

Molecular biology of *Epichloë* endophyte toxin biosynthesis

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Abstract

Epichloë endophytes are an important group of filamentous fungi that confer on the grass host a range of biological benefits. However, endophyte synthesis of ergopeptine and indole-diterpene mammalian toxins in pasture grasses is detrimental to livestock grazing on that forage. The molecular cloning of the genes involved in the biosynthesis of these toxins will enhance our ability to maximise the beneficial attributes of this mutualistic association through the availability of DNA probes to screen and select for desirable endophytes and through our ability to genetically modify endophytes. Genes involved in the biosynthesis of both classes of alkaloids have recently been cloned from *Claviceps purpurea* and *Penicillium paxilli*. In both cases the genes are organised in large clusters; a feature that will facilitate a complete genetic analysis of each pathway and provide probes for isolating homologous genes from *Epichloë* endophytes. This paper reviews recent research developments on the molecular biology of these two pathways.

Keywords: *Epichloë* endophytes, ergopeptines, gene cloning, gene manipulation, indole-diterpenes

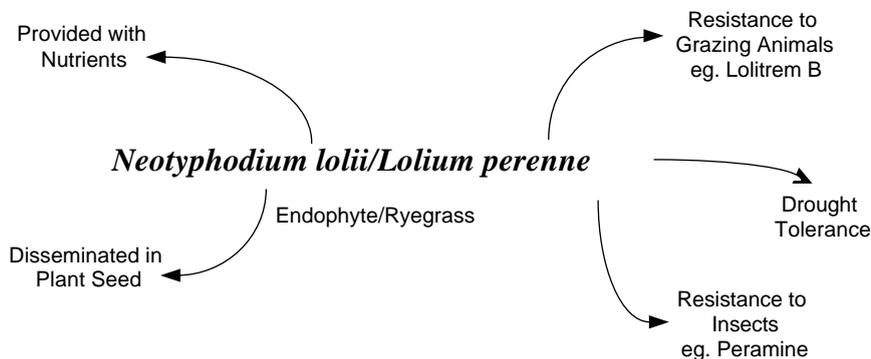
Introduction

Epichloë endophytes are an important group of filamentous fungi that form symbiotic associations with temperate grasses. As a group they comprise both the sexual *Epichloë* species, and their asexual derivatives, the *Neotyphodium* species. This highly evolved association between host and endophyte works to the mutual benefit of each partner. The host provides nutrients for endophyte growth and a means of dissemination through the seed whereas the endophyte provides biological protection to the host (Figure 1).

Biological effects

While the mechanisms responsible for the biological benefits that the endophyte confers on the host are not well understood, the antiherbivore effects are largely attributable to endophyte production of novel alkaloids. Peramine (a pyrrolopyrazine) and *N*-formyllooline (a pyrrolizidine) are potent insect feeding deterrents, whereas lolitrem B (an indole-diterpene) and ergovaline (an ergopeptine) are mammalian toxins (Bush *et al.* 1997). While all four classes of metabolites are ecologically beneficial, from an agricultural perspective endophyte production of mammalian toxins is detrimental to grazing livestock. The lolitrems are potent

Figure 1 Biological benefits for each partner in the *Epichloë* endophyte – grass host association.



tremorgenic neurotoxins implicated in 'ryegrass staggers' in sheep and the ergot alkaloids are toxins implicated in 'fescue toxicosis' in cattle. Both groups of alkaloids have major effects on livestock health and productivity. Consequently, there is considerable interest in maximising the benefits of endophytes to pastoral agriculture by selecting for host-endophyte associations that minimise the detrimental effects to grazing animals yet retain protective benefits, such as field persistence, of the host grass.

The spectrum of alkaloids produced in any given association is dependent on both the genotype of the host and the genotype of the endophyte (Siegel *et al.* 1990). Lolines, ergot alkaloids and peramine predominate in associations between tall fescue (*Festuca arundinacea* Schreb.) and *Neotyphodium coenophialum* (Siegel *et al.* 1990), whereas lolitrems, ergot alkaloids and peramine are commonly found in associations between perennial ryegrass (*Lolium perenne* L.) and *N. lolii* (Christensen *et al.* 1993). However, even within these associations there is considerable variation in both the types of alkaloids found and the levels to which they accumulate. These patterns reflect both environmental effects and interactions between endophyte genotype and the plant host genotype.

A rational approach to selecting the best host-endophyte combination for use in the field requires, among other things, (i) a knowledge of the biochemical steps that contribute to the biosynthesis of each alkaloid, and (ii) a knowledge of the genes involved in these pathways and their organisation within the genome. With this knowledge it will be possible, using molecular probes and the polymerase chain reaction (PCR), to directly select for naturally occurring endophyte isolates that lack the ability to synthesise these toxins, or alternatively, to use gene-replacement techniques to specifically delete key genes that are involved in the biosynthesis of these alkaloids.

This paper reviews recent research developments that contribute toward attaining these goals and, in particular, highlights progress on the molecular cloning of genes involved in the biosynthesis of the ergot alkaloids and the indole-diterpenes.

Proposed biosynthetic pathways

Biosynthesis of ergot alkaloids

The biosynthesis of ergot alkaloids is the best understood of the endophyte alkaloid biosynthetic pathways, principally because of the research carried out on *Claviceps purpurea*, the fungus responsible for St. Anthony's Fire (ergotism) from contaminated rye (Socic & Gaberc-Porekar 1992). The first step in this pathway (Figure 2) is the synthesis of dimethylallyltryptophan

(DMAT), a reaction catalysed by DMAT synthase that uses dimethylallylpyrophosphate (a derivative of mevalonic acid) and tryptophan as the primary precursors (Gebler & Poulter 1992). DMAT is then converted via several intermediates to clavine alkaloids, such as lysergic acid, that are characterised by the presence of an ergolene ring system. The ergolene acids, such as lysergic acid, and ergolene alcohols can be further transformed into more complex ergopeptide derivatives such as ergotamine. The synthesis of ergotamine is catalysed by two non-ribosomal peptide synthetases, LPS1 (lysergyl peptide synthetase 1) and LPS2, that sequentially add the amino acids alanine, phenylalanine (or valine for ergovaline synthesis) and proline to the activated lysergic acid (Figure 2) (Riederer *et al.* 1996; Walzel *et al.* 1997).

Biosynthesis of indole-diterpenes

In contrast to the ergot alkaloid biosynthetic pathway, virtually nothing is known about either the nature of the biochemical intermediates or the enzymology of indole-diterpene biosynthesis in fungi. The biosynthetic schemes proposed are based on [¹³C]-acetate radiolabelling studies and identification of related indole-diterpenoids in other fungi, such as *Penicillium paxilli*, *P. paspali* and the endophytes themselves (Mantle & Weedon 1994; Munday-Finch *et al.* 1996). Based on these schemes geranylgeranyl pyrophosphate (GGPP), derived from mevalonic acid, and indole, derived from tryptophan, are the proposed primary precursors for diterpene biosynthesis. Likely intermediates in the biosynthesis of paxilline, the predominant diterpene found in *P. paxilli* and a proposed intermediate in the biosynthesis of lolitrem B (Weedon & Mantle 1987), are emindole, paspaline, paspaline B and 13-desoxypaxilline (Munday-Finch *et al.* 1996). In endophytes, α -paxitriol and lolitriol are likely intermediates in the conversion of paxilline to lolitrem B (Miles *et al.* 1992). However, the complexity of the intermediates identified would suggest that these pathways are more likely to be organised in metabolic grids (multiple pathways) rather than linear biosynthetic pathways (Munday-Finch *et al.* 1996; Lane *et al.* 1999).

Molecular cloning of alkaloid biosynthetic genes

General strategies

Several approaches are available to clone genes involved in alkaloid biosynthetic pathways. By far the most robust method is to determine the amino acid sequence of peptide fragments from the purified enzyme and to use this sequence data to design degenerate primers to directly amplify the gene from genomic DNA by PCR.

Figure 2 Proposed pathways for the biosynthesis of ergot alkaloids and indole-diterpenes in *Epichloë* endophytes.



However, purification of enzymes for which substrates are not readily available is not a straightforward task. Alternative strategies include: (i) direct screening of a gene library using the corresponding gene (a heterologous probe) from another organism; (ii) direct cloning from genomic DNA using the PCR and degenerate primers designed for conserved domains of functionally similar proteins identified in other organisms; (iii) complementation of a mutation that blocks a step in the biosynthetic pathway; and (iv) by plasmid insertional mutagenesis and rescue of the 'tagged' gene (Timberlake 1991). Each method has its advantages and disadvantages that depend on the biology of the organism, how much is known about the biochemical pathway being targeted, and the level of sophistication of genetic and molecular tools available for that organism. However, one major advantage in targeting genes involved in secondary metabolite biosynthetic pathways is the tendency for these genes to be organised in large gene clusters (Keller & Hohn 1997). This means that once one gene is cloned, the remainder of the genes in the pathway can be isolated by chromosome walking either side of the gene 'in hand'.

Molecular cloning of ergot alkaloid biosynthetic genes

The first ergot alkaloid biosynthetic gene to be cloned was the DMAT synthase, *dmaW* (Tsai *et al.* 1995). This was cloned from *C. fusiformis* by PCR using primers designed to polypeptide sequence determined from the purified protein. More recently a cluster of genes, including the DMAT synthase (*cpd1*) and the LPS1 (*cpss1*), was isolated from *C. purpurea* strain P1 (Tudzynski *et al.* 1999) using the previously cloned *C. fusiformis* DMAT synthase as a probe to isolate the corresponding gene from *C. purpurea*. Chromosome walking was used to isolate the LPS1 gene and several other genes of, as yet, unknown biochemical function. These developments have opened the way for cloning the corresponding genes from grass endophytes.

Using a PCR approach, Wang *et al.* (1999) were recently successful in cloning DMAT synthases from *N. coenophialum*. Similarly, Panaccione (1996) using degenerate primers designed to conserved peptide synthetase domains, succeeded in amplifying three unique products from both *C. purpurea* and *N. coenophialum*; suggesting that these plant-associated fungi contain at least three peptide synthetases. Each *N. coenophialum* gene was present in 2–3 copies which is consistent with the inter-specific hybrid origin of this particular taxonomic group (Tsai *et al.* 1994). One *N. coenophialum* clone hybridised to sequences in both *N. coenophialum* and *C. purpurea* and is, therefore, a strong candidate for an ergovaline peptide synthetase. As ergovaline differs by only one amino acid from

ergotamine, having valine instead of phenylalanine in the second position, the peptide synthetases involved are expected to show sequence conservation in two of the three domains for these two fungi. More recently, one of the *C. purpurea* genes, Cp605, was shown to be on the same cosmid that contains the DMAT synthase, a result consistent with the findings of Tudzynski *et al.* (1999). Using Cp605 as a heterologous probe, the corresponding sequences have been cloned from a gene library of *N. lolii* strain Lp19 (Panaccione *et al.* 1999). As *N. lolii* is haploid, disruption of the gene in this background will provide genetic proof that this gene is indeed involved in ergovaline biosynthesis.

Molecular cloning of indole-diterpene biosynthetic genes

As discussed above, very little is known about the biochemistry of indole-diterpene biosynthesis in fungi, thereby limiting the molecular approaches available to clone genes in this pathway. To add to this difficulty, endophytes grow very slowly in culture, are not particularly amenable to genetic analysis, and conditions for reliable production of these metabolites in culture are yet to be established (Penn *et al.* 1993). In contrast, *Penicillium paxilli* makes large quantities of the indole-diterpene paxilline (Ibba *et al.* 1987), grows relatively quickly, sporulating in 7 days, and is much more amenable to genetic analysis (Young *et al.* 1998). Using the technique of plasmid tagging, three paxilline-negative mutants of *P. paxilli* were isolated and all were shown to contain deletions of the order of 100–200 kb at a single locus (Young *et al.* 1998). Clones spanning ~100 kb across this deleted region have been isolated from a gene library of *P. paxilli* by chromosome walking (Young, McMillan & Scott unpublished results).

In a second round of plasmid mutagenesis using a slightly modified method, called REMI (restriction enzyme mediated integration), a fourth paxilline-negative mutant was isolated. This mutant was found to contain an untagged deletion of 25 kb that maps to the same locus as the original mutants. This deletion defined a minimum gene locus required for paxilline biosynthesis. DNA sequence analysis of this region identified a cluster of genes with similarities to known phenyltransferases and monooxygenases, enzymes expected to be involved in the biosynthesis of paxilline. Targeted deletion of three of the genes in this cluster confirmed that this locus is indeed involved in paxilline biosynthesis.

A major surprise was the identification of a GGPP synthase, an enzyme predicted to be essential for growth of the organism because of its role in primary metabolism. However, further analysis revealed the presence

of a second copy of GGPP synthase in *P. paxilli*. This is presumably the copy required for primary metabolism, whereas that associated with the cluster has been recruited specifically for secondary metabolism. A consequence of this finding is that IPP (a C5) rather than GGPP (a C20) is the most likely immediate carbon precursor for paxilline biosynthesis (Figure 2). Interestingly, two copies of GGPP synthase have also been found in *Gibberella fujikuroi*, one of which has been recruited for gibberellin biosynthesis (Tudzynski & Hölder 1998), suggesting that this may be a conserved mechanism for diterpene biosynthesis in fungi. Extended sequencing of the paxilline (*pax*) biosynthesis locus has identified other genes that are likely to be involved in this pathway, including a putative transcription factor that may control the expression of all the genes in the cluster as does AflR in the sterigmatocystin/aflatoxin biosynthetic pathways present in *Aspergillus nidulans*, *A. flavus* and *A. parasiticus* (Keller & Hohn 1997). To date, 12 genes, extending over a region of about 60 kb on chromosome V of *P. paxilli*, have been identified within the paxilline biosynthetic gene cluster. Work is in progress to identify the biochemical functions of each of these genes, define the pathway and understand how the pathway is regulated.

Our success in cloning the genes for paxilline biosynthesis in *P. paxilli* has opened the way to clone by PCR the corresponding cluster of genes from the grass endophytes. Recently we have succeeded in isolating two copies of GGPPS from *N. lolii* strain Lp19. It is likely that one of these copies is part of a gene cluster involved in indole-diterpene (paxilline and/or lolitrems) biosynthesis in *N. lolii*.

Future opportunities

The cloning of the gene clusters for ergotamine biosynthesis and paxilline biosynthesis from *C. purpurea* and *P. paxilli* respectively, will facilitate the isolation of the corresponding gene clusters from Epichloë endophytes. What will these discoveries reveal and how can we apply the knowledge gained?

The cloning of these alkaloid biosynthetic gene clusters will, for the first time, reveal the identity of the genes involved and the likely biochemical function of the gene products. Having the genes 'in hand' will provide a suite of probes for screening the presence and distribution of these genes in the Epichloë endophytes. This will enable a molecular selection approach to be used to identify strains that lack the genetic capacity to synthesise these toxins, thereby eliminating the uncertainty of current selection processes that rely on detection of the toxin in the plant; which is subject to the confounding effects of host-genotype interactions.

However, many naturally occurring isolates may indeed have pathway genes but still lack the ability to produce the toxins, regardless of the host background. A precedent for this is the presence of genes for the aflatoxin biosynthesis pathway in *Aspergillus sojae* and *A. oryzae*, species which are used in Asian food fermentations (e.g., koji preparation), that are closely related to the aflatoxin-producing *A. parasiticus* and *A. flavus*. The inability of *A. oryzae* isolates to synthesise aflatoxins is due to the absence of one or more of the genes in the pathway, however, in the case of *A. sojae* all the genes are present but are not expressed (Klich *et al.* 1995; Klich *et al.* 1997). The molecular basis for the lack of expression in *A. sojae* is still not known. Using similar molecular techniques, it will be possible to verify that endophyte strains classified as toxin-negative in the host indeed lack a key gene in the pathway or alternatively are blocked in their ability to express those genes. These selection strategies will enhance the robustness of the tests for deciding whether an endophyte is toxin-negative or -positive and overcome the risk of unexpected expression of a particular toxin when a strain is moved from one host background into another.

The cloning of toxin biosynthesis pathway genes also opens up the possibility of using molecular techniques to disrupt a gene at an early step in the biosynthetic pathway. Such methods have already been used to disrupt paxilline biosynthesis in *P. paxilli* (Young, McMillan & Scott, unpublished results). The development of gene transfer techniques for Epichloë endophytes (Murray *et al.* 1992; Tsai *et al.* 1992) makes it feasible to use the same approach in these organisms. For endophytes that have the capacity to synthesise both ergot alkaloids and indole-diterpenes, it will probably be necessary to disrupt both pathways as disruption of one may result in an increased flux of carbon into the other (see Figure 2), resulting in an even higher level of one of the toxins than was previously found. Also, it will be necessary to examine other biological consequences of disrupting these toxin biosynthesis pathways. For example there are reports that some of the indole-diterpenes are toxic to larvae of Argentine stem weevil (Ball & Prestidge 1993; Prestidge & Ball 1993).

A further outcome of this research will be the opportunity to increase our understanding of the biology of the association between symbiont and host, and the fitness of that association from both biotic and abiotic stresses. The ability to genetically disrupt these pathways will allow us to determine both the qualitative and quantitative effects of the various alkaloids in isogenic fungal backgrounds. Using reporter gene technology, it will be possible to determine both spatial and temporal expression of these fungal gene pathways in the plant,

and the effect of environmental and genetic changes on that expression. We should also expect the presence or absence of the alkaloid biosynthesis pathways to reflect the evolutionary relationships of the endophytes. This knowledge will allow us to better understand the complexities of this important association in both natural ecosystems and in the agricultural context.

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