Neotyphodium lolii induces a limited host defence response by *Lolium perenne*

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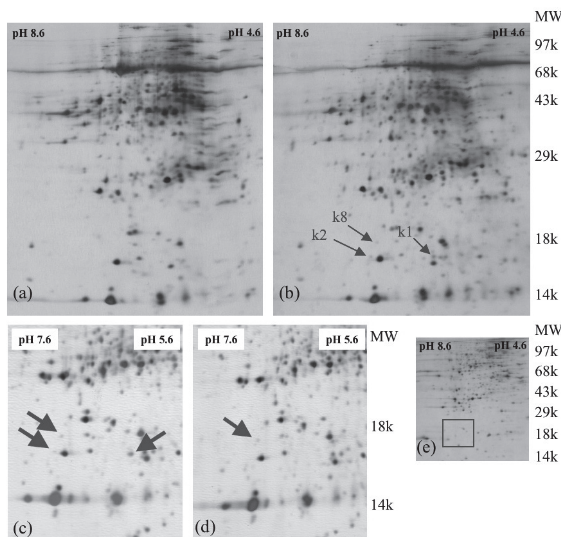
Abstract

We have identified three proteins specifically expressed during the interaction between *N. lolii* and *L. perenne* in natural compatible associations. Two are pathogenesis-related PR-10 proteins, part of a ubiquitous plant response to pathogens. We have identified seven different *L. perenne PR-10* genes and found that endophyte infection increases *PR-10* transcript levels, but only mildly. Furthermore, *PR-10* mRNA levels also increase in a dysfunctional symbiosis, but the two identified PR-10 proteins are absent. This suggests that part of the regulation of protein expression is at the level of translation and that PR-10 proteins could be required for the symbiosis to be functional. A third symbiosis-specific protein is a very highly expressed *N. lolii* superoxide dismutase. Such enzymes neutralise superoxide formed by plants as a defence mechanism. Our results suggest that *N. lolii* elicits a limited host defence, comparable to that elicited by arbuscular mycorrhiza.
Keywords: *Neotyphodium lolii*, *Lolium perenne*, host response, proteomics, gene expresion, pathogenesis related protein PR-10, fungal superoxide dismutase

Introduction

Fungal endophytes can trigger host defence responses comparable to those induced by fungal pathogens, including localised plant cell death and hyphal death; these have been observed in human-generated incompatible associations (Christensen 1995; Christensen *et al.* 1997; Koga *et al.* 1993; Zhang *et al.* 2006). Visual and microscopic assessment to date has suggested the absence or suppression of such defence reactions in compatible natural associations between *Neotyphodium lolii* and *Lolium perenne* (Schardl *et al.* 2004; Schmid & Christensen 1999). However, mechanisms must be in place for controlling fungal growth. Proliferation of the endophyte only occurs during plant growth, and fungal biomass is restricted to <0.2% of infected tissues (Schmid & Christensen 1999; Tan *et al.* 2001). It has been observed that other fungal endophytes, such as the arbuscular mycorrhiza fungi (AMF), trigger a limited host defence and that this response may be part of the signalling required for successful mutualistic interaction (Garcia-Garrido & Ocampo 2002; Kogel *et al.* 2006). We show here that *N. lolii* induces a limited defence response in natural compatible associations with *L. perenne*.

Figure 1 Proteins *L. perenne* NuiD plants, uninfected (a), and infected with KS1 (b), Lp19 (c) and Lp21 (d). Arrows mark k1, k2 and k8. (e). Total protein extract of cultured endophyte mycelium. The area of the gel in which k8 would be visible if it were expressed in culture is marked. PH and molecular weights (in kDa) are marked at the top and the sides of gels.



Methods

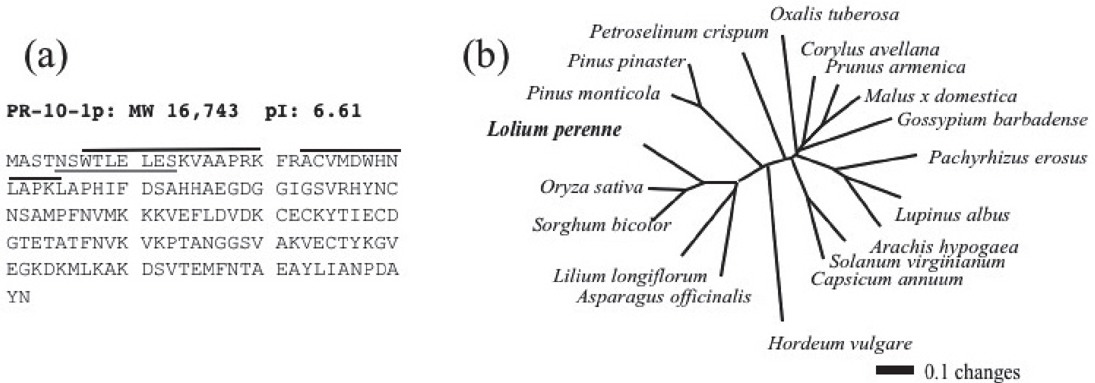
Uninfected *L. perenne* plants (genotypes NuiD, NuiUIII and NuiUIV (Spiering *et al.* 2005)) and plants infected with strains Lp19, Lp21 (Christensen *et al.* 1993; Zhang *et al.* 2004) and KS1(Tan *et al.* 2001) were maintained in a growth cabinet (Tan *et al.* 2001). Protein was extracted (Desfrancs *et al.* 1985) from the sheaths of the second-oldest mature leaves of 3-leaf tillers of exponentially growing plants and from endophyte laboratory cultures. Two-dimensional electrophoresis (Berkelman & Stenstedt 1998) was followed by silver staining (Coligan *et al.* 2003). Proteins of interest were cut out of gels, for tandem mass spectroscopy (MS/MS) at the Bioanalytical Mass Spectrometry Facility of the University of New South Wales, Sydney, Australia. Degenerate primers were used to amplify cDNA sequences encoding parts of the proteins (Ausubel *et al.* 2006) and a RACE kit (Invitrogen) to sequence the entire transcript. Total RNA and mRNA were extracted using TRIzol reagent (Invitrogen) and mRNA was isolated using Sigma GenElute mRNA isolation kit. Each sample was a combination of 10 sheaths from a plant. Northern hybridisation was carried out using standard methods (Ausubel *et al.* 2006). For reverse transcriptase real-time PCR, mRNA was reverse transcribed (Transcriptor Reverse Transcriptase,Roche) and 400 bp amplicons of *PR10-1* and *tub1* were amplified in a LightCycler 2.0 apparatus. *PR10-1* expression was assessed using calibrator-normalised relative quantification (Roche Technical Note LC16/2005).

Results and Discussion

Three proteins, k1, k2 and k8 appear in leaf sheaths when plants are endophyte-infected.

Three proteins, k1, k2 and k8, were detectable in *L. perenne* genotypes NuiD, NuiUIII and NuiIV infected with *N. lolii* strain KS1, a reporter gene-expressing derivative of *N. lolii* Lp19 (Spiering *et al.* 2005; Tan *et al.* 2001), absent in uninfected tissues of each genotype (Fig. 1a,b). Because endophyte transformation can alter the symbiosis (Zhang *et al.* 2006), we confirmed that the three proteins also appeared in NuiD infected with Lp19 (Fig. 1c). All of these findings were reproducible in three biological replicates.

Figure 2 (a) Protein encoded by one of the *PR-10* ORFs. Black and grey lines mark peptide sequences matching those obtained by MS/MS of tryptic digests of k1 and k2, respectively. (b) Neighbour-joining tree showing the relationship between the protein and other PR-10 proteins.



Proteins k1 and k2 are pathogenesis-related (PR-10) proteins, appearing in functional but not in dysfunctional associations.

Peptide sequences, obtained by MS/MS of k1 and k2 showed homology to pathogenesis-related PR-10 proteins (Fig. 2a). Sequencing identified three open reading frames (ORFs), which encode proteins that contained these peptides and roughly matched k1 and k2 in size (PR10-1, 16.7kD; PR10-2, 16.8kD; PR10-3, 16,6kD; predicted pIs of 6.61, 6.33 and 7.33, respectively). Comparison with PR-10 proteins from other species confirmed them as PR-10 proteins (Fig. 2b). We also found four additional *PR-10* ORFs in *L. perenne*. One is truncated by a stop codon half way through the coding sequence. We have not completely sequenced the remaining three.

PR-10 proteins may not only be part of a pathogen defence but also part of the communication between fungal symbionts and their host plants (Garcia-Garrido & Ocampo 2002; Kogel *et al.* 2006). We therefore also tested if k1 and k2 were present in a dysfunctional association, characterised by lack of suppression of endophyte branching and significantly reduced alkaloid levels, between NuiD and a hyperbranching mutant of Lp19, altered in the expression or posttranslational modification of a heat shock protein (Zhang *et al.* 2004). Lack of induction could suggest a role of PR-10 in establishing a functional interaction. Both k1 and k2 were absent in Lp21-containing associations (three biological replicates; Fig. 1d).

Regulation of *PR-10* genes

Because the silver-stained gels did not allow us to quantify the differences in the concentration of the PR-10 proteins k1 and k2 between Lp19-infected and uninfected plants, we carried out Northern hybridisation and found an increase in *PR-10* gene expression, but only by a factor of 2-3 (Fig. 3a). Because Northern hybridisation will only provide information on the concentration of all *PR-10* transcripts, including possibly transcripts derived from the truncated ORF, we also developed a reverse-transcriptase real-time PCR assay specifically for *PR-10-1*. No up-regulation in response to endophyte infection was observed (ratio *PR-10* to *tub1* transcript of 1.25 ± 0.45 in Lp19-infected leaf sheaths and 1.97 ± 1.07 in uninfected sheaths; material from three sampling dates). These results indicate that, as a group, *PR-10* genes are mildly endophyte-induced, but not every *PR-10* gene is. *PR-10* mRNA levels, as assessed by Northern hybridisation, were also increased in Lp21- infected plants (Fig. 3b), suggesting

Figure 3 (a) Expression of *PR-10* transcripts and *tub1* transcripts in Lp19-infected and uninfected plants assessed by Northern hybridisation. Various concentrations of total RNA from infected plants were loaded (marked on top of each lane). (b) Northern hybridisation of 900 ng mRNA from uninfected plants and plants infected with Lp19 and Lp21.

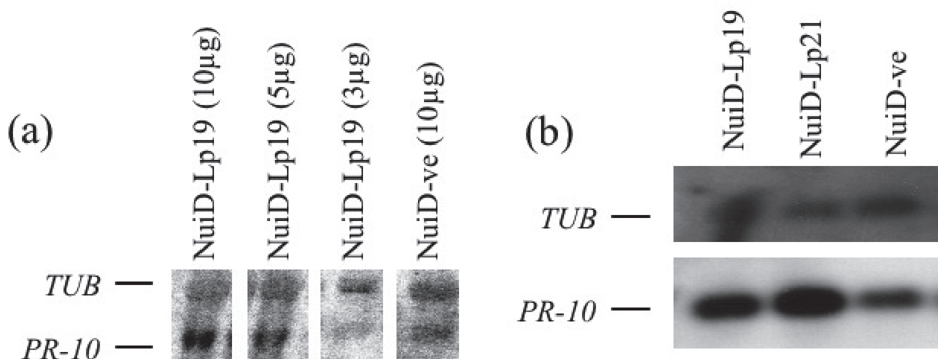
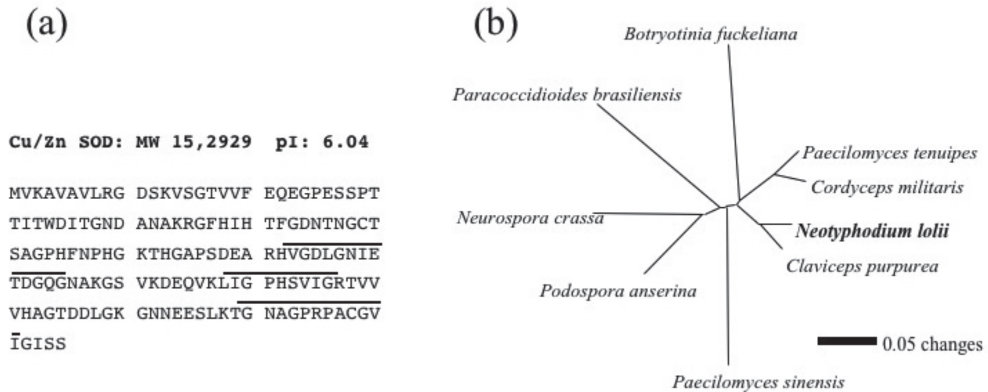


Figure 4 (a) Cu/Zn *N. lolii* superoxide dismutase. Black lines mark peptide sequences matching those obtained by tandem mass spectroscopy of tryptic digests of k8. (b) A neighbour-joining tree showing the relationship between the predicted protein and other fungal superoxide dismutases.



that mRNA levels as detectable by Northern hybridisation are not well correlated with PR-10 protein k1 and k2 levels, and the possibility of regulation at the level of translation.

Protein k8 is a highly induced *N. lolii* Cu/Zn superoxide dismutase

Three short peptide sequences, obtained from k8 by MS/MS, showed a high degree of homology to fungal Cu/Zn superoxide dismutases. We sequenced the entire transcript from *N. lolii* cDNA to predict the amino acid of the protein it encodes (Fig. 4a). The extent of sequence homology to other fungal Cu/Zn superoxide dismutases provided evidence that k8 is a Cu/Zn superoxide dismutase (Fig. 4b). Because the endophyte only accounts for less that 0.2% of plant biomass (Tan *et al.* 2001), and k8 is not present in protein extracts of cultured mycelium (Fig. 1e), k8 must be very highly induced in plants in order to be detectable in extracts prepared from infected plants. This resembles the situation in AMF where induction of a Cu/Zn superoxide dismutase also occurs (Lanfranco *et al.* 2005), possibly to protect the fungus from reactive oxygen species (ROS) in the host environment that are generated by the host as part of defence mechanisms against pathogens and which may, in an attenuated form serve as signals and fungal growth control mechanisms in symbiosis (Dempsey & Klessig 1995; Garcia-Garrido & Ocampo 2002; Hamilton & Holdom 1999; Kogel *et al.* 2006; Lanfranco *et al.* 2005). The superoxide dismutase could also be involved in control of the level of superoxide produced by the endophyte itself *in planta* and which is apparently part of the signals that enable successful symbiotic interaction (Tanaka *et al.* 2006).

Parallels between AMF and ryegrass endophytes

Our results indicate parallels between endophytes and AMF. A weak induction of host responses and induction of a fugal superoxide dismutase occur in both systems (Garcia-Garrido & Ocampo 2002; Lanfranco *et al.* 2005). The superoxide dismutase induction also adds to growing evidence of the importance of ROS balance for successful interaction between endophytes and their hosts (Tanaka *et al.* 2006).

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