Characterisation of a novel endophyte NRPS gene and its role in endophyte-grass symbioses

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Abstract

Symbiotic grass associations with fungal endophytes (genera *Neotyphodium* and *Epichloë*) display enhanced fitness as well as prolonged field persistence over their endophyte free equivalents. Perennial ryegrass, an important agronomic grass, is typically associated with the *N. lolii* endophyte. The endophyte lives within the intercellular spaces without inducing any symptoms in the plant. The aim of this study is to elucidate the biosynthetic function of fungal secondary metabolite gene clusters. Non-ribosomal peptide synthetase genes (NRPSs) of unknown function were targeted, as these genes are commonly associated with the production of bioactive peptides some of which are ecologically important. Some novel endophyte NRPS genes have been identified using a degenerate PCR screen; one of these, NRPS5 will be discussed here. Clones were obtained by screening a fosmid *Epichloë festucae* genomic DNA library and we are currently determining gene function by using targeted gene replacement followed by an assessment *in vitro* and *in planta* using metabolomics and appropriate bioassay screens.
Keywords: endophyte, NRPS, secondary metabolism

Introduction

Endophytic fungi of the genus *Epichloë* and related asexual *Neotyphodium* species form symbioses with cool season grasses such as perennial ryegrass (*Lolium perenne*), an important pasture grass in New Zealand. Hyphal growth is confined to the intercellular space of the aerial parts of the plant - leaf sheaths and blades - and is synchronised with that of leaf growth. The infection is both symptomless and mutualistic, i.e. is beneficial to both partners with the host receiving protection from a number of biotic and abiotic factors (Schardl *et al.* 2004).

Some of the endophytes secondary metabolites are produced by non-ribosomal peptide synthetases (NRPS). Two agronomically important NRPS derived metabolites produced by the endophyte *in planta* are ergovaline (Panaccione *et al*. 2001) and peramine (Tanaka *et al.* 2005). NRPS enzymes can provide a greater variety of peptides compared with the ribosomal system by utilising numerous unusual features, such as the ability to incorporate non-proteineous amino acids. NRPSs are large, multifunctional enzymes with a modular structure. Typically, one module consists of three core domains - an adenylation domain (A), a peptidyl carrier domain (PCP) and a condensation domain (C), which is collectively responsible for the incorporation of one amino acid into a growing polypeptide chain.

Three different types of NRPSs are known; linear (type A), iterative (type B) and nonlinear (type C). In linear NRPSs, the three core domains in an elongation module are arranged in the order C-A-PCP. The number and order of the modules determine the sequence of the linear product. Iterative NRPSs use their modules or domains more than once while assembling the product on the terminal C-domain which is most typically found in fungi or on the reductase domain (R). The R-domain functions in an analogous way to Thioesterase domains which are present almost exclusively in bacterial NRPSs. These domains catalyse release of the product by hydrolysis, cyclisation or oligomerisation (Finking & Marahiel 2004). Thus, the peptide chain is built up by a number of repeated, smaller sequences. After oligomerisation, the final product is released, usually through cyclisation. Nonlinear NRPSs contain at least one unusual arrangement of the core domains and it is very difficult to predict their possible products (Mootz *et al.* 2002).

The aim of this work was to functionally characterise a novel NRPS gene, NRPS5, by using targeted gene replacement. We investigated possible phenotypic changes *in vitro* and *in planta* to functionally characterise the role of NRPS5 in the symbioses and carried out a chemical analysis to determine the end product of NRPS5.

Results

Degenerate primers to conserved motives (YGPTE and YKTGDL) within adenylation domains were designed to establish a library of endophyte NRPS sequences (Johnson *et al.* 2007). One of these NRPSs, termed NRPS5, was investigated further in this study. An *E. festucae* fosmid library was screened using gene specific primers to identify several positive clones. Approximately 12 kb of sequence, including the promoter and the open reading frame (ORF) of NRPS5 has been obtained so far. No introns are present in the ORF of NRPS5. Four more ORFs have been identified within the 12 kb region containing NRPS5; three are located upstream and one immediately downstream of NRPS5. The most 5' ORF contains a protein kinase motive and by BlastX analysis is similar to a CAM kinase-like 1 protein from *homo sapiens*, *mus musculus* and *rattus norvegicus* (E-values of 4e-05). The next 5' ORF is most similar to a cystathionine gamma synthetase (XM_001217572.1, 7e-42), followed by an ORF with a top BlastX hit to a hypothetical protein from *Giberealla zeae* (XM_389085.1, 2e-11). The only ORF found so far that is 3' of NRPS5 is similar to hypothetical proteins from *Magnaporthe grisea* (XM_360930.1, 5e-51), *Gibberella zeae* (XM_388046.1, 3e-49) and *Aspergillus fumigatus* (XM_726439.1, 9e-23). These ORFs do no appear to have an obvious role in the biosynthesis of the NRPS5 derived end product.

A BlastX analysis of the NRPS5 ORF showed the highest similarity to peramine which is a fungal secondary metabolite that protects the host plant from insect herbivory (Tanaka *et al.* 2005). To be able to determine the function of NRPS5, a targeted gene replacement was performed by homologous recombination (Fig. 1). PCR was used to screen for the knock-out event using one primer outside of the knock-out construct and a second primer located in the hygromycin resistance gene (which was inserted into the A-domain of NRPS5 so that a small deletion of the gene also occurred). After screening 75 hygromycin resistant transformants, three knock-out transformants were obtained (Fig. 2). A southern analysis will be performed to confirm a single insertion event.

Based on extensive sequence data, we predict that NRPS5 consists of just one module, containing an A-, a PCP- and an R-domain. Conserved motives were manually found for all of these domains (Konz & Marahiel 1999). To predict which amino acid could be activated by the A-domain, we manually

Figure 1 Chromosomal locus and modular organisation of the NRPS5 gene in the wild type (1) and in the deletion mutant (2). A-domain: adenylation domain, P-domain: peptidyl carrier protein, R-domain: reductase domain.

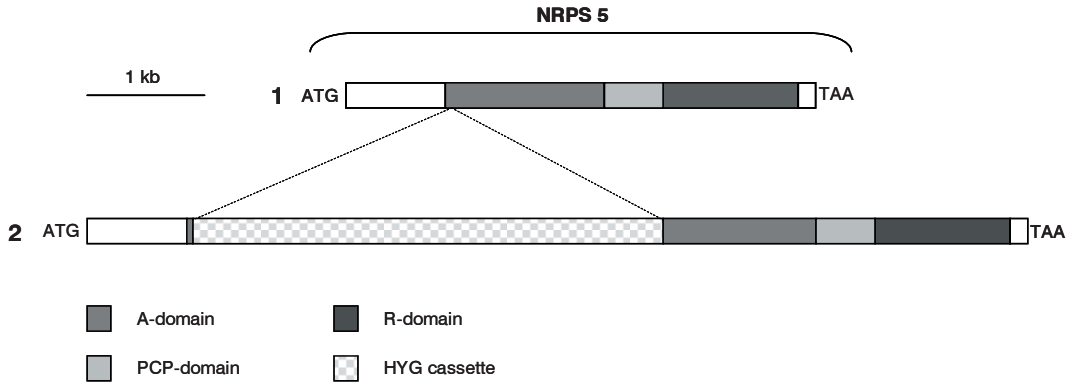
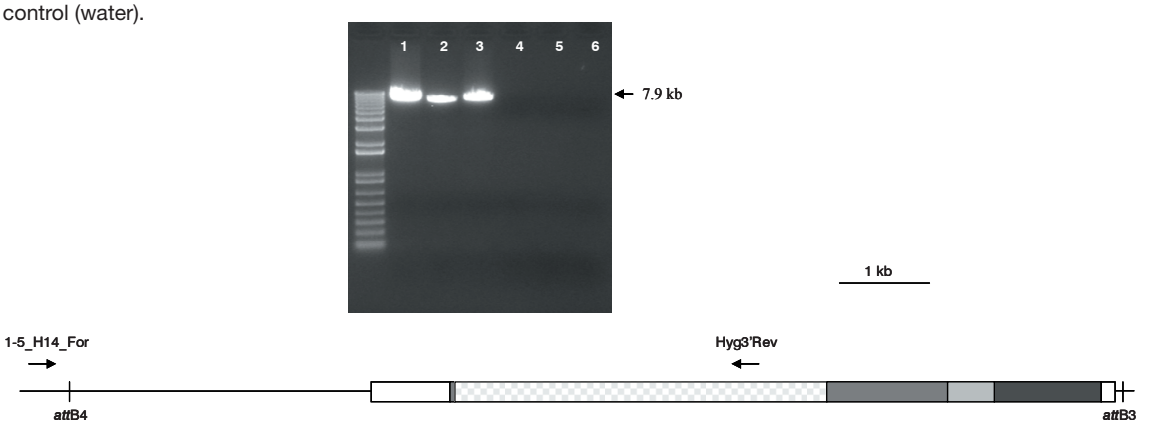


Figure 2 PCR was used to screen for the knock-out event using one primer outside of the knock-out construct (1-5_H14_For) and a second primer located in the hygromycin resistance gene (Hyg3'Rev). *Att*B4 and *att*B3 are primers which were used to amplify the knock-out construct. 1, 2, 3: deletion of NRPS5, 4: ectopic, 5: FL1 wild-type, 6: negative control (water).



identified the eight critical residues necessary using the method described by Challis *et al.* (2000). Essentially, the eight residues lining the binding pocket of NRPS5 were found by alignment of the region spanning from the A-domain motifs A3 to A6 of NRPS5 and compared with the same amino acid region of Gramicidin S synthetase GrsA. The pocket lining residues were most similar, but not identical to those required for incorporating phenylalanine from GrsA suggesting that an aromatic amino acid may be incorporated into the NRPS5 derived peptide product.

In addition, expression studies using RT-PCR were carried out to determine the expression level of the NRPS5 transcript *in planta* and *in vitro*. NRPS5 is expressed *in planta* (outer leaf sheaths and in seedlings). Expression was also detected *in vitro* when the fungus was grown on defined medium (Mantle & Nisbet 1976, yeast extract replaced with 0.6M thymine), but only very weakly when grown on potato dextrose media.

Discussion

Determining the function of NRPS5 is still in progress. Targeted gene replacement has been performed and analysis of the mutants is underway. NRPS5 is expressed *in planta* and the highest BlastX hit corresponds to the gene peramine, an insect deterrent (Tanaka *et al.* 2005). There is therefore a possibility that NRPS-5 could have a similar function. We will investigate both the knock-out and wild-type strains *in vitro* grown under different media conditions. We will also inoculate plants with wild-type and knock-out strains to analyse any visible effects deletion of the NRPS5 gene has on the symbiosis. Metabolic fingerprinting will be carried out to discover any differences between the wild-type and mutant strains, leading to the identification or characterisation of the metabolic end product of NRPS5. We also plan to do feeding studies if appropriate to check if NRPS5 produces a product that may affect insects.

NRPS5 is a one module NRPS composed of an A-domain, a PCP-domain and an R-domain. NRPS5 could function in connection with another NRPS. An example of this comes from the ergotamine biosynthetic pathway from *Claviceps purpurea* where two NRPS genes, *lps1* and *lps2* work together to produce a four amino acid peptide product (Riederer *et al.* 1996). Alternatively, the A-domain may function iteratively; where it uses just one amino acid more than once. Although NRPS5 is a fungal gene, alignment of the A-domain against the A-domain of a bacterial gene *GrsA* showed a good match. The sequence we found for the binding pocket does not match any other bacterial binding pocket amino acid sequences. However, it is similar to the GrsA residues that line the phenylalanine-binding pocket. This indicates that NRPS5 might bind an aromatic amino acid.

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