A gene identified from *Neotyphodium* lolii is expressed only *in planta* and regulates the biosynthesis of a putative oligopeptide secondary metabolite

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Abstract

Endophytes, belonging to the genus *Neotyphodium,* live symptomlessly within the intercellular spaces of cool-season grasses, and confer a number of biotic and abiotic advantages to their hosts. We identified a novel endophyte gene (designated Nc25) that is expressed preferentially *in planta*, is one of the most abundant fungal transcripts in endopyte-infected grasses and which is distributed and highly expressed in a wide range of endophyte/ grass associations. Nc25 is novel and shows no homology to sequence databases or fungal genome initiatives. Characterisation indicates that it encodes a small secreted protein. Re-introduction of a Nc25 deletion strain into perennial ryegrass showed no visible effect on the symbiosis but an unknown oligopeptide, detected only in infected plants, was eliminated. Surprisingly, the oligopeptide is unrelated to the predicted peptide product of Nc25. We hypothesise that Nc25 may regulate the oligopeptide biosynthetic pathway and are investigating this using Affymetrix gene chips to determine how Nc25 affects global gene expression. In addition we are interested in the biological function of this secondary metabolite during symbiosis and in particular whether it has bioactivity that may confer abiotic or biotic advantages to the host plant.

Keywords: *Neotyphodium*, *in planta* expressed gene, oligopeptide, EGFP

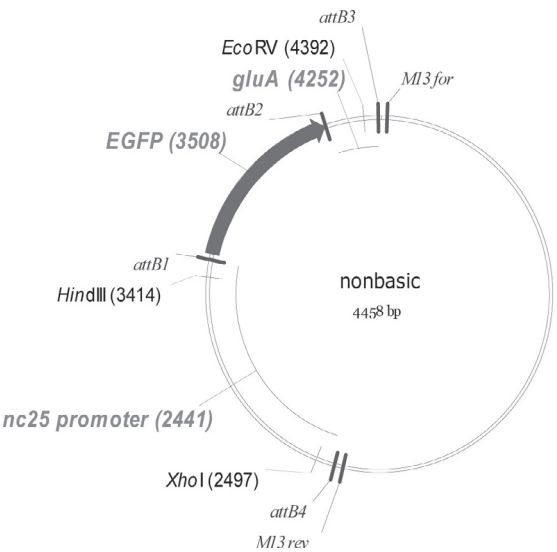
Introduction

Forage grasses belonging to the sub-family Pooideae, including several important forage and turf species, often harbour endophytic fungi belonging to the genus *Neotyphodium* and *Epichloë* (Clavicipitaceae, Ascomycota). These endophytes live entirely within the intercellular spaces of their grass hosts and infection is symptomless, with the endophyte relying entirely on the host plant for dissemination via the seed or through vegetative structures (Schardl *et al*. 2004).

The association is mutually beneficial since the endophyte confers a number of biotic and abiotic advantages to the host, including enhanced plant growth, protection from certain mammalian and insect herbivores, enhanced resistance to nematodes, resistance to some fungal pathogens and in some associations, enhanced drought tolerance (Johnson *et al*. 2003). Some of these benefits are due to the production of fungal secondary metabolites such as the pyrrolopyrazine (peramine) and aminopyrrolizidine (loline) alkaloids (Bush *et al.* 1997; Blankenship *et al*. 2001; Spiering *et al*. 2005; Tanaka *et al*. 2005). However, endophytes also produce additional secondary metabolites such as ergopeptine (ergovaline) and indole diterpene (lolitrem) alkaloids, which cause mammalian toxicosis (Bacon *et al*. 1977; Fletcher & Harvey 1981; Lane *et al.* 2000; Panaccione *et al*. 2001; Easton *et al*. 2002; Wang *et al*. 2004; Gallagher *et al.* 1982; Young *et al*. 2006). Evidence has also accumulated showing that the host plant has a significant effect on the regulation of fungal secondary metabolites (Lane *et al.* 2000) and more recently it has been shown that the expression of fungal genes involved in alkaloid production are up-regulated *in planta* (Tanaka *et al*. 2005; Young *et al.* 2005).

Apart from the characterised role of the above mentioned fungal secondary metabolites during symbiosis, many of the other observed endophyte effects on their host plants have not been elucidated. In this paper we describe the characterisation of a novel fungal gene (Nc25) that was identified by Suppressive Subtractive Hybridisation (SSH) of endophyte infected versus endophyte free ryegrass, and show that it may regulate the biosynthesis of a small oligopeptide secondary metabolite.

Figure 1A Nc25 5' UTR fused to EGFP.



Materials and Methods

Endophyte strains and plant infection

An asexual wild type *N. lolii* strain, Lp19 (Christensen *et al*. 1993) isolated from Nui perennial ryegrass was chosen for this study because it synthesises three of the four important known symbiosis associated secondary metabolites (peramine, ergovaline and lolitrem), has a small genome size (~35 Mb), is stable in culture and is typical of endophytes isolated from perennial ryegrass pastures. Isogenic ryegrass plants infected (G1056) or uninfected (G1057) with *N. lolii* strain Lp19 were obtained as previously described (Tanaka *et al.* 2005). Plants of G1056 and G1057 plants were grown under identical conditions under glass until harvest.

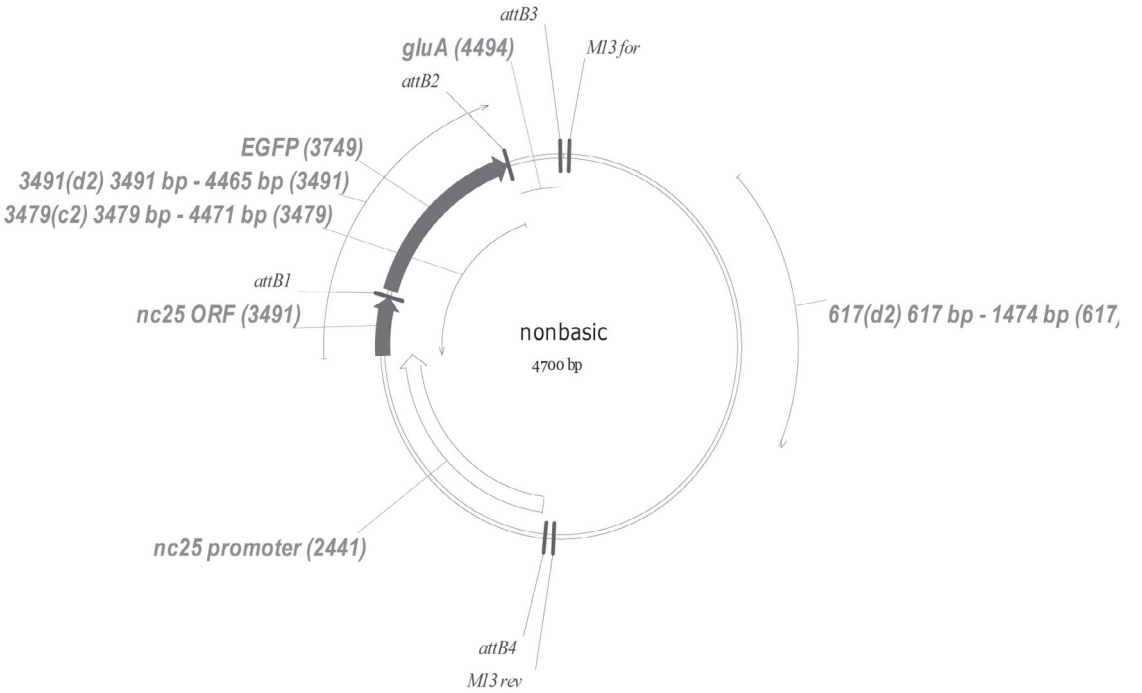
Suppression Subtractive Hybridisation

SSH (Diachenko *et al.* 1996) was carried out using G1056 and G1057 plants as previously described (Young *et al.* 2005) using the PCR-select cDNA subtraction kit (Clontech).

Library screening

A lambda Zap genomic library of *N. lolii* strain Lp19 was screened for clones containing the Nc25 gene using standard procedures. Positive clones were sequenced, using universal forward and

Figure 1B Nc25 translational fusion with EGFP.



reverse M13 primers and Big Dye 3.1 (ABI) cycle sequencing reagents on an ABI 3100 automated sequencer.

Gene replacement

A gene replacement of Nc25 was performed using homologous recombination. Deletion strains of Nc25 were inoculated back into ryegrass using the method of Latch & Christensen (1985) and grown under controlled conditions to assess if symbiosis was affected.

Metabolomic analysis

Metabolomic analysis (LCMS) was performed directly on guttation fluid, collected from plants infected with either wild type or Nc25 deletion strains, as described in Koulman *et al*. (in press).

Green fluorescent protein fusions

To examine expression and regulation of the NC25 gene two constructs were designed using Gateway (Invitrogen) technology. One construct had the Nc25 promoter fused to EGFP (Fig.1A) and a second construct had the Nc25 promoter plus open reading frame fused in frame to EGFP (Fig. 1B). Both constructs were ectopically co-transformed into wild type *E. festucae* strain Fl1 and infected into ryegrass essentially as described in Tanaka *et al.* (2005).

Affymetrix chip analysis

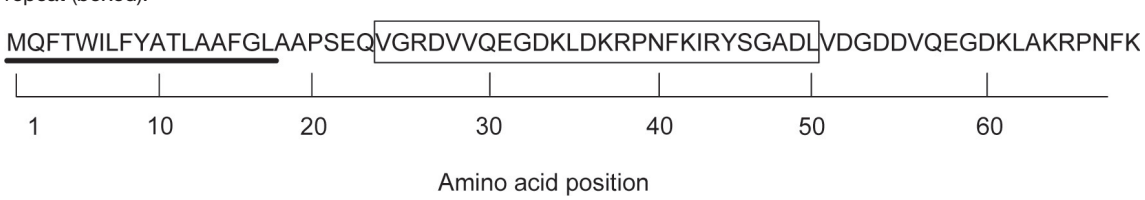
Nimblegen Affymetrix gene chips were designed and probed as described by Johnson *et al.* and Voisey *et al.* (2007).

Results and Discussion

Sequence analysis and expression

Nc25 is novel and shows no homology to sequences in public databases or fungal genome initiatives. Interestingly, SignalP (Bendtsen *et al*. 2004) strongly predicts it encodes a small secreted protein (Fig. 2A) containing a 27 amino acid repeat (boxed). Surprisingly, the number of repeat units appears to differ between strains (Fig. 2B). Nc25 shows exceptionally high levels of expression *in planta* and Affymetrix gene chip (Nimblegen) analysis indicates that transcript levels are similar to, or greater than, corresponding grass housekeeping genes despite the endophyte comprising less than 0.5% (at the DNA level) of the total symbiotum. Interestingly, when we compared the promoter of Nc25 to that of genes in other endophyte alkaloid genes also up-regulated *in planta*, a putative common motif was identified (D. Fleetwood pers. comm.).

Figure 2A Nc25 is strongly predicted to be secreted (signal peptide underlined) and contains a 27 amino acid repeat (boxed).



Effect of Nc25 gene deletion on symbiosis

Re-introduction of the deletion strain into perennial ryegrass showed no visible effect on the symbiosis but an oligopeptide detected only in guttation fluid of infected plants was eliminated (Fig. 3). Unexpectedly, partial characterisation of the oligopeptide suggests that it is not the predicted product of Nc25 but comprises eight amino acids, some of which are modified. Compounds such as these are typically synthesised by non ribosomal peptide synthetases (NRPSs). We hypothesise that Nc25 may regulate a putative NRPS biosynthetic pathway and are investigating this using Affymetrix chips arrayed with endophyte and symbiosis enriched ryegrass ESTs to determine how Nc25 affects global gene expression.

GFP fusions

No fluorescence was expected in culture but surprisingly transformants with the Nc25 promoter::GFP fusion showed bright fluorescence (Fig. 4A). However, transformants with the Nc25 promoter plus ORF::GFP translational fusion showed no fluorescence (Fig. 4A).

In planta, transformants of both fusions showed bright fluorescence but differences in the EFGP localisation were visible (Fig. 4B). For the Nc25 promoter fusion, EGFP was localised in cytoplasmic vesicles, whereas for the Nc25 promoter+ plus ORF translational fusion, EGFP was localised and concentrated at the cell wall and septa (Fig. 4B). This suggests that the NC25 protein is being secreted and confirms the prediction of SignalP. However, due to the large size of the translational fusion product in comparison to the predicted Nc25 protein, the true localisation may be different to what is observed.

Since both fusion constructs fluoresce *in planta* they are both functional. The differential expression seen between the Nc25 promoter fusion and the Nc25 promoter plus ORF translational fusion, in culture, suggests that the ORF is important in regulating its own expression.

Figure 2B Strain distribution study of Nc25. Products were amplified by PCR with primers designed to the 5' and 3' ends of the Nc25 ORF. The product sizes correspond to the number of amino acid repeats (boxed in Fig 2A) within different NC25 genes. **1**. DNA ladder. **2**. *N. lolii* Lp6. **3**. *N. lolii* Lp5. **4**. *N. lolii* AR59. **5**. *N. lolii* AR56. **6**. *N. lolii* AR12. **7**. *N. lolii* AR1. **8**. *E. festucae* FI1. **9**. *N. lolii* AR40. **10**. FaTG3 Tf15 **11**. FaTG3 Tf13.

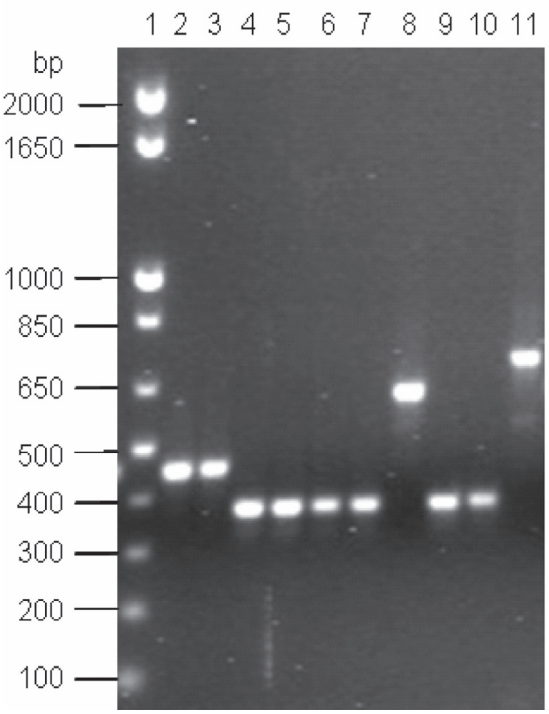


Figure 3 LCMS on extracts of ryegrass infected with either the wild type *E. festucae* FI1 or a mutant with a deletion in the Nc25 gene. Deletion of Nc25 leads to the specific elimination of an oligopeptide *in planta*.

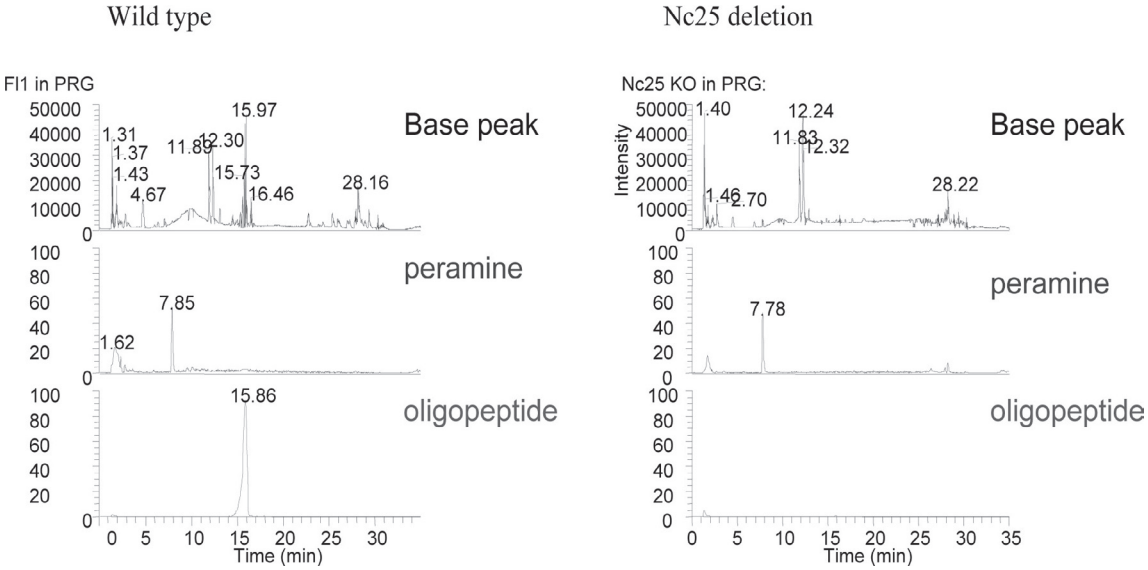
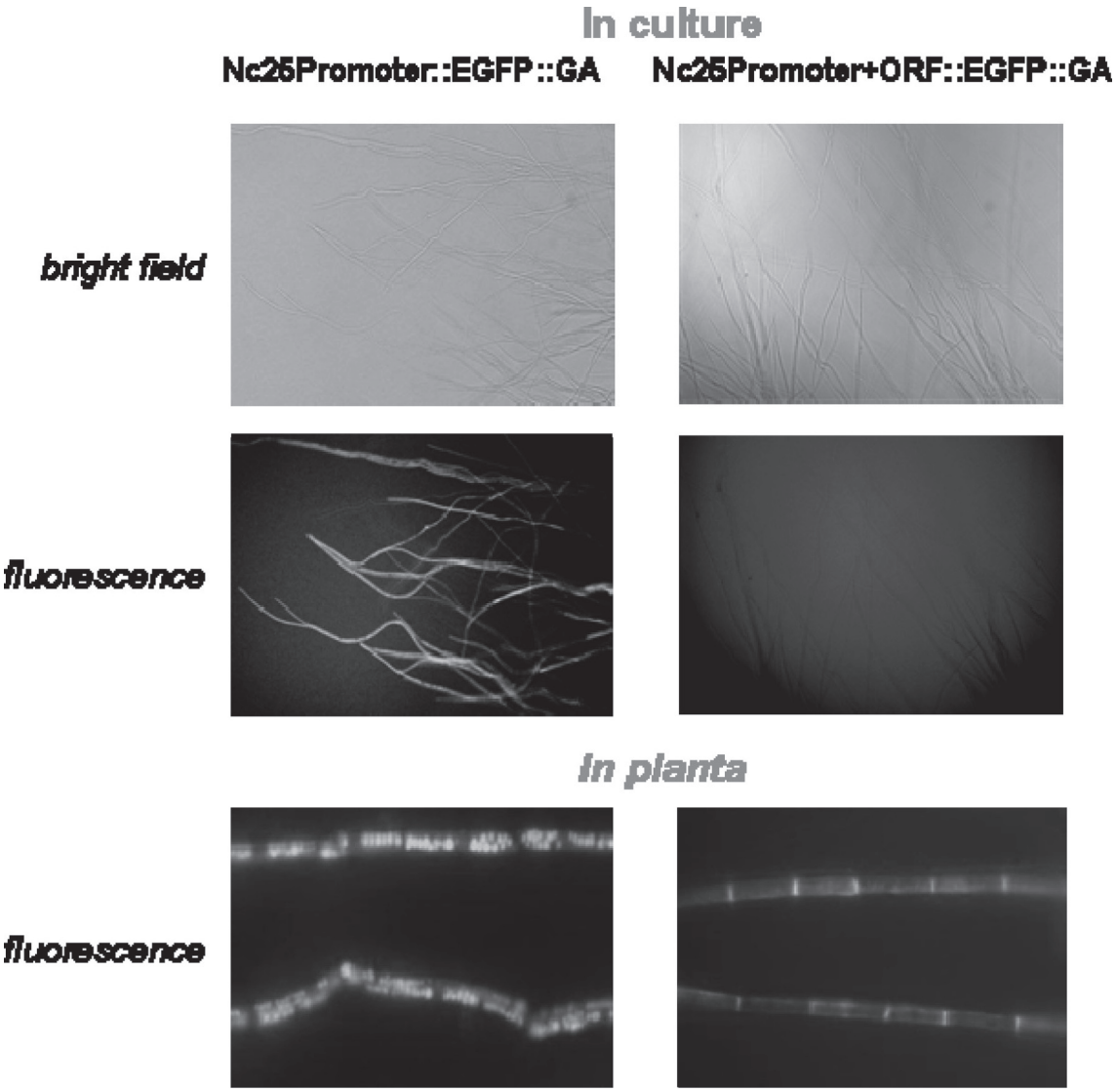


Figure 4 Analyses of protein localisation of GFP-fusions with Nc25. Bright field and fluorescence microscopy of Nc25 promoter and translational fusions with EGFP.



Conclusions

Nc25 encodes a novel small secreted protein that appears to regulate its own expression as well as that of an unrelated oligopeptide. Affymetrix gene chip analysis indicates that Nc25 affects the expression of a number of other genes which is currently being investigated. We are also interested in the biological function of this oligopeptide secondary metabolite during symbiosis and in particular whether it has bioactivity that may confer abiotic or biotic advantages to the host plant.

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