High Levels of a Fungal Superoxide Dismutase and Increased Concentration of a PR-10 Plant Protein in Associations Between the Endophytic Fungus *Neotyphodium lolii* and Ryegrass

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Neotyphodium lolii is a fungal endosymbiont of the ryegrass *Lolium perenne*. Its growth is tightly controlled and synchronized with that of the plant. How the symbionts communicate is largely unknown but failure of the endophyte to elicit a defense response is considered crucial for successful symbiosis. In silver-stained two-dimensional gels of protein extracts from endophyte-infected ryegrass, a fungal Cu/Zn superoxide dismutase was detectable, even though the fungus accounts for only <1/500 of the biomass, indicating that it is an abundant fungal protein and that the fungus needs protection against reactive oxygen species. The plant increased levels of a pathogenesis-related class 10 (PR-10) protein; when equal amounts of protein from infected and uninfected plants were loaded, PR-10 was only detectable in extracts from infected plants. Presence of the endophyte did not lead to a significant increase in *PR10* transcript levels. In protein extracts from a symbiosis containing an *N. lolii* variant with an abnormal in planta growth pattern, the fungal Cu/Zn superoxide dismutase but not PR-10 protein was detectable. The correlation between increased PR-10 levels and presence of a normally growing endophyte is suggestive of a role of a very limited host defense in the interaction between grass and endophyte.

The fungus *Neotyphodium lolii* grows as a symbiont in the intercellular spaces of the ryegrass *Lolium perenne*. The association is apparently mutualistic, at least in agronomic ecosystems (Saikkonen et al. 2006; Schardl et al. 2004), with the grass providing the endophyte with nutrition and a means of dispersal and the endophyte increasing the persistence of in-

fectured plants, in particular through increasing drought and herbivore resistance (Schardl et al. 2004).

In functional associations between grasses and *N. lolii* or related epichloae, no defense response appears to be triggered in the plant, judging by the absence of hyphal and plant cell death (Christensen et al. 2002; Schardl et al. 2004; Schmid and Christensen 1999; Scott 2001). Indeed, in several functional associations between epichloae and their host grasses, presence of the endophyte was found to reduce levels of transcripts with homology to a pathogenesis-related (*PR*) plant gene of class 10, *PR-10*, although this did not occur in the *N. lolii*–*L. perenne* association included in these experiments (Johnson et al. 2003). *PR-10* is part of a set of *PR* genes that appear to contribute to plant defense against fungal infections, although the PR-10 protein's mode of action in this defense remains to be defined; sequence homology and experimental evidence indicate that PR-10 proteins have ribonuclease activity. (Chen et al. 2006, 2010; El-kereamy et al. 2009; Johnson et al. 2003; Katile et al. 2010; McGee et al. 2001; Moiseyev et al. 1997). That epichloae are, in principle, capable of inducing an increase of *L. perenne PR-10* (as well as *PR-1*, *PR-4*, and *PR-5*) transcript levels was recently demonstrated in an association containing a mutant strain that acts as a pathogen (Eaton et al. 2010). Thus, at least some epichloae may be able to actively suppress the host PR response (Johnson et al. 2003).

Although widely held to be crucial (Christensen et al. 2002; Schardl et al. 2004; Schmid and Christensen 1999; Scott 2001), the mere absence of a defense response is apparently not sufficient for establishing a successful symbiosis between *N. lolii* and its host. A number of observations suggest that the plant environment regulates the growth and metabolism of the endophyte: The growth of the endophyte is tightly controlled so that the distribution of a sparsely branched *N. lolii* mycelium in the plant follows basal-apical gradients and reaches reproducible plant genotype-dependent levels (<0.2% of that of the biomass of infected plant) (Spiering et al. 2005; Tan et al. 2001). The rate of hyphal extension is synchronized with that of the plant and extension ceases when the emerging leaves of the grass stop growing (Tan et al. 2001). The cessation of hyphal growth occurs even though the endophyte remains metabolically highly active and, thus, is apparently not merely due to a restriction of resources required for growth (Tan et al. 2001). In functional symbioses, the fungus synthesizes the anti-herbivore alkaloids peramine, lolitrem, and ergovaline, but alkaloid levels are only partially determined by endophyte biomass concentration, with plant genotype and surrounding plant tis-

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sue being significant determinants (Rasmussen et al. 2007; Spiering et al. 2005); this suggests that the plant exerts some control over these biosynthetic pathways.

It is possible to artificially generate novel epichloae–grass associations and, in some of these, the fungus and its host are incompatible. Typical features of incompatible associations include browning of tissue, stunting, reduced vigor, or even death of the infected plants. In these dysfunctional symbioses, control of the endophyte breaks down. Depending on the severity of the incompatibility, one or several of the following can occur: increased branching of the endophyte mycelium, lack of synchronization of hyphal and plant growth, extensive invasion of vascular bundles (uncommon in functional associations), altered alkaloid production, death of hyphae, and a PR response (Christensen et al. 1997; Christensen et al. 2002; Eaton et al. 2010; Koga et al. 1993; Tanaka et al. 2006, 2008; Zhang et al. 2006). The impact of incompatibility on plant phenotype has been used to screen for mutations in *Epichloë festucae* which interfere with its interaction with *L. perenne*. In this way, the reactive oxygen species (ROS)-producing NADPH oxidase (Nox) complex, (Takemoto et al. 2006; Tanaka et al. 2006, 2008) and a stress-induced mitogen-activated protein kinase (Eaton et al. 2010) have been identified as crucial for a successful interaction. Although these results are a major advance, there can be little doubt that a large number of additional plant and endophyte factors involved in establishing a successful association remain to be discovered.

Transcriptomic data on epichloae–grass symbioses are becoming increasingly available but these need to be complemented by proteomics because there is often no strong correlation between transcript and protein levels in biological samples (Maier et al. 2009). To our knowledge, the proteomes of these symbioses have not yet been investigated. Therefore, we undertook an exploratory screen for proteins that may play a role in establishing a successful interaction between *N. lolii* and *L. perenne* by proteomic analysis of uninfected *L. perenne*, functional *N. lolii*–*L. perenne* association, and an association between *L. perenne* and an *N. lolii* variant with an abnormal in planta growth pattern.

RESULTS

Three proteins (k1, k2, and k8) are present in higher concentrations in functional symbioses between *L. perenne* and *N. lolii* than in uninfected plants.

Because we expected the impact of the endophyte on plant patterns to be subtle in functional symbioses and to differ between different plant tissues, we analyzed plants grown under controlled conditions (discussed below) and restricted our analysis to protein extracts from one tissue type, the second leaf sheath of three-leaf tillers. Sheath tissue is mature (i.e., non-growing) and contains the highest concentration of endophyte hyphae (Tan et al. 2001). We used three clonal lineages of ryegrass (NuiD, NuiUIII, and NuiIV). All were infected with KS1, a transformant of *N. lolii* strain Lp19 expressing the β -glucuronidase (GUS) reporter gene. Uninfected clones of the same lineages were used as control replicates. All symbioses are fully functional and extensively characterized in terms of endophyte mycelium distribution, alkaloid profiles, and the impact of the endophyte on plant growth and photosynthesis (Spiering et al. 2005, 2006; Tan et al. 2001).

The overall impact of endophyte infection on protein patterns was indeed subtle, in comparison with the effect of plant genotype (Fig. 1A to H). However, among the well-separated proteins in the lower molecular weight range, three protein spots—k1, k2, and k8—were present in each of three biological replicates of each infected *L. perenne* genotype but not de-

tectable in uninfected plants. We confirmed that none of these differences were caused by the introduction of a reporter gene into KS1. All three spots were also present in plants of NuiD when infected with KS1's parent strain Lp19 (Fig. 1I).

High levels of a *N. lolii* Cu/Zn superoxide dismutase in planta.

Three peptide sequences, obtained from spot k8 by tandem mass spectrometry of tryptic digests, all showed a high degree of similarity to fungal Cu/Zn superoxide dismutases (Cu/Zn-SOD) in SwissProt (top hit, *Claviceps purpurea* Cu/Zn-SOD: Q96VL0.3, *E* value = 1×10^{-14}). Degenerate primers based on one of the peptide sequences and on conserved regions in fungal Cu/Zn-SOD were used in rapid amplification of cDNA ends (RACE). In silico translation of the resulting cDNA sequence indicated that it corresponded to a 15.3-kDa protein with an isoelectric point (pI) of 6.04, roughly matching the gel-based estimates for k8 (18.3 kDa, pI 6.04). The predicted protein contained all three k8 peptides (Fig. 2A, underlined in the peptide sequence) and was 93% identical with the *C. purpurea* Cu/Zn-SOD (*E* value = 8×10^{-78}). Thus, k8 represents an *N. lolii* Cu/Zn-SOD. We also sequenced the entire gene, which we have named *sodA* (GenBank accession number HQ229938).

Because the endophyte accounts for less than $1/500$ of the biomass of the infected tissue in the associations used for these analyses (Tan et al. 2001), the Cu/Zn-SOD had to be a very abundant fungal protein in planta to be detectable in two-dimensional (2D) gels of protein extracts from infected tissues, which only resolved a few hundred proteins. To assess whether the *N. lolii* Cu/Zn-SOD protein was constitutively synthesized at high levels, we analyzed protein extracts from exponentially growing laboratory cultures of Lp19 and KS1 (Fig. 2B). No highly abundant protein matching k8 was visible when extracts were analyzed by 2D polyacrylamide gel electrophoresis, indicating that it is not constitutively present in high concentrations.

The high level of Cu/Zn-SOD in planta suggested that the enzyme might be crucial for the functioning of the symbiosis. To test this, we wanted to assess the effect of its disruption. Because *N. lolii* is difficult to transform, the strain F11 of the related species *E. festucae* was used, which is more amenable to genetic manipulation. A query, with the *N. lolii* *sodA* cDNA sequence, of a transcriptomics database generated from a wild-type *E. festucae*–*L. perenne* association (Eaton et al. 2010) showed that the *E. festucae* *sodA* homologue was among the 3% known *E. festucae* genes most highly expressed in planta. This suggests that, like *N. lolii*, *E. festucae* synthesizes the protein at high levels in planta. We cloned the *E. festucae* homologue of the gene (*sodA*, GenBank HQ229939) from a F11 cosmid library (Tanaka et al. 2005). The sequence of the cloned open reading frame (ORF) was 94.5% identical to that of the *N. lolii* ORF, and it contained sequences that were 100% identical to each of the *N. lolii* ORF's exons. Disruption of the *E. festucae* *sodA*, by replacing most of the ORFs with a hygromycin resistance gene, had no readily apparent effect. The knockout strain, verified by Southern hybridization, had no obvious morphological or growth defects in culture or in planta and the morphology of infected plants was indistinguishable from that of plants infected with the wild type (data not shown).

The concentration of PR-10 proteins is elevated in endophyte-infected *L. perenne*.

For protein k1, tandem mass spectrometry produced two short peptide sequences (Fig. 3). A BLASTP search with these peptides produced significant hits with members of the PR-10 family. The best match was a PR-10 protein of *Sorghum bicolor* (GenBank accession number AAW83208.1, *E* value =

1 e⁻⁸; the top hit in SwissProt was a PR-10 protein from *Asparagus officinalis*, Swiss-Prot Q05736.1, *E* value = 0.014). Both peptides showed homology to an expressed sequence tag (EST) from *N. lolii*-infected plant tissue (Khan et al. 2010), which also showed homology to PR-10 proteins. We used degenerate primers based on the peptide sequences, the EST, and regions in the 3' end of PR-10 protein-encoding ORFs conserved among other species to amplify part of the ORF. Using RACE, we obtained the sequence of an entire transcript, predicted to encode a protein that contained both of the peptides obtained from k1 (Fig. 3). Its predicted size (16.7 kDa) and pI (6.6) roughly matched the properties of k1 estimated from gels (18.1 kDa, pI 5.5). The best match of the predicted protein in a BLASTP search against the National Center for Biotechnology Information (NCBI) nonredundant protein data base was a PR-10 protein from *Oryza sativa* (NCBI reference sequence NP_001049857.1, 66% identity, *E* value = 3 e⁻⁵³; its best hit in SwissProt was again the *A. officinalis* PR-10 protein, *E* value = 3 e⁻²⁷, 40% identity). The extent of sequence identity between the predicted protein and PR-10 proteins from other species provided evidence that k1 is a PR-10 protein. A peptide obtained from k2, a protein of a molecular weight comparable with k1, matched part of this protein (Fig. 3), identifying it likewise as a PR-10 protein.

In our attempts to characterize the genes encoding k1 and k2 through cDNA and genomic DNA sequencing, we found eight *L. perenne* PR-10 ORFs (*PR-10-1* to *PR-10-8*; GenBank accession numbers HQ229927 to HQ229934). Three of them encoded very similar predicted PR-10 proteins (*PR-10-1*, 16.7 kDa; *PR-10-2*, 16.8 kDa; and *PR-10-3*, 16.6 kDa with predicted pIs of 6.61, 6.33, and 7.33, respectively). We did not completely characterize the remaining five genes but one was a truncated ORF with a stop codon halfway through the coding sequence. Judging from the peptide sequence and size, k1 is encoded by *PR-10-1* and k2 could be encoded by either *PR-10-1* or *PR-10-2*.

PR 10 transcript levels do not increase significantly in a functional symbiosis.

To study in more detail the mechanisms leading to an increase of PR-10 protein levels, we developed a reverse-transcriptase real-time polymerase chain reaction (PCR) assay for expression of *PR-10-1*, the gene that encoded the transcript matching the upregulated PR-10 protein represented by spot k1 (with *L. perenne tub1* as a reference gene). In NuiD plants, grown and harvested under the same conditions under which the increased PR-10 protein levels had been observed, no increase in transcript steady-state levels of the *PR-10* gene in response to Lp19 endophyte infection were detectable. Indeed, in most samples from endophyte-infected grass, the ratios of *PR-10-1* transcript/*tub1* transcript measured were slightly lower than in uninfected plants (averages 1.25 ± 0.45 and 1.97 ± 1.0, respectively) (Fig. 4A).

We used semiquantitative reverse-transcription PCR to determine whether transcript levels of *PR-10* genes in general and those of other classes of PR genes with demonstrated or likely roles in the host defense response against fungal pathogens (*PR-1*, *PR-2*, *PR-3*, *PR-4*, and *PR-5*) (Manandhar et al. 1999; Selitrennikoff 2001; van Loon et al. 2006) were increased

in Lp19–NuiD associations. As controls, we included three *L. perenne* housekeeping genes (*tub1*, *act1*, and *ubq5*), encoding β-tubulin, actin, and ubiquitin (Fig. 4B). Under growth conditions identical to those used for the proteomic analyses, there was no evidence of a significant increase in *PR-10* transcript levels. Levels of transcripts of some other classes of PR genes increased, most notably levels of *PR-1* and *PR-2*, but the significance of this is difficult to interpret because levels of two housekeeping genes increased to a comparable degree and, as a set, the *PR* genes did not behave differently from the housekeeping genes (Mann-Whitney test applied to the ranking of the ratio of transcript levels between infected and uninfected plants).

In protein extracts from associations containing an *N. lolii* variant with an abnormal in planta growth pattern, the fungal Cu/Zn-SOD but not the PR-10 protein is detectable.

ROS has been implicated in the regulation of the endophyte's in planta growth (Tanaka et al. 2006), and one of the

Fig. 2. Identification of k8 as a Cu/Zn superoxide dismutase, which is not constitutively expressed by *Neotyphodium lolii*. **A**, Translation of *N. lolii* cDNA with regions corresponding to k8-derived peptides underlined. **B**, Two-dimensional gel of 200 µg of protein extract from Lp19 mycelium growing exponentially in potato dextrose broth. The pH range is indicated on top and molecular weights at the right side (in kDa, based on 14.3- to 200-kDa protein standards loaded on the left side of the gels). The frame indicates the region of the gel where k8 is located in gels loaded with protein extracts from endophyte-infected plant tissues.

MASTNSWTLE LESKVAAPRK FRACVMDWHN LAPKLAPHIF
 DSAHHAEGDG GIGSVRHYN C NSAMPFNVMK KKVEFLDVK
 CECKYTIECD GTETATFNVK VKPTANGGSV AKVECTYKGV
 EGDKDKMLKAK DSVTEMFNTA EAYLIANPDA YN

Fig. 3. Predicted amino acid sequence of the pathogenesis-related class 10 (*PR-10*).1 protein, based on translation of the *PR-10.1* open reading frame. Amino acids corresponding to peptides isolated from k1 are underlined in black and those corresponding to a peptide isolated from k2 are underlined in gray.

Fig. 1. Total protein extracts (200 µg), resolved by two-dimensional electrophoresis and visualized by silver staining, of the second-oldest mature leaf sheath of three-leaf tillers of exponentially tillering *Lolium perenne* plants of genotype NuiUIII **A**, uninfected and **B**, infected with KS1; NuiUIV **C**, uninfected and **D**, infected with KS1; and NuiD **E**, uninfected and **F**, infected with KS1. The pH range is indicated on top and molecular weights at the right side (in kDa, based on 14.3- to 200-kDa protein standards loaded on the left side of the gels). **G** and **H**, Magnified parts of gels indicated by brackets in **A** and **B**. Magnifications of the same gel regions from protein extracts of NuiD plants infected with **I**, Lp19 and **J**, Lp21. Protein spots k1, k2, and k8 are marked in **G** to **J**.

possible functions of a mild host defense could be restriction of the proliferation of endophyte mycelium in planta. Therefore, to further clarify the functional significance of differences in Cu/Zn-SOD and PR-10 levels observed between uninfected and endophyte-infected plants, we inoculated NuiD plants with strain Lp21, a hyperbranching host colonization variant of Lp19, obtained after benomyl treatment (Christensen et al. 1993). The Lp21 variant has an abnormal colony phenotype in culture (Christensen et al. 1993), and a proteomic com-

parison of Lp19 and Lp21 cultures revealed that either the level or the posttranscriptional modification of a 30-kDa heat-shock protein is altered in the variant (Supplementary Fig. S1). In planta, Lp21 hyphae are more frequently branched than those of the wild type. Also, the number of hyphae in older leaf sheaths is higher than that in young leaf sheaths (Christensen et al. 2002), indicating that, unlike in the wild type (Tan et al. 2001), growth continues after leaf extension ceases. We confirmed the highly branched mycelial phenotype in the Lp21-containing associations that we had generated (Fig. 5).

We analyzed three protein extracts from three independently obtained second-leaf sheath samples from the Lp21-NuiD associations grown under the same conditions as the Lp19-containing associations. In each of these, protein spot k8, corresponding to the *N. lolii* Cu/Zn-SOD, was visible (Fig. 1J). Spots k1 and k2, corresponding to the *L. perenne* PR-10 proteins, were not visible (Fig. 1J).

DISCUSSION

The most important result of our analysis is that, in compatible functional symbioses, the presence of *N. lolii* correlates with an increase in the level of a protein, PR-10, which is part of the host defense of plants against pathogens. This correlation suggests that the endophyte elicits a (very limited) host response. There was no demonstrable significant increase in PR-10 transcript levels, possibly because the levels of PR-10 protein did not increase very much. We did not quantify the increase in PR-10 protein levels but k1 and k2 were certainly not among the major proteins visualized in 2D gels from extracts of endophyte-infected plants. Given that the correlation between transcript and protein levels in complex biological

Fig. 4. A, Reverse-transcriptase real-time polymerase chain reaction (PCR) of *PR-10-1* expression, relative to that of *tub1*, in the second-oldest leaf sheaths of ryegrass NuiD plants infected with Lp19 (filled bars) and uninfected plants (unfilled bars). Plants were grown under controlled conditions in a growth cabinet and sampled at three different dates. Pairs of bars indicate duplicate real-time PCR results obtained for the same cDNA preparation from the same plant. **B**, Semiquantitative reverse transcriptase PCR of *PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, and *PR-10* and housekeeping genes *tub1*, *act1*, and *ubq5*. cDNA (2 ng/μl) generated from RNA extracted from the second mature leaf sheath of infected or uninfected ryegrass NuiD was serially diluted and the dilutions used as PCR templates. The amount of cDNA from infected and uninfected plants required as template to produce comparable amounts of PCR products was used to estimate expression differences (see Materials and Methods). Averages and standard deviations of ratios obtained from at least two biological replicates are shown.

Fig. 5. A, Infrequently branching hyphae of Lp19 and **B**, frequently branching hyphae of variant Lp21 in leaf sheaths of ryegrass NuiD. Hyphae were stained with aniline blue (bar = 100 μm).

samples is affected by numerous factors and generally not strong (Maier et al. 2009), our *PR-10* transcription data are within the range of what could be expected given the *PR-10* protein level differences observed, as are the transcription data obtained for an *N. lolii*–*L. perenne* association by Johnson and coworkers (2003).

Transcript levels of some other *PR* genes known to respond to fungal pathogens were elevated in endophyte-infected plants. However, the biological interpretation of these data is complicated by a parallel transcript level increase for some of the housekeeping genes we intended to use as a reference; significant changes of transcript levels of housekeeping genes have also been reported in compatible associations between tall fescue and its epichloid endophyte, *N. coenophialum* (Dinkins et al. 2010).

SOD are a means by which cells protect themselves from the damaging effects of ROS. Detection of *N. lolii* Cu/Zn-SOD in 2D gels of extracts from infected plants in which the endophyte accounts for less than 0.2% of the biomass indicates that the Cu/Zn-SOD must be highly abundant compared with other *N. lolii* proteins present in the symbiosis. In contrast, the Cu/Zn-SOD was not a major protein in extracts from cultured mycelium. This could also be interpreted as evidence for a defense response, of which the formation of ROS is a part (Miguel Angel 2010). Increased expression in association with the plant host of Cu/Zn-SOD-encoding genes was observed in the mycorrhizal fungi *Paxillus involutus* (Johansson et al. 2004) and *Gigaspora margarita* (Lanfranco et al. 2005) and has been interpreted by these authors as a mechanism for protecting the symbionts from a host response. Alternatively, high expression levels may be necessary because the fungus itself generates ROS as part of establishing a successful interaction with the plant (Amrit et al. 2010), as has been shown in associations between *N. lolii*'s close relative *E. festucae* and *L. perenne*. (Tanaka et al. 2006). Yet a third possibility (which does not exclude the previous one) stems from the observation that *N. lolii* grows rapidly and remains metabolically highly active even after hyphal extension has ceased in planta but grows extremely slowly in culture (Tan et al. 2001). It has been proposed that detoxifying ROS generated by its respiratory chain may be the predominant function of SOD in *Aspergillus fumigatus* (Lambou et al. 2010). Thus, higher *N. lolii* Cu/Zn-SOD levels in planta than in culture may simply be a result of corresponding differences in metabolic activity.

The lack of a readily detectable effect of deletion of a highly expressed homologue in the related *E. festucae* argues against the importance of the Cu/Zn-SOD, at least for epichloae in general. However, this does not exclude the possibility that SOD activity may still be essential. Fungi typically have several SOD, and other SOD might compensate for the effect of the disruption. The phenotypic impact of elimination of Cu/Zn-SOD by gene disruption is also fairly mild in *A. fumigatus* (Lambou et al. 2010), and the gene is not indispensable for successful interaction of the mycorrhizal fungus *Oidiodendron maius* and the pathogen *C. purpurea* with their hosts (Abba et al. 2009; Moore et al. 2002).

Experiments with the *N. lolii* Lp21 colonization variant did not implicate the Cu/Zn-SOD in the establishment of a normal growth pattern, because the Lp21 Cu/Zn-SOD was also sufficiently abundant in planta for detection in extracts from infected tissue. The *PR-10* proteins represented by k1 and k2 were not detectable in Lp21-infected plants, suggesting that they could have a role in establishing the hyphal growth pattern typical of a functional symbiosis. The situation might resemble that in arbuscular mycorrhiza, where the fungus also triggers a weak activation of defense mechanisms, which some authors believe to play a role in establishing the mutualistic

association between arbuscular mycorrhiza fungi and plant (Campos-Soriano et al. 2010; Garcia-Garrido and Ocampo 2002; Johansson et al. 2004). However, we have no direct evidence that *PR-10* or other components of the host defense play a role in controlling the growth of the endophyte. Further experimentation is necessary to test whether the elicitation of a weak defense response, rather than its complete avoidance, is not only part of functional *N. lolii*–*L. perenne* symbioses but may even be essential for optimal interaction between the partners.

MATERIALS AND METHODS

Strains and associations.

Most experiments were carried out with three previously established associations between *N. lolii* strain KS1, a transformant of strain Lp19 carrying a constitutively expressed GUS reporter gene, and three clonal lineages of *L. perenne* (NuiD, NuiUIII, and NuiUIV) (Spiering et al. 2005; Tan et al. 2001) as well as uninfected clones of the same lineages. Additional associations were generated by infecting plantlets derived from micropropagated NuiD meristems with mycelium from strains Lp19 and Lp21 (Christensen et al. 1993). The micropropagation method was loosely based on Dale (1975). Excised tillers were surfaced-sterilized with sodium hypochlorite (5% available chlorine) followed by several washes in sterile distilled water. The meristematic regions of tillers were dissected and placed onto a solid medium of Murashige and Skoog (MS) salts (Murashige and Skoog 1962) supplemented with cefotaxime (500 µg/ml) and benomyl (20 µg/ml). Tillers growing aseptically after 2 to 3 weeks of culture were transferred to an MS medium containing 3% (wt/vol) sucrose and supplemented with benzyladenine (2.0 µg/ml). Tillers were inoculated by introducing endophyte mycelium from a 4-week-old colony into a basal incision. Inoculated tillers were then cultured on MS basal medium for an additional 4 weeks before transfer to the greenhouse (Simpson 2009).

Associations were grown in a growth cabinet at 15°C with a 12 out of 24 h light cycle (650 ± 50 µmol photons m⁻² s⁻¹ at canopy height) (Spiering et al. 2006).

E. festucae strain Fl1 (Leuchtmann 1994; Moon et al. 1999) and derivatives of the strain with a disrupted *sodA* gene were inoculated into ryegrass Nui seedlings (Latch and Christensen 1985) and the resulting plants were maintained in the greenhouse.

Proteomics analyses.

All plant protein extracts were prepared from 500 mg of second mature leaf sheaths of three-leaf tillers of exponentially growing plants, minimizing the potential impact of differences in nutrient status between plants harvested at different times. Extraction was carried out by a modification of the method of Desfrances and associates (1985). Material was dry crushed in liquid nitrogen. The powder was resuspended at 0°C in 4 ml of 30 mM Tris-HCl (pH 8.7) buffer containing 1 mM dithiothreitol (Life Technologies, Carlsbad, CA, U.S.A.), 1 mM ascorbic acid, 1 mM EDTA-Na₂, 5 mM MgCl₂, 1 g of insoluble polyvinylpyrrolidone (Sigma-Aldrich, St. Louis), and protease inhibitor (1 complete mini protease inhibitor tablet [Roche Diagnostics New Zealand Ltd. Auckland, New Zealand] in 10 ml of Tris-HCl buffer). After centrifugation at 35,300 × g at 4°C for 15 min, 3 ml of supernatant was taken and an equal volume of sodium dodecyl sulfate (SDS) solution (4% SDS, 5% 2-mercaptoethanol, 5% sucrose) was added. The mixture was then boiled for 3 min in a water bath. Afterward, 8 ml of ice-cold acetone with 10 mM 2-mercaptoethanol was added to 2.5 ml of the mixture followed by precipitation for 1 h at –18°C. After

centrifugation at $35,300 \times g$ for 10 min at 4°C, the pellet was air dried and then resuspended in 400 µl of O'Farrell lysis buffer (9.5 M urea, 5% 2-mercaptoethanol, 2% Nonidet P40) (O'Farrell 1975), to which one complete miniprotease inhibitor tablet (Roche) was added per every 10 ml. Samples were stored at -80°C for up to 12 weeks before electrophoresis.

The same extraction procedure was also used on early (20 to 50× increase in dry weight compared with that of the inoculum) exponentially growing laboratory potato dextrose broth endophyte cultures, set up and harvested as described by Zhang and coworkers (2006).

For 2D electrophoresis (Berkelman and Stenstedt 1998), 150 to 200 µg of protein were loaded on 18-cm IPG strips (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), pH 3 to 10. After isoelectric focusing (40,000 Vh at 20°C), IPG strips were equilibrated (Yan et al. 1999) and transferred onto vertical-slab 15% SDS-polyacrylamide gels. A molecular weight ladder (14.3- to 200-kDa molecular weight range) (Life Technologies) was loaded beside the strip using a filter paper wick (Coligan et al. 2003). Electrophoresis was carried out at room temperature for 4 to 5 h at 30 mA per gel until the dye front reached the bottom of the gel. Gels were stained using Coomassie blue or silver stain (Coligan et al. 2003). Differences in protein patterns were detected by visual inspections of silver-stained gels. Differences were considered reproducible if they were detectable in three of three biological replicates, with each biological replicate harvested at a different date. Proteins of interest were cut out of gels and sent to the Bioanalytical Mass Spectrometry Facility of the University of New South Wales, Sydney, Australia for analysis by tryptic digestion followed by fragmentation of peptides and determination of fragment masses by mass spectroscopy. Masses were used to determine amino acid sequences of peptides (Coligan et al. 2003) for BLAST searches.

RNA extraction.

From samples consisting of 10 sheaths of the second-oldest mature leaves of three-leaf tillers from exponentially growing plants, total RNA was isolated using TRI Reagent-RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.) following the manufacturer's instructions. RNA (1 µg, concentration determined by absorption at 260 nm) was then incubated at room temperature with DNase I (1 U/µg) (Invitrogen, Carlsbad, CA, U.S.A.) followed by thermal inactivation of the enzyme (65°C for 10 min). The purity of the RNA was estimated by the ratio of the absorbance at 260 or 280 nm and verified by 2% SDS-agarose gel electrophoresis separation. Poly-A tail mRNA was obtained from total RNA using the Sigma GenElute mRNA isolation kit.

Characterization of ORFs.

To clone ORFs encoding differentially expressed proteins, primers described in Table 1 were used to generate cDNAs from RNA or to amplify ORFs from genomic DNA (Al-Samarrai and Schmid 2000), using standard methods (Ausubel et al. 2006). Reverse-transcription reactions were carried out in a total volume of 20 µl of 1× reverse-transcriptase buffer containing 1 mM each dNTP, 6.5 µM oligo(dT)₁₅, 20 U of protector RNase inhibitor, and 10 U of Transcriptor Reverse Transcriptase (Roche). To this mixture, we added 1 µg of total RNA treated with DNase I. The reaction was left to proceed for 30 min at 55°C, followed by 5 min of heating at 85°C and rapid cooling on ice. The cDNA was stored at -20°C until use. A RACE kit (Invitrogen) was used to amplify entire transcripts.

To identify *L. perenne* *PR-2*, *PR-3*, and *PR-4* sequences for expression analyses, primers (Table 1) were designed against

conserved regions of these genes as identified by aligning published sequences from other species. PCR products obtained using these primers on *L. perenne* cDNA as a template were sequenced to verify that they represented *L. perenne* homologues of *PR-2*, *PR-3*, and *PR-4* (best hits for *PR-2*, *PR-3*, and *PR-4* in a BLASTX search were BAA77783, 1 e⁻¹⁸; BAB82472, 9 e⁻⁶³; and AAT67050, 3 e⁻⁴⁵, respectively).

Reverse transcriptase real-time PCR.

Primers PR10-1RTpf, PR10-1RTpr, Tub1pf, and Tub1pr (Table 1) used in the analysis of *PR10-1* and β-tubulin (*tub1*, accession number: AY742902) expression were designed across an intron and in a nonconserved region of the *PR-10* genes and the *tub1* gene to amplify only *PR-10-1* cDNA and *tub1* cDNA. The PCR assays were performed in a LightCycler 2.0, using LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics) following the manufacturer's instructions. The reaction volume of 10 µl contained 1 µl of cDNA sample (diluted 1:2), 0.5 µM each primer, and 2 µl of LC-Fast Start DNA Master^{PLUS} SYBR Green I/Enzyme (including *Taq* DNA polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP, SYBR Green I dye, and MgCl₂). The cycling conditions were as follows: one hold for 10 min at 95°C, followed by 45 cycles of 95°C for 10 s, 55°C for 5 s, and 72°C for 20 s. The response of *PR-10-1* transcript levels to infection was assessed by calibrator-normalized relative quantitation with efficiency correction (the latter was calculated using serial dilutions of cDNAs).

Semiquantitative reverse-transcriptase PCR.

RNA extracted from second mature leaf sheaths was used to synthesize cDNA after the treatment with Turbo DNase (Ambion Inc., Austin, TX, U.S.A.) by following the manufacturer's protocol. The cDNA concentration was measured using Quant-IT Oligreen single-stranded DNA Reagent in a Qubit fluorometer and adjusted to 2 ng/µl. Serial dilutions of this cDNA were used as template in PCR using primers shown in Table 1, and the products were visualized on ethidium bromide-stained agarose gels. For some of the genes (*PR-1*, *PR-2*, *PR-5*, and *ubq5*), the primers would amplify a product from genomic DNA indistinguishable from that derived from cDNA. For *PR-3*, *PR-4*, *PR-10*, *act1*, and *tub1*, products of different size were formed from cDNA and gDNA. In PCR assays in which cDNA samples were used as templates, only the cDNA-specific products were formed using primers for the latter genes, indicating absence of genomic DNA contamination that would interfere with the expression assays.

To semiquantitatively assess expression levels, band intensity of PCR products formed using cDNA templates from infected and uninfected tissue samples were visually compared. If products were formed in both samples, dilutions were identified where intensity was comparable in infected and uninfected samples. The differential between these dilutions was used to estimate how much higher a gene was expressed in one sample compared with the other (for instance, if a 10-fold dilution of cDNA from infected tissue produced a PCR product band comparable with that produced using a 2-fold dilution of cDNA from uninfected tissue as a template, this was scored as a 5-fold increase of transcript levels in infected tissue). If two samples to be compared did not have bands of closely matched intensity, averages of the two dilutions that produced band intensities slightly higher and slightly lower in one samples than a band in the second sample were used in these calculations. These evaluations were carried out using dilutions that gave the brightest bands. In some cases, only one of the samples yielded a product. In this case, the differential between the lowest dilution of the sample giving no product and the highest

Table 1. Primers used in this study

Primer name	Primer sequence	Gene ^a	Purpose ^b
LpPR1 forw ^c	ACGGCGAGAACATCTTCTGG	PR-1	Semi-quantitative RT-PCR
LpPR1 rev ^c	CGCCGAGGTTGTTGTCGAG	PR-1	Semi-quantitative RT-PCR
RT-PR2 forw	AGGTGAACGGCAGCGATCC	PR-2	Semi-quantitative RT-PCR
RT-PR2 rev	ACCCGTCTTGGCAAGGTACAG	PR-2	Semi-quantitative RT-PCR
RT-PR3.1 forw	ACCCGACTCTTCCCTT	PR-3	Semi-quantitative RT-PCR
RT-PR3 rev	ATGATGTTGGTGATCACGCCG	PR-3	Semi-quantitative RT-PCR
RT-PR4 forw	CCCGCGGCCAGGAATCG	PR-4	Semi-quantitative RT-PCR
RT-PR4 rev	GGCCCTGCTGCACTCCCA	PR-4	Semi-quantitative RT-PCR
PR5RACEpf	GCAGCTGAACAGCGGCGAGACGTGGAAC	PR-5	Semi-quantitative RT-PCR
PR5RACEpr	GCCGGTGCTGCAGGAGAAGCCCATGC	PR-5	Semi-quantitative RT-PCR
RT-PR10 forw	GACTGGCACAATCTGGCACCC	PR-10	Semi-quantitative RT-PCR
RT-PR10 rev	CTTGCCACACTCCCACCG	PR-10	Semi-quantitative RT-PCR
Tub1pf	ACTACAACGAGGCTCGTGC	tub1	Semi-quantitative RT-PCR
Tub1pr	TCAACCAACTGGTGGACTGAG	tub1	Semi-quantitative RT-PCR
SZ_Lp_act1f	ATGGAACGGCATGGTCAAG	act1	Semi-quantitative RT-PCR
SZ_Lp_act1r	GTTAAGTCGCGTCCAGCAAG	act1	Semi-quantitative RT-PCR
SZ_Lp_UBQ5_1	CTGCGGGCAAGACCATCAC	ubq5	Semi-quantitative RT-PCR
SZ_Lp_UBQ5_2	CTGCTGGTTGTAGACGTAGG	ubq5	Semi-quantitative RT-PCR
K1 ORFpf	TCCTCTAGGTACAATACTCTC	PR-10	ORF identification
K1 ORFpr	GCAAGTGACAACAACCTTCATAC	PR-10	ORF identification
K2ORFpr	TCTTATTTAG ATCCACGAGG	PR-10	ORF identification
K2ORFpr2	ACACCCAGCTT CCTTTTATTT A	PR-10	ORF identification
K1depr	ACNGTIATGGAYTGGCAYAC	PR-10	cDNA identification
K1depr	TANGCYTCIGCIGTYTTRAA	PR-10	cDNA identification
K8 depr	TTYGGYGAYAAAYACAAAYGG	sodA	cDNA identification
K8 depr	CCRATRACICRCARGCRGG	sodA	cDNA identification
PR10-1RTpf	CACTACAAC GCAACTCAGC C	PR-10-1	Real-time RT-PCR
PR10-1RTpr	TAACGGTTAC AAATACTCGC GG	PR-10-1	Real-time RT-PCR
PR10GSpf4	AGTGCCACC ACGTTGAGGG AGAT	PR-10-4	RACE
PR10GSp2	CACAATTGTC CACATCCAGG TACTCA	PR-10-4	RACE
PR10GSp3	CGCATTTGTC CACATCGAGA AACTCA	PR-10-3	RACE
PR10GSp4	CGCATTTGTC CACATCAAGG AACTCA	PR-10-4	RACE
PR10GSp5	TACTTGTTCCA CATCGAGGAA CTGGAT	PR-10-5	RACE
PR10-3gpf	ATCCTCTTGG TACGATGGTC	PR-10-3	RACE
PR10-2gpf	ATCACATCTC CCCAGCGTAC	PR-10-3	RACE
HSPf	TCCCAAGACAACAAACAAATC	hspA	ORF identification
HSPr	ATGCACTTATACTCGACAGC	hspA	ORF identification
BSGSpf	CCGGACAGCAGGGCTCGAAACGA	hspA	RACE
BSGSp2	AGGGAATTCACCGTGGAGCTCGTA	hspA	RACE
BSpf	YTIGAYGAYTTYGAYAAAYTA	hspA	cDNA identification
BSPr2	TCRATRTCRGGVARYTCRCC	hspA	cDNA identification
PR2forw	ATHGCNGTNGGIAAYGARGT	PR-2	cDNA identification
PR2rev	AARTANGGRTANACRTTIAC	PR-2	cDNA identification
PR3forw	GGYTAAGTCTWCAARSAGGAG	PR-3	cDNA identification
PR3rev	CCRTTGATGATRTTGGTGA	PR-3	cDNA identification
PR4forw	GGMGMAGAWCAACTGGGACCT	PR-4	cDNA identification
PR4rev	GTCGTCGAATCSGTAGYYGA	PR-4	cDNA identification

^a Plant genes, with the exception of the *Neotyphodium lolii* genes *sodA* and *hspA*; PR = pathogenesis-related.

^b RT-PCR = reverse-transcription polymerase chain reaction, RACE = rapid amplification of cDNA ends, and ORF = open reading frame.

^c Designed by Tanaka and associates (2006).

dilution of the other sample that still yielded a product were used to estimate the expression differential. Occasionally, band intensity of PCR products from a sample template did not decline with progressive dilution of template. Such series were omitted from the analysis.

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