Functional analysis of the *Epichloë festucae*-perennial ryegrass symbiosis

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Abstract

Although much is known about the beneficial and detrimental effects of the interaction between *Neotyphodium lolii* and *Lolium perenne* in the field comparatively little is known about the molecular and cellular events that underlie these effects and how mutualism is maintained. Because *N. lolii* is quite intractable to genetic analysis we have developed the synthetic association between *Epichloë festucae* and perennial ryegrass as our model experimental system to study the epichloë endophyte-grass symbiotic interaction. Using this system we have recently shown that reactive oxygen species (ROS) produced by a specific endophyte NADPH oxidase (NoxA) have a critical role in regulating hyphal growth and development in perennial ryegrass. We have also shown that two additional components, NoxR and RacA, are required to regulate ROS production to maintain a mutualistic interaction. Using a combined molecular and genetic approach we have recently cloned and characterised genes for peramine and lolitrem biosynthesis. An overview of these advances and the opportunities now available to better understand and exploit this important fungal-grass interaction is presented.

Keywords: *Epichloë festucae*, *Neotyphodium lolii*, *Lolium perenne*, peramine, lolitrems, reactive oxygen species

Introduction

Neotyphodium lolii is an obligate seed-borne biotrophic fungus that colonises the intercellular spaces of perennial ryegrass aerial tissues, including the vegetative and reproductive tillers, as well as the seed (Christensen *et al.* 2002; Philipson & Christey 1986). Hyphae ramify within the meristematic zone of the grass from where they colonise axillary buds and leaf primordia. The hyphae in the leaves are infrequently branched, grow parallel to the axis of the leaf, and have a growth pattern that is synchronised with that of the host throughout the life cycle of the grass (Christensen *et al.* 2002; Tan *et al.* 2001). Strict control of hyphal growth is critical for maintenance of the mutualistic interaction between endophyte and grass host (Tanaka *et al.* 2006).

N. lolii is a haploid asexual derivative of *Epichloë festucae*, a

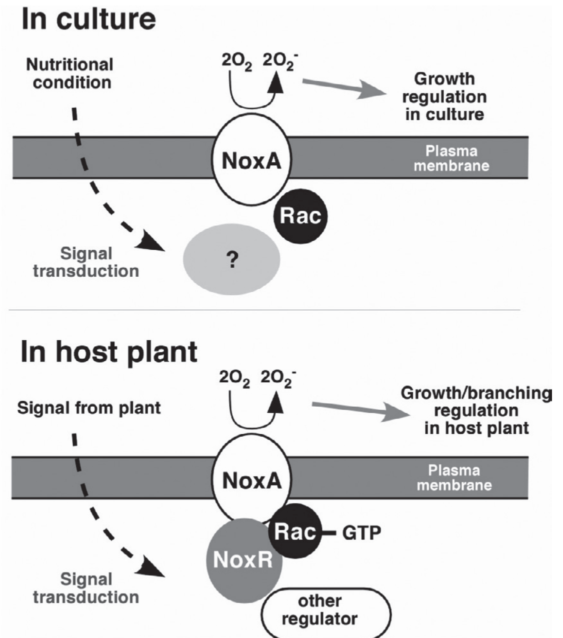
Table 1 Frequency of homologous gene replacement in *E. festucae*

GENE	5' flank (kb)	3' flank (kb)	% KO	Number	Reference
ltmM	2.7	2.7	3.1	5 of 159	Young *et al.* (2005)
noxB[1]	1.8	1.2	5.0	1 of 20	Tanaka *et al.* (2006)
noxB[2]	1.8	1.2	2.5	1 of 40	Tanaka *et al.* (2006)
noxA	2.5	0.8	3.7	2 of 54	Tanaka *et al.* (2006)
perA	2.6	2.5	0.8	1 of 120	Tanaka *et al.* (2005)
sakA	3.2	1.5	8.3	1 of 12	Eaton unpublished results
gcnA	2.8	2.8	10.0	2 of 20	Bryant *et al.* (2007)
ltmJ	1.4	1.0	8.3	3 of 36	Takemoto unpublished results
ltmF	1.3	3.5	8.3	1 of 12	Takemoto unpublished results
ltmE	1.2	1.5	1.0	1 of 96	Takemoto unpublished results
ltmK	1.6	1.2	16.7	2 of 12	Takemoto unpublished results
ltmQ	1.8	1.7	1.3	1 of 80	Takemoto unpublished results
noxR	1.5	2.5	25.0	6 of 24	Takemoto *et al.* (2005)
racA	1.8	0.9	1.0	1 of 100	Tanaka unpublished results
EF108	1.1	1.7	6.6	2 of 30	Tanaka *et al.* (2005)
ltm[3]	2.7	2.7	4.0	4 of 100	May unpublished results

[1]In wt background
[2]In *noxA* background
[2]Knock-in

Figure 1 Symbiotic phenotype of *E. festucae noxA*. Phenotypes of perennial ryegrass infected with *E. festucae* wild-type Fl1 and *noxA* symbiotic mutant.

Figure 2 Models for regulation of ROS production by Nox complex in culture and *in planta*. (A) Model for regulation of NoxA-catalysed ROS production in culture by recruitment of RacA and other unidentified proteins to the endophyte plasma membrane in response to nutritional or environmental signals. (B) Model for regulation of NoxA-catalysed ROS production *in planta* by recruitment of RacA and NoxR to the endophyte plasma membrane in response to a signal from the host grass.



natural symbiont of *Festuca* spp. that is also capable of forming compatible associations with perennial ryegrass (Christensen *et al.* 1997; Leuchtmann *et al.* 1994). Numerous studies have established that *N. lolii*, *E. festucae* and related epichloë endophytes are mutualists that provide a wide range of important benefits to their host plants, particularly under conditions of biotic or abiotic stress (Clay 1990; Easton 1999; Schardl & Clay 1997). The most well documented benefit of *N. lolii* to the perennial ryegrass host is protection from insect herbivory (Ball *et al.* 1997; Prestidge & Gallagher 1988; Rowan & Gaynor 1986), a biological effect ascribed to endophyte synthesis of various alkaloids *in planta*.

Three main classes of biologically active metabolites have been identified in *N. lolii* infected perennial ryegrass: peramine, the only known pyrrolopyrazine; ergot alkaloids, principally ergovaline; and indole-diterpenes, principally lolitrem B (Lane *et al.* 2000; Rowan 1993). Peramine has been shown to be a potent feeding deterrent of adult Argentine stem weevil (ASW; *Listronotus bonariensis*) (Rowan *et al.* 1990; Rowan & Gaynor 1986), an economically important exotic pest of perennial ryegrass in New Zealand. Lolitrem B has biological activity against ASW larvae (Prestidge & Gallagher 1988) but is better known as the causative agent of the neuromuscular disorder known as ryegrass staggers, associated with sheep grazing ryegrass dominant pastures after long periods of water stress or following fresh re-growth after periods of water stress (Fletcher & Easton 1997; Keogh 1973). The importance of ergot alkaloids toward ecological fitness of the host grass has not been well defined. The presence of ergopeptines in perennial ryegrass potentially exacerbates ryegrass staggers, as they induce heat stress in grazing livestock (Fletcher & Easton 1997). Alkaloid levels in endophyte-infected perennial ryegrass are affected by season (Ball *et al.* 1995; di Menna & Waller 1986), environment (Barker *et al.* 1993; Lane *et al.* 1997; Rasmussen *et al.* 2006) and plant genotype (Easton *et al.* 2002; Latch 1994; Spiering *et al.* 2005).

Genes for the biosynthesis of ergot alkaloids (Panaccione *et al.* 2001; Wang *et al.* 2004), indole-diterpenes (Young *et al.* 2005; Young *et al.* 2006) and peramine (Tanaka *et al.* 2005) have now been cloned. Expression analysis has shown that the genes for all three pathways are preferentially and highly expressed *in planta* (Tanaka *et al.* 2005; Young *et al.* 2006), suggesting that plant-specific signalling is required for expression of these pathways. The molecular cloning of these genes now allows the biosynthetic pathways to be elucidated, the mechanisms for plant-regulated expression to be explored, and the functional distribution of these genes within the epichloë endophytes to be determined.

The recent demonstration that endophyte production of reactive oxygen species (ROS) is critical in maintaining the mutualistic interaction between *E. festucae* and perennial ryegrass provides important insights into potential signalling mechanisms that operate between endophyte and host (Takemoto *et al.* 2006; Tanaka *et al.* 2006). The isolation and characterisation of the fungal components of the NADPH oxidase complex and associated regulators and effectors now allows key signalling pathways to be elucidated.

Figure 3 Design of *perA* replacement construct pPN66.

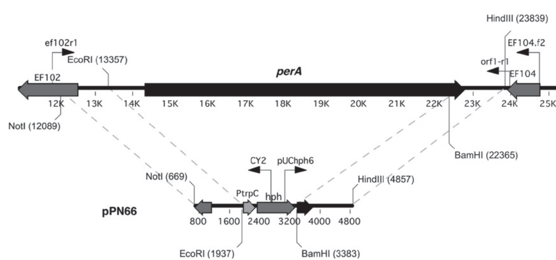


Figure 4 A proposed scheme for peramine biosynthesis. Adaptated from Tanaka *et al*. (2005).

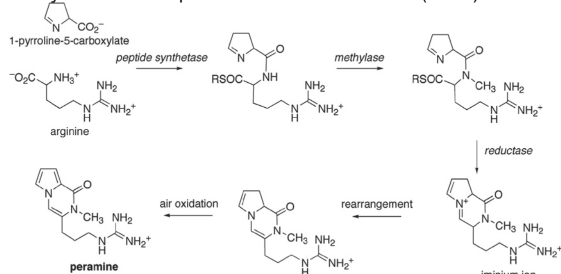
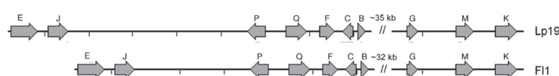


Figure 5 Physical map of the *N. lolii* (Lp19) and *E. festucae* (Fl1) *LTM* locus. The map of the Lp19 *LTM* locus was generated from sequence accession numbers AY742903 and DQ443465. The map of the Fl1 LTM locus was generated from sequence accession number AY742905 and from Southern analysis.



This review provides an overview of recent advances in our understanding of (i) endophyte signalling mechanisms that maintain the mutualistic symbiotic interaction between *E. festucae* and perennial ryegrass and (ii) the endophyte genes and gene products required for biosynthesis of peramine and lolitrems.

Methods

Fungal strains and growth conditions

Cultures of *Epichloë festucae* wild-type strain Fl1 and mutant strains were grown on 2.4% potato dextrose (PD) agar at 22°C.

Plant growth and endophyte inoculation conditions

Inaoculation of endophyte-free seedlings of perennial ryegrass (*Lolium perenne*) was performed by the method of Latch & Christensen (1985). Plants were grown and tested for the presence of endophyte as described previously (Tanaka *et al.* 2005).

Microscopy

Fungal hyphae were examined using the Zeiss Axiophot microscope with DIC optics. Images were captured using IM50 (version 4.0) image capture software. The presence of hyphae in endophyte-infected leaves was examined by staining of epidermal peels from outer leaf sheaths with an aniline blue stain. An Olympus BX51 light microscope was used to examine the hyphal structure, with images captured by an Optronics digital camera using the MagnaFire 2.1C image capture software.

GUS analysis

Plants inoculated with *E. festucae* isolates transformed with the P*ltmM*-*gus*A and P*gpdA*-*gus*A reporter constructs were stained for GUS activity as described previously (Bryant *et al.* 2007).

Preparation of constructs

Plasmid pPN63 was prepared by sequentially ligating into pPN1688 (Young *et al.* 2005) a 1.8-kb *Kpn*I/*Sac*I (3') and a 2.0-kb *Pst*I/*Sal*I (5') fragment from gene EF101. The two fragments were prepared by cloning 1.8 kb *Kpn*I/*Not*I and 2.0-kb *Eco*RV fragments from pPN60 into pBlueScript and redigesting to capture appropriate restriction enzyme sites that were part of the multiple cloning site.

Plasmid pPN66 was prepared by sequentially ligating into pPN1688 (Young *et al.* 2005) 1.94-kb *Not*I/EcoRI (5') and a 1.47-kb *Bam*HI/HindIII (3') fragments from the 5' end of EF102 and the 3' end of *perA*, respectively.

Results and Discussion

***Epichloë festucae* as a model experimental system for genetic analysis of endophytes**

Although *N. lolii* is the natural epichloë endophytic species found in perennial ryegrass it is quite intractable to genetic analysis principally because of its extremely slow growth rate in axenic culture. In contrast, *E. festucae* is relatively fast growing, forming large (1.5-cm) colonies on potato dextrose agar plates within a period of 2 weeks, compared to six-ten weeks for *N. lolii*. Hyphae of *E. festucae* readily form protoplasts when treated with Glucanex and as long as the protoplast viable count is at least 10^6/ml, good transformation rates are usually obtained (Tanaka *et al.* 2005; Young *et al.* 2005). Using *E. festucae* strain Fl1, an isolate from *Festuca longifolia* seedline SR3000 (Siegel *et al.* 1990), targeted replacements can be generated in the genome of this strain by homologous recombination at frequencies of between 1 and 25% depending on the locus targeted and the size of the flanking sequences used to prepare the construct (Table 1). We generally use flanking sequences in the range of 1.5 to 2.5-kb to maximise the yield of recombinants. *E. festucae* is naturally haploid with a genome size of approximately 29 Mb (Kuldau *et al.* 1999), and has a heterothallic mating system (Leuchtmann *et al.* 1994). Synthetic associations between *E. festucae* strain Fl1 and perennial ryegrass can be readily established, thereby allowing genetic manipulation of this strain in culture and subsequent reintroduction into the host to study the symbiotic phenotype (Bryant *et al.* 2007; Takemoto *et al.* 2006; Tanaka *et al.* 2006; Tanaka *et al.* 2005; Young *et al.* 2005). In contrast to *N. lolii*, *E. festucae* is relatively easy to inoculate into perennial ryegrass seedlings with frequencies of infection in the range of 80 to 90% being routinely obtained with the wild-type strain. Although the growth of *E. festucae* Fl1 in these synthetic associations is slightly more vigorous than *N. lolii*, the hyphae still grow parallel to the axis of the leaf, are infrequently branched, and the pattern of growth is synchronised with that of the host throughout the life cycle of the grass (Takemoto *et al.* 2006; Tanaka *et al.* 2006). An important difference between *N. lolii* and *E. festucae* is the ability of the latter to form epiphyllous hyphae on the surface of perennial ryegrass leaves, but the growth of these hyphae are still tightly regulated by the host (Tanaka *et al.* 2006). Because of the above attributes, we have adopted *E. festucae* Fl1 and *L. perenne* as our model experimental system to study the epichloë endophyte-grass symbiotic interaction.

Figure 6 Physical maps of the *N. lolii* (Lp19) and *E. festucae* (Fl1) *ltm* cluster 1. The functional genes, abbreviated to a single letter, are represented by arrows indicating the direction they are transcribed. The microsatellite is represented by an asterisk. Tahi and Rua are relic retrotransposon sequences.

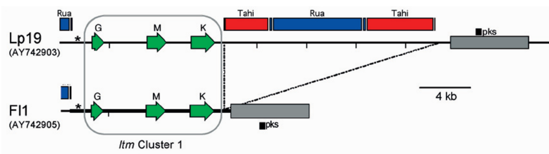
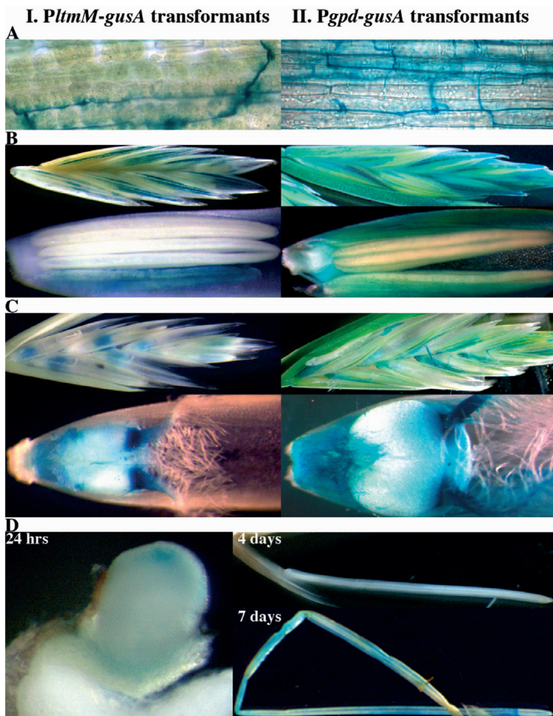


Figure 8 Analysis of GUS activity patterns of *E. festucae* hyphae infecting perennial ryegrass tillers using bright field microscopy. GUS expression patterns observed in infected, **A**, leaf sheath of vegetative tillers; **B**, pre-anthesis spikelets and individual florets; **C**, post-anthesis spikelets and individual florets; and, **D**, germinating seeds and seedlings. In panels A-C, photographs represent either P*ltmM-gusA* transformants (column I) or P*gpd-gusA* transformants, the positive control (column II). Panel D are representative photographs of P*ltmM-gusA* transformants infecting perennial ryegrass seeds and seedlings.

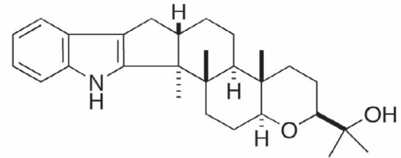


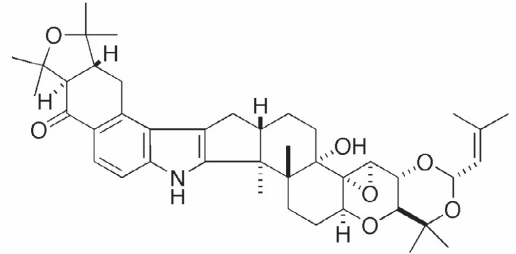
Isolation of *Epichloë festucae* symbiotic mutants

Epichloë festucae insertional mutagenesis

To identify *E. festucae* genes that are required for the establishment and maintenance of a mutualistic interaction with perennial ryegrass we have initiated a forward genetics approach (mutant phenotype to gene) to isolate mutants that disrupt this highly regulated symbiosis. Two insertional mutagenesis methods are being used, restriction enzyme-mediated plasmid integration

Figure 7 Structures of paspaline and lolitrem B.





(REMI) (Schiestl & Petes 1991) and *Agrobacterium tumefaciens* mediated T-DNA integration (Bundock *et al.* 1995; de Groot *et al.* 1998). The first method involves polyethylene glycol mediated uptake of linearised pAN7-1 plus restriction enzyme, into protoplasts of *E. festucae* (Tanaka *et al.* 2006). The presence of the restriction enzyme generates double stranded DNA breaks that promote recombination and repair of the plasmid into the fungal genome. The potential sites for integration are limited only by the number of sites in the genome for the restriction enzyme used. The major disadvantages of this method are the generation of multiple-copy and multi-site integrations and the generation of untagged deletions in the fungal genome (Michielse *et al.* 2005; Young *et al.* 2001). The second method involves acetosyringone activated conjugal transfer of T-DNA, engineered to contain a hygromycin resistant (*hph*) cassette, from *A. tumefaciens* to fungal cells (Bundock *et al.* 1995; de Groot *et al.* 1998). The development of this method using mycelial fragments of *E. festucae* is described in an accompanying paper (Tanaka *et al.* 2007). While this method preferentially results in single-site, single-copy integrations, there is a bias toward insertions into promoters of the fungal genes (Walton *et al.* 2005).

The use therefore of the combined approaches of REMI and T-DNA mediated insertional mutagenesis should maximise our ability to isolate mutations throughout the genome of *E. festucae*. The long-term goal is to generate sufficient number of mutants to provide widespread coverage of the *E. festucae* genome.

Isolation and functional analysis of noxA

Using REMI plasmid pAN7-1 mutagenesis we recently isolated a mutant of *E. festucae* that was symbiotically defective (Tanaka *et al.* 2006). This mutant was shown to have a single-copy insertion of pAN7-1 in the coding region of an NADPH oxidase gene, designated *noxA*, that is a homologue of the mammalian gp91phox, an NADPH oxidase that catalyses the conversion of molecular oxygen to superoxide. Plants infected with the *noxA* mutant are severely stunted and undergo precocious senescence (Fig. 1). Inactivation of this endophyte gene resulted in unregulated growth of the fungal hyphae in meristematic and mature leaf tissue resulting in a dramatic increase in fungal

biomass in all tissues (Tanaka *et al.* 2006). In contrast to wild-type cells, hyphal cells of the *noxA* mutant showed an increase in vacuolation. Introduction of a wild-type copy of the gene into the *noxA* mutant restored the ability of this strain to form a wild-type symbiotic interaction, confirming that the plasmid insertion in *noxA* was responsible for the mutant phenotype. Deletion of a second NADPH oxidase gene, *noxB*, had no effect on the *E. festucae*-perennial ryegrass symbiotic interaction phenotype. Using transmission electron microscopy to locate electron-dense cerium perhydroxide deposits, we were able to show that H_2O_2 production was significantly reduced in the endophyte extracellular matrix and associated plant cell walls of meristematic tissue infected with the *noxA* mutant, compared to wild-type. These results demonstrate that endophyte production of ROS, catalysed by a specific NADPH oxidase isoform, is critical for maintaining the mutualistic interaction between *E. festucae* and perennial ryegrass.

Isolation and functional analysis of noxR and racA

Generation of ROS by the NADPH oxidase found in mammalian neutrophils requires formation of a multi-enzyme complex composed of the catalytic subunit gp91phox and the regulatory subunits p22phox, p40phox, p47phox, p67phox and the small GTPase Rac (Diebold & Bokoch 2001; Lambeth 2004). Bioinformatic analysis of fungal genome sequences identified a gene encoding a protein with an N-terminal domain very similar to p67phox, which we have designated *noxR*, with motifs for both RAC and gp91phox binding (Takemoto *et al.* 2006). This gene was cloned from *E. festucae* and a deletion mutant generated by targeted gene replacement (Table 1). The plant interaction phenotype of symbiota containing the *noxR* mutant was very similar to that observed for the *noxA* mutant i.e. plants were stunted and underwent rapid senescence (Takemoto *et al.* 2006). Light and confocal microscopic examination of leaf tissues revealed that associations containing the *noxR* mutant had an increase in hyphal biomass. In order to understand the role of NoxA and NoxR in regulating hyphal branching *in planta*, two experiments were carried out in culture that recapitulated the hyperbranching phenotype observed for the *noxR* mutant in the plant. Overexpression of *noxR*, under the control of a TEF promoter, or depletion of ROS by growing wild-type cultures in the presence of diphenylene iodonium, disrupted normal apical tip growth and induced hyphal hyperbranching. These results indicate that NoxR has a crucial regulatory role in controlling hyphal tip growth and branching in the grass host. However, NoxR alone is insufficient to activate NoxA *in planta*. By analogy with the mammalian phagocytic system, which requires both p67phox and Rac2 for gp91phox activation, *E. festucae* also requires a small GTP binding protein, RacA, for ROS production. Yeast two-hybrid and pull-down assays showed NoxR interacts with RacA. A single amino acid substitution in the predicted RacA binding site of NoxR (R101E) abolished the ability of NoxR to complement a *noxR* mutation *in planta*, indicating that both NoxR and RacA are required to activate NoxA in the host plant.

These results suggest that localised ROS production, catalysed by NoxA, is critical for controlling the highly regulated growth pattern observed for *E. festucae* in the meristematic and vegetative tissue of the perennial ryegrass host. The genetic evidence suggests that NoxR and RacA control the spatial and temporal activation of NoxA *in planta*, and by extension hyphal tip growth and branching.

Model for ROS regulation of hyphal growth in planta

Based on our functional analysis of NoxA and NoxR we have developed a model to explain how these proteins control polarised growth of *E. festucae* in culture and *in planta* (Fig. 2). Maintenance of polarised growth of *E. festucae* in axenic culture is proposed to involve bursts of ROS catalysed by NoxA following recruitment of RacA and other potential regulators of Nox in response to environmental/nutritional cues. Growth of *E. festucae* in the symbiosis is proposed to involve recruitment of NoxR and RacA to the plasma membrane and activation of NoxA in response to plant signals.

Although we have established a role for ROS in regulating hyphal growth in the *E. festucae*-perennial ryegrass association many questions remain unanswered. Are there unique components of the Nox complex yet to be identified that are required for Nox activation in response to plant signalling? What is the mechanism for activation and recruitment of NoxR and RacA to the plasma membrane? What is the molecular form of the ROS signal and how is that signal perceived and transduced within the cell? The goal of current research is to provide answers to some of these questions.

Isolation and functional analysis of genes for the synthesis of bioprotective metabolites

Molecular cloning and functional analysis of a peramine biosynthetic gene

The structure of peramine, a pyrrolopyrazine, suggests that it is the product of a reaction catalysed by a two-module non-ribosomal peptide synthetase (NRPS) that utilises proline and arginine as substrates. The conservation of polypeptide sequence in the adenylation (A) domain of known NRPSs has made it possible to use PCR strategies to clone various NRPSs from clavicipitalean fungi, including the lysergyl peptide synthetase (*lpsA*) required for ergovaline biosynthesis (Panaccione 1996; Panaccione *et al.* 2001). Using a similar strategy, candidate sequences for a peramine synthetase were amplified by RT-PCR using total RNA from *N. lolii*-infected perennial ryegrass (Tanaka *et al.* 2005). Four unique NRPS products were identified, two of which were weakly expressed in axenic culture but strongly expressed *in planta*. One of these sequences, clone ps9, cross-hybridised to genomic DNA from epichloë endophytes known to synthesise peramine *in planta*. This probe was used to isolate a genomic clone from a pMOcosX cosmid library to *E. festucae*, strain Fl1. Sequence analysis of pPN60, a representative cosmid from the set isolated, identified a gene, which we have designated *perA* (EF103), predicted to encode a two-module NRPS. Nine additional genes (EF100-EF102, EF104-EF109), which show striking conservation of microsynteny with *Fusarium graminearum* and other filamentous fungal genome sequences, were identified on the *perA*-containing cosmid. The absence of *perA* from the other fungal genomes analysed suggests that *perA* has been acquired in *E. festucae* by an insertional event in the intergenic region between EF102 and EF104. The presence of 12 bp direct repeats flanking *perA* supports this hypothesis. A replacement construct (pPN61) containing 1.68-kb of the T1 and half of the C1 domains of *perA* replaced with a hygromycin resistance cassette was prepared and recombined into the genome of Fl1. PCR analysis of the isolated transformants, identified one mutant (strain PN2323) out of 120 that contained the desired gene replacement (Table 1). Perennial ryegrass symbiota containing the *perA* mutant lacked detectable levels of peramine. In contrast, the levels of lolitrem B and ergovaline were similar to those found in wild-type symbiota. A wild-type copy of *perA*

complemented the deletion mutant. Taken together these results confirm that *perA* is a NRPS for peramine biosynthesis.

In the process of doing these experiments it was observed that the perennial ryegrass infection rate for the *perA* mutant was considerably less than that observed for wild-type inoculations, and plants that were infected with the *perA* mutant frequently showed a stunted phenotype. However, some plants infected with the *perA* mutant had a wild-type symbiotic interaction phenotype. Re-isolates (e.g. PN2425) from these plants retained the wild-type symbiotic interaction phenotype when reinoculated into perennial ryegrass seedlings. However, these isolates were shown in axenic culture growth tests to be hygromycin sensitive. Molecular analysis revealed that PN2425 was missing the hygromycin resistance cassette and approximately 4-kb of *perA* sequence, indicating that the original mutant, strain PN2323, had undergone a secondary recombination event that resulted in deletion of the selectable marker and additional flanking *perA* sequences. This analysis suggests that either the insertional (multiple copies of the *hph* cassette) and/or deletional (around the T1 domain of *perA*) event associated with the PN2323 gene replacement lead to a break-down in the mutualistic interaction between *E. festucae* and its perennial ryegrass host. To test this hypothesis a new gene replacement construct (pPN66), that removes almost the entire *perA* was prepared and recombined into the genome of Fl1 (Fig. 3). PCR analysis of these transformants identified one, strain PN2468, out of 30 screened that contained a replacement. Infection rates for this mutant on perennial ryegrass seedlings were similar to that of Fl1. Symbiota containing the new *perA* mutant had a wild-type symbiotic interaction phenotype and were unable to synthesise peramine.

To determine whether additional genes at the *perA* locus were required for peramine biosynthesis, replacement constructs were prepared to EF101 (pPN63) and EF108 (pPN64), genes encoding putative reductase and dioxygenase functions, respectively. Symbiota containing an *E. festucae* EF108 replacement mutant had levels of peramine that were comparable to wild-type and ectopic controls, indicating that EF108 is not required for peramine biosynthesis. Attempts to isolate a replacement of EF101 were unsuccessful, despite screening 205 Hyg^R transformants. These results suggest that EF101 is essential for growth of *E. festucae*. Bioinformatic analysis predicts that EF101 encodes a 3-ketosteroid reductase for sterol biosynthesis, a function that would be indispensable for *E. festucae* growth.

Based on these results a scheme for the biosynthesis of peramine has been proposed that involves PerA alone (Tanaka *et al.* 2005). The primary substrates for peramine biosynthesis are proposed to be 1-pyrroline-5-carboxylate, a proline intermediate, and arginine (Fig. 4). We do not favour the use of proline as a substrate as additional enzyme functions would be needed for formation of the pyrrole ring. The condensation domain of PerA is proposed to catalyse formation of the peptide bond between 1-pyrroline-5-carboxylate and arginine. The methylation domain of PerA is proposed to catalyse the *N*-methylation of the amino group of arginine. The reductase domain is proposed to reduce the thioester and cyclise the dipeptide to form an iminium ion that is concomitantly released. Deprotonation of this intermediate and oxidation of the pyrroline ring would give rise to peramine.

The cloning of *perA* from *E. festucae* now makes it possible to investigate how this gene is regulated in the plant. The availability of an Fl1 *perA* isogenic mutant will allow us to test directly the role of peramine as a feeding deterrent to a range of insects, as has already been demonstrated for Argentine stem weevil (Tanaka *et al.* 2005), and carry out contained greenhouse or field trials to test the ecological benefits of this gene to the symbiotium. Using primers designed to different regions of the *perA* gene, a PCR test is now available to assess the peramine biosynthetic capability of epichloë endophytes (Scott *et al.* 2007).

Molecular cloning and functional analysis of genes for indole-diterpene biosynthesis

A complex lolitrem biosynthetic locus (*LTM*) of at least 10 genes, organised in three mini-clusters, has been cloned and characterised from *N. lolii* strain Lp19 and *E. festucae* strain Fl1 (Young *et al.* 2005; Young *et al.* 2006) (Fig. 5). The first cluster contains three genes, *ltmG*, *ltmM* and *ltmK*, two of which appear to be functional orthologues of *paxG* and *paxM* from *Penicillium paxilli*, genes shown to encode enzymes required for early steps in paxilline biosynthesis (Fig. 6). The right-hand side of *ltm* cluster one in *N. lolii* is flanked by a 17.2-kb relic of a retrotransposon sequence comprised of one retro-element (Rua) inserted within another (Tahi) (Young *et al.* 2005). The presence of long terminal repeats flanking each element together with the presence of 5-bp direct repeats at the target sites provide strong evidence that these elements are relics of Type 1 retrotransposons. This block of retrotransposon sequence is absent from Fl1 (Fig. 6). Instead *ltmK* is linked directly to a polyketide synthetase pseudogene that is also present in Lp19. The presence of this pseudogene and additional AT-rich sequence in both strains suggests that *ltmK* defines the right-hand boundary of the *ltm* gene cluster.

The second *ltm* gene cluster contains five genes, *ltmP*, *ltmQ*, *ltmF*, *ltmC* and *ltmB*, four of which appear to be orthologues of the functionally characterised *paxP*, *paxQ*, *paxC* and *paxB* genes from *P. paxilli* (McMillan *et al.* 2003; Saikia *et al.* 2006; Young *et al.* 2001). Cluster two is separated from cluster one by a block of AT-rich retrotransposon relic sequence of approximately 35- and 32-kb respectively, in Lp19 and Fl1 (Fig. 5).

The third *ltm* gene cluster contains just two genes, *ltmE* and *ltmJ*, that appear to be unique to the epichloë endophytes, and therefore lolitrem biosynthesis. A 16-kb AT-rich sequence, that includes two imperfect direct repeats, separates cluster two from cluster three. The left-hand side of cluster three is comprised of a further AT-rich sequence. Whether *ltmE* defines the left-hand boundary of the *LTM* locus remains to be determined, as no additional linked sequence has been cloned for analysis.

Based on the demonstration that just four gene products, PaxG, PaxM, PaxB and PaxC, are required for the biosynthesis of paspaline, the first stable indole-diterpene intermediate in paxilline biosynthesis, Young *et al*. (2006) proposed that the gene products of *ltmG*, *ltmM*, *ltmB* and *ltmC*, are likely to catalyse identical steps in *N. lolii* and *E. festucae*. The fact that both *ltmM* and *ltmC* have been shown by complementation experiments to be functional orthologues of *paxM* and *paxC* supports this hypothesis. The inability of a symbiotum containing an *E. festucae ltmM* mutant to synthesise lolitrem B or any other indole-diterpenes confirmed that *ltmM* is required for lolitrem B biosynthesis (Young *et al.* 2005). LtmG is proposed to catalyse the synthesis of geranylgeranyl diphosphate, the first step in lolitrem biosynthesis. GGPP then condenses with indole-3-glycerol phosphate to form 3-geranylgeranylindole, a linear intermediate that has been shown by radio-labelling feeding experiments to be incorporated into paxilline (Fueki *et al.* 2004). LtmM is proposed to catalyse the epoxidation of the two terminal alkenes of the geranylgeranyl moiety, which is then cyclised by LtmC, to paspaline.

A comparison of the structure of lolitrem B with paxilline would suggest that LtmP and LtmQ, homologues of PaxP and

PaxQ, catalyse analogous biosynthetic steps in *N. lolii* and *E. festucae*. LtmP is proposed to catalyse the demethylation of C-12 of paspaline and subsequent hydroxylation of C-10, and LtmQ is proposed to hydroxylate the C-13 position of the paspaline ring (McMillan *et al.* 2003; Young *et al.* 2006). Given the diversity of indole-diterpenes identified in *N. lolii*-infected perennial ryegrass tissue (Parker & Scott 2004), several compounds including 13-desoxypaxilline, terpendole E, and lolicine A, are all potential substrates for LtmQ.

Formation of the A- and B-rings of lolitrem B requires prenylation of positions 20 and 21 of the indole ring of paspaline (Fig. 7). A candidate enzyme for one or both of these prenylations is LtmE, given the domain structure of this protein, which appears to be a fusion of two prenyl transferases (Young *et al.* 2006). To complete the oxidation and closure of ring-A of lolitrem B requires two additional catalytic steps. LtmJ, a P450 monooxygenase is a candidate enzyme for this step (Takemoto *et al*. unpublished results). At least two additional catalytic steps are required to form the epoxide between C-11 and C-12 of paspaline, and to oxidize and prenylate C-10 to allow formation of ring-I of lolitrem B (Fig. 7). Candidate enzymes for the latter reactions are LtmF and LtmK (Takemoto *et al*. unpublished results).

The chemical diversity of indole-diterpenes identified in *N. lolii*-infected perennial ryegrass seed indicates that the A and B rings can form independently of the I ring (Gatenby *et al.* 1999; Munday-Finch *et al.* 1998). This observation led these authors to propose that lolitrem biosynthesis is modular, proceeding by way of a metabolic grid rather than a linear pathway. Chemical analysis of the metabolites that accumulate in *E. festucae*-perennial ryegrass symbiota containing deletions of each of the Fl1 *ltm* genes will identify the major and minor biosynthetic pathways that comprise this metabolic grid. The cloning of these genes has also allowed us to develop a PCR-based test to predict the indole-diterpene biosynthetic capability of all epichloë endophytes (Scott *et al.* 2007).

Regulation of ltm gene expression in planta

The *ltmM* gene was selected to examine *ltm* gene expression patterns *in planta* using deletion analysis of the *ltmM* promoter region. Five different *ltmM* promoter deletion fragments were generated via PCR and the fragments translationally fused to *gusA*. The deletion constructs were then transformed into *E. festucae* protoplasts and the transformants tested for *gusA* expression using a seedling assay. The minimum promoter length required for *gusA* expression *in planta* was found to be 800 bp.

In mature vegetative tillers, *gusA* was expressed in all infected aerial plant tissues, including epiphyllous hyphae (Fig. 8A). The *gusA* expression pattern of the positive control, a P*gpd-gusA* transformant, was similar to the P*ltmM-gusA* transformants, confirming that *ltmM* is expressed at all times in vegetative tillers. At pre-anthesis, *gusA* expression was observed in all floral organs except the immature gynoecium (Fig. 8B). Similar GUS activity patterns were observed in control plants, except the ovule was colonised, with hyphae restricted to the nucellar tissue. In post-anthesis florets, gene expression occurred almost exclusively in the anthers and the gynoecium with dense staining observed in the stigma (Fig. 8C). In contrast, for the control plants, GUS activity was observed in all tissues of the spikelet. In germinating seeds and seedlings, P*ltmM* expression was observed in hyphae of seeds 24 h post-imbibition and from 6 days post-germination in seedlings (Fig. 8D). From 6 days, GUS activity was observed in the mesocotyl to the tip of the emerging first leaf. No GUS activity was observed in seedlings 2 to 5 days post-imbibition. These results suggest that during seed germination, only hyphae in the shoot apex have a significant role in further colonisation of the seedling.

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