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A multidisciplinary approach to dissect the molecular basis 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 will present an overview of the multidisciplinary approach we are using at AgResearch to dissect the molecular basis of this symbiosis. Topics covered will include how we intend 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 in combination with a suite of molecular biology tools, including Expressed Sequence Tags (ESTs), Microarray analysis, 2D-gel electrophoresis (to identify novel proteins associated with symbiosis), and metabolic profiling. To aid our proteomics approach, we have also generated both fungal genomic and fungal EST resources.

By linking these approaches we hope to identify genes which are important in both the establishment and maintenance of symbiosis. In addition, by combining transcriptomic with metabolomic approaches, we intend to elucidate how endophyte infection influences host secondary metabolism. We hypothesise the latter is correlated with many of the endophyte-conferred enhancements to its host.

Introduction

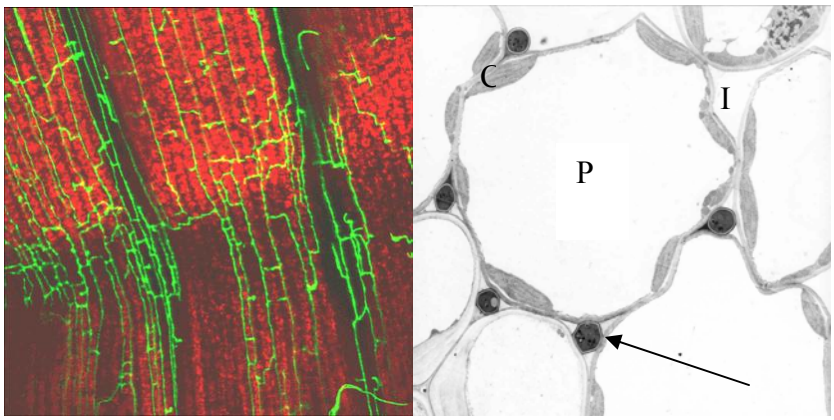
Neotyphodium and *Epichloë* spp (phylum Ascomycota, family Clavicipitaceae) are closely related asexual and sexual endophytic fungi, respectively, that form mutualistic associations with temperate grasses belonging to the family Poaceae. For example, *N. lolii* and *N. coenophialum* are fungal endophytes that live entirely within the intercellular spaces of perennial ryegrass (Figure 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 and Christey 1986 reviewed by Schardl, Leuchtmann, and Spiering, 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 Gwinn and Bernard 1990, Gwinn and Gavin 1992, Schardl and Phillips 1997, Clay and 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 and Belesky 1999, Malinowski *et al.*1999). Some of these benefits are due to the production of fungal secondary metabolites (Figure 2) 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 and 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*. 2005). 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). Although the endophyte comprises a very small amount of the total grass biomass (~0.5%), 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). In addition, the endophytes 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 affects 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), Microarray 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 multidisciplinary approach 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.

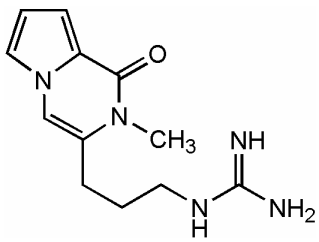
Figure 1: Longitundinal section of emerging leaf of perennial ryegrass infected with *Epichloë festucae* expressing green fluorescent protein (A). Transverse section through leaf sheath of perennial ryegrass plant infected with *N. lolii* (B). Arrow indicates fungal hypha (diameter 2µm); P indicates plant mesophyll cell; I indicates intercellular space; C indicates chloroplast.



A **B**

Figure 2: Secondary metabolites produced by endophytes when in association with their host plants. A, Peramine; B, N-Formyl Loline; C, Lolitrem B; D, Ergovaline. (Courtesy of Brian Tapper, AgResearch).

A



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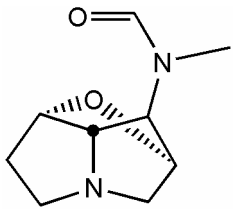

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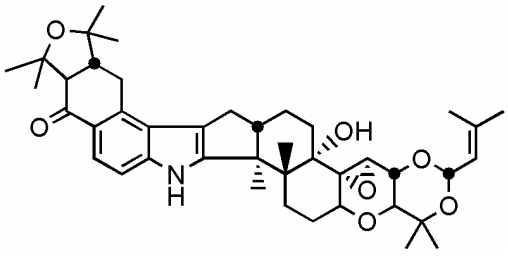

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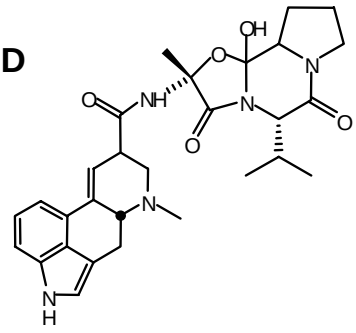
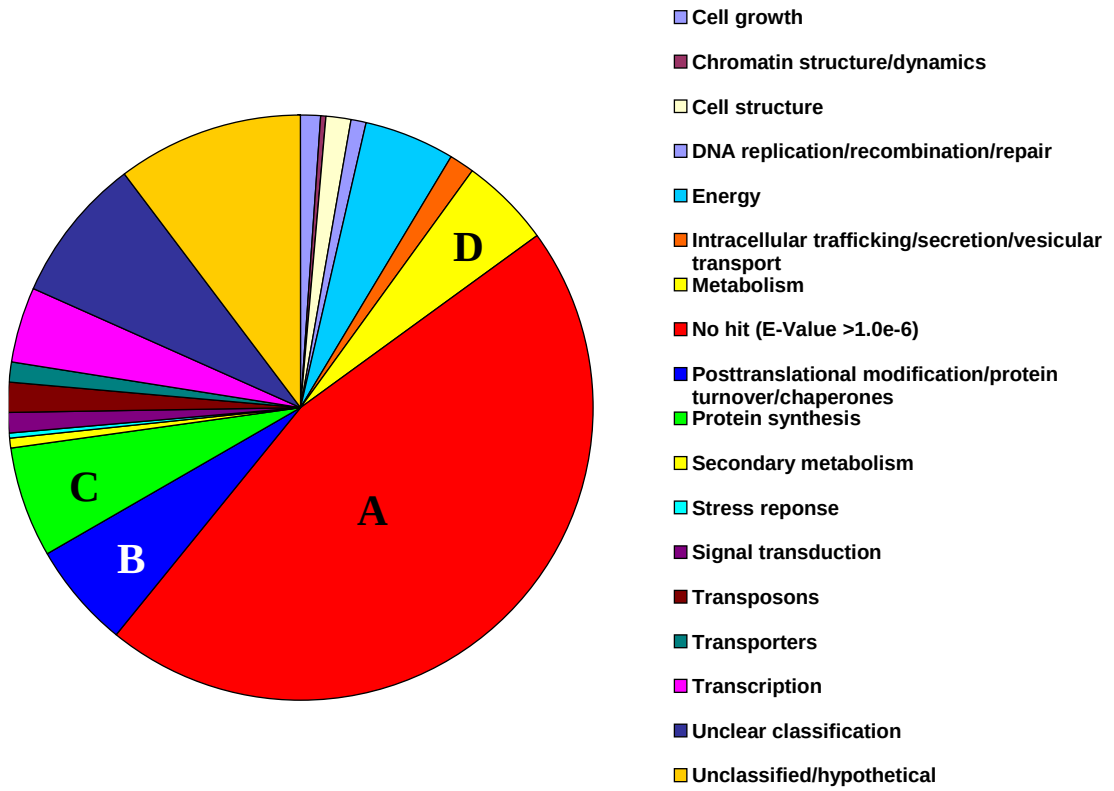


Results and discussion
This research was initiated to provide extensive molecular and biochemical resources around the ryegrass endophyte association and we intend to link the data obtained to provide novel insights into this important symbiosis.

Genomics resources
We have created the following fungal genomic resources for *N. lolii*. A small insert (3-5 Kb) genomic library was prepared for gene identification, and progress towards genome sequencing. Additionally we have genomic libraries in Lambda Zap (Stratagene) and a large insert (~140 Kb) Bacterial Artificial Chromosome (BAC) genomic library with a seven fold redundancy (Amplicon Express). In a targeted approach we also used degenerate PCR to conserved domains of both non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) enzymes (Finking and 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 genomic libraries to identify at least eight NRPS and 10 PKS genes in the genome of *N. lolii*. In addition we have identified several gene clusters on associated fungal BAC clones, and have performed gene disruptions in two NRPS genes producing uncharacterised peptides. We 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.

Transcriptomics resources
Transcriptomics is the study of gene expression in a particular organism under defined conditions. The identification of genes alone (genomics) does not provide any information on their expression (transcription). Genes can be switched on (induced), switched off (repressed), or be expressed somewhere between these two extremes, and this can be due to many factors such as, for example, physiological state, tissue type, and stress. We have generated extensive transcriptomic capabilities that comprise EST databases of both fungal and plant origin. For the former case, fungal mycelia were harvested from *in vitro* cultures, grown in either minimal (Blankenship *et al*. 2001) or complete (potato dextrose broth) media, and RNA harvested for reverse transcription into cDNA. In the later 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. Subtraction was carried out in both the forward and reverse direction in order to create six libraries containing both up- and down-regulated ESTs for three physiological states (nine day old seedlings, immature expanding leaf tissue, and mature sheath and blade tissue). Bioinformatic analysis was performed on 5575 SSH ESTs which fell into ~710 contigs and 3065 singletons. For the fungal ESTs, 7300 sequences were obtained, with 4000 unique contigs representing approximately 30-40% of the predicted 10 000 genes in this species. Functional categories were assigned to the fungal ESTs (Figure 3) based on matches to the NCBI conserved domain database (Marchler-Bauer and Bryant 2004).

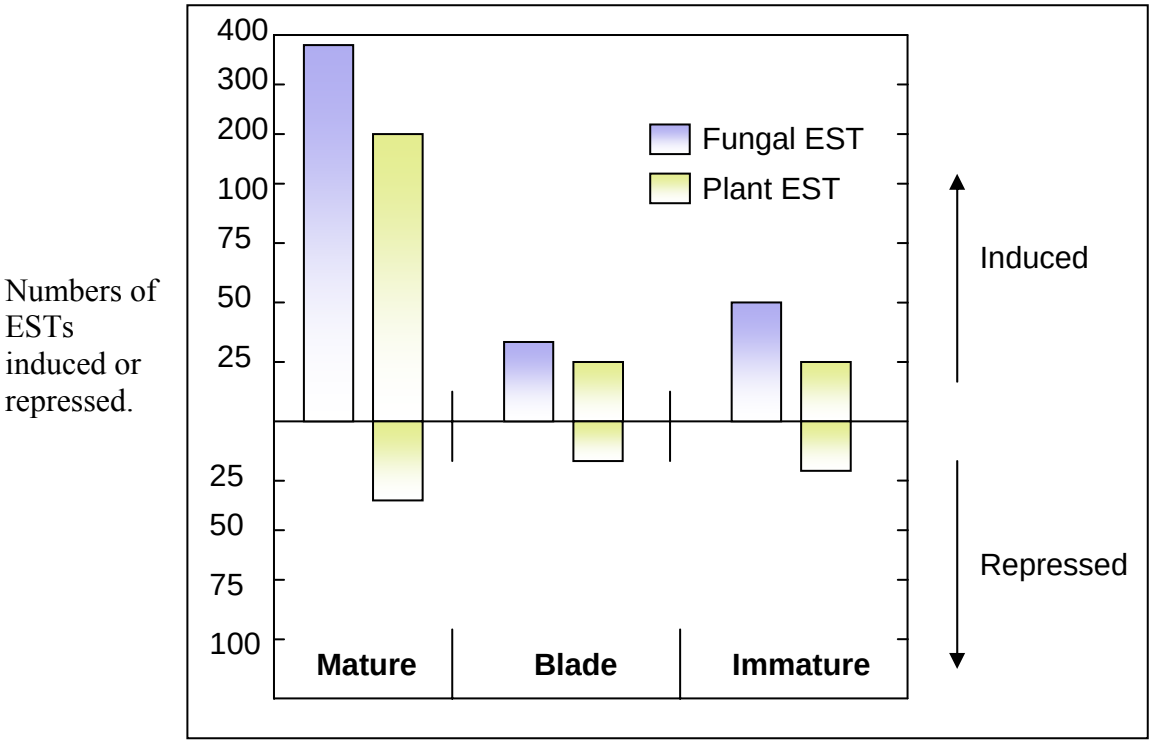
Figure 3: Functional categories assigned to Fungal ESTs obtained from *N. lolii* grown in axenic culture. Several categories are labelled to aid readers with black and white reproductions. A, no hits to database; B, post-translational modification of proteins; C, protein synthesis; D, Metabolism.



Analysis of the SSH libraries identified several fungal genes that have a known role in the symbiosis, for example lolitrem biosynthesis (Young *et al*. 2005, Young 2005), 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 some genes, from the tall fescue/*N. coenophialum* association, in common with this study.

We have also performed cDNA microarray analysis on our entire set of fungal ESTs and SSH subtracted libraries. Microarray analysis has identified both induced (fungal and plant) and repressed (plant) ESTs, including a sub-set of fungal genes that appear significantly up-regulated *in planta* (Figure 4). These include a number of genes also identified in the SSH libraries (for example those involved in lolitrem biosynthesis), verifying the subtractive hybridisation procedure, as well as many hypothetical proteins identified from *in vitro* fungal ESTs. Realtime PCR is being performed on a sub-set of ~ fifty genes to quantify *in planta* expresson levels and validate the microarray dataset. Functional analysis, by gene disruption, is in progress to elucidate their possible involvement in the symbiosis.

Figure 4: Numbers of candidate differentially expressed genes determined from microarray analysis and log2 ratios based on t-tests.

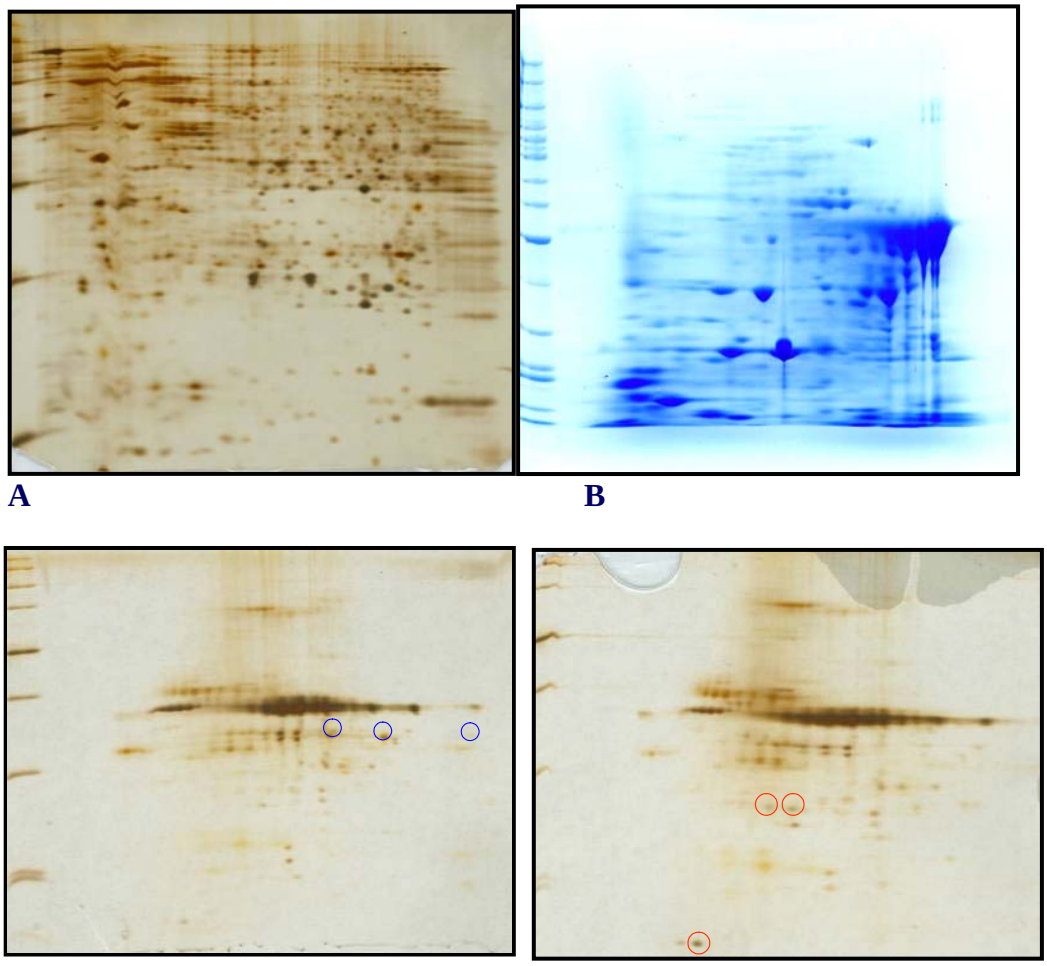


Both fungal and plant ESTs were shown to be induced, most abundantly in mature tissue. Only plant ESTs were identified as repressed in the study illustrated here.

Proteomics Resources

Proteomics is the identification and quantification of proteins under given conditions. Proteomics is also the study of protein localisation, modification, interaction, activity, and, ultimately, function. We have developed significant proteomic capabilities around the ryegrass-endophyte symbiosis. We have begun characterising, by 2D-gel electrophoresis (Figure 5), the fungal proteome by identifying total and secreted proteins from *in vitro* cultures in order to determine the overlap between proteins expressed in culture and those present in infected plants. In order 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). Interrogation of this database with peptide fragmentation data from MALDI-TOF and a SEQUEST database (Yates *et al*. 1995) with MS/MS mass spectrometry has so far identified over 30 proteins of plant and fungal origin. These include a Chitinase, HSP70, Phosphoglycerate kinase, Peptidyl-prolyl cis-trans isomerase, malate dehydrogenase and many hypothetical proteins. 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 and RT-PCR analysis confirms that this gene is highly induced *in planta*.

Figure 5: Example broad range (pH3-pH9) 2-D gels of total (A) and secreted (B) *N. lolii* proteins expressed in culture. Proteins isolated from intercellular fluid of infected (C) and uninfected (D) isogenic ryegrass plants. Circles in panel C indicate examples of fungal or plant proteins induced during symbiosis, and circles in panel D indicate examples of plant proteins repressed during symbiosis. .



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 and Pichersky 2005).

We are using a metabolomics approach to understand the metabolic relationship between *N. lolii* and its host grass during symbiosis. Our EST and transcriptomic resources 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. 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 focused our current analysis on carbohydrates, amino acids, phenolics, polar compounds, and endophyte alkaloids. Tissue specific differences were apparent for the different metabolite classes looked at, emphasising the importance of discriminating between different tissue types in metabolomic studies. Significant differences were identified between endophyte-infected and un-infected 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.

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.

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