m/z* = 153 to 159), the major fragment ion (*m/z* = 82 to 88), and other fragment ions predicted to contain all carbon atoms from the proline ring (Schardl et al., 2007). These results imply that loline alkaloid biosynthesis, including formation of the ether bridge, never involves desaturation of, nor epoxide formation on, the Pro-derived ring, because in that case a +5 *m/z* enrichment in parent ions of lolines would have been expected instead of the observed +6 *m/z* enrichment.

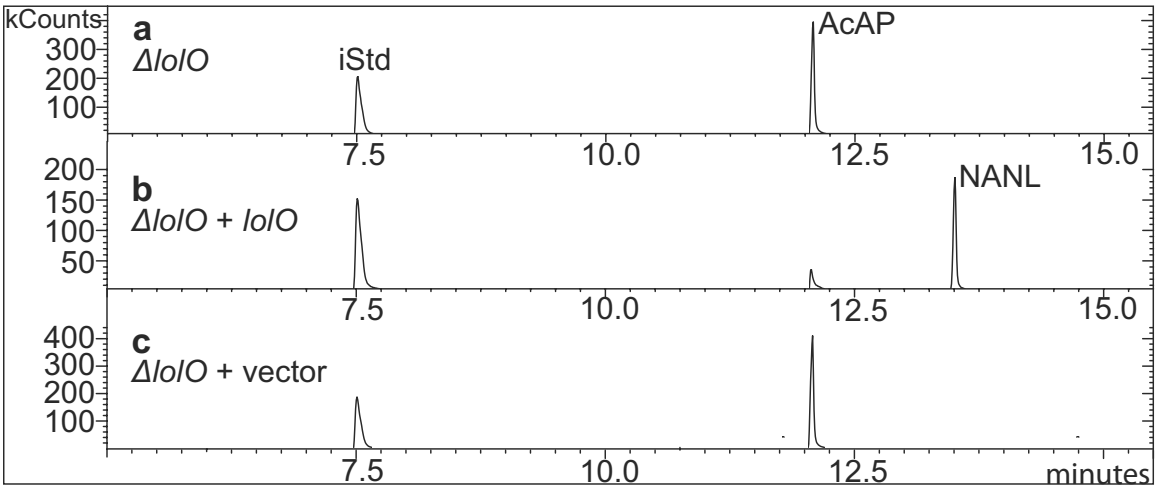


Figure 2.5. Complementation of *Epichloë canadensis* e4814 with wild-type *lolO*. Shown are GC-MS total ion chromatograms of loline alkaloids from the host grass *Elymus canadensis,* when symbiotic with: (a) e4814, which has a partially deleted *lolO* (Δ*lolO*); (b) e4814 transformed with a copy of wild-type *lolO*; (c) e4814 transformed with the empty vector (i.e., without *lolO*).

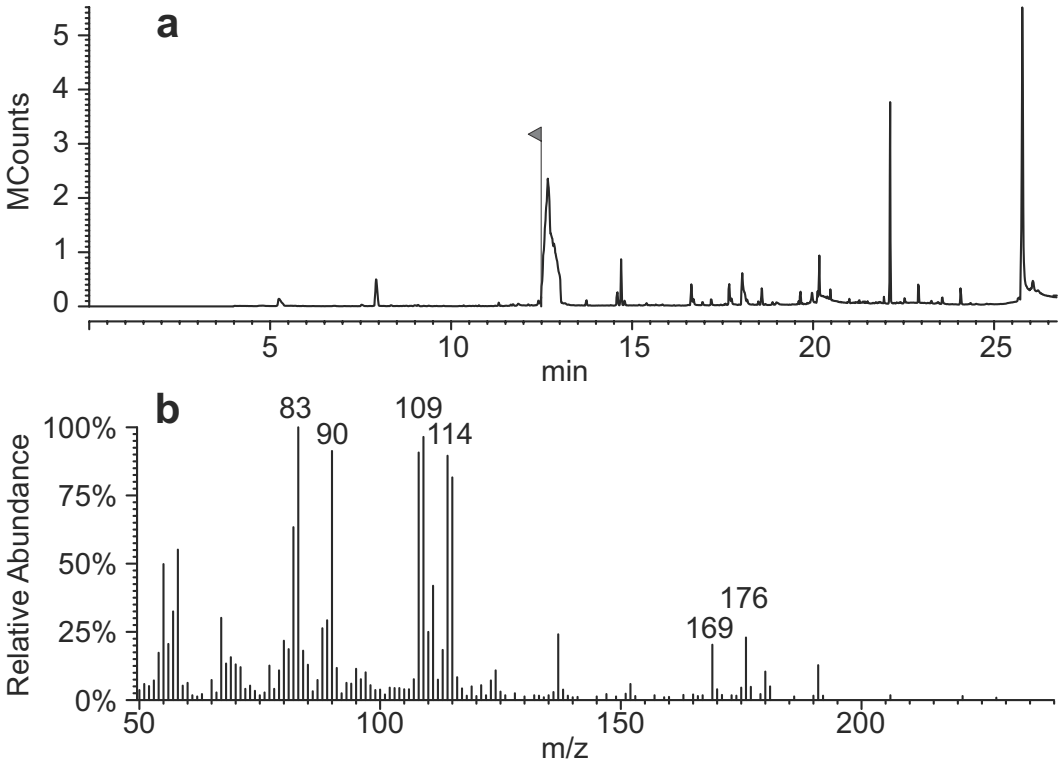


Figure 2.6. Enrichment of deuterated AcAP from application of L-[U-$^{2}H_{7}$]Pro to *El. canadensis* plants that host symbiotic *E. canadensis* e4814. (a) GC-MS total ion chromatogram of chloroform extract. (b) Mass spectrum of the front edge of the AcAP peak (flagged).

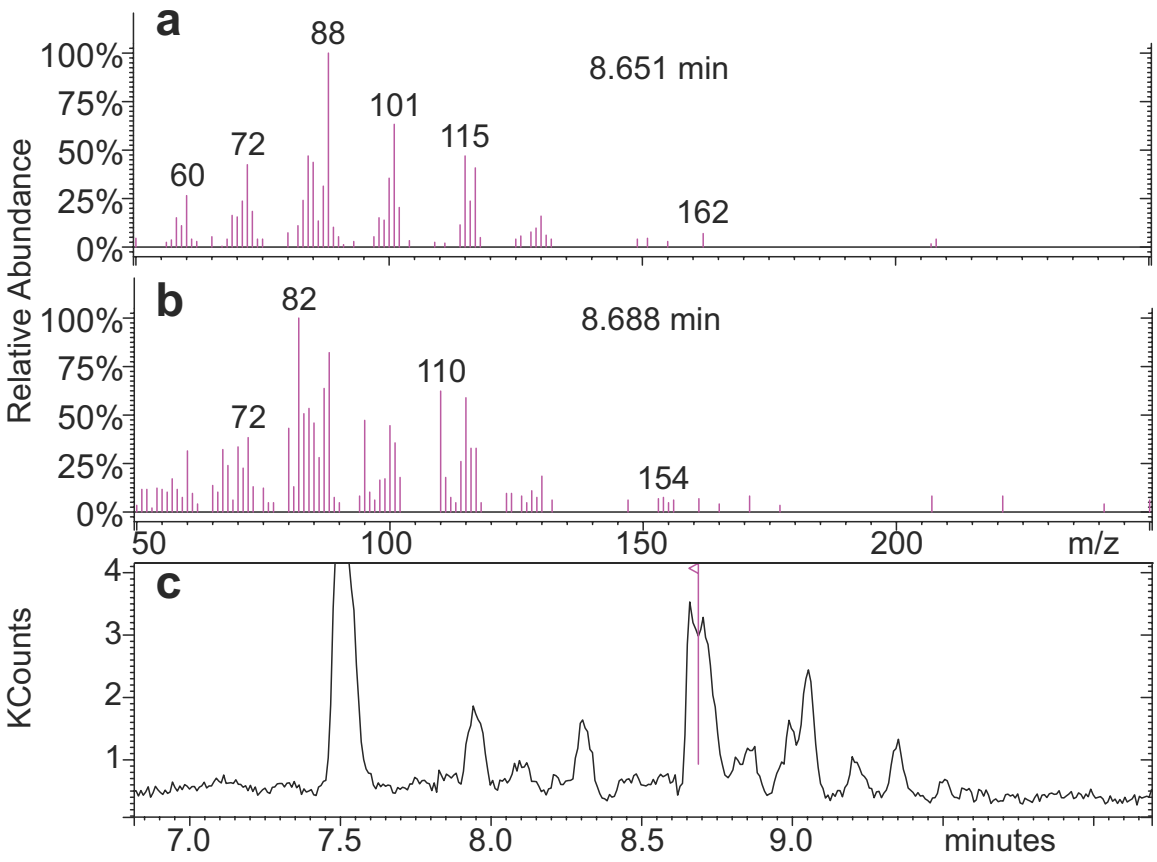


Figure 2.7. GC-MS total ion chromatogram and mass spectrum of lolines extracted from culture of *E. uncinata* e167 after application of L-[U-$^{2}H_{7}$]Pro. The enrichment of deuterated loline is observed as a shoulder peak separated from its non-deuterated form. (a) Mass spectrum of the peak at 8.651 min. This spectrum is consistent with the predicted spectrum for hexadeuterated loline. Although the parent ion is not obvious, the retention time and +6 *m/z* shifts of the fragments are expected for hexadeuterated loline. (b) Mass spectrum of the peak at 8.688 min (flagged in panel c) consistent with the spectrum of all-proton loline. (c) Chromatogram of total ion chromatogram of chloroform extract from L-[U-$^{2}H_{7}$]Pro application, flagged at the loline peak.

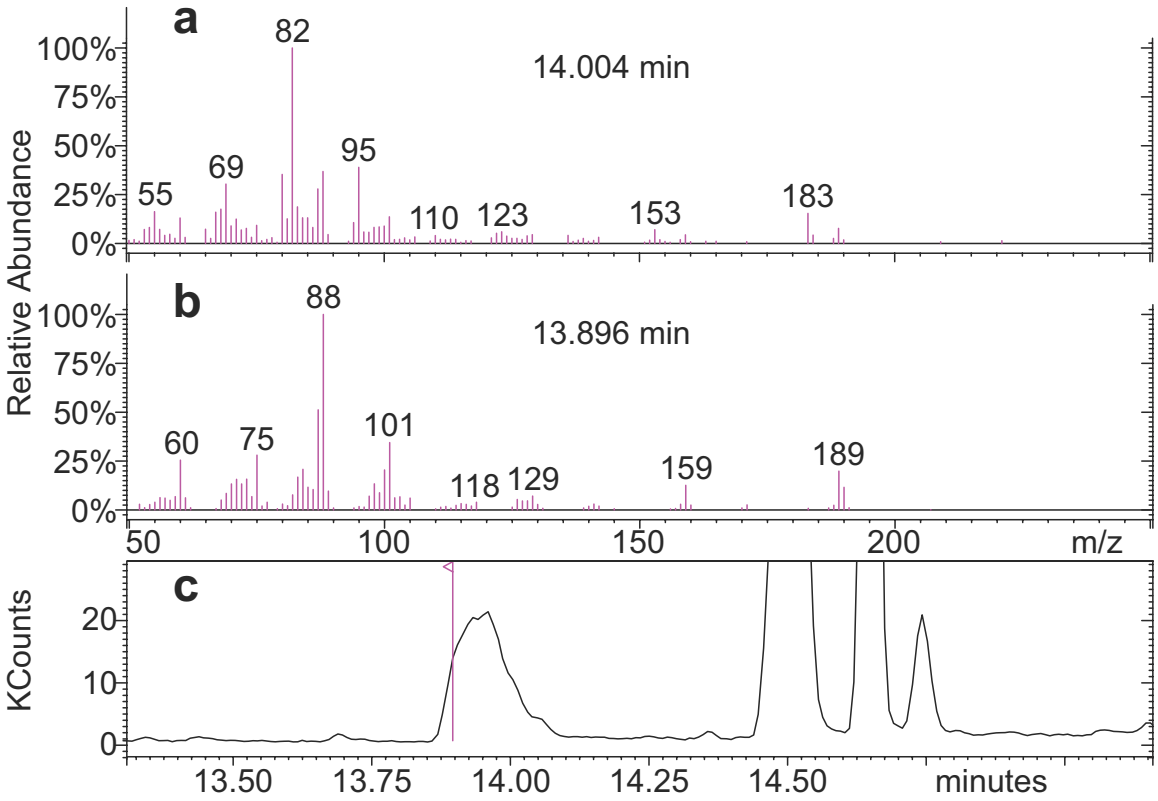


Figure 2.8. GC-MS total ion chromatogram and mass spectrum of *N*-acetylnorloline (NANL) extracted from culture of *E. uncinata* e167 after application of L-[U-$^{2}H_{7}$]Pro.. The enrichment of deuterated NANL is observed as a shoulder peak but not separated from its non-deuterated form. (a) Mass spectrum of the peak at 14.004 min, which is consistent with the mass spectrum of unlabeled NANL. (b) Mass spectrum of the peak at 13.896 min (flagged in panel c). This spectrum is consistent with the predicted spectrum for hexadeuterated NANL, with +6 *m/z* shifts of the parent ion and several fragment ions. (c) Chromatogram of total ion chromatogram of chloroform extract from L-[U-$^{2}H_{7}$]Pro application, flagged at the hexadueterated NANL peak.

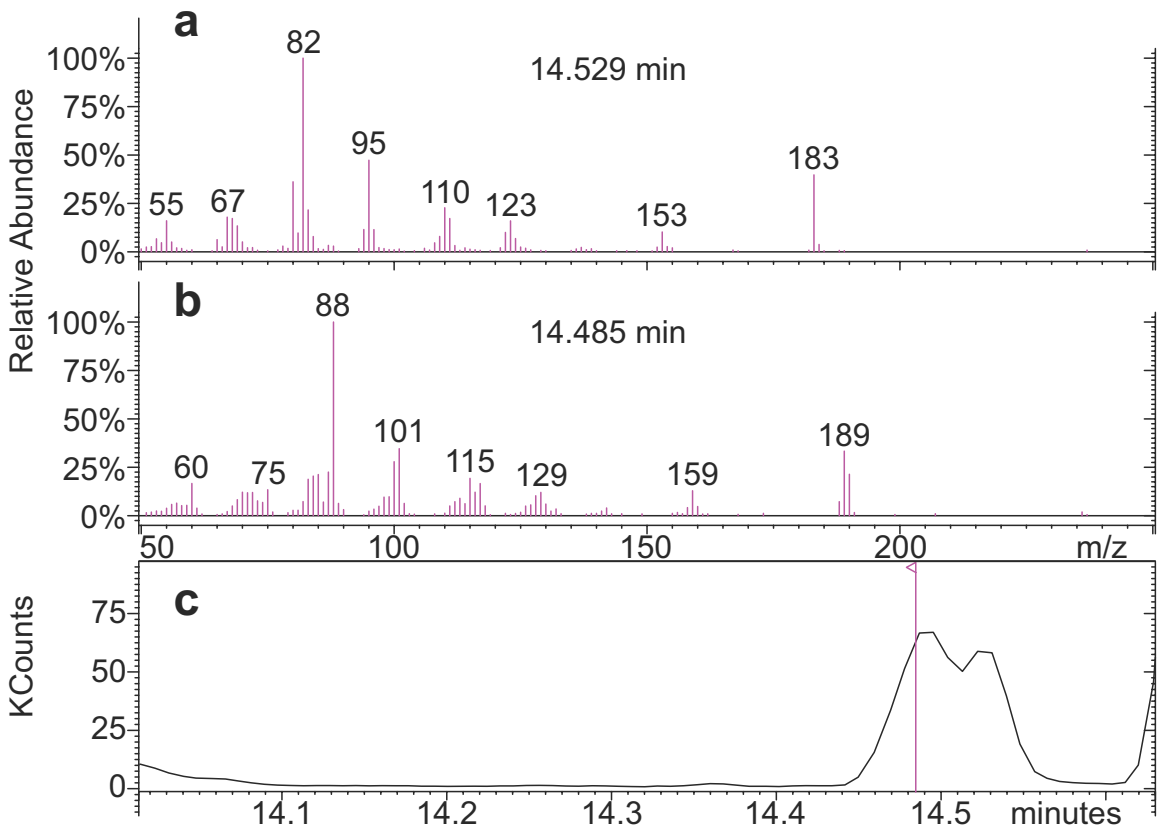


Figure 2.9. GC-MS total ion chromatogram and mass spectrum of *N*-formylloline (NFL) extracted from culture of *E. uncinata* e167 after application of L-[U-$^{2}H_{7}$]Pro. The enrichment of deuterated NFL is observed as a shoulder peak separated from its non-deuterated form. (a) Mass spectrum of the peak at 14.529 min, which is consistent with the spectrum of unlabeled NFL. (b) Mass spectrum of the peak at 14.485 min. This spectrum is consistent with the predicted spectrum for hexadeuterated NFL, with +6 *m/z* shifts of the parent ion and several fragment ions. (c) Chromatogram of total ion chromatogram of chloroform extract from L-[U-$^{2}H_{7}$]Pro feeding, flagged at the hexdeuterated NFL peak.

2.2.6. Retention of the deuterium atoms from tetradeuterium-labeled AcAP ([$^2H_4$]AcAP) in loline alkaloid products

As AcAP accumulated in *lolO* knockdown and *lolO* mutant strains, and AcAP is also detected along with loline alkaloids in natural *Epichloë*-grass symbiota, it is reasonable to hypothesize that AcAP is a pathway intermediate, and probably serves as the direct precursor of loline alkaloids. Alternatively, it is also possible that *exo*-1-aminopyrrolizidine (1-AP) is the real precursor to be oxidized to form norloline, whereas AcAP and NANL are both shunt products through acetylation of 1-AP and norloline, respectively. To test these alternative possibilities, [$^2H_4$]AcAP was prepared with one deuterium atom at C3 of the pyrrolizidine ring system and three deuterium atoms on the acetyl group, and was applied to culture of *Epichloë uncinata* e167 under loline alkaloid production conditions (Blankenship et al., 2001). If the acetylation of 1-AP occurs before ether bridge formation, a four-deuterium retention is expected in NANL, whereas if AcAP is first deacetylated to 1-AP before ether bridge formation, only one deuterium is expected to be retained in NANL. A compound that was determined by MS to be tetradeuterated NANL was observed at the front edge of the NANL peak, with parent ion *m/z* = 187 (*m/z* = 183+4), and major ions *m/z* = 83 (*m/z* = 82+1) and *m/z* = 157 (*m/z* = 153+4) (Figure 2.10). Thus we conclude that AcAP is not deacetylated in the pathway, and that AcAP and NANL are true intermediates.

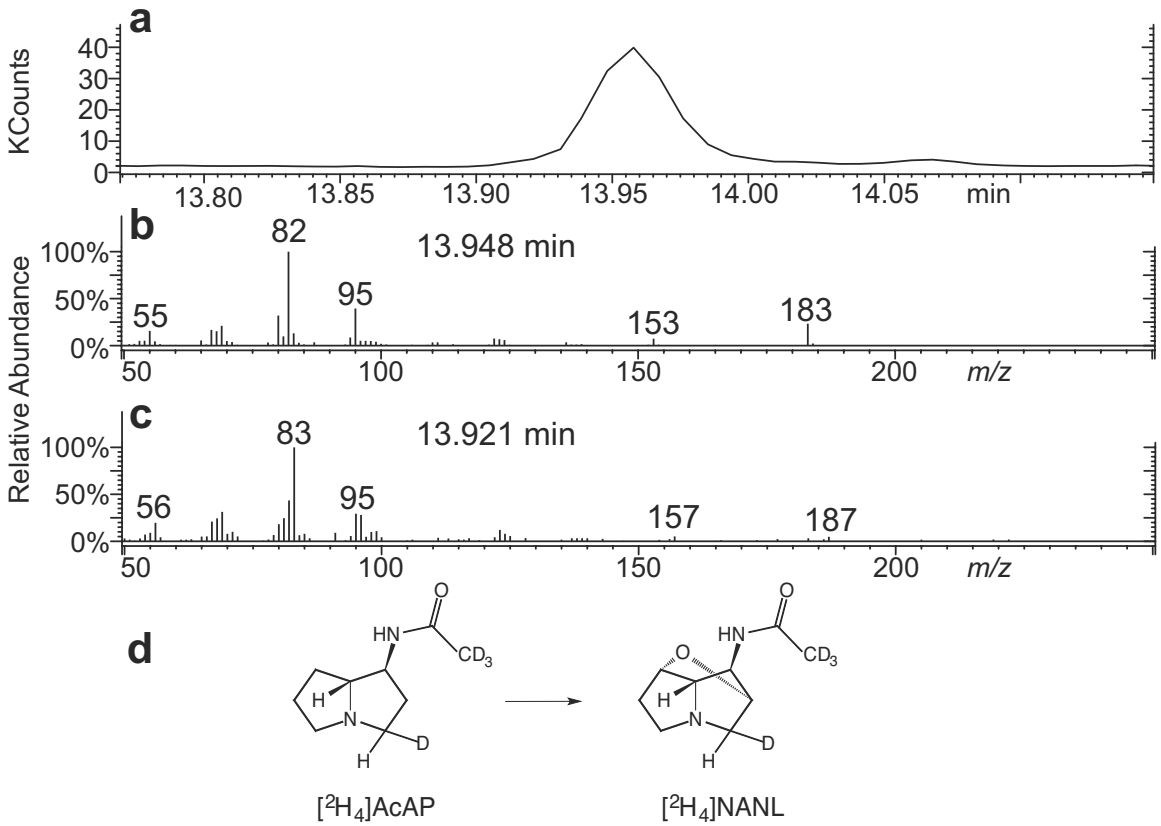


Figure 2.10. Enrichment of deuterated NANL from application of [$^2H_4$]AcAP to loline alkaloid producing culture. Shown are (a) GC-MS total ion chromatogram, (b) mass spectrum at retention time 13.948 min and (c) 13.921 min, and (d) the proposed scheme of [$^2H_4$]NANL formation from [$^2H_4$]AcAP.

2.2.7. LolO heterologously expressed in yeast catalyzed conversion of AcAP to NANL

To further test if LolO or LolE, or both are responsible for oxygenation of AcAP to produce NANL, each of these genes was expressed in yeast by placing each under the control of the yeast GAL10 promoter (Faulkner, 2011), and induced the expression in defined medium with galactose as the sole carbon source. There was no *lolE* transcription detected in yeast for its heterologous expression (Jerome Faulkner and CLS, unpublished data), so I did not carry out further tests with the *lolE* construct and proceeded only with the *lolO* construct. Compound AcAP was applied to yeast transformed with the *lolO* expression construct. A small amount of NANL was detected after incubation (Figure 2.11). Similarly, when [$^2H_4$]AcAP was applied to the yeast in inducing medium, tetradeuterated NANL was detected with parent ion *m/z* = 187 (*m/z* = 183+4), and major ions *m/z* = 83 (*m/z* = 82+1) and *m/z* = 157 (*m/z* = 153+4)) (Figure 2.12). Application of AcAP or [$^2H_4$]AcAP to the empty-vector transformants of the yeast failed to produce all-proton or deuterated NANL. These results clearly indicated that *lolO* is solely responsible for conversion of AcAP to NANL in the loline alkaloid biosynthesis pathway, and also supported the conclusion that AcAP and NANL are true pathway intermediates.

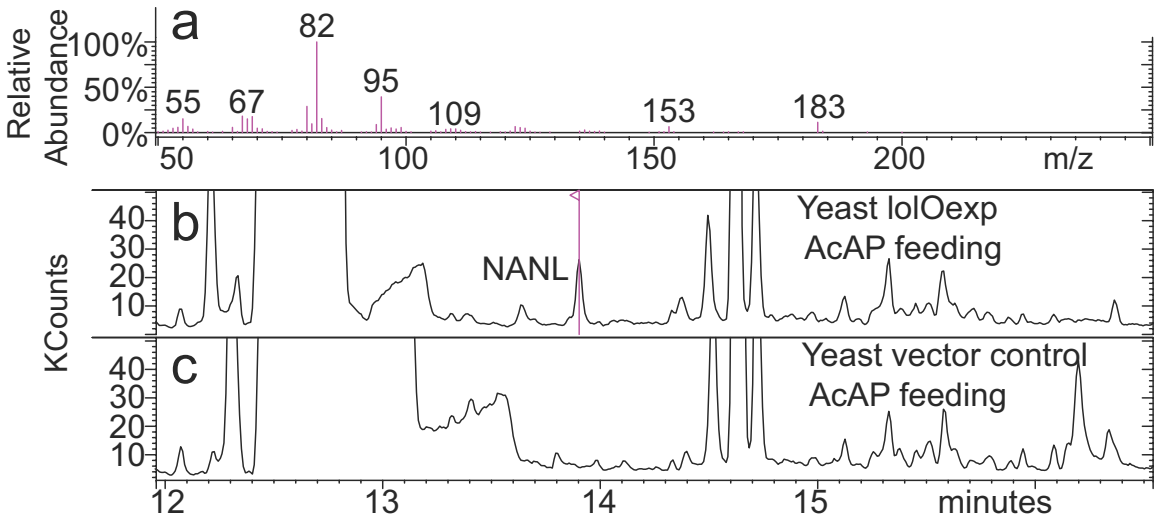


Figure 2.11. Production of NANL from AcAP feeding to yeast that expresses *lolO*. (a) Mass spectrum of NANL (flagged in panel b) from application of AcAP to yeast that expresses *lolO*. (b) and (c) Total ion chromatograms of extract from application of AcAP to yeast transformed with *lolO* expression construct and empty-vector, respectively.

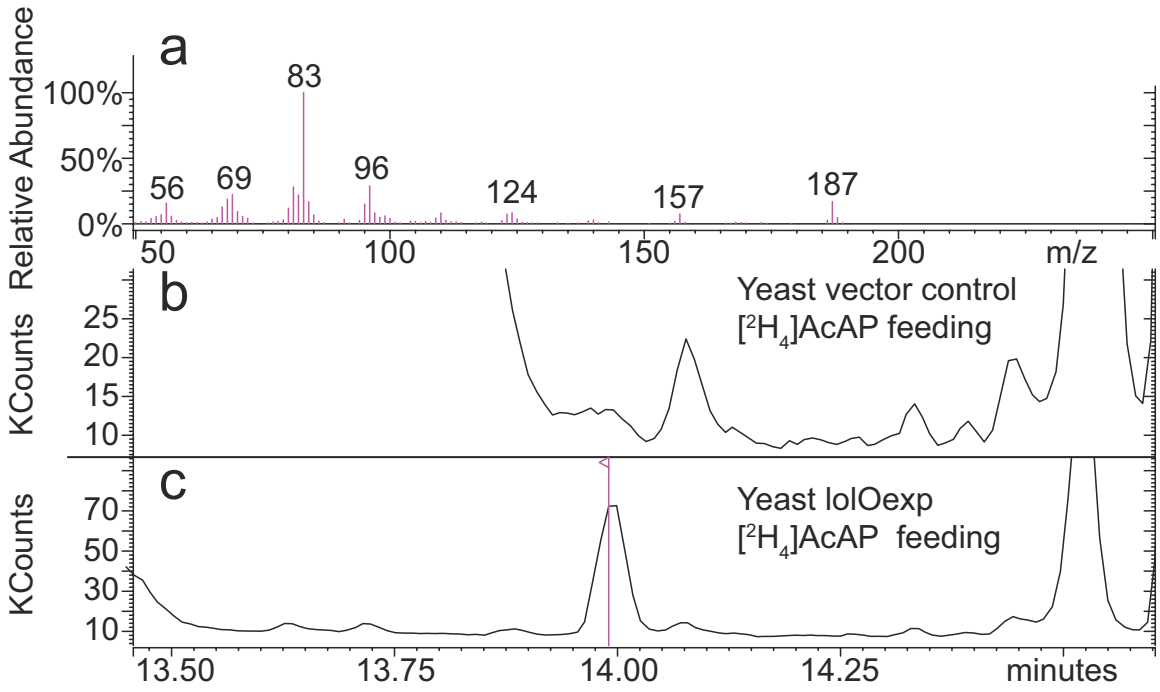


Figure 2.12. Production of [$^2H_4$]NANL from application of [$^2H_4$]AcAP to yeast that expresses *lolO*. (a) Mass spectrum of [$^2H_4$]NANL (flagged in panel c) extracted from the *lolO*-expressing yeast culture after application of [$^2H_4$]AcAP. (b and c) Total ion chromatograms of extract from yeast transformed with (b) empty-vector and (c) *lolO* expression construct, respectively, after application of [$^2H_4$]AcAP.

2.2.8. Knocking down or knocking out **lolE** *did not change the loline alkaloid profile*

LolE is the other predicted non-heme iron α-ketoacid-dependent dioxygenase encoded by the *LOL* cluster. The finding that yeast with *lolO* expression was able to convert AcAP to NANL countered the hypothesis that another oxygenase, such as LolE, was required in this step (Faulkner et al., 2006), although it did not exclude the possibility that both enzymes could be involved in natural situations. To test its function in loline alkaloid biosynthesis, *lolE* was knocked down through RNAi in *E. uncinata* e167. No obvious change of loline alkaloid profile was observed between *lolE* RNAi strains and controls in both inducing medium (data not shown) and when inoculated back to E- meadow fescue (Figure 2.13). This result contrasted with the obvious accumulation of AcAP in meadow fescue symbiotic with *lolO* RNAi strains (Figure. 2.2).

Deletion of *lolE* in *Epichloë festucae* E2368 was carried out through homologous recombination. Two knockout strains were obtained from 200 transformants, and both strains were inoculated to E- meadow fescue to check the loline alkaloid profile. Both *lolE* knockout strains were confirmed to lack *lolE* by Southern blot (Figure. 2.14), and PCR tests indicated that the adjacent *LOL* genes were not disrupted (data not shown). Meadow festucae plants infected with the knockout strain Eko20 showed similar loline alkaloid profiles as plants with the wild-type strain (Figure 2.15). (Plants with the other knockout strain, Eko17, were not yet ready for loline alkaloid profile analysis.) This indicated that *lolO* is solely responsible for the ether bridge formation in loline alkaloid biosynthesis in *Epichloë*. No new loline alkaloid-like compound was observed in Eko20-infected plants, thus giving no clue to the function of LolE in the biosynthesis pathway.

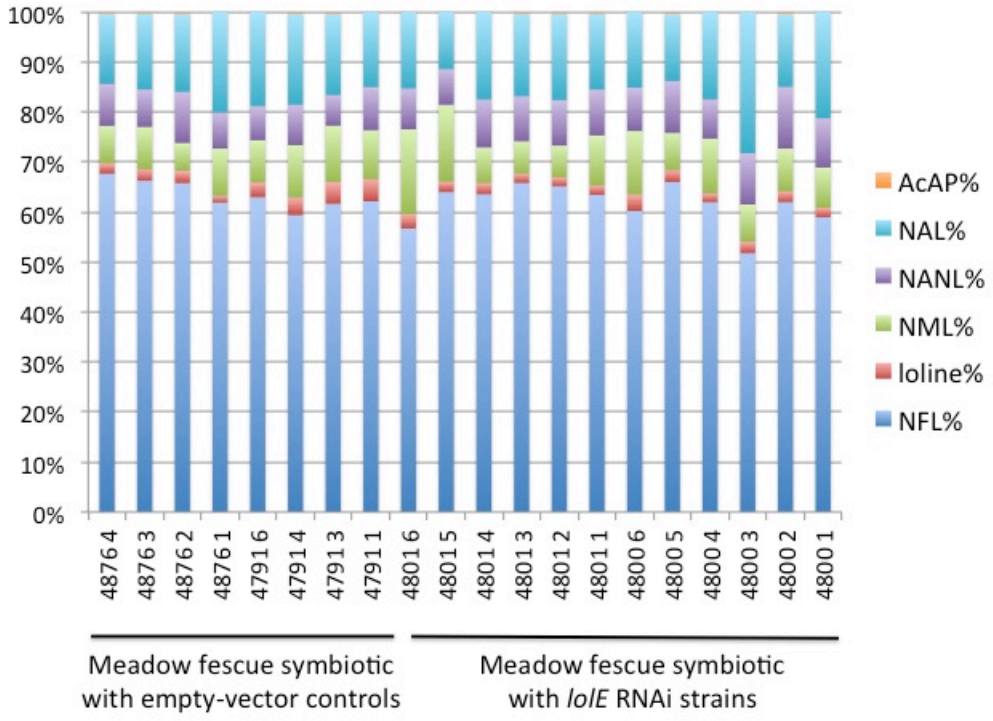


Figure 2.13. Loline alkaloid profiles of meadow fescue symbiotic with different *E. uncinata* strains. Meadow fescue plants were inoculated with vector only control transformants (e4876 and e4791) or *lolE* RNAi transformants (e4800 and e4801). Each alkaloid level is indicated as a percentage of total loline alkaloids detected in the symbiotum. Different numbers after each plant designation indicate independent plant lines infected with the same fungal isolate.

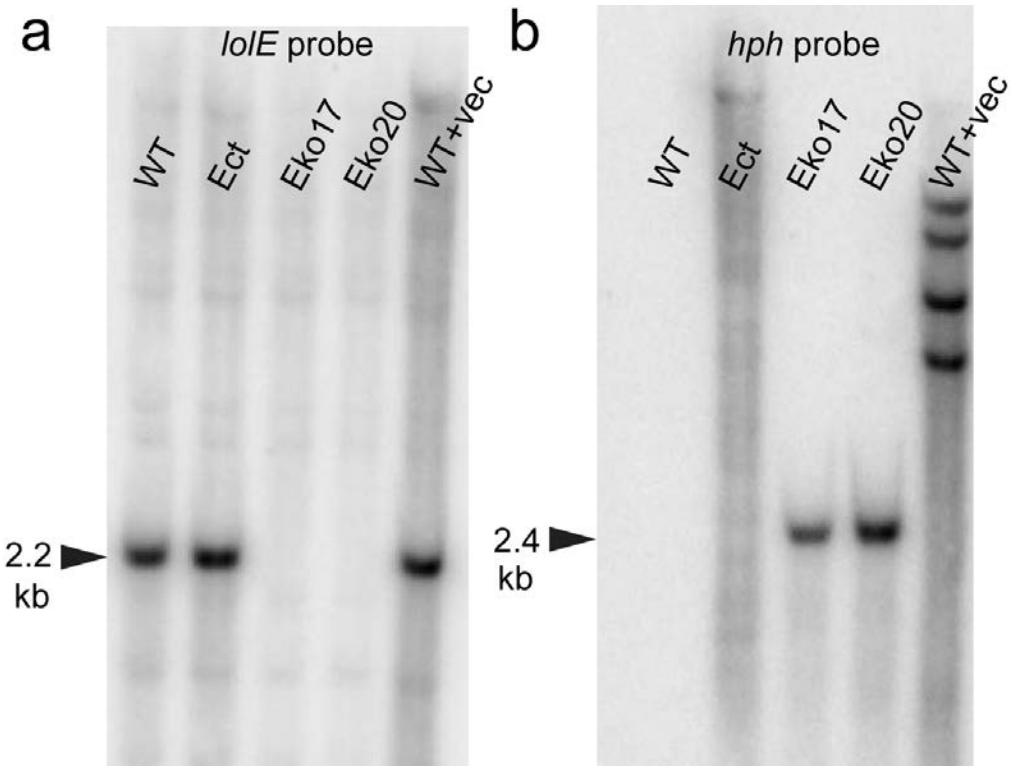


Figure 2.14. Southern-blot analysis of *E. festucae* E2368 wild type and transformants. The membrane was (a) probed with the *lolE* gene amplified from E2368, and (b) probed with *hph* amplified from pKAES173. Lanes contain *Xba*I-digested genomic DNA from E2368 (WT), *lolE* knockout transformants (Eko17 and Eko20), an ectopic transformant of E2368 with pKEAS322 (Ect), and E2368 transformed with the empty vector pKAES173 (WT+vec).

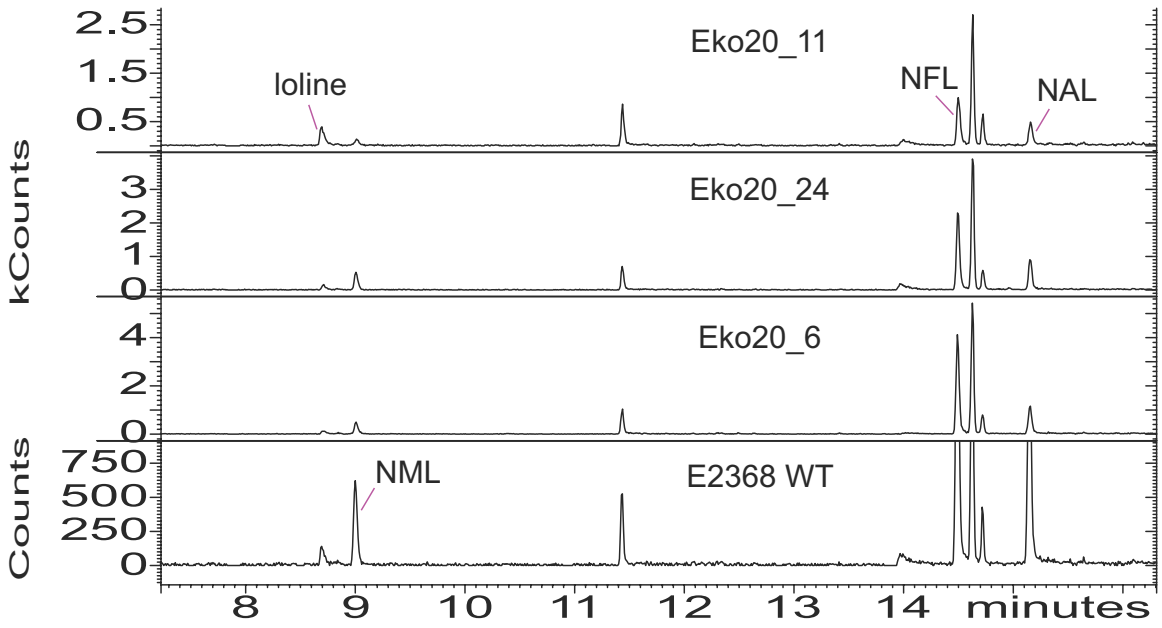


Figure 2.15. Loline alkaloid profiles of meadow fescue symbiotic with *lolE* knockout strain Eko20. Different numbers following the strain designation (Eko20) represent independently inoculated plants. The unlabeled peaks are impurities and non-loline alkaloid compounds extracted from plant.

2.3. Discussion

Loline alkaloids produced by endophytic fungi protect host grasses by affecting a large range of insects, so elucidation of the biosynthetic pathway aids the application of lolines in insect control in forage grasses. My evidence supports the hypothesis that LolO is responsible for ether bridge formation in biosynthesis of loline alkaloids. The lack of a functional *lolO* gene consistently correlated with accumulation of AcAP and the apparent lack of any loline alkaloids. Independent mutations in *lolO* were evident in *E. brachyelytri* E4804, *E. canadensis* e4814, *E. amarillans* E722, E721 and E862, and *A. hypoxylon* B4728, and the plants symbiotic with each of these accumulated AcAP. In the *lolO* RNAi experiment, AcAP consistently accumulated to high levels relative to the lolines. Furthermore, a copy of wild-type *lolO* complemented the natural *lolO* mutation in e4814, giving substantial levels of NANL. Yeast expressing *lolO* converted AcAP to NANL, and [$^2H_4$]AcAP applied to loline alkaloid-producing culture was converted to [$^2H_4$]NANL. All of these findings indicate that LolO is the only enzyme required to oxidize AcAP to form the ether bridge on NANL, and that both AcAP and NANL are true pathway intermediates.

Lolines have an unusual ether linkage of two unactivated carbons in the pyrrolizidine ring system. The previous finding that 1-AP forms prior to the ether bridge (Faulkner et al., 2006), and that six deuterium atoms from L-[U-$^2H_7$]Pro are retained in lolines (Pan et al., 2014 supplementary data) (Faulkner, 2011), eliminate several possible mechanisms commonly responsible for biological ether formation — including addition to double bonds and opening of epoxides (Dominguez de Maria et al., 2010) — suggesting an unusual mechanism for loline ether formation. We propose that the mechanism of ether bridge formation in lolines involves hydroxylation of one of the bridgehead carbons, abstraction of a hydride ion or hydrogen atom from the other, and then ether bridge formation. Figure 2.16 shows possible routes via radical intermediates; carbocation intermediates are also possible.

Considering that the proposed mechanism should require two successive oxidation/oxygenation steps, I tested the possibility that two oxidizing enzymes are involved. Another predicted non-heme iron oxygenase, LolE, seemed a likely candidate to function along with LolO in this process. Yeast expression of *lolO*, however, resulted in conversion of AcAP to NANL, indicating that LolO is probably capable of catalyzing the ether formation by itself, although this result does not exclude the possible involvement of two enzymes in natural conditions. Knocking down *lolE* expression did not change the loline alkaloid profile in culture or in symbiota, and the *lolE* knockout strain showed the same loline alkaloid profile as the wild-type strain, indicating that *lolE* is not required in ether bridge formation in natural conditions. In summary, it is likely that LolO is the only enzyme that catalyzes ether formation in loline alkaloid biosynthesis. Such a finding has precedence. An example of one enzyme that catalyzes several oxidizing steps in a pathway is clavaminate synthase, which is also a non-heme iron oxygenase. This enzyme catalyzes three oxidative steps, two of which function to form the ether linkage in clavaminic acid (Zhang et al., 2000). The function of LolE is currently a mystery.

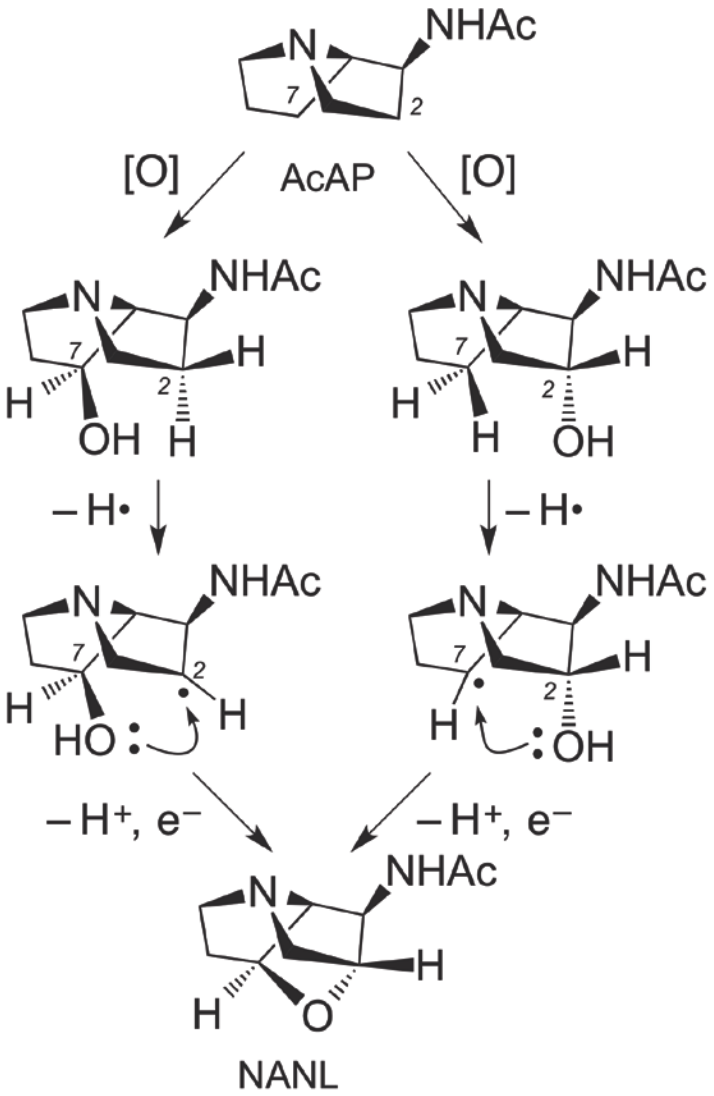


Figure 2.16. Possible pathways of ether bridge formation in loline alkaloid biosynthesis. An alternative mechanism (not shown) would involve carbocation at C2 or C7.

Conversion of AcAP to NANL by the LolO-expressing yeast, and conversion of [$^2H_4$]AcAP to [$^2H_4$]NANL in the grass-*Epichloë* symbiota and loline alkaloid-producing culture demonstrated that both AcAP and NANL are true pathway intermediates. Considering the previous evidence that 1-AP is a pathway intermediate, it is reasonable to predict that AcAP is generated by acetylation of 1-AP (Figure 2.17). Based on the observation that loline, norloline, NANL, NML**,** and NFL are found in many grasses, and the predicted functions of *LOL* genes in the cluster, we hypothesize that, after formation of AcAP, the loline biosynthetic pathway proceeds through deacetylation of NANL to form norloline by LolN, the predicted acetamidase (Figure 2.17). According to this hypothesis, serial methylations of norloline by LolM, the predicted methyltransferase, then produce loline and NML, the latter of which is known to be oxidized to NFL by the action of the cytochrome P450 enzyme, LolP (Spiering et al., 2008).

Neither of the alkaloids *exo*-AcAP or *endo*-AcAP has been previously reported. I purified AcAP from *El. canadensis* plants symbiotic with *E. canadensis* e4814 to compare it with synthetic (±)-*exo*-AcAP (Pan et al., 2014). The NMR spectra (^1H, ^{13}C, COSY, NOESY, and HSQC) and GC-MS of the synthetic (±)-*exo*-AcAP and the isolated AcAP were very similar, and the GC and the ^1H and ^{13}C NMR spectra of an equimolar mixture of synthetic and isolated material showed only a single compound, establishing with high confidence that the isolated natural compound was *exo*-AcAP. This assignment is consistent with both the known configuration of the loline alkaloids and previous work showing that *exo*-1-AP is an intermediate in the biosynthetic pathway of loline (Faulkner et al., 2006).

Lolines are approximately as insecticidal as nicotine (Riedell et al., 1991), so they are probably a major factor in the protective effects of certain endophytes. For example, the loline-producing endophytes, *Epichloë occultans* in *L. multiflorum* and *E. coenophiala* in tall fescue, have documented activity on herbivorous insects as well as their parasitoids, playing an important role in arthropod diversity and food-web structures (Omacini et al., 2001, Rudgers & Clay, 2008). Notably, different lolines vary in specificity and impact on different insects (Riedell et al., 1991, Jensen et al., 2009, Popay et al., 2009). Considering that AcAP is the pathway end product that originated independently for several natural species of grass endophytes, it is possible that this compound is specifically selected in some environments, perhaps by variation against the dominant herbivore species. Hence, the biological activity of AcAP merits further study.

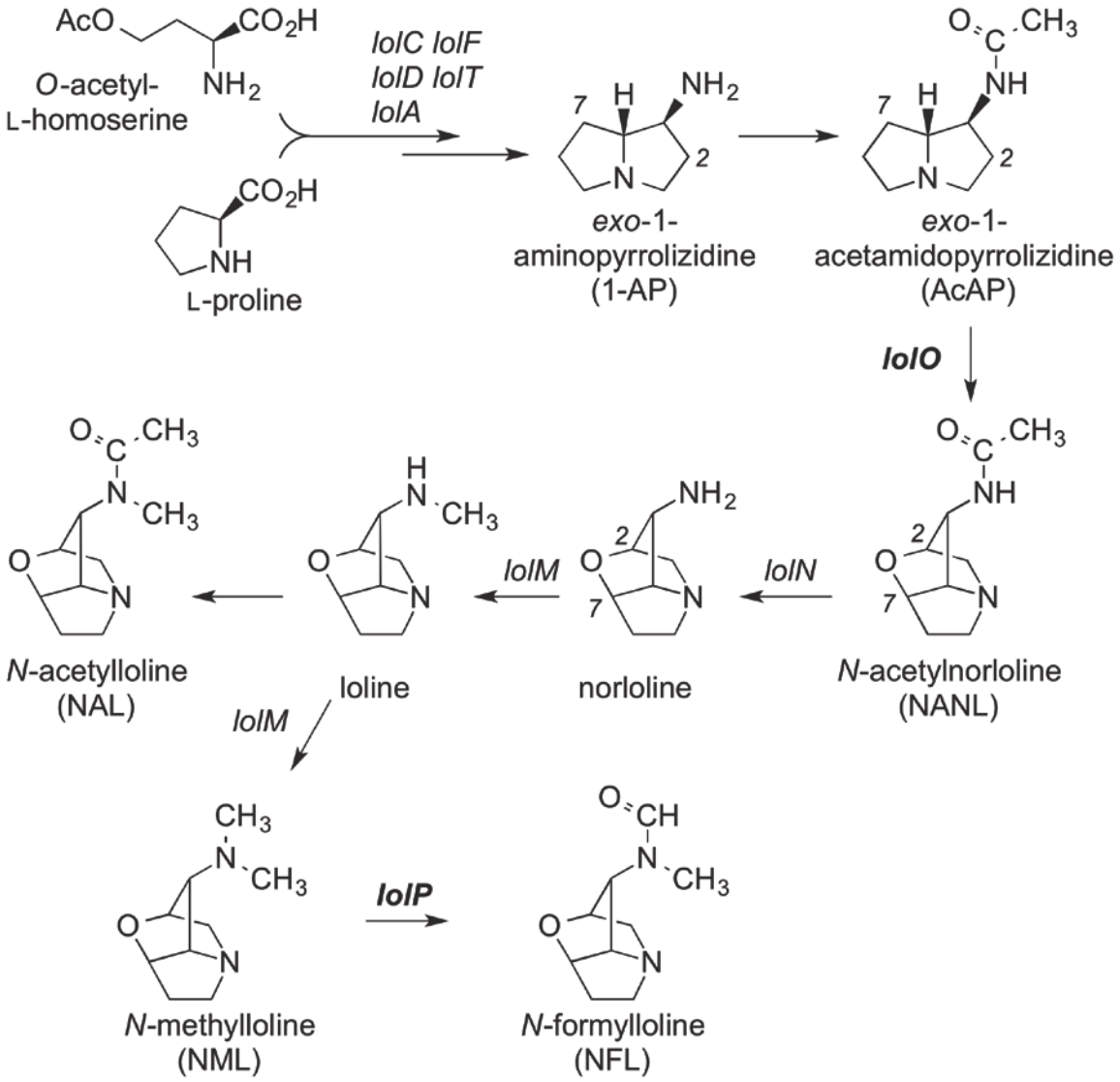


Figure 2.17. Proposed loline alkaloid biosynthetic pathway. Compounds shown are the known precursors, proposed intermediates, and known loline alkaloids produced *in symbio*. *LOL* genes with predicted functions are shown with arrows at hypothesized steps. Genes in bold indicate that functions have been established.

2.4. Concluding Remarks

Through genome sequencing and molecular genetic methods, LolO, a non-heme iron oxygenase, was revealed to be responsible for loline ether bridge formation. The mechanism of the reaction is not yet determined, but the finding that six deuterium atoms from L-[U-$^{2}H_{7}$]Pro were retained in loline, NANL, and NFL, together with the finding that the pyrrolizidine rings form before the ether bridge, ruled out several common biosynthetic mechanisms, such as those proceeding through epoxides. Thus, it is likely that formation of the ether bridge of lolines occurs through an unusual route, probably through a free radical or carbocation at C2 or C7. A novel natural product described here, *exo*-1-acetamidopyrrolizidine (AcAP), is established to be a pathway intermediate that is oxidized on C2 and C7 to form the ether bridge, giving rise to NANL, the first loline alkaloid in the pathway.

2.5. Materials and Methods

2.5.1. General experimental procedures. GC-MS was conducted using a Varian CP-3800 GC (Agilent Technologies, Santa Clara, CA, USA) and a Varian Saturn 2200 MS (Agilent Technologies). GC was equipped with an Agilent J&W DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 um thickness). Helium at 1 ml/min was used as the carrier gas with an injection temperature of 250 °C. Column temperature was initially 75 °C, increased to 225 °C at 8 °C/min, then increased to 300 °C at 25 °C/min, and held for 5 min for a total run time of 27 min. For GC-MS detection, an electron ionization system was used with ionization energy of 70 eV. Mass range was set at 50-250 m/z with a filament delay of 4 min.

Fourier transform ion cyclotron resonance (FTICR) mass spectrometry was performed using a Thermo Electron LTQ-FT with Advion Nanomate nanoelectrospray source operating in the positive mode. Fragmentation in the ICR cell used infrared multi-photon dissociation (IRMPD) with 80 msec pulse following a 2 msec delay. Spectra were acquired using a resolving power of 100,000 @ 400 m/z, resulting in resolution of much better than 1 ppm across the measured m/z range. The instrument was externally calibrated to protonated reserpine.

DNA cloning was by standard methods, and plasmids were grown in *Escherichia coli* XL1-Blue (Agilent Technologies). All plasmid DNA was isolated from bacterial cultures (LB medium at 37 °C for 20 h on a rotary shaker at 200 rpm) with ZR Plasmid Miniprep-Classic kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. Fungal genomic DNA was isolated with ZR Fungal/Bacterial DNA MiniPrep (Zymo Research) following the manufacturer's instructions. Gene screenings by polymerase chain reaction (PCR) were conducted with AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) in manufacturer-provided PCR buffer with 1.5 mM $MgCl_2$. For gene cloning, PCR was performed with Phusion Hot Start High-Fidelity DNA Polymerase (Thermo Scientific, Ratastie, Vantaa, Finland) with provided HF buffer with 1.5 mM $MgCl_2$. All oligonucleotide primers were ordered from Integrated DNA Technologies (Coralville, Iowa, USA), and are listed in Table 2.1.

Table 2.1. Oligonucleotides used in this chapter. [a]

Primer name	Sequence[a]
lolOrnaiF1	GCGATATCATGACGGTAACAAACAAGCCTG
lolOrnaiR1	CCTCTAGAAATGCAGCCAGGCGAATGCTTACCTCGAGCG
lolOrnaiF2	ATTTCTAGAGGCACACAAGATCAATTAGCGATCC
lolOrnaiR2	ACTACTAGTATGACGGTAACAAACAAGCCTG
lolAkoupf	GCGCGGCCGCGAGCTAACCATGCATGGTGT
lolAkoupr	GCTCTAGATTCATCGAGCATCGTTAGAATA
lolOs1	ACCTGCCTCTGGCGGTCAAG
lolOr	CTTGCGCTCATACTCAAGAGC
lolO cDNA 3-5	CTCCGCCATCTGCCGTTG
lolO-F1	GTGAACTGGCAGTAGTCCGTATG
lolO-R2	AATCCATGCCAGTGTCGGGAATG
upFLKf	GCTCTAGATTTAAATGTTCTCCGTAAGGATAAGCATC
upFLKr	GCTCTAGAGTGGTGGACTGGTGAGGACT
hphf	TCTCGTGATTCTTTCCATC
hphr	CGGATCGGACGATTGCGT
lolEkoupf	CGCTCGAGCTAGTGGACAGGTTATTA
lolEkoupr	CGCCCGGGATGGATTACGTGAAACA
lolT35s	ACGGTGAAGATACCAG
Eexpr2	GCACGCGTCAAGTCTGCGCTCCCACT

[a] Underlined segments indicate restriction-endonuclease cleavage sites incoporated in the primers to facilitate cloning.

2.5.2. Biological materials. Fungal isolates were cultured from infected plants as previously described (Blankenship et al., 2001), and are listed in Table 2.2. The strain designations refer to the plant numbers from which they were isolated, with prefix of "E" for sexual *Epichloë* species, "e" for asexual epichloae, and "B" for *Atkinsonella* (*Balansia*) *hypoxylon*.

2.5.3. Loline alkaloid analysis. Grass materials used in this study were all clipped from the crown, lyophilized and ground. For GC-MS analysis, 100 mg of ground plant material was extracted with NaOH (100 µl, 1 M) and loline-alkaloid extraction solution (1 ml, 99% chloroform with 1% quinoline as internal standard). The mixture was agitated for 1 h at room temperature, then centrifuged at 16 x 10^3 *g* for 10 to 15 min. The organic phase was transferred to a capped glass vial for GC-MS analysis as previously described (Faulkner et al., 2006).

Loline alkaloid production by *E. uncinata* e167 and the transformed strains was induced in minimum medium (MM) as in Blankenship et al. (2001), with 15 mM urea as the nitrogen source and 20 mM sucrose as the carbon source. Fungal isolates were ground in sterile water and inoculated to 30 ml of MM in 100 x 25 mm polystyrene Petri dishes. After 13-20 days shaking at 21 °C, the medium was sampled for loline alkaloids analysis by GC-MS, as previously described (Blankenship et al., 2005).

2.5.4. Application of [$^2H_4$]AcAP to loline inducing medium. The labeled compound [$^2H_4$]AcAP was applied at the onset of loline alkaloid production (9th day post inoculation) at a final concentration of 3.75 mM. L-[U-$^2H_7$]Pro (4 mM) or Pro (4 mM) was applied following same procedure as control. To each of the treatment DMSO (1.5 %) was also added to increase permeability of the cells. The cultures were shaken at 55 rpm, 22 °C, for another 35 days and then checked loline alkaloids.

2.5.5. Plasmid construction. Plasmid construction for *lolO* RNAi is described in Pan et al. (2014), and plasmid preparation for *lolE* RNAi is described in Faulkner, 2011.

To knockout *lolE*, a plasmid with flanking regions of *lolE* in *E. festucae* E2368 and a modified *hph* maker gene (Tsai et al., 1992) in the middle was transformed to E2368 to replace *lolE* with *hph* marker through homologous recombination. Using E2368 as the template, a 2.5 kb 3' flanking region of *lolE* was amplified with primers upFLKf (*Xba*I) and upFLKr (*Xba*I), digested with *Xba*I, and ligated with pKAES173 (Spiering et al., 2008) digested with *Xba*I. The ligated product was introduced by transformation into *Escherichia coli* XL1-blue competent cells, and transformants were screened for *hph* by colony-PCR with primers hphf and hphr. Positive colonies were extracted for plasmid DNA, which was then tested by restriction-endonuclease digestions and Sanger sequencing to identify the construct that had the correct orientation. The proper construct was then digestion with *Xma*I and *Xho*I. A 2.4 kb 5' flanking region of *lolE* was amplified with lolEkoupf (*Xho*I) and lolEkoupr (*Xma*I). The digested plasmid and PCR product were gel purified and ligated to generate pKAES322, which has the flanking regions of *lolE* in the same orientation and *hph* expression construct in the middle.

Table 2.2. Origins and source information for fungal strains used in this chapter.

Fungus	**Isolates**	**Host**	**Origin**
Atkinsonella hypoxylon	B4728	*Danthonia spicata*	Lexington, North Carolina, USA
Epichloë amarillans	E57	*Agrostis hyemalis*	Brazoria Co., Texas, USA
Epichloë amarillans	E721, E722, E862	*Sphenopholis obtusata*	Georgia, USA
Epichloë brachyelytri	E4804	*Brachyelytrum erectum*	Edmonson Co., Kentucky, USA
Epichloë canadensis	e4814[a]	*Elymus canadensis*	Nuevo León State, Mexico
Epichloë canadensis	e4815[a]	*Elymus canadensis*	Throckmorton Co., Texas, USA
Epichloë coenophiala	e19	*Lolium arundinaceum*	Lexington, Kentucky, USA
Epichloë festucae	E2368	*Lolium pratense*	Lexington, Kentucky, USA
Epichloë uncinata	e167	*Lolium pratense*	Nyon, Switzerland

[a] Strains e4815 and e4814 are the equivalent of CWR 5 and CWR 34/36 in Charlton et al. (2012).

For *lolO* complementation, a DNA segment including *lolO* and the entire flanking intergenic regions was amplified from DNA of *E. festucae* E2368, by PCR with primers lolAkoupf and lolAkoupr (98 °C for 5 min, 35 cycles of 98 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min). The PCR product was purified and digested with *Xba*I, the site for which was incorporated in the primer lolAkoupr. Plasmid pKAES215 was digested with *Xba*I and *Sma*I. The two fragments were then gel purified and ligated to produce plasmid pKAES309, which has *lolO* of *E. festucae* E2368 under its own promoter.

2.5.6. Fungal transformation. *Epichloë canadensis* e4814 was grown in 50 ml of potato dextrose broth for 5 days at 22 °C with rotary shaking (200 rpm). Fungal mycelium was harvested by centrifugation at 4885 *g,* resuspended in 3.75 mg/ml vinoflow (Novozymes, Bagsvaerd, Denmark), 5 mg/ml driselase (InterSpex products, San Mateo, CA), 0.7 mg/ml zymicase I (InterSpex Products), 5 mg/ml lysing enzymes (Sigma, St. Louis, MO), and 2.5 mg/ml bovine serum albumin (Sigma) in osmotic solution (1.2 M $MgSO_4$, 50 mM sodium citrate, pH 6.0), and gently agitated 3 h at 30 °C. Protoplasts isolation and electroporation were performed as described by (Tsai et al., 1992).

After electroporation, the protoplasts were mixed with 5 ml of regeneration medium (Panaccione et al., 2001) and plated onto potato dextrose agar (PDA) (20 ml) with 125 µg/ml hygromycin B, so the final concentration of hygromycin B over the whole plate (25 ml) was 100 µg/ml. Plates were kept at 22 °C and checked after ca. 2 wk. Fungal colonies were transferred to PDA plates with 100 µg/ml hygromycin B and then single-spore isolated two times before further analysis. Transformation of E2368 to knock out *lolE*, and selection of the transformants, were following the same procedure except that 400 µg/ml of hygromycin B was used.

Transformants were introduced into host plants by the method of Latch and Christensen (1985), by inoculation of seedlings from endophyte-free seed lots. When the plants had at least three tillers, a tiller from each was sacrificed for tissue-print immunoblot as previously described (An et al., 1993).

2.5.7. Purification of* exo-*1-acetamidopyrrolizidine (*exo-*AcAP). Approximately 10 g lyophilized *Elymus canadensis* plant 4814 tillers were used to extract AcAP with the loline extraction method described in part 5.3. The extract was chromatographed twice on 1 mm thick silica gel prep. TLC plates (EMD Chemicals, Darmstadt, Germany) (Blankenship et al., 2005). Purified AcAP was recovered in deuterated chloroform and analyzed by NMR. Compound AcAP was named *N*-[(1*S*,7a*R*)-hexahydro-1*H*-pyrrolizin-1-yl]acetamide according to IUPAC nomenclature and was given the common name, *exo*-1-acetamidopyrrolizidine. Purified AcAP showed ^{1}H NMR (600 MHz, $CDCl_3$, 0.10 M): d 1.59 (dq, J_d = 12.6 Hz, J_q = 7.4 Hz, 1H), 1.72 (dq, J_d = 13.8 Hz, J_q = 7.1 Hz, 1H), 1.73 (dq, J_d = 13.8 Hz, J_q = 7.1 Hz, 1H), 1.83 (m, 1H), 1.96 (s, 3H), 1.95-2.01 (m, 1H), 2.17 (dq, J_d = 12.5 Hz, J_q = 6.2 Hz, 1H), 2.61 (dq, J_d = 11.0 Hz, J_q = 6.7 Hz, 2H), 3.03 (dt, J_d = 10.6 Hz, J_t = 6.4 Hz, 1H), 3.23 (dt, J_d = 11.0 Hz, J_t = 6.0 Hz, 1H), 3.27 (~q, ~6.4 Hz, 1H), 4.12 (~quintet, 6.5 Hz, 1H), 6.10 (bs, 1H). ^{13}C{H} NMR (100 MHz, $CDCl_3$, 0.10 M): d 23.6, 25.5, 30.7, 32.7, 53.3, 55.1, 55.3, 70.8, 170.2. EI-MS (70 eV) *m/z* (rel. int.) M^+ 169 (12.5), 109 (77.5), 108 (100), 83 (97), 82 (52.5), 55 (42.5).

2.5.8. Genome sequencing. All genome sequencing and assembly was conducted at the University of Kentucky Advanced Genetic Technologies Center. Genome assemblies of *E. festucae* E2368, *E. amarillans* E57, and *E. brachyelytri* E4804 were described previously (Schardl et al., 2013b). The genome of *A. hypoxylon* B4728 was sequenced on a Roche/454 Titanium pyrosequencer configured for extended reads (average read length 722 nt; 32-fold coverage). Assembly was as described previously (Schardl et al., 2013b). Assemblies were uploaded to the National Center for Biotechnology Information (NCBI) (GenBank ID: ADFL01000000 for E2368, AFRB01000000 for E4804, and AFRF01000000 for E57, and PRJNA221544 for B4728), and are provided with annotations on GBrowse web sites (www.endophyte.uky.edu). Loline gene sequences are deposited in NCBI (GenBank ID: JF830812, JF830813, JF800659, JF800661, JF800660, JF830815, JF830814, JF830816, and KF056806).

2.5.9. Analysis of* lolO *gene. DNA was purified from endophytes and a fragment of *lolO* was amplified by PCR with primer pairs: lolO-F1 and lolO-R2 (Table 2). PCR was performed at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. The products were sequenced with the PCR primers.

2.5.10. RNA extraction and RT-PCR. RNA from plant materials were extracted with RNeasy Plant Minikit (Qiagen), removed contaminating DNA using DNA-free™ kit (Applied Biosystems), and reverse transcribed with high-capacity cDNA Reverse Transcription kit (Applied Biosystems). The resulting cDNA was used as templates to amplify a *lolO* fragment by PCR with primers lolOs1 and lolOr. The PCR products were then sequenced with primers lolOs1 and lolO cDNA 3-5.

2.5.11. Application of L-[U-$^2H_7$]Pro to plants. A single tiller of *El. canadensis* plant 4814, cut above the first node, was placed in a test tube with 600 µl of the Murashige and Skoog Medium (MS medium) (MP Biomedicals, Solon, OH, USA), pH 7.4 with 4 mM L-[U-$^2H_7$]Pro in a 1.7 ml microcentrifuge tube, and maintained at 25 °C, 16 h light, until all the medium was consumed. A total of 10 tillers were used for both L-[U-$^2H_7$]Pro and control (Pro) feeding. The tillers from each experiment were pooled at the end of the feeding period and checked for loline alkaloids following the standard procedure described in section 5.3.

***2.5.12. Yeast expression of* lolO.** To express *lolO*, yeast strain YPH499 (MATa *ura3*-52 *lys2*-801^{amber} *ade*2-101^{ochre} *trp*1-Δ63 *his*3-Δ200 *leu*2-Δ1)(Sikorski & Hieter, 1989) (Agilent Technologies) was transformed of plasmid pKAES205, which has *lolO* under Gal10 promoter and a *N*-terminal FLAG tag. Yeast competent cell preparation and transformation was performed according to the manufacture's protocol, and transformants were selected on SD/-His minimal medium. The growing colonies were then streaked twice on the same medium before feeding experiment.

2.5.13. Application of AcAP or [$^2H_4$]AcAP to yeast. The yeast transformants of pKAES205, or vector pESC-HIS were grown in SD/-His minimal medium at 30 °C overnight, washed twice with inducing SG/-His minimal medium, and resuspended in the

same medium to achieve an OD_{600} of 0.5. AcAP or [$^2H_4$]AcAP was applied to the suspension at the final concentration of 4 mM. DMSO (1.5 %) was also added to increase permeability of yeast cell. The cultures was grown at 30 °C, 250 rpm for 3 days before sampling. The yeast cells were centrifuged at 1000 × g for 5 min and the supernatant was freeze-dried and extracted of loline alkaloids with routine extraction method and applied on GC-MS to check the profile.

Chapter Three

Enzymes from fungal and plant origin required for chemical diversification of loline alkaloids in grass-*Epichloë* symbiota

3.1. Introduction

The *Epichloë* species, symbionts of cool-season grasses, produce up to four known classes of alkaloids — ergot alkaloids, indolediterpenes, loline alkaloids, and peramine — which help the plant defend against herbivores. The asexual seed-transmitted *Epichloë* species are often found to be inter- or intra-species hybrids (Moon et al., 2004, Ghimire et al., 2011, Kang et al., 2011), which (among other benefits) can pyramid the alkaloid biosynthesis genes and promote diversification of alkaloid profiles (Schardl et al., 2013b, Schardl et al., 2013c). A likely benefit of diversifying defensive alkaloids is to reduce the potential for herbivores to develop resistance, provided that different alkaloids have different modes of specificity (Georghiou, 1972). Another advantage would be that different alkaloids may affect different herbivores and, thus, the chemotypic diversity would provide the host with broad-spectrum protection. The most prominent example would be that ergot alkaloids and indole-diterpenes are toxic to vertebrates, although some studies have also shown that ergots alkaloids might also be toxic to invertebrates, whereas loline alkaloids and peramine are specific against invertebrates (Schardl et al., 2013a). The ability of grass-*Epichloë* symbiota to produce a variety of protective alkaloids greatly enhances the plant defensive arsenal (Schardl & Chen, 2010).

Lolines are a class of saturated 1-aminopyrrolizidines with an unusual ether bridge on unactivated carbons 2 and 7, and differ in modifications of the 1-amino group: -NH_2 (norloline), -$NHCH_3$ (loline), -$N(CH_3)_2$ (*N*-methylloline = NML), -NCH_3Ac (*N*-acetylloline = NAL), -NHAc (*N*-acetylnorloline = NANL), and -NCH_3CHO (*N*-formylloline = NFL). Among loline alkaloids, differently modified forms have been reported to exert different effects on insects (Riedell et al., 1991). For example, at high concentrations (800 and 1600 μg/g), NFL reduces the growth and development of larvae of the Argentine stem weevil (*Listronotus bonariensis*), whereas NANL causes high mortality of Argentine stem weevil larvae but with little effect on their growth or development (Popay et al., 2009). These interesting observations raise the question of how the diverse loline alkaloids are synthesized and how profiles of different loline alkaloids have evolved in this grass-fungus symbiota.

Several genomes of loline alkaloid-producing *Epichloë* species have been sequenced, and a *LOL* cluster that encodes enzymes involved in loline biosynthesis has been found in all of them. The endophytes that produce NFL, as well as the other loline alkaloids, have a total of 11 *LOL* genes positioned in the gene cluster in the order *lolF*, *lolC*, *lolD*, *lolO*, *lolA*, *lolU*, *lolP*, *lolT*, *lolE*, *lolN* and *lolM* (Schardl et al., 2013b). Others that produce NANL or the bicyclic *exo*-1-acetamidopyrrolizidine (AcAP) as the end product have varied gene contents within the 11 aforementioned genes, and either lack or have inactivating mutations in late pathway genes (Schardl et al., 2013b). Functions of

enzymes encoded by *LOL* genes have been suggested based on bioinformatic analysis (Schardl et al., 2007). LolC has been proposed to catalyze an unusual γ-substitution reaction that condenses proline and homoserine, and, by means of an RNA-interference (RNAi) experiment, has been established to be involved in the loline alkaloid pathway (Spiering et al., 2005). A predicted cytochrome P450 enzyme, LolP, is required for oxygenation of NML to produce NFL, and is not involved in earlier biosynthetic steps (Spiering et al., 2008). Recently, it has been established that ether-bridge formation requires the 2-oxoglutarate-dependent non-heme iron oxygenase, LolO (Pan et al., 2014) (Chapter 2). In addition to the LolO and LolP steps, there are other loline alkaloid diversification steps that have yet to be identified. Here we establish the basis for the diversity of loline alkaloids, demonstrating that LolN and LolM are required for biosynthetic steps from NANL to NML, and also showing that the host plant converts loline to NAL.

3.2. Results and Discussion

3.2.1. The lolN *knockdown strains accumulated NANL both* in vitro *and* in symbio.

In the *LOL* gene cluster, *lolN* is predicted to encode an acetamidase, which we have predicted to deacetylate NANL (Chapter 2), the first loline alkaloid formed in the pathway, to produce norloline, which is then subjected to further modification. The function of *lolN* was tested by introducing an RNA-interference (RNAi) construct into *E. uncinata* strain e167. The construct was designed in such a way that it would be transcribed to give a double-strand RNA partially complementary to *lolN* mRNA. Three independent transformants with the *lolN* RNAi plasmid were cultured in loline alkaloid-inducing medium (Blankenship et al., 2001) and monitored the loline alkaloid profile. The wild-type strain e167 and the empty-vector transformant produced NFL as the major loline alkaloid, whereas the *lolN* RNAi transformants produced NANL as the major, or even the sole alkaloid produced in culture (Figure 3.1). These profiles were consistent during the loline-inducing period (Figure 3.2).

The *lolN* RNAi transformants and empty-vector control transformants were introduced back to endophyte-free meadow fescue, loline alkaloids were extracted, and profiles were analyzed by GC-MS. *N*-acetylnorloline constituted a substantially higher proportion of lolines in plants infected with the *lolN* RNAi transformants compared with plants infected with the empty-vector or wild-type strains (Figure 3.3). These results supported my hypothesis that the function of LolN is to catalyze deacetylation of NANL to produce norloline.

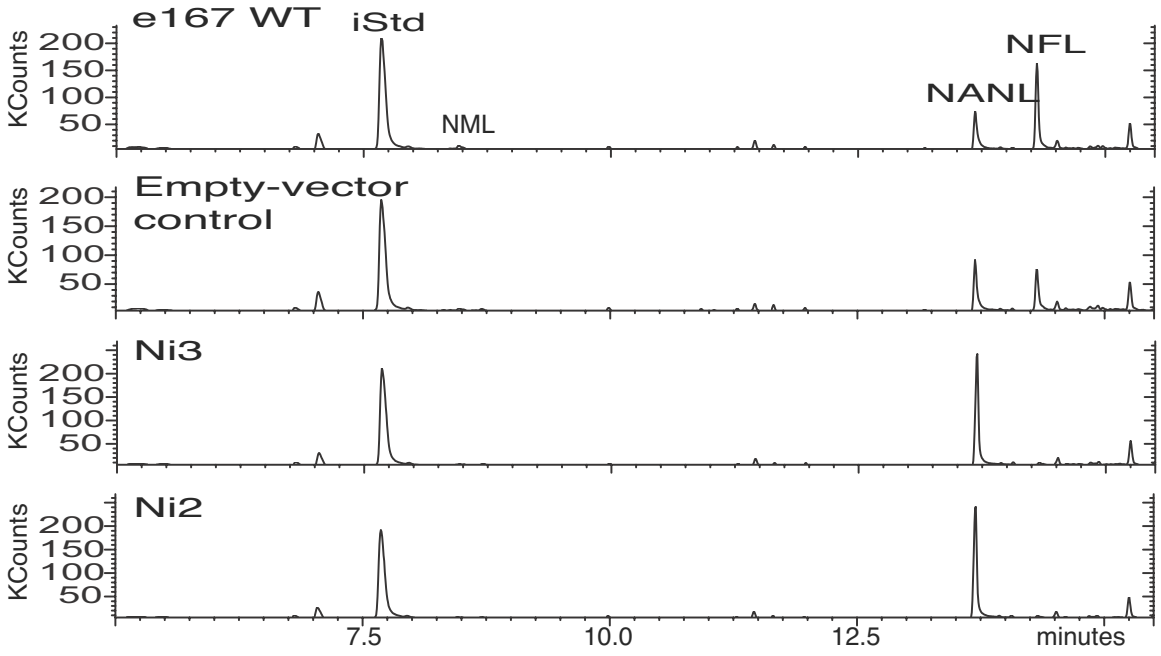


Figure 3.1. Chromatograms of loline alkaloids of *E. uncinata* strains in culture. Shown are loline alkaloid profiles of *E. uncinata* strain e167 wild-type control, empty-vector transformant of e167, and *lolN* RNAi strains Ni2 and Ni3. Quinoline was used as internal standard (iStd). Peaks that are not labeled are non-loline compounds.

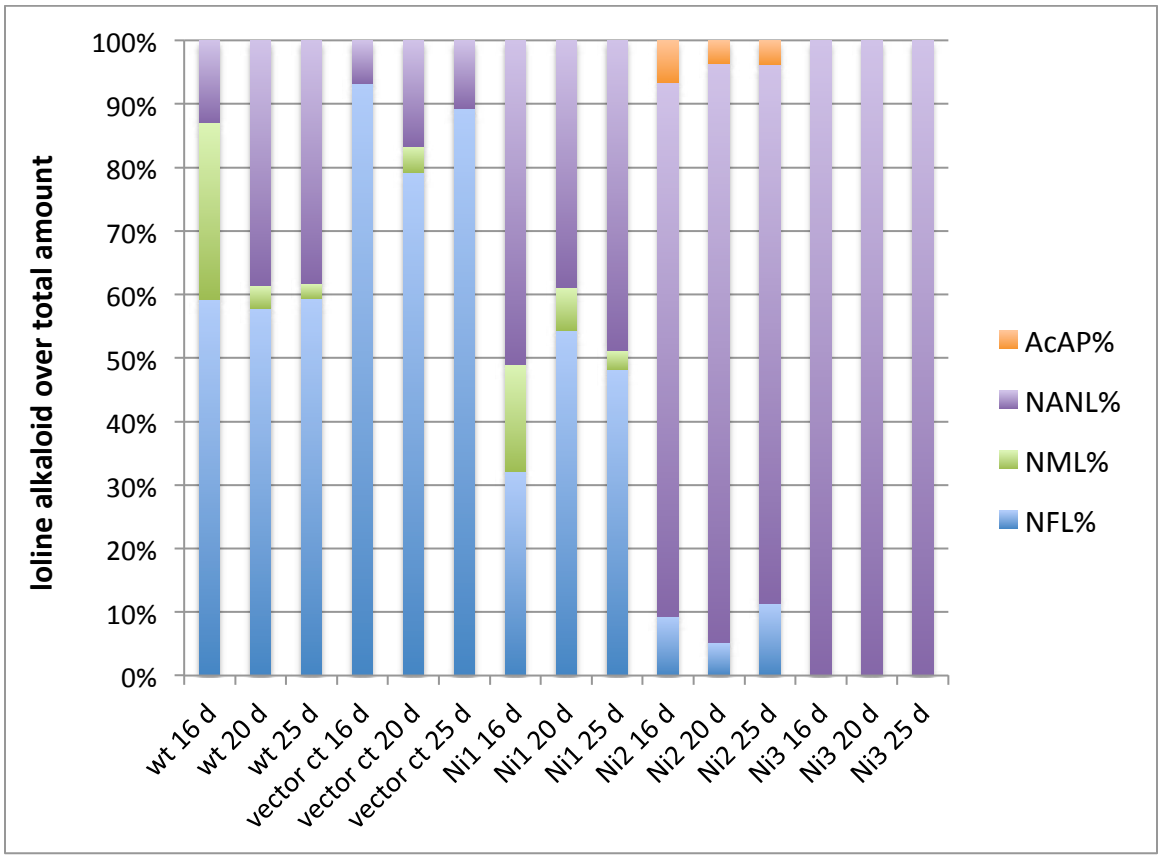


Figure 3.2. Loline alkaloid profiles in loline alkaloid-inducing culture at different time points. The *lolN* RNAi strains are shown as Ni, the numbers after them represent independent transformants; empty-vector control is shown as vector ct, and wild-type control is e167. The alkaloids were checked at three time points, 16 d, 20 d, and 25 d after inoculation, and the profiles are shown as contributions of each alkaloid relative to total loline alkaloids detected.

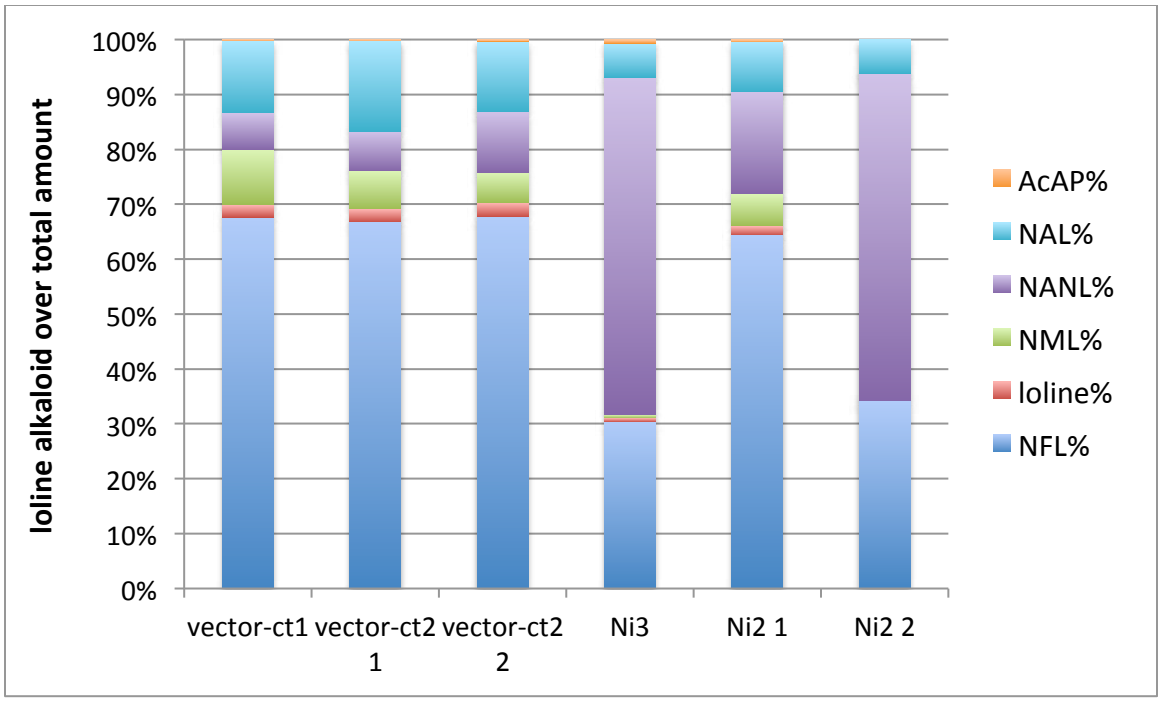


Figure 3.3. Loline alkaloid profiles of meadow fescue symbiotic with different *E. uncinata* strains. Shown are meadow fescue plants symbiotic with empty-vector control transformants (vector ct), or with *lolN* RNAi transformant Ni3 or Ni2. The different numbers after the plant designations represent independent inoculations with the same transformants. The loline alkaloid profiles are shown as contributions of each alkaloid relative to total loline alkaloids detected.

3.2.2 Deletion of the **lolN** *and* **lolM** *genes resulted in accumulation of NANL and eliminated NFL production.*

The *lolN* and *lolM* genes are found in all NFL-producing *Epichloë* species, and in the genome assembly of *Epichloë festucae* E2368 they are linked with the known *LOL* cluster (Schardl et al., 2013b). Bioinformatic analyses of these genes suggested that LolN is an acetamidase, and LolM is a methyltransferase. These functions fit well into our hypothesis that NANL must be deacetylated to produce norloline, which is then methylated to form loline and NML, the precursor of NFL (Spiering et al., 2008). Hence, it appears likely that LolN and LolM are involved in biosynthesis of NFL from NANL. In the *E. festucae* E2368 genome assembly, *lolN* and *lolM* shared a common, divergent promoter, so in order to investigate functions of *lolN* and *lolM*, I constructed a plasmid to replace both genes with the hygromycin-resistance marker gene, *hph*, through homologous recombination (Figure 3.4).

One *lolN* and *lolM* double-knockout transformant was obtained from 150 transformants. This knockout strain was analyzed by PCR with specific primers and the PCR products were sequenced. The expected fragments flanking the replaced *hph* marker gene, including the 3' *lolN* sequence and *lolT* and *lolE*, were intact (Figure 3.5a). Furthermore, PCR analysis of different *E. festucae* strains with primer pair aamAup1 and lolNMkops gave the 1.8 kb product expected from the *lolN-lolM* knockout strain, the 3.5 kb product expected from the wild-type strain, and both the 1.8 kb and 3.5 kb products expected from the ectopic controls (Figure 3.5b). The single PCR products from the knockout strain and wild-type control were sequenced and results confirmed that the knockout strain lacked *lolM* and the 5' portion of *lolN*. The knockout transformant was further confirmed by Southern-blot analysis to have a deletion affecting both genes (Figure 3.6).

The knockout strain, an ectopic transformant, and an empty-vector transformant were inoculated back into endophyte-free meadow fescue seedlings to check their loline-alkaloid profiles (*E. festucae* only produces lolines when symbiotic with host plants). Plants with the knockout mutant contained NANL as the only loline alkaloid detected, whereas plants with the control and wild-type strains contained NFL, NAL, and NANL (Figure 3.7). To confirm that the change of loline alkaloid profile was due to loss of the two genes, a complementation plasmid that had *lolN* and *lolM* under their native promoter was introduced into the knockout mutant. Three independent complementation strains were obtained and inoculated into meadow fescue to check the loline alkaloid profile. All three transformants had restored production of NFL and NAL *in planta* (Figure 3.7). These results indicate that *lolN* and *lolM* are required in the biosynthetic pathway from NANL to the end product, NFL.

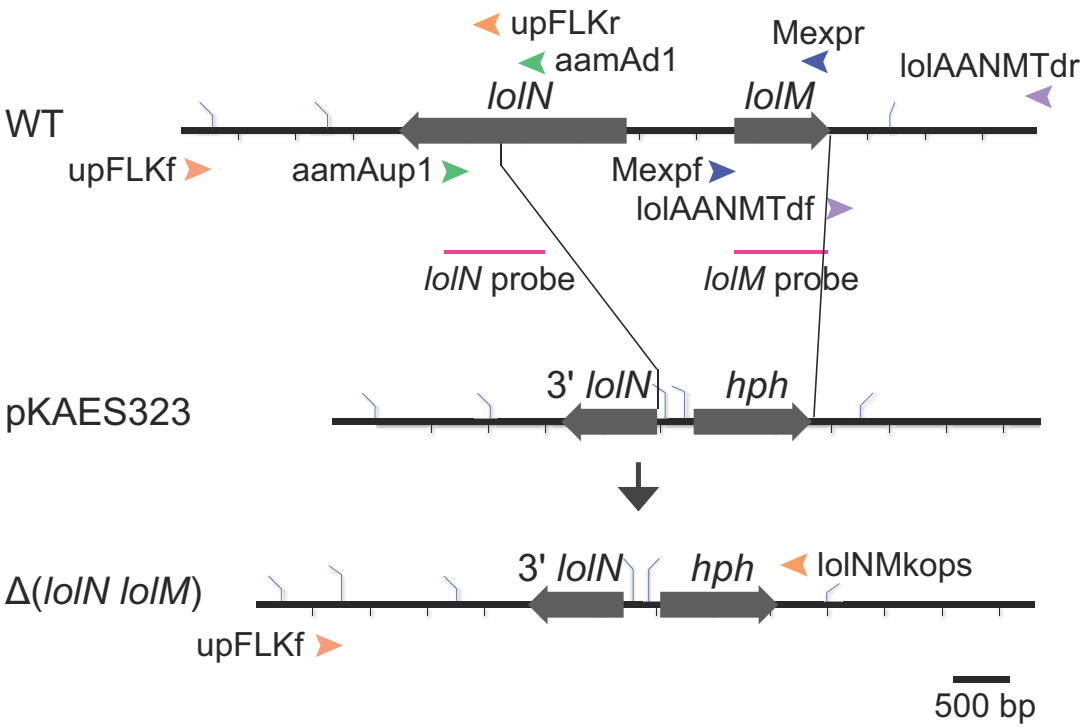


Figure 3.4. Schematic representation of *lolN*-*lolM* replacement by the *hph* marker gene via homologous recombination. Shown are the maps of the wild-type *lolN* and *lolM* in *E. festucae* E2368 (WT), targeting vector (pKAES323), and the locus after homologous recombination [Δ(*lolN lolM*)]. Black bars represent DNA sequence, and filled arrows represent genes. Bent blue lines on the bars represent *Hin*dIII digestion sites. Colored arrowheads represent primers used to amplify the flanking regions (upFLKf and upFLKr, and lolAANMTdf and lolAANMTdr) to generate pKAES323, for negative screening of the transformants and amplification of *lolN* probe (aamAd1 and aamAup1), for amplification of *lolM* probe (Mexpf and Mexpr), and for positive screening of the transformants (upFLKf and lolNMkops).

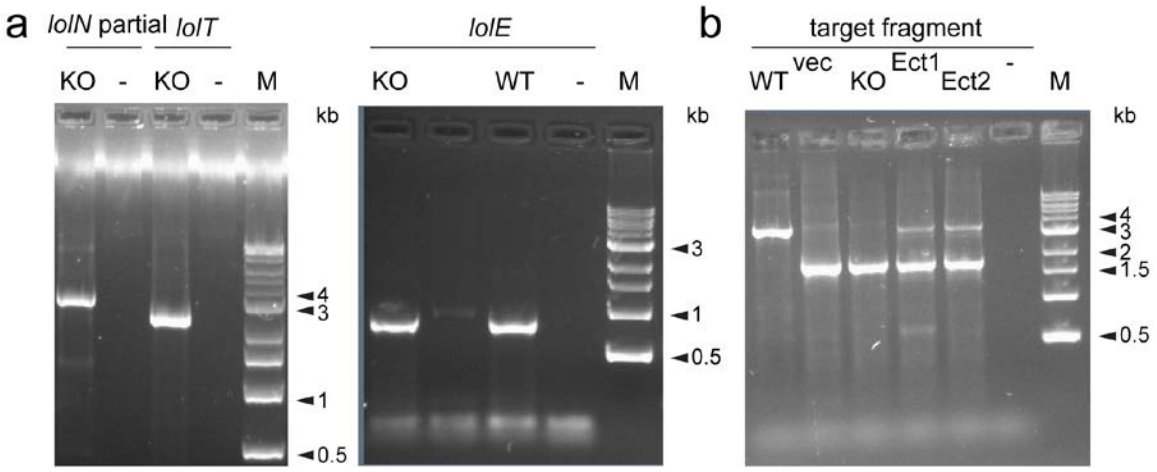


Figure 3.5. Confirmation of *lolN-lolM* knockout transformant by PCR. (a) PCR test of *E. festucae* E2368 *lolN-lolM* knockout strain for the presence of the expected 3'-*lolN* fragment (*lolN* partial), and presence of *lolT* and *lolE* genes that are linked with the *lolN-lolM* fragment in the *LOL* cluster. The *lolN* partial fragment was amplified with primers hph1u and lolEkosr, the *lolT* gene was amplified with lolEkoupf and lolEkoupr, *lolE* gene was amplified with lolEexpf and lolEexpr. (b) PCR amplification of the intact *lolN-lolM* fragment or *hph*-replaced fragment in *E. festucae* strains with primers aamAup1 and lolNMkops. PCR-analysis of the wild-type strain (WT) is expected to generate a fragment of 3.5 kb, PCR-analysis of the knockout strain (KO) and vector (vec) DNA is expected to generate a fragment of 1.8 kb, and PCR-analysis of the ectopic strains (Ect1 and Ect2) is expected to generate both fragments.

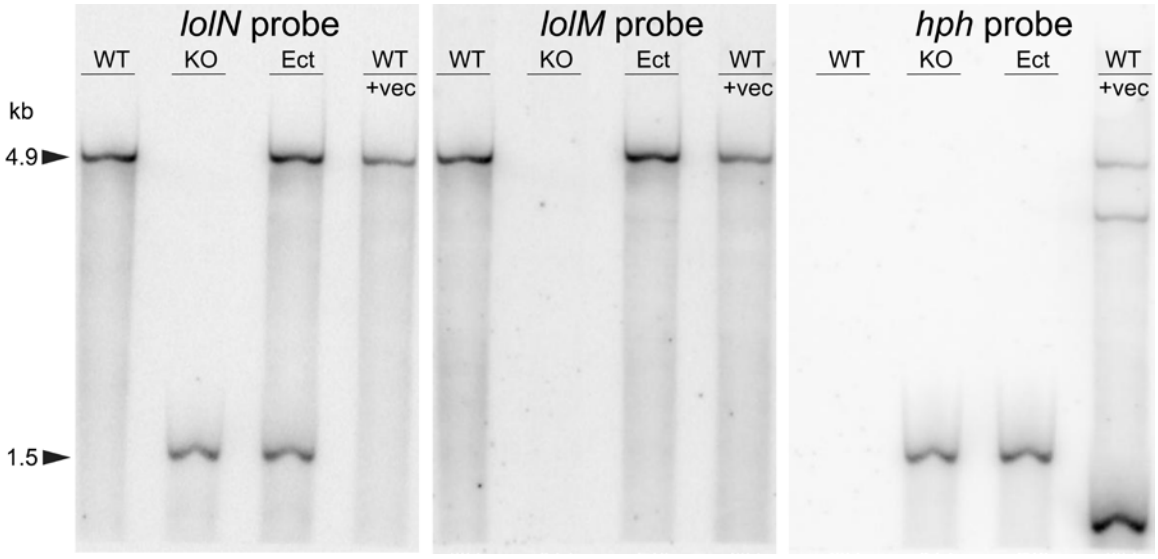


Figure 3.6. Southern-blot analysis of *E. festucae* strains. Wild-type strain E2368 and transformants were probed with a *lolN* fragment or *lolM* gene amplified from E2368 genomic DNA, or an *hph* segment amplified from vector pKAES173. Lanes contained *Hin*dIII-digested genomic DNA from E2368 (WT), *lolN*-*lolM* knockout transformant (KO), an ectopic transformant of E2368 with pKAES323 (Ect), and E2368 transformed with the empty vector pKAES173 (WT+vec).

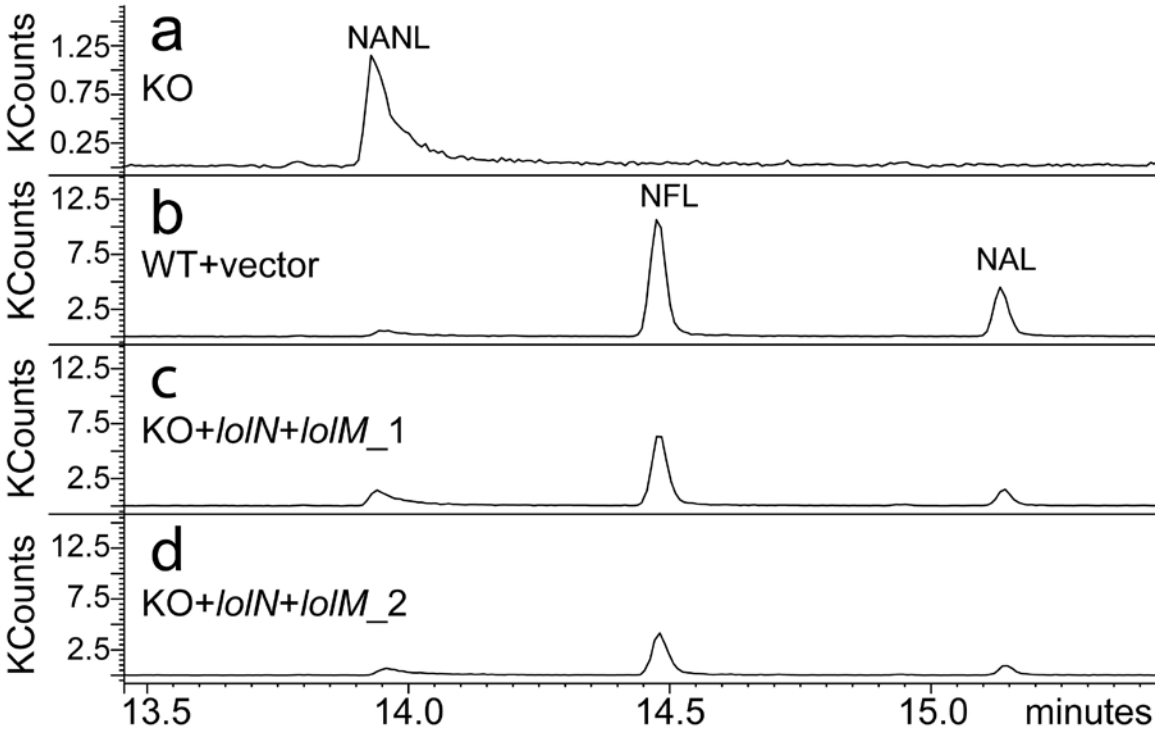


Figure 3.7. GC-MS total ion traces showing loline-alkaloid profiles of meadow fescue symbiotic with different *E. festucae* strains. The *lolN*-*lolM* knockout (KO), an empty-vector control transformant (WT+vector), and complementation strains (KO+*lolN*+*lolM*) were inoculated to E- meadow fescue and checked for loline alkaloid profiles. The numbers after complementation strains represent different meadow fescue plants inoculated with independent transformants.

3.2.3. Natural NANL accumulators have mutations in or deletions of lolN.

If *lolN* and *lolM* are indeed involved in the production of NFL from NANL as I hypothesize, it is conceivable that endophytes that produce NANL as the end product would have mutations in or deletions of *lolN*. In order to test this, *LOL* clusters of several loline-alkaloid producers were compared. Endophytes showing the three chemotypes, NANL, AcAP, or NFL as the end product, all shared most of the *LOL* genes except for *lolO*, *lolP, lolN* or *lolM* (Table 3.1). Plants symbiotic with strains that had mutations in, or complete lack of, *lolO* accumulated AcAP, consistent with the previously reported function of *lolO* (Pan et al., 2014) as discussed in Chapter 2 of this thesis. Interestingly, these strains also lacked functional copies of *lolN*, *lolM*, and *lolP*. Strains that had a functional *lolO* but lacked or had a mutated *lolN* all accumulated NANL. Conversely, those strains from plants that had NANL but not NFL, NML or NAL had lost or obviously defective *lolN*, *lolM* and *lolP* genes, with the sole exception of *E. coenophiala* strain e4309.

In contrast to other NANL accumulators, the *LOL* cluster of e4309 had the complete set of 11 genes with no obvious null mutation in any of them. Therefore, we aligned and compared *lolN* gene sequences and predicted amino acid sequences of e4309 to those of known NFL producers. LolN of e4309 had two nonsynonymous changes, G495D and E551K, compared to LolN of strains that produced NFL (Figure 3.8). Moreover, comparing strain e4309 to two other *E. coenophiala* strains that produce NFL (e19 and e4163), amino acid sequences of all *LOL* genes were identical except for the aforementioned G495D and E551K changes in *lolN* of e4309. Reverse transcription-PCR (RT-PCR) results indicated that *lolN* and *lolM* were expressed by e4309 *in symbio*. Thus, one or both of the two alterations in LolN observed in e4309 might have rendered the protein dysfunctional for deacetylation of NANL.

Results from experiments with the *lolN* and *lolM* double knockout, and comparison of *LOL* clusters of various species and strains, consistently showed that NANL accumulation was associated with *lolN* dysfunction, supporting its predicted function as an acetamidase that would deacetylate NANL to form norloline in the biosynthesis pathway (Figure 2.16).

Table 3.1. Loline alkaloid profiles and *LOL*-gene screening results for endophyte isolates.[a]

	Alkaloids						***LOL* genes**			
Endophyte isolates	AcAP	Loline	NANL	NAL	NML	NFL	*lolO*	*lolP*	*lolN*	*lolM*
E2368	tr	+	+	+	+	+	+	+	+	+
e167	tr	+	+	+	+	+	+	+	+	+
e19	tr	+	+	+	+	+	+	+	+	+
e4163	tr	+	+	+	+	+	+	+	+	+
E57	-	-	+	-	-	-	+	Ψ	-	-
E2772	-	-	+	-	-	-	+	-	Ψ	-
E4815	-	-	+	-	-	-	+	Ψ	-	-
e4309	-	-	+	-	-	-	+	+	+[b]	+
e4814	+	-	-	-	-	-	Ψ	Ψ	nd	nd
E4804	+	-	-	-	-	-	Ψ	-	Ψ	Ψ
E722	+	-	-	-	-	-	Ψ	-	Ψ	Ψ
B4728	+	-	-	-	-	-	Ψ	Ψ	-	-

[a] Abbreviations are: tr = trace amount; + = alkaloid detected or full gene present; - = alkaloid not detected or gene not present; Ψ = pseudogene; nd = gene not detected in PCR screen.

[b] Two nonsynonymous mutations found in otherwise conserved sites G495D and E551K.

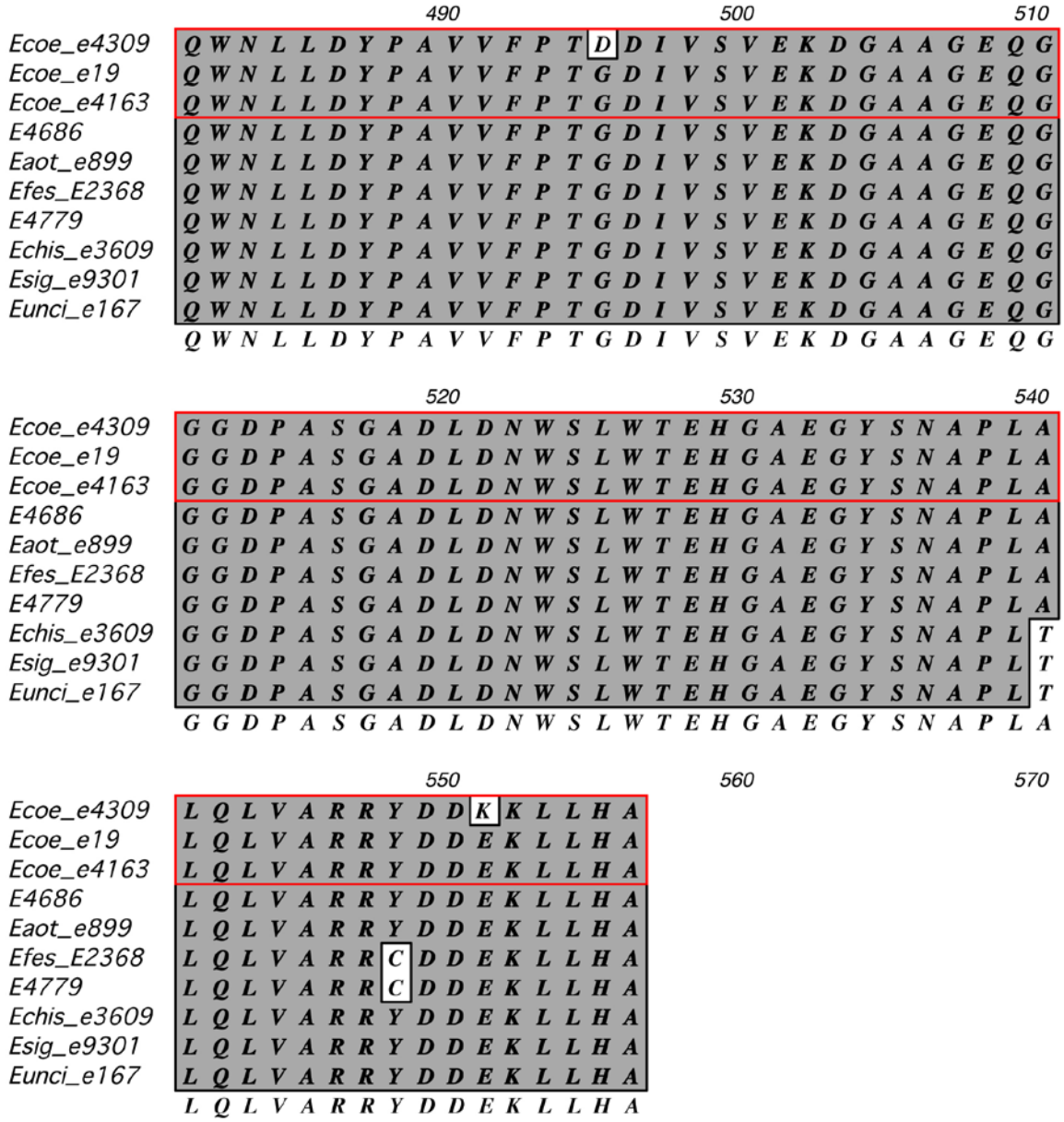


Figure 3.8. Partial LolN amino-acid sequence alignment of *E. coenophiala* e4309 and *N*-formylloline producers. Red-framed sequences are three different *E. coenophiala* isolates. *Ecoe = Epichloë coenophiala*, *Eaot = Epichloë aotearoae*, *Efes = Epichloë festucae*, *Echis = Epichloë chisosa*, *Esig = Epichloë siegelii*, *Eunci = Epichloë uncinata*.

3.2.4. LolM expressed in yeast catalyzed methylation of norloline and loline.

To test if the *lolM* gene product functions as a methyltransferase to methylate norloline in consecutive steps to form loline and NML, I expressed *lolM* in yeast. Heterologous expression of FLAG-tagged LolM in yeast was confirmed by western blot using antibody against the FLAG epitope (data not shown). Interestingly, the N-terminal FLAG-tagged LolM showed a strong signal by western-blot analysis, but the C-terminal tagged protein showed a much weaker signal. Yeast strains were incubated with loline in inducing medium (galactose as C source), and the alkaloid profiles were checked. A substantial amount of NML was produced in the yeast expressing LolM compared to the empty-vector control (Figure 3.9). Further, crude protein extract from LolM-expressing yeast was prepared and incubated with *S*-adenosylmethionine (AdoMet) plus either loline or norloline. The norloline was methylated to produce loline and a smaller amount of NML (Figure 3.10). Extract of control yeast with the empty-vector failed to catalyze methylation of norloline. Similarly, the protein extract from LolM-expressing yeast catalyzed the conversion of almost half of the added loline to NML, whereas no increase in NML profile was observed after incubating loline and AdoMet with protein extract from yeast transformed with empty vector (Figure 3.10). Again, it was found that although LolM tagged on either end catalyzed methylation of the applied norloline or loline, more conversion was observed with the N-terminal tagged LolM than the C-terminal tagged enzyme (data not shown). These results demonstrated that LolM was responsible for methylation of norloline to loline, and loline to NML.

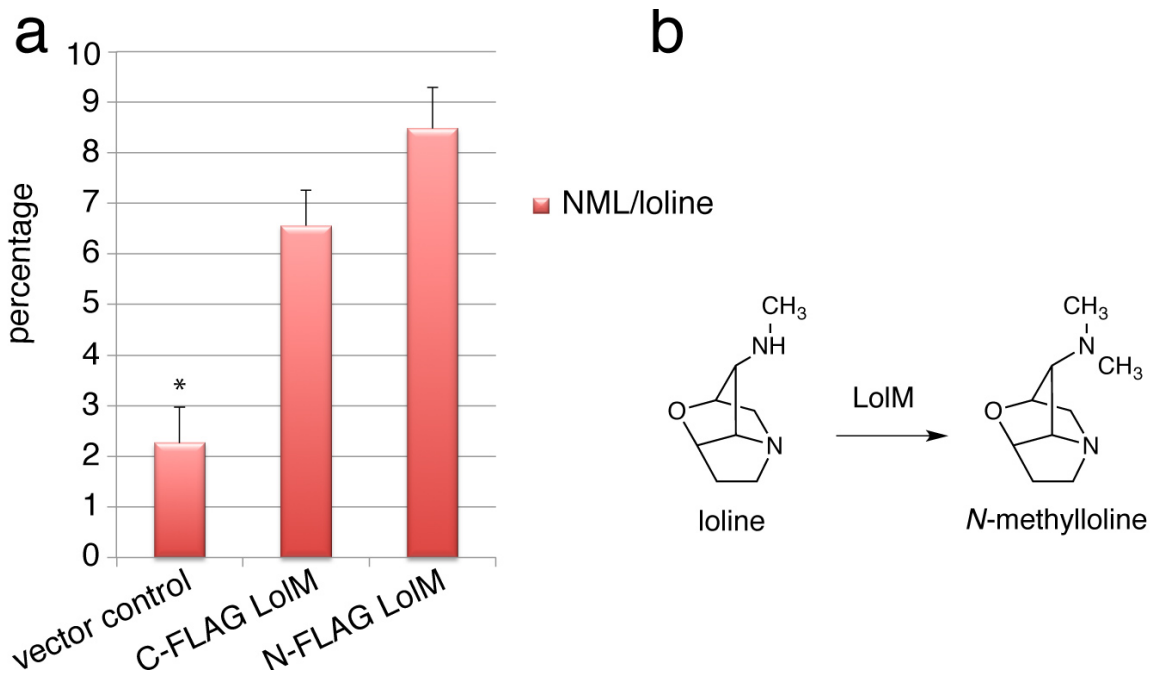


Figure 3.9. Methylation of loline by LolM-expressing yeast. (a) Ratio of NML and loline in the loline alkaloid extract after incubation. Yeast was transformed with empty-vector, or *lolM* expression construct which had a C-terminal or N-terminal FLAG tag. The alkaloid preparation used for this assay contained mainly loline, but also a small amount of NML. Therefore, production of NML in the yeast system is indicated as a ratio of NML over loline amounts. Error bars are standard error of the mean. (b) Scheme of the proposed reaction catalyzed by LolM that converts loline to NML.

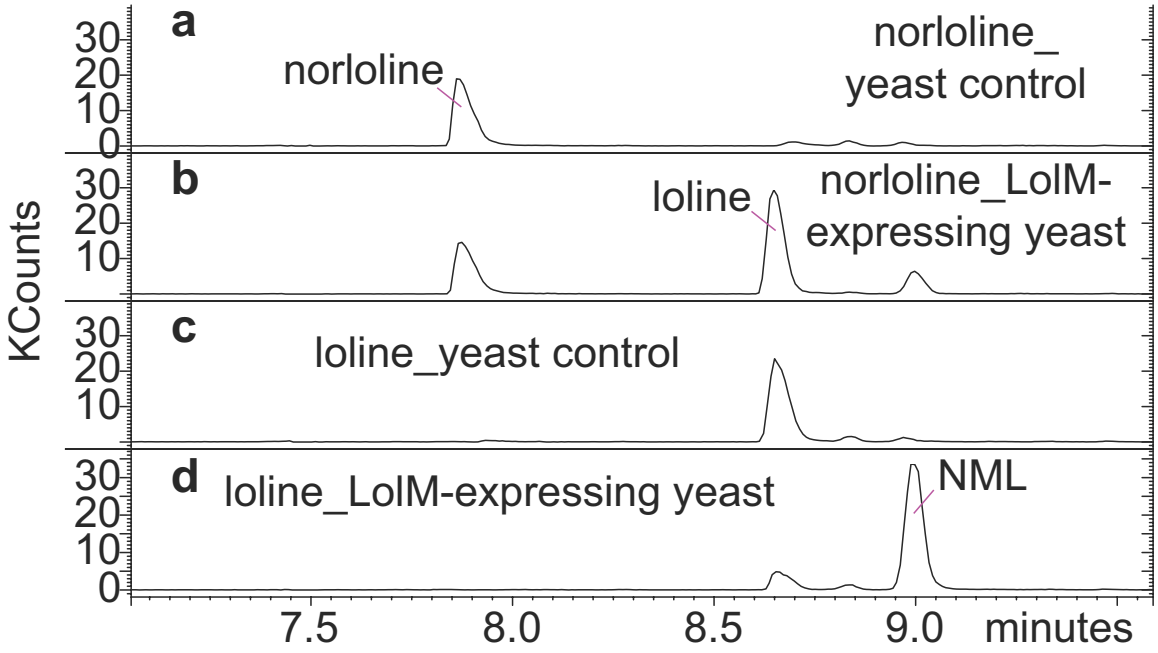


Figure 3.10. Loline alkaloid profile from incubation of yeast crude protein with *S*-adenosylmethionine plus norloline or loline. (a) Chromatogram of loline alkaloids from incubation of norloline and *S*-adenosylmethionine with protein extract of yeast transformed with empty vector. (b) Chromatogram of loline alkaloids from incubation of norloline and *S*-adenosylmethionine with crude protein extract from yeast that expresses LolM with an N-terminal FLAG tag. (c) Chromatogram of loline alkaloids from incubation of loline and *S*-adenosylmethionine with protein extract of yeast transformed with empty vector. (b) Chromatogram of loline alkaloids from incubation of loline and *S*-adenosylmethionine with crude protein extract from yeast that expresses LolM with an N-terminal FLAG tag.

3.2.5. Plant acetyltransferase activity is responsible for NAL production

The acetylated form of loline, *N*-acetylloline (NAL), is also a common loline alkaloid observed together with NANL and NFL in many infected grasses. It has been reported that symbiota comprising *Epichloë* sp. FaTG-3 with its natural host, tall fescue (*Lolium arundinaceum*), contain NFL and NAL, but when FaTG-3 was inoculated to perennial ryegrass, the resulting symbiota contained NFL but lacked NAL (Heist et al., accepted 2001, Ball & Tapper, 1999). Furthermore, we observed that tall fescue and meadow fescue symbiotic with *Epichloë siegelii* accumulated NFL, NANL and NAL, whereas some perennial ryegrass plants symbiotic with the same fungus contained NFL and NANL, but lacked NAL (Padmaja Nagabhyru and Juan Pan, unpublished data), indicating that plant genotype affects production of NAL. In addition, I detected no NAL in loline-alkaloid producing cultures of *E. uncinata* e167, consistent with a previously reported observation (Blankenship et al., 2001), whereas meadow fescue symbiotic with e167 possessed NAL and other loline alkaloids. Incidentally, in addition to NFL, NANL and other lolines, cultures of e167 sometimes gave a small peak that migrated in GC near, but not coincident with, NAL, when compared to extracts from meadow fescue-*E. uncinata* e167 symbiota (Figure 3.11). This peak was identified as *N*-propionylnorloline (also known as decorticasine) based on comparison with extracts from *Adenocarpus decorticans* (Figure 3.11), in which decorticasine is the major pyrrolizidine alkaloid (Ribas & Alonso De Lama 1953, Veen et al., 1992).

I hypothesize that a plant enzyme is required to acetylate loline to form NAL. To test this hypothesis, loline was applied to asymbiotic meadow fescue and perennial ryegrass, and the alkaloid profile of which were then analyzed. Compound NAL was observed in extracts from loline-fed meadow fescue, but not from loline-fed perennial ryegrass (Figure 3.12), suggesting that a grass acetyltransferase enzyme catalyzes the conversion of loline to NAL. This was surprising considering that most of the known loline biosynthesis pathway is catalyzed by enzymes encoded in the fungal *LOL*-cluster (Spiering et al., 2002, Spiering et al., 2008, Pan et al., 2014). This interesting finding raises questions for future investigations such as, does the conversion of loline to NAL occur within or outside of the plant cells, what is the evolutionary origin of this plant acetyltransferase, and is it specific for NAL production or does it catalyze other acetylation reactions as well? An acetyltransferase is also needed to produce AcAP from 1-AP, but this enzyme does not seem to be of plant origin, because AcAP is produced in *E. uncinata* cultures outside the plant system. It will be intriguing to learn how the two partners of the symbiosis communicate with each other and regulate the possible trafficking of loline alkaloids between the two systems.

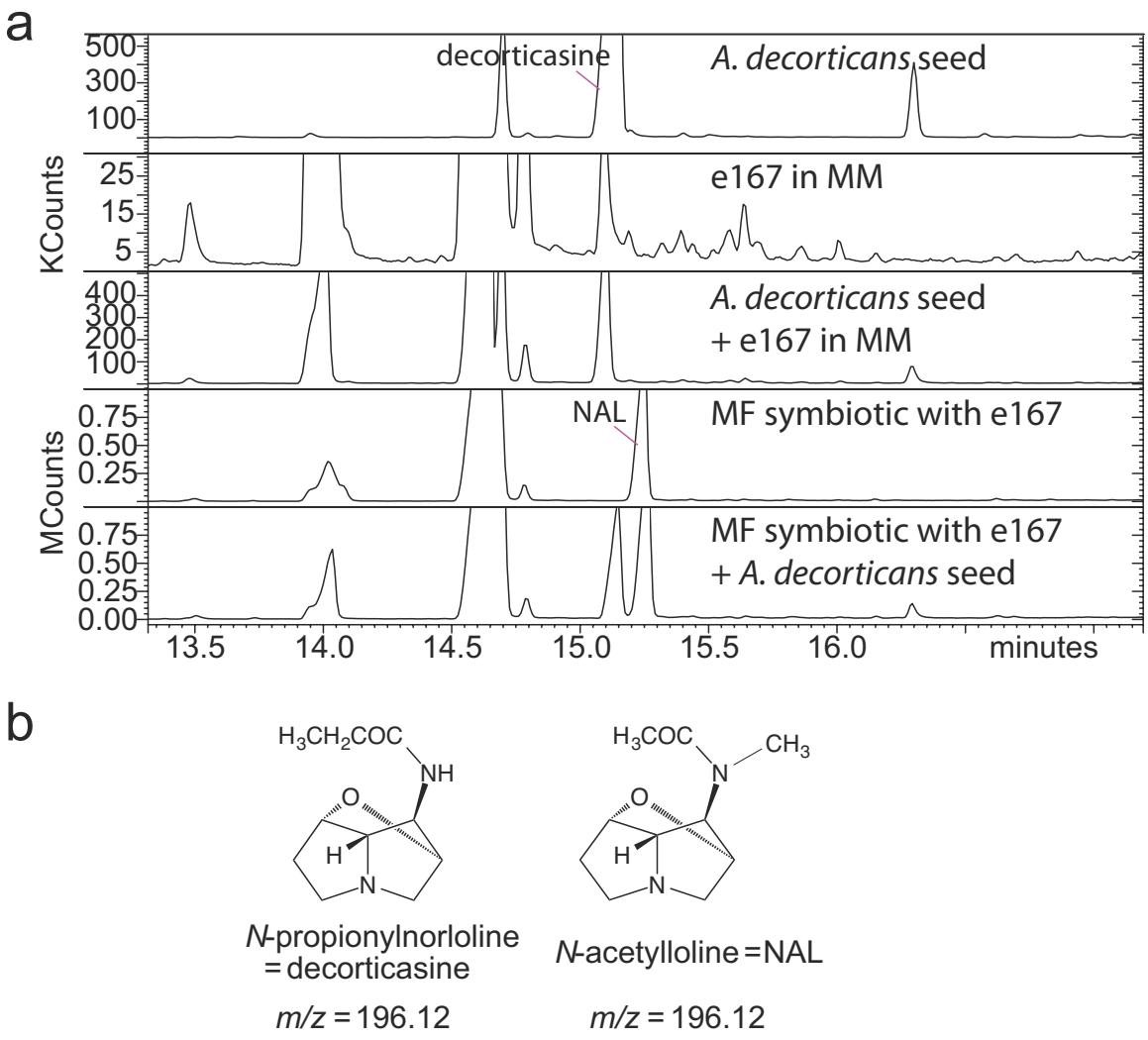


Figure 3.11. Chromatograms of *N*-propionylnorloline (decorticasine) and *N*-acetylloline (NAL) in loline-alkaloid-producing systems. (a) Comparison of loline alkaloids from *Adenocarpus decorticans* seed, *E. uncinata* e167 in inducing culture, and meadow fescue symbiotic with e167. Only decorticasine and NAL peaks are labeled. (b) Structures of decorticasine and NAL.

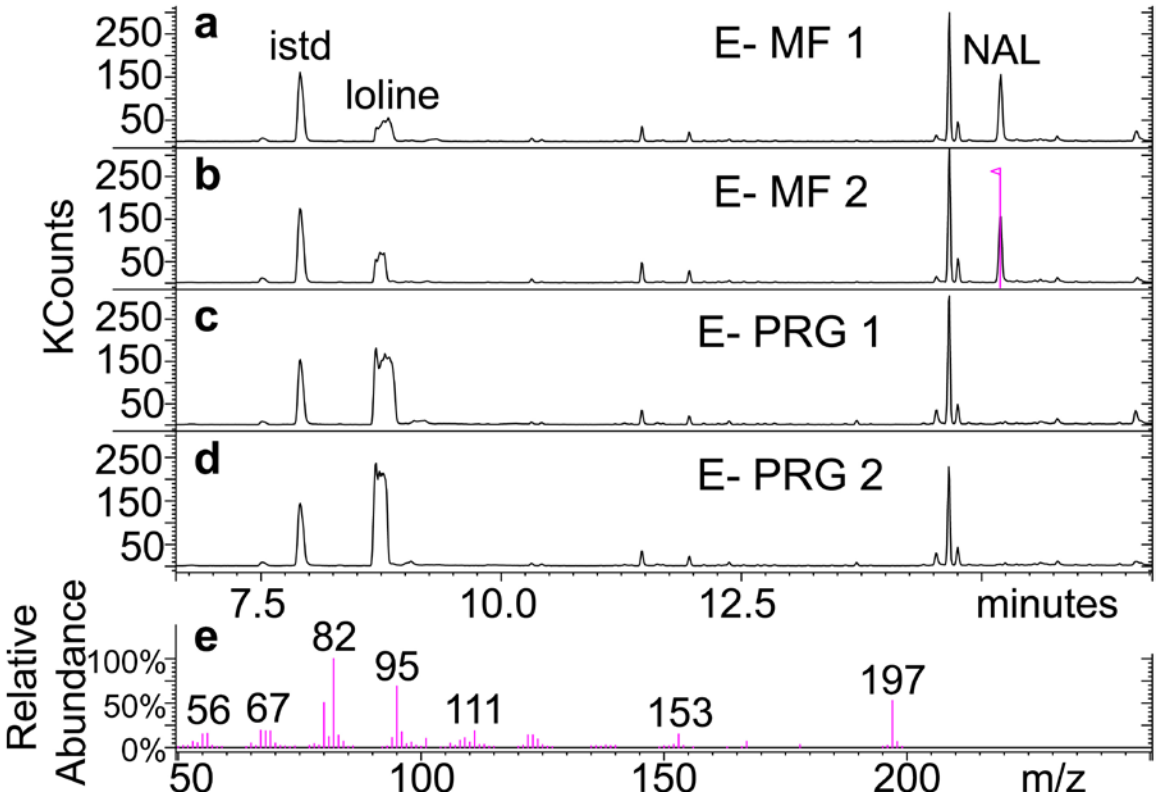


Figure 3.12. GC-MS total ion chromatogram of loline alkaloids after application of loline to asymbiotic plants. Shown are loline alkaloids extracted from loline applications to (a and b) endophyte-free (E-) meadow fescue (MF), and (c and d) E- perennial ryegrass (PRG), and (e) mass spectrum of NAL from application of loline to E- meadow fescue. Quinoline was added as internal standard (istd). Unlabeled peaks are non-loline alkaloid compounds. Numbers after MF or PRG indicate independent trials.

3.2.6. Evolution and implications of loline alkaloid diversity

Alkaloid profiling showed that *Epichloë* species produced different loline alkaloids depending on the gene content of their *LOL* clusters (Table 3.1). When there was a full complement of the 11 *LOL* genes, NFL, NAL, and NANL were produced *in symbio*, but when there was mutation in, or deletion of, *lolO* or *lolN*, AcAP or NANL, respectively, accumulated as the end product. Remnants of *lolN*, *lolO*, *lolM* or *lolP* were often found in strains with NANL or AcAP as end-products, indicating that evolution of these strains involved loss of genes from the ancestral 11-gene *LOL* cluster. In strains for which NANL was the end product, *lolN* genes showed nonsynonymous point mutations (e4309), a partial deletion (E2772), or complete absence (E57 and e4815). Among strains with AcAP end-product, *lolO* had been inactivated by apparently independent mutations (Pan et al., 2014). The multiple inactivations and losses of late pathway genes that alter loline alkaloid profiles suggest that loline alkaloid production may be under diversifying selection.

In this study I found that the plant could directly contribute to diversity of these fungal metabolites. An acetyltransferase activity associated with the host plant is responsible for conversion of loline to NAL, adding a layer of chemotypic complexity to this close plant-fungus symbiotic interaction. Although it is well established that symbiotic organisms may be interdependent in production of indispensible metabolites (Zientz et al., 2004), I am unaware of any other report of one partner modifying specialized (secondary) metabolites produced by the other in a mutualistic symbiosis.

Loline alkaloids show broad-spectrum anti-insect activity, and interestingly, different lolines may have different biological activities. *In vitro* tests of NFL, NAL, NML, and semi-synthetic loline derivatives with long carbon-chain acylations on the 1-amine have shown that many are effective against both fall armyworm (*Spodoptera frugiperda* Smith) larvae and European corn borer (*Ostrinia nubilalis* Hübner) larvae, but the effects seem to differ depending on the modifications (Riedell et al., 1991). *N*-formylloline reduces weight gain of fall armyworm by deterring feeding, and does not significantly affect corn borers. In contrast, NAL reduces corn borer larval weight gain without changing larval feeding behavior, indicating that its effect is due to metabolic toxicity (Riedell et al., 1991). *N*-formylloline, NAL and NML are almost as potent as nicotine in insecticidal activity against green bug aphid (*Schizaphis graminum* Rondani). Furthermore, NML shows a similar regression curve to nicotine sulfate, indicating a similar pharmacological mechanism (Riedell et al., 1991). It has also been shown that lolines (mainly NFL and NAL) reduce oviposition of adult Argentine stem weevil (Jensen et al., 2009). Another study found that NFL reduces the growth, development and survival of the Argentine stem weevil larvae at high concentrations (800 and 1600 μg/g), whereas NANL causes high mortality of Argentine stem weevil larvae, but with little effect on the growth or development of the larvae (Popay et al., 2009). These findings clearly show that the chemically diverse loline alkaloids exert complex and multi-mode effects on grass herbivores. Moreover, loline alkaloids also affect insect parasitoids (Bultman et al., 2009, Bultman et al., 1997), thereby contributing to multi-trophic endophyte effects. Thus, the diversification of loline alkaloid structures produced by

Epichloë species probably reflects selection imposed by the complex and variable biotic environments of their plant hosts.

3.3 Concluding Remarks

The *Epichloë*-grass symbiota produce a variety of loline alkaloids that differ in the modification of the 1-amine group. Through comparison of *LOL* clusters in genomes of endophyte strains that contain different loline alkaloids, it was found that the accumulation of NANL is consistently associated with mutations in, or deletion of, *lolN*, a gene encoding a putative acetamidase. The function of *lolN* was further supported by gene knockdown and knockout experiments. In the sequenced genomes that contain *lolN* and *lolM*, *lolN* is linked to and divergently transcribed from the putative methyltransferase gene, *lolM*. Feeding norloline to yeast that expressed *lolM* resulted in production of both loline and NML, supporting the function of LolM as an *N*-methyltransferase in the pathway. Finally, evidence indicated that the host plant was required to catalyze acetylation of loline to form NAL. Hence the chemical diversification of loline alkaloids involves both fungal and plant enzymes.

3.4 Materials and Methods

3.4.1. ***Biological materials and general experimental procedures.*** Fungal strains (Table 3.2) were isolated and cultured on potato dextrose agar (PDA) at 22 °C as previously described (Blankenship et al., 2005). Infection of the grass with or without the endophyte was checked by tissue print immunoblot (An et al., 1993), grow-out of the fungus from surface-sterilized grass tissue, and confirmation of the endophyte strain with specific primers using polymerase chain reaction (PCR). General experimental procedures and reagents were as previously reported (Pan et al., 2014). Primers used in this study were listed in Table 3.3.

Loline alkaloids were extracted with chloroform, using quinoline as internal standard, and analyzed by GC-MS as previously reported (Faulkner et al., 2006). Extraction from plant and minimal-medium cultures followed previously reported methods (Pan et al., 2014).

Table 3.2. Fungal isolates used in this chapter.

Fungus	Isolate	Host	Origin
Epichloë festucae	E2368	*Lolium s*pp.	Lexington, KY, USA
Epichloë uncinata	e167	*Lolium pratense*	Nyon, Switzerland
Epichloë coenophiala	e19	*Lolium arundinaceum*	Lexington, KY, USA
Epichloë amarillans	E57	*Agrostis hiemalis*	Brazoria Co., Texas, USA
Epichloë glyceriae	E2772	*Glyceria striata*	Canastota, New York, USA
Epichloë canadensis	e4815	*Elymus canadensis*	Throckmorton County, Texas
Epichloë canadensis	e4814	*Elymus canadensis*	Nuevo León State, Mexico
Epichloë brachyelytri	E4804	*Brachyelytrum erectum*	Edmonson Co., Kentucky, USA
Epichloë amarillans	E722	*Sphenopholis obtusata*	Georgia, USA
Atkinsonella hypoxylon	B4728	*Danthonia spicata*	Lexington, North Carolina, USA
Epichloë siegelii	ATCC 74483	*Lolium pratense*	PI 237707, Western Regional Plant Introduction Station,
Epichloë coenophiala	e4309	*Lolium arundinaceum*	PI 598903, Western Regional Plant Introduction Station
Epichloë coenophiala	e4163	*Lolium arundinaceum*	PI 422777, Western Regional Plant
Epichloë sp.	E4686	*Poa autumnalis*	Texas, USA
Epichloë sp.	e4779	*Festuca versuta*	Taber, Austin, Texas, USA

Table 3.3. Oligonucleotides used in this chapter. [a]

Primer	Sequence
upFLKf	GCTCTAGATTTAAATGTTCTCCGTAAGGATAAGCATC
upFLKr	GCTCTAGAGTGGTGGACTGGTGAGGACT
lolAANMTdf	CGCCCGGGATGCACAAGGTCCAGATACATG
lolAANMTdr	CGCTCGAGTTATTGCTACTCTATTACCTCCT
aamAd1	GCTGAGGAAGATGCGCTGAT
aamAup1	CAGCGTTTAGCTGAGCACC
Mexpf	CGACTAGTATGACGGTGAATAGCAGCG
Mexpr	CTATTCCACCTTGCCCATC
Nexpf	CGACTAGTATGTCGAACATGAGAGCTACG
Nexpr	GCACGCGTCATCCAACGAGCTCCGTG
hphf	TCTCGTGATTCTTTCCATC
hphr	CGGATCGGACGATTGCGT
lolNMkops	ATGTAGCCCGGTGCACAGA
hph1u	AGGTCGGAGACGCTGTCGAA
lolEkosr	CCATGGAGTGGAGAGATATGT
lolEkoupf	CGCTCGAGCTAGTGGACAGGTTATTA
lolEkoupr	CGCCCGGGATGGATTACGTGAAACA
MexpYNfBglII	CGAGATCTTTATGACGGTGAATAGCAGCG
MexpYNrBglII	CCGAGATCTcTTCCACCTTGCCCATCTTC
MexpYCfSpeI	CGACTAGTATGACGGTGAATAGCAGCG
MexpYCrSpeI	CCGACTAGTAATTCCACCTTGCCCATCTTC

[a] Underlined segments indicate restriction-endonuclease cleavage sites incoporated in the primers to facilitate cloning.

3.4.2. ***Construction of gene-replacement and complementation plasmids.*** In *Epichloë festucae* E2368, *lolN* and *lolM* are adjacent and divergently transcribed. A plasmid was constructed with a modified *hph* marker gene (Tsai et al., 1992) to replace *lolM* and most of *lolN* via homologous recombination (Figure 3.4). Using E2368 genomic DNA as the template, a 2.5-kb fragment upstream of the target *lolN*-*lolM* segment was amplified by PCR with primers upFLKf (*Xba*I) and upFLKr (*Xba*I), digested with *Xba*I, and ligated with pKAES173 (Spiering et al., 2008) digested with *Xba*I. The ligated product was introduced by transformation into *Escherichia coli* XL1-blue competent cells, and transformants were screened for *hph* by colony-PCR with primers hphf and hphr. Positive colonies were extracted for plasmid DNA, which was then tested by restriction-endonuclease digestions and Sanger sequencing to identify the construct that had the correct orientation. The proper construct was then digestion with *Xma*I and *Xho*I. A 1.9-kb fragment downstream of the *lolN*-*lolM* segment was amplified by PCR with primers lolAANMTdf (*Xma*I) and lolAANMTdr (*Xho*I), and then digested with *Xma*I and *Xho*I. The digested plasmid and PCR product were gel purified and ligated to generate plasmid pKAES323, which has the flanking regions of target *lolN*-*lolM* on either side of the *hph* expression construct.

To complement the *lolN* and *lolM* double-knockout strain, *lolN* and *lolM* of E2368, together with their common, divergent promoter, were amplified by PCR with primers upFLKf and lolNMkops using Phusion Hot Start High-Fidelity DNA Polymerase (Thermo Scientific, Ratastie, Vantaa, Finland) with HF buffer provided from the manufacturer, and 1.5 mM $MgCl_2$. The temperature conditions were 98 °C for 3 min, 35 cycles of 98 °C for 10 s, 62 °C for 10 s, and 72 for °C 3 min, then a final 5 min incubation at 72 °C. The PCR product was purified and digested with *Xba*I. Plasmid pKAES215 (Faulkner, 2011) was digested with *Xba*I and *Eco*RV. The two fragments were gel purified and ligated to generate pKAES341, which confers hygromycin resistance, and expresses *lolN* and *lolM* driven by their native, divergent promoter. All plasmid constructs were confirmed by Sanger sequencing.

3.4.3. ***Fungal transformation and screening for knockouts.*** Protoplast preparation and transformation procedures were performed as previously described (Panaccione et al., 2001) with minor changes (Pan et al., 2014). After electroporation, *lolN* and *lolM* knockout transformants of E2368 were selected on regeneration medium with 450 µg/ml hygromycin B. The same selection was also used in single spore isolation and fungal isolate maintenance on PDA. After two times of single spore isolation, the transformants were extracted for DNA with DNeasy 96 Plant Kit (Qiagen, Valencia, CA, USA), and screened for the *lolN* gene by PCR with primers aamAup1 and aamAd1. The transformants that were negative in the *lolN* screening were then positively screened with primers upFLKf and lolNMkops, which produced a 4.0 kb product from the knockout transformant and a 5.7 kb product from the wild-type strain. As controls, an ectopic transformant with pKAES323 and an empty-vector transformant with plasmid pKAES173 were also generated and confirmed by PCR.

Transformation of the knockout strain with pKAES341 for complementation of *lolN* and *lolM* was performed by co-transforming linearized pKAES341 with pII99

conferring geneticin resistance (Inoue et al., 2002) at a molar ratio of 5:1. Selection of transformants and single spore isolations were carried out on PDA plates with 800 µg/ml G418. The transformants were single-spore isolated twice and extracted for DNA to screen for *lolN* by PCR with primers aamAup1 and aamAd1, and for *lolM* with primers aamAup1 and lolNMkops.

Inoculation of the strains to endophyte-free meadow fescue (*Lolium pratense*) was carried out as described by (Latch & Christensen, 1985). Loline-alkaloid profiles of the plants were checked approximately 6 months after inoculation.

3.4.4. *Southern-blot hybridization.* Genomic DNA was digested with *Hin*dIII and 1 µg of each DNA was electrophoresed in 0.7% agarose gel (PHENIX Research, Candler, NC, USA) in 0.5 x TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH 8.2) for 20 hr in a 4 °C cold room. The DNA was then transferred to Hybond H^+-nylon membrane (GE Healthcare, Piscataway, NJ, USA) with a GENIE electroblotter (Idea Scientific, Minneapolis, MN, USA). The immobilized DNA on membrane was denatured in 0.4 M NaOH for 10 min and neutralized in 2x SSC for 10 min. The DNA was cross-linked to the membrane with UV light using a Spectrolinker (Spectronics, Westbury, NY, USA). Probes for *lolN* and lolM were prepared by PCR with primers Nexpf and Nexpr, and Mexpf and Mexpr, respectively, using E2368 DNA as template. A 974-bp PCR product for *hph* probe was prepared by PCR with primers hphf and hphr using pKAES173 as template. The PCR products were purified and labeled with α[^{32}P]dCTP (GE Healthcare) using the Prime-a-Gene Labelling System (Promega, Madison, WI, USA). Hybridization was performed as in Starnes et al. (2012).

3.4.5. *Yeast expression of lolM.* The full-length *lolM* was amplified from cDNA of e167 that produces lolines in MM (about 20 days). Primer pair MexpYNfBglII and MexpYNrBglII and primer pair MexpYCfSpeI and MexpYCrSpeI were used for *lolM* expression with N-terminal and C-terminal FLAG tag (Hopp et al., 1988), respectively. The PCR products were purified and digested with *Bgl*II or *Spe*I, and ligated with *Bgl*II or *Spe*I digested pESC-LEU (Agilent Technologies, Santa Clara, CA, USA). Correct orientation of the resulting plasmids was chosen through digestion and confirmed by sequencing. The resulting plasmid was named pKAES350, which has *lolM* under inducing promoter GAL10, with an N-terminal FLAG tag, and pKAES351, which has *lolM* under GAL10 promoter and has a C-terminal FLAG tag. Yeast strain YPH499 (MATa *ura3*-52 *lys2*-801^{amber} *ade2*-101^{ochre} *trp*1-Δ63 *his*3-Δ200 *leu*2-Δ1)(Sikorski & Hieter, 1989) (Agilent Technologies) was used to express *lolM* with the aforementioned constructs. Yeast competent cell preparation and transformation was performed according to the manufacture's protocol, and transformants were selected on SD/-Leu minimal medium. The growing colonies were then streaked twice on the same medium before feeding experiment.

3.4.6. *Loline feeding to yeast and yeast crude protein extract.* To apply loline to yeast transformants of pKAES350, pKAES351, or empty vector pESC-LEU, the yeast were grown in SD/-Leu minimal medium at 30 °C overnight, washed twice with inducing SG/-Leu minimal medium, and resuspended in the same medium to achieve an OD_{600} of 0.5.

Loline was applied to the suspension at the final concentration of 4 mM. DMSO (1.5 %) was also added to increase permeability of yeast cell. The cultures were grown at 30 °C, 250 rpm for 3 days before sampling. The yeast cells were centrifuged at 1000 × g for 5 min and the supernatant was freeze-dried and extracted of loline alkaloids with routine extraction method and applied on GC-MS to check the profile.

Yeast for crude protein extraction used a large volume (200 ml) of SG/-Leu minimal medium (1/5 v/v of the container) with similar inoculation methods as described above for direct yeast feeding. Yeast cells were collected after 2 days of induction and 300 mg of yeast pellet was used for each extraction. Cell-free extract was prepared by following the procedure as previously described with modifications (Pogany et al., 2008). Fastprep machine (done twice for 20 sec at 4 m/s) was used to break the cells in a Tris-glycine buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 10 mM DTT), with proteinase (cOmplete ULTRA Tablets, Mini, EDTA-free, Roche) added right before extraction. Either loline or norloline (4 mM), and AdoMet (1 mM), were added to 90 µl of crude extract to get a final volume of around 100 µl. The feeding mixture was incubated at 30 °C overnight and the reaction was stopped by adding 100 µl of 1M NaOH. Then the loline alkaloids were extracted following the routine method and applied on GC-MS.

Loline used in this study was prepared as previously reported (Petroski et al., 1989). Norloline was prepared from the *Adenocarpus decorticans* seeds (100 g). The ground seed was basified by 1 M NaOH to pH 11 and extracted with chloroform (5 x, 10 ml/g seed). Then the alkaloid mixture was concentrated by rotor vapor, hydrolyzed, and purified by column to obtain around 10 mg of norloline. Interestingly, I found that only the loline-like alkaloids from the *Adenocarpus* seeds seem to have been hydrolyzed to produce norloline, whereas the AcAP-like alkaloids remained intact.

3.4.7. ***Genome sequencing and phylogenetic analysis.*** Fungal DNA was isolated and sequenced by pyrosequencing on a Roche/454 GS FLX+ platform, and assembled as previously described (Schardl et al., 2013c, Schardl et al., 2013b). Genes were annotated as previously described (Schardl et al., 2013b) from sequenced genomes available at www.endophyte.uky.edu. Alignment of DNA sequences and amino acid sequences was performed on www.phylogeny.fr (Dereeper et al., 2008), and edited using MacVector v. 12.7.5.

3.4.8. ***Western blot analysis.*** Yeast crude protein was extracted as in Rajendran et al. (2004). Then the proteins were fractioned by electrophoresis in a SDS-PAGE gel (10%). For Western blot analysis, α-FLAG (Sigma-Aldrich, St. Louis, MO) was used as primary antibody, and immunoblots were developed using an ECL detection kit (Roche Applied Science, Basel, Switzerland).

Chapter Four

Loline alkaloids in *Adenocarpus decorticans* and *Adenocarpus telonensis* plants

4.1. Introduction

Other than the cool-season grasses that are symbiotic with *Epichloë* and *Atkinsonella* species, loline alkaloids are also present in *Argyreia mollis* (Convolvulaceae) (Tofern et al., 1999) and *Adenocarpus* spp. (Fabaceae) (Ribas & Alonso De Lama 1953, Veen et al., 1992). In the grass-fungus symbiota, it is well known that the endophytic fungus is responsible for loline alkaloid biosynthesis. No endophyte, however, has been identified in either *Argyreia* or *Adenocarpus* species to account for loline alkaloid production. Interestingly, some *Argyreia* species also contain ergot alkaloids, which in related morning glories have been attributed to fungal symbionts (*Periglandula* spp.) (Schardl et al., 2006, Kucht et al., 2004, Leistner & Steiner, 2009). Although no endophytes are reported in the *Argyreia* species, it is intriguing to speculate that the ergot alkaloids in these plants are also produced by a fungal symbiont instead of the plant. Likewise, it is conceivable that the loline alkaloids detected in *Argyreia mollis*, as well as *Adenocarpus* spp., could be products of so-far uncharacterized symbionts. This study is an attempt to search for a possible fungal symbiont that may be responsible for production of loline alkaloids in *Adenocarpus* spp.

Laburnamine, 1-(2-methylbutyryl)aminopyrrolizidine, which aside from the acyl group is structurally similar to the loline alkaloid intermediate, 1-acetamidopyrrolizidine (AcAP) (Chapter 2), has been reported in seeds of *Laburnum anagyroides* Medik. (Fabaceae) (Tasso et al., 2013). Preliminary tests of alkaloid profiles from *Adenocarpus decorticans* and *Adenocarpus telonensis* seeds by GC-MS showed peaks with similar fragment ions as those reported for laburnamine [1-(2-methyl)butanamidopyrrolizidine] (Tasso et al., 2013) and AcAP (Pan et al., 2014), as well as loline alkaloids (Petroski et al., 1989). Conceivably, if the loline alkaloids in *Adenocarpus* species are indeed synthesized by a fungal symbiont, their biosynthesis may be catalyzed by enzymes similar to those encoded by *LOL* clusters of grass-symbiotic fungi. Considering that AcAP is probably formed by acetylation of 1-aminopyrrolizidine in *Epichloë* species (Chapter 3), laburnamine and the related compounds in *Adenocarpus* species may be produced from 1-aminopyrrolizidine by similar acyl-transferase reactions. Taking a step further, the resulting acylamidopyrrolizidine alkaloids could then also be oxygenated, perhaps by one or more enzymes similar to LolO, to form the loline alkaloids with a C2-C7 ether bridge. The loline alkaloid profiles of *A. decorticans* and *A. telonensis* were similar on GC-MS, so in this study, I analyzed the alkaloid profile from *A. decorticans* by HRMS to gain insight into the possible biosynthesis pathway in *Adenocarpus* species.

4.2. Results and Discussion

4.2.1. Deep sequencing of Adenocarpus decorticans *mRNA did not show fungal reads*

To test if there is any fungal endophyte associated with *Adenocarpus decorticans*

plants, we used Illumina mRNA deep sequencing (also known as RNA-seq). A total of 190,936,340 reads were generated, each 100 nucleotides in length. Of these, 161,444,132 were in matched pairs from both ends of cDNA fragments. Sequences were assembled into contigs using CLCBio Genome Workbench to give 282,322 contigs totaling 78,877,046 bp in length. The contigs were used as queries for BLAST (Basic Local Alignment Search Tool) searches of GenBank sequences for flowering plants and for fungi. The searches were conducted against nucleotide sequences (BLASTn) and protein sequences (BLASTp) (J. Jaromcyzk and C.L. Schardl, unpublished). No fungal contigs were detected in the assembled sequences. In contrast, RNA-seq of morning glory *Ipomoeae asarifolia* symbiotic with *Periglandula ipomoeae* gave a substantial fungal reads (J. Pan and C.L.Schardl, unpublished).

A further attempt to search for fungal symbiont employed PCR. Primers were designed against well-assembled contigs of *A. decorticans* sequences. Using *A. decorticans* DNA that was extracted from leaves and stem as template, PCR was performed with fungal β-tubulin gene (*tubB*) primers, or *LOL* gene specific primers, in an effort to detect a possible fungal endophyte and *LOL* genes potentially similar to those present in *Epichloë* and *Atkinsonella* spp. The primers matching *A. decorticans* plant sequences were used for positive-control PCR reactions. There was no *LOL* gene or fungal *tubB* amplification from the *A. decorticans* DNA (Figure 4.1).

I then tried to remove the possible endophytic fungus from *A. decorticans* and *A. telonensis* by soaking the seeds overnight in a solution of systemic fungicide before germinating them. Plants grown from fungicide-treated seeds were checked for loline alkaloids. There was no change of the alkaloid profile compared to plants grown from untreated seeds. Hence, so far there is no indication of a fungal symbiont associated with *A. decorticans*, and whatever genes in that system that determine biosynthesis of loline alkaloids and loline-related pyrrolizidine alkaloids are probably not similar enough for detection by PCR with primers based on the known fungal *LOL* gene sequences.

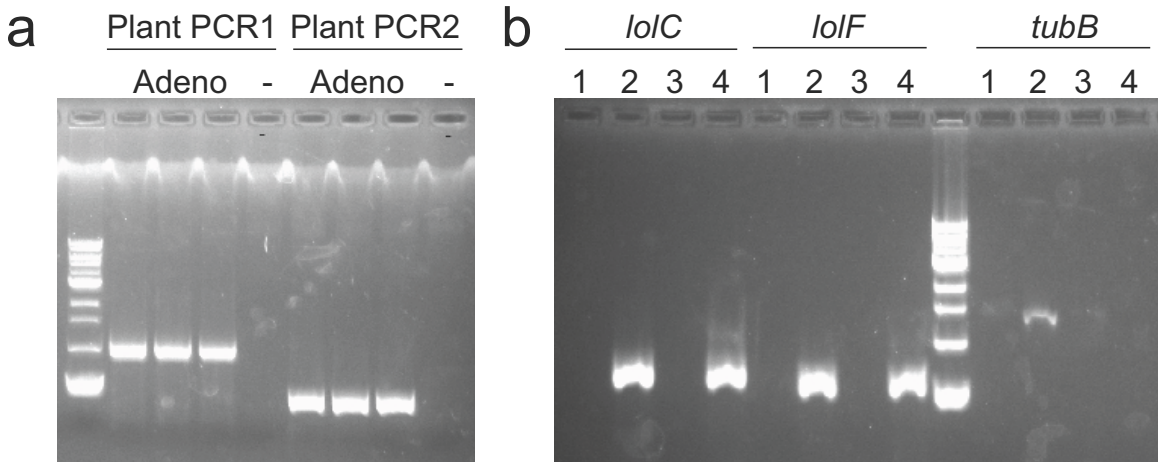


Figure 4.1. Gel image of PCR test for fungal *tubB* and *LOL* genes. (a) Positive control of PCR. A 200x dilution of DNA extracted from *Adenocarpus decorticans* was used as template and water as negative control. Specific primers designed from mRNA sequencing, AdeF1 and AdeR1, were used in "Plant PCR1", and AdeF3 and AdeR3 were used in "Plant PCR2". (b) Loaded to lanes are PCR products using genomic DNA of 1- *Adenocarpus decorticans*, 2- *Atkinsonella hypoxylon* B4728, 3- e144, an *Epichloë* endophyte that does not have *LOL* genes, and 4- *Epichloe uncinata* e167 that has *LOL* genes, as template.

4.2.2 Loline alkaloids from **Adenocarpus** *spp. showed ions characteristic of lolines and acetamidopyrrolizidines*

Analysis by GC-MS, some alkaloids extracted from *A. decorticans* and *A. telonensis* showed fragment ions characteristic of loline alkaloids (*m/z* = 82, 95, and 123), and parent ion with *m/z* values incrementing by 14 units: *m/z* = 183, 197, 211, and 225. There was also a number of chromatograph peaks showing fragment ions that were characteristic of AcAP (*m/z* = 83 and 108), with parent ion peaks also incrementing by 14 units: *m/z* = 169, 183, 197, and 211. I hypothesized that the *m/z* = 14 difference is likely due to variation in the number of CH_2 units in the acyl group on 1-amine. Thus, I propose that *A. decorticans* and *A. telonensis* produce a group of loline- and AcAP-like compounds that differ in the acyl modification on the 1-amine. The predicted compounds are shown in Figure 4.2.

To further investigate the identities of the putative loline and pyrrolizidine alkaloids in *Adenocarpus* spp., the alkaloid extract from *A. decorticans* was air dried and eluted in methanol and run on HPLC-HRMS at Dr. Andrew J. Morris's laboratory, with the help of Ms. Manjula Sunkara, at College of Medicine, University of Kentucky. All of the hypothesized compounds were supported by the HPLC-HRMS spectra, as shown in Figures 4.3 to 4.13. Some of the compounds did not separate well in HPLC, so the extract was further run on LCMS-HRMS to separate the isobaric species. Based on structures of known acylamidopyrrolizidines and lolines from legumes (Tasso et al., 2013, Ribas & Alonso De Lama 1953), compounds **1** (m/z = 225) and 1-acetamidopyrrolizidine (*m/z* = 169) did not separate, although they could be differentiated (Figure 4.14). The compound **4** and NANL (*m/z* = 183) (Figure 4.15), compound **3** and decorticasine (*m/z* = 197) (Figure 4.16), and laburnamine and compound **2** (*m/z* = 211) (Figure 4.17) were separated by LCMS. These results support the hypothesis that *A. decorticans* produces a series of lolines and their corresponding pyrrolizidines with which they differ by possession of an ether bridge. However, more chemical analyses are required to establish the configurations of these hypothesized alkaloids.
The occurrence of 1-aminoacylated lolines (e.g., decorticasine) and 1-aminoacylated pyrrolizidines (e.g., laburnamine) that potentially differ only in the presence and absence of ether bridge, respectively, in *A. decorticans* and related species suggests that the legume lolines may be derived from the pyrrolizidine backbone by addition of the ether bridge, similar to the pathway I established for the lolines produced by *Epichloë* species (Chapter 2).

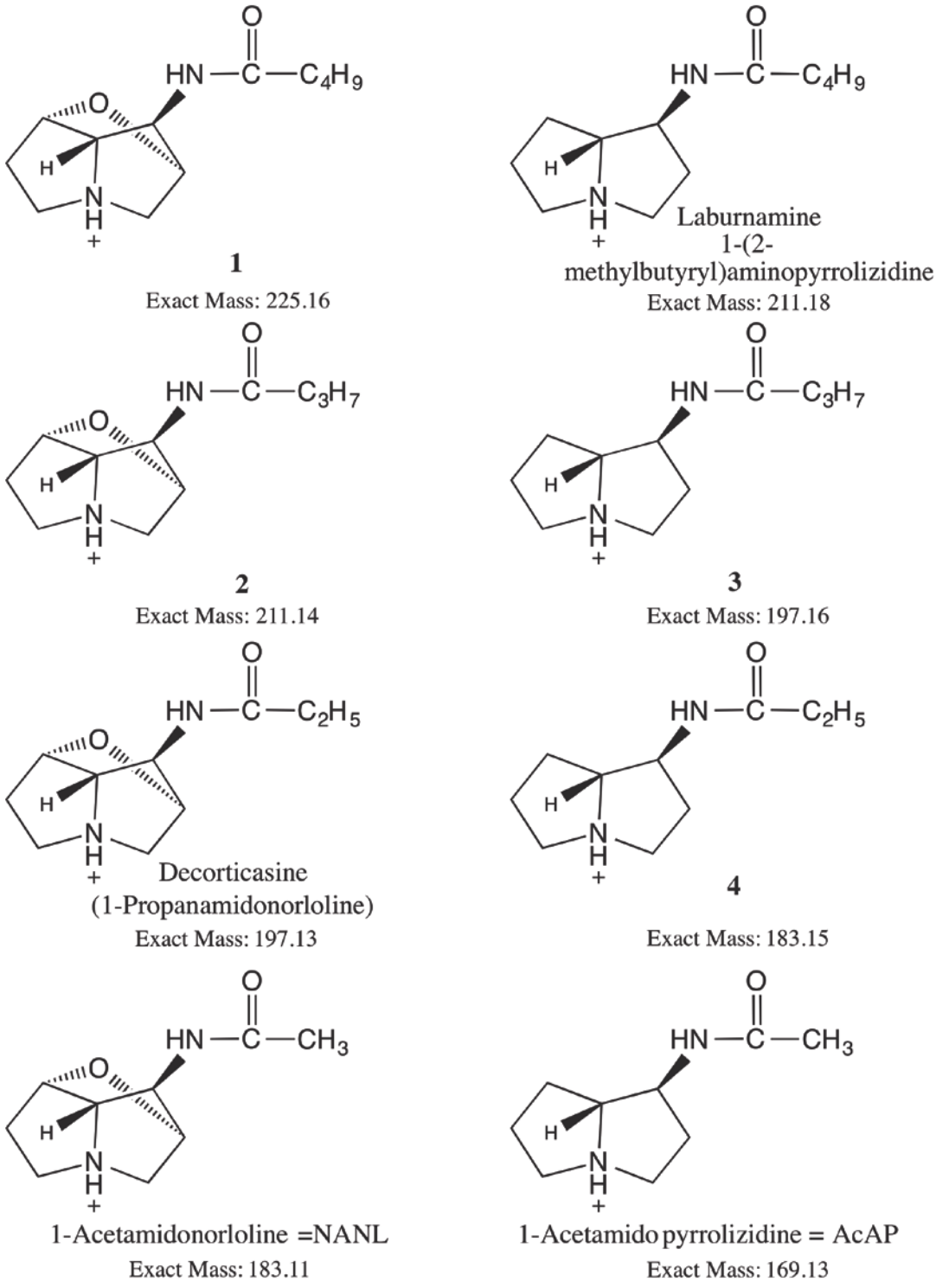


Figure 4.2. Proposed structures of loline alkaloids and related pyrrolizidine alkaloids in *Adenocarpus* species.

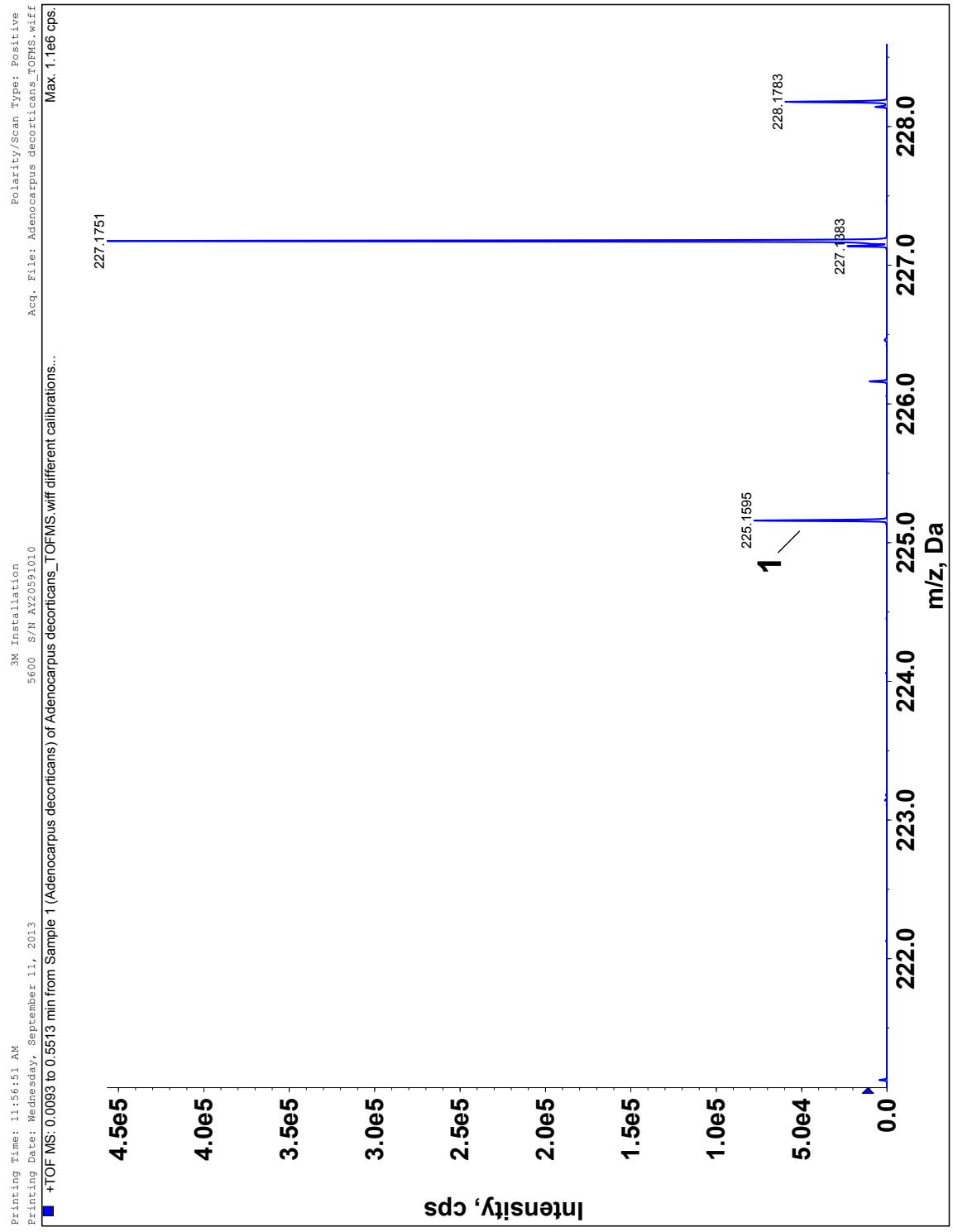


Figure 4.3. HRMS of compound **1**.

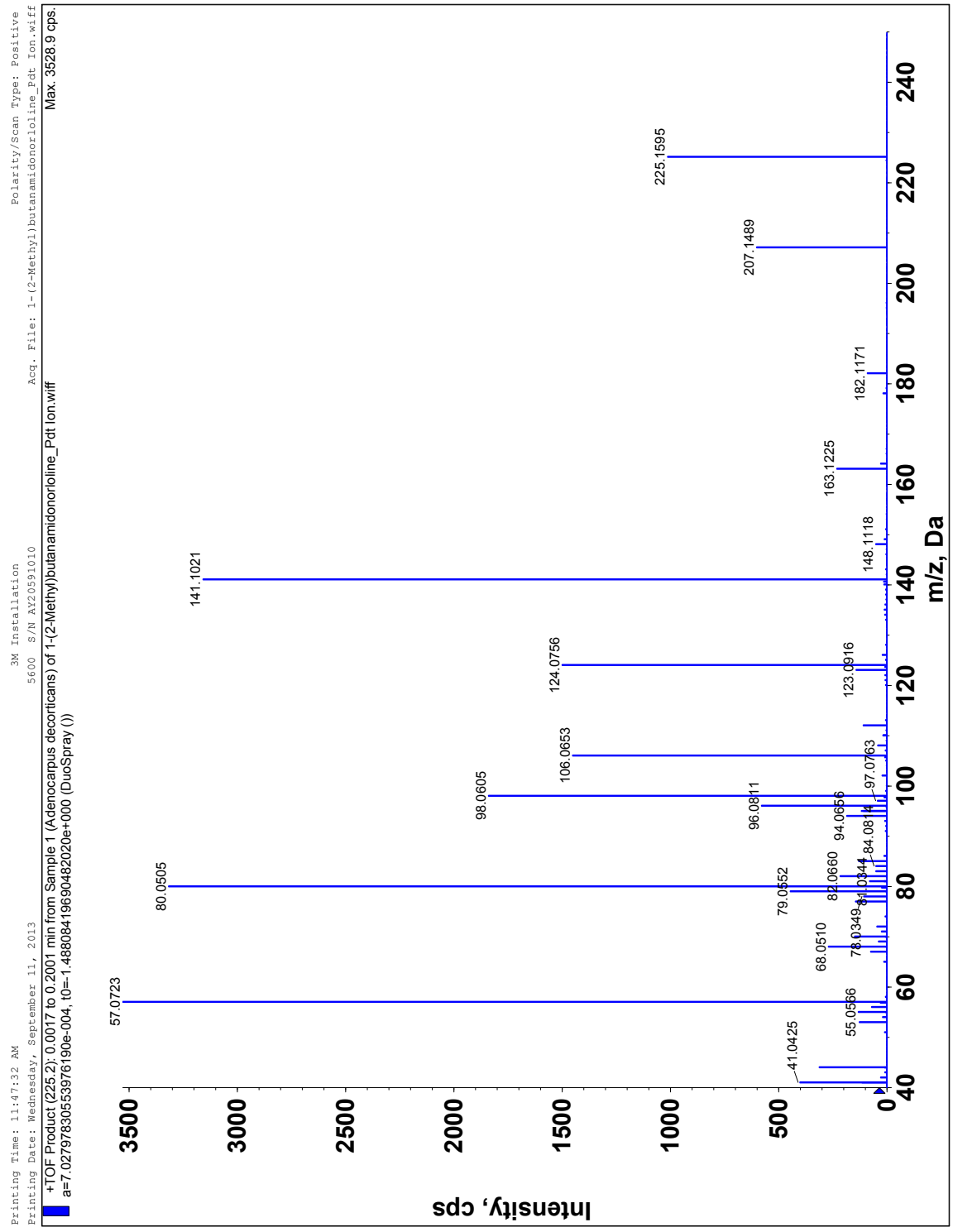


Figure 4.4. Fragment ions of compound **1**.

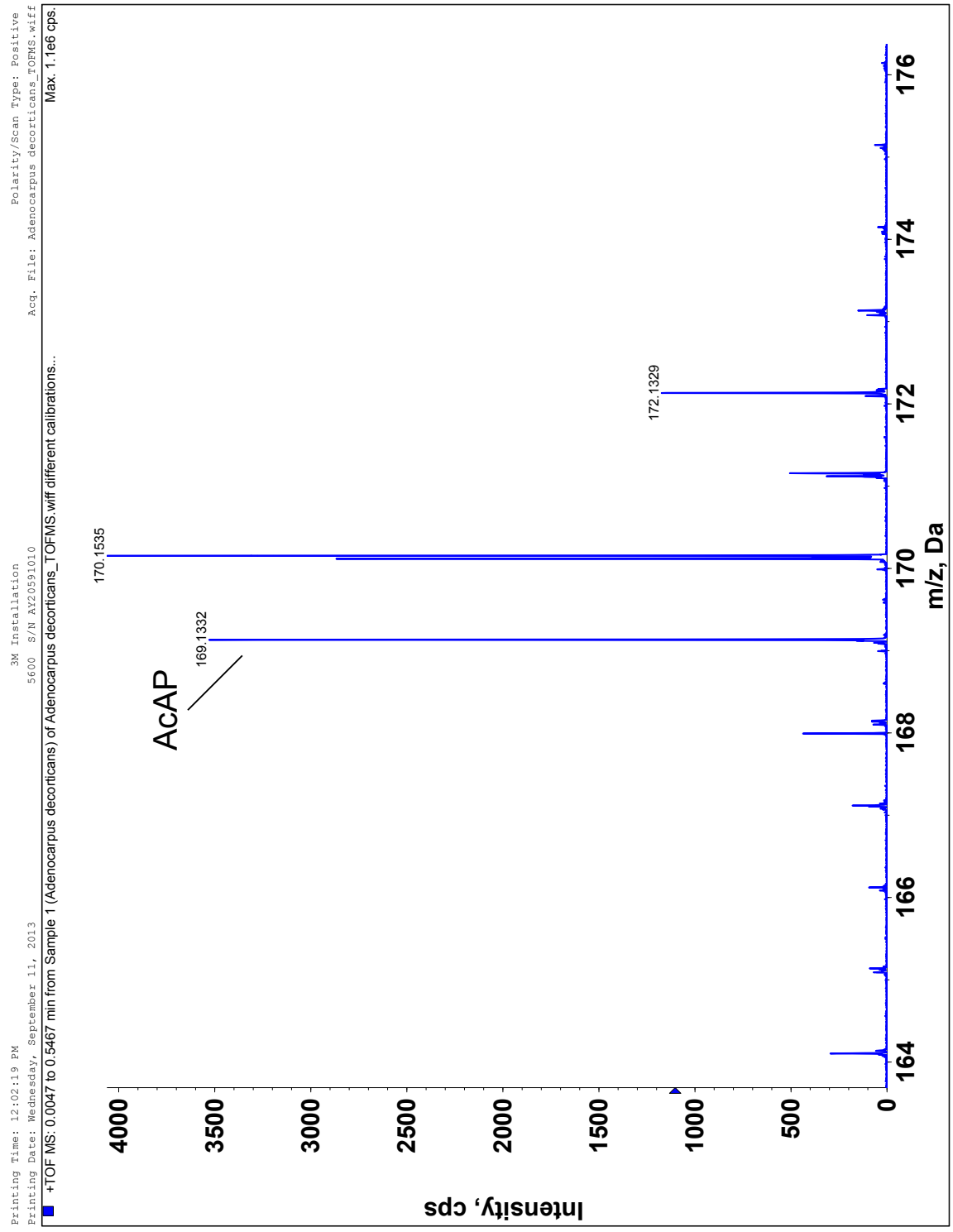


Figure 4.5. HRMS of AcAP.

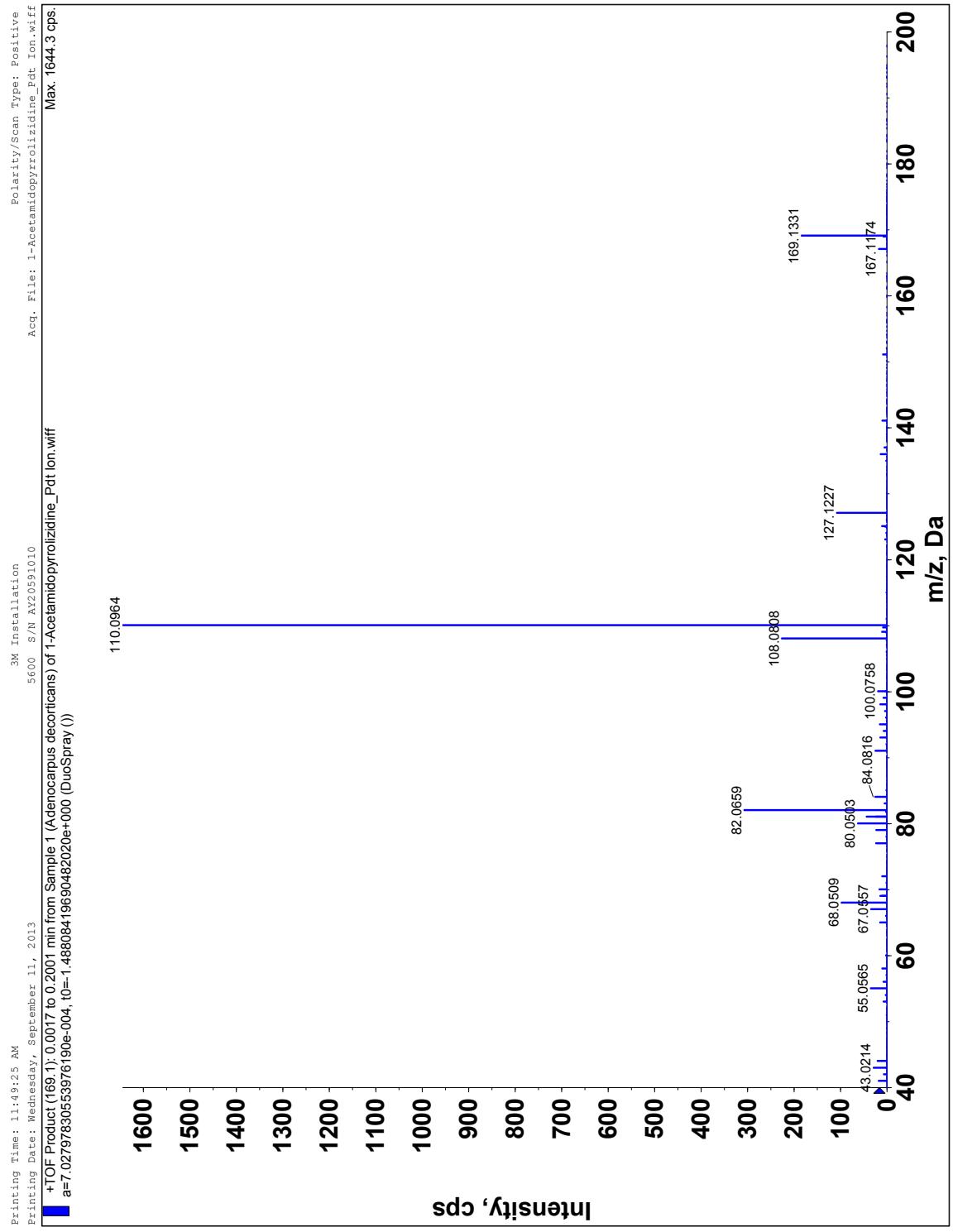


Figure 4.6. Fragment ions of AcAP.

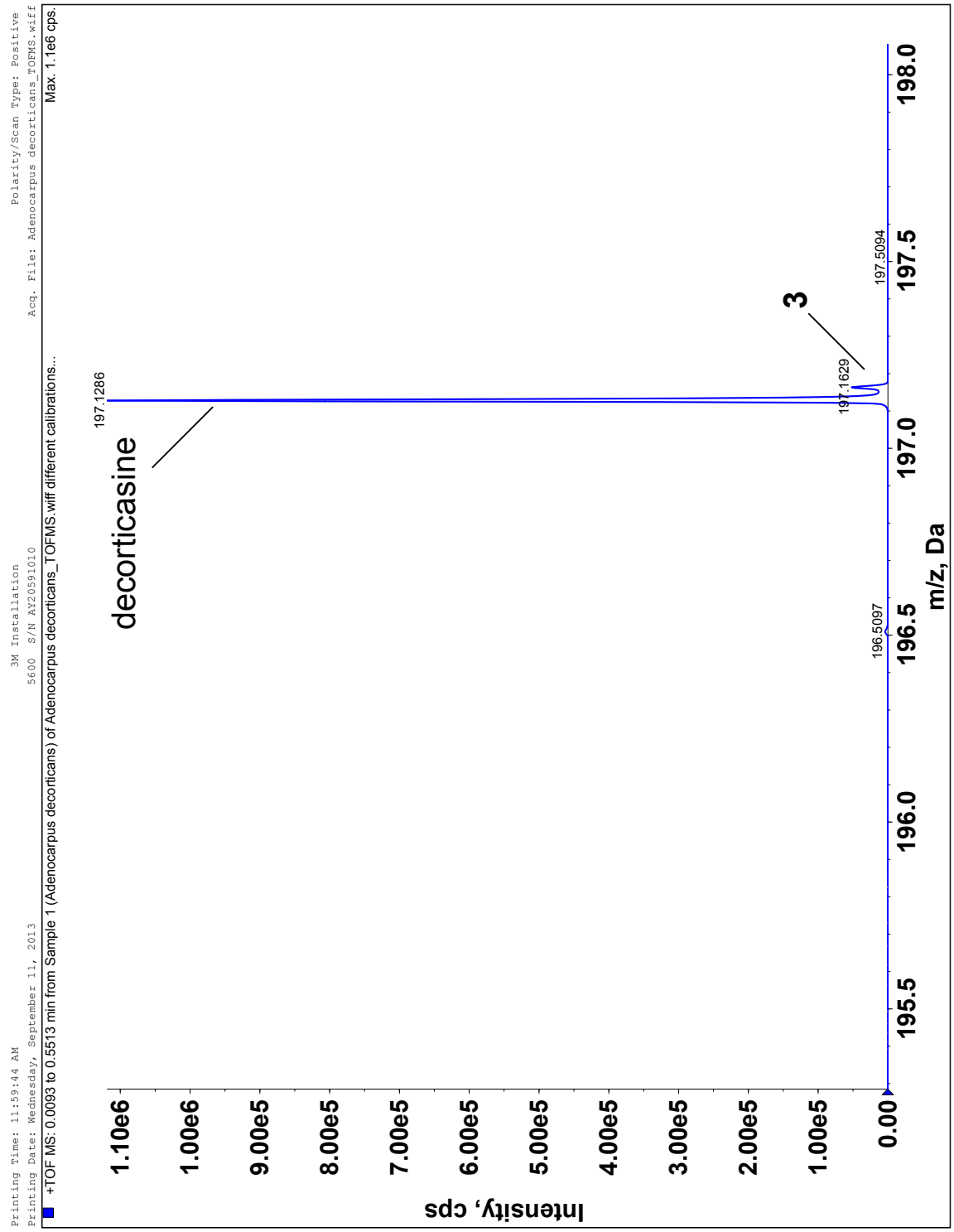


Figure 4.7. HRMS of decorticasine and compound **3**.

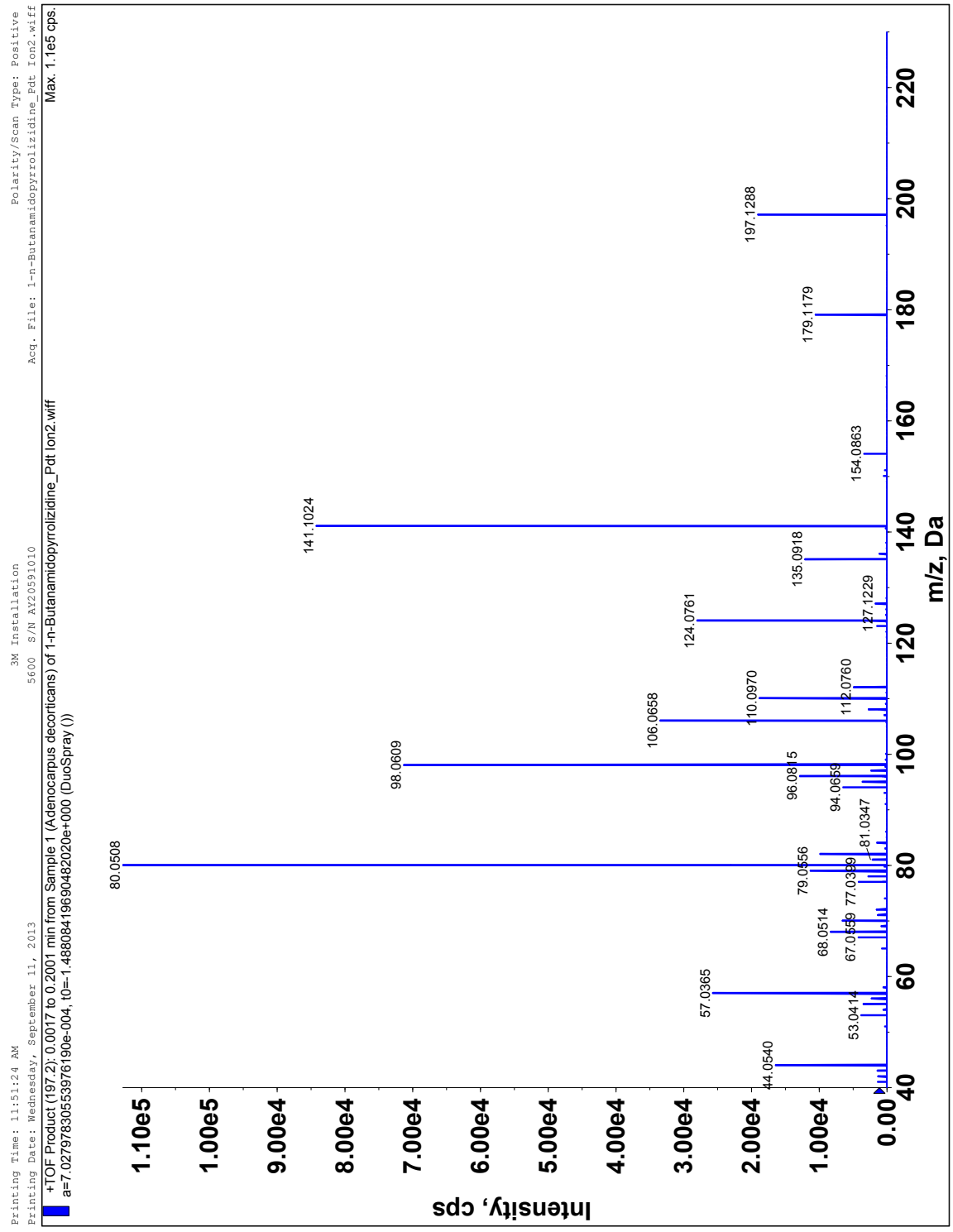


Figure 4.8. Fragment ions of compound **3**.

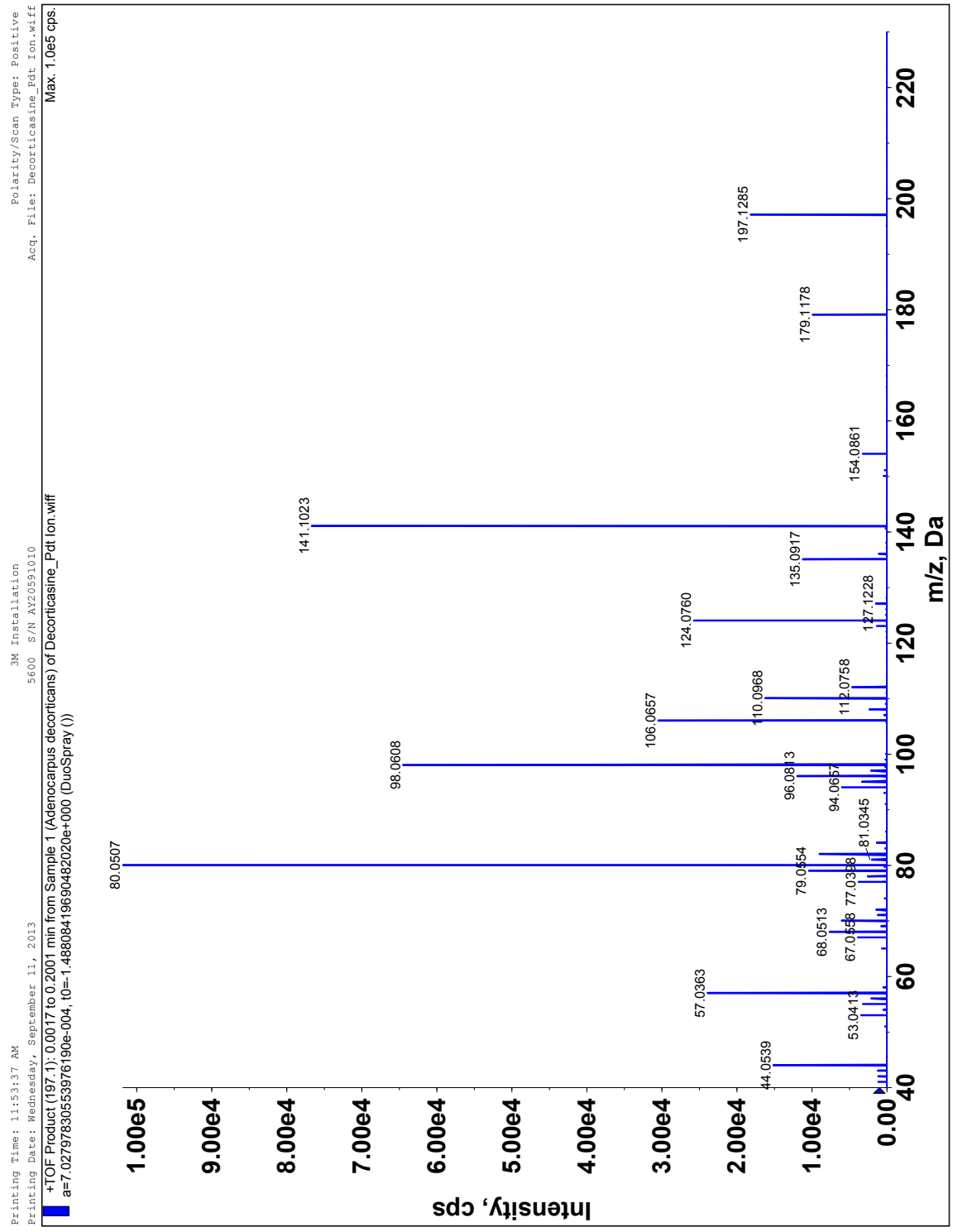


Figure 4.9. Fragment ions of decorticasine.

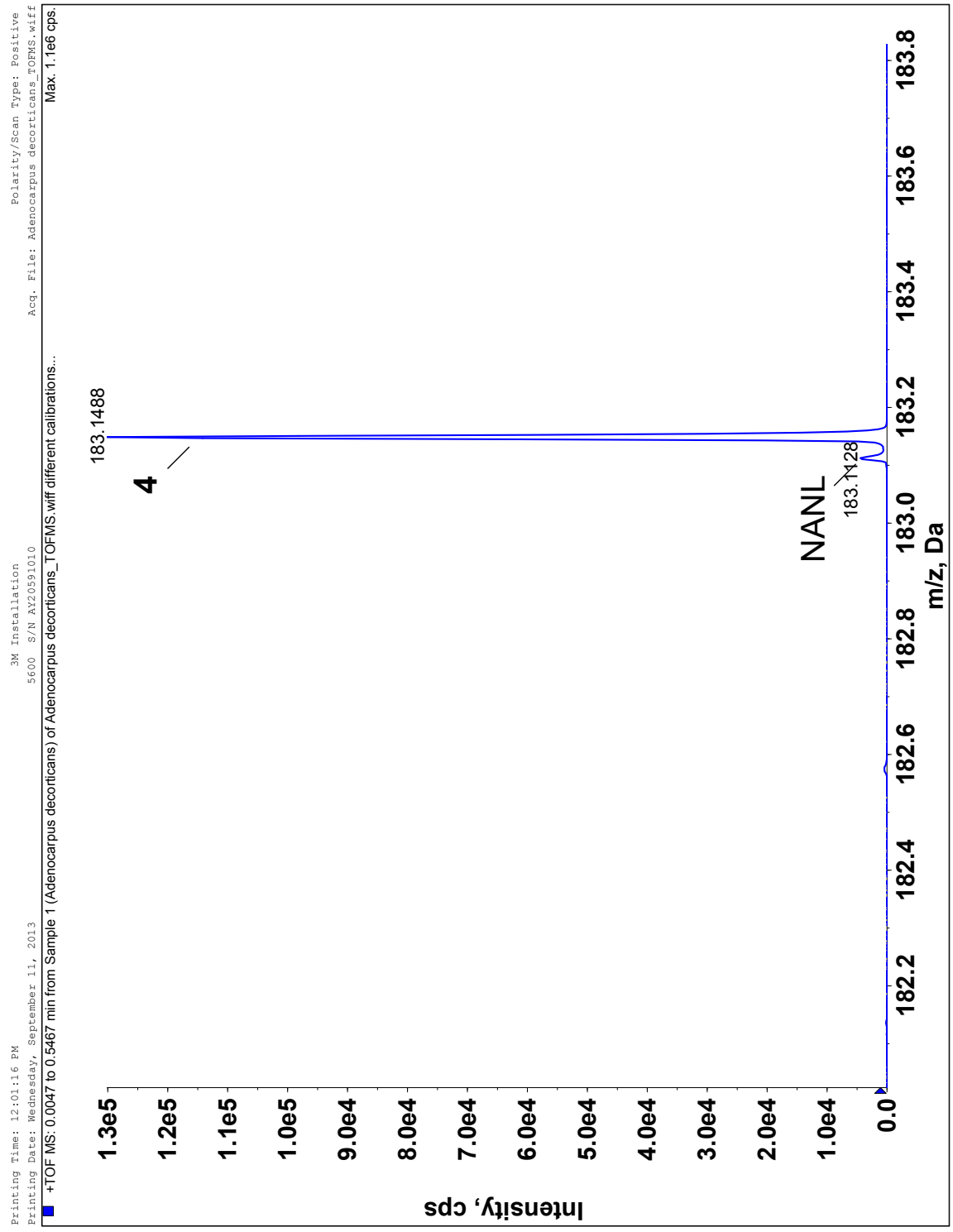


Figure 4.10. HRMS of NANL and compound **4**.

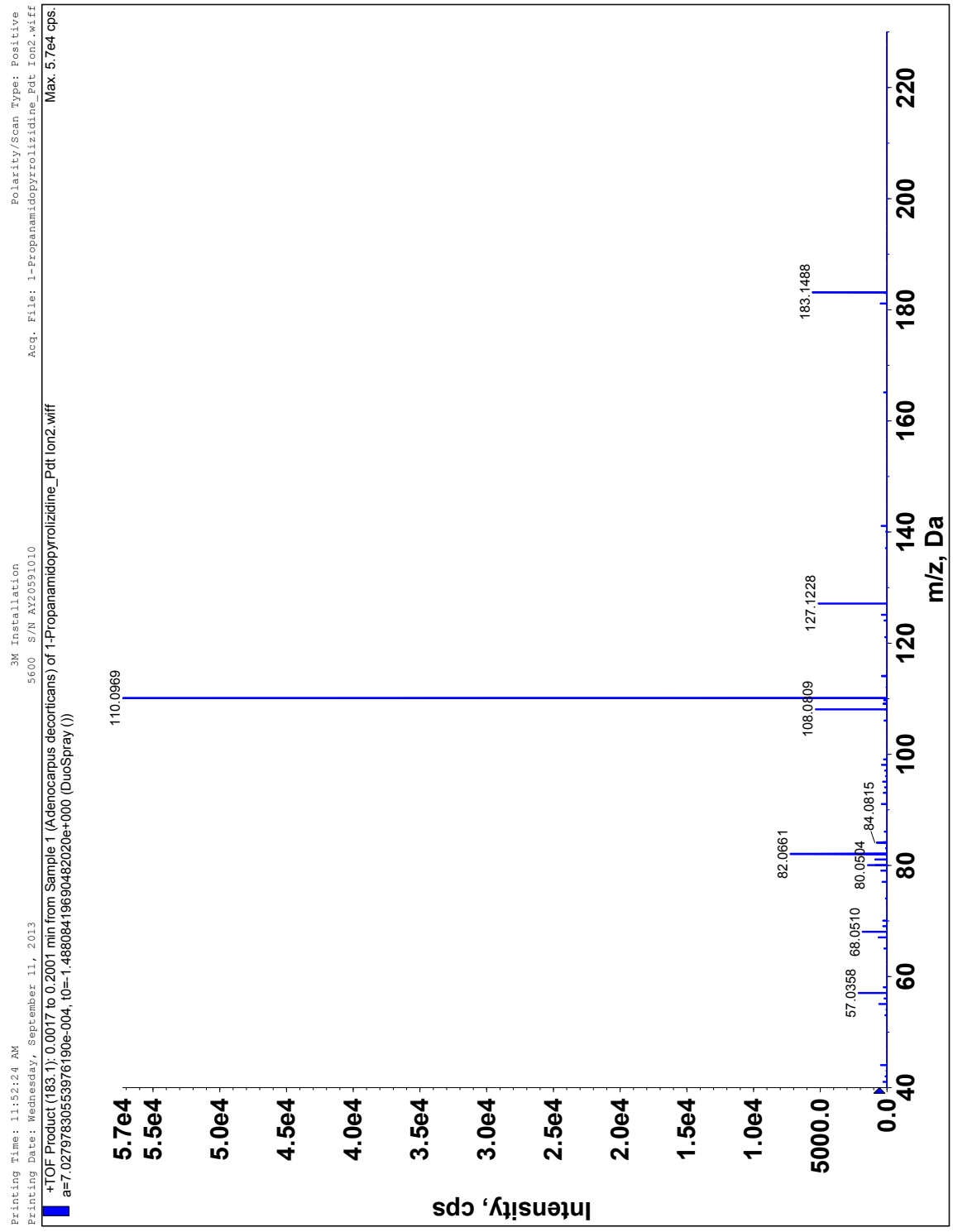


Figure 4.11. Fragment ions of compound **4**.

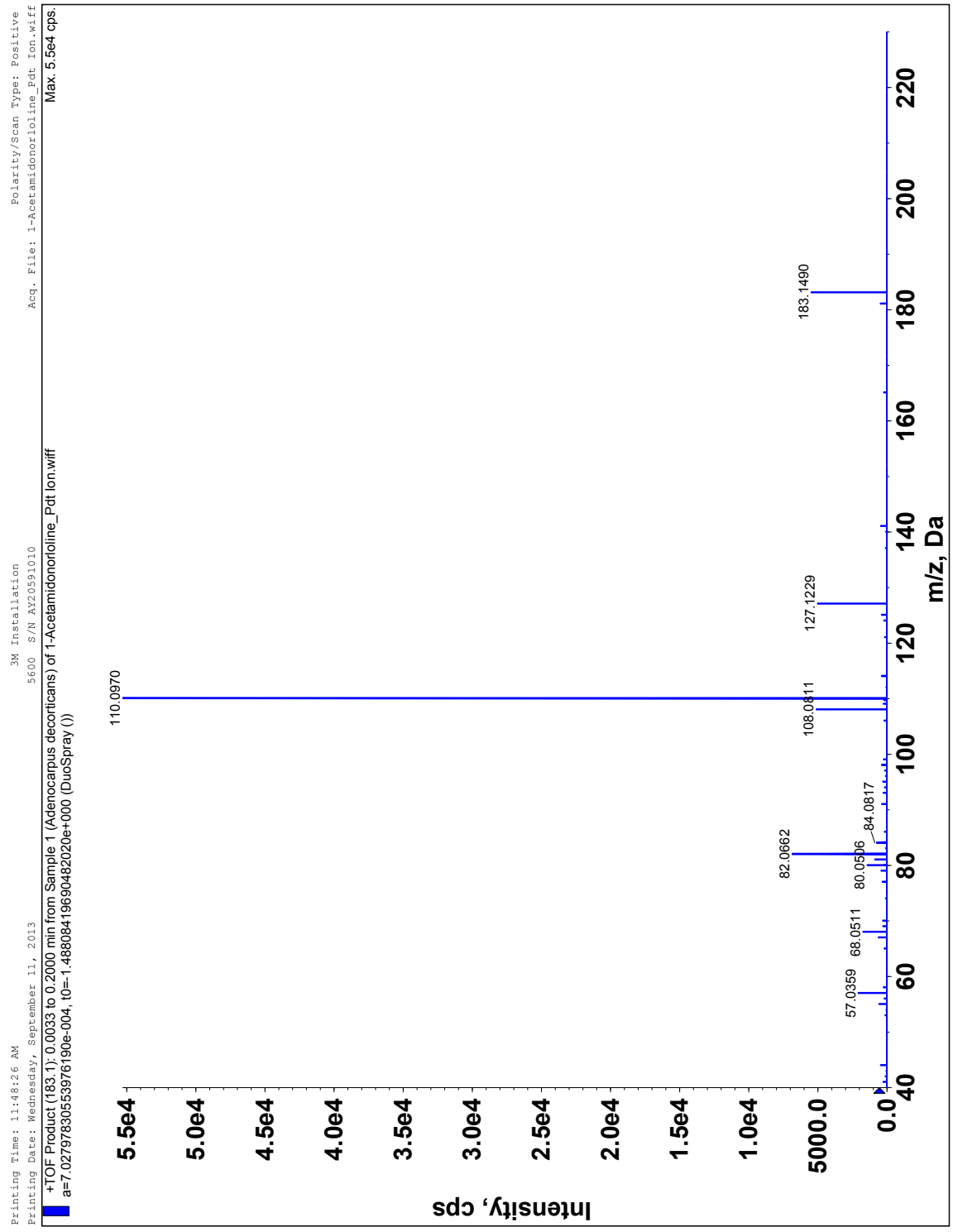


Figure 4.12. Fragment ions of NANL.

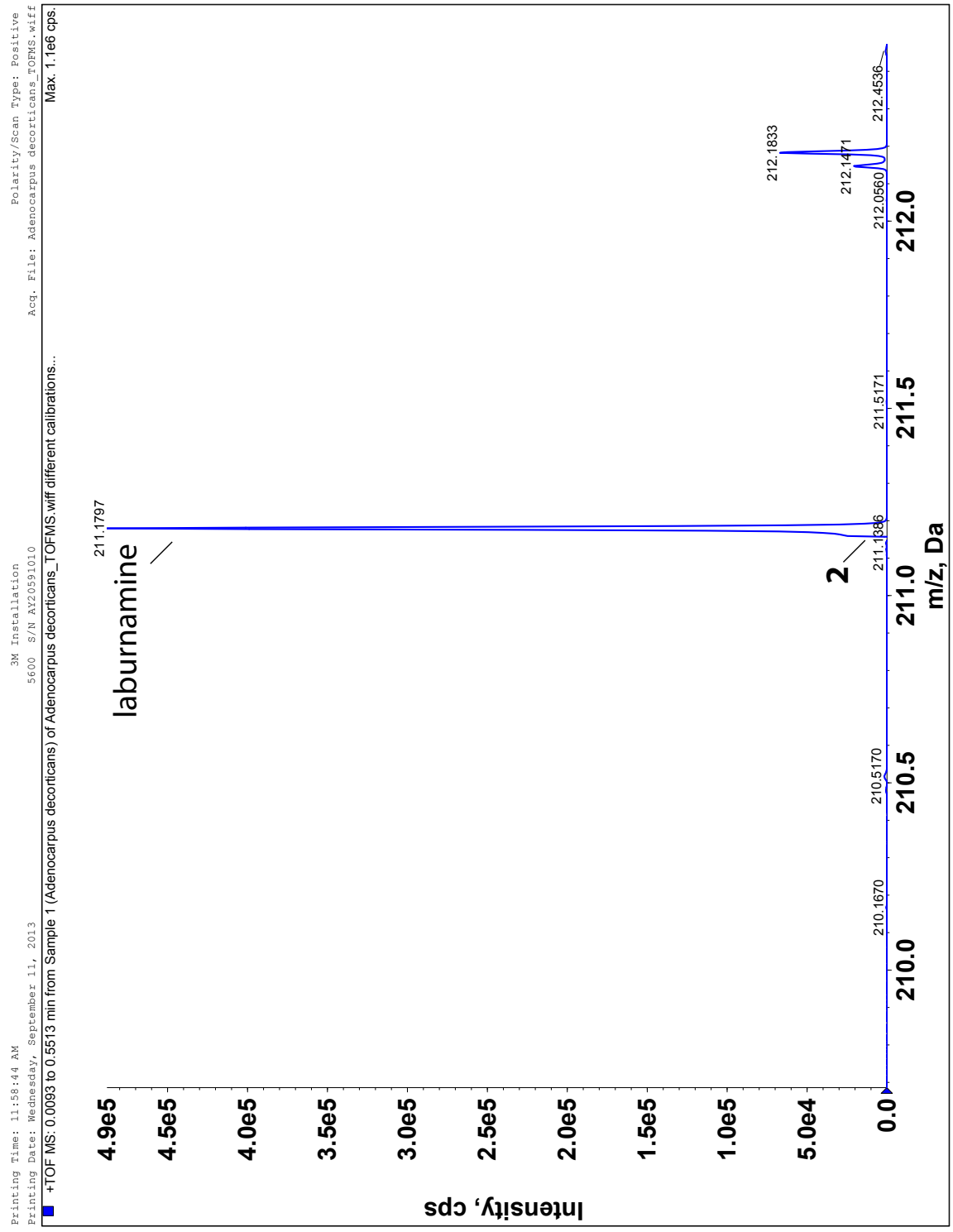


Figure 4.13. HRMS of laburnamine and compound **2**.

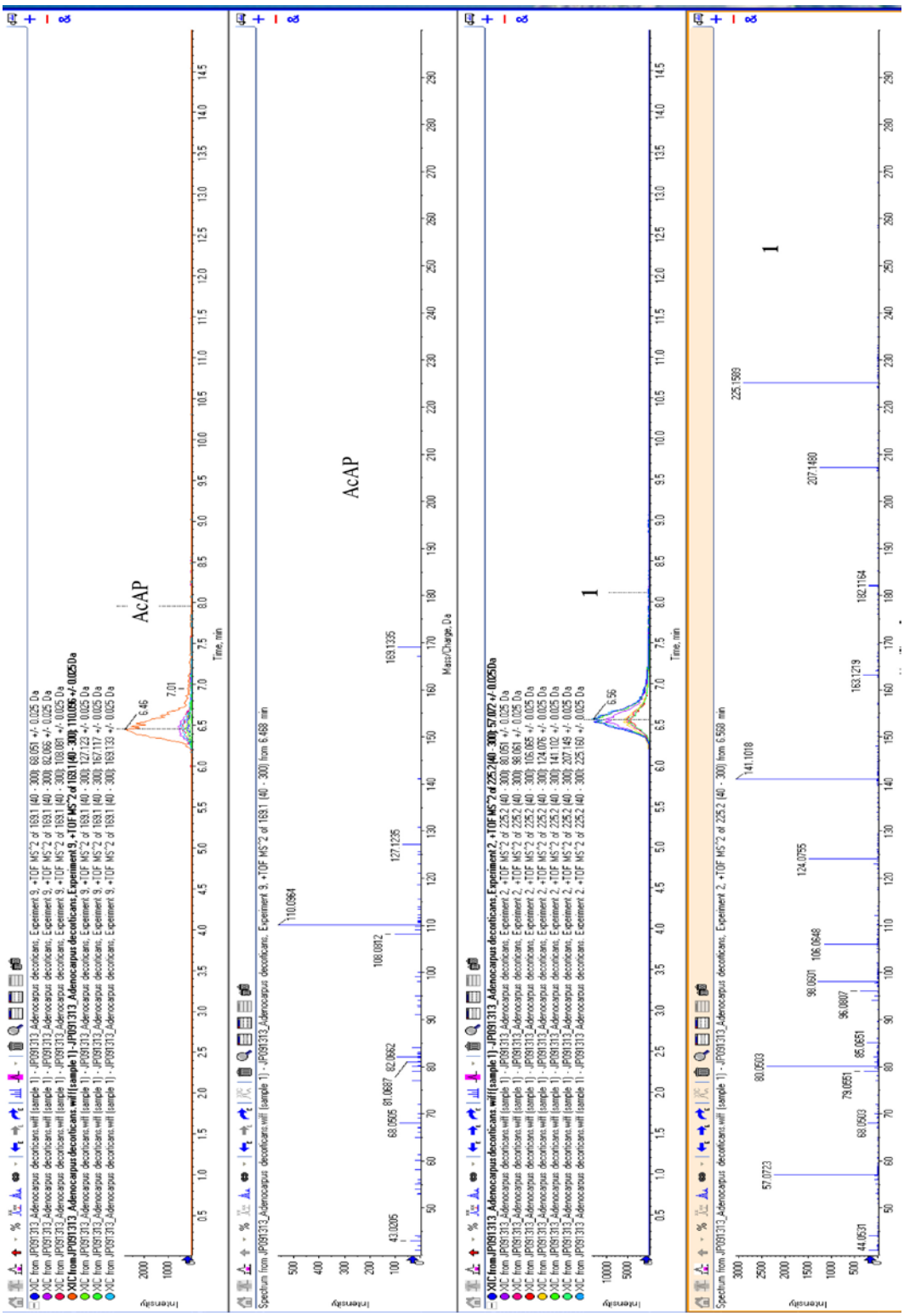


Figure 4.14. LCMS-HRMS of extract from *Adenocarpus decorticans*, showing separation of AcAP and compound **1**.

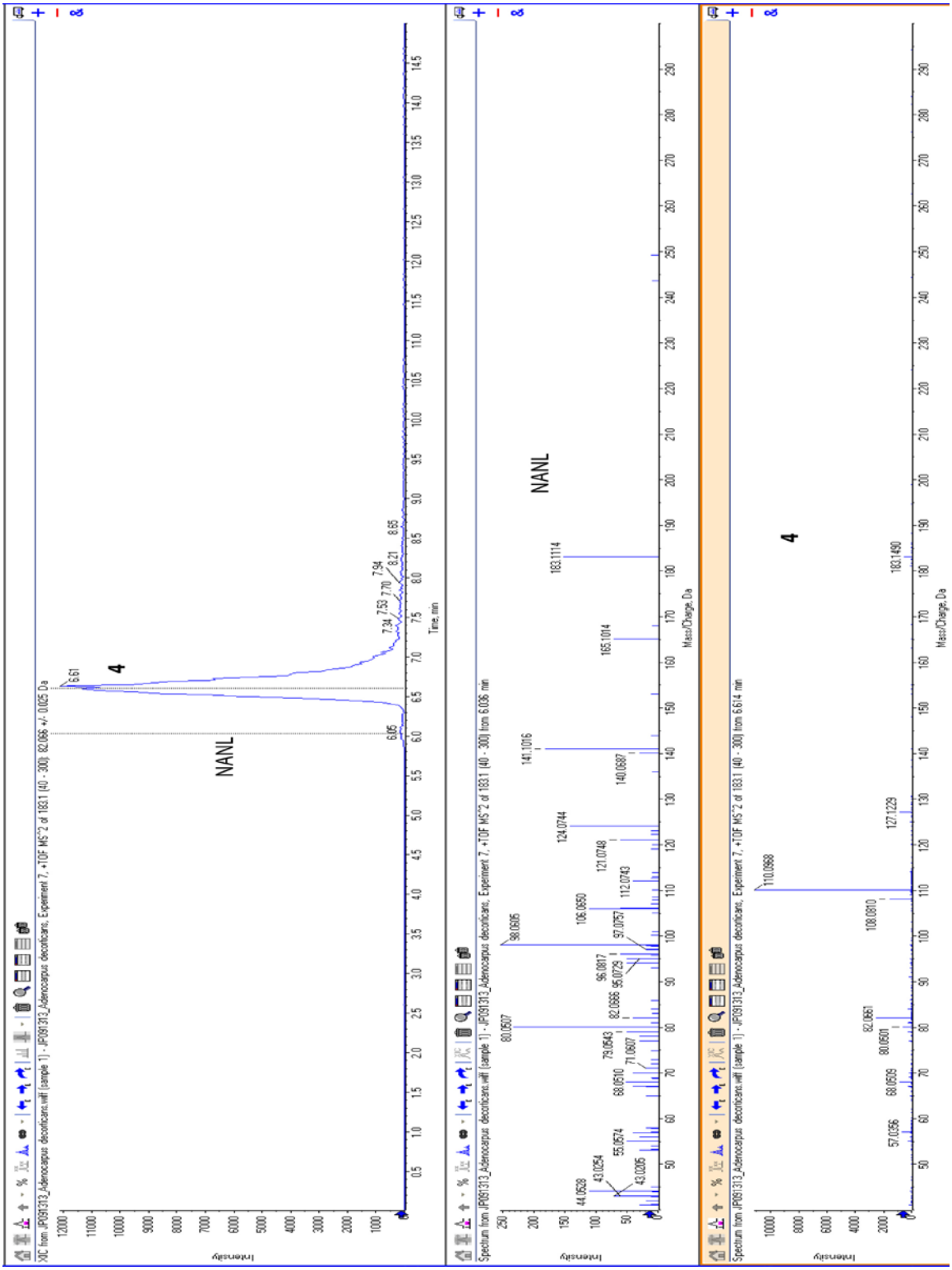


Figure 4.15. LCMS-HRMS of extract from *Adenocarpus decorticans*, showing separation of NANL and compound **4**.

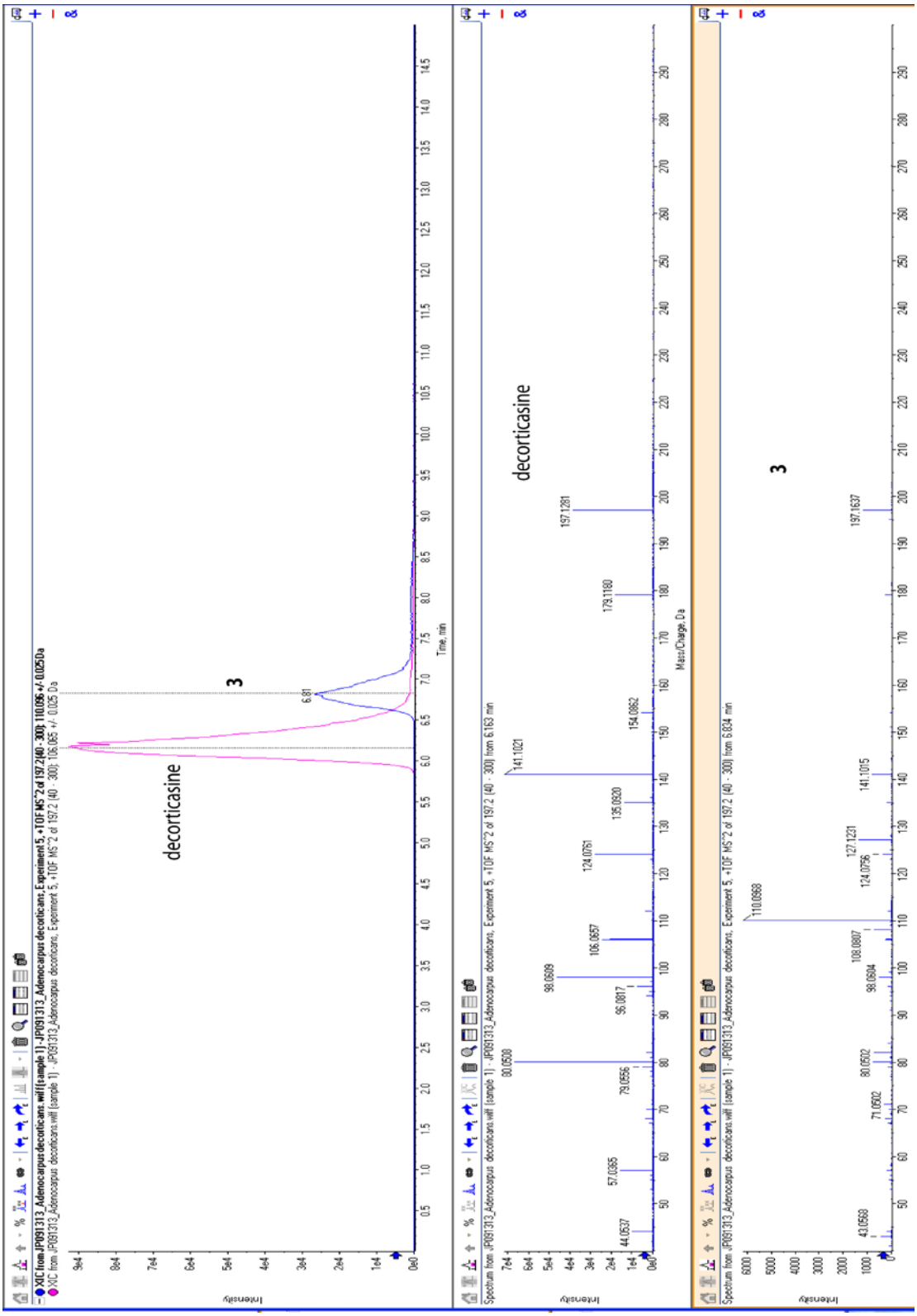


Figure 4.16. LCMS-HRMS of extract from *Adenocarpus decorticans*, showing separation of decorticasine (1-propanamidonorloline) and compound **3**.

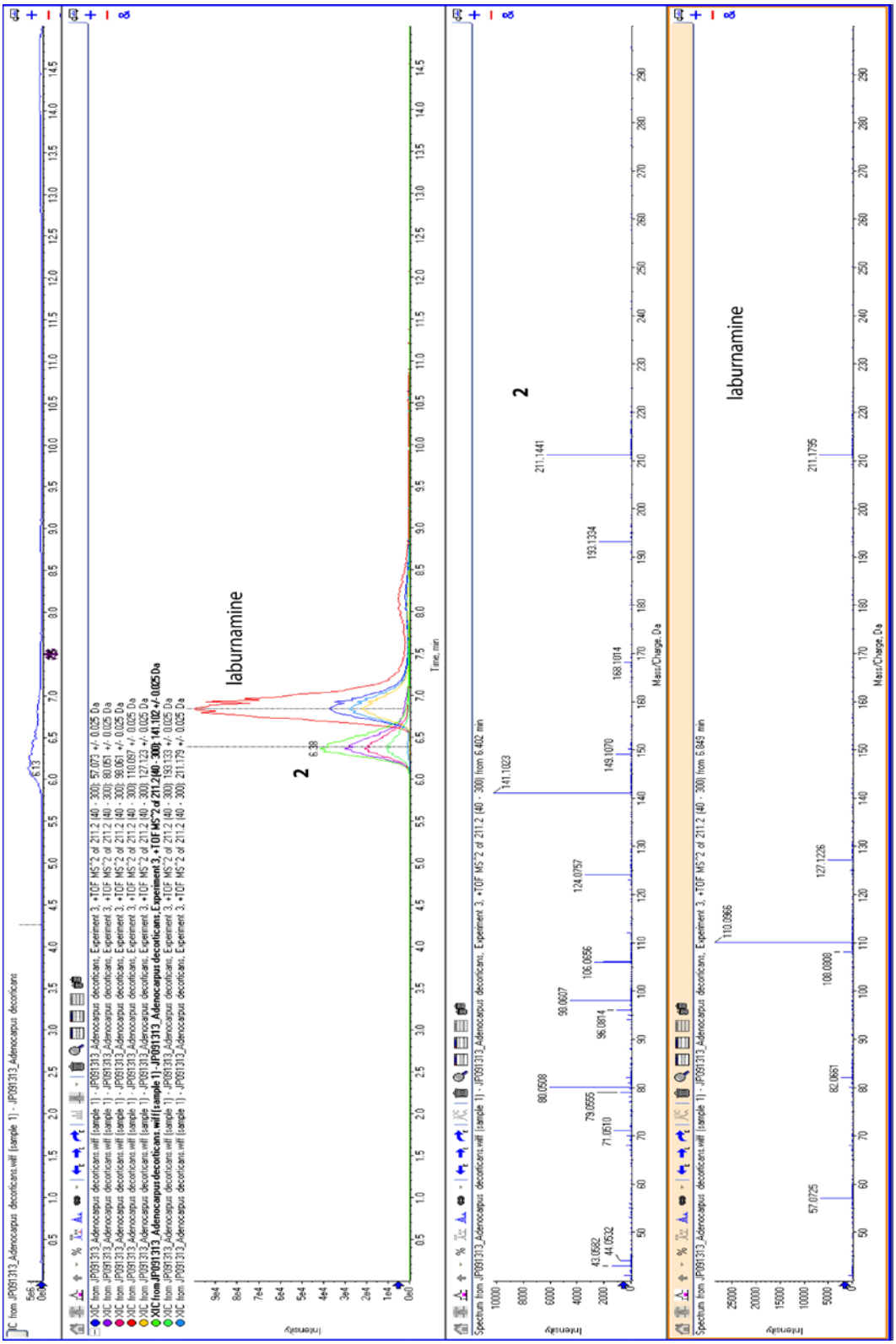


Figure 4.17. LCMS-HRMS of extract from *Adenocarpus decorticans*, showing separation of laburnamine and compound **2**.

4.3. Concluding Remarks

The *Adenocarpus* species and *Argyreia mollis* are the only plants outside the grasses reported to contain loline alkaloids. The loline alkaloids found in cool-season grasses are attributed to the symbiotic Clavicipitaceous fungi. Therefore, I hypothesized that a fungal symbiont associated with *Adenocarpus* species might contribute to loline alkaloids in this system. The various methods used in search for the fungus — deep sequencing of *A. decorticans* mRNA, attempted PCR amplification of *LOL* genes, and fungicide-treatment of *A. decorticans* and *A. telonensis* seeds — all failed to give a positive indication of a possible fungal symbiont in *A. decorticans*. Through HPLC-HRMS and LCMS-HRMS, I was able to obtain evidence of the presence of a series of loline- and pyrrolizidine alkaloid-like compounds that potentially differ only in the presence or absence of the ether bridge. This is in parallel to the scenario of ether formation in grass-Clavicipitaceae symbiota, in which the ether bridge forms on 1-acetamidopyrrolizidine to produce *N*-acetylnorloline (Chapter 2). It is intriguing to propose that the ether-containing loline alkaloids found in *Adenocarpus* species are formed by the similar route, potentially catalyzed by an enzyme functionally similar to LolO. In conclusion, it is likely that the loline alkaloids in *Adenocarpus* species are produced by the plant, although the possibility of a fungal symbiont associated with the root, or a bacterial symbiont, cannot be ruled out as potential sources of the loline and pyrrolizidine alkaloids. The biosynthesis of loline alkaloids in *Adenocarpus* species, at least in the ether formation step, is likely similar to what occurs in Clavicipitaceae fungi that are symbiotic with grasses.

4.4. Materials and Methods

4.4.1. Growth of* Adenocarpus *plants. *Adenocarpus decorticans* and *Adenocarpus telonensis* seeds were surface sterilized by soaking in 50% bleach for 30 min, washed with sterile water 5 times, and scarified by wounding with a scalpel. The seeds were then put in a Petri dish on top of two layers of damp filter paper to keep the dish moist. The petri dish was incubated at 22 °C in dark until seeds germinated (approximately one week). The seedlings were then transferred to moist sandy soil and maintained in a greenhouse. The plants tend to get *Phytophthora* infections if watered too much, so watering was restricted to a minimum. For fungicide treatment, seeds of both species were scarified and soaked in 0.9% pyraclostrobin overnight as described in Cook et al. (2013). After washing with sterile water 5 times, the seeds were germinated in Petri dish as described above.

4.4.2. Preparation of Illumina mRNA sequencing library. RNA of *A. decorticans* was extracted from a mixture of both leaves and stem, and the library for Illumina mRNA sequencing prepared according to the sample preparation guide: mRNA was purified and fragmented using divalent cations under high temperature, cDNA was synthesized and ends repaired, and adenylated at the 3' end, then ligated to adaptors. The ligation products was separated on a 2% agarose gel to select 200-bp fragments, and the fragments were enriched by PCR amplification and submitted for sequencing. Sequencing was carried out on Illumina HiSeq 2000 platform, and 100-bp paired-end

reads were generated. Newly forming buds of morning glory (*Ipomoeae asarifolia*) symbiotic with *Periglandula ipomoeae* were found to be rich in fungal growth. So, as a positive control to cover both fungal and plant transcriptome, new buds of symbiotic morning glory were extracted for mRNA and the library prepared for Illumina mRNA sequencing following the same procedure.

4.4.3. PCR tests for endophyte* LOL *genes. *Adenocarpus decorticans* DNA was prepared from both leaves and stem with the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research) following the manufacture's instructions. For *lolC* PCR, lolC-3a and lolC-5b were used. For *lolF* PCR, lolF-5 and lolF-3 were used, and for *tubB*, 1140 and 1235 were used (Table 4.1). All PCR were performed at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min.

4.4.4. Loline alkaloid extraction and characterization. Loline alkaloid extraction and application on GC-MS were following the methods described in Chapter 2.

Table 4.1. Oligonucleotides used in this chapter.

Primer	**Sequence**
AdenoF3	AGCCTTCGCAGTAGCTTCAT
AdenoR3	TCCTGCTCCTGCCACGACA
AdenoF1	TACGTCTGGAAGATTTGCGA
AdenoR1	GCAGCTAATTCAGGACTCCA
lolC-3a	GGTCTAGTATTACGTTGCCAGGG
lolC-5b	GTTGCCCACGGTGCGCGTCTTC
lolF-5	GCATACTGACAGCGTCATGAGG
lolF-3	TCGTCGTTGGCGCTGGTTTTTC
11140	GAGAAAATGCGTGAGATTGT
1235	GTTTCGTCCGAGTTCTCGAC

Chapter Five

Evolution of *LOL* cluster in cool-season grass endophyte

5.1. Introduction

The distribution of loline alkaloids is limited to cool season grasses (Poaceae, subfamily Poöideae) that are symbiotic with Clavicipitaceae species of fungal endophytes, with the only known exceptions being the dicotyledonous plants, *Adenocarpus* spp. and *Argyreia mollis*. In the grass-fungus symbiota, the *Epichloë* and *Atkinsonella* species are responsible for the biosynthesis of loline alkaloids. Whether the production of the loline alkaloids in the dicotyledonous plants is related to possible fungal symbionts in those systems is not known yet. Consistent with the observation that genes involved in specialized metabolites tend to be clustered in the fungi, the biosynthesis of loline alkaloids in the *Epichloë* spp. is catalyzed by enzymes that are encoded by a cluster of up to 11 genes, designated as the *LOL* cluster. Following genome sequencing of several *Epichloë* and *Atkinsonella* species, I have annotated a *LOL* cluster in each of the isolates that produces loline alkaloids and the related alkaloid, *exo*-1-acetamidopyrrolizide (AcAP). Through comparison of the *LOL* clusters of both sexual and asexual *Epichloë* species, I attempt to infer the origins and evolutionary history of *LOL* clusters in *Epichloë* species, and to assess the congruence between *LOL* gene phylogenies and housekeeping gene phylogenies.

5.2. Results and Discussion

5.2.1. LOL *clusters in sexual* Epichloë *species*

Genomes of six sexual *Epichloë* species have been sequenced, and I found variations in presence and absence of the *LOL* cluster in different isolates of the same species, and also variations in *LOL* gene content between and within species (Table 5.1). The *Epichloë festucae* strain E2368 was well sequenced with various sequencing methods, and each gene in the genome was manually annotated (Schardl et al., 2013b), C.L. Schardl, unpublished). This *E. festucae* strain had a complete *LOL* cluster with most of the *LOL* genes assembled on one scaffold and *lolF* on another, and produces the pathway end-product, *N*-formylloline (NFL), as well as the other loline alkaloids. The other *E. festucae* isolate that was also sequenced, strain Fl1, did not have a *LOL* cluster.

Three isolates of *Epichloë bromicola* were sequenced, of which two had *LOL* clusters, the gene contents of which, however, were different. One strain, *E. bromicola* AL0434, had the complete set of 11 *LOL* genes, although they were not assembled on one scaffold. Genes *lolF*, *lolC*, and *lolD* formed a partial cluster, *lolO*, *lolA*, *lolU*, *lolP*, *lolT*, and *lolE* formed another partial cluster, and neither of them were on the same contig as *lolN* or *lolM*, which were on separate contigs as well. Although the AL0434 *LOL* genes were not assembled as one cluster, there was no indication of any difference in the gene orders compared with the *E. festucae* E2368 or *Epichloë uncinata* e167 *LOL* clusters (Spiering et al., 2005, Schardl et al., 2013b). The *E. bromicola* isolate AL0426_2

had *LOL* genes that formed a cluster but lacked *lolN*, *lolM* and *lolP*, and also had a mutated *lolO*. The grasses symbiotic with these *E. bromicola* strains were not available to check the loline-alkaloid profiles, but judging from the *LOL* gene composition, and based on my studies of late-pathway genes (Chapters 2 and 3), AL0434 is likely to be an NFL producer, whereas AL0426_2 should produce AcAP.

Similar to the variation between the two *LOL* clusters found in the two *E. bromicola* isolates, the *LOL* cluster in each of the two strains of *Epichloë amarillans* showed variation as well. *Epichloë amarillans* E57 *LOL* cluster had a mutated *lolP*, and lacked *lolN* and *lolM*, which rendered it an *N*-acetylnorloline (NANL) producer (Chapter 3). The other *E. amarillans* isolate, E4668, had only *lolF* and *lolC*, which were divergently transcribed as in E2368, but in E4668 there was a nucleotide insertion in one exon of lolC, rendering it nonfunctional. *Epichloë baconii* E1031 also had only *lolC* and *lolF*, plus a linked remnant of *lolM*, suggesting that these genes were probably derived from an ancestral complete *LOL* cluster.

Epichloë glyceriae E277 and *E. brachyelytri* E4804 both had *LOL* genes that were not assembled on one contig, but again there was no indication of change in gene order compared with the complete *LOL* cluster in E2368 or the two *LOL* clusters in e167. Isolate E277 lacked *lolM* and *lolP*, and had a mutated *lolN*, which stops the loline alkaloid biosynthesis at NANL (Chapter 3). Isolate E4804 lacked *lolN* and *lolP*, and had mutated *lolM* and *lolO*, which stops the loline alkaloid biosynthesis at AcAP (Chapter 2) (Pan et al., 2014).

Table 5.1. Loline-alkaloid profiles and *LOL*-gene content in each cluster of plant-symbiotic Clavicipitaceae.[a]

Isolates	***LOL* genes**										**Loline alkaloids**
	lolF	*lolC*	*lolD*	*lolO*	*lolU*	*lolP*	*lolT*	*lolE*	*lolN*	*lolM*	
***E. amarillans* E57**	+	+	+	+	+	Δ	+	+	-	-	NANL
***E. amarillans* E4668**	+	Δ	-	-	-	-	-	-	-	-	nt
***E. brachyelytri* E4804**	+	+	+	Δ	+	-	+	+	-	Δ	AcAP
***E. bromicola* AL0434**	+	+	+	+	+	+	+	+	+	+	nt
***E. bromicola* AL0426_2**	+	+	+	Δ	+	-	+	+	-	-	nt
***E. baconii* E1031**	+	+	-	-	-	-	-	-	-	Δ	nt
***E. canadensis* e4815**	+	+	+	+	+	Δ	+	+	-	-	NANL
***E. chisosa* e3609_Ebro copy**	+	+	+	+	+	+	+	+	+	+	NFL
***E. chisosa* e3609_Eama copy**	+	+	+	+	+	Δ	+	+	-	-	
***E. coenophiala* e4163**	+	+	+	+	+	+	+	+	+	+	NFL

Table 5.1. (continued)

***E. coenophiala* e4309**	+	+	+	+	+	+	+	+	Δ	+	NANL
***E. festucae* E2368**	+	+	+	+	+	+	+	+	+	+	NFL
***E. glyceriae* E277**	+	+	+	+	+	-	+	+	Δ	-	AcAP
***E. aotearoae* e899**	+	+	+	+	+	+	+	+	+	+	NFL
***E. uncinata* e167_Ebro copy**	+	+	+	+	+	+	+	+	+	+	NFL
***E. uncinata* e167_Ety copy**	+	+	+	+	+	Δ	+	+	-	-	
***A. hypoxylon* B4728**	+	+	+	Δ	+	Δ	+	+	-	-	AcAP

[a] Abbreviations: Eama = *Epichloë amarillans*, Ebro = *Epichloë bromicola*, Ety = *Epichloë typhina*, NANL= *N*-acetylnorloline, NFL = *N*-formylloline, AcAP= *exo*-1-acetamidopyrrolizidine, nt = not tested, and Δ is used to indicate inactivating deletions in the gene. More detailed description of the isolates can be found at (Schardl et al., 2013c).

Genomes of related *Atkinsonella* species were also sequenced. The *LOL* genes of *A. hypoxylon* B4728 (the prefix "B" refers to the synonym, *Balansia hypoxylon*) were assembled on one contig, and the order of the *LOL* genes was consistent with that of *Epichloë* species. The *LOL* cluster of B4728, however, lacked *lolN* and *lolM*, and had only remnants of *lolP* and *lolO*, which made it an AcAP producer (Chapter 2)(Pan et al., 2014). On the other hand, another sequenced *Atkinsonella* species, *A. texensis* (isolates B6155 and B6156), did not have a *LOL* cluster, again showing presence/absence polymorphism of the *LOL* cluster between species.

The presence and absence polymorphism of *LOL* clusters and the polymorphisms of the *LOL* gene content in *Epichloë* and the related *Atkinsonella* species suggested that the *LOL* cluster in these plant symbionts is under diversifying selection. The late pathway genes that are involved in modifications of loline alkaloids, *lolN*, *lolM*, and *lolP*, as well as the *lolO* gene involved in loline ether formation from AcAP, showed presence/absence polymorphism or mutations in many of the species. The mutations or deletions of these genes terminate the loline biosynthesis pathway in intermediate steps. This correlation of the disruption of a certain gene and the accumulation of a specific metabolite as the end product makes the prediction of loline-alkaloid profile possible (Takach & Young, 2014, Schardl et al., 2012, Schardl et al., 2013c). For the isolates that accumulate a certain intermediate as the end product, remnants of the late pathway genes were often found (Table 5.1), indicating that the evolutionary trajectory involved independent deletions of these genes from an ancestral complete *LOL* cluster, rather than gains of these genes on lineages to species and strains that bear a complete *LOL* cluster. Furthermore, comparing the mutations of the late pathway genes, the mutations were different (Pan et al., 2014), indicating that independent mutation events caused these variations. The presence of complete *LOL* clusters in *Epichloë* species and the presence of a *LOL* cluster that is likely derived from a complete *LOL* cluster in the related *Atkinsonella* species suggest that a complete *LOL* cluster was already assembled in common ancestor of *Epichloë* and *Atkinsonella* species, i.e., that the formation of the complete *LOL* cluster predates the divergence of *Atkinsonella* and *Epichloë* species.

5.2.2. LOL *clusters in hybrids*

Most of the asexual *Epichloë* species (formerly named *Neotyphodium* spp.) have been found to be inter- or intra-species hybrids of two or more ancestral sexual species (Moon et al., 2004, Schardl, 2010, Ghimire et al., 2011). Size estimates of hybrids suggest that they have retained most of the genomes contributed by the sexual ancestors (Kuldau et al., 1999). The sources of *LOL* clusters in the hybrids were obvious from comparison of the phylogenies of selected *LOL* genes, *lolC* (Figure 5.1), *lolP*, and *lolN* (Figure 5.2) with the housekeeping gene tree (Figure 5.3). *Epichloë amarillans* contributed a *LOL* cluster to *Epichloë canadensis* and *Epichloë chisosa*. Both *E. amarillans* E57 and *E. canadensis* e4815 produce NANL as the end product, and both had a mutated *lolP* pseudogene. Interestingly, the sequences of *lolP* in *E. amarillans* E57, *E. canadensis* e4815, and the *E. amarillans* copy of *lolP* (also a pseudogene) in *E. chisosa* e3609 all shared a 383-bp deletion, suggesting that the deletion in *lolP* predates hybridization events that gave rise to *E. canadensis* e4815 and *E. chisosa* e3609. The

observation that the *lolP* pseudogene in *Epichloë uncinata* e167 did not share this common deletion found in the aforementioned species argues against the alternative possibility that the specific region of *lolP* might be a mutational hotspot that could easily generate the same mutation independently.

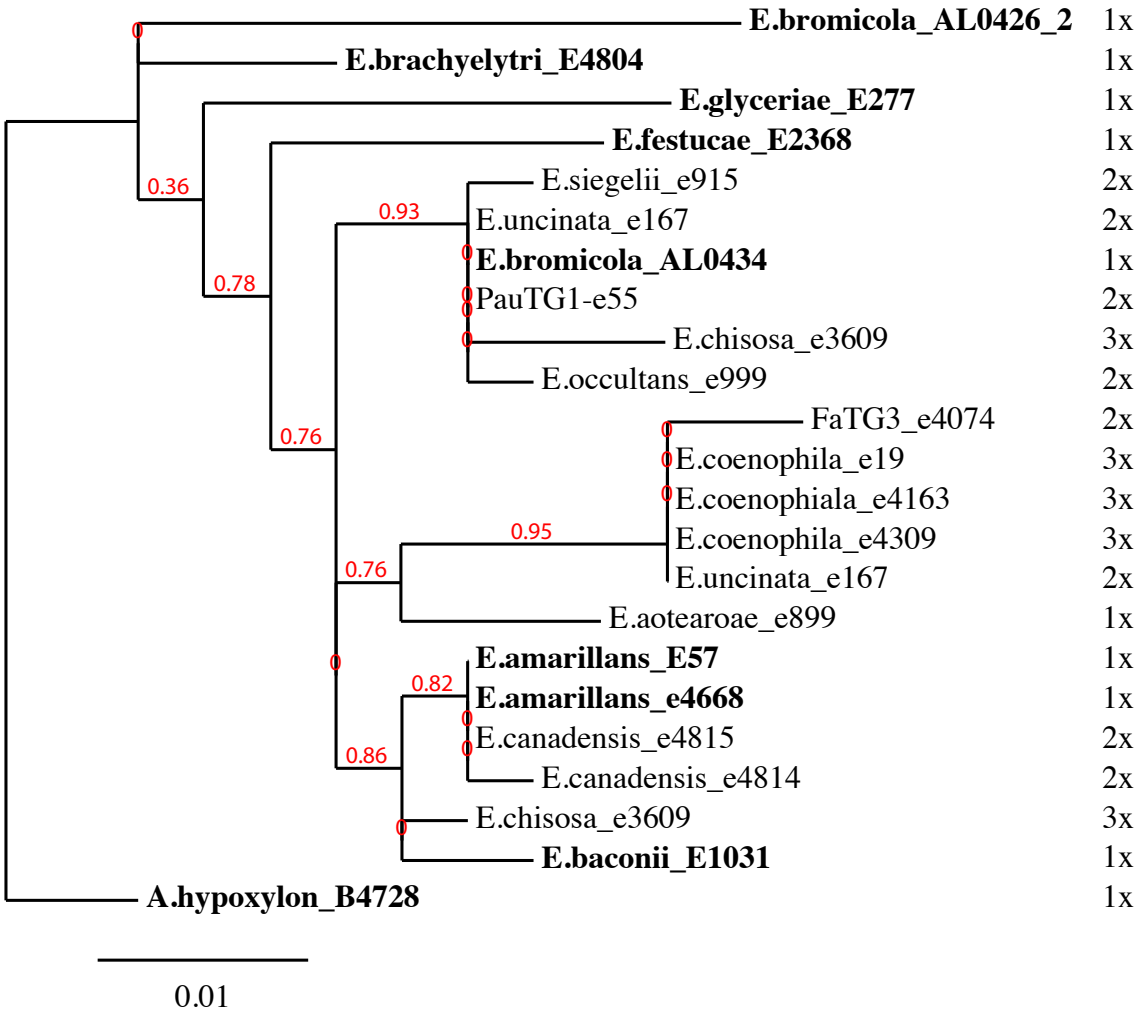
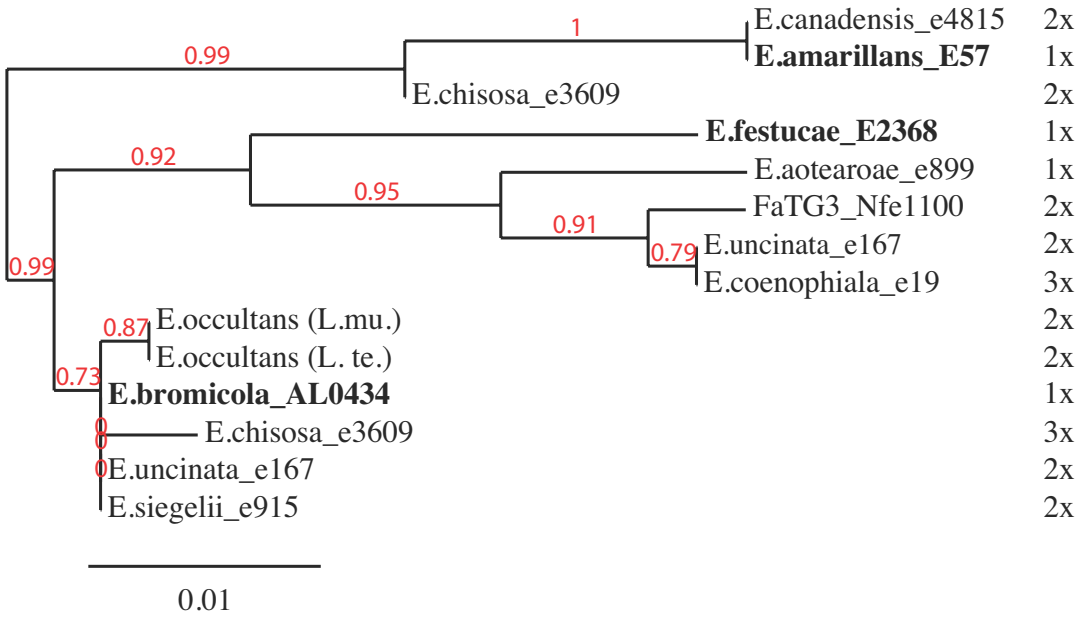


Figure 5.1. Phylogenetic tree inferred from *lolC* sequences. If the genome sequence is available, whole gene sequence was used, and for the ones that do not have the genome sequences, partial sequences of *lolC* were obtained from sequencing of PCR products. The phylogeny was inferred by maximum likelihood estimate (MLE) with PhyML implemented in Phylogeny.fr. (Dereeper et al., 2008). Species names are followed by isolation numbers. Isolates in bold indicate sexual species. The numbers on the right represent ploidy of the isolates, where hybrids have ploidy greater than one. Scale bar indicates 0.01 substitutions per site.

lolP



lolN

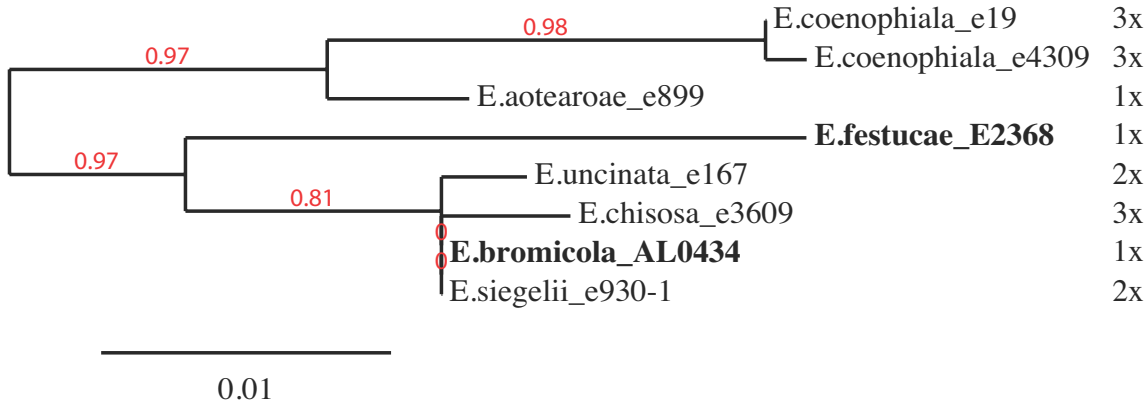


Figure 5.2. Phylogenetic trees inferred by MLE from *lolP* and *lolN* sequences. Isolates in bold indicates sexual species (nonhybrid). The numbers on the right represent ploidy of the isolates, where hybrids have ploidy greater than one. Scale bars indicate 0.01 substitutions per site.

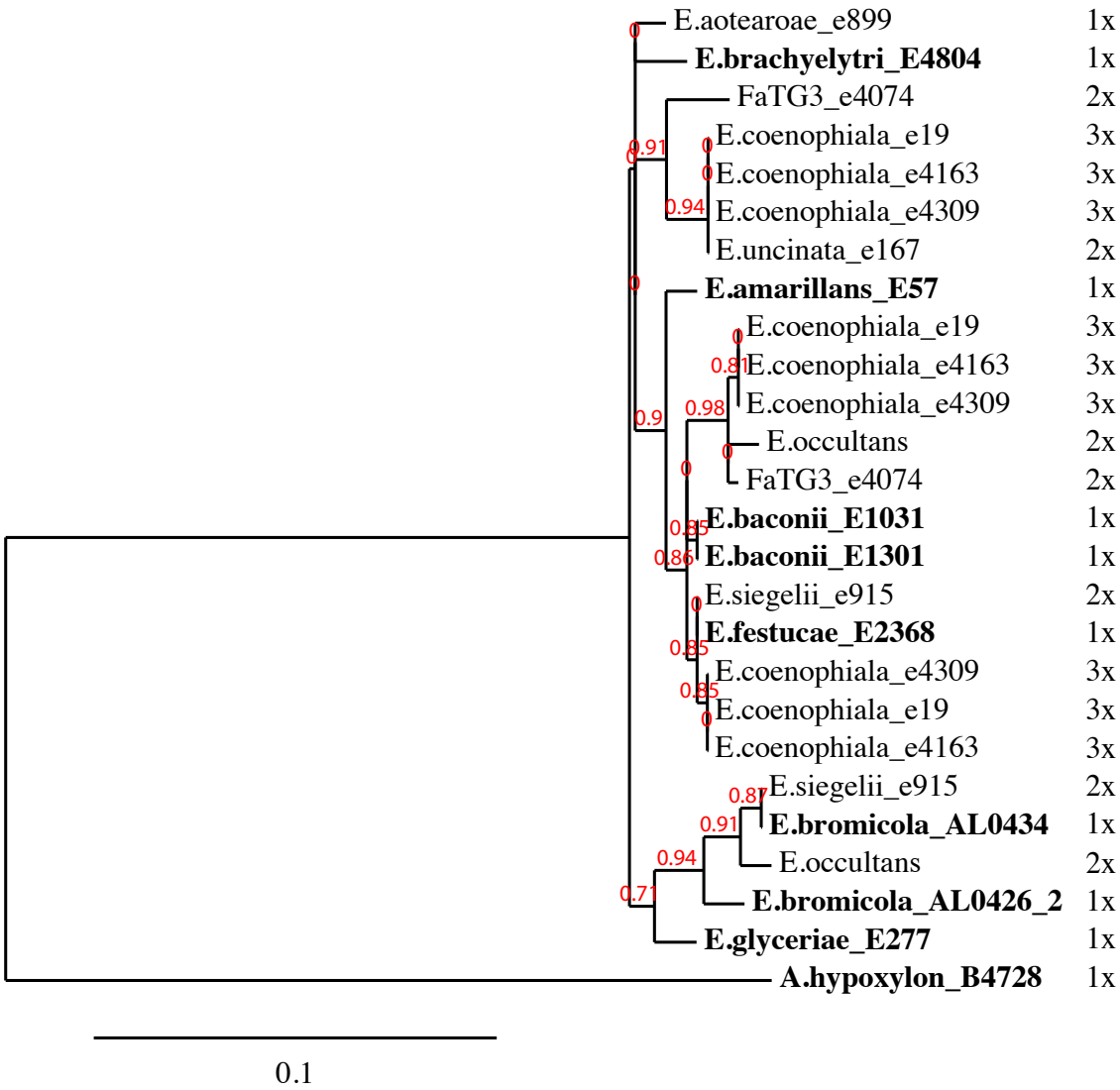


Figure 5.3. Phylogenetic tree inferred by MLE from the *tubB* β-tubulin gene sequences. Isolates in bold indicates sexual species (nonhybrid). The numbers on the right represent ploidy of the isolates, where hybrids have ploidy greater than one. Scale bar indicates 0.1 substitutions per site.

Both *E. chisosa* and *E. uncinata* obtained one complete *LOL* cluster from *Epichloë bromicola*, hence they should produce NFL as the end product (confirmed for *E. uncinata,* but *E. chisosa*-symbiotic plants were unavailable to test). The other *LOL* cluster in *E. uncinata* was derived from *Epichloë typhina* subsp. *poae,* which also contributed a *LOL* cluster to *Epichloë coenophiala, E. aotearoae*, and the uncharacterized loline producing strain PauTG1 isolated from the grass, *Poa autumnalis*. Surprisingly, contrary to the widespread occurrence of the *E. typhina* var. *poae* copy of the *LOL* cluster in the hybrids, no isolate of *E. typhina* var. *poae* has been obtained to date that has a *LOL* cluster, which may be due to losses of *LOL* cluster in the *E. typhina* var. *poae* populations, or simply that the ones bearing a *LOL* cluster have not been sampled yet.

In both *E. uncinata* and *E. chisosa*, two copies of the *LOL* clusters were found, and were inferred by phylogenies to have been donated to those hybrids by different ancestral species. An interesting observation was that in both isolates, one copy of the *LOL* cluster was complete, and the other copy had mutations in late pathway genes. This is similar to cases with other alkaloid biosynthesis gene clusters, which are sometimes found in multiple copies in hybrid *Epichloë* species (Schardl et al., 2013c). It has been hypothesized that this situation is probably selected in hybrids for optimal defense because different loline alkaloids, and probably the other kinds of alkaloids found in the *Epichloë*-grass symbioses as well, tend to be specific against different herbivores (Riedell et al., 1991, Popay et al., 2009). Hence, mutations that cause accumulations of intermediates would increase the variations in the alkaloid combinations rather than predominant accumulation of end products, and may therefore contribute to better defense against the herbivores (Panaccione, 2005). Another interesting observation is that meadow fescue plants symbiotic with hybrid *E. uncinata* e167 accumulate much higher amount of loline alkaloids than the ones symbiotic with sexual species such as *E. festucae* E2368. I assume this is contributed by the two functional copies of *LOL* genes present in the genome. Conceivably, higher alkaloids amounts probably would more strongly deter herbivores and enhance the protective effect conferred to the host by the endophyte. Whether higher alkaloid amount is a common outcome of hybrids that bear multiple copies of functional alkaloid gene clusters merits further study.

5.2.3. Variation of LOL *cluster locations*

The *LOL* genes were arranged in the same order when the genes were assembled in a cluster, and even in the species for which a complete cluster did not assemble there was no indication of change of gene order. So it seems that the *LOL* gene orders remained the same across the species. The orientation and the location of the *LOL* cluster, however, showed variation between species. The *LOL* cluster of *E. festucae* E2368 was between two housekeeping genes *nsfA* and *lteA*, with the *nsfA* linked to *lolF* and *lolM* on the other end of the cluster linked with *lteA* (Figure 5.4). In *E. amarillans* E57, *lolF* was similarly linked with *nsfA*. However, in *E. glyceriae* E277, the whole *LOL* cluster showed an inverted orientation compared with *E. festucae* E2368, with the *nsfA* linked to a remnant of *lolN* proximal to *lolE*, and *lteA* linked to *lolF* (Figure 5.4). In *E. bromicola* AL0426_2, the *LOL* cluster showed the similar orientation as in *E. glyceriae* (data not

shown). Comparing the orientation of the *LOL* cluster across *Epichloë* species, I found that the orientation of the *LOL* cluster have changed at least twice in *LOL* cluster evolution, one at the divergence of *E. festucae* and *E. amarillans*, and another at the divergence of *Epichloë typhina* subsp. *poae* (Figure 5.5a). The proposed two changes could be easily deduced from comparing the housekeeping genes proximal to *LOL* clusters with the *LOL* cluster distribution, represented by *lolF* phylogeny (Figure 5.5b).

Furthermore analysis of the housekeeping genes proximal to *LOL* clusters in *Epichloë* species and *A. hypoxylon* B4728 revealed that the whole cluster has been translocated in chromosome at least once. Unlike what was in *Epichloë* species, the *LOL* cluster of *A. hypoxylon* B4728 was found between another two housekeeping genes, with *lolE* linked to a phosphoketolase gene, and *lolF* linked to an MFS transporter gene, which in turn was beside a mitochondrial protein gene. The *nsfA* and *lteA* genes in B4728, in contrast, were linked and located apart from the *LOL* cluster, as were the phosphoketolase and mitochondrial protein genes in the *Epichloë* species (Figure 5.4). This indicated that translocation of the whole cluster has occurred in *LOL* cluster evolutionary history. It is even possible that the ancestral *LOL* cluster was located at neither locations found in *Epichloë* and *Atkinsonella* and has experienced multiple translocations.

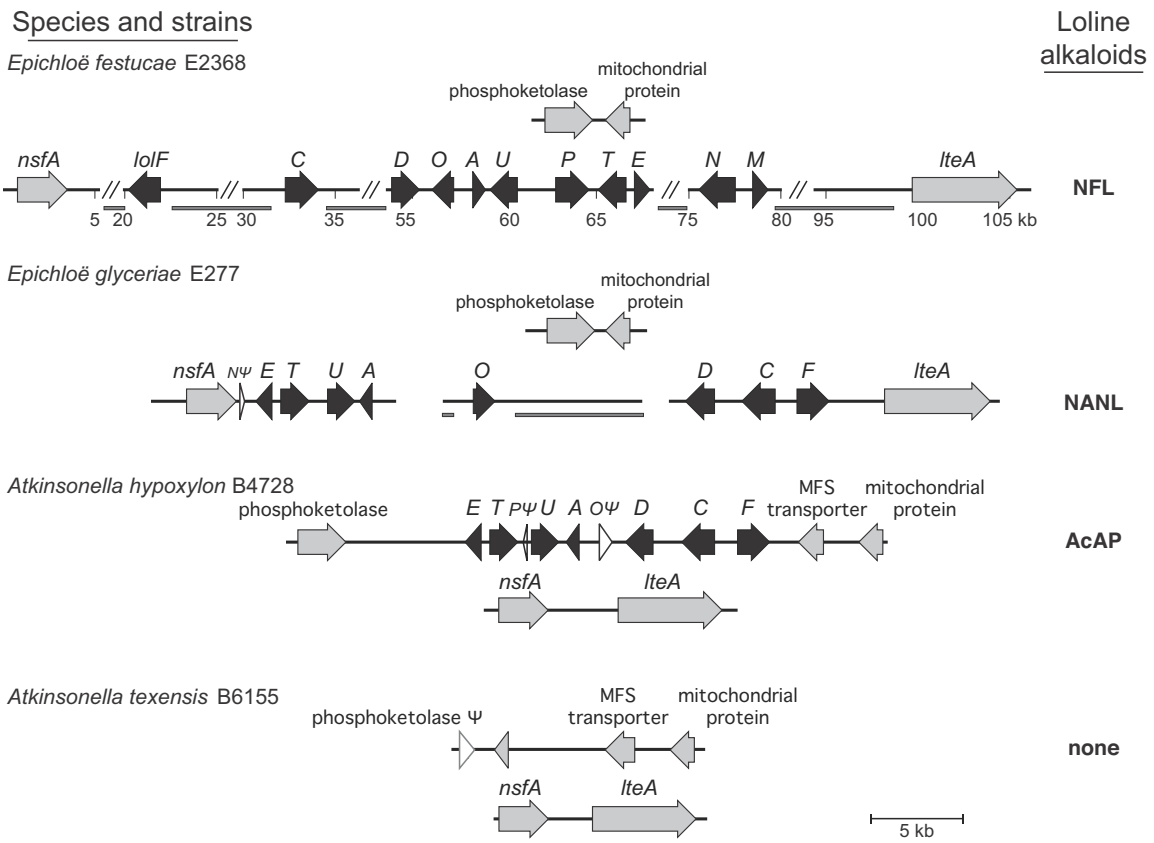


Figure 5.4. Comparison of *LOL* cluster locations. The *LOL* clusters of *Epichloë festucae* E2368 and *Epichloë glyceriae* E277 are between *nsfA* and *lteA*, but with opposite orientations. The *LOL* cluster in *Atkinsonella hypoxylon* B4728 is between a phosphoketolase gene and an MFS transporter gene, which is proximal to a mitochondrial protein gene. The *nsfA* and *lteA* genes in B4728 are linked with each other, and are apart from the *LOL* cluster. Reprinted from Schardl et al. (2014).

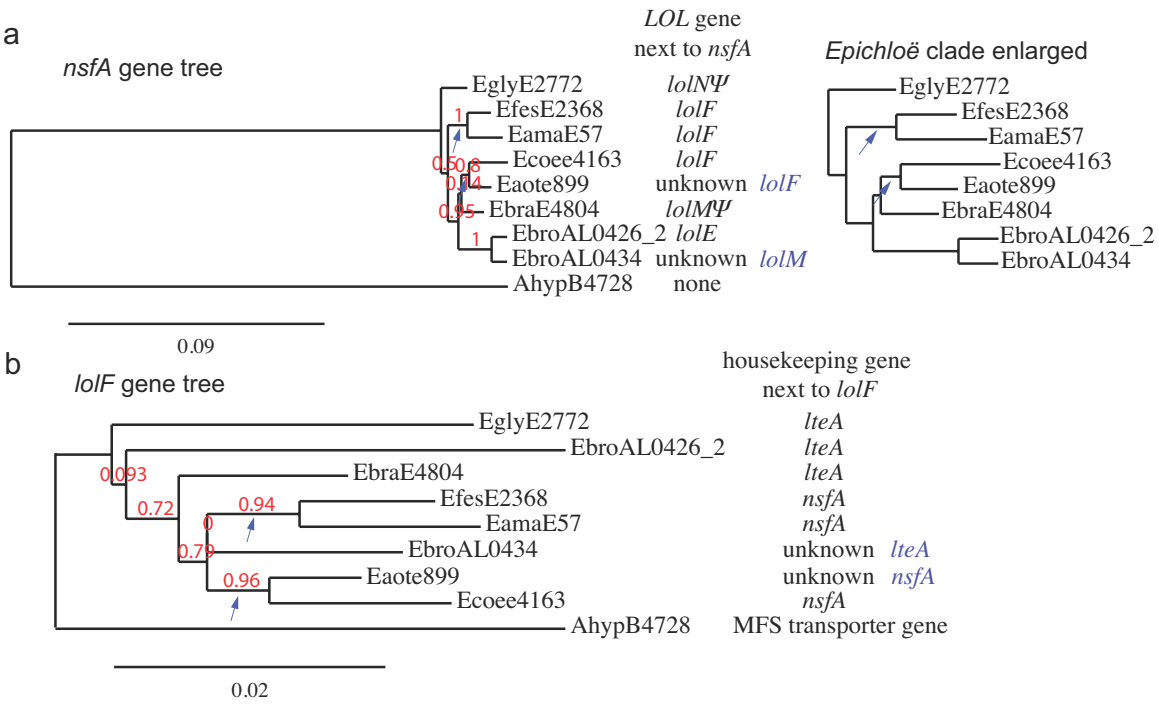


Figure 5.5. Changes of *LOL* cluster orientations. (a) MLE tree of housekeeping gene *nsfA*, with *LOL* gene proximal to *nsfA* in each isolate. Cases where the assembly failed to link the *LOL* cluster to housekeeping genes are listed as "unknown," and for those cases genes are listed in blue text to indicate the hypothesized condition that would require the least number of *LOL* cluster orientation changes assuming the *nsfA* gene phylogeny. (b) MLE tree of *lolF*, with housekeeping gene proximal to *lolF* in each isolate. The genes in blue next to "unknown" indicate the conditions that would fit the hypothesized two orientation changes of the *LOL* cluster in panel a. Arrowheads indicate the branches with orientation changes that might have occurred under the most parsimonious hypothesis given the *nsfA* phylogeny. Egly = *Epichloë glyceriae*, Ebro = *Epichloë bromicola*, Ebra = *Epichloë brachyelytri*, Efes = *Epichloë festucae*, Eama = *Epichloë amarillans*, Eaot= *Epichloë aotearoae*, Ecoe= *Epichloë coenophiala*, and Ahyp= *Atkinsonella hypoxylon*. For the hybrid, *E. coenophiala* e4163, which has copies of most housekeeping genes from its three ancestors, the only copy included here was from *E. typhina* var. *poae,* which is the source of its *LOL* cluster.

5.2.4. Evidence for transspecies polymorphism and recombination in the **LOL** *cluster*

Visual inspections of the phylogenies of *LOL* genes suggested largely similar topologies as the housekeeping gene tree (Figures 5.1, 5.2, and 5.3), with the obvious exception of *E. bromicola* AL0426_2. Instead of being in the same clade as the other *E. bromicola* isolate, AL0434, it grouped with *E. brachyelytri*. To test if the two *E. bromicola* isolates were separated with all of the *LOL* genes, phylogenetic trees of all *LOL* genes generated by MLE were compared with housekeeping gene trees. Surprisingly, phylogenies of *lolC* (Figure 5.1) and *lolF* (Figure 5.6) placed the two *E. bromicola* isolates in separate clades, obviously incongruent with the housekeeping gene phylogenies (Figure 5.7). Phylogenies of the rest of the *LOL* genes (with the possible exception of *lolE*, addressed below) paired the genes from the two isolates as sister taxa in the same clade (Figure 5.8).

In order to test if the tree topologies of *lolF*, *lolC*, and *lolE* were significantly different from those of the rest of the available *LOL* genes (*lolO* was not included in the analysis because it is a pseudogene in AL0426_2), I performed a Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa, 1999) of the MLE tree topology against the corresponding constrained tree topology of every *LOL* gene. The constraint was that the *LOL* genes of the two *E. bromicola* isolates were to constitute one clade. The *p*-values of the SH tests for *lolC* and *lolF* were significant (<0.05) (Table 5.2), leading me to reject the null hypothesis that the two topologies explain the data equally well, and to accept the alternative that the tree topology from the MLE tree, in which the two *E. bromicola* genes do not group together, explains the data better.

The *lolE* MLE tree and constrained tree were not significantly different in SH tests (Table 5.2), in keeping with the observation that relevant branches in the *lolE* tree had poor statistical support (Figure 5.6). This ambiguity in the *lolE* tree may have been due to the small size of the gene.

In summary, the phylogenies of *lolF* and *lolC* grouped the two *E. bromicola* isolates into distinct clades, whereas the phylogenies of *lolD, lolA, lolU,* and *lolT* paired the *E. bromicola* isolates as sisters. These results suggest that, in the evolution of the *E. bromicola LOL* clusters, recombination might have occurred within the *LOL* cluster in a cross between parental strains that had highly divergent *LOL* clusters, as reflected in the deep divergence of *lolC* and *lolF* of the two *E. bromicola* isolates. Thus, the obvious incongruence between phylogenies of *lolC* and *lolF* and the housekeeping genes suggests transspecies polymorphism of *LOL* genes, in which case the *LOL* gene polymorphisms represent lineages that diverged at a time that predates speciation events.

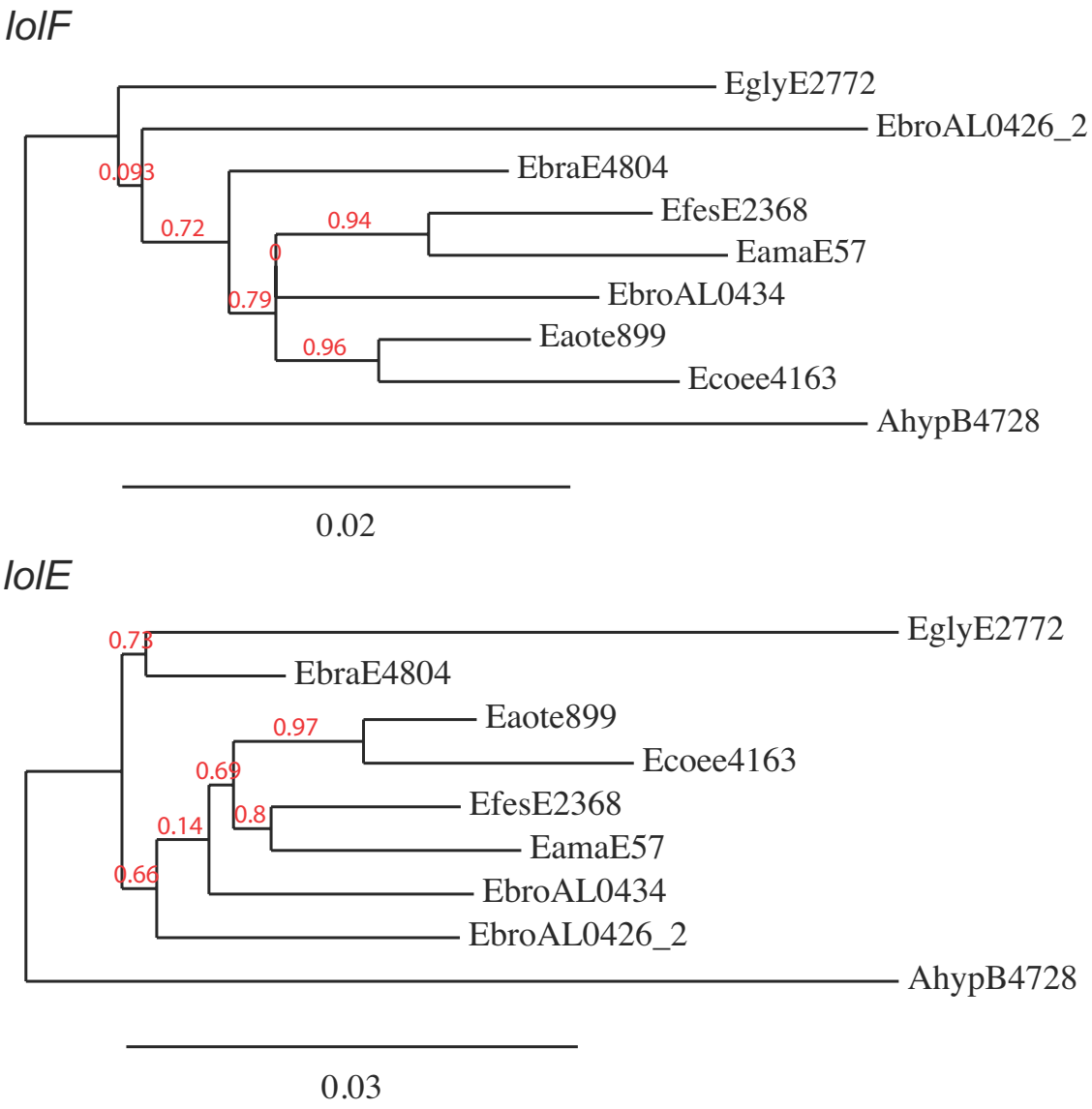


Figure 5.6. Phylogenetic tree inferred by MLE from *lolF* and *lolE* sequences. All isolates indicated here have sequenced genomes. Species names are abbreviated as in Fig. 5.5. For *E. coenophiala* e4163, the only gene copy included here was from *E. typhina* var. *poae,* which is the source of its *LOL* cluster.

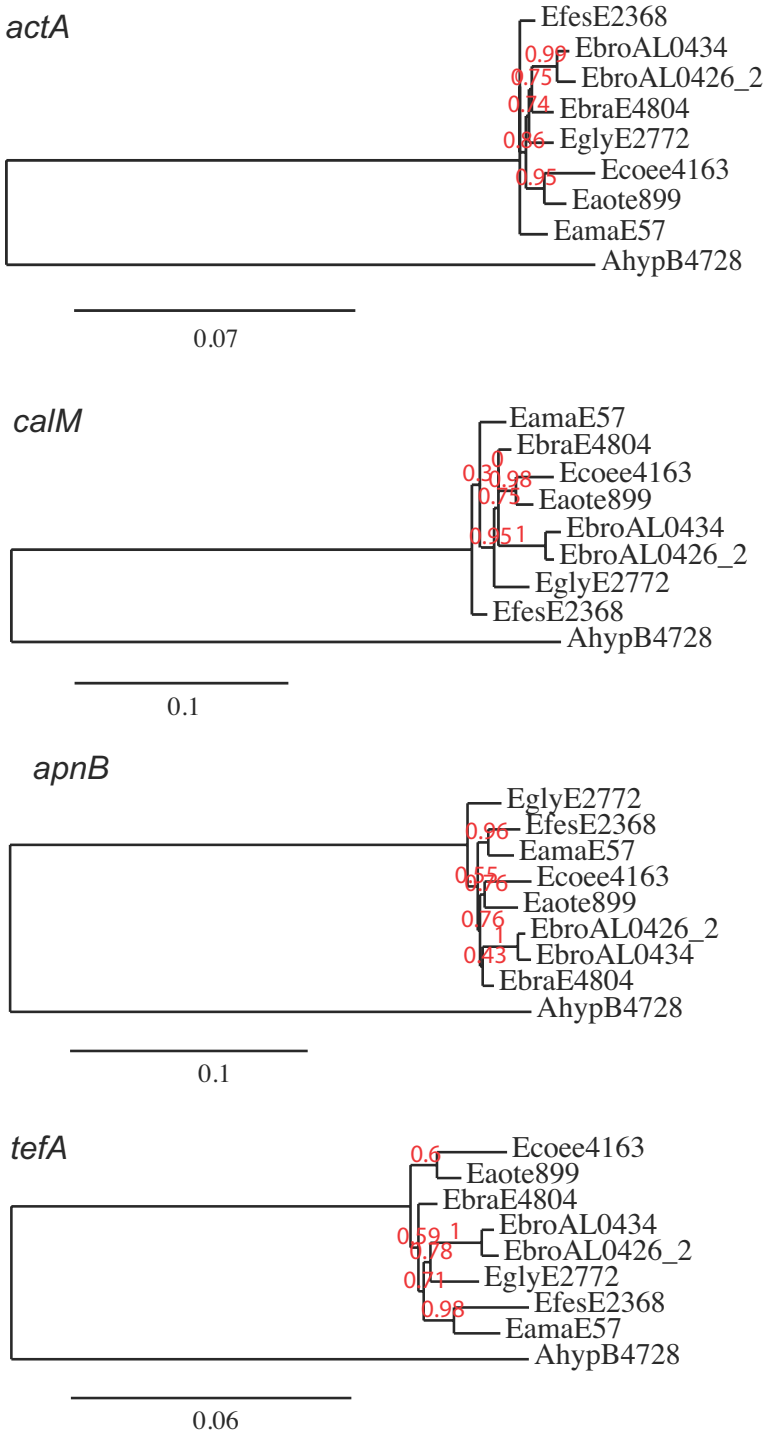


Figure 5.7. Phylogenetic trees of housekeeping genes. Phylogenetic trees were inferred by MLE from sequences of α-actin gene *actA,* calmodulin gene *calM,* DNA lyase gene *apnB*, and translation elongation factor 1-α gene *tefA*. Species names are abbreviated as in Fig. 5.5. For *E. coenophiala* e4163, the only gene copy included here was from *E. typhina* var. *poae,* which is the source of its *LOL* cluster.

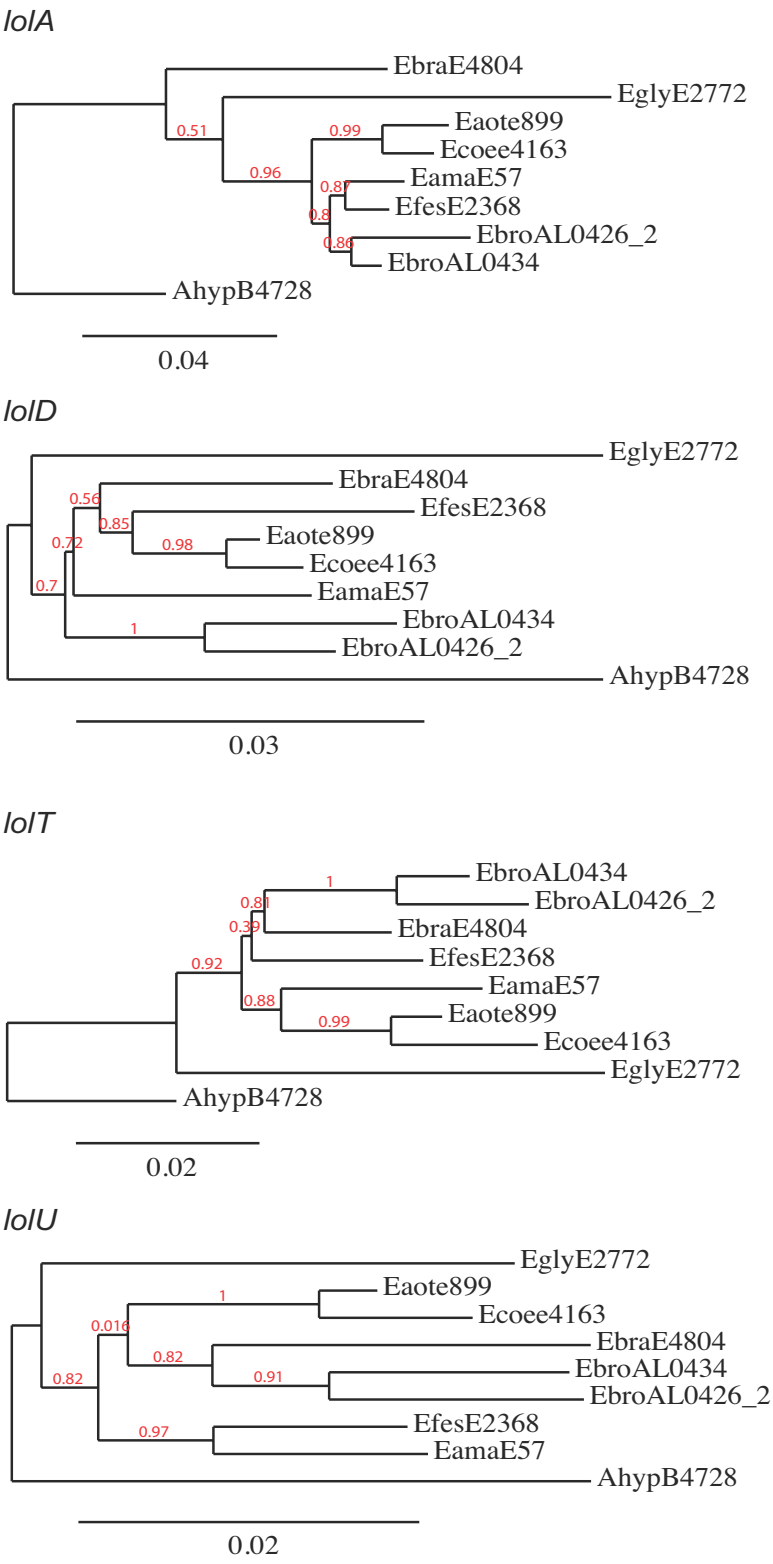


Figure 5.8. Phylogenetic tree inferred by MLE from *lolA*, *lolD, lolT* and *lolU* sequences. Species names are abbreviated as in Fig. 5.5. For *E. coenophiala* e4163, the only gene copy included here was from *E. typhina* var. *poae,* which is the source of its *LOL* cluster.

Table 5.2. SH test of *LOL* gene topologies.

***LOL* gene**	**p-value of SH test**
lolC	0.0248
lolF	0.0001
lolE	0.1700

The other *LOL* genes showed $p = 1$, because the constrained tree was the same as the MLE tree.

To further test the congruence of *LOL* and housekeeping gene phylogenies, the distributions of *LOL* gene trees and housekeeping gene trees from a total of nine isolates were compared using GeneOut. Geneout is a nonparametric goodness-of-fit test to compare the congruence of multiple sets of trees using a support vector machine (SVM) to map the separation (Haws et al., 2012). The *LOL* gene trees and housekeeping gene trees consistently showed significantly different distributions when various combinations of taxa were used in the test (p-value ≤0.01) (Table 5.3). The test was also run with some taxa excluded, such as removal of AL0426_2, removal of B4728, and removal of both *E. bromicola* isolates. In each case the set of *LOL* gene trees was also separated from the set of housekeeping gene trees. These results suggest that the *LOL* gene trees were significantly different, or incongruent, with the housekeeping gene trees. As a positive control for the test, the housekeeping genes were randomly divided into two sets (6 taxa each) and were tested by GeneOut. The *p*-value between the two sets of housekeeping genes was 0.93 (Table 5.3), indicating that the two sets of trees were congruent as expected. The GeneOut and SH test results, taken together, indicate that the divergence of the *LOL* genes predates speciation events of *Epichloë* and *Atkinsonella*.

This significant difference between *lolF* and *lolC* gene topologies and the rest of the *LOL* genes is not consistent with a single-locus model of balancing selection of the *LOL* cluster, and suggests that recombination within the cluster between two transspecies polymorphic *LOL* clusters might have occurred. I propose a possible recombination scenario that would explain the data (Figure 5.9).

The incongruence of *LOL* gene phylogenies with the housekeeping genes suggests that the polymorphism of the *LOL* clusters predates the divergence of *Epichloë* and *Atkinsonella*, and the transspecies polymorphism was maintained after the divergence of *Epichloë* spp. Transspecies polymorphism is the passage of allelic genes from ancestor to different species during speciation events, and is thought to be maintained by balancing selection (Miura et al., 2010), as each phenotype might be desirable under certain conditions and hence neither is purged during evolution. For example, the genes for major histocompatibility complex proteins, which are involved in antigen recognition in mammals, have been under balancing selection for a long time and found to show transspecies polymorphism (Su & Nei, 1999). The genes for enzymes in the fungal specialized metabolic pathway, trichothecene biosynthesis, have also been found to show transspecies polymorphism, possibly due to balancing selection for the different chemotypes (Ward et al., 2002). In the *Epichloë* and related *Atkinsonella* species, mutations in late pathway genes of the *LOL* cluster stop loline biosynthesis at intermediate steps, whereas a full cluster allows completion of the pathway to produce NFL. The diversifying selection on loline alkaloid biosynthesis genes is obvious across the species as indicated by various independent mutations and deletions of the *LOL* genes (Chapters 2 and 3; Pan et al. 2014). This selection probably acted very early on in evolution before the divergence of the different *Epichloë* species extant today. Therefore, the *LOL* cluster transspecies polymorphism seems to represent another example of balancing selection for chemotype differences.

Table 5.3. GeneOut test results. [a]

Test conditions	Final p-value
All isolates	0
Without *E. bromicola* AL0426_2	<0.01
Without *E. bromicola* AL0426_2 and *E. bromicola* AL0434	0
Without *A. hypoxylon* B4728	<0.01
Without *E. bromicola* AL0426_2 and *A. hypoxylon* B4728	0.01
Without *E. bromicola* AL0426_2, *E. amarillans* E57, and Ecoee4163	<0.01
Without *E. bromicola* AL0426_2, *E. festucae* E2368, and *A. hypoxylon* B4728	0.01
Without *A. hypoxylon* B4728, *E. coenophiala* e4163, and *E. glyceriae* E2772	<0.01
LOL genes without *lolF*, *lolC*, and *lolE*	<0.01
6 housekeeping genes against another 6 housekeeping genes	0.93

[a] A total of nine isolates were used in GeneOut test: *A. hypoxylon* B4728, *E. coenophiala* e4163, *E. bromicola* AL0434 and AL0426_2, *E. aotearoae* e899, *E. brachyelytri* E4804, *E. amarillans* E57, *E. glyceriae* E2772, and *E. festucae* E2368. The housekeeping gene dataset includes a total of 12 genes: α-actin gene *actA,* γ-actin gene *actG,* calmodulin gene *calM,* DNA lyase gene *apnB*, translation elongation factor 1-α gene *tefA*, β-tubulin genes *tubB* and *tubP,* α-tubulin genes *tbaA* and *tbaB*, γ-tubulin gene, and two genes that flank *LOL* loci in *Epichloë* species, namely *nsfA*, *lteA*. The seven *LOL* genes in this analysis were those shared by all producers of lolines or aminopyrrolizidines: *lolF, lolC, lolD, lolA, lolU, lolT*, and *lolE*.

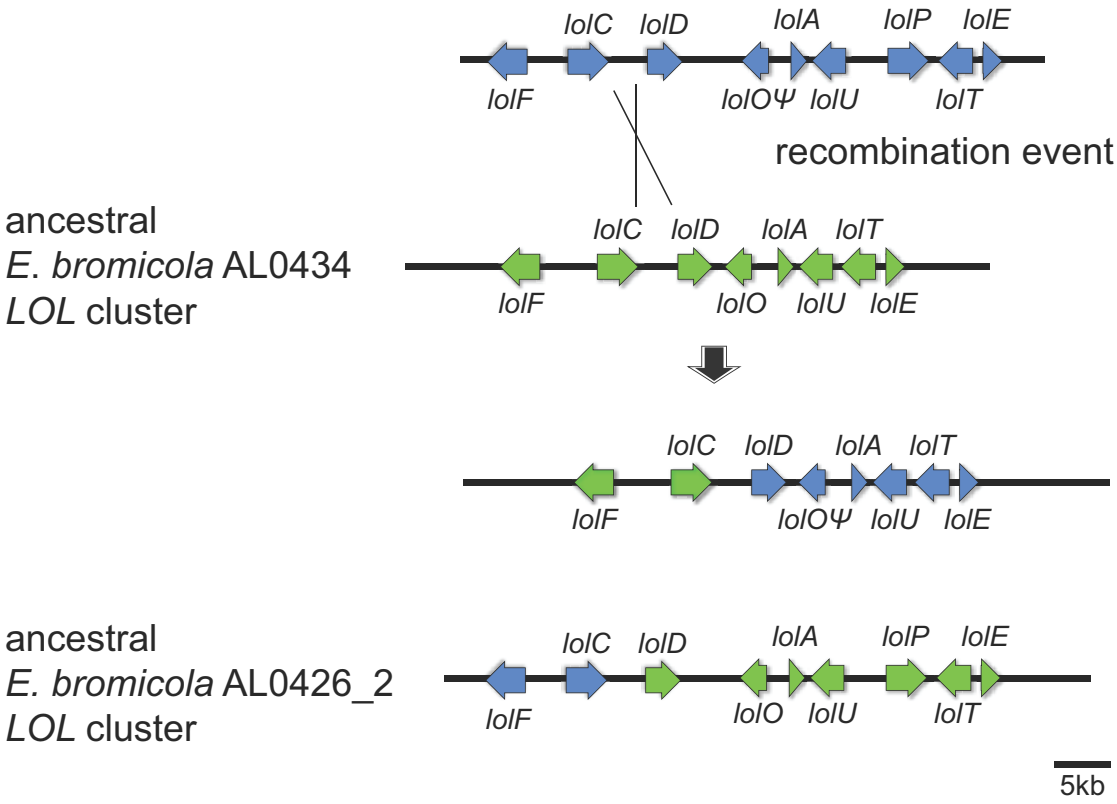


Figure 5.9. Hypothesized recombination event between ancestral transspecies polymorphic *LOL* clusters. The recombination event between two polymorphic *LOL* clusters before speciation events could explain the incongruence of *lolF* and *lolC* phylogenies with those of the rest of the *LOL* genes in the two *E. bromicola* strains. Alternative scenarios can be envisaged.

5.3. Concluding Remarks

Many grass-symbiotic fungi belonging to genera *Epichloë* and *Atkinsonella* (family Clavicipitaceae) produce loline alkaloids, but their specific chemotypes do not correlate with taxonomic groups. Genome sequencing of loline alkaloid-producing *Epichloë* and *Atkinsonella* species enabled analysis of the whole *LOL* cluster to elucidate the basis for this discordance. Repeated deletions and mutations of the *LOL* genes, especially the late-pathway genes, were responsible for most of the chemotype variations between and within species. The independent mutations and deletions in late-pathway genes and the apparent losses of the whole cluster in many isolates indicated selection for diversification of the alkaloids. Furthermore, the distribution of *LOL* gene phylogenies was significantly different from that of the housekeeping genes according to results of the GeneOut support vector machine test. This discordance of *LOL* gene phylogenies and species phylogeny (assumed to be reflected by housekeeping gene phylogenies) indicated that polymorphism of *LOL* clusters predates the divergence of *Epichloë* and *Atkinsonella* species. In addition, incongruence of *lolF* and *lolC* gene phylogenies with those of the rest of the *LOL* genes in the two *Epichloë bromicola* isolates indicated that recombination events might have occurred between two deeply diverged *LOL* loci in *E. bromicola LOL* cluster evolution. The *LOL* cluster dynamics are also reflected by changes in the orientation of the whole cluster on the chromosome. In summary, the *LOL* cluster is likely to have been under sustained diversifying selection, probably maintained by selection for chemotype diversity for optimal defense in the grass-Clavicipitaceae symbiota.

5.4. Materials and Methods

5.4.1. Biological materials. The strains selected for study are listed in table 5.1. The description of the strains, sequencing methods and assembly are described in (Schardl et al., 2013c) (Schardl et al., 2013b).

5.4.2. Phylogenetic analysis. The genes analyzed were annotated according to gene depositions in public database and the well-annotated genome of *Epichloë festucae* strain E2368 (Schardl et al., 2013b). The phylogenies were inferred by maximum likelihood estimate with PhyML implemented by Phylogeny.fr. (Dereeper et al., 2008). Constrained phylogenetic trees were generated with paup4b10-x86-linux.

5.4.3. GeneOut test. Libsvm 2.9 (Chang & Lin, 2011) was used in GeneOut test (Haws et al., 2012). Each time two sets of alignments were uploaded for SVM separation test (Noble, 2006). Bootstrapping was performed on the alignments. Bootstrapping was also used for sampling from the distribution of phylogenetic histories of the alignments, with 10 columns taken one time and repeating 1000 times for a new alignment of 10000. A total of 100 permutations were performed for separation of the two sets of alignments to get the final p-value. The null hypothesis of the SVM separation is that the two sets of alignments were from the same distribution of phylogenetic histories, and the alternative hypothesis is that the two sets of alignments were from different distribution of

phylogenetic histories. If p-value of GeneOut test is smaller than the critical value (0.05), the null-hypothesis is rejected.

5.4.4. Analysis of phylogeny congruence with Shimodaira-Hasegawa (SH) test. The SH test was performed to compare the topologies of phylogenetic tree generated by MLE and a constrained tree (Shimodaira & Hasegawa, 1999), in which the two *Epichloë bromicola* isolates AL0434 and AL0426_2 were given the constraint to group in one clade. The null hypothesis of the SH test is that the phylogenetic tree generated by MLE and the one generated with the constraint are equally good explanations of the data, and the alternative hypothesis is that the phylogenies are not equally good explanations of the data in which case the MLE tree is better. R version 3.0.3 was used to get the p-value.

Chapter Six

Conclusion and Discussion:

Loline alkaloid diversity and evolution of the *LOL* cluster

Plants produce diverse alkaloids for chemical defense. Other than producing alkaloids themselves, many grasses also host fungal endophytes that produce various anti-herbivore alkaloids for protection. The loline alkaloids are one class of the alkaloids produced by endophytic *Epichloë* species (Clavicipitaceae) that are symbiotic with the cool-season grasses. The lolines all share a saturated pyrrolizidine ring with a 1-amine and an ether bridge linking C2 and C7. Different modifications of the 1-amine group define the different loline alkaloids. The lolines are unusual biogenic molecules in that their characteristic ether bridge links two unactivated carbons. Biologically, lolines show very potent broad-spectrum insecticidal effects, and evidence indicates that the different modifications of the 1-amine affect the bioactivity and toxicity of loline alkaloids (discussed in Chapter One). Therefore, I have focused my research on the biosynthesis and diversification of loline alkaloids and on the analysis of the evolutionary history of the loline alkaloid gene cluster to generate the chemical diversity.

Isotopic labeling experiments have established that proline and homoserine are the precursors of loline alkaloids produced by *Epichloë* spp. (reviewed in Chapter One). Biosynthetic steps from these precursors to 1-aminopyrrolizidines and lolines are catalyzed by genes encoded in the *LOL* cluster, identified in genomes of *Epichloë* species and the related fungus, *Atkinsonella hypoxylon*. The functions of the *LOL* gene products have been predicted based on bioinformatic studies. This dissertation describes advances in identification and characterization of *LOL* genes that are responsible for formation of the ether bridge and the subsequent diversification steps that generate different loline alkaloids. I have also analyzed loline-alkaloid profiles of plants in genus *Adenocarpus* (Fabaceae), and have found evidence for a series of loline alkaloids and related acylamidopyrrolizidine alkaloids that differ only in the presence and absence, respectively, of the ether bridge. These results suggest that the loline alkaloid biosynthetic pathway in *Adenocarpus* species, at least at the ether-formation step, is similar to that of the *Epichloë* species. Finally, following the genome sequencing of *Epichloë* and *Atkinsonella* species that produce different loline alkaloids and 1-aminopyrrolizidines, I annotated the *LOL* clusters in each strain and examined the basis for evolution of loline alkaloid diversity.

My investigation of loline alkaloid ether bridge formation is presented in Chapter Two of this dissertation, where I provide evidence that LolO, a non-heme iron oxygenase, is solely responsible for catalyzing the reaction. The mechanism of the reaction is not yet determined, but the findings that six deuterium atoms from L-[U-$^{2}H_{7}$]Pro were retained in loline, NANL, and NFL, and that the pyrrolizidine ring system forms before the ether bridge, rule out several possible common mechanisms, such as epoxide formation and opening. Hence, it is likely that formation of the ether bridge of lolines occurs through an unusual route, probably through a free radical or carbocation at C2 or C7. Endophytes that lack or have a mutated *lolO* accumulate *exo*-1-acetamidopyrrolizidine (AcAP) in

grasses. I confirmed the structure and configuration of the metabolite that I identified as AcAP, and I established that AcAP is the pathway intermediate that is oxidized on C2 and C7 to form the ether bridge and give rise to NANL, the first fully cyclized loline alkaloid in the pathway. Many symbiotic grasses are found to contain AcAP as the pathway end product, instead of fully cyclized loline alkaloids, which suggests that AcAP production is biologically selected under certain conditions. The bioactivity of AcAP is worth investigation in the future. Furthermore, the LolO-catalyzed oxidation of two unactivated carbons is unusual, and work to elucidate the enzymatic mechanism in this reaction is underway.

Having established that NANL is the first loline alkaloid with an ether bridge, I addressed the question of how *N*-methylated forms of lolines (loline and NML) arise, since methylation of an amide seems to be biologically unprecedented and chemically difficult. For this reason, I predicted a role for an acetamidase, which produces norloline from NANL, followed by a methyltransferase, which produces loline and NML from norloline, in the loline alkaloid diversification pathway. Those steps are discussed in Chapter Three of this dissertation. Through comparison of *LOL* clusters in genomes of endophyte strains that exhibit different loline alkaloid profiles, and through molecular genetic methods, I have identified an acetamidase, LolN, responsible for removal of the acetyl group of NANL to form norloline. Subsequently, norloline was converted to loline and NML, and loline was converted to NML, in reactions with AdoMet catalyzed by extract of yeast expressing LolM, confirming the function of LolM as a loline alkaloid *N*-methyltransferase. In the symbiotic grasses, lolines with 1-amine groups (norloline, loline, and NML) are typically much less abundant than those with the formyl amide (NFL) or acetyl amide (NANL and NAL), suggesting that selection favors the amide over the amine forms of lolines. So far, every natural endophyte isolate known to produce lolines and to possess a functional *lolN* gene for deacetylation of NANL also has functional *lolM* and *lolP* genes, which specify the steps to NFL. However, the amine forms of lolines do not appear to be overtly toxic, since meadow fescue plants symbiotic with our *lolP* knockout strain of *E. uncinata* accumulate NML while persisting and growing normally in the greenhouse. However, the ecological consequence of this mutation has not been tested, and it is conceivable that the amide forms of lolines are more beneficial to the plant or endophyte than are the amine forms.

Also in Chapter Three, I investigated the origin of the acetylated loline alkaloid, NAL. Some but not all plants that are symbiotic with loline-producing *Epichloë* species accumulate NAL in addition to NFL and other lolines. I showed that grasses that contain NAL when symbiotic with endophyte could convert loline to NAL even without the symbiont. Furthermore, NAL is not found in cultures of *Epichloë uncinata* e167, even though meadow fescue symbiotic with e167 contains NAL. These findings indicate that a plant acetyltransferase activity is involved in NAL production. Hence, although most of the loline biosynthesis pathway is catalyzed by enzymes encoded in the *LOL* cluster of the endophytic fungi, the chemical diversification of loline alkaloids involves both fungal and plant enzymes, adding more interest to the evolution of the loline alkaloid biosynthesis pathway in this close fungus-plant symbiosis. It would be interesting in

future to characterize the potential plant acetyltransferase that is responsible for NAL production, and to identify the evolutionary history and the specificity of the enzyme.

Outside of grass-Clavicipitaceae symbiota, loline alkaloids are only reported in two distantly related plant taxa, *Adenocarpus* species (Fabaceae) and *Argyreia mollis* (Convolvulaceae). The basis for such a sparse, yet taxonomically broad distribution of lolines, is of interest, and I considered the possibilities of related fungal symbionts being responsible in these dicots, as well as whether the biosynthetic pathways are likely to be similar in these plants and the grass-*Epichloë* symbiota. Chapter Four of this dissertation describes my efforts to identify a potential fungal symbiont responsible for loline alkaloid biosynthesis in the *Adenocarpus* species, and partial characterization of putative aminopyrrolizidine and loline alkaloids in these plants. The various methods used in searching for a fungal symbiont — deep sequencing of *Adenocarpus decorticans* plant mRNA, attempted PCR amplification of *LOL* genes, and fungicide-treatment of *A. decorticans* and *Adenocarpus telonensis* seeds — all failed to give a positive indication of a possible fungal symbiont. Analyses of the alkaloid profile of *A. decorticans* revealed a series of putative pyrrolizidines with various acyl groups, and a corresponding series of lolines that probably differ from those pyrrolizidines only in the presence of the ether bridge. This is reminiscent of AcAP and NANL in grass-Clavicipitaceae symbiota. Given that NANL is formed from oxidization of AcAP catalyzed by LolO, it is intriguing to propose that the differently modified loline alkaloids in *A. decorticans* are produced from the corresponding acylamidopyrrolizidines by a similar route, perhaps catalyzed by one or more enzymes similar to LolO.

Grass-symbiotic fungi of the *Epichloë* and *Atkinsonella* species produce various loline alkaloids, and the chemotypes do not correlate with taxonomic groups. In Chapter Five of this dissertation I describe analyses of the evolution of *LOL* clusters in *Epichloë* and *Atkinsonella* species to elucidate the basis of this discordance between chemotypes and taxonomy. My analysis reveals that repeated deletions and mutations of the *LOL* genes, especially the late-pathway genes, are responsible for most of the chemotypic variation between and within species. Independent mutations and deletions in late-pathway genes are evident (as are losses of the whole cluster in some cases), indicating that the diversification of the alkaloids is selected. Compared to phylogenies of the housekeeping genes, I found that the *LOL* gene phylogenies are inconsistent with the taxonomic groups, which indicates that polymorphism of *LOL* clusters predates the divergence of *Epichloë* and *Atkinsonella* species. Transspecies polymorphism of the *LOL* cluster has probably been maintained by balancing selection for chemotype differences. In addition, incongruence of *lolF* and *lolC* gene phylogenies with those of the rest of the *LOL* genes in the two *Epichloë bromicola* isolates indicates a history of recombination between two deeply diverged *LOL* loci in *E. bromicola*. The *LOL* cluster dynamics are also reflected by changes in the orientation of the whole cluster on the chromosome and its change of location in the genome as evidenced by comparing the adjacent housekeeping genes in genomes of *Epichloë* species and *A. hypoxylon*. In summary, the *LOL* cluster is probably under sustained diversifying selection, which I suggest is maintained by the selection for chemotype diversity for optimal defense in the grass-Clavicipitaceae symbiota.

Cool-season grasses probably invest considerable resources in hosting *Epichloë* sp. endophytes in return for the chemical defenses produced by those fungi. One could argue that this might be a strategy that is more costly to the grasses than if they were to invest more in biosynthesis of their own defensive chemicals. However, there may be additional advantages of being allied to these symbiotic fungi. The endophyte could also confer benefits to the host through various ways other than chemical defense, such as promoting biomass production and increased resistance to biotic and abiotic stresses (reviewed in Chapter One). Even considering just the benefits of chemical defense, one can readily envisage reasons that utilizing the endophyte-derived alkaloids is sometimes favored over the potential for the plant to evolve new or augmented defenses. One may be the ease of generating a diversity of defensive metabolites by capturing endophytes compared with evolving new secondary biosynthetic pathways and the necessary regulatory and compartmentalization systems to prevent conflicts with primary metabolism. Also, in the symbiotic systems there is the potential for the plant to get rid of the endophyte if environment disfavors the extra burden of hosting it, for example when all available resources are needed for plant survival and dispersal. In keeping with this scenario, some studies have indicated that the frequencies of endophyte symbiosis may be higher in moderately stressful environments than in very high stress or low stress environments (Iannone et al., 2013). On the other hand, considering that loline alkaloids could affect insect parasitoids, thereby contributing to multi-tropic level endophyte effects, and the possibility that different kinds of alkaloids produced by both endophyte and host may exert synergic effects on herbivores, it would be interesting to look at the symbiota as a whole in natural ecosystems to see how they evolve and adapt to the environment.

Appendix A

Work on functional characterization of *lolA* and *lolU*

The *lolA* gene is consistently found in the *LOL* cluster of endophyte strains that produce loline alkaloids, and *lolA* transcription shows upregulation during loline alkaloid production in loline inducing medium (Spiering et al., 2005). The product of *lolA* is predicted to be an amino acid binding protein related to the allosteric domain of aspartate kinase, but lacking the kinase domain. So LolA has been proposed to be involved in loline alkaloid regulation (Spiering et al., 2005). I tried to test the function of *lolA* through RNAi. The plasmid pKAES306 was constructed to contain two *lolA* fragments in inverted orientations, driven by the *ToxA* promoter. The plasmid was further confirmed by sequencing.

The *lolU* gene is also found in the *LOL* cluster of endophyte strains that produce loline alkaloids, indicating its involvement in alkaloid biosynthesis. Sequence alignment of *lolU* showed match to an acetyltransferase, so I propose that its product may catalyze the acetylation of 1-AP to produce AcAP. I attempted to test the function of *lolU* by RNAi. The plasmid pKAES307 was constructed to contain two *lolU* fragments in inverted orientations, driven by the *ToxA* promoter. Plasmid sequence was confirmed by sequencing.

Plasmids pKAES306 and pKAES307 were transformed to *E. uncinata* e167 independently. Transformants were cultured in loline alkaloid-inducing medium to check the alkaloid profile. No obvious change of loline alkaloid profile was observed in either *lolA* or *lolU* RNAi transformants.

Functions of *lolA* and *lolU* were also tested by overexpression. Each of the two genes was incorporated in pKAES215 under the *TOXA* promoter from *Pyrenophora tritici-repentis,* to give plasmids pKAES340 to overexpress *lolA*, and pKAES342 to overexpress *lolU*. *Epichloë festucae* E2368 was transformed with the plasmids to give transformants E7491, E7492 and E7493 for *lolA* overexpression, and E7494, E7495, and E7496 for *lolU* overexpression. The transformants were inoculated to meadow fescue. No obvious change of loline alkaloid profile was observed for any of these plants. A quantitative analysis of the profile is required to further test if there is a change of loline alkaloid profile.

The overexpression plasmid for *lolA* or *lolU* was also constructed with the respective gene placed under the *Aspergillus nidulans trpC* promoter, based on the modified gateway plasmid pKAES269. The transformants of these were also inoculated into meadow fescue, but loline alkaloid profiles were not checked. I was concerned (but never determined) that the genes might not be expressed, judging from my experience with *lolO* overexpression plasmid based on the same construct.

A functional test of LolU as a putative acetyltransferase was also performed by placing *lolU* in yeast expression plasmid pESC-LEU under the yeast *GAL10* promoter,

with a C-terminal FLAG tag. The expression plasmid is pKAES360. The expression of the enzyme was not confirmed by western blot. An experimental feeding of the putative substrate 1-AP to yeast transformed with pKAES360 is underway.

Appendix B

Heterologous expression of *lolF*, *lolD* and *lolT* in yeast

A plasmid for heterologous expression of *lolT* in yeast was constructed based on plasmid pESC-LEU. Plasmid pKAES355 was constructed in such a way that LolT would be expressed with an N-terminal FLAG tag, and pKAES356 was constructed in such a way that LolT would be expressed with a C-terminal FLAG tag. Expression of LolT in yeast with the N-terminal FLAG tag has been confirmed by western blot with anti-FLAG antibody. The expression of the C-terminal tagged protein has not yet been tested by western blot.

A plasmid for heterologous expression of *lolD* in yeast was constructed based on plasmid pESC-LEU. Plasmid pKAES357 was constructed in such a way that LolD would be expressed with an N-termnal FLAG tag, and pKAES358 was constructed in such a way that LolD would be expressed with a C-terminal FLAG tag. Expression of the plasmids in yeast has not yet been confirmed by western blot.

Heterologous expression of *lolF*, *lolD*, and *lolT* together in yeast was prepared by co-transforming pKAES360, which has *lolF* and *lolD* expression construct based on plasmid pESC-HIS, and pKAES355, which has *lolT* expression construct based on plasmid pESC-LEU. The yeast transformant with both pKAES360 and pKAES355 was grown in Leu and His deficient medium to induce the expression of *lolF*, *lolD*, and *lolT*. The expression of *lolT* in the yeast was not confirmed by western blot (LolD and LolF were not tagged and we did not have antibody to test their expression with western blot). We hypothesize that if LolF catalyzes oxidative decarboxylation of NACPP, and subsequently, LolD or LolT, or the combination of the latter two are required for the other decarboxylation and ring closure, then application of NACPP to yeast expressing the three genes would take the loline biosynthetic pathway to 1-AP. Preliminary test of the yeast supplied with deuterated NACPP did not give the expected deuterated 1-AP.

Appendix C

Functional characterization of *lolD*

The *lolD* gene is predicted to encode a PLP-dependent decarboxylase. In the loline biosynthesis pathway, after condensation of Pro and homoserine to form NACCP, the two carboxyl groups on NACCP are eliminated during formation of 1-AP. Hence, LolD is predicted to catalyze one or both of these decarboxylation reactions. One hypothesized pathway from NACCP to 1-AP is oxidative decarboxylation by LolF to remove the carboxyl group on Pro of NACCP, then a second decarboxylation catalyzed by LolD would form 1,5-diazabicyclo[4.3.0]nonane (DDBN), which is further cyclized by another PLP-dependent enzyme LolT to form 1-AP. In this case, a *LOL* cluster lacking *lolD* will completely eliminate the production of loline alkaloids. Furthermore, application of DDBN to the symbiotum that has a *lolD* knockout transformant should chemically complement the loline alkaloid biosynthesis pathway. To test these hypotheses, I eliminated *lolD* in *E. festucae* E2368 through homologous recombination, and then applied DDBN to meadow fescue harboring *lolD* knockout strain.

Materials and Methods

Construction of gene replacement plasmid. To knock out *lolD* through homologous recombination, using E2368 DNA as template, a 2.9-kb 5'-flanking region (amplified by PCR with primers lolDkoupf and lolDkoupr) (Table C.1) and a 2.9 kb 3' flanking region of E2368 *lolD* (amplified by PCR with primers lolDkodf and lolDkodr) (Table C.1)were amplified by PCR and cloned into pKAES173. The resulting plasmid was named pKAES321, with *lolD* flanking regions in the same orientation and a modified *hph* maker gene in the middle. Linearized pKAES321 was transformed into E2368 by electroporation. The transformants that tested negative in PCR for *lolD,* but positive for *hph,* were further subjected to a positive screen with primers lolDexpf and lolO53s (Table C.1). The wild-type strain is expected to produce a 2.6 kb fragment, and true *lolD* knockout is expected to show a 2.2 kb fragment.

Synthesis of DDBN and [^{2}H]DDBN (performed by Minakshi Bhardwaj at the Department of Chemistry, University of Kentucky). Synthesis of DDBN was carried out by reduction of commercially available 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) by $LiAlH_4$ as reported previously (Brandänge, Eriksson and Rodriguez, 1984). [^{2}H]DDBN, 6-deutero-1,5-diazabicyclo[4.3.0]nonane, was prepared by the reduction of DBN with $LiAlD_4$ following the similar procedure (Figure C.1).

Table C.1. Oligonucleotides used in this appendix. [a]

Primer name	Sequence
lolDkoupf	GCGCGGCCGCTACATTGTCCGCTCCATCCTAG
lolDkoupr	GCTCTAGACGGGACTTCCCAGCTTAGTGA
lolDkodf	GCGGTACCTTGGCGACAATCGCAATGAGA
lolDkodr	GCGGTACCATTTAAATCGGGAAAGGATTGAGGCCATAC
lolDexpf	AAAAAGCAGGCTTAATGGCCACAGCCGTACGAG
lolO53s	ATACGGACTACTGCCAG

[a] Underlined segments indicate restriction-endonuclease cleavage sites incoporated in the primers to facilitate cloning.

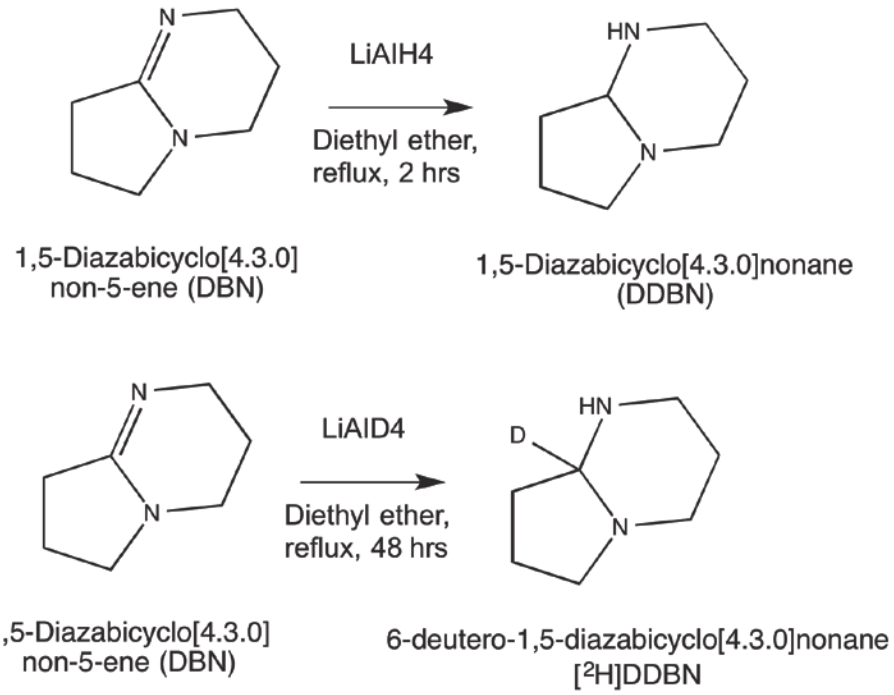


Figure C.1. Synthesis of DDBN and [^{2}H]DDBN.

Application of DDBN to grasses infected with* lolD *knockout strains. A single tiller of meadow fescue plant symbiotic with the *lolD* knockout strains was cut just above the soil line and placed into a 1.7 ml test tube with 600 µl of the Murashige and Skoog Medium (MS medium) (MP Biomedicals, Solon, OH, USA), pH 7.4. Compound DDBN was added at the specified concentration. Two control experiments were included. One control used infected meadow fescue tillers in MS medium with diethylether as a control for the carrier solvent for DDBN, at corresponding concentrations for the DDBN feedings. The other control used endophyte-free meadow fescue with DDBN at the same concentrations. A total of 10 plants were used for each feeding group. To each tube was also added DMSO (1.5%) to increase permeability of plant cells. Feeding tubes with plant tillers were kept at 25 °C, 16 h light, until the entire MS medium was consumed. At the end of the feeding period the tillers in each feeding group were pooled and freeze-dried, and loline alkaloids were extracted .

Results

lolD *knockout strains eliminated loline alkaloid production.*

Two *lolD* knockout isolates were generated from 230 transformants. The presence of *LOL* genes, except for *lolD*, was confirmed by PCR in transformant E7485 (Figure C.2) (only analysis of *lolC* is shown; analysis of the other *LOL* genes is not shown), and E7486 (data not shown). Positive screening of the knockouts was also confirmed by the size difference between true knockouts (E7485 and E7486) and the wild-type strain E2368 (Figure C.3). The knockout transformants were further confirmed by Southern-blot analysis (Figure C.4). Both knockout strains were inoculated to endophyte-free meadow fescue to check the loline alkaloid profile. No known loline alkaloids were detected in grasses infected with either of the knockout strains, which is consistent with the hypothesized *lolD* function.

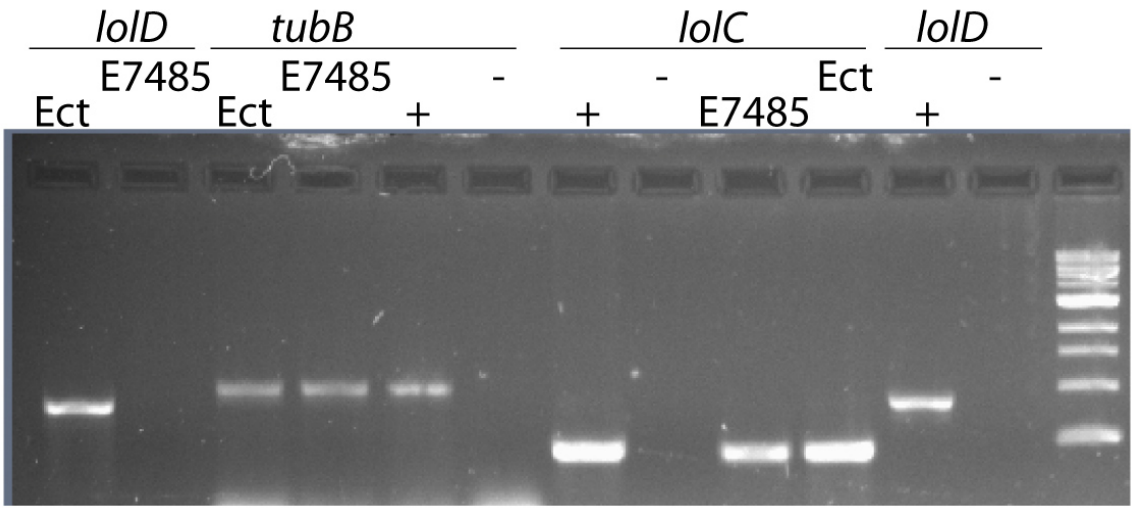


Figure C.2. Negative screening of *lolD* knockout strain E7485 by PCR. An ectopic transformant DNA was used as template in "Ect", *Epichloe festucae* E2368 DNA was used as template in "+", and H_2O was used in "–".

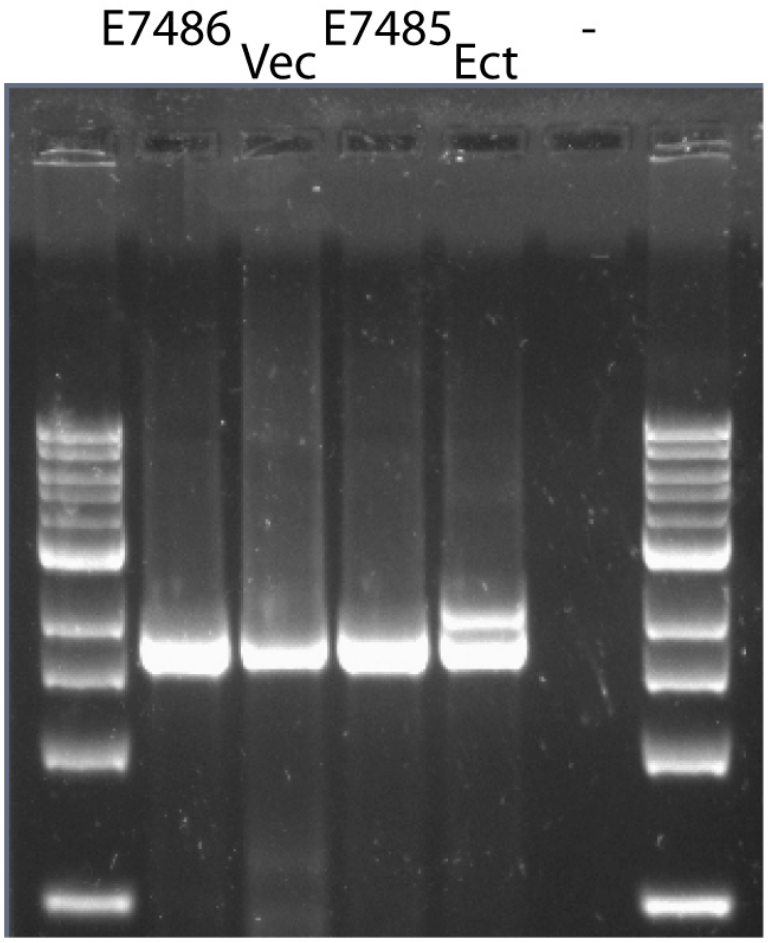


Figure C.3. Screening of *lolD* knockouts E7485 and E7486 with positive screening primers. Vector DNA was used as template in "Vec", an ectopic transformant DNA was used in "Ect", and H_2O was used in "-".

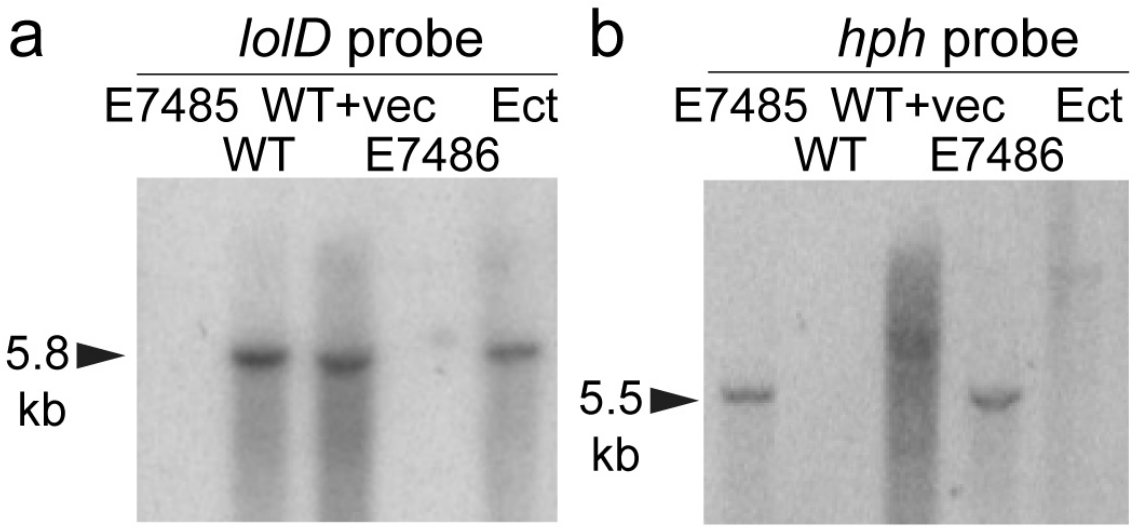


Figure C.4. Southern-blot analysis of *lolD* knockout strains. All DNA samples were digested with *Bgl*II. *E. festucae* E2368 DNA was used as wild-type (WT), DNA of empty-vector transformant of E2368 was used in "WT+vec", and an ectopic transformant DNA was used in "Ect".

Application of DDBN to meadow fescue infected with* lolD *knockout strains restored NFL production.

When DDBN was applied to meadow fescue infected with *lolD* knockout strains 4938 (meadow fescue symbiotic with E7485) and 4939 (meadow fescue symbiotic with E7486), a small amount of NFL, but none of any other loline alkaloids, was produced. In the control experiment, in which DDBN was applied to meadow fescue infected with vector-only transformant, no detectible NFL was observed. This suggests that DDBN is able to chemically complement *lolD* knockout, indicating that DDBN is likely an intermediate in loline biosynthesis, and probably the product of LolD, or at least downstream of the step *lolD* is involved in loline-alkaloid biosynthesis. The observation of NFL production only is understandable. NFL is the most abundant loline alkaloid observed in meadow fescue infected with wild-type E2368, so it would be quite plausible that when small amounts of loline alkaloids are produced, we may only observe the most highly abundant compound.

To further test chemical complementation, I applied [^{2}H]DDBN to 4938 and 4939 plants in the same way. [^{2}H]NFL is expected if the results are consistent. However, I did not observe any deuterium labeled NFL, but all-proton NFL was observed in some cases. Therefore, it was never demonstrated that DDBN is a pathway intermediate.

The inconsistent results in application of DDBN and dideuteridum labeled DDBN to 4939 and 4938 plants failed either to support or refute our hypothesis that DDBN is a pathway intermediate. The NFL we observed in application of DDBN could originate from contamination, or could be due to indirect induction of the *LOL* pathway by DDBN, rather than itself being involved in the reactions directly.

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