Insights into the molecular biology of epichloë endophyte alkaloid biosynthesis

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Abstract
The production of secondary metabolite alkaloids by epichloë endophytes of grasses is of great interest to agriculture due to the opposing effects of pasture protection against insect herbivory and concomitant toxicity to stock. Recent molecular genetic analysis, that has greatly enhanced our understanding of secondary metabolite biosynthesis, is reviewed and potential outcomes for pastoral agriculture are discussed.
Keywords: endophyte, *Epichloë*, *Neotyphodium*, alkaloid, secondary metabolite, gene cluster

Introduction
The interaction between the fungal endophyte *Neotyphodium lolii* and the forage grass *Lolium perenne* (perennial ryegrass) is vital to New Zealand agriculture. Alkaloids produced by the endophyte during biotrophic growth can protect host grasses from herbivory from a range of insects, including Argentine stem weevil (*Listronotus bonariensis*), which would devastate New Zealand ryegrass pastures without the production of alkaloids such as peramine. Despite the net positive effects of endophytes however, some secondary metabolites are toxic to grazing stock, most famously causing the neurotoxicosis ryegrass staggers in livestock by ingestion of endophyte-produced lolitrems.

Strain selection based on chemistry has been successful in identifying *N. lolii* strains (such as AR1) that have low toxicity while retaining resistance to insect herbivory (Tapper & Latch 1999). However, there are many questions remaining about endophyte secondary metabolism. Compounds such as ergovaline for example, which can cause heat-stress and fescue toxicosis, are also implicated in protection against insects (Ball *et al.* 1997). Could understanding the biochemical pathways of such compounds lead to identification of non-toxic intermediate compounds that retain insect-protection? Other than the already characterised endophyte-produced alkaloids it is likely that a number of other novel compounds are also produced: could these have positive or negative effects for agriculture? Our aim is to answer these and many other questions using molecular biology – studying secondary metabolism at the gene level. Here we present a review of recent work which has markedly enhanced our understanding of the molecular basis of endophyte secondary metabolite biosynthesis. Possible outcomes for agriculture are discussed.

Secondary metabolite biosynthetic gene clusters – the *LTM* gene cluster for indole diterpene biosynthesis
Four alkaloid groups are produced by endophytes with well-characterised effects on the symbiotum. These are peramine, indole diterpenes (such as lolitrem B), lolines and ergot alkaloids (such as ergovaline). Genes for secondary metabolite biosynthesis are often clustered in fungal genomes and genes or gene clusters have been cloned and characterised for each of the four identified endophyte alkaloids.

Indole diterpenes are frequently tremorgenic mycotoxins responsible for neurotoxicoses such as ryegrass staggers (Gallagher *et al.* 1984). A gene cluster for lolitrem indole diterpene biosynthesis was the first characterised in endophytes and was obtained by first cloning genes for a related indole diterpene compound from the model organism *Penicillium paxilli* (McMillan *et al.* 2003; Young *et al.* 1998, 2001). A geranylgeranyl diphosphate synthase gene from this cluster, *paxG*, was used as a probe to find the equivalent gene in *N. lolii* (Young *et al.* 2005). Chromosome walking (sequencing step by step along the chromosome from a gene of interest), combined with the use of sequences identified in a gene library enriched for endophyte genes expressed *in planta*, identified further clustered *pax* gene homologues at a locus labelled *LTM* proposed to be required for endophyte indole diterpene biosynthesis (Young *et al.* 2005, 2006).

The biochemical pathway for the synthesis of paxilline has been well characterised in *P. paxilli*, in which it is the end-point indole diterpene (Saikia *et al.* 2006, 2007, 2008). Complementation of *P. paxilli* gene deletion mutants with *ltm* genes along with analysis of an *ltmM* deletion mutant in *E. festucae,* which does not synthesise any indole diterpenes, supports at least most of the shared genes between the two clusters being responsible for the synthesis of paxilline (Saikia *et al.* 2008; Young *et al.* 2005). Genes unique to the epichloë endophyte *LTM* cluster, *ltmE, ltmF, ltmJ* and *ltmK,* are likely to be responsible for the steps leading to lolitrem B, which are unique to epichloë (Scott *et al.* 2007).

The *perA* gene for peramine biosynthesis
Peramine is a pyrrolopyrazine feeding deterrent to

Argentine stem weevil (Rowan 1993). Peramine is proposed to be synthesised from arginine and either proline (Rowan 1993) or 1-pyrroline-5-carboxylate (Tanaka *et al.* 2005). A non-ribosomal peptide synthetase (NRPS) for peramine synthesis, *perA*, was cloned from *N. lolii* using degenerate polymerase chain reaction (PCR) – a method of amplifying sequence based on conserved enzyme motifs. It is possible that *perA* is the only gene required for peramine synthesis (Tanaka *et al.* 2005). Deletion of this gene blocked peramine production, leaving the symbiotum more susceptible to herbivory by Argentine stem weevil (Tanaka *et al.* 2005). Although production of all alkaloids by endophytes is speculated to confer an ecological advantage to the grass/endophyte symbiota, this study provided rare experimental evidence of an adaptive advantage for production of a secondary metabolite by a fungus.

The *EAS* gene cluster for ergot alkaloid biosynthesis

Ergot alkaloids include clavines derived from dimethylallyl tryptophan (DMAT), lysergic acid amides and ergopeptines. The main ergopeptine product synthesised by endophytes is ergovaline, which has been implicated in fescue toxicosis, effects of which may include poor weight gain, hyperthermia, convulsions, reduced fertility, gangrene of the extremities and death (Bacon 1995). The gene for the first step in the ergot alkaloid pathway, the DMAT synthase gene *dmaW*, was cloned by degenerate PCR based on the DmaW sequences in the ergot alkaloid-producing pathogenic fungi *Claviceps fusiformis* and *C. purpurea* (Wang *et al.* 2004), similar to the approach taken to identify the *ltm* genes based on *P. paxilli* sequences. The gene encoding the NRPS LpsA, one of two NRPSs responsible for addition of a tripeptide to lysergic acid to yield lysergyl peptide lactam, the final precursor to ergovaline, was identified by cloning an NRPS from a DNA fragment containing the *dmaW* gene (Panaccione *et al.* 2001). Subsequently, one of the NRPS fragments cloned in the *perA* study was shown to be part of the second NRPS LpsB required for ergovaline synthesis (Fleetwood *et al.* 2007). Genome walking from this gene identified five other genes clustered with *lpsB* that were predicted to be required for ergot alkaloid production based on similarity with genes from the *C. purpurea* ergot alkaloid cluster (Fleetwood *et al.* 2007).

Deletion of the *lpsB* gene in *E. festucae* confirmed its role in the synthesis of ergovaline, causing elimination of ergovaline and lysergic acid amides and accumulation of lysergic acid and other clavine intermediates (Fleetwood *et al.* 2007). Ergovaline has been implicated in pasture-deterrence of African black beetle (*Heteronychus arator*) (Ball *et al.* 1994, 1997). However, insect feeding studies with perennial ryegrass infected with the Δ*lpsB* mutant surprisingly showed that ergovaline was not necessary for black beetle feeding deterrence in these symbiota (Fleetwood 2007). Whether clavine intermediate ergot alkaloids or a different alkaloid altogether are responsible for black beetle deterrence in this mutant remains an interesting area of future study.

The *LOL* gene cluster for loline biosynthesis

Lolines have potent insecticidal and feeding-deterrent properties and can accumulate up to 2% of the dry weight of the infected plant's dry mass, far exceeding the biomass of the fungus and the amounts of other alkaloids (Spiering *et al.* 2002). Until relatively recently, it was not known conclusively if lolines were produced by the plant, the endophyte or both. Blankenship *et al.* (2001) showed that axenic cultures of *N. uncinatum*, a common meadow fescue endophyte, can produce lolines in minimal media, demonstrating that these products can be solely synthesised by the fungus. However, other strains of endophyte that produce lolines *in planta* could not be induced to produce lolines in culture (Blankenship *et al.* 2001). Mendelian analysis of genetic crosses of Loline^{+} and Loline^{-} *E. festucae* showed that loline synthesis segregated as a single genetic locus, designated *LOL* (Wilkinson *et al.* 2000). Genes expected to be involved in loline synthesis were subsequently identified in *N. uncinatum* using suppression subtractive hybridisation (Spiering *et al.* 2002) and genome walking (Spiering *et al.* 2005). The *lol* genes are organised as two almost identical (~93% gene nucleotide identity) gene clusters of nine genes each in the *N. uncinatum* genome, the taxonomic distribution of which strictly correlated with loline production. The role of *lolC*, a putative pyridoxal phosphate-containing enzyme, was confirmed by RNA interference (RNAi) (Spiering *et al.* 2005).

Identification of novel secondary metabolite genes

Non-ribosomal peptide synthetases, such as those involved in peramine and ergovaline biosynthesis, are commonly involved in the synthesis of a variety of secondary metabolites. NRPSs are large multifunctional proteins (Finking & Marahiel 2004) that synthesise a diverse range of bioactive compounds, some of which serve as pathogenicity factors (Walton 2006; Johnson *et al*. 2000; Gardiner *et al.* 2004; Lee *et al*. 2005), in addition to suggested roles in niche adaptation (Lee *et al*. 2005). Johnson *et al.* (2007) sought to survey the numbers of NRPS genes from endophytes of the *Neotyphodium/ Epichloë* genus in addition to the three NRPS genes involved in the biosynthesis of peramine and ergovaline. A degenerate PCR approach to conserved motifs within NRPS enzymes similar to that used for *perA* identification was used to identify at least 12 NRPS genes amongst the collective genomes of epichloë species. This is likely to

be a conservative estimate of the true number of these genes in epichloë endophytes. Based on comparison with characterised genes from other fungi in public databases and the classifications of Lee *et al*. (2005), the 12 NRPS fragments were predicted to correspond with full-length NRPSs involved in the biosynthesis of siderophores, toxins involved in pathogenesis, N-methylated peptides, and several that could not be classified. This study with NRPSs and a preliminary analysis that has identified at least 10 genes for polyketide synthases, another major class of enzymes involved in secondary metabolite synthesis (C. Voisey, unpublished), suggests that *Neotyphodium/Epichloë* species are likely to synthesise a much greater number of secondary metabolites than has currently been identified. Some of these may confer additional protection to the host grass against pests and diseases. Gene knockout experiments combined with recently developed unbiased approaches to chemical analysis in endophytes (Cao *et al.* 2008; Koulman *et al.* 2007) should enable relatively rapid identification of these novel metabolites.

Impact on the farm – screening and beyond

The identification of a suite of genes responsible for secondary metabolite production, provides an opportunity to predict the secondary metabolic potential of different endophyte strains by correlating the presence or absence of different genes with known and unknown chemistry. For example, we can already predict the ability of a particular endophyte to synthesise the known toxic or protective alkaloids (Johnson *et al.* 2007; Tanaka *et al.* 2005; Young 2005) and in the future it may be possible to correlate unidentified bioactivity with the presence of additional secondary metabolite genes with currently unknown function.

A further benefit of identifying endophyte secondary metabolite genes is the ability to completely dissect biochemical pathways. For example, the analysis of mutants deleted for genes responsible for different steps in the pathway (similar to the *ltmM*, *lpsB* and *perA* analyses described above) will allow the precise identification of intermediate and end-point compounds with anti-mammalian and anti-invertebrate properties. This will ultimately enable genetic modification of endophytes to achieve ideal gene sets for maximum pasture protection with minimum animal toxicity, for which no naturally occurring strain has yet been identified.

In conclusion, the molecular dissection of endophyte secondary metabolism is entering an exciting era, with the predicted genes for the synthesis of well-known natural products – indole diterpenes, ergot alkaloids, lolines and peramine – identified. Research focus can now shift to manipulating these known pathways and analysing new predicted secondary metabolite genes with unknown products. These are particularly exciting for future research and may lead to the identification of new bioactivities.

ACKNOWLEDGEMENTS

We thank Geoff Lane, Brian Tapper, Wade Mace, Karl Fraser and Emily Parker for ongoing chemistry analysis and guidance. This work was supported by FRST grant C10X0203.

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