Functional genomics of the *Neotyphodium lolii* / ryegrass symbiosis

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Abstract

Neotyphodium lolii is a fungal endophyte that lives entirely within the intercellular spaces of its grass host, perennial ryegrass (*Lolium perenne*, L.). Infection is symptomless and the endophyte relies on the host plant for dissemination via the seed. The association is mutually beneficial since the endophyte confers a number of biotic and abiotic advantages to the host. This paper presents an overview of the functional genomics approaches we are using at AgResearch to dissect the molecular basis of this symbiosis and will broadly describe the fields of genomics, transcriptomics, proteomics and metabolomics, as applied to this system. We have used isogenic ryegrass lines infected or uninfected with endophyte in combination with a suite of molecular biology tools, including Expressed Sequence Tags (ESTs), cDNA and Affymetrix GeneChip® microarray analysis, 2D-gel electrophoresis (to identify novel proteins associated with symbiosis), and metabolic profiling. By using a multidisciplinary approach we aim to identify genes which are important in both the establishment and maintenance of symbiosis, as well as elucidate how endophyte confers enhancements to its host.
Keywords: *Neotyphodium*, *Epichloë*, symbiosis, functional genomics

Introduction

Neotyphodium and *Epichloë* spp. (phylum Ascomycota, family Clavicipitaceae) are closely related asexual and sexual endophytic fungi, respectively that form associations with temperate grasses belonging to the family Poaceae. *Epichloë* endophytes encompass antagonistic through to mutualistic interactions that cause no disease, whereas the *Neotyphodium* endophytes are exclusively mutualistic (Glenn *et al.* 1996; reviewed in Schardl *et al.* 2004). For example, *N. lolii* and *N. coenophialum* are fungal endophytes that live entirely within the intercellular spaces of perennial ryegrass (Fig. 1) and tall fescue, respectively. Infection is symptomless and the endophyte relies entirely on the host plant for dissemination via the seed or through vegetative structures (Philipson & Christey 1986; reviewed by 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 (Arechevaleta *et al*. 1989; Kimmons *et al.* 1990; Gwinn & Gavin 1992; Schardl & Phillips 1997; Clay & Holah 1999; Scott 2001; Schardl 2001; Johnson *et al*. 2003). Endophyte infection has also been implicated in modification of root morphology, osmotic adjustment and mineral uptake (Malinowski & Belesky 1999; Malinowski *et al*. 1999). Some of these benefits are due to the production of fungal secondary metabolites such as the pyrrolopyrazine (peramine), aminopyrrolizidine (loline), ergopeptine (ergovaline) and indole diterpene (lolitrem) alkaloids (Bush *et al*. 1997; Blankenship *et al*. 2001; Panaccione *et al*. 2001; Spiering *et al*. 2005; Tanaka *et al*. 2005; Young *et al*. 2006), some of which can also 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). In addition, certain fungal-produced secondary metabolites have been shown to accumulate to very high levels; lolines for example can accumulate to concentrations of up to 2% dry weight (Craven *et al*. 2001; Spiering *et al*. 2002). The endophytes also remain metabolically active throughout the growth of the host grass (Tan *et al.* 2001) and compounds associated with endophyte infection are therefore continually produced during the life cycle of its host plant.

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. To gain a better understanding of these processes during endophyte grass associations, we have initiated a multidisciplinary study to link the knowledge gained from basic biology and cytology with the fields of genomics, transcriptomics, proteomics and metabolomics. To achieve this we are using isogenic ryegrass lines infected or uninfected with endophyte and a combination of Expressed Sequence Tags (ESTs), Affymetrix GeneChip® analysis, 2D-gel electrophoresis (to identify novel proteins associated with symbiosis), and metabolic profiling (Fiehn 2002). To aid our proteomics approach (which requires gene sequence information specific to the endophyte), we have also generated both fungal genomic and fungal EST resources.

This paper will present an overview of the functional genomics approaches we are using at AgResearch to identify genes which are important in both the establishment and maintenance of symbiosis. In addition, by combining transcriptomics with metabolomics, we intend to elucidate how endophyte infection influences host secondary metabolism, which we hypothesise is correlated with many of the endophyte conferred enhancements to its host.

Genomics Resources

Genomic libraries are listed in Table 1. These include an *N. lolii* small insert plasmid library, an *N. lolii* Lambda Zap (Stratagene) phagemid library, an *N. lolii* Bacterial Artificial Chromosome (BAC) library with a 7-fold redundancy (Amplicon Express), as well as a Fosmid library of *E. festucae* strain Fl1. In a targeted approach we have also used degenerate PCR to conserved domains of both non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) enzymes (Finking & Marahiel 2004; Gaffoor *et al*. 2005) to generate mini-libraries of these gene families, which are commonly involved in secondary metabolite biosynthetic pathways. We have used these in conjunction with our other libraries to identify at least 13 NRPS (Johnson *et al*. 2007a) and 10 PKS genes in the *Neotyphodium/ Eplichloë* complex.

Functional Analysis by Gene Disruption

Functional analysis, by gene disruption or deletion, is commonly employed with filamentous fungi to determine the role of genes

Table 1 Genomic and cDNA libraries for *N. lolii* and *E. festucae*.

Library	Type	Species	Insert size	Vector	Number unigenes
Small insert	genomic	*N. lolii*	3-5 kb	pBluscript	na
Lambda Zap	genomic	*N. lolii*	4-12 kb	λZapII	na
Fosmid	genomic	*E. festucae*	30-35 kb		na
BAC	genomic	*N. lolii*	~140 kb	pECBAC1	2
NRPS	genomic	*Neotyphodium/Epichloe* spp.	300 bp	pCR2.1Topo	8
PKS	genomic	*Neotyphodium/Epichloe* spp.	300 bp	pCR2.1Topo	10
CS32	cDNA	*N. lolii*	716 bp	λTriplEx2	2011
CS36	cDNA	*N. lolii/ L. perenne*	377 bp	pCR2.1Topo	2285

that have unknown function, or to confirm the roles for those with tentative assignments based on bioinformatics. Studies in the grass-endophyte symbiosis have already provided important information for genes which are either essential for stable symbioses, for example *NoxA* encoding NADPH oxidase (Tanaka *et al*. 2006) and *SidF* encoding a novel extracellular siderophore (Johnson *et al*. 2007b) (Fig. 2), or involved in fungal secondary metabolism, such as those for the peramine (Tanaka *et al*. 2005), lolitrem (Young *et al*. 2006) and ergovaline (Panaccione *et al*. 2001; Fleetwood *et al*. 2006) biosynthetic pathways. We have also performed gene disruptions in two NRPS genes involved in the production of uncharacterised secondary metabolites (L. Johnson *et al*. unpublished results; Harzar *et al*. 2007) and are currently comparing these disrupted pathway mutants to wild-type endophyte using both metabolomics and microarray analysis to determine which compounds these pathways are likely to synthesise (Lane *et al*. 2007; Johnson *et al*. 2007c). Additionally, we have disrupted genes that have a role in fungal signalling such as *acyA* encoding adenylate cyclase from *E. festucae* (Voisey *et al*. 2007a) (Fig. 3) and other genes with unknown function, for example Nc25, which is one of the most abundantly expressed transcripts in the symbiosis (Johnson *et al*. 2007d) (Fig. 4).

The identification of further genes, differentially expressed during the symbiosis, through Affymetrix GeneChip® experiments (Johnson *et al*. 2007c; Voisey *et al*. 2007b) will provide many more candidates for future functional analysis and elucidation of their roles in this symbiosis.

Transcriptomics Resources

Expressed sequence tag (EST) libraries are listed in Table 1. We have generated extensive transcriptomic capabilities that comprise EST databases of both fungal and plant origin. For fungal libraries mycelia were harvested from *in vitro* cultures, grown in either minimal or complete media, and RNA harvested for reverse transcription into cDNA as described in Johnson *et al*. (2007e). In the latter case, ESTs were generated using Suppression Subtractive Hybridisation (SSH) (Diachenko *et al*. 1996) technology in order to enrich for transcripts differentially expressed during the symbiosis (Johnson *et al*. 2007e). Subtraction was carried out in both the forward and reverse directions in order to create six libraries containing both up- and down-regulated ESTs for three physiological states (9-day-old seedlings, immature expanding leaf tissue, and mature sheath and blade tissue).

We generated eight independent EST libraries. Two were from *N. lolii* grown *in vitro* (together designated CS32; Table 1) and six were from suppressive subtractive hybridisations between endophyte infected and endophyte free perennial ryegrass (together designated CS36; Table 1). A total of 5493 and 3896 ESTs were sequenced for CS32 and CS36, respectively. For library CS32, ESTs assembled into 412 contigs and 1599 singletons to provide a set of 2011 unigenes with an average length of 716 base pairs. For library CS36, ESTs assembled into 654 contigs and 1631 singletons to provide a set of 2285 unigenes with an average length of 377 base pairs. Overlap of expression between the two *in vitro* fungal derived libraries that comprise CS32 and between the combined up-regulated and down-regulated SSH libraries was determined on the basis of contig membership. In both cases the majority of contigs or singletons showed no overlap indicating that most unisequences were library specific.

Functional categories were assigned using the MIPS Functional Catalogue (FunCat) (Ruepp *et al*. 2004) and are represented as pie charts for CS32 and CS36, respectively (Figs. 5A & 5B).

Analysis of the SSH libraries identified several fungal genes that have a known role in the symbiosis, for example lolitrem biosynthesis (Young *et al*. 2006), β-1, 6-glucanase (Moy *et al*. 2002), a proteinase (Reddy *et al.* 1996), a chitinase (Li *et al*. 2004), as well as others with no obvious homology to other genes. Johnson *et al*. (2003) also identified two fungal genes (Nc12 and Nc25), from the tall fescue/*N. coenophialum* association in common with this study. To obtain accurate data on the expression of these and other candidate genes we have performed both cDNA microarray (Johnson *et al*. 2006) and Affymetrix GeneChip® analyses (Voisey *et al*. 2007b; Johnson *et al*. 2007c).

Affymetrix GeneChip® Analysis

To further unravel the complex molecular interplay between endophytes and their grass hosts we performed a transcriptomic study using an Affymetrix (NimbleExpress™) dual species (*N. lolii*/*L. perenne*) GeneChip® to analyse gene expression from perennial ryegrass in association with either a *Neotyphodium* species (*N. lolii*) or an *Epichloë* species (*E. festucae*), as well as from these endophytes grown *in vitro* (Johnson *et al*. 2007c; Voisey *et al*. 2007b). Additionally, we have hybridised our GeneChips® with perennial ryegrass plants associated with mutant endophyte strains that contain a single gene deletion of interest (Johnson *et al*. 2007b; Johnson *et al*. 2007d; Voisey *et al*. 2007a). These analyses have enabled us to identify candidate 'symbiotic' genes; an important subset of fungal or ryegrass genes that are only induced during the symbiotic stage. To date we have discovered 90 symbiotic fungal genes where expression of these was only detected on chips hybridised with endophyte-infected perennial ryegrass. The most abundantly expressed gene from FL1-infected perennial ryegrass is Nc25; a novel fungal gene previously shown to be highly expressed in different plant-fungal associations, but absent in cultures of *N. coenophialum* (Johnson *et al.* 2003; Johnson *et al*. 2007d). Other genes shown to be

Figure 1 Cross section of perennial ryegrass tillers infected with *E. festucae* strain Fl1 expressing green fluorescent protein (EGFP).

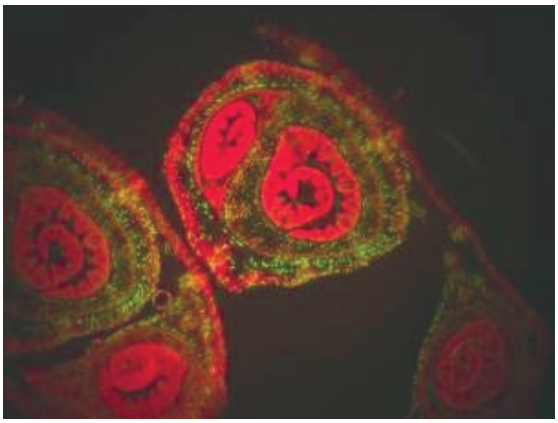
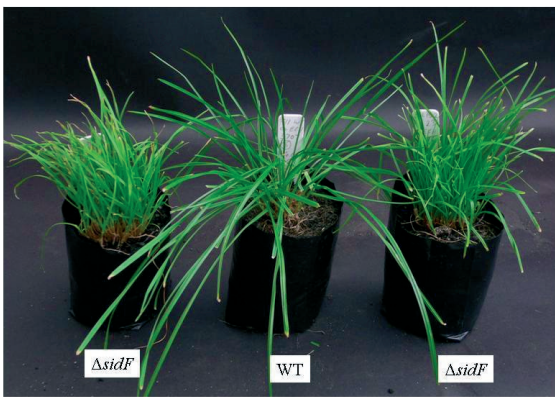


Figure 2 Comparison of wild-type (WT) and individual siderophore minus mutants (Δ*sidF*) associated with perennial ryegrass (Johnson et al. 2007b).



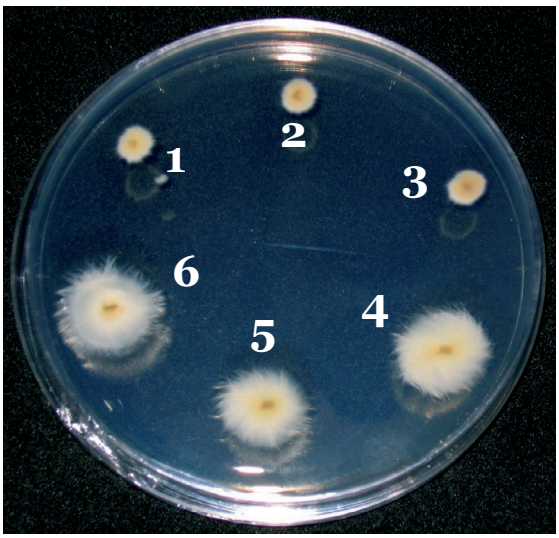
abundantly expressed are the lolitrem and ergovaline biosynthetic pathway genes. These genes are expressed abundantly *in planta* and lowly or not at all in culture (Fleetwood *et al*. 2006; Young *et al*. 2006), validating the results obtained here. Of greatest interest are the number of highly expressed fungal genes that have no annotations (using BLASTX, InterproScan, or Gene Ontology), indicating that they are novel. Future investigation of these genes should yield informative insights into the molecular regulation of grass-endophyte symbioses.

We have also identified approximately 800 or 1200 endophyte genes from Fl1 or Lp19, respectively, that are significantly up-regulated *in planta* versus *in vitro* growth conditions (Fig. 6). Following validation of a subset of our differentially expressed genes by Real-Time RT-PCR, we will be in a position to explore in more detail the function of these genes during the symbiosis.

Proteomics Resources

We have also utilised proteomic approaches to identify both plant and fungal proteins involved in the symbiotic interaction. This approach faces the challenge of trying to identify a small specific subset of proteins within infected plants from among the large number of highly abundant proteins present in plant tissues that are not specifically involved in the symbiotic interaction. We adopted

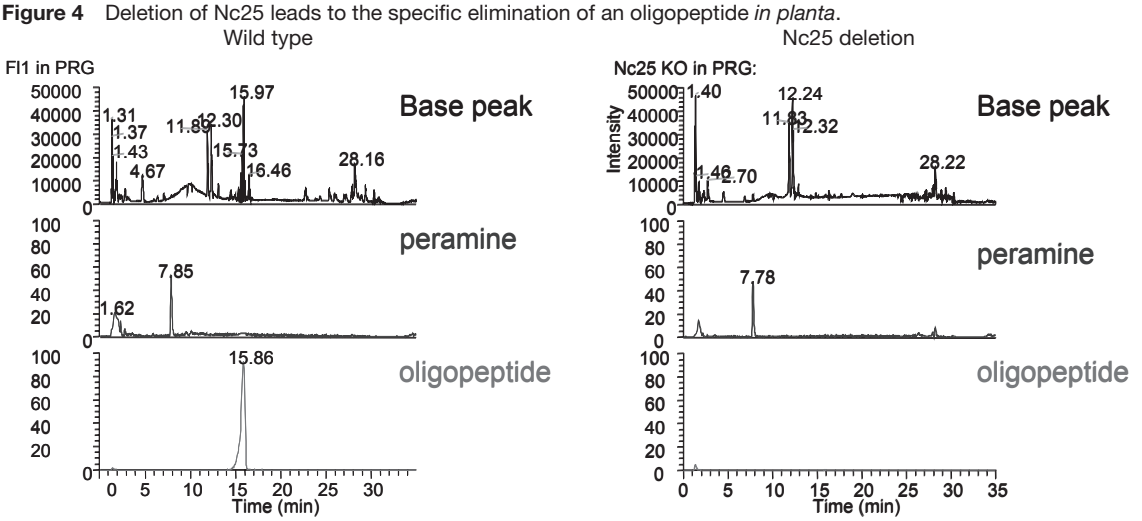
Figure 3 Growth in culture of *E. festucae* wildtype and adenylate cyclase (*acyA*) disruption mutants. 1. *E. festucae* Δ*acyA*34 (disrupted); 2. *E. festucae* Δ*acyA*42 (disrupted); 3. *E. festucae* Δ*acyA*47 (disrupted); 4. *E. festucae* E19 (Ectopic insertion); 5. *E. festucae* Fl1 (Wild-type); 6. *E. festucae* E49 (Ectopic insertion)



two main approaches to overcome this: differential purification of proteins from within the intercellular spaces of infected leaves and focusing on secreted fungal proteins. We began by characterising the fungal proteome using 2D gel electrophoresis to identify total (Fig. 7) and secreted proteins from *in vitro* fungal cultures in order to determine the overlap between proteins expressed in culture and those present in infected plants. To assist our proteomics approach, all of the fungal EST sequences were translated *in silico* into all possible reading frames and imported into a MASCOT database, which uses mass spectrometry data to identify proteins from primary sequence databases (Perkins *et al*. 1999). By interrogating this database with peptide fragmentation data obtained from MALDI-TOF and MS/MS mass spectrometry, we can now routinely identify 50% of protein spots isolated from 2D electrophoresis gels. The likelihood of identifying a protein spot correlates directly with the size and coverage of the EST database. Therefore the availability of the genome sequence should lead to significant improvements in identification of proteins using proteomics.

Differential purification of proteins from within the intercellular space has proven to be a valuable approach for identifying fungal proteins that may play a role in symbiosis. We have also been able to successfully identify a number of plant proteins, reflecting the fact that the ryegrass EST database contains an estimated 15,000 non-redundant sequences. An abundant fungal protein found exclusively in the intercellular fluid of infected ryegrass was characterised as a trypsin-like serine protease. An EST corresponding to this gene was also identified in the SSH subtractive libraries (Johnson *et al*. 2007e), and RT-PCR as well as GeneChip® analysis confirms that this gene is highly induced *in planta*. We over-expressed this gene in the fungus using a highly expressed constitutive promoter and the over-expressing strains have been infected into plants. Analysis of this symbiota is currently underway. We have also identified and characterised a number of fungal proteins with putative roles in cell wall

Figure 4 Deletion of Nc25 leads to the specific elimination of an oligopeptide *in planta*.



LCMS on ryegrass extracts infected with wild type *N. lolii* or a mutant deleted for Nc25.

biogenesis. An additional benefit of the proteomic analysis of secreted fungal proteins has been the identification of the mature processed peptides. We have found that the program SignalP (Bendtsen *et al*. 2004) is effective at identifying cleavage sites associated with secretion from predicted amino acid sequences (see for example Johnson *et al*. 2007d).

Metabolomics

Studies based on gene expression profiling and proteomics have in the past assumed simple correlations between gene expression, protein expression and metabolic states. However, the recent field of metabolomics (Fiehn 2002; Bhalla et al. 2005) has revealed that the end result of gene expression most often results in complex metabolic profiles that are difficult to interpret in terms of simple gene expression data alone (Gygi *et al*. 1999). To date few groups have combined the fields of transcriptomics, proteomics, and metabolomics in an integrated approach to correlate the metabolic phenotype with that of transcription and protein expression (Sumner *et al*. 2003; Fridman & Pichersky 2005).

We are using a metabolomics approach to understand the metabolic relationship between *N. lolii* and its host grass during symbiosis. Our Affymetrix dual species GeneChips® provide the ideal opportunity to link gene expression with the metabolism of both the plant and the endophyte. The combination of metabolomics with microarray gene expression data will enable us to annotate cellular function rather than inferring molecular function from sequence homology alone, and is more informative with regard to phenotype from a systems biology perspective (Cao *et al*. 2007). We intend to gain an overview of the major metabolites affected by endophyte infection and are developing models to describe metabolic interactions.

To compare metabolite profiles against our transcriptomic resources, tissues were dissected which overlapped those originally taken for the SSH libraries, except that blade tissue was treated separately from mature (sheath) tissue, and we did not look at seedlings. We have focussed our current analysis on carbohydrates, amino acids, phenolics, polar compounds, and endophyte alkaloids. Tissue specific differences were apparent for the different metabolite classes, emphasising the importance of discriminating between different tissue types in metabolomic studies. Significant differences were identified between endophyte-infected and uninfected ryegrass for some of the compounds analysed. These included an increase of 10-20% for some high molecular weight and low molecular weight carbohydrates in endophyte-infected grass, the presence of mannitol (a sugar alcohol) only in infected ryegrass, and a reduced level of total free amino acids in endophyte-infected ryegrass, which included large reductions in asparagine, aspartate, pipecolinic acid and gamma aminobutyric acid.

In addition to studying the symbiosis as a whole, we are also using metabolomics to try and determine the function of unknown genes. Indeed, one of the original goals of metabolomics was to determine the metabolic outcome of gene deletions (Raamsdonk *et al.* 2001). We are comparing the metabolite profiles of wild-type endophyte with those of endophytes containing specific gene disruptions. These have so far included uncharacterised NRPS genes (L. Johnson *et al*. unpublished), Nc25 (Johnson *et al*. 2007d), and adenylate cyclase (Voisey *et al*. 2007a).

Integration of Approaches

In summary, we have used a multidisciplinary approach to obtain novel information on the *N. lolii*/ryegrass symbiosis. We have identified plant and fungal differentially expressed genes, some of which may be important in this association, and have also shown that endophyte infection has a profound affect on host plant metabolism. The combination of functional analysis of gene expression and plant metabolic pathways, that appear to be directly manipulated by the ryegrass endophyte, will allow us in the future to develop metabolic models that may assist in better understanding how symbiotic and pathogenic fungi interact with plants.

The longer term benefits will be an improved ability to discover or develop superior endophyte types for pasture protection and animal production through application of the knowledge generated from the molecular interaction between the plant and endophyte. In addition, understanding how compatible symbioses are established and maintained will ultimately allow us to create new associations between endophytes and novel hosts such as cereal and rice crops.

Figure 5 Functional categories assigned to ESTs in libraries CS32 (A) and CS36 (B) based on selected categories from the MIPS Functional Catalogue (FunCat). 42% and 64% of ESTs annotated with at least one category in CS32 and CS36, respectively, are displayed

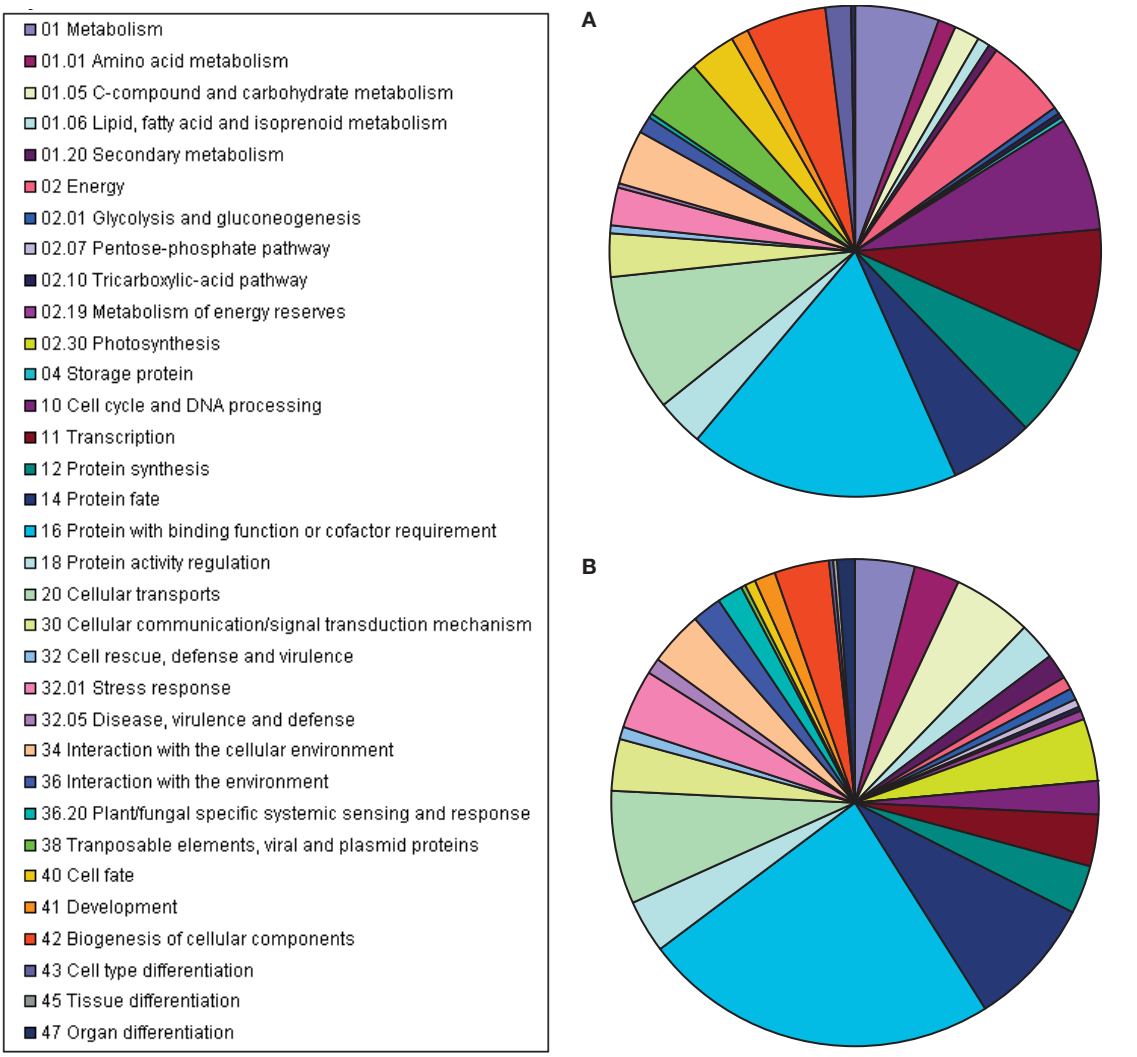


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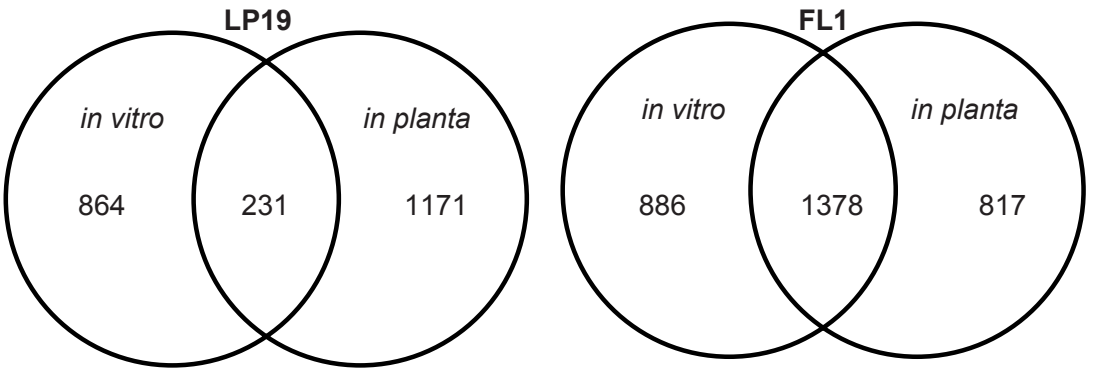
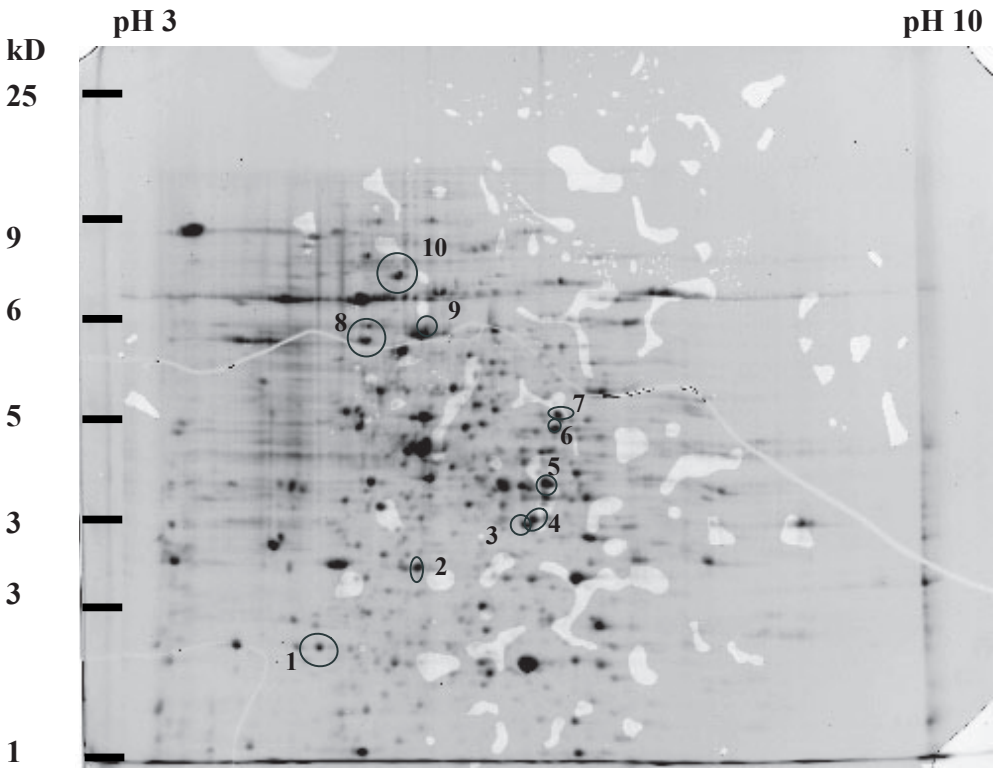
Figure 6 Fungal differentially expressed genes (FDR ≤ 0.05) subgroups with log2-ratio cut-offs.



Log ratio	Fungal gene subgroups
< -1	UP-regulated *in-vitro*
> 1	UP-regulated *in-planta*
[-1,1]	Non-differentially expressed

Figure 7 2-DE analysis of total proteins from *N. lolii* strain Lp19 cultures grown for 14 days in potato dextrose broth. Samples were separated in the first dimension using 24 cm IPG strips (pI 3-10) and in the second dimension on 12% SDS PAGE gels. The proteins were detected with colloidal Coomassie blue stain. Spots were manually excised from the gels as indicated and subjected to in-gel digestion with trypsin prior to MALDI-TOF MS analysis.



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