

Chemical diversity of bioprotective alkaloids of endophytic fungi in cool season grasses

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Abstract

Epichloë endophytes (Epichloë and Neotyphodium species) are important fungal symbionts that form mutualistic symbioses with cool season grasses. The asexual *Neotyphodium* species have been utilized in agriculture to enhance the persistence of grasses such as tall fescue and perennial ryegrass. These fungi are known to produce a range of bioprotective alkaloids, peramine, lolines, ergot alkaloids and lolitrems (indole-diterpenes) that provide anti-insect and anti-mammalian properties that protect their host. Of most interest in the agricultural setting are endophyte/grass associations that produce only the beneficial anti-insect alkaloids. Methodologies are well established to detect and quantify the presence of these alkaloids. More recently the genes required for the biosynthesis of the four bioprotective alkaloids have been cloned and characterized from a number of endophytes present in agriculturally important grasses. As found with other fungal secondary metabolite biosynthesis genes, the genes for three of the four alkaloids (ergot alkaloids, indole-diterpenes and lolines) are present in co-regulated gene clusters. This genomic organization enabled the identification of sets of genes that are likely to be involved in the biosynthesis of each alkaloid. The cloning and characterization of these alkaloid biosynthesis genes now provides an opportunity to understand the biochemical pathways by means of targeted mutagenesis and determine metabolite diversity across naturally occurring endophytes based on the presence and absence of genes.

Introduction

Endophytes (microorganisms that colonize plants) are found everywhere in nature, but many of these associations are not fully understood (Rodriguez & Redman, 2008). However, clavicipitaceous fungi are known to inhabit cool season grasses and these fungal endophytes have been well studied for a number of decades in agriculturally important forages (Bacon et al., 1977; Sampson, 1933). The Epichloae broadly consists of the sexual *Epichloë* and asexual *Neotyphodium* species that systemically infect the aerial parts of the grass plant. They are known to aid with the general persistence of the plant and have been deemed akin to a “supergene” because of the growth advantage they provide their host.

In the 1930s a tall fescue cultivar “Kentucky 31” had shown great promise with persistence especially during times of drought (reviewed in Bacon, 1995). What was not understood at the time was that a systemic infection of the fungus *Neotyphodium coenophialum* provided the grass with persistence (Bacon et al., 1977). Unfortunately, this same fungus also gave Kentucky 31 a bad name due to the poor animal performance and reproductive problems that it caused grazing livestock (reviewed in Bacon, 1995). Kentucky 31 had an even greater effect on horses in Kentucky – a state that prides itself on an extensive thoroughbred industry – triggering a great

deal of investigation into the causes of the disease symptoms. Scientists eventually established that not only was tall fescue infected with an endophyte but that the fungus could produce an ergot alkaloid now known as ergovaline and this alkaloid was responsible for the effects on cattle such as fescue foot and fescue toxicosis (Bacon et al., 1977; Porter et al., 1981). A similar endophyte, *Neotyphodium lolii*, was found in perennial ryegrass (Fletcher & Harvey, 1981) and this fungus is known to cause ryegrass staggers due to the production of the indole-diterpene lolitrem B (Gallagher et al., 1984).

Phylogenetic analysis using the genomic sequences of *tefA* and *tubB* from *N. coenophialum* indicated it was an interspecific hybrid that consisted of the progenitor species *Epichloë festucae*, *E. typhina* and the *Lolium*-associated endophyte closely related to *E. baconii* (Tsai et al., 1994; Moon et al., 2004). The endophyte from perennial ryegrass is less complex and appears to be an asexual *E. festucae* (Schardl et al., 1994). Subsequently it has been shown that many asexual epichloë endophytes are interspecific hybrids and many of these have an *E. festucae* progenitor (Craven et al., 2001a; Craven et al., 2001b; Iannone et al., 2009; Moon et al., 2004; Moon et al., 2007).

Alkaloid Production by Endophytes

One well documented aspect of the cool season grass endophytes is the ability to produce secondary metabolites (also known as natural products) that provide anti-insect and anti-mammalian properties (Popay & Bonos, 2005; Siegel et al., 1990). The epichloae are able to produce four classes of alkaloids, ergot alkaloids, lolines (pyrrolizidines), peramine (pyrrolopyrazine) and lolitrems (indole-diterpenes) with known biological activities. The production of lolines and peramine are considered positive traits because of their anti-insect properties while the ergot alkaloids and lolitrems are best known for anti-mammalian activities that have negative effects on livestock. Prior to the cloning and characterization of the genes required for the biosynthesis of these compounds much work was done on establishing methodologies for detecting and quantifying the alkaloids using techniques such as thin layer chromatography (TLC) (Fannin et al., 1990), high performance liquid chromatography (HPLC) (Lodge-Ivey et al., 2006; Rottinghaus et al., 1991; Spiering et al., 2002b) and enzyme-linked immunosorbent assay (ELISA) (Hill & Agee, 1994). These techniques were also useful for identifying isolates that only provided beneficial alkaloids to the association (Latch et al., 2000a; Latch et al., 2000b). Identification of proposed intermediates identified within the plant extracts of endophyte infected material also provided insight into the predicted biosynthetic pathways for some of the alkaloids (Gatenby et al., 1999; Munday-Finch et al., 1996; Munday-Finch et al., 1997).

The asexual life cycle of the *Neotyphodium* species contain the endophyte within the plant. During the inflorescence development the fungus is able to colonize the developing seed and embryo (Sampson, 1933). Fungal/grass associations can be manipulated in order to replace a native, common toxic endophyte with a strain that might cause less mammalian toxicity while still retaining the positive benefits. The lifestyle of the asexual endophytes that are utilized in forage grasses allow for replacing these endophytes from the “hot” mammalian toxic variety to one known to be mammalian friendly such as MaxQ (Bouton et al., 2002) and AR1 (Fletcher, 1999). The naturally infecting fungus can be selectively killed off using heat and humidity to

produce a seed that will germinate and become an endophyte free seedling. This seedling can then be artificially inoculated with a different, more mammalian friendly, strain (Latch & Christensen, 1985).

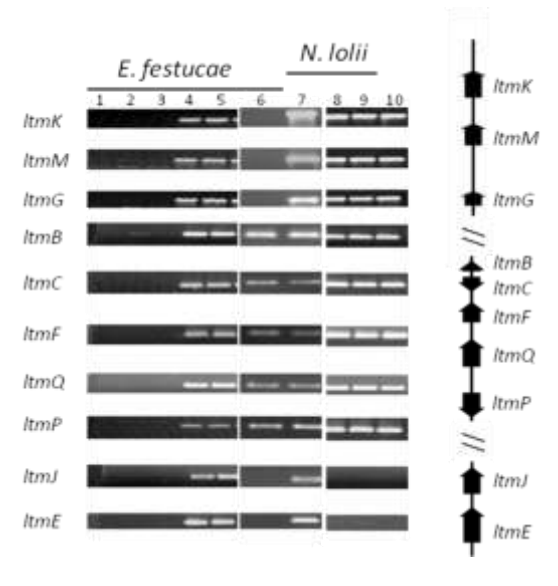
In more recent years, with the advancement of molecular biology methodologies, researchers also focused on the identification of DNA sequences encoding gene products required for the biosynthesis of the alkaloids. As found with other fungal secondary metabolite biosynthesis genes (Keller & Hohn, 1997), the genes for three of the four alkaloids (ergot alkaloids, indole-diterpenes and lolines) are present in co-regulated gene clusters (Fleetwood et al., 2007; Spiering et al., 2005; Young et al., 2006). The genomic organization of a co-regulated gene cluster helps define the boundaries of the cluster and subsequent identification of potential gene products that would be essential for alkaloid biosynthesis. Targeted gene deletions, gene silencing and heterologous gene expression have allowed for the characterization of many genes and provided evidence of specific pathway steps for all four of the known alkaloids (Panaccione et al., 2001; Spiering et al., 2005; Tanaka et al., 2005; Wang et al., 2004; Young et al., 2005; Young et al., 2006).

Using PCR with primers specific to each alkaloid biosynthetic pathway gene, we can now rapidly profile isolates for their ability to produce specific alkaloids. Therefore, the presence of biosynthetic genes helps define the potential biochemical pathways of the symbiotum (association of the endophyte and plant). In other words, if the gene cluster for a specific alkaloid is missing from an isolate, the compound cannot be synthesized as the gene products (the enzymes required for alkaloid biosynthesis) are not produced. This also means that if key pathway genes are missing (or non-functional) then critical pathway steps and resulting metabolites will not be produced. Alternatively, later pathway steps which are missing or non-functional can still result in the biosynthesis of pathway intermediates that may have biological activity.

The most extensive analysis of metabolite genes has been performed for the lolitrem B biosynthesis genes (*ltm* genes) across a number of sexual and asexual epichloae and these data helped define the pathway as a complex biosynthetic grid (Young et al., 2009). These data concluded that many sexual species lack all 10 *ltm* genes and presents the reason they are unable to produce lolitrems. Spiering et al (2002) also showed a correlation with the presence of two genes, *lolC* and *lolP*, with *Epichloë* and *Neotyphodium* isolates that are able to produce lolines (Spiering et al., 2002a).

The *E. festucae* and asexual isolates with an *E. festucae* progenitor showed the greatest variation with respect to the presence of *ltm*

Figure 1. PCR profiling for the lolitrem biosynthesis genes (*ltm*). The *ltm* gene cluster is shown to the right of the PCR analysis

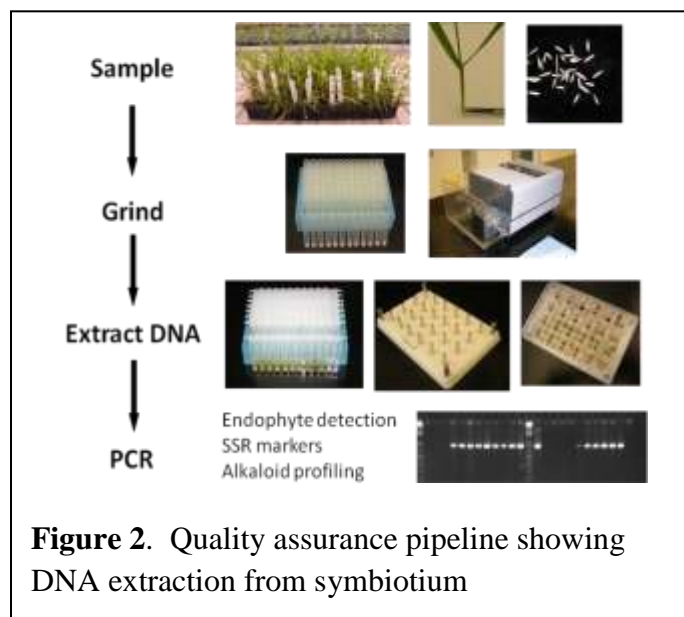


genes and subsequent ability to produce lolitrem B or indole-diterpene intermediates (Young et al., 2009) (Fig. 1). The variation across these isolates ranged from the presence of no genes to the presence of five, eight, nine or all 10 *ltm* genes (Fig. 1). Isolates with only five *ltm* genes were still unable to synthesis indole-diterpenes as two of the early essential pathway genes, *ltmG* and *ltmM*, were missing. It was perhaps surprising to find that the mammalian friendly *N. lolii* isolate AR1 contains eight of the 10 *ltm* genes and is able to produce lolitrem intermediates such as the terpendoles (Young et al., 2009). As evidenced by animal performance trials and feeding studies, the toxicity of the terpendoles produced by AR1 is much lower than that of lolitrem B and does not cause problems to grazing animals (Bluett et al., 2005a; Bluett et al., 2005b; Gatenby et al., 1999).

The lolitrem biosynthesis pathway also presents an opportunity to generate metabolite diversity. It appears that some biosynthesis enzymes such as P450 monooxygenases and aromatic prenyl transferases are more promiscuous and are capable of accepting more than one substrate. This in turn provides a complex biosynthetic grid resulting in the production of more metabolites than can be produced by a linear pathway (Young et al., 2009).

Epichloë festucae is known to generate the greatest range of alkaloids as this species can produce ergot alkaloids, lolitremes (indole-diterpenes), peramine and lolines (reviewed in Clay & Schardl, 2002). However, at present, there has been no single isolate that has produced all four classes of alkaloids. It is therefore, no surprise that asexual endophytes with an *E. festucae* progenitor are also likely to produce these compounds and will represent the alkaloid variation as well.

Quality assurance for grass/endophyte associations



We have developed a quality assurance pipeline that allows us to rapidly screen grass tillers and seeds for the presence of an epichloë endophyte from a range of cool season grasses. The pipeline involves the isolation of genomic DNA from the symbiotium followed by PCR with primers specific to the endophyte for detection of the fungus, identification of isolates using SSR markers and profiling of the alkaloid genes (Mittal, Hopkins and Young unpublished) (Fig. 2). However, it is equally useful for marker assisted selection for host traits (Young, Mittal, Saha and Hopkins, pers. com). We have successfully used this method to identify

contamination of “hot” (mammalian toxic) endophytes within seed batches and plot trials, profiling endophyte alkaloid potential of material from collection trips, and for endophyte infected grasses such as Canada wildrye (Saha et al., 2009), western wheatgrass and orchard grass. The development and utilization of novel (mammalian friendly) endophyte/grass

associations requires additional testing throughout the cultivar development process, providing quality assurance of low level (or no) contamination.

The focus of this paper has been on the alkaloids we are knowledgeable of, but genome sequencing can provide a snapshot of an endophyte's capability with regards to areas that we know little about. The sequenced *E. festucae* genome (www.endophyte.uky.edu) is known to contain other potential alkaloid biosynthesis genes of which we are unsure of the resulting products (Schardl et al, unpublished) and research has begun characterizing a number of these genes. As the cost of genome sequencing comes down, we will soon be able to rapidly screen endophytes to determine their full potential and dissect other important traits that these fungi supply to the symbiotum using comparative genomics approaches.

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